IMPERIAL

GUT HORMONE LEAP2 IN HUMAN METABOLISM AND EATING BEHAVIOUR

Thesis submitted for the degree of the Doctor of Philosophy from Imperial College London

Author: Dr Raghav Bhargava MB BS, MRCP

Submission date: April 2025

Supervisors: Dr Tony Goldstone
Professor Anne Lingford-Hughes

Department: Division of Psychiatry
Department of Brain Sciences
Faculty of Medicine

TABLE OF CONTENTS

ABSTRACT **9**
ACKNOWLEDGMENTS **11**
STATEMENT OF ORIGINALITY **13**
COPYRIGHT DECLARATION **15**
ABBREVIATIONS **16**
INDEX OF FIGURES **19**
INDEX OF TABLES **21**
DISSEMINATION OF RESEARCH **24**
DECLARATION OF CANDIDATE'S ROLE **26**
CHAPTER 1: GENERAL INTRODUCTION **27**
1.1. OBESITY 28
1.2. GHRELIN 29
1.2.1. Ghrelin Physiology 29
1.2.2. Plasma Acyl Ghrelin in Obesity 31
1.2.3. Ghrelin Administration to Humans 33
1.2.4. Ghrelin-based Therapies for Obesity 33
1.2.5. Ghrelin and Addiction 35
1.3. LIVER-EXPRESSED ANTIMICROBIAL PEPTIDE 2 36
1.3.1. LEAP2 Hormone 36
1.3.2. LEAP2 Peptide Pharmacology 38
1.3.3. LEAP2 Activity at GHSR 39
1.3.4. Reciprocal Regulation of Plasma LEAP2 and AG 40
1.3.5. Influences on Plasma LEAP2 and mRNA Expression in Rodents 42
1.3.6. Effects of LEAP2 on Neural Activity 48
1.3.7. Effects of LEAP2 on Growth Hormone and Glucose Metabolism in Rodents 51
1.3.8. Effects of LEAP2 on Food Intake in Rodents 56
1.3.9. Effects of LEAP2 on Alcohol-related Behaviour in Rodents 64
1.4. HUMAN LEAP2 STUDIES 64
1.4.1. Human Plasma LEAP2 Assay 64
1.4.2. Greater Plasma LEAP2 in Obesity 65
1.4.3. Plasma LEAP2 Falls with Weight Loss 70
1.4.4. Plasma LEAP2 Correlates with Markers of Glucose and Fat Metabolism 71
1.4.5. Plasma LEAP2 Falls with Ketonaemia 76
1.4.6. Plasma LEAP2 Increases with Food Intake 76

1.4.7. Plasma LEAP2 in Health Conditions 79
1.4.8. Sex Differences in Plasma LEAP2 84
1.5. EATING BEHAVIOUR 85
1.5.1. Mediators of Appetite 86
1.5.2. Acyl Ghrelin and Eating Behaviour 87
1.5.3. LEAP2 and Eating Behaviour 88
1.6. FUNCTIONAL MAGNETIC RESONANCE IMAGING 91
1.7. GHRELIN SIGNALLING AND FOOD REWARD 92
1.7.1. Exogenous Ghrelin and fMRI Studies 93
1.7.2. Correlations of Endogenous Plasma Ghrelin with Food Cue Reactivity 93
1.7.3. Correlations of Endogenous Plasma LEAP2 with Food Cue Reactivity 95
1.7.4. Ghrelin and Food Memory 97
1.8. HUMAN LEAP2 INFUSION STUDIES 97
1.8.1. Effect of Exogenous LEAP2 (38-47) Fragment 97
1.8.2. Effect of Exogenous LEAP2 (38-77) 98
1.9. RATIONAL FOR CURRENT THESIS 100
1.10. THESIS HYPOTHESES AND AIMS 100
1.10.1. Hypotheses 100
1.10.2. Aims 101
CHAPTER 2: POST-PRANDIAL EFFECT OF PLASMA LEAP2 ON HUMAN EATING BEHAVIOUR IN ADULTS WITHOUT OBESITY 103
2.1. ABSTRACT 104
2.2. INTRODUCTION 105
2.3. AIMS AND HYPOTHESIS 108
2.4. MATERIAL AND METHODS 109
2.4.1. Blood Sampling and Hormone Assays 110
2.4.2. Appetite Visual Analogue Scale Ratings 111
2.4.3. Food Picture Evaluation fMRI Task 111
2.4.4. MRI Scanning Protocol and fMRI Pre-Processing 111
2.4.5. Whole Brain Analysis 112
2.4.6. Energy Intake 112
2.4.7. Statistical Analyses 113
2.5. RESULTS 115
2.5.1. Participant Characteristics 115
2.5.2. Post-Prandial Increase in Plasma LEAP2 115
2.5.3. Post-Prandial Increase in LEAP2 is Unaffected by Exogenous AG Administration 119

2.5.4. Post-Prandial Decrease in Plasma AG 122
2.5.5. Post-Prandial Decrease in Plasma AG/LEAP2 Molar Ratio 122
2.5.6. Correlations Between Change in Post-Prandial Plasma LEAP2 and AG Concentrations 123
2.5.7. Post-Prandial Decreases in Appetite and Increases in Fullness 123
2.5.8. Food Cue Reactivity 126
2.5.9. Food Appeal Ratings 139
2.5.10. Food Intake at *Ad Libitum* Meal 142
2.5.11. Correlations of Post-Prandial Changes in Gut Hormones and Markers Of Glucose and Lipid Metabolism 146
2.6. DISCUSSION 150
2.6.1. Post-Prandial Increase in Plasma LEAP2 151
2.6.2. Role of GHSR-Related Hormones in Appetite and Food Intake 152
2.6.3. Role of GHSR-Related Hormones in Food Cue Reactivity 154
2.6.4. Inter-Relationship of Post-Prandial Plasma LEAP2 with Plasma AG 157
2.6.5. Relationship of Post-Prandial Plasma LEAP2 with Glucose and Fat Metabolism 157
2.6.6. Relationship of Post-Prandial Plasma AG with Glucose and Fat Metabolism 158
2.6.7. Limitations 159
2.6.8. Conclusions 160
2.7. ACKNOWLEDGEMENTS 161
CHAPTER 3: EFFECT OF LOW-CALORIE DIET WITH/WITHOUT DUODENAL-JEJUNAL BYPASS LINER INSERTION ON PLASMA LEAP2 IN ADULTS WITH OBESITY AND TYPE 2 DIABETES MELLITUS 163
3.1. ABSTRACT 164
3.2. INTRODUCTION 165
3.3. METHODS 169
3.3.1. Study Design 169
3.3.2. Fixed Test Meal 171
3.3.3. Inclusion and Exclusion Criteria for Endobarrier Study 171
3.3.4. Blood Collection, Storage and Assay 171
3.3.5. Appetite Visual Analogue Scale Ratings 172
3.3.6. Statistical Analysis 172
3.3.7. Longitudinal Analysis of Study Outcomes 173
3.3.8. Variables Correlating with Absolute and Changes in Plasma LEAP2 174
3.3.9. Sweet Taste Task Adjustment 175

3.4. RESULTS 178
3.4.1. Demographics Of Participants 178
3.4.2. Longitudinal Outcomes 184
3.4.3. Correlations of Fasting Plasma LEAP2 with Fasting Outcomes 203
3.4.4. Correlation Analysis of Fasting Plasma LEAP2 with Outcome Variables after DJBL Explant 206
3.4.5. Longitudinal Study of Post-Prandial Variables 225
3.5. DISCUSSION 240
3.5.1. Decreases in Plasma LEAP2 During Weight Loss 242
3.5.2. Fasting Plasma LEAP2 and Glucose and Fat Metabolism 244
3.5.3. Plasma LEAP2 after DJBL Insertion 245
3.5.4. Post-Prandial Increases in Plasma LEAP2 248
3.5.5. Plasma LEAP2 and Appetite 249
3.5.6 Strengths and Limitations 250
3.5.7. Future Directions 251
3.5.8. Conclusion 251
3.6. ACKNOWLEDGEMENTS 252
CHAPTER 4: SAFETY AND METABOLIC EFFECTS OF EXOGENOUS LEAP2 INFUSIONS IN ADULTS WITHOUT OBESITY 253
4.1. ABSTRACT 254
4.2. INTRODUCTION 256
4.2.1. Acyl Ghrelin and LEAP2 256
4.2.2. Safety of Ghrelin Administration in Humans 257
4.2.3. Safety of GHSR Inverse Agonist PF-5190457 in Humans 258
4.2.4. Safety of LEAP2(38-47) Fragment in Humans 258
4.2.5. Safety of LEAP2 in Humans 259
4.3. AIMS 259
4.4. HYPOTHESES 260
4.5. METHODS 261
4.5.1. Participants 261
4.5.2. Study Design 264
4.5.3. LEAP2 Peptide Preparation 270
4.5.4. LEAP2 Peptide Dosing 271
4.5.5. Test Meals 273
4.5.4. Blood collection, Storage and Assay 273
4.5.5. Visual Analogue Scale ratings 274
4.5.6. Virtual Portion size Creation Task 275

4.5.7. Ad libitum Test Meal 279
4.5.8. Bio-electrical Impedance Analysis 281
4.5.9. Eating Behaviour Tasks 281
4.5.10. Computer-based Cognitive Tasks 282
4.5.11. Questionnaires 282
4.5.12. Safety Measures 283
4.5.13. Statistical Analysis 287
4.5.14. Ethical Approval 289
4.6. RESULTS 290
4.6.1. Participant characteristics 290
4.6.2. Visit summary 294
4.6.3. Safety Outcomes 296
4.6.4. Effects of LEAP2 298
4.7. DISCUSSION 331
4.7.1. Safety Outcomes 333
4.7.2. Effect of Exogenous LEAP2 on Markers of Glucose Metabolism 333
4.7.3. Effect of Exogenous LEAP2 on Markers of Fat Metabolism 336
4.7.4. Effect of Exogenous LEAP2 on Hormones and Biochemical Metabolites 338
4.7.5. Effect of Exogenous LEAP2 on Plasma LEAP2 339
4.7.6. Effect of Exogenous LEAP2 on Appetite 341
4.7.7. Effect of Exogenous LEAP2 on Desired and Actual Food Intake 343
4.7.8. Effect of Exogenous LEAP2 on Temporal Impulsivity and Cognitive Tasks 344
4.7.9. Effect of Exogenous LEAP2 on Food Liking and Wanting 346
4.7.10. Strengths and Limitations 347
4.7.11. Future Work 347
4.8. CONCLUSIONS 348
CHAPTER 5: GENERAL DISCUSSION 349
5.1. THESIS AIMS 350
5.2. THESIS HIGHLIGHTS 351
5.3. LEAP2 AND GLUCOSE METABOLISM 357
5.4. LEAP2 AND FAT METABOLISM 358
5.5. LEAP2 AND EATING BEHAVIOUR 359
5.6. RESEARCH IMPACT 361
5.7. STRENGTHS AND LIMITATIONS 363
5.8. FUTURE DIRECTIONS 365
5.9. CONCLUSIONS 372

REFERENCES **374**
APPENDICES **405**
Appendix 1: Consent Form for Participants 405
Appendix 2: Participant Information Sheet 407
Appendix 3: LEAP2 (38-77) Bio-efficacy Testing in Mice 423
Appendix 4: CANTAB Tasks 424
Appendix 5: Original Abstract 427

ABSTRACT

Introduction: Obesity is a global epidemic with limited therapeutic options. Appetitive gut hormones such as acyl ghrelin (AG) drive weight regain after stopping weight-loss interventions. LEAP2, a novel liver-foregut satiety hormone, act as inverse agonist at growth hormone secretagogue receptor (GHSR) and is reciprocally regulated to AG. Plasma LEAP2 increases post-prandially, after weight gain and decreases after dietary/surgical weight-loss. LEAP2 antagonises orexigenic effects of AG involved in regulating appetite and metabolism. The role of LEAP2 in human eating behaviour and metabolic regulation remains incompletely understood.

Methods: Measurement of plasma LEAP2 from previous human studies examining the effects of: food intake and/or exogenous AG administration in adults without obesity; long-term caloric restriction without or with duodenal-jejunal bypass liner (DJBL) insertion in adults with obesity and T2DM. LEAP2 was correlated with markers of body weight, glucose/fat metabolism, appetite, food intake, food reward and food cue reactivity using fMRI. In pilot studies, safety and metabolic changes were examined during exogenous LEAP2 intravenous infusion in adults without obesity.

Results: Post-prandial increases in plasma LEAP2 correlated with decreases in appetite, high-energy food cue reactivity in brain reward regions and *ad libitum* food intake. Plasma LEAP2 was unaffected by AG administration; decreased after long-term dietary weight loss without or with DJBL insertion (delayed and attenuated in the latter) and correlated with improvements in weight and glucose/fat metabolism. LEAP2 infusions upto 25pmol/kg/min over 5.7h were safe and dose-dependently reduced plasma glucose and venous beta-hydroxybutyrate independent of serum insulin and GH.

Conclusions: These results support an anorexigenic role for post-prandial LEAP2 in human eating behaviour via GHSR antagonism; independent of AG; suggesting that decreases in plasma LEAP2 contribute to weight regain and acute infusions improve glucose/fat metabolism. This lays the foundation for future studies to examine the therapeutic effects of LEAP2 administration for obesity, weight maintenance, and addiction.

ACKNOWLEDGMENTS

I would first like to express my deepest gratitude to my supervisors, Dr Tony Goldstone and Professor Anne Lingford-Hughes.

Tony, I could never have imagined that my first interview (in my new flat on the floor with upside down moving boxes on mobile internet!) with you would have changed the course of my career. I cannot express enough how much I have appreciated your patience and teaching throughout this process. You have amazing insight and work ethic. Thank you for your time and working with you has been a total pleasure.

Anne, I have felt so immensely proud to have been affiliated with you and the department. I have learnt more cross specialty practice than I imagined. Thank you for looking after me and for your ongoing guidance.

To my internal assessors Professor Dasha Nicolls and Dr Gregory Scott, thank you for your constructive feedback and insights on this work throughout the course.

This work would not have been possible without the support the NIHR Imperial Clinical Research Facility (ICRF) and their talented staff.

To my esteemed colleagues within the PsychoNeuroEndocrinology research group, Dr Marcela Rodriguez Flores, Sandra Luur, Mimoza Emini, Wanqian Li, Ceci Zhang, Jiaxun Liu, Jialin Guo and Tasya Parastika thank you for your advice and support throughout this process and for your assistance and encouragement. It has been great to have gone through this experience with you all. I hope all our paths will continue to cross in the coming years.

Ananya, thank you for being my rock and giving me space to rant, commiserate and celebrate the joys during this period. Thank you for your unconditional support through all the twists and turns that have led me here, and for your love. You have shaped this project in more ways than you know.

To both sets of Mummy and Papa, this project would not have been possible without the sacrifices you have made and continue to make for us. Thank you for always encouraging me.

To Swati and Arjun for all the happiness you bring. Madhav, thank you for being my almost twin brother.

And finally, to all the participants who generously gave their time and energy participating in this research. It goes without saying that this kind of research is not possible without people like you.

This thesis is dedicated to my grandfather, Professor KP Bhargava, to adding more stories in the family.

STATEMENT OF ORIGINALITY

All the work presented in this thesis is my own except where the work of others has been referenced in the text or is described below.

Protocol Development

The Ghrelin fMRI study design and protocol was determined by Dr Tony Goldstone. This study has been supported through the Core Medical Research Council (MRC) funding to the Metabolic and Molecular Imaging Group, MRC Clinical Sciences Centre (Chapter 2).

The Duodenal-Jejunal Bypass Liner study design and protocol were determined by Professor Julian Teare and Dr Tony Goldstone. This study has been funded through the Efficacy and Mechanism Evaluation Programme, an MRC and National Institute for Health Research (NIHR) partnership (EME 12/10/04) (Chapter 3).

The exogenous LEAP2 infusion study design and protocol were determined by Dr Tony Goldstone and Raghav Bhargava. This study has been funded through the MRC Population and System Medicine Board (MR/T017279/1) (Chapter 4).

Data Analysis

The majority of the work described in this thesis was undertaken by the author.

All collaboration and assistance is detailed below:

1. The pre-processing and first level analysis of the raw fMRI data for the Exogenous ghrelin administration fMRI study (Chapter 2) was conducted by my supervisor Dr Tony Goldstone.

2. Data handling and analysis of the raw data from the Endobarrier clinical trial (Chapter 3) was done with assistance of Mimoza Emini from the PsychoNeuroEndocrinology research group, Division of Psychiatry, Department of Brain Sciences, Imperial College London, UK

3. Clinical study visits involving exogenous LEAP2 infusions (Chapter 4) were

performed in collaboration with Dr Tony Goldstone (supervisor), and PsychoNeuroEndocrinology lab members, Division of Psychiatry, Department of Brain Sciences, Imperial College London, UK: Dr Marcela Rodriguez Flores, Sandra Luur, Mimoza Emini, Wanqian Li, Ceci Zhang, Jiaxun Liu, Jialin Guo and Tasya Parastika.

4. Preparation and bio-efficacy testing of the LEAP2 peptide (Chapter 4) was performed with the assistance of Dr James Minnion, Division of Diabetes, Endocrinology & Metabolism, Department of Metabolism, Digestion and Reproduction, Imperial College London, UK.

5. Data processing for the Leeds Food Picture Questionnaire (Chapter 4) was done by Professor Graham Finlayson, School of Psychology, Faculty of Medicine and Health, University of Leeds, UK.

COPYRIGHT DECLARATION

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution Non-Commercial 4.0 International Licence (CC BY-NC).

Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose.

When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes.

Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

ABBREVIATIONS

ACC	Anterior cingulate cortex
AG	Acyl ghrelin
AGHD	Adult growth hormone deficiency
AgRP	Agouti-related peptide
ANOVA	Analysis of variance
ARC	Arcuate nucleus
AUC	Area under curve
AUD	Alcohol use disorder
AUDIT	Alcohol Use Disorders Identification Test
BAS/BIS	Behavioural Activation System / Behavioural Inhibition System
BDI-II	Beck Depression Inventory
BES	Binge Eating Scale
BHB	Beta-hydroxy butyrate
BIA	Bioelectrical impedance analysis
BMI	Body mass index
BOLD	Blood oxygen level dependent
CANTAB	Cambridge Neuropsychological Test Automated Battery
CCK	Cholecystokinin
CHO	Carbohydrate
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DAG	Desacyl ghrelin
DDT	Delay discounting task
DEBQ	Dutch Eating Behaviour Questionnaire
DIO	Diet induced obesity
DJBL	Duodenal-jejunal bypass liner
dlPFC	Dorsolateral prefrontal cortex
DMH	Dorsomedial hypothalamus
DSM-5	Diagnostic and Statistical Manual of Mental Disorders (5th edition)
EDE-Q	Eating disorders examination questionnaire
FDR	False discovery rate
FEAT	FMRI Expert Analysis Tool
FFA	Free fatty acids
fMRI	Functional magnetic resonance imaging
fMRIB	Functional magnetic resonance imaging of the brain
fROI	Functional region of interest
FSL	FMRIB Software Library
FSH	Follicular stimulating hormone

FWE	Family wise error
GABA	Gamma aminobutyric acid
GH	Growth hormone
GHADD	Gut Hormones in Addiction
GHSR	Growth hormone secretagogue receptor-1a
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLP-1RA	Glucagon-like peptide-1 receptor agonist
GLM	General linear model
GOAT	Ghrelin O-acyl transferase
HbA1c	Glycated haemoglobin
HE	High-energy
HOMA-IR	Homeostatic Model Assessment-Insulin Resistance
kcal	kilocalorie
KO	Knock out
LCD	Low calorie diet
LE	Low energy
LEAP2	Liver-expressed antimicrobial peptide 2
LFPQ	Leeds food picture questionnaire
LH	Luteinising hormone
LSD	Least significant difference
MAFLD	Metabolic dysfunction-associated fatty liver disease
MFG	Middle frontal gyrus
MRC	Medical research council
mRNA	Messenger RNA
NAcc	Nucleus accumbens
NIHR	National Institute for Health Research
NPY	Neuropeptide-Y
NTS	Nucleus of solitary tract
OFC	Orbitofrontal cortex
OGTT	Oral glucose tolerance test
PANAS	Positive and Negative Affect Schedule
PAL	Paired associate learning
PCOS	Polycystic ovarian syndrome
PFC	Prefrontal cortex
PFS	Power of Food Scale
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
PWS	Prader-Willi syndrome
PYY	Peptide YY
REE	Resting energy expenditure

RYGB	Roux-en-Y gastric bypass
SAE	Serious adverse event
SEM	Standard error of mean
SFG	Superior frontal gyrus
SMA	Supplementary motor area
SMM	Standard medical management
SPSS	Statistical package for the social sciences
SSAI	Spielberger State Anxiety Inventory
SST	Stop signal task
SWM	Spatial working memory
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TFEQ	Three Factor Eating Questionnaire
TSH	Thyroid stimulating hormone
UK	United Kingdom
UPPS-P	Urgency, Premeditation, Perseverance, Sensation seeking, and Positive urgency
USA	United States of America
VAS	Visual analogue scale
VLCD	Very low-calorie diet
VPCT	Virtual portion creation task
VSG	Vertical sleeve gastrectomy
VTA	Ventral tegmental area
WHO	World Health Organisation
WTAR	Wechsler Test of Adult Reading
YFAS	Yale Food Addiction Scale

INDEX OF FIGURES

Figure 1.1. Components of the ghrelin system and potential therapeutic agents
Figure 1.2. Structure of human LEAP2
Figure 1.3. Reciprocal regulation and effects of acyl ghrelin and LEAP2 gut hormones
Figure 1.4. Correlation of post-prandial plasma LEAP2 with meal size consumed across different studies

Figure 2.1. Study Protocol
Figure 2.2. Plasma LEAP2, AG and AG/LEAP2 molar ratio concentrations at study visits
Figure 2.3. Correlation of post-prandial plasma LEAP2 with meal size consumed across different studies
Figure 2.4. Plasma LEAP2 at study visits
Figure 2.5. Appetite and fullness ratings at fed and fasted visits and correlations of post-prandial plasma LEAP2, AG and AG/LEAP2 molar ratio with appetite and fullness ratings
Figure 2.6. Whole-brain analysis of correlation between post-prandial changes in plasma LEAP2 and BOLD signal during food picture evaluation fMRI task
Figure 2.7. Whole-brain analysis of correlation between post-prandial changes in plasma AG and BOLD signal during food picture evaluation fMRI task
Figure 2.8. Food appeal ratings between visits for high-energy and low-energy foods
Figure 2.9. Correlations of post-prandial change in appeal ratings for high-energy food and high-energy vs. low-energy food with post-prandial plasma LEAP2, AG and AG/LEAP2 molar ratio
Figure 2.10. Energy intake at fed and fasted visits and correlations of post-prandial plasma LEAP2, AG and AG/LEAP2 molar ratio with energy intake
Figure 2.11. Correlations of post-prandial plasma LEAP2, AG and AG/LEAP2 molar ratio with BMI
Figure 2.12. Plasma glucose, serum insulin and triglyceride concentrations at study visits
Figure 2.13. Correlations of post-prandial plasma LEAP2, AG and AG/LEAP2 molar ratio with post-prandial markers of glucose and lipid metabolism

Figure 3.1. Study design: outline of randomised controlled trial
Figure 3.2. Distribution of medication use among both SMM and DJBL groups across visits
Figure 3.3. Longitudinal analysis of BMI and change in: % weight, HbA1c, fasting glucose, HOMA-IR, fasting triglycerides, fasting plasma LEAP2 and appetite ratings across visits
Figure 3.4. Longitudinal analysis of absolute fasting plasma LEAP2 across visits
Figure 3.5. Correlations of absolute and change in fasting plasma LEAP2 with absolute and change in weight, fasting glucose, HOMA-IR and appetite ratings across visits in the SMM group

Figure 3.6. Correlations of absolute and change in fasting plasma LEAP2 with absolute and change in weight, fasting glucose, HOMA-IR and appetite ratings across visits in the DJBL group
Figure 3.7. Correlations of absolute and change in fasting plasma LEAP2 with absolute and change in HbA1c, fasting insulin, triglycerides and hunger ratings across visits in SMM group
Figure 3.8. Correlations of absolute and change in fasting plasma LEAP2 with absolute and change in HbA1c, fasting insulin, triglycerides and hunger ratings across visits in DJBL group
Figure 3.9. Correlations of absolute fasting plasma LEAP2 with absolute fasting HOMA-IR and triglycerides in SMM and DJBL groups
Figure 3.10. Correlations of change in fasting plasma LEAP2 with change in weight, HbA1c, fasting glucose, insulin, HOMA-IR, triglycerides, appetite and hunger ratings after DJBL explant
Figure 3.11. Changes in post-prandial plasma LEAP2
Figure 3.12. Longitudinal analysis of post-prandial appetite and fullness ratings across visits and correlations with post-prandial plasma LEAP2

Figure 4.1. Schematic diagram of study visit timeline
Figure 4.2. Pictures in virtual portion size creation task
Figure 4.3. Screenshot showing one sample question in the VPCT
Figure 4.4. Nutritional information of food in the virtual portion size creation task
Figure 4.5. Safety parameters during exogenous LEAP2 infusion
Figure 4.6. Longitudinal representation of change in plasma glucose, serum insulin and venous beta-hydroxybutyrate
Figure 4.7. Correlation of LEAP2 dose with markers of glucose and fat metabolism
Figure 4.8. Plasma LEAP2 concentrations during LEAP2 infusions
Figure 4.9. Correlation of LEAP2 dose with desired and actual food intake
Figure 4.10. Correlation of LEAP2 dose with discounting rate for high-energy food, low-energy food, monetary and alcohol rewards
Figure 4.11. Comparison of RIA vs. ELISA assay to measure plasma LEAP2

Figure 5.1. Effects of interventions on plasma LEAP2 and mRNA expression in rodents
Figure 5.2. Effects of exogenous LEAP2 administration on metabolism in rodents and humans
Figure 5.3. Effects of exogenous LEAP2 administration on ingestive behaviour in rodents and humans
Figure 5.4. Effects on plasma LEAP2 and its correlations with markers of glucose and fat metabolism in humans
Figure 5.5. Correlations of plasma LEAP2 with eating behaviour in humans
Figure 5.6. Proposed LEAP2 fMRI study visit

INDEX OF TABLES

Table 1.1. LEAP2 amino acid sequence and fragment pharmacology
Table 1.2. Pre-clinical studies assessing effects of interventions on plasma AG, LEAP2, AG/LEAP2 ratio and organ Leap2 mRNA expression
Table 1.3. Pre-clinical studies assessing effects of LEAP2 on brain c-fos expression
Table 1.4. Pre-clinical studies assessing the effects of exogenous LEAP2 on serum growth hormone
Table 1.5. Pre-clinical studies assessing the effects of exogenous LEAP2 on plasma glucose
Table 1.6. Pre-clinical studies assessing the effects of exogenous LEAP2 on serum insulin
Table 1.7. Pre-clinical studies assessing the effects of central exogenous LEAP2 on ingestive behaviour in rodents
Table 1.8. Pre-clinical studies assessing the effects of peripheral exogenous LEAP2 on ingestive behaviour in rodents
Table 1.9. Human studies assessing disease states and manipulations on plasma AG, LEAP2 and AG/LEAP2 ratio
Table 1.10. Correlations of markers of glucose and fat metabolism with plasma LEAP2 in humans
Table 1.11. Correlations of eating behaviour with plasma LEAP2 in humans

Table 2.1. Participant characteristics
Table 2.2. Whole brain analysis for correlations of post-prandial changes in BOLD signal to high-energy foods with plasma hormones
Table 2.3. Whole brain analysis for correlations of post-prandial changes in BOLD signal to low-energy foods with plasma hormones
Table 2.4. Whole brain analysis for correlations of post-prandial changes in BOLD signal to high- or low-energy foods with plasma hormones
Table 2.5. Summary of results

Table 3.1. Main effects on absolute: fasting plasma LEAP2, glucose, insulin, HOMA-IR and appetite ratings across weeks
Table 3.2. Post-hoc analysis of absolute: fasting plasma LEAP2, glucose, insulin, HOMA-IR and appetite ratings across weeks
Table 3.3. Participant characteristics at baseline visit
Table 3.4. Description of medication use in both SMM and DJBL groups across weeks
Table 3.5. Main effects of group and weeks on medication use in both SMM and DJBL groups
Table 3.6. Main effect of weeks on absolute BMI and change in: % weight, fasting appetite ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 in SMM group
Table 3.7. Post-hoc analysis of absolute BMI and change in: % weight, fasting appetite ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2, in SMM group

Table 3.8. Main effects of weeks on absolute BMI and change in: % weight, fasting appetite ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 in DJBL group
Table 3.9. Post-hoc analysis of effect of absolute BMI and change in: % weight, fasting appetite ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2, in DJBL group
Table 3.10. Main effects of group, weeks and sweet taste task on absolute fasting LEAP2 between SMM and DJBL groups
Table 3.11. Post-hoc analysis of absolute fasting LEAP2 in SMM and DJBL groups.
Table 3.12. Main effects on absolute BMI and change in: % weight, fasting appetite ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 between SMM and DJBL groups
Table 3.13. Post-hoc analysis of absolute BMI and change in: % weight, fasting appetite ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 in SMM and DJBL groups
Table 3.14. Effect of covariates on absolute and change in fasting plasma LEAP2 and appetite ratings across weeks in the SMM group
Table 3.15. Effect of covariates on absolute and change in fasting plasma LEAP2 and hunger ratings across weeks in the SMM group
Table 3.16. Effect of covariates on absolute and change in fasting plasma LEAP2 and appetite ratings across weeks in the DJBL group
Table 3.17. Effect of covariates on absolute and change in fasting plasma LEAP2 and hunger ratings across weeks in the DJBL group
Table 3.18. Effect of covariates HOMA-IR and triglycerides on absolute fasting plasma LEAP2 in SMM and DJBL groups
Table 3.19. Effect of covariates on absolute fasting plasma LEAP2, appetite and hunger ratings across weeks between SMM and DJBL groups
Table 3.20. Effect of covariates on change in fasting plasma LEAP2, appetite and hunger ratings across weeks between SMM and DJBL groups
Table 3.21. Effect of covariates on change in fasting plasma LEAP2, appetite and hunger ratings across weeks in DJBL group after device explant
Table 3.22. Main effects of weeks on change in % weight and post-prandial LEAP2 in SMM and DJBL groups
Table 3.23. Post-hoc analysis of change in % weight and post-prandial LEAP2 in SMM and DJBL groups
Table 3.24. Post-prandial LEAP2 at -2 weeks across SMM and DJBL groups
Table 3.25. Post-hoc analysis of post-prandial LEAP2 at -2 weeks across SMM and DJBL groups
Table 3.26. List of studies illustrating correlation of post-prandial plasma LEAP2 with meal size consumed
Table 3.27. Main effects on post-prandial LEAP2 across weeks in SMM and DJBL groups
Table 3.28. Post-hoc analysis of post-prandial LEAP2 in SMM and DJBL groups
Table 3.29. Main effects on post-prandial appetite and fullness across weeks in SMM and DJBL groups
Table 3.31. Effect of covariates on post-prandial appetite and fullness across weeks in SMM and DJBL groups
Table 3.32. Summary table of correlational results

Table 4.1. Nutritional composition of foods items in the ad libitum meal
Table 4.2. Participant characteristics
Table 4.3. Eating behaviour questionnaires
Table 4.4. Impulsivity, mood and other questionnaires
Table 4.5. Effect of LEAP2 dose on change in total, fasting and post-prandial markers of glucose and fat metabolism
Table 4.6. Effect of LEAP2 dose on change in hormones and biochemical metabolites
Table 4.7. Effect of LEAP2 dose with covariates on VAS and mood ratings.
Table 4.8. Main effects of fatness, sweetness and LEAP2 dose on desired food intake
Table 4.9. Effect of LEAP2 dose with covariates on desired food intake
Table 4.10. Main effects of fatness, sweetness and LEAP2 dose on ad libitum food intake
Table 4.11. Effect of LEAP2 dose with covariates on ad libitum food intake
Table 4.12. Main effects of reward size and LEAP2 dose on temporal impulsivity for alcohol, food and monetary rewards
Table 4.13. Effect of LEAP2 dose with covariates on temporal impulsivity for alcohol, food and monetary rewards
Table 4.14. Effect of LEAP2 dose with covariates on cognitive outcomes
Table 4.15. Main effects of fatness, sweetness and LEAP2 dose on food reward
Table 4.16. Effect of LEAP2 dose on fat or sweet bias
Table 4.17. Effect of LEAP2 dose on baseline (fasting at -10min) markers of glucose and fat metabolism, pituitary hormones and biochemical metabolites
Table 4.18. Effect of LEAP2 administration on baseline (fasting at -10min) markers of glucose and fat metabolism, pituitary hormones and biochemical metabolites
Table 4.19. Effect of LEAP2 administration on change in total markers of glucose and fat metabolism, pituitary hormones and biochemical metabolites, VAS ratings, desired and ad libitum food intake, temporal impulsivity for alcohol, food and monetary rewards and cognitive outcomes
Table 4.20. Results of paired t-test comparing visits with LEAP2 administration (24/25pmol/kg/min) vs. without (saline) on change in total markers of glucose and fat metabolism, pituitary hormones and biochemical metabolites, VAS ratings, desired and ad libitum food intake, temporal impulsivity for alcohol, food and monetary discounting rates, food bias and cognitive outcomes
Table 4.21. Summary of results

DISSEMINATION OF RESEARCH

Publications incorporated into thesis

Chapter 2: Bhargava R, Luur S, Rodriguez Flores M, Emini M, Prechtl CG and Goldstone AP. Post-prandial increases in liver-gut hormone LEAP2 correlate with attenuated eating behaviour in adults without obesity. *Journal of the Endocrine Society.* Jun 5 2023;7(7):bvad061.

Chapter 3: Emini M*, **Bhargava R*,** Aldhwayan M, Chhina N, Rodriguez Flores M, Aldubaikhi G, Al Lababidi M, Al-Najim W, Miras AD, Ruban A, Glaysher MA, Prechtl CG, Byrne JP, Teare JP and Goldstone AP. Satiety hormone LEAP2 after low-calorie diet with/without Endobarrier insertion in obesity and type 2 diabetes mellitus. *Journal of the Endocrine Society.* Dec 4 2024;9(1):bvae214. *Joint first author

Publications completed during this PhD but not included in thesis

Kharbanda KK, Farokhnia M, Deschaine SL, **Bhargava R**, Rodriguez Flores M, Casey CA, Goldstone AP, Jerlhag E, Leggio L and Rasineni K. Role of the ghrelin system in alcohol use disorder and alcohol-associated liver disease: A narrative review. *Alcohol Clinical and Experimental Research*. Dec 2022;46(12):2149-2159.

Conference and research presentations associated with thesis

1. Exogenous satiety liver/gut hormone LEAP2 dose dependently reduces blood ketones without changes in serum insulin or GH in adults without obesity. **Bhargava R**, Rodriguez-Flores M, Luur S, Emini M, Li W, Ng WI, Zhang X, Guo J, Liu J, Minnion J, Goldstone AP. 31st Annual Meeting of Society for the Study of Ingestive Behavior, Chicago, Illinois, USA, 9-13th July 2024 (Poster).

2. Fasting satiety liver/foregut hormone LEAP2 negatively correlates with high energy versus low energy food cue reactivity in adults with obesity. Emini M, **Bhargava R**, Rodriguez-Flores M, Chhina N, Aldubaikhi G, Al Lababidi M, Flores L, Ruban A, Prechtl, CG, Teare JP, Goldstone AP. 31st Annual Meeting of Society for the Study of Ingestive Behavior, Chicago, Illinois, USA, 9-13th July 2024 (Oral communication).

3. Fasting plasma satiety liver/gut hormone LEAP2 temporarily falls with weight loss and improved glycaemic control from low-calorie diet in adults with obesity and type 2 diabetes mellitus. Emini M, **Bhargava R**, Aldhwayan M, Chhina N, Rodriguez-Flores M, Aldubaikhi G, Al Lababidi M, Al-Najim W, Flores L, Miras AD, Ruban A, Glaysher MA, Prechtl CG, Byrne JP, Teare JP, Goldstone AP. 30th Annual Meeting of Society for the Study of Ingestive Behavior, Portland, Oregon, USA, 11-15th July 2023 (Oral communication).

4. Satiety Gut Hormone LEAP2 and Human Eating Behaviour. **Bhargava R**, Rodriguez-Flores M, Emini M, Luur S, Prechtl CG, Goldstone AP. European Congress of Neuropsychopharmacology, Girona, Spain, 26-27th April 2023 (Oral communication).

5. Investigating metabolic and hormonal influences on post-prandial increase of plasma satiety liver/intestinal hormone LEAP2 in adults without obesity. **Bhargava R**, Rodriguez-Flores M, Emini M, Luur S, Prechtl CG, Goldstone AP. 29th Annual Meeting of Society for the Study of Ingestive Behavior, Porto, Portugal, 12-16th July 2022 (Oral communication).

6. Correlations of post-prandial changes in plasma appetitive gut hormones LEAP2 and acyl ghrelin with post-prandial decreases in appetite, and food cue reactivity, appeal and intake in adults without obesity. Luur S, **Bhargava R**, Rodriguez-Flores M, Emini M, Prechtl CG, Goldstone AP. 29th Annual Meeting of Society for the Study of Ingestive Behavior, Porto, Portugal, 12-16th July 2022 (Oral communication).

DECLARATION OF CANDIDATE'S ROLE

Chapter 1: General introduction

The candidate (Raghav Bhargava) conceptualised and drafted the chapter. The literature search was conducted by the candidate. The candidate authored the chapter with constructive feedback from his supervisor (Dr Tony Goldstone).

Chapter 2: Post-prandial effect of plasma LEAP2 on human eating behaviour in adults without obesity

The study was conceptualised and designed by the candidate's supervisor. The candidate drafted the chapter, measured the LEAP2 hormone, conducted the secondary analysis of data and received constructive feedback from his supervisor, manuscript co-authors and from peer reviewers from *Journal of the Endocrine Society.*

Chapter 3: Effect of low-calorie diet with/without duodenal-jejunal bypass liner insertion on plasma LEAP2 in adults with obesity and type 2 diabetes mellitus

The study was conceptualised and designed by the candidate's supervisor. The candidate drafted the chapter, conducted the secondary analysis of data and received constructive feedback from his supervisor, manuscript co-authors and from peer reviewers from *Journal of the Endocrine Society.*

Chapter 4: Safety and metabolic effects of exogenous LEAP2 infusions in adults without obesity

The study was conceptualised and designed by the candidate and his supervisor. The candidate and his supervisor obtained ethical approval from the NHS Research Ethics Committee and Health Research Authority. The candidate performed all aspects of recruitment, delivery of the trial interventions, data collection, data entry, data analysis and was the study on-call doctor. The candidate drafted the chapter with constructive feedback from his supervisor.

Chapter 5: General discussion

The candidate conceptualised and drafted the chapter with constructive feedback from his supervisor.

CHAPTER 1: GENERAL INTRODUCTION

1.1. OBESITY

Obesity is a chronic, relapsing condition that carries substantial societal and economic consequences. It is one of the primary causes of early mortality and represents a significant health challenge (WHO 2022; Rubino et al. 2025). This condition is defined by a body mass index (BMI) of >30kg/m^2 with more than 890 million or 13% of adults meeting the criteria globally (WHO 2024). The prevalence of obesity has nearly tripled from 1975 and is expected to affect over 1 billion adults by 2030 (Lingvay et al. 2024).

Obesity is associated with decreased quality of life and social wellbeing and development of metabolic (e.g. cardiovascular disease, hypertension, type 2 diabetes mellitus (T2DM), hyperlipidaemia, cancer, infertility, metabolic dysfunction associated fatty liver disease (MAFLD), gout, polycystic ovarian syndrome (PCOS)), mechanical (osteoarthritis, obstructive sleep apnoea, gastroesophageal reflux, respiratory failure and urinary incontinence) and behavioural and mental health complications (Lingvay et al. 2024; Rubino et al. 2025).

Various approaches to managing weight have been attempted with differing levels of success, including calorie restriction, bariatric surgery, and the use of newer approved oral and injectable treatments such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) receptor agonists (Perdomo et al. 2023). The interacting core behavioural components of eating and addictive behaviours (appetite, food and drug hedonics and craving, reward sensitivity, impulsivity, stress sensitivity) are among the most relevant novel therapeutic targets to aid weight loss and maintenance in obesity, and abstinence and prevention of relapse in alcohol and drug dependence. Peripheral signals of energy balance and food intake such as gut hormones, including stomach-derived orexigenic ghrelin, regulate eating behaviour, food intake, and glucose metabolism, not only through hunger modulation via vagal-brainstem-hypothalamic pathways, but also through reward processing, stress responsivity influence and addictive behaviours (Sumithran et al. 2011; Schalla et al. 2019; Srivastava et al. 2018; Jerlhag 2019; Kojima et al. 2006; Korbonits et al. 2004; Stengel et al. 2011).

There is a need to identify additional therapeutic targets that enhance weight loss and,

more importantly, help sustain these reductions in obesity. This is particularly important for individuals who are unwilling to undergo surgery, unable to tolerate certain medications especially GLP-1 analogues due to gastrointestinal side effects or achieve weight loss that fails to normalize body composition. Now, combination therapies such as triple hormone receptor agonists (GLP-1/GIP/glucagon) (Jastreboff et al. 2023) and amylin/GLP-1 analogues (Frias et al. 2023) that focus on gut hormones are increasingly being utilized to improve weight loss outcomes. Moreover, targeting the gut hormones active at the growth hormone secretagogue receptor (GHSR) may provide novel interventions for treatment of obesity and addictive behaviours.

1.2. GHRELIN

1.2.1. Ghrelin Physiology

Ghrelin is a 28 amino acid stomach-derived peptide hormone, whose acylation by the ghrelin O-acyltransferase (GOAT) enzyme instils biological activity, converting desacyl ghrelin (DAG) to acyl ghrelin (AG), active at the GHSR (Kojima et al. 2006; Korbonits et al. 2004; Stengel et al. 2011; Romero et al. 2010; Muller et al. 2015). Plasma AG increases during fasting, stress, chronic negative energy balance and weight loss, and falls after food intake. AG acts in the hypothalamus to stimulate food intake and hunger through hypothalamic orexigenic neuropeptides such as NPY (Muller et al. 2015). In rodents, central ghrelin signalling also mediates stress-induced overeating, increased impulsivity towards food and reward-related eating (Dickson et al. 2011; Skibicka et al. 2011a; Tong et al. 2011; Chuang et al. 2011; Anderberg et al. 2016), including actions via dopaminergic, plus opioid and GABA, circuits (Dickson et al. 2011; Chuang et al. 2011; Muller et al. 2015). AG also reduces insulin secretion, and increases insulin resistance and plasma glucose (Poher et al. 2018), and pituitary growth hormone (GH) secretion via GHSR on pituitary somatotrophs, to maintain plasma glucose during starvation by increasing hepatic gluconeogenesis (Korbonits et al. 2004; Goldstein et al. 2011).

DAG, is more abundant in circulation and can be converted from AG through the action of serum and tissue esterase enzymes, such as butyrylcholinesterase (De Vriese et al. 2004). Initially, DAG was considered biologically inactive because it lacks the acyl

side chain required for full activation of the GHSR receptor (Delhanty et al. 2013, 2014). However, recent research has shown that DAG has biological functions, though the exact receptor and pathway through which it exerts these actions are still unclear. While DAG does not function as an antagonist at GHSR under normal conditions, it can counteract AG's effects on hypothalamic neural activity, glucose metabolism, and lipolysis when both peptides are administered together (Stevanovic et al. 2014; Gauna et al. 2004; Broglio et al. 2004). To date, there are only a few clinical studies examining the effects of exogenous DAG or DAG analogues on food intake or body weight with inconsistent and sometimes contradictory results (Stengel et al. 2010).

GHSR is a seven-transmembrane G-protein-coupled receptor (GPCR) that is widely distributed across various tissues, with the highest levels found in the central nervous system (CNS) (Zigman et al. 2006). It is particularly abundant in the hypothalamus and the nucleus of the solitary tract (NTS), highlighting its involvement in regulating feeding behaviour and energy balance (Mason et al. 2014). Additionally, both AG and DAG influence the CNS, and their transport across the blood-brain barrier can occur independently of GHSR activity (Rhea et al. 2018). The GHSR displays a strikingly high, physiologically relevant, constitutive activity ensuring continuous signalling even in the absence of ghrelin (Holst et al. 2004; Mason et al. 2014). This activity is thought to help maintain basal metabolic processes, appetite regulation, and energy homeostasis during periods when AG levels are low, such as after meals. Heterodimerization of GHSR with other receptors is a key mechanism that expands its functional capabilities and influences signalling pathways, such as functional heterodimers with dopamine receptor D1, which modulates dopaminergic signalling. This interaction is believed to influence reward-seeking behaviours and regulate food reward and addictive behaviours (Jiang et al. 2006; Kern et al. 2012; Skibicka et al. 2011a; Skibicka et al. 2011b).

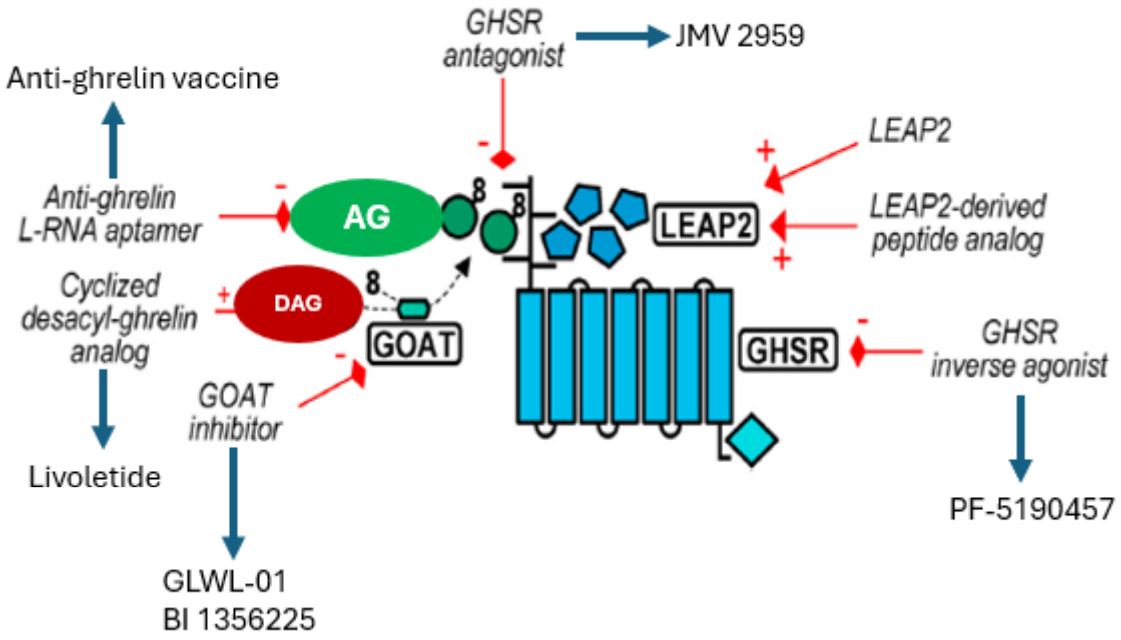


Figure 1.1. Components of the ghrelin system and potential therapeutic agents. Abbreviations: GHSR, Growth hormone secretagogue receptor; GOAT, ghrelin O-acyltransferase; +, stimulate; - inhibit. Figure adapted from (Gupta et al. 2021b).

1.2.2. Plasma Acyl Ghrelin in Obesity

Fasting and post-prandial plasma total ghrelin and AG are reduced in obesity (Muller et al. 2015; Wang et al. 2022), in part related to increased insulin which suppresses ghrelin secretion (Goldstone et al. 2004; Goldstone et al. 2005; Goldstone et al. 2012), but also due to increased insulin resistance, increased plasma leptin which in turn signals the brain to reduce hunger, contributing to a decrease in ghrelin secretion (Cummings et al. 2002b),chronic positive energy balance leads to reduction in AG as the body's mechanism to further limit weight gain (Muller et al. 2015). and altered ghrelin sensitivity in obesity (Camina et al. 2003).

Increases in plasma AG with weight loss could counteract negative energy balance by increasing appetite, food reward and intake, potentially contributing to long-term failure of dietary energy restriction to maintain weight loss.

In both rodents and humans, conditions of negative energy balance such as fasting or weight loss resulting from caloric dietary restriction, including low-calorie (LCD) or very low-calorie diets (VLCD) lead to an increase in plasma AG, while food intake and obesity are associated with reductions in plasma AG levels (Muller et al. 2015; Wang

et al. 2022; Mani et al. 2019; Aukan et al. 2023; Nymo et al. 2017; Nymo et al. 2018; Lyngstad et al. 2019; Coutinho et al. 2018; Peos et al. 2021). Notably, plasma total ghrelin levels remain elevated even one year after completing an eight-week VLCD for obesity (Sumithran et al. 2011).

Furthermore, insertion of duodenal-jejunal bypass liner (DJBL, Endobarrier device), a non-surgical endoscopic intervention, where a 60cm plastic tube lines the proximal small bowel, mimicking the foregut effects of Roux-en-Y gastric bypass (RYGB) surgery, as food is not in contact with the foregut after ingestion. DJBL insertion has greater weight loss and often superior improvement in glycaemic control (vs. LCD and standard medical management alone), in adults with obesity and inadequately-controlled T2DM (Betzel et al. 2017a; Koehestanie et al. 2014; de Jonge et al. 2015), and in recent clinical trials (Glaysher et al. 2017; Ruban et al. 2020; Glaysher et al. 2021; Ruban et al. 2022; Aldhwayan et al. 2022), and improvement in MAFLD (de Jonge et al. 2013; Karlas et al. 2022).

Rodent studies support a role for the increase in plasma AG induced by weight loss in weight regain after cessation of caloric restriction, this is supported by reduced weight regain in ghrelin knock out mice (vs. wild type) after caloric restriction (Briggs et al. 2013).

However in humans, in adults with obesity and T2DM, weight regain after diet-induced weight loss increased with plasma AG (Thom et al. 2020; Thom et al. 2021), however other studies suggest variable evidence that increases in plasma total ghrelin or AG after diet-induced weight loss predicts subsequent weight regain (Puklin et al. 2021; Garcia et al. 2006; Buso et al. 2021). Similarly, in adults with obesity and T2DM, weight loss caused by DJBL insertion leads to an increase in fasting total ghrelin 4 weeks (Koehestanie et al. 2014) and 12 months post implant (Vilarrasa et al. 2017), however, there were no correlations of ghrelin increases with associated weight loss.

Reductions in plasma AG after laparoscopic vertical sleeve gastrectomy (VSG), and relative attenuation of the weight-loss associated rise in AG after RYGB surgery for obesity, may contribute to long-term weight loss and reductions in food cue reactivity

food intake, in addition to the large increase in satiety gut hormones PYY and GLP-1 after RYGB (Yousseif et al. 2014; Li et al. 2019; Miras et al. ; Mathes et al. 2012; Stefater et al. 2012; Goldstone et al. 2016).

1.2.3. Ghrelin Administration to Humans

Exogenous intravenous or subcutaneous administration of AG in humans increased GH secretion, appetite and food intake in adults with normal weight and obesity (Druce et al. 2005; Druce et al. 2006; Wren et al. 2001). In functional magnetic resonance imaging (fMRI) studies, acute AG administration to adults without obesity in the fed state increased amygdala, OFC, anterior insula, and striatum responses to food cues (Malik et al. 2008), and mimicked the effects of fasting induced endogenous hyperghrelinaemia, with increased appeal ratings and cue reactivity to high-energy (HE) food pictures, and increases in blood oxygen level-dependent (BOLD) signal in the orbitofrontal cortex (OFC), a region encoding salience, and hippocampus (Goldstone et al. 2014). Chronic subcutaneous AG administration improved body weight and food intake in cachexia from cancer and renal failure (Ashby et al. 2009; Neary et al. 2004).

1.2.4. Ghrelin-based Therapies for Obesity

Prader-Willi syndrome (PWS) is a genetic obesity syndrome due to the deletion of paternally inherited genes on chromosome 15 that is associated with hyperphagia, weight gain, hypogonadism and GH deficiency (Goldstone 2004) Fasting and post-prandial plasma total ghrelin and AG concentrations are raised in PWS (Cummings et al. 2002a; Haqq et al. 2002; Goldstone et al. 2004) which could be responsible for their hyperphagia and body weight gain.

Administration of a DAG analogue (Livoletide), a functional AG antagonist, reduced body weight, and plasma glucose over 2 weeks, in human obesity and T2DM, and hyperphagia in genetic obesity Prader-Willi syndrome (PWS) (Allas et al. 2013; Allas et al. 2018).However, the subsequent trial did not yield statistically significant improvements in hyperphagia-related food behaviours and led to the discontinuation of any further development (https://clinicaltrials.gov/study/NCT03790865).

The markedly elevated plasma total ghrelin and AG in the PWS (Haqq et al. 2002; Cummings et al. 2002a; Goldstone et al. 2004; Goldstone et al. 2005; Goldstone et al. 2012; Kuppens et al. 2015), led to a Phase 2 clinical trial of a GOAT inhibitor (GLWL-01) to inhibit conversion of DAG to AG to treat their hyperphagia, but 28 days treatment had no effect on hyperphagia-related behaviour, body weight or metabolic markers (Miller et al. 2022). However, it has been postulated that the high plasma ghrelin in PWS may represent elevations of proghrelin due to defective processing of the ghrelin precursor preproghrelin to mature ghrelin from deficiency of the enzyme prohormone convertase 1 (Burnett et al. 2017), since commercial antibodies against total ghrelin and AG may cross react with proghrelin, which does circulate in plasma (Pemberton et al. 2003; Bang et al. 2007).

However, this GOAT inhibitor (GLWL-01) was also administered to adults with obesity and T2DM with reductions in fasting plasma AG without any effect on body weight (clinicaltrials.gov/ct2/show/results/NCT02377362).

Another recent, GOAT inhibitor (BI 1356225) given orally in men and post-menopausal women with overweight and obesity without diabetes over 28 days, did reduce plasma AG concentrations, but did not reduce body weight, appetite, food cravings, *ad libitum* food intake or change markers of glucose metabolism, and so has not been progressed as a therapy for obesity, and has not been studied for weight loss maintenance (Bianzano et al. 2023).

In preclinical models, GHSR antagonists, including non-peptide variants such as JMV 2959, JMV 3002, AZ-GHS-38 have shown promising results in reducing weight loss, enhancing glucose tolerance and suppressing appetite (Esler et al. 2007; Giorgioni et al. 2022). However, none of these have been trialled in humans to date.

In rodent studies, an anti-ghrelin vaccines focussing on passive immunization through transferring of anti-ghrelin antibodies showed reduction in food intake (Andrade et al. 2013). Subsequent preclinical studies of anti-ghrelin vaccines to generate anti-ghrelin antibodies, also reduced food intake with increased energy expenditure (Zorrilla et al. 2006; Azegami et al. 2017; Altabas et al. 2015). However, in humans an anti-ghrelin

vaccine, CYT009-GhrQb, developed by Cytos Biotechnology, was administered once weekly, in adult humans with obesity over a course of 4 weeks (https://adisinsight.springer.com/drugs/800023624)(Rubinic et al. 2024). This human vaccine was safe and well tolerated but further development was discontinued as it did not demonstrate additional weight loss in comparison to the control group, despite a strong generation of ghrelin autoantibodies (Altabas et al. 2015).

There is much interest in developing targets in the ghrelin pathway for disease treatment, including obesity, T2DM and metabolic syndrome (decreasing AG signalling) e.g. GHSR antagonists, and for cancer cachexia, sarcopenia and chronic renal failure (stimulating AG signalling) e.g. GHSR agonists (Collden et al. 2017; Ashby et al. 2009; Neary et al. 2004).

1.2.5. Ghrelin and Addiction

Another major target for manipulating the ghrelin system for therapeutic benefit has been within the addiction spectrum. In recent years, important roles for gut-hormone-brain signals, including AG, GLP-1 and amylin, have been identified in modulating consumptive and reward behaviour to alcohol, nicotine, and other drugs of abuse in rodents, mediated by the mesocorticolimbic dopamine system (Addolorato et al. 2006; Dickson et al. 2011; Edwards et al. 2016; Jerlhag et al. 2006; Jerlhag et al. 2011b; Leggio et al. 2011; Farokhnia et al. 2019; Jerlhag 2019). In particular, AG signalling increases consumption and reward behaviours to alcohol in rodents (Abizaid et al. 2011; Egecioglu et al. 2013; Engel et al. 2014; Jerlhag et al. 2011a; Jerlhag et al. 2011b; Suchankova et al. 2013; Vadnie et al. 2014; Vallof et al. 2016). In humans, plasma AG correlates with alcohol craving in alcohol-dependent, current drinkers, and in early abstinence (Addolorato et al. 2006; Koopmann et al. 2012), striatal responses to alcohol cues in AUD (Koopmann et al. 2018), and alcohol consumption in non-dependent adults (Wittekind et al. 2018). Alcohol abstinence also increases plasma total ghrelin (Kim et al. 2005).

In humans with alcohol dependence, acute AG administration increased alcohol consumption and craving, alcohol self-administration, and amygdala reactivity in an alcohol incentive delay fMRI task (Leggio et al. 2014; Farokhnia et al. 2018).

In recent studies, twice daily oral administration of PF-5190457, a GHSR inverse agonist developed by Pfizer pharmaceutical company (but no longer being pursued for licensing), in adults with AUD, attenuated alcohol cravings, had no acute adverse effects and had no effect on plasma concentrations of plasma glucose, serum insulin, cortisol, thyroid function tests, leptin, amylin, gastric inhibitory polypeptide (GIP), glucagon-like peptide 1 (GLP-1), nor pancreatic polypeptide or peptide YY (PYY) (Lee et al. 2020a; Farokhnia et al. 2020). Over 3 days in adults with alcohol dependence, oral PF-5190457 administration lowered plasma AG/DAG ratio, principally through a reduction in plasma AG and reduced craving for alcohol, without any effect on serum GH or insulin-like growth factor-1 (IGF-1) (Lee et al. 2020b). More recently, oral PF-5190457 in adults with AUD and overweight/obesity there was no reduction in alcohol craving during an alcohol cue reactivity fMRI task (Faulkner et al. 2024), however participants selected fewer calories in a virtual buffet task. Hence it is hypothesised that LEAP2 administration by downregulation of the GHSR may lower craving for alcohol and reduce caloric intake.

Further identification of drug targets to influence AG signalling are therefore of potential wide clinical benefit not only in obesity and metabolic disease, but also in AUD.

In 2018, another metabolite was discovered in mice, LEAP2, a gut hormone that is an endogenous GHSR antagonist / inverse agonist at GHSR, providing a tool to further understand ghrelin physiology and identifying a novel therapy to attenuate AG action in humans (Ge et al. 2018; Al-Massadi et al. 2018).

1.3. LIVER-EXPRESSED ANTIMICROBIAL PEPTIDE 2

1.3.1. LEAP2 Hormone

Mature liver-expressed antimicrobial peptide 2 (LEAP2 38-77) is a 40-amino-acid bicyclic peptide, with two disulphide bridges (Krause et al. 2003; Henriques et al. 2010) See Figure 1.2 and Table 1.1. Named due to its homology with other antimicrobial peptides, it has weak anti-bacterial properties (Krause et al. 2003). In humans, the effective antimicrobial concentration of LEAP2 is 3000 times higher than its plasma

concentration (Krause et al. 2003; Howard et al. 2010). The molecular weight of mature LEAP2 (38-77) is 4.585 kDa (Krause et al. 2003). LEAP2 is the second liver expressed antimicrobial peptide, after LEAP1 (now called hepcidin, involved in iron metabolism) (Krause et al. 2000). LEAP2 has identical amino acid sequence in mouse and humans, expressed predominantly in the liver, then jejunum, duodenum, ileum; in addition to detection in the stomach, kidney, pituitary and lungs, that was originally isolated from human monocytes (Krause et al. 2003; Henriques et al. 2010; Howard et al. 2010; Englund et al. 2024). The biosynthesis of LEAP2 involves several sequential steps. *Leap2* mRNA encodes a precursor protein called prepro-LEAP2 (1-77), which comprises 77 residues in humans and 76 in mice (Krause et al. 2003). This precursor is processed by a signal peptidase into pro-LEAP2 (LEAP2 23–77 in humans or LEAP2 22–76 in mice). Pro-LEAP2 is then cleaved, by furin (a member of the protein conversion enzyme family endoprotease in the liver), to produce the mature 40-residue form of LEAP2 38–77 in humans or LEAP2 37–76 in mice (Krause et al. 2003; Henriques et al. 2010).

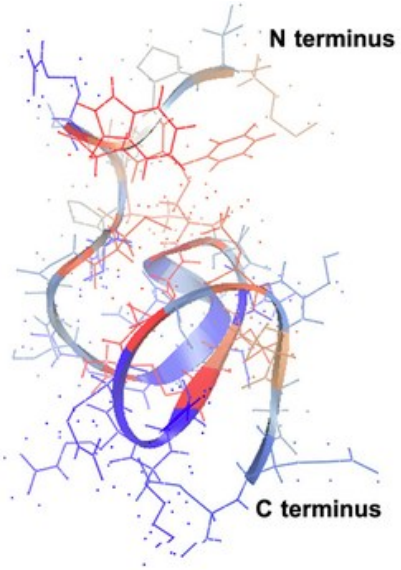


Figure 1.2. Structure of human LEAP2.
Structure of mature human LEAP2(38-77) with N and C terminals at opposite ends. Figure taken from (Wang et al. 2019).

The sequence of mature LEAP2 is highly conserved with identical sequence in humans and mice. Mature LEAP2 includes an N-terminal hydrophobic region with a central cationic domain, and a C-terminal domain (Henriques et al. 2010; Mani et al.

2019). The N-terminal region is critical for receptor binding and microbial membrane interaction (Wang et al. 2019; Henriques et al. 2010). The binding determinant lies in the N-terminal sequence (residues 1–8), while a longer segment including residues 1–12 increases binding strength and efficacy (M'Kadmi et al. 2019). Conversely, the C-terminal region appears to have no role in antibacterial activity or GHSR binding but contributes to LEAP2 stability in plasma via two disulphide bonds (Cys54-Cys65 and Cys60-Cys70) (Krause et al. 2003; Henriques et al. 2010; M'Kadmi et al. 2019).

1.3.2. LEAP2 Peptide Pharmacology

As a peptide, LEAP2 is inherently unstable and undergoes rapid degradation into fragments, with a half-life of approximately 10-15 minutes (Ge et al. 2018; Hagemann et al. 2021). Some LEAP2 fragments retain functionality due to their preserved N-terminal bioactive region. See table 1.1. below. Moreover, the hydrophobicity of the N-terminal makes it essential for the peptide to bind with the phospholipid bilayer of the target cell membrane and interact with the GHSR. This effect is less important with the C-terminal (Sosinski et al. 2025). However, this effect is lost with a potency of 0.2-6.2% with 8-10 amino acid LEAP2 fragments (M'Kadmi et al. 2019; Hagemann et al. 2021). Furthermore, addition of a fatty moiety to LEAP2(38-51) fragment at its C-terminus with palmitic acid enhanced its affinity to the GHSR (Hola et al. 2022).

```
1-37  MWHLKLCAVL MIFLLLLGQI DGSPIPEVSS AKRRPRR
                        ------------ disulphide bridge
                        ------------ disulphide bridge
38-77 MTP FWRGVSLRPI GASCRDDSEC ITRLCRKRRC SLSVAQE  40 aa Hagemann 2022 (mature peptide)
38-54 MTP FWRGVSLRPI GASC                          17 aa fluorescent Barille 2019
38-51 MTP FWRGVSLRPI G                             14 aa M'Kadmi 2019
38-54 MTP FWRGVSLRPI G-Palmitic                    14 aa Hola 2022
38-49 MTP FWRGVSLRPI                               12 aa M'Kadmi 2019
38-47 MTP FWRGVSL                                  10 aa Hagemann 2021
38-45 MTP FWRGV                                     8 aa M'Kadmi 2019
```

Reference	LEAP2 N-terminal fragment	EC50 nM fragment Inositol phosphate	EC50 nM LEAP2(38-77)	Fraction of LEAP2(38-77) Potency	Ki binding nM fragment (binding affinity)	Ki binding nM LEAP2(38-77) (binding affinity)	Fraction of LEAP2(38-77) affinity	E max % basal inhibition fragment	E max % basal inhibition LEAP2(38-77)
Barille 2019 17 aa	38-54-NH2				3.9	1.3	33.3%		
M'Kadmi 2019 14 aa	38-51-NH_2	48	22.8	47.5%	3.05	1.26	41.3%	50.3	52.3
Hola 2022 14 aa	38-51-Palmitic acid-NH2	52.43	46.46	88.61%	1.91	10.74	562.3%		
M'Kadmi 2019 12 aa	38-49-NH_2	46	22.8	49.6%	2.7	1.26	46.7%	49.3	52.3
M'Kadmi 2019 10 aa	38-47-NH_2	134	22.8	6.2%	3.4	1.26	37.1%	50.3	52.3
Hagemann 2021 10 aa	38-47-NH_2	2200	4.88	0.2%					
M'Kadmi 2019 8 aa	38-45-NH_2	368	22.8	6.2%	6.45	1.26	19.5%	45.3	52.3

Table 1.1. LEAP2 amino acid sequence and fragment pharmacology.

1.3.3. LEAP2 Activity at GHSR

The GHSR maintains high constitutive activity at about 50% of its maximum capacity in the absence of AG (Holst et al. 2003). This activity allows the receptor to maintain GH secretion without AG stimulation (Fernandez et al. 2018). LEAP2 acts as a competitive antagonist (competes for AG binding site) of orexigenic AG (Wang et al. 2019) and inverse agonist (reduces constitutive GHSR activity in the absence of AG) (M'Kadmi et al. 2019; Ge et al. 2018; Wang et al. 2019; Mani et al. 2019) at the constitutively active GHSR. In GHSR-null mice, exogenous AG administration did not suppress liver *Leap2* mRNA expression in vivo and ex vivo (Islam et al. 2020).

LEAP2 acts as a GHSR inverse agonist using whole cell patch clamp recordings of hypothalamic arcuate NPY neurons in mouse brain sections by prevention of AG

stimulated NPY hyperpolarisation (Mani et al. 2019). Indeed, *in vitro* LEAP2(38-49) fragment (0.5 uM) administration in cell cultures, also reduced both ghrelin dependent and independent evoked GHSR inhibition of voltage-gated calcium channel 2.2 (Cav2.2) currents using patch-clamp recordings, suggesting it is both a GHSR antagonist and inverse agonist (Mustafa et al. 2021). LEAP2 (38-49) fragment, retains biological activity to that of full length LEAP2 (38-77) (Wang et al. 2019), blocking AG action to increase food intake, and in vitro acting as an antagonist to AG, and an inverse agonist at the constitutively active GHSR (M'Kadmi et al. 2019). Moreover, in direct examination of the electrophysiological effects on ARC (orexigenic arcuate nucleus) NPY neurons, LEAP2 on its own acts as a GHSR inverse agonist by destabilizing the constitutive activity and also as a GHSR antagonist by reversing AG induced activation of NPY neurons (Mani et al. 2019).

LEAP2 functions as a GHSR inverse agonist by suppressing the receptor's constitutive activity and stabilizing it in a distinct inactive conformation (M'Kadmi et al. 2019). This property highlights LEAP2's superior potential for obesity treatment compared to other synthetic GHSR antagonists. Unlike AG, which induces GHSR endocytosis upon binding, LEAP2 does not cause receptor internalization (Barrile et al. 2019). This was demonstrated using fluorescently labelled LEAP2, which localized specifically to the cell membrane in GHSR-expressing cells, whereas fluorescently labelled AG was predominantly observed in the cytoplasm (Barrile et al. 2019).

In summary, LEAP2 acts on and inhibits GHSR function by reducing its constitutive activity, displacing AG from binding sites, and minimizing intracellular signalling triggered by receptor internalization. This makes LEAP2 a specific endogenous GHSR antagonist.

1.3.4. Reciprocal Regulation of Plasma LEAP2 and AG

Plasma LEAP2 is regulated in a reciprocal manner to AG, i.e. decreases with fasting/weight loss, and increases with refeeding/weight gain in rodents and humans (Ge et al. 2018; M'Kadmi et al. 2019; Wang et al. 2019; Fernandez et al. 2022; Mani et al. 2019; Hagemann et al. 2021; Hagemann et al. 2022). See Figure 1.3 and Tables 1.2 and 1.9 below.

In both rodents and humans, plasma levels of AG and LEAP2 exhibit a reciprocal relationship in response to nutritional status. After food intake, plasma AG typically decreases, while plasma LEAP2 increases, with the opposite pattern observed during acute fasting (Liu et al. 2008; Ge et al. 2018; Mani et al. 2019; Islam et al. 2020; Hagemann et al. 2021; Hagemann et al. 2022). In obesity, plasma LEAP2 levels are elevated, whereas plasma AG levels are reduced (Mani et al. 2019; Ma et al. 2020; Shen et al. 2021; Holm et al. 2022).

Conversely, dietary-induced weight loss is associated with an increase in plasma AG (Coutinho et al. 2018; Lyngstad et al. 2019), while plasma LEAP2 decrease following weight loss through dietary restriction in rodents and bariatric surgery in humans (Ge et al. 2018; Mani et al. 2019; Fernandez et al. 2022; Holm et al. 2022; Tezenas du Montcel et al. 2023).

Chronic peripheral LEAP2 administration in mice using adeno-associated virus (AAV) delivery resulted in weight loss, and loss of protection against starvation-induced hypoglycaemia, rescued by GH. Interestingly, after chronic starvation, plasma AG increased less in LEAP2-overexpressing mice, suggesting that LEAP2 might inhibit the production or secretion of AG (Ge et al. 2018). These findings suggest that the plasma AG/LEAP2 ratio, which varies with nutritional status, may play a key role in regulating appetite and food intake as part of energy homeostasis (Mani et al. 2019).

The reciprocal effects of AG and LEAP2 in rodents and humans on food intake and metabolism are further illuminated in the following sections below (Sections 1.3.7, 1.3.8, 1.4.2, 1.4.3, 1.4.4, 1.4.6) with results of individual studies summarised in Tables 1.2 and 1.9.

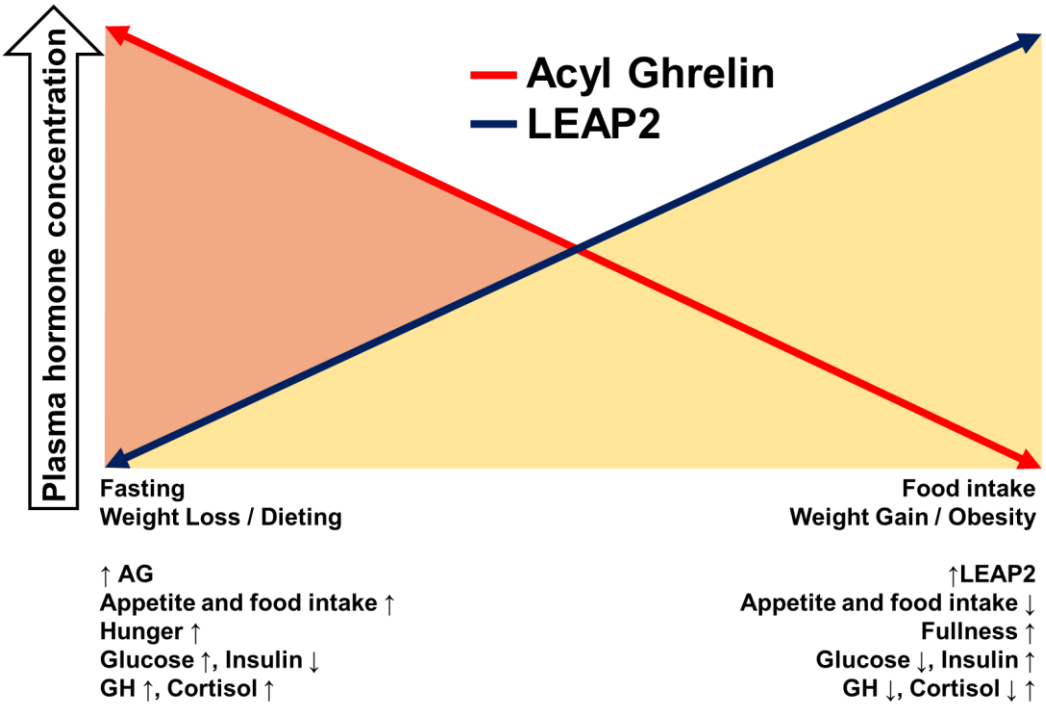


Figure 1.3. Reciprocal regulation and effects of acyl ghrelin and LEAP2 gut hormones.

1.3.5. Influences on Plasma LEAP2 and mRNA Expression in Rodents

The source of nutritional state induced changes in plasma LEAP2 appears to be the liver and small intestine. In rodents, plasma LEAP2 reduced after fasting (24-48 hours) (Ge et al. 2018; Mani et al. 2019; Fernandez et al. 2022). Furthermore, fasting decreased *Leap2* mRNA expression in liver (Islam et al. 2020; Holm et al. 2022; Borchers et al. 2022). However, in other studies fasting did not decrease *Leap2* mRNA in the liver (Mani et al. 2019), duodenum and jejunum (Holm et al. 2022), nor jejunum and ileum (Islam et al. 2020).

In rodents, plasma LEAP2 is increased in the fed state (Ge et al. 2018; Mani et al. 2019; Shankar et al. 2021a) and in diet-induced obesity (DIO) in mice (Mani et al. 2019; Shankar et al. 2021a; Casado et al. 2024). While DIO also increases liver *Leap2* expression in most studies (Ma et al. 2020; Hola et al. 2023; Holm et al. 2022; Casado et al.) but not in liver or jejunum in one study (Mani et al. 2019). Similarly, a mixed meal ingestion (240 kcal/100 mL, carbohydrate [49%], protein [16%], fat [35%] increased liver *Leap2* expression (Gradel et al. 2023).

Increased ileal *Leap2* expression was seen in obese *ob/ob* (leptin gene mutation) and *db/db* (leptin receptor mutation) mice (Shen et al. 2021) with no change in duodenal and jejunal *Leap2* mRNA expression, suggesting that ileal *Leap2* mRNA expression increases in obesity independent of leptin signalling.

In DIO mice that are then given standard chow, expected weight loss induces a decrease in plasma LEAP2 (Mani et al. 2019; Islam et al. 2024) and decrease in liver *Leap2* (Hola et al. 2023) and duodenal and jejunal *Leap2* expression(Islam et al. 2024).

In DIO mice with vertical sleeve gastrectomy (VSG), expected weight loss induced an increase in stomach *Leap2* mRNA expression, and reduction in duodenum *Leap2* expression (Ge et al. 2018). Interestingly, VSG in chow fed mice (vs. sham) increases plasma LEAP2 and liver *Leap2* mRNA expression (Islam et al. 2024), with an expected reduction in plasma LEAP2 in diet induced obese mice (60% fat diet) that have VSG (vs. diet induced obese mice- sham procedure) (Islam et al. 2024). Similarly, weight loss by caloric restriction (40-50%) also reduced plasma LEAP2 in mice (Ge et al. 2018; Tezenas du Montcel et al. 2023).

Similarly, in 24h fasted mice, both oral and intraperitoneal glucose (2g/kg) increased plasma LEAP2, liver *Leap2* mRNA expression (Mani et al. 2019; Islam et al. 2024) and jejunal *Leap2* (Gradel et al. 2023). Plasma LEAP2 is elevated in a mouse streptozotocin-induced model of raised plasma glucose (Mani et al. 2019). Glucose stimulation in mouse hepatocytes increased liver *Leap2* expression (Islam et al. 2024). Oral glucose administration in mice, increased plasma LEAP2 in the hepatic vein and abdominal aorta but not the portal vein (Islam et al. 2024), suggesting that liver is the main organ that secretes post-prandial LEAP2 in response to a glucose load.

In mice fed a ketogenic diet over 3 weeks (75% fat, 3% carbohydrates) increased plasma β-hydroxybutyrate (BHB, a ketone byproduct of fatty acid oxidation) with reduction in body weight, plasma glucose and liver *leap2* expression, but increased duodenal *leap2* expression, compared to standard chow diet (Holm et al. 2022), while in lean mice, oral BHB administration, reduced plasma LEAP2, liver and duodenal

Leap2 mRNA expression after 2 hours, without any change in jejunal *Leap2* mRNA expression nor plasma glucose (Holm et al. 2022). Lastly, ex vivo BHB administration in mouse hepatocytes reduced *Leap2* expression suggesting a direct hepatic action of ketone bodies to reduce LEAP2 secretion (Holm et al. 2022).

Lipid ingestion with olive oil (10ml/kg) increased plasma LEAP2 and jejunal *Leap2* mRNA expression in fasted male mice without effecting liver mRNA expression (Gradel et al. 2023). Furthermore, intestinal contribution of this increase, with increased portal vein *Leap2* mRNA expression after 4 hours of ingestion (Gradel et al. 2023) suggests that fatty acids may be upregulating LEAP2 in the small intestine.

On comparing the effect of different fatty acids on plasma LEAP2 and its expression, all three oils; olive (primarily containing oleic acid), lard (primarily saturated fats) and fish oil (primarily containing n-3 polyunsaturated fatty acids) increased plasma LEAP2 with olive oil and lard (but not fish oil) also increasing jejunal *Leap2* expression (Gradel et al. 2023). Furthermore, in murine jejunal organoids, stimulation with oleic acid (olive oil), but not docosahexanoic acid (fish oil) or linoleic acid (lard/saturated fat) increased jejunal *Leap2* expression in these organoids, suggesting that LEAP2 is regulated by lipid digestion (Gradel et al. 2023).

Confirmation of these findings in another study revealed, in fasted mice, oral corn oil ingestion (30% oleic acid) increased plasma LEAP2 levels and expression of *Leap2* in the duodenum, jejunum and liver, but not stomach (Islam et al. 2024). This fat ingestion also increased plasma LEAP2 in the hepatic, portal vein and abdominal aorta (Islam et al. 2024). Similarly, in fasted mice, oral palmitate (saturated fat) ingestion increased plasma LEAP2 and liver and duodenum *Leap2* (Islam et al. 2024).

The concentration of plasma LEAP2 is up to 10 times more than AG in the fed state in rodents (Cornejo et al. 2021b). This relatively high concentration along with the fact that LEAP2 is not stored in any cell for immediate release, suggest that the plasma levels fluctuate more gradually and hence, LEAP2 likely plays a more sustained role in energy homeostasis.

These results suggest the regulation of LEAP2 differs by meal composition and diet challenges. Food ingestion, obesity and higher plasma glucose upregulate LEAP2 especially from the liver. The regulation of post-prandial increase in LEAP2 is significantly contributed by the jejunum, though dependent on fatty acid composition. Fat and glucose ingestion leads to plasma LEAP2 increases in the hepatic vein and abdominal aorta. These reports are summarised in Table 1.2. Human studies examining the influences on plasma LEAP2 and mRNA expression are in Table 1.9. These pre-clinical experiments suggest that the liver and intestine are the major organs secreting LEAP2 after mixed meal, glucose and/or fat ingestion.

Table 1.2. Pre-clinical studies assessing effects of interventions on plasma AG, LEAP2, AG/LEAP2 ratio and organ Leap2 mRNA expression. Abbreviations: DIO, Diet induced obesity; h, Hours; F, Female; GhIR-KO, Ghrelin insulin receptor knockout; M, Male; m, Months; NC, Normal chow; STZ-DM, Streptozotocin diabetes mellitus model; VSG, Vertical sleeve gastrectomy; (a), Abdominal aorta; (b), Hepatic vein; (c), Portal vein; ↑, Increased; ↓, Decreased; o, No effect.

Author	Animal	Age (weeks)	Sex	Intervention	Plasma AG	Plasma LEAP2	Plasma AG/LEAP2 ratio	Leap2 mRNA					Leap2 peptide
								Gastric	Liver	Duodenum	Jejunum	Ileum	Jejunal organoid
Ge 2018	Mice C57BL/6	17	M	Fasting (24h)	↑	↓	↑						
Mani 2019	Mice C57BL/6N	9-13	M	Fasting (24h)	↑	↓	↑		o				
Holm 2022	Mice C57BL/6	8	M	Fasting (24h)					↓	o	o		
Fernandez 2022	Mice C57BL/6	10-14	M	Fasting (48h)	↑	↓							
Shen 2021	Mice C57BL/6N *ob/ob*	4-13	M	Fasting (12h)					↓	o	o	↑	
Shen 2021	Mice C57BL/6N *db/db*	4-13	M	Fasting (12h)					↑	o	o	↑	
Ge 2018	Mice C57BL/6	17	M	Fed (start light phase)	↓	↑	↓						
Mani 2019	Mice C57BL/6N	9-13	M	Fed (start light phase)	↓	↑	↓						
Shankar 2021	Mice C57BL/6N GhIR-KO	16-20	M/F	Fed (after 2h fasting)	o	↑	↓						
Mani 2019	Mice C57BL/6N	16	M	DIO (60% fat)	↓	↑	↓		o		o		
Ma 2020	Mice C57BL/6	8-10	F	DIO (60% fat)					↑				
Holm 2022	Mice C57BL/6	8	M	DIO (60% fat)					↑				
Hola 2023	Mice C57BL/6	16-20	M	DIO (60% fat)	↓		o		↑				
Casado 2024	Mice C57BL/6	15	M	DIO (60% fat)		↑			↑				
Shankar 2021	Mice C57BL/6N GhIR-KO	16-20	M/F	DIO (60% fat)	o	↑	↓						
Gradel 2023	Mice C57BL/6	12-14	M	Mixed meal					↑				
Mani 2019	Mice C57BL/6N	12	M	DIO then NC	o	↓	↑						
Hola 2023	Mice C57BL/6	16-20	M	DIO then NC	↑				↓				
Islam 2024	Mice C57BL/6J	16	M/F	DIO then NC		↓		o	↓	↓	↓		
Ge 2018	Mice C57BL/6	17	M	Caloric restriction 40% (14 d)	↑	↓							
Tezenas du Montcel 2023	Mice C57BL/6	7	F	Caloric restriction 50% (14 d)	↑	↓	↑						
Ge 2018	Mice C57BL/6	17	M	VSG surgery in DIO	↓			↑		↓			
Islam 2024	Mice C57BL/6J	16	M/F	VSG surgery in DIO		↓							
Islam 2024	Mice C57BL/6J	16	M/F	VSG surgery in NC					↑				
Mani 2019	Mice C57BL/6N	8-12	M	STZ-DM	↑	↑	o						
Mani 2019	Mice C57BL/6N	8-12	M	Oral glucose	↓	↑	↓						
Gradel 2023	Mice C57BL/6	12-14	M	Oral glucose							↑		
Islam 2024	Mice C57BL/6J	16	M/F	Oral glucose		↑ a,b; o c			↑				
Islam 2024	Mice C57BL/6J	16	M/F	Intraperitoneal glucose		↑			↑				
Holm 2022	Mice C57BL/6	8	M	Ketogenic diet (75% fat)					↓	↑			
Holm 2022	Mice C57BL/6	16-22	M	Oral BHB		↓			↓	↓	o		
Islam 2024	Mice C57BL/6	16	M/F	Oral corn oil (oleic acid)		↑ a,b,c			↑	↑	↑		
Islam 2024	Mice C57BL/6	16	M/F	Oral palmitate (saturated fat)		↑			↑	↑			
Gradel 2023	Mice C57BL/6	12-14	M	Oral olive oil (oleic + linoleic acid)		↑			o		↑		↑
Gradel 2023	Mice C57BL/6	12-14	M	Oral lard (linoleic acid + saturated fat)		↑			o		↑		o
Gradel 2023	Mice C57BL/6	12-14	M	Oral fish oil (docosahexanoic acid)		↑			o		o		o
Casado 2024	Mice C57BL/6J	130	M	Aging (30 m vs 15 m)		↑			↑				
Gupta 2024	Mice C57BL/6N	3-4	F	Pregnancy	↓	↑	↓						
Islam 2020	Rats Wistar	8	M	Fasting (overnight)					↓		o	o	
Borchers 2022	Rats Sprague-Dawley	8	M/F	Fasting (overnight)					↓				
Islam 2020	Rats Wistar	8	M	Fed (start light phase)					↑		o	o	
Garces 2022	Rats Wistar	3	F	Pregnancy	↑	↓	↑						

1.3.6. Effects of LEAP2 on Neural Activity

The sites of action of LEAP2 in the brain remain to be confirmed. Central administration of a fluorescent-labelled N-terminal LEAP2 fragment demonstrated binding to GHSR expressing brain regions including hypothalamic nuclei such as ARC and PVH, as well as hippocampal dentate gyrus and medulla (Barrile et al. 2019). These reports are summarised in Table 1.3.

The hypothalamus is implicated as a potential site of action for LEAP2, since while intracerebroventricular (i.c.v.) LEAP2 administration in rats alone did not block *c-fos* expression (a marker of neuronal activity) in hypothalamic arcuate nucleus (ARC) and dorsomedial hypothalamus (DMH), i.c.v. LEAP2 did block the increase in c-fos immunostaining when co-administered with central AG (Islam et al. 2020).

Furthermore, a chronic peripheral infusion of LEAP2 (38-49) fragment over 2 days in fasted mice attenuated the increase in *c-fos* immunostaining in the ARC and olfactory bulb (Fernandez et al. 2022), while in *Leap2*-KO mice, peripheral AG administration increased c-fos peptide, in the ARC and olfactory bulb (vs. wild type mice) (Shankar et al. 2021a).

In cultured mice hippocampal neurons, the constitutive activity of GHSR impairs neurotransmission (Martinez Damonte et al. 2018). Hence, LEAP2 administration in an increasing dose dependent manner (0.2, 1 and 5uM) inhibited the acyl-ghrelin (1uM) mediated increases in synaptic density in cultured murine hippocampal neurons (Tian et al. 2023). LEAP2 (1uM) also attenuated the increase in c-fos immunostaining in the hippocampus by AG (1uM) (Tian et al. 2023). Similar findings were seen with lipidization of LEAP2 fragment with palmitic acid (Palm- LEAP2 38-51), where in this molecule with a greater stability and affinity at the GHSR (Hola et al. 2022) attenuated the ghrelin induced increase in *c-fos* activity at the arcuate nucleus, area postrema and nucleus tractus solitarius (Petelakova et al. 2024).

In fasted mice, adeno-associated virus (AAV) delivery of LEAP2 overexpression increases anorexigenic POMC (Proopiomelanocortin) mRNA/protein in the hypothalamus and central delivery of i.c.v 0.02pmol LEAP2 (10nM in 2uL) increases *c-fos* activity in anorexigenic POMC neurons in the hypothalamus with reduction in food intake (Chu et al. 2022).

This suggests that LEAP2 may be involved in the anorexigenic signalling pathway in the hypothalamus, via the GHSR, though further investigations are warranted.

Author	Animal	Age (weeks)	Sex	LEAP2 Dose	Route	Ghrelin dose	Route	Nutritional state	Location	c-fos expression
Chu 2022	Mice C57BL/6J	8	M	0.02 nmol	icv	-	-	2h fasted	POMC neurons	↑
Fernandez 2022	Mice C57BL/6J	10-14	M	0.78 nmol/g over 48h (LEAP2 38-49)	sc	-	-	48h fasted	ARC	↓
Fernandez 2022	Mice C57BL/6J	10-14	M	0.78 nmol/g over 48h (LEAP2 38-49)	sc	-	-	48h fasted	PVN	↓
Petelakova 2024	Mice C57BL/6N	8	M	-	sc	1mg/kg	sc	16h fasted	ARC, NTS, AP	↑↑
Petelakova 2024	Mice C57BL/6N	8	M	78.2 nmol (Palm-LEAP2 38-51)	sc	1mg/kg	sc	16h fasted	ARC, NTS, AP	↑
Tian 2023	Mice C57BL/6J	16-20	M/F	-	-	1000nmol	ex vivo	n/a	Cultured hippocampus	↑↑
Tian 2023	Mice C57BL/6J	16-20	M/F	1000 nmol	ex vivo	1000nmol	ex vivo	n/a	Cultured hippocampus	↑
Shankar 2021	Mice C57BL/6N *Leap2-KO*	16-20	M/F	-	-	0.5mg/kg	sc	12h fasted	ARC, OLB	↑↑
Islam 2020	Rat Wistar	8	M	-	-	0.1nmol	icv	12h fasted	ARC, DMN	↑↑
Islam 2020	Rat Wistar	9	M	1 nmol	icv	0.1nmol	icv	12h fasted	ARC, DMN	↑
Islam 2020	Rat Wistar	8	M	1 nmol	icv	-	-	12h fasted	ARC, DMN	o

Table 1.3. Pre-clinical studies assessing effects of LEAP2 on brain c-fos expression.
Abbreviations: AP, Area postrema; ARC, Arcuate nucleus; DMN, Dorsomedial nucleus; F, Female; h, Hours; icv, Intracerebrovascular; M, Male; NTS, Nucleus tractus solitarius; OLB, Olfactory bulb; POMC, Proopiomelanocortin; PVN, Paraventricular nucleus; sc, Subcutaneous; ↑↑, Greatly increased; ↑, Increased; ↓, Decreased; o, No effect.

1.3.7. Effects of LEAP2 on Growth Hormone and Glucose Metabolism in Rodents

In rodents, administration of peripheral and/or central LEAP2 either antagonises the effects of exogenous AG to increase blood GH and/or increase in blood glucose or reduce insulin, and/or when administered alone LEAP2 may have the opposite effects to AG to lower blood GH, glucose and increase insulin secretion. In rodent pancreatic islets, AG also inhibits pancreatic polypeptide (PP) release (Qader et al. 2008). These reports are summarised in Tables 1.4, 1.5, 1.6.

In most rodent studies, exogenous LEAP2 inhibited the release of AG induced GH release. Similarly, lipidization of LEAP2(38-51) fragment with palmitic acid to increase the potency and half-life of LEAP2, attenuated AG induced GH release. Not only did LEAP2 on its own either as a continuous infusion or its fragments inhibit glucose release, LEAP2 also inhibited ghrelin induced glucose release. While in *Leap2*-KO mice, peripheral AG increased GH increase (Shankar et al. 2021a). However, peripherally administered fragments of LEAP2 did not affect glucose secretion (Petelakova et al. 2024). Furthermore, this effect was lost in GHSR-KO mice, confirming that LEAP2 is dependent on GHSR. AG reduces insulin sensitivity and insulin secretion, and increases GH release and plasma glucose (including via GH action to increase gluconeogenesis) (Gray et al. 2019). Hence, as part of a homeostatic feedback loop, insulin is known to reduce plasma AG, apparently via direct actions of insulin on ghrelin-secreting stomach cells, which may also contribute to the post-prandial decreases in plasma AG. In rodent pancreatic islets, LEAP2 in varying doses increased insulin secretion and increased glucose stimulated insulin secretion and antagonised the insulinostatic effects of AG (Bayle et al. 2021; M'Kadmi et al. 2019; Hewawasam et al. 2024). However, despite elevations in AG in Ghrelin insulin receptor KO mice (GhIR-KO) after DIO, plasma LEAP2 was similar to wild-type mice, suggesting that LEAP2 was preventing the orexigenic activity of AG in GhIR-KO mice (Gupta et al. 2021a).

In one study, GHSR expression is prominent in gamma (PP-expressing) and delta (SST-expressing) cells in mice (Gupta et al. 2021a) with no differences in plasma PP between wild-type and Ghrelin-KO mice (Gupta et al. 2021a). In the same study, in *ad libitum* fed mice, peripheral ip LEAP2 administration (72 nmol/kg) increased plasma PP contributing to increased insulin sensitivity (Gupta et al. 2021a); there was also a positive correlation observed between plasma LEAP2 and PP concentrations. Similarly, in mice pancreatic islets, LEAP2 reversed AG-induced somatostatin (SST) secretion (Hewawasam et al. 2024).

This suggests that PP-expressing pancreatic cells and SST-expressing cells are targets of LEAP2 via actions on GHSR, and there may be overlapping metabolic actions of PP, SST and LEAP2, and that blockade of GHSR may counteract the decrease in plasma PP associated with fasting.

Therefore, the acute effects of exogenous full length LEAP2 administration to humans would be hypothesised to reduce appetite and food intake, increase serum insulin and PP, lower plasma glucose, lower plasma AG, and lower serum GH, especially under conditions of negative energy balance such as fasting where endogenous plasma AG is elevated (and endogenous plasma LEAP2 is reduced). Lastly, further validation of LEAP2 lipidised analogues is needed in both rodents and humans.

Author	Animal	Age (weeks)	Sex	Nutritional state	LEAP2 dose	Route	Ghrelin Dose	Route	Serum GH
Ge 2018	Mice C57BL/6	12-16	M	Fasting (12h)	-	-	6 nmol/kg	iv	↑↑
Ge 2018	Mice C57BL/6	12-16	M	Fasting (12h)	LEAP2 antibody	ip	-	-	↑
Ge 2018	Mice C57BL/6	12-16	M	Fasting (12h)	7.7 nmol/kg	ip	6 nmol/kg	iv	↑
Ge 2018	Mice C57BL/6	12-16	M	Fasting (12h)	72 nmol/kg	ip	6 nmol/kg	iv	↑
Ge 2018	Mice C57BL/6	12-16	M	Fasting (12h)	360 nmol/kg	ip	6 nmol/kg	iv	↑
Hola 2022	Mice C57BL/6	8	M	Fasting (12h)	-	-	1mg/kg	sc	↑↑
Hola 2022	Mice C57BL/6	8	M	Fasting (12h)	156.4 nmol (LEAP2 38-51)	sc	-	-	↓
Hola 2022	Mice C57BL/6	8	M	Fasting (12h)	156.4 nmol (LEAP2 38-51)	sc	1mg/kg	sc	↑
Hola 2022	Mice C57BL/6	8	M	Fasting (12h)	156.4 nmol (Palm-LEAP2 38-51)	sc	-	-	↓
Hola 2022	Mice C57BL/6	8	M	Fasting (12h)	156.4 nmol (Palm-LEAP2 38-51)	sc	1mg/kg	sc	↑
Ge 2018	Mice C57BL/6	12-16	M	Fasting (12h), caloric restriction 40%	LEAP2 over expression by AAV	-	-	-	↑
Shankar 2021	Mice C57BL/6N *Leap2-KO*	16-20	M/F	Fed (light phase start)	-	-	0.1mg/kg	sc	↑↑
Islam 2020	Rats Wistar	8	M	Fasting (12h)	-	-	5 nmol iv	iv	↑↑
Islam 2020	Rats Wistar	8	M	Fasting (12h)	15 nmol	iv	5 nmol iv	iv	↑
Islam 2020	Rats Wistar pituitary cells	8	M	n/a	-	-	100 nM	ex vivo	↑↑
Islam 2020	Rats Wistar pituitary cells	8	M	n/a	1000 nmol	ex vivo	-	-	↓
Islam 2020	Rats Wistar pituitary cells	8	M	n/a	1000 nmol	ex vivo	100 nM	ex vivo	↑

Table 1.4. Pre-clinical studies assessing the effects of exogenous LEAP2 on serum growth hormone.
Abbreviations: AAV, Adeno-associated virus; F, Female; GH, Growth hormone; h, Hours; ip, Intraperitoneal; iv, Intravenous; M, Male; n/a, Not applicable; Palm, Palmitoylated; sc, Subcutaneous; ↑↑, Greatly increased; ↑, Increased; ↓, Decreased; o, No effect.

Author	Animal	Age (weeks)	Sex	Nutritional state	LEAP2 dose	Route	Ghrelin Dose	Route	Plasma Glucose
Lugilde 2022	Mice C57BL/6J	12	M	Fasting (12h)	1.5 nmol	icv	-	-	o
Hagemann 2022	Mice C57BL/6	14-24	F	Fasting (12h)	3000 nmol/kg	sc	-	-	o
Fernandez 2022	Mice C57BL/6	10-14	M	Fasting (48h)	0.78 nmol/g/day (LEAP2 38-49) (continuous, 2d	sc	-	-	o
Ge 2018	Mice C57BL/6	12-16	M	Fasting (12h), caloric restriction 40%	-	-	-	-	↓
Ge 2018	Mice C57BL/6	12-16	M	Fasting (12h), caloric restriction 40%	LEAP2 over expression by AAV	-	-	-	↓↓
Islam 2022	Mice C57BL/6J	16	M/F	Fasting (12h), caloric restriction 60%	85 nmol/kg/day (continuous, 6d)	sc	-	-	↓
Gupta 2021	Mice C57BL/6N	8-10	M	Fed (light phase start)	72 nmol/kg	ip	-	-	↓
Islam 2022	Mice C57BL/6J	12	M/F	Fed (light phase start)	600 nmol/kg (BD, 3d)	sc	-	-	o
Islam 2022	Mice C57BL/6J	12	M/F	Fed (light phase start)	85 nmol/kg/day (continuous, 6d)	sc	-	-	o
Hagemann 2022	Mice C57BL/6J	15	F	Fed (light phase start)	3000 nmol/kg	sc	-	-	↓
Hagemann 2021	Mice NMRI	5-6	M	Fed (light phase start)	3000 nmol/kg	sc	-	-	o
Hagemann 2021	Mice NMRI	5-6	M	Fed (light phase start)	3000 nmol/kg (LEAP2 38-47)	sc	-	-	o
Hagemann 2021	Mice NMRI	5-6	M	Fed (light phase start)	300 nmol/kg (LEAP2 23-47)	sc	-	-	o
Hagemann 2021	Mice NMRI	5-6	M	Fed (light phase start)	3000 nmol/kg (LEAP2 49-77)	sc	-	-	o
Hagemann 2022	Mice C57BL/6J *Ghsr-KO*	15	F	Fed (light phase start)	3000 nmol/kg	sc	-	-	o
Petelakova 2024	Mice C57BL/6N DIO (60% fat)	16-20	M	Fasting (6h)	156.4 nmol (LEAP2 38-51) (OD, 6w)	sc	-	-	o
Petelakova 2024	Mice C57BL/6N DIO (60% fat)	16-20	M	Fasting (6h)	156.4 nmol (Palm-LEAP2 38-51) (OD, 6w)	sc	-	-	o
Lugilde 2022	Mice C57BL6J DIO (60% fat)	12	M	Fasting (12h)	1.5 nmol	icv	-	-	↓
Lugilde 2022	Mice C57BL/6J *ob/ob*	12	M	Fasting (12h)	1.5 nmol	icv	-	-	↓
Islam 2022	Mice C57BL/6J *Ghsr-KO*	30	M/F	Fasting (12h), caloric restriction 60%	85 nmol/kg/day (continuous, 6d)	sc	-	-	↓
Islam 2020	Rats Wistar	8	M	Fasting (12h)	-	-	100 pmol	icv	↑
Islam 2020	Rats Wistar	8	M	Fasting (12h)	1 nmol	icv	100 pmol	icv	↓
Islam 2020	Rats Wistar	8	M	Fasting (12h)	1 nmol	icv	-	-	o
Lugilde 2022	Rats Sprague-Dawley	12	M	Fasting (12h)	8 nmol	icv	-	-	o

Table 1.5. Pre-clinical studies assessing the effects of exogenous LEAP2 on plasma glucose.

Abbreviations: AAV, Adeno-associated virus; BD, Twice daily; d, Days; F, Female; Ghsr-KO, Ghrelin receptor knockout; h, Hours; ip, Intraperitoneal; iv, Intravenous; M, Male; OD, Once daily; Palm, Palmitoylated; sc, Subcutaneous; w, Weeks; ↑↑, Greatly increased; ↑, Increased; ↓↓, Greatly decreased; ↓, Decreased; o, No effect.

Author	Animal	Age (weeks)	Sex	Nutritional state	LEAP2 dose	Route	Ghrelin Dose	Route	Serum Insulin
Hagemann 2022	Mice C57BL/6	15	F	Fasting (12h)	3 umol/kg	sc	-	-	o
Islam 2022	Mice C57BL/6J	16	M/F	Fasting (12h), caloric restricted 60%	85 nmol/kg/day (continuous, 6d)	sc	-	-	o
Islam 2022	Mice C57BL/6J *Ghsr-KO*	30	M/F	Fasting (12h), caloric restricted 60%	85 nmol/kg/day (continuous, 6d)	sc	-	-	o
Islam 2022	Mice C57BL/6J	12	M/F	Fed (start light phase)	85 nmol/kg/day (continuous, 6d)	sc	-	-	o
Hewawasam 2024	Mice CD-1 pancreatic islets	8-12	M/F	n/a	-	-	100nmol/L	ex vivo	↓↓
Hewawasam 2024	Mice CD-1 pancreatic islets	8-12	M/F	n/a	100 nmol/L	ex vivo	100nmol/L	ex vivo	↓
Hewawasam 2024	Mice CD-1 pancreatic islets	8-12	M/F	n/a	100 nmol/L	ex vivo	-	-	↑
Bayle 2021	Rat pancreas islets	?	M	n/a	-	-	10nmol	ex vivo	↓
Bayle 2021	Rat pancreas islets	?	M	n/a	100 nmol	ex vivo	10nmol	ex vivo	↑
Bayle 2021	Rat pancreas islets	?	M	n/a	100 nmol	ex vivo	-	-	↑
M'Kadmi 2019	Rat Wistar pancreas islets	12-16	M	n/a	-	-	10nmol	ex vivo	↓
M'Kadmi 2019	Rat Wistar pancreas islets	12-16	M	n/a	1000 nmol	ex vivo	10nmol	ex vivo	↑
M'Kadmi 2019	Rat Wistar pancreas islets	12-16	M	n/a	1000 nmol	ex vivo	-	-	o

Table 1.6. Pre-clinical studies assessing the effects of exogenous LEAP2 on serum insulin.
Abbreviations: d, Days; F, Female; h, Hours; M, Male; n/a, Not applicable; sc, Subcutaneous; ?, Not known; ↑↑, Greatly increased; ↑, Increased; ↓↓, Greatly decreased; ↓, Decreased; o, No effect.

1.3.8. Effects of LEAP2 on Food Intake in Rodents

In rodents, administration of peripheral and/or central LEAP2 (38-77), representing the full length 40 amino acid peptide, either antagonises the orexigenic effects of exogenous AG and/or when administered alone LEAP2 has the opposite effects to AG administration to reduce food intake in most studies (either by antagonising endogenous AG action at GHSR and/or as an inverse agonist attenuating constitutive activity of the GHSR even in the absence of AG). These reports are summarised in Tables 1.7, 1.8.

Central administration of LEAP2 blocked the orexigenic effects of AG in rodents with LEAP2 doses at 0.17 of AG dose (Barrile et al. 2019), 3.3 times in mice with or without *ob/ob* mutation (Lugilde et al. 2022), in rats at an equivalent AG dose (Wald et al. 2023) and in fed rats at 10 and 30 times the AG dose (Lin et al. 2024). But not in studies where in rats were given central LEAP2 at 3,10 and 30 times the AG dose in a time restricted feed pattern (Lin et al. 2024) and daily injections in aging mice (Casado et al. 2024).

Central administration of LEAP2 in several rodent models including DIO mice, *ob/ob* and binge-like eating reduced food intake on its own (Cornejo et al. 2019; Lugilde et al. 2022; Chu et al. 2022; Wald et al. 2023; Tufvesson-Alm et al. 2024b; Lin et al. 2024; Tufvesson-Alm et al. 2024a), but not in others when rodents were given LEAP2 alone including DIO, GHSR-KO mice and GH-deficient dwarf mice (Barrile et al. 2019; Casado et al. 2024; Lugilde et al. 2022; Lin et al. 2024; Islam et al. 2020), suggesting evidence of the anorexigenic effect of central LEAP2 administration in rodents..

Chronic central LEAP2 administration in mice arcuate (ARC) nucleus using AAV-LEAP2 overexpression in normal chow fed resulted in weight loss (over 11 weeks), reduction in food intake, fat mass (over 11 weeks), white adipose tissue, adipocyte size and a smaller body size, but no changes in lean mass, brown adipose tissue, glucose tolerance or plasma total cholesterol, triglycerides or LEAP2, body temperature (Chu et al. 2022). Similar reduction in food intake were seen after chronic central AAV-LEAP2 delivery to the ARC in high fat diet (60% calories from fat) fed mice, additionally there was reduction in plasma total cholesterol and reduction in glucose intolerance suggesting improved glucose utilisation (Chu et al. 2022). This suggests that the anorexigenic effects of LEAP2 overexpression in the ARC may be independent of fat content in diet and protects mice against the adverse metabolic consequences of obesity.

This suggests that mice with obesity are may be more sensitive to the hypophagic effects of central LEAP2 administration, and that LEAP2 reduction in food intake are mediated via GHSR, and ability to block orexigenic action of AG are independent of leptin signalling and GH (Lugilde et al. 2022; Casado et al. 2024). In mice that were restricted to eat for 8h/day there was no attenuation of the orexigenic effect of AG by central LEAP2, possibly because ghrelin is higher after time restricted eating and LEAP2 is not able to inhibit it, or due to the mutual antagonism of AG and LEAP2 at the GHSR, or the higher half-life of AG (27-31min) vs. that of LEAP2 (9min) (Lin et al. 2024).

Peripheral administration of LEAP2 blocked the orexigenic effects of AG at 3-30 times the AG equivalent dose (Ge et al. 2018; Islam et al. 2020; Islam et al. 2022; M'Kadmi et al. 2019; Shankar et al. 2024). Peripheral LEAP2 (including fragments) on its own reduced food intake (Ge et al. 2018; Hola et al. 2022; Hagemann et al. 2022), but not in those experiments including rodents in the fed state, DIO, GHSR-KO models as either bolus or continuous doses over 3-12 days (M'Kadmi et al. 2019; Islam et al. 2020; Islam et al. 2022; Hagemann et al. 2021; Hagemann et al. 2022; Cornejo et al. 2019; Ferreira-Junior et al. 2024; Petelakova et al. 2024).

In GHSR-KO mice exogenous LEAP2 did not increase food intake (Islam et al. 2022; Hagemann et al. 2022; Lugilde et al. 2022) suggesting that the effects of LEAP2 are mediated via the GHSR.

There were no differences in food intake after a 24 hour fast in either lean or DIO *Leap2*-KO mice (Shankar et al. 2021b), but over a longer duration of 20 weeks, food intake was increased in DIO *Leap2*-KO mice (Shankar et al. 2021b). In lean *Leap2*-KO mice, peripheral AG administration increased food intake, compared to a double dose required in wild-type mice to increase food intake (Shankar et al. 2021b). In DIO *Leap2*-KO mice, AG administration increased food intake, but not in wild-type mice indicating ghrelin resistance in obesity (Shankar et al. 2021a). Exogenous AG increased food intake in *Leap2*-KO mice (Shankar et al. 2021b) suggesting that LEAP2 deletion sensitises mice to the orexigenic effects of AG. Thus, it is suggested that both lean and obese *Leap2*-KO mice are more sensitive to the effects of AG on increasing food intake, and that LEAP2 deletion may alleviate ghrelin resistance in obesity.

In rodents, *Leap2* mRNA expression was detected in several areas of the brain including reward related areas, although in lower levels compared to the liver or small intestine. *Leap2* mRNA expression was 2.7 times higher in the nucleus accumbens and 4.4 times higher in the laterodorsal tegmental area vs. the hypothalamus (Tufvesson-Alm et al. 2024b). However, LEAP2 peptide was not measured in this study. Furthermore, although brain *Leap2* mRNA expression was seen in one LEAP2 study in areas including hypothalamic nuclei such as ARC and PVH, as well as hippocampal dentate gyrus and medulla (Barrile et al. 2019), regional brain expression has yet to be reported by any other groups.

In mice, icv administration of LEAP2 1.2 nmol attenuated the reward-related memory of highly palatable foods (e.g. peanut butter and Nutella) (Tufvesson-Alm et al. 2024b). In mice, icv LEAP2 (1.2 nmol) administration reduced the accumbal dopamine release in mice pre-exposed to highly palatable food (Tufvesson-Alm et al. 2024b). However, in mice with access to highly palatable food, there was a reduction in *Leap2* mRNA expression in areas associated with reward (nucleus accumbens, VTA and laterodorsal tegmental area) and memory (hippocampus) but not the hypothalamus, which is an area associated with appetitive homeostasis hypothalamus (Tufvesson-Alm et al. 2024b). These results show that central LEAP2 has a profound effect on dopaminergic reward signalling associated with food and effects several aspects of feeding. This highlights LEAP2's effect on reward, which may have applications for obesity and other reward-related addictive disorders.

GHSR receptors are found in the locus coeruleus, part of the pons in the brainstem (Maric et al. 2023). This area is involved in feeding behaviour in rodents (Sciolino et al. 2022 24813). Acute intra-locus coeruleus administration of AG (1ug) stimulated acute food intake and food motivation in *ad libitum* fed rats and conversely, intra-locus coeruleus administration of LEAP2 (1.1nmol) reduced acute food intake in fasted mice (Maric et al. 2023).

Lipidization of LEAP2(38-51) fragment at its C-terminus with palmitic acid enhanced its affinity to the GHSR, amplified the anorexigenic effect (vs. non-palmitoylated LEAP2(38-51) fragment) to attenuate AG induced food intake in mice and increased stability in plasma and reduce food intake on its own (Hola et al. 2022). However, peripherally administered palmitoylated-LEAP2 (38-51) fragment, on its own did not reduce food intake in DIO mice,

nor did it attenuate the orexigenic effect of AG suggesting resistance, but on switching to normal chow, this resistance was reversed (Hola et al. 2023). Similarly, chronic peripheral administration of palmitoylated-LEAP2 (38-51) fragment over two weeks in DIO mice or simultaneously with a high fat diet over six weeks did not reduce food intake (Petelakova et al. 2024).

Similarly, addition of a fatty moiety to full length LEAP2 (38-77) (exact structure of fatty acid not known), was recently synthesised that increased its affinity at GHSR (Shankar et al. 2024), now referred to as long-acting LEAP2 (LA-LEAP2). LA-LEAP2 had a half-life of 5.19 hours (longer than 10-15mins without any fatty moiety (Ge et al. 2018)) in rodents and was slightly more potent than LEAP2 in acutely reducing food intake in mice (without fatty molecule), with similar attenuation of the orexigenic effect of AG (Shankar et al. 2024).

Furthermore, the chronic effect of LA-LEAP2 on hepatic function by modulating GHSR signalling, was studied in a mouse model of MAFLD created by feeding a GAN (Gubra-amylin-NASH; 40% fat) diet. Peripheral subcutaneous LA-LEAP2 administered daily 2000 nmol/kg over 4 weeks reduced body weight, liver weight, hepatic triglycerides, plasma total cholesterol, plasma ALT, hepatic steatosis and inflammation and liver fibrosis in *Leap2*-KO and wild type mice fed a GAN diet over 10 weeks (Shankar et al. 2024). Similarly, LA-LEAP2 administered daily 2000 nmol/kg over 10 days reduced body weight, plasma ALT and liver fibrosis, but increased liver macrovesicular steatosis in *Leap2*-KO and wild type mice fed a GAN diet over 40 weeks (Shankar et al. 2024). However, in majority of the mice (85%), ulcerative dermatitis skin lesions developed at the subcutaneous injection sites, necessitating to shorten the duration of administration (from 4 weeks to 10 days) (Shankar et al. 2024). This further adds that GHSR activation either by ghrelin administration or *Leap2*-KO increases liver steatosis and fibrosis (Shankar et al. 2021a; Shankar et al. 2024). This suggests the effects of chronic, peripheral LA-LEAP2 improves MAFLD in mice by reducing body weight and improves markers of lipid metabolism. This opens the therapeutic scope of exogenous LEAP2 administration in humans with obesity and MAFLD.

Table 1.7. Pre-clinical studies assessing the effects of central exogenous LEAP2 on ingestive behaviour in rodents.

Abbreviations: M, Male; F, Female; h, Hours; d, Days; HFD, High fat diet; GH, Growth hormone; icv, Intracerebrovascular; LDTg, Lateral dorsal tegmental nucleus; LC, Locus coeruleus; AAV, Adeno-associated virus; ARC, Arcuate nucleus; OD, Once daily; N, No; *, Alcohol intake; n/a, Not applicable; ?, Not known; ↑↑, Greatly increased; ↑, Increased; ↓↓, Greatly decreased; ↓, Decreased; o, No effect.

Author	Animal	Age (weeks)	Sex	Nutritional state	LEAP2 dose (nmol)	Route	Chronic	AG dose (nmol)	Route	Chronic	Food intake
Barrile 2019	Mice C57BL/6	8-12	M	Fasting (12h)	-	-	-	0.06	icv	N	↑
Barrile 2019	Mice C57BL/6	8-12	M	Fasting (12h)	0.01	icv	N	0.06	icv	N	↓
Barrile 2019	Mice C57BL/6	8-12	M	Fasting (12h)	0.01	icv	N	-	-	-	o
Casado 2024	Mice C57BL/6J	15	M	Fasting (12h)	2 (OD)	icv	Yes (7d)	5 (OD)	icv	Yes (7d)	o
Casado 2024	Mice C57BL/6J	15	M	Fasting (12h)	-	-	-	5 (OD)	icv	Yes (7d)	↑
Casado 2024	Mice C57BL/6J	130	M	Fasting (12h)	2 (OD)	icv	Yes (7d)	5 (OD)	icv	Yes (7d)	o
Casado 2024	Mice C57BL/6J	130	M	Fasting (12h)	-	-	-	5 (OD)	icv	Yes (7d)	o
Casado 2024	Mice C57BL/6J	15	M	Fasting (12h)	2 (OD)	icv	Yes (7d)	-	-	-	o
Casado 2024	Mice C57BL/6J	130	M	Fasting (12h)	2 (OD)	icv	Yes (7d)	-	-	-	o
Richardson 2023	Mice C57BL/6J	8	M/F	Fasting (12h)	1.1	icv	N	-	-		↓*
Lugilde 2022	Mice C57BL/6J	12	M	Fasting (12h)	1.5	icv	N	-	-	-	↓↓
Chu 2022	Mice C57BL/6J	8	M	Fasting (12h)	10	icv	N	-	-	-	↓↓
Chu 2022	Mice C57BL/6J	8	M	Fasting (12h)	100	icv	N	-	-	-	↓↓
Chu 2022	Mice C57BL/6J	8	M	Fasting (12h)	10 (OD)	icv	Yes (10d)	-	-	-	↓↓
Chu 2022	Mice C57BL/6J	8	M	Fasting (12h)	LEAP2 over expression by AAV in ARC	icv	N	-	-	-	↓↓
Tufvesson-Alm 2024	Mice NMRI	?	M	Fasting (12h)	1.2	icv	N	-	-	-	↓↓
Tufvesson-Alm 2024	Mice NMRI	?	M	Fasting (12h)	1.2	intra-LDTg	N	-	-	-	↓↓
Lugilde 2022	Mice C57BL/6J DIO (60% fat)	12	M	Fasting (12h)	1.5	icv	N	-	-	-	↓↓
Casado 2024	Mice C57BL/6J DIO (60% fat)	15	M	Fasting (12h)	2 (OD)	icv	Yes (7d)	-	-	-	o
Chu 2022	Mice C57BL/6J DIO (60% fat)	8	M	Fasting (12h)	LEAP2 over expression by AAV in ARC	icv	N	-	-	-	↓↓
Lugilde 2022	Mice C57BL/6J *Ghsr-KO*	12	M	Fasting (12h)	1.5	icv	N	-	-	-	o
Lugilde 2022	Mice C57BL/6J	12	M	Fed (start light phase)	-	-	-	0.45	icv	N	↑
Lugilde 2022	Mice C57BL/6J	12	M	Fed (start light phase)	1.5	icv	N	0.45	icv	N	↓
Lugilde 2022	Mice C57BL/6J	12	M	Fed (start light phase)	1.5	icv	N	-	-	-	↓↓
Cornejo 2019	Mice C57BL/6 (HFD 2h/d for 4d	9-12	M	Fasting (12h)	-	-	-	0.06	icv	N	↑
Cornejo 2019	Mice C57BL/6 (HFD 2h/d for 4d	9-12	M	Fasting (12h)	0.6 (LEAP2 38-51)	icv	N	-	-	-	↓↓
Lugilde 2022	Mice C57BL/6J *ob/ob*	12	M	Fasting (12h)	1.5	icv	N	-	-	-	↓↓
Lugilde 2022	Mice C57BL/6J *ob/ob*	12	M	Fed (start light phase)	-	-	-	0.45	icv	N	↑
Lugilde 2022	Mice C57BL/6J *ob/ob*	12	M	Fed (start light phase)	1.5	icv	N	0.45	icv	N	↓
Lugilde 2022	Mice C57BL/6J *ob/ob*	12	M	Fed (start light phase)	1.5	icv	N	-	-	-	↓↓
Lugilde 2022	Rats Sprague-Dawley	12	M	Fasting (12h)	8	icv	N	-	-	-	↓↓
Wald 2023	Rats Sprague-Dawley	?	M	Fasting (12h)	0.15	icv	N	-	-	-	↓↓
Wald 2023	Rats Sprague-Dawley	?	M	Fasting (12h)	-	-	-	0.15	icv	N	↑
Wald 2023	Rats Sprague-Dawley	3	M	Fasting (12h)	0.15	icv	N	0.15	icv	N	↓
Wald 2023	Rats Sprague-Dawley	?	M	Fasting (12h)	1.5	icv	N	-	-	-	↓↓
Maric 2023	Rats Sprague-Dawley	8	M/F	Fasting (12h)	1.1	intra-LC	N	-	-	-	↓↓
Lin 2024	Rats Sprague-Dawley	3	M	Fed (start light phase)	1	icv	N	0.1	icv	N	↓
Lin 2024	Rats Sprague-Dawley	3	M	Fed (start light phase)	3	icv	N	0.1	icv	N	↓
Lugilde 2022	Rats Sprague-Dawley	12	M	Fed (start light phase)	-	-	-	1.5	icv	N	↑
Lin 2024	Rats Sprague-Dawley	3	M	Fed (start light phase)	3	icv	N	-	-	-	↓↓
Lugilde 2022	Rats Sprague-Dawley	12	M	Fed (start light phase)	8	icv	N	-	-	-	o
Lugilde 2022	Rats Sprague-Dawley	12	M	Fed (start light phase)	-	-	-	1.5	icv	N	↑
Maric 2023	Rats Sprague-Dawley	8	M/F	Fed (start light phase)	-	-	-	0.3	intra-LC	N	↑
Lin 2024	Rats Sprague-Dawley	3	M	Fed (8h)	3	icv	N	-	-	-	o
Lin 2024	Rats Sprague-Dawley	3	M	Fed (8h)	0.3	icv	N	0.1	icv	N	o
Lin 2024	Rats Sprague-Dawley	3	M	Fed (8h)	1	icv	N	0.1	icv	N	o
Lin 2024	Rats Sprague-Dawley	3	M	Fed (8h)	3	icv	N	0.1	icv	N	o
Lin 2024	Rats Sprague-Dawley	3	M	Fed (8h)	-	-	-	0.1	icv	N	↑
Tufvesson-Alm 2024	Rats Wistar	?	M/F	Fasting (12h)	1.2	icv	N	-	-		↓*
Tufvesson-Alm 2024	Rats Wistar	?	M/F	Fasting (12h)	1.2	icv	N	-	-	-	↓↓
Islam 2020	Rats Wistar	8	M	Fasting (4h)	1	icv	N	-	-	-	o
Islam 2020	Rats Wistar	8	M	Fasting (14h)	1	icv	N	100	icv	N	↓
Islam 2020	Rats Wistar	8	M	Fasting (14h)	1	icv	N	-	-	-	o
Islam 2020	Rats Wistar	8	M	Fasting (14h)	-	-	-	100	icv	N	↑
Lugilde 2022	Rats Lewis	104	M	Fasting (12h)	8	icv	N	-	-	-	o
Lugilde 2022	Rats Lewis	104	M	Fasting (12h)	-	-	-	1.5	icv	N	↑
Lugilde 2022	Rats Lewis	104	M	Fasting (12h)	8	icv	N	1.5	icv	N	↓
Lugilde 2022	Rats dwarf (GH-deficient)	12	M	Fasting (12h)	8	icv	N	1.5	icv	N	↓
Lugilde 2022	Rats dwarf (GH-deficient)	12	M	Fasting (12h)	8	icv	N	-	-	-	o
Lugilde 2022	Rats dwarf (GH-deficient)	12	M	Fasting (12h)	-	-	-	1.5	icv	N	↑

Table 1.8. Pre-clinical studies assessing the effects of peripheral exogenous LEAP2 on ingestive behaviour in rodents.
Abbreviations: BD, Twice daily DIO, Diet induced obesity; F, Female; h, Hours; HFD, High fat diet; ip, Intraperitoneal; LA, Long acting; M, Male; N, No; n/a, Not applicable; NC, Normal chow; OD, Once daily; Palm, Palmitoylated; sc, subcutaneous; *, Alcohol intake; ?, Not known; ↑↑, Greatly increased; ↑, Increased; ↓↓, Greatly decreased; ↓, Decreased; o, No effect.

Author	Animal	Age (weeks)	Sex	Nutritional state	LEAP2 dose	Route	Chronic	Ghrelin dose	Route	Chronic	Food intake
Ge 2018	Mice C57BL/6	12-16	M	Fasting (12h)	-	-	-	150 nmol/kg	sc	N	↑
Ge 2018	Mice C57BL/6	12-16	M	Fasting (12h)	150 nmol/kg	sc	N	150 nmol/kg	sc	N	↓
Ge 2018	Mice C57BL/6	12-16	M	Fasting (12h)	3000 nmol/kg	sc	N	150 nmol/kg	sc	N	↓
Ge 2018	Mice C57BL/6	12-16	M	Fasting (12h)	3000 nmol/kg	sc	N	-	-	-	↓↓
Shankar 2021	Mice C57/BL6N	16-20	M/F	Fasting (12h)	-	-	-	1mg/kg	sc	N	↑
Current thesis	Mice C57BL/6	12-16	M	Fasting (12h)	0.3 mg/kg	sc	N	-	-	-	↓↓
Current thesis	Mice C57BL/6	12-16	M	Fasting (12h)	1 mg/kg	sc	N	-	-	-	↓↓
Current thesis	Mice C57BL/6	12-16	M	Fasting (12h)	3 mg/kg	sc	N	-	-	-	↓↓
Hola 2022	Mice C57BL/6	14	M	Fasting (12h)	78.2 nmol (LEAP2 38-51)	sc	N	-	-	-	↓↓
Hola 2022	Mice C57BL/6	14	M	Fasting (12h)	78.2 nmol (Palm-LEAP2 38-51)	sc	N	-	-	-	↓↓
Ge 2018	Mice C57BL/6	12-16	M	Fasting (12h)	150 nmol/kg	sc	N	-	-	-	o
Islam 2022	Mice C57BL/6J	12-27	M	Fasting (24h)	600 nmol/kg	ip	N	-	-	-	o
Hagemann 2022	Mice C57BL/6	14-24	F	Fasting (4h)	3000 nmol/kg	sc	N	-	-	-	↓↓
Hola 2023	Mice C57BL/6 DIO then NC	16-20	M	Fasting (12h)	78.2 nmol (Palm-LEAP2 38-51)	sc	N	-	-	-	↓↓
Hola 2023	Mice C57BL/6 DIO (60% fat)	16-20	M	Fasting (12h)	-	-	-	1mg/kg	sc	N	o
Hola 2023	Mice C57BL/6 DIO (60% fat)	16-20	M	Fasting (12h)	78.2 nmol (Palm-LEAP2 38-51)	sc	N	-	-	-	o
Petelakova 2024	Mice C57/BL6N DIO (60% fat)	16-20	M	Fasting (12h)	78.2 nmol (Palm-LEAP2 38-51)	sc	Yes (14d)	-	-	-	o
Petelakova 2024	Mice C57/BL6N DIO (60% fat)	16-21	M	Fasting (12h)	156.4 nmol (Palm-LEAP2 38-51)	sc	Yes (42d)	-	-	-	o
Shankar 2021	Mice C57/BL6N DIO (60% fat)	16-20	M/F	Fasting (12h)	-	-	-	10mg/kg	sc	N	o
Cornejo 2019	Mice C57BL/6 (HFD 2h/d for 4d)	9-12	M	Fasting (12h)	-	-	-	0.06nmol/kg	sc	N	↑
Cornejo 2019	Mice C57BL/6 (HFD 2h/d for 4d)	9-12	M	Fasting (12h)	0.6 nmol/kg (LEAP2 38-51)	sc	N	-	-	-	o
Islam 2022	Mice C57BL/6J *Ghsr-KO*	12-28	M	Fasting (24h)	600 nmol/kg	ip	N	-	-	-	o
Richardson 2023	Mice C57BL/6J	8	M/F	Fasting (12h)	10 nmol/kg	ip	N	-	-	-	o*
Richardson 2023	Mice C57BL/6J	8	M/F	Fasting (12h)	100 nmol/kg	ip	N	-	-	-	o*
Richardson 2023	Mice C57BL/6J	8	M/F	Fasting (12h)	1000 nmol/kg	ip	N	-	-	-	o*
Shankar 2021	Mice C57BL/6N *Leap2-KO*	16-20	M/F	Fed (start light phase)	-	-	-	0.5mg/kg	sc	N	↑↑
Shankar 2021	Mice C57BL/6N DIO (60% fat) *Leap2-KO*	16-20	M/F	Fed (start light phase)	-	-	-	10mg/kg	sc	N	↑
Current thesis	Mice C57BL/7	12-16	M	Fed (start light phase)	-	-	-	150 nmol/kg	sc	N	↑
Current thesis	Mice C57BL/7	12-16	M	Fed (start light phase)	150 nmol/kg	sc	N	150 nmol/kg	sc	N	↓
Islam 2022	Mice C57BL/6J	12-23	M	Fed (start light phase)	-	-	-	60 nmol/kg	ip	N	↑
Hola 2022	Mice C57BL/6J	14	M	Fed (start light phase)	-	-	-	1 mg/kg	sc	N	↑
Islam 2022	Mice C57BL/6J	12-23	M	Fed (start light phase)	600 nmol/kg	ip	N	60 nmol/kg	ip	N	↓
Hola 2022	Mice C57BL/6J	14	M	Fed (start light phase)	78.2 nmol (LEAP2 38-51)	sc	N	1 mg/kg	sc	N	↓
Hola 2022	Mice C57BL/6J	14	M	Fed (start light phase)	78.2 nmol (Palm-LEAP2 38-51)	sc	N	1 mg/kg	sc	N	↓
M'Kadmi 2019	Mice C57BL/6J	12-16	M	Fed (start light phase)	-	-	-	60 nmol/kg	sc	N	↑
M'Kadmi 2019	Mice C57BL/6J	12-16	M	Fed (start light phase)	600 nmol/kg	sc	N	60 nmol/kg	sc	N	↓
Shankar 2024	Mice C57BL/6J	16	M/F	Fed (start light phase)	-	-	-	600 nmol/kg	sc	N	↑
M'Kadmi 2019	Mice C57BL/6J	12-16	M	Fed (start light phase)	600 nmol/kg (LEAP2 38-49)	sc	N	60 nmol/kg	sc	N	↓
Shankar 2024	Mice C57BL/6J	16	M/F	Fed (start light phase)	2000 nmol/kg (LA-LEAP2)	sc	N	600 nmol/kg	sc	N	↓↓
Shankar 2024	Mice C57BL/6J	16	M/F	Fed (start light phase)	2000 nmol/kg	sc	N	600 nmol/kg	sc	N	↓
M'Kadmi 2019	Mice C57BL/6J	12-16	M	Fed (start light phase)	600 nmol/kg	sc	N	-	-	-	o
M'Kadmi 2019	Mice C57BL/6J	12-16	M	Fed (start light phase)	600 nmol/kg (LEAP2 38-49)	sc	N	-	-	-	o
Current thesis	Mice C57BL/6	12-16	M	Fed (start light phase)	150 nmol/kg	sc	N	-	-	-	o
Current thesis	Mice C57BL/6	12-16	M	Fed (start light phase)	0.3 mg/kg	sc	N	-	-	-	o
Current thesis	Mice C57BL/6	12-16	M	Fed (start light phase)	1 mg/kg	sc	N	-	-	-	o
Current thesis	Mice C57BL/6	12-16	M	Fed (start light phase)	3 mg/kg	sc	N	-	-	-	o
Islam 2022	Mice C57BL/6J	12-29	M	Fed (start light phase)	600 nmol/kg (BD)	sc	Yes (3d)	-	-	-	o
Islam 2022	Mice C57BL/6J	12-30	M	Fed (start light phase)	85 nmol/kg/day (continuous)	sc	Yes (6d)	-	-	-	o
Islam 2022	Mice C57BL/6J (caloric restriction 60%)	12-31	M	Fed (start light phase)	85 nmol/kg/day (continuous)	sc	Yes (6d)	-	-	-	o
Islam 2022	Mice C57BL/6J (caloric restriction 60%) *GHSR-KO*	12-32	M	Fed (start light phase)	85 nmol/kg/day (continuous)	sc	Yes (6d)	-	-	-	o
Hagemann 2022	Mice C57BL/6 *Ghsr-KO*	14-24	F	Fed (start light phase)	3000 nmol/kg	sc	N	-	-	-	o
Hagemann 2021	Mice NMRI	5-6	M	Fed (start light phase)	3000 nmol/kg	sc	N	-	-	-	o
Hagemann 2021	Mice NMRI	5-6	M	Fed (start light phase)	3000 nmol/kg (LEAP2 38-47)	sc	N	-	-	-	o
Hagemann 2021	Mice NMRI	5-6	M	Fed (start light phase)	3000 nmol/kg (LEAP2 23-47)	sc	N	-	-	-	o
Hagemann 2021	Mice NMRI	5-6	M	Fed (start light phase)	3000 nmol/kg (LEAP2 49-77)	sc	N	-	-	-	o
Islam 2020	Rats Wistar	8	M	Fasting (14h)	-	-	-	5 nmol	ip	N	↑
Islam 2020	Rats Wistar	8	M	Fasting (14h)	15 nmol	ip	N	5 nmol	ip	N	↓
Ferreira-Junior 2024	Rats Wistar	10	M/F	Fasting (12h)	100 nmol/kg (LEAP2 38-51) (OD)	sc	Yes (12d)	-	-	-	o
Ferreira-Junior 2024	Rats Wistar	10	M/F	Fasting (12h)	1000 nmol/kg (LEAP2 38-51) (OD)	sc	Yes (12d)	-	-	-	o
Ferreira-Junior 2024	Rats Wistar (pregnant)	10	M/F	Fasting (12h)	72 nmol/kg (LEAP2 38-51) (OD)	sc	Yes (10d)	-	-	-	o

1.3.9. Effects of LEAP2 on Alcohol-related Behaviour in Rodents

The rewarding properties of alcohol are mediated by dopamine release from the nuclueus accumbens (Jerlhag et al. 2009) and the underlying neurobiology of AUD involves AG and the GHSR (Kharbanda et al. 2022). LEAP2 is found mainly in the liver and small intestine (Krause et al. 2003; Islam et al. 2020), it is also found in reward related areas in the brain such as nucleus accumbens and laterodorsal tegmental area although in lower concentrations (Tufvesson-Alm et al. 2024b). Exogenous effects on alcohol ingestion are summarised in Tables 1.7 and 1.8.

