Glucagon analogue therapy for obesity and metabolic disease; Implications of amino acid metabolism

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i. Abstract

Obesity and its related comorbidities pose a significant challenge to the individual and global healthcare systems. Bariatric surgery has provided a benchmark for weight loss outcomes however is limited by supply of resources, side effects and patient preference. Over the last two decades, significant progress has been made in the development of pharmacotherapies for the treatment of obesity and metabolic disease. GLP-1-based analogues have emerged as a safe and effective anti-obesity medicines (AOM), for example, Semaglutide (Novo Nordisk) and Tirzepetide (Eli Lilly).

'Multi-agonists' targeting multiple hormone receptors are actively being developed and have the potential to achieve substantial weight loss. A key component of many of these is glucagon receptor activity, which leverages metabolic benefits of glucagon including enhanced energy expenditure and improved lipid metabolism. However, glucagon enhances hepatic amino acid catabolism and can lead to hypoaminoacidemia in excess. Emerging evidence also suggests that hypoaminoacidemia leads to a divergent activation of energy expenditure, which raises the possibility that glucagon-mediated EE may be a response to low plasma amino acid availability. This thesis aims to determine whether a long-acting analogue active at the glucagon receptor results in chronic suppression of amino acids and the implications of this on whole-body physiology.

In this thesis, a long-acting glucagon analogue, G108, is used to explore the effect on amino acid metabolism. In an obese mouse model, a high protein diet 'rescues' plasma amino acids and mitigates against functional muscle loss in G108-treated mice. Glucagon-mediated energy expenditure and body weight loss are also linked to its action on hepatic amino acid catabolism. This was confirmed with mechanistic studies in obese mice. Despite a high protein diet, key benefits of glucagon receptor signalling remained in G018-treated mice, including improved hepatic lipid metabolism and blood glucose control. A phase 1 study was carried out using a potent GLP-1R/GCGR co-agonist, G3215, which led to a broad reduction in plasma amino acids and highlighting the importance of screening for changes in protein metabolism in further development. Finally, strategies are suggested to optimise glucagon-receptor targeted multi-agonists for the treatment of obesity and metabolic disease.

ii. Declaration of contributors

Most of the work in this thesis was performed by the author. Individuals who kindly contributed are listed below.

Chapter 2 – Peptide G108 was kindly provided by S. R Bloom and J. Minnion. The cAMP receptor accumulation studies were undertaken under by B. Jones with D. C. D Hope assisting. The G108 PK profile and single-dose G108 glucose profile was undertaken by C. Hinds. Transgenic mice were kindly provided by B. Owen.

Chapter 3 – Individual amino acids were measured using a gas-chromatography-mass spectrometry platform by Professor N. Dalton and Dr C. Turner at the WellChild Laboratory, Evelina Children's Hospital, London. Western blot analysis was carried out with the assistance of M. Vincent. Targeted PCR analysis of hepatic lipid metabolism was carried out by A. T. C Yu. Metabolic variable analysis was performed using specifically designed metabolic cages, provided by D. Carling. Studies were carried out with assistance by A. Sardini and J. Grant. Glucose tolerance testing was carried out with assistance from B. Owen, M. Norton, Y. Patel and T. Lopes.

Chapter 4 – Analysis of metabolic variables were performed as described above. Transgenic mice were provided by B. Owen. I. Davies

Chapter 5 – The Phase 1 clinical trial was overseen by the CI Professor S. R Bloom and PI Professor T Tan. Several individuals from Professor T Tan's research group contributed to this programme of work from the trial design and management to the clinical running of the study. These include but are not limited to: J Minnion, R. Malviya, Y. Ilesanmi, K. Alexiadou, C. Ling, LJ. Ball, S. Choudhury, B. Reglinska, A. Kasim, D. Meredith, Y. Tabakh, K. Lazarus.

Chapter 6 – Peptides Q and H were provided by S.R Bloom with peptide characterisation performed by B Jones.

iii. Declaration of originality

The work in this thesis was conceived and designed by the author, D. C. D Hope. Previous research is referenced throughout the text and found at the end of the thesis. The author gratefully acknowledges the prior research by others which formed the basis of this thesis.

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vi. Abbreviations

4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
AA	Amino acid
AAR	Amino acid response
ADP	Adenosine diphosphate
AE	Adverse event
AgRP	Agouti-related protein
AMPK	AMP-activated protein kinase
AOM	Anti-obesity medication
AP	Area postrema
ARC	Arcuate nucleus
ARG	Arginase
ASL	Arginosuccinate lyase
ASS1	Arginosuccinate synthase 1
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BCAA	Branched chain amino acid
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
CIDEA	Cell Death Inducing DFFA Like Effector A
CNS	Central nervous system
CPS-1	Carbomyl phosphate synthetase 1
CREB	cAMP-response element binding protein
DIO	Iodothyronine diodinase
DIO	Diet-induced obese
DMH	Dorsal medial hypothalamus
EAA	Essential amino acid
ECG	Electrocardiogram
EE	Energy expenditure
EIF2alpha	Eukaryotic translation initiation factor 2 subunit 1
EMA	European Medicines Agency
EU	European union
FBPase2	Phosphofructokinase-2
FDA	US Food and Drug Administration
FFA	Free Fatty acid
FGF21	Fibroblast growth factor 21
FITH	First in human clinical trial
FXR	Farsenoid X receptor
G6P	Glucose 6-phosphate
G6Pase	Glucose 6-phosphatase
GABA	Gamma aminobutyric acid
GCGR	Glucagon receptor
GCMS	Gas-chromatography/Mass-spectrometry
GCN2	General control nonderepressible 2
GCP	Good Clinical Practice

GCPR	G-coupled protein receptor
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon like peptide - 1
GLP-2	Glucagon like peptide - 2
GMP	Good Manufacturing Practice
Gs	G-stimulatory protein
GTT	Glucose tolerance test
HP	High protein
ICH	International Conference on Harmonisation
IFG	Impaired fasting glucose
IGF1	Insulin-like growth factor 1
IGT	Impaired glucose tolerance
IMP	Investigational medicinal product
IP	Intraperitoneal
IP	Inositol phosphate
IR	Insulin receptor
Kcal	Kilocalorie
kJ	Kilojoule
KO	Knock-out
LA	Long-acting
LAA	Low amino acid
LepRb	Leptin receptor long isoform
LH	Lateral hypothalamus
MCR4	Melanocortin 4 receptor
MRI	Magnetic resonance imaging
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
MuRF1	Muscle RING-finger protein 1
NAG	N-acetyl glutamate
NAGS	N-Acetylglutamate synthase
NEAA	Non-essential amino acid
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
OXM	Oxyntomodulin
PD	Pharmacodynamic
PEPCK	Phosphoenol pyrivate carboxykinase
PEPCK	Phosphoenolpyruvate carboxykinase
PK	Pharmacokinetic
PKA	Protein kinase A
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus
PYGL	Glycogen phosphorylase
PYY	Peptide YY
RER	Respiratory exchange ratio
RIA	Radioimmunoassay
S6K	Ribosomal protein S6 kinase
SA	Short-acting

SC	subcutaneous
SDS	Serine dehydratase
SNP	Single nucleotide polymorphism
SP	Standard protein
SPAA	Standard protein amino acid
T2DM	Type 2 diabetes mellitus
UCP1	Uncoupling protein 1
UCP1	Uncoupling protein 1
UPP	Ubiquitin proteosome pathway
VDCC	Voltage-gates calcium channels
VMH	Ventral medial hypothalamus
VTA	Ventral tegmental area
WAT	White adipose tissue
WT	Wild type

Table of Contents

1 Chapter 1 – General Introduction - Glucagon in an obesity strategy and implications of glucagon-stimulated protein metabolism 14

1.1 Obesity; a global epidemic and an emerging treatment gap 14

1.2 Body weight homeostasis in an obesogenic environment 15

1.3 Central regulation of energy balance 16

1.4 The need for a safe and effective anti-obesity medication (AOM) 18

1.5 Previous challenges and current obesity pharmacotherapeutics 19

1.6 Novel and combination AOM's in various research stages 21

1.7 Glucagon as an anti-obesity agent - overview 23

1.8 Glucagon – discovery of the protein 24

1.9 Glucagon secretion 26

1.10 Hepatic glucagon signalling 27

1.11 Glucagon in the pathophysiology of type 2 diabetes mellitus (T2DM) 30

1.12 The effect of glucagon on food intake 31

1.13 The effect of glucagon on EE 32

1.14 Rationale for GLP-1/glucagon co-agonism as an anti-obesity strategy 33

1.15 Glucagon mediated EE – Mechanisms 34

1.15.1 Glucagon mediated EE and BAT 34

1.15.2 Alternative possible mechanisms for glucagon-mediated EE 35

1.16 Glucagon and protein metabolism 37

1.16.1 The urea cycle and amino acid catabolism 37

1.16.2 Early experiments exploring the interaction between native glucagon and nitrogen balance 39

1.16.3 Protein metabolism in the glucagonoma syndrome 41

1.16.4 Plasma amino acid availability and amino acid sensing; Recent insights from the liver-alpha axis 41

1.17 The effect of amino acid availability on amino acid sensing and lean mass; implications for long-term glucagon treatment? 43

1.18 The effect of amino acid availability on whole body energy balance – insight from models of dietary protein and amino acid restriction 47

1.18.1 Hepatic FGF21, a key endocrine signal of protein restriction 48

1.18.2 Restriction or depletion of individual amino acids can alter energy balance 50

1.18.3 Methionine deprivation or restriction alters energy balance 51

1.18.4 Threonine deprivation or restriction alters energy balance 52

1.18.5 Lysine deprivation or restriction alters energy balance 53

1.18.6 Leucine deprivation or restriction alters energy balance 53

1.18.7 Isoleucine or valine restriction alters energy balance 54

1.18.8 Histidine restriction alters energy balance 54

1.18.9 Phenylalanine deprivation or restriction alters energy balance 55

1.18.10 Tryptophan deprivation or restriction alters energy balance 55

1.19 Aims of thesis - overview 55

2 Chapter 2– A long-acting glucagon analogue, G108, stimulates canonical glucagon signalling pathways causing chronic hypoaminoacidemia and lean mass loss. 57

2.1 Introduction 57
2.1.1 Early evidence that native glucagon enhances hepatic amino acid catabolism leading to reduced plasma amino acids 57
2.1.2 Mechanisms of hepatic GCGR-stimulated ureagenesis and hypoaminoacidemia 58
2.1.3 Evidence that native glucagon increases lean mass loss and potential mechanisms involved 59
2.1.4 Implications of using a long-acting GCGR agonist for metabolic disease, on plasma amino acids and lean mass 62
2.1.5 Implications of the pharmacodynamic and pharmacokinetic properties of GCGR-targeted agonists on hepatic amino acid catabolism and plasma amino acids 63
2.1.6 G108 as a research tool to explore the effects of sustained GCGR agonism on hepatic amino acid catabolism, plasma amino acids and lean mass 64

2.2 Chapter summary and aims 66

2.3 Methods 66
2.3.1 Peptide G108 66
2.3.2 Animal husbandry 66
2.3.3 Diets 67
2.3.4 Murine receptor potency experiments 67
2.3.5 Pharmakokinetic profile study 67
2.3.6 *In vivo* single dose administration studies 68
2.3.7 Chronic dose finding study 69
2.3.8 Chronic 5 nmol/kg G108 administration in lean mice 69
2.3.9 Chronic 5 nmol/kg G108 administration in hepatic specific glucagon knockout mice (*Gcgr*$^{\Delta liver}$) 70
2.3.10 Food intake, body weight and body composition (EchoMRI) 70
2.3.11 Glucose tolerance tests 70
2.3.12 Forelimb grip strength 71
2.3.13 Biochemical analysis of plasma 71
2.3.14 Quantitative polymerase chain reaction (qPCR) analysis. 71