In rodent experiments, peripheral administration of LEAP2 did not reduce alcohol intake in either sex, but improved motor coordination in both sexes (Richardson et al. 2023). However, central administration of LEAP2 decreased alcohol intake in heavy-drinking mice of both sexes (Richardson et al. 2023).

Similarly, central administration of LEAP2 into the third ventricle prevented the ability of alcohol to cause locomotor stimulation, attenuate the dopamine release in the nucleus accumbens and suppressed the memory of alcohol reward in male mice (not done in females) (Tufvesson-Alm et al. 2024a). Central LEAP2 also reduced alcohol consumption in alcohol consuming rats of both sexes, without altering alcohol preference, with no correlation between alcohol consumption and serum LEAP2 concentrations (Tufvesson-Alm et al. 2024a).

Taken together, this suggests that LEAP2 modulates reward and responses related to alcohol, leading to reduced consumption of alcohol by its central actions. Future studies should involve exogenous administration of LEAP2 in humans with AUD.

1.4. HUMAN LEAP2 STUDIES

1.4.1. Human Plasma LEAP2 Assay

Plasma LEAP2 can be measured using a commercial LEAP2 immunoassay (Phoenix Pharmaceuticals Inc, CA, USA, EK-075-40), to gain information on LEAP2 physiology in humans (Mani et al. 2019). This ELISA assay has been validated in several other studies (Tables 1.9, 1.10). Some studies have been using assays that are not validated or used only in one study and may not correctly represent the findings seen (Ma et al. 2020; Ezquerro et al. 2023; Liu et al. 2024; Aslanipour et al. 2020; Garces et al. 2022).

1.4.2. Greater Plasma LEAP2 in Obesity

In obesity and a positive energy balance AG is reduced and elevated during weight loss and caloric restriction (Muller et al. 2015). AG and LEAP2 are reciprocally regulated in humans acting on the GHSR. The results of different effects/ comparisons on plasma LEAP2 are summarised in Table 1.9.

Circulating fasting plasma LEAP2 is elevated with adults with overweight/obesity including T2DM and/or MAFLD (Mani et al. 2019; Nabekura et al. 2023; Andreoli et al. 2024a; Li et al. 2022; Andreoli et al. 2024c).

However, conflicting evidence is seen from studies, with no change in plasma LEAP2 (Barja-Fernandez et al. 2021; Liu et al. 2024) or lower in women with PCOS (Aslanipour et al. 2020) however, this was using a different LEAP2 assay that has not been used in any other study (SRB-T-81190, SunRed Biotechnology, Shanghai, China) and in children with overweight/ obesity (Fittipaldi et al. 2020).

In humans, plasma LEAP2 concentrations not only is increased in obesity, fasting/post-prandial LEAP2 also positively correlated with BMI in several studies with or without T2DM and/or MAFLD (Mani et al. 2019; Andreoli et al. 2024a; Andreoli et al. 2024c; Emini et al. 2024; Ma et al. 2020; Byberg et al. 2023), but not in studies including participants with only normal weight (Bhargava et al. 2023; Andreoli et al. 2024c; Li et al. 2022) or in different populations such as lactating women (Kruger et al. 2024) or adults with and without rheumatoid arthritis (Francisco et al. 2020).

Although there is a positive correlation for fasting plasma LEAP2 in overweight participants with metabolic dysfunction-associated fatty liver disease (MAFLD) (Ma et al. 2020), in people with rheumatoid arthritis without obesity (n=101, 77 female), there was no correlation of fasting plasma LEAP2 with BMI (Francisco et al. 2020), nor in people with T2DM (Li et al. 2022), while there was negative correlation in children with obesity (Fittipaldi et al. 2020) and in women with PCOS with overweight/obesity (Aslanipour et al. 2020). In patients with bacterial meningitis, there was no correlation of cerebrospinal LEAP2 with BMI (Sakai et al. 2021). Given the heterogeneity of current data, we suggest the correlation of post-prandial increments in plasma LEAP2, and BMI may be a U-shaped curve, with a negative correlation in participants without obesity and then a positive correlation in participants with obesity. In

subsequent studies, recruitment should include participants across BMI spectrums and in different disease states.

In participants with obesity and MAFLD with and without T2DM (n=146), fasting plasma LEAP2 was higher than normal weighted participants (n=33) (Ezquerro et al. 2023). Plasma LEAP2 in participants with obesity, positively correlated with biopsy proven liver fibrosis (Ezquerro et al. 2023). However, the assay used was Cat no. CSB-EL012853HU; Cusabio Biotech, Wuhan, China for measuring plasma LEAP2. Opposite results were seen for plasma AG in participants with obesity and MAFLD (vs. lean participants) (Ezquerro et al. 2023).

Identification of single nucleotide polymorphisms (SNP) in the *LEAP2* gene have been investigated in a UK Biobank to demonstrate associations with obesity related traits. Five independent SNPs of the LEAP2 gene (rs803221, rs57880964, rs1634959, rs708459 and rs765760) were identified. Homozygous rs57880964 (G allele) or rs803221 (C allele) and heterozygous rs765760 was positively associated with waist-to-hip ratio (with and without adjustment for BMI) in men and women. While rs57880964 (G allele), rs1634959 (T allele) and rs708459 (C allele) was associated with higher BMI in men and women (Andreoli et al. 2024b). Overall, the rs57880964 G allele was consistently associated with higher WHR, BMI (Andreoli et al. 2024b). Fasting plasma LEAP2 demonstrated a positive correlation with rs803223 G allele and rs765760 T alleles (Andreoli et al. 2024c) independent of BMI status or sex further suggesting that variants may influence *LEAP2* gene expression. However, there was no correlation of fasting plasma LEAP2 and *LEAP2* methylation status suggesting a lack of relevant epigenetic signatures on gene expression (Andreoli et al. 2024c).

Following on from rodent studies, in adults with obesity, liver *LEAP2* mRNA expression was higher (vs. lean controls), with no difference in liver *GHRL* or *GHSR* expression (Englund et al. 2024). In overweight adults ± T2DM (Type 2 diabetes mellitus), intestinal expression of *LEAP2* mRNA is highest in jejunum followed by the duodenum with minimal expression in the large intestine with no difference in expression between overweight adults with or without T2DM (Englund et al. 2024). These findings are consistent with the first publication of LEAP2's isolation with higher jejunal expression compared to the duodenum and lower expression in the colon (Krause et al. 2003). The mechanisms underlying elevated plasma LEAP2 concentrations in T2DM and obesity remain incompletely understood.

This suggests that some people with obesity may benefit from raising plasma LEAP2 to further protect against AG actions to increase appetite, food intake and reward, and during weight loss when plasma AG increases (Sumithran et al. 2011; Neseliler et al. 2019).

Table 1.9. Human studies assessing disease states and manipulations on plasma AG, LEAP2 and AG/LEAP2 ratio.

Abbreviations: aGHD, Adult growth hormone deficiency; ALD, Alcohol associated liver disease; BHB, Beta-hydroxy butyrate; CDGP, Constitutional delay of growth and puberty; CSF, Cerebro-spinal fluid; DJBL, Duodenal-jejunal bypass liner; GD, Gambling disorders; HC, Healthy control; LCD, Low calorie diet; m, months; MAFLD, Metabolic dysfunction-associated fatty liver disease; NW, Normal weight (BMI <24.9 kg/m^2); OW, Overweight (BMI 25.0-29.9 kg/m^2); OB, Obese (BMI >30.0 kg/m^2); PCOS, Polycystic ovarian syndrome; RYGB, Roux-en-Y Gastric Bypass; T2DM, Type 2 Diabetes Mellitus; TG, Total ghrelin; VSG, Vertical Sleeve Gastrectomy; n/a, Not applicable; ?, Not known; ↑, Increased; ↓, Decreased; → No effect; (a), 1200-1500kcal/day, females; 1500-1800kcal/day, males; (b), 1200kcal/day; (c), 50min/day; (d), 60min/day over 14 days; (e), Testosterone (1mg/kg/month) or Letrozole (2.5mg/day) over 12 months. Assays used: Cusabio Biotiotech, Wuhan, China (Cat no. CSB-EL012853HU); My Bio Source, CA, USA (MBS-917663); Phoenix Pharmaceuticals Inc, CA, USA (EK-075-40); Sun Red Biotech, Shangai, China (SRB-T-81190). Results in bold are from this thesis; [3], results from Chapter 3.

Author	Population type	Study population	N Study population	Female (%)	Control population	N control	Female (%)	Effect of / Comparison:	Fasting plasma AG	Fasting plasma LEAP2	Plasma AG/LEAP2 ratio	Assay
Mani 2019	Adults	OB	42	64.3	NW/OW	63	63.5	OB	↑	↑	↑	Phoenix
Holm 2022	Adults	OB	20	75.0	NW	9	0.0	OB		↑		Phoenix
Andreoli 2023	Adults	OW/OB	23	39.1	NW	21	57.1	OW/OB	↓	↑	↓	Phoenix
Andreoli 2024	Adults	OW/OB	131	30.5	NW	196	44.9	OW/OB		↑		Phoenix
Barja-Fernández 2020	Adults	OB	102	52.9	NW	69	42.0	OB		→		Phoenix
Fittipaldi 2020	Children	OW/OB	46	50.0	NW	36	52.8	OW/OB		↓		Phoenix
Nabekura 2023	Adults	OB + T2DM	39	53.8	NW	8	50.0	OB + T2DM		↑		Phoenix
Ma 2020	Adults	NW/OW + MAFLD +T2DM	80	33.8	NW/OW + T2DM	80	33.8	MAFLD		↑		Cusabio
Ezquerro 2023	Adults	OB + MAFLD ± T2DM	146	61.6	NW	33	0.0	OB + MAFLD ± T2DM	↓	↑	↓	Cusabio
Liu 2024	Adults	OB + MAFLD	14	42.9	NW	22	63.6	Obesity + MAFLD		→		Cusabio
Liu 2024	Adults	OB + MAFLD	14	42.9	NW +ALD	12	16.7	OB + MAFLD (vs. ALD)		↓		Cusabio
Aslanipour 2019	Adults	OW/OB + PCOS	64	100.0	OW/OB	64	100.0	PCOS		↓		Sun Red
Tezenas du Montcel 2023	Adults	Anorexia nervosa	30	100.0	-	-	n/a	Weight regain in Anorexia nervosa	↓	↓	↓	Phoenix
Barja-Fernández 2020	Children	NW/OW/OB - F	83	100.0	NW/OW/OB -M	88	0.0	Female vs. Male	→ (TG)	↑		Phoenix
Andreoli 2024	Adults	NW/OW/OB - F	128	100.0	NW/OW/OB -M	199	0.0	Female vs. Male		↑		Phoenix
Kruger 2024	Infants	NW/OW/OB - F	?	?	NW/OW/OB -M	?	?	Female vs. Male		→		Phoenix
Li 2022	Adults	NW + T2DM	29	27.6	NW	27	29.6	T2DM	↓	↑	↓	Cusabio
Byberg 2023	Adults	OB +pre-diabetes	115	55.7	-	-	n/a	Metformin (1700mg) or Dapagliflozin (10mg) (OD, 13 weeks)		→ (13 weeks)		Phoenix
Mani 2019	Adults	OB	8-14	0.0	-	-	n/a	RYGB at 3m	↓	→ (3m)	→	Phoenix
Mani 2019	Adults	OB	8-14	0.0	-	-	n/a	RYGB at 24m	↓	↓ (24m)	→	Phoenix
Hagemann 2020	Adults	OB	20	75.0	-	-	n/a	RYGB at 1 week		→		Phoenix
Ezquerro 2023	Adults	OB + MAFLD ± T2DM	61	73.8	-	-	n/a	RYGB at 6m	→ (6m)	→ (6m)	→ (6m)	Cusabio
Mani 2019	Adults	OB	7	71.4	-	-	n/a	VSG at 12-18m	↓ (12-18m)	↓ (12-18m)	↓ (12-18m)	Phoenix
Nabekura 2023	Adults	OB + T2DM	39	53.8	-	-	n/a	VSG at 12m		→ (12m)		Phoenix
Emini 2024 [3]	Adults	OW/OB + T2DM	37	48.6	-	-	n/a	DJBL 0-12m		↓ (24m)		Phoenix
Emini 2024 [3]	Adults	OW/OB + T2DM	30	50.0		-	n/a	LCD 0-24m (a)		↓ (24m)		Phoenix
Ragland 2023	Adults	OB	13	100.0	-	-	n/a	LCD 0-2 weeks (b)	→	↓	→	Phoenix
Holm 2022	Adults	NW	9	0.0	-	-	n/a	Exercise (c) (Venous BHB ~0.37mM)		↓		Phoenix
Ragland 2023	Adults	OB	12	100.0	-	-	n/a	LCD (b) + Exercise (d)	→	↓	→	Phoenix
Pedersen 2023	Adults	NW/OW	8	0.0	NW/OW	37	0.0	Fasting (36h vs. 10h)		↓		Phoenix
Holm 2023	Adults	NW/OW	10	0.0	-	-	n/a	Oral ketone (BHB-ester)(Venous BHB ~5.5mM)	↓	↑	↓	Phoenix
Pedersen 2023	Adults	NW/OW	8	0.0	-	-	n/a	Oral ketone (Sodium-BHB) (Venous BHB ~2.5mM)	↓	↓	↓	Phoenix
Pedersen 2023	Adults	NW/OW	8	0.0	-	-	n/a	Intravenous ketone (Sodium-BHB)(Venous BHB ~2.5mM)	↓	↓	↓	Phoenix
Pedersen 2023	Adults	NW/OW	11	0.0	-	-	n/a	Oral glucose		↑		Phoenix
Ragland 2023	Adults	OB	13	100.0	-	-	n/a	Oral glucose	→	→		Phoenix
Pedersen 2023	Adults	NW/OW	11	0.0	-	-	n/a	Intravenous glucose		↓		Phoenix
Garces 2022	Adults	NW/OW	25	100.0	-	-	n/a	Pregnancy	↓ (TG,each trimester)	↓(each trimester)	↑	My Bio Source
Francisco 2020	Adults	NW/OW	101	76.2	NW	26	50.0	Rhematoid arthritis (vs. HC)		↑		Phoenix
Sakai 2021	Adults	NW/OW	5	80.0	NW/OW	5	60.0	Meningitis (vs. HC)		↑ CSF LEAP2		Phoenix
Sakai 2021	Adults	NW/OW	1	100.0	-	-	n/a	Antibiotics for meningitis		↓ CSF LEAP2		Phoenix
Vergani 2021	Adults	NW/OW/OB	15	53.3	NW/OW	15	66.7	aGHD (vs. HC)	↓(TG)	↑		Phoenix
Vergani 2022	Adults	OW/OB	18	38.9	NW	20	60.0	aGHD (vs. HC)	↓ (TG)	↑		Phoenix
Etxandi 2022	Adults	NW/OW	297	6.7	NW/OW	79	10.1	Gambling disorders (GD) (vs. HC)	↑ (TG)	↓		Phoenix
Etxandi 2023	Adults	NW/OW/OB	23	13.0	NW/OW/OB	274	5.8	GD + Food addiction (vs. GD)	→ (TG)	→		Phoenix
Tian 2023	Adults	NW/OW	19	63.2	-	-	n/a	Age	↓	↑	↓	Phoenix
Varshney 2024	Adults	?	23	0.0	?	32	0.0	Cancer cachexia (vs. without cancer cachexia)	→ (TG)	→	→	Phoenix
Varshney 2024	Adults	?	55	0.0	?	25	0.0	Cancer (vs. HC)	→ (TG)	→	→	Phoenix
Kruger 2024	Adults	OW/OB	26	100.0	NW	26	100.0	Breast feeding (6m) (vs. HC)		↑		Phoenix
Kruger 2024	Adults	OW/OB	26	100.0	NW	26	100.0	Breast feeding (6m) (vs. HC)		→ Breast milk LEAP2		Phoenix
Barja-Fernández 2020	Children	NW/OW	44	0.0	NW/OW/OB	44	0.0	Pre-pubertal vs. pubertal (boys)	↓(TG)	→		Phoenix
Barja-Fernández 2020	Children	NW/OW	42	100.0	NW/OW/OB	41	100.0	Pre-pubertal vs. pubertal (girls)	↓(TG)	↑		Phoenix
Barja-Fernández 2020	Children	NW/OW	86	48.8	NW/OW/OB	85	48.2	Pre-pubertal vs. pubertal	↓ (TG)	↑		Phoenix
Varimo 2022	Children	NW	28	0.0	-	-	n/a	CDGP treatment (e) (boys)	→	↓ (12 m)		Phoenix

1.4.3. Plasma LEAP2 Falls with Weight Loss

Given the reciprocal relationship with AG, in conditions of weight loss and negative energy balance plasma LEAP2 concentrations fall. Specifically, surgically induced weight loss by either Roux-en-Y gastric bypass (RYGB) or vertical sleeve gastrectomy surgery (VSG). However, no difference in plasma LEAP2 was seen after 3-6months of RYGB (Mani et al. 2019; Hagemann et al. 2021; Ezquerro et al. 2023) but a reduction at 24 months after the procedure (Mani et al. 2019) in participants with obesity. Similarly, plasma LEAP2 fell after VSG surgery at 12-18months (Mani et al. 2019), but not after 12 months in those with T2DM and obesity (Nabekura et al. 2023). Though the change in fasting plasma LEAP2 as a percentage (post-VSG/pre-VSG) positively correlated with change in BMI (Nabekura et al. 2023), suggesting that higher fasting post-VSG plasma LEAP2 correlates with less weight loss post-VSG in those with T2DM and obesity.

Confirmation of these findings, are seen after non-surgical weight loss, wherein fasting plasma LEAP2 falls after a low-calorie diet (LCD) in women with obesity over 2 weeks with or without exercise (1200kcal/day) (Ragland et al. 2023) and long-term calorie restriction in adults with obesity and T2DM over 24 months (1200-1500kcal/d, females; 1500-1800kcal/day, males) (Emini et al. 2024). Regarding mechanisms for this reduction in fasting plasma LEAP2 after LCD ± exercise, there was reduction in fasting glucose concentrations and post-prandial serum insulin, with no change in post-prandial glucose or fasting serum insulin suggesting that improved hepatic insulin sensitivity contributing to these LEAP2 reductions after weight loss by diet and exercise. Another mechanism for reduction in LEAP2 may be due to elevation in fasting plasma free fatty acids or ketone bodies.

A fall in LEAP2 with weight loss may hinder maintenance of weight loss from lifestyle modification, combined with rise in plasma AG (Sumithran et al. 2011; Neseliler et al. 2019), by increasing appetite and food reward (especially when there are no beneficial appetitive hormone changes such as large increase in plasma PYY/GLP-1 and fall in plasma AG seen after RYGB and/or VSG (Yousseif et al. 2014; Li et al. 2019; Miras et al. ; Mathes et al. 2012; Stefater et al. 2012; Goldstone et al. 2016). This supports the possibility that increasing LEAP2 by exogenous administration may be a potential treatment to help maintain non-bariatric surgery produced weight loss from energy restriction (Mani et al. 2019). The results of different manipulations on plasma LEAP2 are summarised in Table 1.9.

1.4.4. Plasma LEAP2 Correlates with Markers of Glucose and Fat Metabolism

In humans, fasting/ post-prandial plasma LEAP2 consistently correlates with obesity and BMI but also other metabolic associations of increased adiposity including, fasting/ post-prandial plasma glucose, insulin resistance (HOMA-IR), serum insulin and triglycerides across several studies. The results of different comparisons on plasma LEAP2 are summarised in Table 1.10.

In adults with obesity, plasma LEAP2 concentrations positively correlated other metabolic associations of increased adiposity including body fat, fasting plasma glucose, insulin resistance (HOMA-IR), fasting serum triglycerides, visceral adipose tissue volume, and intrahepatocellular lipid, while plasma AG concentrations and the AG/LEAP2 molar ratio are negatively correlated with BMI, body fat and HOMA-IR (Mani et al. 2019). In adults without obesity with T2DM ± MAFLD plasma LEAP2 positively correlated fasting serum insulin and HOMA-IR but not with fasting plasma glucose or serum triglycerides (Ma et al. 2020). While, in adults with overweight and obesity, fasting plasma LEAP2 positively correlated with fasting insulin, total insulin secretion, 1st phase and 2nd phase insulin secretion after hyperinsulinemic-euglycemic clamp (Stark et al. 2023). Also, plasma LEAP2 positively correlates with insulin resistance, but this result disappeared after adjustment for body fat, suggesting obesity status as a mediator. Also, this is supported by lack of correlations of fasting plasma LEAP2 with fasting and post-prandial glucose concentrations after an oral glucose tolerance test (Stark et al. 2023). This relationship between LEAP2 and insulin secretion may be useful in the future for the treatment of T2DM and metabolic syndrome characterised by increased insulin resistance.

Similarly, in paediatric populations, plasma LEAP2 was also positively correlated with insulin, HOMA-IR and serum triglycerides in girls, and with leptin in boys (Barja-Fernandez et al. 2021), and in boys with constitutional delay of growth and puberty, plasma LEAP2 positively correlated with fasting serum triglycerides, fasting insulin and HOMA-IR (Varimo et al. 2022). Furthermore, changes in plasma LEAP2 over 6 months positively correlated with changes in fat mass, body fat percentage and serum fasting triglycerides, while changes in plasma LEAP2 over 12 months positively correlated with changes in insulin and HOMA-IR (Varimo et al. 2022). This would suggest that higher plasma LEAP2 at puberty may be predictive of impaired glucose and lipid metabolism in adulthood.

In adults with obesity and pre-diabetes there was a positive correlation with fasting plasma LEAP2 fasting plasma glucose and serum insulin and triglycerides, body weight and fat mass but not with cholesterol (Byberg et al. 2023). In adults with normal weight and overweight/obesity, fasting plasma LEAP2 positively correlated with fat mass and plasma glucose after adjusting for sex and BMI (Andreoli et al. 2024a). However, in only in adults with normal weight, fasting plasma LEAP2 positively correlate with fasting plasma glucose (Andreoli et al. 2024c).

By contrast, in women diagnosed with PCOS with overweight/obesity, plasma LEAP2 were negatively correlated with BMI, insulin, HOMA-IR and glucose levels, but this is the only study in adults showing this opposite relationship (Aslanipour et al. 2020). However, this was using a different LEAP2 assay that has not been used in any other study (SRB-T-81190, Sun Red Biotechnology, Shanghai, China). Similarly, in a small study involving children with obesity, plasma LEAP2 negatively correlated with BMI Z-score but not with fasting insulin, triglycerides or HOMA-IR which may suggest differential regulation in children and adults (Fittipaldi et al. 2020). Unexpectedly, in 30 women diagnosed with anorexia nervosa (AN), 4 months after an inpatient food rehabilitation programme, mean BMI increased (14.22±0.44 to 20.11±0.09 kg/m^2), a decrease in fasting plasma LEAP2 (17.55 to 14.19 ng/mL) after weight regain, but expected increases in plasma AG. There was no correlation between participants BMI and fasting plasma LEAP2 or AG/LEAP2 molar ratio. This elevated baseline LEAP2 may be inhibiting the orexigenic potential of AG and hence there is inadequate weight gain and metabolic changes observed in AN. These changes in women with AN are different from physiological regulation of AG and LEAP2 with weight gain/loss

LEAP2 gene is found on chromosome 5 (5q31.1), SNP's in the *LEAP2* gene viz. rs57880964, rs1634959, rs708459 or rs803221 (all homozygous) were associated with higher serum triglycerides concentration in adults with/without obesity (Andreoli et al. 2024b). Overall, the rs57880964 G allele was consistently associated with higher serum triglycerides concentrations suggesting that carriers of this allele are more susceptible to obesity (Andreoli et al. 2024b).

Post-prandial increases in plasma glucose and serum insulin (calculated as absolute change 1.5h after a 337kcal meal) showed positive correlation with fasting plasma LEAP2 in adults across all BMI categories (Mani et al. 2019). However, this thesis will further elaborate these

findings by correlating post-prandial changes in markers of glucose and fat metabolism with changes in post-prandial plasma LEAP2.

This summary suggests that plasma LEAP2 is likely regulated by metabolic factors such as glucose, insulin and triglyceride levels furthermore, post-prandial increases in and regulation of plasma LEAP2 is summarised in section 1.4.6.

Table 1.10. Correlations of markers of glucose and fat metabolism with plasma LEAP2 in humans.

Abbreviations: Abbreviations: ALD, Alcohol associated liver disease; BMI, Body mass index; DJBL, Duodenal-jejunal bypass liner; h, Hours; HC, Healthy control; HOMA-IR, Homeostatic model assessment for insulin resistance; LCD, Low calorie diet; MAFLD, Metabolic dysfunction-associated fatty liver disease; m, Months; NW, Normal weight (BMI <24.9 kg/m^2); OW, Overweight (BMI 25.0-29.9 kg/m^2); OB, Obese (BMI <30.0 kg/m^2); PCOS, Polycystic ovarian syndrome; PP, Post-prandial; RYGB, Roux-en-Y Gastric Bypass; T2DM, Type 2 Diabetes Mellitus; VSG, Vertical Sleeve Gastrectomy; +ve, Positive correlation; -ve, Negative correlation; o; No correlation. Assays used: Cusabio Biotiotech, Wuhan, China (Cat no. CSB-EL012853HU); Phoenix Pharmaceuticals Inc, CA, USA (EK-075-40); Sun Red Biotech, Shangai, China (SRB-T-81190). Results in bold are from this thesis; [2], results from Chapter 2; [3], results from Chapter 3.

Author	Number	Female (%)	Population type	BMI category	Disease / intervention	Nutritional state variable	Outcome variable	Nutritional state plasma LEAP2	Correlation with plasma LEAP2	Assay
Mani 2019	105	63.8	Adults	NW/OW/OB	-	Fasting	BMI	Fasting	+ve	Phoenix
Andreoli 2023	44	47.7	Adults	NW/OW/OB	-	Fasting	BMI	Fasting	+ve	Phoenix
Mani 2019	20	100.0	Adults	NW/OW/OB	-	Fasting	BMI	PP (1.5h post-337kcal)	+ve	Phoenix
Fittipaldi 2020	82	51.2	Children	NW/OW/OB	-	Fasting	BMI	Fasting	-ve	Phoenix
Barja-Fernández 2020	171	48.5	Adolescents	NW/OW/OB	-	Fasting	BMI	Fasting	o	Phoenix
Kruger 2024	52	100.0	Adults	NW/OW/OB	Lactating 6m	Fasting	BMI	Fasting	o	Phoenix
Andreoli 2024	131	30.5	Adults	OW/OB	-	Fasting	BMI	Fasting	+ve	Phoenix
Stark 2023	65	36.9	Adults	OW/OB	-	Fasting	BMI	Fasting	o	Phoenix
Emini 2024 [3]	30	50.0	Adults	OW/OB	+ T2DM, LCD 0-24m	Fasting	BMI	Fasting	+ve	Phoenix
Emini 2024 [3]	37	48.6	Adults	OW/OB	+ T2DM, DJBL 0-12m	Fasting	BMI	Fasting	+ve	Phoenix
Aslanipour 2019	128	100.0	Adults	OW/OB	±PCOS	Fasting	BMI	Fasting	-ve	Sun Red
Bhargava 2023 [2]	22	22.7	Adults	NW/OW	-	Fasting	BMI	PP (2.5h post 730kcal)	-ve	Phoenix
Ma 2020	160	33.8	Adults	NW/OW	+ T2DM ± MAFLD	Fasting	BMI	Fasting	+ve	Cusabio
Francisco 2020	101	76.2	Adults	NW/OW	± Rheumatoid arthritis	Fasting	BMI	Fasting	o	Phoenix
Byberg 2023	115	55.7	Adults	OB	Pre-diabetes	Fasting	BMI	Fasting	+ve	Phoenix
Mani 2019	16	81.3	Adults	OB	+ RYGB at 0 and 24m	Fasting	BMI	Fasting	+ve	Phoenix
Andreoli 2024	196	44.9	Adults	NW	-	Fasting	BMI	Fasting	o	Phoenix
Li 2022	56	28.6	Adults	NW	± T2DM	Fasting	BMI	Fasting	o	Cusabio
Mani 2019	90	60.0	Adults	NW/OW/OB	-	Fasting	Plasma glucose	Fasting	+ve	Phoenix
Andreoli 2023	44	47.7	Adults	NW/OW/OB	-	Fasting	Plasma glucose	Fasting	+ve	Phoenix
Barja-Fernández 2020	88	0.0	Adolescents	NW/OW/OB	-	Fasting	Plasma glucose	Fasting	+ve	Phoenix
Barja-Fernández 2020	83	100.0	Adolescents	NW/OW/OB	-	Fasting	Plasma glucose	Fasting	o	Phoenix
Mani 2019	20	100.0	Adults	NW/OW/OB	-	PP (1.5h post 337kcal)	Plasma glucose	Fasting	+ve	Phoenix
Kruger 2024	52	100.0	Adults	NW/OW/OB	Lactating 6m	Fasting	Plasma glucose	Fasting	o	Phoenix
Stark 2023	65	36.9	Adults	OW/OB	-	Fasting	Plasma glucose	Fasting	o	Phoenix
Andreoli 2024	131	30.5	Adults	OW/OB	-	Fasting	Plasma glucose	Fasting	o	Phoenix
Emini 2024 [3]	30	50.0	Adults	OW/OB	+ T2DM, LCD 0-24m	Fasting	Plasma glucose	Fasting	+ve	Phoenix
Emini 2024 [3]	37	48.6	Adults	OW/OB	+ T2DM, DJBL 0-12m	Fasting	Plasma glucose	Fasting	o	Phoenix
Aslanipour 2019	128	100.0	Adults	OW/OB	±PCOS	Fasting	Plasma glucose	Fasting	-ve	Sun Red
Bhargava 2023 [2]	22	22.7	Adults	NW/OW	-	PP (1.5h post 337kcal)	Plasma glucose	PP (2.5h post 730kcal)	o	Phoenix
Ma 2020	160	33.8	Adults	NW/OW	+ T2DM ± MAFLD	Fasting	Plasma glucose	Fasting	o	Cusabio
Mani 2019	16	81.3	Adults	OB	+ RYGB at 0 and 24m	Fasting	Plasma glucose	Fasting	+ve	Phoenix
Byberg 2023	115	55.7	Adults	OB	Pre-diabetes	Fasting	Plasma glucose	Fasting	+ve	Phoenix
Andreoli 2024	196	44.9	Adults	NW	-	Fasting	Plasma glucose	Fasting	+ve	Phoenix
Li 2022	56	28.6	Adults	NW	± T2DM	Fasting	Plasma glucose	Fasting	o	Cusabio
Fittipaldi 2020	82	51.2	Children	NW/OW/OB	-	Fasting	Serum insulin	Fasting	o	Phoenix
Mani 2019	20	100.0	Adults	NW/OW/OB	-	PP (1.5h post 337kcal)	Serum insulin	Fasting	o	Phoenix
Barja-Fernández 2020	83	100.0	Adolescents	NW/OW/OB	-	Fasting	Serum insulin	Fasting	+ve	Phoenix
Barja-Fernández 2020	88	0.0	Adolescents	NW/OW/OB	-	Fasting	Serum insulin	Fasting	o	Phoenix
Andreoli 2023	44	47.7	Adults	NW/OW/OB	-	Fasting	Serum insulin	Fasting	o	Phoenix
Kruger 2024	52	100.0	Adults	NW/OW/OB	Lactating 6m	Fasting	Serum insulin	Fasting	+ve	Phoenix
Stark 2023	65	36.9	Adults	OW/OB	-	Fasting	Serum insulin	Fasting	+ve	Phoenix
Emini 2024 [3]	30	50.0	Adults	OW/OB	+ T2DM, LCD 0-24m	Fasting	Serum insulin	Fasting	+ve	Phoenix
Emini 2024 [3]	37	48.6	Adults	OW/OB	+ T2DM, DJBL 0-12m	Fasting	Serum insulin	Fasting	o	Phoenix
Aslanipour 2019	128	100.0	Adults	OW/OB	±PCOS	Fasting	Serum insulin	Fasting	-ve	Sun Red
Bhargava 2023 [2]	22	22.7	Adults	NW/OW	-	PP (2.5h post 730kcal)	Serum insulin	PP (2.5h post 730kcal)	o	Phoenix
Ma 2020	160	33.8	Adults	NW/OW	+ T2DM ± MAFLD	Fasting	Serum insulin	Fasting	+ve	Cusabio
Li 2022	56	28.6	Adults	NW	± T2DM	Fasting	Serum insulin	Fasting	o	Cusabio
Mani 2019	90	60.0	Adults	NW/OW/OB	-	Fasting	HOMA-IR	Fasting	+ve	Phoenix
Barja-Fernández 2020	171	48.5	Adolescents	NW/OW/OB	-	Fasting	HOMA-IR	Fasting	+ve	Phoenix
Andreoli 2023	44	47.7	Adults	NW/OW/OB	-	Fasting	HOMA-IR	Fasting	o	Phoenix
Fittipaldi 2020	82	51.2	Children	NW/OW/OB	-	Fasting	HOMA-IR	Fasting	o	Phoenix
Emini 2024 [3]	30	50.0	Adults	OW/OB	+ T2DM, LCD 0-24m	Fasting	HOMA-IR	Fasting	+ve	Phoenix
Emini 2024 [3]	37	48.6	Adults	OW/OB	+ T2DM, DJBL 0-12m	Fasting	HOMA-IR	Fasting	+ve	Phoenix
Aslanipour 2019	128	100.0	Adults	OW/OB	±PCOS	Fasting	HOMA-IR	Fasting	-ve	Sun Red
Ma 2020	160	33.8	Adults	NW/OW	+ T2DM ± MAFLD	Fasting	HOMA-IR	Fasting	+ve	Cusabio
Mani 2019	90	60.0	Adults	NW/OW/OB	-	Fasting	Serum triglycerides	Fasting	+ve	Phoenix
Fittipaldi 2020	82	51.2	Children	NW/OW/OB	-	Fasting	Serum triglycerides	Fasting	o	Phoenix
Barja-Fernández 2020	83	100.0	Adolescents	NW/OW/OB	-	Fasting	Serum triglycerides	Fasting	+ve	Phoenix
Barja-Fernández 2020	88	0.0	Adolescents	NW/OW/OB	-	Fasting	Serum triglycerides	Fasting	o	Phoenix
Emini 2024 [3]	30	50.0	Adults	OW/OB	+ T2DM, LCD 0-24m	Fasting	Serum triglycerides	Fasting	+ve	Phoenix
Emini 2024 [4]	37	48.6	Adults	OW/OB	+ T2DM, DJBL 0-12m	Fasting	Serum triglycerides	Fasting	o	Phoenix
Bhargava 2023 [2]	22	22.7	Adults	NW/OW	-	PP (2.5h post 730kcal)	Serum triglycerides	PP (2.5h post 730kcal)	-ve (trend)	Phoenix
Ma 2020	160	33.8	Adults	NW/OW	+ T2DM ± MAFLD	Fasting	Serum triglycerides	Fasting	o	Cusabio
Byberg 2023	115	55.7	Adults	OB	Pre-diabetes	Fasting	Serum triglycerides	Fasting	+ve	Phoenix
Li 2022	56	28.6	Adults	NW	± T2DM	Fasting	Serum triglycerides	Fasting	o	Cusabio

1.4.5. Plasma LEAP2 Falls with Ketonaemia

In a negative energy state, especially starvation, there is a buildup of ketone bodies (e.g. acetoacetate, BHB) as byproduct of fatty acid oxidation to provide energy in such circumstances. Such an increase in endogenous plasma ketones also occurs after exercise, during prolonged fasting and adopting a diet deficient of carbohydrates (ketogenic diet). A state of negative energy balance then stimulates food intake, but that mechanism is not fully understood (Vestergaard et al. 2021).

Consistent with preclinical literature (Holm et al. 2022), endogenous ketonaemia in lean men, achieved after a bike exercise at 70% VO2 peak for 50 mins reduced plasma LEAP2 with an exercise-induced increase in venous BHB to 0.37±0.7mM (Holm et al. 2022).

Similarly, in men without obesity both oral (sodium-BHB) and matched intravenous infusion of sodium-BHB, achieving venous BHB levels to ~ 2.5mM, reduced fasting plasma LEAP2 (Pedersen et al. 2023). However, in men without obesity a BHB-ester ingestion increased venous BHB to 5.5mM and led to increases in plasma LEAP2 (Holm et al. 2023). This discrepancy is likely due to the nature of the ketone molecule ingested as in men without obesity prolonged fasting of 36 hours significantly reduced plasma LEAP2 vs. a 10 hour fast (Pedersen et al. 2023).

A role for fasting-induced increase in blood ketones has been implicated in lowering hepatic LEAP2 secretion and thus plasma LEAP2, to increase GHSR signalling as a counter-regulatory response (in addition to the fasting-induced increase in AG itself) to increase food intake and plasma glucose.

1.4.6. Plasma LEAP2 Increases with Food Intake

In rodent studies, post-prandial increases plasma LEAP2 is influenced by both the size and macronutrient composition of meals, with fat inducing greater increases compared to carbohydrates (Gradel et al. 2023). In human ingestion studies, despite variations in macronutrient composition and the timing of post-prandial sampling, there is evidence suggesting that post-prandial LEAP2 elevations are generally proportional to the caloric content of the pre-load meal, although a peak elevation has not yet been achieved due to short post-prandial sampling times. These results are summarised in Figure 1.4.

Lack of a post-prandial increase in LEAP2 was seen in a minority of studies. There was no obvious post-prandial increase in plasma LEAP2 after a 300 kcal liquid meal in obesity (Hagemann et al. 2021), nor in men without obesity after 575kcal (Hagemann et al. 2022). However, OGTT (oral glucose tolerance test) increased 2-hour post-prandial plasma LEAP2 by 25% in men without obesity (n=11) (Pedersen et al. 2023), but not in women with obesity (n=13) (Ragland et al. 2023).

Interestingly, iso-glycaemic intravenous glucose infusion in men without obesity (n=11) reduced fasting plasma LEAP2 suggesting the 'incretin' effect with LEAP2 (Pedersen et al. 2023). There were sex and BMI differences observed after a fixed breakfast (20% of resting energy expenditure, 400-600 kcal, 56% carbohydrate, 34% fat, 12% protein), in only one study wherein only women with overweight/obesity showed a 58% increase in post-prandial plasma LEAP2 after 60min of meal ingestion (Andreoli et al. 2024a), but no similar post-prandial increase in men or women with normal weight nor in men with overweight/obesity (Andreoli et al. 2024a).

In adults without obesity (mean BMI 23.5 ± 0.9 kg/m^2, n=11, women=4) and adults with obesity and T2DM (mean BMI 28.3 ± 1.7 kg/m^2, n=11, women=9) (mean HbA1c 7.9%), post-prandial ingestion of a mixed meal of 592 kcal (50.8% carbohydrate, 43.7% fat, 5.5 % protein) increased plasma LEAP2 by 36% and 117% respectively after 2 hours (Islam et al. 2024). However, in adults with T2DM this post-prandial increase in LEAP2 was delayed as due to a lack of post-prandial increase at 1h, suggesting disrupted signalling in participants with obesity and diabetes (Islam et al. 2024).

Across a variety of studies (Figure 1.4) including participants without and with obesity, using different pre-load meal sizes (though of similar macronutrient composition) and at different post-prandial time points, there is a suggestion of a positive correlation between pre-load meal size and the post-prandial increase in plasma LEAP2. Therefore, it is suggested that meals of higher energy intake may have greater effects to increase plasma LEAP2, as also seen for post-prandial decreases in plasma total ghrelin (le Roux et al. 2005). However, this has yet to be examined within a single study in a repeated visit design.

Interestingly, fasting and post-prandial plasma glucose, but not serum insulin, positively correlated with fasting plasma LEAP2 in adults without/with obesity suggesting that it may

be differences in circulating glucose that are promoting post-prandial LEAP2 secretion, but post-prandial LEAP2 was not measured (Mani et al. 2019). By contrast, decreases in plasma ghrelin may be more related to hyperinsulinaemia than increases in plasma glucose (Flanagan et al. 2003; Mohlig et al. 2002; Schaller et al. 2003; Ali et al. 2013)(McCowen et al. 2002).

These studies suggest that LEAP2 secretion is sensitive to intestinal exposure to specific nutrients. The stimulus driving reciprocal changes in LEAP2 compared to AG, are unclear, but might be related to reciprocal changes in glucose or appetitive gut hormones, rather than insulin.

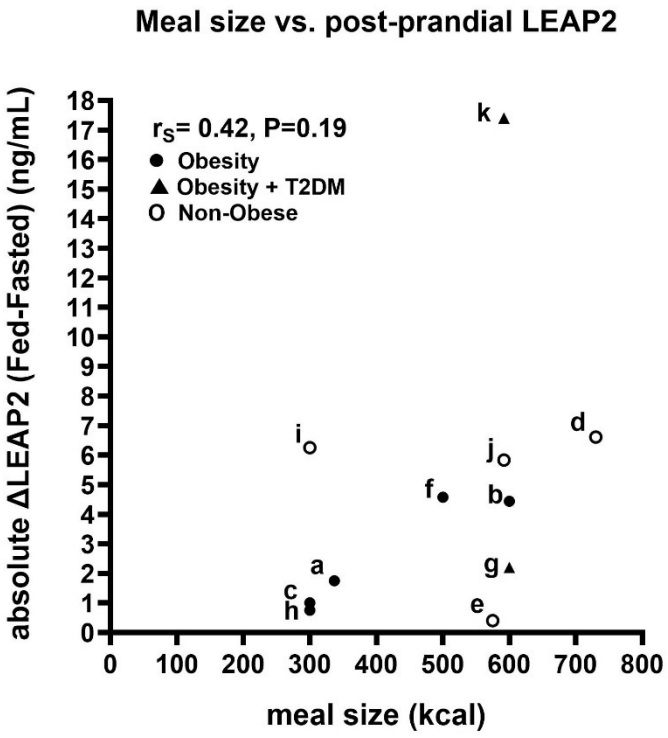


Figure 1.4. Correlation of post-prandial plasma LEAP2 with meal size consumed across different studies.

Correlation of post-prandial change in absolute plasma LEAP2 concentrations between fed and fasted time points (Δ Fed-Fasted) and meal size (kcal) consumed across different studies made using Spearman's (r_S) correlation coefficient. Black dots indicate participants with obesity (BMI >30kg/m^2), open dots participants without obesity (BMI <30kg/m^2) and black triangles participants with obesity and T2DM. Meals are liquid nutritional supplements with similar macronutrient composition: carbohydrates 49-55%, fat 30-35% and protein 14-18%.

Table at bottom indicates details of meal and time of post-prandial blood sample for each study indicated by letters a-k. Abbreviations: BMI, body mass index; CHO carbohydrate; kcal, kilocalorie;; NW, Normal weight (BMI <24.9 kg/m^2); OW, Overweight (BMI 25.0-29.9 kg/m^2); OB, Obese (BMI <30.0 kg/m^2); OGTT, Oral glucose tolerance test; REE, Resting energy expenditure;T2DM, Type 2 diabetes mellitus. All studies used Phoenix Pharmaceuticals Inc, CA, USA (EK-075-40) assay; Results in bold are from this thesis; [2], results from Chapter 2; [3], results from Chapter 3.

Study	Author	Obesity status	N	Female %	kcal	Mixed meal macronutrients (% energy)	Post-prandial sampling (mins)
A	Mani 2019	NW/OW/OB	20	100.0	337	52% CHO, 30% fat, 18% protein	90
B	Mani 2019	OB	20	90.0	600	49% CHO, 35% fat, 16% protein	120
C	Hagemann 2021	OB	18	72.2	300	50% CHO, 35% fat, 15% protein	120
D	**Bhargava 2023** [2]	NW/OW	22	22.7	730	55% CHO, 31% fat, 14% protein	150
E	Hagemann 2022	NW/OW	20	0.0	575	49% CHO, 35% fat, 16% protein	120
F	Andreoli 2023	OW/OB	9	100.0	400-600 (20% REE)	56% CHO, 34% fat, 12% protein	60
G	**Emini 2024** [3]	OW/OB + T2DM	15	60.0	600	49% CHO, 35% fat, 16% protein	120
H	Ragland 2023	OB	13	100.0	300	100% CHO, 0% fat, 0% protein (OGTT)	60
I	Pedersen 2023	NW	9	0.0	300	100% CHO, 0% fat, 0% protein (OGTT)	120
J	Islam 2024	NW/OW	11	36.4	592	51% CHO, 44% fat, 5% protein	120
K	Islam 2024	OW/OB + T2DM	11	81.8	592	51% CHO, 44% fat, 5% protein	120

1.4.7. Plasma LEAP2 in Health Conditions

The results of circulating plasma LEAP2 in different diseased states and populations have been summarised in Table 1.9 above. Lack in consistency of results is due to differing populations, incorrectly matched, without controlling for BMI or plasma glucose, done in a single participant, single study or not able to replicate the findings.

1.4.7.1. Elevated plasma LEAP2 in growth hormone deficiency

In adult growth hormone deficiency (aGHD), serum LEAP2 was not significantly higher than healthy controls (groups were not matched with aGHD group having more men, being older (53 vs. 47 years) and with higher BMI (27.1 vs. 22.9 kg/m^2), and there was no correlation between serum LEAP2 and BMI or HOMA-IR in both groups (Vergani et al. 2021). A repeat analysis by the same authors, showed that in patients with aGHD (vs. healthy controls) had significantly higher serum LEAP2 (Vergani et al. 2022). However, these aGHD patients were older and had higher BMI (age 59.7 years, BMI 30.2 kg/m^2) compared to their previous study (Vergani et al. 2021) which may account for a higher plasma LEAP2 and not necessarily growth hormone deficiency.

1.4.7.2. Elevated plasma LEAP2 in inflammation

In humans, plasma LEAP2 have been found to be increased in inflammatory states. In patients with rheumatoid arthritis plasma LEAP2 (in unspecified nutritional state) was increased compared to health controls (mean 7.66 vs. 3.04 ng/mL), though this patient group was unmatched, was older and with higher BMI (Francisco et al. 2020). Plasma LEAP2 positively correlated with serum C-reactive protein (CRP) concentrations, an inflammatory marker, but not age or BMI (Francisco et al. 2020). In another small study, cerebrospinal fluid (CSF) LEAP2 was higher (9.32 vs. 0.56 ng/mL) in patients diagnosed with bacterial

meningitis (vs. patients with psychosomatic disorders as controls) though serum LEAP2 was similar, and CSF LEAP2 positively correlated with serum glucose and CRP, but not BMI (Sakai et al. 2021). The suggestion that increased CSF LEAP2 was related to brain inflammation/infection was supported by finding that in one patient with bacterial meningitis, CSF LEAP2 fell after antibiotic treatment, while CSF/serum LEAP2 ratio was elevated (0.53 vs. 0.08) in one patient with bacterial meningitis compared to another with bacteraemia without central nervous system inflammation.

LEAP2 neutralising antibodies blocked endogenous LEAP2 function to enhance AG action in vivo (Ge et al. 2018), raising the possibility of inhibiting LEAP2 action to treat cachexia (Neary et al. 2004; Ashby et al. 2009). However, studies lacking increased plasma LEAP2 in inflammation include, comparing male participants with cancer and weight loss (>5% over the last 6 months) vs. those with cancer and stable weight, with no difference in fasting plasma LEAP2 or total ghrelin (Varshney et al. 2024). Similarly, no difference in fasting plasma LEAP2 or total ghrelin comparing male participants with or without cancer (Varshney et al. 2024).

Another study lacking increased plasma LEAP2 in inflammation, demonstrated no difference in *LEAP2* mRNA expression in in inter-vertebral disc cells between healthy deceased donors (n=12, mean age 54 ± 15 years) and those with age-related degenerative changes (n=37, mean age 55 ± 13 years) (Francisco et al. 2023). Further studies are need in different states of inflammation controlling for obesity status to confirm the above findings.

1.4.7.3. Plasma LEAP2 and puberty

Puberty is characterized by increased energy demand with increased height velocity and increase in muscle and bone mass. In boys with constitutional delay of growth and puberty (CDGP) treated with either exogenous testosterone (1mg/kg/month im) or an aromatase inhibitor letrozole (2.5mg/day) plasma LEAP2 fell over 6 and 12 months without any change in plasma AG (Varimo et al. 2022).

On treatment with exogenous testosterone, decrease in plasma LEAP2 positively correlated with increase in growth velocity and serum testosterone concentrations over 6 months (and change in plasma AG negatively correlated with change in insulin concentrations and HOMA-IR) (Varimo et al. 2022). By contrast, there was no change in plasma AG/LEAP2

molar ratio, nor did it correlate with any blood marker.

However, this contradicts the only other study of LEAP2 in puberty (Barja-Fernandez et al. 2021), where there was no difference in serum LEAP2 between pre-pubertal and pubertal boys, although it must be compared with caution as this is a cross-sectional study and that boys with CDGP have enhanced metabolic demands as compared with age and height matched children (Han et al. 2006; Barja-Fernandez et al. 2021).

In girls, plasma LEAP2 increases in puberty with reductions plasma total ghrelin (Barja-Fernandez et al. 2021). There is a suggestion of sexual dimorphism in plasma LEAP2 being higher in in girls than in boys independent of BMI status, but this comparison includes children with and without those going through puberty (Barja-Fernandez et al. 2021).

1.4.7.4. Plasma LEAP2 and gambling disorders

In a recent case control study adult patient with gambling disorders (GD) (n=297) (vs. healthy controls, n=79) had higher fasting plasma total ghrelin and lower plasma LEAP2 concentrations (after adjusting for age, sex and BMI) (Etxandi et al. 2022). In a further analysis by the same research group, amongst participants with GD (n=297), a sub-group of participants (n=54) that were older, had more women with a higher BMI (vs. mean of all gambling disorder participants (n=194)) had expected elevated fasting plasma LEAP2 (6.39 vs. 5.05 ng/mL) (Baenas et al. 2023) and an increased likelihood of GD severity (Mora-Maltas et al. 2024).This subset of female participants had poorer inhibitory control (more impulsive) with higher fasting plasma LEAP2, which may influence behaviours towards food and gambling rewards (Baenas et al. 2023).

Furthermore, when these adults with gambling disorders were evaluated for food addiction using the Yale Food Addiction Scale (YFAS), 23 participants (7.7% of n=297) were found to have addictive like eating behaviour (Etxandi et al. 2023). However, these GD participants with food addiction (vs. those without food addiction but with gambling disorders), did not have differing fasting total ghrelin or plasma LEAP2 nor any correlation with any psychological task (Etxandi et al. 2023).

Further studies are required to explore the role of exogenous LEAP2 and the GHSR signalling pathway as a therapeutic target for gambling and food addiction disorders.

1.4.7.5. Plasma LEAP2 in pregnancy

Human pregnancy is a stage of metabolic change and weight gain to ensure foetal growth and prevent adverse perinatal and maternal outcomes. In pregnant women without diabetes and obesity serum total ghrelin peaks in the second trimester and reaches its nadir towards the end of pregnancy when the maximum weight has been gained and there is greater insulin resistance (Fuglsang et al. 2005).

LEAP2 is visualised by immunohistochemistry in both pregnant human and rat liver, cytotrophoblast and syncytiotrophoblast placental tissues (Garces et al. 2022). In pregnant rats, plasma LEAP2 reduced and total ghrelin increased (Garces et al. 2022). However, in direct contradiction to these findings, in pregnant mice, there was an increase in plasma LEAP2 and reduction in AG (Gupta et al. 2024). Differences include species of rodents and assay. With Garces et al. using rats with an assay that has not been used in any other study (MBS-917663, My Bio Source, CA, USA and hence, has not been validated.

In pregnant women without obesity there was a reduction in serum LEAP2 each trimester (1.38 ± 0.16, 1.14 ± 0.17, 0.99 ± 0.16 ng/mL) and similar reduction in serum total ghrelin with nadir levels in the 3rd trimester, however the AG/LEAP2 ratio peaks in the second trimester (1.49 ± 0.14, 1.66 ± 0.22, 1.50 ± 0.19) and gradually came back to baseline 3 months post-partum. The proposed mechanism is that reductions in circulating LEAP2 during pregnancy may favour the orexigenic, diabetogenic and lipogenic actions of AG at the GHSR. Additionally, in a separate cohort of non-pregnant women without obesity (n=20, BMI= 21.96 ±2.45 (18.37 – 25.54)) there was no difference in the serum AG (5.16 vs. 5.24 ng/mL, P=0.71), serum LEAP2 (1.49 vs. 1.60 ng/mL, P=0.09) or AG/LEAP2 ratio (1.47 vs. 1.43, P=0.23) between the follicular and luteal phases of the menstrual cycle (Garces et al. 2022).

Further longitudinal studies are required as there is an unexplored role of AG and LEAP2 in the metabolic adaptations including weight gain in pregnancy.

1.4.7.6. LEAP2 in human milk

LEAP2 has been detected in human milk from 52 lactating women at 6 months post-partum (n=26 with overweight/ obesity or normal weight each) however, concentrations are 1/5th of that seen in plasma (Kruger et al. 2024). No *LEAP2* mRNA was detected in human milk or

mammary cells, suggesting that LEAP2 is selectively transported to human milk from the blood. Consistent with other cross-sectional studies, fasting plasma LEAP2 is higher in women with overweight/obesity (vs. normal weight) at 6 months post-partum with no differences in human milk LEAP2 (Kruger et al. 2024).

In lactating women, plasma LEAP2 positively correlated with insulin, but not with BMI, glucose or human milk LEAP2. In infants at 6 months age, there were no sex differences in plasma LEAP2; while in infants of both sexes, infant plasma LEAP2 positively correlated with BMI, but not with insulin or glucose, nor with maternal plasma/milk LEAP2 (Kruger et al. 2024). In summary, this one study identifies LEAP2 as a novel bioactive component in human milk and is associated with infant adiposity, suggesting a role in nutritional programming during early life.

1.4.7.7. Plasma LEAP2 in aging and cognitive decline

Age related cognitive disorders have been suggested to be deregulated by the ghrelin pathway via GHSR signalling in the hippocampus (Buntwal et al. 2019; Langenberg et al. 2005; Diano et al. 2006).

In one study, plasma LEAP2 is higher in aging mice (age 30 months) vs. 8-months old, with similar AG concentrations (Tian et al. 2023). To further characterise these age-related hormonal changes on hippocampal pathology, these aging mice (vs. 8-month-old mice) showed suppressed GHSR activation, reduced synaptic density and impaired neurogenesis in the hippocampus, a brain area involved in age-related cognitive decline (Tian et al. 2023). Furthermore, these aging mice were treated with lentiviral small hairpin RNA (shRNA) vectors to silence hepatic *Leap2* expression that restored synaptic loss, neurogenesis in the hippocampus and attenuated neuroinflammation seen with aging. Similarly, fasting plasma LEAP2 and liver *Leap2* mRNA is elevated in aging mice (age 30 months) vs. 15 months old (Casado et al. 2024).

In human participants over the age of 60 without obesity or T2DM (n=19, 12 female), increasing age was positively correlated with lower fasting plasma AG, higher fasting plasma LEAP2, and hence a lower AG/LEAP2 ratio (Tian et al. 2023). Furthermore, in this cohort higher plasma LEAP2 and lower AG/LEAP2 ratio positively correlated with lower mini-mental state examination scores (MMSE, a validated questionnaire of cognitive function) though

these findings were likely driven by one outlier participant; and no such correlations were seen with plasma AG (Tian et al. 2023).

There is a suggestion especially in pre-clinical studies that age-related increases in LEAP2 contribute cognitive changes, especially in the hippocampus and that these cognitive deficits are reversed by downregulation of LEAP2 (via *Leap2* shRNA silencing). Further studies are required in humans to confirm pre-clinical findings.

1.4.8. Sex Differences in Plasma LEAP2

There are suggestions that LEAP2 may be regulated differently in males and females, however, studies remain limited as most are only in one sex (males), under powered to find differences, opposite findings across studies or lack reports on sex differences.

In preclinical studies, in *ad libitum* fed rats at baseline, circulation levels of liver *Leap2* mRNA expression was 50% lower in female vs. male rats (Borchers et al. 2022). When these rats were challenged to an overnight fast, there was a significant reduction in liver *Leap2* mRNA expression only in male and not in female rats (Borchers et al. 2022). Despite these sex differences, there may be a floor effect in female rats where a further reduction in liver *Leap2* mRNA after an overnight fast cannot be seen as fed levels were already low.

In *Leap2*-KO mice, only females were hyperphagic and gained more weight with greater liver fat after a high fat diet (Shankar et al. 2021a). However, lentiviral *Leap2* knock down showed the opposite findings where in DIO female mice had reduced liver fat (Ma et al. 2020). This discrepancy is likely explained by different experimental approaches; lentivirus causes *Leap2* knockdown primarily in the liver while *Leap2*-KO mice have whole body LEAP2 deficiency. Similarly, there no sex differences seen in the anorexigenic effect of exogenous LEAP2 in mice (Islam et al. 2022; Maric et al. 2023; Shankar et al. 2024). Furthermore, there were no differences between sexes on the reduction of alcohol intake in mice (Richardson et al. 2023) or rats (Tufvesson-Alm et al. 2024a) after central delivery of LEAP2.

In the few clinical studies exclusively looking at sex differences, opposite findings to rodents are observed, girls had higher circulating fasting plasma LEAP2 than boys (Barja-Fernandez et al. 2021) but this study included children with and without puberty, and across BMI

categories. Similarly, fasting plasma LEAP2 was higher in women (vs. men) across BMI categories (Andreoli et al. 2024c), but no differences observed in infant girls (vs. boys) at 6 months of age (Kruger et al. 2024).

There was an increase in post-prandial plasma LEAP2 after OGTT in men without obesity (Pedersen et al. 2023), but not in women with obesity (Ragland et al. 2023), however population BMI status was different, and numbers were small (n=9-13). Opposite findings are seen after a mixed meal (400-600kcal) where in women with overweight/obesity showed an increase in post-prandial plasma LEAP2 after 60min (Andreoli et al. 2024a), but not women or men with normal weight nor in men with overweight/obesity (Andreoli et al. 2024a).

It seems there is no conclusive evidence of sex differences in circulating or regulation of fasting / post-prandial plasma LEAP2, however further work is required in larger populations controlling for BMI category and glucose to look for (any) differences.

1.5. EATING BEHAVIOUR

Humans have developed biological and behavioural adaptations to regulate energy balance, ensuring that food consumption aligns with the needs for health and growth (Hall et al. 2012). The term eating behaviour encompasses the biological and social factors influencing actions, attitudes and physiological responses related to food intake and preference (Grimm et al. 2011; DeCosta et al. 2017).

This behaviour is regulated by two interconnected central nervous systems (DiLeone et al. 2012) wherein the brainstem-hypothalamic circuits are responsible for control of appetite and the mesocorticolimbic reward system that is associated with pleasure, hedonic and motivation for food consumption (Cornejo et al. 2021a; Berthoud et al. 2017). The connectivity of this system is altered by gut hormones (Guerrero-Hreins et al. 2021) and controlled by communication between gut-brain axis, and subject to homeostatic modulation by peripheral hormonal signals such as insulin and ghrelin (Serrenho et al. 2019).

Maladaptive eating habits such as food addiction is correlated with obesity (Horsager et al. 2023) and shows sensitisation in the ventral striatum similar to other addictions (Mestre-Bach et al. 2023). Cognitive changes associated with obesity include attentional bias (Liu et

al. 2022), increased impulsivity (Han et al. 2022), and compulsivity (Kakoschke et al. 2018) that may affect executive function and decision making to promote eating behaviour to gain weight (Voon 2015).

Eating behaviour can be assessed through various methods, such as questionnaires on appetite, food preferences, and intake; providing test meals to evaluate food consumption; and conducting behavioural tasks to measure the hedonic value of food. However, these approaches have limitations, the main one being that research settings often fail to replicate real-life environments, typical food choices, or familiar portion sizes. As a result, the eating behaviours observed in short-term studies may not accurately reflect usual patterns (Emilien et al. 2017).

1.5.1. Mediators of Appetite

Appetite refers to the desire to eat, which is influenced by physiological hunger signals, such as gut hormones like AG (Kojima et al. 1999). Satiation describes the sensation of fullness that arises during a meal, while satiety refers to the feeling of fullness that delays the initiation of the next meal (Blundell et al. 1987). Satiety is regulated by various metabolites, including glucose, as well as hormones like leptin, cholecystokinin (CCK), GIP, GLP-1 and peptide YY (PYY) (de Graaf et al. 2004; Mela 2006; Bilman 2017; Polak 1973).

The corticolimbic system encompasses extensive cortical regions, the basal ganglia, hippocampus, and amygdala. This system integrates sensory information and contributes to the emotional, cognitive, and executive regulation of eating behaviour by influencing the hypothalamus and hindbrain. Additionally, nutritional states play a key role in shaping eating behaviour, primarily through the modulation of endocrine hormones following food intake. (Berthoud et al. 2017).

Both external and internal factors influence the development of satiation and satiety over time. At the beginning of a meal, perceived meal quality, including expectations, reward, and enjoyment, plays a significant role in shaping satiation. As food enters the stomach, gastric volume increases, causing the stomach to stretch and signal the brain while releasing gastrointestinal hormones. In the post-ingestive phase, nutrient absorption stimulates the release of satiety signals, which are detected by the hypothalamus (Bilman 2017).

The mechanisms for regulating energy intake are controlled by the interactions between the actions of the gastrointestinal system, pancreas, adipose tissue, and the autonomic nervous system in the brain. Various appetitive hormones released in the absence, deficit, presence or excess of energy, signal to the hypothalamus, brainstem, and midbrain, to either initiate, stimulate, or stop food intake and therefore regulate energy balance.

These regulating hormones are further sub-classified as orexigenic peptides, meaning that they influence increasing appetite, such as AG and anorexigenic peptides, which stimulate satiety, such as intestinal-derived glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (Muller et al. 2015; Coll et al. 2007) and the recently discovered GHSR inverse agonist / competitive agonist LEAP2 (M'Kadmi et al. 2019; Ge et al. 2018; Wang et al. 2019; Mani et al. 2019).

1.5.2. Acyl Ghrelin and Eating Behaviour

A role of endogenous GHSR-related hormones in stimulating human appetite and food intake, as opposed to meal preparation and initiation, remains debated. While exogenous acyl-ghrelin (AG) increases appetite ratings and food intake in individuals with and without obesity, this only occurs when plasma AG concentrations are markedly supraphysiological (Druce et al. 2005; Druce et al. 2006; Wren et al. 2001). In contrast, low-dose exogenous AG administration to achieve physiological or mildly supraphysiological plasma AG concentrations had no impact on appetite ratings or food intake in normal-weight, fed men (Lippl et al. 2012). Similarly, chronic administration of an oral GOAT inhibitor (GLWL-01) over 28 days, which significantly reduced plasma AG in adults with obesity and T2DM, did not affect body weight. However, this result is complicated by already low plasma AG levels in obesity (https://clinicaltrials.gov/ct2/show/results/NCT02377362). An oral GHSR inverse agonist, PF-5190457, reduced alcohol craving in adults with AUD without affecting glucose metabolism (Lee et al. 2020b; Lee et al. 2020a; Farokhnia et al. 2020) and when given to adults with AUD and overweight/obesity a reduction in virtual calories selected in food buffet task was noted (Faulkner et al. 2024).

A definitive role for endogenous ghrelin in driving appetite remains uncertain. Studies examining the relationship between postprandial plasma ghrelin levels and eating behaviour, such as appetite ratings and food intake, have produced inconsistent results. Variability may stem from small sample sizes, differences in study design, obesity status,

specificity of ghrelin immunoassays for AG, DAG or total ghrelin, use of protease inhibitors to prevent AG breakdown, reliance on absolute or relative changes in plasma ghrelin, and failure to adjust food intake for body size or resting energy expenditure (Monteleone et al. 2003; Moran et al. 2004; Doucet et al. 2004; Erdmann et al. 2004; Cummings et al. 2004; Blom et al. 2005; Stock et al. 2005; Bowen et al. 2006; Di Francesco et al. 2006; Mackelvie et al. 2007; Akhavan et al. 2007; Erdmann et al. 2007; Doucet et al. 2008; Frecka et al. 2008; Blom et al. 2009; Lioger et al. 2009; Serra-Prat et al. 2009; Unick et al. 2010; Massolt et al. 2010; Larson-Meyer et al. 2010; Cooper et al. 2011; Rosen et al. 2011; Kroemer et al. 2013; Monteleone et al. 2012; Martens et al. 2012; Bryant et al. 2013; Gibbons et al. 2013; Monteleone et al. 2013; St-Onge et al. 2014; Deighton et al. 2014; Goldstone et al. 2014; Andarini et al. 2017; Giezenaar et al. 2018b; Nymo et al. 2018; Costabile et al. 2018; Giezenaar et al. 2018a; Zhu et al. 2019; McVeay et al. 2019; Sinha et al. 2019; Japur et al. 2019; Duszka et al. 2020). Most studies do not show consistent positive correlations between postprandial ghrelin levels (absolute or changes) and appetite, hunger, fullness, or food intake.

1.5.3. LEAP2 and Eating Behaviour

There is a paucity of human studies looking at the interaction of satiety gut hormone LEAP2 and eating behaviours in humans. The results have been summarised in Table 1.11.

In a cross-sectional study of adults with normal weight and overweight/obesity (n=44), fasting plasma LEAP2 negatively correlated with hunger VAS ratings after adjusting for sex and BMI, but no such correlation was seen with post-prandial plasma LEAP2 (1 hours after a 400-600kcal meal) (Andreoli et al. 2024a). Similarly, there were a lack of positive correlations between fasting/post-prandial plasma LEAP2 with fullness VAS ratings (Andreoli et al. 2024a). The lack of results may be due to the meal size being insufficient to completely suppress hunger and hence unmask post-prandial increases in LEAP2. As these were only 1-hour post-prandial samples there may have been incomplete fullness.

In another study by the same authors, fasting plasma LEAP2 positively correlated with fullness VAS ratings in adults with normal weight but not in adults with overweight/obesity (Andreoli et al. 2024c), there was no interaction between fasting plasma LEAP2 and BMI categories for hunger VAS ratings (Andreoli et al. 2024c). The limitations of this study include using discrete VAS scales which are less accurate than continuous scales and not

using composite appetite scores. However, even after using composite appetite scores there was lack of a correlation with fasting plasma LEAP2 from two abstracts recently presented by the research group in adults across all weight categories (Goldstone et al. 2022) and in adults with obesity with/without T2DM (Guo 2024).

In the first-in human infusion study of full length LEAP2 peptide at a fixed dose over 290min in men without obesity, induced a ~ 12% decrease of food intake and a faster fullness sensation, without significant effects on hunger, satiety or fullness VAS scores (Hagemann et al. 2022).

This thesis aims to link the effects of circulating fasting/ post-prandial LEAP2 and exogenous LEAP2 infusions with measures of eating behaviour including VAS ratings, desired and actual food intake, food picture evaluation using a functional MRI paradigm and food liking and wanting.

Author	Number	Female (%)	Population type	BMI category	Disease / intervention	Nutritional state variable	Outcome variable	Nutritional state plasma LEAP2	Correlation with plasma LEAP2
Emini 2024 [3]	30	50.0	Adults	OW/OB	+ T2DM, LCD 0-24m	Fasting	Appetite	Fasting	o
Emini 2024 [3]	37	48.6	Adults	OW/OB	+ T2DM, DJBL 0-12m	Fasting	Appetite	Fasting	o
Goldstone 2022*	90	60.0	Adults	NW/OW/OB		Fasting	Appetite	Fasting	o
Guo 2024*	46	63.0	Adults	OB	± T2DM	Fasting	Appetite	Fasting	o
Emini 2024 [3]	17	64.7	Adults	OW/OB	+ T2DM, LCD 0-24m	PP (2h post 600kcal)	Appetite	PP (2h post 600kcal)	o
Emini 2024 [3]	23	39.1	Adults	OW/OB	+ T2DM, DJBL 0-12m	PP (2h post 600kcal)	Appetite	PP (2h post 600kcal)	o
Bhargava 2023 [2]	22	22.7	Adults	NW/OW	-	PP (2.5h post 730kcal)	Appetite	PP (2.5h post 730kcal)	-ve
Andreoli 2023	44	47.7	Adults	NW/OW/OB	-	Fasting	Hunger	Fasting	-ve
Andreoli 2023	44	47.7	Adults	NW/OW/OB	-	PP (1h post 400-600kcal)	Hunger	PP (1h post 400-600kcal)	o
Guo 2024*	46	63.0	Adults	OB	± T2DM	Fasting	Hunger	Fasting	o
Emini 2024 [3]	30	50.0	Adults	OW/OB	+ T2DM, LCD 0-24m	Fasting	Hunger	Fasting	o
Emini 2024 [4]	37	48.6	Adults	OW/OB	+ T2DM, DJBL 0-12m	Fasting	Hunger	Fasting	o
Andreoli 2024	327	39.1	Adults	NW/OW/OB	-	Fasting	Hunger	Fasting	o
Andreoli 2023	44	47.7	Adults	NW/OW/OB	-	Fasting	Fullness	Fasting	o
Andreoli 2023	44	47.7	Adults	NW/OW/OB	-	PP (1h post 400-600kcal)	Fullness	PP (1h post 400-600kcal)	o
Andreoli 2024	131	30.5	Adults	OW/OB	-	Fasting	Fullness	Fasting	o
Emini 2024 [3]	17	64.7	Adults	OW/OB	+ T2DM, LCD 0-24m	PP (2h post 600kcal)	Fullness	PP (2h post 600kcal)	o
Emini 2024 [3]	23	39.1	Adults	OW/OB	+ T2DM, DJBL 0-12m	PP (2h post 600kcal)	Fullness	PP (2h post 600kcal)	o
Bhargava 2023 [2]	22	22.7	Adults	NW/OW	-	PP (2.5h post 730kcal)	Fullness	PP (2.5h post 730kcal)	o
Andreoli 2024	196	44.9	Adults	NW	-	Fasting	Fullness	Fasting	+ve
Bhargava 2023	22	22.7	Adults	NW/OW	-	PP (2.5h post 730kcal)	Food appeal HE, LE, HE vs. LE	PP (2.5h post 730kcal)	o
Guo 2024*	46	63.0	Adults	OB	± T2DM	Fasting	Food appeal HE, LE, HE vs. LE	Fasting	o
Goldstone 2022*	90	60.0	Adults	NW/OW/OB		Fasting	Food cue reactivity HE, HE vs. LE [a]	Fasting	+ve
Bhargava 2023 [2]	22	22.7	Adults	NW/OW	-	PP (2.5h post 730kcal)	Food cue reactivity HE, LE, HE or LE [b]	PP (2.5h post 730kcal)	-ve
Guo 2024*	46	63.0	Adults	OB	± T2DM	Fasting	Food cue reactivity LE [c]	Fasting	+ve
Guo 2024*	46	63.0	Adults	OB	± T2DM	Fasting	Food cue reactivity HE vs. LE [c]	Fasting	-ve
Goldstone 2022*	90	60.0	Adults	NW/OW/OB		Fasting	Food intake	Fasting	-ve
Guo 2024*	46	63.0	Adults	OB	± T2DM	Fasting	Food intake	Fasting	-ve
Bhargava 2023 [2]	22	22.7	Adults	NW/OW	-	PP (2.5h post 730kcal)	Food intake	PP (2.5h post 730kcal)	-ve (trend)

Table 1.11. Correlations of eating behaviour with plasma LEAP2 in humans.

Abbreviations: Abbreviations: BMI, Body mass index; DJBL, Duodenal-jejunal bypass liner; h, Hours; HE, High energy; HOMA-IR, Homeostatic model assessment for insulin resistance; kcal, kilocalorie; LCD, Low calorie diet; LE, Low energy; m, Months; NW, Normal weight (BMI <24.9 kg/m^2); OW, Overweight (BMI 25.0-29.9 kg/m^2); OB, Obese (BMI <30.0 kg/m^2); PP, Post-prandial; T2DM, Type 2 Diabetes Mellitus; +ve, Positive correlation; -ve, Negative correlation; o; No correlation; *A bstract from our research group; [a] Posterior cingulate cortex; [b] Anterior/posterior cingulate cortex, paracingulate cortex, frontal pole, middle frontal gyrus; [c] Nucleus accumbens, caudate. All studies used Phoenix Pharmaceuticals Inc, CA, USA (EK-075-40) assay. Results in bold are from this thesis; [2], results from Chapter 2; [3], results from Chapter 3.

1.6. FUNCTIONAL MAGNETIC RESONANCE IMAGING

Functional magnetic resonance imaging (fMRI) has become a widely used tool in humans to explore how food-related tasks, such as viewing or evaluating food images or words (food cue reactivity), stimulate specific brain regions, particularly those associated with reward processing and decision-making (Smeets et al. 2019; Carnell et al. 2012).

During an fMRI brain scan, a magnetic field aligns the protons in water molecules within the brain along a longitudinal axis. An external radiofrequency (RF) pulse is then applied, disrupting this alignment by transferring energy in a process called "resonance." This disturbance generates a new transverse MRI signal as the protons rotate in synchrony, which can be recorded. When the RF pulse is turned off, the protons relax and realign longitudinally. During this relaxation, longitudinal magnetization gradually returns to its original state (characterized by the T1 time constant), while transverse magnetization simultaneously decreases to zero (characterized by the T2 time constant). Variations in the number of protons across different brain tissues result in differences in T1 and T2. By selecting appropriate TR (time to repeat) and TE (time to echo) values, repeated RF pulses (pulse sequences) can reveal differences in MRI signal intensity between brain tissues, producing T1- or T2-weighted images of the entire brain over time (Schild 1990).

Additionally, the fMRI blood oxygen level-dependent (BOLD) signal adds a dynamic component to these MRI images. The BOLD signal measures regional cerebral blood flow, which serves as an indirect indicator of neural activity and energy demand. Following a stimulus, such as viewing a food image, there is typically a delay of about 2 seconds before the onset of the hemodynamic response, representing the time required for blood to travel through the arteries to the capillaries and veins (Logothetis 2003). Increased neural activity after viewing an image, leads to increases in regional cerebral blood flow and decreases to the deoxy-haemoglobin (paramagnetic) to oxy-haemoglobin (diamagnetic) ratio. This results in less perturbance to magnetic field and higher BOLD signal.

This BOLD signal then reaches a peak between 6–12 seconds before returning to

baseline. This increase in BOLD signal generally reflects heightened cerebral blood flow that overcompensates the oxygen supply to the activated brain region (Logothetis 2003). Overall, the rise in the BOLD signal during fMRI tasks is reflects enhanced functionality of the brain region through neurovascular coupling.

Advantages of using fMRI is its ability to pair quantitative changes in BOLD signals during tasks with hormonal blood analyses, helping to establish connections within the gut-brain axis. fMRI neuroimaging serves as a non-invasive method for connecting the extensive body of animal research on obesity and addiction with studies conducted in humans. However, fMRI faces challenges in drug development due to difficulties in creating reproducible paradigms, susceptibility of BOLD signals to artifacts and drug-induced non-specific neurovascular changes, variability in analysis methods leading to inconsistent results, and challenges in linking BOLD signal changes to clinically relevant outcomes (Carmichael et al. 2018).

Nutritional state affects food cue reactivity, with food consumption reducing the appeal of food and the BOLD signal response to food images in various brain regions involved in reward processing, emotional regulation, and decision-making. These effects may depend on factors such as the type, salience, palatability, and energy density of the food cues, as well as an individual's BMI status (Fuhrer et al. 2008; Bruce et al. 2010; Goldstone et al. 2009; Siep et al. 2009; Frank et al. 2010; Goldstone et al. 2014; Cameron et al. 2014). Peripheral signals that mediate the impact of nutritional state on food cue reactivity likely involve circulating appetite-related hormones that influence the brain (Coll et al. 2007), such as a reduction in plasma AG levels following food intake, an increase in plasma AG during acute fasting (Muller et al. 2015), and reciprocal changes in plasma LEAP2.

1.7. GHRELIN SIGNALLING AND FOOD REWARD

Ghrelin plays a significant role in regulating food reward by influencing brain regions involved in reward and motivation, such as the amygdala, hippocampus, orbitofrontal cortex (OFC), and striatum (Goldstone et al. 2009; Goldstone et al. 2014; Scholtz et al. 2014; Carnell et al. 2012; Goldstone et al. 2016). Ghrelin enhances the appeal of high-energy foods, increases the hedonic value of food-related stimuli, and stimulation

of ghrelin receptors in the mouse brain modulates ingestive behaviour through its action on the GHSR (Zigman et al. 2006).

1.7.1. Exogenous Ghrelin and fMRI Studies

In human fMRI studies, exogenous intravenous ghrelin administration (1mcg/kg) in fed adults without obesity increased BOLD to high energy (HE) food pictures in the amygdala, hippocampus, pulvinar, orbitofrontal cortex (OFC), anterior and mid-dorsal insula, and substantia nigra brain regions (Malik et al. 2008). Similarly, in adults without obesity, subcutaneous AG administration (3.6 nmol/kg) when fed mimicked fasting to increase BOLD signal during evaluation of HE food pictures in the OFC, and to HE and LE food pictures in the hippocampus, but not nucleus accumbens, OFC, anterior insula and amygdala in functional ROI analysis, and increase appeal of HE food pictures (Goldstone et al. 2014). In the fed state, an intravenous bolus of ghrelin (0.3nmol/kg vs. saline) given to adults without obesity, reduced resting state BOLD signal in the medulla, midbrain, pons, cerebellum, insula, parahippocampal gyrus, post central gyrus and thalamus (ventral anterior nucleus) but with no reported changes in the occipital cortex (Jones et al. 2012).

Therefore, the effects of exogenous AG administration, mimics the effect of fasting to increase neural reward system activation during the evaluation of HE food pictures and their appeal ratings. This recent human research therefore now supports a role for LEAP2 to modulate feeding, with reciprocal changes in LEAP2 and AG, by increasing/decreasing the AG/LEAP2 ratio and signalling at GHSR under negative/positive energy balance. However, to date there have been no studies, to study the effect of exogenous LEAP2 on human eating behaviour using fMRI paradigms.

1.7.2. Correlations of Endogenous Plasma Ghrelin with Food Cue Reactivity

Numerous studies have investigated the relationship between fasting and/or post-prandial increments/reductions in endogenous plasma ghrelin and changes in food cue reactivity (such as to pictures or taste) or resting BOLD signals. However, the findings have been highly inconsistent, likely due to variations in study design, participant characteristics (including obesity status), sample size, methods for

measuring total ghrelin or AG, fMRI analysis techniques, and statistical thresholds.

In adults with normal weight, fasting DAG positively correlated with BOLD signal in occipital/temporal and fusiform gyrus, caudate, pallidum, midbrain, rolandic operculum, amygdala, thalamus and anterior cingulate gyrus and negative correlation of fasting DAG with fasting appetite (Kroemer et al. 2013).

1.7.2.1. Fasting plasma ghrelin and food cue reactivity

In adults without obesity, there was no correlation for AG with BOLD signal for HE, LE or HE vs. LE food pictures in the OFC or hippocampus in a ROI fMRI analysis (Goldstone et al. 2014). Similarly, in women with obesity, there was no correlation of total ghrelin with BOLD signal for food images in a whole brain fMRI analysis, however after a 4 week LCD there was a positive correlation of fasting total ghrelin with BOLD signal for food images in the parahippocampal gyrus, parietal lobe, cuneus, putamen and frontal gyrus (Jakobsdottir et al. 2016). Likewise, in adults across all BMI categories, total ghrelin positively correlated with BOLD signal to HE and LE food images in the inferior occipital gyrus, superior occipital gyrus and supramarginal gyrus (Wever et al. 2021).

In adults with normal weight and low obesity risk FTO gene variant (rs9939609), plasma AG in positively correlated with BOLD signal increase to HE vs. LE food pictures in the nucleus accumbens, hypothalamus, thalamus, OFC, parahippocampal and cingulate cortex, with opposite findings in carriers with high risk FTO gene variant (rs9939609) (Karra et al. 2013).

While, in adults with obesity who had RYGB and LAGB there was no correlation between fasting AG and BOLD signal when viewing HE vs LE foods in the reward system (Scholtz et al. 2014).

1.7.2.2. Post-prandial plasma ghrelin and food cue reactivity

In adults with normal weight, post-prandial decrease in plasma total ghrelin positively correlated with decrease in resting BOLD signal in the amygdala after protein intake, BOLD signal in the insula and OFC after glucose intake BOLD signal in the amygdala,

insula and OFC after fat intake (Li et al. 2012). In adults with and without obesity, post-prandial reduction in plasma total ghrelin positively correlated with decrease in BOLD signal to palatable milkshakes (vs. tasteless solution) in midbrain, amygdala, pallidum, hippocampus and insula in region of interest (ROI) analysis (Sun et al. 2014). In women with obesity, post-prandial total ghrelin positively correlated with BOLD signal to food pictures in the amygdala in whole brain analysis, as well as correlating with BOLD signal in the insula after a 4-week LCD (Jakobsdottir et al. 2016). In adults with obesity, post-VSG, there was a positive correlation with reduction in total ghrelin and BOLD signal in the dorsolateral prefrontal cortex to HE vs. LE food images (Li et al. 2019).

In adults with normal weight and low obesity risk FTO gene variant (rs9939609), the post-prandial decrease in plasma AG negatively correlated with the post-prandial BOLD signal to HE vs. LE food pictures in the fusiform gyrus, postcentral gyrus and cuneus, with opposite correlations in carriers with the high obesity risk FTO gene variant (Karra et al. 2013).

In adults with normal weight, post-prandial decrease in total ghrelin after an OGTT did not correlate in whole brain analysis with BOLD signal change to palatable food images (Kroemer et al. 2013), nor in participants with and without obesity after a liquid breakfast (Wever et al. 2021). Similarly, in adults without obesity, post-prandial decreases in plasma AG did not correlate with post-prandial decreases in BOLD signal during evaluation of HE, LE or HE vs. LE food pictures in the OFC or hippocampus in ROI analysis (Goldstone et al. 2014).

The clinical importance of BOLD signal changes in brain reward system pathways lies in their ability to provide insights into the neural mechanisms underlying behaviours and conditions related to feeding behaviour reward, motivation, and decision-making in individuals with obesity. These findings provide a neural basis for interventions targeting the GHSR pathway by using LEAP2.

1.7.3. Correlations of Endogenous Plasma LEAP2 with Food Cue Reactivity

To the best of our knowledge no other research group apart from ours has examined

the role of LEAP2 and eating behaviour using food cue reactivity in a fMRI paradigm. These findings are summarised in Table 1.11, though other than the results from Chapter 2 in this thesis, these have only been presented in abstract form to date (Goldstone et al. 2022; Guo 2024).

Our findings suggest that in adults with or without obesity BOLD signal in posterior cingulate cortex (PCC) to HE foods (but not LE food) and HE vs. LE foods positively correlated with fasting plasma LEAP2, and negatively with plasma AG/LEAP2 ratio, but not plasma AG alone (Goldstone et al. 2022). Higher PCC BOLD signal with higher LEAP2 and lower AG/LEAP2 ratio would be consistent with less deactivation of this part of the default mode network (DMN). The PCC deactivates during attention to tasks, suggesting reduced attention to HE food pictures when plasma LEAP2 is elevated (Leech et al. 2014).

Similarly, in adults with obesity with or without T2DM, fasting plasma LEAP2 negatively correlated with BOLD signal to HE vs. LE food cue reactivity averaged across all fROIs (amygdala, anterior OFC, anterior insula, nucleus accumbens, putamen, caudate), driven by a positive correlation with BOLD signal to LE food rather than a negative correlation with HE food (Guo 2024). Furthermore, in exploratory analysis of individual fROIs, similar findings were seen in nucleus accumbens with a negative correlation between fasting plasma LEAP2 and BOLD signal to HE vs. LE food cue reactivity driven by a positive correlation with BOLD signal to LE food rather than a negative correlation with HE food (Guo 2024).

These findings are consistent with a preclinical study, where central exogenous LEAP2 administration decreased dopamine release to highly palatable foods (e.g. peanut butter and Nutella) in nucleus accumbens (Tufvesson-Alm et al. 2024b). This implicates endogenous GHSR signalling in stimulating nucleus accumbens responses to HE food-related stimuli in rodents/humans.

This recent human research therefore now supports a role for LEAP2 to modulate feeding, with reciprocal changes in LEAP2 and AG, by increasing/decreasing the AG/LEAP2 ratio and signalling at GHSR under negative/positive energy balance.

1.7.4. Ghrelin and Food Memory

Peripheral endocrine signals, such as ghrelin, play a role in regulating food intake and memory through their action on the hippocampus (Kanoski et al. 2017). In rodent studies, ghrelin signalling within the hippocampus has been shown to counteract anorexigenic signals from peripheral hormones, including cholecystokinin, exendin-4 (a GLP-1 receptor agonist), amylin, as well as signals from gastric mechanical distension, thereby promoting increased food consumption (Suarez et al. 2020; Suarez et al. 2019).

Hyperghrelinemia by exogenous intravenous total ghrelin administration (1mcg/kg) (vs. saline) in fed humans without obesity (n=12) increased recall of HE food (vs. neutral) pictures shown 30 mins after fMRI scan (Malik et al. 2008), without any change in HE food picture hedonic ratings. However, there was no effect of ghrelin on cognitive testing including nonverbal fluid reasoning task, working memory task, creativity task, perceptual speed tests and tests for reaction times using non-food related stimuli (Kunath et al. 2016). Furthermore, fasting, increased memory recall of food (vs. non-food) pictures in humans with and without obesity (n=10) and increased cerebral blood flow in the OFC and amygdala and consumption of HE sweet and savoury food to satiation reduced memory for food recall (Morris et al. 2001).

Ghrelin signalling appears to be involved in recall for food cues in rodents, but gaps remain on the effect of nutritional state and ghrelin signalling on in humans. Therefore, increase in plasma LEAP2 or decrease in plasma AG/LEAP2 ratio after meal consumption would be hypothesised to reduce memory to HE food cues, especially over LE foods, and this may correlate with the degree of plasma LEAP2 elevation or AG/LEAP2 ratio suppression by different meal sizes.

1.8. HUMAN LEAP2 INFUSION STUDIES

1.8.1. Effect of Exogenous LEAP2 (38-47) Fragment

A 10 amino-acid LEAP2 (38-47) fragment was administered to n=10 healthy men without obesity with no adverse reactions reported, nor any effects on appetite ratings, fasting plasma glucose, serum insulin, plasma AG, resting energy expenditure (REE) or respiratory quotient (RQ) (Hagemann et al. 2021). Although in rodent studies, AG

reduces REE and physical activity, increases RQ indicating reduced fat oxidation (Tschop et al. 2000). This LEAP2 (38-47) fragment has only ~0.2-6.2% of the potency of LEAP2(38-77) at GHSR in *in vitro* studies men (Hagemann et al. 2021; M'Kadmi et al. 2019). Thus, this infusion rate would not have been expected to produce any clinically relevant responses.

1.8.2. Effect of Exogenous LEAP2 (38-77)

The first-in-human full length 40 amino acid LEAP2 (38-77) infusion was administered to men without obesity (Hagemann et al. 2022). Exogenous LEAP2 was initially administered intravenously to n=8 men without obesity to study its pharmacokinetics in the fasted state. It was given as a bolus infusion for 10 min (at 120 pmol/kg/min) followed by 30pmol/kg/min for 110 mins (total infusion time 2 hours).

Approximate half-life was 9 min, with an estimated volume of distribution of 14 litres and a metabolic clearance rate of 0.86 litres/min. Peak plasma LEAP2 concentrations achieved were 30-40 ng/mL measured using an in-house radioimmunoassay (not the commercial assays used in rodent/human studies). There were no side-effects encountered with median [IQR] nausea ratings of 3 [0, 9] (max 100mm VAS).

Furthermore, full-length exogenous LEAP2 was administered intravenously in n=20 men without obesity in a double-blind, randomised, crossover design as a 290 min infusion at ~25 pmol/kg/min or 115 ng/kg/min (total infusion time 4 hr 50min) (Hagemann et al. 2022). Participants ate a standardized meal overnight (753kCal, 45.6% carbohydrate, 38.3% fat, 16.1% protein) and arrived fasted till infusion commenced. Peak mean plasma LEAP2 concentrations achieved were 41.2 ng/mL. Participants also received a liquid meal of 575kcal liquid meal (49.3% carbohydrate, 34.8% fat, 15.9% protein) at 65min into the infusion. Thus, exogenous LEAP2(38-77) (vs. saline)

(i) reduced *ad libitum* food intake by 12% at *ad libitum* meal (after a pre-load) and reduced meal duration;
(ii) had no significant effect on VAS ratings of fasting or post-prandial hunger, fullness, satiety, nausea, comfort, thirst nor prospective food intake;
(iii) attenuated the post-prandial increase in plasma glucose (without

hypoglycaemia) but did not effect fasting glucose;

(iv) increased fasting and post-prandial serum/plasma insulin, C-peptide and glucagon, suggesting insulinotropic effects;

(v) lowered fasting and post-prandial serum glycerol suggesting decreased lipolysis; however there was no effect on serum triglycerides.

(vi) suppressed post-prandial but not fasting serum GH;

(vii) no effect on fasting or post-prandial plasma AG;

(viii) had no effect on REE nor RQ (by indirect calorimetry), urinary urea excretion (measure of protein turnover), supraclavicular brown adipose tissue thermogenesis (using thermal imaging) nor gastric emptying (using plasma paracetamol concentrations as a surrogate marker);

(ix) had no side effects reported and no effect on nausea (highest mean ~15mm at both saline and LEAP2 visits) or discomfort (lowest mean comfort ~70mm at both saline and LEAP2 visits) ratings (max 100mm VAS).

In another study by the same research group, recently presented as an abstract, (Englund et al. 2023) (Englund et al., Abstracts American Diabetes Association 2023,https://diabetesjournals.org/diabetes/article/72/Supplement_1/285-LB/149524/285-LB-LEAP2-Reduces-Preprandial-and-Postprandial), LEAP2 (38-77) was infused intravenously to n=20 healthy men with obesity (mean BMI 36.3 ± 0.8 kg/m^2) at ~40 pmol/kg/min over ~5 hours with a ~13% reduction in fasting/fed plasma glucose and *ad libitum* food intake without any serious gastrointestinal or cardiac adverse events.

Hence, exogenous LEAP2 infusion was safe and reduced *ad libitum* food intake and lowered post-prandial plasma glucose in men with or without obesity in addition to increased fasting serum insulin in men without obesity.

This thesis aims to administer LEAP2 to re-confirm its safety and tolerability, collect pilot data on efficacy, as well as giving different doses to examine a dose response effect on human metabolism and eating behaviour.

1.9. RATIONAL FOR CURRENT THESIS

Obesity is a chronic relapsing disease and the leading cause of morbidity and premature death including T2DM, cardiovascular disease, obstructive sleep apnoea, osteoarthritis and certain cancers associated with significant economic and societal burden (WHO 2024; Rubino et al. 2025). Treatment options are limited and the discovery of new targets and pharmacological therapies to provide novel interventions for obesity is needed. Appetitive gut hormones, such as AG, an important peripheral signal of weight loss and dietary energy restriction from the stomach to the brain, can regulate food intake and reward, and glucose metabolism. Increases in plasma AG after dietary-induced weight loss or cessation of weight loss medications likely contributes to rebound weight gain and contributes to the failure of weight loss maintenance. The recent discovery that LEAP2 is a gut peptide hormone and endogenous antagonist / inverse agonist of AG has revealed a previously unknown player in energy balance. Therefore, this thesis aims to better understand the role of LEAP2 in human eating behaviour and metabolism. This includes understanding factors coordinating the reciprocal regulation of LEAP2 with AG under changing energy balance, antagonising GHSR signalling to alter eating behaviour, metabolism and other hormones, and investigate the safety of exogenous administration of LEAP2 in humans as a first step to help the development of GHSR-targeted interventions to therapeutically treat obesity and T2DM.

1.10. THESIS HYPOTHESES AND AIMS

1.10.1. Hypotheses

In adults without obesity, post-prandial increases in plasma LEAP2, and/or post-prandial decreases in plasma AG or AG/LEAP2 ratio will:

(i) positively correlate with post-prandial decreases in appetite ratings, food cue reactivity (especially to HE foods), food intake, while

(ii) the post-prandial changes in these appetitive hormones would be positively correlated with post-prandial increases in plasma glucose, serum insulin and triglycerides, and

(iii) exogenous AG administration would lower plasma LEAP2 when fed and/or the post-prandial increase in plasma LEAP2 would be positively correlated with the post-prandial decrease in plasma AG.

In adults with obesity, long term weight loss through caloric restriction or DJBL insertion will:

(iv) decrease fasting and/or post-prandial plasma LEAP2,

(v) which will positively correlate with decreases in weight, improvements in glucose and/or fat metabolism, while

(vi) weight regain will increase fasting plasma LEAP2, and

(vii) DJBL insertion will exaggerate the decrease in fasting and/or post-prandial plasma LEAP2, due to loss of contact of ingested food with the small intestine.

In adults without obesity, acute infusion of LEAP2, to achieve supra-physiological plasma LEAP2 concentrations will:

(x) be safe and without nausea, vomiting, abdominal pain, cardiac rhythm abnormalities,

(xi) reduce appetite and, desired and actual food intake,

(xii) reduce plasma glucose via stimulation of insulin secretion and decreasing GH secretion, and

(xiii) reduce craving for alcohol,

(xiv) reduce food liking and wanting, preferred food portion size, and temporal impulsivity to food,

(xv) impair working and episodic memory and reduce motor response inhibition.

1.10.2. Aims

Plasma LEAP2 using a commercial EIA assay will be measured in secondary analysis of our previous studies comparing effects of:

(i) Exogenous and endogenous hyperghrelinaemia on eating behaviour, in adults without obesity, after an overnight fast or after consuming a meal without or with prior subcutaneous AG administration, with measurement of appetite ratings, food cue reactivity, food intake, plasma AG and glucose and serum insulin and triglycerides (Goldstone et al. 2014).

(ii) Weight loss induced by caloric restriction and increased physical activity, without or with DJBL insertion, with a follow-up after DJBL removal, in adults with obesity and T2DM with measurement of fasting and post-prandial plasma glucose, serum insulin and triglycerides (Glaysher et al. 2017; Ruban et al. 2020; Ruban

et al. 2022; Glaysher et al. 2021; Aldhwayan et al. 2022).

Using a single-blind, randomised cross-over design pilot study, to investigate the effects of acute administration of LEAP2 in adults without obesity on:

(iii) safety and tolerability of infusions and paradigms, appetite ratings, craving for food and alcohol, food liking and wanting, temporal impulsivity to food, money and alcohol, *ad libitum* food intake, plasma glucose, serum insulin, triglycerides, GH, cortisol, thyroid function tests, venous beta-hydroxybutyrate and renal/bone/liver biochemistry, and non-food related memory and motor response inhibition.

CHAPTER 2: POST-PRANDIAL EFFECT OF PLASMA LEAP2 ON HUMAN EATING BEHAVIOUR IN ADULTS WITHOUT OBESITY

2.1. ABSTRACT

Background: The novel liver-gut hormone LEAP2 is a centrally acting inverse agonist, and competitive antagonist of orexigenic acyl ghrelin (AG), at the growth hormone secretagogue receptor, reducing food intake in rodents. In humans, the effects of LEAP2 on eating behaviour and mechanisms behind the post-prandial increase in LEAP2 are unclear, though this is reciprocal to the post-prandial decrease in plasma AG.

Methods: Plasma LEAP2 was measured in a secondary analysis of a previous study. Twenty-two adults without obesity attended after an overnight fast (Fasted-saline), consuming a 730kcal meal without (Fed-saline) or with (Fed-ghrelin) subcutaneous AG administration. Post-prandial changes in plasma LEAP2 were correlated with post-prandial changes in appetite, high-energy (HE) or low-energy (LE) food cue reactivity using functional MRI, ad libitum food intake, and plasma/serum AG, glucose, insulin and triglycerides.

Results: Post-prandial plasma LEAP2 increased by 24.5-52.2% at 70-150 min, but was unchanged by exogenous AG administration. Post-prandial increases in LEAP2 correlated positively with post-prandial decreases in appetite, and cue reactivity to HE/LE and HE food in anterior/posterior cingulate cortex, paracingulate cortex, frontal pole, middle frontal gyrus, with similar trend for food intake. Post-prandial increases in LEAP2 correlated negatively with body mass index, but did not correlate positively with increases in glucose, insulin or triglycerides, nor decreases in AG.

Conclusions: These correlational findings are consistent with a role for post-prandial increases in plasma LEAP2 in suppressing human eating behaviour in adults without obesity. Post-prandial increases in plasma LEAP2 are unrelated to changes in plasma AG and the mediator(s) remain uncertain.

2.2. INTRODUCTION

Acyl ghrelin (AG) is a 28 amino acid orexigenic hormone principally secreted by the oxyntic cells of the fundus and body of the stomach that stimulates preparation for feeding and food intake via actions as an agonist at the growth hormone secretagogue receptor (GHSR) in a number of brain regions, including the hypothalamus, midbrain and hippocampus, as well as indirectly via the vagus nerve (Kojima et al. 1999; Muller et al. 2015). Ghrelin also exists in an inactive form des-acyl ghrelin (DAG) which is not a GHSR agonist, and is acylated to AG by the ghrelin-O-acyl transferase (GOAT) enzyme (Yang et al. 2008).

Liver enriched anti-microbial peptide-2 (LEAP2) is a recently discovered circulating anorexigenic hormone (Ge et al. 2018), originally isolated from human monocytes (Krause et al. 2003), that acts as a competitive antagonist of orexigenic acyl ghrelin (AG) (Wang et al. 2019) and inverse agonist (M'Kadmi et al. 2019; Ge et al. 2018) at the constitutively active GHSR. Mature LEAP2 is a 40 amino acid peptide (Krause et al. 2003; Henriques et al. 2010), with identical structure in rodents and humans. It is expressed principally in the liver and small intestine (Krause et al. 2003; Islam et al. 2020). In rodents, peripheral and/or central administration of LEAP2 blocks the orexigenic actions of peripheral and/or central administration of AG (Ge et al. 2018; Islam et al. 2020; Islam et al. 2022; Lugilde et al. 2022), and LEAP2 may reduce food intake when administered alone (Ge et al. 2018; Cornejo et al. 2019; Lugilde et al. 2022; Hagemann et al. 2022).

Study of *Ghrl*, *Ghsr* and *Goat* knockout mice and use of GHSR antagonists or inverse agonists in rodents, supports a direct role for endogenous AG and GHSR signalling to stimulate food intake and impulsivity in animals, especially under conditions of stress and after food restriction (Albarran-Zeckler et al. 2011; Dickson et al. 2011; Skibicka et al. 2011a; Tong et al. 2011; Chuang et al. 2011; Muller et al. 2015; Anderberg et al. 2016; Briggs et al. 2013). However, there is limited direct evidence for a role of endogenous plasma AG in human eating behaviour, including in the fed state, as increases in appetite and food intake are not seen after physiological, as opposed to pharmacological, exogenous AG administration (Druce et al. 2005; Druce et al. 2006; Wren et al. 2001; Lippl et al. 2012). Correlations of the post-prandial decreases in

endogenous plasma AG with post-prandial decreases in appetite and food intake are infrequently seen.
In the only human study to date, exogenous full-length LEAP2 infusion in men without obesity when fed to produce mildly supraphysiological plasma LEAP2 concentrations acutely reduced food intake but not appetite ratings, supporting a stimulatory role of GHSR signalling on human food intake (Hagemann et al. 2022).

In rodents and humans, both plasma AG and LEAP2 have a reciprocal relationship with nutritional status, with plasma AG decreasing, and in most studies to date plasma LEAP2 increasing, after food intake, with reciprocal responses in the acute fasted state (Liu et al. 2008; Ge et al. 2018; Mani et al. 2019; Islam et al. 2020; Hagemann et al. 2021; Hagemann et al. 2022). Plasma LEAP2 is increased, while plasma AG is decreased, in obesity (Mani et al. 2019; Ma et al. 2020; Shen et al. 2021; Holm et al. 2022),. Plasma AG increases with dietary-induced weight loss (Coutinho et al. 2018; Lyngstad et al. 2019), while LEAP2 falls after weight loss by both dietary restriction in rodents, and bariatric surgery in humans (Ge et al. 2018; Mani et al. 2019; Fernandez et al. 2022; Holm et al. 2022). Therefore, changes in the plasma AG/LEAP2 ratio by nutritional status may be an important driver to regulate appetite and food intake during energy homeostasis (Mani et al. 2019).

Post-prandial decreases in plasma ghrelin may be via increases in circulating insulin or changes in autonomic nervous system innervation of stomach (Shankar et al. 2021b; Muller et al. 2015; Zhao et al. 2010; Williams et al. 2003; Mohlig et al. 2002; Flanagan et al. 2003; Kamegai et al. 2004; Lippl et al. 2004; Cummings et al. 2001; Caixas et al. 2002; Maffeis et al. 2006; Karschin et al. 2015; Japur et al. 2019). However, any role for circulating insulin in increasing plasma LEAP2 is currently unknown.

Furthermore, it is incompletely understood how and from where plasma LEAP2 increases after food intake. In rodents, *Leap2* mRNA expression decreased with fasting and increased after refeeding only from the liver but not small intestine (Islam et al. 2020; Holm et al. 2022), though this has yet to be examined in humans. In mice without obesity and humans with obesity, plasma glucose positively correlated with

plasma LEAP2 across fasting and post-prandial time points suggesting that glucose might stimulate LEAP2 secretion after meals in humans (Mani et al. 2019). Post-prandial increases in plasma LEAP2 were also positively correlated with BMI across a combined group of adults with normal weight and obesity (Mani et al. 2019). Another possible mediator of the post-prandial increase in plasma LEAP2 might be the reciprocal post-prandial decrease in plasma AG, as well as circulating, direct intraluminal intestinal or indirect hepatic portal vein effects of glucose or other nutrients.

The sites of action of LEAP2 in the human brain remain to be determined but animal studies have implicated actions on hypothalamic orexigenic NPY and anorexigenic POMC neurons (Islam et al. 2020; Fernandez et al. 2022; Shankar et al. 2021a; Mani et al. 2019; Chu et al. 2022).

In humans, functional magnetic resonance imaging (fMRI) has been increasingly used to understand how food-related tasks, such as looking at or rating food pictures or words (food cue reactivity), activate specific areas of the brain, especially those involved in reward processing and decision making (Smeets et al. 2019; Carnell et al. 2012). Nutritional state influences food cue reactivity with food intake decreasing food appeal and BOLD signal to food pictures in several brain regions including those involved in reward processing, emotional responses and decision making, which may depend on the nature, salience, palatability and energy density of the food cues, as well as obesity status (Fuhrer et al. 2008; Bruce et al. 2010; Goldstone et al. 2009; Siep et al. 2009; Frank et al. 2010; Goldstone et al. 2014; Cameron et al. 2014).

The peripheral signals that mediate these effects of nutritional state on food cue reactivity likely include circulating appetitive hormones that act on the brain (Coll et al. 2007), such as the decrease in plasma AG with food intake, and increase in plasma AG with acute fasting (Muller et al. 2015), and potentially the reciprocal changes in plasma LEAP2.

However, results from fMRI studies investigating correlations of the post-prandial decrease in endogenous plasma ghrelin with food cue reactivity in brain regions

involved in reward, motivation and emotion have been inconsistent, likely related to often small samples sizes, differences in study design and obesity status, and specificity of ghrelin immunoassays to AG, DAG or both (Li et al. 2012; Karra et al. 2013; Kroemer et al. 2013; Wever et al. 2021; Goldstone et al. 2014; Sun et al. 2014 17414; Scholtz et al. 2014; Jakobsdottir et al. 2016; Roth et al. 2019; Li et al. 2019; Sewaybricker et al. 2020).

2.3. AIMS AND HYPOTHESIS

To our knowledge, to date there have been no published studies of the effects of exogenous LEAP2 administration on or correlations of plasma LEAP2 with food cue reactivity using fMRI or food hedonics. We therefore hypothesized that as AG stimulates appetite and food intake, cue reactivity and hedonics, the opposite would be seen for LEAP2, and so post-prandial increases in plasma LEAP2, and/or post-prandial decreases in plasma AG or AG/LEAP2 ratio would be: (i) positively correlated with post-prandial decreases in appetite ratings, (ii) positively correlated with food cue reactivity (especially to HE foods), and/or (iii) positively correlated with food intake, while (iv) the post-prandial changes in these appetitive hormones would be positively correlated with post-prandial increases in potential metabolic or hormonal mediators, and (v) if AG directly suppresses LEAP2 secretion, exogenous AG administration would lower plasma LEAP2 when fed and/or the post-prandial increase in plasma LEAP2 would be positively correlated with the post-prandial decrease in plasma AG.

Plasma LEAP2 was therefore measured in a secondary analysis of our previous study comparing effects of exogenous and endogenous hyperghrelinaemia on eating behaviour (Goldstone et al. 2014), in which adults without obesity had visits after an overnight fast, or after consuming a breakfast meal without or with prior subcutaneous AG administration, with measurement of appetite ratings, food cue reactivity using a food picture evaluation fMRI task measuring BOLD signal from whole brain analysis, food intake at an *ad libitum* lunch meal, and plasma glucose, serum insulin and triglycerides.

2.4. MATERIAL AND METHODS

Healthy adults without obesity of either sex aged 18-50 years participated in a randomized, placebo-controlled crossover design study with three visits at least six days apart: (i) after a 16 hour overnight fast (Fasted-saline), or after consuming mixed breakfast (at t= 0 min at ~10:00) 95 min before scanning (730kcal, 14% protein, 31% fat and 55% carbohydrate) and 40 min before receiving a subcutaneous (ii) saline injection (Fed-saline) or (iii) AG injection at 3.6nmol/kg (Fed-ghrelin) before scanning, followed by an *ad libitum* lunch meal 150 min after breakfast, as previously described (Goldstone et al. 2014) (Figure 2.1. (Bhargava et al. 2023)). Participants were in the MRI scanner between t= +75 and +135 min, with the food picture evaluation fMRI task between t= +95 and +115 min. Further details of the study design are available in the earlier publication (Goldstone et al. 2014). All women attended in the first 14 days of their menstrual cycle.

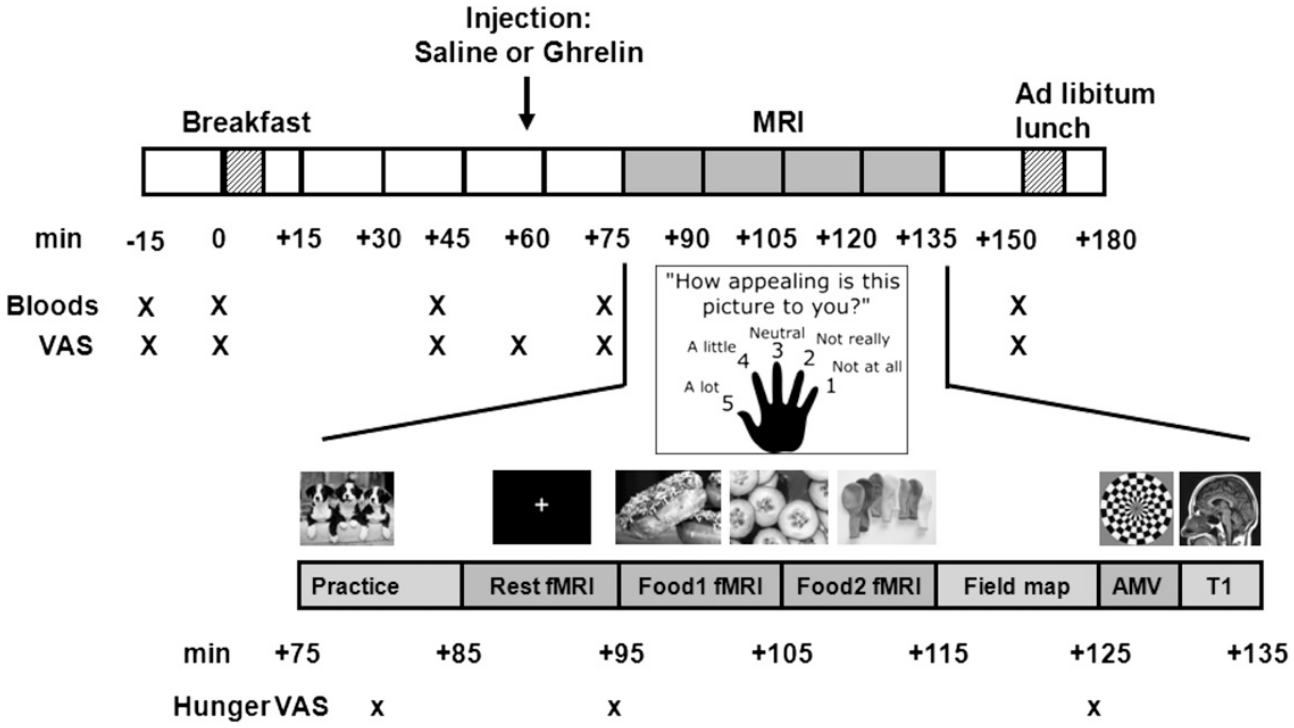


Figure 2.1. Study Protocol.
Breakfast consumption at t=+0min and saline or ghrelin injection at t=+55min. Blood and VAS measurements used for the analysis were at t=-15, 0, 40, 70 and 150 min, fMRI food evaluation task between t=+75 and +135min. *Ad libitum* lunch given at t=+150min. Adapted from Goldstone AP et al. Am J Clin Nutr. 2014; 99(6):1319-30.

The study was approved by the local Hammersmith and Queen Charlotte's & Chelsea

Research Ethics Committee (07/Q0406/19) and was performed in accordance with the principles of the Declaration of Helsinki. All participants provided written informed consent.

2.4.1. Blood Sampling and Hormone Assays

At each visit, blood samples were taken at t= -15, 0, +40, +70 and +150 min. Plasma glucose, and serum insulin and triglycerides were determined with routine laboratory techniques as reported previously (Goldstone et al. 2014). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula: Fasting insulin (mU/L) x fasting glucose (mmol/L)/22.5 (Matthews et al. 1985), averaging the results from the t= -15 and 0 min time points at Fasted-saline and Fed-saline visits. Plasma samples were stored at -80°C for future measurement of hormones. Plasma AG was measured at t= -15, 0, +40, +70 and +150 min from lithium heparin samples containing AEBSF protease inhibitor (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride) (A8456; Sigma-Aldrich) with acidification of plasma using HCl, with a previously validated enzyme linked immunoassay, University of Virginia (Liu et al. 2008; Prudom et al. 2010), as reported previously (Goldstone et al. 2014).

For this study, plasma LEAP2 concentrations were measured at time points t= -15, 0, +70 and +150 min using a commercial enzyme immunoassay (EK-075-40, Phoenix Pharmaceuticals Inc., CA, USA) according to the manufacturer's instructions using lithium heparin samples containing AEBSF protease inhibitor (without acidification of plasma). This LEAP2 assay has been previously validated using human samples (Mani et al. 2019). Plasma samples were diluted 10 times in the assay buffer. The inter-assay and intra-assay coefficients of variation were 12.5% and 8.2% respectively, both within the manufacturer guidance of <15% and <10%.

The plasma AG/LEAP2 molar ratio was calculated by dividing the results for each assay after converting concentrations to pmol/L. For plasma LEAP2, AG and AG/LEAP2 molar ratio, within-participant post-prandial changes were calculated by subtracting the value for the Fasted-saline from the Fed-saline visit. Due to the timing of individual outcome measures over the study visit, the timing of hormone values used for correlations with eating behaviour were as follows - appetite and food cue reactivity: average of t= +70 and +150 min time points (pre-MRI, and post-MRI/pre-lunch

respectively), food intake at *ad libitum* lunch: t= +150 min (post-MRI/pre-lunch). For correlations within appetitive hormones, and between appetitive hormones and plasma glucose, serum insulin and triglycerides, the difference in the incremental area under curve (iAUC) between 0 and 150 min ($iAUC_{0\text{-}150\ min}$) was calculated between Fed-saline and Fasted-saline visits.

2.4.2. Appetite Visual Analogue Scale Ratings

Serial visual analogue scale (VAS) ratings (0-10cm) were used to assess hunger, pleasantness to eat, prospective volume able to eat and fullness, as previously reported (Goldstone et al. 2014). For data reduction and to reduce issues with multiple comparisons, these VAS results were used to calculate a composite appetite VAS rating as: appetite = [hunger + pleasantness + volume + (10 - fullness)] / 4 for the two time-points (t= +70 and +150 min). Within-participant post-prandial changes of composite appetite and fullness VAS ratings were calculated using the average of t= +70 and +150min at each visit and then subtracting the value for Fasted-saline from the Fed-saline visit.

2.4.3. Food Picture Evaluation fMRI Task

As previously described, during the fMRI task, participants were requested to rate the picture appeal while simultaneously viewing pictures of high-energy (HE) foods, low-energy (LE) foods, household objects, and blurred pictures (Goldstone et al. 2014). The total fMRI task lasted 20 min using a block design in two runs. Within each run, pictures were presented in a randomised order with a pseudorandomised order of 5 blocks of high-energy-density foods, 5 blocks of low-energy-density foods and 5 blocks of objects, with 16 blocks of blurred images shown between the other blocks. Picture appeal was rated simultaneously using a keypad with five buttons. Ratings ranged from one to five, with 1 = not at all, 2 = not really, 3 = neutral, 4 = a little, and 5 = a lot on the scale of picture appeal (Goldstone et al. 2014). Picture appeal for HE food vs. objects and LE food vs objects was calculated as the difference between Fed-saline and Fasted-saline visits for this analysis.

2.4.4. MRI Scanning Protocol and fMRI Pre-Processing

This is given in detail in the original publication (Goldstone et al. 2014) and included

motion correction, field-map-based unwarping, spatial smoothing, high-pass temporal filtering, and inclusion of temporal derivative and motion variables as covariates (Goldstone et al. 2014). For this analysis, the scans were reprocessed to now include boundary-based registration (BBR) to improve spatial registration between the low-resolution echoplanar fMRI images and high-resolution T1 anatomical scans in subject space.

2.4.5. Whole Brain Analysis

FMRI data processing was carried out using FEAT (FMRI Expert Analysis Tool) Version 6.00, part of FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl). Whole-brain analysis was used to first combine the two runs of the fMRI task, and then to determine differences in BOLD signal between Fed-saline and Fasted-saline visits using a paired t-test with fixed effects analyses for each participant. This was done separately for HE food vs. object, LE food vs. object, HE/LE food vs. object, and HE vs. LE food contrasts.

For whole brain analysis, to examine correlations between the post-prandial changes in BOLD signal for each contrast with post-prandial changes in each plasma hormone (LEAP2, AG, AG/LEAP2 molar ratio), the demeaned hormone value for Fed-saline minus Fasted-saline visits for the average of t= +70 and +150 min time points were entered into the general linear model as an explanatory variable using FEAT, using a mixed effects analysis with threshold cluster-wise family-wise error (FWE) $Z>2.3$, $P<0.05$, to correct for multiple comparisons.

2.4.6. Energy Intake

During the Fasted-saline visit, participants did not receive breakfast at t= +0min. At the Fed-saline and Fed-ghrelin visits, a 730-kcal standardised fixed breakfast was given to the participants.

After the fMRI session at t= +150min, participants were served an excess amount of savoury lunch, consisting of macaroni cheese (per 100 g: 205 kcal, 6.5g protein, 19.9g carbohydrate, 11.5g fat), or (if the former was not liked having been tested at the screening visit, n=5) chicken tikka masala (per 100 g: 150 kcal, 6.6g protein, 13g

carbohydrate, 8g fat). Participants were told to consume the lunch until they felt adequately full. The total amount of food presented varied between sexes: men were given 2000 g and women 1500 g.

At each visit, participants had their body fat percentage determined by bioelectrical impedance analysis (BIA) (Bodystat 1500, Bodystat Ltd., UK). Estimated resting energy expenditure (REE) in kcal was calculated for each participant: (i) incorporating fat free mass (kg) from bio-impedance analysis (BIA) using the Cunningham equation: (22 x free fat mass) + 500 (equating lean body mass with fat-free mass) (Cunningham 1980); and (ii) incorporating weight (kg) adjusting for age and sex using the Schofield equation - males (ages 18-29 years): (15.057 x weight) + 692.2; males (ages 30-59 years): (11.472 x weight) + 873.1; females (ages 18-29 years): (14.818 x weight) + 486.6; females (ages 30-59 years): (8.126 x weight) +845.6 (Schofield 1985).

Total energy intake was expressed as total kcal consumed expressed as a percentage of 24-hour estimated REE using Cunningham equation as primary outcome. Correlations of plasma hormones with total energy intake also used REE-adjusted values from Schofield equation to avoid any potential confounds from BIA % body fat measurements, for example variability in hydration status (though participants had free access to water throughout the study visits). The original publication demonstrated no significant difference in body mass index (BMI) or % body fat between Fed-saline and Fasted-saline visits. Percentage body fat was also compared between these visits using intraclass correlation coefficients to further exclude any factors that may have influenced the calculation of fat free mass using BIA between visits.

Reported BMI and % body fat was calculated from measurements of height and weight and averaged from Fed-saline and Fasted-saline visits.

2.4.7. Statistical Analyses

Data is presented as mean ± SEM or median [interquartile range, IQR] where it was not normally distributed. Data was checked for normal distribution using the Shapiro-Wilk test.

Comparisons between visits (Fed-saline vs. Fasted-saline, or Fed-saline vs. Fed-ghrelin) using multiple time points for appetitive hormones was made using a two-way repeated measures ANOVA including visit and time as within-participant factors, with post-hoc Tukey's test. Between visit comparison for single measurements [$iAUC_{0\text{-}150min}$ for hormones, appetite/fullness VAS ratings, food intake (as % REE)] were made using paired Student's t-test for normally distributed data or Wilcoxon matched-pairs signed rank test where data were not normally distributed. Comparisons between visits (Fed-saline vs. Fasted-saline) for food appeal rating was made using a two-way repeated measures ANOVA including visit and energy density (HE food, LE food) as within-participant factors, with post-hoc Tukey's test. Effect sizes were reported from these ANOVA and t-test analyses.

Correlation analysis was performed to examine the relationships between outcome variables (without any covariates) reporting Pearson's (r_P) if data was normally distributed, or Spearman's (r_S) correlation coefficients if ether variable was not normally distributed. This was used to correlate relationships between plasma AG, LEAP2 and AG/LEAP2 molar ratio (dependent variable) and appetite/fullness VAS ratings, food intake (as % REE), plasma glucose, serum insulin and triglycerides concentrations, HOMA-IR and BMI values (independent variable), and also between plasma LEAP2 and AG. Statistical significance was set to $P<0.05$. Statistical analysis used IBM SPSS v27 (Armonk, NY, USA) and GraphPad Prism v9.5.0 (San Diego, CA, USA).

2.5. RESULTS

2.5.1. Participant Characteristics

As previously reported (Goldstone et al. 2014), twenty-two adults without obesity, predominately men, completed all 3 study visits, with time between visits median [IQR] 16 [7–35] days (Table 2.1). Intraclass correlation coefficient for % body fat using BIA between Fasted-saline and Fed-saline visits was 0.98, P<0.001.

	Mean ± SD	**Range**
N	22	
Age (y)	25.9 ± 8.0	19-44
Male, n (%)	17 (72.3%)	
Ethnicity white, n (%)	15 (68.1%)	
Height (m)	1.78 ± 0.09	1.61-1.93
Weight (kg)	76.0 ± 13.6	51.6-100.9
BMI (kg/m^2)	23.9 ± 2.8	19.1-29.9
Body fat (%)	16.1 ± 6.6	7.8-30.7

Table 2.1. Participant characteristics.
Abbreviations: BMI, body mass index.

2.5.2. Post-Prandial Increase in Plasma LEAP2

In repeated measures ANOVA, including visit (Fed-saline, Fasted-saline) and time (t=0, 70, 150 min) as within-participant factors, there was a significant time x visit interaction for plasma LEAP2 [F(3,63)=6.05, P=0.001]. Plasma LEAP2 increased after food intake at both t=70 and 150 min time points (Figure 2.2.A).

In post-hoc analysis, at t=70 min after food intake, plasma LEAP2 was mean ± SEM 24.5 ± 7.8% higher (P<0.001) at the Fed-saline (mean ± SEM 12.87 ± 0.91 ng/mL) than the Fasted-saline (10.90 ± 0.84 ng/mL) visit [effect size mean ± SEM 1.96 ± 0.63 ng/mL (95% CI 0.38, 3.55), P=0.014]. At t=150 min after food intake, plasma LEAP2

was 52.2 ± 13.8% higher (P=0.002) at the Fed-saline visit (15.15 ± 1.55 ng/mL) than the Fasted-saline visit (10.56 ± 0.80 ng/mL) [effect size 4.59 ± 1.31 ng/mL (95% CI 1.29, 7.90), P=0.006].

Plasma LEAP2 $iAUC_{0\text{-}150\ min}$ was significantly higher at the Fed-saline (119.37 ± 45.68 ng/mL.min) than Fasted-saline (-72.23 ± 46.65 ng/mL.min) visit [effect size 191.61 ± 77.13 ng/mL.min (95% CI 28.35, 340.10), t= 2.48, P=0.021, paired Student's t-test] (Figure 2.2.B).

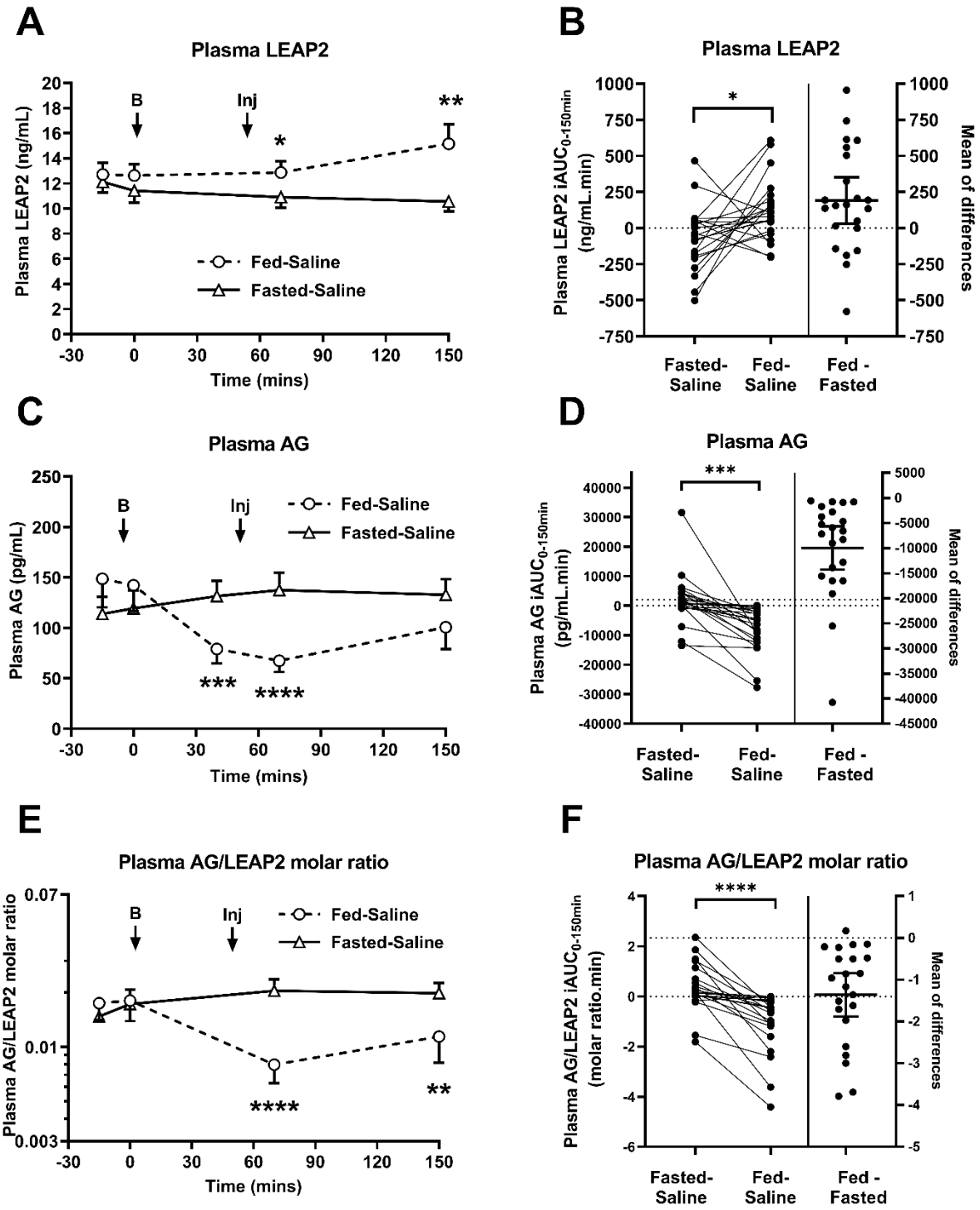


Figure 2.2. Plasma LEAP2, AG and AG/LEAP2 molar ratio concentrations at study visits. (A,C,E) Plasma hormone concentrations over study visits for (A) LEAP2, (C) AG and (E) AG/LEAP2 molar ratio at Fasted-saline and Fed-saline visits. Arrows indicate (B) time of breakfast (t=0min) and (Inj) subcutaneous saline/AG injection (t= +55min). Comparisons made using 2-way repeated measures ANOVA with post-hoc Tukey's test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 Fed-saline vs. Fasted-saline, n=22. (B,D,F) Comparison of incremental value of area under the curve (iAUC) from t=0 to +150 min for plasma (B) LEAP2,

(D) AG and (F) AG/LEAP2 molar ratio between Fed-saline and Fasted-saline visits with right-hand part of graph showing mean differences between visits (Δ fed-fasted). Comparisons made using (B) paired students t-test and (D,F) Wilcoxon matched-pairs signed rank test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, n=22. All data given as mean ± SEM. Abbreviations: AG, acyl ghrelin.

When comparing the absolute post-prandial increase in plasma LEAP2 across this study (Goldstone et al. 2014), and the four analyses from three previously published studies (Mani et al. 2019; Hagemann et al. 2021; Holm et al. 2022) (overall two without obesity, three with obesity), there was a suggestion that the post-prandial increase in plasma LEAP2 was greater with increasing energy intake at the meal, although with the small sample size this did not reach statistical significance (r_s= +0.70, P=0.23) (Figure 2.3. (Bhargava et al. 2023)).

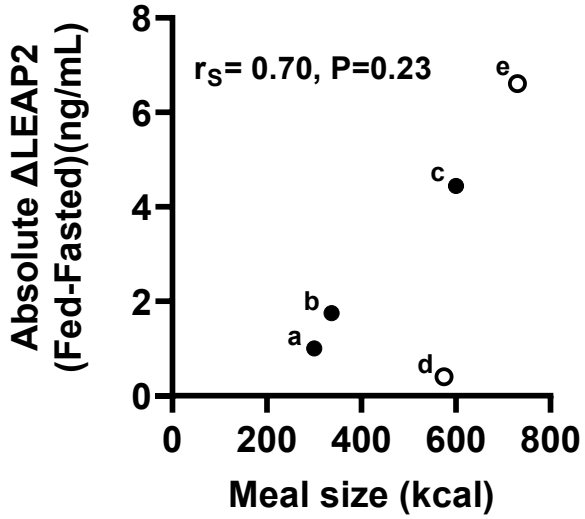


Figure 2.3. Correlation of post-prandial plasma LEAP2 with meal size consumed across different studies.
Correlation of post-prandial change in absolute plasma LEAP2 concentrations between fed and fasted time points (Δ Fed-Fasted) and meal size (kcal) consumed across different studies made using Spearman's (r_S) correlation coefficient. Black dots indicate participants with obesity (BMI >30kg/m^2) and open dots participants without obesity (BMI <30kg/m^2). Meals are liquid nutritional supplements with similar macronutrient composition: carbohydrates 49-55%, fat 30-35% and protein 14-18%. Table at bottom indicates details of meal and time of post-prandial blood sample for each study indicated by letters a-e: [a] Hagemann CA et al. J Clin Endocrinol Metab. 2021;106(2):e966-e981; [b,c] Mani BK et al. J Clin Invest. 2019;129(9):3909-3923; [d] Hagemann CA et al. Cell Rep Med. 2022;3(4):100582. Abbreviations: BMI, body mass index; CHO carbohydrate; kcal, kilocalorie.