2.4 Results 72
Key results for Chapter 2 72
2.4.1 G108 is a selective GCGR agonist *in vitro* 72
2.4.2 G108 has an extended pharmacokinetic profile in plasma 73
2.4.3 Single dose administration of G108 in lean and obese mice reduces total plasma amino acids. 74
2.4.4 Single dose administration of G108 leads to upregulation of hepatic amino acid catabolism and gluconeogenesis. 76
2.4.5 Single dose administration of G108 leads to acute dynamic changes in blood glucose. 78
2.4.6 G108-mediated hepatic amino acid catabolism and hypoaminoacidemia is dependent on the presence of the hepatic glucagon receptor. 79
2.4.7 Chronic dose finding with G108 reveals the sub-anorectic dose range 82
2.4.8 Chronic dose finding with G108 reveals dose-dependent effects on plasma amino acid levels, lean mass, and muscle strength 83
2.4.9 Chronic dose finding with G108 reveals bidirectional changes in glucose tolerance. 85
2.4.10 Chronic sub-anorectic administration of G108 leads to hypoaminoacidemia and lean mass loss 86
2.4.11 Chronic sub-anorectic administration of G108 leads to reduced muscle weight and function 90
2.4.12 Chronic sub-anorectic administration of G108 leads to improved glucose tolerance. 91
2.4.13 Loss of lean mass with chronic sub-anorectic administration of G108 is dependent on the presence of the hepatic glucagon receptor. 92

2.5 Discussion 94
2.5.1 G108 is a long-acting glucagon analogue which acutely enhances hepatic AA catabolism and reduces plasma amino acids 94
2.5.2 G108 causes a chronic reduction in plasma amino acids and lean mass 95
2.5.3 The hepatic GCGR is required for G108-stimulated hypoaminoacidemia and lean mass loss 97
2.5.4 Evidence of metabolic benefits with chronic G108 administration 97

2.6 Conclusions 98

3 Chapter 3 – A high protein diet protects lean mass in G108-treated obese mice and reveals whole-body effects of amino acid availability on energy expenditure and body weight loss. 100

3.1 Introduction 100
3.1.1 Hypoaminoacidemia and lean mass loss in response to a long-acting glucagon analogue may occur in both lean and obese rodents 100
3.1.2 Previous evidence for lean mass loss in obese rodents in response to glucagon receptor active analogues 100
3.1.3 Glucagon-stimulated hepatic amino acid catabolism resulting in hypoaminoacidemia is predicted to enhance lean mass loss in obese rodents 102
3.1.4 Using G108 and a high protein diet intervention to explore effects on glucagon-stimulated amino acid catabolism and lean mass loss 102

3.2 Chapter summary and aims 103

3.3 Methods 104
3.3.1 *In vivo* studies 104
3.3.2 Animal husbandry 104
3.3.3 Diets 104
3.3.4 Chronic DIO study 105
3.3.5 Food intake, body weight and body composition (EchoMRI) 105
3.3.6 Glucose and insulin tolerance tests 106
3.3.7 Metabolic cages 106
3.3.8 Biochemical assessments 107
3.3.9 Western blots 107
3.3.10 Histology 108
3.3.11 Quantitative polymerase chain reaction (qPCR) analysis 108
3.3.12 Forelimb grip strength tests 108

3.4 Results 109
3.4.1 Chronic daily G108 administration results in food-intake independent loss of body weight in diet-induced-obese mice 110
3.4.2 Chronic daily G108 administration results in lean and fat mass loss in obese mice, prevented with a high protein diet 112
3.4.3 Chronic daily G108 administration results in a decline in muscle weight and function, mitigated with a high protein diet 114
3.4.4 Chronic daily G108 administration in obese mice increases hepatic catabolism of amino acids resulting in suppressed plasma amino acids, rescued by a high protein diet 116
3.4.5 Chronic daily G108 administration suppresses plasma individual amino acids in obese mice which are rescued by a high protein diet 118
3.4.6 G108-stimulated hypoaminoacidemia in obese mice is associated with markers of proteolysis in skeletal muscle, mitigated with a high protein diet. 123
3.4.7 G108-stimulated hypoaminoacidemia is associated with increased hepatic expression and circulating levels of fibroblast growth factor 21 (FGF21) 126
3.4.8 The effect of 7.5 nmol/kg G108 and a high protein diet on whole-body energy expenditure .. 126
3.4.9 Chronic G108 administration leads to improved glucose tolerance independent to dietary protein content 131
3.4.10 Chronic G108 administration leads to a reduction in hepatic lipid accumulation independent to dietary protein content 133
3.4.11 Chronic G108 administration activates a multi-organ energy consuming response associated with hypoaminoacidemia 134

3.5 Discussion 140
3.5.1 G108 stimulates chronic hepatic amino acid catabolism leading to low plasma amino acids and lean mass loss in DIO mice 140
3.5.2 A high protein diet rescues G108-stimulated hypoaminoacidemia and limits lean mass loss .. 143

3.5.3 The effect of a high protein diet on molecular markers of muscle protein synthesis and proteolysis. ... 145
3.5.4 G108 leads to hepatic FGF21 induction and fat mass loss ... 147
3.5.5 Plasma amino acid availability; Implications for understanding glucagon-stimulated EE ... 149
3.5.6 Mechanisms of EE in response to G108-stimulated hypoaminoacidemia ... 151
3.5.7 G108 increases protein turnover ... 151
3.5.8 G108 increases markers of physical activity ... 152
3.5.9 G108 increases circulating FGF21 and thermogenic markers ... 154
3.5.10 Other possible amino acid centric mechanisms of G108-mediated EE ... 156
3.5.11 Effects on glucose tolerance and hepatic fat ... 156

3.6 Conclusions ... 157

4 Chapter 4 – Hypoaminoacidemia underpins chronic G108-mediated energy expenditure and body weight loss ... 159

4.1 Introduction ... 159
4.1.1 Dietary protein supplementation prevents G108-mediated EE and total body weight loss; Similarities to dietary protein or amino acid restriction paradigms ... 159
4.1.2 Hepatic amino acid sensing and FGF21 induction in response to dietary protein or amino acid restriction ... 159
4.1.3 Mechanisms of EE in response to dietary protein or amino acid restriction ... 160
4.1.4 Using custom amino acid-based diets to determine whether G108-stimulated hypoaminoacidemia underpins glucagon-mediated EE and body weight loss ... 163

4.2 Chapter summary and aims ... 164

4.3 Methods ... 164
4.3.1 Animal husbandry ... 164
4.3.2 Diets ... 164
4.3.3 Acute metabolic cage study ... 167
4.3.4 Chronic G108 administration in DIO mice with total individual amino acid dietary 'add-back' 169
4.3.5 Chronic G108 administration in DIO mice with either EAA or NEAA 'add-back' ... 171
4.3.6 Custom low amino acid profile diet study in obese mice ... 173
4.3.7 Dietary restriction of amino acids in hepatic specific *Fgf21* (*Fgf21*$^{\Delta liver}$) knockout mice ... 175
4.3.8 Food intake, body weight and body composition (EchoMRI) ... 175
4.3.9 Metabolic cages ... 175
4.3.10 Glucose time-course profile ... 175
4.3.11 Biochemical analysis of plasma ... 176

4.4 Results ... 177
4.4.1 Acute metabolic cage study ... 177
4.4.2 Chronic G108 administration in DIO mice with dietary total amino acid 'add-back' ... 182
4.4.3 Chronic G108 administration in DIO mice with dietary 'add-back' of either EAA or NEAA ... 186
4.4.4 Custom low amino acid profile diet study in obese mice ... 189
4.4.5 Increased circulating FGF21 is not required to enhance EE in response to a custom low amino acid diet 194

4.5 Discussion ... 196
4.5.1 G108 enhances EE acutely and is prevented by a high protein diet ... 196
4.5.2 Hypoaminoacidemia underpins chronic G108-mediated EE and total body weight loss ... 197
4.5.3 Replacing EAA or NEAA separately does not prevent EE effect ... 199
4.5.4 The EE response to a rationally designed low amino acid diet suggests an FGF-21-independent effect. 200

4.6 Conclusions ... 202

5 Chapter 5– A novel long-acting GLP-1/glucagon co-agonist (G3215) results in markers of increased protein turnover in humans in a Phase 1 trial ... 204

5.1 Introduction 204
5.1.1 GCGR-targeted multi-agonists as a treatment for obesity and metabolic disease in humans .. 204
5.1.2 Emerging pre-clinical evidence for amino acid catabolism and lean mass loss in GCGR-targeted multi-agonists 207
5.1.3 Evidence for hypoaminoacidemia in humans treated with GCGR-targeted agonists 208
5.1.4 Infusion of a novel GLP1/GCGR co-agonist, G3215, in humans to determine the effect on plasma amino acids and lean mass 209

5.2 Chapter summary and aims 210

5.3 Methods 210
5.3.1 Study design and participants 210
5.3.2 Peptide G2315 211
5.3.3 Inclusion and exclusion criteria 212
5.3.4 Intervention 212
5.3.5 Randomisation process and IMP preparation 215
5.3.6 Subcutaneous pump and dose-adjustments 215
5.3.7 Body weight and body composition 215
5.3.8 Grip strength test 215
5.3.9 Oral glucose tolerance test 216
5.3.10 Food intake studies 216
5.3.11 Safety blood tests, ECG, vital signs, and visual analogue scales 216
5.3.12 Physical examination 217
5.3.13 PK and Biochemical analysis 217

5.4 Results 218
5.4.1 G3215 infusion reduces plasma alanine over 14 days 218
5.4.2 G3215 infusion reduces individual plasma amino acids over 14 days 219
5.4.3 Effects of G3215 infusion on body composition and functional strength 226

5.5 Discussion 230
5.5.1 Infusion of a novel GLP1/GCGR co-agonist, G3215, over 14-days in humans causes a broad reduction in plasma amino acids 230
5.5.2 A 14-day infusion of G3215 is associated with changes in body composition including lean mass loss 235
5.5.3 A 14-day infusion of G3215 is associated with positive changes in functional strength tests ... 237

5.6 Conclusions 237

6 Chapter 6– Future treatment strategies to mitigate against glucagon-stimulated hypoaminoacidemia and lean mass loss 239

6.1 Introduction 239
6.1.1 A high protein diet prevents lean mass loss *in vivo* during chronic G108 administration 239
6.1.2 Alternative strategies to prevent lean mass loss during chronic glucagon analogue administration - Timing of glucagon administration 240
6.1.3 Alternative strategies to prevent lean mass loss during chronic glucagon analogue administration - Partial agonism of a GLP1R/GCGR co-agonist 240
6.1.4 Alternative strategies to prevent lean mass loss during chronic glucagon analogue administration - Increasing dietary protein availability to prevent lean mass loss during chronic glucagon analogue administration 241
6.1.5 Investigating drug duration of GCG analogues and biased agonism in a GLP1/GCGR co-agonist on plasma amino acids and metabolic outcomes 242

6.2 Chapter summary and aims 243

6.3 Methods 243
6.3.1 Peptide selection 243
6.3.2 Acute dose finding study of SA GCG (G395) in DIO mice 244
6.3.3 Chronic daily dosing study of SA GCG (G395) vs LA GCG (G108) in DIO mice 244

6.3.4 Biased-partial agonism by a GLP1/GCGR co-agonist at both the GLP1R and GCGR to optimise metabolic benefits whilst minimising amino acid catabolism 245

6.4 Results 245

6.4.1 Acute dose-finding study of the SA GCG analogue G395 246

6.4.2 Chronic daily dosing of the short acting glucagon analogue G395 does not cause plasma amino acid suppression or lean mass loss 248

6.4.3 Chronic daily dosing of the short-acting glucagon analogue G395 confers similar metabolic benefits to the long-acting glucagon analogue G108 251