Study	Study	N	kcal	Macronutrient composition of meal	Post-prandial sampling (mins)
a	Hagemann et al. 2021	18	300	50% CHO, 35% fat, 15% protein	120
b	Mani et al. 2019	20	337	52% CHO, 30% fat, 18% protein	90
c	Mani et al. 2019	20	600	49% CHO, 35% fat, 16% protein	90
d	Hagemann et al. 2022	20	575	49% CHO, 35% fat, 16% protein	120
e	Current study	22	730	55% CHO, 31% fat, 14% protein	150

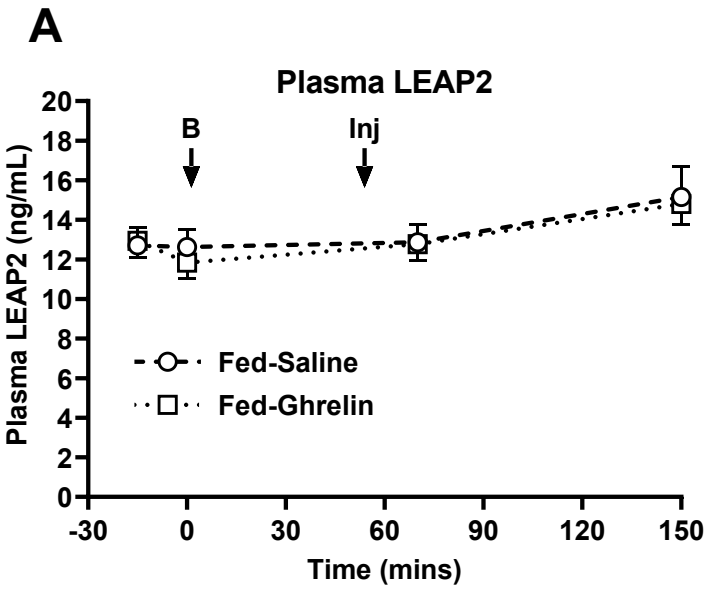
2.5.3. Post-Prandial Increase in LEAP2 is Unaffected by Exogenous AG Administration

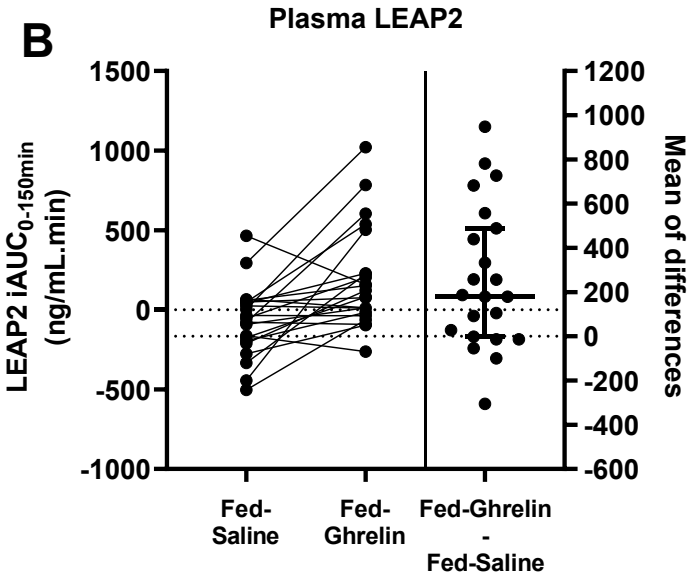
In repeated measures ANOVA, including visit (Fed-saline, Fed-ghrelin) and time (t=0, 70, 150 min) as within-participant factors, there was no significant time x visit interaction [F(3,63)=0.323, P=0.81] on plasma LEAP2 (Figure 2.4.A (Bhargava et al. 2023)). There was also no significant overall effect of visit (independent of time) [F(1,21)=0.09, P=0.77], but there was an overall significant effect of time (independent of visit) [F(3,63)=11.54, P=<0.0001].

Plasma LEAP2 $iAUC_{0\text{-}150\ min}$ was similar at the Fed-saline (median 92.38 ng/mL.min [IQR -23.2,196.8]) and Fed-ghrelin (median 101.2 ng/mL.min [IQR -21.3,297.9]) visit (W-statistic 55, P=0.38, Wilcoxon matched-pairs signed rank test) (Figure 2.4.B (Bhargava et al. 2023)).

At t=70 min, plasma LEAP2 was significantly higher by 25.7 ± 7.6% (P<0.001) when averaged across both Fed visits (12.82 ± 0.75 ng/mL) compared to the Fasted-saline visit (10.90 ± 0.84 ng/mL) [effect size 1.92 ± 0.63 ng/mL (95% CI 0.61, 3.22), t=3.05, P=0.006, paired Student's t-test]. At t=150 min, plasma LEAP2 was higher by 51.6 ± 11.4% (P<0.001) when averaged across both Fed visits (14.98 ± 1.10 ng/mL) compared to the Fasted-saline visit (10.56 ± 0.80 ng/mL) [effect size 4.42 ± 0.88 ng/mL (95% CI 2.58, 6.26), t=5.00, P<0.0001, paired Student's t-test].

Averaged across both Fed visits, plasma LEAP2 was significantly higher by 22.5 ± 4.8% ($P<0.001$) at t=150 min (14.98 ± 1.10 ng/mL), than at t=0 min (12.23 ± 0.76 ng/mL) [effect size 2.75 ± 0.50 (95% CI 1.42, 4.08), t=4.76, $P<0.0001$, paired Student's t-test]. However, plasma LEAP2 at t=70 min (12.82 ± 0.75 ng/mL) was not significantly different (higher by 5.68 ± 3.17%, $P=0.15$) from t=0 min [effect size 0.60 ± 0.50 (95% CI -0.73, 1.92), t=1.74, $P=0.64$, paired Student's t-test].


A
Plasma LEAP2
B
Inj
Plasma LEAP2 (ng/mL)
20
18
16
14
12
10
8
6
4
2
0
-30
0
30
60
90
120
150
Time (mins)
Fed-Saline
Fed-Ghrelin


B
Plasma LEAP2
LEAP2 iAUC0-150min (ng/mL.min)
1500
1000
500
0
-500
-1000
1200
1000
800
600
400
200
0
-200
-400
-600
Mean of differences
Fed-Saline
Fed-Ghrelin
Fed-Ghrelin - Fed-Saline

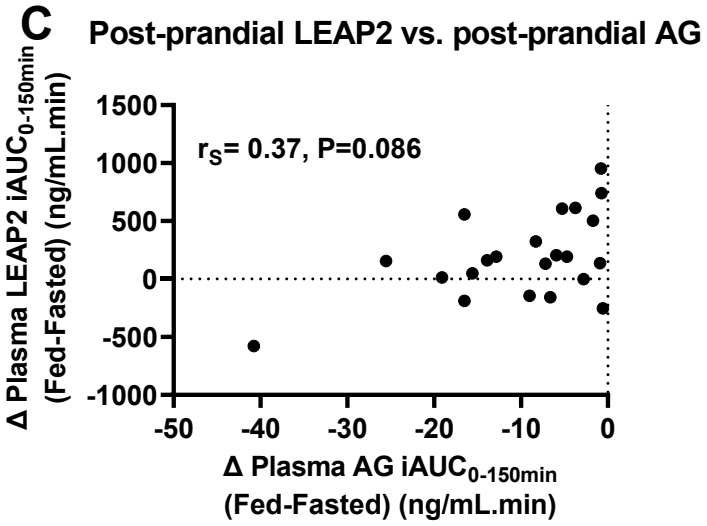

C
Post-prandial LEAP2 vs. post-prandial AG
Δ Plasma LEAP2 iAUC0-150min (Fed-Fasted) (ng/mL.min)
rS= 0.37, P=0.086
1500
1000
500
0
-500
-1000
-50
-40
-30
-20
-10
0
Δ Plasma AG iAUC0-150min (Fed-Fasted) (ng/mL.min)

Figure 2.4. Plasma LEAP2 at study visits.
(A) Plasma LEAP2 concentration over study visits at Fed-Ghrelin and Fed-Saline visits. Arrows indicate (B) time of breakfast (t=0min) and (Inj) subcutaneous saline/AG injection (t= +55min). Comparisons made using 2-way repeated measures ANOVA with post-hoc Tukey's test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 Fed-Ghrelin vs. Fed-Saline, n=22. (B) Comparison of incremental value of area under the curve (iAUC) from t=0 to +150 min for plasma LEAP2 between Fed-ghrelin and Fed-saline visits with right-hand part of graph showing mean differences between visits (Δ Fed-ghrelin minus Fed-saline). Comparisons made using Wilcoxon matched-pairs signed rank test: P>0.05, n=22. (C) Correlation of change in incremental value of area under the curve (iAUC) from t=0 to +150 min between Fed-saline and Fasted-saline visits (Δ Fed-Fasted) for plasma LEAP2 with change in incremental value of area under the curve (iAUC) from t=0 to +150 min between Fed-saline and Fasted-saline visits (Δ Fed-Fasted) for plasma AG using Spearman's (r_S) correlation coefficient. Abbreviations: AG, acyl ghrelin.

2.5.4. Post-Prandial Decrease in Plasma AG

As previously reported (Goldstone et al. 2014), in repeated measures ANOVA, including visit (Fed-saline, Fasted-saline) and time (t=0, 40, 70, 150 min) as within-participant factors, there was a significant time x visit interaction on plasma AG [F(4,84)=13.17, P<0.0001].

In post-hoc analysis, plasma AG decreased after food intake at both t=70 and 150 min time points (Figure 1C). At t=70 min, plasma AG was significantly lower by 47.3 ± 4.3% (P<0.001) at the Fed-saline (67.36 ± 11.03 pg/mL) than the Fasted-saline (137.38 ± 17.44 pg/mL) visit [effect size -70.02 ± 12.73 pg/mL (95% CI -94.97, -45.06), t=-5.5, P<0.0001]. However, at t=150 min, plasma AG was lower by 23.4 ± 8.9% (P=0.08) at the Fed-saline (100.63 ± 21.70 pg/mL) than the Fasted-saline (132.76 ±15.51 pg/mL) visit [effect size -32.14 ± 18.06 pg/mL (95% CI -69.69,5.42), t=-2.54, P=0.089].

Plasma AG $iAUC_{0\text{-}150\ min}$ was significantly lower at the Fed-saline (median -5713 pg/mL.min, IQR[-12194,-2125]) than Fasted-saline (median 1391 pg/mL.min, IQR[-524,4254]) visit (W-statistic -253, P<0.0001, Wilcoxon matched-pairs signed rank test) (Figure 2.2.D).

2.5.5. Post-Prandial Decrease in Plasma AG/LEAP2 Molar Ratio

In repeated measures ANOVA, including visit (Fed-saline, Fasted-saline) and time (t=0, 70, 150 min) as within-participant factors, there was a significant time x visit interaction on plasma AG/LEAP2 molar ratio [F(2,42)=15.56, P<0.0001].

In post-hoc analysis, plasma AG/LEAP2 molar ratio decreased after food intake at both t=70 and 150 min time points (Figure 1E). At t=70 min, plasma AG/LEAP2 molar ratio was 53.7 ± 5.2% lower (P<0.001) at the Fed-saline (0.0079 ± 0.0016) than the Fasted-saline (0.0205 ± 0.0032) visit [effect size -0.0125 ± 0.0017 (95% CI -0.0168, -0.0082), P<0.0001]. At t=150 min, plasma AG/LEAP2 molar ratio was 43.9 ± 7.2% lower (P<0.001) at the Fed-saline (0.0114 ± 0.0032) than the Fasted-saline (0.0198 ± 0.0028) visit [effect size -0.0084 ± 0.0017 (95% CI -0.0127, -0.0042), P<0.0001].

Plasma AG/LEAP2 molar ratio $iAUC_{0\text{-}150\ min}$ was significantly lower at the Fed-saline (median -0.51 molar ratio.min, [IQR -1.29, -0.20]) than Fasted-saline (median 0.17 molar ratio.min, IQR [0.04,.081]) visit (W-statistic -247, P<0.0001, Wilcoxon matched-pairs signed rank test) (Figure 2.2.F).

2.5.6. Correlations Between Change in Post-Prandial Plasma LEAP2 and AG Concentrations

The post-prandial increase in plasma LEAP2 $iAUC_{0\text{-}150\ min}$ in fact, showed a trend to negatively correlate with the post-prandial decrease in plasma AG $iAUC_{0\text{-}150\ min}$ (r_s= -0.37, P=0.086) (Figure 2.4.C (Bhargava et al. 2023)).

2.5.7. Post-Prandial Decreases in Appetite and Increases in Fullness

There was a significant decrease in the average t=+70/150 min composite appetite VAS rating at Fed-saline (4.65 ± 0.22) vs. Fasted-saline (7.87 ± 0.30) visit [effect size mean ± SEM -3.22 ± 0.34 (95% CI -2.51, -3.92), t=9.46, P<0.0001, paired Student's t-test] (Figure 2.5.A).

As reported previously (Goldstone et al. 2014), there was a significant increase in the average t=+70/150min fullness VAS rating at Fed-saline (3.64 ± 0.34) vs. Fasted-saline (0.78 ± 0.17) visit [effect size 2.86 ± 0.33 (95% CI 2.16,3.55), t=8.55, P<0.0001, paired Student's t-test] (Figure 2.5.B).

2.5.7.1 Correlations with plasma LEAP2

The post-prandial increase in plasma LEAP2 (∆ fed-fasted average t= +70/150min)

positively correlated with the post-prandial decrease in appetite VAS rating (Δ fasted-fed average t= +70/150min) (r_s= +0.47, P=0.026) (Figure 2.5.C).

However, the post-prandial increase in plasma LEAP2 (Δ fed-fasted average t= +70/150min) did not correlate with the post-prandial increase in fullness VAS rating (Δ fed-fasted average t= +70/150min) (r_s= +0.09, P=0.69) (Figure 2.5.D).

2.5.7.2. Correlations with plasma AG

The post-prandial decrease in plasma AG (Δ fed-fasted average t=+70/150min) did not correlate significantly with the post-prandial decrease in appetite VAS rating (Δ fasted-fed average t= +70/150min) (r_p= +0.16, P=0.47) (Figure 2.5.E).

Similarly, the post-prandial decrease in plasma AG (Δ fed-fasted average t=+70/150min) did not correlate significantly with the post-prandial increase in fullness VAS rating (Δ fed-fasted average t=+70/150min) (r_p= -0.30, P=0.18) (Figure 2.5.F).

2.5.7.3. Correlations with plasma AG/LEAP2 molar ratio

The post-prandial decrease in plasma AG/LEAP2 molar ratio (Δ fed-fasted average t= +70/150min) did not correlate significantly with the post-prandial decreases in appetite VAS rating (Δ fasted-fed average t=+70/150min) (r_p= +0.18, P=0.41) (Figure 2.5.G).

The post-prandial decrease in plasma AG/LEAP2 molar ratio (Δ fed-fasted average t= +70/150min) showed a trend towards a negative correlation with post-prandial increase in fullness VAS rating (Δ fed-fasted average t=+70/150min) (r_p= -0.38, P=0.082) (Figure 2.5.H).

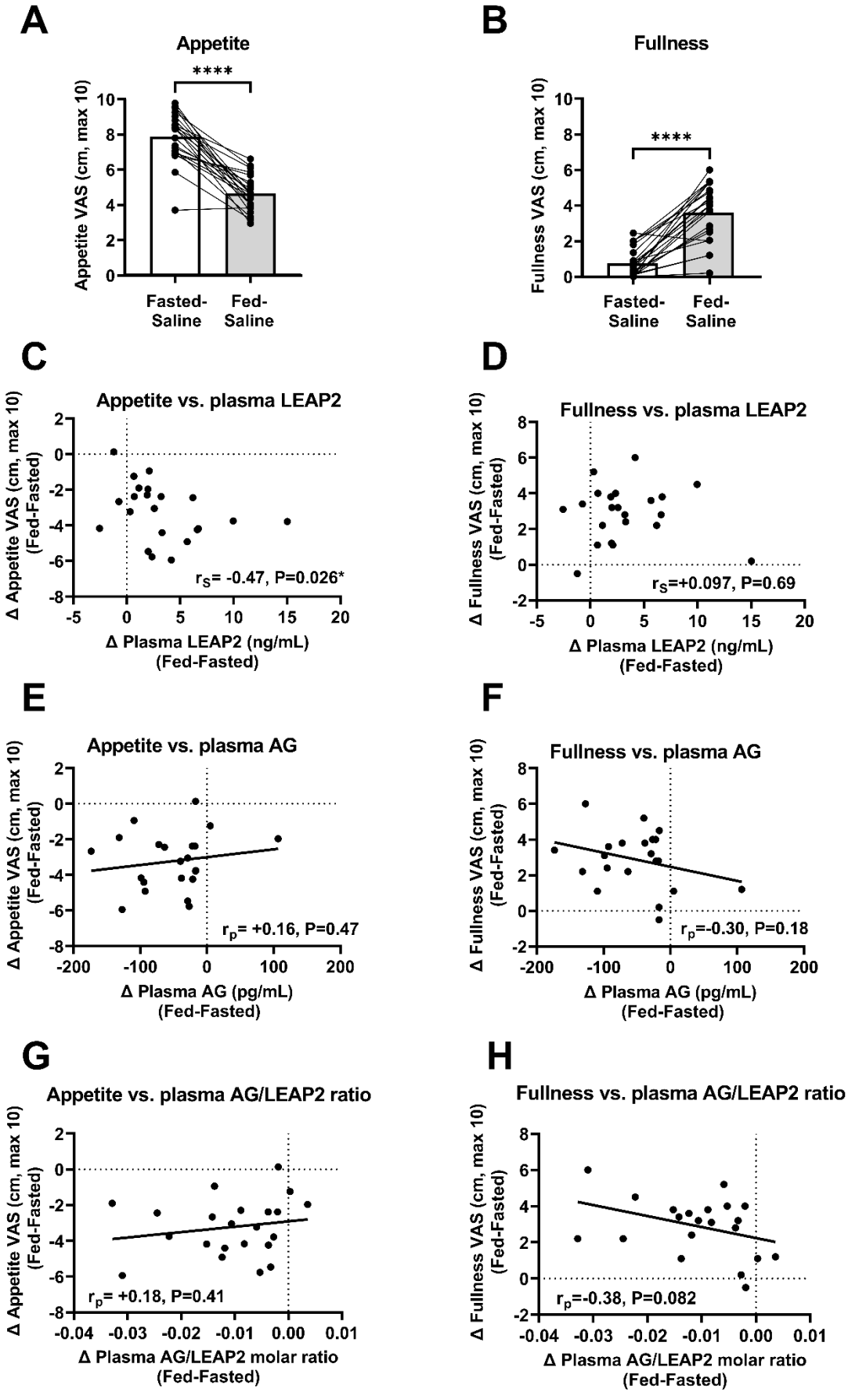

A
Appetite
****
Appetite VAS (cm, max 10)
Fasted-Saline
Fed-Saline
B
Fullness
****
Fullness VAS (cm, max 10)
Fasted-Saline
Fed-Saline
C
Appetite vs. plasma LEAP2
Δ Appetite VAS (cm, max 10) (Fed-Fasted)
rS= -0.47, P=0.026*
Δ Plasma LEAP2 (ng/mL) (Fed-Fasted)
D
Fullness vs. plasma LEAP2
Δ Fullness VAS (cm, max 10) (Fed-Fasted)
rS=+0.097, P=0.69
Δ Plasma LEAP2 (ng/mL) (Fed-Fasted)
E
Appetite vs. plasma AG
Δ Appetite VAS (cm, max 10) (Fed-Fasted)
rp= +0.16, P=0.47
Δ Plasma AG (pg/mL) (Fed-Fasted)
F
Fullness vs. plasma AG
Δ Fullness VAS (cm, max 10) (Fed-Fasted)
rp=-0.30, P=0.18
Δ Plasma AG (pg/mL) (Fed-Fasted)
G
Appetite vs. plasma AG/LEAP2 ratio
Δ Appetite VAS (cm, max 10) (Fed-Fasted)
rp= +0.18, P=0.41
Δ Plasma AG/LEAP2 molar ratio (Fed-Fasted)
H
Fullness vs. plasma AG/LEAP2 ratio
Δ Fullness VAS (cm, max 10) (Fed-Fasted)
rp=-0.38, P=0.082
Δ Plasma AG/LEAP2 molar ratio (Fed-Fasted)

Figure 2.5. Appetite and fullness ratings at fed and fasted visits and correlations of post-prandial plasma LEAP2, AG and AG/LEAP2 molar ratio with appetite and fullness ratings.
Visual analogue scale (VAS) ratings of (A) appetite and (B) fullness averaged at t=+70 and +150min between Fed-saline (730kcal breakfast) and Fasted-saline (overnight fast) visits with comparisons made using Paired students t-test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, n=22. (C-H) Correlation between (C,E,G) appetite and (D,F,H) fullness VAS ratings and changes in incremental value of area under the curve (iAUC) from t= 0 to +150 min between Fed-saline and Fasted-saline visits (Δ fed-fasted) for plasma (C,D) LEAP2, (E,F) AG, and (G,H) AG/LEAP2 molar ratio using using Pearson's (r_P, linear regression line) or Spearman's (r_S) correlation coefficients, n=22. Abbreviations: AG, acyl ghrelin.

2.5.8. Food Cue Reactivity

2.5.8.1. Correlations with plasma LEAP2

High-energy food pictures

In whole brain analysis, there was a significant negative correlation between post-prandial changes in BOLD signal to HE food (vs. object) pictures with post-prandial increases in plasma LEAP2 in the superior frontal gyrus, paracingulate gyrus, anterior division cingulate gyrus, posterior division cingulate gyrus, precuneus cortex, juxta positional lobule cortex, inferior frontal gyrus pars triangularis, inferior frontal gyrus pars opercularis, frontal pole, middle frontal gyrus, superior division lateral occipital cortex, superior occipital lobule, angular gyrus and superior parietal lobule (Table 2.2. (Bhargava et al. 2023), Figure 2.6.A).

Low-energy food pictures

In whole brain analysis, there was a significant negative correlation between post-prandial changes in BOLD signal to LE food (vs. object) pictures with post-prandial decreases in plasma LEAP2 in the postcentral gyrus, precentral gyrus, and superior parietal lobule (Table 2.3. (Bhargava et al. 2023), Figure 2.6.B).

High-energy or low-energy food pictures

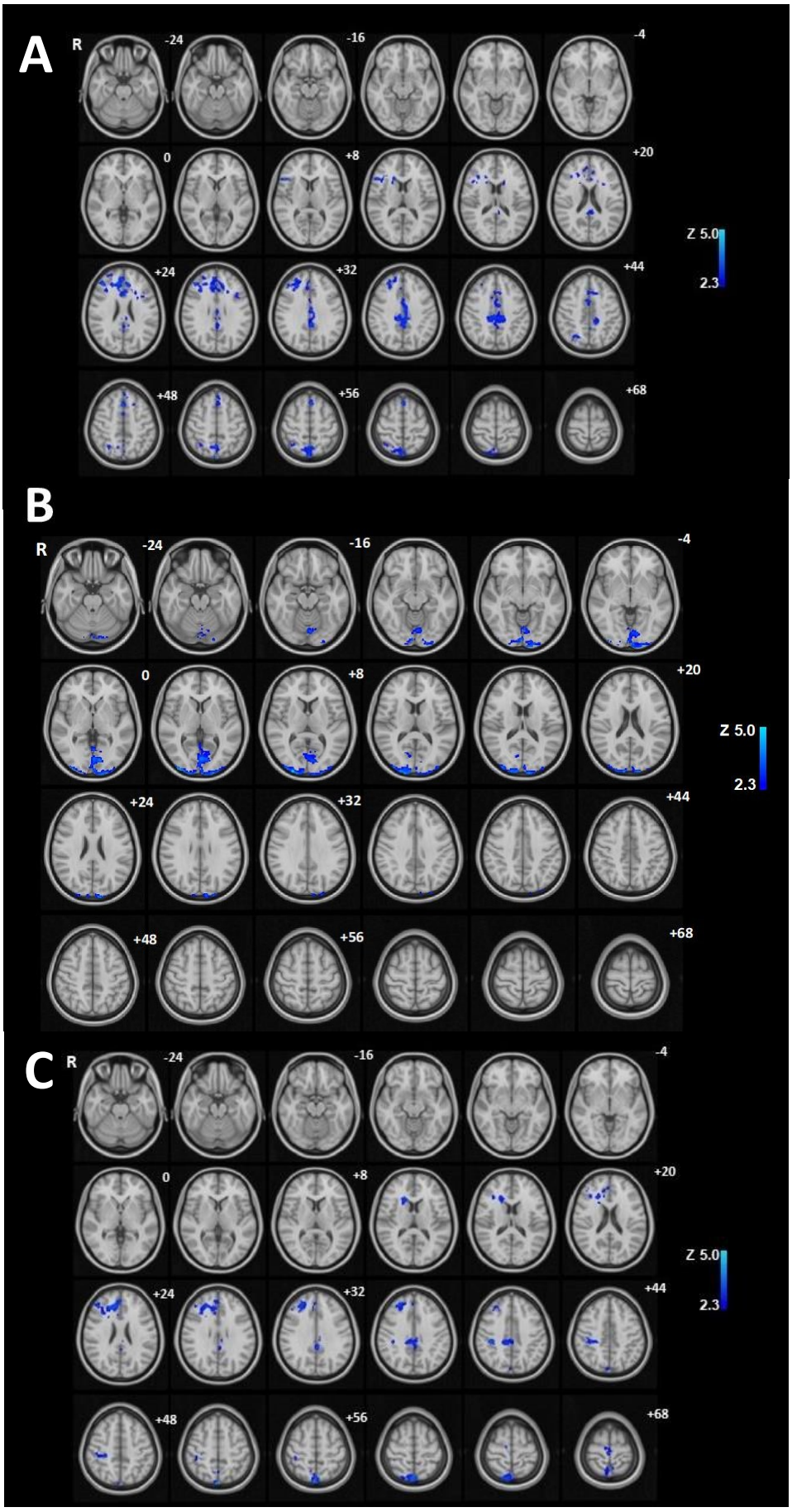
In whole brain analysis, there was a significant negative correlation between post-prandial changes in BOLD signal to HE/LE food (vs. object) pictures with post-prandial decreases in plasma LEAP2 in the paracingulate gyrus, anterior division cingulate gyrus, middle frontal gyrus, frontal pole, precuneus cortex, superior division lateral occipital cortex, postcentral gyrus, precentral gyrus, anterior division supramarginal

gyrus and posterior division cingulate gyrus (Table 2.4. (Bhargava et al. 2023), Figure 2.7.C).

High-energy vs. low-energy food pictures

In whole brain analysis, there were no significant correlations between post-prandial changes in BOLD signal to HE vs. LE food pictures with post-prandial decreases in plasma LEAP2.

Figure 2.6. Whole-brain analysis of correlation between post-prandial changes in plasma LEAP2 and BOLD signal during food picture evaluation fMRI task.
Brain regions (blue) showing positive correlations between post-prandial increases in plasma LEAP2 (Δ fed-fasted, average t=+70/+150min) and post-prandial decreases (Δ fasted-fed) in BOLD signal during evaluation of pictures of (A) high-energy food vs. objects (B) low-energy foods (vs. objects), and (C) high-energy or low-energy foods (vs. objects) in whole brain analysis (n=22, cluster-wise family wise error (FWE) Z>2.3, P<0.05). Colour bar indicates Z statistic, z coordinates in Montreal Neurological Institute (MNI) space. Abbreviations: R, right.


A
R
-24
-16
-4
0
+8
+20
+24
+32
+44
+48
+56
+68
Z 5.0
2.3
B
R
-24
-16
-4
0
+8
+20
+24
+32
+44
+48
+56
+68
Z 5.0
2.3
C
R
-24
-16
-4
0
+8
+20
+24
+32
+44
+48
+56
+68
Z 5.0
2.3

2.5.8.2. Correlations with plasma AG

High-energy food pictures

In whole brain analysis, there was a significant negative correlation between post-prandial changes in BOLD signal to HE food (vs. object) pictures with post-prandial decreases in plasma AG in the angular gyrus, superior division lateral occipital cortex and temporooccipital part of middle temporal gyrus (Table 2.2. (Bhargava et al. 2023), Figure 2.7.A).

Low-energy food pictures

In whole brain analysis, there was a significant positive correlation between post-prandial changes in BOLD signal to LE food (vs. object) pictures with post-prandial decreases in plasma AG in the occipital pole, intra-calcarine cortex, lingual gyrus, occipital fusiform gyrus, supracalcarine cortex and inferior division lateral occipital cortex (Table 2.3. (Bhargava et al. 2023), Figure 2.7.B).

High-energy or low-energy food pictures

In whole brain analysis, there were no significant correlations between post-prandial changes in BOLD signal to HE/LE food (vs. object) pictures with post-prandial decreases in plasma AG concentrations (Table 2.4. (Bhargava et al. 2023))

High-energy vs. low-energy food pictures

In whole brain analysis, there were no significant correlations between post-prandial changes in BOLD signal to HE vs. LE food pictures with post-prandial decreases in plasma AG.

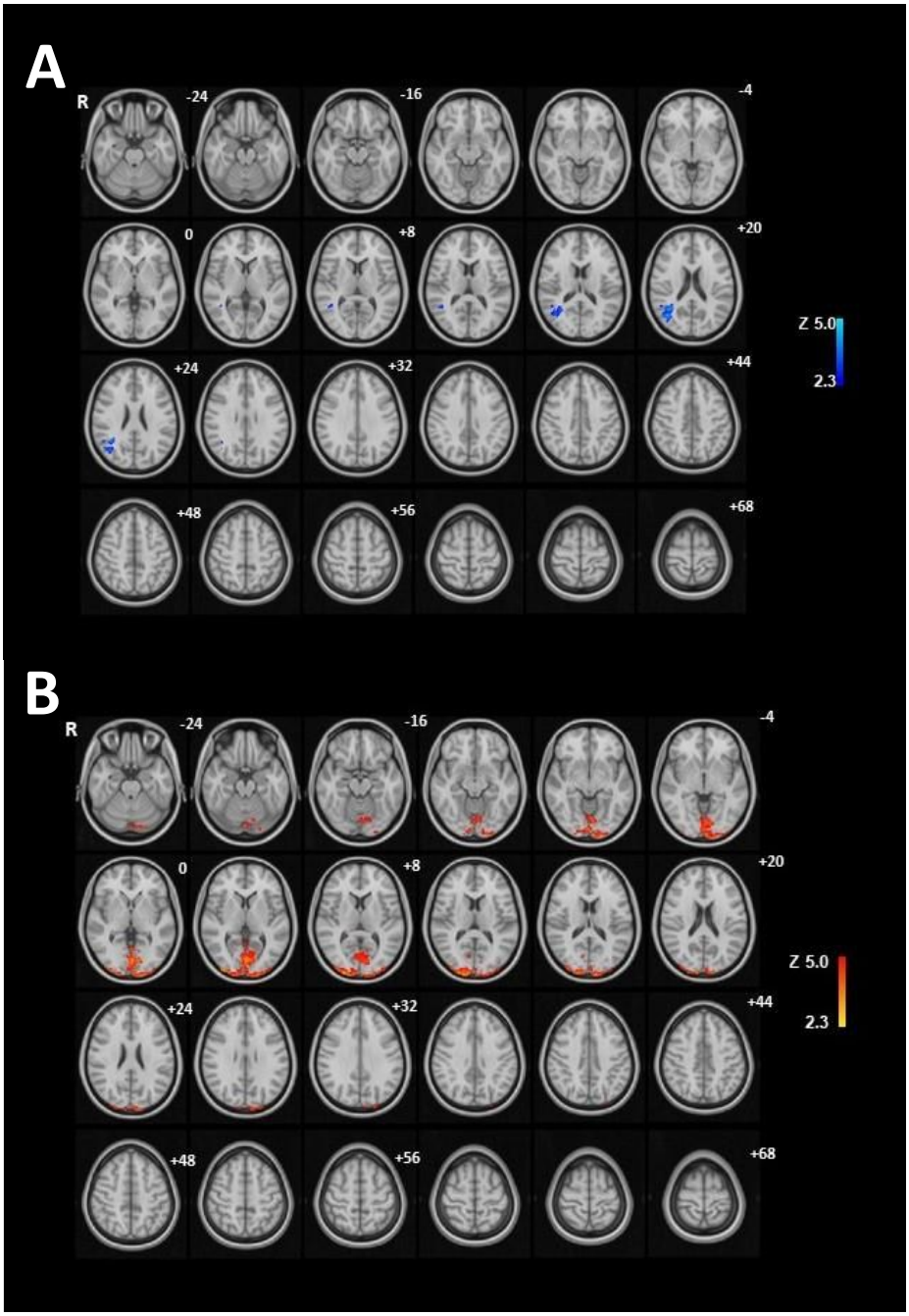


Figure 2.7. Whole-brain analysis of correlation between post-prandial changes in plasma AG and BOLD signal during food picture evaluation fMRI task.
(A) Brain regions (blue) showing negative correlations between post-prandial decreases in plasma AG (Δ fasted-fed, average t=+70/+150min) and post-prandial decreases (Δ fasted-fed) in BOLD signal during evaluation of high-energy food pictures (vs. objects), and (B) brain regions (red) showing positive correlations between post-prandial decreases in plasma AG (Δ fasted-fed, average t=+70/+150min) and post-prandial decreases (Δ fasted-fed) in BOLD signal during evaluation of low-energy food pictures (vs. objects) in whole brain analysis (n=22, cluster-wise family wise error (FWE) Z>2.3, P<0.05). Colour bar indicates Z statistic, z coordinates in Montreal Neurological Institute (MNI) space. Abbreviations: AG, acyl ghrelin; R, right.

2.5.8.3. Correlations with plasma AG/LEAP2 molar ratio

In whole brain analysis, there were no significant correlations between post-prandial changes in BOLD signal to HE food (vs. object), LE food (vs. object), HE or LE food (vs. object) or HE vs. LE food pictures with post-prandial decreases in plasma AG/LEAP2 molar ratios (Table 2.2-2.4 (Bhargava et al. 2023)).

Table 2.2. Whole brain analysis for correlations of post-prandial changes in BOLD signal to high-energy foods with plasma hormones.

Hormone	Correlation	Cluster	Number of voxels	Z	x	y	z	Laterality	Brain region
LEAP2	Positive	None							
	Negative	1	1462	3.70	0	24	58	B	43% superior frontal gyrus
				3.49	2	20	46	R	65% paracingulate gyrus, 5% anterior division cingulate gyrus, 5% superior frontal gyrus
				3.35	-4	-12	34	L	22% anterior division cingulate gyrus, 10% posterior division cingulate gyrus
				3.22	10	-32	38	R	31% posterior division cingulate gyrus, 5% precuneus
				3.18	2	2	46	R	44% anterior division cingulate gyrus, 27% juxta positional lobule cortex
				3.17	-2	-42	32	L	98% posterior division cingulate gyrus
		2	1445	3.55	6	34	26	R	48% paracingulate gyrus, 42% anterior division cingulate gyrus
				3.45	50	24	10	R	35% inferior frontal gyrus pars triangularis, 19% inferior frontal gyrus pars opercularis
				3.43	42	38	26	R	41% frontal pole, 24% middle frontal gyrus
				3.32	12	38	24	R	49% paracingulate gyrus, 8% anterior division cingulate gyrus
				3.29	6	48	26	R	48% paracingulate gyrus, 28% superior frontal gyrus

				3.27	20	36	24	R	99% right cerebral white matter
		3	612	3.72	4	-82	54	R	8% precuneus
				3.31	2	-62	54	R	72% precuneus
				3.19	30	-60	50	R	52% superior division lateral occipital cortex, 11% superior parietal lobule, 8% angular gyrus
				3.18	28	-64	46	R	52% superior division lateral occipital cortex
				3.11	18	-72	62	R	54% superior division lateral occipital cortex
				3.03	36	-56	58	R	35% superior division lateral occipital cortex, 25% superior parietal lobule, 5% angular gyrus
AG	Positive	None							
	Negative	1	403	3.59	40	-54	22	R	22% angular gyrus
				3.52	40	-68	22	R	49% superior division lateral occipital cortex
				3.49	34	-56	18	R	98% cerebral white matter
				2.88	52	-52	22	R	72% angular gyrus
				2.84	46	-52	10	R	24% temporooccipital part middle temporal gyrus
AG/LEAP2 ratio	Positive	None							

	Negative	None							

Group level spatial co-ordinates for correlations of post-prandial Δ BOLD signal (Fed-Fasted) to high-energy foods (vs. objects) with post-prandial changes (Fed-Fasted) in plasma hormones (average 70/150 min) from whole-brain analysis. n=22 adults without obesity, cluster-wise family-wise error (FWE) Z>2.3, P<0.05. Abbreviations: B: bilateral, L: left, R: right, Z: Z-statistic. x, y, z coordinates given in Montreal Neurological Institute (MNI) space.

Table 2.3. Whole brain analysis for correlations of post-prandial changes in BOLD signal to low-energy foods with plasma hormones.

Hormone	Correlation	Cluster	Number of voxels	Z	x	y	z	Laterality	Brain region
LEAP2	Positive	None							
	Negative	1	637	3.82	12	-30	80	R	32% postcentral gyrus, 16% precentral gyrus
				3.64	12	-46	76	R	35% postcentral gyrus, 21% superior parietal lobule
				3.49	-10	-34	82	L	10% postcentral gyrus, 7% precentral gyrus
				3.46	10	-42	78	R	58% postcentral gyrus, 8% superior parietal lobule
				3.36	-14	-42	78	L	57% postcentral gyrus, 7% superior parietal lobule
				3.18	-22	-22	76	L	48% precentral gyrus
AG	Positive	1	444	3.65	14	-90	2	R	41% occipital pole, 17% intra calcarine cortex, 9% lingual gyrus
				3.63	24	-74	-8	R	55% occipital fusiform gyrus, 10% lingual gyrus
				3.04	2	-82	-2	R	56% lingual gyrus, 25% intra calcarine cortex, 5% supra calcarine cortex
				2.93	2	-84	2	R	39% intra calcarine cortex, 26% lingual gyrus, 23% supra calcarine cortex
				2.86	26	-82	4	R	7% inferior division lateral occipital

									cortex
				2.86	12	-92	10	R	52% occipital pole
	Negative	None							
AG/ LEAP2 ratio	Positive	None							
	Negative	None							

Group level spatial co-ordinates for correlations of post-prandial Δ BOLD signal (Fed-Fasted) to low-energy foods (vs. objects) with post-prandial changes (Fed-Fasted) in plasma hormones (average 70/150 min) from whole-brain analysis. n=22 adults without obesity, cluster-wise family-wise error (FWE) Z>2.3, P<0.05. Abbreviations: L: left, R: right, Z: Z-statistic. x, y, z coordinates given Montreal Neurological Institute (MNI) space.

Table 2.4. Whole brain analysis for correlations of post-prandial changes in BOLD signal to high- or low-energy foods with plasma hormones.

Hormone	Correlation	Cluster	Number of voxels	Z	x	y	z	Laterality	Brain region
LEAP2	Positive	None							
	Negative	1	1012	3.55	18	36	24	R	99% cerebral white matter
				3.42	12	38	24	R	49% paracingulate gyrus, 8% anterior division cingulate gyrus
				3.25	32	24	26	R	12% middle frontal gyrus
				3.24	28	36	30	R	23% frontal pole, 17% middle frontal gyrus
				3.21	42	38	24	R	48% frontal pole, 16% middle frontal gyrus
				3.21	18	24	12	R	70% right cerebral white matter
		2	818	4.13	4	-82	54	R	8% precuneus
				3.66	2	-72	58	R	42% superior division lateral occipital cortex
				3.59	12	-30	80	R	32% postcentral gyrus, 16% precentral gyrus
				3.27	20	-72	62	R	48% superior division lateral occipital cortex
				3.24	8	-36	80	R	36% postcentral gyrus, 9% precentral gyrus
				3.23	10	-40	76	R	64% postcentral gyrus
		3	581	3.50	40	-32	40	R	27% anterior division supramarginal gyrus, 27% postcentral gyrus

				3.22	-2	-42	32	R	98% posterior division cingulate gyrus
				3.22	10	-32	38	R	31% posterior division cingulate gyrus, 5% precuneus
				3.17	2	-30	38	R	98% posterior division cingulate gyrus
				3.16	32	-30	46	R	19% postcentral gyrus
				3.11	28	-30	46	R	91% right cerebral white matter
AG	Positive	None							
	Negative	None							
AG/LEAP2 ratio	Positive	None							
	Negative	None							

Group level spatial co-ordinates for correlations of post-prandial Δ BOLD signal (Fed-Fasted) to high- or low-energy foods (vs. objects) with post-prandial changes (Fed-Fasted) in plasma hormones (average 70/150 min) from whole-brain analysis. n=22 adults without obesity, cluster-wise family-wise error (FWE) $Z>2.3$, $P<0.05$. Abbreviations: L: left, R: right, Z: Z-statistic. x, y, z coordinates given Montreal Neurological Institute (MNI) space.

2.5.9. Food Appeal Ratings

As previously reported using a different analysis method (Goldstone et al. 2014), in repeated measures ANOVA, including visit (Fed-saline, Fasted-saline) and food energy density (HE food vs. objects, LE food vs. objects) as within-participant factors, there was a trend for a significant energy density x visit interaction on food appeal scores [F(1,21)=3.57, P=0.073]. However, there was a significant overall effect of visit (independent of energy density) [F(1,21)=8.61, P=0.008], and a significant overall effect of energy density (independent of visit) [F(1,21)=7.28, P=0.014] (Figure 2.8. (Bhargava et al. 2023)).

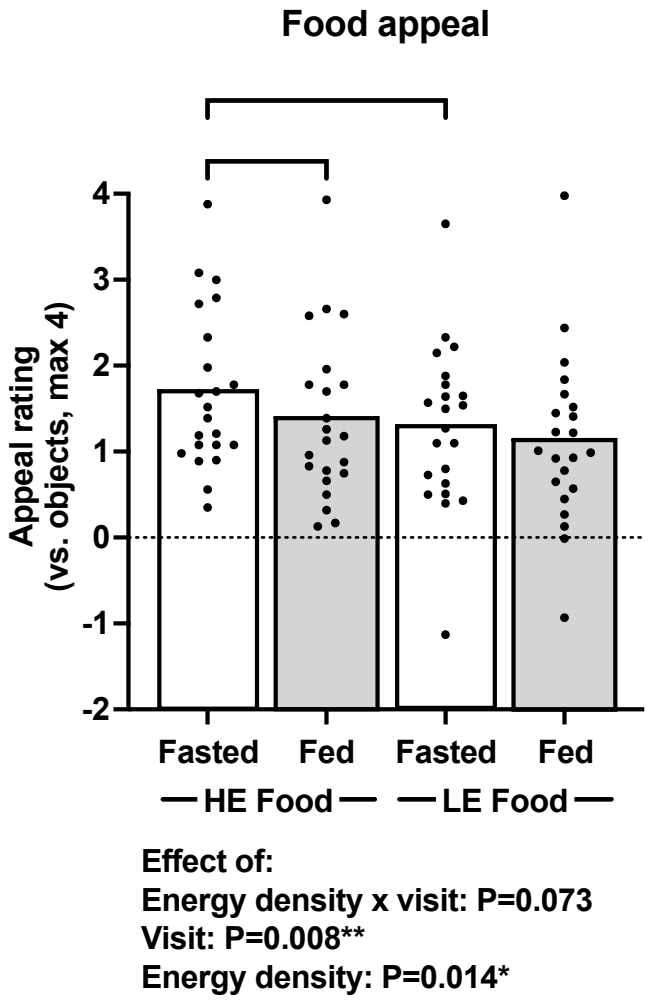


Figure 2.8. Food appeal ratings between visits for high-energy and low-energy foods. Ratings of food picture appeal (averaged at t=+70 and +150min) between Fed-saline (730 kcal breakfast, grey bars) and Fasted-saline (overnight fast, white bars) visits for high-energy and low-energy foods. Comparisons made using 2-way repeated measures ANOVA with post-hoc Tukey's test: *P<0.05, **P<0.01, n=22. Data given as mean (bar). Abbreviations: HE, high-energy; LE, low-energy.

In post-hoc analysis, HE food appeal ratings were significantly lower at Fed-saline (1.36 ± 0.20) than Fasted-saline (1.69 ± 0.20) visit [effect size -0.33 ± 0.06 (95% CI -0.47, -0.18), P<0.015] (Figure 2.8. (Bhargava et al. 2023)). Food appeal rating was significantly greater for HE food compared to LE food at the Fasted-saline visit [effect size 0.41 ± 0.18 (95% CI 0.08, 0.73), P=0.012], but not at the Fed-saline visit [effect size 0.25 ± 0.14 (95% CI -0.14, 0.63), P=0.31].

In direct comparison, the impact of food intake on food appeal rating tended to be greater for HE food (∆ Fed-Fasted) (-0.33 ± 0.09) than for LE food (∆ Fed-Fasted) (-0.17 ± 0.09) [effect size -0.16 ± 0.08 (95% CI -0.018,0.34), t=1.86, P=0.076, paired Student's t-test].

2.5.9.1. Correlations with plasma LEAP2, AG and AG/LEAP2 molar ratio

There were no significant correlations between post-prandial increases in plasma LEAP2 (∆ Fed-Fasted average t= +70/150min) , nor post-prandial decreases in plasma AG and plasma AG/LEAP2 molar ratio (∆ Fasted-Fed average t= +70/150min) with post-prandial decreases (∆ Fasted-Fed) in appeal rating of HE food (vs. objects) (r_s= +0.12, P=0.59; r_p=+0.06, P=0.80; r_P= +0.20, P=0.37 respectively) (Figure 2.9.A,C,E (Bhargava et al. 2023)).

There were no significant correlations between post-prandial increases in plasma LEAP2 nor post-prandial decrease in plasma AG with post-prandial decreases in appeal ratings of HE vs. LE food (r_s = +0.08, P=0.71; r_p = +0.27, P=0.22 respectively) (Figure 2.9.B,D (Bhargava et al. 2023)). Though there was a trend for a positive correlation between post-prandial decreases in plasma AG/LEAP2 molar ratio with post-prandial decreases in appeal ratings of HE vs. LE food (r_p= +0.40, P=0.064) (Figure 2.9.F (Bhargava et al. 2023)).

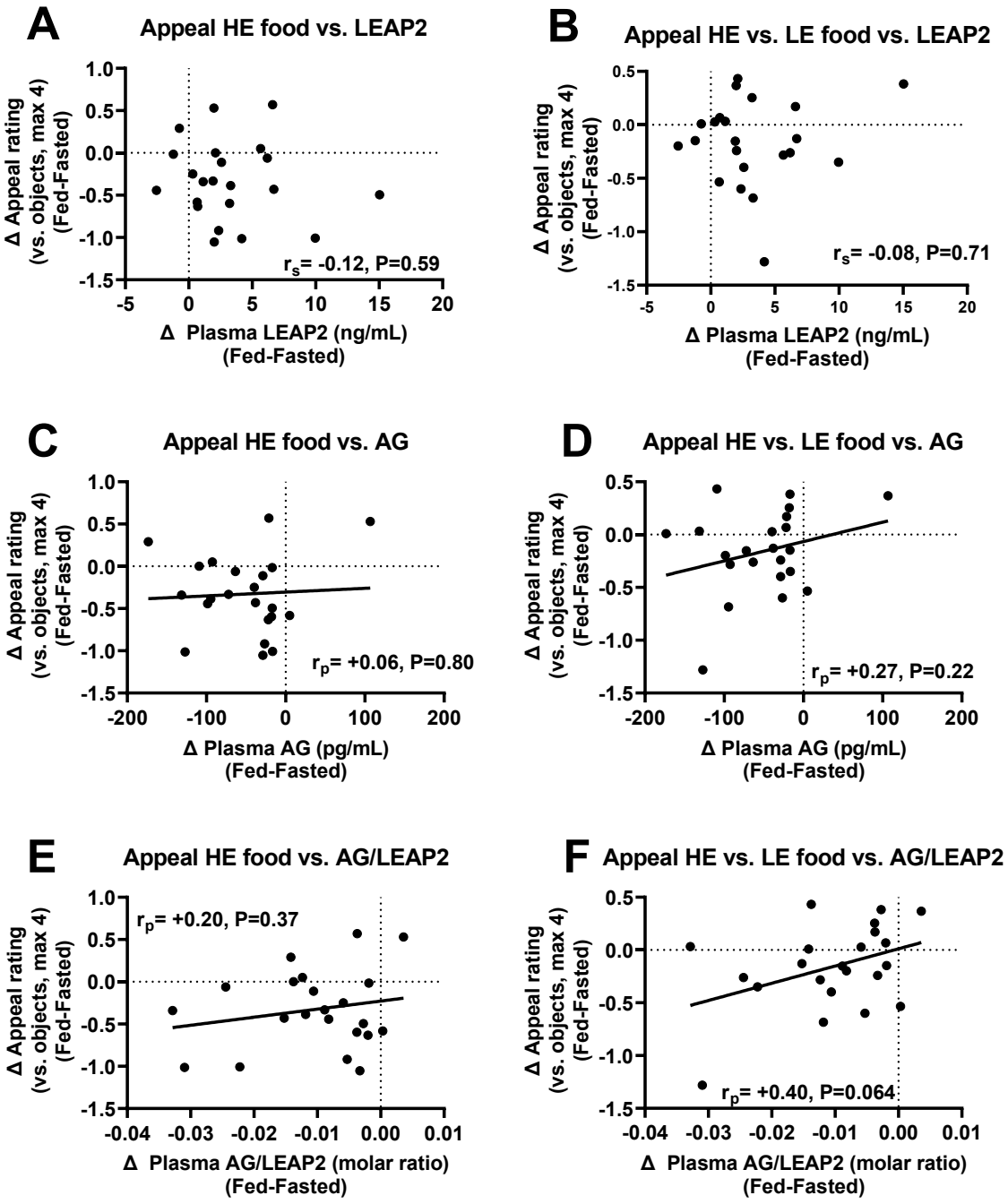


Figure 2.9. Correlations of post-prandial change in appeal ratings for high-energy food and high-energy vs. low-energy food with post-prandial plasma LEAP2, AG and AG/LEAP2 molar ratio.

Correlations of post-prandial change in appeal ratings (average of t=+70 and +150 min time points) between Fed-saline and Fasted-saline visits (Δ Fed-Fasted) for (A,C,E) high-energy food picture appeal (vs. objects) and (B,D,F) high-energy vs. low-energy food picture appeal with change in incremental value of area under the curve (iAUC) from t= 0, +40, +70 and +150 min time points (except AG and AG/LEAP2 molar ratio missing t=+40 min time point) between Fed-saline and Fasted-saline visits (Δ Fed-Fasted) or plasma (A,B) LEAP2, (C,D) AG and (E,F) AG/LEAP2 molar ratio, using Pearson's (r_P, linear regression line) or Spearman's (r_S) correlation coefficients, n=22. Abbreviations: AG, acyl ghrelin; HE, high-energy; LE, low-energy.

2.5.10. Food Intake at *Ad Libitum* Meal

As previously reported using a different analysis method using unadjusted values (Goldstone et al. 2014), there was a significant decrease in REE-adjusted energy intake at Fed-saline (67.19 ± 3.50 %REE) vs. Fasted-saline (79.48 ± 4.11 %REE) visits using the Cunningham equation [effect size -12.28 ± 2.94 %REE (95% CI -18.39, -6.18), t= -4.18, P=0.0004, paired Student's t-test] (Figure 2.10.A). Similar decreases in REE-adjusted energy intake between the Fed-saline (70.07 ± 3.66 %REE) vs. Fasted-saline visits (84.34 ± 4.47 %REE) were seen using the Schofield equation [effect size -13.31 ± 3.25 %REE (95% CI -20.08, -6.55), t= -4.09, P=0.0005, paired Student's t-test].

2.5.10.1. Correlations with plasma LEAP2

The post-prandial decrease in food intake (Δ Fasted-Fed kcal as %REE using Cunningham equation) showed a trend towards a positive correlation with the post-prandial increase in plasma LEAP2 (Δ Fed-Fasted t=+150min) (r_s= +0.37, P=0.088) (Figure 2.10.B).

A similar correlation coefficient was seen for post-prandial increase in plasma LEAP2 (Δ Fed-Fasted t=+150min) with post-prandial decrease in food intake (Δ Fed-Fasted) expressed as %REE using the Schofield equation (r_s= +0.35, P=0.11).

2.5.10.2. Correlations with plasma AG and AG/LEAP2 molar ratio

The post-prandial decrease in food intake (Δ Fasted-Fed kcal as %REE using Cunningham equation) did not correlate significantly with the post-prandial decrease (Δ Fasted-Fed t= +150min) in plasma AG (r_s= -0.32, P=0.14) (Figure 2.10.C) nor plasma AG/LEAP2 molar ratio (r_s= -0.19, P=0.41) (Figure 2.10.D).

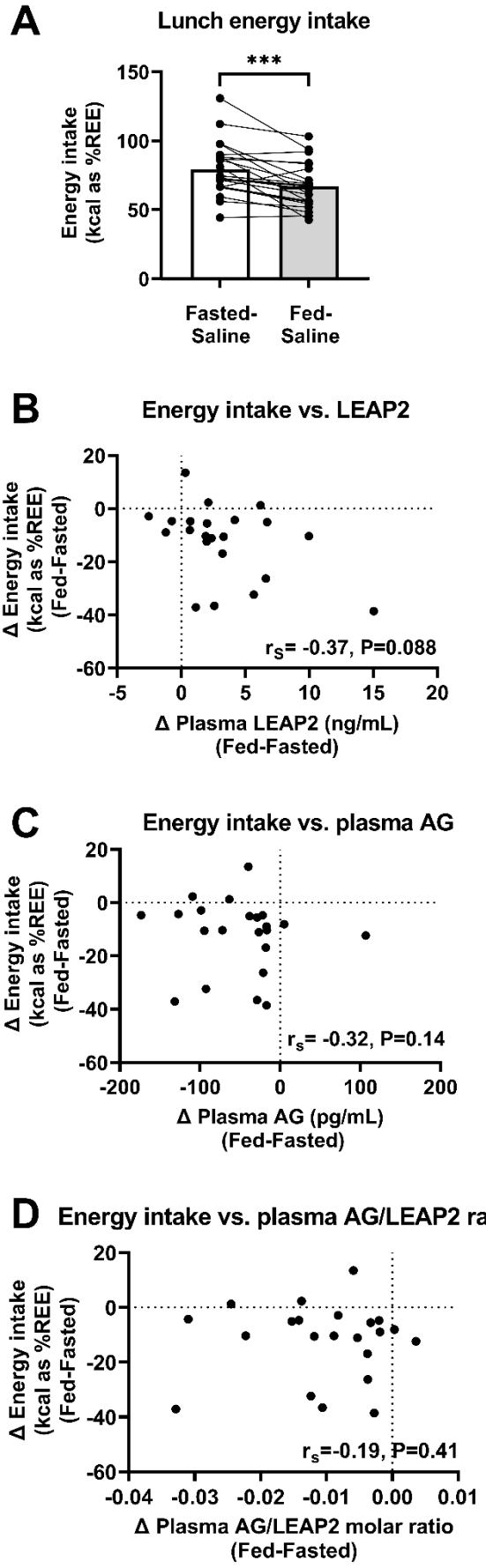

A
Lunch energy intake
***
Energy intake (kcal as %REE)
150
100
50
0
Fasted-Saline
Fed-Saline
B
Energy intake vs. LEAP2
Δ Energy intake (kcal as %REE) (Fed-Fasted)
20
0
-20
-40
-60
-5
0
5
10
15
20
rS= -0.37, P=0.088
Δ Plasma LEAP2 (ng/mL) (Fed-Fasted)
C
Energy intake vs. plasma AG
Δ Energy intake (kcal as %REE) (Fed-Fasted)
rs= -0.32, P=0.14
-200
-100
0
100
200
Δ Plasma AG (pg/mL) (Fed-Fasted)
D
Energy intake vs. plasma AG/LEAP2 ratio
Δ Energy intake (kcal as %REE) (Fed-Fasted)
rs=-0.19, P=0.41
-0.04
-0.03
-0.02
-0.01
0.00
0.01
Δ Plasma AG/LEAP2 molar ratio (Fed-Fasted)

Figure 2.10. Energy intake at fed and fasted visits and correlations of post-prandial plasma LEAP2, AG and AG/LEAP2 molar ratio with energy intake.
(A) Energy intake adjusted for kcal as percentage of resting energy expenditure between Fed-saline (730kcal breakfast) and Fasted-saline (overnight fast) visits, paired students t-test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, n=22. (B-D) Correlation of change in energy intake between Fed-saline and Fasted-saline visits (Δ fed-fasted) with change in incremental value of area under the curve (iAUC) from t=0 to +150 min between Fed-saline and Fasted-saline visits (Δ fasted-fed) for plasma (B) LEAP2, (C) AG, and (D) AG/LEAP2 molar ratio using Spearman's (r_S) correlation coefficients, n-22. Abbreviations: AG, acyl ghrelin; kcal, kilocalorie; REE, resting energy expenditure.

2.5.10.3. Correlations of post-prandial changes in gut hormones with BMI

BMI was negatively correlated with post-prandial increase in plasma LEAP2 $iAUC_{0\text{-}150\ min}$ (Δ Fed-Fasted) (r_p=-0.54, P=0.009) (Figure 2.11.A). However, BMI was not correlated significantly with post-prandial decreases in either plasma AG $iAUC_{0\text{-}150min}$ (Δ Fasted-Fed) (r_s= +0.06, P=0.76), nor plasma AG/LEAP2 molar ratio $iAUC_{0\text{-}150\ min}$ (Δ Fasted-Fed) (r_p= -0.07, P=0.73) (Figure 2.11.B, C).

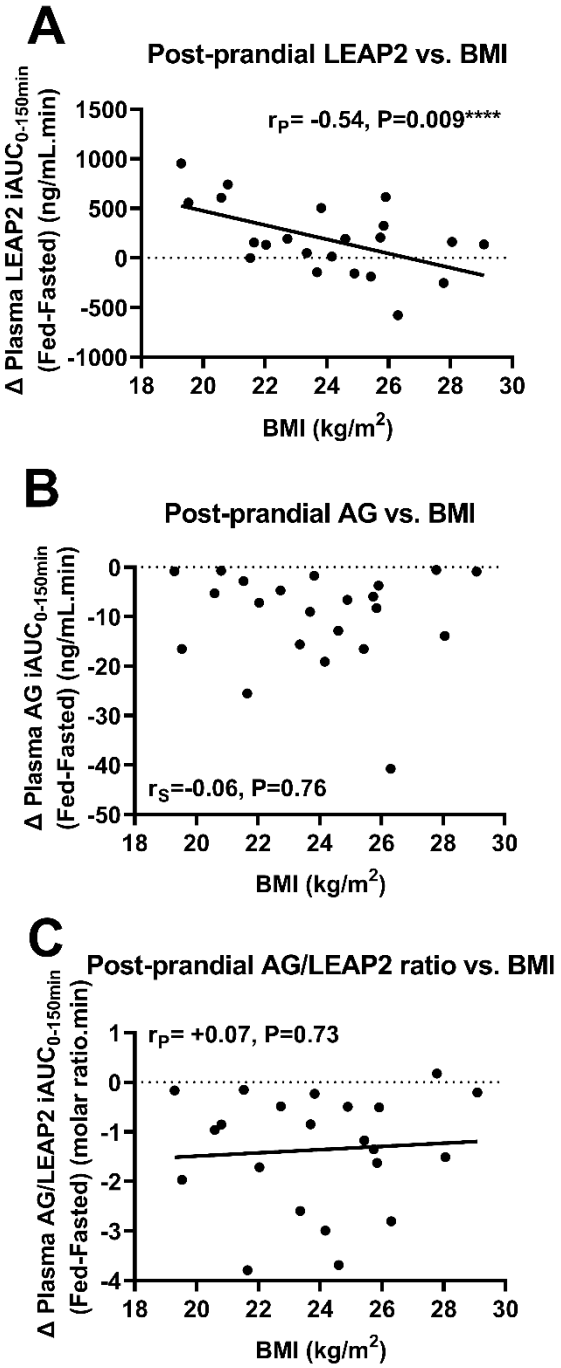


Figure 2.11. Correlations of post-prandial plasma LEAP2, AG and AG/LEAP2 molar ratio with BMI.

Correlation of change in incremental value of area under the curve (iAUC) from t=0 to +150 min between Fed-saline and Fasted-saline visits (Δ fed-fasted) for plasma (A) LEAP2, (B) AG, and (C) AG/LEAP2 molar ratio with BMI using Pearson's (r_P, linear regression line) or Spearman's (r_S) correlation coefficients. Abbreviations: AG, acyl ghrelin; BMI, body mass index.

2.5.11. Correlations of Post-Prandial Changes in Gut Hormones and Markers Of Glucose and Lipid Metabolism

2.5.11.1. Post-prandial plasma glucose

As previously reported using a different analysis method (Goldstone et al. 2014), there was a significant increase in plasma glucose $iAUC_{0\text{-}150min}$ at the Fed-saline (20.13 ± 15.35 mmol/L.min) vs. Fasted-saline (-13.93 ± 3.52 mmol/L.min) visit [effect size 34.07 ± 14.97 (95% CI 2.93, 65.20), t=2.28, P=0.033, paired Student's t-test] (Figure 2.12.A,B (Bhargava et al. 2023)).

The post-prandial increase in plasma glucose $iAUC_{0\text{-}150min}$ (Δ Fed-Fasted) was not significantly correlated with the post-prandial increase in plasma LEAP2 $iAUC_{0\text{-}150min}$ (Δ Fed-Fasted) (r_p= +0.03, P=0.91), nor the post-prandial decrease in plasma AG $iAUC_{0\text{-}150min}$ (Δ Fasted-Fed) (r_s= +0.31, P=0.15), but was positively correlated with the post-prandial decrease in plasma AG/LEAP2 molar ratio (Δ fasted-fed) (r_p= +0.44, P=0.041) (Figure 2.13.A-C).

Similar results were seen when excluding the t= +40 min time point for plasma glucose for the correlations of post-prandial increase in plasma glucose $iAUC_{0\text{-}150\ min}$ (Δ Fed-Fasted) with post-prandial increase in plasma LEAP2 $iAUC_{0\text{-}150\ min}$ (Δ Fed-Fasted) (r_p= +0.01, P=0.93), or the post-prandial decrease in plasma AG/LEAP2 molar ratio (Δ Fasted-Fed) (r_p= +0.49, P=0.022), performed as t= +40 min time point was missing for plasma LEAP2 values.

2.5.11.2. Post-prandial serum insulin

As previously reported using a different analysis method (Goldstone et al. 2014), there was a significant increase in serum insulin $iAUC_{0\text{-}150\ min}$ at the Fed-saline (median 2379 mU/L.min, IQR [1482.5, 3726.5]) vs. Fasted-saline (median 477 mU/L.min, IQR [303, 601]) visit (W-statistic= 253, P<0.0001, Wilcoxon matched-pairs signed rank test) (Figure 2.12.C,D (Bhargava et al. 2023)).

However, there were no significant correlation between the post-prandial increase in serum insulin $iAUC_{0\text{-}150\ min}$ (Fed-Fasted) and post-prandial changes in plasma LEAP2, AG and AG/LEAP2 molar ratio (r_s= -0.03 to +0.04, P=0.88-0.97) (Figure 2.13.D-F).

2.5.11.3. Post-prandial serum triglycerides

As previously reported using a different analysis method (Goldstone et al. 2014), there was a significant increase in serum triglycerides $iAUC_{0\text{-}150\ min}$ at the Fed-saline (median 21.93 mmol/L.min, IQR 14.35, 34.16) vs. Fasted-saline (median 10.71 mmol/L.min, IQR 4.21, 16.49) visit (Z-statistic -3.04, W-statistic 33, P=0.002, Wilcoxon matched-pairs signed rank test) (Figure 2.12.E,F (Bhargava et al. 2023)).

The post-prandial increase in serum triglycerides $iAUC_{0\text{-}150\ min}$ (Δ Fed-Fasted) showed a trend towards a negative correlation with the post-prandial increase in plasma LEAP2 $iAUC_{0\text{-}150\ min}$ (Δ Fed-Fasted) (r_s= -0.38, P=0.079) (Figure 2.13.G). However, there was no correlation of the post-prandial increase in serum triglycerides with the post-prandial decrease in plasma AG $iAUC_{0\text{-}150\ min}$ (Δ Fasted-Fed) (r_s= -0.07, P=0.76), nor post-prandial decrease in plasma AG/LEAP2 molar ratio (Δ Fasted-Fed) (r_s= -0.18,P=0.42) (Figure 2.13.H,I).

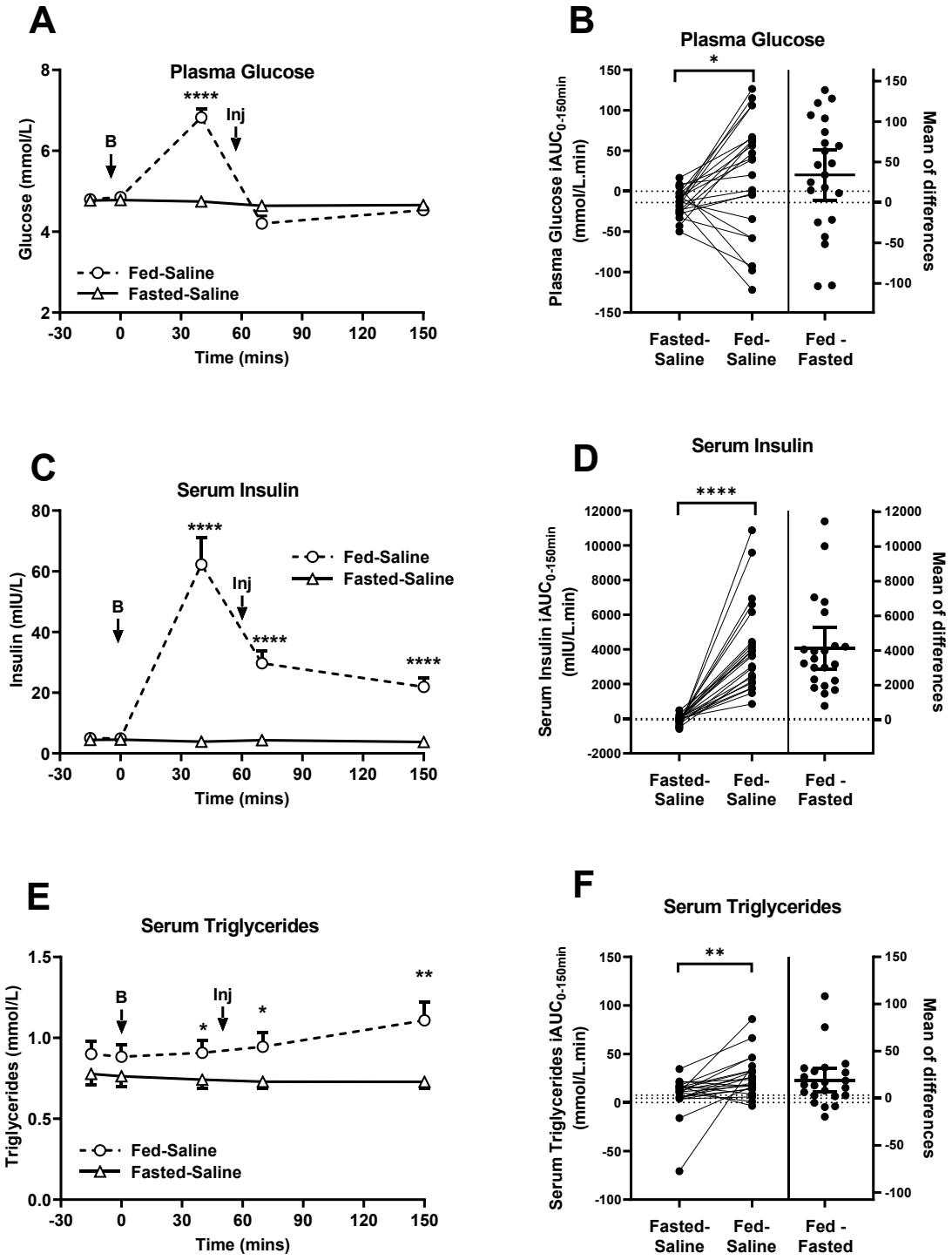


Figure 2.12. Plasma glucose, serum insulin and triglyceride concentrations at study visits. (A,C,E) Concentrations over study visits for (A) plasma glucose, and serum (C) insulin and (E) triglycerides at Fasted-saline and Fed-saline visits. Arrows indicate (B) time of breakfast (t=0min) and (Inj) subcutaneous saline/acyl ghrlein injection (t= +55min). (B,D,F) Comparison of incremental value of area under the curve (iAUC) from t=0 to +150 min for (B) plasma glucose, and serum (D) insulin and (F) triglycerides between Fed-saline and Fasted-saline visits with right-hand part of graph showing mean differences between visits (Δ Fed-Fasted). Comparisons made using (A,C,E) 2-way repeated measures ANOVA with post-hoc Tukey's test, (B) paired Students t-test, and (D,F) Wilcoxon matched-pairs signed rank test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 Fed-Saline vs. Fasted-Saline, n=22. All data given as mean ± SEM.

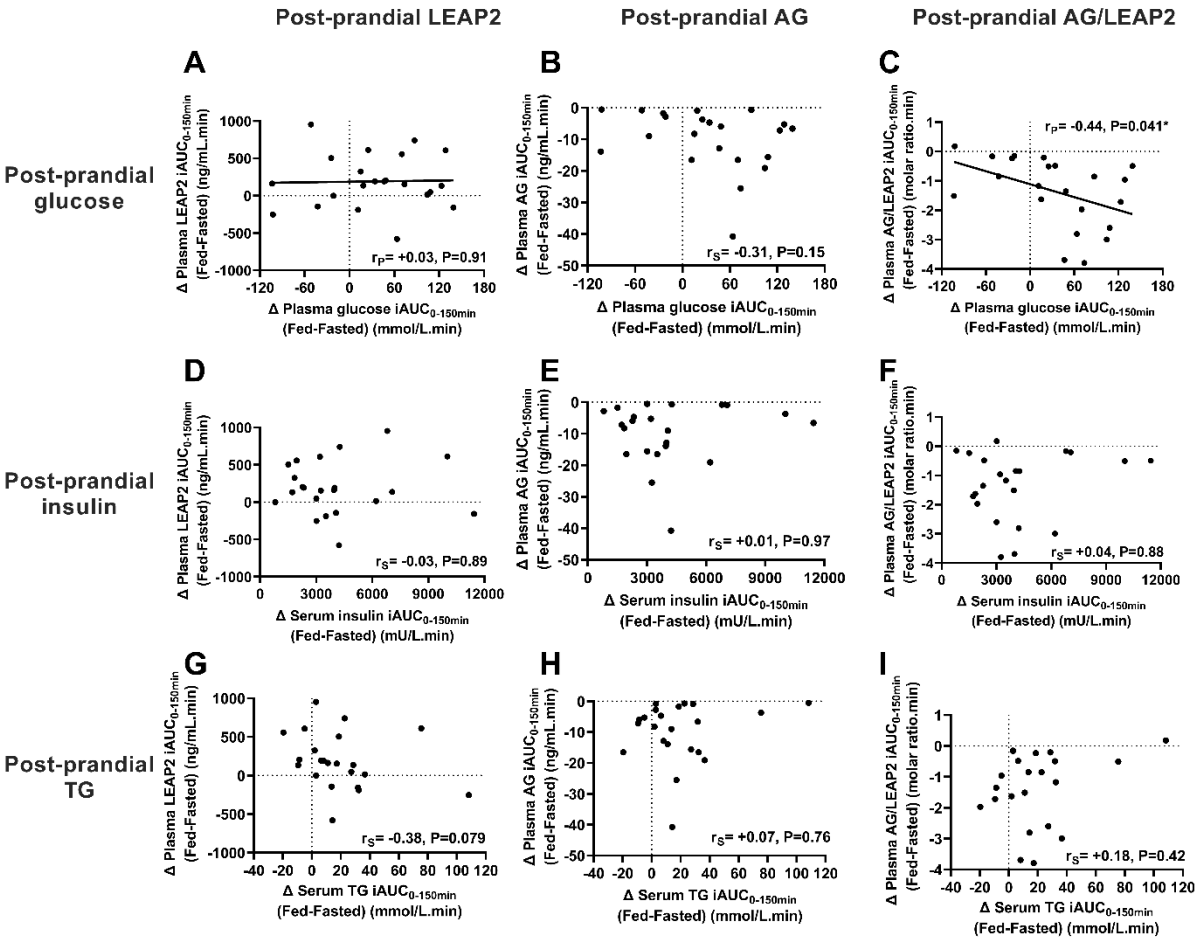


Figure 2.13. Correlations of post-prandial plasma LEAP2, AG and AG/LEAP2 molar ratio with post-prandial markers of glucose and lipid metabolism.

Correlations of change in incremental value of area under the curve (iAUC) from t= 0, +40, +70 and +150 min timepoints (except AG and AG/LEAP2 molar ratio missing t=+40 min timepoint) between Fed-saline and Fasted-saline visits (Δ fed-fasted) for (A-C) plasma glucose, (D-F) serum insulin and (G-I) serum triglycerides with plasma (A,D,G) LEAP2, (B,E,H) AG, and (C,F,I) AG/LEAP2 molar ratio, using Pearson's (r_P, linear regression line) or Spearman's (r_S) correlation coefficients, n=22. Abbreviations: AG, acyl ghrelin; TG, triglycerides.

2.6. DISCUSSION

This human study in adults without obesity demonstrated that increases in plasma LEAP2 after food intake were related to post-prandial suppression of eating behaviour since (Table 2.5): (i) as hypothesised, post-prandial increases in plasma LEAP2 positively correlated with the post-prandial decrease in appetite ratings, though there was no correlation with the post-prandial decrease in plasma AG or AG/LEAP2 ratio; (ii) as hypothesised, the post-prandial increase plasma LEAP2 tended to positively correlate with the post-prandial reduction in energy intake at an *ad libitum* lunch, though there was no trend for plasma AG or AG/LEAP2 ratio; (iii) as hypothesized, post-prandial increases in plasma LEAP2 positively correlated with post-prandial decreases in food cue reactivity (using food picture evaluation fMRI task) to HE, LE and HE/LE foods, while post-prandial decreases in plasma AG positively correlated with the post-prandial decrease in food cue reactivity to LE foods, though contrary to our hypothesis post-prandial decreases in plasma AG negatively correlated with the post-prandial decrease in food cue reactivity to HE foods, and there were no correlations of post-prandial changes in food cue reactivity with post-prandial decreases in plasma AG/LEAP2; (iv) contrary to that hypothesised, the post-prandial increase in plasma LEAP2 does not appear to be due to the post-prandial decreases in plasma AG, as these post-prandial hormone changes were not positively correlated with each other, and especially as exogenous AG administration did not reduce post-prandial plasma LEAP2; (v) contrary to that hypothesised, the post-prandial increase in plasma LEAP2 was not positively correlated with post-prandial increases in glucose, insulin or triglycerides, while the post-prandial decrease in plasma AG/LEAP2 ratio was negatively correlated with the postprandial increase in plasma glucose (mainly driven by the post-prandial decrease in plasma AG), though not serum insulin as would be predicted from the literature; and (vi) the post-prandial increase in plasma LEAP2 was negatively correlated with BMI, in contrast to that previously reported in the literature, with no correlations seen between post-prandial decrease in plasma AG or AG/LEAP2 ratio and BMI.

Outcome Variable (Δ Fed-Fasted)	LEAP2 (Δ Fed-Fasted)	AG (Δ Fed-Fasted)	AG/LEAP2 (Δ Fed-Fasted)	Results vs. Hypothesis
Energy intake	Negative (trend)	o	o	Y, C, C
Food Cue Reactivity				
HE food	**Negative**	**Negative**	o	Y, C, C
LE food	**Negative**	**Positive**	o	Y, Y, C
HE or LE food	**Negative**	o	o	Y, C, C
HE vs. LE food	o	o	o	C, C, C
Appetite VAS ratings	**Negative**	o	o	Y, C, C
Fullness VAS ratings	o	o	o	C, C, C
Appeal ratings				
HE food	o	o	o	C, C, C
HE vs. LE food	o	o	Positive (trend)	C, C, Y
BMI	**Negative**	o	o	C, C, C
Plasma Glucose	o	o	**Negative**	C, C, Y
Serum Insulin	o	o	o	C, C, C
Serum Triglycerides	Negative (trend)	o	o	Y, C, C

Table 2.5. Summary of results.
Abbreviations: AG, Acyl Ghrelin; BMI, body mass index; HE, High-Energy; LE, Low-Energy; VAS, Visual Analogue Scale; Y, as per hypothesis; C, Contrary to hypothesis; o, No correlation.

2.6.1. Post-Prandial Increase in Plasma LEAP2

Consistent with previous literature (Ge et al. 2018; Mani et al. 2019; Islam et al. 2020; Hagemann et al. 2021; Holm et al. 2022). Interestingly, when examining these multiple human studies, there is a suggestion that the post-prandial increase plasma LEAP2 is proportional to the energy content of meal, though there were some slight differences in macronutrient content of the fixed meals (carbohydrates 49-55%, fat 30-35% and protein

14-18%), nature of meals (four out of five meals were liquid meal supplements, but mixed meal in current study), and variation in the timing of post-prandial blood sampling between the studies (90, 120 and 150 mins). However, all studies used the same LEAP2 assay (Phoenix Pharmaceuticals Inc., CA, USA, cat. No. EK-075-40). This influence of meal size on plasma LEAP2 has previously been demonstrated for other appetitive gut hormones such as orexigenic total ghrelin (negative relationship) and anorexigenic PYY (positive relationship) (le Roux et al. 2006; le Roux et al. 2005). This suggests that plasma LEAP2 may be a further peripheral anorexigenic signal of recent energy intake to the brain to attenuate human eating behaviour, especially when integrated with post-prandial decreases in plasma AG to decrease the plasma AG/LEAP2 ratio. However, this needs to be examined in future within participant studies measuring plasma LEAP2 and AG after consumption of fixed meals of varying caloric value. Furthermore, the influence of intake of different macronutrients on plasma LEAP2 needs to be examined, as previously reported for plasma AG and total ghrelin (Muller et al. 2015; Foster-Schubert et al. 2008).

In the current study, post-prandial plasma LEAP2 increased further from 70 to 150 min after food intake, but later time points were unavailable, which should be examined in future studies, since peak concentrations might be achieved later. This compares to peak suppression of plasma AG and AG/LEAP2 ratio at 70 min in this study. Unfortunately, due to technical problems with between assay variation we were not able to report exact plasma LEAP2 concentrations at 40 min after the meal, but there was no suggestion that plasma LEAP2 peaked at this earlier time point using quality control-adjusted corrected values. This would be consistent with the hepatic (rather than intestinal) source of post-prandial circulating LEAP2 suggested from pre-clinical studies (Islam et al. 2020; Holm et al. 2022).

2.6.2. Role of GHSR-Related Hormones in Appetite and Food Intake

A role of endogenous GHSR-related hormones in stimulating human appetite and food intake, as opposed to meal preparation and initiation, is disputed. While exogenous AG simulates appetite ratings and food intake in humans without and with obesity, this is only with markedly supraphysiological plasma AG concentrations (Druce et al. 2005; Druce et al. 2006; Wren et al. 2001). By contrast, low dose exogenous AG administration to achieve physiological or mildly supraphysiological plasma AG concentrations had no effect on appetite ratings nor food intake in men of normal weight when fed (Lippl et al.

2012). Chronic administration of an oral GOAT inhibitor (GLWL-01) over 28 days which markedly decreased plasma AG in adults with obesity and T2DM also had no effect on body weight (though this is complicated by plasma AG being low already in obesity) (https://clinicaltrials.gov/ct2/show/results/NCT02377362). Although an oral GHSR inverse agonist, PF-5190457, reduced alcohol craving in adults with alcohol use disorder with no effect on glucose metabolism, it has not been examined in normal weight or obesity, and effects on appetite and food intake have not been reported (Lee et al. 2020b; Lee et al. 2020a; Farokhnia et al. 2020).

In the current study, there was a clear post-prandial decrease in appetite ratings and *ad libitum* meal energy intake compared to the fasted state. Post-prandial increases in plasma LEAP2 positively correlated with post-prandial decreases in appetite ratings (though not fullness), with a trend for post-prandial decreases in energy intake. However, there were no correlations with post-prandial decreases in AG nor AG/LEAP2 molar ratio. These novel findings thus do provide support a role for GHSR signalling in stimulating human appetite and food intake, either through antagonism of endogenous AG or inverse agonism at the constitutively active GHSR (Wang et al. 2019; M'Kadmi et al. 2019; Ge et al. 2018). These results are consistent with the recent finding that administration of exogenous LEAP2 to men without obesity (to achieve mildly supraphysiological plasma LEAP2 concentrations) reduced food intake at an *ad libitum* test lunch, although no decreases in appetite ratings were seen (Hagemann et al. 2022).

Other than plasma LEAP2, post-prandial increases in circulating metabolites and other hormones, including glucose, triglycerides, insulin and cholecystokinin, might also contribute to post-prandial decreases in appetitive behaviour, this was not compatible with the time course and duration of post-prandial increases in plasma glucose, serum insulin and triglycerides in this study, while at the time of the *ad libitum* test meal, plasma glucose was in fact lower at the Fed-saline than Fasted-saline visit. Furthermore, there were no significant post-prandial increases in anorexigenic peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) hormones in this study (Goldstone et al. 2014).

The lack of correlations of post-prandial decreases in plasma AG or AG/LEAP2 ratio with post-prandial decreases in appetite or food intake in this study suggest that post-prandial increases in plasma LEAP2 may be more important than decreases in plasma AG in

contributing to post-prandial reductions in appetitive behaviour. Although this might be related to small sample size (type 2 error), as discussed above a definitive role for endogenous ghrelin (as opposed to GHSR signalling) in stimulating appetitive behaviour is uncertain. There is great variability in the results of studies correlating endogenous post-prandial plasma ghrelin concentrations and measures of eating behaviour including appetite ratings and food intake, likely related to often small samples sizes, differences in study design and obesity status, specificity of ghrelin immunoassays to AG, DAG or both, use of protease inhibitors to prevent breakdown of AG to DAG, use of absolute or changes in plasma ghrelin, and lack of correction of food intake for body size or resting energy expenditure (Monteleone et al. 2003 22585; Moran et al. 2004 22586; Doucet et al. 2004; Erdmann et al. 2004; Cummings et al. 2004; Blom et al. 2005; Stock et al. 2005; Bowen et al. 2006; Di Francesco et al. 2006; Mackelvie et al. 2007; Akhavan et al. 2007; Erdmann et al. 2007; Doucet et al. 2008; Frecka et al. 2008; Blom et al. 2009; Lioger et al. 2009; Serra-Prat et al. 2009; Unick et al. 2010; Massolt et al. 2010; Larson-Meyer et al. 2010; Cooper et al. 2011; Rosen et al. 2011; Kroemer et al. 2013; Monteleone et al. 2012; Martens et al. 2012; Bryant et al. 2013; Gibbons et al. 2013; Monteleone et al. 2013; St-Onge et al. 2014; Deighton et al. 2014; Goldstone et al. 2014; Andarini et al. 2017; Giezenaar et al. 2018b; Nymo et al. 2018; Costabile et al. 2018; Giezenaar et al. 2018a; Zhu et al. 2019; McVeay et al. 2019; Sinha et al. 2019; Japur et al. 2019; Duszka et al. 2020). Indeed, the majority of published studies do not show positive correlations between post-prandial absolute or decreases in plasma total ghrelin/AG with post-prandial absolute or decreases in appetite or hunger ratings, post-prandial absolute or increases in fullness, nor post-prandial food intake.

2.6.3. Role of GHSR-Related Hormones in Food Cue Reactivity

2.6.3.1. Correlations of plasma LEAP2 with food cue reactivity

Interestingly, the current study had the novel finding that there was also a negative correlation between post-prandial increases in plasma LEAP2 and post-prandial decreases in food cue reactivity during evaluation of HE food, LE food and HE vs. LE food pictures in the following brain regions: paracingulate gyrus, anterior and posterior cingulate gyrus, precuneus, juxtapositional lobule cortex, superior and inferior frontal gyrus, frontal pole, middle frontal gyrus, lateral occipital cortex, superior occipital lobule, angular gyrus and superior parietal lobule.

It is however difficult to assign specific tasks to these brain regions in the context of this study, but they include areas involved in reward processing, decision making and executive function. The relevance of some of these brain regions is highlighted by the findings from several fMRI meta-analyses. A meta-analysis of food cue reactivity found increased BOLD signal to HE food (vs. non-food) stimuli in the following regions that overlapped with the findings of the current study: inferior frontal gyrus and anterior cingulate gyrus (Yang et al. 2021). A meta-analysis of brain responses to taste found increased BOLD signal to sweet taste (vs. water or tasteless solution) in these overlapping regions: frontal pole, anterior cingulate gyrus and inferior frontal gyrus (Chen et al. 2020). Furthermore, a meta-analysis of obesity-related differences in food cue reactivity found increased BOLD signal in obesity to passive viewing of food (vs. non-food) stimuli in the inferior frontal gyrus (Morys et al. 2020), a region that has shown lower food cue reactivity in those with lower desire to eat (Lopez et al. 2016).

The clinical importance of several of these brain regions is also revealed from post-weight loss reductions BOLD signal to food stimuli in the precuneus, inferior and middle frontal gyrus, posterior cingulate cortex and lateral occipital cortex from longitudinal fMRI studies of Roux-en-Y gastric bypass (RYGB) surgery for obesity (Ochner et al. 2011; Ochner et al. 2012b; Ochner et al. 2012a; Salem et al. 2021). Furthermore, reductions in the BOLD signal to HE vs. LE food stimuli after RYGB surgery have been correlated with reductions in the preference for HE vs. LE food in the precuneus (Zoon et al. 2018), and reductions in the liking of HE vs. LE food in the superior frontal gyrus and anterior cingulate cortex (Ochner et al. 2012b).

In addition, a physiological role for some of these brain regions in mediating effects of nutritional state on behaviour is supported by findings that several of these brain regions also show decreases in food cue reactivity in the post-prandial fed compared to fasted state. In men and children with normal weight, food intake decreased BOLD signal to HE and LE food pictures in the anterior cingulate cortex, superior occipital sulcus, posterior cingulate cortex and superior frontal gyrus (regions that overlapped with the findings of the current study), and also the pregenual cingulate cortex, amygdala, subcallosal gyrus, orbitofrontal cortex (OFC) (Fuhrer et al. 2008; Bruce et al. 2010). In adults without obesity, food intake decreased BOLD signal HE vs. LE food pictures in the anterior and posterior cingulate cortex, middle and superior frontal lobe (regions that overlapped with the

findings of the current study), and also the nucleus accumbens, caudate/putamen, amygdala, anterior insula, OFC and fusiform gyrus (Goldstone et al. 2009; Siep et al. 2009; Frank et al. 2010).

Therefore, these findings provide novel evidence supporting a role for post-prandial increases in endogenous plasma LEAP2 in altering food-related brain activity in humans. However, no correlations of post-prandial plasma LEAP2 were seen with post-prandial changes in food cue reactivity in ventral/dorsal striatum, amygdala, OFC and hippocampus, regions involved in food reward processing, emotional reactivity, salience and memory (Goldstone et al. 2009; Goldstone et al. 2014; Scholtz et al. 2014; Carnell et al. 2012; Goldstone et al. 2016). Future studies will need to examine the effect of exogenous LEAP2 administration on food cue reactivity in humans.

2.6.3.2. Correlations of plasma AG with food cue reactivity

The post-prandial decrease in plasma AG positively correlated with post-prandial decreases in BOLD signal to LE foods in the occipital pole, intra-calcarine cortex, lingual gyrus, occipital fusiform gyrus, supracalcarine cortex and inferior division lateral occipital cortex, areas of the visual pathway, a direction that would be hypothesised by a role for AG in stimulating visual attention and processing to food. By contrast, there was an unexplained negative correlation between the post-prandial decrease in plasma AG and decrease in food cue reactivity to HE food in the angular gyrus, superior division of lateral occipital cortex and temporooccipital part of middle temporal gyrus. However, no correlations were seen between post-prandial decreases in plasma AG/LEAP2 ratio and changes in food cue reactivity.

Several previous studies have examined correlations of post-prandial decreases in endogenous plasma ghrelin with changes in food cue reactivity (pictures or taste) or resting BOLD signal with highly variable results, likely related to differences in study design, participant characteristics including obesity status, sample size, assay of total ghrelin or AG, fMRI analysis methods and statistical thresholds. However, none of these studies have reported correlations of plasma ghrelin with BOLD signal to food cues in the visual pathways, as seen in the current study (Sun et al. 2014; Jakobsdottir et al. 2016; Li et al. 2019; Karra et al. 2013; Kroemer et al. 2013; Wever et al. 2021; Roth et al. 2019).

Studies of the effects of exogenous ghrelin administration on brain function using fMRI have also found varied results with a number of different tasks (including passive or active viewing of food pictures, encoding, consolidation or recall of memory to food words, food incentive delay task, Pavlovian conditioning to food-odors), with no changes in seen in the occipital lobe in any studies (Malik et al. 2008; Goldstone et al. 2014; Kunath et al. 2016; Farokhnia et al. 2018; Han et al. 2018). Changes in resting BOLD signal in the occipital lobe have been seen in one study (Kunath et al. 2016), but not others (Jones et al. 2012).

2.6.4. Inter-Relationship of Post-Prandial Plasma LEAP2 with Plasma AG

The current study found no evidence for inhibition of plasma LEAP2 by plasma AG to explain their reciprocal fasting and post-prandial regulation. Exogenous supraphysiological AG administration did not decrease post-prandial plasma LEAP2, and furthermore there was no positive correlation between the post-prandial decrease in plasma AG and post-prandial increase in plasma LEAP2 (and in fact there was a trend for a negative correlation).

This conclusion is supported by the finding that plasma LEAP2 and liver *Leap2* mRNA expression are unaltered in *Ghsr* knockout mice (Islam et al. 2020). By contrast, there is a suggestion in rodents that LEAP2 may suppress AG, as in food-restricted mice introduction of adeno-associated virus (AAV) expressing *Leap2*, increasing plasma LEAP2 to three times normal, attenuated the expected rise in plasma AG (Ge et al. 2018). However, acute exogenous LEAP2 administration in men without obesity, to increase plasma LEAP2 to ~2.6 times normal when fed, did not alter plasma AG, though this could be a dose or duration effect rather than species difference (Hagemann et al. 2022).

2.6.5. Relationship of Post-Prandial Plasma LEAP2 with Glucose and Fat Metabolism

A previous study suggested that post-prandial increases in plasma LEAP2 may be regulated by post-prandial increases in plasma glucose due to their positive correlation in obesity, though absolute values at multiple time points were correlated in one regression model rather than incremental changes, with no accounting for within-participant factors (Mani et al. 2019). However, in the current study of adults without obesity, the lack of positive correlations suggests that post-prandial increases in plasma

LEAP2 are not driven by post-prandial increases in plasma glucose, serum insulin, or triglycerides. Decreases in AG/LEAP2 molar ratio did correlate negatively with post-prandial increases in plasma glucose, but this appeared to be driven primarily by post-prandial decreases in plasma AG. If glucose is stimulating LEAP2 secretion it remains unknown if this is via changes in portal vein to liver, arterial glucose to liver or small intestine, or an effect of glucose on the luminal surface of the small intestine whose subsequent absorption into the circulation produces an indirect association.

In a previous study, post-prandial increases in plasma LEAP2 positively correlated with BMI across a combined cohort of women with normal weight and obesity, although this did not appear to be present in those without obesity alone, and no adults with overweight were included (Mani et al. 2019). However, in the current study of men/women without obesity, higher BMI was associated with lower post-prandial increases in plasma LEAP2. This may suggest that there is a U-shaped relationship between post-prandial increases in plasma LEAP2 and BMI, though this needs confirmation in larger studies across a range of BMI and both sexes.

2.6.6. Relationship of Post-Prandial Plasma AG with Glucose and Fat Metabolism

Ghrelin reduces insulin sensitivity and insulin secretion and increases GH release and plasma glucose (including via GH action to increase gluconeogenesis) (Gray et al. 2019). Hence, as part of a homeostatic feedback loop, insulin is known to reduce plasma AG, apparently via direct actions of insulin on ghrelin-secreting gastric cells, which may also contribute to the post-prandial decreases in plasma AG. Insulin perfused into the rat stomach inhibits ghrelin secretion (Kamegai et al. 2004; Lippl et al. 2004). Furthermore, peripheral insulin administration suppressed plasma AG in wild-type mice but not mice with insulin receptor knockout in ghrelin-expressing cells (GhIR-knockout), while similarly hyperinsulinaemic-hypoglycaemic and hyperinsulinaemic-euglycemic clamps reduced plasma AG in wild type mice but not in GhIR-knockout mice (Shankar et al. 2021b). Furthermore, in 24 hour fasted mice, refeeding reduced plasma AG in wild-type mice but not in GhIR-knockout mice, despite no difference in the post-prandial increases in plasma LEAP2 (Shankar et al. 2021b). Similar results were seen in mice with diet induced obesity, where in reductions in plasma AG were seen in wild-type mice but not in GhIR-knockout mice suggesting that hyperinsulinaemia is mediating the reduction in plasma AG in obesity (Shankar et al. 2021b). This suggests that post-prandial increases in insulin

mediates post-prandial decreases in AG but not increases in plasma LEAP2 in rodents.

In humans, euglycaemic hyperinsulinaemic clamps inhibits secretion of total ghrelin in adults without obesity (Mohlig et al. 2002; Flanagan et al. 2003), while over a 24 hour period plasma total ghrelin changes reciprocally with serum insulin in adults without obesity (Cummings et al. 2001). Furthermore, correlational studies implicate a role for post-prandial increases in circulating insulin to decrease plasma ghrelin post-prandially in humans. In boys with obesity, post-prandial insulin AUC negatively correlated with post-prandial AUC plasma total ghrelin (Maffeis et al. 2006).

However, acute administration of subcutaneous insulin bolus with intravenous glucose infusion in adults with normal weight did not suppress plasma AG, unlike oral glucose or liquid meal ingestion, suggesting a lack of a role for post-prandial insulin secretion in directly suppressing ghrelin secretion (Caixas et al. 2002). In support of this conclusion, in our study of adults without obesity the post-prandial decrease in plasma AG was not correlated with the post-prandial increase in insulin. These differences between studies may be explained by assay of AG rather than total ghrelin, and the methodology of correlating post-prandial changes in insulin with changes in AG rather than absolute values.

2.6.7. Limitations

Small samples may also have contributed to type 1 and type 2 errors and so confirmation of the study findings with larger samples sizes are needed. Furthermore, as the current study was only correlational, to further examine the influences of LEAP2 on eating behaviour in humans, the effects of exogenous LEAP2 administration on appetite and food intake need to be investigated in obesity, and on food cue reactivity in both adults without and with obesity.

Due to the limited duration of blood sampling post-prandially, we were not able to determine the timing of the post-prandial peak in plasma LEAP2, and so longer duration post-prandial or 24-hour studies are required to ascertain the temporal dynamics of LEAP2 secretion. Furthermore, this study did not report plasma LEAP2 concentrations at the earlier timepoint at 40 mins after food intake, and so an earlier peak of plasma LEAP2 cannot be definitively excluded. This missing timepoint is unlikely to have affected the

correlational results with hormonal-metabolic mediators of the post-prandial increase in plasma LEAP2, since results excluding the t=+40min time point for plasma AG, glucose, serum insulin and triglycerides gave similar lack of significant correlations.

The sample size was small and sex ratio unbalanced and so it was not possible to examine whether correlations of post-prandial plasma LEAP2 with eating behaviour differed between sexes. Indeed, sex differences have been seen in body weight and food intake in *Leap2* knockout mice with greater increases seen in females (Shankar et al. 2021a). The current study did not include adults with obesity that have higher fasting and post-prandial plasma LEAP2 (Mani et al. 2019), and so future examination of relationships between plasma LEAP2 and eating behaviour needs to be conducted across all BMI categories, as well as children/adolescents since this study only included adults.

2.6.8. Conclusions

Taken together, these novel findings are consistent with the hypothesis that post-prandial increases in plasma LEAP2 (through its actions to decrease GHSR signalling as shown in published pre-clinical studies) is involved in homeostatic human energy balance by contributing to post-prandial reductions in appetite, food intake and food cue reactivity. Furthermore, these post-prandial changes in plasma LEAP2 may be more important than post-prandial decreases in plasma AG. The metabolic mechanisms underlying this post-prandial increase in plasma LEAP2 remains incompletely understood with no evidence found for a relationship with post-prandial changes in AG, glucose, insulin nor triglycerides.

2.7. ACKNOWLEDGEMENTS

We would like to acknowledge the authors of the original paper (Goldstone et al. 2014) from the Metabolic and Molecular Imaging Group, MRC Clinical Sciences Centre, Imperial College London, and Section of Investigative Medicine, Division of Diabetes, Endocrinology and Metabolism, Imperial College London, who performed the original study including participant recruitment, study visits, data collection and analysis, and fMRI scanning (Samantha Scholtz, Alexander Miras, Navpreet Chhina, Giuliana Durighel, Seyedeh Deliran), provision of AG peptide (Damian Ashby, Mohammad Ghatei, Stephen Bloom) and student supervision (Jimmy Bell, Gary Frost), and from Department of Endocrinology, University of Virginia, USA who performed the plasma AG assay (Bruce Gaylinn, Michael Thorner).

This research was funded by UK Medical Research Council (MRC) core funding to Metabolic and Molecular Imaging Group, MRC Clinical Sciences Centre; UK Research and Innovation MRC Population and Systems Biology Board project grant (MR/T017279/1); Wellcome Trust Research Training Fellowship (to S.S.); MRC Clinical Training Fellowship (to A.D.M.); Imperial College Healthcare Charity Fellowship (to N.C.); European Union Marie-Curie Fellowship (NuSISCO, to C.G.P). Infrastructure support was provided by the National Institute for Health Research (NIHR) Imperial Biomedical Research Centre and the NIHR Imperial Clinical Research Facility. The views expressed are those of the author(s) and not necessarily those of the National Health Service (NHS), the NIHR or the Department of Health and Social Care (DHSC).

CHAPTER 3: EFFECT OF LOW-CALORIE DIET WITH/WITHOUT DUODENAL-JEJUNAL BYPASS LINER INSERTION ON PLASMA LEAP2 IN ADULTS WITH OBESITY AND TYPE 2 DIABETES MELLITUS

3.1. ABSTRACT

Introduction: The liver/foregut satiety hormone LEAP2 is an inverse agonist at the acyl ghrelin receptor (GHSR), increasing after food intake and decreasing after bariatric surgery and short-term non-surgical weight loss, but effects of long-term dietary weight loss are unknown.

Methods: Plasma LEAP2 was measured in a previously published two-year trial comparing standard medical management (SMM) (including 600 kcal/day deficit) with duodenal-jejunal bypass liner (DJBL, Endobarrier) insertion (explanted after one year) in adults with obesity and inadequately controlled type 2 diabetes mellitus (T2DM). The objective of this study was to examine and compare the effects of these interventions on fasting and post-prandial plasma LEAP2 and investigate potential metabolic mediators of change in plasma LEAP2.

Results: In SMM group (n=25-37), weight decreased by 4.3%, 8.1%, 7.8%, 6.4% at 2, 26, 50, 104 weeks and fasting plasma LEAP2 decreased from baseline mean ± SD 15.3 ± 0.9 ng/mL by 1.7, 3.8, 2.1, 2.0 ng/mL respectively. Absolute/decreases in fasting plasma LEAP2 positively correlated with absolute/decreases in BMI, HbA1c, fasting plasma glucose, serum insulin, HOMA-IR and serum triglycerides.

Despite greater weight loss in DJBL group (n=23-30) at 26-50 weeks (10.4-11.4%), the fall in fasting plasma LEAP2 was delayed and attenuated (vs. SMM), which may contribute to greater weight loss by attenuating GHSR signalling. Plasma LEAP2 did not increase with weight regain from 50-104 weeks after DJBL explant, suggesting a new set-point with weight loss maintenance.

Increases in plasma LEAP2 after 600kcal meal (10.8-16.1% at 1-2h) were unaffected by weight loss, improved glucose metabolism or DJBL insertion (n=9-25), suggesting liver rather than duodenum/jejunum may be the primary source of post-prandial LEAP2 secretion.

Conclusions: These findings add to our understanding of the regulation and potential physiological role of plasma LEAP2.

3.2. INTRODUCTION

Obesity is a chronic relapsing disease, associated with significant societal and economic effects. It is a leading cause of premature death and serious health burden (WHO 2022). Several methods to address weight management have been tried with varied success, including caloric restriction, bariatric surgery and newer licensed oral/injectable medications such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) receptor analogues (Perdomo et al. 2023). Identification of further therapeutic targets that improve weight loss and more importantly maintain this reduction in obesity are needed, especially as some people do not wish to have surgery, cannot tolerate these medications, have weight loss that may not normalise body composition, and increasingly combination therapies targeting gut hormones are being used to improve weight loss outcomes.

Ghrelin is a stomach-derived orexigenic hormone that stimulates appetite and food intake as its active form acyl ghrelin (AG) via the growth hormone secretagogue receptor (GHSR) in the vagus nerve, hypothalamus, brainstem and brain reward circuits (Sumithran et al. 2011; Schalla et al. 2019; Srivastava et al. 2018; Jerlhag 2019; Kojima et al. 2006; Korbonits et al. 2004; Stengel et al. 2011). AG also increases plasma growth hormone, cortisol and glucose, and reduces insulin secretion (Muller et al. 2015; Poher et al. 2018; Goldstein et al. 2011).

In rodents and humans, states of negative energy balance, including fasting and weight loss from caloric dietary restriction (low-calorie (LCD) or very low-calorie diet (VLCD)), increase plasma AG, while food intake and obesity reduce plasma AG (Muller et al. 2015; Wang et al. 2022; Mani et al. 2019; Aukan et al. 2023; Nymo et al. 2017; Nymo et al. 2018; Lyngstad et al. 2019; Coutinho et al. 2018; Peos et al. 2021). Indeed, plasma total ghrelin remains elevated even at 1 year after cessation of an eight-week VLCD for obesity (Sumithran et al. 2011). Rodent studies support a role for the increase in plasma AG induced by weight loss in weight regain after cessation of caloric restriction (Briggs et al. 2013). In humans, there is variable evidence that increases in plasma total ghrelin or AG after diet-induced weight loss predicts subsequent weight regain (Thom et al. 2020; Thom et al. 2021; Puklin et al. 2021; Garcia et al. 2006; Buso et al. 2021).

Liver-expressed antimicrobial peptide 2 (LEAP2) is a hormone with identical structure in mice and humans, expressed in liver, jejunum and duodenum (Krause et al. 2003; Henriques et al. 2010; Howard et al. 2010; Englund et al. 2024). Recently, in pre-clinical studies, LEAP2 has been identified as a competitive AG antagonist and inverse agonist at the constitutively active GHSR (Ge et al. 2018; M'Kadmi et al. 2019; Wang et al. 2019; Mani et al. 2019). Plasma LEAP2 is regulated in a reciprocal manner to AG, i.e. decreases with fasting/weight loss, and increases with refeeding/weight gain in rodents and humans (Ge et al. 2018; M'Kadmi et al. 2019; Wang et al. 2019; Fernandez et al. 2022; Mani et al. 2019; Hagemann et al. 2021; Hagemann et al. 2022). In mice, peripheral and central LEAP2 administration antagonises orexigenic effects of AG administration, and on its own reduces food intake (Barrile et al. 2019; Cornejo et al. 2019; Islam et al. 2020; Hagemann et al. 2022), and a long-acting LEAP2 peptide reduces weight hepatic steatosis over four weeks in diet-induced obesity (Shankar et al. 2024). *Leap2* gene deletion enhances the orexigenic effects of exogenous AG in mice (Shankar et al. 2021a).

Intravenous LEAP2 administration to men without obesity reduced *ad libitum* food intake without side effects, though without any reduction in appetite ratings (Hagemann et al. 2022). In adults without obesity, post-prandial increases in LEAP2 correlated positively with post-prandial decreases in appetite and food cue reactivity to high-energy foods, with a trend for decreases in *ad libitum* food intake (Bhargava et al. 2023), while in adults with overweight/obesity fasting plasma LEAP2 negatively correlated with hunger ratings (Andreoli et al. 2024a). These data point to plasma LEAP2 being an important satiety signal by modifying GHSR signalling in humans.

Regulation of fasting and post-prandial increases in plasma LEAP2 is not fully understood, and it remains unclear from rodent, human and organoid studies whether liver, duodenum or jejunum are differential sources of plasma LEAP2, especially post-prandially (Islam et al. 2020; Holm et al. 2022; Gradel et al. 2023; Englund et al. 2024). Similarly, human studies correlating fasting and post-prandial increases in plasma LEAP2 with fasting and post-prandial increases in metabolic markers, often suggest potential roles for blood glucose, insulin and/or triglycerides in stimulation of LEAP2

secretion (Mani et al. 2019; Andreoli et al. 2024a; Bhargava et al. 2023; Li et al. 2022; Ma et al. 2020; Stark et al. 2023), while effects of exercise and ketone administration in rodents and humans have suggested that beta-hydroxybutyrate may inhibit hepatic LEAP2 secretion (Pedersen et al. 2023; Holm et al. 2022; Holm et al. 2023). Other possible mediators for post-prandial increases in plasma LEAP2 could be direct contact of nutrients with intraluminal surfaces of the duodenum/jejunum or portal venous delivery of glucose, triglycerides or other metabolites to the liver. Indeed, the greater post-prandial release of plasma LEAP2 after oral ingestion than intravenous administration of glucose implicates an 'incretin' effect (Pedersen et al. 2023).

In humans, weight loss after Roux-en-Y gastric bypass (RYGB) and vertical sleeve gastrectomy (VSG) surgery for obesity reduced fasting and/or postprandial plasma LEAP2 (Mani et al. 2019). Short-term 2 weeks LCD also reduced fasting plasma LEAP2 in obesity, though plasma LEAP2 tended to increase post-glucose ingestion (Ragland et al. 2023). However, the effects of long-term dietary caloric restriction for obesity causing weight loss on plasma LEAP2, and the potential metabolic mediators, are unknown in humans.

Insertion of duodenal-jejunal bypass liner (DJBL, Endobarrier device), is a non-surgical endoscopic intervention, where a 60cm plastic tube lines the proximal small bowel, mimicking the foregut effects of RYGB surgery, as food is not in contact with the foregut after ingestion. DJBL insertion has greater weight loss and often superior improvement in glycaemic control (vs. LCD and standard medical management alone), in adults with obesity and inadequately-controlled T2DM (Betzel et al. 2017a; Koehestanie et al. 2014; de Jonge et al. 2015), and in our recent phase 2 clinical trial (Glaysher et al. 2017; Ruban et al. 2020; Glaysher et al. 2021; Ruban et al. 2022; Aldhwayan et al. 2022), and improvement in non-alcoholic steatohepatitis (NASH) (de Jonge et al. 2013; Karlas et al. 2022). Examining the effects of DJBL insertion on plasma LEAP2 enables comparison of the role of duodenal/jejunal vs. hepatic origins of LEAP2 as food is excluded from contact with the former.

Therefore, the hypotheses for the current study were that in adults with obesity, long-term weight loss through caloric restriction will: (i) decrease fasting and/or post-

prandial plasma LEAP2, (ii) which will positively correlate with decreases in weight, improvements in glucose and/or fat metabolism, while (iii) weight regain will increase fasting plasma LEAP2, and (iv) DJBL insertion will exaggerate the decrease in fasting and/or post-prandial plasma LEAP2, due to loss of contact of ingested food with the duodenum/jejunum.

Hence, plasma LEAP2 was measured in a secondary analysis of our previously published Phase 2 clinical trial comparing effects of standard medical management (SMM) including caloric restriction and increased physical activity, without or with DJBL insertion over 12 months, with a further 12 month follow-up after DJBL removal, in adults with obesity and T2DM (Glaysher et al. 2017; Ruban et al. 2020; Ruban et al. 2022; Glaysher et al. 2021; Aldhwayan et al. 2022). Participants had multiple visits after an overnight fast, with a sub-group also consuming a fixed meal, with measurement of fasting and post-prandial plasma LEAP2 and glucose, serum insulin and triglycerides, and HbA1c.

3.3. METHODS

3.3.1. Study Design

Participant data is taken from a randomised, controlled interventional clinical trial, in a cohort of patients with obesity (body mass index (BMI) 30-50 kg/m^2) and inadequately controlled T2DM (HbA1c 7.7-11.1% or 58-97 mmol/mol, on oral hypoglycaemic medication only) (Glaysher et al. 2017; Ruban et al. 2020; Ruban et al. 2022). Participants recruited received standard medical management (SMM) for obesity and T2DM including dietary restriction, increased physical activity, and escalation of T2DM medication following American Diabetes Association (ADA) guidelines (Chamberlain et al. 2017), alone or in addition to an endoscopic insertion of DJBL Endobarrier device, as in the original protocol (Glaysher et al. 2017). This involved advice to follow a daily low-calorie diet of 1200-1500 kcal for women and 1500-1800 kcal for men, eat regularly every day (5 times/ day), control portion sizes, and intake of carbohydrates/starchy foods, increase intake of low glycemic index and high protein foods, and vegetables, and reduce intake of foods high in fat, sugar, and alcohol. Participants were advised to include more physical activity in their daily routine for example to walk more every day, with a goal of 2.5 hours a week of moderate intensity and 75 minutes a week of vigorous intensity aerobic activity, and muscle strengthening activities >2 days a week.

Participants were further split into mechanistic sub-groups, included in this analysis, undergoing fMRI or taste experimental visits (Figure 3.1).

Data is taken from clinical visits at -2, 2, 26, 50 and 104 weeks. Participants arrive after an overnight fast for each visit. With implant of DJBL device at week 0 and explant at 52 weeks. Visit at -2 weeks consisted of baseline measurements including a bloodwork, anthropometry (BMI, body fat percentage) and record of eating habits, diet, and lifestyle (Figure 3.1). Visits at 2, 26, 50 and 104 weeks are follow-ups to the treatment intervention (Figure 3.1).

A sweet taste detection task was performed in the taste mechanistic sub-group only at weeks -2, 2 and 26, where seven incremental sucrose concentrations were used to examine sweet taste detection thresholds, before the fasting blood samples were

taken (group included as covariate in correction of fasting and post-prandial outcomes) (Glaysher et al. 2017; Ruban et al. 2020; Ruban et al. 2022).

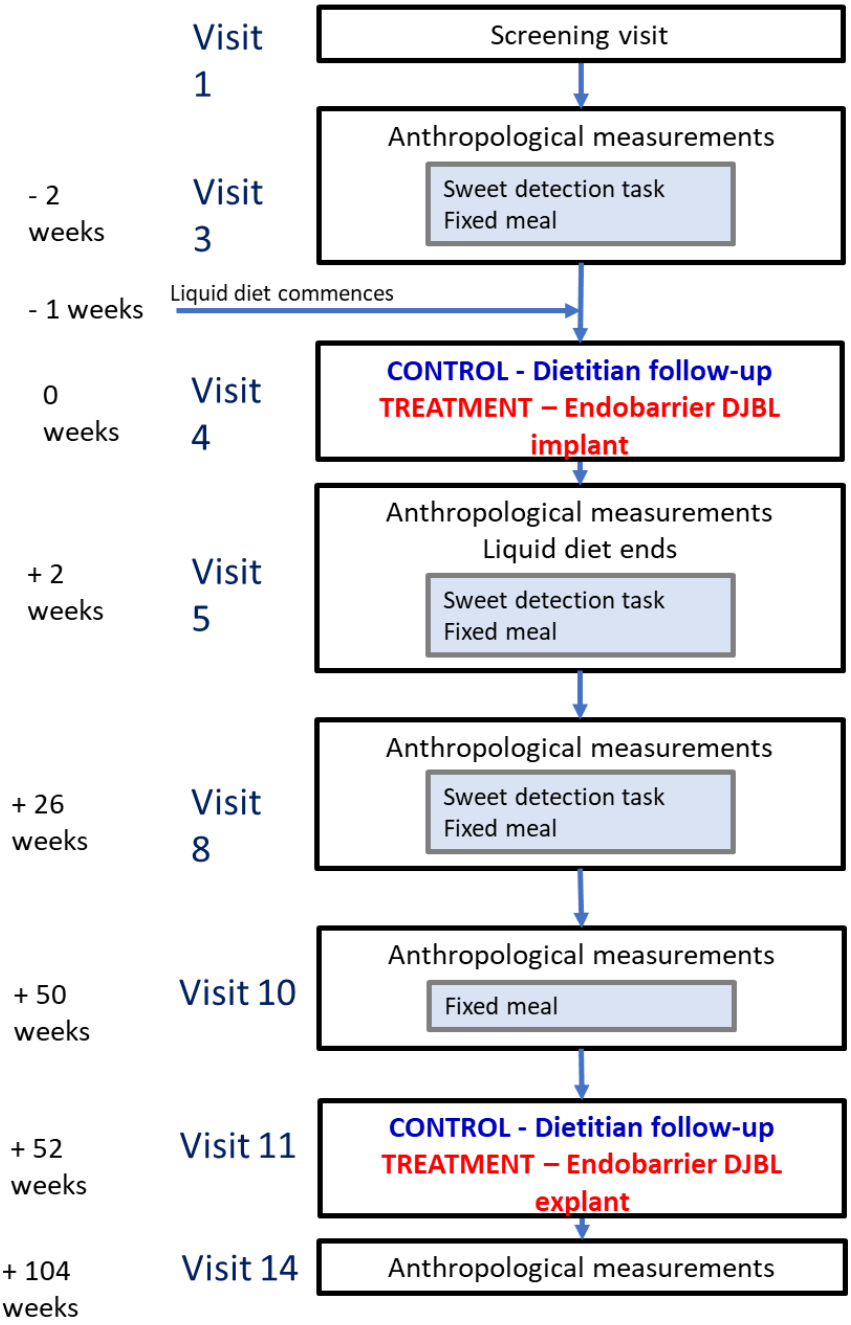


Figure 3.1. Study design: outline of randomised controlled trial.
Participants at the screening visit, were randomised into duodenal-jejunal bypass liner (DJBL) insertion or standard medical management (SMM) alone and further divided into a mechanistic taste subgroup (blue box). Visits at -2, 2, 26 weeks are similar, where data used for analysis: fasting bloods (both sub-groups) and post-prandial bloods (taste sub-group only); anthropological measurements (weight, height, body fat percentage, fasting bloods) and appetite ratings (visits at -2, 2, 26, 50, 104 weeks); sweet detection task (-2, 2, 26 weeks) and fixed meal consumption (-2, 2, 26, 50 weeks) in taste sub-group only (blue box). Liquid diet of 1300 kcal (females) or 1500 kcal (males) was taken by all participants across both groups from -1 to 2 weeks. At 52 weeks, the DJBL device was removed, with 104 weeks being the 1-

year follow-up after the DJBL implant was removed. Adapted from (Glaysher et al. 2017).

3.3.2. Fixed Test Meal

In the sub-group examining post-prandial changes, participants were given a fixed meal consisting of 250mL vanilla Fortisip Compact milkshake (Nutricia, Trowbridge, Wiltshire, UK), containing 600 kcal (carbohydrate 49%, fat 35%, protein 16%), at 1.5h after starting the sweet taste task, at -2, 2, 26 and 50 week visits, with post-prandial blood samples taken at t=0, 60 and 120 mins.

3.3.3. Inclusion and Exclusion Criteria for Endobarrier Study

Inclusion criteria for participants were diagnosis of type 2 diabetes mellitus (T2DM) for at least one year, age 18–65 years, male or female, HbA1c 7.7%–11.0% (equivalent to 58–97mmol/mol), on oral glucose lowering medications only, and body mass index (BMI) 30–50 kg/m^2.

Exclusion criteria included use of insulin or GLP-1 analogues at recruitment, previous bariatric surgery, history of anaemia, liver or kidney impairment, severe mental illness, current depression, and (for the fMRI sub-group only) current smokers and gluten or lactose intolerance, being vegan or vegetarian. For complete list see published protocol paper (Glaysher et al. 2017; Ruban et al. 2020).

All participants gave written informed consent, and the study was approved by the NHS Research Ethics Committee London - Fulham (REC 14/LO/0871) and conducted in accordance with the principles of the Declaration of Helsinki.

3.3.4. Blood Collection, Storage and Assay

Fasting blood samples were taken at -2, 2, 26, 50 and 104 week visits, with post-prandial samples collected at the same visits excluding week 104. Plasma glucose, and serum insulin, triglycerides and HbA1c were assayed using routine clinical laboratory assays as reported previously (Glaysher et al. 2017; Ruban et al. 2020). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula: Fasting insulin (mU/L) x fasting glucose (mmol/L)/22.5 (Matthews et al. 1985). Blood was collected in lithium heparin tubes containing protease inhibitors

aprotinin (Trasylol, Nordic Pharma Ltd., UK) and AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride) (A8456; Sigma-Aldrich, Dorset, UK), as previously described (Mani et al. 2019; Bhargava et al. 2023). Plasma samples were stored at -80°C for future measurement of gut hormones.

Plasma LEAP2 concentrations were measured using a commercial enzyme immunoassay (EK-075-40, Phoenix Pharmaceuticals Inc., CA, USA) according to the manufacturer's instructions. This LEAP2 assay has been previously validated using human samples (Mani et al. 2019; Bhargava et al. 2023). Plasma samples were diluted 10 times in the assay buffer. The detectable assay range was 0-100 ng/mL, and inter-assay and intra-assay coefficients of variation were both within the manufacturer guidance at 14.9% and 4.6% respectively.

3.3.5. Appetite Visual Analogue Scale Ratings

Serial ratings of hunger, fullness, pleasantness to eat, volume of food able to eat were recorded on a 100 mm visual analogue scale (VAS) using a tablet computer at each visit. Composite appetite was calculated by combining these ratings: appetite = [hunger + pleasantness + volume + (100 - fullness)] / 4 (Bhargava et al. 2023).

3.3.6. Statistical Analysis

All statistical analysis was performed using IBM SPSS version 29 (Armonk, NY, USA) and GraphPad Prism v9.5.1 (San Diego, CA, USA) with effects considered significant at $P<0.05$ and a trend at $P<0.10$.

Results for some of the clinical and appetite outcomes, with comparison between groups, from the trial have already been published in several papers, including for BMI, weight loss, HbA1c, fasting plasma glucose, serum insulin and triglycerides, fasting and post-prandial hunger, fullness and composite appetite ratings (Ruban et al. 2020; Ruban et al. 2022; Glaysher et al. 2021; Aldhwayan et al. 2022). These longitudinal results are also included in the current analysis, as the included participants differ from that previously published, since in this report outcomes were only analysed from trial participants in the functional neuroimaging and taste mechanistic sub-groups 1 and 3, and only in those who had plasma LEAP2 measurements available at baseline and at

least at one follow-up visit (n=67 out of n=113 total in primary analysis population). This allows combination of results across mechanistic sub-groups 1 and 3, and the appropriate direct visualisation, comparison and statistical corelation of these clinical outcomes with plasma LEAP2.

3.3.7. Longitudinal Analysis of Study Outcomes

Anthropological measures: Demographic data is presented as mean ± SEM or median [interquartile range, IQR] (Table 3.1). Data was checked for normal distribution using the Shapiro-Wilk test (Table 3.1). A mixed-effect models was performed with group (SMM vs. DJBL) as between-participant factor, and weeks as a within-participant factor, to examine significant changes in BMI, % weight loss, HbA1c, appetite, hunger and fullness ratings, encoding 'Yes' as 1 and 'No' as 0, at each visit (-2 to 104 weeks) (Table 3.4, 3.5, Figure 3.2). Post-hoc analyses using Fisher's LSD test examined differences from baseline visit (-2 weeks) in each group and between groups at each visit. A mixed-effects model was performed on the medication participants took throughout the study duration to examine significant changes within drug classes at each visit (-2, 2, 26, 50, 104 weeks). The drug classes investigated were: metformin, sulphonylureas (SU), sodium-glucose co-transporter 2 inhibitors (SGLT2i), dipeptidyl peptidase 4 inhibitors (DPP4i), glucagon-like peptide-1 receptor agonists (GLP1RA), thiazolidinediones (TZD), insulin, meglitinide and beta adrenoreceptor antagonists (beta-blockers) (Figure 3.2, Tables 3.4, 3.5).

Fasting analysis: Longitudinal data from the Endobarrier study were analysed using mixed-effects model to examine changes in dependent variables: absolute fasting plasma LEAP2, BMI, changes in: fasting plasma LEAP2, % weight, HbA1c, fasting plasma glucose, insulin, HOMA-IR, plasma triglycerides, and fasted VAS appetite, hunger and fullness ratings across all visits (vs. -2 week visit). Analysis included the following between-participant factors: participant ID, group (SMM vs. DJBL) and weeks as a within-participant factor. Post-hoc analyses using Fisher's LSD test to examine differences from baseline visit (-2 weeks) and between groups at each visit. This analysis was also run separately for each group to identify any within-group changes in fasting plasma LEAP2, BMI, % weight, HbA1c, fasting plasma glucose, HOMA-IR, plasma triglycerides, and fasted appetite, hunger and fullness VAS ratings.

Sweet taste task was additionally included only for fasting plasma LEAP2, plasma glucose, insulin and HOMA-IR (Tables 3.1, 3.2).

Post-prandial analysis: In the taste cohort, the incremental area under the curve (iAUC0-120min) was calculated using the trapezoid rule for post-prandial plasma LEAP2 and fed appetite VAS ratings, by subtracting fasting (t=0mins) from fed (t=+60 to +120mins after food intake) measures for each participant. Identical analyses were performed in the taste sub-group of the Endobarrier study for % weight change, post-prandial plasma LEAP2 $iAUC_{0\text{-}120min}$, appetite $iAUC_{0\text{-}120min}$, fullness $iAUC_{0\text{-}120min}$, but without the sweet taste task correction. Comparisons between time points (t=0,60 and 120mins) for increase in post-prandial plasma LEAP2 was made using a general linear model including time as within-participant factor, with post-hoc Fisher's LSD test at baseline visit (-2 weeks). Comparisons of post-prandial plasma LEAP2 response between visits (-2,2,26,50 weeks) within-group (SMM, DJBL) was performed using a mixed-effects model with post-hoc Fisher's LSD test.

3.3.8. Variables Correlating with Absolute and Changes in Plasma LEAP2

Fasting analysis: Linear mixed-effect model analysis was performed to examine the relationships between absolute and changes from baseline visit (-2 weeks) in outcome variables (BMI, % weight, glucose, HbA1c, insulin, HOMA-IR, triglycerides, appetite and hunger ratings) with absolute and changes from baseline visit (-2 weeks) of fasting plasma LEAP2. Bivariate analysis was conducted separately for the DJBL group from -2 to 50 weeks and SMM group from -2 to 104 weeks. The effect of the variable on the absolute and change from baseline visit (-2 weeks) of fasting plasma LEAP2 were analysed as the absolute and change from week -2 of variable: BMI, % weight, HbA1c, fasting plasma glucose, insulin, HOMA-IR, plasma triglycerides, and fasted VAS appetite and hunger ratings. Fasting plasma LEAP2, plasma glucose, insulin and HOMA-IR had values adjusted accordingly due to the sweet taste task. Bivariate analysis was conducted using a mixed-effects model the pseudo-r^2 value, slope (β) and P-value. This was also performed with both DJBL and SMM groups combined to look for significant group*independent variable interactions indicating differences in the slopes between groups.

DJBL explant sub-group: To examine the impact of weight regain and changes in metabolism with changes in plasma LEAP2 after DJBL explant, correlation analysis was performed to examine the relationships between outcome variables (weight, fasting glucose, HbA1c, insulin, HOMA-IR, triglycerides, hunger and appetite) and changes in fasting plasma LEAP2, for the DJBL group from 50 to 104 weeks, after the explant of the DJBL device, the relationships between change from 50-104 weeks in plasma LEAP2 with change from 50-104 weeks in % weight, HbA1c, fasting plasma glucose, insulin, HOMA-IR, plasma triglycerides, and fasted VAS appetite and hunger ratings. Correlation analysis was performed to examine the relationships between outcome variables (without any covariates) in the DJBL group from 50-104 weeks, reporting Pearson's (r_P) if data was normally distributed, or Spearman's (r_S) correlation coefficients if ether variable was not normally distributed and P-value

Post-prandial analysis: Identical analyses to the fasting analyses were performed in the sub-group having the fixed meal to examine the relationships between absolute and changes from baseline visit (-2 weeks) in outcome variables (BMI, % weight, HbA1c, post-prandial $iAUC_{0\text{-}120min}$ glucose, appetite and fullness ratings) with absolute and changes from baseline visit (-2 weeks) of post-prandial $iAUC_{0\text{-}120min}$ plasma LEAP2. Bivariate analysis was conducted separately for the DJBL group from -2 to 50 weeks and SMM group from -2 to 104 weeks. The effect of absolute and change from baseline visit (-2 week) of variable post-prandial plasma LEAP2 $iAUC_{0\text{-}120min}$ on the absolute and change from baseline visit (-2 week) of post-prandial appetite $iAUC_{0\text{-}120min}$, post-prandial hunger $iAUC_{0\text{-}120min}$ was investigated. Bivariate analysis was conducted using a mixed-effects model to report r^2 value, slope (β) and p-value. For correlations between post-prandial plasma LEAP2 and appetite, the difference in the incremental area under curve (iAUC) between 0 and 120 min ($iAUC_{0\text{-}120\ min}$) was calculated at -2, 2, 26 and 50 weeks, when the participants were given fixed test meals. Additional correlational analysis was performed across different studies using absolute difference in post-prandial (fed-fasted) plasma LEAP2 and meal size consumed in kilocalories.

3.3.9. Sweet Taste Task Adjustment

Data from weeks -2, 2 and 26 weeks from the taste sub-group were analysed using

mixed-effects model to examine the effect of sweet taste task on absolute variables: fasting plasma LEAP2, HbA1c, plasma glucose, serum insulin, HOMA-IR, plasma triglycerides, and fasting VAS appetite ratings across all visits, as this task was performed prior to blood collection and VAS ratings in this taste mechanistic sub-group (Table 3.1). Analysis included group (SMM vs. DJBL) and sweet taste task (yes, no) as between participant factors, and weeks as a within participant factor. For dependent variables with an overall main effect of sweet taste task, or sweet taste task*weeks interaction, adjusted values were calculated using the mean effect size for -2, 2, 26 weeks to remove the effects of sweet taste task. Manually adjusted dependent variables were then used in correlational analysis if the variable was significantly affected by sweet taste task.

There were no significant sweet taste task*weeks interactions, but there were significant main effects of sweet taste task on fasting plasma LEAP2, plasma glucose, serum insulin and HOMA-IR (P=0.022 to <0.001) (Tables 3.1, 3.2). The mean effect size of participating in the sweet taste task at individual visits for each group was calculated from post-hoc analysis and used to adjust fasting plasma LEAP2, plasma glucose, serum insulin, and HOMA-IR with adjusted values.

Main effect of sweet taste task on dependent variable				
Dependent variable	**Parameters**	**Group**	**Sweet taste task**	**Sweet taste task*Weeks**
LEAP2	Numerator df	1	1	2
	Denominator df	68.09	255.00	209.09
	F	0.40	15.78	2.93
	P value	0.53	**<0.001******	0.055
Appetite	Numerator df	1	1	2
	Denominator df	68.07	254.00	209.45
	F	0.11	2.27	0.28
	P value	0.74	0.13	0.75
Glucose	Numerator df	1	1	2
	Denominator df	68.59	265.00	219.93
	F	8.57	12.68	1.92
	P value	**0.005*****	**<0.001******	0.15
Insulin	Numerator df	1	1	2
	Denominator df	68.09	258.00	212.67
	F	0.22	5.33	1.51
	P value	0.64	**0.022***	0.22
HOMA-IR	Numerator df	1	1	2
	Denominator df	68.41	256.18	215.35
	F	1.49	10.62	0.44
	P value	0.23	**0.001*****	0.65

Table 3.1. Main effects on absolute: fasting plasma LEAP2, glucose, insulin, HOMA-IR and appetite ratings across weeks.