6.4.4 Biased-partial agonism by a GLP1/GCGR co-agonist at both the GLP1R and GCGR to optimise metabolic benefits whilst minimising amino acid catabolism 254

6.5 Discussion 256

6.5.1 The short-acting GCG analogue, G395, does not stimulate hypoaminoacidemia or lean mass loss 256

6.5.2 The short-acting GCG analogue, G395, provides metabolic benefits also attributed to the long-acting GCG analogue G108 257

6.5.3 Biased-partial agonism by a GLP1/GCGR to optimise metabolic benefits whilst minimising amino acid catabolism 258

6.6 Conclusions 259

7 Chapter 7 – General discussions 261

7.1 100 years of glucagon research highlight the diverse actions of this intriguing hormone 261

7.2 The actions of G108, a long-acting GCG analogue, are consistent with the key amino acid catabolic action of glucagon 262

7.3 Implications of G108-stimulated chronic hypoaminoacidemia on lean mass and a preventative strategy 265

7.4 Implications of G108-stimulated chronic hypoaminoacidemia on EE and total body weight loss in a pre-clinical model of obesity 268

7.5 Implications of G108-stimulated chronic hypoaminoacidemia for the use of GCGR-targeted multi-agonists in humans? 271

7.6 Future directions to harness the metabolic benefits of chronic glucagon therapy whilst mitigating potential side effects of excess amino acid catabolism 274

7.7 Conclusions 277

8 REFERENCES 279

1 Chapter 1 – General Introduction - Glucagon in an obesity strategy and implications of glucagon-stimulated protein metabolism

1.1 Obesity; a global epidemic and an emerging treatment gap

We are currently facing a global epidemic of overweight and obesity. More than half of European adults were classified as overweight or obese in 2016 (World Health Organisation). Globally, in 2016 1.9 billion adults globally were classified as overweight with 650 million adults classified as obese (World Health Organisation). The co-morbidities associated with obesity represent major burdens to the healthcare system and economy and include type 2 diabetes, ischaemic heart disease, hypertension and many types of cancer (Guh, Zhang et al. 2009). The Covid-19 pandemic has also highlighted the link between obesity and illness severity (Gao, Piernas et al. 2021). The burden of overweight and obesity go beyond health impacts and are also reflected in the economic costs to society, estimated at 27 billion pounds per year in the United Kingdom (England 2017).

Both genetic and environmental factors play a role in the development of obesity with genetic factors accounting for up to 50-90% of variance of BMI in twin studies (Maes, Neale et al. 1997). Numerous environmental factors are implicated including increased availability to energy dense food (McAllister, Dhurandhar et al. 2009), reduced physical activity (Cassidy, Chau et al. 2017), chronic stress (Jackson, Kirschbaum et al. 2017), disturbed sleep (Cassidy, Chau et al. 2017), and drug side effects (Ness-Abramof and Apovian 2005). The rapid change in our 'built environment' exposing humans to many of these environmental factors explains the rapid increase in the prevalence of obesity over the last 50 years (Lam, Vaartjes et al. 2021).

Due to the rapid increase in the prevalence of obesity, associated with both health and economic burdens, increased demand for anti-obesity strategies subsequently developed. The gold-standard weight loss strategy which has proved itself since inception in the 1960's, is bariatric surgery. Recent meta-analysis has found that long-term outcomes after gastric bypass surgery show a mean weight loss of 56.7% (O'Brien, Hindle et al. 2019). However, despite this success, surgical options for obesity are not suitable for everyone due to either availability, preference, or side effects. Furthermore,

it is not conceivable for surgery to meet the current demand driven by the obesity epidemic globally. Despite the need for a highly efficacious anti-obesity medicine (AOM), a 'treatment gap' has however emerged between ineffective lifestyle interventions and these invasive surgical procedures (Hope, Tan et al. 2018). Over the last two decades there has been an increasing effort to develop pharmacotherapeutics to fill this 'treatment gap' with newer drugs continuously raising the bar in terms of weight loss achieved. Many of these newer treatments are based on one or more gut hormones, known to regulate energy balance. For example, at higher doses, injectable GLP-1 analogues are licensed and routinely used to treat obesity and include Liraglutide 3 mg daily (Saxenda) (Pi-Sunyer, Astrup et al. 2015) and (recently approved in the US) Semaglutide 2.4 mg weekly (Wegovy) (Wilding, Batterham et al. 2021). Over the time-course of these trials, these result in more impressive 8 % and 14.9 % body weight loss, respectively. Demonstrating the rapidly moving field and push for these greater degrees of body weight loss, the novel GLP-1/GIP combined analogue Tirzepetide showed an impressive, combined effect on glycaemia (-2.37 % HbA1C) and body weight loss (12.4 kg) in a recent phase 3 SURPASS trial (Ludvik, Giorgino et al. 2021). More recently, in the SURMOUNT-1 phase 3 trial, Tirzepetide showed an impressive average 16% (16 kg) body weight loss over 72 weeks in participants with overweight or obesity (Jastreboff, Aronne et al. 2022); Tirzepetide is currently filed for regulatory approval in the US.

To understand the challenges of previous/historic obesity pharmacotherapeutics and the role of current and novel research pharmacotherapeutics, the regulation of body weight and mechanisms of energy balance will be discussed. It is by understanding these mechanisms that the rationale design of AOM's has allowed the progression of novel treatments through to clinical practice.

1.2 Body weight homeostasis in an obesogenic environment

Body weight is controlled within a narrow margin, and this prevents diseases associated with under- or over-nutrition. Central signalling pathways have evolved to maintain energy balance and potently protect against times or famine or starvation. The regulation of these pathways is underpinned by the human genotype and in particular gene products of central signalling hubs of the brain. Indeed, many studies have demonstrated that single-nucleotide-polymorphisms (SNP) in the brain contribute towards polygenic

obesity (Loos and Yeo 2022). However, in our current society the readily available calorie dense foods coupled with increased sedentary behaviour, have meant a thrifty genotype predisposes to increased weight gain. To complicate matters, the body defends against loss of weight to restore its previous energy balance. For example, decreased expression of anorexigenic peptides in the hypothalamus occurs in response to weight loss, reducing the threshold for weight re-gain (Yu, South et al. 2008). Furthermore, long-term signals of adiposity, in particular leptin, exhibit resistance in obesity resulting in a dampened effect on satiety (Scarpace and Zhang 2009).

1.3 Central regulation of energy balance

The central homeostatic control of feeding and energy balance comprises of several signalling pathways located mainly in the hypothalamus and the brainstem. The two major hubs implicated in this highly regulated homeostatic machinery is the hypothalamic arcuate nucleus (ARC) in addition to the nucleus tractus solitarius (NTS) and area postrema (AP) in the brainstem (Figure 1.1). Once activated, these highly integrated areas of the brain communicate with each other through GABAergic signalling, resulting in either increased or suppression of hunger. A plethora of hormones released from the gut, pancreas, liver and adipose tissue signal directly to the ARC or AP via the circulation or via vagal afferents to the NTS. These hormones respond both in the short term to food intake or hunger, or to the longer-term degree of adiposity in the body. Short term gut hormones include for example Ghrelin, GLP-1, peptide tyrosine tyrosine (PYY), glucose-dependent insulinotropic polypeptide (GIP) and oxyntomodulin (OXM). Longer term signals of adipose tissue storage include leptin, insulin and amylin. In addition to these homeostatic control signals in the hypothalamus and brainstem, hedonic centres including the mesolimbic areas provide a strong drive to consume highly palatable food.

Circulating hormones which signal directly to the hypothalamus or activated pathways from the NTS and AP brainstem regions, converge on a key neuronal signalling pathways within the hypothalamic ARC (Figure 1.1). These pathways comprise of the orexigenic and anorexigenic neurons. Orexigenic neurons express neuropeptide Y (NPY) and agouti-related peptide (AgRP), whilst anorexigenic neurons express pro-opiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART). Alpha-melanocyte stimulating hormone is released from POMC/CART neurons and activate MCR4 receptor,

leading to suppression of food intake. In contrast, AgRP released by the orexigenic NPY/AgRP neurons leads to inhibition of MCR4 and stimulation of food intake.

Intestinal derived hormones which regulate food intake include GLP-1, PYY, CCK, Ghrelin and GIP. GLP-1, secreted from the intestinal L-cells, suppresses food intake by activating a number of brain regions including vagal afferents, the anorexigenic POMC/CART neurons in the ARC, the NTS and AP in the brainstem, and dopaminergic reward centres (Chen, Chen et al. 2021). The multi-faceted effects on satiety pathways may be a reason to why GLP-1 has emerged as the current forerunner anti-obesity medication. PYY, in its activated form of PYY_{3-36} is also secreted from the intestinal L-cells and reduces food intake by binding to Y2 receptors and inhibiting the orexigenic NPY/AgRP neurons (Batterham, Cowley et al. 2002). PYY also regulates food intake by signalling via vagal afferents to the brainstem NTS (Koda, Date et al. 2005). CCK is secreted from intestinal I-cells and by binding to CCK1 receptors in vagal afferents, is able to enhance the vagal-brainstem-hypothalamic NTS pathway to suppress food intake (Raybould 2007). Ghrelin is secreted from enteroendocrine cells of the stomach and enhances food intake by signalling directly from the circulation to activate the orexigenic NPY/AgRP neurons at the hypothalamic ARC (Chen, Trumbauer et al. 2004). Glucose-dependent insulinotropic polypeptide (GIP), is released from the intestinal K-cell. The role of GIP in the control of food intake is not fully understood, however it appears to signal directly to the hypothalamic ARC to suppress food intake as shown in acute and chronic studies *in vivo* (Zhang, Delessa et al. 2021). The pancreatic signals, insulin, amylin and glucagon are also able to regulate food intake via central mechanisms. Insulin, secreted from the pancreatic beta-cell in response to nutrient ingestion, inhibits the orexigenic NPY/AgRP neurons in the ARC to suppress food intake (Dodd, Kim et al. 2021). Amylin, which is co-secreted with insulin, suppresses food intake by signalling through the circulation to the AP and ARC (Lutz, Coester et al. 2018). Glucagon, secreted from the pancreatic alpha-cell in response to fasting suppresses food intake through vagal afferent signalling to the NTS (Weatherford and Ritter 1988). Leptin secreted from adipose tissue informs the brain of the long-term degree of adiposity. It signals via the leptin receptor (LepRb) in the ARC to both activate the anorexigenic POMC/CART neurons and inhibit the orexigenic NPY/AgRP neurons (Elias, Aschkenasi et al. 1999).