This table describes the F statistic, numerator and denominator of degrees of freedom for the main effects of group, sweet taste task and sweet taste task*weeks on each dependent variable (plasma LEAP2, appetite ratings, glucose, insulin and HOMA-IR). Weeks includes all 5 visits at -2, 2, 26, 50 and 104 weeks, with the sweet taste task done only at weeks -2, 2 and 26. Group includes the SMM and DJBL groups. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: HOMA-IR, homeostatic model assessment for insulin resistance; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

Post-hoc analysis showing difference of sweet taste task (Taste - Non-taste)										
		SMM			DJBL			DJBL vs. SMM		
Dependent variable	Parameters	-2 w	2 w	26 w	-2 w	2 w	26 w	-2 w	2 w	26 w
LEAP2	Mean	-4.46	-2.57	-0.05	-3.51	-4.56	-2.38	-0.20	1.89	1.06
	SE	1.44	1.55	1.51	1.34	1.48	1.39	1.39	1.59	1.46
	P-value	**0.002*** **	0.097	0.97	**0.009** **	**0.002*** **	0.089	0.89	0.24	0.47
	95 CI Lower	-7.30	-5.62	-3.03	-6.15	-7.48	-5.12	-2.94	-1.25	-1.81
	95 CI Upper	-1.63	0.47	2.93	-0.87	-1.63	0.36	2.54	5.02	3.93
Appetite	Mean	5.93	-6.53	6.75	9.26	12.28	5.36	3.46	9.61	-0.28
	SE	7.36	7.77	7.62	6.75	7.45	6.97	6.89	7.88	7.18
	P-value	0.42	0.40	0.38	0.17	0.101	0.44	0.62	0.22	0.97
	95 CI Lower	-8.56	-21.84	-8.26	-4.03	-2.40	-8.37	-10.11	-5.91	-14.43
	95 CI Upper	20.43	8.78	21.76	22.55	26.95	19.08	17.03	25.13	13.87
Glucose	Mean	1.84	0.47	1.06	2.07	0.77	2.01	1.52	2.39	1.32
	SE	0.77	0.84	0.80	0.72	0.80	0.75	0.73	0.84	0.75
	P-value	**0.018***	0.57	0.19	**0.004*** **	0.34	**0.008** **	**0.038*** **	**0.005** **	0.082
	95 CI Lower	0.32	-1.17	-0.52	0.65	-0.81	0.53	0.08	0.74	-0.17
	95 CI Upper	3.36	2.12	2.64	3.50	2.35	3.50	2.95	4.04	2.80
Insulin	Mean	1.20	0.27	2.53	1.36	3.27	5.70	-1.15	2.83	2.94
	SE	2.03	2.25	2.08	1.90	2.12	1.98	2.01	2.35	2.05
	P-value	0.55	0.90	0.23	0.47	0.12	**0.004*** **	0.57	0.23	0.15
	95 CI Lower	-2.80	-4.16	-1.57	-2.38	-0.91	1.81	-5.10	-1.80	-1.09
	95 CI Upper	5.20	4.70	6.63	5.11	7.44	9.59	2.81	7.45	6.97
HOMA-IR	Mean	1.69	0.59	0.96	1.89	1.59	2.79	0.19	1.76	1.66
	SE	0.98	1.09	1.01	0.92	1.03	0.96	0.93	1.10	0.95
	P-value	0.086	0.59	0.34	**0.040***	0.12	**0.004*** **	0.84	0.11	0.081
	95 CI Lower	-0.24	-1.56	-1.03	0.09	-0.43	0.90	-1.63	-0.41	-0.20
	95 CI Upper	3.63	2.73	2.95	3.70	3.61	4.67	2.01	3.94	3.52

Table 3.2. Post-hoc analysis of absolute: fasting plasma LEAP2, glucose, insulin, HOMA-IR and appetite ratings across weeks.

This table describes the post-hoc analysis of results in Table 3.1. Mean difference (taste – non-taste), standard error, upper and lower confidence intervals for fasting plasma LEAP2, glucose, insulin, HOMA-IR and appetite VAS ratings at each visit. Weeks include the sweet taste task done at weeks -2, 2 and 26. Group includes the SMM and DJBL groups. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: VAS, visual analogue scale; HOMA-IR, homeostatic model assessment for insulin resistance; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

3.4. RESULTS

3.4.1. Demographics Of Participants

Participant demographics are presented in Table 3.3. Analysis in the entire Endobarrier study cohort used fasted outcomes, while analysis on the taste sub-group cohort used post-prandial outcomes. Reported outcomes differ from the previously published Endobarrier papers (Ruban et al. 2020; Glaysher et al. 2021; Ruban et al. 2022; Aldhwayan et al. 2022), as participants are only included who have plasma LEAP2 measurements available.

For the Endobarrier study, at baseline the SMM and DJBL groups had similar sex distribution, ethnicity, BMI, HbA1c, fasting insulin and HOMA-IR, however fasting glucose was higher in the DJBL than SMM group in the whole Endobarrier study (but not taste sub-group) (Tables 3.4, 3.5).

There was no visit*group interaction (P=0.34-0.64), nor main effect of group (P=0.14-0.96), on use of any glucose lowering medication group or beta-blockers, though there was the expected greater use of proton pump inhibitors from 2 to 50 weeks in the DJBL group (Tables 3.4, 3.5; Figure 3.2), as per study protocol (Glaysher et al. 2017; Ruban et al. 2020; Ruban et al. 2022).

Within and between group effects of the SMM and DJBL interventions on BMI, body weight, HbA1c, fasting plasma glucose, HOMA-IR, serum triglycerides and hunger, fullness and appetite VAS ratings are essentially identical in direction for the sub-group in the current analysis with plasma LEAP2 available, as for the complete primary analysis population and individual mechanistic sub-group analyses (Ruban et al. 2020; Ruban et al. 2022; Glaysher et al. 2021; Aldhwayan et al. 2022), and are included to enable direct comparison with plasma LEAP2.

		Fasting cohort					Post-prandial sub-cohort				
					DJBL vs. SMM					DJBL vs. SMM	
		All	SMM	DJBL	Test statistic	P	All	SMM	DJBL	Test statistic	P
N		67	30	37			40	17	23		
Age (years)	**Mean ± SD Median [IQR]**	54 [48, 58]	54 [49, 59]	52 [47, 58]	U 759	0.81	53 ± 6.9	55 ± 4.8	52 ± 8.0	t -1.08	0.29
	Range	32 - 64	31 - 64	33 - 64			34 - 64	48 - 64	34 - 64		
Female	**n (%)**	33 (43.4%)	15 (44.1%)	18 (42.9%)	χ2 0.06	0.80	20 (43.5%)	11 (55.0%)	9 (33.3%)	χ2 2.74	0.098
Caucasian	**n (%)**	55 (72.4%)	23 (67.6%)	32 (76.2%)	χ2 1.03	0.31	38 (82.6%)	16 (84.2%)	22 (81.5%)		>0.99
Weight (kg)	**Mean ± SD**	106.0 ± 16.9	102.5 ± 14.0	108.9 ± 18.6	t 1.43	0.16	105.9 ± 17.9	99.4 ± 13.9	110.5 ± 19.2	t 1.72	0.093
	Range	66 - 153	66 - 132	73 - 153			66 - 150	66 - 123	73 - 150		
BMI (kg/m2)	**Median [IQR]**	35.4 [32.6, 39.4]	34.9 [31.8, 38.8]	36.3 [32.8, 39.8]	U 661	0.45	34.9 [32.2, 39.0]	35.1 [31.9, 38.5]	34.0 [32.3, 39.3]	U 266	0.83
	Range	29 - 51	29 - 44	29 - 51			29 - 48	29 - 44	29 - 48		
T2DM	**n (%)**	76 (100%)	34 (100%)	42 (100%)			46 (100%)	19 (100%)	27 (100%)		
HbA1c	**Median**	61 [53, 73]	59 [52, 67]	63 [55, 75]	U 12214	**0.005*** **	73 [62, 82]	64 [58, 87]	75 [66, 82]	U 226	0.29

(mmol/mol)	**[IQR]**										
Fasting glucose (mmol/L)	**Mean ± SD**	10.1 ± 2.7	9.3 ± 2.4	10.7 ± 2.9	t 2.09	**0.040***	10.9 ± 2.6	10.2 ± 2.5	11.5 ± 2.6	t 1.45	0.15
Fasting insulin (mU/L)	**Median [IQR]**	9.7 [6.7, 15.9]	9.7 [6.7, 18.1]	9.2 [6.7, 14.6]	U 738	0.39	9.7 [6.7, 16.2]	9.9 [6.1, 18.7]	9.7 [7.0, 15.4]	U 232	0.87
HOMA-IR	**Median [IQR]**	4.7 [3.2, 6.5]	4.2 [2.7, 7.8]	4.9 [6.1, 3.6]	U 602	0.52	4.9 [3.3, 5.9]	4.6 [2.2, 9.1]	4.9 [3.8, 5.9]	U 203	0.59

Table 3.3. Participant characteristics at baseline visit.
Participants characteristics at baseline visit (-2 weeks) in both DJBL and SMM with plasma samples available when fasting and in post-prandial sub-cohort. Characteristics of participants with continuous variables denoted as mean ± standard deviation (SD) or median and interquartile range (IQR) if not normally distributed with range, or n (%). Comparisons made using, independent samples t-test, t test statistic; Mann-Whitney U test, U statistic and Chi-squared test, $\chi 2$ statistic. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: BMI, body mass index; DJBL, duodenal-jejunal bypass liner; HOMA-IR, homeostasis model assessment for insulin resistance; kg, kilogram; SMM, standard medical management; T2DM, type 2 diabetes mellitus. Ranges: HbA1c normal 20-41 mmol/mol; fasting glucose >7.0 mmol/L diagnostic of T2DM; fasting insulin normal 3-15 mU/L; HOMA-IR optimal <1, >1.9 early insulin resistance, >2.9 marked insulin resistance.

Medications for every visit															
	-2 weeks			2 weeks			26 weeks			50 weeks			104 weeks		
	All	SMM	DJBL	All	SMM	DJBL	All	SMM	DJBL	All	SMM	DJBL	All	SMM	DJBL
N	67	30	37	52	25	27	67	30	37	66	30	36	67	30	37
Metformin (n,%)	65 (97.0)	29 (96.7)	36 (97.3)	48 (92.3)	23 (92.0)	25 (92.6)	59 (88.1)	26 (86.7)	33 (89.2)	57 (86.4)	27 (90.0)	30 (83.3)	41 (61.2)	16 (53.3)	25 (67.6)
Sulphonylurea (n,%)	27 (40.3)	11 (36.7)	16 (43.2)	17 (32.7)	8 (32.0)	9 (33.3)	18 (26.9)	6 (20.0)	12 (32.4)	14 (21.2)	5 (16.7)	9 (25.0)	10 (14.9)	2 (6.7)	8 (21.6)
SGLT2i (n,%)	15 (22.4)	5 (16.7)	10 (27.0)	12 (23.1)	4 (16.0)	8 (29.6)	17 (25.4)	8 (26.7)	9 (24.3)	24 (36.4)	10 (33.3)	14 (38.9)	23 (34.3)	7 (23.3)	16 (43.2)
DPP4i (n,%)	20 (29.9)	9 (30.0)	11 (29.7)	14 (26.9)	8 (32.0)	6 (22.2)	18 (26.9)	6 (20.0)	12 (32.4)	17 (25.8)	6 (20.0)	11 (30.6)	13 (19.4)	5 (16.7)	8 (21.6)
GLP1RA (n,%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	1 (2.7)	1 (1.5)	0 (0.0)	1 (2.8)	4 (6.0)	0 (0.0)	4 (10.8)
Thiazolidinedione (n,%)	3(4.5)	1 (3.3)	2 (5.4)	3 (5.8)	1 (4.0)	2 (7.4)	3 (4.5)	1 (3.3)	2 (5.4)	1 (1.5)	1 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Insulin (n,%)	1 (1.5)	1 (3.3)	0 (0.0)	1 (1.9)	1 (4.0)	0 (0.0)	1 (1.5)	1 (3.3)	0 (0.0)	1 (1.5)	1 (3.3)	0 (0.0)	1 (1.5)	1 (3.3)	0 (0.0)
Meglitinide (n,%)	1 (1.5)	0 (0.0)	1 (2.7)	1 (1.9)	0 (0.0)	1 (3.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Beta-blockers (n,%)	3 (4.5)	1 (3.3)	2 (5.4)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.5)	1 (3.3)	0 (0.0)	1 (1.5)	1 (3.3)	0 (0.0)	2 (3.0)	1 (3.3)	1 (2.7)
PPI (n,%)	10 (14.9)	5 (16.7)	5 (13.5)	18 (34.6)	5 (20.0)	13 (48.1)	28 (41.8)	4 (13.3)	24 (64.9)	13 (19.7)	3 (10.0)	10 (27.8)	4 (6.0)	4 (13.3)	0 (0.0)

Table 3.4. Description of medication use in both SMM and DJBL groups across weeks.
This table describes medication use across visits. Visits includes all 5 visits at -2, 2, 26, 50 and 104 weeks. Group includes the SMM and DJBL groups. Medication use by category is represented in both groups at each visit as N (%) indicating number of participants.
Abbreviations: SU, sulphonylureas; DPP4i, Dipeptidyl peptidase 4 inhibitors; SGLT2i, Sodium-glucose co-transporter 2 inhibitors; TZD, Thiazolidinediones; GLP1RA, glucagon-like peptide-1 receptor agonists; PPI, proton pump inhibitors; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

Main effect of weeks on medication uptake				
Medication	**Parameters**	**Weeks**	**Group**	**Weeks*Group**
Metformin	Numerator df	4	1	4
	Denominator df	213.47	69.71	213.47
	F	0.92	0.71	0.80
	P value	0.45	0.40	0.53
Sulphonylurea	Numerator df	4	1	4
	Denominator df	211.93	67.42	211.93
	F	6.12	2.27	0.64
	P value	**<0.001****	0.14	0.64
DPP4i	Numerator df	4	1	4
	Denominator df	211.93	67.41	211.93
	F	0.47	0.18	1.13
	P value	0.76	0.67	0.34
SGLT2i	Numerator df	4	1	4
	Denominator df	214.90	68.67	214.90
	F	8.67	1.31	0.63
	P value	**<0.001****	0.26	0.64
Thiazolidinedione	Numerator df	4	1	4
	Denominator df	213.03	68.62	213.03
	F	1.67	0.01	1.58
	P value	0.16	0.94	0.18
Beta-blockers	Numerator df	4	1	4
	Denominator df	202.94	59.15	202.94
	F	1.02	0.00	0.87
	P value	0.40	0.96	0.48
PPI	Numerator df	4.00	1.00	4.00
	Denominator df	170.10	50.02	170.10
	F	15.40	5.27	20.03
	P value	**<0.001****	**0.026***	**<0.001****

Table 3.5. Main effects of group and weeks on medication use in both SMM and DJBL groups.

This table describes the F statistic, numerator and denominator of degrees of freedom for the main effects of group, weeks and group*weeks on each medication. Weeks includes all 5 visits at -2, 2, 26, 50 and 104 weeks. Group includes the SMM and DJBL groups. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: SU, sulphonylureas; DPP4i, Dipeptidyl peptidase 4 inhibitors; SGLT2i, Sodium-glucose co-transporter 2 inhibitors; TZD, Thiazolidinediones; PPI, proton pump inhibitors; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

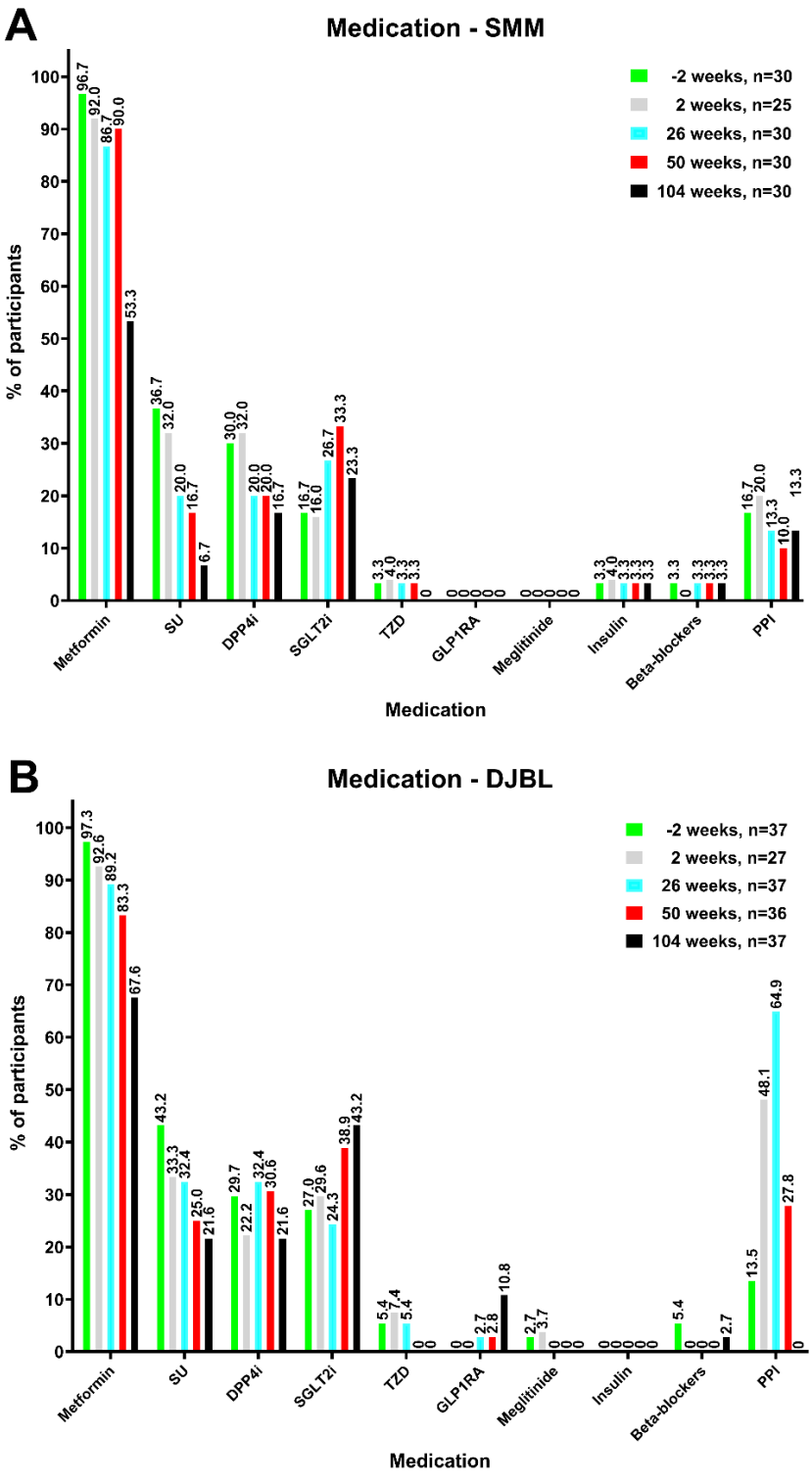


Figure 3.2. Distribution of medication use among both SMM and DJBL groups across visits.

Bar graphs describing prevalence of medication use in (A) SMM and (B) DJBL groups at -2, 2, 26, 50 and 104 weeks as % of participants. Abbreviations: SU, sulphonylureas; DPP4i, dipeptidyl peptidase 4 inhibitors; SGLT2i, sodium-glucose co-transporter 2 inhibitors; TZD, thiazolidinediones; GLP1RA, glucagon-like peptide-1 receptor agonists; PPI, proton pump inhibitors; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

3.4.2. Longitudinal Outcomes

3.4.2.1. Longitudinal changes in BMI and weight

SMM group

Including weeks (-2, 2, 26, 50, 104) as within-participant factor, there was a significant effect of weeks on *absolute* BMI (P<0.001) and *change* in % weight (P<0.001) (Table 3.6; Figure 3.3A,B). In post-hoc analysis, *absolute* BMI decreased and remained lower than baseline at 104 weeks (effect size -2.04 ±0.41 kg/m^2, P<0.001). The greatest loss in % weight occurred at 26 weeks (effect size -8.09 ± 0.99 %, P<0.001), and remained below baseline at 104 weeks (effect size -6.35 ± 1.04%, P<0.001) (Figure 3.3A,B, Table 3.7)

DJBL group

Including weeks (-2, 2, 26, 50) as within-participant factor, there was a significant effect of weeks on *absolute* BMI (P<0.001) and *change* in % weight (P<0.001) (Table 3.8, Figure 3.3A,B), with *absolute* BMI decreasing and remaining lower than baseline at 50 weeks (effect size -4.23 ± 0.30 kg/m^2, P<0.001). The greatest % weight loss occurred at 50 weeks (effect size -11.35 ± 0.70 %, P<0.001), with subsequent weight regain after DJBL explant (Figure 3.3A,B, Table 3.9)

DJBL vs. SMM group

Including weeks (-2, 2, 26, 50, 104) as within-participant factor and group as between-participant factor, there was a significant group*weeks interaction for BMI (P=0.004) and % weight change (P=0.002) (Table 3.10, 3.12; Figure 3.3A,B). DJBL participants lost significantly more weight than SMM participants at only weeks 26 and 50 (DJBL vs. SMM; 26 weeks: effect size -2.20 ± 1.04%, P=0.036; 50 weeks: effect size -3.44 ± 1.09 %, P=0.002) (Figure 3.3A,B, Tables 3.11, 3.12, 3.13).

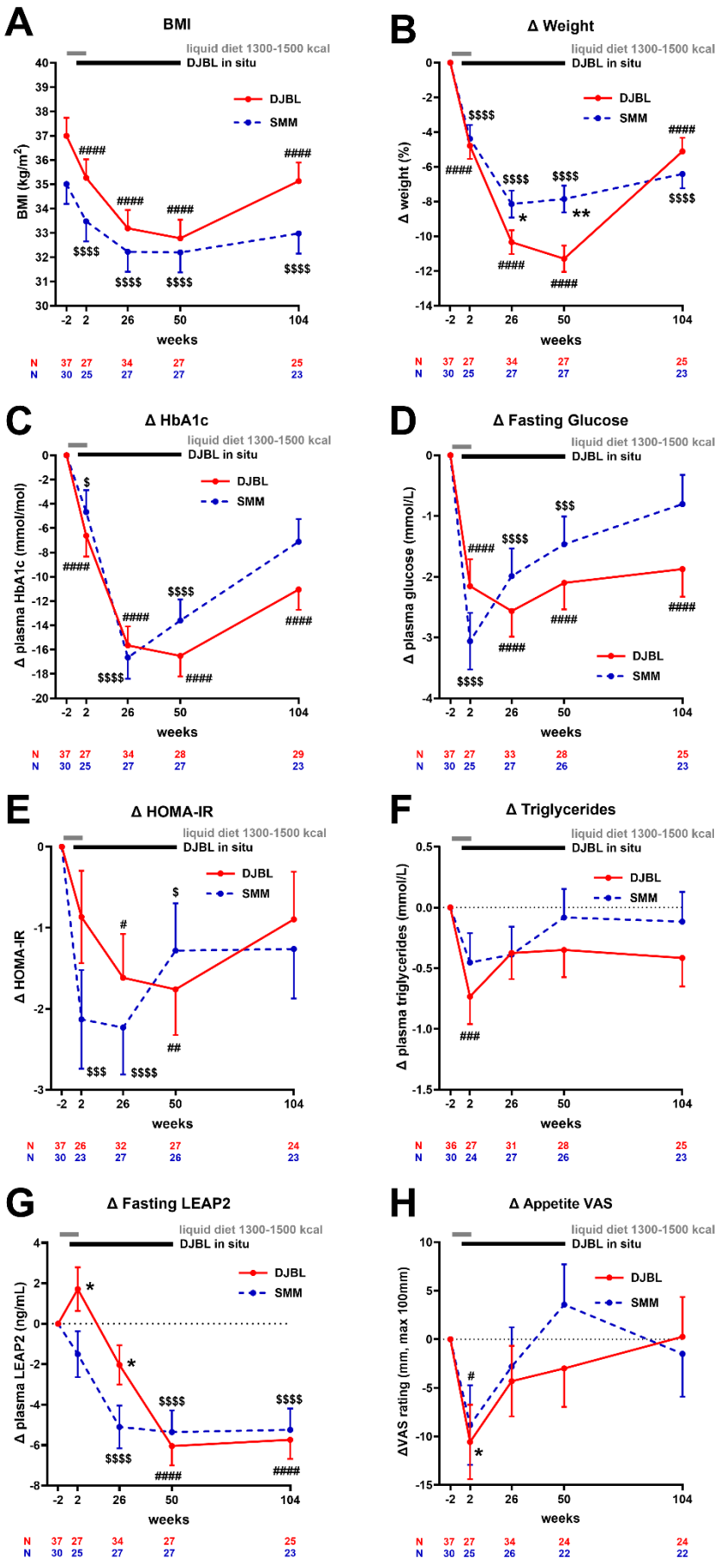

A
BMI
liquid diet 1300-1500 kcal
DJBL in situ
DJBL
SMM
BMI (kg/m²)
weeks
B
Δ Weight
Δ weight (%)
C
Δ HbA1c
Δ plasma HbA1c (mmol/mol)
D
Δ Fasting Glucose
Δ plasma glucose (mmol/L)
E
Δ HOMA-IR
F
Δ Triglycerides
Δ plasma triglycerides (mmol/L)
G
Δ Fasting LEAP2
Δ plasma LEAP2 (ng/mL)
H
Δ Appetite VAS
ΔVAS rating (mm, max 100mm)

Figure 3.3. Longitudinal analysis of BMI and change in: % weight, HbA1c, fasting glucose, HOMA-IR, fasting triglycerides, fasting plasma LEAP2 and appetite ratings across visits.

(A) BMI, change in (B) % weight loss, (C) HbA1c, (D) fasting glucose, (E) HOMA-IR, (F) fasting triglycerides, (G) fasting plasma LEAP2, (H) fasting appetite VAS ratings vs. -2 weeks, across time for participants undergoing standard medical management alone (SMM, blue dashed line) and duodenal-jejunal bypass liner (DJBL, red solid line) groups. Black bar indicates duration of DJBL being in situ (0-52 weeks). N indicates number of participants at each time point. Grey bar indicates duration of liquid diet (-1 to 2 weeks). Statistical results below graph indicates linear mixed model analysis including group (SMM, DJBL), sweet taste detection task (done in the taste sub-group at weeks -2, 2 and 26 only, for fasting glucose, HOMA-IR and fasting LEAP2 only) as between participant, and weeks (-2, 2, 26, 50 and 104) as within participant fixed factors, to examine effects of interventions on dependent variables over time. DJBL vs. SMM: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001; DJBL vs. baseline week -2: # P<0.05, ## P<0.01, ### P<0.005, #### P<0.001; SMM vs. baseline week -2: $ P<0.05, $$ P<0.01, $$$ P<0.005, $$$$ P<0.001. All data given as mean ± SEM. Abbreviations: HOMA-IR, homeostatic model assessment for insulin resistance; kcal, kilocalorie; VAS, visual analogue scale.

3.4.2.2. Longitudinal changes in fasting glucose metabolism

There was no significant group*weeks interaction nor effect of group, but a significant effect of weeks on *changes* in HbA1c, fasting plasma glucose and HOMA-IR (Figure 3.3C-E, Tables 3.12, 3.13)

SMM group

Over 104 weeks, there was a significant effect of weeks on *changes* in: HbA1c [F(4, 97.38)=21.60, P<0.001], HOMA-IR [F(4, 108.19)=6.34, P<0.001], fasting plasma glucose [F(4, 109.22) = 11.39, P<0.001] and fasting plasma insulin [F(4, 108.19)=4.45, P=0.003) (Figure 3.3C-E, Table 3.6). In post-hoc analysis, HbA1c decreased the most from baseline at 26 weeks (effect size -16.56 ± 2.06 mol/mmol [95% CI -20.64, -12.46], P<0.001), remain below baseline at 104 weeks (effect size -6.89 ± 2.17 mol/mmol [95% CI -11.20, -2.59], P=0.002) (Figure 3.3C, Table 3.7). HOMA-IR decreased the most from baseline at 26 weeks (effect size -2.19 ± 0.49 [95% CI -3.16, -1.21], P<0.001) (Figure 3.3E, Table 3.7). Fasting plasma glucose decreased the most from baseline at 2 weeks (effect size -3.01 ± 0.47 mmol/L [95% CI -3.95, -2.07], P<0.001) (Figure 3.3D, Table 3.7). Fasting plasma insulin decreased the most from baseline at 26 weeks (effect size -3.77 ± 0.91 mIU/L [95% CI -5.57, -1.97], P<0.001), and remained below baseline at 104 weeks (effect size -2.36 ± 0.97 mIU/L [95% CI -4.28, -0.43], P=0.017) (Table 3.7).

DJBL group

Over 50 weeks, there was a significant effect of weeks on *changes* in HbA1c [F(3, 94.37) = 46.733, P<0.001] and fasting plasma glucose [F(3, 99.51)=12.13, P<0.001], but weeks had no significant effect on *changes* in fasting plasma insulin [F(3, 94.93)=0.54, P=0.65] and HOMA-IR [F(3, 98.17) = 1.73, P=0.17] (Figure 3.3C-E, Table 3.8). In post-hoc analysis, HbA1c decreased the most from baseline at 50 weeks (effect size -16.42 ± 1.67 mol/mmol [95% CI -19.74, -13.11], P<0.001) (Figure 3.3C, Table 9). HOMA-IR decreased the most from baseline at 26 weeks (effect size -1.70 ± 0.73 [95% CI -3.15, -0.24], P=0.023) (Figure 3.3E, Table 3.9). Fasting plasma glucose decreased most from baseline at 26 weeks (effect size -2.45 ± 0.44 mmol/L [95% CI -3.33, -1.57], P<0.001), and remained below baseline at 50 weeks (-1.99 ± 0.46 mmol/L [95% CI -2.90, -1.08], P<0.001) (Figure 3.3D, Table 3.9).

DJBL vs. SMM groups

Over 50 weeks, there was no significant effect of group*weeks interaction for *changes* in: HbA1c [F(4, 229.79)=0.99, P = 0.41], HOMA-IR [F(4, 215.68) = 1.054, P=0.38], fasting plasma glucose [F(4, 220.89)=1.831, P=0.12] and fasting plasma insulin [F(4, 216.75)=1.43, P=0.22] (Table 3.10). There was a significant effect of weeks on HbA1c [F(4, 229.79)=44.967, P<0.001], HOMA-IR [F(4, 229.17)=5.452, P<0.001] and fasting plasma glucose [F(4, 234.82)=19.42, P<0.001], but not on fasting serum insulin [F(4, 231.71)=2.41, P=0.05] (Table 3.10). There was no significant effect of group on HbA1c [F(1, 69.49)=1.026, P=0.31], HOMA-IR [F(1, 66.94)=0.450, P=0.50] or fasting serum insulin [F(1, 66.39)=2.34, P=0.13], but a significant group effect on fasting plasma glucose [F(1, 66.44)=7.737, P=0.007] (Table 10). In post-hoc analysis, there were no significant group difference in *changes* in HbA1c, HOMA-IR, fasting plasma glucose or fasting serum insulin from baseline (Figure 3.3C-E, Table 3.11).

3.4.2.3. Longitudinal changes in fasting triglycerides

There was no significant group*weeks interaction nor effect of group, but a significant effect of weeks on *changes* serum triglycerides (Figure 3.3F, Tables 3.12, 3.13).

SMM group

There was a significant effect of weeks on *changes* in serum triglycerides [F(4, 95.34)=2.49, P=0.048] (Figure 3.3F, Table 3.6). In post-hoc analysis, serum triglycerides decreased the most from baseline at 2 weeks (-0.452 ± 0.175 mol/mmol, P=0.011) (Figure 3.3F, Table 3.7).

DJBL group

There was a non-significant main effect of visit on *changes* in serum triglycerides [F(3, 78.25)=1.651, P=0.19] (Figure 3.3F, Table 3.8). In post-hoc analysis, serum triglycerides decreased from baseline at 2 weeks (-0.734 ± 0.331 mol/mmol, P=0.030) (Figure 3.3F, Table 3.8).

DJBL vs. SMM groups

There was no significant effect of group*visit interaction for *changes* in serum triglycerides [F(4, 173.92)=0.53, P=0.71] (Table 3.12). There was a significant effect

of visit on *changes* in serum triglycerides [F(4, 173.92)=2.79, P=0.028] (Table 3.12). There was no significant effect of group on *changes* in serum triglycerides [F(1, 57.88)=0.96, P=0.33] (Table 3.12). In post-hoc analysis, there was no significant between group differences in *changes* in serum triglycerides (Figure 3.3F, Table 3.13).

3.4.2.4. Longitudinal changes in appetite

There was no significant group*weeks interaction nor effect of group, but a significant effect of weeks on *changes* appetite VAS ratings (Figure 3.3H, Tables 3.12, 3.13).

SMM group

There was no significant effect of weeks on *changes* in fasting appetite ratings [F(4, 96.04)=1.022, P=0.40] (Figure 3.3H, Table 3.6). In post-hoc analysis, there were no significant *changes* in appetite ratings (Figure 3.3H, Table 3.7).

DJBL group

There was a significant effect of weeks on *changes* in fasted appetite ratings [F(3, 88.79)=4.156, P=0.008] (Figure 3H, Table 8). In post-hoc analysis, appetite ratings decreased from baseline at 2 weeks (effect size -10.23 ± 3.89 mm [95% CI -17.96, -2.49], P=0.010) (Figure 3.3H, Table 3.9,).

DJBL vs. SMM groups

There was no significant effect of group*visit interaction for *changes* in appetite ratings [F(4, 207.02)=0.40, P=0.81] (Table 3.12). There was a significant effect of weeks on *changes* in appetite ratings [F(4, 220.04)=2.90, P=0.02] (Table 3.12). There was no significant effect of group on *changes* in appetite ratings [F(1, 63.46)=0.18, P=0.67] (Figure 3.3H, Table 3.12). In post-hoc analysis, there was no significant between group differences in change in appetite ratings (Figure 3.3H, Table 3.13).

3.4.2.5. Longitudinal changes in fasting plasma LEAP2

SMM group

There was a significant effect of weeks on *change* in plasma LEAP2 (P<0.001) (Table 3.6; Figure 3.4), with the largest *change* in plasma LEAP2 at week 26 (effect size -3.78 ± 0.81 ng/mL, P<0.001), remaining below baseline at week 104 (effect size -1.83

± 0.86ng/mL, P=0.019) (Figures 3.3G, Figure 3.4, Table 3.7).

DJBL group

There was a significant effect of weeks on *changes* in plasma LEAP2 (P<0.001) (Table 3.8, Figure 3.4), with the largest *increase* in plasma LEAP2 at week 2 (effect size +1.98 ± 0.96 ng/mL, P=0.042). The largest *decrease* in plasma LEAP2 occurred at week 50 (effect size -2.44 ± 1.00 ng/mL, P=0.017) (Figure 3.3G, Figure 3.4, Table 3.9).

DJBL vs. SMM group

There was a significant group*weeks interaction for *absolute* plasma LEAP2 (P=0.003) (Table 3.10; Figure 3.4), with *absolute* plasma LEAP2 significantly higher in DJBL vs. SMM at week 2 (effect size +2.88 ± 1.23 ng/mL, P=0.021) (Figure 3.3G, Figure 3.4, Table 3.11).

There was a significant group*weeks interaction for *change* in plasma LEAP2 (P=0.039) (Figure 3.3G, Table 3.12). *Change* in plasma LEAP2 was significantly higher in DJBL vs. SMM at 2 weeks (effect size +3.22 ± 1.56 ng/mL, P=0.041) and 26 weeks (effect size +3.07 ± 1.1.44 ng/mL, P=0.034) (Figure 3.3G, Table 3.13).

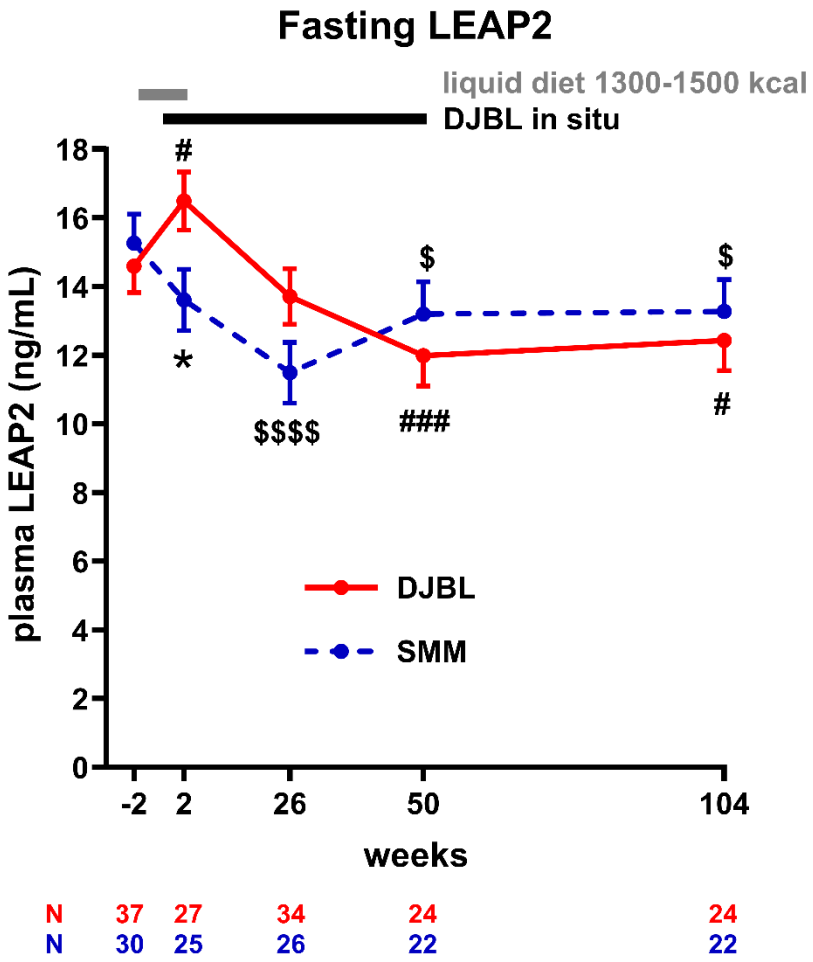


Figure 3.4. Longitudinal analysis of absolute fasting plasma LEAP2 across visits. Absolute fasting plasma LEAP2 across time for participants undergoing standard medical management alone (SMM, blue dashed line) and duodenal-jejunal bypass liner (DJBL, red solid line) groups. Black bar indicates duration of DJBL being in situ (0-52 weeks). N indicates number of participants at each time point. Grey bar indicates duration of liquid diet (-1 to 2 weeks). Statistical results below graph indicates linear mixed model analysis including group (SMM, DJBL) and sweet taste detection task (present for taste sub-group at weeks -2, 2 and 26 only) as between participant factors, and weeks (-2, 2, 26, 50, 104) as within participant fixed factor, to examine effects of interventions on fasting plasma LEAP2 over time. DJBL vs. SMM: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001; DJBL vs. baseline week -2: # P<0.05, ## P<0.01, ### P<0.005, #### P<0.001; SMM vs. baseline week -2: $ P<0.05, $$ P<0.01, $$$ P<0.005, $$$$ P<0.001. All data given as mean ± SEM. Abbreviations: kcal, kilocalorie.

Main effect of weeks on dependent variable - SMM		
Dependent variable	**Parameters**	**Weeks**
BMI	Numerator df	4
	Denominator df	101.66
	F	18.040
	P value	**<0.001******
Δ %Weight	Numerator df	4
	Denominator df	105.44
	F	22.524
	P value	**<0.001******
Δ Appetite	Numerator df	4
	Denominator df	96.04
	F	1.022
	P value	0.40
Δ HbA1c	Numerator df	4
	Denominator df	97.38
	F	21.60
	P value	**<0.001******
Δ Glucose	Numerator df	4
	Denominator df	109.22
	F	11.39
	P value	**<0.001******
Δ Insulin	Numerator df	4
	Denominator df	108.19
	F	4.45
	P value	**0.002*****
Δ HOMA-IR	Numerator df	4
	Denominator df	108.19
	F	6.34
	P value	**<0.001******
Δ LEAP2	Numerator df	4
	Denominator df	99.16
	F	5.39
	P value	**<0.001******
Δ Triglycerides	Numerator df	4
	Denominator df	104.93
	F	2.76
	P value	**0.031***

Table 3.6. Main effect of weeks on absolute BMI and change in: % weight, fasting appetite ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 in SMM group.
This table describes the F statistic, numerator and denominator of degrees of freedom for the main effect of weeks on each dependent variable (absolute BMI, changes in: % weight, fasting appetite VAS ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 between weeks 2,26,50 and 104 vs. -2 weeks) in the SMM group. Between-participant factors including sweet taste detection task (done at weeks -2, 2 and 26 for fasting glucose, insulin, HOMA-IR and LEAP2). Within-participant factors include weeks at -2, 2, 26, 50 and 104 weeks. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: VAS, visual analogue scale; HOMA-IR, homeostatic model assessment for insulin resistance; SMM, standard medical management.

Post-hoc analysis of weeks on dependent variable - SMM					
Dependent variable	**Parameters**	**Δ 2 vs. -2 w**	**Δ 26 vs. -2 w**	**Δ 50 vs. -2 w**	**Δ 104 vs. -2 w**
BMI	Mean difference	-1.54	-2.79	-2.83	-2.04
	SE	0.39	0.38	0.39	0.41
	P-value	**<0.001******	**<0.001******	**<0.001******	**<0.001******
	95 CI Lower	-2.31	-3.55	-3.60	-2.84
	95 CI Upper	-0.76	-2.02	-2.06	-1.23
Δ %Weight	Mean difference	-4.31	-8.09	-7.79	-6.35
	SE	1.01	0.99	0.99	1.04
	P-value	**<0.001******	**<0.001******	**<0.001******	**<0.001******
	95 CI Lower	-6.31	-10.05	-9.76	-8.41
	95 CI Upper	-2.31	-6.13	-5.82	-4.29
Δ Appetite	Mean difference	-9.34	-2.84	3.46	2.01
	SE	5.64	5.57	5.72	5.99
	P-value	0.10	0.61	0.55	0.74
	95 CI Lower	-20.53	-13.89	-7.90	-9.88
	95 CI Upper	1.85	8.21	14.82	13.90
Δ HbA1c	Mean difference	-4.55	-16.55	-13.45	-6.89
	SE	2.11	2.06	2.07	2.17
	P-value	**0.033***	**<0.001******	**<0.001******	**0.002*****
	95 CI Lower	-8.73	-20.64	-17.56	-11.20
	95 CI Upper	-0.37	-12.46	-9.34	-2.59
Δ Glucose	Mean difference	-3.01	-1.94	-1.35	-0.75
	SE	0.47	0.46	0.47	0.49
	P-value	**<0.001******	**<0.001******	**0.0047*****	0.13
	95 CI Lower	-3.95	-2.86	-2.27	-1.72
	95 CI Upper	-2.07	-1.02	-0.42	0.22
Δ Insulin	Mean difference	-1.87	-3.77	-1.64	-2.36
	SE	0.95	0.91	0.91	0.97
	P-value	0.052	**<0.001******	0.075	**0.017***
	95 CI Lower	-3.76	-5.57	-3.45	-4.28
	95 CI Upper	0.02	-1.97	0.17	-0.43
Δ HOMA-IR	Mean difference	-2.07	-2.19	-1.19	-1.20
	SE	0.51	0.49	0.49	0.52
	P-value	**<0.001******	**<0.001******	**0.017***	**0.022***
	95 CI Lower	-3.08	-3.16	-2.17	-2.22
	95 CI Upper	-1.05	-1.21	-0.22	-0.18
Δ LEAP2	Mean difference	-1.50	-3.76	-1.94	-1.83
	SE	0.82	0.81	0.86	0.86
	P-value	0.071	**<0.001******	**0.026***	**0.036***
	95 CI Lower	-3.14	-5.37	-3.64	-3.54
	95 CI Upper	0.13	-2.14	-0.24	-0.12
Δ Triglycerides	Mean difference	-0.45	-0.38	-0.07	-0.11
	SE	0.17	0.16	0.17	0.17
	P-value	**0.008****	**0.023***	0.67	0.51
	95 CI Lower	-0.79	-0.70	-0.40	-0.45
	95 CI Upper	-0.12	-0.05	0.26	0.23

Table 3.7. Post-hoc analysis of absolute BMI and change in: % weight, fasting appetite ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2, in SMM group.

This table describes the post-hoc analysis of results in Table 3.6. Mean difference, standard error, upper and lower confidence intervals for absolute BMI and change in: % weight, fasting appetite VAS ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 between weeks 2,26,50 and 104 vs. -2 weeks in the SMM group. Between-participant factors including sweet taste detection task (done in the taste sub-group at weeks -2, 2 and 26 only for fasting glucose, insulin, HOMA-IR and LEAP2 only). Within-participant factors include visits at -2, 2, 26, 50 and 104 weeks. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: VAS, visual analogue scale; HOMA-IR, homeostatic model assessment for insulin resistance; SMM, standard medical management.

Main effect of weeks on dependent variable - DJBL		
Dependent variable	**Parameters**	**Weeks**
BMI	Numerator df	3
	Denominator df	88.24
	F	92.449
	P value	**<0.001****
Δ %Weight	Numerator df	3
	Denominator df	96.29
	F	121.038
	P value	**<0.001****
Δ Appetite	Numerator df	3
	Denominator df	88.79
	F	4.156
	P value	**0.008****
Δ HbA1c	Numerator df	3
	Denominator df	94.37
	F	46.733
	P value	**<0.001****
Δ Glucose	Numerator df	3
	Denominator df	99.51
	F	12.13
	P value	**<0.001****
Δ Insulin	Numerator df	3
	Denominator df	95.93
	F	0.54
	P value	0.65
Δ HOMA-IR	Numerator df	3
	Denominator df	98.17
	F	1.73
	P value	0.17
Δ LEAP2	Numerator df	3
	Denominator df	98.67
	F	6.08
	P value	**<0.001****
Δ Triglycerides	Numerator df	3
	Denominator df	78.25
	F	1.65
	P value	0.18

Table 3.8. Main effects of weeks on absolute BMI and change in: % weight, fasting appetite ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 in DJBL group.
This table describes the F statistic, numerator and denominator of degrees of freedom for the effect of weeks on each dependent variable (absolute BMI and change in: % weight, fasting appetite VAS ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 between weeks 2,26, and 50 vs. -2 weeks) in the DJBL group. Between-participant factors including sweet taste detection task (done at weeks -2, 2 and 26 for fasting glucose, insulin, HOMA-IR and LEAP2). Within-participant factors include visits at -2, 2, 26, and 50 weeks. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: VAS, visual analogue scale; HOMA-IR, homeostatic model assessment for insulin resistance; DJBL, duodenal-jejunal bypass liner.

Post-hoc analysis of weeks on dependent variable - DJBL				
Dependent variable	**Parameters**	**Δ 2 vs. -2 w**	**Δ 26 vs. -2 w**	**Δ 50 vs. -2 w**
BMI	Mean difference	-1.77	-3.81	-4.23
	SE	0.30	0.27	0.30
	P-value	**<0.001******	**<0.001******	**<0.001******
	95 CI Lower	-2.37	-4.35	-4.82
	95 CI Upper	-1.18	-3.26	-3.64
Δ %Weight	Mean difference	-4.89	-10.35	-11.35
	SE	0.70	0.65	0.70
	P-value	**<0.001******	**<0.001******	**<0.001******
	95 CI Lower	-6.28	-11.64	-12.74
	95 CI Upper	-3.50	-9.06	-9.96
Δ Appetite	Mean difference	-10.23	-4.23	-3.42
	SE	3.89	3.66	3.99
	P-value	**0.010**	0.25	0.39
	95 CI Lower	-17.96	-11.50	-11.35
	95 CI Upper	-2.49	3.04	4.51
Δ HbA1c	Mean difference	-6.56	-15.67	-16.42
	SE	1.69	1.57	1.67
	P-value	**<0.001******	**<0.001******	**<0.001******
	95 CI Lower	-9.91	-18.79	-19.74
	95 CI Upper	-3.20	-12.55	-13.11
Δ Glucose	Mean difference	-2.03	-2.45	-1.99
	SE	0.46	0.44	0.46
	P-value	**<0.001******	**<0.001******	**<0.001******
	95 CI Lower	-2.95	-3.33	-2.90
	95 CI Upper	-1.12	-1.57	-1.08
Δ Insulin	Mean difference	0.59	-1.17	-2.11
	SE	1.53	1.46	1.52
	P-value	0.70	0.43	0.17
	95 CI Lower	-2.46	-4.08	-5.12
	95 CI Upper	3.63	1.74	0.91
Δ HOMA-IR	Mean difference	-0.79	-1.50	-1.70
	SE	0.74	0.70	0.73
	P-value	0.29	**0.037***	**0.023***
	95 CI Lower	-2.26	-2.90	-3.15
	95 CI Upper	0.68	-0.09	-0.24
Δ LEAP2	Mean difference	1.98	-0.93	-2.44
	SE	0.96	0.92	1.00
	P-value	**0.042***	0.32	**0.017***
	95 CI Lower	0.07	-2.75	-4.43
	95 CI Upper	3.89	0.90	-0.46
Δ Triglycerides	Mean difference	-0.73	-0.36	-0.34
	SE	0.33	0.32	0.33
	P-value	**0.030***	0.25	0.31
	95 CI Lower	-1.39	-0.99	-0.99
	95 CI Upper	-0.07	0.27	0.32

Table 3.9. Post-hoc analysis of effect of absolute BMI and change in: % weight, fasting appetite ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2, in DJBL group.
This table describes the post-hoc analysis of results in Table 3.8. Mean difference, standard error, upper and lower confidence intervals for absolute BMI and change in: % weight, fasting appetite VAS ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 between weeks 2,26 and 50 vs. -2 weeks in the DJBL group. Between-participant factors including sweet taste detection task (done weeks -2, 2 and 26 for fasting glucose, insulin, HOMA-IR and LEAP2). Within-participant factors include visits at -2, 2, 26, and 50 weeks. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: VAS, visual analogue scale; HOMA-IR, homeostatic model assessment for insulin resistance; DJBL, duodenal-jejunal bypass liner.

Main effect of weeks, group and sweet taste task on dependent variable - DJBL vs SMM								
Dependent variable	**Parameters**	**Group**	**Week**	**Taste task**	**Group*Week**	**Group*Taste task**	**Week*Taste task**	**Week*Group*Taste task**
LEAP2	Numerator df	1	4	1	4	1	2	2
	Denominator df	68.09	221.29	255.00	221.29	255.00	209.09	209.09
	F	0.40	10.99	15.78	4.06	0.58	2.93	1.13
	P-value	0.53	**0.000**	**0.000**	**0.003**	0.45	0.055	0.32

Table 3.10. Main effects of group, weeks and sweet taste task on absolute fasting LEAP2 between SMM and DJBL groups.
This table describes the F statistic, numerator and denominator of degrees of freedom for the effect of group, weeks and sweet taste task on fasting plasma LEAP2 across both groups. Between-participant factors including sweet taste detection task (done at weeks -2, 2 and 26). Within-participant factors include weeks at -2, 2, 26, 50 and 104 weeks. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

Post-hoc analysis of weeks, group and sweet taste task on dependent variable - DJBL vs SMM														
		SMM				DJBL				DJBL vs SMM				
Dependent variable	**Parameters**	**Δ2 vs. -2 w**	**Δ26 vs. -2 w**	**Δ 50 vs. -2 w**	**Δ 104 vs. -2 w**	**Δ2 vs. -2 w**	**Δ26 vs. -2 w**	**Δ 50 vs. -2 w**	**Δ 104 vs. -2 w**	**-2 w**	**2 w**	**26 w**	**50 w**	**104 w**
LEAP2	Mean difference	-1.65	-3.78	-2.07	-2.00	1.90	-0.88	-2.60	-2.16	-0.68	2.88	2.22	-1.21	-0.84
	SE	0.90	0.89	0.94	0.94	0.85	0.81	0.89	0.89	1.15	1.23	1.20	1.29	1.29
	P-value	0.067	**0.000**	**0.029**	**0.035**	**0.027**	0.28	**0.004**	**0.017**	0.56	**0.021**	0.066	0.35	0.51
	95 CI Lower	-3.43	-5.53	-3.92	-3.85	0.22	-2.48	-4.36	-3.92	-2.95	0.44	-0.15	-3.75	-3.37
	95 CI Upper	0.12	-2.02	-0.22	-0.14	3.58	0.73	-0.84	-0.40	1.59	5.31	4.59	1.33	1.69

Table 3.11. Post-hoc analysis of absolute fasting LEAP2 in SMM and DJBL groups.
This table describes the post-hoc analysis of results in Table 3.10. Mean difference, standard error, upper and lower confidence intervals fasting appetite VAS ratings, LEAP2 between weeks 2,26, 50 and 104 vs. -2 weeks in and between both groups. Between-participant factors including sweet taste detection task (done weeks -2, 2 and 26). Within-participant factors include visits at -2, 2, 26,50 and 104 weeks. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001.
Abbreviations: SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

Main effect of weeks, group and sweet taste task on dependent variable - DJBL vs SMM							
Dependent variable	**Parameters**	**Group**	**Week**	**Taste task**	**Group*Week**	**Week*Taste task**	**Week*Group*Taste task**
BMI	Numerator df	1	4		4		
	Denominator df	68.10	214.80		214.80		
	F	1.94	69.51		3.64		
	P value	0.17	**0.000**		**0.007**		
Δ %weight	Numerator df	1	4		4		
	Denominator df	71.87	226.25		226.25		
	F	1.73	84.47		4.24		
	P value	0.19	**0.000**		**0.002**		
Δ HbA1c	Numerator df	1	4		4		
	Denominator df	69.49	229.80		229.80		
	F	1.03	44.97		0.99		
	P value	0.31	**0.000**		0.41		
Δ Glucose	Numerator df	1	4	1	4	2	3
	Denominator df	67.24	234.81	265.00	221.08	220.02	235.56
	F	0.25	17.44	0.22	2.10	2.12	0.19
	P value	0.62	**0.000**	0.64	0.082	0.12	0.90
Δ HOMA-IR	Numerator df	1	4	1	4	2	3
	Denominator df	66.94	229.17	259.00	215.68	215.20	231.82
	F	0.45	5.45	1.41	1.05	0.59	0.72
	P value	0.50	**0.000**	0.24	0.38	0.56	0.54
Δ Triglycerides	Numerator df	1	4		4		
	Denominator df	48.99	198.28		198.28		
	F	0.56	2.96		0.40		
	P value	0.46	**0.021**		0.81		
Δ LEAP2	Numerator df	1	4	1	4	2	3
	Denominator df	66.57	266.88	301.95	250.98	251.02	269.40
	F	1.46	15.22	0.08	2.56	2.15	0.27
	P value	0.23	**0.000**	0.78	**0.039**	0.12	0.85
Δ Appetite	Numerator df	1	4		4		
	Denominator df	62.99	207.93		207.93		
	F	0.36	3.05		0.25		
	P value	0.55	**0.018**		0.91		

Table 3.12. Main effects on absolute BMI and change in: % weight, fasting appetite ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 between SMM and DJBL groups. This table describes the F statistic, numerator and denominator of degrees of freedom for the main effects of group, weeks and sweet taste task on each dependent variable (absolute BMI and change in: % weight, fasting appetite VAS ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 between weeks 2,26,50 and 104 vs. -2 weeks) between SMM and DJBL groups. Between-participant factors including sweet taste detection task (done at weeks -2, 2 and 26 for fasting glucose, insulin, HOMA-IR and LEAP2). Within-participant factors include visits at -2, 2, 26, 50 and 104 weeks. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: BMI, body mass index; VAS, visual analogue scale; HOMA-IR, homeostatic model assessment for insulin resistance; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

Post-hoc analysis of weeks, group and sweet taste task on dependent variable - DJBL vs SMM														
		SMM				DJBL				Δ DJBL vs. SMM				
Dependent variable	**Parameters**	**Δ 2 vs. -2 w**	**Δ 26 vs. -2 w**	**Δ 50 vs. -2 w**	**Δ 104 vs. -2 w**	**Δ 2 vs. -2 w**	**Δ 26 vs. -2 w**	**Δ 50 vs. -2 w**	**Δ 104 vs. -2 w**	**-2 w**	**2 w**	**26 w**	**50 w**	**104 w**
BMI	Mean difference	-1.60	-2.76	-2.80	-2.04	-1.78	-3.82	-4.24	-1.88	1.99	1.79	0.96	0.58	2.16
	SE	0.35	0.35	0.35	0.36	0.33	0.31	0.33	0.34	1.11	1.13	1.12	1.12	1.13
	P-value	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.077	0.12	0.39	0.61	0.060
	95 CI Lower	-2.30	-3.44	-3.49	-2.75	-2.44	-4.43	-4.90	-2.55	-0.22	-0.45	-1.26	-1.65	-0.10
	95 CI Upper	-0.91	-2.08	-2.12	-1.32	-1.13	-3.22	-3.59	-1.21	4.20	4.03	3.19	2.82	4.41
											Δ 2 vs. -2 w	Δ 26 vs. -2 w	Δ 50 vs. -2 w	Δ 104 vs. -2 w
Δ %Weight	Mean difference	-4.33	-8.09	-7.80	-6.36	-4.78	-10.34	-11.30	-5.12		-0.40	-2.20	-3.44	1.30
	SE	0.90	0.88	0.88	0.93	0.85	0.79	0.85	0.87		1.10	1.04	1.09	1.14
	P-value	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**		0.71	**0.036**	**0.002**	0.26
	95 CI Lower	-6.10	-9.83	-9.55	-8.18	-6.45	-11.89	-12.97	-6.83		-2.57	-4.25	-5.58	-0.95
	95 CI Upper	-2.55	-6.36	-6.06	-4.54	-3.11	-8.79	-9.63	-3.40		1.77	-0.15	-1.30	3.55
Δ HbA1c	Mean difference	-4.58	-16.56	-13.52	-7.03	-6.62	-15.66	-16.52	-11.05		-1.95	0.99	-2.91	-3.93
	SE	2.11	2.06	2.07	2.17	1.98	1.84	1.96	1.94		2.49	2.34	2.43	2.51
	P-value	**0.031**	**0.000**	**0.000**	**0.001**	**0.001**	**0.000**	**0.000**	**0.000**		0.43	0.67	0.23	0.12
	95 CI Lower	-8.73	-20.62	-17.60	-11.30	-10.52	-19.29	-20.38	-14.87		-6.85	-3.62	-7.70	-8.87
	95 CI Upper	-0.43	-12.50	-9.44	-2.76	-2.71	-12.02	-12.65	-7.23		2.96	5.60	1.88	1.00
Δ Glucose	Mean difference	-3.01	-1.94	-1.42	-0.76	-2.05	-2.46	-2.00	-1.77		0.91	-0.58	-0.64	-1.07
	SE	0.50	0.49	0.49	0.51	0.47	0.45	0.47	0.49		0.64	0.62	0.63	0.66
	P-value	**0.000**	**0.000**	**0.004**	0.14	**0.000**	**0.000**	**0.000**	**0.000**		0.16	0.36	0.32	0.11
	95 CI Lower	-3.99	-2.90	-2.39	-1.77	-2.98	-3.35	-2.92	-2.73		-0.36	-1.80	-1.89	-2.38
	95 CI Upper	-2.03	-0.98	-0.44	0.26	-1.12	-1.56	-1.07	-0.81		2.17	0.65	0.62	0.24
Δ HOMA-IR	Mean difference	-2.08	-2.18	-1.23	-1.21	-0.75	-1.50	-1.64	-0.78		1.26	0.62	-0.48	0.37
	SE	0.65	0.62	0.63	0.65	0.61	0.58	0.60	0.63		0.84	0.79	0.81	0.85
	P-value	**0.002**	**0.001**	0.050	0.065	0.22	**0.011**	**0.007**	0.22		0.13	0.44	0.56	0.67
	95 CI Lower	-3.36	-3.40	-2.47	-2.50	-1.94	-2.64	-2.83	-2.01		-0.38	-0.95	-2.08	-1.31
	95 CI Upper	-0.80	-0.96	0.00	0.07	0.45	-0.35	-0.45	0.46		2.91	2.18	1.12	2.04
Δ Triglycerides	Mean difference	-0.45	-0.38	-0.08	-0.11	-0.73	-0.38	-0.35	-0.42		-0.28	0.01	-0.27	-0.30
	SE	0.26	0.25	0.26	0.27	0.25	0.23	0.24	0.25		0.33	0.32	0.32	0.34
	P-value	0.089	0.13	0.77	0.68	**0.003**	0.11	0.15	0.10		0.40	0.97	0.41	0.38
	95 CI Lower	-0.96	-0.88	-0.58	-0.64	-1.22	-0.84	-0.83	-0.91		-0.93	-0.61	-0.91	-0.97
	95 CI Upper	0.07	0.12	0.43	0.41	-0.25	0.09	0.13	0.08		0.37	0.64	0.37	0.37
Δ LEAP2	Mean difference	-1.64	-5.24	-5.49	-5.39	1.65	-2.09	-6.11	-5.80		3.22	3.07	-0.69	-0.49
	SE	1.30	1.23	1.24	1.23	1.23	1.13	1.13	1.12		1.56	1.44	1.43	1.42
	P-value	0.21	**0.000**	**0.000**	**0.000**	0.18	0.066	**0.000**	**0.000**		**0.041**	**0.034**	0.63	0.73
	95 CI Lower	-4.20	-7.67	-7.94	-7.81	-0.77	-4.32	-8.33	-8.01		0.14	0.24	-3.52	-3.28
	95 CI Upper	0.91	-2.81	-3.05	-2.97	4.07	0.14	-3.88	-3.59		6.29	5.91	2.13	2.30
Δ Appetite	Mean difference	-9.26	-2.75	3.51	1.93	-10.38	-4.22	-3.07	-0.16		-1.12	-1.46	-6.57	-2.09
	SE	4.97	4.91	5.04	5.28	4.61	4.33	4.74	4.88		5.91	5.64	6.07	6.37
	P-value	0.064	0.58	0.49	0.71	**0.025**	0.33	0.52	0.97		0.85	0.80	0.28	0.74
	95 CI Lower	-19.06	-12.43	-6.44	-8.48	-19.48	-12.75	-12.41	-9.77		-12.77	-12.57	-18.54	-14.65
	95 CI Upper	0.53	6.92	13.45	12.34	-1.29	4.31	6.28	9.45		10.53	9.65	5.39	10.46

Table 3.13. Post-hoc analysis of absolute BMI and change in: % weight, fasting appetite ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 in SMM and DJBL groups.
This table describes the post-hoc analysis of results in Table 3.12. Mean difference, standard error, upper and lower confidence intervals for absolute BMI and change in: % weight, fasting appetite VAS ratings, HbA1c, glucose, insulin, HOMA-IR and LEAP2 between weeks 2,26,50 and 104 vs. -2 weeks) between SMM and DJBL groups. Between-participant factors including sweet taste detection task (done weeks -2, 2 and 26 for fasting glucose, insulin, HOMA-IR and LEAP2). Within-participant factors include visits at -2, 2, 26, 50 and 104 weeks. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: VAS, visual analogue scale; HOMA-IR, homeostatic model assessment for insulin resistance; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

3.4.3. Correlations of Fasting Plasma LEAP2 with Fasting Outcomes

3.4.3.1. Correlations with weight

SMM group

Over 104 weeks, *absolute* plasma LEAP2 *positively* correlated with BMI (Figure 3.5A, Table 3.1), and *changes* in plasma LEAP2 vs. baseline also *positively* correlated with change in % weight (Figure 3.5B, Table 3.14).

DJBL group

Over 50 weeks, *absolute* plasma LEAP2 *positively* correlated with BMI (Figure 3.6A, Table 3.16), and *changes* in plasma LEAP2 vs. baseline also *positively* correlated with change in % weight (Figure 3.6B, Table 3.14).

DJBL vs. SMM group

In comparison of correlational analysis between groups (i.e. difference in β parameters) over 50 weeks, there was no significant difference in correlations of *absolute* or *changes* in plasma LEAP2 with BMI between groups (P=0.38-0.97) (Tables 3.19, 3.20).

3.4.3.2. Correlations with fasting glucose metabolism

SMM group

Absolute plasma LEAP2 *positively* correlated with plasma glucose, HOMA-IR (Figure 3.5C,E, Figure 3.9A, Tables 3.14, 3.18), HbA1c and serum insulin (Figure 3.7A,C, Table 3.15). *Changes* in plasma LEAP2 also *positively* correlated with *changes* in plasma glucose, HOMA-IR (Figure 3.5D,F, Tables 3.14, 3.18), HbA1c, but not with

changes in serum insulin (Figure 3.7B,D, Table 3.15).

DJBL group

Absolute plasma LEAP2 *positively* correlated with HOMA-IR and HbA1c (Figure 3.6E, Figures 3.8A, 3.9B, Table 3.16, 3.17, 3.18). *Absolute* plasma LEAP2 *tended* to *positively* correlate with *absolute* plasma glucose and serum insulin (Figure 3.6C, Figure 3.8C, Tables 3.16, 3.17) *Changes* in plasma LEAP2 did not correlate with *changes* in plasma glucose, HOMA-IR nor serum insulin (Figure 3.6D,F, Figure 3.8D, Tables 3.16, 3.17), but *positively* correlated with *changes* in HbA1c (Figure 3.8B, Table 3.17)

DJBL vs. SMM group

Over 50 weeks, there was a significant between-group difference in correlation of *absolute* plasma LEAP2 with *absolute* HOMA-IR, with beta parameter being *higher* in SMM than DJBL group (Figures 3.9A,B, Table 3.19), but not for *change* in plasma LEAP2 and HOMA-IR (Table 3.20). There were no between group differences in correlations of *absolute* or *changes* in plasma LEAP2 with plasma glucose, serum insulin nor HbA1c (Tables 3.19, 3.20).

3.4.3.3. Correlations with fasting triglycerides

SMM group

Absolute plasma LEAP2 *positively* correlated with serum triglycerides (Figures 3.7E, 3.9C, Tables 3.15, 3.18). *Change* in plasma LEAP2 did not correlate with *change* in serum triglycerides (Figure 3.7F, Table 3.15).

DJBL group

Absolute or *changes* in plasma LEAP2 did not correlate with *absolute* or *changes* in serum triglycerides (Figures 3.8E,F, 3.9D, Tables 3.17, 3.18).

DJBL vs. SMM group

Over 50 weeks, there was a significant between-group difference in *absolute* plasma LEAP2 correlation with *absolute* serum triglycerides (Table 3.19), but not in *changes* in plasma LEAP2 with *changes* in serum triglycerides (Table 3.20).

3.4.3.4. Correlations with fasting appetite ratings

SMM group

Over 104 weeks, *absolute* fasting plasma LEAP2 did not correlate with *absolute* fasted appetite ratings (Table 3.14; Figure 3.5G), even when including *absolute* glucose as a covariate (Table 3.14). *Changes* in fasting plasma LEAP2 also did not correlate with *changes* in fasted appetite ratings (Table 3.14; Figure 3.5H), and when including *changes* in glucose as a covariate (Table 3.14).

DJBL group

Over 50 weeks, *absolute* fasting plasma LEAP2 did not correlate with *absolute* fasted appetite ratings (Figure 3.6G, Table 3.16), but, there was a significant *negative* correlation of *absolute* appetite with *absolute* LEAP2, when adjusting for *absolute* plasma glucose (r^2=0.074, P=0.048) as a covariate (Table 3.16). *Changes* in fasting plasma LEAP2 did not correlate with *changes* in fasted appetite ratings (P=0.85-0.93; Figure 3.6H, Table 3.16), even when including *changes* in fasting plasma glucose as a covariate (Table 3.16).

DJBL vs. SMM group

There was a more negative correlation in DJBL vs. SMM for correlation of *absolute* fasting plasma LEAP2 with *absolute* fasted appetite ratings (P=0.042, Table 3.19), with a trend for group effect when including *absolute* fasting plasma glucose as a covariate (P=0.063, Table 3.19). There was no significant between-group difference in correlation of *changes* in fasting plasma LEAP2 with *changes* in fasted appetite ratings, even when including *changes* in fasting plasma glucose as a covariate (P=0.85-0.93, Table 3.20).

3.4.3.5. Correlations with fasting hunger ratings

SMM group

Absolute fasting plasma LEAP2 did not correlate with *absolute* fasted hunger ratings (Figure 3.7G, Table 3.19). *Changes* in fasting plasma LEAP2 also did not correlate with *changes* in fasted hunger ratings (Figure 3.7H, Table 3.20).

DJBL group

Absolute fasting plasma LEAP2 did not correlate with *absolute* fasted hunger ratings (Figure 3.8G, Table 3.19). *Changes* in fasting plasma LEAP2 also did not correlate with *changes* in fasted hunger ratings (Figure 3.8H, Table 3.20).

DJBL vs. SMM group

There was a more negative correlation in DJBL vs. SMM for correlation of *absolute* fasting plasma LEAP2 with *absolute* fasted hunger ratings (P=0.046, Table 3.19). There were no significant between group differences in correlations of *changes* in hunger with *changes* in plasma LEAP2 (P=0.91, Table 3.20).

3.4.4. Correlation Analysis of Fasting Plasma LEAP2 with Outcome Variables after DJBL Explant

3.4.4.1. Correlations with weight

Changes in fasting plasma LEAP2 did not correlate with *change*s in weight between 50 and 104 weeks after DJBL explant (Figure 3.10A, Table 3.21).

3.4.4.2. Correlations with glucose metabolism markers

*Change*s in fasting plasma LEAP2 did not correlate with *changes* in: HbA1c, HOMA-IR, fasting plasma glucose and serum insulin between 50 and 104 weeks after DJBL explant (Figure 3.10B-E, Table 3.21).

3.4.4.3. Correlations with triglycerides

*Change*s in fasting plasma LEAP2 did not correlate with *changes* in fasting serum triglycerides between 50 and 104 weeks after DJBL explant (Figure 3.10F, Table 3.21).

3.4.4.4. Correlations with appetite and hunger ratings

Changes in fasting plasma LEAP2 did not correlate with *changes* in fasting appetite ratings or fasting hunger ratings between 50 and 104 weeks after DJBL explant (Figure 3.10G,H, Table 3.21).

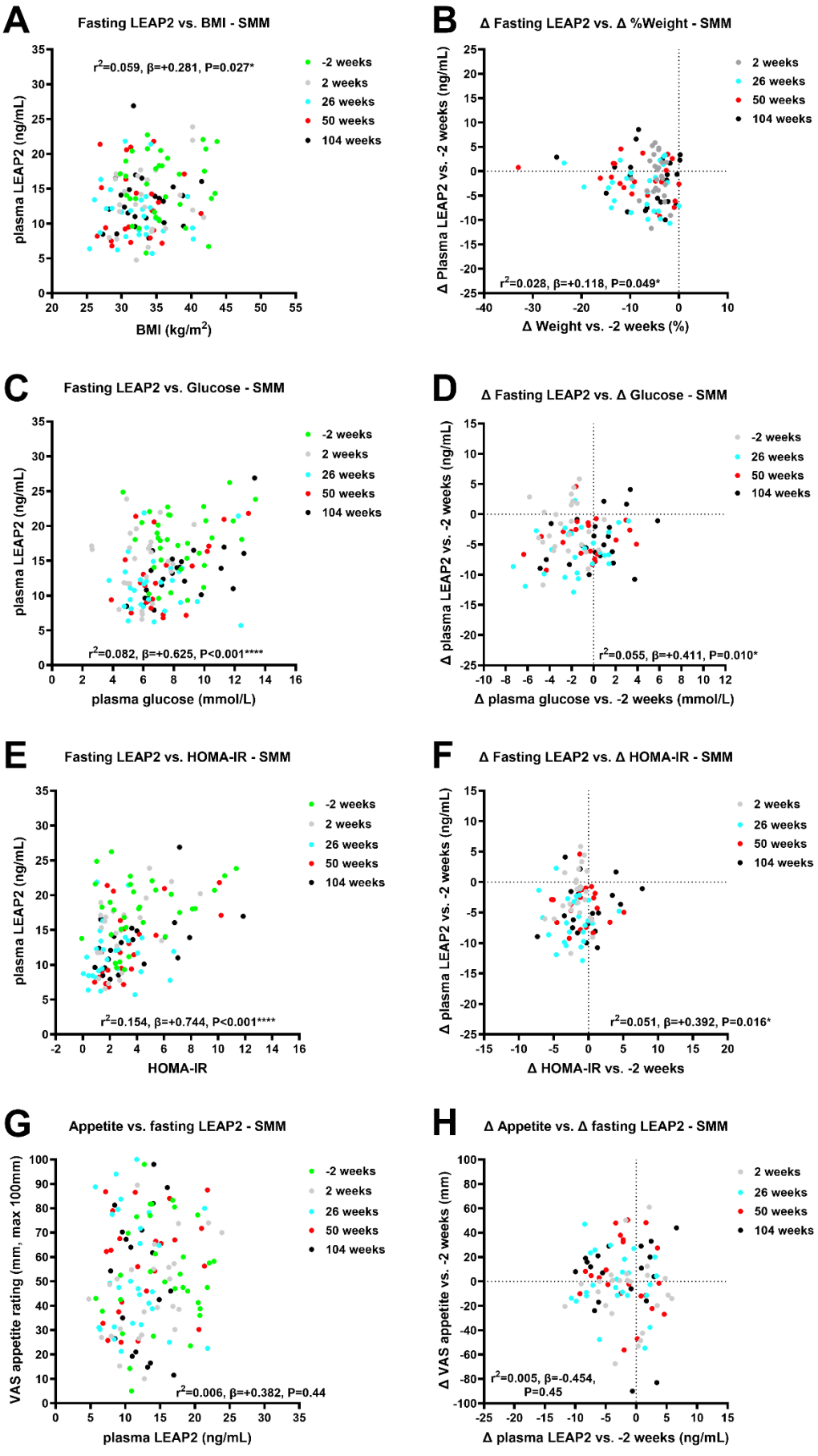

A
Fasting LEAP2 vs. BMI - SMM
r²=0.059, β=+0.281, P=0.027*
plasma LEAP2 (ng/mL)
BMI (kg/m²)
-2 weeks
2 weeks
26 weeks
50 weeks
104 weeks
B
Δ Fasting LEAP2 vs. Δ %Weight - SMM
Δ Plasma LEAP2 vs. -2 weeks (ng/mL)
Δ Weight vs. -2 weeks (%)
r²=0.028, β=+0.118, P=0.049*
2 weeks
26 weeks
50 weeks
104 weeks
C
Fasting LEAP2 vs. Glucose - SMM
plasma LEAP2 (ng/mL)
plasma glucose (mmol/L)
r²=0.082, β=+0.625, P<0.001****
-2 weeks
2 weeks
26 weeks
50 weeks
104 weeks
D
Δ Fasting LEAP2 vs. Δ Glucose - SMM
Δ plasma LEAP2 vs. -2 weeks (ng/mL)
Δ plasma glucose vs. -2 weeks (mmol/L)
r²=0.055, β=+0.411, P=0.010*
-2 weeks
26 weeks
50 weeks
104 weeks
E
Fasting LEAP2 vs. HOMA-IR - SMM
plasma LEAP2 (ng/mL)
HOMA-IR
r²=0.154, β=+0.744, P<0.001****
-2 weeks
2 weeks
26 weeks
50 weeks
104 weeks
F
Δ Fasting LEAP2 vs. Δ HOMA-IR - SMM
Δ plasma LEAP2 vs. -2 weeks (ng/mL)
Δ HOMA-IR vs. -2 weeks
r²=0.051, β=+0.392, P=0.016*
2 weeks
26 weeks
50 weeks
104 weeks
G
Appetite vs. fasting LEAP2 - SMM
VAS appetite rating (mm, max 100mm)
plasma LEAP2 (ng/mL)
r²=0.006, β=+0.382, P=0.44
-2 weeks
2 weeks
26 weeks
50 weeks
104 weeks
H
Δ Appetite vs. Δ fasting LEAP2 - SMM
Δ VAS appetite vs. -2 weeks (mm)
Δ plasma LEAP2 vs. -2 weeks (ng/mL)
r²=0.005, β=-0.454, P=0.45
2 weeks
26 weeks
50 weeks
104 weeks

Figure 3.5. Correlations of absolute and change in fasting plasma LEAP2 with absolute and change in weight, fasting glucose, HOMA-IR and appetite ratings across visits in the SMM group.

Correlations of absolute fasting plasma LEAP2 with (A) BMI, (C) absolute fasting plasma glucose, (E) HOMA-IR, (G) absolute fasting appetite VAS ratings across visits -2, 2, 26, 50 and 104 weeks; and correlations of change in fasting plasma LEAP2 with (B) % weight change; change in (D) fasting plasma glucose, (F) HOMA-IR and (H) fasting appetite VAS ratings between weeks 2,26,50 and 104 vs. -2 weeks using linear mixed model analysis including sweet taste detection task (done in the taste sub-group at weeks -2, 2 and 26 only, for fasting glucose, HOMA-IR and LEAP2 only) as between participant, and weeks (-2, 2, 26, 50, 104) as within participant fixed factors, to examine effects of interventions on absolute and change in fasting plasma LEAP2 over time with pseudo r^2 and beta parameters. * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=22-30. Abbreviations: HOMA-IR, homeostatic model assessment for insulin resistance; SMM, standard medical management; VAS, visual analogue scale.

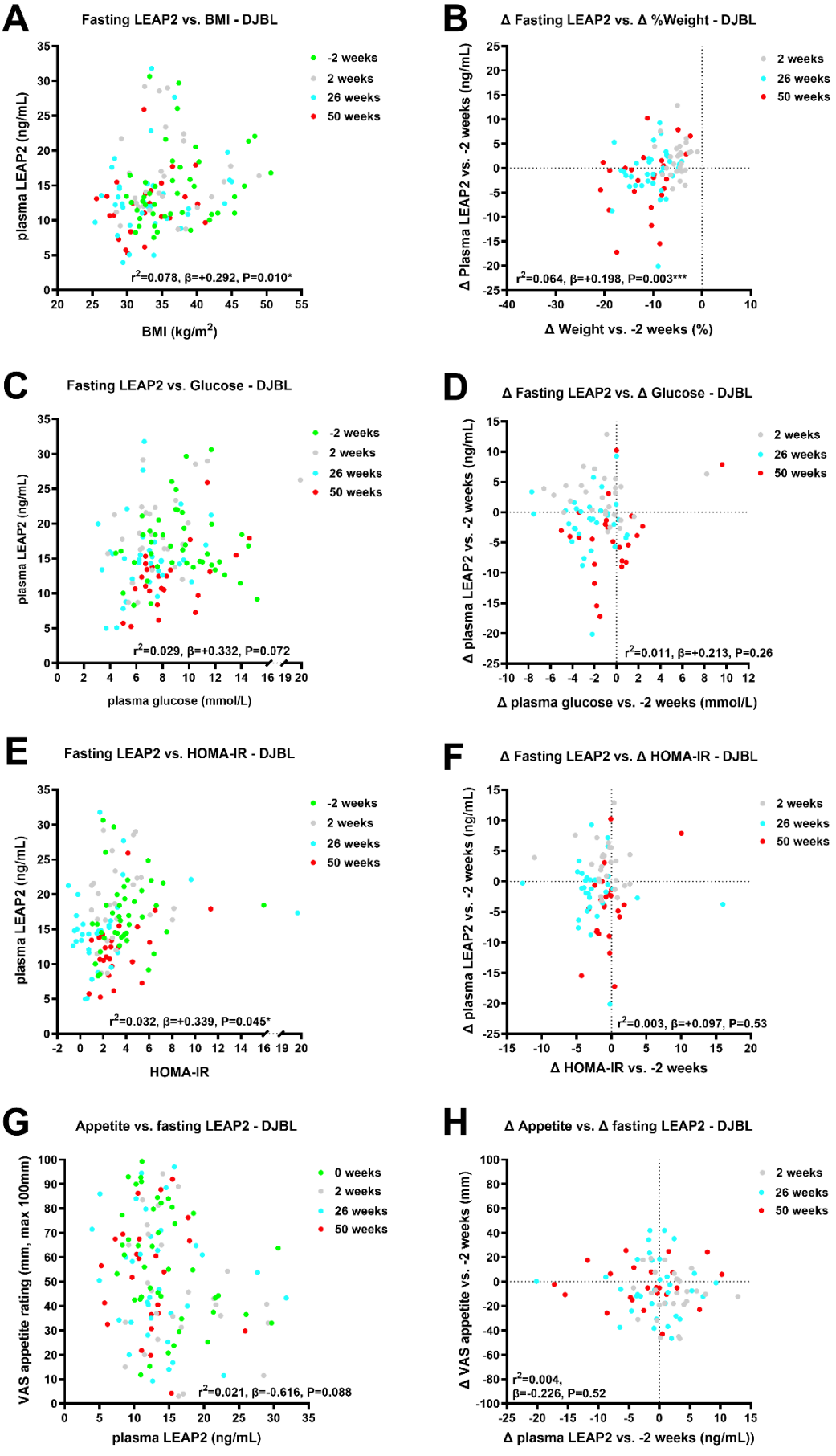
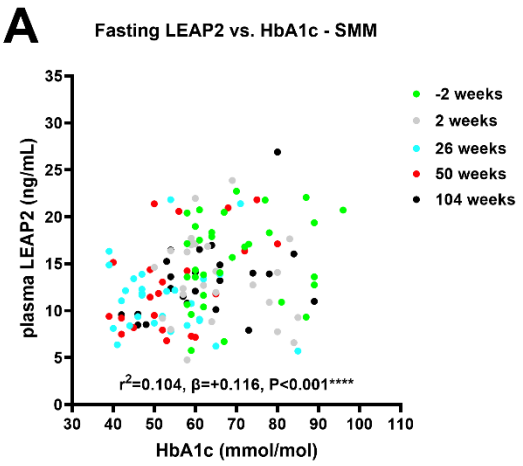
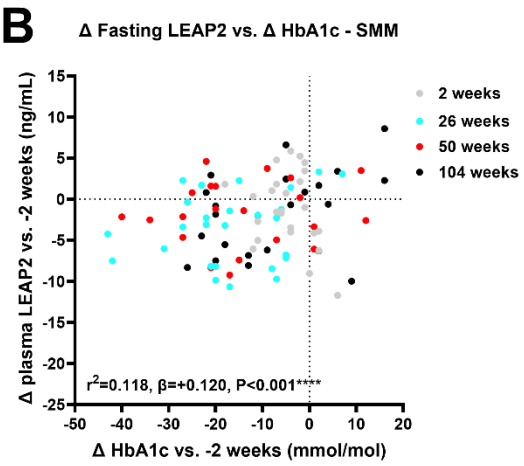
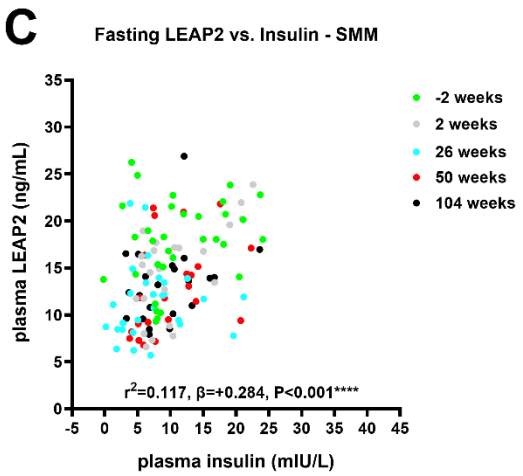

A
Fasting LEAP2 vs. BMI - DJBL
plasma LEAP2 (ng/mL)
BMI (kg/m2)
r2=0.078, β=+0.292, P=0.010*
-2 weeks
2 weeks
26 weeks
50 weeks
B
Δ Fasting LEAP2 vs. Δ %Weight - DJBL
Δ Plasma LEAP2 vs. -2 weeks (ng/mL)
Δ Weight vs. -2 weeks (%)
r2=0.064, β=+0.198, P=0.003***
2 weeks
26 weeks
50 weeks
C
Fasting LEAP2 vs. Glucose - DJBL
plasma LEAP2 (ng/mL)
plasma glucose (mmol/L)
r2=0.029, β=+0.332, P=0.072
-2 weeks
2 weeks
26 weeks
50 weeks
D
Δ Fasting LEAP2 vs. Δ Glucose - DJBL
Δ plasma LEAP2 vs. -2 weeks (ng/mL)
Δ plasma glucose vs. -2 weeks (mmol/L)
r2=0.011, β=+0.213, P=0.26
2 weeks
26 weeks
50 weeks
E
Fasting LEAP2 vs. HOMA-IR - DJBL
plasma LEAP2 (ng/mL)
HOMA-IR
r2=0.032, β=+0.339, P=0.045*
-2 weeks
2 weeks
26 weeks
50 weeks
F
Δ Fasting LEAP2 vs. Δ HOMA-IR - DJBL
Δ plasma LEAP2 vs. -2 weeks (ng/mL)
Δ HOMA-IR vs. -2 weeks
r2=0.003, β=+0.097, P=0.53
2 weeks
26 weeks
50 weeks
G
Appetite vs. fasting LEAP2 - DJBL
VAS appetite rating (mm, max 100mm)
plasma LEAP2 (ng/mL)
r2=0.021, β=-0.616, P=0.088
0 weeks
2 weeks
26 weeks
50 weeks
H
Δ Appetite vs. Δ fasting LEAP2 - DJBL
Δ VAS appetite vs. -2 weeks (mm)
Δ plasma LEAP2 vs. -2 weeks (ng/mL))
r2=0.004,
β=-0.226, P=0.52
2 weeks
26 weeks
50 weeks

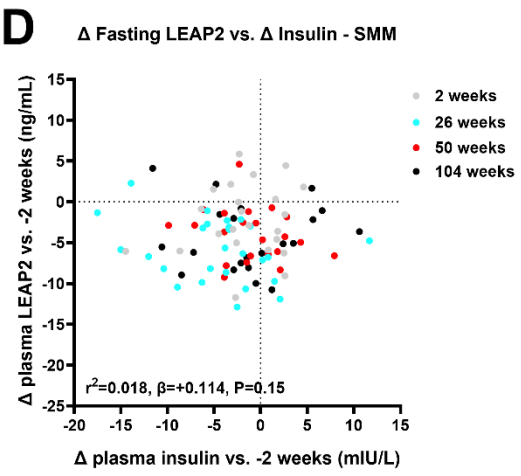
Figure 3.6. Correlations of absolute and change in fasting plasma LEAP2 with absolute and change in weight, fasting glucose, HOMA-IR and appetite ratings across visits in the DJBL group.

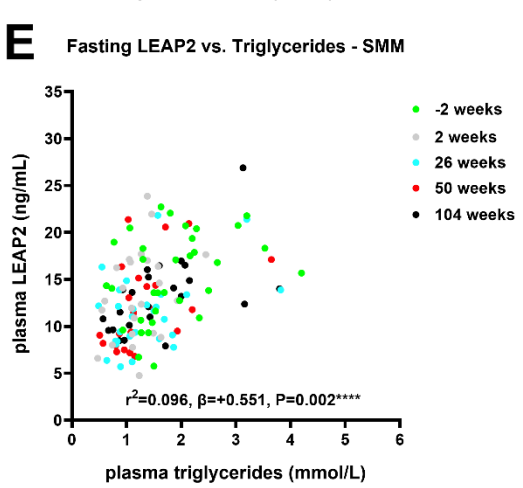
Correlations of absolute fasting plasma LEAP2 with (A) BMI, (C) absolute fasting plasma glucose, (E) HOMA-IR, (G) absolute fasting appetite VAS ratings across visits -2, 2, 26 and 50 weeks; and correlations of change in fasting plasma LEAP2 (B) % weight change; change in (D) fasting plasma glucose, (F) HOMA-IR and (H) fasting appetite VAS ratings between weeks 2,26 and 50 vs. -2 weeks using linear mixed model analysis including sweet taste detection task (done in the taste sub-group at weeks -2, 2 and 26 only, for fasting glucose, HOMA-IR and LEAP2 only) as between participant, and weeks (-2, 2, 26, 50) as within participant fixed factors, to examine effects of interventions on absolute and change in fasting plasma LEAP2 over time with pseudo r^2 and beta parameters. * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=22-30. Abbreviations: DJBL, duodenal-jejunal bypass liner; HOMA-IR, homeostatic model assessment for insulin resistance; VAS, visual analogue scale.

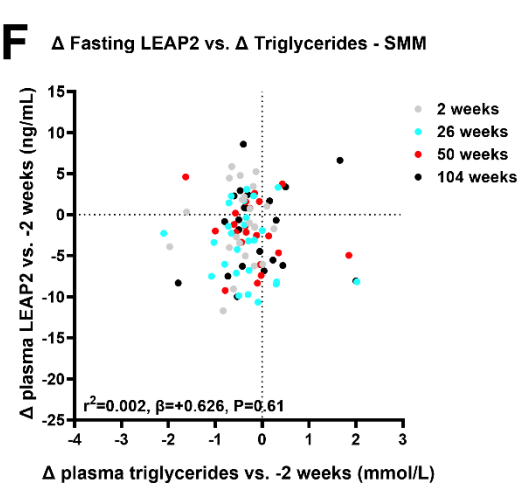

A
Fasting LEAP2 vs. HbA1c - SMM
plasma LEAP2 (ng/mL)
HbA1c (mmol/mol)
r²=0.104, β=+0.116, P<0.001****
-2 weeks
2 weeks
26 weeks
50 weeks
104 weeks

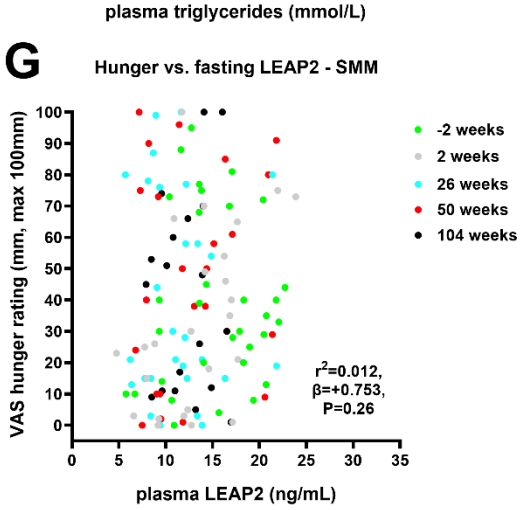

B
Δ Fasting LEAP2 vs. Δ HbA1c - SMM
Δ plasma LEAP2 vs. -2 weeks (ng/mL)
Δ HbA1c vs. -2 weeks (mmol/mol)
r²=0.118, β=+0.120, P<0.001****
2 weeks
26 weeks
50 weeks
104 weeks


C
Fasting LEAP2 vs. Insulin - SMM
plasma LEAP2 (ng/mL)
plasma insulin (mIU/L)
r²=0.117, β=+0.284, P<0.001****
-2 weeks
2 weeks
26 weeks
50 weeks
104 weeks


D
Δ Fasting LEAP2 vs. Δ Insulin - SMM
Δ plasma LEAP2 vs. -2 weeks (ng/mL)
Δ plasma insulin vs. -2 weeks (mIU/L)
r²=0.018, β=+0.114, P=0.15
2 weeks
26 weeks
50 weeks
104 weeks


E
Fasting LEAP2 vs. Triglycerides - SMM
plasma LEAP2 (ng/mL)
plasma triglycerides (mmol/L)
r²=0.096, β=+0.551, P=0.002****
-2 weeks
2 weeks
26 weeks
50 weeks
104 weeks


F
Δ Fasting LEAP2 vs. Δ Triglycerides - SMM
Δ plasma LEAP2 vs. -2 weeks (ng/mL)
Δ plasma triglycerides vs. -2 weeks (mmol/L)
r²=0.002, β=+0.626, P=0.61
2 weeks
26 weeks
50 weeks
104 weeks


G
Hunger vs. fasting LEAP2 - SMM
VAS hunger rating (mm, max 100mm)
plasma LEAP2 (ng/mL)
r²=0.012,
β=+0.753,
P=0.26
-2 weeks
2 weeks
26 weeks
50 weeks
104 weeks

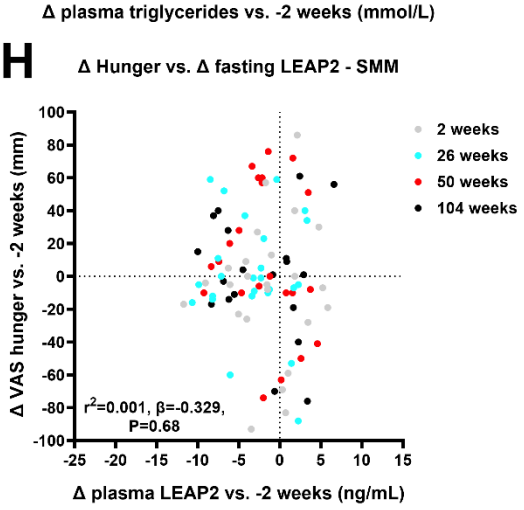

H
Δ Hunger vs. Δ fasting LEAP2 - SMM
Δ VAS hunger vs. -2 weeks (mm)
Δ plasma LEAP2 vs. -2 weeks (ng/mL)
r²=0.001, β=-0.329,
P=0.68
2 weeks
26 weeks
50 weeks
104 weeks

Figure 3.7. Correlations of absolute and change in fasting plasma LEAP2 with absolute and change in HbA1c, fasting insulin, triglycerides and hunger ratings across visits in SMM group.

Correlations of absolute fasting plasma LEAP2 with absolute fasting (A) HbA1c, (C) serum insulin, (E) serum triglycerides, (G) hunger VAS ratings across visits -2, 2, 26, 50 and 104 weeks; and correlations of change in fasting plasma LEAP2 with change in fasting (B) HbA1c, (D) serum insulin, (F) serum triglycerides and (H) hunger VAS ratings between weeks 2, 26, 50 and 104 vs. -2 weeks. Statistical analysis used linear mixed model analysis with weeks (-2, 2, 26, 50, 104) as within participant fixed factor, to examine relationships between absolute and changes in fasting plasma LEAP2 over time, and metabolic parameters and hunger ratings (using values corrected for sweet taste detection task for fasting serum insulin and plasma LEAP2 in taste sub-group at weeks -2, 2 and 26 only). Results given as pseudo r^2 and beta parameters. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, n=22-30. Abbreviations: SMM, standard medical management, VAS, visual analogue scale.

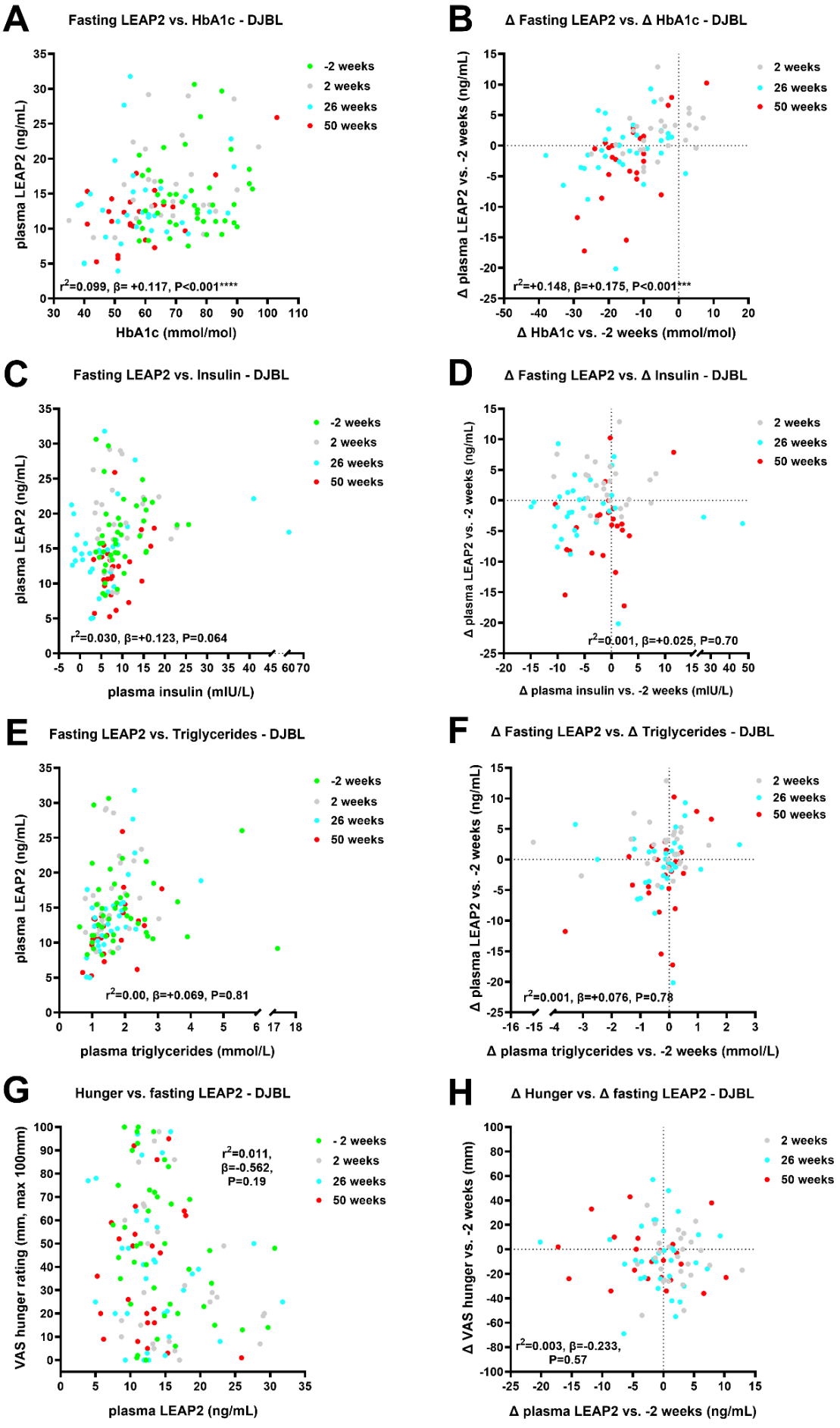

A
Fasting LEAP2 vs. HbA1c - DJBL
plasma LEAP2 (ng/mL)
HbA1c (mmol/mol)
r²=0.099, β= +0.117, P<0.001****
-2 weeks
2 weeks
26 weeks
50 weeks
B
Δ Fasting LEAP2 vs. Δ HbA1c - DJBL
Δ plasma LEAP2 vs. -2 weeks (ng/mL)
Δ HbA1c vs. -2 weeks (mmol/mol)
r²=+0.148, β=+0.175, P<0.001***
2 weeks
26 weeks
50 weeks
C
Fasting LEAP2 vs. Insulin - DJBL
plasma LEAP2 (ng/mL)
plasma insulin (mIU/L)
r²=0.030, β=+0.123, P=0.064
-2 weeks
2 weeks
26 weeks
50 weeks
D
Δ Fasting LEAP2 vs. Δ Insulin - DJBL
Δ plasma LEAP2 vs. -2 weeks (ng/mL)
Δ plasma insulin vs. -2 weeks (mIU/L)
r²=0.001, β=+0.025, P=0.70
2 weeks
26 weeks
50 weeks
E
Fasting LEAP2 vs. Triglycerides - DJBL
plasma LEAP2 (ng/mL)
plasma triglycerides (mmol/L)
r²=0.00, β=+0.069, P=0.81
-2 weeks
2 weeks
26 weeks
50 weeks
F
Δ Fasting LEAP2 vs. Δ Triglycerides - DJBL
Δ plasma LEAP2 vs. -2 weeks (ng/mL)
Δ plasma triglycerides vs. -2 weeks (mmol/L)
r²=0.001, β=+0.076, P=0.78
2 weeks
26 weeks
50 weeks
G
Hunger vs. fasting LEAP2 - DJBL
VAS hunger rating (mm, max 100mm)
plasma LEAP2 (ng/mL)
r²=0.011, β=-0.562, P=0.19
- 2 weeks
2 weeks
26 weeks
50 weeks
H
Δ Hunger vs. Δ fasting LEAP2 - DJBL
Δ VAS hunger vs. -2 weeks (mm)
Δ plasma LEAP2 vs. -2 weeks (ng/mL)
r²=0.003, β=-0.233, P=0.57
2 weeks
26 weeks
50 weeks

Figure 3.8. Correlations of absolute and change in fasting plasma LEAP2 with absolute and change in HbA1c, fasting insulin, triglycerides and hunger ratings across visits in DJBL group.

Correlations of absolute fasting plasma LEAP2 with absolute fasting (A) HbA1c, (C) serum insulin, (E) serum triglycerides, (G) hunger VAS ratings across visits -2, 2, 26, 50 and 104 weeks; and correlations of change in fasting plasma LEAP2 with change in fasting (B) HbA1c, (D) serum insulin, (F) serum triglycerides and (H) hunger VAS ratings between weeks 2, 26, 50 and 104 vs. -2 weeks. Statistical analysis used linear mixed model analysis with weeks (-2, 2, 26, 50 and 104) as within participant fixed factor, to examine relationships between absolute and changes in fasting plasma LEAP2 over time, and metabolic parameters and hunger ratings (using values corrected for sweet taste detection task for fasting serum insulin and plasma LEAP2 in taste sub-group at weeks -2, 2 and 26 only). Results given as pseudo r^2 and beta parameters. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, n=22-30. Abbreviations: DJBL, duodenal-jejunal bypass liner; VAS, visual analogue scale.

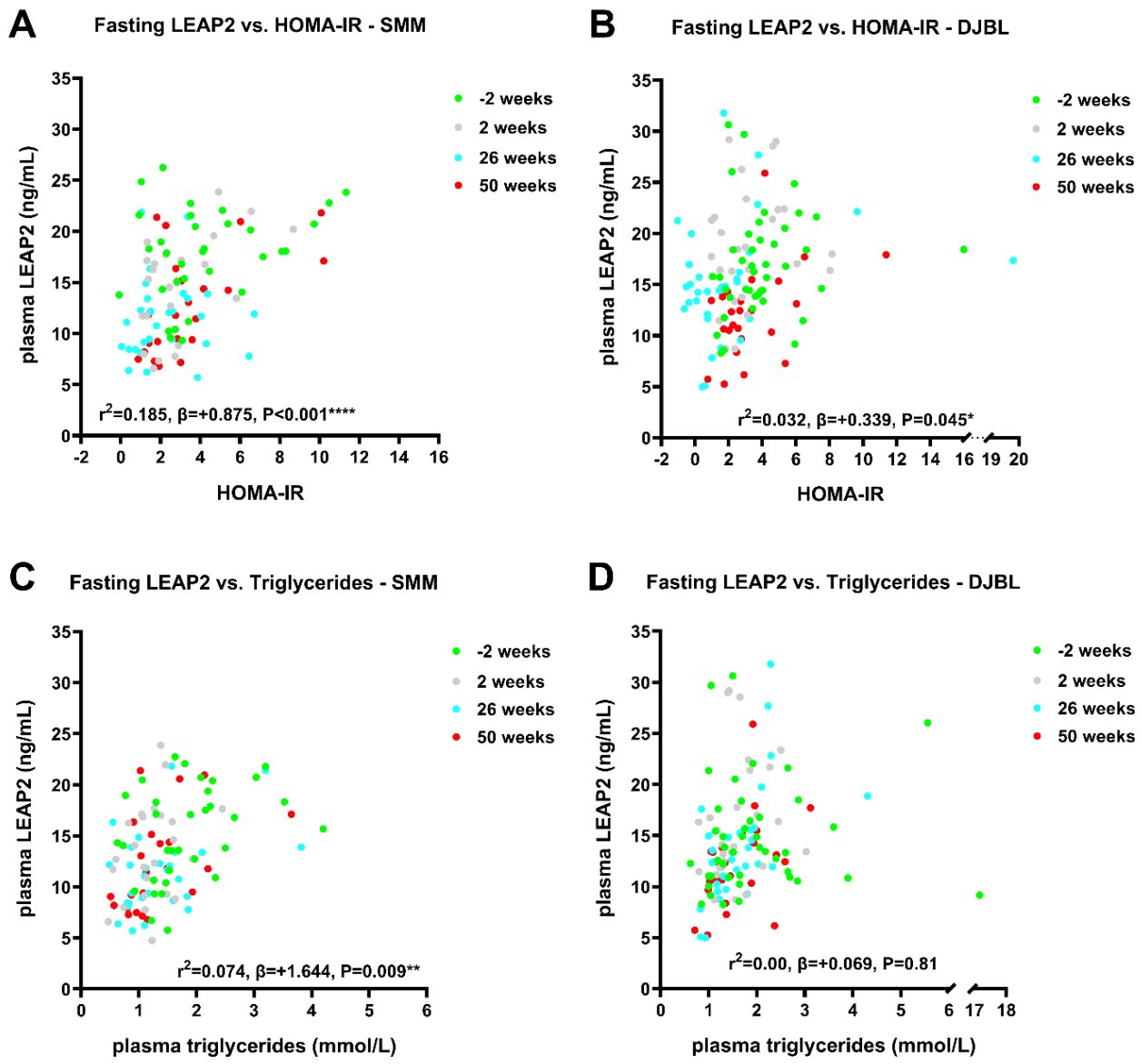


Figure 3.9. Correlations of absolute fasting plasma LEAP2 with absolute fasting HOMA-IR and triglycerides in SMM and DJBL groups.

Correlations of absolute fasting plasma LEAP2 with (A,B) absolute fasting HOMA-IR and (C,D) absolute fasting serum triglycerides in (A,C) SMM and (B,D) SMM groups, across visits -2, 2, 26 and 50 weeks. Statistical analysis used linear mixed model analysis with weeks (-2, 2, 26 and 50) as within participant fixed factor, to examine relationships between fasting plasma LEAP2 and metabolic parameters over time using values corrected for sweet taste detection task for fasting serum insulin and plasma LEAP2 in taste sub-group at weeks -2, 2 and 26 only). Results given as pseudo r^2 and beta parameters. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, n=22-30. Abbreviations: DJBL, duodenal-jejunal bypass liner; HOMA-IR, homeostatic model assessment for insulin resistance; SMM, standard medical management.

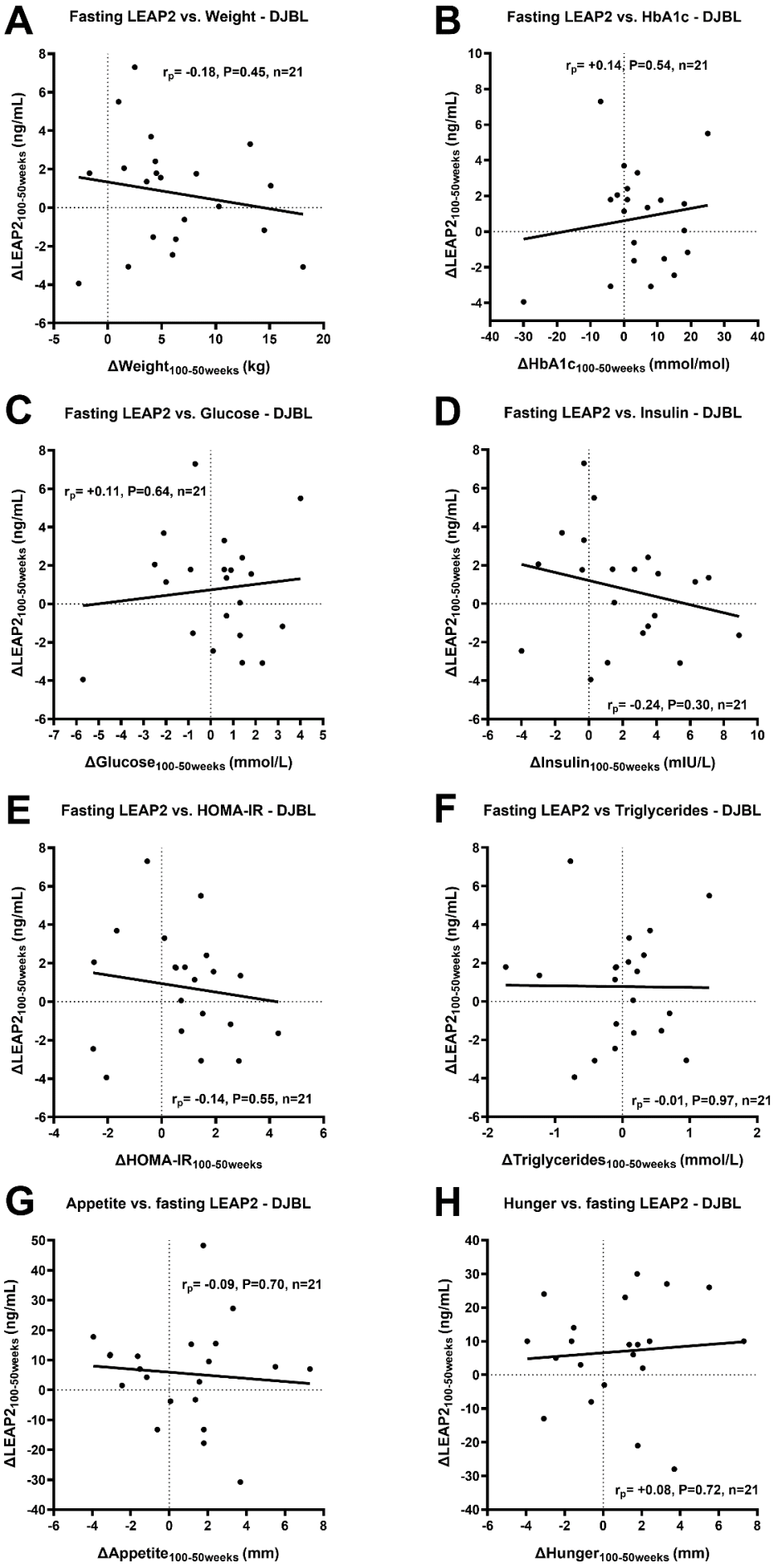

A
Fasting LEAP2 vs. Weight - DJBL
r_p= -0.18, P=0.45, n=21
ΔLEAP2100-50weeks (ng/mL)
ΔWeight100-50weeks (kg)
B
Fasting LEAP2 vs. HbA1c - DJBL
r_p= +0.14, P=0.54, n=21
ΔLEAP2100-50weeks (ng/mL)
ΔHbA1c100-50weeks (mmol/mol)
C
Fasting LEAP2 vs. Glucose - DJBL
r_p= +0.11, P=0.64, n=21
ΔLEAP2100-50weeks (ng/mL)
ΔGlucose100-50weeks (mmol/L)
D
Fasting LEAP2 vs. Insulin - DJBL
r_p= -0.24, P=0.30, n=21
ΔLEAP2100-50weeks (ng/mL)
ΔInsulin100-50weeks (mIU/L)
E
Fasting LEAP2 vs. HOMA-IR - DJBL
r_p= -0.14, P=0.55, n=21
ΔLEAP2100-50weeks (ng/mL)
ΔHOMA-IR100-50weeks
F
Fasting LEAP2 vs Triglycerides - DJBL
r_p= -0.01, P=0.97, n=21
ΔLEAP2100-50weeks (ng/mL)
ΔTriglycerides100-50weeks (mmol/L)
G
Appetite vs. fasting LEAP2 - DJBL
r_p= -0.09, P=0.70, n=21
ΔLEAP2100-50weeks (ng/mL)
ΔAppetite100-50weeks (mm)
H
Hunger vs. fasting LEAP2 - DJBL
r_p= +0.08, P=0.72, n=21
ΔLEAP2100-50weeks (ng/mL)
ΔHunger100-50weeks (mm)

Figure 3.10. Correlations of change in fasting plasma LEAP2 with change in weight, HbA1c, fasting glucose, insulin, HOMA-IR, triglycerides, appetite and hunger ratings after DJBL explant.

Correlations of change in fasting plasma LEAP2 with change in (A) weight, (B) HbA1c, fasting (C) plasma glucose, (D) serum insulin, (E) HOMA-IR, (F) serum triglycerides, (G) appetite and (H) hunger VAS ratings between weeks 50 and 100 (Δ 50-100weeks) from the period of DJBL explant (at 52 weeks) using Pearson's (r_P, linear regression line) correlation coefficients. * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001, n=10-21. Abbreviations: DJBL, duodenal-jejunal bypass liner; HOMA-IR, homeostatic model assessment for insulin resistance; kg, kilogram; VAS, visual analogue scale.

SMM, from -2 to 104 weeks		Covariate effect on the dependent variable							
Dependent	Covariate	r^2	df	F	P value	β parameter	β SEM	95% CI of β	β units
LEAP2	**BMI**	0.059	1, 90.05	5.057	**0.027**	0.281	0.125	+0.033, +0.530	ng/mL.kg/m2
Δ LEAP2	**Δ %weight**	0.028	1, 121.56	3.933	**0.050**	0.118	0.060	+0.000, +0.237	ng/mL.%
LEAP2	**Glucose**	0.082	1, 121.38	12.313	**0.001**	0.625	0.178	+0.272, +0.978	ng/mL.mmol/L
Δ LEAP2	**Δ Glucose**	0.055	1, 115.73	6.794	**0.010**	0.411	0.158	+0.99, +0.723	ng/mL.mmol/L
LEAP2	**HOMA-IR**	0.154	1, 118.12	19.727	**0.000**	0.744	0.167	+0.412, +1.076	ng/mL.arbitrary units
Δ LEAP2	**Δ HOMA-IR**	0.051	1, 110.83	5.957	**0.016**	0.392	0.161	+0.074, +0.711	ng/mL.arbitrary units
Appetite	**LEAP2**	0.006	1, 112.53	0.596	0.44	0.382	0.494	-0.598, +1.361	mm.ng/mL
	incl. Glucose	0.017	1, 111.73	0.216	0.64	0.235	0.506	-0.768, +1.239	mm.ng/mL.mmol/mL
Δ Appetite	**Δ LEAP2**	0.005	1, 118	0.572	0.45	-0.454	0.600	-1.643, +0.735	mm.ng/mL
	incl. Δ Glucose	0.007	1, 117	0.678	0.41	-0.501	0.609	-1.708, +0.705	mm.ng/mL.mmol/mL

Table 3.14. Effect of covariates on absolute and change in fasting plasma LEAP2 and appetite ratings across weeks in the SMM group.

This table shows the effects of covariates on absolute and change in fasting plasma LEAP2 and appetite VAS ratings across -2, 2, 26, 50 and 104 weeks (change is weeks vs. -2 weeks) using linear mixed model analysis including sweet taste detection task (done in the taste sub-group at weeks -2, 2 and 26 only, for fasting glucose, HOMA-IR and LEAP2) as between participant, and weeks (-2, 2, 26, 50 and 104) as within participant fixed factors. Covariates include absolute and change in BMI, weight, glucose, HOMA-IR and LEAP2 to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=22-30. Abbreviations: BMI, body mass index; VAS, visual analogue scale; HOMA-IR, homeostatic model assessment for insulin resistance; SMM, standard medical management.

SMM, from -2 to 104 weeks		Covariate effect on the dependent variable							
Dependent	Covariate	r2	df	F	P value	β parameter	β +/-SEM	95% CI of β	β units
LEAP2	HbA1c	0.104	1, 121.38	17.198	**0.000**	0.116	0.028	+0.061, +0.172	ng/mL.mmol/L
Δ LEAP2	Δ HbA1c	0.118	1, 121.89	18.336	**0.000**	0.120	0.028	+0.064, +0.175	ng/mL.mmol/L
LEAP2	insulin	0.117	1, 111.42	13.490	**0.000**	0.284	0.077	+0.131, +0.438	ng/mL.mIU/L
Δ LEAP2	Δ insulin	0.018	1, 114.77	2.083	0.15	0.114	0.079	-0.042, +0.271	ng/mL.mIU/L
LEAP2	TG	0.096	1, 99.07	10.500	**0.002**	1.786	0.551	+0.692, +2.879	ng/mL.mmol/L
Δ LEAP2	Δ TG	0.002	1, 121	0.26	0.61	0.319	0.626	-0.921, +1.559	ng/mL.mmol/L
Hunger	LEAP2	0.012	1, 107.66	1.273	0.26	0.753	0.667	-0.570, +2.076	mm.ng/mL
Δ Hunger	Δ LEAP2	0.001	1, 118	0.175	0.68	-0.329	0.787	-1.887, +1.228	mm.ng/mL

Table 3.15. Effect of covariates on absolute and change in fasting plasma LEAP2 and hunger ratings across weeks in the SMM group.

This table shows the effects of covariates on absolute and change in fasting plasma LEAP2 and hunger VAS ratings across -2, 2, 26, 50 and 104 weeks (change is weeks vs. -2 weeks) using linear mixed model analysis including sweet taste detection task (done in the taste sub-group at weeks -2, 2 and 26 only, for fasting glucose, HOMA-IR and LEAP2) as between participant, and weeks (-2, 2, 26, 50 and 104) as within participant fixed factors. Covariates include absolute and change in HbA1c, insulin, triglycerides and LEAP2 to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=22-30. Abbreviations: VAS, visual analogue scale; TG, triglycerides; SMM, standard medical management.

DJBL, from -2 to 50 weeks		Covariate effect on the dependent variable							
Dependent	Covariate	r2	df	F	P value	β parameter	β +/-SEM	95% CI of β	β units
LEAP2	**BMI**	0.078	1. 78.23	6.899	**0.010**	0.292	0.111	+0.071, +0.513	ng/mL.kg/m2
Δ LEAP2	**Δ %weight**	0.064	1, 108.93	9.184	**0.003**	0.198	0.065	+0.116, +0.354	ng/mL.%
LEAP2	**Glucose**	0.029	1, 115.93	3.304	0.072	0.332	0.183	-0.030, +0.695	ng/mL.mmol/L
Δ LEAP2	**Δ Glucose**	0.011	1, 119	1.285	0.26	0.213	0.188	-0.159, +0.586	ng/mL.mmol/L
LEAP2	**HOMA-IR**	0.032	1, 115	4.102	**0.045**	0.339	0.168	+0.007, +0.671	ng/mL.arbitrary units
Δ LEAP2	**Δ HOMA-IR**	0.003	1, 116	0.399	0.53	0.097	0.154	-0.207, +0.401	ng/mL.arbitrary units
Appetite	**LEAP2**	0.021	1, 117.63	2.965	0.088	-0.616	0.358	-1.325, +0.092	mm.ng/mL
	incl. Glucose	0.074	1, 114.81	3.982	**0.048**	-0.711	0.356	-1.416, -0.005	mm.ng/mL.mmol/mL
Δ Appetite	**Δ LEAP2**	0.004	1, 118	0.421	0.52	-0.226	0.348	-0.915, +0.463	mm.ng/mL
	incl. Δ Glucose	0.047	1, 116	0.600	0.44	-0.265	0.342	-0.942, +0.412	mm.ng/mL.mmol/mL

Table 3.16. Effect of covariates on absolute and change in fasting plasma LEAP2 and appetite ratings across weeks in the DJBL group.

This table shows the effects of covariates on absolute and change in fasting plasma LEAP2 and appetite VAS ratings across -2, 2, 26 and 50 weeks (change is weeks vs. -2 weeks) using linear mixed model analysis including sweet taste detection task (done in the taste sub-group at weeks -2, 2 and 26 only, for fasting glucose, HOMA-IR and LEAP2) as between participant, and weeks (-2, 2, 26 and 50) as within participant fixed factors. Covariates include absolute and change in BMI, weight, glucose, HOMA-IR and LEAP2 to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=24-37. Abbreviations: BMI, body mass index; VAS, visual analogue scale; HOMA-IR, homeostatic model assessment for insulin resistance; DJBL, duodenal-jejunal bypass liner.

DJBL, from -2 to 50 weeks		Covariate effect on the dependent variable							
Dependent	Covariate	r2	df	F	P value	β parameter	β +/-SEM	95% CI of β	β units
LEAP2	HbA1c	0.099	1, 118.10	11.884	**0.001**	0.117	0.034	+0.050, +0.184	ng/mL.mmol/L
Δ LEAP2	Δ HbA1c	0.148	1, 118.10	21.930	**0.000**	0.175	0.037	+0.101, +0.249	ng/mL.mmol/L
LEAP2	insulin	0.030	1, 115	3.505	0.064	0.123	0.066	-0.007, +0.253	ng/mL.mIU/L
Δ LEAP2	Δ insulin	0.001	1, 116	0.153	0.70	0.025	0.065	-0.103, +0.154	ng/mL.mIU/L
LEAP2	TG	0.000	1, 116	0.055	0.81	0.069	0.294	-0.513, +0.652	ng/mL.mmol/L
Δ LEAP2	Δ TG	0.001	1, 116	0.080	0.78	0.076	0.268	-0.456, +0.607	ng/mL.mmol/L
Hunger	LEAP2	0.011	1, 114.82	1.702	0.19	-0.562	0.431	-1.416, +0.291	mm.ng/mL
Δ Hunger	Δ LEAP2	0.003	1, 118	0.331	0.57	-0.233	0.405	-1.035, +0.569	mm.ng/mL

Table 3.17. Effect of covariates on absolute and change in fasting plasma LEAP2 and hunger ratings across weeks in the DJBL group.
This table shows the effects of covariates on absolute and change in fasting plasma LEAP2 and hunger VAS ratings across -2, 2, 26 and 50 weeks (change is weeks vs. -2 weeks) using linear mixed model analysis including sweet taste detection task (done in the taste sub-group at weeks -2, 2 and 26 only, for fasting glucose, HOMA-IR and LEAP2) as between participant, and weeks (-2, 2, 26 and 50) as within participant fixed factors. Covariates include absolute and change in HbA1c, insulin, triglycerides and LEAP2 to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=24-37. Abbreviations: VAS, visual analogue scale; TG, triglycerides; DJBL, duodenal-jejunal bypass liner.

	from -2 -to 50 weeks		Covariate effect on the dependent variable							
	Dependent	Covariate	r2	df	F	P value	β parameter	β +/-SEM	95% CI of β	β units
SMM	LEAP2	HOMA-IR	0.185	1, 95.43	19.804	**0.000**	0.875	0.197	+0.485, +1.265	ng/mL.arbitary units
	LEAP2	Triglycerides	0.074	1, 91.80	7.021	**0.009**	1.644	0.621	+0.412, +2.877	ng/mL.mmol/L
DJBL	LEAP2	HOMA-IR	0.032	1, 115	4.102	**0.045**	0.339	0.168	+0.007, +0.671	ng/mL.arbitrary units
	LEAP2	Triglycerides	0.000	1, 116	0.055	0.81	0.069	0.294	-0.513, +0.652	ng/mL.mmol/L

Table 3.18. Effect of covariates HOMA-IR and triglycerides on absolute fasting plasma LEAP2 in SMM and DJBL groups.
This table shows the effects of covariates on absolute fasting plasma LEAP2 across -2, 2, 26 and 50 weeks using linear mixed model analysis including sweet taste detection task (done in the taste sub-group at weeks -2, 2 and 26, for fasting glucose, HOMA-IR and LEAP2) as between participant, and weeks (-2, 2, 26 and 50) as within participant fixed factors. Covariates include HOMA-IR and triglycerides to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

DJBL vs SMM, from -2 to 50 weeks		Group*covariate effect on the dependent variable							
Dependent	Covariate	r^2	df	F	P value	β parameter	β +/- SEM	95% CI of β	β units
LEAP2	BMI	0.078	1, 166.98	0.002	0.97	-0.008	0.178	-0.360, +0.345	ng/mL.kg/m2
	Glucose	0.068	1, 219	1.736	0.19	0.381	0.290	-0.189, +0.952	ng/mL.mmol/L
	HbA1c	0.099	1, 221	0.044	0.83	-0.010	0.047	-0.102, +0.082	ng/mL.mmol/L
	Insulin	0.086	1, 202.60	2.669	0.10	0.183	0.112	-0.038, +0.404	ng/mL.mIU/L
	HOMA-IR	0.108	1, 213	4.034	**0.046***	0.543	0.270	+0.010, +1.076	ng/mL.arbitrary units
	Triglycerides	0.035	1, 207.09	4.014	**0.046***	1.527	0.762	+0.024, +3.029	ng/mL.mmol/L
Appetite	LEAP2	0.022	1, 219	4.170	**0.042***	1.267	0.621	+0.044, +2.490	mm.ng/mL
	incl. Glucose	0.062	1, 215	3.498	0.063	1.168	0.625	-0.063, +2.399	mm.ng/mL. mmol/L
Hunger	LEAP2	0.021	1. 219	4.014	**0.046***	1.651	0.824	+0.027, +3.275	mm.ng/mL

Table 3.19. Effect of covariates on absolute fasting plasma LEAP2, appetite and hunger ratings across weeks between SMM and DJBL groups.

This table shows the effects of covariates on absolute fasting plasma LEAP2, appetite and hunger VAS ratings across -2, 2, 26 and 50 using linear mixed model analysis including sweet taste detection task (done in the taste sub-group at weeks -2, 2 and 26 only, for fasting glucose, HOMA-IR and LEAP2) as between participant, and weeks (-2, 2, 26 and 50) as within participant fixed factors between SMM and DJBL groups. Covariates include absolute BMI, glucose, HbA1c, insulin, HOMA-IR, triglycerides and LEAP2 to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: BMI, body mass index; VAS, visual analogue scale; HOMA-IR, homeostatic model assessment for insulin resistance; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

DJBL vs SMM, from -2 to 50 weeks		Group*covariate effect on the dependent variable							
Dependent	Covariate	r^2	df	F	P value	β parameter	β +/-SEM	95% CI of β	β units
Δ LEAP2	Δ BMI	0.071	1, 212.94	0.769	0.38	-0.213	0.243	-0.691, +0.265	ng/mL.kg/m2
	Δ Glucose	0.092	1, 218.41	1.029	0.31	0.272	0.268	-0.256, +0.799	ng/mL.mmol/L
	Δ HbA1c	0.146	1, 218.52	2.104	0.15	-0.070	0.048	-0.165, +0.025	ng/mL.mmol/L
	Δ Insulin	0.074	1, 215	0.834	0.36	0.106	0.116	-0.123, +0.335	ng/mL.mIU/L
	Δ HOMA-IR	0.088	1, 209.84	2.172	0.14	0.385	0.261	-0.130, +0.900	ng/mL.aribitrary units
	Δ Triglycerides	0.029	1, 215	0.029	0.87	-0.139	0.818	-1.752, +1.474	ng/mL.mmol/L
Δ Appetite	Δ LEAP2	0.005	1, 217	0.034	0.85	0.123	0.670	-1.197, +1.444	mm.ng/mL
	incl. Δ Glucose	0.031	1, 214	0.007	0.93	0.056	0.669	-1.263, +1.375	mm.ng/mL. mmol/L
Δ Hunger	Δ LEAP2	0.007	1, 217	0.013	0.91	0.103	0.898	-1.668, +1.873	mm.ng/mL

Table 3.20. Effect of covariates on change in fasting plasma LEAP2, appetite and hunger ratings across weeks between SMM and DJBL groups.

This table shows the effects of covariates on change in fasting plasma LEAP2, appetite and hunger VAS ratings between weeks 2,26 and 50 vs. -2 using linear mixed model analysis including sweet taste detection task (done in the taste sub-group at weeks -2, 2 and 26 only, for fasting glucose, HOMA-IR and LEAP2) as between participant, and weeks (-2, 2, 26 and 50) as within participant fixed factors between SMM and DJBL groups. Covariates include change in BMI, glucose, HbA1c, insulin, HOMA-IR, triglycerides and LEAP2 to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: BMI, body mass index; VAS, visual analogue scale; HOMA-IR, homeostatic model assessment for insulin resistance; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

DJBL, from 50 to 104 weeks		Covariate effect on the dependent variable							
Dependent	**Covariate**	**r2**	**df**	**F**	**P value**	**β parameter**	**β +/-SEM**	**95% CI of β**	**β units**
Δ LEAP2	**Δ Weight**	0.031	1, 19	0.607	0.45	-0.093	0.119	-0.3413 to 0.1561	ng/mL/kg
	Δ HbA1c	0.035	1, 19	0.385	0.54	0.035	0.056	-0.08187 to 0.1509	ng/mL/mmol/L
	Δ Glucose	0.012	1, 19	0.223	0.64	0.145	0.308	-0.4989 to 0.7894	ng/mL/mmol/L
	Δ Insulin	0.057	1, 19	1.141	0.30	-0.210	0.196	-0.6206 to 0.2012	ng/mL/mUI/L
	Δ HOMA-IR	0.019	1, 19	0.374	0.55	-0.221	0.361	-0.9761 to 0.5348	ng/mL/arbitrary units
	Δ Triglycerides	0.000	1, 19	0.002	0.97	-0.041	0.959	-2.049 to 1.966	ng/mL/mmol/L
Δ Appetite	**Δ LEAP2**	0.008	1, 19	0.157	0.70	-0.520	1.311	-3.263 to 2.224	mm/ng/mL
Δ Hunger		0.007	1, 19	0.137	0.71	0.449	1.211	-2.085 to 2.983	mm/ng/mL

Table 3.21. Effect of covariates on change in fasting plasma LEAP2, appetite and hunger ratings across weeks in DJBL group after device explant.
This table shows the effects of covariates on change in fasting plasma LEAP2, appetite and hunger VAS ratings between weeks 50 vs. 104 using linear mixed model analysis including sweet taste detection task (done in the taste sub-group at weeks -2, 2 and 26 only, for fasting glucose, HOMA-IR and LEAP2) as between participant, and weeks (50 and 104) as within participant fixed factors. Covariates include change in weight, glucose, HbA1c, insulin, HOMA-IR, triglycerides and LEAP2 to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: VAS, visual analogue scale; HOMA-IR, homeostatic model assessment for insulin resistance; DJBL, duodenal-jejunal bypass liner.

3.4.5. Longitudinal Study of Post-Prandial Variables

3.4.5.1. Longitudinal analysis of weight in post-prandial sub-group

There was a significant group*weeks interaction for weight [F(3, 90.95)=4.51, P=0.005] (Table 3.22). In post-hoc analysis, weight loss was significantly greater in DJBL vs. SMM at week 26 (effect size -3.53 ± 1.01 % [95% CI -5.53, -1.52], P=0.001) and week 50 (effect size -3.41 ± 1.16 % [95% CI -5.70, -1.11], P=0.004) (Figure 3.11A, Table 3.23).

3.4.5.2. Post-prandial response of plasma LEAP2

At -2 weeks, post-prandial plasma LEAP2 response after a fixed test meal *increased* up to 2-hours after eating (Figure 3.11B, Tables 3.24, 3.25). In post-hoc analysis, after food intake, plasma LEAP2 was 10.8% higher at timepoint t=60min (mean ± SEM, 15.10 ± 0.71 ng/mL) than at t= 0min (13.63 ± 0.59 ng/mL) [effect size 1.47 ± 0.42 ng/mL (95% CI 0.63, 2.31), P<0.001], and 16.1% higher at t=120min (15.83 ±0.72ng/mL) [effect size 2.20 ± 0.34 ng/mL (95% CI 1.50, 2.89), P<0.001] (Figure 3.11B, Table 3.25).

Comparing results across different human studies (overall three without obesity, six with obesity, including the current study) (Mani et al. 2019; Hagemann et al. 2021; Holm et al. 2022; Hagemann et al. 2022; Bhargava et al. 2023; Pedersen et al. 2023; Andreoli et al. 2024a; Ragland et al. 2023), there was a suggestion that the *absolute* post-prandial increase in plasma LEAP2 is proportional to the pre-load fixed meal energy intake (r_S=+0.59, P=0.17 (Figure 3.11F, Table 3.26).

3.4.5.3. Longitudinal analysis of post-prandial plasma LEAP2 response

SMM group

Over 50 weeks, there was no significant post-prandial timepoint*visit interaction on post-prandial plasma LEAP2 (Table 3.27). There was a significant main effect of timepoint (P<0.001), but no significant main effect of visit (P=0.81) on post-prandial plasma LEAP2 (Table 3.27). In post-hoc analysis, there was a trend for the *change* in post-prandial plasma LEAP2 response at t=120min to be greater at 50 weeks vs. baseline (effect size +0.96 ± 0.50 ng/mL, P=0.092) (Figure 3.11C, Table 3.28).

DJBL group

Over 50 weeks, there was a significant timepoint*visit interaction (P=0.036) on post-prandial plasma LEAP2 (Table 3.27). In post-hoc analysis, the *change* in post-prandial plasma LEAP2 response was significantly *lower* at t=60min at 50 weeks vs. baseline (effect size -2.24 ± 0.85 ng/mL, P=0.024) (Figure 3.11D, Table 3.28).

3.4.5.4. Longitudinal analysis of post-prandial LEAP2 incremental area under curve

SMM group

Over 50 weeks, there was no significant effect of visit on plasma LEAP2 $iAUC_{0\text{-}120min}$ (Table 3.22). In post-hoc analysis, there was no significant difference in plasma LEAP2 $iAUC_{0\text{-}120min}$ between visits (Figure 3.11E, Table 3.23). Across all visits, there was no significant correlation between the plasma LEAP2 $iAUC_{0\text{-}120min}$ and BMI (mean ± SEM β = +7.72 ± 7.73 ng/mL.min, P=0.32).

DJBL group

Over 50 weeks, there was no significant effect of visit on plasma LEAP2 $iAUC_{0\text{-}120min}$ (Table 3.22). In post-hoc analysis, the largest *decrease* in plasma LEAP2 $iAUC_{0\text{-}120min}$ was at week 50 (-134.05 ± 65.11 ng/mL.min, P=0.042) (Figure 3.11E, Table 3.23). Across all visits, there was no significant correlation between the plasma LEAP2 $iAUC_{0\text{-}120min}$ and BMI (mean ± SEM β = -4.06 ± 4.22 ng/mL.min, P=0.34).

DJBL vs. SMM group

There was no significant group*visit interaction nor significant main effect of visit or group on plasma LEAP2 $iAUC_{0\text{-}120min}$ (Table 3.22). In exploratory post-hoc analysis, there was no group difference in plasma LEAP2 $iAUC_{0\text{-}120min}$ at any visit, nor any significant *change* in plasma LEAP2 $iAUC_{0\text{-}120min}$ from baseline in either SMM or DJBL group, other than a significant *decrease* in the DJBL group at 50 weeks when there was a 9.6% weight loss (Figure 3.11E, Table 3.23). In the DJBL group, % weight loss from baseline to 50 weeks did not positively correlate with the *fall* in plasma LEAP2 $iAUC_{0\text{-}120min}$ from baseline to 50 weeks (P=0.38).

3.4.5.5. Longitudinal analysis of post-prandial appetite and fullness ratings

SMM group

There was no significant effect of weeks on post-prandial appetite $iAUC_{0\text{-}120min}$ [F(3, 29.6)=0.61, P=0.61] or post-prandial fullness $iAUC_{0\text{-}120min}$ [F(3, 30.06)=1.65, P=0.20] (Figures 3.12A,B,Table 3.29). In post-hoc analysis, there were no significant differences in appetite $iAUC_{0\text{-}120min}$ between weeks (Figure 3.12A, Table 3.30). The largest decrease in post-prandial fullness $iAUC_{0\text{-}120min}$ was at week 50 (-2064.26 ± 957.42 mm.min, P=0.039) (Figure 3.12B, Table 3.30).

DJBL group

There was no significant effect of weeks on post-prandial appetite $iAUC_{0\text{-}120min}$ [F(3, 47.34)=1.421, P=0.25] but a significant effect of weeks on post-prandial fullness $iAUC_{0\text{-}120min}$ [F(3, 42.46)=3.63, P=0.020] (Figure 3.12A,B, Table 3.29). In post-hoc analysis, there were no significant differences in appetite $iAUC_{0\text{-}120min}$ between visits (Figure 3.12A, Table 3.30). Post-prandial fullness $iAUC_{0\text{-}120min}$ tended to be lower at 26 vs -2 weeks (-1277.12 ± 590.72 mm.min, P=0.036) (Figure 3.12B, Table 3.30).

DJBL vs. SMM groups

There was no significant group*weeks interaction for post-prandial appetite $iAUC_{0\text{-}120min}$ [F(3, 74.96)=1.12, P=0.35], but a significant interaction for post-prandial fullness $iAUC_{0\text{-}120min}$ [F(3, 72.52)=3.97, P=0.011] (Table 3.29). There was no significant effect of visit on post-prandial appetite $iAUC_{0\text{-}120min}$ [F(3, 74.96)=0.60, P=0.61] or effect of group on post-prandial appetite $iAUC_{0\text{-}120min}$ [F(1, 32.63)=0.05, P=0.83] (Table 3.29). However, in post-hoc analysis, there were no group differences in post-prandial appetite $iAUC_{0\text{-}120min}$ or post-prandial fullness $iAUC_{0\text{-}120min}$ at any visit (Figure 3.12A,B, Table 3.30).

3.4.5.6. Correlation with post-prandial appetite ratings

SMM group

Absolute plasma LEAP2 $iAUC_{0\text{-}120min}$ did not correlate with *absolute* appetite $iAUC_{0\text{-}120min}$ (Table 3.31, Figure 3.12C), even when including *absolute* plasma glucose $iAUC_{0\text{-}120min}$ as a covariate (Table 3.31, Figure 3.12C). *Changes* in plasma LEAP2 $iAUC_{0\text{-}120min}$ did not correlate with *changes* in appetite $iAUC_{0\text{-}120min}$ (Table 3.31, Figure 3.12D),

even when including *changes* in plasma glucose $iAUC_{0\text{-}120min}$ as a covariate (Table 3.31).

DJBL group

Absolute plasma LEAP2 $iAUC_{0\text{-}120min}$ did not correlate with *absolute* appetite $iAUC_{0\text{-}120min}$ (Table 3.31, Figure 3.12E), even when including *absolute* plasma glucose $iAUC_{0\text{-}120min}$ as a covariate (Table 3.31). *Changes* in plasma LEAP2 $iAUC_{0\text{-}120min}$ did not correlate with *changes* in appetite $iAUC_{0\text{-}120min}$ (Table 3.31, Figure 3.12F), even when including *changes* in plasma glucose $iAUC_{0\text{-}120min}$ as a covariate (Table 3.31).

DJBL vs. SMM groups

There were no significant between group differences in correlation of *absolute* or *changes* in LEAP2 $iAUC_{0\text{-}120min}$ correlation with *absolute* and *changes* in appetite $iAUC_{0\text{-}120min}$ (P=0.79-0.96), even when including *absolute* and *changes* in plasma glucose $iAUC_{0\text{-}120min}$ (P=0.25-0.60) (Table 3.31).

3.4.5.7. Correlation with post-prandial fullness ratings

SMM group

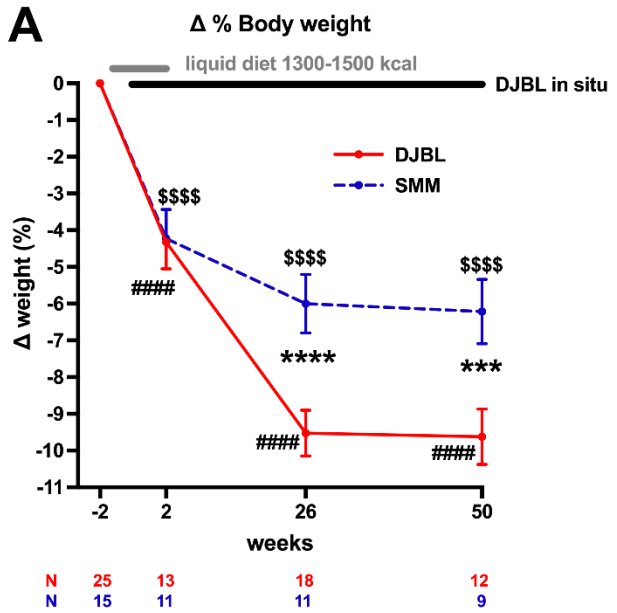
Absolute plasma LEAP2 $iAUC_{0\text{-}120min}$ did not correlate with *absolute* fullness $iAUC_{0\text{-}120min}$ (Table 3.31). *Change*s in plasma LEAP2 $iAUC_{0\text{-}120min}$ did not correlate with *changes* in fullness $iAUC_{0\text{-}120min}$ (Table 3.31).

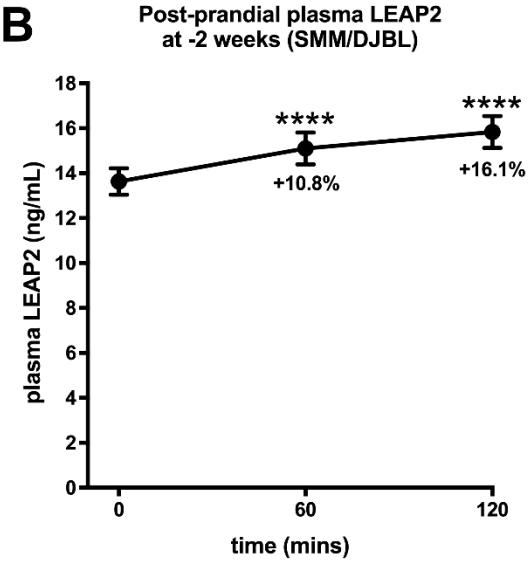
DJBL group

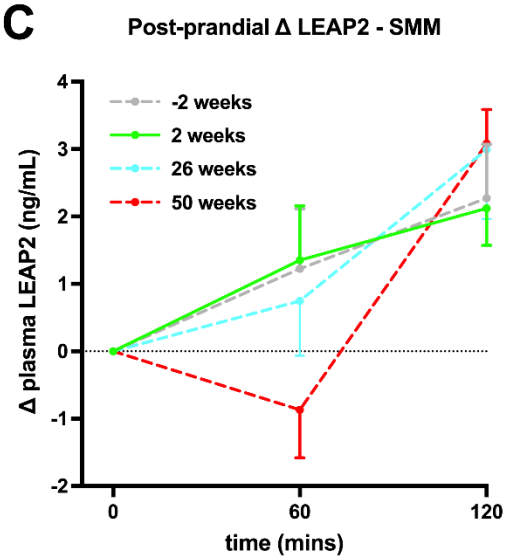
Absolute plasma LEAP2 $iAUC_{0\text{-}120min}$ did not correlate with *absolute* fullness $iAUC_{0\text{-}120min}$ (Table 3.31). *Changes* in plasma LEAP2 $iAUC_{0\text{-}120min}$ did not correlate with *changes* in fullness $iAUC_{0\text{-}120min}$ (Table 3.31).

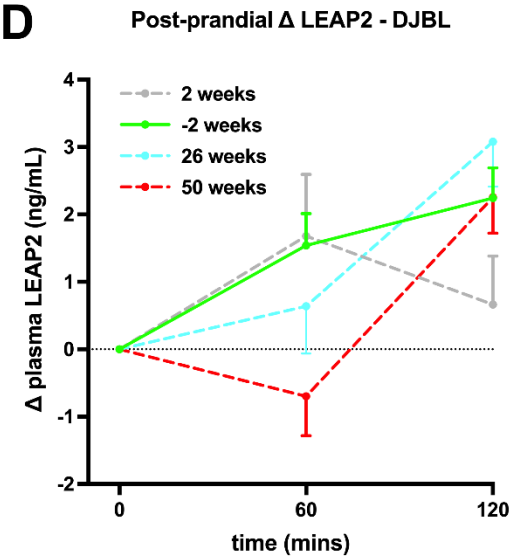
DJBL vs. SMM groups

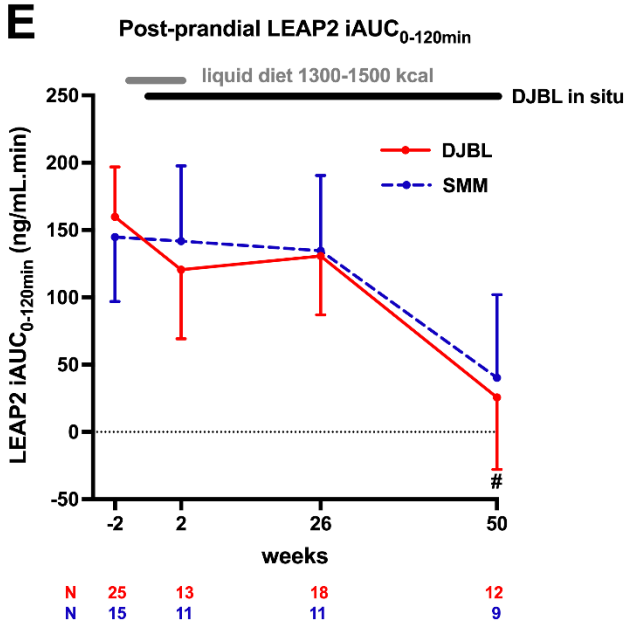
There were no significant between group differences in correlations of *absolute* and *changes* in plasma LEAP2 $iAUC_{0\text{-}120min}$ with *absolute* and *change*s in fullness $iAUC_{0\text{-}120min}$ (P=0.24-0.75) (Table 3.31).


A
Δ % Body weight
liquid diet 1300-1500 kcal
DJBL in situ
DJBL
SMM
Δ weight (%)
$$$$
$$$$
$$$$
####
****
***
####
####
weeks
N 25 13 18 12
N 15 11 11 9


B
Post-prandial plasma LEAP2
at -2 weeks (SMM/DJBL)
plasma LEAP2 (ng/mL)
****
****
+10.8%
+16.1%
time (mins)


C
Post-prandial Δ LEAP2 - SMM
-2 weeks
2 weeks
26 weeks
50 weeks
Δ plasma LEAP2 (ng/mL)
time (mins)


D
Post-prandial Δ LEAP2 - DJBL
2 weeks
-2 weeks
26 weeks
50 weeks
Δ plasma LEAP2 (ng/mL)
time (mins)


E
Post-prandial LEAP2 iAUC0-120min
liquid diet 1300-1500 kcal
DJBL in situ
DJBL
SMM
LEAP2 iAUC0-120min (ng/mL.min)
#
weeks
N 25 13 18 12
N 15 11 11 9

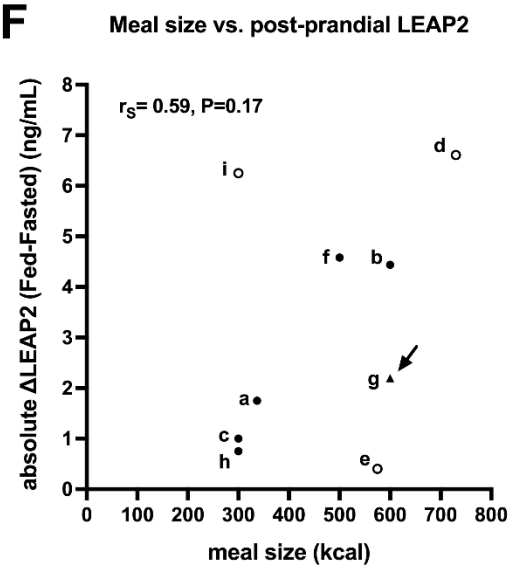

F
Meal size vs. post-prandial LEAP2
rS= 0.59, P=0.17
absolute ΔLEAP2 (Fed-Fasted) (ng/mL)
meal size (kcal)

Figure 3.11. Changes in post-prandial plasma LEAP2.
(A,E) Longitudinal analysis of (A) % weight loss and (E) post-prandial plasma LEAP2 iAUC from t=0 min to t=120 min after consumption of a fixed 600kcal test meal (49% carbohydrate, 35% fat, 16% protein), across visits for participants undergoing standard medical management alone (SMM, blue dashed line) and duodenal-jejunal bypass liner (DJBL, red solid line) groups. Black bar indicates duration of DJBL being in situ (0-52 weeks). Grey bar indicates duration of liquid diet (-1 to 2 weeks). N indicates number of participants at each time point. Statistical results below graph indicates linear mixed model analysis including group (SMM, DJBL), sweet taste detection task (done at weeks -2, 2 and 26 only), as between participant, and weeks (-2, 2, 26, 50) as within participant fixed factors, to examine effects of interventions on dependent variables over time: DJBL vs. SMM * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001; DJBL vs. baseline week 0: # P<0.05, ## P<0.01, ### P<0.005, #### P<0.001; SMM vs. baseline week 0: $ P<0.05, $$ P<0.01, $$$ P<0.005, $$$$ P<0.001.

(B) Post-prandial plasma LEAP2 concentrations after 600 kcal fixed test meal (49% carbohydrate, 35% fat, 16% protein) at baseline -2 week visit. Comparisons made using one-way repeated measures ANOVA including time (t=0, 60, 120min) as within participant factors with post-hoc Fisher LSD: *P<0.05, **P<0.01, ***P<0.005, ****P<0.001, t=60 or 120min vs. 0min, n=40.

(C,D) Change in absolute post-prandial plasma LEAP2 response at time points t=60 and 120min vs. t=0min in (C) DJBL group and (D) SMM group, after 600 kcal fixed test meal at t=0min across visits. Statistical results given in Supplemental Tables S26-27, made using linear mixed model analysis including time (t=0, 60, 120min) and weeks (-2, 2, 26, 50) as within participant fixed factors, to examine effects of interventions on post-prandial plasma LEAP2 over time. n=9-25.

(F) Correlation of change in absolute plasma LEAP2 (ng/mL) concentrations between fed (+60 to +150min) and fasted (0min) time points (∆ fed-fasted) and meal size (kcal) consumed across different studies using Spearman's (r_S) correlation coefficient. Black dots indicate participants with obesity (BMI ≥30kg/m^2), black triangle with arrow indicates obesity with T2DM in current stud, open dots indicate participants without obesity (BMI <30kg/m^2). Meals are nutritional supplements with similar macronutrient composition: carbohydrate: 49-55%, fat 30-35%, protein 14-18% energy. List of individual studies given in Table 3.26. Graph modified from Bhargava et al. 2023 (Bhargava et al. 2023). All data given as mean ± SEM. Abbreviations: BMI, body mass index; kcal, kilocalorie; iAUC, incremental area under the curve; OGTT, oral glucose tolerance test.

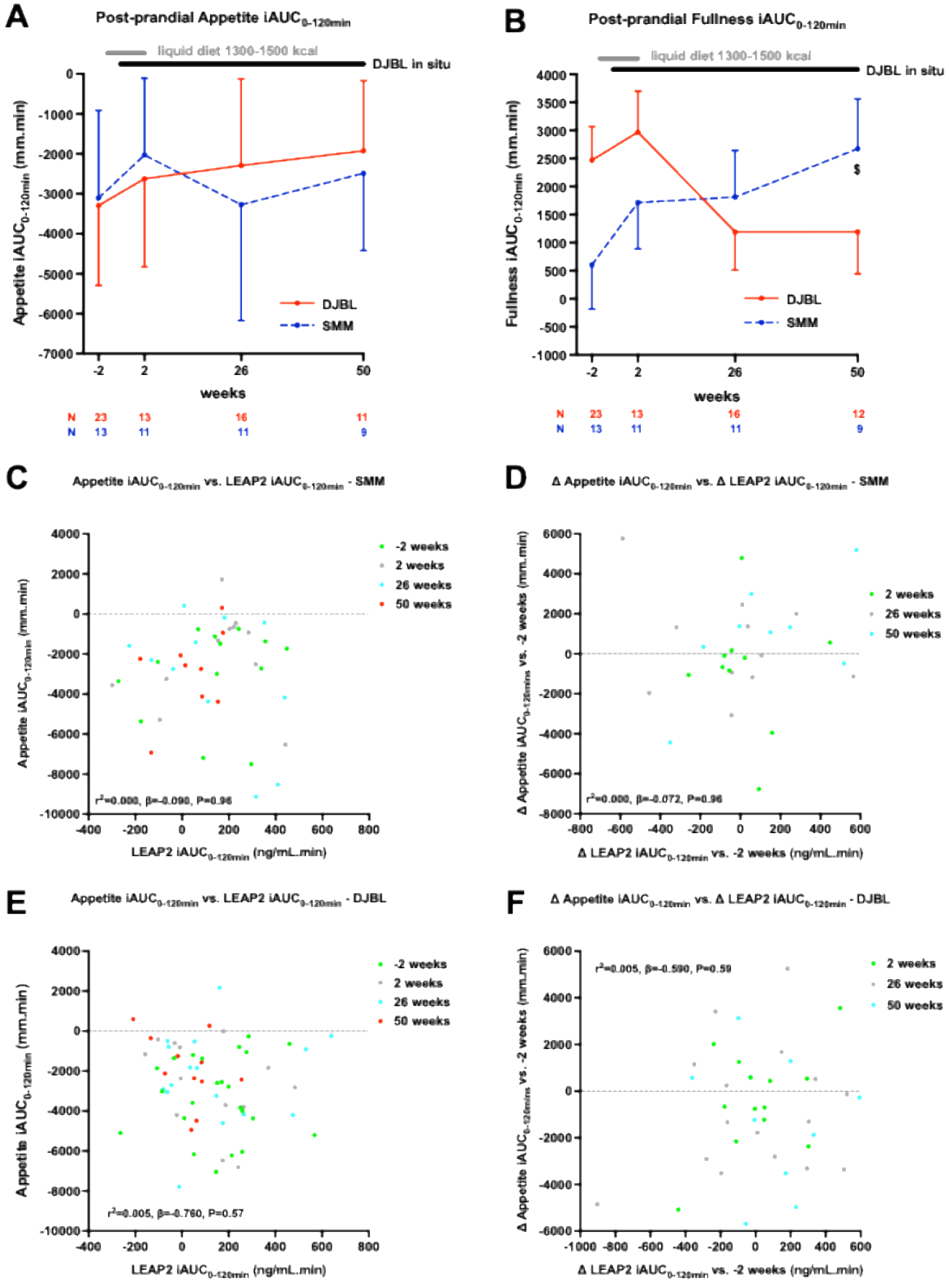

A
Post-prandial Appetite iAUC0-120min
liquid diet 1300-1500 kcal
DJBL in situ
Appetite iAUC0-120min (mm.min)
DJBL
SMM
weeks
N 23 13 16 11
N 13 11 11 9
B
Post-prandial Fullness iAUC0-120min
liquid diet 1300-1500 kcal
DJBL in situ
Fullness iAUC0-120min (mm.min)
$
DJBL
SMM
weeks
N 23 13 16 12
N 13 11 11 9
C
Appetite iAUC0-120min vs. LEAP2 iAUC0-120min - SMM
-2 weeks
2 weeks
26 weeks
50 weeks
r²=0.000, β=-0.090, P=0.96
LEAP2 iAUC0-120min (ng/mL.min)
D
Δ Appetite iAUC0-120min vs. Δ LEAP2 iAUC0-120min - SMM
2 weeks
26 weeks
50 weeks
r²=0.000, β=-0.072, P=0.96
Δ LEAP2 iAUC0-120min vs. -2 weeks (ng/mL.min)
E
Appetite iAUC0-120min vs. LEAP2 iAUC0-120min - DJBL
-2 weeks
2 weeks
26 weeks
50 weeks
r²=0.005, β=-0.760, P=0.57
LEAP2 iAUC0-120min (ng/mL.min)
F
Δ Appetite iAUC0-120min vs. Δ LEAP2 iAUC0-120min - DJBL
r²=0.005, β=-0.590, P=0.59
2 weeks
26 weeks
50 weeks
Δ LEAP2 iAUC0-120min vs. -2 weeks (ng/mL.min)

Figure 3.12. Longitudinal analysis of post-prandial appetite and fullness ratings across visits and correlations with post-prandial plasma LEAP2.
(A,B) Post-prandial (A) appetite iAUC and (B) fullness VAS iAUC ratings from t=0 min to t=120 min after consumption of a fixed 600 kcal test meal (49% carbohydrate, 35% fat, 16% protein) for participants undergoing standard medical management alone (SMM, blue dashed line) or with additional duodenal-jejunal bypass liner insertion (DJBL, red solid line) groups. Black bar indicates duration of DJBL being in situ (0-52 weeks). N indicates number of participants at each time point. Grey bar indicates duration of liquid diet (-1 to 2 weeks). Statistical analysis used linear mixed model analysis including group (SMM, DJBL) as between participant factor, and weeks (-2, 2, 26 and 50) as within participant fixed factor, to examine effects of interventions on dependent variables over time. DJBL vs. SMM: nil significant; DJBL vs. baseline week -2: nil significant; SMM vs. baseline week -2: $ P<0.05. All data given as mean ± SEM.

(C-F) Correlations of (C,E) *absolute* post-prandial plasma LEAP2 iAUC with *absolute* post-prandial appetite VAS iAUC ratings from t=0 min to t=120 min after consumption of a fixed 600kcal test meal (49% carbohydrate, 35% fat, 16% protein) in the taste sub-group across visits -2, 2, 26 and 50 weeks; and (D,F) *change* in post-prandial plasma LEAP2 iAUC from t=0 min to t=120 min with *change* in post-prandial appetite iAUC VAS ratings between weeks 2, 26 and 50 vs. -2 weeks, in (C,D) SMM group and (E,F) DJBL group. Statistical analysis used linear mixed model analysis with weeks (-2, 2, 26 and 50) as within participant fixed factor, to examine relationships between post-prandial plasma LEAP2 and appetite over time. Results reported as pseudo r^2 and beta parameters, n=9-13. Abbreviations: DJBL, duodenal-jejunal bypass liner; iAUC, incremental area under the curve; kcal, kilocalorie SMM, standard medical management; VAS, visual analogue scale.

Main effect of weeks and group - SMM, DJBL, DJBL vs SMM, -2 to 50 weeks						
		SMM	DJBL	DJBL vs SMM		
Dependent variable	**Parameters**	**Weeks**	**Weeks**	**Group**	**Weeks**	**Group*Weeks**
Δ %weight	Numerator df	3	3	1	3	3
	Denominator df	36.92	54.43	44.59	90.95	90.95
	F	19.16	68.56	7.87	72.92	4.51
	P value	**0.000**	**0.000**	**0.007**	**0.000**	**0.005**
LEAP2 iAUC (0-120mins)	Numerator df	3	3	1	3	3
	Denominator df	42.00	64.00	106.00	106.00	106.00
	F	0.68	1.49	0.03	1.96	0.05
	P value	0.57	0.23	0.87	0.12	0.98

Table 3.22. Main effects of weeks on change in % weight and post-prandial LEAP2 in SMM and DJBL groups.
This table describes the F statistic, numerator and denominator of degrees of freedom for the effect of weeks on change in % weight (between weeks 2,26, and 50 vs. -2 weeks) and post-prandial LEAP2 iAUC from t=0 min to t=120 min in SMM and DJBL groups. Between-participant factors including sweet taste detection task (done at weeks -2, 2 and 26 for LEAP2). Within-participant factors include visits at -2, 2, 26, and 50 weeks. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: iAUC, incremental area under the curve; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

Post-hoc analysis of weeks, group and sweet taste task on dependent variable - SMM, DJBL, DJBL vs SMM											
		SMM			DJBL			DJBL vs SMM			
Dependent variable	Parameters	Δ 2 vs. -2 w	Δ 26 vs. -2 w	Δ 50 vs. -2 w	Δ 2 vs. -2 w	Δ 26 vs. -2 w	Δ 50 vs. -2 w	-2 w	2 w	26 w	50 w
Δ %Weight	Mean difference	-4.22	-6.00	-6.22	-4.32	-9.53	-9.62	0.00	-0.10	-3.53	-3.41
	SE	0.96	0.96	1.02	0.84	0.75	0.86	0.87	1.08	1.01	1.16
	P-value	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	1.00	0.93	**0.001**	**0.004**
	95 CI Lower	-6.13	-7.90	-8.25	-5.98	-11.01	-11.33	-1.72	-2.24	-5.53	-5.70
	95 CI Upper	-2.32	-4.10	-4.18	-2.66	-8.04	-7.91	1.72	2.04	-1.52	-1.11
LEAP2 iAUC (0-120mins)	Mean difference	-3.09	-10.19	-104.52	-39.13	-29.05	-134.05	14.93	-21.11	-3.93	-14.61
	SE	73.60	73.60	78.17	63.40	57.31	65.11	60.55	75.95	70.96	81.76
	P-value	0.97	0.89	0.18	0.54	0.61	**0.042**	0.81	0.78	0.96	0.86
	95 CI Lower	-149.00	-156.11	-259.50	-164.82	-142.68	-263.14	-105.12	-171.70	-144.60	-176.69
	95 CI Upper	142.82	135.72	50.47	86.56	84.58	-4.96	134.98	129.47	136.75	147.48

Table 3.23. Post-hoc analysis of change in % weight and post-prandial LEAP2 in SMM and DJBL groups.

This table describes the post-hoc analysis of results in Table 3.22. Mean difference, standard error, upper and lower confidence intervals for the effect of weeks on change in % weight (between weeks 2,26, and 50 vs. -2 weeks) and post-prandial LEAP2 iAUC from t=0 min to t=120 in SMM and DJBL groups. Between-participant factors including sweet taste detection task (done at weeks -2, 2 and 26 for LEAP2). Within-participant factors include visits at -2, 2, 26, and 50 weeks. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: iAUC, incremental area under the curve; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

Post-prandial LEAP2 at -2weeks, SMM + DJBL					
time	Mean	Std. Deviation	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0 min	13.63	3.73	0.59	12.44	14.82
60 min	15.10	4.50	0.71	13.66	16.54
120 min	15.83	4.52	0.72	14.38	17.28

Table 3.24. Post-prandial LEAP2 at -2 weeks across SMM and DJBL groups.

This table describes the post-prandial LEAP2 concentrations at -2 weeks (baseline) across SMM and DJBL groups at time points 0,60 and 120 mins. Comparisons made using one-way repeated measures ANOVA including time (t=0,60 and 120min) as within subject factor. Abbreviations: SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

Main effect of timepoint, SMM + DJBL		-2 weeks		
Dependent variable	Parameters	60 min - 0 min	120 min - 0 min	120 min - 60 min
Post-prandial LEAP2	Mean difference	1.47	2.20	0.73
	SE	0.42	0.34	0.45
	P-value	**0.001**	**0.000**	0.12
	95 CI Lower	0.63	1.50	-0.19
	95 CI Upper	2.31	2.89	1.65

Table 3.25. Post-hoc analysis of post-prandial LEAP2 at -2 weeks across SMM and DJBL groups.

This table describes the post-hoc analysis of results in Table 3.24. Mean difference, standard error, upper and lower confidence intervals for effects of time on post-prandial LEAP2 at -2 weeks (baseline) across SMM and DJBL groups. Within-participant factors include time points 0,60 and 120min. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

Study	Author	Obesity status	N	Female (%)	kcal	Mixed meal macronutrients (% energy)	Post-prandial sampling (mins)	Absolute Δplasma LEAP2 (Fed-Fasted)(ng/mL)
A	Mani 2019	NW/OW/OB	20	100.0	337	52% CHO, 30% fat, 18% protein	90	1.75
B	Mani 2019	OB	20	90.0	600	49% CHO, 35% fat, 16% protein	120	4.44
C	Hagemann 2021	OB	18	72.2	300	50% CHO, 35% fat, 15% protein	120	1.00
D	Bhargava 2023	NW/OW	22	22.7	730	55% CHO, 31% fat, 14% protein	150	6.61
E	Hagemann 2022	NW/OW	20	0.0	575	49% CHO, 35% fat, 16% protein	120	0.40
F	Andreoli 2023	OW/OB	9	100.0	400-600 (20% REE)	56% CHO, 34% fat, 12% protein	60	4.58
G	Current study	OW/OB + T2DM	15	60.0	600	49% CHO, 35% fat, 16% protein	120	2.20
H	Ragland 2023	OB	13	100.0	300	100% CHO, 0% fat, 0% protein (OGTT)	60	0.75
I	Pedersen 2023	NW	9	0.0	300	100% CHO, 0% fat, 0% protein (OGTT)	120	6.25

Table 3.26. List of studies illustrating correlation of post-prandial plasma LEAP2 with meal size consumed.

Correlation of change in absolute plasma LEAP2 (ng/mL) concentrations from fed (+60 to +150min) and fasted (0min) time points (Δ fed-fasted) and meal size (kcal) consumed across different studies. Meals are nutritional supplements with similar macronutrient composition: carbohydrates 49-55%, fat 30-35%, protein 14-18%.Abbreviations: BMI, body mass index; CHO carbohydrate; kcal, kilocalorie; NW, Normal weight (BMI <24.9 kg/m^2); OW, Overweight (BMI 25.0-29.9 kg/m^2); OB, Obese (BMI <30.0 kg/m^2); OGTT, Oral glucose tolerance test; kcal, kilocalorie; NW, normal N indicating number of participants; OGTT, oral glucose tolerance test; REE, Resting energy expenditure; T2DM, Type 2 diabetes mellitus.

Main effect of weeks and group on dependent variable - SMM, DJBL, -2 to 50 weeks							
		SMM			DJBL		
Dependent variable	Parameters	Timepoint	Weeks	Timepoint*Weeks	Timepoint	Weeks	Timepoint*Weeks
Post-prandial LEAP2	Numerator df	1.6	2.6	3.1	1.6	2.4	3.3
	Denominator df	21.83	35.73	21.58	37.66	58.22	26.02
	F	19.46	0.28	1.38	17.05	1.19	3.21
	P value	**<0.001**	0.81	0.28	**<0.001**	0.32	**0.036**

Table 3.27. Main effects on post-prandial LEAP2 across weeks in SMM and DJBL groups.

This table describes the F statistic, numerator and denominator of degrees of freedom for the main effects of timepoint, weeks and timepoint*weeks on post-prandial LEAP2. Weeks includes -2, 2, 26 and 50 weeks, timepoints include t=0,60 and 120min. Group includes the SMM and DJBL groups. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=40. Abbreviations: SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

Post-hoc analysis of weeks and group on dependent variable - SMM, DJBL													
	Group	SMM						DJBL					
	Comparison	Δ 2 vs. -2 w		Δ 26 vs. -2 w		Δ 50 vs. -2 w		Δ 2 vs. -2 w		Δ 26 vs. -2 w		Δ 50 vs. -2 w	
Dependent variable	**Parameters**	**60 min - 0 min**	**120 min - 0 min**	**60 min - 0 min**	**120 min - 0 min**	**60 min - 0 min**	**120 min - 0 min**	**60 min - 0 min**	**120 min - 0 min**	**60 min - 0 min**	**120 min - 0 min**	**60 min - 0 min**	**120 min - 0 min**
Post-prandial LEAP2	Mean difference	-0.13	0.15	-0.61	0.87	-2.22	0.96	0.14	-1.58	-0.90	0.84	-2.24	0.01
	SE	0.68	0.77	1.38	1.01	1.56	0.50	0.78	1.02	1.03	0.82	0.85	0.81
	P-value	0.86	0.85	0.67	0.41	0.19	0.092	0.86	0.15	0.39	0.32	**0.024***	0.99
	95 CI Lower	-1.63	-1.56	-3.67	-1.38	-5.83	-0.20	-1.56	-3.79	-3.08	-0.90	-4.12	-1.77
	95 CI Upper	1.38	1.86	2.46	3.13	1.38	2.12	1.83	0.63	1.27	2.57	-0.36	1.79

Table 3.28. Post-hoc analysis of post-prandial LEAP2 in SMM and DJBL groups.

This table describes the post-hoc analysis of results in Table 3.27. Mean difference, standard error, upper and lower confidence intervals for post-prandial LEAP2 at t=0,60 and 120 min timepoints between weeks 2, 26 and 50 vs. -2 weeks. Group includes the SMM and DJBL groups. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

Main effect of weeks and group - SMM, DJBL, DJBL vs SMM, -2 to 50 weeks						
		SMM	DJBL	DJBL vs SMM		
Dependent variable	Parameters	Week	Week	Group	Week	Group*Week
Appetite iAUC (0-120mins)	Numerator df	3	3	1	3	3
	Denominator df	29.64	47.34	32.63	74.96	74.96
	F	0.61	1.42	0.05	0.60	1.12
	P value	0.61	0.25	0.83	0.61	0.35
Fullness iAUC (0-120mins)	Numerator df	3	3	1	3	3
	Denominator df	30.06	42.46	35.73	72.52	72.52
	F	1.65	3.63	0.09	1.01	3.97
	P value	0.20	**0.020**	0.76	0.39	**0.011**

Table 3.29. Main effects on post-prandial appetite and fullness across weeks in SMM and DJBL groups.
This table describes the F statistic, numerator and denominator of degrees of freedom for the main effects of group, weeks and group*weeks on post-prandial appetite and fullness iAUC from t=0 min to t=120. Weeks includes -2, 2, 26 and 50 weeks. Group includes the SMM and DJBL groups. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: iAUC, incremental area under the curve; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

Post-hoc analysis of weeks, group and sweet taste task on dependent variable - SMM, DJBL, DJBL vs SMM											
		SMM			DJBL			DJBL vs SMM			
Dependent variable	Parameters	Δ 2 vs. -2 w	Δ 26 vs. -2 w	Δ 50 vs. -2 w	Δ 2 vs. -2 w	Δ 26 vs. -2 w	Δ 50 vs. -2 w	-2 w	2 w	26 w	50 w
Appetite iAUC (0-120mins)	Mean difference	783.87	-152.47	-221.13	516.18	955.24	1177.05	-433.81	-701.50	673.90	964.37
	SE	773.88	764.59	828.56	663.97	616.55	712.27	767.36	889.61	855.62	965.76
	P-value	0.31	0.84	0.79	0.44	0.13	0.10	0.57	0.43	0.43	0.32
	95 CI Lower	-758.00	-1677.43	-1870.77	-805.64	-272.87	-239.59	-1958.54	-2466.69	-1024.20	-951.91
	95 CI Upper	2325.75	1372.49	1428.51	1838.01	2183.34	2593.69	1090.92	1063.69	2371.99	2880.65
Fullness iAUC (0-120mins)	Mean difference	1109.72	1204.49	2066.32	498.31	-1276.86	-1275.34	1863.81	1252.40	-617.54	-1477.85
	SE	805.01	787.56	867.26	694.07	642.96	727.45	992.28	1105.56	1075.77	1161.48
	P-value	0.17	0.13	**0.020**	0.48	0.051	0.084	0.065	0.26	0.57	0.21
	95 CI Lower	-494.80	-366.55	338.50	-884.58	-2558.12	-2723.69	-117.59	-946.44	-2759.10	-3785.21
	95 CI Upper	2714.23	2775.53	3794.15	1881.19	4.40	173.01	3845.22	3451.25	1524.02	829.51

Table 3.30. Post-hoc analysis of post-prandial appetite and fullness in SMM and DJBL groups.
This table describes the post-hoc analysis of results in Table 3.29. Mean difference, standard error, upper and lower confidence intervals for post-prandial appetite and fullness iAUC from t=0 to t= 120 min between weeks 2, 26 and 50 vs. -2 weeks. Group includes the SMM and DJBL groups. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: iAUC, incremental area under the curve; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

Covariate effect on the dependent variable, -2 to 50 weeks										
Comparison	Dependent	Covariate	r2	df	F	P value	β parameter	β +/-SEM	95% CI of β	β units
SMM	Appetite iAUC	LEAP2 iAUC	0.000	1, 33.64	0.003	0.96	-0.090	1.648	-3.440, +3.260	mm.min.ng/mL.min
		incl. Glucose iAUC	0.036	1, 35.41	0.315	0.58	-0.954	1.698	-4.400, +2.492	mm.min.ng/mL.min. mmol/L.min
	Δ Appetite iAUC	Δ LEAP2 iAUC	0.000	1, 42	0.002	0.96	0.072	1.449	-2.853, +2.996	mm.min.ng/mL.min
		incl. Glucose iAUC	0.010	1, 40	0.323	0.57	-0.860	1.514	-3.920, +2.200	mm.min.ng/mL.min. mmol/L.min
	Fullness iAUC	LEAP2 iAUC	0.001	1, 34.12	0.036	0.85	0.365	1.932	-3.56, +4.290	mm.min.ng/mL.min
	Δ Fullness iAUC	Δ LEAP2 iAUC	0.004	1, 42	0.160	0.69	0.674	1.686	-2.729, +4.077	mm.min.ng/mL.min
DJBL	Appetite iAUC	LEAP2 iAUC	0.005	1, 57.18	0.335	0.57	-0.760	1.314	--3.391, +1.870	mm.min.ng/mL.min
		incl. Glucose iAUC	0.002	1, 52.90	0.006	0.94	0.114	1.417	-2.728, +2.956	mm.min.ng/mL.min. mmol/L.min
	Δ Appetite iAUC	Δ LEAP2 iAUC	0.005	1, 62	0.290	0.59	0.590	1.095	-1.598, +2.778	mm.min.ng/mL.min
		incl. Glucose iAUC	0.033	1, 53.31	1.839	0.18	1.494	1.102	-0.715, +3.704	mm.min.ng/mL.min. mmol/L.min
	Fullness iAUC	LEAP2 iAUC	0.001	1, 48.99	0.086	0.77	-0.424	1.446	-3.331, +2.482	mm.min.ng/mL.min
	Δ Fullness iAUC	Δ LEAP2 iAUC	0.044	1, 51.52	2.859	0.097	-1.612	0.953	-3.524, +0.301	mm.min.ng/mL.min
Covariate*dependent variable interaction -2 to 50 weeks										
DJBL vs SMM	Appetite iAUC	LEAP2 iAUC	0.002	1, 89.12	0.072	0.79	0.561	2.092	-3.596, +4.719	mm.min.ng/mL.min
		incl. Glucose iAUC	0.026	1, 89.22	0.276	0.60	-1.155	2.201	-5.529, +3.218	mm.min.ng/mL.min. mmol/L.min
	Δ Appetite iAUC	Δ LEAP2 iAUC	0.014	1, 104	0.073	0.79	-0.483	1.785	-4.022, +3.055	mm.min.ng/mL.min
		incl. Glucose iAUC	0.032	1, 96	1.452	0.23	-2.230	1.851	-5.904, +1.444	mm.min.ng/mL.min. mmol/L.min
	Fullness iAUC	LEAP2 iAUC	0.007	1, 82.82	0.106	0.75	0.106	0.745	-3.918, +5.456	mm.min.ng/mL.min
	Δ Fullness iAUC	Δ LEAP2 iAUC	0.106	1, 105	1.399	0.24	2.186	1.848	-1.478, +5.849	mm.min.ng/mL.min

Table 3.31. Effect of covariates on post-prandial appetite and fullness across weeks in SMM and DJBL groups.
This table shows the effects of covariates on absolute post-prandial appetite and fullness iAUC ratings (from t=0 to t=120min) and change in post-prandial appetite and fullness between weeks 2, 26 and 50 vs. -2 weeks using linear mixed model analysis with weeks (-2, 2, 26 and 50) as within participant fixed factors between SMM and DJBL groups. Covariates include absolute and change in glucose and LEAP2 iAUC to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: iAUC, incremental area under the curve; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner.

3.5. DISCUSSION

In this longitudinal human study of adults with obesity and inadequately controlled T2DM, the following results were found (Table 3.32): (i) fasting plasma LEAP2 was reduced over 2 years with weight loss and improved glycaemic control via LCD, increased physical activity and standard pharmacological management of T2DM (with rare use of GLP-1 analogues); (ii) fasting plasma LEAP2 over this 2 year period through lifestyle modification was positively correlated with absolute BMI, fasting plasma glucose, HbA1c, serum insulin and triglycerides, and HOMA-IR for absolute plasma LEAP2, and positively correlated with decreases in weight, fasting plasma glucose, HbA1c and HOMA-IR for decreases in plasma LEAP2; (iii) after DJBL insertion fasting plasma LEAP2 had a delayed and smaller decrease up to 26 weeks, and at 50 weeks was similar to the control group, despite the greater weight loss and similar decreases in fasting plasma glucose, HbA1c, serum insulin, HOMA-IR and serum triglycerides; (v) the fasting plasma LEAP2 over this 1 year period through DJBL insertion in addition to lifestyle modification was positively correlated with absolute BMI, HbA1c and HOMA-IR for absolute plasma LEAP2, and positively correlated with decreases in weight and HbA1c for decreases in plasma LEAP2; (vi) there was no significant differences in the relationships between absolute or decreases in fasting plasma LEAP2 with BMI, weight, fasting plasma glucose or HbA1c, serum insulin, though there were more positive correlations of absolute plasma LEAP2 with absolute HOMA-IR and fasting serum triglycerides (though not for decreases in variables) for the DJBL group than the standard medical management alone group; (vii) weight regain 1 year after explant of DJBL was not associated with any significant increase in fasting plasma LEAP2; (viii) although post-prandial plasma LEAP2 increased after a fixed 600 kcal liquid meal at baseline, with 16.1% increase at 2 hours, there were no changes in the post-prandial increase in plasma LEAP2 with weight loss and improved glycaemic control over time in either group, nor any significant difference in the post-prandial increase in plasma LEAP2 between groups; (ix) there were no significant correlations between absolute or changes in fasting or post-prandial plasma LEAP2 and absolute or changes in hunger, fullness or appetite in either group, nor differences in these relationships between the groups.

Summary of results							
Outcome variable		**LEAP2**	**SMM**	**DJBL**	**DJBL vs. SMM**	**Post-DJBL explant**	**Results vs. hypothesis**
Fasting	**BMI**	**Absolute**	**+**	**+**	o	n/a	Y, Y, C, n/a
	% Weight	**Delta (Δ)**	**+ [a]**	**+ [b]**	o	o [c]	Y, Y, C, C
	Plasma glucose	**Absolute**	**+**	o	o	n/a	Y, C, C, n/a
		Delta (Δ)	**+ [a]**	o [b]	o [b]	o [c]	Y, C, C, C
	Serum insulin	**Absolute**	**+**	o	o	n/a	Y, C, C, n/a
		Delta (Δ)	o [a]	o [b]	o [b]	o [c]	C, C, C, C
	Serum triglycerides	**Absolute**	**+**	o	↑	n/a	Y, C, Y, n/a
		Delta (Δ)	o [a]	o [b]	o [b]	o [c]	C, C, C, C
	HbA1c	**Absolute**	**+**	**+**	o	n/a	Y, Y, C, n/a
		Delta (Δ)	**+ [a]**	**+ [b]**	o [b]	o [c]	Y, Y, C, C
	HOMA-IR	**Absolute**	**+**	**+**	↑	n/a	Y, Y, Y, n/a
		Delta (Δ)	**+ [a]**	o [b]	o [b]	o [c]	Y, C, C, C
	Appetite VAS ratings	**Absolute**	o	o	o	n/a	C, C, C, n/a
		Delta (Δ)	o [a]	o [b]	o [b]	o [c]	C, C, C, C
	Hunger VAS ratings	**Absolute**	o	o	o	n/a	C, C, C, n/a
		Delta (Δ)	o [a]	o [b]	o [b]	o [c]	C, C, C, C
Post-prandial	**Appetite VAS ratings**	**Absolute [d]**	o	o	o	n/a	C, C, C, n/a
		Delta (Δ) [d]	o [b]	o [b]	o [b]	n/a	C, C, C, n/a
	Fullness VAS ratings	**Absolute [d]**	o	o	o	n/a	C, C, C, n/a
		Delta (Δ) [d]	o [b]	o [b]	o [b]	n/a	C, C, C, n/a

Table 3.32. Summary table of correlational results.
This table shows the correlation of absolute and changes in fasting or post-prandial plasma LEAP2 with absolute and changes in BMI, plasma glucose, serum insulin, triglycerides, HbA1c, HOMA-IR, appetite, hunger and fullness VAS ratings for SMM and DJBL groups and the differences between groups (DJBL vs. SMM) across the visits. All changes expressed as delta (Δ). + indicates significant positive correlation, o indicates no correlation, ↑ indicates significantly greater correlation in DJBL vs. SMM groups. Abbreviations: BMI, body mass index; HbA1c, glycated haemoglobin; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; iAUC, incremental area under the curve; SMM, standard medical management; DJBL, duodenal-jejunal bypass liner; VAS, visual analogue scale; n/a, not applicable. Footnotes: [a] Δ 104 vs. -2 weeks, [b] Δ 50 vs. -2 weeks, [c] Δ 104 vs. 50 weeks, [d] Post-prandial variables expressed as iAUC from t=0 min to t=120 min; Y, as per hypothesis; C, Contrary to hypothesis.

3.5.1. Decreases in Plasma LEAP2 During Weight Loss

To our knowledge, this is the longest study finding a decrease in fasting plasma LEAP2 with LCD (500 kcal per day deficit) with increased physical activity–induced weight loss in obesity (with T2DM) over two years in the standard medical management group. These findings are consistent with previous literature of shorter-term caloric restriction and weight loss. Acute overnight fasting lowers fasting plasma LEAP2 in mice and humans (Mani et al. 2019) and 36 hour fasting in humans (Pedersen et al. 2023). In mice with normal weight, (i) a 40% caloric restriction over 2 weeks tended to decrease fasting plasma LEAP2 (Ge et al. 2018), while (ii) weight loss in diet-induced obesity (high fat diet for 12 weeks) after return to normal chow for 4 weeks (Mani et al. 2019), (iii) a 50% caloric restriction over 14 days in mice eating normal chow (Tezenas du Montcel et al. 2023), and (iv) a ketogenic diet with 75% dietary fat over 3 weeks leading to ~14% weight loss in chow-fed mice (Holm et al. 2022), all decreased fasting plasma LEAP2. In humans, short-term two weeks LCD (1200 kcal/day) without and with interval exercise (60 min/day) in women with obesity lowered fasting plasma LEAP2, and this was likely due to caloric restriction and weight loss, since exercise had no additive effect (Ragland et al. 2023).

Weight loss from bariatric surgery also lowers fasting plasma LEAP2. In mice, duodenal *Leap2* mRNA expression decreased after VSG surgery (Ge et al. 2018). In humans, weight loss after VSG and RYGB surgery for obesity reduced fasting and/or post-prandial plasma LEAP2 at 3-24 months after 16-31% weight loss (Mani et al. 2019; Hagemann et al. 2021).

In the current study, post-prandial plasma LEAP2 increased after a 600kcal liquid meal at baseline in adults with obesity and T2DM, consistent with previous literature (Mani et al. 2019; Bhargava et al. 2023; Pedersen et al. 2023; Andreoli et al. 2024a). In mice, the magnitude of post-prandial increases in plasma LEAP2 increased with greater meal size (Gradel et al. 2023). Adding the current study to our previous analysis across different human studies (Bhargava et al. 2023), there is a suggestion that post-prandial increases in plasma LEAP2 are proportional to the caloric value of the pre-load meal (Figure 3.11F), despite slight differences in macronutrient content and timing of post-prandial sampling.

Surprisingly, in the current study, there was no decrease in post-prandial increases in plasma LEAP2 with weight loss and improved glycaemic/metabolic control from LCD and increased physical activity–induced weight loss in obesity and T2DM over two years in the standard medical management group; however, this was only examined in a smaller sub-set of the cohort. Similarly, there was only a trend for the post-prandial increase in plasma LEAP2 to be attenuated at 12-18 month after VSG surgery, and no significant change in the post-prandial increase in plasma LEAP2 at 3 months after RYGB surgery (Mani et al. 2019).

This suggests that decreases in plasma LEAP2 may be driven by weight loss or reduced food intake, rather than a secondary consequence of the specific and different anatomical gut manipulations from RYGB and VSG surgery. However, it cannot be excluded that the decreases in fasting plasma LEAP2 is due to improvement in glycaemic control, metabolic syndrome and insulin resistance. Fasting plasma/serum LEAP2 did not fall significantly after RYGB surgery in obesity with metabolic dysfunction–associated fatty liver disease (MAFLD) at 6 months with ~29% weight loss despite marked improvements in insulin resistance and fasting plasma glucose (Ezquerro et al. 2023), nor after VSG surgery in obesity with T2DM at 12 months with ~28% weight loss despite marked improvements in glycaemic control and markers of metabolic syndrome (Nabekura et al. 2023). However, after VSG surgery for T2DM, the decreases in BMI, fasting serum triglycerides, but not HbA1c, did correlate with decreases in serum LEAP2 (Nabekura et al. 2023).

In the current study, in the standard medical management group with obesity and T2DM, BMI positively correlated with fasting plasma LEAP2 across all visits, both for absolute values and decreases from baseline visit. Several single time point cross-sectional studies have also found positive correlations between fasting plasma LEAP2 and BMI, in adults across a range of BMI from normal weight to obesity, mainly without T2DM (Mani et al. 2019), overweight/obesity and T2DM (Nabekura et al. 2023; Andreoli et al. 2024a), overweight with MAFLD (Ma et al. 2020), obesity and pre-diabetes (Byberg et al. 2023), and with % body fat, visceral adiposity and liver fat (Mani et al. 2019; Byberg et al. 2023; Ma et al. 2020), though these positive correlations with

BMI or % body fat were not seen in one study of adults with overweight/obesity (Stark et al. 2023). No previous studies of weight loss after bariatric surgery or LCD have reported correlations of changes in BMI or weight with changes in fasting plasma LEAP2 (Mani et al. 2019; Ragland et al. 2023).

In the current study in obesity and T2DM, there was no correlation between BMI and the post-prandial increase in plasma LEAP2 across all visits over 50 weeks in the sub-group having the fixed meal, in contrast to other studies reporting that higher BMI is associated with lower post-prandial increase in plasma LEAP2 in adults without obesity or T2DM (Bhargava et al. 2023), but with higher post-prandial plasma LEAP2 across two discreet groups with normal weight and obesity without T2DM (Mani et al. 2019).

3.5.2. Fasting Plasma LEAP2 and Glucose and Fat Metabolism

In the current study of obesity with T2DM, fasting plasma LEAP2 over the 2 year period in the standard medical management group was positively correlated with fasting plasma glucose, HbA1c, serum insulin and triglycerides, and HOMA-IR for absolute plasma LEAP2, and decreases in plasma LEAP2 positively correlated with decreases in fasting plasma glucose, HbA1c and HOMA-IR,

In support of these findings, in both mice without obesity, and in humans with obesity, fasting/post-prandial plasma LEAP2 positively correlated with fasting/post-prandial plasma glucose (Mani et al. 2019), while fasting plasma LEAP2 positively correlated with fasting plasma glucose, serum insulin and/or HOMA-IR, in adults with normal weight/overweight/obesity (Mani et al. 2019), with obesity (Andreoli et al. 2024a; Stark et al. 2023), in overweight/obesity with pre-diabetes (Byberg et al. 2023), in adults with MAFLD without obesity (Ma et al. 2020), though not in adults without obesity, without T2DM (Bhargava et al. 2023) or with T2DM (Li et al. 2022). Furthermore, fasting plasma LEAP2 positively correlated with fasting serum triglycerides in adults with normal weight/overweight/obesity (Mani et al. 2019), and in overweight/obesity with pre-diabetes (Byberg et al. 2023), but not in adults with T2DM without obesity (Li et al. 2022), while post-prandial increases in plasma LEAP2 tended to negatively correlate with post-prandial increases in triglycerides in adults without obesity or T2DM

(Bhargava et al. 2023).

This suggests that metabolic factors including blood glucose, insulin and triglycerides may be mediators of plasma LEAP2 regulation. However, medications to lower plasma glucose and improve insulin resistance (including metformin and dapagliflozin) in adults with overweight/obesity and pre-diabetes, did not decrease fasting plasma LEAP2 over 13 weeks (Byberg et al. 2023).

Another potential mediator of weight-loss associated falls in plasma LEAP2 is elevations of blood ketones as a result of lipolysis. In men without obesity, 60 mins endurance exercise reaching 70% VO2 lowered fasting plasma LEAP2, associated with increases in plasma ketone beta-hydroxybutyrate (Holm et al. 2022). Beta-hydroxybutyrate has been postulated to directly lower plasma LEAP2 through hepatic action, via studies examining effects on LEAP2 secretion from murine hepatocytes *ex vivo*, while ketogenic diets and exogenous ketone administration lowered plasma LEAP2 in animal studies and men without obesity (Holm et al. 2022; Pedersen et al. 2023), though the opposite effect on plasma LEAP2 was seen in another study of exogenous ketone administration in men without obesity (Holm et al. 2023). Oral ingestion of the ketone beta-hydroxybutyrate also suppresses plasma total ghrelin and AG (Stubbs et al. 2018; Vestergaard et al. 2021), while in the ketogenic phase before weight maintenance, VLCD suppresses or attenuates weight-loss associated increases in fasting plasma AG or total ghrelin (Sumithran et al. 2013; Dunn et al. 2017; Pop et al. 2018). Future work should examine correlations between plasma beta-hydroxybutyrate and plasma LEAP2 in the current study, though the intervention was only an LCD not a VLCD.

3.5.3. Plasma LEAP2 after DJBL Insertion

Endoscopic DJBL insertion mimics the exclusion of food from the duodenum and jejunum seen after RYGB surgery (Glaysher et al. 2017; Ruban et al. 2020; Ruban et al. 2022), though after RYGB there is also exclusion of food from the stomach remnant, and also surgical interference with vagal nerve signalling, and effects on bile salt flow, increases in satiety gut hormones PYY and GLP-1, and changes in gut microbiome (Zakeri et al. 2018; Akalestou et al. 2022).

Weight loss after RYGB surgery for obesity without/with T2DM reduced fasting and/or post-prandial plasma LEAP2 at 3-24 months after 16-31% weight loss (Mani et al. 2019; Hagemann et al. 2021).

However, in the current study of obesity with T2DM, the decrease in fasting plasma LEAP2 with weight loss was delayed and attenuated after DJBL insertion compared to the SMM group. Indeed at the first two week time point after DJBL insertion, fasting plasma LEAP2 had increased relative to baseline, while by 26 weeks fasting plasma LEAP2 had decreased relative to baseline, but the fall was less than in the SMM group, while at 50 weeks the fall in plasma LEAP2 was similar in DJBL and SMM groups, despite weight loss being greater at 26 and 50 weeks in the DJBL than SMM groups, though improvements in HbA1c were similar. This suggests a dissociation between weight loss and fasting plasma LEAP2 in the DJBL versus SMM groups, although there was no difference in the slopes of the relationship between absolute or change in fasting plasma LEAP2 and absolute BMI or % weight loss between the groups. However, there was a more positive correlation of fasting plasma LEAP2 with fasting serum triglycerides and HOMA-IR in the DJBL versus SMM groups, which could contribute to the attenuated fall in fasting plasma LEAP2 in the DJBL group, despite the similar falls in fasting serum triglycerides and HOMA-IR between the groups. However, the exact mechanisms behind this delayed and attenuated fall in fasting plasma LEAP2 in the DJBL group remains unclear. It is important to appreciate that both groups had a three-week 1300-1500 kcal per day diet (depending on sex) before the DJBL insertion or commencement of LCD.

One potential confound is that the DJBL group, but not SMM group, received proton pump inhibitors (PPI) and Helicobacter pylori eradication therapy prior to the DJBL insertion, and the DJBL group had a greater prevalence of PPI usage at all visits from 2 to 50 weeks. This could theoretically influence plasma LEAP2, though any possible mechanism is unclear given that LEAP2 is released from duodenum/ileum and liver rather than stomach. There is no published data available on the effects of PPI use or Helicobacter pylori presence or eradication on plasma LEAP2. However, there was no difference in fasting plasma LEAP2 at baseline (-2 weeks) across both groups

between those not using PPI (n=33) or using PPI (n=10), without any covariates (P=0.30), or including BMI or HOMA-IR as covariates (P=0.63-0.68). Of note, no differences have been seen in fasting serum total ghrelin in adults with vs. without PPI use for 6 months (Kim et al. 2009), or in men including overweight/obesity in those with or without Helicobacter pylori infection (Roper et al. 2008).

In the whole cohort, as with the two mechanistic sub-groups presented in this paper, from the clinical trial of DJBL insertion vs. SMM of obesity with inadequately controlled T2DM, there was greater weight loss in the DJBL group at 26 and 50 weeks, which was lost after DJBL explant at 104 weeks, with similar improvements in HbA1c and fasting plasma glucose at 26, 50 and 104 weeks (Ruban et al. 2020; Ruban et al. 2022). The reason behind the greater weight loss in the DJBL group remains uncertain. There were no between groups differences in: (i) the decrease in total food intake and macronutrient selection from three day food diaries, one day food recall, though there appeared to be a greater decrease in total energy intake and carbohydrate intake in the DJBL than SMM group from food frequency questionnaires (though this was higher at baseline in the DJBL than SMM group), nor changes in (ii) total food intake, intake of low/high fat, savoury/sweet foods, or macronutrient intake at an *ad libitum* lunch meal when fasted overnight, (iii) sweet taste detection or hedonics, (iv) motivations for sweets from a progressive ratio task, (v) high-energy or low-energy food cue reactivity using functional magnetic resonance imaging, (vi) explicit liking/wanting and implicit wanting of low/high fat, savoury/sweet foods using Leeds Food Preference Questionnaire, (vii) pleasantness ratings of low/high fat, savoury/sweet foods, (viii) eating behaviour questionnaires assessing dietary restraint, hunger-related, external, disinhibited and emotional eating, “food addiction” and binge-eating related behaviours, (ix) fasting and post-prandial visual analogue scale ratings of hunger, fullness, pleasantness to eat, desired food intake and nausea (x) fasting and post-prandial satiety hormones PYY, GLP-1 and FGF-19, though sample sizes were low in some of these mechanistic sub-group outcomes (Aldhwayan et al. 2022; Ruban et al. 2020).

The delay in and attenuation of the fall in fasting plasma LEAP2 with weight loss in the DJBL group could theoretically contribute to greater weight loss, by attenuating the increase in GHSR signalling that will result both from increased constitutive GHSR

activity (with any fall of LEAP2) and increase in plasma AG that occurs with weight loss from dietary restriction (Muller et al. 2015; Wang et al. 2022; Mani et al. 2019; Aukan et al. 2023; Nymo et al. 2017; Nymo et al. 2018; Lyngstad et al. 2019; Coutinho et al. 2018; Peos et al. 2021). However, this attenuation of GHSR signalling would be expected to result in blunting of weight-loss associated increases in appetite, food cue reactivity or other eating behaviours, and greater reductions in energy intake, in the DJBL vs SMM groups, none of which were seen in this study, other than a possible greater reduction in total and carbohydrate intake from food frequency questionnaires, but not other measures (Ruban et al. 2020).

One year after DJBL explant, there was no increase in fasting plasma LEAP2, despite rebound increases in weight from nadir of 11.3% weight loss at 52 weeks to 5.1% at 104 weeks (though still remaining significantly below baseline). Similar weight rebound was seen in the whole DJBL cohort after explant in the current study (Ruban et al. 2020; Ruban et al. 2022), and in a previous study of adults with T2DM and obesity at one year after DJBL (Betzel et al. 2017b). This suggests that there may be establishment of a new set point in fasting plasma LEAP2 after weight loss with DJBL insertion, that may impair weight loss maintenance, since plasma LEAP2 did not increase with weight regain to assist with suppression of eating behaviour. However, the effects of weight regain on plasma LEAP2 after dietary-induced weight loss alone needs to be examined, since in the SMM group weight loss was maintained between 52 and 104 weeks.

3.5.4. Post-Prandial Increases in Plasma LEAP2

In the current study, post-prandial LEAP2 increased after a 600 kcal liquid meal at baseline in adults with obesity and T2DM (across both groups), consistent with previous literature (Mani et al. 2019; Bhargava et al. 2023; Pedersen et al. 2023; Andreoli et al. 2024a). In pre-clinical studies, the magnitude of post-prandial increases in plasma LEAP2 depends on the size and macronutrient content of meals, with greater increases with fat than carbohydrate (Gradel et al. 2023). Across human ingestion studies, despite differences in macronutrient content and timing of post-prandial sampling, there is a suggestion that post-prandial increases in LEAP2 are proportional to the caloric value of the pre-load meal with outliers including studies

with adults without obesity, see (Bhargava et al. 2023), and further expanded in the current study (Figure 3.11F).

Contrary to the decrease in fasting plasma LEAP2 with weight loss in the SMM group, there was no significant change in the post-prandial increase in plasma LEAP2 after a fixed 600 kcal meal in the SMM group at 2, 26 or 50 weeks. Furthermore, despite the exclusion of food from the duodenum/jejunum, and their greater weight loss, there was also no change in the post-prandial increase in plasma LEAP2 in the DJBL group at 2 nor 26 weeks, nor any difference from the SMM group. These negative findings may be complicated by the smaller sample sizes for those participants receiving the fixed meal. However, in exploratory analysis at 50 weeks in the DJBL group, the post-prandial increase in plasma LEAP2 was attenuated compared to baseline when average weight loss across the two groups was at its maximum of 9.6%. This finding is consistent with the positive correlation of post-prandial LEAP2 with BMI in a previous cross-sectional study in obesity (Mani et al. 2019), suggesting that post-prandial increases in plasma LEAP2 are influenced by degree of obesity, being lower with lower BMI and after weight loss.

The lack of differences in the post-prandial increases in plasma LEAP2 between the DJBL and SMM groups may suggest that the duodenum/jejunum is not vital in eliciting the post-prandial increase in plasma LEAP2, with the liver being more important. In mice, both liver and jejunum/ileum *Leap2* mRNA expression decreased with fasting, but only increased in the liver after standard chow refeeding (Islam et al. 2020; Holm et al. 2022), while mixed macronutrient ingestion (49% carbohydrate, 35% fat) increased both liver and jejunal *Leap2* mRNA expression (Gradel et al. 2023). Furthermore, in mice, olive, lard and fish oil increased plasma LEAP2 though not liver L*eap2* mRNA, while only olive oil and lard increased jejunal L*eap2* mRNA, and olive oil but not fish oil increased LEAP2 secretion from jejunal organoids (Gradel et al. 2023).

3.5.5. Plasma LEAP2 and Appetite

Contrary to our hypothesis in the current study, there were no correlations between absolute/changes in fasting or post-prandial plasma LEAP2 with absolute/changes in

fasting hunger/appetite or post-prandial fullness/appetite ratings in adults with obesity and T2DM in either the SMM or DJBL groups at baseline or across all visits, nor any differences between the groups. In previous studies, acute intravenous LEAP2 administration to men without obesity did not alter various appetite ratings, despite reducing *ad libitum* savoury food intake (Hagemann et al. 2022); in adults without obesity, post-prandial increases in LEAP2 correlated positively with post-prandial decreases in appetite but not increases in fullness ratings (Bhargava et al. 2023); in adults with overweight/obesity fasting plasma LEAP2 negatively correlated with fasting hunger ratings, but there was no correlation between absolute post-prandial plasma LEAP2 and post-prandial hunger ratings (Andreoli et al. 2024a). The lack of any correlations in the current study may be contributed to by the longitudinal nature of the analysis and the fact that all participants had T2DM, though including plasma glucose as a covariate did not produce any significant findings.

3.5.6 Strengths and Limitations

The novelty of the current study is the long duration of follow-up using two different mechanisms to achieve weight loss. Despite the expected participant dropouts over a longitudinal study, final sample sizes were reasonable although numbers for the post-prandial plasm LEAP2 results were more limited. There was no sham endoscopic procedure in the SMM group, though the SMM group still received a stricter LCD intervention for two weeks as in the DJBL group around the time of implant, though the SMM group did not receive H. pylori eradication therapy and long-term PPI medication.

A sub-set of the cohort had a sweet taste detection task prior to blood sampling, which had a significant cross-sectional effect to decrease fasting plasma LEAP2, but adjusted values to remove this effect were included in all analyses. Glycaemic control was poor in all the T2DM participants at baseline due to the inclusion criteria of the trial, and so results may be different in those with obesity without T2DM or with well-controlled T2DM.

Participants had their pharmacological treatment for T2DM changed during the trial following international guidelines, but there was no difference in use of medications between the groups. Furthermore, there were very few participants who started

medications that might alter their appetite including GLP1-RA and insulin.

3.5.7. Future Directions

Given the increases in plasma total ghrelin and AG with dietary-induced weight loss (Muller et al. 2015; Nymo et al. 2017; Nymo et al. 2018; Lyngstad et al. 2019; Coutinho et al. 2018; Peos et al. 2021; Sumithran et al. 2011), which is also seen for total ghrelin in obesity and T2DM after DJBL insertion (though there was no dietary control group) (Vilarrasa et al. 2017), and the potential importance of the plasma AG/LEAP2 ratio in human eating behaviour (Mani et al. 2019; Bhargava et al. 2023), it will be important to measure plasma AG in this study. The effects of dietary induced weight loss on fasting and plasma LEAP2 will also need to be assessed in people with obesity without T2DM and well-controlled T2DM.

3.5.8. Conclusion

Dietary-induced weight loss with increased physical activity in participants with obesity and inadequately controlled T2DM, fasting plasma LEAP2 falls. Whether this may be due to weight loss and/or improved glycaemic control and insulin sensitivity is uncertain, since absolute/decreases in fasting plasma LEAP2 positively correlated with absolute/decreases in BMI, HbA1c, fasting plasma glucose, serum insulin, HOMA-IR and serum triglycerides. DJBL insertion in obesity/T2DM delayed and attenuated the weight-loss associated decrease in fasting plasma LEAP2 through uncertain mechanisms, which might have contributed to the greater weight loss after DJBL insertion, through attenuation of GHSR signalling, although differences in eating behaviour or dietary intake between the two groups have not been identified. The lack of any differences in post-prandial increases in plasma LEAP2 between SMM and DJBL groups may implicate the liver rather than the duodenum/jejunum as the main source for post-prandial secretion of LEAP2. The lack of any increase in fasting plasma LEAP2 following DJBL explant might represent establishment of a novel set-point after weight loss maintenance that may contribute to weight regain. These results add to our understanding of the role for plasma LEAP2 in human body weight regulation, though further longitudinal studies of weight loss are needed in adults with obesity without T2DM.

3.6. ACKNOWLEDGEMENTS

We thank the authors of the original papers from the Department of Surgery and Cancer, Department of Metabolism, Digestion and Reproduction, and Department of Brain Sciences, Imperial College London, and Division of Surgery, University Hospital Southampton NHS Foundation Trust, Southampton, UK, who performed the original clinical trial (Glaysher et al. 2017; Ruban et al. 2020; Ruban et al. 2022). We thank staff at the NIHR Imperial Clinical Research Facility, Imperial College Healthcare NHS Trust, Hammersmith Hospital, London, and NIHR Southampton Clinical Research Facility, University Hospital Southampton NHS Foundation Trust, UK. We thank Departments of Clinical Biochemistry at Imperial College Healthcare NHS Trust, London, and University Hospital Southampton NHS Foundation Trust, Southampton, for performing routine clinical blood assays, and Phoenix Pharmaceuticals Inc., CA, USA for performing the plasma LEAP2 assay.

This research was funded by the UK Research Institute (UKRI) Medical Research Council (MRC) Population and Systems Medicine Board (PSMB) project grant to APG (MR/T017279/1), and National Institute for Health Research (NIHR) and MRC Efficacy and Mechanism Evaluation Programme grant to JPT (12/10/04). The duodenal-jejunal bypass liner (Endobarrier) devices and nutritional supplements were kindly provided free of charge by GI Dynamics Inc. and Nutricia Advanced Medical Nutrition respectively. Infrastructure support was provided by the NIHR Imperial Biomedical Research Centre and the NIHR Imperial Clinical Research Facility, London, and NIHR Southampton Biomedical Research Centre UK and NIHR Southampton Clinical Research Facility, Southampton, UK. The views expressed are those of the author(s) and not necessarily those of the National Health Service (NHS), the NIHR or the Department of Health and Social Care (DHSC).

CHAPTER 4: SAFETY AND METABOLIC EFFECTS OF EXOGENOUS LEAP2 INFUSIONS IN ADULTS WITHOUT OBESITY

4.1. ABSTRACT

Background: Liver/gut satiety hormone LEAP2, inverse agonist at the growth hormone secretagogue receptor (GHSR), is reciprocally regulated to acyl ghrelin (AG). Fasting increases blood AG, ketones/free fatty acids (FFA) and GH, and lowers serum insulin, mimicked by supraphysiological exogenous AG administration (Garin et al. 2013). Intravenous LEAP2 infusion has only been administered once in men without obesity (Hagemann et al. 2022), without any adverse event. Exogenous LEAP2 infusion (25 pmol/kg/min) decreased post-prandial glucose, increased fasting insulin, and reduced post-prandial GH, fasting and/or post-prandial glycerol and FFA in men without obesity (Hagemann et al. 2022). This suggests GHSR signalling reduces glucose, increasing lipolysis, but this may be direct or indirectly via changes in insulin and GH.

Methods: Single-blind, placebo-controlled, within-participant study in adults without obesity who received 5.7h intravenous infusion of saline or LEAP2 (12, 24, 25, 36 pmol/kg/min) after an overnight fast, with 71kcal snack at 195min. Plasma AG, glucose, serum insulin, GH, cortisol, lipids and venous beta-hydroxybutyrate (BHB) assayed at 4-7 time points, and pituitary hormones at baseline and 310min. Linear mixed model analysis for correlation of LEAP2 dose with changes in blood measures.

Results: Adults without obesity (age 22-55 years, BMI 18.7-27.1 kg/m^2) attended for total of 22 infusions (n=7, 4 men). LEAP2 dose negatively correlated with plasma glucose incremental AUC (iAUC) (overall iAUC $_{-10\ to\ 310min}$: r^2=0.27, β=-2.79 mmol/L.min, P=0.007; fasting iAUC $_{-10\ to195min}$ r^2=0.37, β=-1.61 mmol/L.min P=0.001) and also with venous BHB (overall iAUC $_{-10\ to\ 310min}$: r^2=0.17, β=-0.79 mmol/L.min, P=0.054; post-prandial iAUC $_{195\ to\ 310min}$ r^2=0.09, β=-0.27 mmol/L.min P=0.042). There were no significant correlations of LEAP2 dose with iAUC serum insulin, GH, cortisol, triglycerides overall or just for fasting and post-prandial periods, nor change in serum free thyroxine, TSH, prolactin. There were no significant correlations of LEAP2 dose with temporal impulsivity for alcohol, food or monetary reward, for memory outcomes in cognitive tasks or food reward (P>0.05). One male participant had an unexpected serious adverse effect of bradycardia and hypotension (out of 4 participants that

received LEAP2 at 36 pmol/kg/min), but all other infusions were otherwise well tolerated without adverse effects.

Conclusion: Intravenous LEAP2 infusion dose dependently reduced fasting plasma glucose and post-prandial venous BHB, independent of changes in serum insulin and GH. These preliminary findings support an effect of GHSR signalling to reduce plasma glucose and directly stimulate lipolysis in adults without obesity. Future studies can now examine the effects of LEAP2 administration on human eating and addictive behaviours using functional MRI using the dose of 25 pmol/kg/min.

4.2. INTRODUCTION

4.2.1. Acyl Ghrelin and LEAP2

Acyl ghrelin (AG, active form of total ghrelin after acylation from desacyl ghrelin) is produced by the cells in the oxyntic gland in the mucosa of the gastric fundus (Yanagi et al. 2018). AG is an endogenous agonist of the growth hormone secretagogue receptor (GHSR), stimulating food intake, appetite (Sumithran et al. 2011; Schalla et al. 2019; Srivastava et al. 2018; Jerlhag 2019; Kojima et al. 2006; Korbonits et al. 2004; Stengel et al. 2011), gastrointestinal motility and increasing plasma glucose, serum growth hormone, cortisol and reducing serum insulin (Muller et al. 2015; Poher et al. 2018; Goldstein et al. 2011).

Mature full-length liver-expressed antimicrobial peptide 2 (LEAP2), is a 40 amino-acid long hormone with identical structure in rodent and humans another (Krause et al. 2003 18912; Ge et al. 2018). LEAP2 is expressed in the liver and proximal small intestine (Krause et al. 2003; Henriques et al. 2010; Howard et al. 2010; Englund et al. 2024; Islam et al. 2024). LEAP2 has been identified as both an inverse agonist (reduces GHSR's baseline activity) and a competitive antagonist (blocks ghrelin's action on GHSR) (Ge et al. 2018; M'Kadmi et al. 2019; Wang et al. 2019; Mani et al. 2019).

Plasma LEAP2 concentrations are inversely regulated compared to acyl ghrelin (AG), decreasing with fasting or weight loss and increasing with refeeding or weight gain in both rodents and humans (Ge et al. 2018; M'Kadmi et al. 2019; Wang et al. 2019; Fernandez et al. 2022; Mani et al. 2019; Hagemann et al. 2021; Hagemann et al. 2022). In mice, exogenous administration of LEAP2, either peripherally or centrally, counters the orexigenic effects of AG and exogenous LEAP2 independently reduces food intake (Barrile et al. 2019; Cornejo et al. 2019; Islam et al. 2020; Hagemann et al. 2022). Conversely, deletion of the *Leap2* gene in mice amplifies the orexigenic effects of exogenous AG (Shankar et al. 2021a). Lipidisation of LEAP2(1-14) fragment at its C-terminus enhanced its affinity to the GHSR, amplified the anorexigenic effect to attenuate ghrelin induced food intake in (Hola et al. 2022). Additionally, lipidisation of mature LEAP2 peptide has a half-life of 5.19hours in rodents (longer than 10-15mins

of mature LEAP2 (Ge et al. 2018)) and has been shown to reduce weight and hepatic steatosis over four weeks in diet-induced obese mice (Shankar et al. 2024).

The mechanisms behind fasting and post-prandial increases in plasma LEAP2 remain incompletely understood. However, from rodent, human, and organoid studies that the liver, duodenum, and jejunum contribute to plasma LEAP2, particularly post-postprandially after oral glucose and fat administration plasma LEAP2 is found in the hepatic artery and abdominal aorta (Islam et al. 2020; Holm et al. 2022; Gradel et al. 2023; Englund et al. 2024; Islam et al. 2024). Correlational studies in humans suggest that fasting and postprandial increases in plasma LEAP2 may be associated with changes in blood glucose, insulin, and triglycerides, which may stimulate LEAP2 secretion (Mani et al. 2019; Andreoli et al. 2024a; Bhargava et al. 2023; Li et al. 2022; Ma et al. 2020; Stark et al. 2023). Notably, as with other gut hormone, there may be an “incretin effect” where in there is a larger increase in post-prandial plasma LEAP2 after an oral glucose load (vs. intravenous route) (Pedersen et al. 2023).

Furthermore, in humans, fasting increases blood AG, ketones, free fatty acids (FFA) and growth hormone (GH), and reduces serum insulin, mimicked by supraphysiological exogenous AG administration (Garin et al. 2013). Recent studies in rodents and humans indicates that exercise (increasing endogenous ketonaemia) and exogenous ketone administration may reduce plasma LEAP2 secretion, potentially via beta-hydroxybutyrate (Pedersen et al. 2023; Holm et al. 2022; Holm et al. 2023). This suggests that GHSR signalling may increase lipolysis either directly or indirectly by changes in insulin and GH.

4.2.2. Safety of Ghrelin Administration in Humans

Several studies involving AG and DAG intravenous infusions or subcutaneous injections, that stimulate the GHSR receptor, have been performed without any significant adverse reactions (Wren et al. 2001; Neary et al. 2004; Druce et al. 2005; Wynne et al. 2005; Druce et al. 2006; Nagaya et al. 2001; Miljic et al. 2006; Levin et al. 2006; Goldstone et al. 2014; Malik et al. 2008).

However, some previous studies have shown that AG infusion leads to a decrease in systolic blood pressure, diastolic blood pressure, heart rate, respiratory rate and temperature (Nagaya et al. 2001; Zhang et al. 2017). These effects are seen whether given as a bolus or infusion. This decrement in systemic blood pressure was only up to 6mmHg. These effects were transient and non-severe. As LEAP2 is an inverse agonist at GHSR and antagonist of AG, it is hypothesised that there may be an increment in blood pressure and heart rate. Based on the known effects of AG and LEAP2 from pre-clinical studies, and other studies of appetitive gut hormones in humans, anticipated effects of LEAP2 might include hypoglycaemia, nausea, vomiting, abdominal pain or bloating, reduced appetite, early satiety, and delayed gastric emptying.

4.2.3. Safety of GHSR Inverse Agonist PF-5190457 in Humans

In recent studies, twice daily oral administration of PF-5190457, a GHSR inverse agonist developed by Pfizer pharmaceutical company (but no longer being pursued for licensing), in adults with alcohol dependence, acutely or for up to 3 days, had no serious adverse events or effects on nausea, lethargy, gastrointestinal symptoms, headache, light-headedness or weakness that required discontinuation or dose reductions (Lee et al. 2020b; Lee et al. 2020a; Farokhnia et al. 2020). More recently, this drug reduced calories selected in a virtual buffet task in participants with AUD and overweight/obesity without reduction in alcohol cravings during an alcohol cue reactivity fMRI task (Faulkner et al. 2024).

4.2.4. Safety of LEAP2(38-47) Fragment in Humans

One previous study has infused the N-terminal 10 amino acid fragment LEAP2(38-47) to n=10 healthy non-obese men (Hagemann et al. 2021). No serious adverse events were seen and there were no effects of appetite ratings, glucose regulation or insulin secretion. Estimated plasma concentrations with the infusion rate of 20pmol/kg/min will have been 4208-4596 pmol/L. However, although this is in the range of that seen for endogenous full length LEAP2(38-77), LEAP2(38-47) has only ~0.2-6.2% of the potency of LEAP2(38-77) at GHSR in in vitro studies. Thus, this infusion rate would not have been expected to produce any clinically relevant responses.

4.2.5. Safety of LEAP2 in Humans

However, none of these side effects were not seen in the first in human study of intravenous full length LEAP2(38-77) infusion in (n=20) healthy men without obesity (Hagemann et al. 2022). In the dose escalation phase, LEAP2 was given as a bolus infusion for 10 min at a rate of ~101 pmol/kg/min followed by an infusion for 110 min at ~25 pmol/kg/min (total infusion time 2 hours). There were no side-effects and median [IQR] nausea scores of 3 [0, 9] on 100mm VAS. In the continuous dose infusion study at ~25 pmol/kg/min over 290min (total infusion time 4 hours 50 min), there were no cardiac problems reported and no effects on appetite ratings, nausea (highest mean ~15mm at both saline and LEAP2 visits) or discomfort (lowest mean comfort ~70mm at both saline and LEAP2 visits) ratings (100mm VAS), nor gastric emptying (paracetamol), and while there was a small reduction in post-prandial plasma glucose (vs. saline), there was no hypoglycaemia with the lowest mean plasma glucose 4.9 mmol/L (Hagemann et al. 2022). This down signalling of the GHSR increased fasting insulin, reduced GH, glycerol, FFA suggesting increase in lipolysis.

Therefore, administration of full length 40 amino acid LEAP2(38-77) to non-obese healthy participants to achieve slightly supra-physiological plasma LEAP2 concentrations will have metabolic and hormonal effects that are expected from its effects as an GHSR inverse agonist, which are opposite to the previously identified effects of AG in rodents and humans. This will add to findings from previous studies of oral administration of PF-5190457, a GHSR inverse agonist, in adults with alcohol dependence.

This LEAP2 infusion study will validate the infusion rate needed to achieve target plasma concentrations without problematic or unsafe side effects, building on results from a recently published first-in-human LEAP2 infusion study (Hagemann et al. 2022). This study will also allow practice, confirm acceptability and novel results of effects of LEAP2 on eating behaviour and cognitive tasks.

4.3. AIMS

To determine from a human administration study, the effects of the gut hormone, LEAP2, an endogenous AG inverse agonist, in a within-participant design, the effects

of acute intravenous infusion of full length 40 amino acid LEAP2(38-77) to healthy participants without obesity on:

(i) plasma LEAP2 concentrations to confirm infusion rate to achieve required plasma LEAP2 concentrations within required safety and side effect profile;

and allow practice, initial pilot examination and verification of acceptability to participants of the tasks to examine its effects on:

(ii) appetite visual analogue scale (VAS) ratings of appetite and nausea, food craving, desired (virtual portion size creation task) and actual (*ad libitum)* food intake,
(iii) plasma/serum glucose, insulin, GH concentrations through serial blood sampling
(iv) alcohol craving form Alcohol Urge Questionnaire (AUQ),
(v) food liking and wanting (Leeds Food Picture Questionnaire), and delay discounting rate for food, money and alcohol and,
(vi) motor response inhibition, working and episodic memory (CANTAB cognitive tasks).

4.4. HYPOTHESES

The hypotheses for this study are that acute infusion of LEAP2 to non-obese healthy participants, to achieve slightly supra-physiological plasma LEAP2 concentrations, will:

(i) be safe and without nausea, vomiting, abdominal pain, cardiac rhythm abnormalities,
(ii) reduce appetite and, desired and actual food intake,
(iii) reduce plasma glucose via stimulation of insulin secretion and decreasing GH secretion, and
(iv) reduce craving for alcohol,
(v) reduce food liking and wanting, preferred food portion size, and impulsivity to food,
(vi) impair working and episodic memory and reduce motor response inhibition.

Outcome Measures

Primary: Changes in plasma concentrations of LEAP2, heart rate, blood pressure, ECG, and side effect profile.

Secondary: Changes in plasma/serum concentrations of insulin, glucose, cortisol, growth hormone (GH), visual analogue scale ratings of appetite, nausea, food craving, alcohol urge questionnaire and, energy intake at *ad libitum* test meal.
Exploratory: Food liking and wanting (Leeds Food Picture Questionnaire), preferred food portion size (Virtual portion creation task), temporal impulsivity to food, alcohol and money (delay discounting task), rate of unsuccessful motor response inhibition (stop signal task), working and episodic memory formation (spatial working memory and paired associate learning tasks).

4.5. METHODS

4.5.1. Participants

Participants recruited were healthy adults between the ages of 18 and 60 years without obesity (i.e. body mass index (BMI) 18.0-29.9kg/m^2). Recruitment was facilitated through public advertisements, social media (Facebook), Imperial College London and hospital websites and noticeboards, Imperial Clinical Research Facility (CRF) healthy volunteer database. Interested participants received a link which was directed to an online questionnaire. Upon completion and preliminary eligibility, a telephone screening was arranged to inform potential participants about the study and to verify their eligibility. Eligible participants underwent in-person health assessments and practiced computer-based tasks before participating in the experimental study visits.

4.5.1.1. On-line screening

Interested participants were sent a link to complete an on-line questionnaire using the Alchemer platform. This included brief information about the study and checked for any obvious inclusion and exclusion criteria. If eligible, they were asked to leave their contact information, and were emailed a participant information sheet (see Appendix 2). If interested in participating, a telephone screening was conducted.

4.5.1.2. Telephone screening

Potential participants had undergone a telephone screening interview to inform the potential participant about the study and to check that the basic criteria for the dose confirmation study are met. Upon meeting basic criteria, they were sent an appointment to attend an in-person screening visit. This call lasted between 15-

30mins.

4.5.1.3. Inclusion criteria

(i) Male or female between ages of 18 and 60 years.
(ii) Without obesity with body mass index (BMI) 18.0-29.9 kg/m^2
(iii) Healthy as determined by a responsible physician, based on a medical evaluation including medical history, physical examination, laboratory tests, and ECG.
(iv) Capable of giving written informed consent, which includes compliance with the requirements and restrictions listed in the consent form.
(v) Participant is able to read, comprehend and record information written in English.

4.5.1.4. Exclusion criteria

Volunteers were ineligible for inclusion in this dose-confirming study if any of the following criteria apply:

(i) History of, or current abuse or dependence on alcohol, illegal drugs or disordered gambling.
(ii) Current smoker or less than 2 years since quitting (cigarette, cigars, e-cigarettes) or use of nicotine replacement therapy.
(iii) Significant current or past medical or psychiatric history that, in the opinion of the investigators, contraindicates their participation.
(iv) History of type 1 or type 2 diabetes mellitus.
(v) History of ischaemic heart disease, heart failure, cardiac arrhythmia, peripheral vascular, cerebrovascular disease or uncontrolled hypertension.
(vi) Current diagnosis of anaemia or iron deficiency.
(vii) Body weight instability (change in body weight of more than 5% over the preceding 3 months).
(viii) Unwillingness or inability to follow the procedures outlined in the protocol.
(ix) Use of current regular prescription or over-the-counter medications that in the opinion of the Investigators may affect participant safety or outcome measures.
(x) Clinically significant abnormalities in screening blood tests abnormalities which in the opinion of the study physician, is clinically significant e.g. diabetes mellitus, hypothyroidism, renal impairment, abnormal liver function tests [bilirubin, alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl

transferase (GGT)] >3x upper limit of normal, other than due to fatty liver disease.

(xi) Current pregnancy or breast-feeding in female volunteers (the Investigators will recommend using contraception for the duration of the visits to avoid participant drop-out).

(xii) Pulse rate <40 or >100 beats per minute OR systolic blood pressure >160 and <100 OR diastolic blood pressure >95 and <50 in the semi-supine position.

(xiii) The volunteer has participated in a clinical trial and has received an investigational product within the following time period prior to the first experimental visit in the current study: 90 days, 5 half-lives or twice the duration of the biological effect of the investigational product (whichever is longer).

(xiv) Exposure to more than 3 new investigational medicinal products within 12 months prior to the scan.

(xv) Vegan, gluten or lactose-intolerant (as test meals in the paradigms may include animal products, dairy and wheat products).

(xvi) Volunteers who have donated, or intend to donate, blood within three months before the screening visit or following study visit completion.

(xvii) History of sensitivity to LEAP2 peptide, or components of infusion thereof, or a history of drug or other allergy that, in the opinion of the investigators, contraindicates their participation.

(xviii) Clinically significant abnormalities in screening electrocardiogram (ECG) which in the opinion of the study physician, is clinically significant and represents a safety risk.

(xix) Positive drug screens on screening visit. A minimum list of drugs that will be screened for include amphetamines, barbiturates, cocaine, opiates, cannabinoids and benzodiazepines.

(xx) Known history of SARS-CoV-2 infection (Covid-19) in the last 4 weeks.

(xxi) Ongoing symptoms suggestive of complications from previous SARS-CoV-2 infection (‘long Covid-19’) such as loss or change in sense of smell or taste, shortness of breath, palpitations, lethargy.

(xxii) SCOFF questionnaire score >1/5 indicating eating disorders (Luck et al. 2002).

(xxiii) DSM-V criteria for alcohol use disorder (AUD) >2/11 indicating mild AUD (American-Psychiatric-Association 2013).

(xxiv) Participants who have had previous obesity surgery or are on medications for

obesity.

(xxv) Dutch Eating Behaviour Questionnaire (DEBQ) restraint score >3/5 indicating highly restrained eating behaviour (Van Strien et al. 1984).

4.5.1.5. Withdrawal criteria

Withdrawal from the study was entirely up to each participant to initiate. Each participant recruited had the right to withdraw from the study for any reason at any time.

4.5.2. Study Design

This study was a single-blinded LEAP2 peptide or saline (placebo) infusion study in healthy volunteers who meet the eligibility criteria above. The purpose of this dose-confirmation phase was to validate the final LEAP2 infusion rate with acceptable safety and side effect profile, and confirm acceptability of protocol and obtain pilot data on efficacy.

This was a single-blind dose-confirmation phase. Participants were be blinded but study researchers and doctors were not blinded in this phase. Initially there was no randomisation of the volunteers to the order of LEAP2 or placebo infusions as this was an ascending dose finding study, but there was randomisation to order in subsequent visits once the target infusion rate was confirmed. Participants did not receive all the peptide infusion rates and placebo over their study visits.

On the morning of each experimental visit, participants had one intravenous cannula placed in the vein of each forearm for peptide infusion and one for venous blood sampling. Full length 40 amino acid LEAP2(38-77) peptide was administered as an infusion of up to 6 hours. Infusions will be performed at the NIHR Imperial Clinical Research Facility (CRF), Hammersmith Hospital, London.

Peptide infusions followed protocols established after long experience investigating the physiological role of numerous peptides in humans by the Division of Diabetes, Endocrinology and Metabolism, Imperial College London (Goldstone et al. 2014; Druce et al. 2005; Wren et al. 2001; Druce et al. 2006; de Silva et al. 2011).

After telephone screening each eligible participant attended an in-person screening visit (described below). If they are successful, then each participant was be pseudo-randomised and attended up to 6 study visits (described below). The procedure during one study visit is presented in the flowchart (Figure 4.1).

4.5.2.1. Visit 1: screening visit

All volunteers had undergone consent, enrolment, and routine clinical assessments at the NIHR Imperial Clinical Research Facility, Hammersmith Hospital, London. During this session, the purpose of this study was fully explained to the volunteer. All questions were answered, and consent obtained before any procedures are performed. Participants did not need to fast for this visit. This visit takes 2-3 hours to complete. The screening visit included:

- Informed consent (see Appendix 1)
- Medical and psychiatric history and physical examination
- Measurement of height and weight
- Measurement of vital signs (blood pressure, heart rate)
- 12- lead electrocardiogram (ECG)
- Blood sampling (including full blood count, urea and electrolytes, liver function tests, clotting, glucose, thyroid function test, HbA1c, iron studies, DNA)
- Urinary pregnancy test for females
- Urine drug test
- Bio-electrical impedance analysis to calculate total fat-free and fat mass
- Questionnaires about mood, eating behaviour, alcohol use
- Practice of eating behaviour tasks: Leeds food picture questionnaire, Virtual portion size creation task, Delay discounting task

4.5.2.2. Visits 2-7: experimental dose confirmation study visits

Up to 6 visits per participant over 4-10 weeks, 8 hours per visit at least 2 days apart, including a 5.7 hour LEAP2 peptide or placebo infusion, conducted at the NIHR Imperial Clinical Research Facility, Hammersmith Hospital, London. These study visits included the following:

Day before admission: fast after dinner (evening meal) at 8pm

t (time) = -75 min: arrival at ~09:00
t = -70 min: health check
t = -65 min: pregnancy test for females
t= -60 min: 2 x cannula inserted into peripheral veins
t= -45 min: Measurement of height and weight, bio-electrical impedance analysis (MC-780MA, Tanita, Tokyo, Japan), ECG baseline
t= -15 min to +400 min: serial venous blood sampling
t= -15 min to +400 min: continuous cardiac rhythm monitoring, serial pulse and blood pressure monitoring
t= -10 min: PANAS and SSAI questionnaires about mood
t= -10 min to +400 min: visual analogue scales ratings of appetite, nausea, sleepiness, anxiety, stress
t= 0 to +340 min: LEAP2 peptide infusion may include initial double rate for 30 min (starts ~10:15, ends ~15:55)
t= +40 min: ECG #1
t= +45 min to +60 min: Stop Signal Task (SST, 14min)
t= +60 min: water (500mL) (~11:15)
t= +190 min: ECG #2
t= +195 min: small snack (71 kcal) (~13:30)
t= +225 min to +250 min: Leeds Food Picture Questionnaire (LFPQ, 25 min)
t= +260 min to +295 min: delay discounting task (DDT, 15 min), virtual portion size creation task (5 min)
t= +300 to + 315 min: paired associate learning task (PAL, 8 min), spatial working memory task (SWM, 4 min)
t= +315 min: *ad libitum* meal (~15:30)
t= +335 min: ECG #3
t= +340 min: LEAP2 infusion stops
t= +400 min: safety monitoring stops
t= +405 min: participant departs at ~17:00

4.5.2.3. Duration of the study period

The duration of the study period for each participant was 4-10 weeks, during which the initial clinical assessment screening visit, and up to 6 further doses confirming peptide

or placebo infusion study visits, were conducted per participant (with a maximum of 2 visits in one week with at least 2 days between any infusion visit).

4.5.2.4. Definition of completion of study for an individual participant

The end of the study is defined as 2 days after the last peptide infusion visit, which consequently means the end of data collection as it is defined in ethics and regulatory terms. Follow up of this cohort will not take place.

4.5.2.5. Participant reimbursement

Participants received compensation for taking part in the research to cover their time. They received £25 for the screening visit and £100 per completed experimental visit, totalling a maximum of £625 for completion of 6 infusion visits. Payment was made by bank transfer. Reasonable travel costs were also reimbursed.

4.5.2.6. Summary of study visits

Up to six identical visits: single-blind, experimental medicine evaluations

- LEAP2 peptide (at varying infusion rates) or Placebo (Saline)

Each visit duration was be separated by at least 2 days.

Total duration of each visit including regular breaks: up to 8 hours

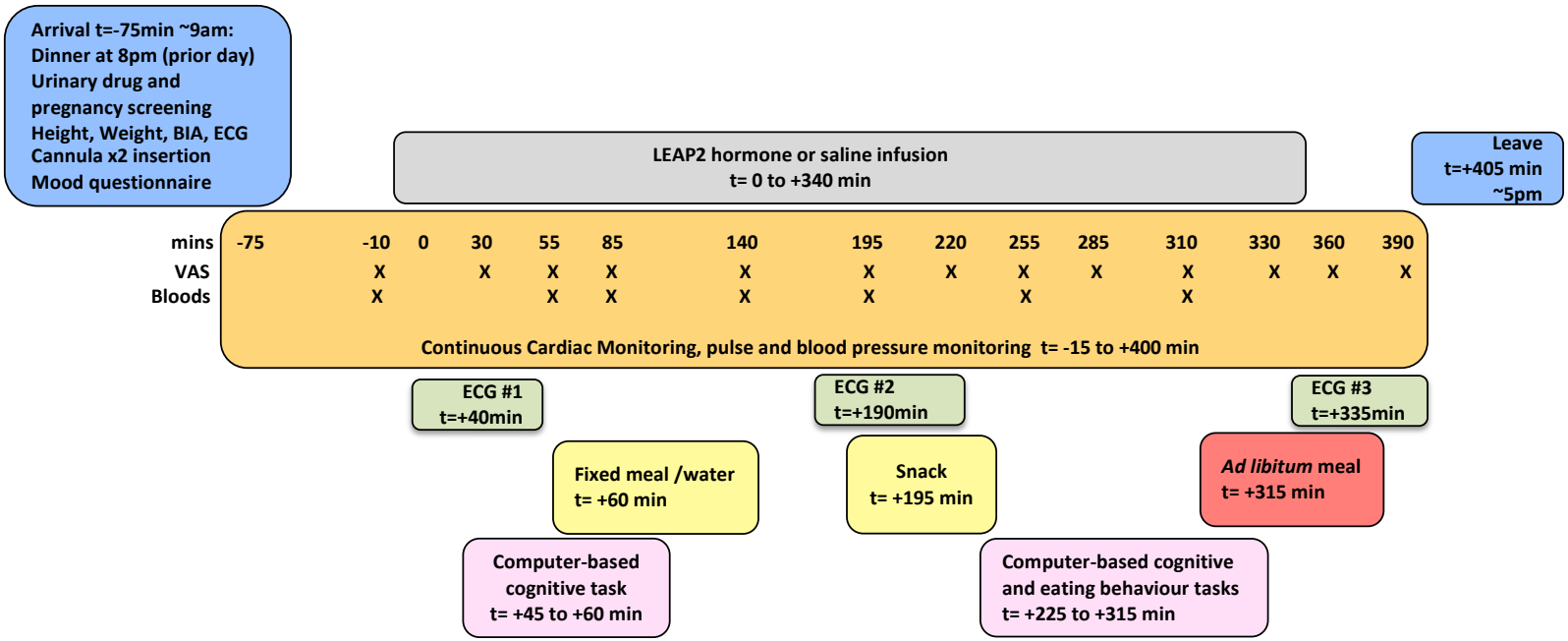


Figure 4.1. Schematic diagram of study visit timeline.

Participants were asked to avoid strenuous exercise and alcohol, and refrain from eating or drinking from 8pm on the day before the visit. When participants arrived, height and weight were measured. Bio-electrical impedance analysis (BIA) was used to estimate their body composition and vital signs including blood pressure and pulse rate were recorded. Urinary pregnancy test was required for females. After cannulation in each arm at t=-75min, participants were attached to continuous cardiac rhythm monitoring, Dexcom G7 continuous glucose monitoring and first ECG was done. Venous blood samples were collected through one cannula at t=-10, +55, +85, +140, +195, +255, and +310 min for analysing fasting and post-prandial metabolites. Visual analogue scale (VAS) ratings of fullness and composite appetite ratings taken at -10, +30, +55, +85, +140, +195, +220, +255, +285, +310, +330, +360, and +390 min. LEAP2/ Placebo infusion was delivered from the other cannula from t=0 to +340min. Participants had ECG measurements at t= +40, +190 and +335min. At t=60min participants received a fixed preload drink (750 mL) with 0 kcal

and a snack at t=+195min. From t=+45 to +60min participants engaged in the Stop Signal Task (SST). From t= +225 to +315 min participants engaged in computer based cognitive and eating behaviour tasks including, virtual portion creation task (VPCT), Leeds Food Picture Questionnaires, Delay Discounting Task, Paired Associate Learning and Spatial Working Memory, followed by an *ad libitum* test meal at t=+315 min.

4.5.3. LEAP2 Peptide Preparation

GMP-grade LEAP2(38-77) peptide was obtained from a commercial supplier (AmbioPharm Inc., South Carolina, USA) at >98% purity, and tested for peptide content, endotoxin and microbial analysis, toxicity in mice, bio-efficacy in mice (e.g. by confirming ability to block AG- or fasting-induced food intake or GH secretion after intraperitoneal administration (Ge et al. 2018; M'Kadmi et al. 2019), see Appendix 3), prior to administration to humans. This follows protocols as previously performed for studies of peptide gut hormone administration in humans, at Imperial College London and first in human studies, e.g. AG, PYY, GLP-1 (Wren et al. 2001; Druce et al. 2005; Druce et al. 2006; de Silva et al. 2011; Goldstone et al. 2014), and DAG in GHADD study (https://clinicaltrials.gov/ct2/show/NCT02690987).

LEAP2(38-77) peptide was prepared and bio-efficacy testing conducted by by Dr James Minnion, Division of Diabetes, Endocrinology & Metabolism, Department of Metabolism, Digestion and Reproduction, Imperial College London, complying with their standard preparation procedures used for human peptide infusion studies (see Appendix 3).

Steps are listed below:

(i) GMP grade LEAP2 peptide (human natural sequence) was purchased from AmbioPharm Inc., South Carolina, USA. This confirms absence of organic solvents and endotoxin, purity and sterility.

(ii) LEAP2 peptide was aliquoted into sterile glass vials under aseptic conditions and freeze-dried. Although the material is packed and stored under vacuum, which destroys any active (aerobic) micro-organisms, samples of freeze-dried peptide in aliquoted vials were also be sent for culture of common pathogenic micro-organisms as a further safety check, and absence of pyrogen contamination of every batch of LEAP2 peptide was confirmed by highly sensitive measurement of endotoxin content using a Limulus Amoebocyte Lyaste (LAL) test, performed by Associates of Cape Cod International, Liverpool, United Kingdom.

(iii) Amino acid content of the LEAP2 peptide was determined by High-performance liquid chromatography (HPLC) to allow correct calculation of dose to be administered.

(iv) Bio-efficacy of the synthesised LEAP2 was confirmed in mice after subcutaneous administration by demonstration of dose-dependent reduction in food intake in the fasted state and ability to block AG-induced food intake (see Appendix 3), as previously described (Ge et al. 2018; M'Kadmi et al. 2019).

Peptide infusions followed protocols established after long experience investigating the physiological role of numerous peptides in humans by the Department of Metabolism, Digestion and Reproduction, Imperial College London. The placebo visit involved infusion of pharmaceutical GMP-grade sterile 0.5% human albumin (dissolved in saline) obtained from Imperial College Healthcare NHS Trust Pharmacy. Peptide solutions were made up with 0.5% human albumin dissolved in saline. To reduce binding of peptides to infusion lines, 0.5% human albumin dissolved in saline was used to prime the infusion lines and syringe for 30 mins prior to infusion of the peptides or placebo solutions.

4.5.4. LEAP2 Peptide Dosing

Human peptide hormone administration study, for which Imperial College London has much experience, including first or early clinical administration of other peptides including AG, DAG, GLP-1, PYY, kisspeptin. After an overnight fast (to maximise endogenous plasma AG, and minimise endogenous plasma LEAP2), participants received a 5.7 hour single-blind intravenous infusion of LEAP2 or saline. Close monitoring with cardiac rate/rhythm, blood pressure, capillary glucose measurement with serial venous blood sampling was performed. Infusion doses were incrementally increased at each visit, with progression to next dose based on tolerability, safety and serial plasma LEAP2 concentrations (ELISA, EK-075-40, Phoenix Pharmaceuticals Inc., CA, USA).

Final LEAP2 infusion rates was based on body weight, for final infusion rate achieving:

(i) fasting plasma LEAP2 concentration of ~50 ng/mL (10,915 pmol/L) in mildly supraphysiological range for non-obese adults from our pilot studies, and
(ii) no marked side effects e.g. nausea, vomiting, abdominal pain, hypoglycaemia.

Based on previous infusion studies of other peptides, the estimated maintenance infusion dose of LEAP2 to achieve target final plasma LEAP2 concentration of 50 ng/mL is expected to be ~36 pmol/kg/min, with an initial 30-minute double dose ramping phase of ~72 pmol/kg/min. As reported in a recent publication describing first-in-human full length LEAP2 infusions (Hagemann et al. 2022), intravenous LEAP2 infusion to n=8 healthy men without obesity at rate of ~101 pmol/kg/min for 10 min, followed by an infusion at ~25 pmol/kg/min for 110 min was safe with achieved mean plasma LEAP2 of 30-40 ng/mL, with no serious adverse events nor adverse cardiac side effects.

In the second part of the same study (Hagemann et al. 2022), LEAP2 was infused intravenously to n=20 healthy men without obesity who were then given a 575 kcal meal, at ~25 pmol/kg/min or 115 ng/kg/min over 290 min with achieved mean plasma LEAP2 of 41.2 ng/mL (vs. 15.8 ng/mL during saline infusion). This LEAP2 infusion dose reduced food intake at an *ad libitum* test meal, though had no effect on appetite ratings, and was safe and well tolerated with no serious adverse events nor adverse cardiac side effects reported, and with no increase in nausea. Thus, to achieve our target plasma LEAP2 concentration of 50 ng/mL it would be estimated to need a maintenance infusion rate of 30.3 pmol/kg/min.

In another study by the same group (Englund et al. 2023) (Englund et al., Abstracts American Diabetes Association 2023, https://diabetesjournals.org/diabetes/article/72/Supplement_1/285-LB/149524/285-LB-LEAP2-Reduces-Preprandial-and-Postprandial), LEAP2 was infused intravenously to n=20 healthy men with obesity (mean BMI 36.3 ± 0.8 kg/m^2) at ~40 pmol/kg/min over ~5 hours with a ~13% reduction in *ad libitum* food intake and fasting/fed plasma glucose without any serious gastrointestinal or cardiac adverse events.

The final target maintenance infusion rate is thus estimated to be 30-36 pmol/kg/min. The dose escalation used an adaptive protocol based on measured plasma LEAP2 with a minimum of two participants receiving each infusion dose before proceeding to the next infusion rate increment.

In this study, participants were planned to sequentially receive constant infusion rates at each study visit, though this was adapted depending on measured plasma LEAP2 concentrations with infusion doses between 12.5 to 36 pmol/kg/min to achieve final plasma LEAP2 of ~50 ng/mL, and included visits using:

(i) infusions with double rate for first 30 mins to more rapidly achieve steady state concentrations,
(ii) higher infusion rates in order to achieve target plasma LEAP2 concentrations,
(iii) 1-2 saline infusion visits (to control for visit order for pilot efficacy outcome variables).

4.5.5. Test Meals

Participants were blinded to the energy content of the preload liquid meal at t=+60min. This vanilla-flavoured water was 0kcal, using Bulk Zero Calorie Barista syrup, sugar free, Vanilla (Sports Supplements Ltd. t/a Bulk, Colchester, UK, 0% carbohydrate, 0% fat, 0% protein) standardized to 750mL (as the original protocol included an option for a fed visit, with participants consuming this volume of a liquid meal of 1200kcal, Vanilla Fortisip Compact, Nutricia, Trowbridge, Wiltshire, UK, 300 kcal per 125 mL, 49% carbohydrate, 35% fat, 16% protein) which participants were asked to consume within 10-15min after receiving the meal.

At each study visit, during the LEAP2 infusion participants also received a small snack consisting of one digestive biscuit (McVitie's Ltd., London, UK, 71kcal, 54% carbohydrate, 40% fat, 6% protein) and one pot of jelly (Histon's sweets spread Ltd., Leeds, UK, 0kcal, 0% carbohydrate, 0% fat, 0% protein) at t=+195min (~71 kcal) to avoid inattention and discomfort due to duration of fasting since dinner (evening meal) at 8pm the previous day.

4.5.4. Blood collection, Storage and Assay

Blood samples were collected at 7 time points at t=-10, +55, +85, +140, +195, +255, and +310 min and assayed for plasma glucose, serum insulin, triglycerides, growth hormone, cortisol (Department of Clinical Biochemistry, Imperial College Healthcare NHS Trust) and stored for assay of gut hormones. Venous beta-hydroxybutyrate (BHB; ketones) were measured at time of blood sample collection using point-of-care

ketone meter (Abbott Freestyle Precision Pro ketone sticks, Abbott House, UK) Incremental area under curve ($iAUC_{10\ to\ 310min}$) for all metabolites (with >2 measurements) was calculated using the trapezoid rule. Change is metabolite concentrations from t=310 to t=10min was used if only 2 measurements were taken during a study visit.

Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula: Fasting insulin (mU/L) x fasting glucose (mmol/L)/22.5 (Matthews et al. 1985). Blood was collected in lithium heparin tubes containing protease inhibitors aprotinin (Trasylol, Nordic Pharma Ltd., UK) and AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride) (A8456; Sigma-Aldrich, Dorset, UK), as previously described (Mani et al. 2019; Bhargava et al. 2023). Plasma samples were stored at -80°C for future measurement of gut hormones.

At baseline and during the LEAP2 infusion at each study infusion visit, full blood count, renal function and electrolytes, HbA1c, bone profile and liver function tests will be measured to confirm the safety profile.Iron profile will also be measured at the last study visit to ensure that participants are not iron deficient requiring iron supplements.

Plasma LEAP2 concentrations were measured using a commercial enzyme immunoassay (EK-075-40, Phoenix Pharmaceuticals Inc., CA, USA) according to the manufacturer's instructions. This LEAP2 assay has been previously validated using human samples (Mani et al. 2019; Bhargava et al. 2023). Plasma samples were diluted 10 times in the assay buffer. The inter-assay (5.4%) and intra-assay (5.2%) coefficients of variation were both within the manufacturer guidance of <15% and <10%.

4.5.5. Visual Analogue Scale ratings

Serial Visual Analogue Scale (VAS) ratings (0-100mm) were used to measure hunger, pleasantness to eat, fullness, perceived volume able to eat, food craving, alcohol urge (using the Alcohol Urge Questionnaire assessing thoughts and feelings about drinking, higher scores reflect greater craving (Bohn et al. 1995)), as well as mood and adverse

symptoms, namely nausea, anxiety, thirst, discomfort, sleepiness and stress at each visit using a tablet computer (Goldstone et al. 2014; Scholtz et al. 2014).

Composite appetite was calculated as appetite = ((hunger + pleasantness + volume + (100 - fullness) / 4) for data reduction and to minimise issues with multiple comparisons. Within-participant changes of appetite, fullness, food craving, alcohol urge and adverse symptoms were calculated using data from -10, +30, +55, +85, +140, +195, +220, +255, +285, +310, +330, +360, and +390 min at each visit to give iAUC using the trapezoid rule. The slider for each question was from ‘not at all’ to ‘extremely’ at each timepoint.

Questions included were: “how hungry do you feel right now?” (Hunger); “how pleasant would it be to eat right now?” (Pleasantness); “how much do you think you could eat right now?” (Volume able to eat); “how full do you feel right now?” (Fullness); “all I want right now is something to eat” and “nothing would be better than eating something right now” (Average score for food craving).

Questions for adverse events were: “how sick do you feel right now?” (Nausea); “how anxious do you feel right now?” (Anxiety); “how thirsty do you feel right now?” (Thirst); “how much discomfort do you feel right now?” (Discomfort); “how stressed do you feel right now?” (Stress); “how sleepy do you feel right now?” (Sleepiness).

Alcohol Urge Questionnaire was based on a composite score of the following statements from a 7 item Likert scale ranging from ‘strongly agree’ to ‘strongly disagree’ at each timepoint: 1. All I want to do now is have a drink.; 2. I do not need to have a drink now.; 3. It would be difficult to turn down a drink this minute.; 4. Having a drink now would make things seem just perfect.; 5. I want a drink so bad I can almost taste it.; 6. Nothing would be better than having a drink right now.; 7. If I had the chance to have a drink, I don’t think I would drink it.; 8. I crave a drink right now.

4.5.6. Virtual Portion size Creation Task

At t=+260min, participants performed a 5-minute VPCT to measure desired food intake for 16 dishes (savoury/sweet, low/high fat) modified from a previous study

(Hamm et al. 2021; Hamm et al. 2020) (Figures 4.2, 4.3). Food pictures selected for this VPCT represent savoury or sweet foods with high-fat (energy from fat >33%, >4.0kcal/g) or low-fat (energy from fat <33%, <3.5kcal/g (Figure 4.4.). Desired food intake (absolute/relative) was averaged across the dishes for all foods and by food category low-/high-fat, sweet/savoury.

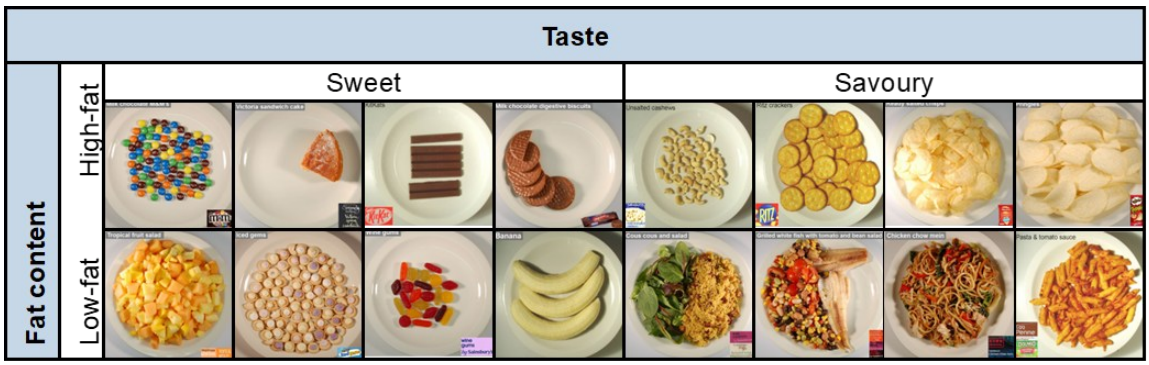


Figure 4.2. Pictures in virtual portion size creation task.
Pictures of food portions shown in the virtual portion creation task are arranged by taste (sweet or savoury) in columns, and fat content (high or low) in rows.



Figure 4.3. Screenshot showing one sample question in the VPCT.
This figure is a screenshot of one question in the VPCT. There is a slider below each image of the 16 dishes that allows participants to select a preferred portion size by increasing or decreasing the amount of food shown on the screen from 0 to 800 kcal, in response to the question “Imagine this is the only food available. How much would you eat right now”.

Low-fat Savoury Foods



ED: 0.8 kcal/g
Carbohydrate:
4.7% (weight), 24.6% (energy)
Of which sugars:
4.1% (weight), 21.7% (energy)
Fat:
0.9% (weight), 11.2% (energy)
Protein:
10.8% (weight), 57.1% (energy)



ED: 0.8 kcal/g
Carbohydrate:
8.9% (weight), 44.0% (energy)
Of which sugars:
3.3% (weight), 16.3% (energy)
Fat:
2.1% (weight), 23.3% (energy)
Protein:
6.6% (weight), 32.6% (energy)



ED: 1.3 kcal/g
Carbohydrate:
18.1% (weight), 56.2% (energy)
Of which sugars:
4.5% (weight), 13.8% (energy)
Fat:
4.4% (weight), 30.5% (energy)
Protein:
4.5% (weight), 13.9% (energy)



ED: 1.8 kcal/g
Carbohydrate:
30.6% (weight), 68.1% (energy)
Of which sugars:
8.9% (weight), 19.7% (energy)
Fat:
4.2% (weight), 20.9% (energy)
Protein:
4.1% (weight), 3.9% (energy)

Low-fat Sweet Foods



ED: 0.5 kcal/g
Carbohydrate:
11.3% (weight), 86.9% (energy)
Of which sugars:
9.6% (weight), 73.9% (energy)
Fat:
0.2% (weight), 3.5% (energy)
Protein:
0.7% (weight), 5.4% (energy)



ED: 1.0 kcal/g
Carbohydrate:
23.3% (weight), 98.1% (energy)
Of which sugars:
12.0% (weight), 50.5% (energy)
Fat:
0.3% (weight), 2.8% (energy)
Protein:
1.2% (weight), 5.0% (energy)



ED: 3.5 kcal/g
Carbohydrate:
79.0% (weight), 91.1% (energy)
Of which sugars:
55.0% (weight), 63.4% (energy)
Fat:
0.9% (weight), 2.3% (energy)
Protein:
5.8% (weight), 6.7% (energy)



ED: 4.0 kcal/g
Carbohydrate:
86.3% (weight), 87.0% (energy)
Of which sugars:
51.4% (weight), 51.8% (energy)
Fat:
3.1% (weight), 7.0% (energy)
Protein:
5.0% (weight), 5.0% (energy)

High-fat Savoury Foods



ED: 5.0 kcal/g
Carbohydrate:
63.5% (weight), 50.8% (energy)
Of which sugars:
8.8% (weight), 7.0% (energy)
Fat:
23.0% (weight), 41.4% (energy)
Protein:
7.7% (weight), 6.2% (energy)



ED: 5.2 kcal/g
Carbohydrate:
51.0% (weight), 39.1% (energy)
Of which sugars:
1.4% (weight), 1.1% (energy)
Fat:
34.0% (weight), 58.6% (energy)
Protein:
3.8% (weight), 2.9% (energy)



ED: 5.3 kcal/g
Carbohydrate:
51.5% (weight), 39.2% (energy)
Of which sugars:
0.4% (weight), 0.3% (energy)
Fat:
31.9% (weight), 54.6% (energy)
Protein:
6.1% (weight), 4.6% (energy)



ED: 6.0 kcal/g
Carbohydrate:
18.7% (weight), 12.6% (energy)
Of which sugars:
4.7% (weight), 3.2% (energy)
Fat:
49.7% (weight), 75.2% (energy)
Protein:
18.3% (weight), 12.3% (energy)

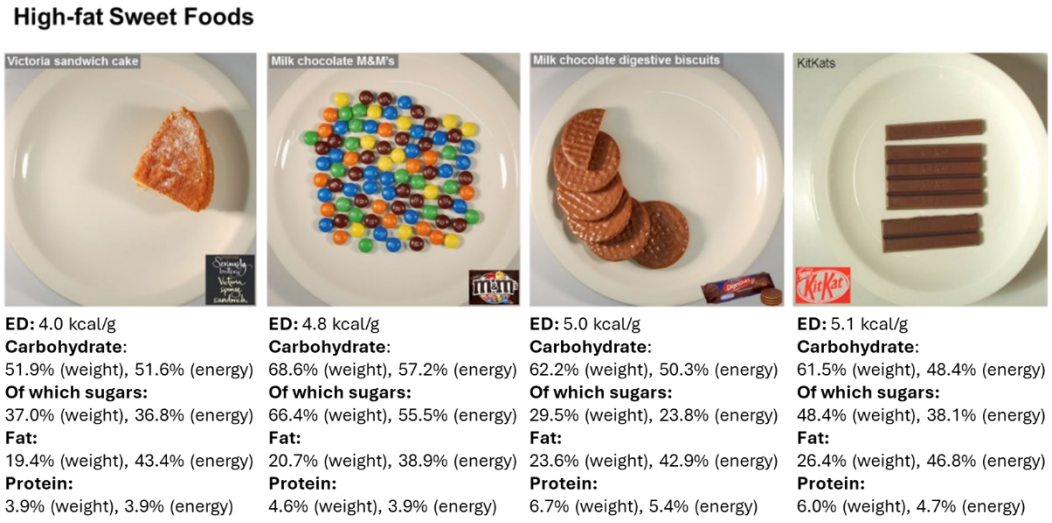


Figure 4.4. Nutritional information of food in the virtual portion size creation task.
Abbreviations: ED, energy density

4.5.7. Ad libitum Test Meal

A buffet with low-fat (LF) and high-fat (HF), savoury and sweet foods (Table 4.1) and water was provided to participants for energy intake measurement at t=+315min. During the screening visit, participants gave Likert scale ratings of liking of chicken broth and soup (from 'dislike extremely' to 'like extremely'), which were the default savoury foods in the test meal. If they disliked chicken broth and soup as determined by ratings lower than 'like slightly', tomato broth and soup were given instead to ensure their acceptability of the foods in the meal. Participants were blinded to the energy content of the test meal. All foods were individually presented in transparent glass bowls.

Food and water intake was the respective weighed difference before and after ingestion to calculate the calories consumed as energy intake. Total calorie intake in kcal at the ad libitum meal and consumed at the pre-load was presented as a percentage of estimated 24-hour resting energy expenditure (REE). REE for each participant was calculated by the Cunningham equation: Basal Metabolic Rate = 500 + 22*Lean Body Mass (LBM) (Cunningham 1980). LBM was equated to fat-free mass (FFM) generated from anthropometric data using bioelectrical impedance analysis (MC-780MA, TANITA, Tokyo, Japan) collected at each visit.

Category	Savoury				Sweet	
	Low fat		High fat		Low fat	High fat
Composition	Chicken Broth	Tomato Broth	Cream of Chicken Soup	Cream of Tomato Soup	Vanilla Yoghurt	Ice Cream
Energy Density (kcal/100g)	36.45	38.22	103.12	111.70	81	251
Fat (g/100g)	0.62	0.80	8.05	8.22	0.00	17.0
of which saturated (g/100g)	0.18	0.18	4.58	4.59	0.00	10.4
Carbohydrates (g/100g)	6.40	6.22	5.46	8.21	14.1	20.0
of which sugars (g/100g)	1.87	2.67	1.29	5.36	14.0	18.8
Fibre (g/100g)	0.62	0.44	0.09	0.18	0	0
Protein (g/100g)	1.42	1.42	2.07	1.06	5.8	4.3
Salt (g/100g)	0.58	0.77	0.63	0.6	0.17	0.18
Amount served (g)	900	900	900	900	900	920
Amount served (kcal)	328	344	928	1005	729	2309

Table 4.1. Nutritional composition of foods items in the *ad libitum* meal.
All food items were provided in excess: (i) chicken broth and (ii) cream of chicken soup with double cream (Baxters, Fochabers, Scotland, UK), (iii) 0% fat vanilla yogurt (Yeo Valley, Belfast, UK), (iv) vanilla ice cream (Haagen Dazs, Uxbridge, UK). Tomato broth and cream of tomato soup (Baxters, Fochabers, Scotland, UK) with double cream (Waitrose, UK) was available as alternatives if participants stated that they did not like chicken soup at the screening visit (not needed). Soups were heated and served at a temperature ≥ 70-80°C.

4.5.8. Bio-electrical Impedance Analysis

Bio-electrical impedance analysis (BIA) (MC-780MA, TANITA, Tokyo, Japan) was used for estimating body composition, in particular body fat, and was performed at screening and all study visits. This method measures body composition by sending a low, safe electrical current through the body. The current passes freely through the fluids contained in muscle tissue, but encounters difficulty/resistance when it passes through fat tissue. This resistance of the fat tissue to the current is termed 'bioelectrical impedance', which can be accurately measured by body fat scales. When set against a person's height, gender and weight, the scales can then compute their body fat percentage. Participants stood on a metal plate and held the handles for up to a minute.

4.5.9. Eating Behaviour Tasks

At the screening visit (for initial practice) and infusion study visits, participants completed computer-based eating behavioural tasks (total duration tasks 60 min):

(i) Leeds Food Preference Questionnaire: to measure explicit liking and wanting, and implicit wanting of savoury/sweet and low/high fat foods using combinations of different types of low fat (LF)/high fat (HF) and savoury/sweet foods (Finlayson et al. 2007; Finlayson et al. 2011; Blundell et al. 2017).

On a computer, participants see individual food photographs, for explicit rating of ‘liking’ and ‘wanting’ at that moment, or pairs of photos and are asked to choose which they would prefer to eat right now for assessment of implicit wanting (determined from reaction time during forced choice adjusted for pooled standard deviation and choice frequency) (Griffioen-Roose et al. 2010; Miguet et al. 2018; Carvalho-Ferreira et al. 2019).

(ii) Delay discounting task: to measure temporal impulsivity to food or money via discounting rate (Perry et al. 2008; Weller et al. 2008; Davis et al. 2010), as AG increases temporal impulsivity to food in rats (Anderberg et al. 2016). No rewards will actually be given.

4.5.10. Computer-based Cognitive Tasks

At the screening visit (for initial practice) and infusion study visits, participants completed the following cognitive tasks on a computer tablet using Cambridge Neuropsychological Test Automated Battery (CANTAB) software (www.cambridgecognition.com/cantab/cognitive-tests/) (see Appendix 4):

(i) stop signal task (SST) to measure motor response inhibition,
(ii) paired associate learning (PAL) task to measure episodic memory,
(iii) spatial working memory (SWM) task to measure working memory.

4.5.11. Questionnaires

Participants answered questionnaires to ensure they meet our eligibility criteria. These questionnaires were performed at the screening visit. The list of questionnaires includes:

(i) Wechsler Test of Adult Reading (WTAR, max score 50) - to measure intellectual functioning and memory abilities. A higher score suggests a higher intelligence quotient and better memory performance (Wechsler 2001),
(ii) Beck Depression Inventory II (BDI-II, max score 63) – to measure levels of depressed mood, exclude if score >28/63 (Beck et al. 1996),
(iii) Alcohol Use Disorders Identification Test (AUDIT, max score 40) – to assess alcohol use disorder (Saunders et al. 1993),
(iv) DSM-V criteria for alcohol use disorder (DSM-V AUD, max 11) – to assess alcohol use disorder, exclude if score >2/11 (American-Psychiatric-Association 2013),
(v) SCOFF questionnaire (max score 5) - to exclude eating disorders, excluded if score >1/5 (Luck et al. 2002),
(vi) Dutch Eating Behaviour Questionnaire (DEBQ, max score 15) - to assess emotional, external and restrained eating (Van Strien et al. 1984),
(i) Three-factor Eating Questionnaire (TEFQ, max score 51) - to evaluate dietary restraint, uncontrolled and emotional eating (Stunkard et al. 1985),
(ii) Eating Disorders Examination Questionnaire (EDE-Q, max score 6) - to evaluate dietary restraint and binge eating (Fairburn et al. 1994),
(iii) Yale Food Addiction Scale (YFAS, max score 7) - to measure features of addiction towards particular foods (Gearhardt et al. 2009),
(iv) Power of Food Scale (PFS, max score 105) - to assess the psychological

influence of the food environment, i.e. the appetite for, rather than consumption of, food (Lowe et al. 2009),

(v) Binge Eating Scale (BES, max score 46) - to assess the presence of binge eating behaviour (Gormally et al. 1982),

(vi) Disgust Scale revised (DS-R, max score 100) - to assess disgust sensitivity that may alter responsivity to unpleasant images (Haidt et al. 1994),

(vii) Urgency, Premeditation, Perseverance and Sensation Seeking Questionnaire (UPPS-P) - to assess impulsivity (max score 104), risk taking (max score 44) and sensation (max score 48) seeking seen in addiction (Cyders et al. 2009),

(viii) Barratt Impulsiveness Scale Questionnaire (BIS-11, max score 120) - to assess the personality/behavioural construct of impulsiveness (Patton et al. 1995),

(ix) Behavioural Activation / Inhibition Scale (BAS/BIS, max score 81) - to assess dispositional sensitivity to the behavioural inhibition system.

At each study visit, participants completed the following questionnaires to ensure consistency of mood between visits:

(x) Positive and Negative Affect Schedule (PANAS, mas score 50 for positive and negative affect each) - to measure tendency to experience positive and negative affect (Watson et al. 1988),

(xi) Spielberger State Anxiety Inventory (SSAI, max score 80) - to measure state anxiety (Spielberger et al. 1983).

4.5.12. Safety Measures

4.5.12.1. Acceptable safety profile for LEAP2 infusion

An *unacceptable* safety profile is defined as any of the following in at least 2 participants, and felt to be related to hormone administration:

(i) serious adverse event,

(ii) skin rash or anaphylaxis,

(iii) significant abnormalities in vital signs, arrythmia or ECG, defined as:

a. heart rate from continuous ECG <= 35 bpm or >= 120 bpm on 3 separate occasions, 5 min apart;

b. heart rate changes by more than 60 bpm from pre-dose baseline on 3 occasions, 5 min apart;

c. systolic blood pressure changes by >30 mmHg and/or diastolic blood pressure

changes by >20 mmHg from pre-dose baseline on three occasions, 5 mins apart;

d. sustained (>= 5 beats for >=30 sec) or non-sustained (>= 5 beats for <30 sec) ventricular arrhythmias;
e. sustained (>= 5 beats for >= 30 sec) supraventricular arrhythmias including atrial fibrillation and flutter; complete bundle branch block;
f. pauses >3 sec;
g. based on average QTc from 3 ECGs: QTcB or QTcF >500 msec (if participant has bundle branch block then criteria is QTcB or QTcF >530 msec), QTc interval change from baseline >60 msec,

(iv) hypoglycaemia (fasting plasma glucose <3.2 mmol/L, <4.0 mmol/L with symptoms), or hyperglycaemia (fasting glucose >7.0 mmol/L),

(v) alanine aminotransferase >= 3x upper limit of normal (ULN) and bilirubin >= 1.5x ULN,

(vi) changes in serum electrolytes outside of the normal range during the infusion that are felt by Investigators to be clinically significant.

(vii) vomiting,

(viii) severe nausea (>20mm assessed using 0-100mm visual analogue scale rating), or

(ix) other symptoms felt by the investigators to be of sufficient severity to necessitate stopping the infusion or interfere with assessment of appetite-related outcomes, such as headache, abdominal pain

4.5.12.2. Safety monitoring

All participants had regular monitoring for symptoms, pulse, blood pressure, cardiac rhythm and blood glucose during LEAP2 peptide infusions. There is a risk of minor discomfort at venous cannulation sites, and a rare risk of infection from cannulae insertion.