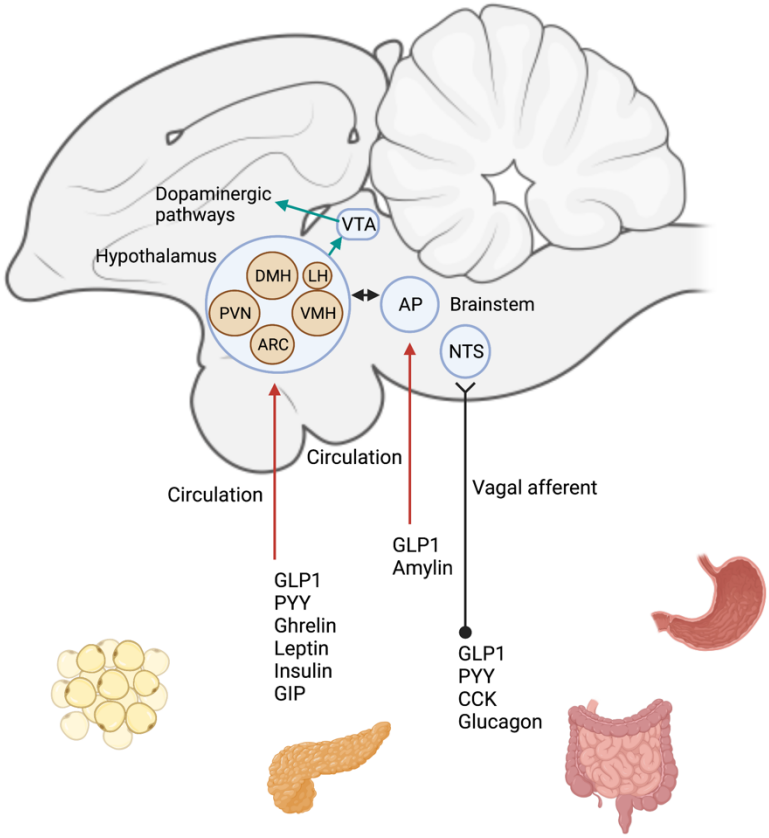


Figure 1.1: Central control of food intake through integration of peripheral signals to the brainstem and hypothalamus. Peripheral hormones derived from the adipose tissue, pancreas, intestines and stomach signal via the circulation to the Arcuate nucleus (ARC) and Area postrema (AP). Peripherally secreted hormones also activate the vagal afferent nerve signalling directly to the Nucleus Tractus Solitarius (NTS). Bidirectional crosstalk allows integrated signalling between these central hubs regulating food intake through activation or inhibition of key orexigenic and anorexigenic neurons in the ARC. Further regulation of hedonic food intake occurs through activation of dopaminergic reward pathways. PVN; Paraventricular nucleus, DMH; Dorsomedial hypothalamus, LH; Lateral hypothalamus, VMH; Ventromedial hypothalamus, VTA; Ventral tegmental area. Image created using Biorender.

1.4 The need for a safe and effective anti-obesity medication (AOM)

Due to the prevalence of obesity in the UK and globally, there is a demand for effective and safe anti-obesity medications (AOM's) which can target specific central pathways of energy balance. However, achieving this has been challenging due to safety and tolerability of centrally acting drugs (See section 1.6). Furthermore, to achieve weight loss effects similar to bariatric surgery and avoid side effects, it is likely that multiple pathways need to be targeted using a pharmacotherapeutic strategy; This approach is not dissimilar to other chronic diseases such as hypertension or type-2 diabetes, where multiple pathways are targeted with polypharmacy. Ultimately, the benchmark for a safe

and effective AOM will be long term outcomes of metabolic disease, quality of life and mortality. Indeed, long term studies following bariatric surgery are emerging showing improved outcomes including long-term mortality (Courcoulas, King et al. 2018, Carlsson, Sjoholm et al. 2020).

1.5 Previous challenges and current obesity pharmacotherapeutics

The history of anti-obesity medications (AOM's) is marked by long-term failures due to significant side effects profiles. One of the first class of AOM's to be used was the sympathomimetics, introduced in the 1950's mainly in the United States (US). A number of these are still approved for treatment (Muller, Bluher et al. 2022) despite no long-term outcome data and troublesome side effects of dry mouth and nausea. Several other drug classes have been tried including mitochondrial uncouplers, inverse agonists of the cannabinoid receptor, serotonergic agonists and monoamine reuptake inhibitors (Muller, Bluher et al. 2022). However, notable failures highlight the unexpected and severe side effect profiles which precluded the long-term use of many of these classes of drugs. For example, Fenfluramine, a serotonergic activator was approved by the US Food and Drug Administration (FDA) in 1996 however withdrawn due to studies demonstrating a higher rate of valvular heart disease and pulmonary hypertension (Connolly, Crary et al. 1997). Lorcaserin, a serotonergic agonist marketed under the brand name Belviq, was licensed for use in the US between 2012 and 2020. However, due to increased rates of cancer in patients treated with the drug, Lorcaserin was subsequently withdrawn by the FDA (Sharretts, Galescu et al. 2020). Silbutramine, a serotonin-norepinephrine re-uptake inhibitor was licensed for use in the US and Europe between 1997 and 2010. However, due to an increased risk of non-fatal myocardial infarction and non-fatal stroke in a large prospective study (SCOUT trial), it was subsequently withdrawn (James, Caterson et al. 2010). In addition to cardiovascular safety, previously used AOM's have also been linked with psychological harm. For example, Rimonabant, an inverse agonist to the endocannabinoid receptor, was approved for use in Europe between 2006 and 2009. However, due to increased risk of anxiety, depression and suicide in the CRESENDO study, the European Medicines Agency (EMA) withdrew the license for Rimonabant in 2008 (King 2010).

The current selection of AOM's approved for use therefore remain limited. In the UK these mainly include the pancreatic lipase inhibitor Orlistat and GLP-1 based treatments, including liraglutide 3mg (Saxenda). Naltrexone/Bupoprion (Mysimba), a combined opioid receptor antagonist/dopamine and noradrenaline reuptake inhibitor is currently licensed for use in the UK, however, is not commonly prescribed or approved for use within the NHS by NICE. In the US, a wider selection of AOM's is available including various sympathomimetics, phentermine/topiramate, naltrexone/bupropion, and more recently one-weekly Semaglutide 2.4mg (Wegovy).