Participants were monitored throughout the LEAP2 peptide infusion and until 60 minutes after the cessation of the infusion. During the visit, at least 1 experienced physician was present at all times to monitor the participant with regular observations and participants were encouraged to report any unusual or unpleasant sensation to the investigator immediately.

Hypoglycaemia was measured by serial venous blood sampling. In the event of significant hypoglycaemia, nausea or vomiting, we would stop the infusion temporarily and restart at a lower maintenance rate .

With serial blood sampling, there is a theoretical risk of hypotension on standing, especially as participants are lying down for 4-8 hours. Water was available throughout the study visit and participants were watched carefully for 5 minutes at the end of the study visit, and blood pressure checked if there are any symptoms e.g. light headedness, dizziness, feeling faint.

Any serious adverse reaction would lead to withdrawal of the individual from the study. Throughout the study, there was at least 1 physician available on 24-hour call via a direct line, and a secondary direct line to the senior physicians. Although we do not anticipate any serious adverse reactions, participants were provided with contact numbers and clear instructions that, if they feel unwell, they should call us.

In the event of any participants experiencing an adverse reaction during study visits, a clinically trained member of the research team would conduct a full clinical assessment. The clinically trained member of the research team would arrange extended stay, including overnight if required, or admission for acute medical management via A&E at St. Mary's or Charing Cross Hospital.

It was made clear to volunteers that they will be free to withdraw from the study at any time without providing any reason. Given the short half-life of the infused peptides, adverse events were only be recorded for up to 2 days after the last study infusion visit.

4.5.12.3. Adverse event definitions

Adverse Event (AE): any untoward medical occurrence in a patient or clinical study participant.

Serious Adverse Event (SAE): any untoward and unexpected medical occurrence or effect that:

- Results in death
- Is life-threatening – refers to an event in which the participant was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe
- Requires hospitalisation, or prolongation of existing inpatients' hospitalisation
- Results in persistent or significant disability or incapacity
- Is a congenital anomaly or birth defect

4.5.12.4. Protocol for dealing with nausea

If iPad VAS rating of nausea at any time point was >=20 mm (max 100) the following protocol was followed:

(i) Do a repeat paper VAS rating for nausea after 5 minutes, and if remains high >=20 reduce the infusion rate (stop for 30 minutes and continue at half original maintenance infusion rate).

(ii) Repeat paper VAS nausea before restarting infusion at lower infusion rate. If VAS still >=20 mm then wait further 15 min and try again.

(iii) If VAS still >=20 mm then wait further 15 min and reduce infusion rate to 1/3 of original maintenance infusion rate.

(iv) Once infusion has been restarted, repeat VAS every 15 minutes for next 30 minutes.

(v) If vomiting occurs at any stage, reduce the infusion rate (stop for 30 minutes and continue at half original maintenance infusion rate. However, if VAS nausea is >= 20 mm, then follow procedure in (ii) above.

4.5.12.5. Protocol for dealing with hypoglycaemia

(i) If capillary glucose < 3.2 mmol/L, or symptomatic with capillary glucose < 4.0 mmol/L, repeat glucose after 5 minutes, and if remains as above, reduce the infusion rate by stopping for 30 minutes, and continue at half original maintenance infusion rate

(ii) If capillary glucose < 3.0 mmol/L, stop the infusion immediately for 30 mins, and continue at half original maintenance infusion rate

(iii) Repeat capillary glucose at 15 mins, and again at 30 mins, before restarting infusion at lower infusion rate.

(iv) If still capillary glucose < 3.2 mmol/L, or symptomatic with capillary glucose < 4.0

mmol/L, then wait further 15 min (i.e. do not start at lower rate) and try again.

(v) If still capillary glucose < 3.2 mmol/L, or symptomatic with capillary glucose < 4.0 mmol/L, then wait further 15 min, and reduce infusion rate to 1/3 of original maintenance infusion rate if sugar back up.

(vi) Once infusion has been restarted, repeat capillary glucose every 15 minutes for next 30 minutes.

(vii) If capillary glucose <3.0 mmol/L at any stage, give 150-200mL orange juice (or other beverage with equivalent sugar content)

4.5.12.6. Protocol for dealing with cardiac monitoring abnormalities

Blood pressure and heart rate recordings were taken every 15-30 minutes throughout the infusion, and participants had continuous cardiac monitoring throughout the infusion and for 60 minutes after stopping the infusion. A 12-lead ECG was also be performed twice during the infusion.

If there are any clinically concerning cardiac parameters (described below) the infusion would be stopped.

(i) sustained atrial fibrillation/flutter or supraventricular tachycardia ≥5 beats for ≥30 secs, or

(ii) ventricular tachycardia for ≥5 beats for ≥30 secs, or

(iii) heart rate >140 or <35 beats per min or change from baseline of more than 60 beats per min, or

(iv) systolic blood pressure >170 or <85 mm Hg or change of more than 30 mm Hg from baseline, or diastolic blood pressure >110 or <40 mm Hg or change of more than 20 mm Hg from baseline.

4.5.13. Statistical Analysis

Demographic data were checked for normality using the Shapiro-Wilk test. For normally distributed data, mean ± standard deviation (SD) was reported, and otherwise, median and interquartile range (IQR).

The area under curve between -10 and 310 min ($AUC_{-10\ to\ 310min}$) of VAS ratings and metabolites (with more>2 measurements) were calculated using the trapezoid rule by summating measures for each participant visit, which further derived the incremental

AUC ($iAUC_{-10\ to\ 310min}$) of these variables by subtracting baseline measure taken before the start of the infusion i.e. t=-10min over the whole visit. They were further subdivided as $iAUC_{-10\ to\ 195\ mins}$ and $iAUC_{195\ to\ 310\ min}$ for fasting and post-prandial periods. For metabolites measured twice during a study visit, change in concentrations from t=310 to t=-10min was used.

Linear mixed effects model analysis was performed to examine the effects of increasing LEAP2 dose (12,24,25,36 pmol/kg/min) (independent factor) or as administration of LEAP2 (all doses vs. saline) on VAS ratings, desired and actual energy intake, plasma glucose and LEAP2, serum insulin, triglycerides, growth hormone, cortisol, thyroid stimulating hormone, free thyroxine, LH, FSH, testosterone, prolactin, venous beta-hydroxybutyrate (venous ketones), biochemical metabolites, outcomes from delay discounting and cognitive memory tasks to account for within participant nature of the study and missing data. The dependent variables were VAS ratings, plasma glucose and LEAP2, serum insulin, triglycerides, growth hormone, cortisol, thyroid stimulating hormone, free thyroxine, venous beta-hydroxybutyrate and LH, FSH, testosterone, prolactin (in men only as study not controlled for menstrual cycle), (both as $AUC_{-10\ to\ 310min}$ and $iAUC_{-10\ to\ 310min}$), and VPCT and *ad libitum* energy intake (both as absolute kcal and kcal as % of estimated REE), overall alcohol, food and monetary reward in the delay discounting task and memory outcomes in the cognitive tasks. Analysis included participant ID as between-participant factor and visits as within-participant factor. To evaluate the effectiveness of LEAP2 administration paired t-tests were conducted to compare the above variables before and after LEAP2 administration (24-25 pmol/kg/min) vs. saline.

For each analysis, marginal value of pseudo-R squared measures was taken as an estimate of the variance of the dependent variable explained by the covariate. Coefficients of determination F statistics and P value were also reported from Type III Tests of Fixed Effects, while mean, SE and 95% CI of β parameter estimate from Estimates of Fixed Effects was reported to examine the relationship between the dependent variable and the covariate (i.e. slope of correlation). For paired t-test results mean difference, standard deviation, t statistic with Cohen's d was reported.

The focus of this human infusion study is to evaluate the safety and tolerability of LEAP2 and to estimate its pharmacokinetic parameters. No formal statistical hypotheses will be tested. No formal power or sample size calculations have been performed. The sample size is based on the requirement of sentinel dosing.

All statistical analysis was performed using IBM SPSS version 29 (Armonk, NY, USA) and GraphPad Prism v10.3.1 (San Diego, CA, USA) with effects considered significant at $P<0.05$, with moderate effect sizes at pseudo-R square values >0.25 and Cohen's d >0.50.

4.5.14. Ethical Approval

Ethical approval for the study was obtained from West London & GTAC Research Ethics Committee (23/LO/0510) and Health Research Authority (HRA) on 30 June 2023. This study was conducted in accordance with the principles of the Declaration of Helsinki, and all participants provided written informed consent. An animal project licence was in place for LEAP2 peptide testing (PB7CFFE7A).

4.6. RESULTS

4.6.1. Participant characteristics

A total of 7 participants without obesity were recruited and analysed with majority of participants being male, of Caucasian ethnicity and of normal weight (BMI 18.0-24.9 kg/m^2) with just over a quarter overweight (BMI 25.0-29.9 kg/m^2). All participants had normal insulin sensitivity (i.e. HOMA-IR<2) and no excessive dietary restraint (i.e. DEBQ-restraint <3/5) at screening visit (Tables 4.2, 4.3, 4.4).

The analysis does not include one male (58 years old, BMI 24.2 kg/m^2) and one female participant (39 years old, BMI 26.1 kg/m^2) both of whom only attended one infusion visit that were otherwise well tolerated (one LEAP2 at 36pmol/kg/min dose and one saline respectively), and hence dropped out of the study because of limited of availability for full-day infusion visits. The analysis does not include oestradiol, progesterone and prolactin concentrations in women as this study was not controlled for menstrual cycle. Average day of menstrual cycle on day of infusion visit was 16.2 ± 6.0 days with the range of oestradiol between 100-1136 pmol/L and progesterone between 2 - 47 nmol/L.

One male participant (51 years old, BMI 25.3 kg/m^2) is also excluded from the analysis as they had a serious adverse reaction at a LEAP2 infusion visit (36 pmol/kg/min) (see section 4.6.3.2. for details).

Participant demographics	
N	7
Gender, n (%)	
Male	4 (57.14%)
Female	3 (42.86%)
Ethnicity, n (%)	
Caucasian	5 (71.42%)
Other	2 (28.58%)
Age (years) [a]	
Mean ± SD	36.43 ± 13.09
Range	22 - 55
Height (m) [a]	
Mean ± SD	1.70 ± 0.08
Range	1.56 - 1.88
Weight (kg) [a]	
Mean ± SD	69.38 ± 10.87
Range	49.80 - 90.65
BMI (kg/m^2) [a]	
Median [IQR]	23.56 [23.20, 24.65]
Range	18.7 - 27.1
Overweight status, n (%)	
BMI ≥18.0, <24.9 kg/m^2	5 (71.42%)
BMI ≥25.0 kg/m^2	2 (28.58%)
Body fat (%) [a]	
Mean ± SD	21.63 ± 3.55
Range	15.0 - 28.9
HOMA-IR [a]	
Mean ± SD	0.67 ± 0.31
Range	0.32 - 1.66

Table 4.2. Participant characteristics.
Categorical variables were presented as numbers and percentage of the population. Normally distributed data were shown as mean ± SD, otherwise median [IQR], along with the range (minimum to maximum). Abbreviations: BMI, Body mass index; HOMA-IR, homeostatic model assessment for insulin resistance. [a] averaged across all study visits (age, height, weight, BMI, body fat %, HOMA-IR).

Dietary restraint questionnaires	
DEBQ: Restraint, max score 5	
Mean ± SD	1.59 ± 0.49
Range	1.0 - 2.4
EDE-Q: Restraint, max score 6	
Mean ± SD	0.17 ± 0.29
Range	0.0 - 0.6
TFEQ: Restraint, max score 21	
Mean ± SD	5.00 ± 1.63
Range	3 - 8
Dutch Eating Behaviour Questionnaire (DEBQ)	
Emotional, max score 5	
Median [IQR]	1.08 [1.0, 1.52]
Range	0.92 - 1.92
External, max score 5	
Mean ± SD	2.16 ± 0.83
Range	1.0 - 3.5
Three Factor Eating Questionnaires (TFEQ)	
Disinhibition, max score 16	
Mean ± SD	2.86 ± 1.77
Range	1 - 6
Hunger, max score 14	
Mean ± SD	2.14 ± 2.41
Range	0 - 7
Yale Food Addiction Score (YFAS), max score 7	
Mean ± SD	0.86 ± 0.69
Range	0 - 2
Power of Food Scale (PFS), max score 105	
Mean ± SD	32.57 ± 10.15
Range	21 - 52
Binge Eating Scale (BES), max score 46	
Mean ± SD	2.57 ± 1.90
Range	0 - 5

Table 4.3. Eating behaviour questionnaires.
Normally distributed data were shown as mean ± SD, otherwise median [IQR], along with the range (minimum to maximum). Demographics on results of dietary restraint questionnaires (i.e. DEBQ, TFEQ and EDEQ) completed at screening visit indicate that participants exhibited normal eating behaviour. Abbreviation: max, maximum.

Urgency, Premeditation, Perseverance and Sensation Seeking Questionnaire (UPPS-P)	
Urgency, max score 104	
Mean ± SD	25.00 ± 4.58
Range	18 - 30
Premeditation, max score 44	
Mean ± SD	21.00 ± 3.21
Range	17 - 25
Perseverance, max score 40	
Mean ± SD	22.86 ± 3.02
Range	18 - 27
Sensation, max score 48	
Mean ± SD	19.57 ± 3.78
Range	15 - 25
Barratt Impulsiveness Scale Questionnaire (BIS-11), max score 120	
Mean ± SD	53.86 ± 8.28
Range	42 - 68
Behavioural Activation / Inhibition Scale (BAS/BIS), max score 81	
Mean ± SD	18.86 ± 3.67
Range	11 - 22
Beck Depression Inventory II (BDI-II), max score 63	
Mean ± SD	2.48 ± 3.10
Range	0 - 8
Positive and Negative Affect Schedule (PANAS) [a]	
Positive affect, max score 50	
Mean ± SD	34.08 ± 8.67
Range	21 - 50
Negative affect, max score 50	
Mean ± SD	12.58 ± 2.52
Range	10 - 21
Spielberger State Anxiety Inventory (SSAI) [a], max score 80	
Mean ± SD	41.25 ± 4.43
Range	34- 49
Alcohol Use Disorders Identification Test (AUDIT), max score 40	
Mean ± SD	3.14 ± 2.41
Range	1 - 7
Weschler Test of Adult Reading (WTAR), max score 50	
Mean ± SD	43.00 ± 7.69
Range	26 - 48

Table 4.4. Impulsivity, mood and other questionnaires.
Normally distributed data were shown as mean ± SD, otherwise median [IQR], along with the range (minimum to maximum). [a] averaged across all study visits (PANAS and SSAI). Abbreviation: max, maximum.

4.6.2. Visit summary

There was a total of 22 infusion visits in 7 participants. The order of the visits was not randomised, with the saline (placebo) infusion being administered on the 1st visit in five participants (3 male, 2 female), and at 2nd visit in one female), while one of the former men had an additional saline visit at their 5th visit.

The LEAP2 infusion doses included:

(i) 12 pmol/kg/min in 5 participants (2 male, 3 female) being administered on the 1st (n=1), 2nd (n=3) and 3rd (n=2) visits, with one man receiving this dose at two separate visits,

(ii) 24-25 pmol/kg/min in 6 participants (4 male, 2 female) being administered on the 1st (n=1), 2nd (n=2), 3rd (n=2), 4th (n=1) visits,

(iii) 36 pmol/kg/min in 2 men being administered on the 4th and 5th visits (n=1 each).

LEAP2 infusion dose was significantly positively correlated with visit number over the whole infusion (r^2=0.27, β= 0.05 visit.min, P=0.011) (Table 4.5), as well as visit number trended to be higher between any visit with LEAP2 (combined 12, 24-25, 36 pmol/kg/min) infusions vs. without (saline) (P=0.10) (Table 4.19), demonstrating that the visit order was not in a randomised order as our primary outcome was safety and majority of the participants received a saline visit as their first visit and sequential increments in LEAP2 dose over the subsequent visits.

Main effect of LEAP2 dose on dependent variable										
Dependent variable	**r2**	**numerator df**	**denominator df**	**F**	**P value**	**β parameter**	**β ± SEM**	**95% CI of β lower limit**	**95% CI of β upper limit**	**β parameter units**
Visit order	**0.27**	1.00	20.00	7.75	**0.011**	0.05	0.02	0.01	0.09	visit.min
Glucose iAUC -10 to 310min	**0.27**	1.00	17.50	9.49	**0.007**	-2.79	0.91	-4.70	-0.88	mmol/L.min
Insulin iAUC -10 to 310min	0.01	1.00	14.83	1.20	0.29	32.61	29.78	-30.92	96.15	mIU/L.min
GH iAUC -10 to 310min	0.00	1.00	17.52	0.02	0.90	2.63	20.13	-39.75	45.00	mIU/L.min
Cortisol iAUC -10 to 310min	0.02	1.00	13.51	0.61	0.45	-407.92	523.54	-1534.63	718.79	ug/L.min
Beta hydroxybutyrate iAUC -10 to 310min	0.17	1.00	20.00	4.20	0.054	-0.80	0.39	-1.61	0.01	mmol/L.min
Triglyceride iAUC -10 to 310min	0.02	1.00	14.95	0.93	0.35	-0.39	0.40	-1.25	0.47	mmol/L.min
LEAP2 iAUC -10 to 310min	**0.90**	1.00	17.21	178.46	**<0.001**	939.24	70.31	791.04	1087.45	ng/mL.min
FASTING										
Glucose iAUC -10 to 195min	0.37	1.00	18.63	13.94	**0.001**	-1.61	0.43	-2.52	-0.71	mmol/L.min
Insulin iAUC -10 to 195min	0.01	1.00	20.00	0.30	0.59	-1.61	2.92	-7.70	4.48	mIU/L.min
GH iAUC -10 to 195min	0.00	1.00	17.63	0.03	0.87	1.93	11.73	-22.75	26.61	mIU/L.min
Cortisol iAUC -10 to 195min	0.01	1.00	12.32	0.21	0.65	-133.32	291.01	-765.58	498.94	ug/L.min
Beta hydroxybutyrate iAUC -10 to 195min	0.08	1.00	16.29	2.14	0.16	-0.37	0.25	-0.91	0.17	mmol/L.min
Triglyceride iAUC -10 to 195min	0.02	1.00	15.32	0.60	0.45	-0.16	0.21	-0.61	0.28	mmol/L.min
LEAP2 iAUC -10 to 195min	**0.93**	1.00	17.32	250.39	**<0.001**	523.45	33.08	453.75	593.14	ng/mL.min
POST-PRANDIAL										
Glucose iAUC 195 to 310min	0.002	1.00	14.85	0.08	0.78	0.08	0.28	-0.52	0.68	mmol/L.min
Insulin iAUC 195 to 310min	0.11	1.00	16.91	2.56	0.13	2.37	1.49	-0.76	5.51	mIU/L.min
GH iAUC 195 to 310min	0.12	1.00	15.78	2.94	0.11	2.60	1.51	-0.62	5.81	mIU/L.min
Cortisol iAUC 195 to 310min	0.04	1.00	19.00	0.91	0.35	-68.58	72.05	-219.38	82.22	ug/L.min
Beta hydroxybutyrate iAUC 195 to 310min	0.09	1.00	16.04	4.90	**0.042**	-0.27	0.12	-0.52	-0.01	mmol/L.min
Triglyceride iAUC 195 to 310min	0.01	1.00	10.16	0.17	0.69	0.04	0.09	-0.16	0.23	mmol/L.min
LEAP2 iAUC 195 to 310min	0.010	1	18.000	0.196	0.66	12.05	27.20	-45.10	69.21	ng/mL.min

Table 4.5. Effect of LEAP2 dose on change in total, fasting and post-prandial markers of glucose and fat metabolism.
This table shows the effects of LEAP2 dose on change in total (iAUC from t=-10 to 310min), fasting (iAUC from t=-10 to 195 min) and post-prandial (iAUC from t=195 to 310min) plasma glucose, serum insulin, GH, cortisol, triglycerides and venous beta hydroxybutyrate using linear mixed model analysis including participant ID as between participant, and LEAP2 dose as within participant fixed factors. Effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=7, visits=22. Abbreviations: iAUC, incremental area under the curve; GH, growth hormone.

4.6.3. Safety Outcomes

4.6.3.1. Blood pressure, heart rate and hypoglycaemia

Increasing intravenous LEAP2 doses did not cause any anaphylactic reaction in any of the participants included in this analysis. LEAP2 infusions did not cause any fluctuations in systolic, diastolic blood pressure, heart rate or hypoglycaemia that were outside the confines of the safety criteria described above (n=7 participants, 22 infusion visits).

4.6.3.2. Serious adverse effect with LEAP2 infusion in one male participant

A 51-year-old man without obesity (ID N216, BMI 25.3 kg/m^2) received LEAP2 peptide intravenous infusion at 72 pmol/kg/min for first 30 minutes, then 36 pmol/kg/min maintenance having fasted overnight (having received saline infusion at his previous 1st study visit). As per protocol, an ECG before starting the infusion and at 0.75 hours into the infusion was normal, and he was on continuous cardiac monitoring. At 2.5 hours after starting the LEAP2 infusion, he developed a symptomatic sinus bradycardia with hypotension at 34 beats per minute (bpm) with blood pressure 53/27 mmHg and then switched to a junctional bradycardia with hypotension 33-40 bpm with blood pressure 68/31 mmHg. He did not lose consciousness. There were no other acute changes on the ECG (acutely or after return to sinus rhythm). The infusion was immediately stopped from the initial development of bradycardia/hypotension. No pharmacological intervention was needed (Figure 4.5A-D).

By 19 mins after stopping the infusion, he had spontaneously returned to sinus rhythm, initially with a sinus bradycardia 48-51 bpm with improved but still low blood pressure 87-97/63 mmHg. His heart rate and blood pressure gradually increased back to baseline while remaining in sinus rhythm, and by 1.5 hours later, his heart rate was 72 bpm and blood pressure 112/64 mmHg. There was no hypoglycaemia (using Dexcom interstitial glucose monitor) nor hypoxia (oxygen saturations > 97% throughout) at any time during the infusion or afterwards. There was no neurological deficit, and he fully recovered. He had no previous cardiac history and baseline ECGs had been normal including PR interval and QTcB intervals at the screening and his previous 1st study visit. His potassium and thyroid function tests at screening, previous study visit, and morning of this study visit were normal. There was no rise in serum troponin.

He was reviewed by the cardiology team at the time of the event, demonstrating normal bedside echocardiogram using V-scan of the heart. He was admitted onto the cardiac ward for overnight cardiac monitoring and had no further cardiac arrhythmia or bradycardia. The participant was taking no medications. He had no significant medical history other than mild hyperlipidaemia with fasting total cholesterol 6.0, LDL 3.9, HDL 0.9 and triglycerides 2.9 mmol/L with total/HDL cholesterol ratio 6.7. He was a non-smoker and had no history of alcohol excess. The cardiology team advised that he start Rosuvastatin 5mg once daily for his hypercholesterolaemia. He was subsequently discharged the following morning by the cardiology team.

This participant was withdrawn from the study in view of this resolved unexpected serious adverse event which is probably related to the LEAP2 peptide infusion.

Subsequent investigations revealed that there were no problems with the LEAP2 peptide infusion given the following:

(i) For this participant, the LEAP2 concentrations in plasma and from infusion syringe and tubing using commercial ELISA was similar to that for previous participants receiving infusion dose 36 pmol/kg/min indicating that there was no error in preparing or aliquoting peptide for infusion, nor excess plasma concentrations of the C-terminal LEAP2 peptide reached, compared to previous infusions at rate 36 pmol/kg/min.

(ii) Tests for presence of bacterial endotoxin were negative for both the LEAP2 peptide infusion from this participant, and for 4 stored aliquot vials of freeze dried LEAP2 peptide that were subsequently used in future infusion preparations (kinetic QCL endotoxin test, Wickham Micro, Gosport, UK).

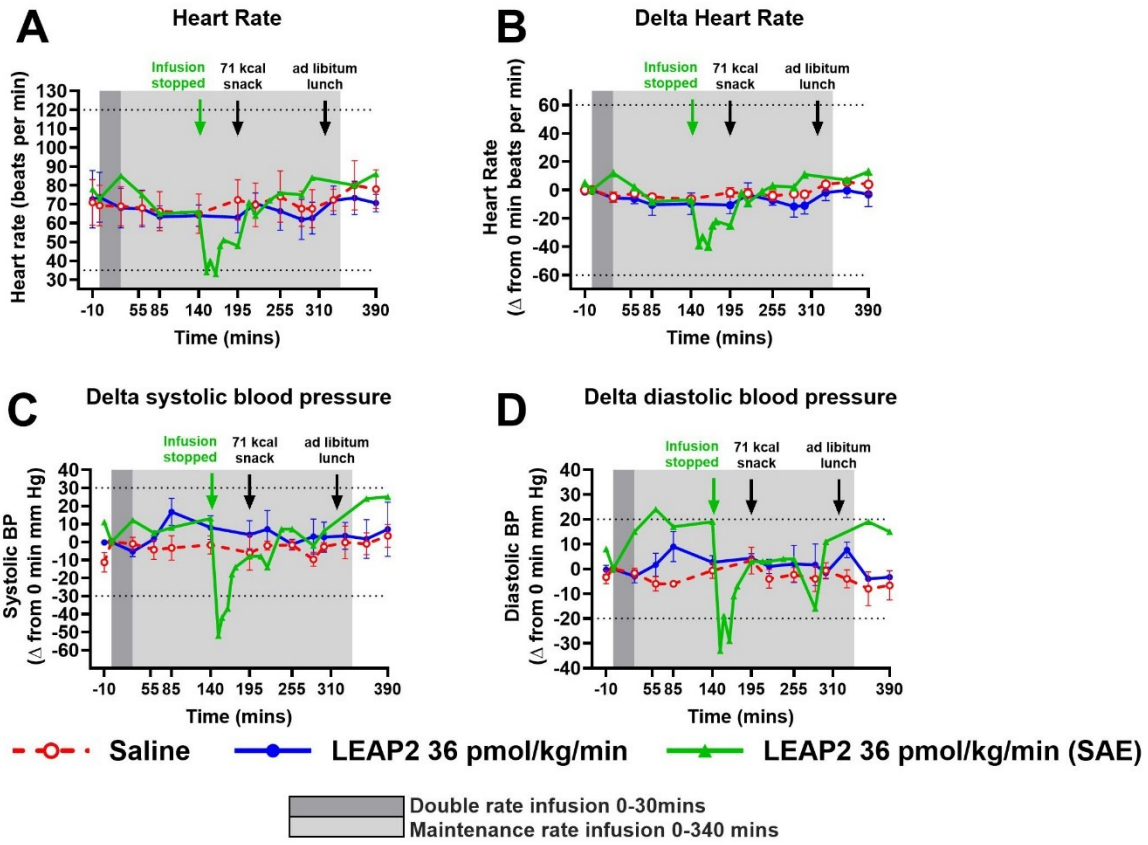


Figure 4.5. Safety parameters during exogenous LEAP2 infusion.
(A) Heart rate, Change in (B) heart rate, (C) systolic blood pressure and (D) diastolic blood pressure over the duration of the study day. Grey bars indicate infusion duration (double rate: 0-30mins and rates in legend: 30-340min). Data presented as mean ± SEM, includes matched participants with saline (LEAP2=0pmol/kg/min) and highest dose LEAP2=36pmol/kg/min from 3 participants, 6 visits. Abbreviations: BP, blood pressure; SAE, serious adverse effect.

4.6.4. Effects of LEAP2

4.6.4.1. Plasma glucose

Baseline plasma glucose (t= -10 min, pre-infusion) did not correlate with subsequent LEAP2 infusion dose (r^2=0.18, P=0.47, n=22 visits)(Table 4.18), and did not differ between visits without (saline) vs. with LEAP2 (combined 12, 24-25, 36 pmol/kg/min) infusions (P=0.61), demonstrating that there was no difference at baseline between visits by chance (Figure 4.6A, Table 4.19).

LEAP2 infusion dose was significantly negatively correlated with plasma glucose: (i) over the whole infusion (iAUC $_{-10 \text{ to } 310 \text{ min}}$, r^2=0.27, β= -2.79 mmol/L.min, P=0.007)

(Figure 4.7A, Table 4.5), (ii) over the fasting period (iAUC $_{-10 \text{ to } 195 \text{ min}}$, r^2=0.37, β=-1.61 mmol/L.min, P<0.001) (Figure 4.7B, Table 4.5), but (iii) not during the post-prandial period (iAUC $_{195 \text{ to } 310 \text{ min}}$, r^2=0.002, β=0.08 mmol/L.min, P=0.78) (Figure 4.7C, Table 4.5).

Plasma glucose over the whole infusion (iAUC $_{-10 \text{ to } 310 \text{ min}}$) was significantly lower between any visit with LEAP2 (12, 24-25, 36 pmol/kg/min) infusions vs. without (saline) (P=0.006) (Table 4.19). When just comparing LEAP2 administration at the final selected dose for future studies (24-25 pmol/kg/min) vs. saline, there was a trend for plasma glucose to decrease over the whole infusion (iAUC $_{-10 \text{ to } 310 \text{ min}}$) (P=0.069, Cohen's d= -0.94, n=6) (Table 4.20).

4.6.4.2. Serum insulin

Baseline serum insulin (t= -10 min. pre-infusion) did not correlate with subsequent LEAP2 infusion dose (r^2=0.01, P=0.56, n=22 visits) (Table 4.17), and did not differ between visits without (saline) vs. with LEAP2 (combined 12, 24-25, 36 pmol/kg/min) infusions (F(1,14.52)=3.04, P=0.11), demonstrating that there was no difference at baseline between visits by chance (Figure 4.6B, Table 4.18).

LEAP2 infusion dose did not correlate with serum insulin (i) over the whole infusion (iAUC $_{-10 \text{ to } 310 \text{ min}}$), (ii) fasting (iAUC $_{-10 \text{ to } 195 \text{ min}}$) or, (iii) post-prandial (iAUC $_{195 \text{ to } 310 \text{ min}}$) period (P=0.13-0.59) (Figure 4.7D-F), Table 4.5).

Serum insulin over the whole infusion (iAUC $_{-10 \text{ to } 310 \text{ min}}$) did not differ between visits without (saline) vs. with LEAP2 (12, 24-25, 36 pmol/kg/min) infusions (P=0.45) (Table 4.19). When just comparing LEAP2 administration at the final selected dose for future studies (24-25 pmol/kg/min) vs. saline, there no difference in serum insulin over the whole infusion (iAUC $_{-10 \text{ to } 310 \text{ min}}$) (P=0.74, Cohen's d= -0.14, n=6) (Table 4.20).

4.6.4.4. Venous beta-hydroxybutyrate and serum triglycerides

Baseline venous BHB and serum triglyceride concentrations (t= -10 min, pre-infusion) did not correlate with subsequent LEAP2 infusion dose (r^2=0.05-0.12, P=0.10-0.28, n=22 visits) (Table 4.17), and did not differ between visits without (saline) vs. with LEAP2 (combined 12, 24-25, 36 pmol/kg/min) infusions (P=0.41-0.75), demonstrating

that there was no difference at baseline between visits by chance (Figure 4.6C, Table 4.18).

LEAP2 infusion dose trended to negatively correlated with venous beta-hydroxybutyrate (i) over the whole infusion (iAUC $_{-10\ to\ 310}$ min, r^2=0.17, β=-0.80 mmol/L.min, P=0.054), (ii) did not correlate over the fasting period (iAUC $_{-10\ to\ 195\ min}$, r^2=0.08, β=-0.37 mmol/L.min, P=0.16) and, (iii) negatively correlated over the post-prandial period (iAUC $_{195\ to\ 310}$ min, r^2=0.09, β=-0.27 mmol/L.min, P=0.042) (Figure 4.7G-I, Table 4.5).

LEAP2 infusion dose did not correlate with serum triglycerides: (i) over the whole infusion (iAUC $_{-10\ to\ 310\ min}$), (ii) fasting (iAUC $_{-10\ to\ 195\ min}$), or (iii) post-prandial (iAUC $_{195\ to\ 310\ min}$) period (P=0.35-0.69) (Table 4.5).

Venous beta-hydroxybutyrate or serum triglycerides over the whole infusion (iAUC $_{-10\ to\ 310\ min}$) did not differ between visits without (saline) vs. with LEAP2 (12, 24-25, 36 pmol/kg/min) infusions (P=0.11-0.59) (Table 4.19). When just comparing LEAP2 administration at the final selected dose for future studies (24-25 pmol/kg/min) vs. saline, there no difference in venous BHB or serum triglycerides over the whole infusion (iAUC $_{-10\ to\ 310\ min}$) (P=0.33-0.40, Cohen's d= -0.37 to -0.44, n=6) (Table 4.20).

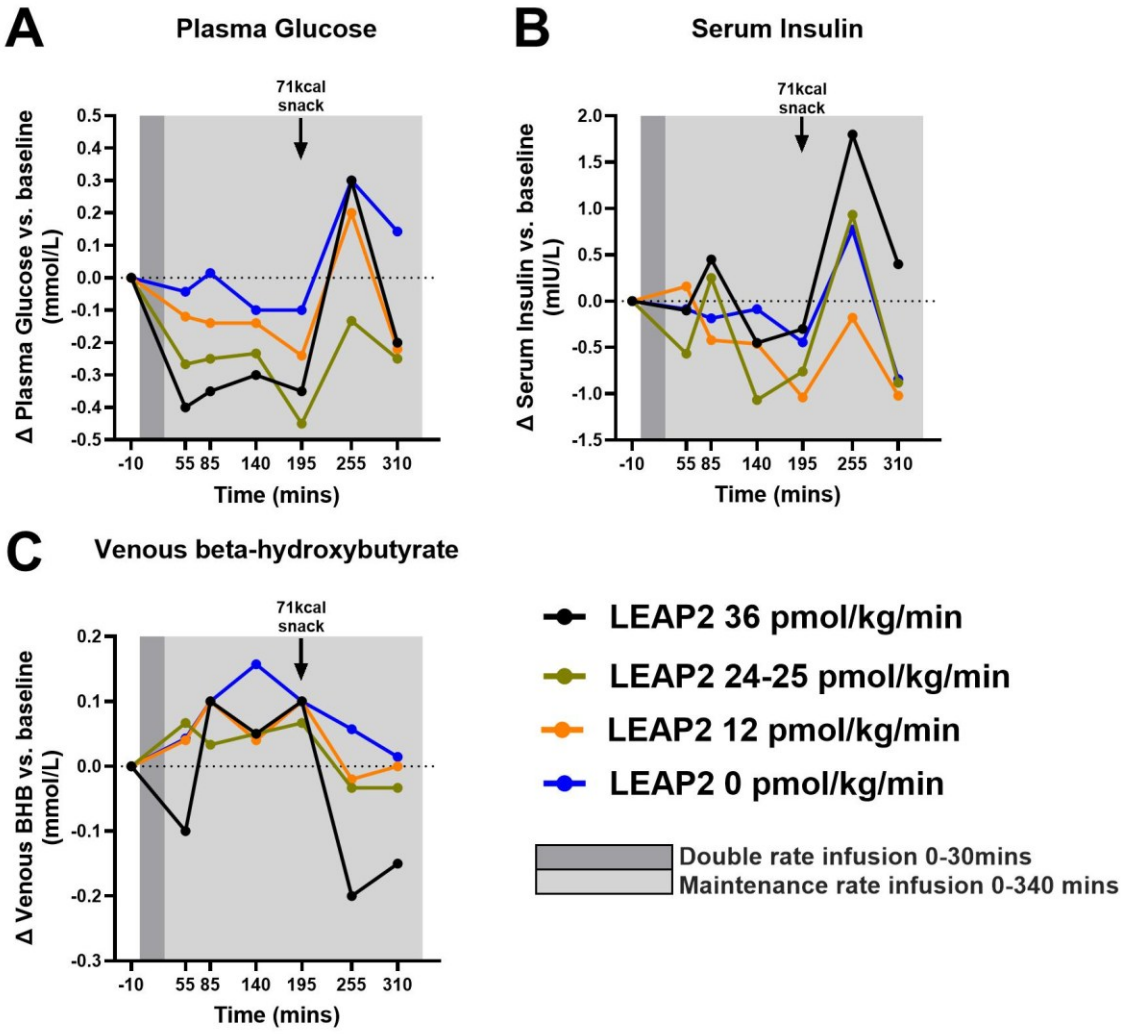


Figure 4.6. Longitudinal representation of change in plasma glucose, serum insulin and venous beta-hydroxybutyrate.

(A) Plasma glucose, (B) serum insulin and (C) venous beta-hydroxybutyrate over the duration of the study day (expressed as mean difference from -10min time point). Grey bars indicate infusion duration (double rate: 0-30mins, see legend for infusion rates: 30-340min). Participants given 71 kcal meal at t=195min. Data from 7 participants, 22 visits. Abbreviations: BHB, beta-hydroxybutyrate.

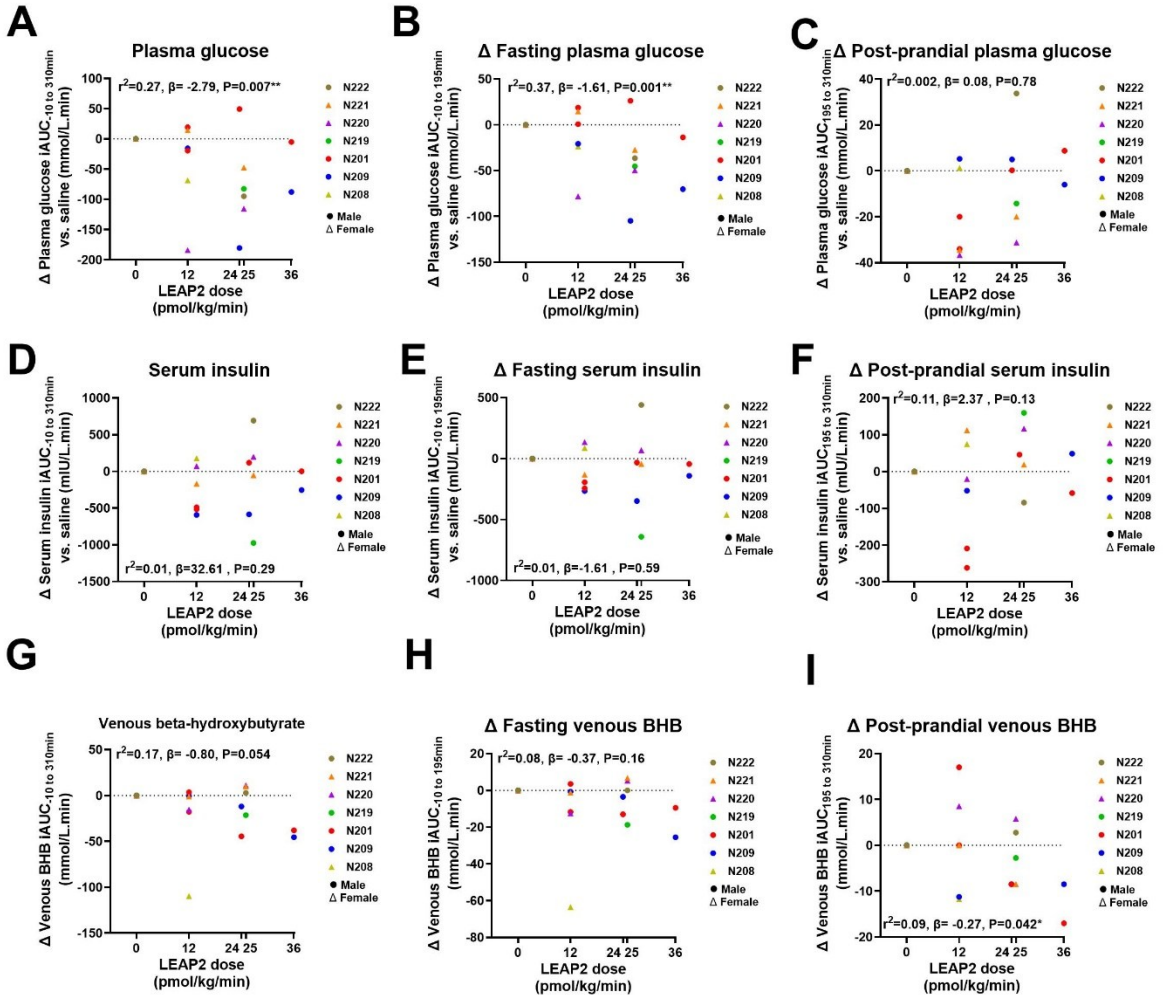


Figure 4.7. Correlation of LEAP2 dose with markers of glucose and fat metabolism. Effects of increasing LEAP2 dose as total $iAUC_{-10\ to\ 310min}$ (A,D,G), fasting $iAUC_{-10\ to\ 195min}$ (B,E,H) and post-prandial $iAUC_{195\ to\ 310min}$ (C,F,I) on plasma glucose (A,B,C), serum insulin (D,E,F) and venous beta-hydroxybutyrate (G,H,I) (expressed as difference from saline visit, LEAP2=0pmol/kg/min) with marginal value of pseudo-R squared (r^2) and estimated mean of β parameter from linear mixed model analysis based on data from 7 participants, 22 visits. Abbreviations: iAUC, incremental area under the curve; BHB, beta hydroxybutyrate. * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001.

4.6.4.2. Serum GH and cortisol

Baseline serum GH and cortisol concentrations (t= -10 min, pre-infusion) did not correlate with subsequent LEAP2 infusion dose (r^2=0.001-0.03, P=0.39-0.90, n=22 visits) (Table 4.17), and did not differ between visits without (saline) vs. with LEAP2 (combined 12, 24-25, 36 pmol/kg/min) infusions (P=0.32-0.59), demonstrating that there was no difference at baseline between visits by chance (Table 4.18).

LEAP2 infusion dose did not correlate with serum GH or cortisol (i) over the whole infusion (iAUC $_{-10 \text{ to } 310 \text{ min}}$), (ii) fasting (iAUC $_{-10 \text{ to } 195 \text{ min}}$) or, (iii) post-prandial (iAUC $_{195 \text{ to } 310 \text{ min}}$) period (P=0.11-0.90) (Table 4.5)

Serum GH and cortisol over the whole infusion (iAUC $_{-10 \text{ to } 310 \text{ min}}$) did not differ between visits without (saline) vs. with LEAP2 (12, 24-25, 36 pmol/kg/min) infusions (P=0.13-0.91) (Table 4.19). When just comparing LEAP2 administration at the final selected dose for future studies (24-25 pmol/kg/min) vs. saline, there was no difference in serum GH or cortisol over the whole infusion (iAUC $_{-10 \text{ to } 310 \text{ min}}$) (P=0.31-0.83, Cohen's d= -0.46 to 0.09, n=6) (Table 4.20).

4.6.4.3. Hormones and biochemical metabolites

Baseline serum TSH, free thyroxine, electrolytes (sodium, potassium, calcium, phosphate), liver biochemistry (ALT, AST, gGT, ALP, Total bilirubin), and in men only prolactin, LH, FSH testosterone (t= -10 min, pre-infusion) did not correlate with subsequent LEAP2 infusion dose (r^2=0.001-0.22, P=0.14-0.99, n=22 visits) (Table 4.17), and did not differ between visits without (saline) vs. with LEAP2 (combined 12, 24-25, 36 pmol/kg/min) infusions (P=0.13-0.91), demonstrating that there was no difference at baseline between visits by chance (Table 4.18).

LEAP2 infusion dose did not correlate with serum TSH, free thyroxine, nor with prolactin, LH, FSH, testosterone (only in men) over the whole infusion from t=-10min to t=310min (P=0.22-0.77) (Table 4.6).

LEAP2 infusion doses did not correlate with changes in serum sodium, potassium, calcium, phosphate AST, ALT, ALP, gGT or total bilirubin over the whole infusion from t=-10min to t=310min (P=0.45-0.72) (Table 4.6).

Serum prolactin, TSH, free thyroxine, LH/FSH/testosterone and renal/bone/liver biochemistry over the whole infusion (from t=10 min to t=310min) did not differ between visits without (saline) vs. with LEAP2 (12, 24-25, 36 pmol/kg/min) infusions (P=0.11-0.99) (Table 4.19). When just comparing LEAP2 administration at the final selected dose for future studies (24-25 pmol/kg/min) vs. saline, there were no

differences in hormones or biochemical metabolites over the whole infusion (iAUC $_{-10 \text{ to } 310 \text{ min}}$) (P=0.32-0.70, Cohen's d= -0.99 to 0.22, n=6) (Table 4.20).

Main effect of LEAP2 dose on dependent variable										
Dependent variable	**r2**	**numerator df**	**denominator df**	**F**	**P value**	**β parameter**	**β ± SEM**	**95% CI of β lower limit**	**95% CI of β upper limit**	**β parameter units**
Sodium Δ -10 to 310min	0.02	1.00	17.20	0.56	0.47	-0.03	0.04	-0.10	0.05	mmol/L.min
Potassium Δ -10 to 310min	0.03	1.00	18.00	0.60	0.45	0.00	0.00	-0.01	0.01	mmol/L.min
Alanine aminotransferase Δ -10 to 310min	0.01	1.00	20.00	0.13	0.72	0.01	0.03	-0.05	0.07	IU/L.min
Aspartate aminotransferase Δ -10 to 310min	0.03	1.00	11.98	0.53	0.48	-0.04	0.06	-0.16	0.08	IU/L.min
Alkaline Phosphatase Δ -10 to 310min	0.01	1.00	17.74	0.31	0.58	-0.05	0.10	-0.26	0.15	IU/L.min
Gamma glutamyl transferase Δ -10 to 310min	0.00	1.00	20.00	0.01	0.93	0.00	0.04	-0.08	0.08	U/L.min
Total bilirubin Δ -10 to 310min	0.02	1.00	13.93	2.85	0.11	-0.03	0.01	-0.06	0.01	umol/L.min
Calcium Δ -10 to 310min	0.02	1.00	20.00	0.36	0.55	0.00	0.00	0.00	0.00	mmol/L.min
Phosphate Δ -10 to 310min	0.00	1.00	14.62	0.35	0.56	0.00	0.00	0.00	0.00	mmol/L.min
TSH Δ -10 to 310min	0.01	1.00	14.45	0.68	0.42	0.00	0.00	-0.01	0.01	mIU/L.min
free thyroxine Δ -10 to 310min	0.03	1.00	20.00	0.63	0.44	0.01	0.02	-0.02	0.05	pmol/L.min
LH Δ -10 to 310min (men)	0.07	1.00	17.58	1.63	0.22	-0.04	0.03	-0.11	0.03	IU/L.min
FSH Δ -10 to 310min (men)	0.00	1.00	18.05	0.09	0.77	0.00	0.02	-0.04	0.03	IU/L.min
Prolactin Δ -10 to 310min (men)	0.04	1.00	12.00	0.52	0.49	-0.61	0.85	-2.46	1.24	mIU/L.min
Testosterone Δ -10 to 310min (men)	0.05	1.00	8.00	0.47	0.51	0.02	0.03	-0.05	0.10	nmol/L.min

Table 4.6. Effect of LEAP2 dose on change in hormones and biochemical metabolites.

This table shows the effects of LEAP2 dose on change in multiple pituitary hormones and biochemical metabolites (change from -10 min to 310 min) using linear mixed model analysis including participant ID as between participant, and LEAP2 dose as within participant fixed factors. Effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=7, visits=22. Abbreviations: IU, International unit; TSH, Thyroid stimulating hormone; LH, Luteinising hormone; FSH, Follicular stimulating hormone.

4.6.4.5. Plasma LEAP2

Baseline plasma LEAP2 (t= -10 min, pre-infusion) did not correlate with subsequent LEAP2 infusion dose (r^2=0.05, P=0.16, n=22 visits), and did not differ between visits without (saline) vs. with LEAP2 (combined 12, 24-25, 36 pmol/kg/min) infusions (P=0.86), demonstrating that there was no difference at baseline between visits by chance (Figure 4.8 A,B, Tables 4.17, 4.18). The coefficient of variation of baseline plasma LEAP2 (-10min, pre-infusion) measured by ELISA assay was 19.8%. This accounted for both inter, intra-assay, kit lot and participant differences across all the baseline samples collected in the study (Figure 4.8E)

LEAP2 infusion dose positively correlated with plasma LEAP2 concentrations (i) over the whole infusion (iAUC $_{\text{-10 to 310 min}}$, r^2=0.90, β=939.24 ng/mL.min, P<0.001), (ii) over the fasting period (iAUC $_{\text{-10 to 195 min}}$, r^2=0.93, β=523.45 ng/mL.min, P<0.001) but, (iii) not over the post-prandial period (iAUC $_{\text{195 to 310 min}}$, r^2=0.01, β=12.05 ng/mL.min, P=0.66) (Figure 4.8 C,D, Table 4.5).

Plasma LEAP2 over the whole infusion (iAUC $_{\text{-10 to 310 min}}$) was significantly greater between visits with LEAP2 (12, 24-25, 36 pmol/kg/min) vs. without (saline) ($F(1,18)$=66.65, β=22547.92 ng/mL.min, P<0.001) (Table 4.19). When just comparing LEAP2 administration at the final selected dose for future studies (24-25 pmol/kg/min) vs. saline, plasma LEAP2 increased over the whole infusion (iAUC $_{\text{-10 to 310 min}}$) (P<0.001, Cohen's d=4.81, n=6) (Table 4.20).

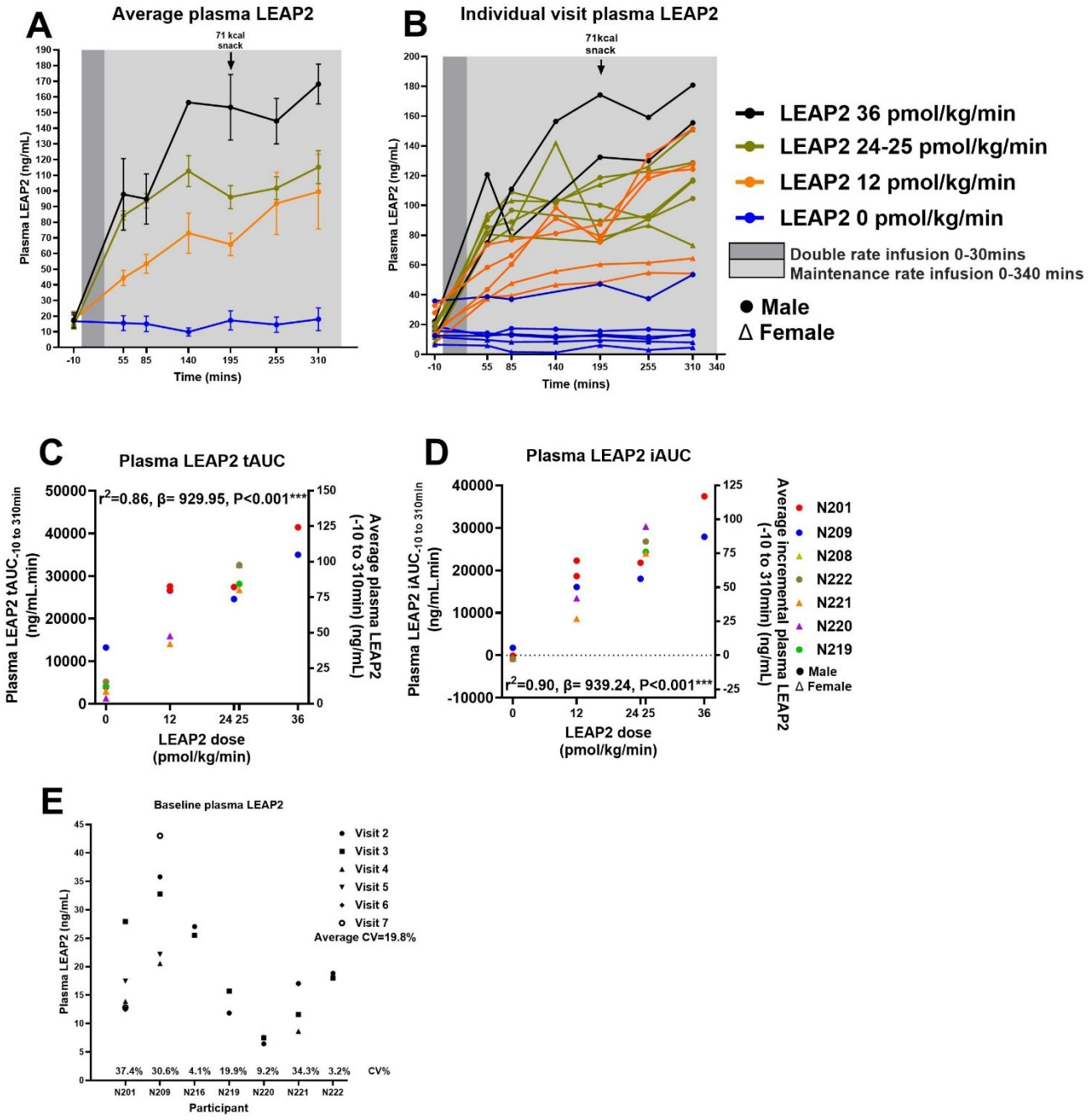


Figure 4.8. Plasma LEAP2 concentrations during LEAP2 infusions.
(A) Average and (B) individual plasma LEAP2 concentrations over the duration of the study day. Grey bars indicate infusion duration (double rate: 0-30mins, see legend for infusion rates: 30-340min). Participants given 71 kcal meal at t=195min. Data presented as mean ± SEM, from 7 participants, 22 visits. Effects of increasing LEAP2 dose on (C) plasma LEAP2 $tAUC_{-10 \text{ to } 310min}$ and (D) plasma LEAP2 $iAUC_{-10 \text{ to } 310min}$ (expressed as difference from saline visit, LEAP2 0 pmol/kg/min) with marginal value of pseudo-R squared (r^2) and estimated mean of β parameter from linear mixed model analysis based on data from 7 participants, 22 visits. (E) Fasting plasma LEAP2 (t=-10min, pre-infusion) by visit and participant ID with % CV. Abbreviations: CV, coefficient of variation; iAUC, incremental area under the curve; tAUC, total area under the curve. * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001.

4.6.4.6. VAS ratings

Baseline appetite, hunger, pleasantness, fullness, food craving, nausea, anxiety, thirst, stress, sleepiness VAS ratings or alcohol urge questionnaire (t= -10 min, pre-infusion) did not correlate with subsequent LEAP2 infusion dose (P=0.10-0.91, n=22 visits), and did not differ between visits without (saline) vs. with LEAP2 (combined 12, 24-25, 36 pmol/kg/min) infusions (P=0.12-0.95), demonstrating that there was no difference at baseline between visits by chance (Tables 4.17, 4.18). Baseline mood ratings viz. PANAS (Positive and Negative Affect Schedule) and SSAI (Spielberger State Anxiety Inventory) scores did not correlate with subsequent LEAP2 infusion dose (P=0.30-0.78, n=22), and did not differ between visits without (saline) vs. with LEAP2 (combined 12, 24-25, 36 pmol/kg/min) infusions (P=0.17-0.63), demonstrating that there was no difference in mood between visits by chance (Tables 4.17, 4.18).

LEAP2 infusion dose over the whole infusion (iAUC $_{\text{-10 to 310 min}}$) did not correlate with appetite, hunger, pleasantness, fullness, food craving VAS ratings or alcohol urge questionnaires (P=0.16-0.92) nor with (ii) nausea, anxiety, thirst, stress or sleepiness VAS ratings (P=0.13-0.87) but (iii) positively correlated with discomfort VAS (r^2=0.19, β=2.69 mm/hr.min, P=0.025) (Table 4.7). Similar results were observed with visit number as a covariate and there was no longer any correlation of LEAP2 dose with discomfort VAS ratings (P=0.12-0.99) (Table 4.7).

VAS ratings over the whole infusion (iAUC $_{\text{-10 to 310 min}}$) did not differ between visits without (saline) vs. with LEAP2 (12, 24-25, 36 pmol/kg/min) infusions (P=0.10-0.97) (Table 4.19). However, discomfort VAS ratings were greater in visits with LEAP2 (12, 24-25, 36 pmol/kg/min) vs. without (saline) (F(1,17.25)=8.39, β=76.23 mm.min P=0.01). When just comparing LEAP2 administration at the final selected dose for future studies (24-25 pmol/kg/min) vs. saline, there was no difference in VAS ratings over the whole infusion (iAUC $_{\text{-10 to 310 min}}$) (P=0.12-0.98, Cohen's d= -0.21 to 0.76, n=6) (Table 4.20).

Main effect of LEAP2 dose on dependent variable											
Dependent variable	**Covariate**	**r2**	**numerator df**	**denominator df**	**F**	**P value**	**β parameter**	**β ± SEM**	**95% CI of β lower limit**	**95% CI of β upper limit**	**β parameter units**
Appetite iAUC -10 to 310min	+visit number	0.13	1.00	15.39	0.49	0.49	1.20	1.71	-2.44	4.84	mm.min
Appetite iAUC -10 to 310min	-visit number	0.00	1.00	15.08	0.10	0.75	0.23	0.73	-1.32	1.79	mm.min
Hunger iAUC -10 to 310min	+visit number	0.03	1.00	15.77	0.16	0.69	-1.29	3.21	-8.10	5.52	mm.min
Hunger iAUC -10 to 310min	-visit number	0.01	1.00	15.10	0.31	0.59	0.61	1.10	-1.73	2.95	mm.min
Fullness iAUC -10 to 310min	+visit number	**0.43**	1.00	16.54	0.49	0.49	-2.10	3.00	-8.44	4.24	mm.min
Fullness iAUC -10 to 310min	-visit number	0.07	1.00	15.99	2.15	0.16	2.25	1.54	-1.00	5.51	mm.min
Food craving iAUC -10 to 310min	+visit number	0.05	1.00	16.20	0.59	0.45	-0.15	0.19	-0.56	0.26	mm.min
Food craving iAUC -10 to 310min	-visit number	0.00	1.00	15.02	0.04	0.84	-0.01	0.07	-0.16	0.13	mm.min
Alcohol Urge Questionnaire iAUC -10 to 310min	+visit number	0.19	1.00	18.00	2.72	0.12	0.19	0.12	-0.05	0.43	arbitrary units.min
Alcohol Urge Questionnaire iAUC -10 to 310min	-visit number	0.00	1.00	15.92	0.01	0.92	0.00	0.04	-0.09	0.08	arbitrary units.min
Nausea iAUC -10 to 310min	+visit number	0.18	1.00	18.00	1.87	0.19	5.24	3.83	-2.81	13.29	mm.min
Nausea iAUC -10 to 310min	-visit number	0.09	1.00	20.00	2.17	0.16	2.04	1.38	-0.85	4.93	mm.min
Anxiousness iAUC -10 to 310min	+visit number	0.07	1.00	18.00	0.20	0.66	1.02	2.29	-3.78	5.82	mm.min
Anxiousness iAUC -10 to 310min	-visit number	0.05	1.00	20.00	1.01	0.33	0.80	0.80	-0.86	2.46	mm.min
Thirst iAUC -10 to 310min	+visit number	0.04	1.00	16.57	0.46	0.51	-2.42	3.56	-9.95	5.11	mm.min
Thirst iAUC -10 to 310min	-visit number	0.01	1.00	16.15	0.19	0.66	0.55	1.24	-2.08	3.17	mm.min
Discomfort iAUC -10 to 310min	+visit number	0.26	1.00	18.00	2.05	0.17	4.36	3.05	-2.04	10.77	mm.min
Discomfort iAUC -10 to 310min	-visit number	0.19	1.00	17.29	6.07	**0.025**	2.69	1.09	0.39	5.00	mm.min
Stress iAUC -10 to 310min	+visit number	**0.33**	1.00	18.00	0.03	0.86	-0.24	1.33	-3.03	2.56	mm.min
Stress iAUC -10 to 310min	-visit number	0.11	1.00	20.00	2.53	0.13	0.84	0.53	-0.26	1.93	mm.min
Sleepyness iAUC -10 to 310min	+visit number	0.00	1.00	18.00	0.00	0.99	-0.02	2.47	-5.20	5.15	mm.min
Sleepyness iAUC -10 to 310min	-visit number	0.00	1.00	17.79	0.03	0.87	-0.14	0.84	-1.90	1.61	mm.min

Table 4.7. Effect of LEAP2 dose with covariates on VAS and mood ratings.

This table shows the effects of LEAP2 dose with covariates on multiple VAS iAUC from t=-10 to 310 min ratings using linear mixed model analysis including participant ID as between participant, and LEAP2 dose as within participant fixed factors. Covariate is visit number to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=7, visits=22. Abbreviations: iAUC, incremental area under the curve; VAS, Visual analogue scale**.**

4.6.4.7. Desired and actual food intake

For both desired (VPCT) and actual (*ad libitum* meal) food intake (as absolute kcal or % estimated REE, averaged across dishes), there were no significant interactions for: (i) sweet*fat*LEAP2 dose, (ii) fat*LEAP2 dose (independent of sweetness), (iii) sweet*LEAP2 dose (independent of fat content), nor (iv) main effects of LEAP2 dose (Tables. 4.8, 4.10).

LEAP2 infusion dose did not correlate with desired food intake (as total kcal or % estimated REE) from VPCT (P=0.58-0.61), Figure 4.9A, B, Table 4.9). LEAP2 infusion dose did not correlate with actual food intake (as total kcal or % estimated REE) from *ad libitum* meals (P=0.69, Figure 4.9C, D, Table 4.11) or with duration of *ad libitum* meal (P=0.37, Table 4.11). Similar results were observed with visit number as a covariate (P=0.12-0.78) (Tables 4.9, 4.11).

For both desired (VPCT) and actual (*ad libitum* meal) food intake (as absolute kcal or % estimated REE, averaged across dishes), over the whole infusion did not differ between visits without (saline) vs. with LEAP2 (12, 24-25, 36 pmol/kg/min) infusions (P=0.31-0.99) (Table 4.19). When just comparing LEAP2 administration at the final selected dose for future studies (24-25 pmol/kg/min) vs. saline, there was no difference in desired (VPCT) or actual (*ad libitum* meal) food intake (as absolute kcal or % estimated REE, averaged across dishes) over the whole infusion ($iAUC_{-10\ to\ 310\ min}$) (P=0.88-0.95, Cohen's d= -0.24 to 0.05, n=6) (Table 4.20).

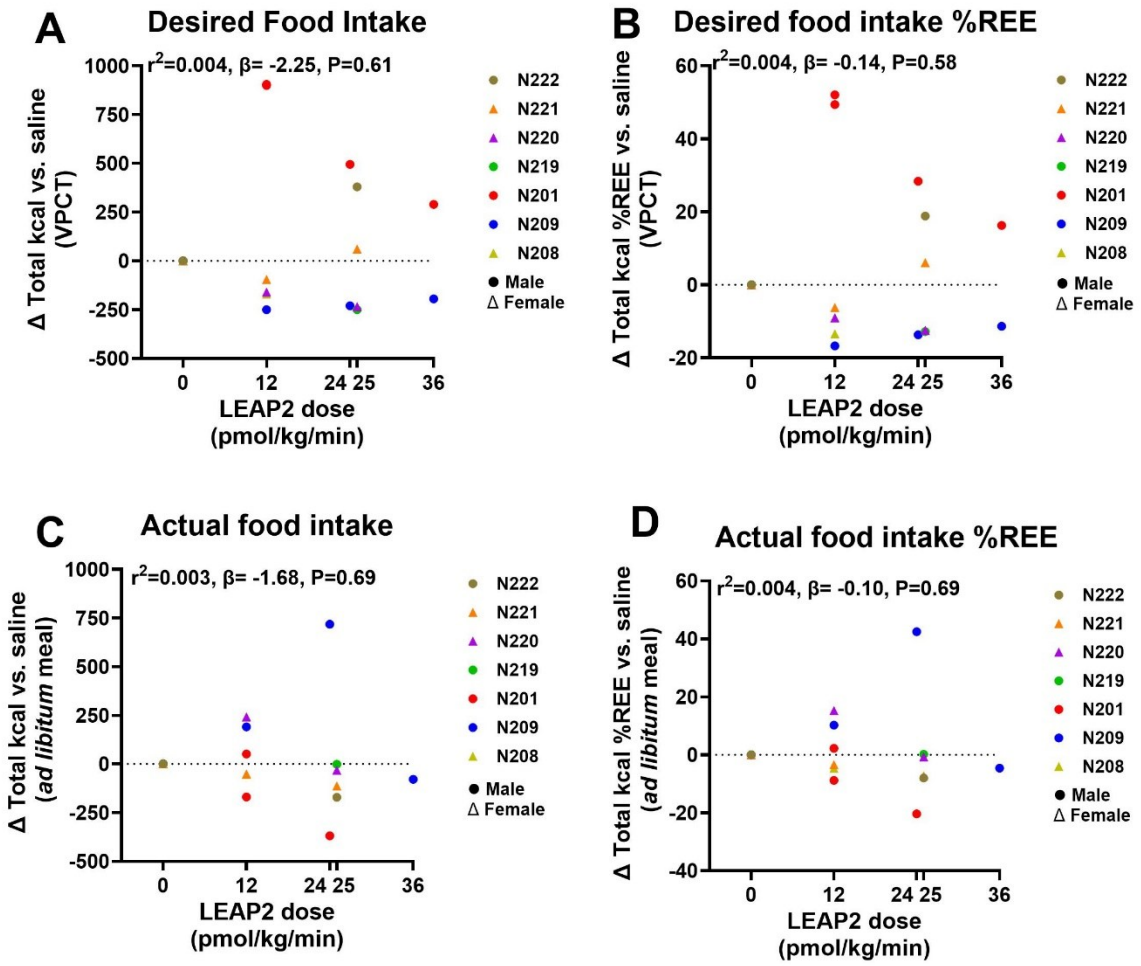


Figure 4.9. Correlation of LEAP2 dose with desired and actual food intake.
Effects of increasing LEAP2 dose as (A,C) absolute and (B,D) relative energy intake (expressed as difference from saline visit, LEAP2=0pmol/kg/min) on absolute/relative (A,B) desired food intake estimated from VPCT task and actual food intake measured with (C,D) *ad libitum* intake with marginal value of pseudo-R squared (r^2) and estimated mean of β parameter from linear mixed model analysis based on data from 7 participants, 22 visits. Abbreviations: REE, resting energy expenditure; VPCT, Virtual portion creation task. * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001.

Main effect of LEAP2 dose on dependent variable									
Dependent variable	**Interactions**	**Numerator df**	**denominator df**	**F**	**P value**	**Estimate**	**SE**	**95 CI lower**	**95 CI upper**
Total kcal VPCT	**LEAP2 dose*sweetness*fatness**	1.00	80.00	0.04	0.85	0.18	0.92	-1.66	2.02
	LEAP2 dose*fatness	1.00	80.00	0.07	0.79	-0.17	0.65	-1.47	1.13
	LEAP2 dose*sweetness	1.00	80.00	0.06	0.80	0.17	0.65	-1.14	1.47
	LEAP2 dose	1.00	80.00	0.15	0.70	-0.18	0.47	-1.11	0.75
	sweetness*fatness	1.00	80.00	1.87	0.18	22.71	16.62	-10.37	55.79
	fatness	1.00	80.00	0.69	0.41	-9.76	11.75	-33.15	13.63
	sweetness	1.00	80.00	11.65	**0.001**	-40.12	11.75	-63.51	-16.73
Total kcal %REE VPCT	**LEAP2 dose*sweetness*fatness**	1.00	80.00	0.04	0.85	0.18	0.92	-1.66	2.02
	LEAP2 dose*fatness	1.00	80.00	0.07	0.79	-0.17	0.65	-1.47	1.13
	LEAP2 dose*sweetness	1.00	80.00	0.06	0.80	0.17	0.65	-1.14	1.47
	LEAP2 dose	1.00	80.00	0.15	0.70	-0.18	0.47	-1.11	0.75
	sweetness*fatness	1.00	80.00	1.87	0.18	22.71	16.62	-10.37	55.79
	fatness	1.00	80.00	0.69	0.41	-9.76	11.75	-33.15	13.63
	sweetness	1.00	80.00	11.65	**0.001**	-40.12	11.75	-63.51	-16.73

Table 4.8. Main effects of fatness, sweetness and LEAP2 dose on desired food intake.
This table describes the F statistic, numerator and denominator of degrees of freedom for the main effects of fatness, sweetness, LEAP2 dose and fatness*sweetness*LEAP2 dose interactions on total and estimated kcal (as %REE) desired food intake using the virtual portion creation task. Covariates include sweetness and fatness content of meal. LEAP2 dose includes 0,12,24,25 and 36 pmol/kg/min. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: kcal, kilocalorie; REE, Resting energy expenditure; VPCT, Virtual portion creation task.

Main effect of LEAP2 dose on dependent variable											
Dependent variable	**Covariate**	**r2**	**numerator df**	**denominator df**	**F**	**P value**	**β parameter**	**β ± SEM**	**95% CI of β lower limit**	**95% CI of β upper limit**	**β parameter units**
Total kcal VPCT	+ Visit number	0.013	1.00	15.76	0.18	0.68	5.44	12.93	-22.00	32.89	kcal
Total kcal VPCT	- Visit number	0.004	1.00	15.41	0.27	0.61	-2.25	4.32	-11.45	6.94	kcal
Total kcal %REE VPCT	+ Visit number	0.012	1.00	15.70	0.09	0.77	0.22	0.74	-1.36	1.79	kcal
Total kcal %REE VPCT	-Visit number	0.005	1.00	15.32	0.32	0.58	-0.14	0.25	-0.67	0.38	kcal

Table 4.9. Effect of LEAP2 dose with covariates on desired food intake.
This table shows the effects of LEAP2 dose with covariates on total and estimated kcal (as %REE) food intake using the virtual portion creation task using linear mixed model analysis including participant ID as between participant, and LEAP2 dose as within participant fixed factors. Covariate is visit number to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=7, visits=22. Abbreviations: kcal: kilocalorie; REE: Resting energy expenditure; VPCT: Virtual portion creation task.

Main effect of LEAP2 dose on dependent variable									
Dependent variable	**Interactions**	**Numerator df**	**Denominator df**	**F**	**P value**	**Estimate**	**SE**	**95 CI lower**	**95 CI upper**
Total kcal ad libitum meal	**LEAP2 dose*sweetness*fatness**	1.00	76.00	0.21	0.65	2.94	6.43	-9.87	15.75
	LEAP2 dose*fatness	1.00	76.00	0.43	0.51	-2.98	4.55	-12.03	6.08
	LEAP2 dose*sweetness	1.00	76.00	0.42	0.52	-2.95	4.55	-12.01	6.11
	LEAP2 dose	1.00	76.00	0.31	0.58	1.79	3.22	-4.63	8.21
Total kcal %REE ad libitum meal	**LEAP2 dose*sweetness*fatness**	1.00	76.00	0.17	0.68	0.15	0.37	-0.59	0.90
	LEAP2 dose*fatness	1.00	76.00	0.45	0.51	-0.18	0.26	-0.70	0.35
	LEAP2 dose*sweetness	1.00	76.00	0.40	0.53	-0.17	0.26	-0.69	0.36
	LEAP2 dose	1.00	76.00	0.29	0.59	0.10	0.19	-0.27	0.47

Table 4.10. Main effects of fatness, sweetness and LEAP2 dose on ad libitum food intake.
This table describes the F statistic, numerator and denominator of degrees of freedom for the main effects of fatness, sweetness, LEAP2 dose and fatness*sweetness*LEAP2 dose interactions on total and estimated kcal (as %REE) *ad libitum* food. Covariates include sweetness and fatness content of meal. LEAP2 dose includes 0,12,24,25 and 36 pmol/kg/min. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: kcal, kilocalorie; REE, Resting energy expenditure.

Main effect of LEAP2 dose on dependent variable											
Dependent variable	**Covariate**	**r2**	**numerator df**	**denominator df**	**F**	**P value**	**β parameter**	**β ± SEM**	**95% CI of β lower limit**	**95% CI of β upper limit**	**β parameter units**
Total kcal ad libitum meal	+ visit number	0.09	1.00	15.30	0.15	0.71	4.78	12.42	-21.66	31.21	kilocalorie
Total kcal ad libitum meal	-visit number	0.00	1.00	14.31	0.16	0.69	-1.68	4.16	-10.59	7.23	kilocalorie
Total kcal %REE ad libitum meal	+ visit number	0.08	1.00	15.27	0.08	0.78	0.21	0.73	-1.35	1.76	kilocalorie
Total kcal %REE ad libitum meal	-visit number	0.00	1.00	14.31	0.17	0.69	-0.10	0.24	-0.62	0.42	kilocalorie
Ad libitum meal duration	+ visit number	0.12	1.00	15.45	2.66	0.12	-0.21	0.13	-0.48	0.06	minutes
Ad libitum meal duration	-visit number	0.01	1.00	15.02	0.86	0.37	-0.05	0.05	-0.16	0.06	minutes

Table 4.11. Effect of LEAP2 dose with covariates on ad libitum food intake.

This table shows the effects of LEAP2 dose with covariates on total and estimated kcal (as %REE) ad libitum food intake and duration of meal using linear mixed model analysis including participant ID as between participant, and LEAP2 dose as within participant fixed factors. Covariate is visit number to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=7, visits=22. Abbreviations: kcal, kilocalorie; REE, Resting energy expenditure.

4.6.4.8. Delay discounting task

For discounting rate for high-energy (HE) food, low-energy (LE) food, monetary and alcohol rewards, there were no significant interactions for reward size*LEAP2 dose (P=0.37-0.94) (Table 4.12), thus correlations were examined between LEAP2 dose and overall discounting rate independent of reward size.

LEAP2 infusion dose did not correlate with overall discounting rate overall for HE food, LE food and alcohol (P=0.40-0.84, Figure 4.10A, B, D, Table 4.13), though there was close to a trend for a positive correlation between LEAP2 infusion dose and temporal impulsivity for monetary reward (P=0.10, Figure 4.10C, Table 4.13). Similar results were observed with visit number as a covariate (P=0.12-0.89) (Table 4.13).

Overall, HE food, LE food, monetary and alcohol overall delay discounting rate did not differ significantly between visits without (saline) vs. with LEAP2 (12, 24-25, 36 pmol/kg/min) infusions (P=0.14-0.62) (Table 4.19).

When just comparing LEAP2 administration at the final selected dose for future studies (24-25 pmol/kg/min) vs. saline, there was no significant difference in overall delay discounting rate for LE food, monetary or alcohol rewards (P=0.19-0.67, Cohen's d= -0.24 to 0.32, n=6), though there was a non-significant moderate effect size (d= -0.63, P=0.19) for HE food delay discounting rate to be lowered by LEAP2 administration (Table 4.20).

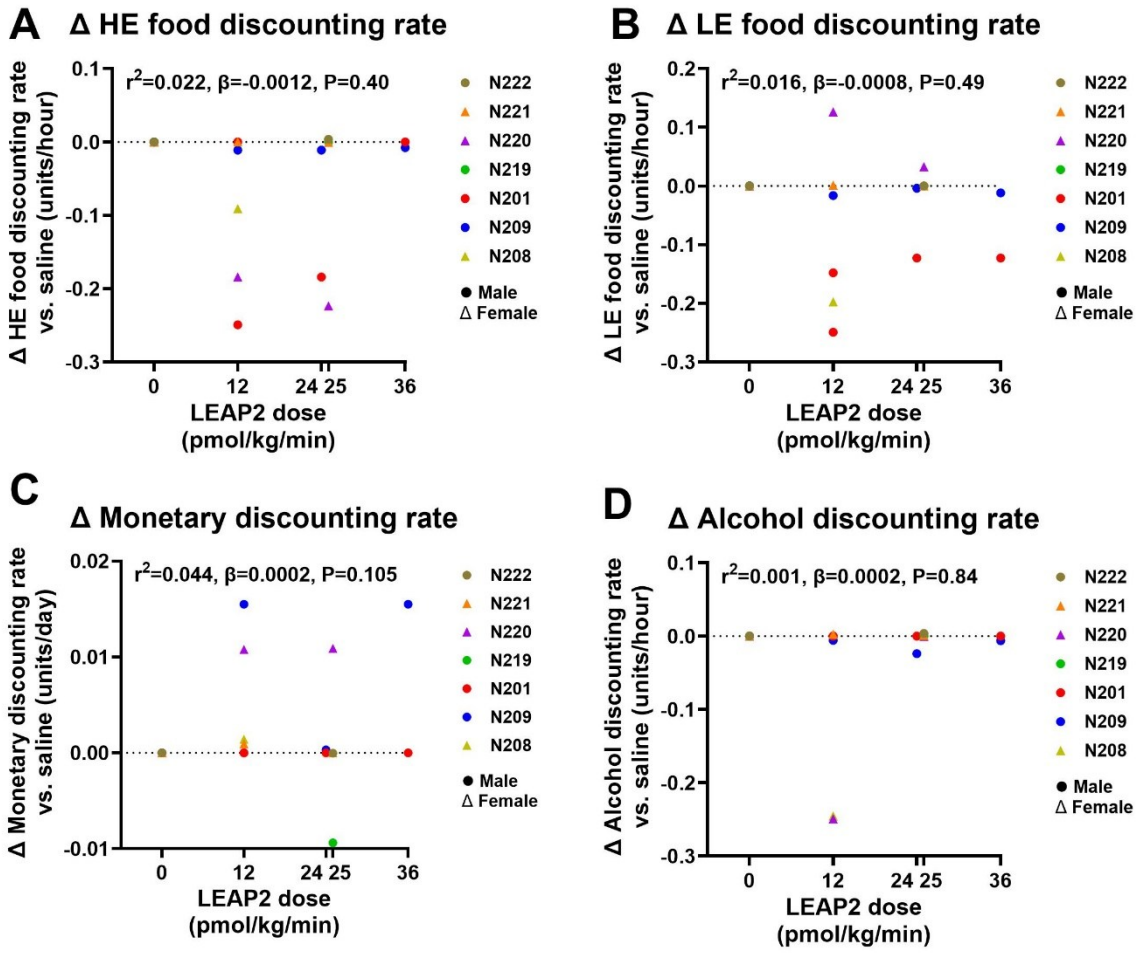


Figure 4.10. Correlation of LEAP2 dose with discounting rate for high-energy food, low-energy food, monetary and alcohol rewards.

Effects of increasing LEAP2 dose on discounting rates for (A) HE food, (B)LE food, (C) Money and (D) Alcohol (expressed as difference from saline visit, LEAP2=0pmol/kg/min) with marginal value of pseudo-R squared (r^2) and estimated mean of β parameter from linear mixed model analysis based on data from 7 participants, 22 visits. Abbreviations: HE, high energy; LE, low energy. * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001.

Main effect of LEAP2 dose on dependent variable									
Dependent variable	**Interactions**	**Numerator df**	**Denominator df**	**F**	**P value**	**Estimate**	**SE**	**95 CI lower**	**95 CI upper**
Alcohol reward	**LEAP2 dose *reward size**	1.000	59.128	0.720	0.40	-0.001	0.001	-0.002	0.001
	LEAP2 dose	1.000	59.167	0.184	0.67	0.001	0.001	-0.002	0.003
	reward size	1.000	59.128	0.254	0.62	-0.006	0.012	-0.029	0.017
HE food reward	**LEAP2 dose *reward size**	1.000	59.006	0.005	0.94	0.000	0.001	-0.002	0.002
	LEAP2 dose	1.000	59.093	0.276	0.60	-0.001	0.002	-0.004	0.003
	reward size	1.000	59.006	0.325	0.57	0.008	0.014	-0.020	0.036
LE food reward	**LEAP2 dose *reward size**	1.000	59.016	0.830	0.37	0.001	0.001	-0.001	0.002
	LEAP2 dose	1.000	59.153	1.851	0.18	-0.002	0.001	-0.005	0.001
	reward size	1.000	59.016	0.067	0.80	-0.003	0.012	-0.028	0.021
Monetary reward	**LEAP2 dose *reward size**	1.000	59.021	0.228	0.64	0.000	0.000	0.000	0.000
	LEAP2 dose	1.000	59.176	0.003	0.96	0.000	0.000	-0.001	0.001
	reward size	1.000	59.021	8.572	**0.005**	-0.009	0.003	-0.016	-0.003

Table 4.12. Main effects of reward size and LEAP2 dose on temporal impulsivity for alcohol, food and monetary rewards.
This table describes the F statistic, numerator and denominator of degrees of freedom for the main effects of reward size, LEAP2 dose and reward size*LEAP2 dose interaction on alcohol, food and monetary rewards. Reward size includes small, medium and large rewards. LEAP2 dose includes 0,12,24,25 and 36 pmol/kg/min. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: HE, High-Energy; LE, Low-Energy.

Main effect of LEAP2 dose on dependent variable											
Dependent variable	**Covariate**	**r2**	**numerator df**	**denominator df**	**F**	**P**	**β parameter**	**β ± SEM**	**95% CI of β lower limit**	**95% CI of β upper limit**	**β parameter units**
Alcohol discounting rate	+visit number	0.01	1.00	15.57	0.02	0.89	-0.0004	0.003	-0.006	0.006	units/hour
Alcohol discounting rate	-visit number	0.001	1.00	15.49	0.04	0.84	0.0002	0.001	-0.002	0.002	units/hour
HE food discounting rate	+visit number	0.18	1.00	16.65	6.61	0.20	-0.0096	0.004	-0.017	-0.002	units/hour
HE food discounting rate	-visit number	0.02	1.00	15.66	0.76	0.40	-0.0012	0.001	-0.004	0.002	units/hour
LE food discounting rate	+visit number	0.09	1.00	17.33	2.70	0.12	-0.0054	0.003	-0.012	0.002	units/hour
LE food discounting rate	-visit number	0.02	1.00	15.83	0.50	0.49	-0.0008	0.001	-0.003	0.002	units/hour
Monetary discounting rate	+visit number	0.04	1.00	15.79	0.14	0.72	0.0001	0.000	-0.001	0.001	units/day
Monetary discounting rate	-visit number	0.04	1.00	15.26	2.98	0.10	0.0002	0.000	0.000	0.001	units/day

Table 4.13. Effect of LEAP2 dose with covariates on temporal impulsivity for alcohol, food and monetary rewards.
This table shows the effects of LEAP2 dose with covariates on temporal impulsivity for alcohol, food and monetary rewards using linear mixed model analysis including participant ID as between participant, and LEAP2 dose as within participant fixed factors. Covariate is visit number to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=7, visits=22. Abbreviations: HE, High-Energy; LE, Low-Energy.

4.6.4.9. Cognitive memory tasks

LEAP2 dose did not correlate significantly with: (i) either the first attempt memory score or total errors made in the paired associate learning (PAL) task (P=0.11-0.12, Table 4.14), (ii) reaction time in the stop signal task (SST) (P=0.37, Table 4.14), nor (iii) errors made in the spatial working memory (SWM) task (P=0.97, Table 4.14). Similar results were observed with visit number as a covariate (P=0.11-0.72) (Table 4.14).

Outcomes from the cognitive memory tasks did not differ significantly between visits without (saline) vs. with LEAP2 (12, 24-25, 36 pmol/kg/min) infusions (P=0.11-0.66) (Table 4.19).

When just comparing LEAP2 administration at the final selected dose for future studies (24-25 pmol/kg/min) vs. saline, there was no significant or moderate effect size difference in cognitive outcomes over the whole infusion ($iAUC_{-10\ to\ 310\ min}$) (P=0.30-0.92, Cohen's d= -0.47 to 0.28, n=6) (Table 4.20).

Main effect of LEAP2 dose on dependent variable											
Dependent variable	**Covariate**	**r2**	**numerator df**	**denominator df**	**F**	**P value**	**β parameter**	**β ± SEM**	**95% CI of β lower limit**	**95% CI of β upper limit**	**β parameter units**
Paired Associate Learning First attempt memory score	+visit number	0.13	1.00	16.22	0.40	0.54	-0.11	0.18	-0.50	0.27	arbitrary units
Paired Associate Learning First attempt memory score	-visit number	0.07	1.00	15.12	2.68	0.12	0.10	0.06	-0.03	0.23	arbitrary units
Paired Associate Learning Total errors	+visit number	0.30	1.00	16.48	0.94	0.35	0.43	0.44	-0.51	1.37	arbitrary units
Paired Associate Learning Total errors	-visit number	0.09	1.00	15.80	2.80	0.11	-0.30	0.18	-0.68	0.08	arbitrary units
Stop Signal Task Reaction time	+visit number	0.04	1.00	15.83	1.53	0.23	-1.87	1.51	-5.07	1.33	milliseconds
Stop Signal Task Reaction time	-visit number	0.01	1.00	15.66	0.87	0.37	-0.47	0.50	-1.53	0.60	milliseconds
Spatial Working Memory Between errors	+visit number	0.00	1.00	15.84	0.14	0.72	0.11	0.31	-0.54	0.77	arbitrary units
Spatial Working Memory Between errors	-visit number	0.00	1.00	15.06	0.00	0.97	0.00	0.10	-0.22	0.21	arbitrary units

Table 4.14. Effect of LEAP2 dose with covariates on cognitive outcomes.

This table shows the effects of LEAP2 dose with covariates on cognitive outcomes from the CANTAB tasks using linear mixed model analysis including participant ID as between participant, and LEAP2 dose as within participant fixed factors. Covariate is visit number to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=7, visits=22. Abbreviations: CANTAB, Cambridge Neuropsychological Test Automated Battery.

4.6.4.10. Leeds food picture questionnaire

For both explicit liking and wanting, there were no significant interactions for: (i) sweet*fat*LEAP2 dose, (ii) fat*LEAP2 dose (independent of sweetness), (iii) sweet*LEAP2 dose (independent of fat content), nor (iv) main effects of LEAP2 dose. For implicit wanting bias there were no significant (i) interactions for LEAP2 dose*sweet/fat bias, nor (ii) main effects of LEAP2 dose for either high fat or sweet bias (Table 4.15)

LEAP2 dose did not correlate significantly with ratings of implicit wanting bias for high fat or sweet food (P=0.73-0.89) (Table 4.16). Similar results were observed with visit number as a covariate (P=0.58-0.71) (Table 4.16).

Implicit bias for high fat or sweet food did not differ significantly between visits without (saline) vs. with LEAP2 (12, 24-25, 36 pmol/kg/min) infusions (P=0.38-0.54) (Table 4.10).

When just comparing LEAP2 administration at the final selected dose for future studies (24-25 pmol/kg/min) vs. saline, there was no significant difference in implicit wanting bias for high fat or sweet foods (P=0.38-0.54, Cohen's d=-0.44 to -0.06) (Table 4.20).

Main effect of LEAP2 dose on dependent variable									
Dependent variable	**Interactions**	**Numerator df**	**Denominator df**	**F**	**P value**	**Estimate**	**SE**	**95 CI lower**	**95 CI upper**
Explicit liking	**LEAP2 dose*sweetness*fatness**	1.00	80.00	0.23	0.64	0.18	0.38	-0.58	0.95
	LEAP2 dose*fatness	1.00	80.00	0.18	0.67	0.12	0.27	-0.42	0.66
	LEAP2 dose*sweetness	1.00	80.00	0.00	0.96	0.01	0.27	-0.53	0.55
	LEAP2 dose	1.00	80.00	1.02	0.32	-0.19	0.19	-0.58	0.19
Explicit wanting	**LEAP2 dose*sweetness*fatness**	1.00	80.00	0.02	0.90	-0.05	0.39	-0.83	0.72
	LEAP2 dose*fatness	1.00	80.00	0.17	0.68	0.11	0.28	-0.44	0.66
	LEAP2 dose*sweetness	1.00	80.00	0.12	0.73	0.10	0.28	-0.45	0.64
	LEAP2 dose	1.00	80.00	1.23	0.27	-0.22	0.20	-0.61	0.17
Implicit wanting	**LEAP2 dose*sweetness/fatness**	1.00	37.04	0.05	0.82	0.13	0.55	-0.99	1.24
	LEAP2 dose	1.00	37.85	0.00	0.97	0.05	0.39	-0.74	0.85
	sweetness/fatness	1.00	37.03	26.73	**<0.001**	50.98	9.86	31.00	70.96

Table 4.15. Main effects of fatness, sweetness and LEAP2 dose on food reward.
This table describes the F statistic, numerator and denominator of degrees of freedom for the main effects of fatness, sweetness, LEAP2 dose and fatness*sweetness*LEAP2 dose interactions on explicit liking/wanting and implicit bias using the LFPQ. Covariates include sweetness and fatness content of meal. LEAP2 dose includes 0,12,24,25 and 36 pmol/kg/min. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. Abbreviations: LFPQ, Leeds Food Picture Questionnaire.

Main effect of LEAP2 dose on dependent variable											
dependent variable	**Covariate**	**r2**	**numerator df**	**denominator df**	**F**	**P value**	**β parameter**	**β ± SEM**	**95% CI of β lower limit**	**95% CI of β upper limit**	**β parameter units**
Fat bias	+visit number	0.06	1.00	15.52	0.31	0.58	1.81	3.25	-5.08	8.71	arbitrary units
Fat bias	-visit number	0.00	1.00	15.51	0.13	0.73	0.08	0.24	-0.42	0.59	arbitrary units
Sweet bias	+visit number	0.00	1.00	16.10	0.14	0.71	2.70	7.09	-12.32	17.71	arbitrary units
Sweet bias	-visit number	0.00	1.00	15.54	0.02	0.89	-0.07	0.48	-1.10	0.96	arbitrary units

Table 4.16. Effect of LEAP2 dose on fat or sweet bias.
This table shows the effects of LEAP2 dose on implicit bias and fat or sweet bias on food reward using linear mixed model analysis including participant ID as between participant, and LEAP2 dose as within participant fixed factors using the LFPQ. Covariate is visit number to examine their effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=7, visits=22. Abbreviations: LFPQ, Leeds Food Picture Questionnaire.

Main effect of LEAP2 dose on baseline dependent variables										
dependent variable	r2	numerator df	denominator df	F	P value	β parameter	β ± SEM	95% CI of β lower limit	95% CI of β upper limit	β parameter units
Baseline glucose -10min	0.18	1.00	20.00	4.48	0.47	0.01	0.00	0.00	0.02	mmol/L
Baseline insulin -10min	0.01	1.00	14.43	0.35	0.56	0.01	0.02	-0.03	0.05	mIU/L
Baseline GH -10min	0.03	1.00	17.51	0.77	0.39	-0.05	0.06	-0.17	0.07	mIU/L
Baseline cortisol -10min	0.001	1.00	16.68	0.02	0.90	0.15	1.13	-2.24	2.53	ug/L
Baseline beta hydroxybutyrate -10min	0.05	1.00	14.98	3.14	0.10	0.00	0.00	0.00	0.01	mmol/L
Baseline triglyceride -10min	0.12	1.00	14.43	5.93	0.28	-0.01	0.00	-0.01	0.00	mmol/L
Baseline LEAP2 -10min	0.05	1.00	14.03	2.19	0.16	-0.14	0.09	-0.33	0.06	ng/mL
Baseline Appetite -10min	0.06	1.00	14.71	2.78	0.12	-0.33	0.20	-0.76	0.09	mm
Baseline Hunger -10min	0.05	1.00	14.98	2.77	0.12	-0.45	0.27	-1.03	0.13	mm
Baseline Fullness -10min	0.04	1.00	17.12	0.96	0.34	-0.27	0.28	-0.86	0.32	mm
Baseline Food craving -10min	0.00	1.00	15.52	0.10	0.75	0.01	0.02	-0.04	0.05	mm
Baseline Alcohol Urge Questionnaire -10min	0.00	1.00	15.95	0.24	0.63	0.00	0.01	-0.02	0.01	arbitrary units
Baseline Nausea -10min	0.01	1.00	17.61	0.27	0.61	-0.08	0.15	-0.40	0.24	mm
Baseline Anxiousness -10min	0.05	1.00	17.73	1.21	0.29	-0.23	0.21	-0.67	0.21	mm
Baseline Thirst -10min	0.00	1.00	15.48	0.01	0.91	-0.03	0.27	-0.61	0.54	mm
Baseline Discomfort -10min	0.11	1.00	16.48	3.14	0.10	-0.50	0.28	-1.10	0.10	mm
Baseline Stress -10min	0.04	1.00	17.51	1.14	0.30	-0.16	0.15	-0.46	0.15	mm
Baseline Sleepyness -10min	0.06	1.00	15.58	2.44	0.14	-0.37	0.23	-0.86	0.13	mm
Positive affect score	0.00	1.00	15.04	0.77	0.39	-0.03	0.04	-0.12	0.05	arbitrary units
Negative affect score	0.00	1.00	17.10	0.08	0.78	-0.01	0.05	-0.12	0.10	arbitrary units
SSAI score	0.01	1.00	15.21	1.14	0.30	-0.04	0.03	-0.11	0.04	arbitrary units
Baseline Sodium -10min	0.000	1.00	16.93	0.00	0.99	0.00	0.04	-0.09	0.09	mmol/L
Baseline Potassium -10min	0.03	1.00	15.82	1.04	0.32	0.00	0.00	-0.01	0.00	mmol/L
Baseline Alanine aminotransferase -10min	0.03	1.00	15.05	1.32	0.27	-0.04	0.04	-0.12	0.04	IU/L
Baseline Aspartate aminotransferase -10min	0.002	1.00	15.32	0.21	0.66	-0.02	0.05	-0.12	0.08	IU/L
Baseline Alkaline Phosphatase -10min	0.01	1.00	15.04	2.42	0.14	-0.15	0.10	-0.36	0.06	IU/L
Baseline Gamma glutamyl tranferase -10min	0.00	1.00	15.07	1.44	0.25	-0.03	0.03	-0.09	0.03	U/L
Baseline Total bilirubin -10min	0.00	1.00	14.00	0.02	0.89	-0.01	0.04	-0.09	0.08	umol/L
Baseline Calcium -10min	0.00	1.00	14.89	0.23	0.64	0.00	0.00	0.00	0.00	mmol/L
Baseline Phosphate -10min	0.02	1.00	16.59	0.51	0.49	0.00	0.00	-0.01	0.01	mmol/L
Baseline TSH -10min	0.02	1.00	14.18	3.50	0.82	0.01	0.01	0.00	0.02	mIU/L
Baseline free thyroxine -10min	0.02	1.00	14.74	1.63	0.22	-0.02	0.01	-0.04	0.01	pmol/L
Baseline LH -10min	0.003	1.00	16.49	0.07	0.79	0.01	0.04	-0.07	0.09	IU/L
Baseline FSH -10min	0.03	1.00	16.80	1.03	0.32	-0.03	0.03	-0.09	0.03	IU/L
Baseline Prolactin -10min	0.05	1.00	15.20	4.01	0.64	1.77	0.89	-0.11	3.66	mIU/L
Baseline Testosterone -10min	0.04	1.00	11.00	0.53	0.48	0.03	0.05	-0.07	0.14	nmol/L

Table 4.17. Effect of LEAP2 dose on baseline (fasting at -10min) markers of glucose and fat metabolism, pituitary hormones and biochemical metabolites.

This table shows the effects of LEAP2 dose on baseline (fasting at -10 min, pre-infusion) for plasma glucose, serum insulin, GH, cortisol, triglycerides, venous beta hydroxybutyrate, hormones and biochemical metabolites using linear mixed model analysis including participant ID as between participant, and LEAP2 dose as within participant fixed factors. Effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=7, visits=22.

Abbreviations: GH, Growth Hormone; IU, International unit; TSH, Thyroid stimulating hormone; LH, Luteinising hormone; FSH, Follicular stimulating hormone; SSAI, Spielberger State Anxiety Inventory

Main effect of LEAP2 administration (vs. saline) on baseline dependent variables										
dependent variable	r2	numerator df	denominator df	F	P value	β parameter	β ± SEM	95% CI of β lower limit	95% CI of β upper limit	β parameter units
Baseline glucose -10min	0.158	1.00	20.00	3.94	0.61	0.20	0.10	-0.01	0.42	mmol/L
Baseline insulin -10min	0.044	1.00	14.52	3.04	0.11	0.69	0.39	-0.16	1.53	mIU/L
Baseline GH -10min	0.040	1.00	17.18	1.05	0.32	-1.48	1.44	-4.53	1.56	mIU/L
Baseline cortisol -10min	0.011	1.00	16.56	0.30	0.59	15.37	28.14	-44.13	74.86	ug/L
Baseline beta hydroxybutyrate -10min	0.002	1.00	15.04	0.11	0.75	0.02	0.06	-0.10	0.14	mmol/L
Baseline triglyceride -10min	0.097	1.00	14.39	5.05	0.41	-0.13	0.06	-0.25	-0.01	mmol/L
Baseline LEAP2 -10min	0.001	1.00	14.86	0.03	0.86	-0.46	2.64	-6.09	5.17	ng/mL
Baseline Appetite -10min	0.026	1.00	14.58	1.18	0.29	-5.70	5.24	-16.91	5.50	mm
Baseline Hunger -10min	0.022	1.00	14.90	1.13	0.30	-7.62	7.16	-22.88	7.65	mm
Baseline Fullness -10min	0.092	1.00	17.10	2.49	0.13	-10.80	6.85	-25.24	3.64	mm
Baseline Food craving -10min	0.018	1.00	15.44	0.73	0.41	0.41	0.48	-0.61	1.44	mm
Baseline Alcohol Urge Questionnaire -10min	0.004	1.00	15.88	0.20	0.66	-0.10	0.23	-0.59	0.38	arbitrary units
Baseline Nausea -10min	0.032	1.00	17.53	0.81	0.38	-3.37	3.73	-11.21	4.48	mm
Baseline Anxiousness -10min	0.022	1.00	17.78	0.56	0.46	-4.04	5.40	-15.41	7.32	mm
Baseline Thirst -10min	0.000	1.00	15.41	0.00	0.95	-0.43	6.80	-14.88	14.03	mm
Baseline Discomfort -10min	0.147	1.00	16.19	4.88	0.42	-14.98	6.78	-29.34	-0.62	mm
Baseline Stress -10min	0.021	1.00	17.53	0.56	0.47	-2.78	3.73	-10.63	5.07	mm
Baseline Sleepyness -10min	0.000	1.00	15.23	0.01	0.94	-0.48	6.35	-14.00	13.04	mm
Positive affect score	0.001	1.00	15.03	0.24	0.63	0.48	0.99	-1.62	2.58	arbitrary units
Negative affect score	0.034	1.00	16.71	1.01	0.33	-1.27	1.27	-3.95	1.40	arbitrary units
SSAI score	0.026	1.00	15.16	2.12	0.17	-1.19	0.82	-2.93	0.55	arbitrary units
Baseline Sodium -10min	0.001	1.00	16.84	0.02	0.89	-0.15	1.06	-2.38	2.09	mmol/L
Baseline Potassium -10min	0.013	1.00	15.70	0.51	0.49	-0.05	0.07	-0.20	0.10	mmol/L
Baseline Alanine aminotransferase -10min	0.026	1.00	14.97	1.16	0.30	-1.03	0.96	-3.07	1.01	IU/L
Baseline Aspartate aminotransferase -10min	0.011	1.00	15.30	1.14	0.30	-1.18	1.10	-3.53	1.17	IU/L
Baseline Alkaline Phosphatase -10min	0.004	1.00	15.06	0.65	0.43	-2.11	2.62	-7.69	3.47	IU/L
Baseline Gamma glutamyl tranferase -10min	0.001	1.00	15.07	0.34	0.57	-0.43	0.74	-2.00	1.14	U/L
Baseline Total bilirubin -10min	0.010	1.00	13.97	1.78	0.20	-1.22	0.92	-3.19	0.74	umol/L
Baseline Calcium -10min	0.000	1.00	14.82	0.02	0.89	0.00	0.02	-0.05	0.05	mmol/L
Baseline Phosphate -10min	0.006	1.00	16.63	0.17	0.69	-0.02	0.02	-0.12	0.00	mmol/L
Baseline TSH -10min	0.002	1.00	14.16	0.45	0.51	0.11	0.16	-0.24	0.45	mIU/L
Baseline free thyroxine -10min	0.009	1.00	14.65	0.58	0.46	-0.26	0.34	-0.98	0.46	pmol/L
Baseline LH -10min	0.015	1.00	16.46	0.34	0.57	0.55	0.94	-1.44	2.55	IU/L
Baseline FSH -10min	0.068	1.00	16.63	2.50	0.13	-1.12	0.71	-2.61	0.37	IU/L
Baseline Prolactin -10min	0.025	1.00	15.20	1.68	0.21	31.00	23.91	-19.90	81.91	mIU/L
Baseline Testosterone -10min	0.001	1.00	11.00	0.01	0.91	-0.15	1.32	-3.05	2.75	nmol/L

Table 4.18. Effect of LEAP2 administration on baseline (fasting at -10min) markers of glucose and fat metabolism, pituitary hormones and biochemical metabolites.

This table shows the effects of LEAP2 administration on baseline (fasting at -10 min, pre-infusion) for plasma glucose, serum insulin, GH, cortisol, triglycerides, venous beta hydroxybutyrate, hormones and biochemical metabolites using linear mixed model analysis including participant ID as between participant, and LEAP2 administration as within participant fixed factors. Effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=7, visits=22. Abbreviations: GH, Growth Hormone; IU, International unit; TSH, Thyroid stimulating hormone; LH, Luteinising hormone; FSH, Follicular stimulating hormone; SSAI, Spielberger State Anxiety Inventory.

Main effect of LEAP2 administration (vs. saline) on dependent variable										
dependent variable	r2	numerator df	denominator df	F	P value	β parameter	β ± SEM	95% CI of β lower limit	95% CI of β upper limit	β parameter units
Visit order	0.12	1.00	20.00	2.91	**0.104**	0.89	0.52	-0.20	1.99	visit.min
Glucose iAUC -10 to 310min	**0.26**	1.00	16.70	9.89	**0.006**	-70.07	22.29	-117.16	-22.99	mmol/L.min
Insulin iAUC -10 to 310min	0.01	1.00	14.77	0.59	0.45	590.14	766.29	-1045.35	2225.62	mIU/L.min
GH iAUC -10 to 310min	0.00	1.00	17.22	0.01	0.91	55.68	468.43	-931.67	1043.04	mIU/L.min
Cortisol iAUC -10 to 310min	0.08	1.00	13.62	2.58	0.13	-19758.79	12304.25	-46217.37	6699.78	ug/L.min
Beta hydroxybutyrate iAUC -10 to 310min	0.12	1.00	20.00	2.80	0.11	-16.87	10.09	-37.91	4.17	mmol/L.min
Triglyceride iAUC -10 to 310min	0.01	1.00	15.10	0.30	0.59	-5.76	10.49	-28.10	16.58	mmol/L.min
LEAP2 iAUC -10 to 310min	**0.78**	1.00	18.00	66.65	**<0.001**	22547.92	2761.92	16745.35	28350.50	ng/mL.min
Sodium Δ -10 to 310min	0.01	1.00	17.54	0.17	0.69	-0.37	0.92	-2.31	1.56	mmol/L.min
Potassium Δ -10 to 310min	0.00	1.00	18.00	0.02	0.88	0.02	0.10	-0.20	0.23	mmol/L.min
Alanine aminotransferase Δ -10 to 310min	0.01	1.00	20.00	0.16	0.69	-0.30	0.76	-1.89	1.28	IU/L.min
Aspartate aminotransferase Δ -10 to 310min	0.01	1.00	11.45	0.10	0.75	0.47	1.45	-2.70	3.64	IU/L.min
Alkaline Phosphatase Δ -10 to 310min	0.02	1.00	17.51	0.45	0.51	-1.63	2.42	-6.73	3.47	IU/L.min
Gamma glutamyl tranferase Δ -10 to 310min	0.01	1.00	20.00	0.11	0.74	0.32	0.96	-1.68	2.32	U/L.min
Total bilirubin Δ -10 to 310min	0.01	1.00	13.87	0.95	0.35	-0.38	0.39	-1.23	0.46	umol/L.min
Calcium Δ -10 to 310min	0.00	1.00	20.00	0.00	0.97	0.00	0.03	-0.06	0.06	mmol/L.min
Phosphate Δ -10 to 310min	0.00	1.00	14.59	0.00	0.99	0.00	0.04	-0.08	0.08	mmol/L.min
TSH Δ -10 to 310min	0.00	1.00	14.22	0.03	0.87	0.02	0.10	-0.20	0.24	mIU/L.min
free thyroxine Δ -10 to 310min	0.00	1.00	20.00	0.09	0.77	0.12	0.41	-0.73	0.98	pmol/L.min
LH Δ -10 to 310min	0.18	1.00	17.47	4.99	0.84	-1.75	0.78	-3.39	-0.10	IU/L.min
FSH Δ -10 to 310min	0.03	1.00	18.01	0.73	0.41	-0.34	0.40	-1.19	0.50	IU/L.min
Prolactin Δ -10 to 310min	0.10	1.00	12.55	2.93	0.17	-63.30	37.00	-143.52	16.93	mIU/L.min
Testosterone Δ -10 to 310min	0.05	1.00	8.00	0.45	0.52	-0.54	0.81	-2.41	1.33	nmol/L.min
Total kcal ad libitum meal	0.00	1.00	14.27	0.00	0.95	5.75	97.37	-202.72	214.22	kcal
Total kcal %REE ad libitum meal	0.00	1.00	14.26	0.01	0.91	0.62	5.67	-11.52	12.76	kcal
Ad libitum meal time	0.02	1.00	14.94	1.10	0.31	-1.40	1.34	-4.26	1.46	mins
Total kcal VPCT	0.00	1.00	15.37	0.01	0.91	12.47	109.61	-220.67	245.62	kcal
Total kcal %REE VPCT	0.00	1.00	15.18	0.00	0.99	0.08	6.25	-13.23	13.40	kcal
Paired Associate Learning First attempt memory score	0.08	1.00	15.05	2.87	0.11	2.70	1.59	-0.69	6.09	arbitrary units
Paired Associate Learning Total errors	0.13	1.00	16.04	3.97	0.64	-8.98	4.51	-18.54	0.58	arbitrary units
Stop Signal Task Reaction time	0.02	1.00	15.62	1.58	0.23	-15.58	12.39	-41.89	10.73	milliseconds
Spatial Working Memory Between errors	0.00	1.00	14.92	0.20	0.66	42.65	42.65	-170.69	-10.66	arbitrary units
Alcohol reward overall	0.00	1.00	15.41	0.25	0.62	-0.01	0.02	-0.06	0.04	alcohol units
HE food reward overall	0.06	1.00	15.46	2.48	0.14	-0.05	0.03	-0.12	0.02	kilocalorie
LE food reward overall	0.04	1.00	15.62	1.47	0.24	-0.03	0.03	-0.09	0.02	kilocalorie
Monetary reward overall	0.05	1.00	15.19	3.49	0.08	0.01	0.00	0.00	0.01	British pounds
Implicit fat bias	0.023	1.00	15.62	0.80	0.38	-5.18	5.80	-17.49	7.13	arbitrary units
Implicit sweet bias	0.010	1.00	15.46	0.40	0.54	7.62	12.02	-17.93	33.18	arbitrary units
Appetite iAUC -10 to 310min	0.00	1.00	15.09	0.00	0.97	-0.68	18.48	-40.04	38.69	mm.min
Hunger iAUC -10 to 310min	0.01	1.00	15.37	0.25	0.63	-13.86	27.94	-73.29	45.57	mm.min
Fullness iAUC -10 to 310min	0.08	1.00	15.51	2.41	0.14	59.34	38.21	-21.87	140.55	mm.min
Food craving iAUC -10 to 310min	0.06	1.00	14.84	1.76	0.20	-2.24	1.69	-5.84	1.36	mm.min
Alcohol Urge Questionnaire iAUC -10 to 310min	0.00	1.00	15.71	0.04	0.85	-0.20	0.99	-2.30	1.91	arbitrary units.min
Nausea iAUC -10 to 310min	0.13	1.00	15.63	3.03	0.11	59.57	34.21	-13.09	132.22	mm.min
Anxiousness iAUC -10 to 310min	0.02	1.00	20.00	0.41	0.53	12.94	20.33	-29.46	55.35	mm.min
Thirst iAUC -10 to 310min	0.00	1.00	16.03	0.02	0.88	-4.94	31.40	-71.50	61.62	mm.min
Discomfort iAUC -10 to 310min	0.24	1.00	17.25	8.39	**0.010**	76.23	26.32	20.77	131.69	mm.min
Stress iAUC -10 to 310min	0.02	1.00	20.00	0.43	0.52	9.03	13.85	-19.85	37.91	mm.min
Sleepyness iAUC -10 to 310min	0.01	1.00	17.66	0.33	0.58	-11.88	20.80	-55.64	31.88	mm.min

Table 4.19. Effect of LEAP2 administration on change in total markers of glucose and fat metabolism, pituitary hormones and biochemical metabolites, VAS ratings, desired and ad libitum food intake, temporal impulsivity for alcohol, food and monetary rewards and cognitive outcomes.

This table shows the effects of LEAP2 administration on change in total (iAUC from t=-10 to 310min or change from t=-10 to 310 min) for plasma glucose, serum insulin, GH, cortisol, triglycerides, venous beta hydroxybutyrate, hormones, biochemical metabolites, VAS ratings, desired and ad libitum food intake, temporal impulsivity for alcohol, food and monetary rewards and cognitive outcomes using linear mixed model analysis including participant ID as between participant, and LEAP2 administration as within participant fixed factors. Effects on dependent variables over time with pseudo r^2 and beta parameters. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=7, visits=22. Abbreviations: iAUC, incremental area under the curve; GH, Growth Hormone; IU, International unit; TSH, Thyroid stimulating hormone; LH, Luteinising hormone; FSH, Follicular stimulating hormone; VAS, Visual analogue scale; kcal: kilocalorie; REE: Resting energy expenditure; VPCT: Virtual portion creation task; HE, High-Energy; LE, Low-Energy; CANTAB, Cambridge Neuropsychological Test Automated Battery.

Results of Paired t-tests comparing LEAP2 administration (24/25pmol/kg/min) vs. saline, n=6 participants										
Dependent variable	**Mean difference**	**Std. Deviation**	**Std. Error Mean**	**95 CI lower**	**95 CI upper**	**t**	**df**	**P**	**Cohen's d**	**units**
VAS ratings										
Appetite iAUC -10 to 310min	20.39	53.06	21.66	-35.29	76.07	0.94	5	0.39	0.38	mm.min
Hunger iAUC -10 to 310min	-0.84	82.92	33.85	-87.86	86.18	-0.03	5	0.98	-0.01	mm.min
Fullness iAUC -10 to 310min	5.76	85.90	35.07	-84.39	95.90	0.16	5	0.88	0.07	mm.min
Food craving iAUC -10 to 310min	-0.17	4.00	1.63	-4.37	4.03	-0.10	5	0.92	-0.04	mm.min
AUQ iAUC -10 to 310min	0.58	3.29	1.34	-2.87	4.03	0.43	5	0.68	0.18	arbitrary units.min
Nausea iAUC -10 to 310min	89.74	155.98	63.68	-73.95	253.43	1.41	5	0.22	0.48	mm.min
Anxious iAUC -10 to 310min	29.58	38.86	15.86	-11.20	70.37	1.87	5	0.12	0.49	mm.min
Thirst iAUC -10 to 310min	12.68	118.94	48.56	-112.14	137.50	0.26	5	0.80	0.11	mm.min
Discomfort iAUC -10 to 310min	78.02	110.86	45.26	-38.32	194.36	1.72	5	0.15	**0.70**	mm.min
Stress iAUC -10 to 310min	12.23	36.86	15.05	-26.46	50.92	0.81	5	0.45	0.33	mm.min
Sleepyness iAUC -10 to 310min	-13.66	65.63	26.79	-82.53	55.21	-0.51	5	0.63	-0.21	mm.min
Markers of glucose and fat metabolism										
Glucose iAUC -10 to 310min	-88.09	93.61	38.22	-186.33	10.15	-2.31	5	**0.069**	**-0.94**	mmol/L.min
Insulin iAUC -10 to 310min	-83.55	577.38	235.72	-689.48	522.38	-0.35	5	0.74	-0.14	mIU/L.min
GH iAUC -10 to 310min	213.31	2246.36	917.07	-2144.10	2570.72	0.23	5	0.83	0.09	mIU/L.min
Cortisol iAUC -10 to 310min	-11885.00	25702.43	10492.97	-38858.05	15088.05	-1.13	5	0.31	-0.46	ug/L.min
Beta hydroxybutyrate iAUC -10 to 310min	-9.53	21.62	8.83	-32.22	13.15	-1.08	5	0.33	-0.44	mmol/L.min
Triglyceride iAUC -10 to 310min	-8.22	22.03	8.99	-31.34	14.89	-0.92	5	0.40	-0.37	mmol/L.min
LEAP2 iAUC -10 to 310min	24471.21	-5083.02	-2075.13	29805.51	19136.92	11.79	5	**<.001**	**4.81**	ng/mL.min
Hormones										
TSH Δ -10 to 310min	-0.06	-0.35	-0.15	0.37	-0.49	-0.41	4	0.70	-0.19	mIU/L.min
free thyroxine Δ -10 to 310min	0.37	-1.66	-0.68	2.11	-1.38	0.54	5	0.61	0.22	pmol/L.min
LH Δ -10 to 310min	-1.28	-3.74	-1.53	2.64	-5.21	-0.84	5	0.44	-0.34	IU/L.min
FSH Δ -10 to 310min	0.18	-1.11	-0.45	1.35	-0.99	0.40	5	0.70	0.16	IU/L.min
Prolactin Δ -10 to 310min	-79.17	-175.15	-71.50	104.64	-262.98	-1.11	5	0.32	-0.45	mIU/L.min
Testosterone Δ -10 to 310min	-0.53	-0.53	-0.38	4.24	-5.29	-1.40	1	0.40	**-0.99**	nmol/L.min
Desired and actual food intake										
Total kcal VPCT	11.67	361.76	147.69	-367.97	391.31	0.08	5	0.94	0.03	kcal
Total kcal %REE VPCT	0.92	20.08	8.20	-20.15	21.99	0.11	5	0.92	0.05	kcal
Total kcal ad libitum meal	-20.36	314.51	128.40	-350.41	309.70	-0.16	5	0.88	-0.06	kcal
Total kcal %REE ad libitum meal	-0.46	18.14	7.40	-19.50	18.57	-0.06	5	0.95	-0.03	kcal
Ad libitum meal duration	-1.17	4.92	2.01	-6.33	3.99	-0.58	5	0.59	-0.24	mins
Delay discounting rate										
Alcohol reward	0.00	0.00	0.00	0.00	0.00	-0.45	5	0.67	-0.18	units/hour
HE food reward	-0.07	0.11	0.04	-0.18	0.05	-1.54	5	0.19	-0.63	units/hour
LE food reward	-0.01	0.06	0.02	-0.07	0.04	-0.58	5	0.59	-0.24	units/hour
Monetary reward	0.00	0.01	0.00	-0.01	0.01	0.79	5	0.47	0.32	units/day
Cognitive memory task										
Paired Associate Learning First attempt memory scor	1.50	5.36	2.19	-4.12	7.12	0.69	5	0.52	0.28	arbitrary units
Paired Associate Learning Total errors	-3.83	11.72	4.79	-16.13	8.47	-0.80	5	0.46	-0.33	arbitrary units
Stop Signal Task Reaction time	-25.88	54.66	22.32	-83.24	31.49	-1.16	5	0.30	-0.47	milliseconds
Spatial Working Memory Between errors	0.50	11.83	4.83	-11.91	12.91	0.10	5	0.92	0.04	arbitrary units
Leed Food Picture Questionnaire										
Implicit fat bias	-0.09	15.69	6.40	-17.34	15.58	-0.14	5	0.90	-0.06	arbitrary units
Implicit sweet bias	-6.21	14.19	5.79	-21.11	8.68	-1.07	5	0.33	-0.44	arbitrary units

Table 4.20. Results of paired t-test comparing visits with LEAP2 administration (24/25pmol/kg/min) vs. without (saline) on change in total markers of glucose and fat metabolism, pituitary hormones and biochemical metabolites, VAS ratings, desired and ad libitum food intake, temporal impulsivity for alcohol, food and monetary discounting rates, food bias and cognitive outcomes.

This table shows the effects of LEAP2 administration (vs. saline) on change in total (iAUC from t=-10 to 310min or change from t=-10 to 310 min) for plasma glucose, serum insulin, GH, cortisol, triglycerides, venous beta hydroxybutyrate, hormones, biochemical metabolites, VAS ratings, desired and ad libitum food intake, temporal impulsivity for alcohol, food and monetary rewards, food bias and cognitive outcomes using paired t-tests. Metrics reported are mean difference, standard deviation and standard error of mean differences, confidence intervals, t values, degrees of freedom and Cohen's d. P values in bold indicate statistical significance: * P<0.05, ** P<0.01, *** P<0.005, **** P<0.001. n=6. Abbreviations: iAUC, incremental area under the curve; GH, Growth Hormone; IU, International unit; TSH, Thyroid stimulating hormone; LH, Luteinising hormone; FSH, Follicular stimulating hormone; VAS, Visual analogue scale; kcal: kilocalorie; REE: Resting energy expenditure; VPCT: Virtual portion creation task; HE, High-Energy; LE, Low-Energy; CANTAB, Cambridge Neuropsychological Test Automated Battery.

4.7. DISCUSSION

In this small, pilot, within-participant, single-blinded, cross-over study in adults without obesity (n=7 participants, n=4 men, 22 visits), the following results were found: exogenous LEAP2 in a dose-dependent manner (Table 4.21):

(i) was safe at doses up to 25 pmol/kg/min with no adverse changes in blood pressure, heart rate or nausea ratings, or hypoglycaemia with a priori criteria (though one additional participant out of 4 men in total who received a higher dose of 36 pmol/kg/min unexpectedly had a sinus bradycardia, hypotension and junctional rhythm that resolved after cessation of infusion that may have been a vasovagal episode rather than hormone-related),

(ii) *negatively* correlated with fasting/post-prandial and fasting plasma glucose and post-prandial venous beta-hydroxybutyrate, without changes in serum insulin;

(iii) did not affect serum GH or cortisol, prolactin, TSH, free thyroxine, LH/FSH/testosterone and renal/bone/liver biochemistry

(iv) did not affect appetite, hunger, food craving VAS ratings,

(v) did not affect alcohol craving, desired or actual food intake,

(vi) did not affect temporal impulsivity to alcohol, food or monetary rewards,

(vii) did not affect cognitive memory tasks,

(viii) did not reduce food liking or wanting.

Similar results were seen when directly comparing the confirmed dose for future studies (24-25 pmol/kg/min) vs. saline (n=6 pairs), with a trend for plasma glucose to be lower over the whole infusion period at the LEAP2 visit, with no significant differences in any other outcome variables.

A potential caveat regarding the lack of significant results for food intake, impulsivity and memory was that the order of infusion visits was not randomized, with the majority or participants receiving a saline infusion first and then sequential increasing doses of LEAP2 in subsequent visits. This may have introduced order effects, as participants may have become more habituated to the tasks over time. Consequently, improvements in performance or adaptations to the testing environment could have masked or influenced the outcomes, limiting the interpretability of the findings. However, when adjusting for visit order similar results were seen.

Summary of results				
Outcome group	Whole infusion			Results vs. hypothesis
	Outcome variable	LEAP2 dose	LEAP2 administration	
Metabolic markers	Plasma glucose	↓	↓	Y, Y
	Serum insulin	o	o	C, C
	Serum GH	o	o	C, C
	Serum cortisol	o	o	Y, Y
	Venous ketones	↓	o	Y, C
	Serum triglycerides	o	o	Y, Y
	Plasma LEAP2	↑	↑	Y, Y
Hormones and biochemistry	Serum sodium	o	o	Y, Y
	Serum potassium	o	o	Y, Y
	Serum ALT	o	o	Y, Y
	Serum AST	o	o	Y, Y
	Serum ALP	o	o	Y, Y
	Serum gGT	o	o	Y, Y
	Serum Total bilirubin	o	o	Y, Y
	Serum calcium	o	o	Y, Y
	Serum phosphate	o	o	Y, Y
	Serum TSH	o	o	Y, Y
	Serum fT4	o	o	Y, Y
	Serum LH (men)	o	o	Y, Y
	Serum FSH (men)	o	o	Y, Y
	Serum prolactin (men)	o	o	Y, Y
	Serum testosterone (men)	o	o	Y, Y
VAS ratings	Appetite	o	o	C, C
	Hunger	o	o	C, C
	Pleasantness	o	o	C, C
	Fullness	o	o	C, C
	Food craving	o	o	C, C
	Alcohol Urge Questionnaire	o	o	Y, Y
	Nausea	o	o	Y, Y
	Anxiousness	o	o	Y, Y
	Thirst	o	o	Y, Y
	Discomfort	↑	↑	C, C
	Stress	o	o	Y, Y
	Sleepiness	o	o	Y, Y
Desired food intake	Total kcal	o	o	C, C
	Total kcal %REE	o	o	C, C
Actual food intake	Total kcal	o	o	C, C
	Total kcal %REE	o	o	C, C
	Meal duration	o	o	C, C
Delay discounting rate	Alcohol	o	o	Y, Y
	HE food	o	o	C, C
	LE food	o	o	C, C
	Money	o	o	Y, Y
Paired associate learning	First attempt memory score	o	o	Y, Y
	Total errors	o	o	Y, Y
Stop signal task	Reaction time	o	o	Y, Y
Spatial working memory	Between errors	o	o	Y, Y
Food hedonics	Explicit Liking	o	o	C, C
	Explicit Wanting	o	o	C, C
	Implicit Wanting	o	o	C, C
	Implicit fat bias	o	o	C, C
	Implicit sweet bias	o	o	C, C

Table 4.21. Summary of results.
This table shows the effect of exogenous LEAP2 infusion on various outcomes over the whole infusion. Abbreviations: ALT, Alanine amino transferase; AST, Aspartate aminotransferase; ALP, Alkaline phosphatase; gGT, Gamma glutamyl transferase; Growth hormone; LH, Luteinising hormone; FSH, Follicular stimulating hormone; TSH, Thyroid stimulating hormone; fT4, Free thyroxine; HE, High-Energy; LE, Low-Energy; REE, Resting energy expenditure; VAS, Visual Analogue Scale; Y, as per hypothesis; C, Contrary to hypothesis; o, No correlation or effect; ↑ increase; ↓ decrease; bracket is trend (P=0.05-0.10).

4.7.1. Safety Outcomes

Previous exogenous LEAP2 (mature peptide and fragments) infusions in men without obesity have been reported to be safe at 25pmol/kg/min (Hagemann et al. 2022; Hagemann et al. 2021). In the current study, we reported no adverse effects in the 14 LEAP2 infusions at doses 12, 24-25 and 36 pmol/kg/min that were conducted in the current study, other than one man who suffered an episode of bradycardia and hypotension that necessitated stopping the LEAP2 infusion.

It was not expected that inhibiting ghrelin receptor signalling using LEAP2 peptide would produce bradycardia with hypotension suggestive of increased parasympathetic or decreased sympathetic drive, or via a direct effect on cardiac rhythm. Ghrelin receptor expression was not found in the human heart (Ueberberg et al. 2009). Cardiac adverse events have not been reported in previous human studies of exogenous administration of AG (Garin et al. 2013) or intravenous infusions of LEAP2 (Hagemann et al. 2022; Hagemann et al. 2021), though numerical data on changes in heart rate or blood pressure have not been reported. Indeed, the literature if anything would have predicted that LEAP2 would decrease parasympathetic or increase sympathetic drive, since AG appears to have the opposite effects by improving cardiac function (Hosoda 2022).

This episode of sinus bradycardia, junctional cardiac rhythm and resulting hypotension was thus an unexpected adverse event of LEAP2 that was only been seen in 1 of 4 men receiving maintenance infusion rate of 36 pmol/kg/min. This was not due to LEAP2 peptide preparation as similar plasma LEAP2 concentrations were seen in other participants receiving the same dose, there was no endotoxin detected in the peptide and hence likely a vasovagal episode. Thus, the maximum dose was altered to 25pmol/kg/min after REC approval.
Reassuringly, four further participants did not report any adverse effects at LEAP2 infusion dose 25 pmol/kg/min, allowing this dose to be used for future studies.

4.7.2. Effect of Exogenous LEAP2 on Markers of Glucose Metabolism

In the current study, consistent with the hypothesis there was a negative correlation of increasing LEAP2 doses on fasting/post-prandial plasma glucose, but no positive

correlation between increasing LEAP2 doses and serum insulin (fasting nor post-prandial).

In rodents, administration of exogenous peripheral and/or central LEAP2 either antagonises the effects of exogenous AG to increase blood glucose or reduce insulin, and/or when administered alone, exogenous LEAP2 may have the opposite effects to AG to lower blood glucose and increase serum insulin. In *ad libitum* fed mice, intraperitoneal LEAP2 administration reduced blood glucose (Gupta et al. 2021a); while, in *ad libitum* fed mice peripheral subcutaneous AG (vs. saline) increased blood glucose (Gupta et al. 2021a); and in *ad libitum* fed wild-type mice, peripheral subcutaneous LEAP2 (vs. saline), attenuated a rise in post-prandial glucose, but this effect was not seen in *ad libitum* fed *Ghsr*-KO mice (Hagemann et al. 2022). In mice undergoing 40% food restriction, introduction of adeno-associated virus (AAV) expressing Leap2, enhanced food restriction induced fall in blood glucose (Ge et al. 2018); and in rats, central intracerebrovascular LEAP2 blocked the increase in blood glucose induced by central intracerebrovascular AG, though central LEAP2 had no effect alone (Islam et al. 2020).

While, in rat pancreatic islets, LEAP2(38-47) fragment alone increased insulin release (Hagemann et al. 2021), In 48 hour fasted wild-type mice, chronic infusion of subcutaneous LEAP2(1-12) fragment day over 2 days (vs. saline), did not decrease plasma glucose expected in a fasting state (Fernandez et al. 2022). In fasted wild-type female mice, peripheral subcutaneous LEAP2, had no effect on fasting glucose or insulin (Hagemann et al. 2022).

However, in *ad libitum* fed pregnant female mice, there was no difference in serum insulin in *Ghrelin*-KO mice (vs. wild-type) nor any difference in plasma glucose levels (Gupta et al. 2024). In rat pancreatic islets, LEAP2 blocked the suppression of insulin release induced by AG, but LEAP2 alone had no effect (Bayle et al. 2021; M'Kadmi et al. 2019); increasing LEAP2 concentrations alone did not increase insulin release in rodents (Bayle et al. 2021), However, LEAP2 alone stimulates insulin secretion from male mice pancreatic islets (Hewawasam et al. 2024), LEAP2(38-47) N-terminal

fragment alone increased insulin release in human pseudo islets, which was reversed by AG (Hagemann et al. 2021).

This suggests that LEAP2 has an antagonistic role against AG, consistently mitigating AG's effects on increasing blood glucose and reducing insulin but does not always have independent effects. Although incompletely understood the mechanism of this may be increased insulin secretion directly from the pancreas with improved insulin sensitivity and glucose uptake.

In partial agreement with the current findings, exogenous LEAP2 infusion (at 25 pmol/kg/min) in men without obesity reduced post-prandial but not fasting plasma glucose, and increased fasting but not post-prandial serum insulin (Hagemann et al. 2022). Obvious differences between the studies are inclusion of women in the current study (though no established sex differences, in pre-clinical studies, female *Leap2* -KO mice exhibit greater food intake, body weight and hepatic fat accumulation vs. wild type (Shankar et al. 2021a), while there is evidence of higher circulating plasma LEAP2 in women and girls (Andreoli et al. 2024c; Barja-Fernandez et al. 2021)), small sample size in current study, dose-response (current study used doses from 12-36pmol/kg/min) vs effect (at 25pmol/kg/min), and size of fixed meal to calculate post-prandial outcomes (current study 71 vs. 575 kcal).

In addition to LEAP2 lowering plasma glucose, there is evidence from correlation studies of a link between glucose metabolism and plasma LEAP2 though this may be an effect of glucose on LEAP2 secretion rather than vice versa. Fasting plasma LEAP2 positively correlated with fasting plasma glucose (Mani et al. 2019), in adults with normal weight/overweight/obesity (Mani et al. 2019), with obesity (Andreoli et al. 2024a; Stark et al. 2023), in overweight/obesity with pre-diabetes (Byberg et al. 2023), though not in adults without obesity without T2DM (Bhargava et al. 2023) or with T2DM (Li et al. 2022). However, OGTT (oral glucose tolerance test after a 75g oral load of glucose) increased 2 hour post-prandial plasma LEAP2 by 25% in men without obesity (n=11) (Pedersen et al. 2023), but not in women with obesity (n=13) (Ragland et al. 2023). Interestingly, iso-glycaemic intravenous glucose infusion in men without obesity

(n=11) reduced fasting plasma LEAP2 suggesting the ‘incretin’ effect with LEAP2 (Pedersen et al. 2023).

In the current study there was no positive correlation seen with serum insulin and incremental LEAP2 doses. By contrast, fasting plasma LEAP2 positively correlated with HOMA-IR in adults with normal weight/overweight/obesity (Mani et al. 2019), in women diagnosed with polycystic ovary syndrome (PCOS) with overweight/obesity, plasma LEAP2 were negatively correlated insulin, HOMA-IR (Aslanipour et al. 2020). Similarly, in paediatric populations, plasma LEAP2 was positively correlated with fasting serum insulin, HOMA-IR and triglycerides in girls (Barja-Fernandez et al. 2021), and in boys with constitutional delay of puberty, plasma LEAP2 positively correlated with fasting serum insulin, triglycerides and HOMA-IR (Varimo et al. 2022). Lastly, in lactating women with and without obesity plasma LEAP2 positively correlated with insulin (Kruger et al. 2024). In support of our findings, in a small study involving children with obesity, plasma LEAP2 did not correlate with fasting insulin or HOMA-IR (Fittipaldi et al. 2020). The previous chapter demonstrated lack of positive correlations of post-prandial LEAP2 with post-prandial insulin suggesting that post-prandial increases in LEAP2 are not driven by post-prandial increases in serum insulin (Bhargava et al. 2023). The relationship between insulin and LEAP2 is less well established and further studies are warranted.

4.7.3. Effect of Exogenous LEAP2 on Markers of Fat Metabolism

In the current study, dose dependent reduction in post-prandial venous BHB and a trend for lowering BHB throughout the study visit by exogenous LEAP2 infusions supports lipolytic effect of GHSR signalling in adults without obesity. Exogenous AG administration in adults causes lipolysis without alteration in GH concentrations (Lauritzen et al. 2020), suggesting that exogenous LEAP2 should inhibit lipolysis. In support of exogenous LEAP2 decreasing ketone concentrations, as a result of inhibiting lipolysis, in men without obesity, LEAP2 infusion (at 25pmol/kg/min) reduced fasting glycerol and both glycerol and free fatty acids in the post-prandial state (Hagemann et al. 2022). These findings with a lack of any dose-dependent effect on serum insulin or GH supports non-hormonal effect of GHSR activation on lipolysis by exogenous LEAP2. This may be an indirect action via the autonomic nervous system,

or direct action via GHSR on adipocytes, though latter action does not appear to be lipolytic (Granata et al. 2013; Lee et al. 2021).

In mice, oral ketone administration (Sodium-BHB) reduced plasma LEAP2 (Holm et al. 2022), as was also seen in men without obesity with oral or intravenous ketone administration (Sodium-BHB) achieving plasma ketones of ~ 2.5mM (Pedersen et al. 2023), although opposite results were seen in another study in men without obesity with oral ingestion of BHB-ester achieving plasma ketones of ~ 5.5mM (Holm et al. 2023). Differences were observed due to different ketone drinks. Similarly, endogenous ketonemia after prolonged fasting of 36 hours (vs. 10 hour fast) significantly reduced plasma LEAP2 in men without obesity (Pedersen et al. 2023). Similarly, in men without obesity, ketosis of ~ 0.4mM after one hour of endurance cycling reaching 70% VO2 reduced fasting plasma LEAP2 (Holm et al. 2022).

In mice, oral beta-hydroxybutyrate administration, reduced plasma LEAP2 and liver *Leap2* expression without any change in jejunal *Leap2* expression (Holm et al. 2022). While BHB administration reduced *leap2* expression in mouse hepatocytes *ex vivo*, (Holm et al. 2022), suggesting a direct hepatic action of ketone bodies to reduce LEAP2 secretion.

In the current study, there was no negative correlation with triglyceride concentrations (fasting or post-prandial). Consistent with the findings from the previous chapter wherein post-prandial increases in LEAP2 did not correlate with post-prandial increases in serum triglycerides in adults without obesity (Bhargava et al. 2023). By contrast, correlational human studies demonstrate, fasting plasma LEAP2 positively correlated with fasting serum triglycerides in adults with normal weight/overweight/obesity (Mani et al. 2019), and in overweight/obesity with pre-diabetes (Byberg et al. 2023), but not in adults with T2DM without obesity (Li et al. 2022).

All these findings suggest that mediators of LEAP2 regulation includes blood glucose, insulin and triglycerides, with liver as the main site of secretion and supports a LEAP2-GHSR-adipocyte-ketone feedback loop for fat metabolism.

Furthermore, there has been much interest in metabolic effects of long acting LEAP2, by increasing its half-life after addition of a fatty molecule to its C-terminus. Although only administered in pre-clinical studies, in *ad libitum* fed mice, acute peripheral subcutaneous administration of palmitoylated LEAP2(1-14) fragment (vs. non-palmitoylated LEAP2(1-14) fragment) attenuated subcutaneous AG induced GH release (Hola et al. 2022). Furthermore, a long-acting version of LEAP2 (through addition of a fat moiety, increasing the half-life to 5.2hours from 10-15min (Ge et al. 2018)) administered daily over 4 weeks reduced body weight, liver weight, hepatic triglycerides, plasma total cholesterol, plasma ALT, hepatic steatosis and inflammation and liver fibrosis in LEAP2-KO and wild type diet induced obese mice over 10 weeks (Shankar et al. 2024). This suggests the effects of long acting-LEAP2 in reducing body weight, improving markers of lipid metabolism and fatty liver disease.

4.7.4. Effect of Exogenous LEAP2 on Hormones and Biochemical Metabolites

In the current study there was no correlation seen with LEAP2 dose and fasting/post-prandial (71kcal) serum GH or cortisol. In contrast, in rodents, administration of exogenous peripheral and/or central LEAP2 either antagonises the effects of exogenous AG to increase blood growth hormone (GH) and/or when administered alone, exogenous LEAP2 may have the opposite effects to AG to lower GH and cortisol. In mice, peripheral intraperitoneal LEAP2 blocked, the increase in blood GH induced by peripheral intravenous AG, while peripheral administration of a LEAP2 antibody alone increased blood GH (Ge et al. 2018). Indeed, in mice, undergoing 40% food restriction, introduction of adeno-associated virus (AAV) expressing *Leap2*, attenuated food restriction induced rise in blood GH (Ge et al. 2018) and in rats, peripheral intravenous LEAP2 blocked increase in blood GH induced by peripheral intravenous AG (Islam et al. 2020). While in the only other human administration study, there was a decrease in post-prandial serum GH after a 575kcal meal (Hagemann et al. 2022).

Acyl ghrelin and total ghrelin have both been administered via different routes to a heterogeneous population of adults (including with/without obesity, pituitary diseases, diabetes, etc.), a review of all these findings suggests that acute ghrelin administration

in healthy adults increases GH, cortisol and prolactin in a dose dependent manner, but decreased LH and FSH concentrations, without causing any alteration in TSH (Garin et al. 2013). The opposite would be expected with exogenous LEAP2 infusions in humans.

We have demonstrated for the first time that increasing intravenous doses of LEAP2 does not affect, cortisol, prolactin, LH, FSH, TSH, free thyroxine (fT4), or testosterone concentrations (due to lack of correlations). On directly comparing LEAP2 administration at 24/25 pmol/kg/min which is to be used in future studies, the Cohen's d for reduction in serum testosterone is -0.99 suggesting a suppressive effect of LEAP2. This is consistent with another study, in men without obesity serum testosterone reduced at two hours after a 550kcal meal (Lehtihet et al. 2012). The number of participants required for LEAP2 administration to reduce serum testosterone with an effect size of 0.99, with alpha 0.05 and power set to 80%, is n=11.

However, these results are limited by the small sample size of adults without obesity especially when looking at men (n=4) for testosterone, LH, FSH and prolactin; these hormones and TSH, fT4 only being measured twice, and GH and cortisol four times during the infusion visit; inability to assess effects in women due to small sample size (n=3) and lack of control for menstrual cycle.

4.7.5. Effect of Exogenous LEAP2 on Plasma LEAP2

In the current study as expected exogenous LEAP2 infusion increased plasma LEAP2. However, this increase never reached steady state even after 340 mins with a double rate infusion for the first 30 mins. In contrast the only other published first-in-human LEAP2 infusion study at University of Copenhagen achieved steady-state of ~40 ng/mL (at 101 pmol/kg/min for 10 mins followed by 25 pmol/kg/min for 110 mins) in men without obesity (n=20) (Hagemann et al. 2022) (Figure 4.11A). In the same study a pilot infusion of LEAP2 was administered to study the pharmacokinetic parameters of LEAP2, achieved steady state ~ 35ng/mL (at 120 pmol/kg/min for 10 mins with followed by 30pmol/kg/min for 110 mins) in men without obesity (n=8) (Hagemann et al. 2022) (Figure 4.11B).

This discrepancy is likely due to the differences in the assay antibodies used since that study used an in-house radioimmunoassay (RIA) to measure plasma LEAP2, with polyclonal antibodies directed at just the 12 N-terminal amino acids of LEAP2, i.e. LEAP2 (38-49). The current study (n=7) used a commercial ELISA EK-075-40, Phoenix Pharmaceuticals Inc., CA, USA; https://phoenixpeptide.com/products/leap-2-38-77-human-leap-2-37-76-mouse-eia-kit), using polyclonal antibodies raised against full length LEAP2 peptide, i.e. LEAP2(38-77). Indeed, when plasma samples were assayed for LEAP2 using the commercial ELISA kit in a single participant without obesity from the LEAP2 infusion studies at University of Copenhagen there was also failure to achieve steady state (Byberg 2022) (Figure 4.11C).

These differences in assayed plasma LEAP2 concentrations suggests accumulation of C-terminal fragments of the LEAP2 peptide during exogenous infusion of full length LEAP2.

The current study achieved higher plasma LEAP2 concentrations at ~100 ng/mL (at 48-50 pmol/kg/min for 30 mins followed by 24-25 pmol/kg/min for 310mins) than seen at ~50 ng/mL by Byberg et al. (at 120 pmol/kg/min for 10 mins with followed by 30pmol/kg/min for 110 mins) (Figures 4.11C,D), there may be differences in the preparation of the peptide infusions (with current study including priming of infusion syringe and tubing with 0.5% human albumin that was not done in the University of Copenhagen studies) which may reduce peptide binding to equipment. Furthermore, data from Byberg et al. using the Phoenix Pharmaceuticals assay was only available in one participant.

Unfortunately, the antibody against LEAP2 N-terminal 12 amino acids is no longer available at University of Copenhagen, and so further antibody generation and assay development (RIA or ELISA) needs to be made to accurately measure plasma LEAP2 after exogenous administration. An alternative option is to develop mass spectrometry to measure plasma LEAP2 though this is often difficult and time consuming to develop.

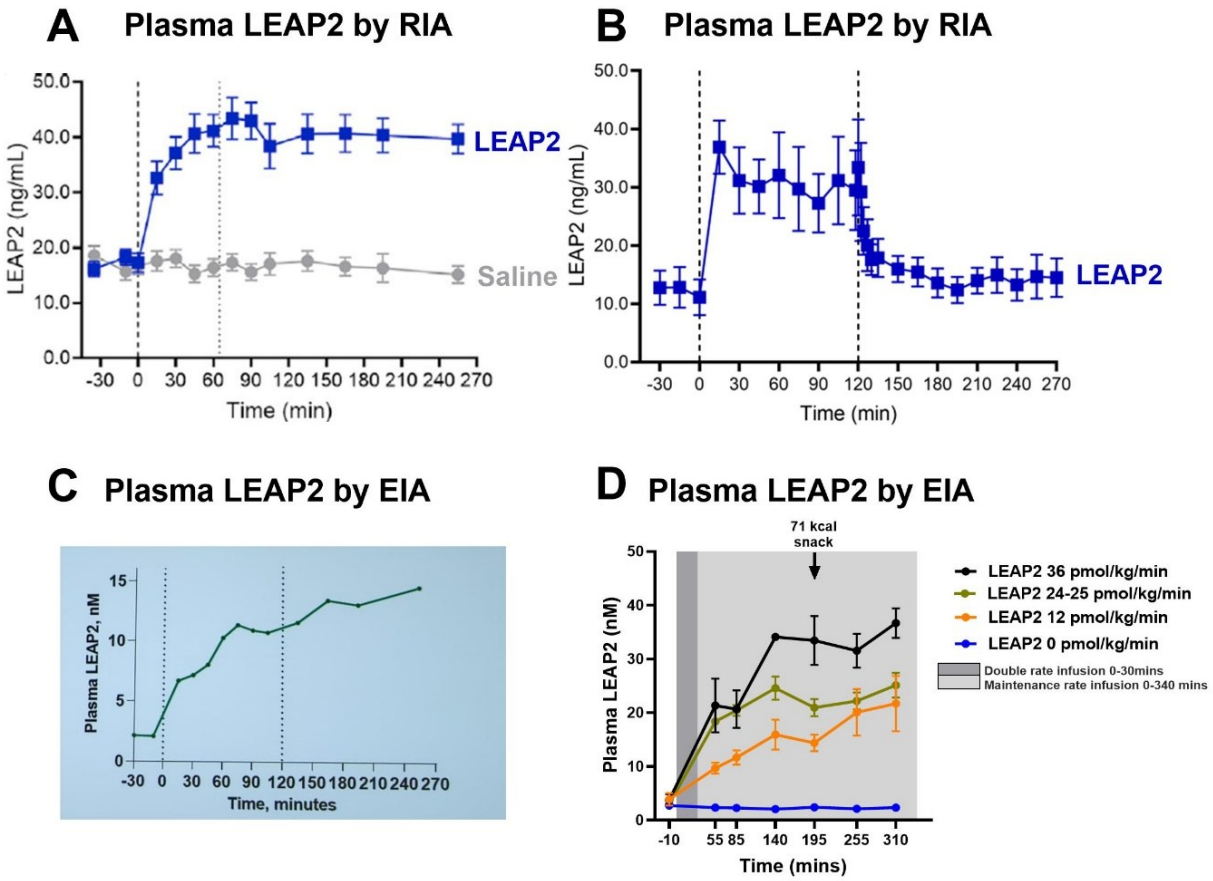


Figure 4.11. Comparison of RIA vs. ELISA assay to measure plasma LEAP2.
Differences between (A) RIA assay by Hagemann et al. 2022 (Hagemann et al. 2022) in n=20 men without obesity at ~25 pmol/kg/min, (B) RIA by Hagemann et al. 2022 (Hagemann et al. 2022) in n=8 men without obesity at ~30 pmol/kg/min, (C) EIA assay by Byberg et al. 2022 (Byberg 2022) in n=1 man without obesity at ~30 pmol/kg/min and (D) EIA in current study in n=7 adults without obesity at doses from 0-36 pmol/kg/min; Grey bars indicate infusion duration (double rate: 0-30mins and rates in legend: 30-340min) and dotted lines indicate duration of infusion. Abbreviations: RIA, Radio immunoassay; EIA, Enzyme immunoassay.

4.7.6. Effect of Exogenous LEAP2 on Appetite

Contrary to our hypothesis, there was no significant dose-responsive or overall effect or LEAP2 administration on composite appetite, hunger and fullness, VAS ratings or food craving. This is consistent with the previous study of intravenous LEAP2 in adult men without obesity (Hagemann et al. 2022) despite reducing *ad libitum* food intake there was no difference in hunger, fullness and satiety.

In support of our findings, from our research group's unpublished findings, in adults with obesity and T2DM (n=46), fasting plasma LEAP2 did not correlate with hunger or

appetite ratings (Guo 2024). Similarly, in adults with normal weight, overweight and obesity (n=90), fasting plasma LEAP2 ratio did not correlate with appetite ratings (Goldstone et al. 2022). This study's negative findings differ from previous cross-sectional correlational studies. In adults with overweight/obesity fasting plasma LEAP2 negatively correlated with fasting hunger ratings, but there was no correlation between absolute post-prandial plasma LEAP2 and post-prandial hunger ratings (Andreoli et al. 2024a). Additionally, in the current study, the consent form for participants suggested that their participation involved the study of gut hormone LEAP2 and its effects on appetite and eating behaviours which could have introduced a bias in the results. However, it is unlikely that such a sentence in the consent form would interfere with the results on appetite and eating behaviour as the protocols/consent form for these studies are established after long experience investigating numerous gut peptide infusions on appetite. Participants were also screened for any eating disorders with the use of validated questionnaires. The most likely reasons why null results on eating behaviour were found in the current study include small sample sizes given that the current study was not adequately powered to measure such a difference, as the primary aim of the study was to demonstrate safety and tolerability of exogenous LEAP2 infusions, and the lack of complete order randomisation.

In lean men (n=10), exogenous consumption of a ketone drink (BHB-ester) achieving ~5.5nM or plasma BHB, increased plasma LEAP2 and negatively correlated with a reduction in hunger and positively with fullness and satiety ratings (Holm et al. 2023) however, the analysis did not account for within-participant factors and used values at multiple time points and correlated into one regression model rather than using incremental changes (as we have done).

Finally, as seen in Chapter 2 in adults without obesity, post-prandial increases in LEAP2 correlated positively with post-prandial decreases in appetite and fullness (Bhargava et al. 2023). This further underscores the role of LEAP2 on GHSR signalling to reduce appetite. In the current study a small sample size and order effects may pose limitations, even though no significant correlations were observed when including visit number as a covariate.

The current study did not find a correlation between LEAP2 administration and alcohol urge. Although all participants consumed alcohol (as per inclusion criteria) but they did not have alcohol use disorder or were heavy drinkers (as per exclusion criteria). Apart from the small sample size, the study was conducted in the morning which is an unusual time for most participants to consume alcohol and may have contributed to the lack of findings.

However, in rodent studies intraperitoneal administration of LEAP2 did not reduce alcohol intake in mice, while central administration of LEAP2 decreased alcohol intake in heavy-drinking mice across both sexes (Richardson et al. 2023). This was replicated in another study where central LEAP2 administration not only reduced food intake, but also alcohol consumption in rats (Tufvesson-Alm et al. 2024a). In humans, oral administration of PF-5190457, a GHSR1a inverse agonist (similar action to LEAP2) in adults with alcohol dependence reduced craving for alcohol without any adverse effects (Lee et al. 2020b).

Taken together, this suggests that downregulating the GHSR modulates reward and responses related to alcohol, leading to reduced consumption of alcohol by its central actions. Future studies of LEAP2 administration in adults with alcohol use disorder are needed.

The current study did not show any correlation between LEAP2 dose and adverse outcomes viz. nausea, stress, sleepiness, anxiousness, thirst, food craving VAS ratings or alcohol urge questionnaire scores. These findings are consistent with the first-in-humans exogenous LEAP2 infusion study where in no differences were seen in nausea, comfort or thirst ratings (vs. placebo arm) (Hagemann et al. 2022). Lastly, a statistically significant increase in discomfort ratings may be contributed by repeated cannulation during the LEAP2 infusion visit in some participants, but this correlation was not seen when including visit number as a covariate.

4.7.7. Effect of Exogenous LEAP2 on Desired and Actual Food Intake

Contrary to our hypothesis, in the current study, intravenous infusion of supraphysiological LEAP2 did not reduce desired or actual food intake (*ad libitum meal*

intake). This lack of results may be contributed by a smaller sample size, although our study was designed to reduce variability as a within-participant trial and there was a lack of randomisation between saline and LEAP2 infusion visits, i.e. there may be an order effect, where in the participants find the food initially stimulating, but then are accustomed to it however, similar results were seen when including visit number as a covariate. The only other human infusion study demonstrated a 12% reduction in *ad libitum* food intake with exogenous LEAP2 at 25 pmol/kg/min. Although this study has greater food choices (four vs. one) than the first-in-human infusion study (Hagemann et al. 2022), our meals consisted of semi-solid food, primarily white in colour, this was done to elicit effects of low/high fat and sweet/savoury on food intake.

Correlation studies in support of the above in adults with obesity, fasting plasma LEAP2 negatively with food intake (estimated as kcal %REE) at an *ad libitum* meal (Goldstone et al. 2022). As seen in chapter 2, in adults without obesity, there was a trend for the post-prandial increase in plasma LEAP2 to negatively correlate with the post-prandial decrease in food intake (Bhargava et al. 2023). However, in adults with obesity and T2DM, consistent with our findings fasting LEAP2 did not correlate with food intake at an *ad libitum* meal (Guo 2024).

Altogether, there is inconsistent evidence supporting a role for LEAP2 to inhibit GHSR signalling in attenuating human eating behaviour and larger studies are required as there are discrepancies in study populations and nutritional state.

4.7.8. Effect of Exogenous LEAP2 on Temporal Impulsivity and Cognitive Tasks

In the current study in adults without obesity, exogenous LEAP2 infusion in increasing doses did not correlate with the hypothesised reduction in alcohol, high-energy/low-energy food or monetary rewards. Neither did increasing doses of LEAP2 correlate with the hypothesised inhibition of motor responses (an impulsivity measure), working or episodic (hippocampal dependent) memory from the CANTAB tasks.

Peripheral endocrine signals including ghrelin acting on the hippocampus are involved in food consumption and memory (Kanoski et al. 2017). In rodents, ghrelin signalling in the hippocampus counteracts anorexigenic peripheral endocrine signals such as

cholecystokinin, exendin-4 (GLP-1 receptor agonist), amylin and mechanical distension of the stomach, to increase food intake (Suarez et al. 2020; Suarez et al. 2019).

In mice, central administration of LEAP2 decreased alcohol intake in heavy-drinking rodents across both sexes (Richardson et al. 2023), and also reduces alcohol consumption in alcohol consuming rats without reduction in body weight (Tufvesson-Alm et al. 2024a). Central administration of LEAP2 into the third ventricle prevented the ability of alcohol to cause locomotor stimulation, attenuate the dopamine release in the nucleus accumbens and suppress the memory of alcohol reward in mice (Tufvesson-Alm et al. 2024a). However, there was no reduction in alcohol intake with peripheral administration of LEAP2 (Richardson et al. 2023). This suggests that LEAP2 modulates reward and responses related to alcohol, leading to reduced consumption of alcohol by its central actions. A systematic review of participants with eating disorders and obesity, suggest increased temporal impulsivity to monetary and food rewards (McClelland et al. 2016).

Exogenous ghrelin administration (1mcg/kg i.v.) (vs. saline) in fed adults without obesity increased recall of HE food (vs. neutral) pictures shown 30 mins after fMRI scan (Malik et al. 2008), without any change in HE food picture hedonic ratings. Furthermore, increasing endogenous ghrelin by fasting, increased memory recall of food (vs. non-food) pictures in humans with and without obesity and consumption of HE sweet and savoury food to satiation reduced memory for food recall (Morris et al. 2001). However, exogenous ghrelin administration (bolus 100mcg twice i.v., vs. saline) to fed participants without obesity had no effect on BOLD signal during encoding or consolidation of memory to HE or LE food words, or on their recall the following day (Kunath et al. 2016). There was no effect of ghrelin administration on cognitive testing including nonverbal fluid reasoning task, working memory task, creativity task, perceptual speed tests and tests for reaction times using non-food related stimuli (Kunath et al. 2016).

The reasons for lack of finding with temporal discounting and memory tasks, in our study is likely due to a small sample size, our participants were adults without obesity,

and none were diagnosed with any eating disorder. Future work should include exogenous LEAP2 administration in larger and more diverse populations.

4.7.9. Effect of Exogenous LEAP2 on Food Liking and Wanting

In the current study exogenous LEAP2 infusions did not influence explicit liking/wanting or implicit wanting for sweet vs. savoury or high-fat vs. low-fat foods.

AG has been shown to significantly influence food intake and preference in rodent and humans. In mice, peripheral total ghrelin (10nmol, intraperitoneally) promotes the intake and preference for sweet foods, regardless of their caloric content (vs. GHSR-KO mice) (Disse et al. 2010).

In humans, using the LFPQ, in 30 men with obesity, implicit wanting and relative preference for savoury foods decreased in the fed state (10mins after a 600kcal mixed meal) vs. fasted, whereas scores for sweet foods increased (Alkahtni et al. 2016). Explicit liking and explicit wanting were also lower for savoury vs. sweet foods in the fed state (Alkahtni et al. 2016). Similarly, in 15 adults (9 men) without obesity; explicit wanting and liking significantly decreased for average across high/low fat, sweet/savoury food categories in the fed state (30min after a 781kcal mixed meal) vs. fasted (Cameron et al. 2014). Furthermore, in 29 adults with obesity (3 men) 2 years after Roux-en-Y gastric bypass (RYGB) surgery, ratings for explicit liking and wanting were lower in the fed state (after a 300kcal mixed meal) vs. fasted (McInnis et al. 2022).

Furthermore, in 14 adults with obesity, comparing the effects of RYGB surgery (2 years post-surgery vs. before surgery), on explicit liking and wanting, there was a decrease for sweet foods and HF foods and, implicit wanting switched towards savoury (vs. sweet) foods and low-fat (vs. high-fat) foods post-surgery (Aldubaikhi et al. 2019). However, in participants with obesity and T2DM there was no differences in the effects of endoluminal duodenal-jejunal bypass liner (DJBL) insertion vs. standard medical management on explicit liking, wanting or implicit wanting after 6 months (Ruban et al. 2020). These findings collectively demonstrate the role of feeding

(elevated endogenous LEAP2, lowered endogenous AG) in modulating food preference and reward-seeking behaviour in human across different patient groups.

To the best of our knowledge, this is the first study to elicit the effect of exogenous LEAP2 on food liking and wanting. The most likely reasons for the lack of findings are that participants in our study were of a relatively small sample size (n=7), were of normal weight without any eating disorders and duration of the LEAP2 infusion may not have been long enough to elicit meaningful results.

4.7.10. Strengths and Limitations

The novelty of this study is that it is only the second time known to us that LEAP2 has been infused in adults without obesity and the first in the world to females without obesity. Despite the small number of participants in this study, this is a repeated measures design, which increases the power of the study. We also reconfirmed the safety profile of the LEAP2 peptide with and infused multiple doses of LEAP2 to demonstrate a dose-response relationship.

Due to the focus of this pilot infusion study on safety, there were limited number of samples for pulsatile hormones such as serum GH, LH and FSH. There were not enough participants by sex to assess changes in gonadotrophins and sex hormones by sex and female participants were not controlled for menstrual cycle or hormonal contraception. Lastly, this was a single-blinded study, and some participants were given LEAP2 in a non-randomised dose order with the saline infusion being administered in their first visit.

4.7.11. Future Work

Given that LEAP2 acts in a reciprocal manner to AG, it will be important to measure the effect of LEAP2 peptide infusion on plasma AG, although no change was observed in the previous publication (Hagemann et al. 2022). Future studies are planned with the confirmed safe dose of 25 pmol/kg/min in a larger sample size (n=24) and assessment of metabolism, eating and addictive behaviours as in this chapter but with addition of fMRI with food cue reactivity to HE and LE foods, food, money and alcohol reward anticipation (incentive delay task) and negative emotional reactivity in adults

without and with obesity (see Chapter 5).

4.8. CONCLUSIONS

In conclusion, we demonstrate that exogenous LEAP2 infusion in adults without obesity reduces fasting plasma glucose and post-prandial venous beta-hydroxy butyrate concentrations in a dose-dependent manner supporting a glycolytic and lipolytic pathway of GHSR signalling. These effects are observed without changes in any other pituitary hormones, serum insulin or GH supporting non-hormonal effect of GHSR on glycolysis and lipolysis. The findings underscore LEAP2's therapeutic potential in managing obesity and T2DM by modulating GHSR activity, but translating these successes to human applications will require further research to fully elucidate its safety profile, pharmacodynamics, and efficacy across different patient groups.

CHAPTER 5: GENERAL DISCUSSION

5.1. THESIS AIMS

This thesis aimed to characterise the regulation, safety and effects of the novel liver/gut hormone LEAP2 in human metabolism and eating behaviour.

This was done by measuring LEAP2 from a biorepository of plasma samples from two previously published clinical studies of (i) examining the effects of food intake, and its correlations with changes in eating behaviour including food cue reactivity using fMRI, and after exogenous AG administration, in adults without obesity (Goldstone et al. 2014) and (ii) comparing the effects of long-term caloric restriction without or with duodenal-jejunal bypass liner (DJBL) insertion in adults with obesity and poorly controlled type 2 diabetes mellitus, on body weight, markers of glucose and fat metabolism (Glaysher et al. 2017; Ruban et al. 2020; Ruban et al. 2022) using a commercial ELISA assay (Phoenix Pharmaceuticals Inc, CA, USA, EK-075-40).

Furthermore, through intravenous administration, in a pilot human experimental study to examine the safety, metabolic and human eating behaviour-related effects of exogenous LEAP2 in adults without obesity in a dose dependent manner.

Aims were as follows:

(i) to examine the metabolic and hormonal influences on plasma LEAP2 in humans;
(ii) to examine correlations of plasma LEAP2, AG and AG/LEAP2 ratio with appetite, food cue reactivity and food intake, from previous studies including acute infusion of AG, and weight loss by LCD without/with DJBL insertion, by assaying stored samples in adults without and with obesity;
(iii) to confirm the safety and dosage of intravenous LEAP2 administration to achieve mildly supraphysiological plasma LEAP2 concentrations and;
(iv) to obtain pilot data on tolerability and examine a dose-response relationship of exogenous LEAP2 on metabolism and eating behaviour, in an experimental medicine study in humans without obesity.

5.2. THESIS HIGHLIGHTS

Gaps in knowledge addressed from this thesis include:

(i) Post-prandial increases in plasma LEAP2 positively correlated with post-prandial decreases in appetite ratings (Chapter 2).

(ii) Post-prandial increases in plasma LEAP2 correlated with post-prandial decreases in food cue reactivity for HE and LE foods and, food intake (trend) in adults without obesity (Chapter 2).

(iii) Post-prandial increases in plasma LEAP2 are unlikely to be driven by post-prandial decreases in plasma AG due to lack of correlations, and exogenous AG administration does not affect post-prandial plasma LEAP2 (Chapter 2).

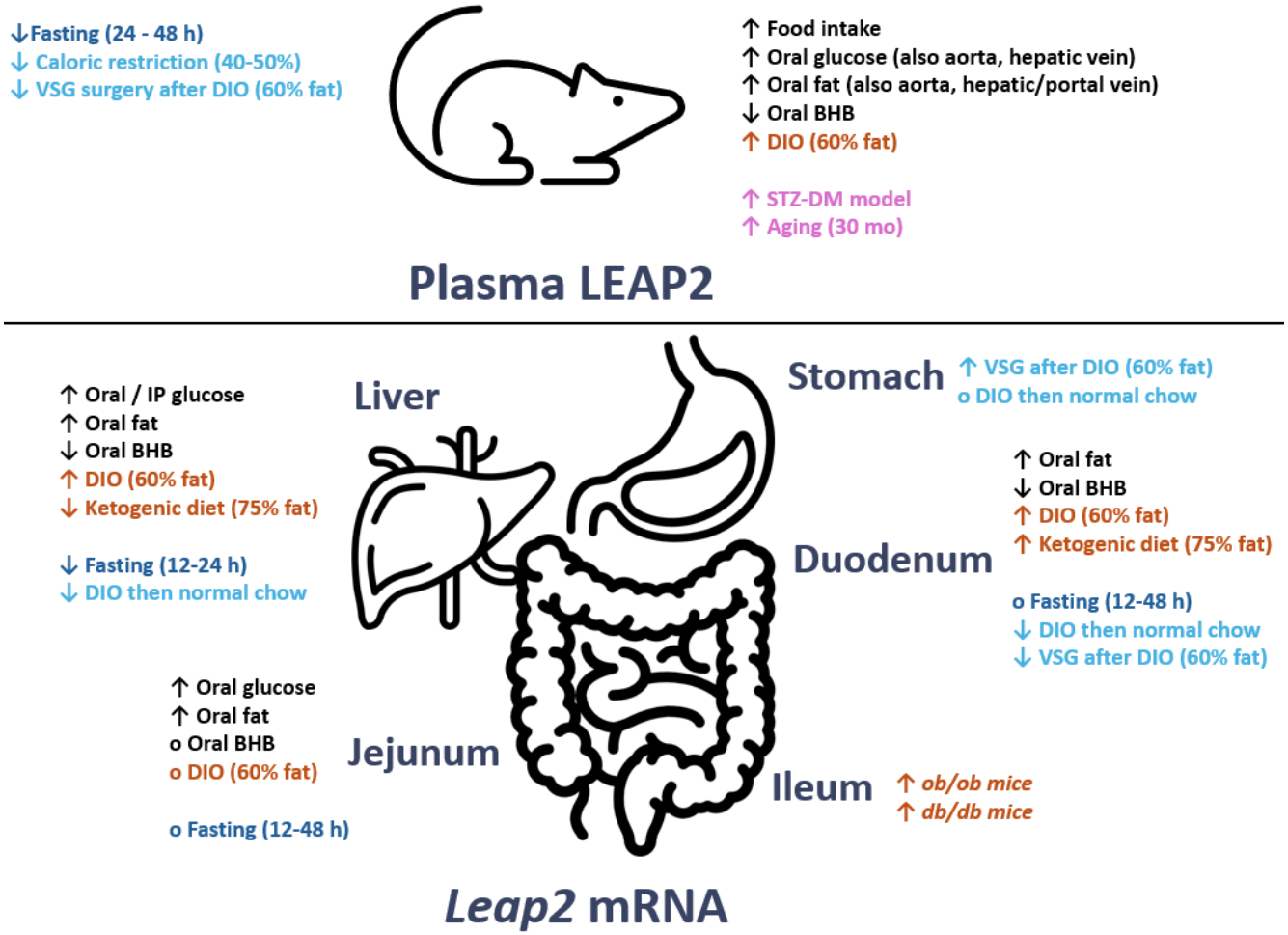
(iv) Long term weight loss, over 2 years, by dietary restriction or duodenal-jejunal bypass liner (DJBL) insertion reduced fasting plasma LEAP2 (delayed and attenuated in the latter) in adults with obesity and type 2 diabetes mellitus (Chapter 3).

(v) Weight loss related improvements in weight, markers of glucose and fat metabolism correlated with decreases in fasting plasma LEAP2 (Chapter 3).

(vi) Lack of differences in post-prandial increases in plasma LEAP2 between diet induced or foregut bypass (via DJBL insertion) suggests liver (rather than foregut) as source of post-prandial LEAP2 secretion (Chapter 3).

(vii) Intravenous exogenous LEAP2 up to 25 pmol/kg/min infused over 5.7 hours was safe in adults without obesity without nausea, hypoglycaemia or cardiac rhythm abnormalities (Chapter 4).

(viii) Intravenous exogenous LEAP2 dose dependently (12-36 pmol/kg/min) reduced fasting plasma glucose and post-prandial beta-hydroxybutyrate without changes in serum insulin, GH, cortisol, thyroid function or renal/bone/liver biochemical metabolites (Chapter 4).



Positive Energy Balance	Acute	Black
	Chronic	Orange
Negative Energy Balance	Acute	Dark blue
	Chronic	Light blue
Disease states	Pink	

Figure 5.1. Effects of interventions on plasma LEAP2 and mRNA expression in rodents.
Abbreviations: BHB, Beta-hydroxy butyrate; DIO, Diet induced obesity; h, Hours; mo, months; STZ-DM, Streptozotocin diabetes mellitus model; VSG, Vertical sleeve gastrectomy. ↑, Increased; ↓, Decreased; o, No effect.

Outcome	State	Treatment	Peripheral LEAP2		Central LEAP2	*Leap2-KO* mouse
			Rodent	Human	Rodent	
Plasma glucose	Baseline	acute	↓ LEAP2 Fasted NW	o LEAP2 (38-47) Fasted NW ↓ LEAP2 Fed NW ↓ LEAP2 Fasted/Fed OB ↓ LEAP2 Fasted/Fed NW/OW [4]	↓ icv LEAP2 Fasted NW	
		chronic	o LEAP2, LEAP2(38-49), LEAP2(38-51) ± palmitic acid Fasted NW/DIO			
	AG induced	acute	↓ LEAP2 Fed NW		↓ icv LEAP2 Fed NW	
		chronic				
Serum insulin	Baseline	acute	↑ LEAP2 Fasted NW	o LEAP2 (38-47) Fasted NW ↑LEAP2 Fasted/Fed NW o LEAP2 Fasted/Fed NW/OW [4]	↑ icv LEAP2 Fasted NW	
		chronic	o LEAP2 Fasted NW/caloric restriction			
	AG induced	acute	↑ LEAP2 Fasted NW		↑ icv LEAP2 Fasted NW	
		chronic				
Serum GH	Baseline	acute	↓ LEAP2 Fasted NW ↓ LEAP2(38-51) ± palmitic acid Fasted NW	↓ LEAP2 Fed NW o LEAP2 Fasted/Fed NW/OW [4]		o Fed NW
	AG induced	acute	↓ LEAP2 Fed NW ↓ LEAP2(38-51) ± palmitic acid Fed NW			↑ Fed NW
BHB (Ketone)	Baseline	acute		↓ LEAP2 Fed NW/OW [4]		

Figure 5.2. Effects of exogenous LEAP2 administration on metabolism in rodents and humans.
Abbreviations: AG, Acyl ghrelin; BHB, Beta-hydroxy butyrate; DIO, Diet induced obesity; GH, Growth hormone; LC, Locus coeruleus; LDTg, Lateral dorsal tegmental area; NW, Normal weight (BMI <25.0 kg/m^2); OW, Overweight (BMI 25.0-29.9 kg/m^2); OB, Obese (BMI ≥30.0 kg/m^2). LEAP2 refers to the full mature peptide (38-77) unless specified. ↑, Increased; ↓, Decreased; o, No effect.
[4] Results from Chapter 4.

Outcome	State	Treatment	Peripheral LEAP2		Central LEAP2	*Leap2-KO* mouse
			Rodent	Human	Rodent	
Food intake	Baseline	acute	↓ LEAP2 ± fat moiety Fasted/Fed NW/DIO ↓ LEAP2 (38-51) ± palmitic acid Fasted/Fed NW/DIO o LEAP2 (38-47), LEAP2 (23-47), LEAP2 (49-77) Fed NW	↓ LEAP2 Fed NW ↓ LEAP2 Fed OB o LEAP2 Fed NW/OW [4]	↓ icv LEAP2 Fasted/Fed NW/DIO ↓ intra-LC LEAP2 Fasted NW ↓ intra-LDTg LEAP2 Fasted NW	o Fasted NW o Fasted DIO
		chronic	o LEAP2 Fed NW/caloric restriction o LEAP2 (38-51) ± palmitic acid Fasted/Fed NW/DIO		↓ icv LEAP2 Fasted NW	o NW ↑ DIO
	AG induced	acute	↓ LEAP2 ± fat moiety Fed NW ↓ LEAP2 (38-51) ± palmitic acid Fed NW		↓ icv LEAP2 Fed NW/DIO	↑ Fed NW/DIO
		chronic				
Appetite	Baseline	acute		o LEAP2 (38-47) Fasted NW o LEAP2 Fasted/Fed NW o LEAP2 Fasted/Fed NW/OW [4]		
Alcohol intake	Baseline	acute	o LEAP2 Fasted NW		↓ icv LEAP2 Fasted NW	

Figure 5.3. Effects of exogenous LEAP2 administration on ingestive behaviour in rodents and humans.
Abbreviations: AG, Acyl ghrelin; BHB, Beta-hydroxy butyrate; DIO, Diet induced obesity; GH, Growth hormone; LC, Locus coeruleus; LDTg, Lateral dorsal tegmental area; NW, Normal weight (BMI <25.0 kg/ m^2); OW, Overweight (BMI 25.0-29.9 kg/ m^2); OB, Obese (BMI >30.0 kg/m m^2). LEAP2 refers to the full mature peptide (38-77) unless specified. ↑, Increased; ↓, Decreased; o, No effect.
[4] Results from Chapter 4.

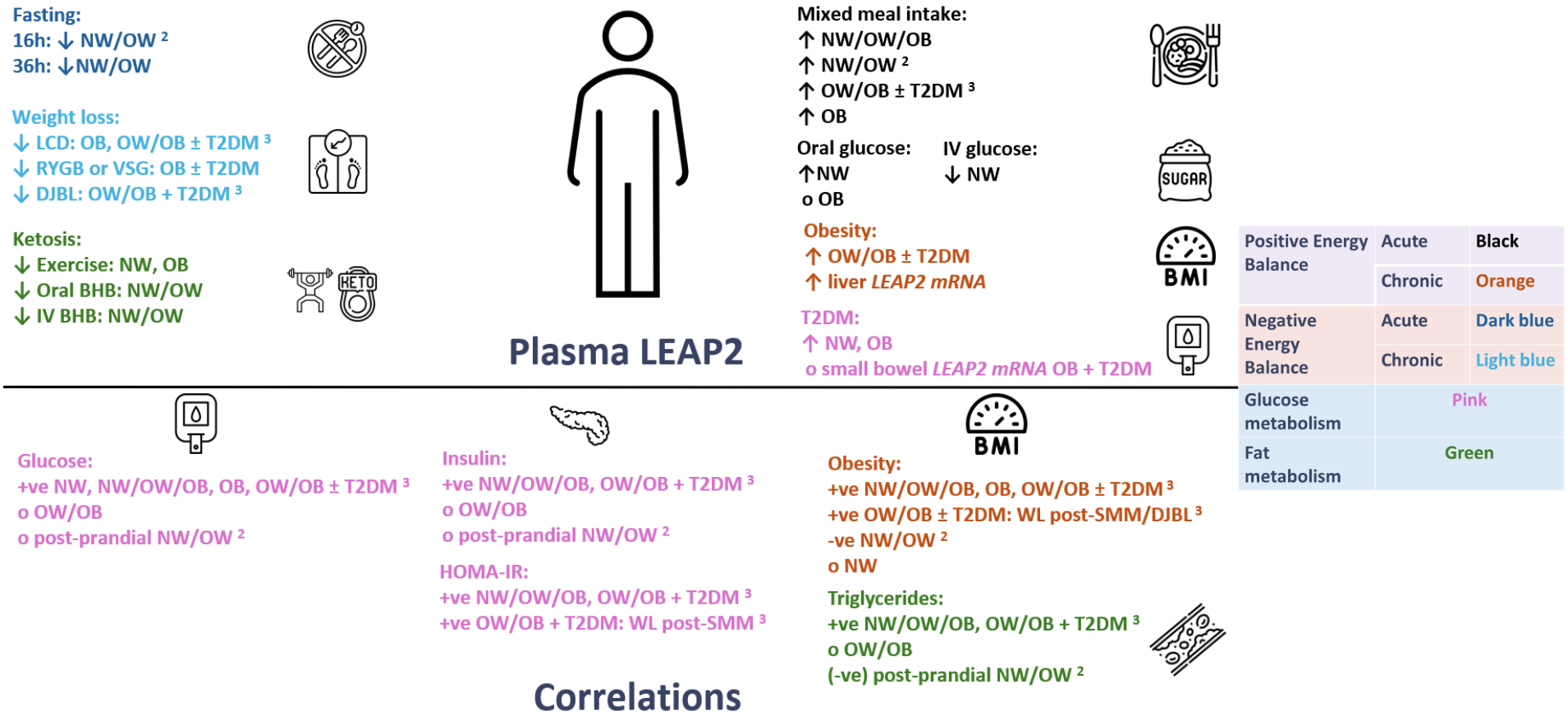


Figure 5.4. Effects on plasma LEAP2 and its correlations with markers of glucose and fat metabolism in humans.
Abbreviations: DJBL, Duodenal-jejunal bypass liner; IV, Intravenous; NW, Normal weight (BMI <25 kg/ m^2); OW, Overweight (BMI 25.0-29.9 kg/ m^2); OB, Obese (BMI >30.0 kg/ m^2); IV, Intravenous; SMM, Standard medical management; T2DM, Type 2 Diabetes Mellitus; WL, Weight loss; ↑, Increased; ↓, Decreased; o, No effect or no correlation seen. All correlations are with fasting plasma LEAP2 unless specified.
[2] Results from Chapter 2; [3] Results from Chapter 3

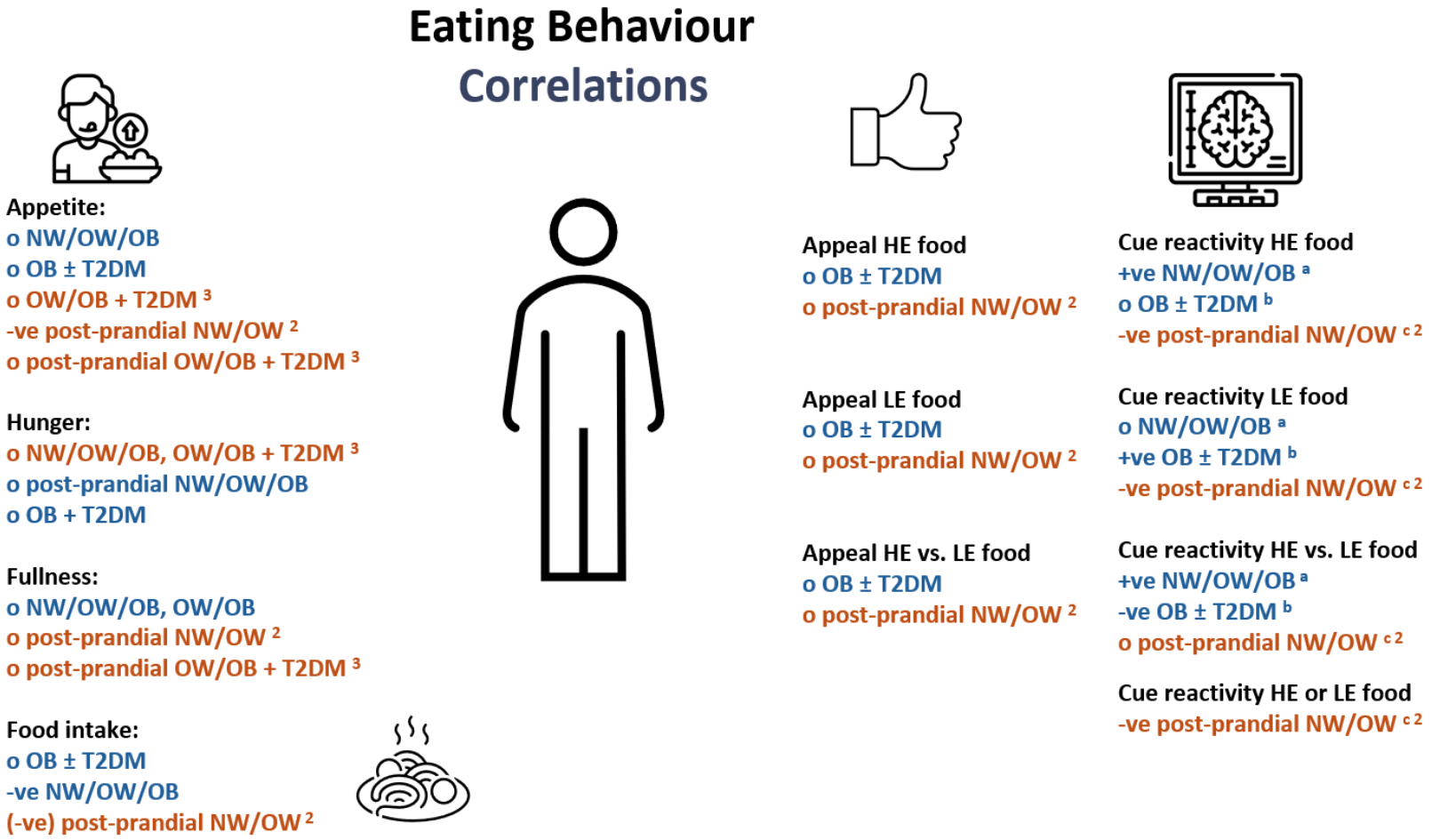


Figure 5.5. Correlations of plasma LEAP2 with eating behaviour in humans.
Abbreviations: HE, High energy; LE, Low energy; NW, Normal weight (BMI <25 kg/m^2); OW, Overweight (BMI 25.0-29.9 kg/ m^2); OB, Obese (BMI >30.0 kg/ m^2); T2DM, Type 2 diabetes mellitus; [a] Posterior cingulate cortex; [b] Nucleus accumbens, caudate; [c] Anterior/posterior cingulate cortex, paracingulate cortex, frontal pole, middle frontal gyrus. o No correlation seen. All correlations are with fasting plasma LEAP2 unless specified. Brackets indicate a trend. Result in orange from this thesis.
[2] Results from Chapter 2, [3] Results from Chapter 3

5.3. LEAP2 AND GLUCOSE METABOLISM

This thesis explores the complex relationship between LEAP2, glucose, and insulin metabolism (see Figures 5.1, 5.2, 5.4). This thesis builds on preclinical findings in rodents that both peripheral and central administration of exogenous LEAP2 reduces glucose and opposes AG's glucose-raising effects (Figure 5.2, Table 1.4). In rodent studies, exogenous LEAP2 increases insulin secretion in specific scenarios, such as in pancreatic islets or through LEAP2 fragments though its effects vary by experimental conditions (Table 1.6).

In humans, correlational studies including this thesis allude to the role of LEAP2 in improving markers of glucose metabolism. Fasting plasma LEAP2 has been positively correlated with fasting glucose in various populations, including those with overweight/obesity, prediabetes and PCOS (Table 1.10). But these correlations are not consistent in all groups, such as individuals without obesity (Chapters 2). However, improvements in fasting glucose in adults with obesity and T2DM correlated with reductions in fasting plasma LEAP2 after dietary weight loss over 2 years suggesting that glucose may be a mediator of LEAP2 regulation (Chapter 3).

LEAP2 levels increase post-OGTT in men without obesity but not in women with obesity (Figure 1.4). Conversely, intravenous glucose infusion but not oral glucose ingestion can reduce fasting LEAP2, suggesting an "incretin-like" effect in men without obesity (Pedersen et al. 2023).

LEAP2 positively correlates with insulin resistance (HOMA-IR) in adults with overweight/obesity, PCOS, and paediatric populations. However, these correlations are inconsistent, with some studies reporting no association between LEAP2 and insulin sensitivity or fasting insulin (Table 1.10). The lack of correlation in several populations including those without or with obesity and T2DM (Chapters 2 and 3) and conditions (e.g. post-prandial states) underscores the complexity of the LEAP2-insulin relationship.

In exogenous full length LEAP2 infusion studies in humans with or without obesity (including Chapter 4), there was a reduction in fasting and post-prandial glucose with

increases in fasting and post-prandial insulin only in men without obesity (Hagemann et al. 2022). Differences in human findings may be attributed to sample size, meal size, dose, obesity status and inclusion of women as in Chapter 4.

Although in humans, LEAP2's independent effects on glucose and insulin are inconsistent, with variations based on context (e.g. fasting vs. fed states, or sex). LEAP2 appears to act as a regulatory antagonist to AG, mitigating AG's glucose-raising and insulin-suppressing effects. Plasma LEAP2 secretion appears to be regulated by glucose, with the liver as a primary source. However, its independent role in modulating glucose and insulin is less consistent and may involve improved insulin sensitivity and glucose uptake. Further research is needed to clarify the mechanisms and context-dependent effects of LEAP2 on glucose and insulin metabolism in humans.

5.4. LEAP2 AND FAT METABOLISM

The thesis highlights the role of LEAP2 in regulating lipolysis and ketone metabolism, primarily through its interaction with the GHSR (Figures 5.1, 5.2, 5.4).

Previous studies in humans have found positive correlations between fasting LEAP2 and triglycerides in individuals with overweight or obesity with or without pre-diabetes, indicating possible context-dependent regulation. In this thesis, post-prandial increases in plasma LEAP2 tended to negatively correlate with post-prandial increases in triglycerides in adults without obesity or T2DM (Chapter 2). In a long-term low-calorie diet intervention in participants with obesity with T2DM, fasting plasma LEAP2 over a 2-year period positively correlated with fasting serum triglycerides (Chapter 3) suggesting that it may mediate plasma LEAP2 regulation. However, in the two exogenous LEAP2 infusion studies in humans (including this thesis), there were no reductions seen (fasting or post-prandial) in serum triglycerides (Chapter 4).

Nevertheless, other markers of fat metabolism demonstrated a role of LEAP2 in improving lipid metabolism. Exogenous ketones and endogenous ketosis (from fasting or exercise) reduce plasma LEAP2 levels in both mice and humans (Holm et al. 2022; Pedersen et al. 2023). In mice, ketone-induced reductions in LEAP2 are associated

with decreased liver *Leap2* expression, suggesting direct hepatic regulation (Holm et al. 2022). Furthermore, in humans exogenous LEAP2 infusions reduce post-prandial beta-hydroxybutyrate (BHB) levels in adults without obesity (Chapter 4) and fasting glycerol concentrations with reductions in post-prandial free fatty acids in men without obesity (Hagemann et al. 2022), supporting its inhibitory effect on lipolysis.

This effect appears to be independent of changes in insulin or GH suggesting a non-hormonal mechanism possibly involving the autonomic nervous system or direct action on adipocytes.

These findings support a proposed feedback loop involving LEAP2, GHSR, adipocytes, and ketone metabolism. Overall, the findings suggest LEAP2 plays a crucial role in fat metabolism through GHSR signalling and may serve as a target for obesity and lipid-related conditions.

5.5. LEAP2 AND EATING BEHAVIOUR

The novelty of this thesis is investigating, confirming and building on from preclinical studies the relationship of satiety gut hormone LEAP2 on various eating behaviour measures (Figures 5.3, 5.5).

In pre-clinical studies, both peripheral and central administration of exogenous LEAP2 and its long-acting analogues attenuate AG induced food intake and reduce food intake on their own (Figure 5.3).

Human studies investigating the relationship between the satiety hormone LEAP2 and eating behaviours are limited. However, correlational studies suggest a role of LEAP2 in altering human eating behaviour. Fasting plasma LEAP2 negatively correlates with hunger after adjusting for BMI and sex in participants across all weight categories, but no such findings seen with post-prandial plasma LEAP2 (Andreoli et al. 2024a).

In this thesis, using composite appetite ratings on a continuous scale, (including hunger, pleasantness, volume and fullness ratings) in adults without obesity, post-prandial increases in LEAP2 correlated positively with post-prandial decreases in

appetite but not increases in fullness ratings (Chapter 2). However, no correlation was seen with fasting plasma LEAP2 and appetite or hunger, nor post-prandial plasma LEAP2 with appetite or fullness in adults with obesity and poorly controlled T2DM without or with DJBL insertion, perhaps due to the longitudinal nature of the study and suboptimal management of diabetes (Chapter 3).

Whereas no correlations were observed with fullness ratings in the post-prandial state across multiple studies potentially due to insufficient meal sizes or incomplete post-prandial fullness assessment (Andreoli et al. 2024a; Bhargava et al. 2023; Emini et al. 2024).

In both the exogenous LEAP2 infusion studies, in men of normal weight (Hagemann et al. 2022) and in men with obesity (Englund et al. 2023) there was no reduction in various appetite ratings, despite men with obesity receiving a higher infusion rate. The lack of reductions in appetite and food intake in men/women without obesity in this thesis (Chapter 4) may be due to smaller sample sizes and problems with order effects due to saline visits usually being given first during the LEAP2 dose escalation.

In Chapter 4, effects of LEAP2 infusion were also assessed om other aspects of eating behaviour, namely *ad libitum* food intake, food liking and wanting, preferred food portion size (desired intake) (all assessed with interactions by food category: savoury/sweet, low/high fat) temporal impulsivity to food, alcohol and monetary rewards, and memory (spatial working, paired associates learning). Again, the null findings may be related to small sample sizes and problems with order effects. This need to be addressed using this platform with larger sample sizes and randomised visit order now that a LEAP2 infusion dose has been selected for future studies (see Section 5.8).

Lastly, using fMRI paradigms, this thesis (Chapter 2) demonstrated a negative correlation of post-prandial food cue reactivity to HE, LE and HE/LE food with post-prandial increases in plasma LEAP2 in several brain areas involved with decision making and executive function (though not the classical brain reward areas viz. amygdala, anterior OFC, anterior insula, nucleus accumbens, putamen, caudate),

although without any change in food appeal ratings, providing evidence supporting a role for LEAP2 in altering food-related brain activity in humans. Furthermore, using the same food cue reactivity paradigms our research group has demonstrated that fasting plasma LEAP2 correlates with HE food in the posterior cingulate cortex (Goldstone et al. 2022) and correlates with LE food and inversely with HE food in the nucleus accumbens (Guo 2024). Differences in the results may be due to different BMI categories without/with T2DM, whole brain vs. fROI analysis and nutritional state. Hence, future studies should not only be correlational in nature, but measure cue reactivity after exogenous LEAP2 administration.

These investigations provide a more comprehensive understanding of LEAP2's role in appetite regulation and eating behaviours in humans and provide a basis for future studies of exogenous LEAP2 in populations of different weight categories using fMRI paradigms.

5.6. RESEARCH IMPACT

The results from this thesis confirm the role of plasma LEAP2 as a satiety signal. We also confirm that LEAP2 after eating may be an important gut-brain signal to reduce appetite, food intake and food cue reactivity, especially to HE foods. Although further studies to validate these findings with larger meals and meals of different macro-nutrient contents on the effects of LEAP2 are needed.

The results from this thesis confirm that long term weight loss by caloric restriction or DJBL device insertion reduces plasma LEAP2 at least in obesity with poorly controlled T2DM, though it has yet to be examined in those without T2DM other than through bariatric surgery. Weight regain did not cause an increase in plasma LEAP2 suggesting an establishment of new baseline (Chapter 3). This suggests that after weight loss, the protective effect of LEAP2 to prevent weight regain is lost and that at this point exogenous administration to increase plasma LEAP2 may aid in suppression of eating behaviour.

This thesis provides additional confirmation that the liver is the main organ of post-prandial LEAP2 secretion rather than the foregut (Chapter 3). This suggests that future

experiments to targeting manipulations of endogenous LEAP2 should involve hepatic organoids, hepatocyte cell lines and/or measurement of portal vein plasma LEAP2 in rodents.

After stopping GLP-1 or GLP-1/GIP receptor agonists (e.g. semaglutide, tirzepatide) people with obesity regain approximately two-third of their weight loss accompanied by similar reversal of cardiometabolic benefits (Wilding et al. 2022; Aronne et al. 2024). Some clinical studies have found absolute or relative reductions in plasma total or AG after use of GLP-1RA (Nonogaki et al. 2013; Topyildiz et al. 2016; Guclu et al. 2018). Difficulty maintaining VLCD and weight regain may be related to weight loss associated increases in plasma AG (Thom et al. 2020; Thom et al. 2021; Puklin et al. 2021; Garcia et al. 2006; Buso et al. 2021). This underscores the chronic nature of obesity and highlights the need for ongoing treatment(s) to sustain weight and health benefits. Future studies are therefore warranted to give exogenous LEAP2 or analogues after VLCD or stopping GLP-1RA to possibly prevent this weight regain in both preclinical models and humans, in addition to examination of the acute effects of LEAP2 infusions on human eating behaviour.

The results from Chapter 4 in this thesis confirmed the safety and tolerability of exogenous LEAP2 infusions in humans at 25 pmol/kg/min allowing further experimental medicine studies of its effects on eating behaviour to proceed including fMRI.

The findings from Chapter 4 and others support its role in lowering plasma glucose. The findings highlight LEAP2's potential as a therapeutic option for managing T2DM by inhibiting GHSR activity either indirectly through decreases in GH secretion or improvements in insulin secretion or sensitivity, independent of its effects on body weight. Furthermore, in preclinical studies reduction in plasma ghrelin either by ghrelin gene knockout or high fat diet feeding, was associated with increased pancreatic islet cell size (Gupta et al. 2023). This suggests that in states of positive energy balance with increased LEAP2 or exogenous supplementation may contribute to reduction in GHSR signalling and elevated pancreatic mass to increase insulin secretion.

The findings from Chapter 4 suggests that acute exogenous LEAP2 infusion reduces venous BHB and inhibits lipolysis, this is in line with the hypothesis that fasting/starvation increases lipolysis with increases plasma AG, serum GH and ketones as a source of energy to maintain plasma glucose levels during periods of negative energy balance. It would be hypothesised that exogenous LEAP2 administration may run the risk of hypoglycaemia although this has not been seen in preclinical or clinical studies.

5.7. STRENGTHS AND LIMITATIONS

The strengths of this thesis are: (i) novelty as it adds to the understanding of the regulation of plasma LEAP2 in (a) both the fasted and fed state, (b) in a range of people with and without obesity, (c) including both sexes, (d) in Chapter 3 over a long period of follow-up using two different mechanisms to elicit the effect of weight loss on plasma LEAP2, (e) supraphysiologic infusion in humans without obesity; (ii) statistical approaches: appropriate linear mixed model analysis was conducted for a repeated measures analysis, unlike in a previous study where multiple timepoints were included in one regression model rather than incremental changes in plasma LEAP2 and not accounting for within participant factors (e.g. correlation of post-prandial plasma glucose with fasting plasma LEAP2 (Mani et al. 2019) and including between-subject factors in the analysis (iii) furthermore, the current thesis confirms the safety of exogenous LEAP2 in humans and to our knowledge the first time demonstrates a dose response relationship on markers of glucose and fat metabolism; (iv) although this thesis had relatively moderate sample sizes in each result chapter, a within participant analysis was adopted that reduced variability and increased power to detect differences compared to a between participant analysis; (v) measurement of plasma LEAP2 was done using a validated assay, and (vi) an extensive literature review amalgamating preclinical and clinical publications was conducted.

Although throughout the thesis, relatively small sample sizes may also have contributed to type 1 (false-positive) and type 2 (false-negative) errors, hence confirmation of thesis findings with larger populations and in people of all weight categories are needed. A post-prandial rise in plasma LEAP2 was confirmed; however, the post-prandial peak in plasma LEAP2 was not found as plasma assays

were only performed up to 150 minutes, and furthermore only relatively small meals were given of 600-730 kcal, with the suggestion from analysis across different studies that post-prandial increases in plasma LEAP2 are proportional to pre-load size (Figure 1.4). Hence, future studies should involve larger meal sizes with prolonged sampling to find the peak increase in post-prandial plasma LEAP2.

In Chapter 3, the participants included only those with poorly controlled T2DM, and so the current findings of weight loss associated improvements in markers of weight, glucose and fat metabolism correlating with decreases in plasma LEAP2 may differ in individuals with obesity without T2DM or those with well-controlled T2DM. Although a small number of participants initiated appetite-modulating medications such as GLP1-RA or insulin there was no difference in the groups over the duration of the study.

Both sexes were included in Chapters 2-4 of this thesis, however due to the even smaller sample sizes and unequal distribution when separated by sex, it was not sufficiently powered to statistically investigate if there are any sex differences in the regulation or effects of LEAP2 (see Section 1.4.8). Future studies are required to examine such differences with larger populations of both sexes in adults with and without obesity.

In addition, the current thesis used correlational paradigms, future studies are needed to examine the effects of exogenous LEAP2 on food cue reactivity in adults with and without obesity. Building on results from chapter 2 and abstracts presented by the research group (Goldstone et al. 2022; Guo 2024) (see section 1.7.3) that LEAP2 is involved with food-reward related brain areas we plan a future study of exogenous LEAP2 administration with our group's collaborators at the University of Copenhagen.

Another limitation which we aim to address in future studies is that the infusion visits were not in a randomised order as our primary outcome was safety with majority of the participants receiving a saline infusion first with sequential increments in LEAP2 dose over the subsequent visits.

Lastly, in Chapter 4, exogenous LEAP2 administration increased plasma LEAP2, but steady state of plasma LEAP2 concentrations was never achieved. The most likely reason being accumulation of C-terminal fragments of the LEAP2 peptide during exogenous infusion of full length LEAP2. This is explained in detail in section 4.7.5. Development of a N-terminal antibody to the LEAP2(38-49) fragment is planned with our collaborators at the University of Copenhagen.

5.8. FUTURE DIRECTIONS

Since LEAP2 functions in a reciprocal relationship with AG, and the plasma AG/LEAP2 ratio may play a critical role in regulating human eating behaviour (Mani et al. 2019; Bhargava et al. 2023; Goldstone et al. 2022; Guo 2024), the next logical step is to measure plasma AG in the weight loss studies (Chapter 3) to examine if there are the expected increases in plasma AG and AG/LEAP2 ratio, and correlate these concentrations with appetite ratings.

Previous research reported no change in plasma AG after exogenous LEAP2 administration in humans (Hagemann et al. 2022), and this needs to be confirmed by measuring plasma AG from Chapter 4, and in planned future studies.

Moreover, a secondary analysis by measurement of plasma LEAP2 is planned in the following studies:

(i) LEAP2-meal size study (https://clinicaltrials.gov/study/NCT06013592) that investigates in a single-blinded, randomized, within participant study, whether consumption of liquid meals of increasing sizes (caloric pre-load 0, 600, 900, 1200 kcal) leads to proportional increases in post-prandial plasma LEAP2 (measured at five time points from 0-180 min) in adults without obesity.

(ii) GHADD study (clinicaltrials.gov/ct2/show/NCT02690987), to examine in a double-blinded, randomized, within participant study, whether in adults with obesity without T2DM, acute exogenous intravenous infusion over 6 hours of exenatide (GLP-1 analogue) or DAG (vs. saline) increases plasma LEAP2, as a potential contribution to the anorexigenic effect of GLP-1 and DAG (Gallwitz 2012; Stengel et al. 2010; Tong et al. 2014).

(iii) to compare fasting plasma LEAP2 in adults with obesity, in a cross-sectional study of adults with obesity after RYGB and gastric banding, and BMI-matched unoperated controls (Scholtz et al. 2014), to examine if there are differences in fasting and post-prandial plasma LEAP2 or its relationship with BMI after these bariatric surgeries, given that RYGB is associated with higher post-prandial ± fasting plasma PYY and GLP-1 and lower plasma total ghrelin / AG than after gastric banding (Lee et al. 2006; Scholtz et al. 2014), and that fasting plasma LEAP2 falls after RYGB surgery (Mani et al. 2019).

(iv) to examine of acute subcutaneous administration of the somatostatin analogue octreotide (vs. saline) suppresses post-prandial plasma LEAP2 (as part of the general inhibitory actions of somatostatin in the enteroendocrine system) in adults with obesity after RYGB and gastric banding (Goldstone et al. 2016).

Additionally, the impact of weight loss on fasting and post-prandial plasma LEAP2 levels should be explored in individuals with obesity without T2DM, as well as in those with well-controlled T2DM.

Furthermore, our research group, in collaboration with the University of Copenhagen (Anders Englund, Asger Bach Lund), is planning to develop an antibody targeting the N-terminal region of the LEAP2 (38-49) fragment to accurately measure plasma LEAP2 concentration after exogenous LEAP2 administration.

The confirmation of a safe LEAP2 infusion dose from Chapter 4 means that experimental medicine studies can now proceed with larger sample sizes to assess effects of acute LEAP2 administration on metabolism, eating and addictive behaviours now with the inclusion a fMRI session in the same protocol used in Chapter 4. Ethical approval for this fMRI study in adults without obesity has already been obtained (REC 23/LO/0510).

Although this was planned during the current thesis delays due to delays in the manufacture of LEAP2 peptide, delays in obtaining ethical approval, additional pilot

studies need to confirm safe LEAP2 infusion dose, meant that it was not feasible to complete this fMRI study within the timeframe of the MRC grant. A more limited fMRI study is planned using just HE and LE food cue reactivity in adults with obesity through the Group's collaboration with the University of Copenhagen (funded by Novo Nordisk Foundation).

Subject to further funding being available, this proposed LEAP2-fMRI study will be a double-blind, within-participant, randomised, cross-over design in participants without obesity, to investigate the ability of acute intravenous infusion of LEAP2(38-77) at dose 25 pmol/kg/min vs. saline over 5.7 hours, when fasted (Figure 5.6):

(i) to attenuate eating and addictive behaviours, including reward sensitivity, impulsivity/compulsivity, and stress sensitivity and,
(ii) to reduce appetite, food intake, preferred food portion size and food preference to high-energy foods.

The hypotheses for this neurobehavioral mechanistic study in healthy participants without obesity, are that both acute infusion of LEAP2 when fasted will:

(i) attenuate reward system cue reactivity to HE foods,
(ii) attenuate reward system responses during anticipation of monetary, food and alcohol rewards,
(iii) attenuate negative emotional reactivity,
(iv) reduce appetite,
(v) reduce food intake, particularly to high fat and sweet foods,
(vi) reduce food liking and wanting, particularly to high fat and sweet foods,
(vii) reduce taste pleasantness of alcohol, high fat and sweet foods,
(viii) reduce preferred meal size,
(ix) reduce temporal impulsivity to food,
(x) reduce serum GH and glucose concentrations,
(xi) reduce plasma AG, and/or increase plasma satiety gut hormones,
(xii) alter hippocampal-dependent memory,
(xiii) reduce risk taking,
(xiv) reduce motor response inhibition.

on the following measures:

(i) whole brain and functional ROI BOLD signal in a food picture evaluation fMRI task,

(ii) whole brain and functional ROI BOLD signal in monetary, food and alcohol incentive delay fMRI task,

(iii) whole brain and functional ROI BOLD signal in an unpleasant images fMRI task,

(iv) appetite visual analogue scale (VAS) rating,

(v) food intake at an *ad libitum* test lunch meal,

(vi) food explicit liking, explicit and implicit wanting of low/high fat and savoury/sweet foods using the Leeds Food Preference Questionnaire (LFPQ),

(vii) currently desired food portion size using virtual portion size creation task (VPCT) for dishes varying in energy density,

(viii) delay discounting rates for low-energy and high-energy density food, alcohol and money using the delay discounting task (DDT),

(ix) serum GH and glucose concentrations,

(x) plasma AG, DAG, PYY and GLP-1 concentrations,

(xi) taste ratings of alcohol, low/high fat and savoury/sweet foods

(xii) memory using paired associate learning (PAL) task,

(xiii) working memory from Spatial Working Memory (SWM) task,

(xiv) measurement of motor response inhibition from Stop Signal Task (SST).

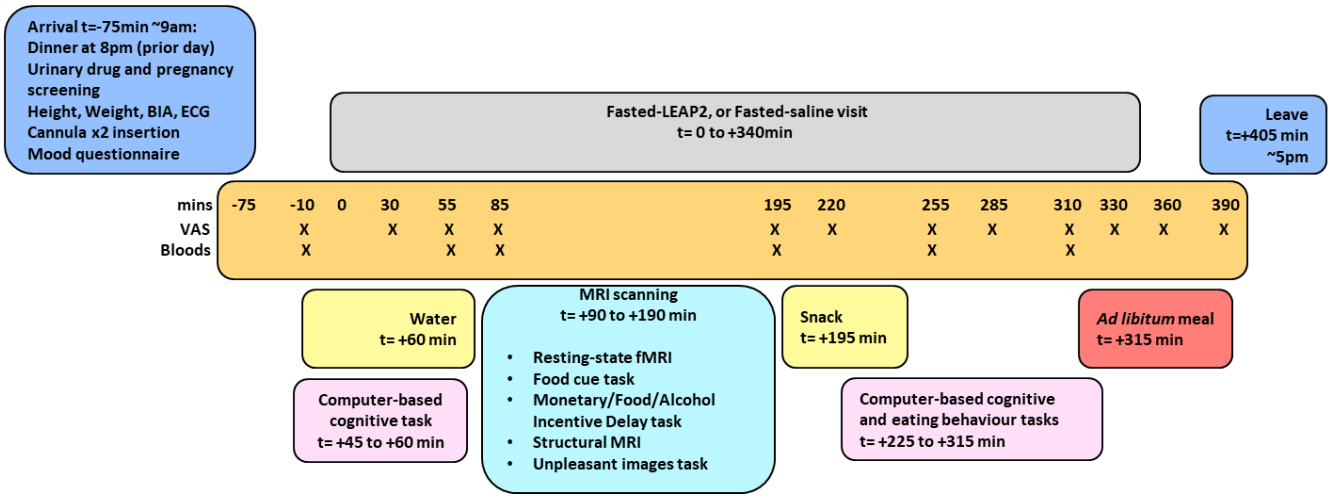


Figure 5.6. Proposed LEAP2 fMRI study visit.

This planned study (Figure 5.6) at Imperial College London in adults without obesity will employ the set of well-characterised appetitive and computer-based paradigms

that assess eating and addictive behaviours and neurocognitive processes used in Chapter 4, with the addition of the following fMRI paradigms:

(i) Food cue evaluation task: participants will view HE food, LE food and control neutral images while simultaneously rating the appeal of the picture using a hand-held 5 button box (Goldstone et al. 2014; Scholtz et al. 2014; Goldstone et al. 2016; Byrne et al. 2016).

(ii) Food incentive delay (FID) task: FID task is a neurocognitive task that measures the reward sensitivity during the anticipation of a food reward and is a modification of the monetary incentive delay (MID) task (Knutson et al. 2001). Participants must press a button whilst in the fMRI scanner during a specified time window when given cues on a screen to win or prevent the loss of HE food points. The task contains win, loss and neutral trials.

In fed adult women without obesity, in an FID task, during food reward anticipation there was increased BOLD signal in the ventral striatum, thalamus, cingulate cortex, insula and occipital cortex, and during food reward receipt in the lateral OFC, prefrontal, parietal and occipitotemporal cortex (Simon et al. 2015). In adults without obesity during an FID task, there was an increase in BOLD signal in the ventral striatum during anticipation of food reward in the fasted state (Simon et al. 2017). In adults with normal weight, total ghrelin nor AG correlated with HE food images or a food incentive delay task to BOLD signal in the ventral striatum, OFC, insula, amygdala or substantia nigra (Roth et al.). Women with obesity that lost weight after a 6 month LCD showed attenuation of BOLD signal in the ventral striatum and medial OFC during the receipt of food rewards in the fed state (Simon et al. 2018).

(iii) Alcohol / Monetary incentive delay (AID/MID) fMRI task: using a task adapted from the ICCAM study at Imperial College London, this task will assess the response to visual cues that predict anticipation of monetary reward (McGonigle et al. 2017; Murphy et al. 2017; Nestor et al. 2017; Paterson et al. 2015) or alcohol (Farokhnia et al. 2018) wins and losses, and the delivery of reward and

punishment outcomes.

In adults without obesity, fasting increased the BOLD signal in the amygdala and putamen during anticipation of monetary reward in a MID fMRI task (Nikolova et al. 2015). While in alcohol dependence, acute AG administration increases alcohol consumption and craving, alcohol self-administration, and amygdala reactivity in an AID fMRI task (Leggio et al. 2014; Farokhnia et al. 2018).

Participants will first be presented with one of three cues: (i) a cue indicating the potential to win the reward, (ii) a cue indicating the potential to lose the reward, or (iii) a cue indicating no potential outcome. Following the cue, participants will wait for a variable period while they anticipate the rapid presentation of a visual target. Upon the presentation of the visual target, subjects will be required to respond as quickly as possible with a single digit button press with their right index finger. Finally, participants will receive feedback regarding the outcome of the trial (win, lose or no outcome), together with a running total of the winnings accumulated up to that point in the task. Subjects will be encouraged to maximise their winnings.

The food (e.g. chocolate), preferred alcohol (e.g. wine, spirit, beer) and monetary reward will be given to participants at the end of their study visit to consume at home, while the monetary reward will either be given to them at the end of this study visit or once they have completed all study visits.

(iv) Unpleasant images task: this ICCAM study platform task uses images taken from the International Affective Picture System (University of Florida). Fasting increased BOLD signal in the OFC, caudate and putamen during viewing of unpleasant images in adults without obesity (Ali et al. 2014). Participants will view blocks of images with negative (aversive) emotional valence and images with neutral emotional valence (McGonigle et al. 2017; Paterson et al. 2015).

We estimate the power for detecting differences in brain function between treatments will vary according to imaging modality employed, hormone and population studied.

The novelty of the experimental design hinders formal power analysis due to lack of directly comparable data. However, previous fMRI studies with acute pharmacological challenge in drug dependent individuals and healthy volunteers (n=20 per group) using both task- related and resting-state have demonstrated significant drug effects as well as significant group-by-drug interactions.

Analysis of pilot data from the group's previous studies (Ali et al. 2014; Chhina et al. 2016) of the effect of feeding in participants with normal weight on the fMRI paradigms (to be used in future planned fMRI studies with exogenous LEAP2) indicate that with final n=24 per group and alpha 0.05 the power to detect the following reductions in % BOLD signal with food intake (estimated to be similar in effect size to the proposed hormonal manipulations) are as follows:

(i) Food cue evaluation fMRI task: 71% power for delta 0.135 with SD 0.251 in OFC during evaluation of HE food vs. neutral pictures.
(ii) Food/alcohol/monetary incentive delay fMRI task: 74% power for delta 0.172 with SD 0.311 in amygdala, and 73% power for delta 0.111 with SD 0.203 in striatum during anticipation of win vs. neutral monetary reward (Ali et al. 2013).
(iii) Unpleasant images fMRI task: 86% power for delta 0.142 with SD 0.218 in OFC, and 84% power for delta 0.089 with SD 0.142 in dorsal striatum during viewing of unpleasant vs. neutral images (Ali et al. 2013).
(iv) Delay discounting task: estimating that the within subject effect size of LEAP2 administration on discounting area under curve (AUC) will be two thirds that seen after ingestion of a 1200 kcal meal, HE food: mean ± SD decrease in AUC 0.24 ± 0.30 effect size 0.80, money: increase in AUC 0.14 +/- 0.22, effect size 0.64), then n=24 will have power of 0.96 with alpha 0.05.

The improved signal / noise ratio of the 32-channel head coil 3T Siemens Verio scanner compared to the 8-channel head coil 3T Philips Achieva scanner used in the pilot studies will increase power even further than indicated above. 28 participants will be recruited per group to allow for the estimated 10% subject drop-out over visits and 10% loss of scans due to excess motion and artefact seen in our previous studies, to give a minimum final n=24 per group.

Following from this, these acute experimental medicine studies with LEAP2 infusions experiments could be done in participants after dietary and drug induced weight loss, given the associated increase in plasma AG (Thom et al. 2020; Thom et al. 2021; Puklin et al. 2021; Garcia et al. 2006; Buso et al. 2021), and in AUD to investigate if LEAP2 suppresses alcohol-related behaviours given the preclinical and clinical evidence for GHSR signalling stimulating alcohol intake and reward (Jerlhag et al. 2009; Kharbanda et al. 2022).

In the longer term, modified LEAP2 and/or LEAP2 analogues need to be developed that are more stable and long-acting (e.g. by addition of a fat moiety as has been shown promise in pre-clinical studies both acutely to decrease food intake, and chronically to improve markers of fatty liver disease) to investigate their safety and chronic effects on eating behaviour, weight regulation and glucose/fat metabolism in humans with obesity and/or T2DM.

5.9. CONCLUSIONS

The findings from this thesis, collectively support the hypothesis that post-prandial increases in plasma LEAP2, by suppressing GHSR signalling, contribute to post-prandial reductions in appetite, food intake, and food cue reactivity. Post-prandial changes in plasma LEAP2 may have greater significance than decreases in plasma AG in this context. Furthermore, in participants with obesity and T2DM weight loss induced by diet and physical activity reduced fasting plasma LEAP2. This reduction in plasma LEAP2, positively correlated with reductions in BMI, HbA1c, fasting plasma glucose, serum insulin, HOMA-IR, and triglycerides, suggesting the regulation of LEAP2 is due to an interaction with glucose and fat metabolism. Insertion of DJBL device resulting in loss of foregut signalling by exclusion of food from the duodenum and jejunum (mimicking this part of RYGB surgery) in obesity and T2DM attenuated the weight loss-associated decreases in fasting but not post-prandial plasma LEAP2, through unclear mechanisms, which might have contributed to greater weight loss with DJBL insertion than after standard medical management.

Interestingly, furthermore the lack of any change in post-prandial plasma LEAP2 after

DJBL device implantation to lose weight suggests that the liver, rather than the foregut is the primary site of post-prandial secretion of plasma LEAP2. The absence of an increase in fasting plasma LEAP2 following DJBL device removal (and subsequent weight regain) may indicate a new set-point potentially influencing weight regain.

The last experiment in this thesis showed that infusing exogenous LEAP2 in adults without obesity is safe (up to 25 pmol/kg/min) and tolerable and reduces fasting plasma glucose and post-prandial BHB concentrations in a dose-dependent manner, suggesting that LEAP2 influences glycolytic and lipolytic pathways via GHSR signalling. These effects occurred without changes in serum insulin, GH, cortisol and thyroid function tests indicating a potential non-hormonal mechanism for GHSR-mediated effects on glycolysis and lipolysis. Altogether, these results highlight the importance of LEAP2 in maintaining human energy equilibrium and emphasise the therapeutic potential of LEAP2 in managing obesity and T2DM through GHSR modulation.

However, the metabolic mechanisms driving the post-prandial rise in plasma LEAP2 remain unclear, with no established link to changes in AG, glucose, insulin or triglycerides. It remains incompletely understood whether the weight loss induced reduction in fasting plasma LEAP2 is directly attributable to weight loss, improved glycaemic control, or enhanced insulin sensitivity.

Finally, additional research is needed to thoroughly understand the safety, pharmacodynamics and effects of exogenous LEAP2 administration in experimental medicine studies of different weight categories, after weight loss, with or without T2DM, of both sexes, in different ethnicities, and also in AUD. This is needed before it can be investigated as pharmacological therapy for obesity, T2DM and AUD, which will need the development of long-acting LEAP2 analogues.

REFERENCES

Abizaid, A., Y. S. Mineur, R. H. Roth, J. D. Elsworth, M. W. Sleeman, M. R. Picciotto, and T. L. Horvath. 2011. 'Reduced locomotor responses to cocaine in ghrelin-deficient mice', *Neuroscience*, 192: 500-06.

Addolorato, G., E. Capristo, L. Leggio, A. Ferrulli, L. Abenavoli, N. Malandrino, S. Farnetti, M. Domenicali, C. D'Angelo, L. Vonghia, A. Mirijello, S. Cardone, and G. Gasbarrini. 2006. 'Relationship between ghrelin levels, alcohol craving, and nutritional status in current alcoholic patients', *Alcohol Clin Exp Res*, 30: 1933-7.

Akalestou, E., A. D. Miras, G. A. Rutter, and C. W. le Roux. 2022. 'Mechanisms of Weight Loss After Obesity Surgery', *Endocr Rev*, 43: 19-34.

Akhavan, T., and G. H. Anderson. 2007. 'Effects of glucose-to-fructose ratios in solutions on subjective satiety, food intake, and satiety hormones in young men', *Am J Clin.Nutr.*, 86: 1354-63.

Al-Massadi, O., T. Muller, M. Tschop, C. Dieguez, and R. Nogueiras. 2018. 'Ghrelin and LEAP-2: Rivals in Energy Metabolism', *Trends Pharmacol Sci*, 39: 685-94.

Albarran-Zeckler, R. G., Y. Sun, and R. G. Smith. 2011. 'Physiological roles revealed by ghrelin and ghrelin receptor deficient mice', *Peptides*, 32: 2229-35.

Aldhwayan, M. M., W. Al-Najim, A. Ruban, M. A. Glaysher, B. Johnson, N. Chhina, G. K. Dimitriadis, C. G. Prechtl, N. A. Johnson, J. P. Byrne, A. P. Goldstone, J. P. Teare, C. W. Le Roux, and A. D. Miras. 2022. 'Does Bypass of the Proximal Small Intestine Impact Food Intake, Preference, and Taste Function in Humans? An Experimental Medicine Study Using the Duodenal-Jejunal Bypass Liner', *Nutrients*, 14: 2141.

Aldubaikhi, G., N. Chhina, T. Parastika, B. Zaki, N. Onokwai, C. W. Le Roux, G. Finlayson, and A. P. Goldstone. 2019. 'Healthier food preference following Roux-en-Y gastric bypass surgery for obesity, and relationship with weight loss and satiety gut hormones PYY, GLP1 and FGF19', *Abstracts Society for Study of Ingestive Behaviour, Utrecht, Netherlands*.

Ali, S. N., R. Bowie, G. Durighel, J. McGonigle, A. R. Lingford-Hughes, D. J. Nutt, Gibson.E.L, J. D. Bell, and A. P. Goldstone. 2014. 'Fasting enhances brain responses to anticipation of monetary reward in humans', *Abstracts 32nd Annual Meeting Obesity Society, Boston, USA*.

Ali, S. N., E. Hennessey, C. Orban, G. Durighel, J. McGonigle, A. R. Lingford-Hughes, D. J. Nutt, J. D. Bell, and A. P. Goldstone. 2013. 'Food intake attenuates brain responses to unpleasant images in humans', *Abstracts 31st Annual Meeting Obesity Society, Atlanta USA*.

Alkahtni, S. A., M. Dalton, O. Abuzaid, O. Obeid, and G. Finlayson. 2016. 'Validation of the Leeds Food Preference Questionnaire in Arabs', *Asia Pac J Clin Nutr*, 25: 257-64.

Allas, S., and T. Abribat. 2013. 'Clinical perspectives for ghrelin-derived therapeutic products', *Endocr Dev*, 25: 157-66.

Allas, S., A. Caixas, C. Poitou, M. Coupaye, D. Thuilleaux, F. Lorenzini, G. Diene, A. Crino, F. Illouz, G. Grugni, D. Potvin, S. Bocchini, T. Delale, T. Abribat, and M. Tauber. 2018. 'AZP-531, an unacylated ghrelin analog, improves food-related behavior in patients with Prader-Willi syndrome: A randomized placebo-controlled trial', *PLoS One*, 13: e0190849.

Altabas, V., and V. Zjacic-Rotkvic. 2015. 'Anti-ghrelin antibodies in appetite suppression: recent advances in obesity pharmacotherapy', *Immunotargets Ther*, 4: 123-30.
American-Psychiatric-Association. 2013. *Diagnostic and Statistical Manual of Mental Disorders, 5th edition* (American Psychiatric Association: Washington DC, USA).
Andarini, S., F. B. Kangsaputra, and D. Handayani. 2017. 'Pre- and postprandial acylated ghrelin in obese and normal weight men', *Asia Pac J Clin Nutr*, 26: S85-S91.
Anderberg, R. H., C. Hansson, M. Fenander, J. E. Richard, S. L. Dickson, H. Nissbrandt, F. Bergquist, and K. P. Skibicka. 2016. 'The stomach-derived hormone ghrelin increases impulsive behavior', *Neuropsychopharmacology*, 41: 1199-209.
Andrade, S., F. Pinho, A. M. Ribeiro, M. Carreira, F. F. Casanueva, P. Roy, and M. P. Monteiro. 2013. 'Immunization against active ghrelin using virus-like particles for obesity treatment', *Curr Pharm Des*, 19: 6551-8.
Andreoli, M. F., A. S. Fittipaldi, D. Castrogiovanni, P. N. De Francesco, S. Valdivia, F. Heredia, C. Ribet-Travers, I. Mendez, M. V. Fasano, H. B. Schioth, S. A. Doi, A. M. Habib, and M. Perello. 2024a. 'Pre-prandial plasma liver-expressed antimicrobial peptide 2 (LEAP2) concentration in humans is inversely associated with hunger sensation in a ghrelin independent manner', *Eur J Nutr*, 63: 751-62.
Andreoli, M. F., M. Gentreau, G. Rukh, M. Perello, and H. B. Schioth. 2024b. 'Genetic variants of LEAP2 are associated with anthropometric traits and circulating insulin-like growth factor-1 concentration: A UK Biobank study', *Diabetes Obes Metab*.
Andreoli, M. F., A. L. Kruger, A. V. Sokolov, G. Rukh, P. N. De Francesco, M. Perello, and H. B. Schioth. 2024c. 'LEAP2 is associated with impulsivity and reward sensitivity depending on the nutritional status and decreases with protein intake in humans', *Diabetes Obes Metab*.
Aronne, L. J., and Surmount- Investigators. 2024. 'Tirzepatide for Maintenance of Weight Reduction in Adults With Obesity-Reply', *JAMA*, 331: 1676.
Ashby, D. R., H. E. Ford, K. J. Wynne, A. M. Wren, K. G. Murphy, M. Busbridge, E. A. Brown, D. H. Taube, M. A. Ghatei, F. W. Tam, S. R. Bloom, and P. Choi. 2009. 'Sustained appetite improvement in malnourished dialysis patients by daily ghrelin treatment', *Kidney Int.*, 76: 199-206.
Aslanipour, B., M. Alan, and I. Demir. 2020. 'Decreased levels of liver-expressed antimicrobial peptide-2 and ghrelin are related to insulin resistance in women with polycystic ovary syndrome', *Gynecol Endocrinol*, 36: 222-25.
Aukan, M. I., S. Coutinho, S. A. Pedersen, M. R. Simpson, and C. Martins. 2023. 'Differences in gastrointestinal hormones and appetite ratings between individuals with and without obesity-A systematic review and meta-analysis', *Obes Rev*, 24: e13531.
Azegami, T., Y. Yuki, S. Sawada, M. Mejima, K. Ishige, K. Akiyoshi, H. Itoh, and H. Kiyono. 2017. 'Nanogel-based nasal ghrelin vaccine prevents obesity', *Mucosal Immunol*, 10: 1351-60.
Baenas, I., B. Mora-Maltas, M. Etxandi, I. Lucas, R. Granero, F. Fernandez-Aranda, S. Tovar, N. Sole-Morata, M. Gomez-Pena, L. Moragas, A. Del Pino-Gutierrez, J. Tapia, C. Dieguez, A. E. Goudriaan, and S. Jimenez-Murcia. 2023. 'Cluster analysis in gambling disorder based on sociodemographic,

neuropsychological, and neuroendocrine features regulating energy homeostasis', *Compr Psychiatry*, 128: 152435.
Barja-Fernandez, S., J. Lugilde, C. Castelao, R. Vazquez-Cobela, L. M. Seoane, C. Dieguez, R. Leis, and S. Tovar. 2021. 'Circulating LEAP-2 is associated with puberty in girls', *Int J Obes (Lond)*, 45: 502-14.
Barrile, F., C. M'Kadmi, P. N. De Francesco, A. Cabral, G. Garcia Romero, E. R. Mustafa, S. Cantel, M. Damian, S. Mary, S. Denoyelle, J. L. Baneres, J. Marie, J. Raingo, J. A. Fehrentz, and M. Perello. 2019. 'Development of a novel fluorescent ligand of growth hormone secretagogue receptor based on the N-Terminal Leap2 region', *Mol Cell Endocrinol*, 498: 110573.
Bayle, M., S. Peraldi-Roux, G. Gautheron, G. Cros, C. Oiry, and J. Neasta. 2021. 'Liver-Expressed Antimicrobial Peptide 2 antagonizes the insulinostatic effect of ghrelin in rat isolated pancreatic islets', *Fundam Clin Pharmacol*.
Beck, A. T., Steer, R. A., and G. K. Brown. 1996. "Manual for the Beck Depression Inventory-II." In.: Psychological Corporation.
Berthoud, H. R., H. Münzberg, and C. D. Morrison. 2017. 'Blaming the Brain for Obesity: Integration of Hedonic and Homeostatic Mechanisms', *Gastroenterology*, 152: 1728-38.
Betzel, B., J. Homan, E. O. Aarts, I. M. C. Janssen, H. de Boer, and P. J. Wahab. 2017a. 'Weight reduction and improvement in diabetes by the duodenal-jejunal bypass liner: a 198 patient cohort study', *Surg Endosc*, 31: 2881-91.
Betzel, B., P. Koehestanie, J. Homan, E. O. Aarts, I. M. Janssen, and H. de Boer. 2017b. 'Changes in glycemic control and body weight after explantation of the duodenal-jejunal bypass liner', *Gastrointest Endosc*, 85: 409-15.
Bhargava, R., S. Luur, M. Rodriguez Flores, M. Emini, C. G. Prechtl, and A. P. Goldstone. 2023. 'Post-prandial increases in liver-gut hormone LEAP2 correlate with attenuated eating behaviour in adults without obesity', *J Endocr Soc*, 7: bvad061.
Bianzano, S., A. Henrich, L. Herich, B. Kalsch, D. Sarubbi, F. Seitz, and T. Forst. 2023. 'Efficacy and safety of the ghrelin-O-acyltransferase inhibitor BI 1356225 in overweight/obesity: Data from two Phase I, randomised, placebo-controlled studies', *Metabolism*, 143: 155550.
Bilman, E., van Kleef, E., & van Trijp, H. 2017. 'External cues challenging the internal appetite control system-Overview and practical implications. ', *Crit Rev Food Sci Nutr*, 57: 2825–34.
Blom, W. A., C. de Graaf, A. Lluch, A. Stafleu, G. Schaafsma, and H. F. Hendriks. 2009. 'Postprandial ghrelin responses are associated with the intermeal interval in time-blinded normal weight men, but not in obese men', *Physiol Behav*, 96: 742-8.
Blom, W. A., A. Stafleu, C. de Graaf, F. J. Kok, G. Schaafsma, and H. F. Hendriks. 2005. 'Ghrelin response to carbohydrate-enriched breakfast is related to insulin', *Am J Clin Nutr*, 81: 367-75.
Blundell, J. E., and V. J. Burley. 1987. 'Satiation, satiety and the action of fibre on food intake', *Int.J.Obes.*, 11 Suppl 1: 9-25.
Blundell, J., G. Finlayson, M. Axelsen, A. Flint, C. Gibbons, T. Kvist, and J. B. Hjerpsted. 2017. 'Effects of once-weekly semaglutide on appetite, energy intake, control of eating, food preference and body weight in subjects with obesity', *Diabetes Obes Metab*, 19: 1242-51.

Bohn, M. J., D. D. Krahn, and B. A. Staehler. 1995. 'Development and initial validation of a measure of drinking urges in abstinent alcoholics', *Alcohol Clin Exp Res*, 19: 600-6.
Borchers, S., J. P. Krieger, I. Maric, J. Carl, M. Abraham, F. Longo, M. Asker, J. E. Richard, and K. P. Skibicka. 2022. 'From an Empty Stomach to Anxiolysis: Molecular and Behavioral Assessment of Sex Differences in the Ghrelin Axis of Rats', *Front Endocrinol (Lausanne)*, 13: 901669.
Bowen, J., M. Noakes, C. Trenerry, and P. M. Clifton. 2006. 'Energy intake, ghrelin, and cholecystokinin after different carbohydrate and protein preloads in overweight men', *J Clin Endocrinol Metab*, 91: 1477-83.
Briggs, D. I., S. H. Lockie, Q. Wu, M. B. Lemus, R. Stark, and Z. B. Andrews. 2013. 'Calorie-restricted weight loss reverses high-fat diet-induced ghrelin resistance, which contributes to rebound weight gain in a ghrelin-dependent manner', *Endocrinology*, 154: 709-17.
Broglio, F., C. Gottero, F. Prodam, C. Gauna, G. Muccioli, M. Papotti, T. Abribat, A. J. Van der Lely, and E. Ghigo. 2004. 'Non-acylated ghrelin counteracts the metabolic but not the neuroendocrine response to acylated ghrelin in humans', *J Clin Endocrinol Metab*, 89: 3062-65.
Bruce, A. S., L. M. Holsen, R. J. Chambers, L. E. Martin, W. M. Brooks, J. R. Zarcone, M. G. Butler, and C. R. Savage. 2010. 'Obese children show hyperactivation to food pictures in brain networks linked to motivation, reward and cognitive control', *Int J Obes*, 34: 1494-500.
Bryant, E. J., N. A. King, Y. Falken, P. M. Hellstrom, J. J. Holst, J. E. Blundell, and E. Naslund. 2013. 'Relationships among tonic and episodic aspects of motivation to eat, gut peptides, and weight before and after bariatric surgery', *Surg Obes Relat Dis*, 9: 802-8.
Buntwal, L., M. Sassi, A. H. Morgan, Z. B. Andrews, and J. S. Davies. 2019. 'Ghrelin-Mediated Hippocampal Neurogenesis: Implications for Health and Disease', *Trends Endocrinol Metab*, 30: 844-59.
Burnett, L. C., C. A. LeDuc, C. R. Sulsona, D. Paull, R. Rausch, S. Eddiry, J. F. Carli, M. V. Morabito, A. A. Skowronski, G. Hubner, M. Zimmer, L. Wang, R. Day, B. Levy, I. Fennoy, B. Dubern, C. Poitou, K. Clement, M. G. Butler, M. Rosenbaum, J. P. Salles, M. Tauber, D. J. Driscoll, D. Egli, and R. L. Leibel. 2017. 'Deficiency in prohormone convertase PC1 impairs prohormone processing in Prader-Willi syndrome', *J Clin Invest*, 127: 293-305.
Buso, M. E. C., R. V. Seimon, S. McClintock, R. Muirhead, F. S. Atkinson, S. Brodie, J. Dodds, J. Zibellini, A. Das, A. L. Wild-Taylor, J. Burk, M. Fogelholm, A. Raben, J. C. Brand-Miller, and A. Sainsbury. 2021. 'Can a Higher Protein/Low Glycemic Index vs. a Conventional Diet Attenuate Changes in Appetite and Gut Hormones Following Weight Loss? A 3-Year PREVIEW Sub-study', *Front Nutr*, 8: 640538.
Byberg, S. 2022. 'LEAP2 Assay Development and Clinical and Preclinical Investigations', *PhD thesis, University of Copenhagen*.
Byberg, S., M. B. Blond, S. Holm, H. Amadid, L. B. Nielsen, K. K. B. Clemmensen, K. Faerch, and B. Holst. 2023. 'LEAP2 is Associated with Cardiometabolic Markers, but is Unchanged by Antidiabetic Treatment in People with Prediabetes', *Am J Physiol Endocrinol Metab*, 325: E244-E51.
Byrne, C. S., E. S. Chambers, H. Alhabeeb, N. Chhina, D. J. Morrison, T. Preston, C. Tedford, J. Fizpatrick, C. Irani, A. Busza, I. Garcia-Perez, S. Fountana, E. Holmes, A. P. Goldstone, and G. S. Frost. 2016. 'Increased colonic propionate

reduces anticipatory reward responses in the human striatum to high-energy foods', *Am J Clin Nutr*, 104: 5-14.
Caixas, A., C. Bashore, W. Nash, F. Pi-Sunyer, and B. Laferrere. 2002. 'Insulin, unlike food intake, does not suppress ghrelin in human subjects', *J Clin Endo Metab*, 87: 1902-06.
Cameron, J. D., G. S. Goldfield, G. Finlayson, J. E. Blundell, and E. Doucet. 2014. 'Fasting for 24 hours heightens reward from food and food-related cues', *PLoS One*, 9: e85970.
Camina, J. P., M. C. Carreira, D. Micic, M. Pombo, F. Kelestimur, C. Dieguez, and F. F. Casanueva. 2003. 'Regulation of ghrelin secretion and action', *Endocrine.*, 22: 5-12.
Carmichael, O., A. J. Schwarz, C. H. Chatham, D. Scott, J. A. Turner, J. Upadhyay, A. Coimbra, J. A. Goodman, R. Baumgartner, B. A. English, J. W. Apolzan, P. Shankapal, and K. R. Hawkins. 2018. 'The role of fMRI in drug development', *Drug Discov Today*, 23: 333-48.
Carnell, S., C. Gibson, L. Benson, C. N. Ochner, and A. Geliebter. 2012. 'Neuroimaging and obesity: current knowledge and future directions', *Obes Rev*, 13: 43-56.
Carvalho-Ferreira, J. P., G. Finlayson, D. T. da Cunha, G. Caldas, D. Bandoni, and V. V. de Rosso. 2019. 'Adiposity and binge eating are related to liking and wanting for food in Brazil: A cultural adaptation of the Leeds food preference questionnaire', *Appetite*, 133: 174-83.
Casado, S., M. Varela-Miguens, T. de Oliveira Diz, C. Quintela-Vilarino, R. Nogueiras, C. Dieguez, and S. Tovar. 2024. 'The effects of ghrelin and LEAP-2 in energy homeostasis are modulated by thermoneutrality, high-fat diet and aging', *J Endocrinol Invest*.
Chamberlain, J. J., W. H. Herman, S. Leal, A. S. Rhinehart, J. H. Shubrook, N. Skolnik, and R. R. Kalyani. 2017. 'Pharmacologic Therapy for Type 2 Diabetes: Synopsis of the 2017 American Diabetes Association Standards of Medical Care in Diabetes', *Ann Intern Med*, 166: 572-78.
Chen, E. Y., and T. A. Zeffiro. 2020. 'Hunger and BMI modulate neural responses to sweet stimuli: fMRI meta-analysis', *Int J Obes (Lond)*, 44: 1636-52.
Chhina, N., C. Coxon, N. Onokwai, S. Scholtz, S. Purkayastha, K. Moorthy, S. Hakky, A. Ahmed, and A.P. Goldstone. 2016. 'Healthier food hedonics and emotional eating link with orbitofrontal cortex and amygdala responses to food and unpleasant images after gastric bypass surgery', *Abstracts 24th Annual Meeting Soc for Study of Ingestive Behavior, Porto*.
Chu, G., H. Peng, N. Yu, Y. Zhang, X. Lin, and Y. Lu. 2022. 'Involvement of POMC neurons in LEAP2 regulation of food intake and body weight', *Front Endocrinol (Lausanne)*, 13: 932761.
Chuang, J. C., M. Perello, I. Sakata, S. Osborne-Lawrence, J. M. Savitt, M. Lutter, and J. M. Zigman. 2011. 'Ghrelin mediates stress-induced food-reward behavior in mice', *J Clin Invest*, 121: 2684-92.
Coll, A. P., I. S. Farooqi, and S. O'Rahilly. 2007. 'The hormonal control of food intake', *Cell*, 129: 251-62.
Collden, G., M. H. Tschop, and T. D. Muller. 2017. 'Therapeutic Potential of Targeting the Ghrelin Pathway', *Int J Mol Sci*, 18.
Cooper, J. A., A. C. Watras, C. M. Paton, F. H. Wegner, A. K. Adams, and D. A. Schoeller. 2011. 'Impact of exercise and dietary fatty acid composition from a high-fat diet on markers of hunger and satiety', *Appetite*, 56: 171-8.

Cornejo, M. P., D. Castrogiovanni, H. B. Schioth, M. Reynaldo, J. Marie, J. A. Fehrentz, and M. Perello. 2019. 'Growth hormone secretagogue receptor signalling affects high-fat intake independently of plasma levels of ghrelin and LEAP2, in a 4-day binge eating model', *J Neuroendocrinol*, 31: e12785.
Cornejo, M. P., E. R. Mustafa, F. Barrile, D. Cassano, P. N. De Francesco, J. Raingo, and M. Perello. 2021a. 'The Intriguing Ligand-Dependent and Ligand-Independent Actions of the Growth Hormone Secretagogue Receptor on Reward-Related Behaviors', *Neurosci Biobehav Rev*, 120: 401-16.
Cornejo, M. P., E. R. Mustafa, D. Cassano, J. L. Baneres, J. Raingo, and M. Perello. 2021b. 'The ups and downs of growth hormone secretagogue receptor signaling', *Febs j*.
Costabile, G., E. Griffo, P. Cipriano, C. Vetrani, M. Vitale, G. Mamone, A. A. Rivellese, G. Riccardi, and R. Giacco. 2018. 'Subjective satiety and plasma PYY concentration after wholemeal pasta', *Appetite*, 125: 172-81.
Coutinho, S. R., E. H. Halset, S. Gasbakk, J. F. Rehfeld, B. Kulseng, H. Truby, and C. Martins. 2018. 'Compensatory mechanisms activated with intermittent energy restriction: A randomized control trial', *Clin Nutr*, 37: 815-23.
Cummings, D. E., K. Clement, J. Q. Purnell, C. Vaisse, K. E. Foster, R. S. Frayo, M. W. Schwartz, A. Basdevant, and D. S. Weigle. 2002a. 'Elevated plasma ghrelin levels in Prader Willi syndrome', *Nature Medicine*, 8: 643-44.
Cummings, D. E., R. S. Frayo, C. Marmonier, R. Aubert, and D. Chapelot. 2004. 'Plasma ghrelin levels and hunger scores among humans initiating meals voluntarily in the absence of time- and food-related cues', *Am.J Physiol Endocrinol Metab*.
Cummings, D. E., J. Q. Purnell, R. S. Frayo, K. Schmidova, B. E. Wisse, and D. S. Weigle. 2001. 'A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans', *Diabetes*, 50: 1714-19.
Cummings, D. E., D. S. Weigle, R. S. Frayo, P. A. Breen, M. K. Ma, E. P. Dellinger, and J. Q. Purnell. 2002b. 'Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery', *New England Journal of Medicine*, 346: 1623-30.
Cunningham, J. J. 1980. 'A reanalysis of the factors influencing basal metabolic rate in normal adults', *Am J Clin Nutr*, 33: 2372-4.
Cyders, M. A., K. Flory, S. Rainer, and G. T. Smith. 2009. 'The role of personality dispositions to risky behavior in predicting first-year college drinking', *Addiction*, 104: 193-202.
Davis, C., K. Patte, C. Curtis, and C. Reid. 2010. 'Immediate pleasures and future consequences. A neuropsychological study of binge eating and obesity', *Appetite*, 54: 208-13.
de Graaf, C., W. A. Blom, P. A. Smeets, A. Stafleu, and H. F. Hendriks. 2004. 'Biomarkers of satiation and satiety', *Am J Clin Nutr*, 79: 946-61.
de Jonge, C., S. S. Rensen, G. H. Koek, M. F. Joosten, W. A. Buurman, N. D. Bouvy, and J. W. Greve. 2013. 'Endoscopic duodenal-jejunal bypass liner rapidly improves plasma parameters of nonalcoholic fatty liver disease', *Clin Gastroenterol Hepatol*, 11: 1517-20.
de Jonge, C., S. S. Rensen, F. J. Verdam, R. P. Vincent, S. R. Bloom, W. A. Buurman, C. W. le Roux, N. D. Bouvy, and J. W. Greve. 2015. 'Impact of Duodenal-Jejunal Exclusion on Satiety Hormones', *Obes Surg*, 26: 672-78.
de Silva, A., V. Salem, C. J. Long, A. Makwana, R. D. Newbould, E. A. Rabiner, M. A. Ghatei, S. R. Bloom, P. M. Matthews, J. D. Beaver, and W. S. Dhillo. 2011.

'The gut hormones PYY 3-36 and GLP-1 7-36 amide reduce food intake and modulate brain activity in appetite centers in humans', *Cell Metab*, 14: 700-06.
De Vriese, C., F. Gregoire, R. Lema-Kisoka, M. Waelbroeck, P. Robberecht, and C. Delporte. 2004. 'Ghrelin degradation by serum and tissue homogenates: identification of the cleavage sites', *Endocrinology*, 145: 4997-5005.
DeCosta, P., P. Moller, M. B. Frost, and A. Olsen. 2017. 'Changing children's eating behaviour - A review of experimental research', *Appetite*, 113: 327-57.
Deighton, K., R. L. Batterham, and D. J. Stensel. 2014. 'Appetite and gut peptide responses to exercise and calorie restriction. The effect of modest energy deficits', *Appetite*, 81: 52-9.
Delhanty, P. J., S. J. Neggers, and A. J. Van der Lely. 2013. 'Des-acyl ghrelin: a metabolically active peptide', *Endocr.Dev.*, 25: 112-21.
———. 2014. 'Should we consider des-acyl ghrelin as a separate hormone and if so, what does it do?', *Front Horm.Res.*, 42: 163-74.
Di Francesco, V., M. Zamboni, E. Zoico, G. Mazzali, A. Dioli, F. Omizzolo, L. Bissoli, F. Fantin, P. Rizzotti, S. B. Solerte, R. Micciolo, and O. Bosello. 2006. 'Unbalanced serum leptin and ghrelin dynamics prolong postprandial satiety and inhibit hunger in healthy elderly: another reason for the "anorexia of aging"', *Am J Clin Nutr*, 83: 1149-52.
Diano, S., S. A. Farr, S. C. Benoit, E. C. McNay, Silva I. da, B. Horvath, F. S. Gaskin, N. Nonaka, L. B. Jaeger, W. A. Banks, J. E. Morley, S. Pinto, R. S. Sherwin, L. Xu, K. A. Yamada, M. W. Sleeman, M. H. Tschop, and T. L. Horvath. 2006. 'Ghrelin controls hippocampal spine synapse density and memory performance', *Nat.Neurosci.*, 9: 381-88.
Dickson, S. L., E. Egecioglu, S. Landgren, K. P. Skibicka, J. A. Engel, and E. Jerlhag. 2011. 'The role of the central ghrelin system in reward from food and chemical drugs', *Mol Cell Endocrinol.*, 340: 80-87.
DiLeone, R. J., J. R. Taylor, and M. R. Picciotto. 2012. 'The drive to eat: comparisons and distinctions between mechanisms of food reward and drug addiction', *Nat.Neurosci.*, 15: 1330-35.
Disse, E., A. L. Bussier, C. Veyrat-Durebex, N. Deblon, P. T. Pfluger, M. H. Tschop, M. Laville, and F. Rohner-Jeanrenaud. 2010. 'Peripheral ghrelin enhances sweet taste food consumption and preference, regardless of its caloric content', *Physiol Behav.*, 101: 277-81.
Doucet, E., M. Laviolette, P. Imbeault, I. Strychar, R. Rabasa-Lhoret, and D. Prud'homme. 2008. 'Total peptide YY is a correlate of postprandial energy expenditure but not of appetite or energy intake in healthy women', *Metabolism*, 57: 1458-64.
Doucet, E., M. Pomerleau, and M. E. Harper. 2004. 'Fasting and postprandial total ghrelin remain unchanged after short-term energy restriction', *J Clin Endo Metab*, 89: 1727-32.
Druce, M. R., N. M. Neary, C. J. Small, J. Milton, M. Monteiro, M. Patterson, M. A. Ghatei, and S. R. Bloom. 2006. 'Subcutaneous administration of ghrelin stimulates energy intake in healthy lean human volunteers', *Int J Obes*, 30: 293-96.
Druce, M. R., A. M. Wren, A. J. Park, J. E. Milton, M. Patterson, G. Frost, M. A. Ghatei, C. Small, and S. R. Bloom. 2005. 'Ghrelin increases food intake in obese as well as lean subjects', *Int J Obes*, 29: 1130-36.
Dunn, J. P., N. N. Abumrad, R. M. Kessler, B. W. Patterson, R. Li, P. Marks-Shulman, and R. A. Tamboli. 2017. 'Caloric Restriction-Induced Decreases in

Dopamine Receptor Availability are Associated with Leptin Concentration', *Obesity*, 25: 1910-15.
Duszka, K., A. Gregor, M. W. Reichel, A. Baierl, C. Fahrngruber, and J. Konig. 2020. 'Visual stimulation with food pictures in the regulation of hunger hormones and nutrient deposition, a potential contributor to the obesity crisis', *PLoS One*, 15: e0232099.
Edwards, A., and A. Abizaid. 2016. 'Driving the need to feed: Insight into the collaborative interaction between ghrelin and endocannabinoid systems in modulating brain reward systems', *Neurosci Biobehav Rev*, 66: 33-53.
Egecioglu, E., P. Steensland, I. Fredriksson, K. Feltmann, J. A. Engel, and E. Jerlhag. 2013. 'The glucagon-like peptide 1 analogue Exendin-4 attenuates alcohol mediated behaviors in rodents', *Psychoneuroendocrinology*, 38: 1259-70.
Emilien, C., and J. H. Hollis. 2017. 'A brief review of salient factors influencing adult eating behaviour', *Nutr Res Rev*, 30: 233-46.
Emini, M., R. Bhargava, M. Aldhwayan, N. Chhina, M. Rodriguez Flores, G. Aldubaikhi, M. Al Lababidi, W. Al-Najim, A. D. Miras, A. Ruban, M. A. Glaysher, C. G. Prechtl, J. P. Byrne, J. P. Teare, and A. P. Goldstone. 2024. 'Satiety hormone LEAP2 after low-calorie diet with/without Endobarrier insertion in obesity and type 2 diabetes mellitus', *J Endocr Soc*, 9: bvae214.
Engel, J. A., and E. Jerlhag. 2014. 'Role of appetite-regulating peptides in the pathophysiology of addiction: implications for pharmacotherapy', *CNS Drugs*, 28: 875-86.
Englund, A., H. Gilliam-Vigh, M. P. Suppli, L. S. Gasbjerg, T. Vilsboll, and F. K. Knop. 2024. 'Intestinal Expression Profiles and Hepatic Expression of LEAP2, Ghrelin and their Common Receptor, GHSR, in Humans', *Peptides*, 177: 171227.
Englund, A., CA. Hagemann, LS. Gasbjerg, and FK. Knop. 2023. 'LEAP2 Reduces Preprandial and Postprandial Glucose Levels and Ad Libitum Food Intake in Obese Men', *Abstracts 84th Annual Meeting of American Diabetes Association, Orlando, USA*.
Erdmann, J., Y. Hebeisen, F. Lippl, S. Wagenpfeil, and V. Schusdziarra. 2007. 'Food intake and plasma ghrelin response during potato-, rice- and pasta-rich test meals', *Eur J Nutr*, 46: 196-203.
Erdmann, J., R. Topsch, F. Lippl, P. Gussmann, and V. Schusdziarra. 2004. 'Postprandial response of plasma ghrelin levels to various test meals in relation to food intake, plasma insulin, and glucose', *J Clin Endocrinol Metab*, 89: 3048-54.
Esler, W. P., J. Rudolph, T. H. Claus, W. Tang, N. Barucci, S. E. Brown, W. Bullock, M. Daly, L. Decarr, Y. Li, L. Milardo, D. Molstad, J. Zhu, S. J. Gardell, J. N. Livingston, and L. J. Sweet. 2007. 'Small-molecule ghrelin receptor antagonists improve glucose tolerance, suppress appetite, and promote weight loss', *Endocrinology*, 148: 5175-85.
Etxandi, M., I. Baenas, B. Mora-Maltas, R. Granero, F. Fernandez-Aranda, S. Tovar, N. Sole-Morata, I. Lucas, S. Casado, M. Gomez-Pena, L. Moragas, A. D. Pino-Gutierrez, E. Codina, E. Valenciano-Mendoza, M. N. Potenza, C. Dieguez, and S. Jimenez-Murcia. 2022. 'Are Signals Regulating Energy Homeostasis Related to Neuropsychological and Clinical Features of Gambling Disorder? A Case-Control Study', *Nutrients*, 14.

Etxandi, M., I. Baenas, B. Mora-Maltas, R. Granero, F. Fernandez-Aranda, S. Tovar, N. Sole-Morata, I. Lucas, S. Casado, M. Gomez-Pena, L. Moragas, A. D. Pino-Gutierrez, J. Tapia, E. Valenciano-Mendoza, M. N. Potenza, A. N. Gearhardt, C. Dieguez, and S. Jimenez-Murcia. 2023. 'Plasma concentration of leptin is related to food addiction in gambling disorder: Clinical and neuropsychological implications', *J Behav Addict*, 12: 1019-31.

Ezquerro, S., C. Tuero, S. Becerril, V. Valenti, R. Moncada, M. F. Landecho, V. Catalan, J. Gomez-Ambrosi, F. Mocha, C. Silva, K. Piper Hanley, J. Escalada, G. Fruhbeck, and A. Rodriguez. 2023. 'Antagonic effect of ghrelin and LEAP-2 on hepatic stellate cell activation and liver fibrosis in obesity-associated NAFLD', *Eur J Endocrinol*, 188: 564-77.

Fairburn, C. G., and S. J. Beglin. 1994. 'Assessment of eating disorders: interview or self-report questionnaire?', *Int.J.Eat.Disord.*, 16: 363-70.

Farokhnia, M., M. L. Faulkner, D. Piacentino, M. R. Lee, and L. Leggio. 2019. 'Ghrelin: From a gut hormone to a potential therapeutic target for alcohol use disorder', *Physiol Behav*, 204: 49-57.

Farokhnia, M., E. N. Grodin, M. R. Lee, E. N. Oot, A. N. Blackburn, B. L. Stangl, M. L. Schwandt, L. A. Farinelli, R. Momenan, V. A. Ramchandani, and L. Leggio. 2018. 'Exogenous ghrelin administration increases alcohol self-administration and modulates brain functional activity in heavy-drinking alcohol-dependent individuals', *Mol Psychiatry*, 23: 2029-38.

Farokhnia, M., J. Portelli, M. R. Lee, G. R. McDiarmid, V. Munjal, K. M. Abshire, J. T. Battista, B. D. Browning, S. L. Deschaine, F. Akhlaghi, and L. Leggio. 2020. 'Effects of exogenous ghrelin administration and ghrelin receptor blockade, in combination with alcohol, on peripheral inflammatory markers in heavy-drinking individuals: Results from two human laboratory studies', *Brain Res*, 1740: 146851.

Faulkner, M. L., M. Farokhnia, M. R. Lee, L. Farinelli, B. D. Browning, K. Abshire, A. M. Daurio, V. Munjal, S. L. Deschaine, S. R. Boukabara, C. Fortney, G. Sherman, M. Schwandt, F. Akhlaghi, R. Momenan, T. J. Ross, S. Persky, and L. Leggio. 2024. 'A randomized, double-blind, placebo-controlled study of a GHSR blocker in people with alcohol use disorder', *JCI Insight*, 9.

Fernandez, G., A. Cabral, M. F. Andreoli, A. Labarthe, C. M'Kadmi, J. G. Ramos, J. Marie, J. A. Fehrentz, J. Epelbaum, V. Tolle, and M. Perello. 2018. 'Evidence Supporting a Role for Constitutive Ghrelin Receptor Signaling in Fasting-Induced Hyperphagia in Male Mice', *Endocrinology*, 159: 1021-34.

Fernandez, G., A. Cabral, P. N. De Francesco, M. Uriarte, M. Reynaldo, D. Castrogiovanni, G. Zubiria, A. Giovambattista, S. Cantel, S. Denoyelle, J. A. Fehrentz, V. Tolle, H. B. Schioth, and M. Perello. 2022. 'GHSR controls food deprivation-induced activation of CRF neurons of the hypothalamic paraventricular nucleus in a LEAP2-dependent manner', *Cell Mol Life Sci*, 79: 277.

Ferreira-Junior, M. D., K. V. N. Cavalcante, C. H. Xavier, E. C. Vanzela, A. C. Boschero, P. Matafome, and R. M. Gomes. 2024. 'GHSR signalling in perinatal phases is involved in liver metabolism at puberty', *J Endocrinol*.

Finlayson, G., A. Arlotti, M. Dalton, N. King, and J. E. Blundell. 2011. 'Implicit wanting and explicit liking are markers for trait binge eating. A susceptible phenotype for overeating', *Appetite*, 57: 722-8.

Finlayson, G., N. King, and J. E. Blundell. 2007. 'Liking vs. wanting food: importance for human appetite control and weight regulation', *Neurosci Biobehav Rev*, 31: 987-1002.
Fittipaldi, A. S., J. Hernandez, D. Castrogiovanni, D. Lufrano, P. N. De Francesco, V. Garrido, P. Vitaux, M. V. Fasano, J. A. Fehrentz, A. Fernandez, M. F. Andreoli, and M. Perello. 2020. 'Plasma levels of ghrelin, des-acyl ghrelin and LEAP2 in children with obesity: correlation with age and insulin resistance', *Eur J Endocrinol*, 182: 165-75.
Flanagan, D. E., M. L. Evans, T. P. Monsod, F. Rife, R. A. Heptulla, W. V. Tamborlane, and R. S. Sherwin. 2003. 'The influence of insulin on circulating ghrelin', *Am J Physiol Endocrinol Metab*, 284: E313-E16.
Foster-Schubert, K. E., J. Overduin, C. E. Prudom, J. Liu, H. S. Callahan, B. D. Gaylinn, M. O. Thorner, and D. E. Cummings. 2008. 'Acyl and total ghrelin are suppressed strongly by ingested proteins, weakly by lipids, and biphasically by carbohydrates', *J Clin Endocrinol Metab*, 93: 1971-9.
Francisco, V., S. Tovar, J. Conde, J. Pino, A. Mera, F. Lago, M. A. Gonzalez-Gay, C. Dieguez, and O. Gualillo. 2020. 'Levels of the Novel Endogenous Antagonist of Ghrelin Receptor, Liver-Enriched Antimicrobial Peptide-2, in Patients with Rheumatoid Arthritis', *Nutrients*, 12.
Frank, S., N. Laharnar, S. Kullmann, R. Veit, C. Canova, Y. L. Hegner, A. Fritsche, and H. Preissl. 2010. 'Processing of food pictures: influence of hunger, gender and calorie content', *Brain Res.*, 1350: 159-66.
Frecka, J. M., and R. D. Mattes. 2008. 'Possible entrainment of ghrelin to habitual meal patterns in humans', *Am J Physiol Gastrointest Liver Physiol*, 294: G699-707.
Frias, J. P., S. Deenadayalan, L. Erichsen, F. K. Knop, I. Lingvay, S. Macura, C. Mathieu, S. D. Pedersen, and M. Davies. 2023. 'Efficacy and safety of co-administered once-weekly cagrilintide 2.4 mg with once-weekly semaglutide 2.4 mg in type 2 diabetes: a multicentre, randomised, double-blind, active-controlled, phase 2 trial', *Lancet*, 402: 720-30.
Fuglsang, J., C. Skjaerbaek, U. Espelund, J. Frystyk, S. Fisker, A. Flyvbjerg, and P. Ovesen. 2005. 'Ghrelin and its relationship to growth hormones during normal pregnancy', *Clin Endocrinol (Oxf)*, 62: 554-9.
Fuhrer, D., S. Zysset, and M. Stumvoll. 2008. 'Brain activity in hunger and satiety: an exploratory visually stimulated fMRI study', *Obesity*, 16: 945-50.
Gallwitz, B. 2012. 'Anorexigenic effects of GLP-1 and its analogues', *Handb Exp Pharmacol*: 185-207.
Garces, M. F., J. D. Buell-Acosta, E. Angel-Muller, A. J. Parada-Banos, J. Acosta-Alvarez, H. F. Saavedra-Lopez, R. Franco-Vega, L. M. Maldonado-Acosta, F. Escobar-Cordoba, K. Vasquez-Romero, E. Lacunza, S. A. Caminos-Cepeda, R. Nogueiras, C. Dieguez, A. I. Ruiz-Parra, and J. E. Caminos. 2022. 'Study of the Ghrelin/LEAP-2 Ratio in Humans and Rats during Different Phases of Pregnancy', *Int J Mol Sci*, 23.
Garcia, J. M., D. Iyer, W. S. Poston, M. Marcelli, R. Reeves, J. Foreyt, and A. Balasubramanyam. 2006. 'Rise of plasma ghrelin with weight loss is not sustained during weight maintenance', *Obesity (Silver Spring)*, 14: 1716-23.
Garin, M. C., C. M. Burns, S. Kaul, and A. R. Cappola. 2013. 'Clinical review: The human experience with ghrelin administration', *J Clin Endocrinol Metab*, 98: 1826-37.

Gauna, C., F. M. Meyler, J. A. Janssen, P. J. Delhanty, T. Abribat, P. van Koetsveld, L. J. Hofland, F. Broglio, E. Ghigo, and A. J. van der Lely. 2004. 'Administration of acylated ghrelin reduces insulin sensitivity, whereas the combination of acylated plus unacylated ghrelin strongly improves insulin sensitivity', *J Clin Endocrinol Metab*, 89: 5035-42.

Ge, X., H. Yang, M. A. Bednarek, H. Galon-Tilleman, P. Chen, M. Chen, J. S. Lichtman, Y. Wang, O. Dalmas, Y. Yin, H. Tian, L. Jermutus, J. Grimsby, C. M. Rondinone, A. Konkar, and D. D. Kaplan. 2018. 'LEAP2 is an endogenous antagonist of the ghrelin receptor', *Cell Metab*, 27: 461-69 e6.

Gearhardt, A. N., W. R. Corbin, and K. D. Brownell. 2009. 'Preliminary validation of the Yale Food Addiction Scale', *Appetite*, 52: 430-6.

Gibbons, C., P. Caudwell, G. Finlayson, D. L. Webb, P. M. Hellstrom, E. Naslund, and J. E. Blundell. 2013. 'Comparison of postprandial profiles of ghrelin, active GLP-1, and total PYY to meals varying in fat and carbohydrate and their association with hunger and the phases of satiety', *J Clin Endocrinol Metab*, 98: E847-55.

Giezenaar, C., K. Lange, T. Hausken, K. L. Jones, M. Horowitz, I. Chapman, and S. Soenen. 2018a. 'Acute Effects of Substitution, and Addition, of Carbohydrates and Fat to Protein on Gastric Emptying, Blood Glucose, Gut Hormones, Appetite, and Energy Intake', *Nutrients*, 10.

Giezenaar, C., N. D. Luscombe-Marsh, A. T. Hutchison, S. Standfield, C. Feinle-Bisset, M. Horowitz, I. Chapman, and S. Soenen. 2018b. 'Dose-Dependent Effects of Randomized Intraduodenal Whey-Protein Loads on Glucose, Gut Hormone, and Amino Acid Concentrations in Healthy Older and Younger Men', *Nutrients*, 10.

Giorgioni, G., F. Del Bello, W. Quaglia, L. Botticelli, C. Cifani, E. Micioni Di Bonaventura, M. V. Micioni Di Bonaventura, and A. Piergentili. 2022. 'Advances in the Development of Nonpeptide Small Molecules Targeting Ghrelin Receptor', *J Med Chem*, 65: 3098-118.

Glaysher, M. A., A. Mohanaruban, C. G. Prechtl, A. P. Goldstone, A. D. Miras, J. Lord, N. Chhina, E. Falaschetti, N. A. Johnson, W. Al-Najim, C. Smith, J. V. Li, M. Patel, A. R. Ahmed, M. Moore, N. Poulter, S. Bloom, A. Darzi, C. Le Roux, J. P. Byrne, and J. P. Teare. 2017. 'A randomised controlled trial of a duodenal-jejunal bypass sleeve device (EndoBarrier) compared with standard medical therapy for the management of obese subjects with type 2 diabetes mellitus', *BMJ Open*, 7: e018598.

Glaysher, M. A., J. Ward, M. Aldhwayan, A. Ruban, C. G. Prechtl, H. L. Fisk, N. Chhina, W. Al-Najim, C. Smith, N. Klimowska-Nassar, N. Johnson, E. Falaschetti, A. P. Goldstone, A. D. Miras, J. P. Byrne, P. C. Calder, and J. P. Teare. 2021. 'The effect of a duodenal-jejunal bypass liner on lipid profile and blood concentrations of long chain polyunsaturated fatty acids', *Clin Nutr*, 40: 2343-54.

Goldstein, J. L., T. J. Zhao, R. L. Li, D. P. Sherbet, G. Liang, and M. S. Brown. 2011. 'Surviving Starvation: Essential Role of the Ghrelin-Growth Hormone Axis', *Cold Spring Harb Symp Quant Biol*, 76: 121-27.

Goldstone, A. P. 2004. 'Prader-Willi syndrome: advances in its genetics, pathophysiology and treatment', *Trends in Endocrinology and Metabolism*, 15: 12-20.

Goldstone, A. P., A. J. Holland, J. V. Butler, and J. E. Whittington. 2012. 'Appetite hormones and the transition to hyperphagia in children with Prader-Willi syndrome', *Int J Obes (Lond)*, 36: 1564-70.
Goldstone, A. P., B. K. Mani, B. Gaylinn, C. G. Prechtl, M. L. Sleeth, S. Scholtz, A. D. Miras, N. M. Daud, C. Pettit, N. Chhina, and J. M. Zigman. 2022. 'Fasting plasma acyl ghrelin to LEAP2 ratio is positively correlated with human appetite and food intake, and negatively correlated with high-energy food cue reactivity in posterior cingulate cortex', *29th Annual Meeting of Society for the Study of Ingestive Behavior, Porto*.
Goldstone, A. P., A. D. Miras, S. Scholtz, S. Jackson, K. J. Neff, L. Penicaud, J. Geoghegan, N. Chhina, G. Durighel, J. D. Bell, S. Meillon, and C. W. le Roux. 2016. 'Link between increased satiety gut hormones and reduced food reward following gastric bypass surgery for obesity', *J Clin Endocrinol Metab*, 101: 599-609.
Goldstone, A. P., M. Patterson, N. Kalingag, M. A. Ghatei, A. E. Brynes, S. R. Bloom, A. B. Grossman, and M. Korbonits. 2005. 'Fasting and post-prandial hyperghrelinemia in Prader-Willi syndrome is partially explained by hypoinsulinemia, and is not due to peptide YY 3-36 deficiency or seen in hypothalamic obesity due to craniopharyngioma', *J Clin Endocrinol Metab*, 90: 2681-90.
Goldstone, A. P., C. G. Prechtl, S. Scholtz, A. D. Miras, N. Chhina, G. Durighel, S. S. Deliran, C. Beckmann, M. A. Ghatei, D. R. Ashby, A. D. Waldman, B. D. Gaylinn, M. O. Thorner, G. S. Frost, S. R. Bloom, and J. D. Bell. 2014. 'Ghrelin mimics fasting to enhance human hedonic, orbitofrontal cortex, and hippocampal responses to food', *Am J Clin Nutr*, 99: 1319-30.
Goldstone, A. P., C. G. Prechtl de Hernandez, J. D. Beaver, K. Muhammed, C. Croese, G. Bell, G. Durighel, E. Hughes, A. D. Waldman, G. Frost, and J. D. Bell. 2009. 'Fasting biases brain reward systems towards high-calorie foods', *Eur J Neurosci*, 30: 1625-35.
Goldstone, A. P., E. L. Thomas, A. E. Brynes, G. Castroman, R. Edwards, M. A. Ghatei, G. Frost, A. J. Holland, A. B. Grossman, M. Korbonits, S. R. Bloom, and J. D. Bell. 2004. 'Elevated fasting plasma ghrelin in Prader-Willi syndrome adults is not solely explained by their reduced visceral adiposity and insulin resistance', *J Clin Endocrinol Metab*, 89: 1718-26.
Gormally, J., S. Black, S. Daston, and D. Rardin. 1982. 'The assessment of binge eating severity among obese persons', *Addict Behav*, 7: 47-55.
Gradel, A. K. J., S. K. Holm, S. Byberg, M. Merkestein, W. F. J. Hogendorf, M. L. Lund, J. A. Buijink, J. Damgaard, J. Lykkesfeldt, and B. Holst. 2023. 'The dietary regulation of LEAP2 depends on meal composition in mice', *Faseb j*, 37: e22923.
Granata, R., and E. Ghigo. 2013. 'Products of the ghrelin gene, the pancreatic beta-cell and the adipocyte', *Endocr Dev*, 25: 144-56.
Gray, S. M., L. C. Page, and J. Tong. 2019. 'Ghrelin regulation of glucose metabolism', *J Neuroendocrinol*, 31: e12705.
Griffioen-Roose, S., G. Finlayson, M. Mars, J. E. Blundell, and C. de Graaf. 2010. 'Measuring food reward and the transfer effect of sensory specific satiety', *Appetite*, 55: 648-55.
Grimm, E. R., and N. I. Steinle. 2011. 'Genetics of eating behavior: established and emerging concepts', *Nutr Rev*, 69: 52-60.

Guclu, M., S. Kiyici, Z. Gul, and S. Cavun. 2018. 'Exenatide treatment causes suppression of serum fasting ghrelin levels in patients with type 2 diabetes mellitus', *Endocr Connect*, 7: 193-98.
Guerrero-Hreins, E., A. P. Goldstone, R. M. Brown, and P. Sumithran. 2021. 'The therapeutic potential of GLP-1 analogues for stress-related eating and role of GLP-1 in stress, emotion and mood: a review', *Prog Neuropsychopharmacol Biol Psychiatry*, 110: 110303.
Guo, J. 2024. 'Do plasma appetitive gut hormones acyl ghrelin and LEAP2 influence appetite, food cue reactivity, hedonics, intake and motivation in adults with obesity', *MRes Clinical Research Human Nutrition Imperial College London*.
Gupta, D., A. W. Burstein, D. C. Schwalbe, K. Shankar, S. Varshney, O. Singh, S. Paul, S. B. Ogden, S. Osborne-Lawrence, N. P. Metzger, C. P. Richard, J. N. Campbell, and J. M. Zigman. 2023. 'Ghrelin deletion and conditional ghrelin cell ablation increase pancreatic islet size in mice', *J Clin Invest*, 133.
Gupta, D., A. W. Burstein, K. Shankar, S. Varshney, O. Singh, S. Osborne-Lawrence, C. P. Richard, and J. M. Zigman. 2024. 'Impact of ghrelin on islet size in non-pregnant and pregnant female mice', *Endocrinology*.
Gupta, D., G. K. C. Dowsett, B. K. Mani, K. Shankar, S. Osborne-Lawrence, N. P. Metzger, B. Y. H. Lam, G. S. H. Yeo, and J. M. Zigman. 2021a. 'High co-expression of the ghrelin and LEAP2 receptor GHSR with pancreatic polypeptide in mouse and human islets', *Endocrinology*.
Gupta, D., S. B. Ogden, K. Shankar, S. Varshney, and J. M. Zigman. 2021b. '"A LEAP 2 conclusions? Targeting the ghrelin system to treat obesity and diabetes"', *Mol Metab*, 46: 101128.
Hagemann, C. A., M. S. Jensen, S. Holm, L. S. Gasbjerg, S. Byberg, K. Skov-Jeppesen, B. Hartmann, J. J. Holst, F. Dela, T. Vilsboll, M. B. Christensen, B. Holst, and F. K. Knop. 2022. 'LEAP2 reduces postprandial glucose excursions and ad libitum food intake in healthy men', *Cell Rep Med*, 3: 100582.
Hagemann, C. A., C. Zhang, H. H. Hansen, T. Jorsal, K. T. G. Rigbolt, M. R. Madsen, N. C. Bergmann, S. M. N. Heimburger, M. Falkenhahn, S. Theis, K. Breitschopf, S. Holm, M. A. Hedegaard, M. B. Christensen, T. Vilsboll, B. Holst, N. Vrang, J. Jelsing, and F. K. Knop. 2021. 'Identification and metabolic profiling of a novel human gut-derived LEAP2 fragment', *J Clin Endocrinol Metab*, 106: e966-e81.
Haidt, Jonathan, Clark McCauley, and Paul Rozin. 1994. 'Individual differences in sensitivity to disgust: A scale sampling seven domains of disgust elicitors', *Personality and Individual Differences*, 16: 701-13.
Hall, K. D., S. B. Heymsfield, J. W. Kemnitz, S. Klein, D. A. Schoeller, and J. R. Speakman. 2012. 'Energy balance and its components: implications for body weight regulation', *Am J Clin Nutr*, 95: 989-94.
Hamm, J. D., J. Dotel, S. Tamura, A. Shechter, M. Herzog, J. M. Brunstrom, J. Albu, F. X. Pi-Sunyer, B. Laferrere, and H. R. Kissileff. 2020. 'Reliability and responsiveness of virtual portion size creation tasks: Influences of context, foods, and a bariatric surgical procedure', *Physiol Behav*, 223: 113001.
Hamm, J. D., R. R. Klatzkin, M. Herzog, S. Tamura, J. M. Brunstrom, and H. R. Kissileff. 2021. 'Recalled and momentary virtual portions created of snacks predict actual intake under laboratory stress condition', *Physiol Behav*, 238: 113479.

Han, J. C., P. Balagopal, S. Sweeten, D. Darmaun, and N. Mauras. 2006. 'Evidence for hypermetabolism in boys with constitutional delay of growth and maturation', *J Clin Endocrinol Metab*, 91: 2081-6.
Han, J. E., J. Frasnelli, Y. Zeighami, K. Larcher, J. Boyle, T. McConnell, S. Malik, M. Jones-Gotman, and A. Dagher. 2018. 'Ghrelin Enhances Food Odor Conditioning in Healthy Humans: An fMRI Study', *Cell Rep*, 25: 2643-52 e4.
Han, X. D., H. W. Zhang, T. Xu, L. Liu, H. T. Cai, Z. Q. Liu, Q. Li, H. Zheng, T. Xu, and T. F. Yuan. 2022. 'How Impulsiveness Influences Obesity: The Mediating Effect of Resting-State Brain Activity in the dlPFC', *Front Psychiatry*, 13: 873953.
Haqq, A. M., J. Q. Purnell, R. G. Rosenfeld, K. L. Pratt, E. Tjoeng, and S. H. LaFranchi. 2002. 'Serum ghrelin levels are markedly increased in children with Prader-Willi syndrome (PWS)', *Abstracts 84th Annual Meeting Endocrine Society, San Francisco*, OR23-6.
Henriques, S. T., C. C. Tan, D. J. Craik, and R. J. Clark. 2010. 'Structural and functional analysis of human liver-expressed antimicrobial peptide 2', *Chembiochem*, 11: 2148-57.
Hewawasam, N., D. Sarkar, O. Bolton, B. Delishaj, M. Almutairi, A. J. F. King, A. S. Dereli, C. Despontin, P. Gilon, S. Reeves, M. Patterson, and A. C. Hauge-Evans. 2024. 'The GHSR1a antagonist LEAP2 regulates islet hormone release in a sex-specific manner', *J Endocrinol*, 263.
Hola, L., T. Tureckiuova, J. Kunes, B. Zelezna, and L. Maletinska. 2023. 'High-Fat Diet Induces Resistance to Ghrelin and LEAP2 Peptide Analogs in Mice', *Physiol Res*, 72: 607-19.
Hola, L., B. Zelezna, A. Karnosova, J. Kunes, J. A. Fehrentz, S. Denoyelle, S. Cantel, M. Blechova, D. Sykora, A. Myskova, and L. Maletinska. 2022. 'A Novel Truncated Liver Enriched Antimicrobial Peptide-2 Palmitoylated at its N-Terminal Antagonizes Effects of Ghrelin', *J Pharmacol Exp Ther*, 383: 129-36.
Holm, S., A. S. Husted, L. J. Skov, T. H. Morville, C. A. Hagemann, T. Jorsal, M. Dall, A. Jakobsen, A. B. Klein, J. T. Treebak, F. K. Knop, T. W. Schwartz, C. Clemmensen, and B. Holst. 2022. 'Beta-hydroxybutyrate suppresses hepatic production of the ghrelin receptor antagonist, LEAP2', *Endocrinology*, 163: bqac038.
Holm, S. K., E. T. Vestergaard, N. B. Zubanovic, S. Byberg, C. Clemmensen, B. Holst, and H. H. Thomsen. 2023. 'Ketone monoester increases circulating levels of LEAP2 and decreases appetite in healthy men', *Diabetes Obes Metab*, 25: 2023-27.
Holst, B., A. Cygankiewicz, Jensen T. Halkjar, M. Ankersen, and T. W. Schwartz. 2003. 'High Constitutive Signaling of the Ghrelin Receptor-Identification of a Potent Inverse Agonist', *Mol.Endocrinol*.
Holst, B., and T. W. Schwartz. 2004. 'Constitutive ghrelin receptor activity as a signaling set-point in appetite regulation', *Trends Pharmacol Sci.*, 25: 113-17.
Horsager, C., J. M. Bruun, E. Faerk, S. Hagstrom, M. B. Lauritsen, and S. D. Ostergaard. 2023. 'Food addiction is strongly associated with type 2 diabetes', *Clin Nutr*, 42: 717-21.
Hosoda, H. 2022. 'Effect of Ghrelin on the Cardiovascular System', *Biology (Basel)*, 11.
Howard, A., C. Townes, P. Milona, C. J. Nile, G. Michailidis, and J. Hall. 2010. 'Expression and functional analyses of liver expressed antimicrobial peptide-2 (LEAP-2) variant forms in human tissues', *Cell Immunol*, 261: 128-33.

Islam, M. N., Y. Mita, K. Maruyama, R. Tanida, W. Zhang, H. Sakoda, and M. Nakazato. 2020. 'Liver-expressed antimicrobial peptide 2 antagonizes the effect of ghrelin in rodents', *J Endocrinol*, 244: 13-23.
Islam, M. N., H. Nabekura, H. Ueno, T. Nishida, A. Nanashima, H. Sakoda, W. Zhang, and M. Nakazato. 2024. 'Liver-expressed antimicrobial peptide 2 is a hepatokine regulated by ghrelin, nutrients, and body weight', *Sci Rep*, 14: 24782.
Islam, M. N., W. Zhang, K. Sakai, Y. Nakazato, R. Tanida, H. Sakoda, T. Takei, T. Takao, and M. Nakazato. 2022. 'Liver-expressed antimicrobial peptide 2 functions independently of growth hormone secretagogue receptor in calorie-restricted mice', *Peptides*, 151: 170763.
Jakobsdottir, S., I. C. van Nieuwpoort, C. C. van Bunderen, M. B. de Ruiter, J. W. Twisk, J. B. Deijen, D. J. Veltman, and M. L. Drent. 2016. 'Acute and short-term effects of caloric restriction on metabolic profile and brain activation in obese, postmenopausal women', *Int J Obes (Lond)*, 40: 1671-78.
Japur, C. C., R. W. Diez-Garcia, F. R. de Oliveira Penaforte, G. das Gracas Pena, L. B. de Araujo, and M. F. S. de Sa. 2019. 'Insulin, ghrelin and early return of hunger in women with obesity and polycystic ovary syndrome', *Physiol Behav*, 206: 252-58.
Jastreboff, A. M., L. M. Kaplan, J. P. Frias, Q. Wu, Y. Du, S. Gurbuz, T. Coskun, A. Haupt, Z. Milicevic, M. L. Hartman, and Investigators Retatrutide Phase 2 Obesity Trial. 2023. 'Triple-Hormone-Receptor Agonist Retatrutide for Obesity - A Phase 2 Trial', *N Engl J Med*, 389: 514-26.
Jerlhag, E. 2019. 'Gut-brain axis and addictive disorders: A review with focus on alcohol and drugs of abuse', *Pharmacol Ther*, 196: 1-14.
Jerlhag, E., E. Egecioglu, S. L. Dickson, M. Andersson, L. Svensson, and J. A. Engel. 2006. 'Ghrelin stimulates locomotor activity and accumbal dopamine-overflow via central cholinergic systems in mice: implications for its involvement in brain reward', *Addict Biol.*, 11: 45-54.
Jerlhag, E., E. Egecioglu, S. L. Dickson, and J. A. Engel. 2011a. 'Glutamatergic regulation of ghrelin-induced activation of the mesolimbic dopamine system', *Addict Biol.*, 16: 82-91.
Jerlhag, E., E. Egecioglu, S. Landgren, N. Salome, M. Heilig, D. Moechars, R. Datta, D. Perrissoud, S. L. Dickson, and J. A. Engel. 2009. 'Requirement of central ghrelin signaling for alcohol reward', *Proc Natl Acad Sci*, 106: 11318-23.
Jerlhag, E., S. Landgren, E. Egecioglu, S. L. Dickson, and J. A. Engel. 2011b. 'The alcohol-induced locomotor stimulation and accumbal dopamine release is suppressed in ghrelin knockout mice', *Alcohol*, 45: 341-47.
Jiang, H., L. Betancourt, and R. G. Smith. 2006. 'Ghrelin amplifies dopamine signaling by cross talk involving formation of growth hormone secretagogue receptor/dopamine receptor subtype 1 heterodimers', *Mol.Endocrinol.*, 20: 1772-85.
Jones, R. B., S. McKie, N. Astbury, T. J. Little, S. Tivey, D. J. Lassman, J. McLaughlin, S. Luckman, S. R. Williams, G. J. Dockray, and D. G. Thompson. 2012. 'Functional neuroimaging demonstrates that ghrelin inhibits the central nervous system response to ingested lipid', *Gut*, 61: 1543-51.
Kakoschke, N., E. Aarts, and A. Verdejo-Garcia. 2018. 'The Cognitive Drivers of Compulsive Eating Behavior', *Front Behav Neurosci*, 12: 338.

Kamegai, J., H. Tamura, T. Shimizu, S. Ishii, H. Sugihara, and S. Oikawa. 2004. 'Effects of insulin, leptin, and glucagon on ghrelin secretion from isolated perfused rat stomach', *Regul Pept*, 119: 77-81.
Kanoski, S. E., and H. J. Grill. 2017. 'Hippocampus Contributions to Food Intake Control: Mnemonic, Neuroanatomical, and Endocrine Mechanisms', *Biol Psychiatry*, 81: 748-56.
Karlas, T., D. Petroff, J. Feisthammel, S. Beer, M. Bluher, T. Schutz, R. Lichtinghagen, A. Hoffmeister, and J. Wiegand. 2022. 'Endoscopic Bariatric Treatment with Duodenal-Jejunal Bypass Liner Improves Non-invasive Markers of Non-alcoholic Steatohepatitis', *Obes Surg*, 32: 2495-503.
Karra, E., O. G. O'Daly, A. I. Choudhury, A. Yousseif, S. Millership, M. T. Neary, W. R. Scott, K. Chandarana, S. Manning, M. E. Hess, H. Iwakura, T. Akamizu, Q. Millet, C. Gelegen, M. E. Drew, S. Rahman, J. J. Emmanuel, S. C. Williams, U. U. Ruther, J. C. Bruning, D. J. Withers, F. O. Zelaya, and R. L. Batterham. 2013. 'A link between FTO, ghrelin, and impaired brain food-cue responsivity', *J Clin Invest*, 123: 3539-51.
Karschin, J., M. Lagerpusch, J. Enderle, B. Eggeling, M. J. Muller, and A. Bosy-Westphal. 2015. 'Endocrine determinants of changes in insulin sensitivity and insulin secretion during a weight cycle in healthy men', *PLoS One*, 10: e0117865.
Kern, A., R. Albarran-Zeckler, H. E. Walsh, and R. G. Smith. 2012. 'Apo-ghrelin receptor forms heteromers with DRD2 in hypothalamic neurons and is essential for anorexigenic effects of DRD2 agonism', *Neuron*, 73: 317-32.
Kharbanda, K. K., M. Farokhnia, S. L. Deschaine, R. Bhargava, M. Rodriguez Flores, C. A. Casey, A. P. Goldstone, E. Jerlhag, L. Leggio, and K. Rasineni. 2022. 'Role of the ghrelin system in alcohol use disorder and alcohol-associated liver disease: A narrative review', *Alcohol Clin Exp Res*, 46: 2149-59.
Kim, B. W., B. I. Lee, H. K. Kim, Y. S. Cho, H. S. Chae, H. K. Lee, H. J. Kim, and S. W. Han. 2009. 'Influence of long-term gastric acid suppression therapy on the expression of serum gastrin, chromogranin A, and ghrelin', *Korean J Gastroenterol*, 53: 84-9.
Kim, D. J., S. J. Yoon, B. Choi, T. S. Kim, Y. S. Woo, W. Kim, H. Myrick, B. S. Peterson, Y. B. Choi, Y. K. Kim, and J. Jeong. 2005. 'Increased fasting plasma ghrelin levels during alcohol abstinence', *Alcohol Alcohol*, 40: 76-9.
Knutson, B., C. M. Adams, G. W. Fong, and D. Hommer. 2001. 'Anticipation of increasing monetary reward selectively recruits nucleus accumbens', *J Neurosci.*, 21: RC159.
Koehestanie, P., C. de Jonge, F. J. Berends, I. M. Janssen, N. D. Bouvy, and J. W. Greve. 2014. 'The effect of the endoscopic duodenal-jejunal bypass liner on obesity and type 2 diabetes mellitus, a multicenter randomized controlled trial', *Ann Surg*, 260: 984-92.
Kojima, M., H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, and K. Kangawa. 1999. 'Ghrelin is a growth-hormone-releasing acylated peptide from stomach', *Nature*, 402: 656-60.
Kojima, M., and K. Kangawa. 2006. 'Drug insight: The functions of ghrelin and its potential as a multitherapeutic hormone', *Nat Clin Pract Endocrinol Metab*, 2: 80-88.
Koopmann, A., P. Bach, R. Schuster, J. M. Bumb, S. Vollstadt-Klein, I. Reinhard, M. Rietschel, S. H. Witt, K. Wiedemann, and F. Kiefer. 2018. 'Ghrelin modulates

mesolimbic reactivity to alcohol cues in alcohol-addicted subjects: a functional imaging study', *Addict Biol*.
Koopmann, A., C. von der Goltz, M. Grosshans, C. Dinter, M. Vitale, K. Wiedemann, and F. Kiefer. 2012. 'The association of the appetitive peptide acetylated ghrelin with alcohol craving in early abstinent alcohol dependent individuals', *Psychoneuroendocrinology*, 37: 980-6.
Korbonits, M., A. P. Goldstone, M. Gueorguiev, and A. B. Grossman. 2004. 'Ghrelin - a hormone with multiple functions', *Front Neuroendocrinol*, 25: 27-68.
Krause, A., S. Neitz, H. J. Magert, A. Schulz, W. G. Forssmann, P. Schulz-Knappe, and K. Adermann. 2000. 'LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity', *FEBS Lett*, 480: 147-50.
Krause, A., R. Sillard, B. Kleemeier, E. Kluver, E. Maronde, J. R. Conejo-Garcia, W. G. Forssmann, P. Schulz-Knappe, M. C. Nehls, F. Wattler, S. Wattler, and K. Adermann. 2003. 'Isolation and biochemical characterization of LEAP-2, a novel blood peptide expressed in the liver', *Protein Sci*, 12: 143-52.
Kroemer, N. B., L. Krebs, A. Kobiella, O. Grimm, M. Pilhatsch, M. Bidlingmaier, U. S. Zimmermann, and M. N. Smolka. 2013. 'Fasting levels of ghrelin covary with the brain response to food pictures', *Addict Biol.*, 18: 855-62.
Kruger, A. L., A. Malpeli, M. Sala, C. Casado, I. Mendez, L. Fotia, M. Lopez, A. Tournier, D. Castrogiovanni, F. Heredia, R. Llovera, H. B. Schioth, M. Perello, and M. F. Andreoli. 2024. 'The Concentration of Liver-Expressed Antimicrobial Peptide 2 in Human Milk and Infant Plasma Is Positively Associated with Adiposity and Body Weight in the First Year of Life', *J Nutr*, 154: 3388-99.
Kunath, N., N. C. Muller, M. Tonon, B. N. Konrad, M. Pawlowski, A. Kopczak, I. Elbau, M. Uhr, S. Kuhn, D. Repantis, K. Ohla, T. D. Muller, G. Fernandez, M. Tschop, M. Czisch, A. Steiger, and M. Dresler. 2016. 'Ghrelin modulates encoding-related brain function without enhancing memory formation in humans', *Neuroimage*, 142: 465-73.
Kuppens, R. J., G. Diene, N. E. Bakker, C. Molinas, S. Faye, M. Nicolino, D. Bernoux, P. J. Delhanty, A. J. van der Lely, S. Allas, M. Julien, T. Delale, M. Tauber, and A. C. Hokken-Koelega. 2015. 'Elevated ratio of acylated to unacylated ghrelin in children and young adults with Prader-Willi syndrome', *Endocrine*, 50: 633-42.
Langenberg, C., J. Bergstrom, G. A. Laughlin, and E. Barrett-Connor. 2005. 'Ghrelin and the metabolic syndrome in older adults', *J Clin Endocrinol Metab*, 90: 6448-53.
Larson-Meyer, D. E., K. S. Willis, L. M. Willis, K. J. Austin, A. M. Hart, A. B. Breton, and B. M. Alexander. 2010. 'Effect of honey versus sucrose on appetite, appetite-regulating hormones, and postmeal thermogenesis', *J Am Coll Nutr*, 29: 482-93.
Lauritzen, E. S., J. O. L. Jorgensen, N. Moller, S. Nielsen, and E. T. Vestergaard. 2020. 'Increased lipolysis after infusion of acylated ghrelin: a randomized, double-blinded placebo-controlled trial in hypopituitary patients', *Clin Endocrinol (Oxf)*, 93: 672-77.
le Roux, C. W., R. L. Batterham, S. J. Aylwin, M. Patterson, C. M. Borg, K. J. Wynne, A. Kent, R. P. Vincent, J. Gardiner, M. A. Ghatei, and S. R. Bloom. 2006. 'Attenuated peptide YY release in obese subjects is associated with reduced satiety', *Endocrinology*, 147: 3-8.
le Roux, C. W., M. Patterson, R. P. Vincent, C. Hunt, M. A. Ghatei, and S. R. Bloom. 2005. 'Postprandial plasma ghrelin is suppressed proportional to meal calorie

content in normal weight but not obese subjects', *J Clin Endo Metab*, 90: 1068-71.
Lee, H., C. Te, S. Koshy, J. A. Teixeira, F. X. Pi-Sunyer, and B. Laferrere. 2006. 'Does ghrelin really matter after bariatric surgery?', *Surg.Obes.Relat Dis.*, 2: 538-48.
Lee, J. H., C. Fang, X. Li, C. S. Wu, J. Y. Noh, X. Ye, R. S. Chapkin, K. Sun, and Y. Sun. 2021. 'GHS-R suppression in adipose tissues protects against obesity and insulin resistance by regulating adipose angiogenesis and fibrosis', *Int J Obes (Lond)*, 45: 1565-75.
Lee, M. R., M. Farokhnia, E. Cobbina, A. Saravanakumar, X. Li, J. T. Battista, L. A. Farinelli, F. Akhlaghi, and L. Leggio. 2020a. 'Endocrine effects of the novel ghrelin receptor inverse agonist PF-5190457: Results from a placebo-controlled human laboratory alcohol co-administration study in heavy drinkers', *Neuropharmacology*, 170: 107788.
Lee, M. R., J. D. Tapocik, M. Ghareeb, M. L. Schwandt, A. A. Dias, A. N. Le, E. Cobbina, L. A. Farinelli, S. Bouhlal, M. Farokhnia, M. Heilig, F. Akhlaghi, and L. Leggio. 2020b. 'The novel ghrelin receptor inverse agonist PF-5190457 administered with alcohol: preclinical safety experiments and a phase 1b human laboratory study', *Mol Psychiatry*, 25: 461-75.
Leech, R., and D. J. Sharp. 2014. 'The role of the posterior cingulate cortex in cognition and disease', *Brain*, 137: 12-32.
Leggio, L., G. Addolorato, A. Cippitelli, E. Jerlhag, A. B. Kampov-Polevoy, and R. M. Swift. 2011. 'Role of feeding-related pathways in alcohol dependence: A focus on sweet preference, NPY, and ghrelin', *Alcohol Clin Exp Res.*, 35: 194-202.
Leggio, L., W. H. Zywiak, S. R. Fricchione, S. M. Edwards, S. M. de la Monte, R. M. Swift, and G. A. Kenna. 2014. 'Intravenous ghrelin administration increases alcohol craving in alcohol-dependent heavy drinkers: a preliminary investigation', *Biol Psychiatry*, 76: 734-41.
Lehtihet, M., S. Arver, I. Bartuseviciene, and A. Pousette. 2012. 'S-testosterone decrease after a mixed meal in healthy men independent of SHBG and gonadotrophin levels', *Andrologia*, 44: 405-10.
Levin, F., T. Edholm, P. T. Schmidt, P. Gryback, H. Jacobsson, M. Degerblad, C. Hoybye, J. J. Holst, J. F. Rehfeld, P. M. Hellstrom, and E. Naslund. 2006. 'Ghrelin stimulates gastric emptying and hunger in normal-weight humans', *J Clin.Endocrinol.Metab*, 91: 3296-302.
Li, G., G. Ji, Y. Hu, L. Liu, Q. Jin, W. Zhang, L. Liu, Y. Wang, J. Zhao, K. M. von Deneen, A. Chen, G. Cui, Y. Han, H. Wang, Q. Zhao, K. Wu, C. E. Wiers, D. Tomasi, L. Leggio, N. D. Volkow, Y. Nie, Y. Zhang, and G. J. Wang. 2019. 'Reduced plasma ghrelin concentrations are associated with decreased brain reactivity to food cues after laparoscopic sleeve gastrectomy', *Psychoneuroendocrinology*, 100: 229-36.
Li, J., R. An, Y. Zhang, X. Li, and S. Wang. 2012. 'Correlations of macronutrient-induced functional magnetic resonance imaging signal changes in human brain and gut hormone responses', *Am J Clin Nutr*, 96: 275-82.
Li, J., P. Huang, J. Xiong, X. Liang, M. Li, H. Ke, C. Chen, Y. Han, Y. Huang, Y. Zhou, Z. Luo, D. Feng, and C. Chen. 2022. 'Serum levels of ghrelin and LEAP2 in patients with type 2 diabetes mellitus: correlation with circulating glucose and lipids', *Endocr Connect*, 27: e220012.

Lin, C. E., and C. Y. Chen. 2024. 'Impacts of Central Administration of the Novel Peptide, LEAP-2, in Different Food Intake Models in Conscious Rats', *Nutrients*, 16.
Lingvay, I., R. V. Cohen, C. W. L. Roux, and P. Sumithran. 2024. 'Obesity in adults', *Lancet*, 404: 972-87.
Lioger, D., A. Fardet, P. Foassert, M. J. Davicco, J. Mardon, B. Gaillard-Martinie, and C. Remesy. 2009. 'Influence of sourdough prefermentation, of steam cooking suppression and of decreased sucrose content during wheat flakes processing on the plasma glucose and insulin responses and satiety of healthy subjects', *J Am Coll Nutr*, 28: 30-6.
Lippl, F., J. Erdmann, A. Steiger, N. Lichter, C. Czogalla-Peter, M. Bidlingmaier, S. Tholl, and V. Schusdziarra. 2012. 'Low-dose ghrelin infusion--evidence against a hormonal role in food intake', *Regul Pept*, 174: 26-31.
Lippl, F., F. Kircher, J. Erdmann, H. D. Allescher, and V. Schusdziarra. 2004. 'Effect of GIP, GLP-1, insulin and gastrin on ghrelin release in the isolated rat stomach', *Regul Pept*, 119: 93-98.
Liu, J., C. E. Prudom, R. Nass, S. S. Pezzoli, M. C. Oliveri, M. L. Johnson, P. Veldhuis, D. A. Gordon, A. D. Howard, D. R. Witcher, H. M. Geysen, B. D. Gaylinn, and M. O. Thorner. 2008. 'Novel ghrelin assays provide evidence for independent regulation of ghrelin acylation and secretion in healthy young men', *J Clin Endocrinol Metab*, 93: 1980-87.
Liu, Y., A. Roefs, and C. Nederkoorn. 2022. 'Fluctuations in attentional bias for food and the role of executive control', *Appetite*, 168: 105761.
Liu, Z., Q. Ren, H. Mu, Y. Zeng, Z. An, and H. He. 2024. 'Preliminary study on the diagnostic value of LEAP-2 and CK18 in biopsy-proven MAFLD', *BMC Gastroenterol*, 24: 182.
Logothetis, N. K. 2003. 'The underpinnings of the BOLD functional magnetic resonance imaging signal', *J Neurosci*, 23: 3963-71.
Lopez, R. B., M. Milyavskaya, W. Hofmann, and T. F. Heatherton. 2016. 'Motivational and neural correlates of self-control of eating: A combined neuroimaging and experience sampling study in dieting female college students', *Appetite*, 103: 192-99.
Lowe, M. R., M. L. Butryn, E. R. Didie, R. A. Annunziato, J. G. Thomas, C. E. Crerand, C. N. Ochner, M. C. Coletta, D. Bellace, M. Wallaert, and J. Halford. 2009. 'The Power of Food Scale. A new measure of the psychological influence of the food environment', *Appetite*, 53: 114-18.
Luck, A. J., J. F. Morgan, F. Reid, A. O'Brien, J. Brunton, C. Price, L. Perry, and J. H. Lacey. 2002. 'The SCOFF questionnaire and clinical interview for eating disorders in general practice: comparative study', *BMJ*, 325: 755-56.
Lugilde, J., S. Casado, D. Beiroa, J. Cunarro, M. Garcia-Lavandeira, C. V. Alvarez, R. Nogueiras, C. Dieguez, and S. Tovar. 2022. 'LEAP-2 Counteracts Ghrelin-Induced Food Intake in a Nutrient, Growth Hormone and Age Independent Manner', *Cells*, 11: 324.
Lyngstad, A., S. Nymo, S. R. Coutinho, J. F. Rehfeld, H. Truby, B. Kulseng, and C. Martins. 2019. 'Investigating the effect of sex and ketosis on weight-loss-induced changes in appetite', *Am J Clin Nutr*, 109: 1511-18.
M'Kadmi, C., A. Cabral, F. Barrile, J. Giribaldi, S. Cantel, M. Damian, S. Mary, S. Denoyelle, S. Dutertre, S. Peraldi-Roux, J. Neasta, C. Oiry, J. L. Baneres, J. Marie, M. Perello, and J. A. Fehrentz. 2019. 'N-Terminal Liver-Expressed

Antimicrobial Peptide 2 (LEAP2) Region Exhibits Inverse Agonist Activity toward the Ghrelin Receptor', *J Med Chem*, 62: 965-73.
Ma, X., X. Xue, J. Zhang, S. Liang, C. Xu, Y. Wang, and J. Zhu. 2020. 'Liver expressed antimicrobial peptide 2 is associated with steatosis in mice and humans', *Exp Clin Endocrinol Diabetes*, 129: 601-10.
Mackelvie, K. J., G. S. Meneilly, D. Elahi, A. C. Wong, S. I. Barr, and J. P. Chanoine. 2007. 'Regulation of appetite in lean and obese adolescents after exercise: role of acylated and desacyl ghrelin', *J Clin Endocrinol Metab*, 92: 648-54.
Maffeis, C., R. C. Bonadonna, A. Consolaro, R. Vettor, C. Banzato, D. Silvagni, G. Bogoni, M. Pellegrino, and L. Tato. 2006. 'Ghrelin, insulin sensitivity and postprandial glucose disposal in overweight and obese children', *Eur J Endocrinol*, 154: 61-8.
Malik, S., F. McGlone, D. Bedrossian, and A. Dagher. 2008. 'Ghrelin modulates brain activity in areas that control appetitive behavior', *Cell Metab*, 7: 400-09.
Mani, B. K., N. Puzziferri, Z. He, J. A. Rodriguez, S. Osborne-Lawrence, N. P. Metzger, N. Chhina, B. Gaylinn, M. O. Thorner, E. L. Thomas, J. D. Bell, K. W. Williams, A. P. Goldstone, and J. M. Zigman. 2019. 'LEAP2 changes with body mass and food intake in humans and mice', *J Clin Invest*, 129: 3909-23.
Maric, I., L. Lopez-Ferreras, Y. Bhat, M. Asker, S. Borchers, L. Bellfy, S. Byun, J. L. Kwapis, and K. P. Skibicka. 2023. 'From the stomach to locus coeruleus: new neural substrate for ghrelin's effects on ingestive, motivated and anxiety-like behaviors', *Front Pharmacol*, 14: 1286805.
Martens, M. J., S. G. Lemmens, J. M. Born, and M. S. Westerterp-Plantenga. 2012. 'Satiating capacity and post-prandial relationships between appetite parameters and gut-peptide concentrations with solid and liquefied carbohydrate', *PLoS One*, 7: e42110.
Martinez Damonte, V., S. S. Rodriguez, and J. Raingo. 2018. 'Growth hormone secretagogue receptor constitutive activity impairs voltage-gated calcium channel-dependent inhibitory neurotransmission in hippocampal neurons', *J Physiol*, 596: 5415-28.
Mason, B. L., Q. Wang, and J. M. Zigman. 2014. 'The central nervous system sites mediating the orexigenic actions of ghrelin', *Annu Rev Physiol*, 76: 519-33.
Massolt, E. T., P. M. van Haard, J. F. Rehfeld, E. F. Posthuma, der V. Van, and D. H. Schweitzer. 2010. 'Appetite suppression through smelling of dark chocolate correlates with changes in ghrelin in young women', *Regul Pept*, 161: 81-86.
Mathes, C. M., and A. C. Spector. 2012. 'Food selection and taste changes in humans after Roux-en-Y gastric bypass surgery: a direct-measures approach', *Physiol Behav*, 107: 476-83.
Matthews, D. R., J. P. Hosker, A. S. Rudenski, B. A. Naylor, D. F. Treacher, and R. C. Turner. 1985. 'Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man', *Diabetologia*, 28: 412-19.
McClelland, J., B. Dalton, M. Kekic, S. Bartholdy, I. C. Campbell, and U. Schmidt. 2016. 'A systematic review of temporal discounting in eating disorders and obesity: Behavioural and neuroimaging findings', *Neurosci Biobehav Rev*, 71: 506-28.
McCowen, K. C., J. A. Maykel, B. R. Bistrian, and P. R. Ling. 2002. 'Circulating ghrelin concentrations are lowered by intravenous glucose or hyperinsulinemic euglycemic conditions in rodents', *Journal of Endocrinology*, 175: R7-11.

McGonigle, J., A. Murphy, L. M. Paterson, L. J. Reed, L. Nestor, J. Nash, R. Elliott, K. D. Ersche, R. S. Flechais, R. Newbould, C. Orban, D. G. Smith, E. M. Taylor, A. D. Waldman, and T. W. Robbins. 2017. 'The ICCAM platform study: An experimental medicine platform for evaluating new drugs for relapse prevention in addiction. Part B: fMRI description', *J Psychopharmacol*, 31: 3-16.

McInnis, K., J. L. Brown, G. Finlayson, R. Dent, and E. Doucet. 2022. 'Appetite Changes in Weight Regain and Weight Maintenance After Roux-en-Y Gastric Bypass', *Obes Surg*, 32: 1-12.

McVeay, C., P. C. E. Fitzgerald, S. S. Ullrich, R. E. Steinert, M. Horowitz, and C. Feinle-Bisset. 2019. 'Effects of intraduodenal administration of lauric acid and L-tryptophan, alone and combined, on gut hormones, pyloric pressures, and energy intake in healthy men', *Am J Clin Nutr*, 109: 1335-43.

Mela, David J. 2006. 'Eating for pleasure or just wanting to eat? Reconsidering sensory hedonic responses as a driver of obesity', *Appetite*, 47: 10–17.

Mestre-Bach, G., and M. N. Potenza. 2023. 'Potential Biological Markers and Treatment Implications for Binge Eating Disorder and Behavioral Addictions', *Nutrients*, 15.

Miguet, M., A. Fillon, M. Khammassi, J. Masurier, V. Julian, and B. Pereira. 2018. 'Appetite, energy intake and food reward responses to an acute high intensity interval exercise in adolescents with obesity', 195: 90-97.

Miljic, D., S. Pekic, M. Djurovic, M. Doknic, N. Milic, F. F. Casanueva, M. Ghatei, and V. Popovic. 2006. 'Ghrelin has partial or no effect on appetite, growth hormone, prolactin, and cortisol release in patients with anorexia nervosa', *J Clin.Endocrinol.Metab*, 91: 1491-95.

Miller, J. L., A. Lacroix, L. M. Bird, A. H. Shoemaker, A. Haqq, C. L. Deal, K. A. Clark, M. H. Ames, J. G. Suico, A. de la Pena, and C. Fortier. 2022. 'The Efficacy, Safety, and Pharmacology of a Ghrelin O-Acyltransferase Inhibitor for the Treatment of Prader-Willi Syndrome', *J Clin Endocrinol Metab*, 107: e2373-e80.

Miras, A. D., and C. W. le Roux. 2013. 'Mechanisms underlying weight loss after bariatric surgery', *Nat Rev Gastroenterol Hepatol.*, 10: 575-84.

Mohlig, M., J. Spranger, B. Otto, M. Ristow, M. Tschop, and A. F. Pfeiffer. 2002. 'Euglycemic hyperinsulinemia, but not lipid infusion, decreases circulating ghrelin levels in humans', *J Endocrinol Invest*, 25: RC36-RC38.

Monteleone, P., R. Bencivenga, N. Longobardi, C. Serritella, and M. Maj. 2003. 'Differential responses of circulating ghrelin to high-fat or high-carbohydrate meal in healthy women', *J Clin Endocrinol Metab*, 88: 5510-4.

Monteleone, P., F. Piscitelli, P. Scognamiglio, A. M. Monteleone, B. Canestrelli, V. Di Marzo, and M. Maj. 2012. 'Hedonic eating is associated with increased peripheral levels of ghrelin and the endocannabinoid 2-arachidonoyl-glycerol in healthy humans: a pilot study', *J Clin Endocrinol Metab*, 97: E917-24.

Monteleone, P., P. Scognamiglio, A. M. Monteleone, D. Perillo, B. Canestrelli, and M. Maj. 2013. 'Gastroenteric hormone responses to hedonic eating in healthy humans', *Psychoneuroendocrinology*, 38: 1435-41.

Mora-Maltas, B., I. Baenas, M. Etxandi, I. Lucas, R. Granero, F. Fernandez-Aranda, S. Tovar, N. Sole-Morata, M. Gomez-Pena, L. Moragas, A. Del Pino-Gutierrez, J. Tapia, C. Dieguez, A. E. Goudriaan, and S. Jimenez-Murcia. 2024. 'Association between endocrine and neuropsychological endophenotypes and gambling disorder severity', *Addict Behav*, 153: 107968.

Moran, L. J., M. Noakes, P. M. Clifton, G. A. Wittert, L. Tomlinson, C. Galletly, N. D. Luscombe, and R. J. Norman. 2004. 'Ghrelin and measures of satiety are altered in polycystic ovary syndrome but not differentially affected by diet composition', *J Clin Endocrinol Metab*, 89: 3337-44.
Morris, J. S., and R. J. Dolan. 2001. 'Involvement of human amygdala and orbitofrontal cortex in hunger-enhanced memory for food stimuli', *J.Neurosci.*, 21: 5304-10.
Morys, F., I. Garcia-Garcia, and A. Dagher. 2020. 'Is obesity related to enhanced neural reactivity to visual food cues? A review and meta-analysis', *Soc Cogn Affect Neurosci*, 18: nsaa113.
Muller, T. D., R. Nogueiras, M. L. Andermann, Z. B. Andrews, S. D. Anker, J. Argente, R. L. Batterham, S. C. Benoit, C. Y. Bowers, F. Broglio, F. F. Casanueva, D. D'Alessio, I. Depoortere, A. Geliebter, E. Ghigo, P. A. Cole, M. Cowley, D. E. Cummings, A. Dagher, S. Diano, S. L. Dickson, C. Dieguez, R. Granata, H. J. Grill, K. Grove, K. M. Habegger, K. Heppner, M. L. Heiman, L. Holsen, B. Holst, A. Inui, J. O. Jansson, H. Kirchner, M. Korbonits, B. Laferrere, C. W. LeRoux, M. Lopez, S. Morin, M. Nakazato, R. Nass, D. Perez-Tilve, P. T. Pfluger, T. W. Schwartz, R. J. Seeley, M. Sleeman, Y. Sun, L. Sussel, J. Tong, M. O. Thorner, A. J. van der Lely, L. H. van der Ploeg, J. M. Zigman, M. Kojima, K. Kangawa, R. G. Smith, T. Horvath, and M. H. Tschop. 2015. 'Ghrelin', *Mol Metab*, 4: 437-60.
Murphy, C. F., N. G. Docherty, and C. W. le Roux. 2017. 'Liraglutide: another reason to target prediabetes?', *Oncotarget*, 8: 99203-04.
Mustafa, E. R., S. Cordisco Gonzalez, M. Damian, S. Cantel, S. Denoyelle, R. Wagner, H. B. Schioth, J. A. Fehrentz, J. L. Baneres, M. Perello, and J. Raingo. 2021. 'LEAP2 Impairs the Capability of the Growth Hormone Secretagogue Receptor to Regulate the Dopamine 2 Receptor Signaling', *Front Pharmacol*, 12: 712437.
Nabekura, H., M. N. Islam, H. Sakoda, T. Yamaguchi, A. Saiki, T. Nabekura, T. Oshiro, Y. Tanaka, S. Murayama, W. Zhang, I. Tatsuno, and M. Nakazato. 2023. 'Liver-Expressed Antimicrobial Peptide 2 is a Hepatokine that Predicts Weight Loss and Complete Remission of Type 2 Diabetes Mellitus After Vertical Sleeve Gastrectomy in Japanese Individuals', *Obes Facts*, 16: 392-400.
Nagaya, N., M. Kojima, M. Uematsu, M. Yamagishi, H. Hosoda, H. Oya, Y. Hayashi, and K. Kangawa. 2001. 'Hemodynamic and hormonal effects of human ghrelin in healthy volunteers', *Am J Physiol Regul Integr Comp Physiol*, 280: R1483-R87.
Neary, N. M., C. J. Small, A. M. Wren, J. L. Lee, M. R. Druce, C. Palmieri, G. S. Frost, M. A. Ghatei, R. C. Coombes, and S. R. Bloom. 2004. 'Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial', *J Clin Endocrinol Metab*, 89: 2832-36.
Neseliler, S., W. Hu, K. Larcher, M. Zacchia, M. Dadar, S. G. Scala, M. Lamarche, Y. Zeighami, S. C. Stotland, M. Larocque, E. B. Marliss, and A. Dagher. 2019. 'Neurocognitive and hormonal correlates of voluntary weight loss in humans', *Cell Metab*, 29: 39-49 e4.
Nestor, L. J., A. Murphy, J. McGonigle, C. Orban, L. Reed, E. Taylor, R. Flechais, L. M. Paterson, D. Smith, E. T. Bullmore, K. D. Ersche, J. Suckling, R. Tait, R. Elliott, B. Deakin, I. Rabiner, A. Lingford-Hughes, D. J. Nutt, B. Sahakian, T. W. Robbins, and ICCAM Consortium. 2017. 'Acute naltrexone does not

remediate fronto-striatal disturbances in alcoholic and alcoholic polysubstance-dependent populations during a monetary incentive delay task', *Addict Biol*, 22: 1576-89.
Nikolova, V., S. N. Ali, R. Bowie, G. Durighel, J. McGonigle, A. R. Lingford-Hughes, D. J. Nutt, E. L. Gibson, J. D. Bell, and A. P. Goldstone. 2015. 'State-trait interaction of hunger on striatal response to anticipatory monetary reward', *J Psychopharmacol*, 29: A102.
Nonogaki, K., and M. Suzuki. 2013. 'Liraglutide suppresses the plasma levels of active and des-acyl ghrelin independently of active glucagon-like Peptide-1 levels in mice', *ISRN Endocrinol*, 2013: 184753.
Nymo, S., S. R. Coutinho, P. H. Eknes, I. Vestbostad, J. F. Rehfeld, H. Truby, B. Kulseng, and C. Martins. 2018. 'Investigation of the long-term sustainability of changes in appetite after weight loss', *Int J Obes*, 42: 1489-99.
Nymo, S., S. R. Coutinho, J. Jorgensen, J. F. Rehfeld, H. Truby, B. Kulseng, and C. Martins. 2017. 'Timeline of changes in appetite during weight loss with a ketogenic diet', *Int J Obes*, 41: 1224-31.
Ochner, C. N., Y. Kwok, E. Conceicao, S. P. Pantazatos, L. M. Puma, S. Carnell, J. Teixeira, J. Hirsch, and A. Geliebter. 2011. 'Selective reduction in neural responses to high calorie foods following gastric bypass surgery', *Ann.Surg.*, 253: 502-07.
Ochner, C. N., B. Laferrere, L. Afifi, D. Atalayer, A. Geliebter, and J. Teixeira. 2012a. 'Neural responsivity to food cues in fasted and fed states pre and post gastric bypass surgery', *Neurosci.Res.*, 74: 138-43.
Ochner, C. N., E. Stice, E. Hutchins, L. Afifi, A. Geliebter, J. Hirsch, and J. Teixeira. 2012b. 'Relation between changes in neural responsivity and reductions in desire to eat high-calorie foods following gastric bypass surgery', *Neuroscience*, 209: 128-35.
Paterson, L. M., R. S. Flechais, A. Murphy, L. J. Reed, S. Abbott, V. Boyapati, R. Elliott, D. Erritzoe, K. D. Ersche, Y. Faluyi, L. Faravelli, E. Fernandez-Egea, N. J. Kalk, S. S. Kuchibatla, J. McGonigle, A. Metastasio, I. Mick, L. Nestor, C. Orban, F. Passetti, E. A. Rabiner, D. G. Smith, J. Suckling, R. Tait, E. M. Taylor, A. D. Waldman, T. W. Robbins, J. F. Deakin, D. J. Nutt, A. R. Lingford-Hughes, and ICCAM Platform. 2015. 'The Imperial College Cambridge Manchester (ICCAM) platform study: An experimental medicine platform for evaluating new drugs for relapse prevention in addiction. Part A: Study description', *J Psychopharmacol*, 29: 943-60.
Patton, J. H., M. S. Stanford, and E. S. Barratt. 1995. 'Factor structure of the Barratt impulsiveness scale', *J Clin Psychol.*, 51: 768-74.
Pedersen, M. G. B., E. S. Lauritzen, M. V. Svart, J. Stoy, E. Sondergaard, H. H. Thomsen, U. Kampmann, M. Bjerre, N. Jessen, N. Moller, and N. Rittig. 2023. 'Nutrient sensing: LEAP2 concentration in response to fasting, glucose, lactate, and beta-hydroxybutyrate in healthy young males', *Am J Clin Nutr*, 118: 1091-98.
Peos, J. J., E. R. Helms, P. A. Fournier, J. Ong, C. Hall, J. Krieger, and A. Sainsbury. 2021. 'Continuous versus Intermittent Dieting for Fat Loss and Fat-Free Mass Retention in Resistance-trained Adults: The ICECAP Trial', *Med Sci Sports Exerc*, 53: 1685-98.
Perdomo, C. M., R. V. Cohen, P. Sumithran, K. Clement, and G. Fruhbeck. 2023. 'Contemporary medical, device, and surgical therapies for obesity in adults', *Lancet*, 401: 1116-30.

Perry, J. L., and M. E. Carroll. 2008. 'The role of impulsive behavior in drug abuse', *Psychopharmacology*, 200: 1-26.
Petelakova, M., B. Neprasova, Z. Smotkova, A. Myskova, L. Hola, A. Petelak, A. Acova, S. Cantel, J. A. Fehrentz, D. Sykora, J. Kunes, B. Zelezna, and L. Maletinska. 2024. 'Simultaneous treatment with palm-LEAP2(1-14) and feeding high-fat diet attenuates liver lipid metabolism but not obesity: Sign of selective resistance to palm-LEAP2(1-14)', *Mol Cell Endocrinol*, 597: 112442.
Poher, A. L., M. H. Tschop, and T. D. Muller. 2018. 'Ghrelin regulation of glucose metabolism', *Peptides*, 100: 236-42.
Polak, J. M., Bloom, S. R., Kuzio, M., Brown, J. C., & Pearse, A. G. E 1973. 'Cellular localization of gastric inhibitory polypeptide in the duodenum and jejunum', *Gut*, 14: 284-88.
Pop, L. M., A. Mari, T. J. Zhao, L. Mitchell, S. Burgess, X. Li, B. Adams-Huet, and I. Lingvay. 2018. 'Roux-en-Y gastric bypass compared with equivalent diet restriction: Mechanistic insights into diabetes remission', *Diabetes Obes Metab*, 20: 1710-21.
Prudom, C., J. Liu, J. Patrie, B. D. Gaylinn, K. E. Foster-Schubert, D. E. Cummings, M. O. Thorner, and H. M. Geysen. 2010. 'Comparison of competitive radioimmunoassays and two-site sandwich assays for the measurement and interpretation of plasma ghrelin levels', *J Clin Endocrinol Metab*, 95: 2351-8.
Puklin, L., B. Cartmel, M. Harrigan, L. Lu, F. Y. Li, T. Sanft, and M. L. Irwin. 2021. 'Randomized trial of weight loss on circulating ghrelin levels among breast cancer survivors', *NPJ Breast Cancer*, 7: 49.
Qader, S. S., R. Hakanson, J. F. Rehfeld, I. Lundquist, and A. Salehi. 2008. 'Proghrelin-derived peptides influence the secretion of insulin, glucagon, pancreatic polypeptide and somatostatin: a study on isolated islets from mouse and rat pancreas', *Regul Pept*, 146: 230-7.
Ragland, T. J., and S. K. Malin. 2023. 'Plasma LEAP-2 Following a Low-Calorie Diet with or without Interval Exercise in Women with Obesity', *Nutrients*, 15: 655.
Rhea, E. M., T. S. Salameh, S. Gray, J. Niu, W. A. Banks, and J. Tong. 2018. 'Ghrelin transport across the blood-brain barrier can occur independently of the growth hormone secretagogue receptor', *Mol Metab*, 18: 88-96.
Richardson, R. S., A. Sulima, K. C. Rice, J. A. Kucharczk, K. D. Janda, K. E. Nisbett, G. F. Koob, L. F. Vendruscolo, and L. Leggio. 2023. 'Pharmacological GHSR (ghrelin receptor) blockade reduces alcohol binge-like drinking in male and female mice', *Neuropharmacology*: 109643.
Romero, A., H. Kirchner, K. Heppner, P. T. Pfluger, M. H. Tschop, and R. Nogueiras. 2010. 'GOAT: the master switch for the ghrelin system?', *Eur J Endocrinol.*, 163: 1-8.
Roper, J., F. Francois, P. L. Shue, M. S. Mourad, Z. Pei, A. Z. Olivares de Perez, G. I. Perez-Perez, C. H. Tseng, and M. J. Blaser. 2008. 'Leptin and ghrelin in relation to Helicobacter pylori status in adult males', *J Clin Endocrinol Metab*, 93: 2350-7.
Rosen, L. A., E. M. Ostman, and I. M. Bjorck. 2011. 'Effects of cereal breakfasts on postprandial glucose, appetite regulation and voluntary energy intake at a subsequent standardized lunch; focusing on rye products', *Nutr J*, 10: 7.
Roth, C. L., S. J. Melhorn, C. T. Elfers, K. Scholz, M. R. B. De Leon, M. Rowland, S. Kearns, E. Aylward, T. J. Grabowski, B. E. Saelens, and E. A. Schur. 2019. 'Central Nervous System and Peripheral Hormone Responses to a Meal in Children', *J Clin Endocrinol Metab*, 104: 1471-83.

Ruban, A., M. A. Glaysher, A. D. Miras, A. P. Goldstone, C. G. Prechtl, N. Johnson, J. Li, M. Aldhwayan, G. Aldubaikhi, B. Glover, J. Lord, O. Onyimadu, E. Falaschetti, N. Klimowska-Nassar, H. Ashrafian, J. Byrne, and J. P. Teare. 2020. 'A duodenal sleeve bypass device added to intensive medical therapy for obesity with type 2 diabetes: a RCT', *Efficacy and Mechanism Evaluation*, doi:10.3310/eme07060.

Ruban, A., A. D. Miras, M. A. Glaysher, A. P. Goldstone, C. G. Prechtl, N. Johnson, N. Chhina, W. Al-Najim, M. Aldhwayan, N. Klimowska-Nassar, C. Smith, J. Lord, J. V. Li, L. Flores, M. Al-Lababidi, G. K. Dimitriadis, M. Patel, M. Moore, H. Chahal, A. R. Ahmed, J. Cousins, G. Aldubaikhi, B. Glover, E. Falaschetti, H. Ashrafian, C. W. L. Roux, A. Darzi, J. P. Byrne, and J. P. Teare. 2022. 'Duodenal-Jejunal Bypass Liner for the management of Type 2 Diabetes Mellitus and Obesity: A Multicenter Randomized Controlled Trial', *Ann Surg*, 275: 440-47.

Rubinic, I., M. Kurtov, and R. Likic. 2024. 'Novel Pharmaceuticals in Appetite Regulation: Exploring emerging gut peptides and their pharmacological prospects', *Pharmacol Res Perspect*, 12: e1243.

Rubino, F., D. E. Cummings, R. H. Eckel, R. V. Cohen, J. P. H. Wilding, W. A. Brown, F. C. Stanford, R. L. Batterham, I. S. Farooqi, N. J. Farpour-Lambert, C. W. le Roux, N. Sattar, L. A. Baur, K. M. Morrison, A. Misra, T. Kadowaki, K. W. Tham, P. Sumithran, W. T. Garvey, J. P. Kirwan, J. M. Fernandez-Real, B. E. Corkey, H. Toplak, A. Kokkinos, R. F. Kushner, F. Branca, J. Valabhji, M. Bluher, S. R. Bornstein, H. J. Grill, E. Ravussin, E. Gregg, N. B. Al Busaidi, N. F. Alfaris, E. Al Ozairi, L. M. S. Carlsson, K. Clement, J. P. Despres, J. B. Dixon, G. Galea, L. M. Kaplan, B. Laferrere, M. Laville, S. Lim, J. R. Luna Fuentes, V. M. Mooney, J. Nadglowski, Jr., A. Urudinachi, M. Olszanecka-Glinianowicz, A. Pan, F. Pattou, P. R. Schauer, M. H. Tschop, M. T. van der Merwe, R. Vettor, and G. Mingrone. 2025. 'Definition and diagnostic criteria of clinical obesity', *Lancet Diabetes Endocrinol*.

Sakai, K., K. Shiomi, H. Mochizuki, M. N. Islam, H. Nabekura, R. Tanida, H. Sakoda, and M. Nakazato. 2021. 'Human liver-expressed antimicrobial peptide 2 elevation in the cerebrospinal fluid in bacterial meningitis', *Brain Behav*: e02111.

Salem, V., L. Demetriou, P. Behary, K. Alexiadou, S. Scholtz, G. Tharakan, A. D. Miras, S. Purkayastha, A. R. Ahmed, S. R. Bloom, M. B. Wall, W. S. Dhillo, and T. M. M. Tan. 2021. 'Weight Loss by Low-Calorie Diet Versus Gastric Bypass Surgery in People With Diabetes Results in Divergent Brain Activation Patterns: A Functional MRI Study', *Diabetes Care*: dc202641.

Saunders, J. B., O. G. Aasland, T. F. Babor, J. R. de la Fuente, and M. Grant. 1993. 'Development of the Alcohol Use Disorders Identification Test (AUDIT): WHO Collaborative Project on Early Detection of Persons with Harmful Alcohol Consumption--II', *Addiction*, 88: 791-804.

Schalla, M. A., and A. Stengel. 2019. 'Pharmacological Modulation of Ghrelin to Induce Weight Loss: Successes and Challenges', *Curr Diab Rep*, 19: 102.

Schaller, G., A. Schmidt, J. Pleiner, W. Woloszczuk, M. Wolzt, and A. Luger. 2003. 'Plasma ghrelin concentrations are not regulated by glucose or insulin: a double-blind, placebo-controlled crossover clamp study', *Diabetes*, 52: 16-20.

Schild, H.H. 1990. *MRI made easy*.

Schofield, W. N. 1985. 'Predicting basal metabolic rate, new standards and review of previous work', *Hum Nutr Clin Nutr*, 39 Suppl 1: 5-41.

Scholtz, S., A. D. Miras, N. Chhina, C. G. Prechtl, M. Sleeth, N. M. Daud, N. Ismail, G. Durighel, A. R. Ahmed, T. Olbers, R. P. Vincent, J. Alaghband-Zadeh, M. A. Ghatei, A. D. Waldman, G. S. Frost, J. D. Bell, C. W. le Roux, and A. P. Goldstone. 2014. 'Obese patients after gastric bypass surgery have lower brain hedonic responses to food than after gastric banding', *Gut*, 63: 891-902.

Sciolino, N. R., M. Hsiang, C. M. Mazzone, L. R. Wilson, N. W. Plummer, J. Amin, K. G. Smith, C. A. McGee, S. A. Fry, C. X. Yang, J. M. Powell, M. R. Bruchas, A. V. Kravitz, J. D. Cushman, M. J. Krashes, G. Cui, and P. Jensen. 2022. 'Natural locus coeruleus dynamics during feeding', *Sci Adv*, 8: eabn9134.

Serra-Prat, M., E. Palomera, P. Clave, and M. Puig-Domingo. 2009. 'Effect of age and frailty on ghrelin and cholecystokinin responses to a meal test', *Am J Clin Nutr*, 89: 1410-7.

Serrenho, D., S. D. Santos, and A. L. Carvalho. 2019. 'The Role of Ghrelin in Regulating Synaptic Function and Plasticity of Feeding-Associated Circuits', *Front Cell Neurosci*, 13: 205.

Sewaybricker, L. E., S. J. Melhorn, M. K. Askren, M. F. Webb, V. Tyagi, M. R. B. De Leon, T. J. Grabowski, W. W. Seeley, and E. A. Schur. 2020. 'Salience network connectivity is reduced by a meal and influenced by genetic background and hypothalamic gliosis', *Int J Obes (Lond)*, 44: 167-77.

Shankar, K., N. P. Metzger, C. Lawrence, D. Gupta, S. Osborne-Lawrence, S. Varshney, O. Singh, C. P. Richard, A. N. Zaykov, R. Rolfts, B. N. DuBois, D. Perez-Tilve, B. K. Mani, S. T. G. Hammer, and J. M. Zigman. 2024. 'A long-acting LEAP2 analog reduces hepatic steatosis and inflammation and causes marked weight loss in mice', *Mol Metab*, 84: 101950.

Shankar, K., N. P. Metzger, O. Singh, B. K. Mani, S. Osborne-Lawrence, S. Varshney, D. Gupta, S. B. Ogden, S. Takemi, C. P. Richard, K. Nandy, C. Liu, and J. M. Zigman. 2021a. 'LEAP2 deletion in mice enhances ghrelin's actions as an orexigen and growth hormone secretagogue', *Mol Metab*, 53: 101327.

Shankar, K., S. Takemi, D. Gupta, S. Varshney, B. K. Mani, S. Osborne-Lawrence, N. P. Metzger, C. P. Richard, E. D. Berglund, and J. M. Zigman. 2021b. 'Ghrelin cell-expressed insulin receptors mediate meal- and obesity-induced declines in plasma ghrelin', *JCI Insight*, 6.

Shen, M., C. Manca, F. Suriano, N. Nallabelli, F. Pechereau, B. Allam-Ndoul, F. A. Iannotti, N. Flamand, A. Veilleux, P. D. Cani, C. Silvestri, and V. Di Marzo. 2021. 'Three of a Kind: Control of the Expression of Liver-Expressed Antimicrobial Peptide 2 (LEAP2) by the Endocannabinoidome and the Gut Microbiome', *Molecules*, 27.

Siep, N., A. Roefs, A. Roebroeck, R. Havermans, M. L. Bonte, and A. Jansen. 2009. 'Hunger is the best spice: an fMRI study of the effects of attention, hunger and calorie content on food reward processing in the amygdala and orbitofrontal cortex', *Behav Brain Res.*, 198: 149-58.

Simon, J. J., A. Becker, M. H. Sinno, M. Skunde, M. Bendszus, H. Preissl, P. Enck, W. Herzog, and H. C. Friederich. 2018. 'Neural Food Reward Processing in Successful and Unsuccessful Weight Maintenance', *Obesity*, 26: 895-902.

Simon, J. J., M. Skunde, M. Wu, K. Schnell, S. C. Herpertz, M. Bendszus, W. Herzog, and H. C. Friederich. 2015. 'Neural dissociation of food- and money-related reward processing using an abstract incentive delay task', *Soc Cogn Affect Neurosci*, 10: 1113-20.

Simon, J. J., A. Wetzel, M. H. Sinno, M. Skunde, M. Bendszus, H. Preissl, P. Enck, W. Herzog, and H. C. Friederich. 2017. 'Integration of homeostatic signaling and food reward processing in the human brain', *JCI Insight*, 2.
Sinha, R., P. Gu, R. Hart, and J. B. Guarnaccia. 2019. 'Food craving, cortisol and ghrelin responses in modeling highly palatable snack intake in the laboratory', *Physiol Behav*, 208: 112563.
Skibicka, K. P., and S. L. Dickson. 2011a. 'Ghrelin and food reward: the story of potential underlying substrates', *Peptides*, 32: 2265-73.
Skibicka, K. P., C. Hansson, M. Alvarez-Crespo, P. A. Friberg, and S. L. Dickson. 2011b. 'Ghrelin directly targets the ventral tegmental area to increase food motivation', *Neuroscience*, 180: 129-37.
Smeets, P. A. M., A. Dagher, T. A. Hare, S. Kullmann, L. N. van der Laan, R. A. Poldrack, H. Preissl, D. Small, E. Stice, and M. G. Veldhuizen. 2019. 'Good practice in food-related neuroimaging', *Am J Clin Nutr*, 109: 491-503.
Sosinski, O., E. Pruszynska-Oszmalek, N. Leciejewska, M. Sassek, and P. A. Kolodziejski. 2025. 'LEAP2 in Physiology-A Narrative Review', *Int J Mol Sci*, 26.
Spielberger, C.D., R.L. Gorssuch, P.R. Lushene, P.R. Vagg, and G.A Jacobs. 1983. 'Manual for the State-Trait Anxiety Inventory', *Consulting Psychologists Press, Inc.*
Srivastava, G., and C. Apovian. 2018. 'Future Pharmacotherapy for Obesity: New Anti-obesity Drugs on the Horizon', *Curr Obes Rep*, 7: 147-61.
St-Onge, M. P., B. Mayrsohn, M. O'Keeffe, H. R. Kissileff, A. R. Choudhury, and B. Laferrere. 2014. 'Impact of medium and long chain triglycerides consumption on appetite and food intake in overweight men', *Eur J Clin Nutr*, 68: 1134-40.
Stark, R., J. Feehan, A. Mousa, Z. B. Andrews, and B. de Courten. 2023. 'LEAP2 associated with improved pancreatic insulin secretion in adults with overweight and obesity', *Diabetes Obes Metab*, 25: 1213-20.
Stefater, M. A., H. E. Wilson-Perez, A. P. Chambers, D. A. Sandoval, and R. J. Seeley. 2012. 'All bariatric surgeries are not created equal: insights from mechanistic comparisons', *Endocr Rev*.
Stengel, A., M. Goebel, L. Wang, and Y. Tache. 2010. 'Ghrelin, des-acyl ghrelin and nesfatin-1 in gastric X/A-like cells: role as regulators of food intake and body weight', *Peptides*, 31: 357-69.
Stengel, A., L. Wang, and Y. Tache. 2011. 'Stress-related alterations of acyl and desacyl ghrelin circulating levels: mechanisms and functional implications', *Peptides*, 32: 2208-17.
Stevanovic, D. M., A. Grefhorst, A. P. Themmen, V. Popovic, J. Holstege, E. Haasdijk, V. Trajkovic, A. J. Van der Lely, and P. J. Delhanty. 2014. 'Unacylated ghrelin suppresses ghrelin-induced neuronal activity in the hypothalamus and brainstem of male rats', *PLoS.ONE.*, 9: e98180.
Stock, S., P. Leichner, A. C. Wong, M. A. Ghatei, T. J. Kieffer, S. R. Bloom, and J. P. Chanoine. 2005. 'Ghrelin, Peptide YY, Glucose-Dependent Insulinotropic Polypeptide, and Hunger Responses to a Mixed Meal in Anorexic, Obese, and Control Female Adolescents', *J Clin Endocrinol Metab*, 90: 2161-68.
Stubbs, B. J., P. J. Cox, R. D. Evans, M. Cyranka, K. Clarke, and H. de Wet. 2018. 'A Ketone Ester Drink Lowers Human Ghrelin and Appetite', *Obesity (Silver Spring)*, 26: 269-73.

Stunkard, A. J., and S. Messick. 1985. 'The three-factor eating questionnaire to measure dietary restraint, disinhibition and hunger', *J Psychosom.Res.*, 29: 71-83.
Suarez, A. N., C. M. Liu, A. M. Cortella, E. E. Noble, and S. E. Kanoski. 2020. 'Ghrelin and Orexin Interact to Increase Meal Size Through a Descending Hippocampus to Hindbrain Signaling Pathway', *Biol Psychiatry*, 87: 1001-11.
Suarez, A. N., E. E. Noble, and S. E. Kanoski. 2019. 'Regulation of Memory Function by Feeding-Relevant Biological Systems: Following the Breadcrumbs to the Hippocampus', *Front Mol Neurosci*, 12: 101.
Suchankova, P., P. Steensland, I. Fredriksson, J. A. Engel, and E. Jerlhag. 2013. 'Ghrelin receptor (GHS-R1A) antagonism suppresses both alcohol consumption and the alcohol deprivation effect in rats following long-term voluntary alcohol consumption', *PLoS One*, 8: e71284.
Sumithran, P., L. A. Prendergast, E. Delbridge, K. Purcell, A. Shulkes, A. Kriketos, and J. Proietto. 2011. 'Long-term persistence of hormonal adaptations to weight loss', *N Engl J Med*, 365: 1597-604.
———. 2013. 'Ketosis and appetite-mediating nutrients and hormones after weight loss', *Eur J Clin Nutr*, 67: 759-64.
Sun, X., M. G. Veldhuizen, A. E. Wray, I. E. de Araujo, R. S. Sherwin, R. Sinha, and D. M. Small. 2014. 'The neural signature of satiation is associated with ghrelin response and triglyceride metabolism', *Physiol Behav*, 136: 63-73.
Tezenas du Montcel, C., P. Duriez, J. Cao, N. Lebrun, N. Ramoz, O. Viltart, P. Gorwood, and V. Tolle. 2023. 'The role of dysregulated ghrelin/LEAP-2 balance in anorexia nervosa', *iScience*, 26: 107996.
Thom, G., S. U. Dombrowski, N. Brosnahan, Y. Y. Algindan, M. Rosario Lopez-Gonzalez, G. Roditi, M. E. J. Lean, and D. Malkova. 2020. 'The role of appetite-related hormones, adaptive thermogenesis, perceived hunger and stress in long-term weight-loss maintenance: a mixed-methods study', *Eur J Clin Nutr*, 74: 622-32.
Thom, G., A. McIntosh, C. M. Messow, W. S. Leslie, A. C. Barnes, N. Brosnahan, L. McCombie, D. Malkova, A. Al-Mrabeh, S. Zhyzhneuskaya, P. Welsh, N. Sattar, R. Taylor, and M. E. J. Lean. 2021. 'Weight loss-induced increase in fasting ghrelin concentration is a predictor of weight regain: Evidence from the Diabetes Remission Clinical Trial (DiRECT)', *Diabetes Obes Metab*, 23: 711-19.
Tian, J., L. Guo, T. Wang, K. Jia, R. H. Swerdlow, J. M. Zigman, and H. Du. 2023. 'Liver-expressed antimicrobial peptide 2 elevation contributes to age-associated cognitive decline', *JCI Insight*, 8.
Tong, J., H. W. Davis, S. Summer, S. C. Benoit, A. Haque, M. Bidlingmaier, M. H. Tschop, and D. D'Alessio. 2014. 'Acute administration of unacylated ghrelin has no effect on Basal or stimulated insulin secretion in healthy humans', *Diabetes*, 63: 2309-19.
Tong, J., E. Mannea, P. Aime, P. T. Pfluger, C. X. Yi, T. R. Castaneda, H. W. Davis, X. Ren, S. Pixley, S. Benoit, K. Julliard, S. C. Woods, T. L. Horvath, M. M. Sleeman, D. D'Alessio, S. Obici, R. Frank, and M. H. Tschop. 2011. 'Ghrelin enhances olfactory sensitivity and exploratory sniffing in rodents and humans', *J Neurosci.*, 31: 5841-46.
Topyildiz, F., S. Kiyici, Z. Gul, D. Sigirli, M. Guclu, G. Kisakol, and S. Cavun. 2016. 'Exenatide Treatment Causes Suppression of Serum Ghrelin Levels following Mixed Meal Test in Obese Diabetic Women', *J Diabetes Res*, 2016: 1309502.

Tschop, M., D. L. Smiley, and M. L. Heiman. 2000. 'Ghrelin induces adiposity in rodents', *Nature*, 407: 908-13.
Tufvesson-Alm, M., C. Aranas, S. Blid Skoldheden, J. Vestlund, C. E. Edvardsson, and E. Jerlhag. 2024a. 'LEAP2, a ghrelin receptor inverse agonist, and its effect on alcohol-related responses in rodents', *Transl Psychiatry*, 14: 401.
Tufvesson-Alm, M., Q. Zhang, C. Aranas, S. B. Skoldheden, C. E. Edvardsson, and E. Jerlhag. 2024b. 'Decoding the influence of central LEAP2 on food intake and its effect on accumbal dopamine release', *Prog Neurobiol*: 102615.
Ueberberg, B., N. Unger, W. Saeger, K. Mann, and S. Petersenn. 2009. 'Expression of ghrelin and its receptor in human tissues', *Horm Metab Res*, 41: 814-21.
Unick, J. L., A. D. Otto, B. H. Goodpaster, D. L. Helsel, C. A. Pellegrini, and J. M. Jakicic. 2010. 'Acute effect of walking on energy intake in overweight/obese women', *Appetite*, 55: 413-9.
Vadnie, C. A., J. H. Park, N. Abdel Gawad, A. M. Ho, D. J. Hinton, and D. S. Choi. 2014. 'Gut-brain peptides in corticostriatal-limbic circuitry and alcohol use disorders', *Front Neurosci*, 8: 288.
Vallof, D., P. Maccioni, G. Colombo, M. Mandrapa, J. W. Jornulf, E. Egecioglu, J. A. Engel, and E. Jerlhag. 2016. 'The glucagon-like peptide 1 receptor agonist liraglutide attenuates the reinforcing properties of alcohol in rodents', *Addict Biol*, 21: 422-37.
Van Strien, T., J. E. R. Frijters, G. Bergers, and P. B. Defares. 1984. 'The Dutch Eating Behavior Questionnaire (DEBQ) for assessment of restrained, emotional, and external eating behavior', *Int J Eat Disord*, 5: 295-315.
Varimo, T., P. J. Miettinen, K. Vaaralahti, J. Toppari, H. Huopio, R. Voutilainen, S. Tenhola, M. Hero, and T. Raivio. 2022. 'Circulating Liver-enriched Antimicrobial Peptide-2 Decreases During Male Puberty', *J Endocr Soc*, 6: bvac013.
Varshney, S., K. Shankar, H. L. Kerr, L. J. Anderson, D. Gupta, N. P. Metzger, O. Singh, S. B. Ogden, S. Paul, F. Pinon, S. Osborne-Lawrence, C. P. Richard, C. Lawrence, B. K. Mani, J. M. Garcia, and J. M. Zigman. 2024. 'The LEAP2 Response to Cancer-Related Anorexia-Cachexia Syndrome in Male Mice and Patients', *Endocrinology*, 165.
Vergani, E., C. Bruno, C. Gavotti, L. S. Aversa, M. Martire, A. Mancini, and D. Curro. 2021. 'LEAP-2/ghrelin interplay in adult growth hormone deficiency: Cause or consequence? A pilot study', *IUBMB Life*, 73: 978-84.
Vergani, E., C. Bruno, C. Gavotti, A. Oliva, D. Curro, and A. Mancini. 2022. 'Increased levels of plasma neudesin in adult growth hormone deficiency and their relationship with plasma liver-expressed antimicrobial peptide-2 levels: a cross-sectional study', *J Endocrinol Invest*.
Vestergaard, E. T., N. B. Zubanovic, N. Rittig, N. Moller, R. E. Kuhre, J. J. Holst, J. F. Rehfeld, and H. H. Thomsen. 2021. 'Acute ketosis inhibits appetite and decreases plasma concentrations of acyl ghrelin in healthy young men', *Diabetes Obes Metab*, 23: 1834-42.
Vilarrasa, N., A. G. de Gordejuela, A. Casajoana, X. Duran, S. Toro, and E. Espinet. 2017. 'Endobarrier in grade I obese patients with long-standing type 2 diabetes: role of gastrointestinal hormones in glucose metabolism', 27: 569-77.
Voon, V. 2015. 'Cognitive biases in binge eating disorder: the hijacking of decision making', *CNS Spectr*, 20: 566-73.

Wald, H. S., M. Y. Ghidewon, M. R. Hayes, and H. J. Grill. 2023. 'Hindbrain ghrelin and LEAP2, ligands for GHSR, bidirectionally control food intake', *Am J Physiol Regul Integr Comp Physiol*.
Wang, J. H., H. Z. Li, X. X. Shao, W. H. Nie, Y. L. Liu, Z. G. Xu, and Z. Y. Guo. 2019. 'Identifying the binding mechanism of LEAP2 to receptor GHSR1a', *Febs j*, 286: 1332-45.
Wang, Y., Q. Wu, Q. Zhou, Y. Chen, X. Lei, Y. Chen, and Q. Chen. 2022. 'Circulating acyl and des-acyl ghrelin levels in obese adults: a systematic review and meta-analysis', *Sci Rep*, 12: 2679.
Watson, D., L. A. Clark, and A. Tellegen. 1988. 'Development and validation of brief measures of positive and negative affect: the PANAS scales', *J Pers Soc Psychol*, 54: 1063-70.
Wechsler, D. 2001. *Wechsler Test of Adult Reading: WTAR.* (Psychological Corporation).
Weller, R. E., E. W. Cook, III, K. B. Avsar, and J. E. Cox. 2008. 'Obese women show greater delay discounting than healthy-weight women', *Appetite*, 51: 563-69.
Wever, M. C. M., F. van Meer, L. Charbonnier, D. R. Crabtree, W. Buosi, A. Giannopoulou, O. Androutsos, A. M. Johnstone, Y. Manios, C. L. Meek, J. J. Holst, P. A. M. Smeets, and consortium Full4Health. 2021. 'Associations between ghrelin and leptin and neural food cue reactivity in a fasted and sated state', *Neuroimage*, 240: 118374.
WHO. 2022. 'World health statistics 2022: monitoring health for the SDGs, sustainable development goals'. https://www.who.int/publications/i/item/9789240051157.
———. 2024. 'Obesity and Overweight'. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight.
Wilding, J. P. H., R. L. Batterham, M. Davies, L. F. Van Gaal, K. Kandler, K. Konakli, I. Lingvay, B. M. McGowan, T. K. Oral, J. Rosenstock, T. A. Wadden, S. Wharton, K. Yokote, R. F. Kushner, and Step Study Group. 2022. 'Weight regain and cardiometabolic effects after withdrawal of semaglutide: The STEP 1 trial extension', *Diabetes Obes Metab*, 24: 1553-64.
Williams, D. L., H. J. Grill, D. E. Cummings, and J. M. Kaplan. 2003. 'Vagotomy dissociates short- and long-term controls of circulating ghrelin', *Endocrinology*, 144: 5184-87.
Wittekind, D. A., J. Kratzsch, R. Mergl, C. Enzenbach, A. V. Witte, A. Villringer, and M. Kluge. 2018. 'Alcohol consumption is positively associated with fasting serum ghrelin in non-dependent adults: Results from the population-based LIFE-Adult-Study', *Psychoneuroendocrinology*, 97: 143-48.
Wren, A. M., L. J. Seal, M. A. Cohen, A. E. Brynes, G. S. Frost, K. G. Murphy, W. S. Dhillo, M. A. Ghatei, and S. R. Bloom. 2001. 'Ghrelin enhances appetite and increases food intake in humans', *J Clin Endo Metab*, 86: 5992-95.
Wynne, K., K. Giannitsopoulou, C. J. Small, M. Patterson, G. Frost, M. A. Ghatei, E. A. Brown, S. R. Bloom, and P. Choi. 2005. 'Subcutaneous ghrelin enhances acute food intake in malnourished patients who receive maintenance peritoneal dialysis: a randomized, placebo-controlled trial', *J Am Soc Nephrol*, 16: 2111-18.
Yanagi, S., T. Sato, K. Kangawa, and M. Nakazato. 2018. 'The Homeostatic Force of Ghrelin', *Cell Metab*, 27: 786-804.

Yang, J., M. S. Brown, G. Liang, N. V. Grishin, and J. L. Goldstein. 2008. 'Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone', *Cell*, 132: 387-96.
Yang, Y., Q. Wu, and F. Morys. 2021. 'Brain Responses to High-Calorie Visual Food Cues in Individuals with Normal-Weight or Obesity: An Activation Likelihood Estimation Meta-Analysis', *Brain Sci*, 11: 1587.
Yousseif, A., J. Emmanuel, E. Karra, Q. Millet, M. Elkalaawy, A. D. Jenkinson, M. Hashemi, M. Adamo, N. Finer, A. G. Fiennes, D. J. Withers, and R. L. Batterham. 2014. 'Differential effects of laparoscopic sleeve gastrectomy and laparoscopic gastric bypass on appetite, circulating acyl-ghrelin, peptide YY3-36 and active GLP-1 levels in non-diabetic humans', *Obes Surg.*, 24: 241-52.
Zakeri, R., and R. L. Batterham. 2018. 'Potential mechanisms underlying the effect of bariatric surgery on eating behaviour', *Curr Opin Endocrinol Diabetes Obes*, 25: 3-11.
Zhang, C. J., M. Bidlingmaier, M. Altaye, L. C. Page, D. D'Alessio, M. H. Tschop, and J. Tong. 2017. 'Acute administration of acyl, but not desacyl ghrelin, decreases blood pressure in healthy humans', *Eur J Endocrinol*, 176: 123-32.
Zhao, T. J., I. Sakata, R. L. Li, G. Liang, J. A. Richardson, M. S. Brown, J. L. Goldstein, and J. M. Zigman. 2010. 'Ghrelin secretion stimulated by {beta}1-adrenergic receptors in cultured ghrelinoma cells and in fasted mice', *Proc Natl Acad Sci USA*, 107: 15868-73.
Zhu, L., Y. Huang, I. Edirisinghe, E. Park, and B. Burton-Freeman. 2019. 'Using the Avocado to Test the Satiety Effects of a Fat-Fiber Combination in Place of Carbohydrate Energy in a Breakfast Meal in Overweight and Obese Men and Women: A Randomized Clinical Trial', *Nutrients*, 11.
Zigman, J. M., J. E. Jones, C. E. Lee, C. B. Saper, and J. K. Elmquist. 2006. 'Expression of ghrelin receptor mRNA in the rat and the mouse brain', *J Comp Neurol.*, 494: 528-48.
Zoon, H. F. A., S. E. M. de Bruijn, P. A. M. Smeets, C. de Graaf, I. M. C. Janssen, W. Schijns, E. O. Aarts, G. Jager, and S. Boesveldt. 2018. 'Altered neural responsivity to food cues in relation to food preferences, but not appetite-related hormone concentrations after RYGB-surgery', *Behav Brain Res*, 353: 194-202.
Zorrilla, E. P., S. Iwasaki, J. A. Moss, J. Chang, J. Otsuji, K. Inoue, M. M. Meijler, and K. D. Janda. 2006. 'Vaccination against weight gain', *Proc Natl Acad Sci U S A*, 103: 13226-31.

APPENDICES

APPENDIX 1: CONSENT FORM FOR PARTICIPANTS

Centre name: Division of Psychiatry, Department of Brain Sciences, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN

Study Protocol number: v1.4.1 28-May-2024

IRAS number: 304210, **REC no.** 23/LO/0510

Full Title of Project:

Gut hormone LEAP2 in metabolism and eating behaviour - dose confirmation study - study 2

Name of Principal Investigator: Dr. Tony Goldstone

	Please initial box
1. I confirm that I have read and understand the participant information sheet version ..... dated ……..... for 'Gut hormone LEAP2 in metabolism and eating behaviour - dose confirmation study - study 2' and have had the opportunity to ask questions which have been answered fully.	
2. I understand that my participation is voluntary, and I am free to withdraw at any time, without giving any reason and without my legal rights nor treatment / healthcare being affected.	
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from Imperial College London, from Imperial College Healthcare NHS Trust or from regulatory authorities where it is relevant to my taking part in this research.	
4. I give permission for my General Practitioner to be contacted for any medical information to ensure my eligibility into this study as part of the screening process, and be informed of the results of medical tests from my visits.	
5. I understand that samples (plasma, tissue, DNA) and / or data collected from me are a gift donated to Imperial College and that I will not personally benefit financially if this research leads to an invention and/or the successful development of a new test, medication treatment, product or service.	
6. I consent to take part in 'Gut hormone LEAP2 in metabolism and eating behaviour - dose confirmation study - study 2'	

7. I give consent for information collected about me to be used to support other research or in the development of a new test, medication, or treatment by an academic institution or commercial company in the future, including those outside of the United Kingdom (which Imperial has ensured will keep this information secure).	YES ❑ NO ❑ (please tick and initial)
8. I give consent for samples (plasma, tissue, DNA) collected about me to be used to support other research or in the development of a new test, medication, medical device or treatment by an academic institution or commercial company in the future, including those outside of the United Kingdom (which Imperial has ensured will keep this information secure).	YES ❑ NO ❑ (please tick and initial)
9. I give consent to being contacted about the possibility to take part in other research studies.	YES ❑ NO ❑ (please tick and initial)
10. I agree to my tissue samples being used to undertake genetic research which may have the potential to generate data that can be tracked back to me.	YES ❑ NO ❑ (please tick and initial)
11. I agree to be sent a summary of the research findings at the end of the study	YES ❑ NO ❑ (please tick and initial)

_____	_____	_____
Name of participant	Signature	Date
_____	_____	_____
Name of person taking consent	Signature	Date

1 copy for participant; 1 copy for Principal Investigator; 1 copy for hospital notes

To ensure confidence in the process and minimise risk of loss, all consent forms must be printed, presented and stored in double sided format

APPENDIX 2: PARTICIPANT INFORMATION SHEET

LEAP2 dose finding study v1.4.1, 28-May-2024. IRAS 304210, REC 23/LO/0510

Department of Brain Sciences
Imperial College London
Hammersmith Hospital
Du Cane Road
London W12 0NN

INFORMATION SHEET FOR RESEARCH PARTICIPANTS (DOSE CONFIRMATION STUDY - STUDY 2)

You will be given a copy of this Information Sheet and a signed copy of your consent form to keep, should you decide to participate in the study.

Principal Investigators:
Dr Tony Goldstone

Study Team:
Dr Raghav Bhargava
Dr Marcela Rodriguez Flores

Study title:
Gut hormone LEAP2 in metabolism and eating behaviour: Dose confirmation study

Invitation
You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information.

If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. You are free to withdraw at any time without explanation. Thank you for reading this.

Overview of study
LEAP2 is a naturally occurring hormone circulating in our blood. LEAP2 blocks the action of the 'hunger' hormone ghrelin to reduce appetite. This study no. 2 aims to infuse LEAP2 into the vein of people without obesity to check the levels of LEAP2 that are achieved in the blood, and gain some preliminary information about its effect on appetite, metabolism and eating behaviour.

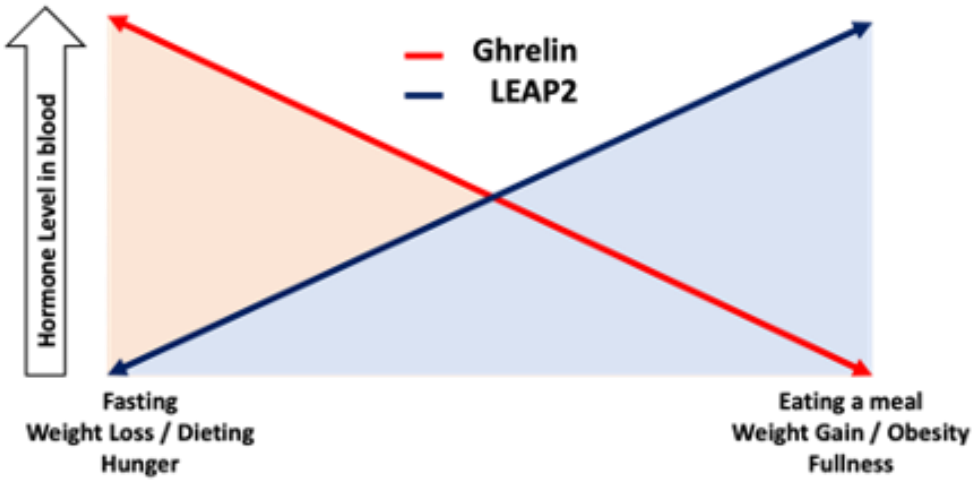
Complimentary studies
Another study (no. 3) will also infuse LEAP2 into a larger number of people without obesity to examine its effect on appetite, metabolism and eating behaviour, but now also including brain scanning.

Another study (no. 1) will also examine the effects of eating different meals on appetite, and blood levels of LEAP2 and ghrelin, in people without and with obesity.

You will **not** be able to take part in these other studies in addition to this study no. 2.

What is the purpose of the study?
LEAP2 is a hormone naturally produced by the intestine that increases in the blood after a meal. It blocks the action of another hormone called ghrelin that is produced by the stomach. Ghrelin levels increase when fasting and fall after eating, the opposite to LEAP2. As ghrelin stimulates appetite, and LEAP2 causes fullness/satiety, the combination of these hormone changes in ghrelin and LEAP2 contributes to people feeling less hungry after they have consumed a meal. Ghrelin also temporarily increases blood sugar and growth hormone levels and so LEAP2 may be expected to lower blood sugar and growth hormone levels.

LEAP2 hormone has been given to humans before and has been found to be safe and tolerable. However, the effects on appetite, eating behaviour, blood sugar and growth hormone levels, are not fully understood. Imperial College London including the head of this study has great experience in giving other gut hormones involved in appetite control to healthy volunteers and patients.



In this study, we will therefore be giving LEAP2 hormone to healthy volunteers to find out:

(i) what levels of LEAP2 hormone are achieved in the blood with different infusion rates/doses;
(ii) what rate/dose to give to achieve blood levels that are in the upper range of those normally seen in humans with obesity;
(iii) what effects LEAP2 has on appetite, eating behaviour and metabolism;
(iv) what effects LEAP2 has on blood sugar and other hormone levels (such as growth hormone, cortisol (stress hormone) and other appetite hormones;
(v) while ensuring that the given rates/doses of LEAP2 are safe and do not cause any side effects of concern.

You will not know whether you are receiving saline (salt water) or LEAP2 infusions, or

what dose of LEAP2 you are having. This study will give us information on the effects of different doses of LEAP2 infusions on appetite and levels of sugar and other hormones in the blood. For some participants in this study, we will also investigate the effects of LEAP2 on metabolism (how much energy is burnt at rest) and eating behaviour (using computer-based tasks).

Why have I been chosen?
As we would like to confirm that this hormone has no side-effects in humans, we need to first give LEAP2 to healthy volunteers, rather than people with health problems such as obesity.

We are therefore recruiting up to 25 healthy volunteers who are not obese (body mass index 18.0-29.9 kg/m^2) to take part in this study. We are recruiting both males and females, aged between 18 and 60 years.

We require people who do **not** have a blood or needle phobia, and do **not** suffer from any serious medical conditions. We require people who do **not** have any history or current dependence on alcohol or illicit drugs. You **cannot** take part if you currently do **not** drink any alcohol since one of the study tasks involves assessing preferences for different amounts of alcohol. You must **not** be a current smoker. If you were previously a smoker, you must have **not** smoked cigarettes for 2 years. This is because nicotine reduces appetite.

You will **not** be able to take part if you are vegan, gluten or lactose intolerant, as during the study visits the meals that people consume and the food pictures that are used in the computer tasks, may include animal products, wheat and dairy products.

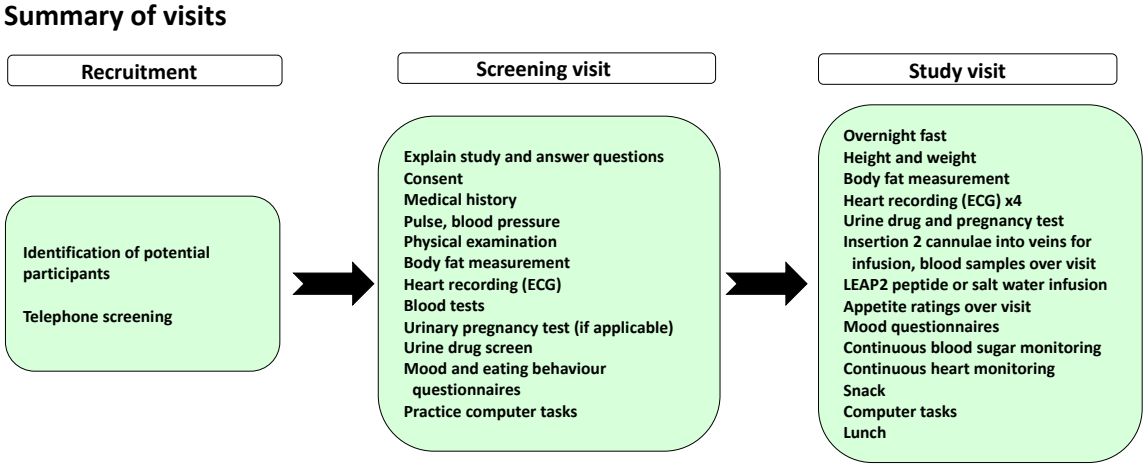
You will **not** be able to take part if you have a history of current diabetes, heart, lung, kidney, brain disease or cancer.

Females: If you are pregnant or breast feeding, you will **not** be able to take part and we will do a pregnancy test at each visit to confirm this.

The research will also form part of PhD, Masters and undergraduate student research projects.

Do I have to take part?
You do not have to take part in this research study. If after reading this information sheet you decide you would like to take part in the study, please email or telephone us (contact details at the bottom of this document) and we will arrange an initial telephone screening session to assess your suitability for the study. If you decide to take part, you are still free to withdraw at any time without giving a reason for your withdrawal.

What will happen to me if I take part?



Should you decide to take part, you will have a total of up to **7 visits** (1 screening visit and up to 6 study visits) at the Hammersmith Hospital in West London (near White City, London W12 0NN) over a period of usually 4 and at most within 10 weeks.

You will initially have a screening visit, lasting up to 2-3 hours, where consent, screening blood tests and body fat measurement will take place. Some participants will also practice computer tasks used at the study visits.

Your experimental study visits will be lasting up to 8 hours (with an approximately 6 hour infusion). Study visits involve being given the LEAP2 hormone or dummy 'placebo' (salt water) infusions. The LEAP2 hormone may be given at different infusion rates/doses so as to achieve different levels of LEAP2 in the blood. The infusions will be administered directly into your veins via a cannula (small plastic tube) connected to an infusion pump. This means you will have to stay in the Clinical Research Facility for the whole duration of the infusion.

You will be asked to attend the Clinical Research Facility at the Hammersmith Hospital (near White City, London W12 0NN), on dates convenient to you and the investigators.

Telephone screening session

This initial screening session is very important as it will initially determine your suitability to take part. We will ask you some questions about your medical and drug use history and general health. This screening session should take approximately 45 minutes to complete. We will review your information following the telephone screening session and let you know whether you may be able to take part.

What will happen if I pass the telephone screen and decide to take part?

If you are eligible and decide to take part, you will be invited to take part in a screening visit at the Hammersmith Hospital, near White City, west London.

Screening visit

The screening visit will take place at the Clinical Research Facility at Imperial College

Healthcare NHS Trust (NIHR Imperial Clinical Research Facility) at the Hammersmith Hospital, West London (see map at the bottom). Screening will take approximately 3 hours to complete.

Upon arrival at the Clinical Research Facility, you will meet with the research team who will go through the details of the study with you to ensure that you fully understand what is involved. This session will be a good time for you to ask questions about the study.

If you are sure you would like to take part, and we are comfortable that you understand what is involved, you will be asked to sign a consent form. Signing the consent form declares that you fully understand the information you have been given about the study. Signing the consent form will not bind you to taking part in the study, and you will be free to withdraw at any time.

You should be prepared to give a urine sample when you arrive at the hospital so we can screen for recent drug use. Recent drug use may interfere with your eligibility to take part in the study so please avoid any illicit substances.

All female participants will also have a urinary pregnancy test at the screening visit. If positive, they will be excluded from the study.

You will be asked questions about your general medical and psychiatric health, medication use, alcohol, drug use and smoking habits.

You will also have a physical examination and vital sign measurements by the research doctor. You will also have your body fat content measured using a “bio-electrical impedance” machine. This is a painless and safe method that involves measuring the electrical current from your body over a period of only 1 minute, which will show us the percentage of body fat. You will also have a heart recording (ECG) to check your heart rate and rhythm.

You will also complete some questionnaires with questions about your mood, current or past use of alcohol, and eating behaviour before you entered the study. The total duration of completing these questionnaires is up to 45 minutes.

You will provide a sample of blood as part of the routine medical examination to check your sugar, kidney, liver, thyroid hormone, iron studies, blood count. We will also take a sample of blood to test your DNA for genes that may be involved in addiction, obesity, brain function and the control of appetite and hormones. You will not receive any feedback regarding your DNA results unless there is something that may alter the management of your health. The total amount of blood taken at the screening visit will be no more than 40mL, equivalent to 3 tablespoons.

You may complete some computer-based eating behaviour tasks at the screening visit as a practice for the later study visits. (The total duration of these tasks is 1 hour).

You will be given a standard ready to eat meal at the end of the screening visit, which is to be consumed as your evening meal (dinner) the evening before the first study visit.

We will also ask for the contact details of your GP (primary care or family doctor). We will need to contact you doctor to inform them that you are taking part. Please bring these contact details with you to the screening visit.

If the results of your blood analysis, physical examination or questionnaires show anything outside of the normal range, such as sugar diabetes, high cholesterol levels, or hormone problems, your doctor will be informed so that they can coordinate your further care, arrange any further tests, and refer you on to hospital doctors if necessary.

Summary of the screening visit:

- Meet the research team
- Talk further about the study and ask any questions
- Sign consent form
- Urine sample for drugs of abuse and pregnancy test (females)
- Physical examination and questions about medical and psychiatric history
- Electrocardiography (ECG) recording the activity of your heart
- Bio-electrical impedance analysis (body fat)
- Routine blood sample for general health check and DNA sample
- Completion of questionnaires about mood, alcohol use and eating behaviour
- Completion of computer-based eating behavioural tasks (practice for study visits)
- Answer any final questions you might have about the study
- Provide contact details for your doctor
- Provision of standardised evening meal

What happens next?

After the screening visit, we will contact you to let you know if you are eligible for the study visits. We will ask you again on each of these study visits if you are willing to continue with the study and come for the next visit. Each of the study visits will be done at least 2 days apart. It is expected that the screening and study visits will be completed over at least 4 and at most 10 weeks, depending on your availability.

Study visits

If eligible, you will be invited to take part in up to a total of 6 study visits. These study visits last up to 8 hours. Visits involve being given the hormone LEAP2 or dummy ‘placebo’ (salt water) infusions for up to approximately 6 hours each visit, which will be administered directly into your veins via a cannula (small plastic tube) connected to an infusion pump.

These study visits will also commence at the Clinical Research Facility at the Hammersmith Hospital. You will be asked to attend each study visit at around 8:45 am. The study visit will last around 8 hours, and you will be free to leave at around 5pm.

You will be asked to abstain from alcohol and strenuous exercise for 24 hours before each study visit. For example, you should not do sporting activities such as running the day before the study and should not cycle to the study visit.

You will be asked not to eat any food or drink other than water after dinner (evening meal) the night before. At 8pm the evening before each study visit, you will be asked to eat the standardised dinner (evening meal) which will be a savoury 'ready' meal e.g. spaghetti bolognese or similar, and dessert e.g. yoghurt or similar. This meal will be provided to take home at the screening visit or previous study visit. You will get small snacks over the morning of the study visit and you will also be given lunch.

As with the screening visit, at each study visit you will need to provide a urine sample for drugs of abuse and pregnancy (females), have measurement of your height, weight, blood pressure and heart rate, and body fat using the impedance machine and an initial ECG recording of your heart.

You will have one cannula (small plastic tube) inserted into a vein in each of your forearms. The hormone or salt water will be administered directly into your vein via one of the cannula connected to a pump. The other cannula is so you can have blood samples taken during the infusion.

The rate of LEAP2 infusion will vary between each visit and sometimes within visits, so that we can achieve the target levels in your blood. The infusion rates will be guided by measurements from previous visits in yourself and others. We will ensure that previous lower doses are safe and well tolerated before giving higher doses. You will not know the dose of the hormone being given but the study investigators will know. On some visits you may be given an infusion of salt water 'placebo' instead of hormone.

Blood samples will be used to measure the levels of sugar, LEAP2, ghrelin, insulin and other hormones, and kidney and liver function during the infusion at regular time intervals. You may also have a finger prick test on several occasions over the study visit to measure the sugar level from a drop of blood. The maximum amount of blood that will be taken at any single study visit will be no more than 180 ml (equal to 13 tablespoons). The total amount of blood taken over all your 6 study visits will not be more than 1200 ml. This is slightly over two pints which is roughly the amount taken when making two donations of blood for blood transfusion.

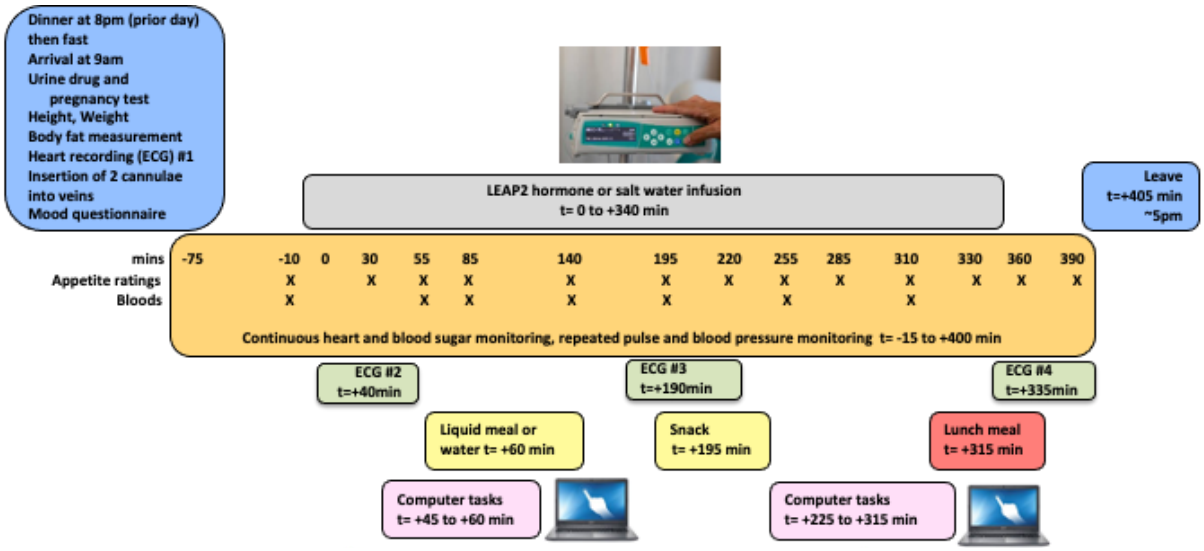
During the study visit, you will be seated or lying on a couch, and can read a newspaper or magazine. Before the infusion starts you will complete a brief questionnaire about your mood. During the infusion you will: (i) have continuous monitoring of your heart rate and rhythm and regular blood pressure measurements, (ii) 3 further ECG's, (iii) repeatedly complete some ratings about your appetite and mood on a computer; and (iv) towards the end of the infusion be given lunch to eat. You will be able to go home 1 hour after the infusion has finished.

In addition to the above, you will also:
(i) be given a liquid meal or water to drink, and a snack to eat, during the infusion;
(ii) do some computer-based tasks to assess your eating behaviour, such as rating and choosing pictures of food on a screen (lasting up to 90 minutes);
(iii) do some computer-based tasks to assess your memory.

Summary of a study visit:

- Avoid alcohol and strenuous exercise the day before study

- Eat the provided dinner (evening meal) at 8pm the evening before and then only have water to eat or drink
- Arrival at the Clinical Research Facility at 8.45am
- Urine sample for drugs of abuse and pregnancy test (females)
- Record of height, weight, blood pressure, heart rate and body fat
- Electrocardiography (ECG) recording (4 times)
- Insertion of 2 cannulae into veins
- Hormone infusion for up to approximately 6 hours
- Routine blood sampling and possible finger prick tests for blood sugar several times during infusion
- Ratings of how you are feeling during infusion
- Liquid meal or water during infusion, and snack
- Computer-based cognitive tasks
- Computer-based eating behaviour tasks
- Lunch meal to taste and eat (towards end of infusion)
- Completion of infusion
- Plan the next study visit
- Provision of standardised evening meal to take home for next study visit
- Leave the hospital at 5pm



What are the possible side effects and risks of taking part?

LEAP2 hormone infusion:

Based on the known effects of ghrelin (the stomach hunger hormone that LEAP2 blocks the action of) from animal and human studies, and of LEAP2 from animal studies, possible anticipated effects of LEAP2 may include: temporary lowering of sugar and growth hormone levels, nausea, vomiting, abdominal pain or bloating, reduced appetite, early fullness/satiety, or delayed emptying of the stomach. However, you will be closely monitored throughout the study visits and such symptoms, if at all occurring, are expected to be usually mild.

LEAP2 hormone has been given as an infusion for 6 hours to 28 healthy men without obesity in one recent study from Denmark without any side effects. There were no

effects on nausea, discomfort or stomach emptying and there were no concerning effects on the heart or blood pressure. As expected, the LEAP2 infusion did reduce appetite slightly and caused a small decrease in blood glucose levels within the normal range.

Another recent study in Europe, infused a small fragment of LEAP2 hormone to 10 healthy non-obese men. No adverse events were seen. A different tablet medication that blocks the action of ghrelin has also been given to healthy adults, and patients with alcohol dependence, in studies in the USA without any serious adverse effects.

In our studies, one participant without obesity (out of 4) had a temporary lowering of their heart rate and blood pressure at a higher LEAP2 infusion dose than will be given to you. This resolved without any intervention after stopping the infusion, and the participant made a full recovery. Going forward we will therefore be giving a lower LEAP2 infusion dose. No concerning falls in blood pressure or heart rate were seen in 28 healthy men without obesity in the recent study from Denmark using this lower LEAP2 infusion dose, nor in 20 healthy men with obesity in another recent study from Denmark at an even higher LEAP2 infusion dose than we have used.

We have not seen any serious adverse events, including low heart rate or blood pressure, at lower LEAP2 infusion doses in 3 participants without obesity at 6 infusion visits.

You will be monitored closely throughout the infusion and for 1 hour afterwards including heart rate and rhythm and blood pressure.

No long-term consequences of the infusions are expected. After the infusion is stopped, the infused LEAP2 hormone will be broken down within minutes, and any effects on appetite, sugar or hormones will be gone over a few hours. At the end of the study, we will ensure you are feeling well, and your observations are satisfactory before you go home.

If at any stage, there are medical concerns about how you are feeling or you become very unwell the infusions will be slowed down or stopped. Higher LEAP2 doses will only be given when we know that lower doses are safe and without any major side effects.

Furthermore, many other studies, including those performed by the head of this project have given infusions or injections of ghrelin to healthy volunteer and patients, without any significant adverse effects.

Blood sugar monitoring:
We may also be monitoring blood sugar levels, without the use of needles using a continuous sugar monitoring disc (such as Dexcom continuous glucose monitoring; Dexcom Inc., CA, USA) which is applied to the skin on the upper arm, abdomen or lower leg. Sugar levels can be read by swiping a dedicated reader or mobile phone and will allow us to know real-time sugar readings as well as previous readings.

With disc application to the skin the following adverse effects are possible: local inflammation, infection, redness, pain or discomfort, bleeding at insertion site, bruising,

itching, scarring, tape irritation, sensor or needle fracture during insertion, wear or removal.

Vein cannulation:
Cannula insertion may cause minor discomfort or superficial bruising.

In case of emergency:
In case of emergencies, throughout the study, there will be a doctor available on 24-hour call via a direct line, and a secondary direct line to the senior doctor. You will be given the contact telephone numbers when you commence the study.

What are the possible benefits of taking part?
There is no direct benefit to the participants taking part in the study. The information that we get from this study will help us to better understand how gut hormones may be used in the study of people who are overweight, and this may help us to better develop treatments for these, as well as other, addictions, as well as obesity.

What if new information becomes available?
Sometimes during the course of a research project, new information becomes available about the topic that is being studied. If this happens, the researchers will tell you about it and discuss with you whether you want to continue in the study. If you decide to continue in the study, you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study.

What happens when the research study stops?
Once the study has finished, the results of the study can be made available to you and/or your GP should you wish. If you have any problems immediately following the study, then you should contact one of the researchers on the numbers provided.

What will happen to the results of the research study?
Data analysis can take a long time, so you should not expect to hear the results of the wider study for a number of months after your participation. Any participant wishing to be informed about the results can do so by emailing the study coordinator.

Your confidentiality will be ensured at all times, and you will not be identified in any publication. At the end of the analysis of the study, a summary of the results of the study can be sent to you should you wish.

Will my taking part in this study be kept confidential?
All information that is collected about you during the course of the research will be kept strictly confidential.

It is a requirement that you consent to letting us inform your GP about your participation in this study, and check with your GP that you are eligible to take part. We will ask your GP for a summary of your medical history and list of your current medications.

If you have private medical insurance, you should notify your insurer if you decide to take part in this study.

If you were to lose capacity to consent at any stage during the course of this study, you will automatically be withdrawn from the study and no further samples or data would be collected from you. However, any samples or data already collected would still be retained and used in the study.

At the end of the study, samples will be destroyed unless you have given consent for them to be used for other research.

What if something goes wrong?
Imperial College London holds insurance policies which apply to this study. If you experience harm or injury as a result of taking part in this study, you will be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator (Dr Tony Goldstone, email: tony.goldstone@imperial.ac.uk). The normal National Health Service mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial College, Research Governance and Integrity Team: RGIT@imperial.ac.uk, Tel: 020 7594 1872.

How will we use information about you?
Imperial College London is the sponsor for this study and will act as the Data Controller for this study. This means that we are responsible for looking after your information and using it appropriately. Imperial College London will keep your personal data for:

- 10 years after the study has finished in relation to data subject consent forms.
- 10 years after the study has completed in relation to primary research data.

The study is expected to finish in June 2025.

For more information / confirmation regarding the end date please contact the study team, see '**Where can you find out more about how your information is used?'** for contact information.

We will need to use information from you, your medical records and your GP for this research project.
This information will include your:

- initials
- NHS number
- hospital number
- name
- contact details
- email address.

People within the College and study team (see section sharing your information with others) will use this information to do the research or to check your records to make sure that research is being done properly and the information held (such as contact details) is accurate.

People who do not need to know who you are will not be able to see your name or contact details. Your data will have a code number instead.
We will keep all information about you safe and secure. Some of your information will be sent to the USA. They must follow our rules about keeping your information safe.

Once we have finished the study, we will keep some of the data so we can check the results. We will write our reports in a way that no-one can work out that you took part in the study.

As a university we use personally-identifiable information to conduct research to improve health, care and services. As a publicly-funded organisation, we have to ensure that it is in the public interest when we use personally-identifiable information from people who have agreed to take part in research. This means that when you agree to take part in a research study, we will use your data in the ways needed to conduct and analyse the research study. Our legal basis for using your information under the General Data Protection Regulation (GDPR) and the Data Protection Act 2018, is as follows:

"performance of a task carried out in the public interest"); Health and care research should serve the public interest, which means that we have to demonstrate that our research serves the interests of society as a whole. We do this by following the UK Policy Framework for Health and Social Care Research."

Where special category personal information is involved (most commonly health data, biometric data and genetic data, racial and ethnic data etc.), Imperial College London relies on "scientific or historical research purposes or statistical purposes".

International transfers
There may be a requirement to transfer information to countries outside the United Kingdom (for example, to a research partner, either within the European Economic Area (EEA) or to other countries outside the EEA. Where this information contains your personal data, Imperial College London will ensure that it is transferred in accordance with data protection legislation. If the data is transferred to a country which is not subject to a UK adequacy decision in respect of its data protection standards, Imperial College London will enter into a data sharing agreement with the recipient research partner that incorporates UK approved standard contractual clauses or utilise another transfer mechanism that safeguards how your personal data is processed.

Sharing your information with others
We will only share your personal data with certain third parties for the purposes referred to in this participant information sheet and by relying on the legal basis for processing your data as set out above.

- Other Imperial College London employees (including staff involved directly with the research study or as part of certain secondary activities which may include support functions, internal audits, ensuring accuracy of contact details etc.), Imperial College London agents, contractors and service providers (for example, suppliers of printing and mailing services, email communication services or web services, or suppliers who help us carry out any of the activities described above). Our third-party service providers are required to enter into data processing

agreements with us. We only permit them to process your personal data for specified purposes and in accordance with our policies.

- the following Research Collaborators / Partners in the study
  - University of Leeds - data from the Leeds Food Preference Questionnaire for purpose of data analysis
  - Phoenix Pharmaceuticals, USA - coded blood samples for measurement of blood hormones
  - Kings College Hospital, London UK - coded blood samples for measurement of blood hormones
  - We will ensure your name and any identifying details will NOT be given to these third parties, instead you will be identified by a unique study number with any sample / data analysis having the potential to generate 'personal data'

Potential use of study data for future research

When you agree to take part in a research study, the information collected either as part of the study or in preparation for the study (such as contact details) may, if you consent, be provided to researchers running other research studies at Imperial College London and in other organisations which may be universities or organisations involved in research in this country or abroad. Your information will only be used to conduct research in accordance with legislation including the GDPR and the UK Policy Framework for Health and Social Care Research.

This information will not identify you and will not be combined with other information in a way that could identify you, used against you or used to make decisions about you.

Commercialisation

Samples / data from the study may also be provided to organisations not named in this participant information sheet, e.g. commercial organisations or non-commercial organisations for the purposes of undertaking the current study, future research studies or commercial purposes such as development by a company of a new test, product or treatment. We will ensure your name and any identifying details will NOT be given to these third parties, instead you will be identified by a unique study number with any sample / data analysis having the potential to generate 'personal data'.

Aggregated (combined) or pseudonymised data sets (all identifying information is removed) may also be created using your data (in a way which does not identify you individually) and be used for such research or commercial purposes where the purposes align to relevant legislation (including the GDPR) and wider aims of the study. Your data will not be shared with a commercial organisation for marketing purposes.

What are your choices about how your information is used?

You can stop being part of the study at any time, without giving a reason, but we will keep information about you that we already have, because some research using your data may have already taken place and this cannot be undone.

- We need to manage your records in specific ways for the research to be reliable. This means that we won't be able to let you see or change the data we hold about you if this could affect the wider study or the accuracy of data collected.

- If you agree to take part in this study, you will have the option to take part in future research using your data saved from this study.

Where can you find out more about how your information is used?
You can find out more about how we use your information:
- at www.hra.nhs.uk/information-about-patients
- by asking one of the research team
- by sending an email to appetite@imperial.ac.uk
- by ringing us on 020 7594 5988

Complaints
If you wish to complain, or about how we have handled your personal data, or have any concerns about any aspect of the way you have been treated during the course of this study then you should inform the Principal Investigator (Dr. Tony Goldstone, Department of Brain Sciences, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN. Email: tony.goldstone@imperial.ac.uk).

Following our response, if you are not satisfied about how we have handled your personal data, please contact Imperial College London's Data Protection Officer via email at dpo@imperial.ac.uk, via telephone on 020 7594 3502 and/or via post at Imperial College London, Data Protection Officer, Faculty Building Level 4, London SW7 2AZ.

If you remain unsatisfied with our response or believe we are processing your personal data in a way that is not lawful you can complain to the Information Commissioner's Office (ICO) via www.ico.org.uk. Please note the ICO does recommend that you seek to resolve matters with the data controller (us) first before involving them.

For someone independent, you can contact the Imperial AHSC Joint Research Office. The normal National Health Service complaints mechanisms are also available to you. The nearest Patient Advice and Liaison Service (*PALS*) is the Hammersmith Hospital: imperial.pals@nhs.net or by telephoning 020 3313 3322. Further details about the NHS PALS can be found at www.nhs.uk.

Who is funding the research?
This study is being funded by the UK Medical Research Council and sponsored by Imperial College London. The main scientific investigator is Dr Tony Goldstone in the Department of Brain Sciences at Imperial College London.

Payment
You will receive a fixed payment to cover expenses and your time. This will be £25 for the screening visit, and £100 for each of up to 6 study visits. In addition, your travel expenses will be covered.

The £25 for the screening visit will be submitted for payment after that visit, and the remainder will be paid when you have completed all your study visits. If you do not have all 6 study visits you will be paid according to how many you have attended.

Therefore, the total amount payable for completion of the screening visit and all 6 study visits is £625.

Who has reviewed the study?
This study has been reviewed by the London - West London & GTAC NHS Research Ethics Committee.

Contact for further information:
Email: appetite@imperial.ac.uk
Tel: 020 7594 5988

Study coordinators and medical doctors:
Dr Raghav Bhargava
Dr Marcela Rodriguez Flores

Principal Investigator:
Dr Tony Goldstone

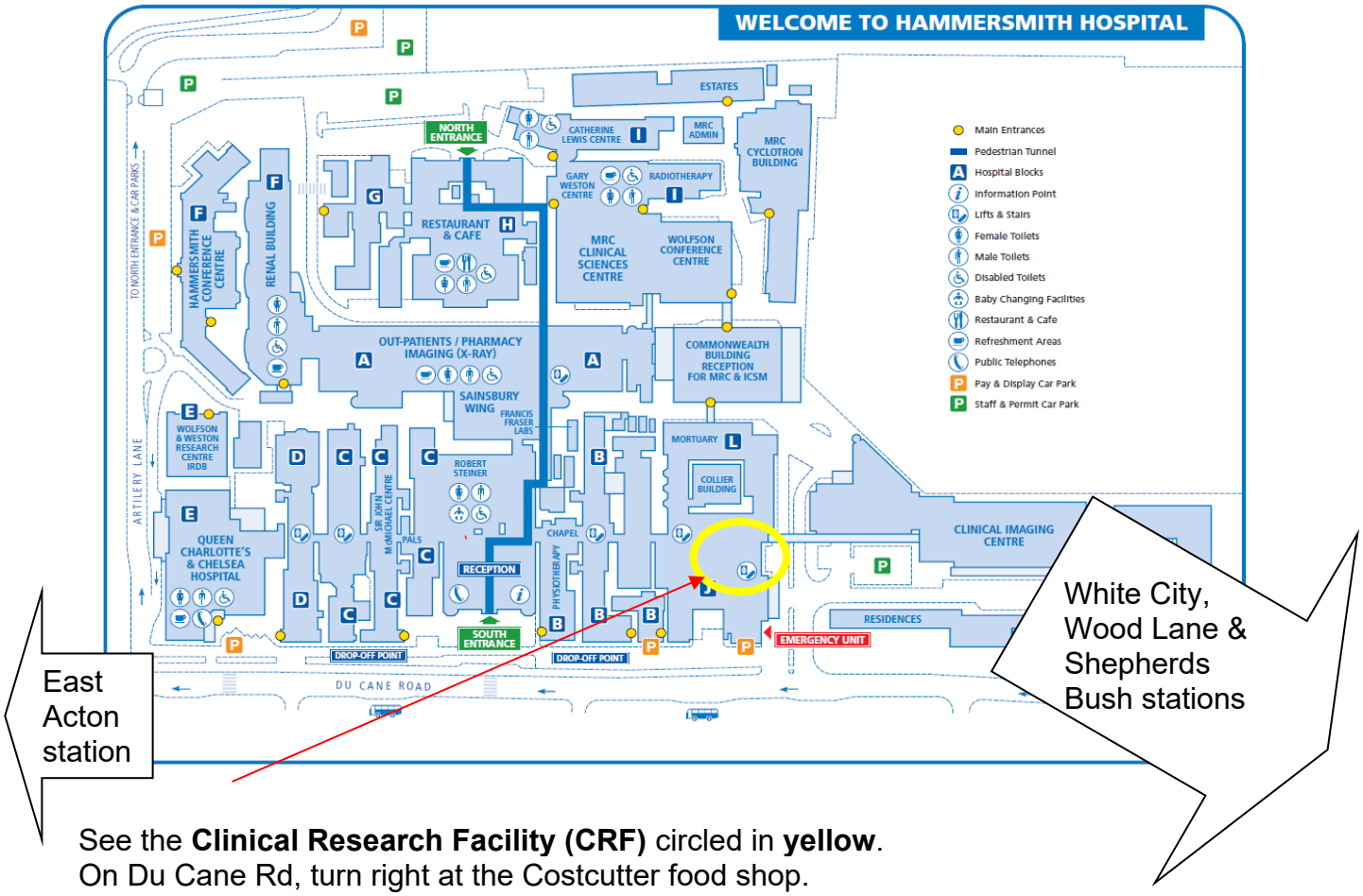
Research Group address:
PsychoNeuroEndocrinology Research Group, Division of Psychiatry, Department of Brain Sciences, Hammersmith Hospital, Imperial College London, Du Cane Road, London, W12 0NN

Out of hours / emergency contact number:
07714 051232 (this is a study mobile held by the researchers not a personal number)

Study centre address for visits:
NIHR Imperial Clinical Research Facility
Hammersmith Hospital
Du Cane Road
London W12 0HS
Tel: 020 3313 8070



Directions:
Nearest underground stations = White City (Central Line) or Wood Lane (Hammersmith and City Line).
From White City underground station, it takes about 12 minutes to walk to us. Turn right on exiting the station; this road is called 'Wood Lane'. Walk along Wood Lane, past the BBC buildings, under the Westway flyover and turn left at Du Cane Rd. Walk past the playing fields and turn right at the Costcutter food shop. The CRF is a large silver and black building with some yellow framing. The entrance to the CRF is a revolving door with a large yellow frame around it.
Alternatively, you can get bus 72 or 272 from the bus stop opposite White City or Wood Lane stations.



See the **Clinical Research Facility (CRF)** circled in **yellow**.
On Du Cane Rd, turn right at the Costcutter food shop.
The CRF is a large black, yellow and silver building ahead and to the left (see photo below). The CRF is on the ground floor of this building. Go through the yellow-framed revolving door.

APPENDIX 3: LEAP2 (38-77) BIO-EFFICACY TESTING IN MICE

Bio-efficacy of the synthesised LEAP2 has been confirmed in mice after subcutaneous administration by demonstration of dose-dependent reduction in food intake when fasted but not when fed (Figure A3A), and ability to block acyl ghrelin-induced food intake (Figure A3B), as previously described (Ge et al. 2018; M'Kadmi et al. 2019).

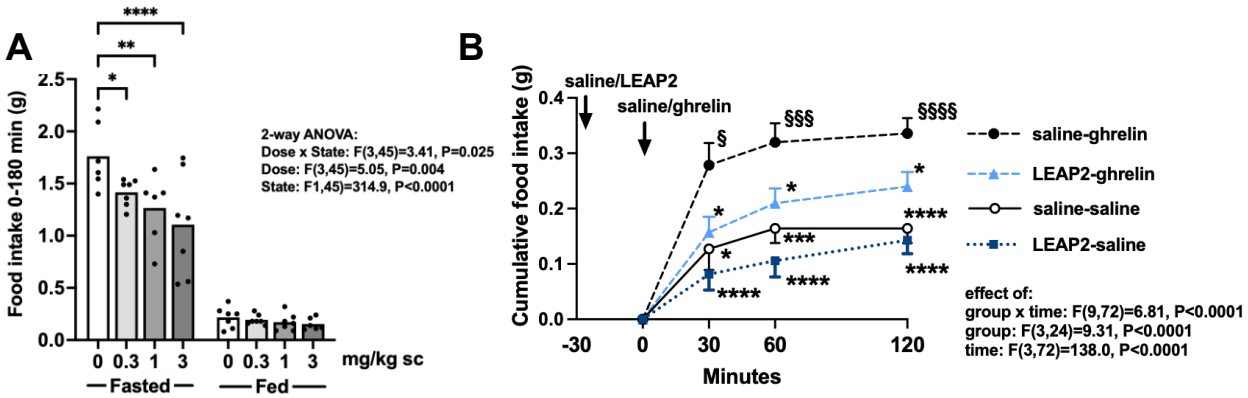


Figure A3. In mice, exogenous LEAP2 decreases food intake in dose-dependent manner when fasted but not when fed, and blocks acyl ghrelin-induced increase in food intake when fed.

Effect of subcutaneous administration of GMP-grade synthesised LEAP2 peptide (AmbioPharm Inc.) being used in LEAP2 infusion study, in mice (male C57BL/6) (A) at doses 0.3, 1.0 and 3 mg/kg on total food intake over 180 min in early light phase when fasted (16 hours) or fed (n=6-7), post-hoc Fisher LSD: *P<0.05, **P<0.01, ***P<0.005, ****P<0.001), (B) given at dose 0.15 umol/kg (or saline) 20 mins before subcutaneous acyl ghrelin at dose 0.15 umol/kg (or saline) on cumulative food intake over 120 min in early light phase when fed (n=7), post-hoc Fisher LSD: vs. saline-ghrelin *P<0.05, **P<0.01, ***P<0.005, ****P<0.001; vs. saline-saline § P<0.05, §§ P<0.01, §§§ P<0.005, §§§§ P<0.001.

APPENDIX 4: CANTAB TASKS

1. PAIRED ASSOCIATE LEARNING

Paired Associates Learning assesses episodic memory.

1.1. Duration

8 mins

1.2. Task format

Boxes are displayed on the screen and are “opened” in a randomised order. One or more of them will contain a pattern. The patterns are then displayed in the middle of the screen, one at a time and the participant must select the box in which the pattern was originally located. If the participant makes an error, the boxes are opened in sequence again to remind the participant of the locations of the patterns. Increased difficulty levels can be used to test high-functioning, healthy individuals (Figure A4A).

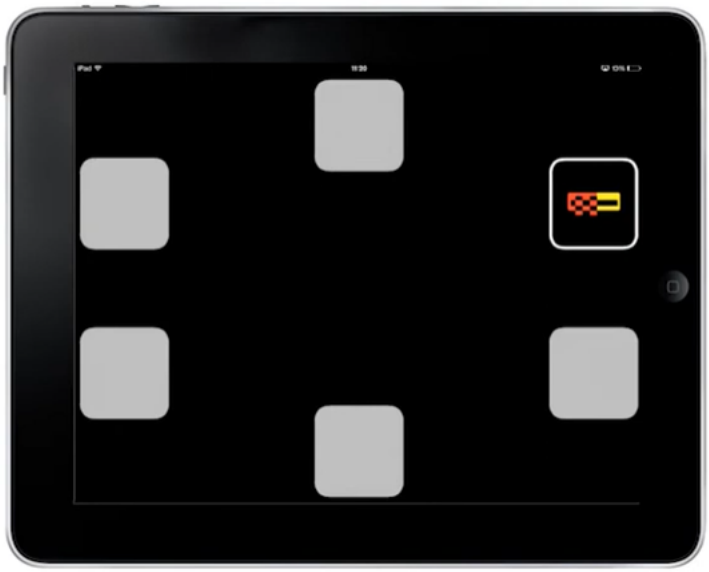


Figure A4A

1.3. Outcome measures

Outcome measures include the total errors made by the participant (lower better) and the number of the number of times a participant chooses the correct box on their first attempt when recalling the pattern locations (higher better).

2. STOP SIGNAL TASK

The Stop Signal Task assesses motor response inhibition (impulse control).

2.1. Duration

14 mins

2.2. Task format

The participant must respond to an arrow stimulus, by selecting one of two options, depending on the direction in which the arrow points. If an audio tone is present, the subject must withhold making that response (inhibition). The test consists of two parts: In the first part, the participant is introduced to the test and told to select the left-hand button when they see a left-pointing arrow and the right-hand button when they see a right-pointing arrow. There is one block of 16 trials for the participant to practice this. In the second part, the participant is told to continue selecting the buttons when they see the arrows but, if they hear an auditory signal (a beep), they should withhold their response and not select the button. The task uses a staircase design for the stop signal delay, allowing the task to adapt to the performance of the participant, narrowing in on the 50% success rate for inhibition (Figure A4B).

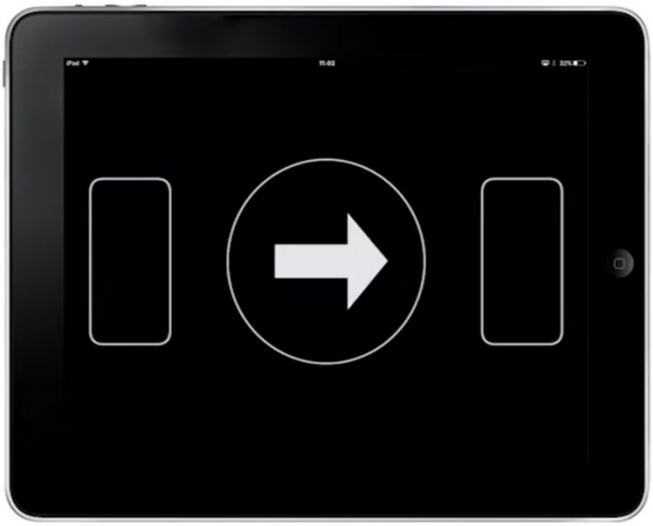


Figure A4B

2.3. Outcome measures

Outcome measures cover stop signal reaction time, i.e. the estimate of time where a participant can successfully inhibit their responses 50% of the time (lower better).

3. SPATIAL WORKING MEMORY

Spatial Working Memory requires retention and manipulation of visuospatial information. This self-ordered test has notable executive function demands and provides a measure of strategy and assesses working memory.

3.1. Duration

4 mins

3.2. Task format

The test begins with a number of coloured squares (boxes) shown on the screen. The aim of this test is that by selecting the boxes and using a process of elimination, the participant should find one yellow ‘token’ in each of a number of boxes and use them to fill up an empty column on the right-hand side of the screen. Depending on the difficulty level used for this test, the number of boxes can be gradually increased until a maximum of 12 boxes are shown for the participants to search. The colour and position of the boxes used are changed from trial to trial to discourage the use of stereotyped search strategies (Figure A4C).

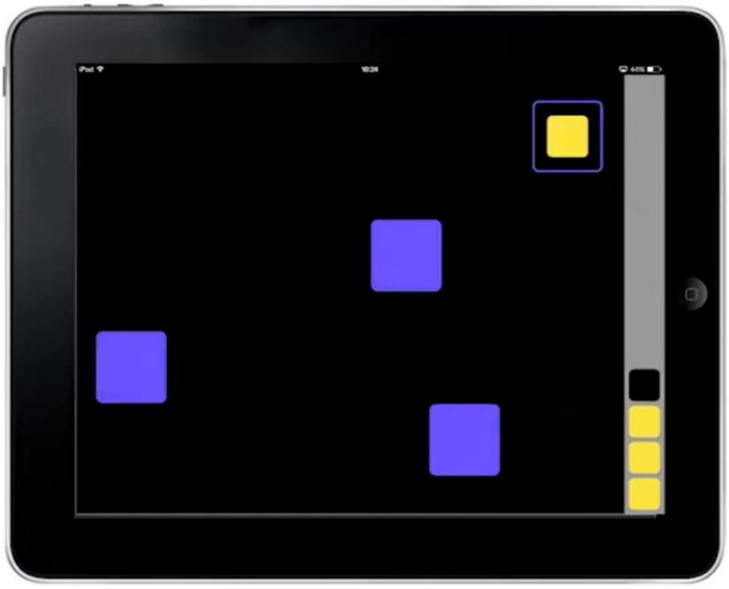


Figure A4C

3.3. Outcome measures

Outcome measures include the total errors made (lower better), i.e. number of times the participant incorrectly revisits a box in which a token has previously been found.by selecting boxes that have already been found to be empty and revisiting boxes which have already been found to contain a token. This is calculated across all four, six and eight token trials.

APPENDIX 5: ORIGINAL ABSTRACT

Original abstract submitted for viva voce

Introduction: Obesity is a global epidemic with limited therapeutic options. Appetitive gut hormones such as acyl ghrelin (AG) drive failure of weight maintenance after cessation of weight loss interventions. The novel liver-foregut satiety hormone LEAP2 is an inverse agonist at the growth hormone secretagogue receptor (GHSR) and is reciprocally regulated to AG. LEAP2 increases after food intake and weight gain and decreases after bariatric surgery and short-term weight loss. LEAP2 antagonises the orexigenic effects of AG and plays a potential role in regulating appetite and metabolism. The role of LEAP2 in human eating behaviour and metabolic regulation, the mechanisms underlying its post-prandial increase, and potential changes in plasma LEAP2 over long-term weight loss remain incompletely understood.

Methods: Plasma LEAP2 was measured in secondary analyses of previous human studies: (i) examining the effects of food intake, and its correlations with changes in eating behaviour including food cue reactivity using functional MRI, (ii) after exogenous AG administration, in adults without obesity (Chapter 2), (iii) comparing the effects of long-term caloric restriction without or with duodenal-jejunal bypass liner (DJBL) insertion in adults with obesity and poorly controlled type 2 diabetes mellitus, on body weight, markers of glucose and fat metabolism (Chapter 3). In pilot studies, safety and metabolic changes were examined during exogenous LEAP2 intravenous infusion at various doses in adults without obesity (Chapter 4).

Results: Post-prandial increases in plasma LEAP2 correlated with post-prandial decreases in appetite and high-energy food cue reactivity in several brain reward regions, with similar decreases (trend) for *ad libitum* food intake. Plasma LEAP2 was unaffected by exogenous AG administration. Plasma LEAP2 decreased after long-term dietary weight loss without or with DJBL insertion (but was delayed and attenuated in the latter) that correlated with decreases in body mass index (BMI) and markers of glucose/fat metabolism. LEAP2 infusion at 25 pmol/kg/min over 5.7 hours was safe and dose dependently reduced plasma glucose and venous beta-hydroxybutyrate independent of changes in serum insulin and growth hormone.

Conclusions: These findings support (i) an anorexigenic role for post-prandial LEAP2 in human eating behaviour, (ii) a lack of any direct influence of AG on LEAP2, (iii) a potential role for decreases in plasma LEAP2 contributing to weight regain, (iv) attenuation of GHSR signalling acutely improving glucose metabolism and attenuating lipolysis. These results lay the groundwork for further experimental medicine studies to examine the acute effects of LEAP2 administration to investigate its potential as a therapeutic agent for obesity and weight loss maintenance, and addiction.