GLP-1 treatment has led the way in reaching higher degrees of weight loss for a safe and effective AOM. Its discovery as an incretin in humans and key central regulator of food intake *in vivo* occurred in the current department at Imperial College London (Kreymann, Williams et al. 1987, Turton, O'Shea et al. 1996). Further studies confirmed its role in regulating food intake in humans (Gutzwiller, Goke et al. 1999) and was subsequently developed and progressed through clinical trials over the next decade for the treatment of type 2 diabetes and obesity. GLP-1 therapy benefits from having predictable gastrointestinal side effects of nausea and vomiting, based on its known mechanism of action. Liraglutide 3mg (Saxenda) was the first GLP-1-based dedicated anti-obesity treatment to be approved by the FDA in 2014, and subsequently EMA. In a randomised control trial, Saxenda was shown to result in approximately 8% body weight loss over placebo (Pi-Sunyer, Astrup et al. 2015). Prior to the approval of Saxenda, Liraglutide 1.8mg (Ozempic), licensed for use in type-2 diabetes was often used for its weight loss properties in overweight patients with dysglycaemia. In addition to weight loss properties, the beneficial cardiovascular outcomes associated with Liraglutide 1.8mg as shown in the LEADER trial (Marso, Daniels et al. 2016) have promoted swift development of more potent GLP-1 based therapies. Recently, once weekly Semaglutide 2.4mg has been shown to achieve 14.9% total body weight loss over 68 weeks, compared to placebo-controlled body weight loss of 2.4% in adults with overweight or obesity (Wilding, Batterham et al. 2021). Furthermore, a third of patients who received Semaglutide achieved an impressive 20% total body weight loss. Semaglutide 2.4mg (Wegovy) has been recently approved for use in the US and Europe and is awaiting introduction for use as an AOM later this year in the UK.

1.6 Novel and combination AOM's in various research stages

Whilst the success of GLP-1 mono-agonist therapies has emerged, in the background there has been a drive to develop gut-hormone-based multi-agonists which target more than one pathway implicated in energy balance. This strategy remains promising due to the potential for synergistic action, therefore reducing the side effect profile and by targeting multiple pathways may maximise weight loss efficacy. A well-known example of this combination strategy is the development of the GLP-1R/GCGR co-agonist, designed to target food intake with GLP-1 action and energy expenditure with glucagon action. The combination approach was first demonstrated as a feasible strategy in human clinical studies where co-infusions of glucagon and GLP-1 led to enhanced energy expenditure and reduced food intake (Tan, Field et al. 2013, Cegla, Troke et al. 2014). This strategy also benefited by being glucose neutral, due to the balanced effects of GLP-1 and glucagon. In a seminal study, Day *et al* developed a unimolecular GLP-1R/GCGR co-agonist which demonstrated superior weight loss effects compared to GLP-1 alone in obese rodents (Day, Ottaway et al. 2009, Day, Gelfanov et al. 2012). This seminal work led to development of several GLP-1R/GCGR co-agonists with a number of successes in pre-clinical trials (Hope, Vincent et al. 2021). However, of these, MEDI0832 (Cotadutide) was the first to progress well through clinical trials. Despite this, in humans Cotadutide appears to have modest weight loss effects and more prominent effects on hepatic lipid metabolism (Nahra, Wang et al. 2021); It is therefore being targeted towards individuals with obesity and NAFLD in later phase clinical trials. Although Cotadutide has progressed well in clinical trials, other have not had such success. A recent report of a Novo Nordisk once weekly administered compound 'NN1177' in Phase 1 highlighted significant tolerability and safety issues, including cardiovascular concerns (PRE PRINT: DIO 10.1101/2022.06.02.22275920). It therefore appears that a careful balance of receptor potency is required to obtain efficacy whilst minimising side effects attributed to either the GLP-1 or GCG pathways (Hope, Vincent et al. 2021). A selection of GLP-1R/GCGR co-agonists in clinical development is listed in Table 1.1.

Several other therapies targeting multiple hormone receptors are in various stages of drug development programmes. These include GLP-1R/GIPR co-agonists, GLP-1R/GIPR/GCGR tri-agonists, Y2R agonists, amylin analogues and GDF analogues (Table 1.1). Others drug classes in pre-clinical development include mitochondrial uncouplers

and compounds which target the ghrelin pathway (Muller, Bluher et al. 2022). Despite the variety of drug classes and hormone combinations, many of these are in early phase clinical trials. However, particular progress has been made with the development of the GLP-1R/GIPR co-agonist, Tirzepetide, achieving an average 15% weight loss in obese adults with once weekly administration over 72 weeks (Jastreboff, Aronne et al. 2022). Furthermore, recently, translational studies have reported promising pre-clinical and phase 1 single dosing studies of GLP-1R/GIPR/GCGR triple agonists (Bossart, Wagner et al. 2021, Coskun, Urva et al. 2022). Therefore, the research and clinical community will await results of whether one or more of these drug candidates will prove safe and efficacious for the primary aim of treating obesity. Furthermore, it remains to be seen whether these novel drug candidates will rival or surpass the current bar set by bariatric surgery in terms of total body weight loss and metabolic outcomes.

Drug Name	**Drug Class**	**Developer**	**Status**	**Reference**
G3215	GLP-1R/GCGR co-agonist	Imperial College London	Phase 1	NCT02692040
NN1177	GLP-1R/GCGR co-agonist	NovoNordisk	Phase 1 discontinued	Pre-print https://doi.org/10.1101/2022.06.02.22275920
MEDI0832/Cotadutide	GLP-1R/GCGR co-agonist	Astrazeneca/Medimmune	Phase 2b	(Nahra, Wang et al. 2021)
BI456906	GLP-1R/GCGR co-agonist	Boehringer Ingelheim	Phase 2	NCT04153929 NCT04771273
Efinopegdutide	GLP-1R/GCGR co-agonist	Hamni Pharmaceutical	Phase 2 (Fatty liver disease)	NCT03486392
ALT-801/Pemvidutide	GLP-1R/GCGR co-agonist	Altimmune	Phase 2	NCT05295875
IBI362 LY3305677	GLP-1R/GCGR co-agonist	Eli Lilly	Phase 2	NCT04904913 (Ji, Gao et al. 2022)

Tirzepetide (SURMOUNT1)	GLP-1R/GIPR co-agonist	Eli Lilly	Phase 3	NCT04184622 (Jastreboff, Aronne et al. 2022)
HM15211	GCGR/GIPR /GLP-1R tri-agonist	Hamni Pharmaceutical	Phase 2	NCT04505436
GCG tri-agonist SAR441255	GCGR/GIPR /GLP-1R tri-agonist	Sanofi	Phase 1	NCT04521738 (Bossart, Wagner et al. 2021)
GCG tri-agonist LY3437943	GCGR/GIPR /GLP-1R tri-agonist	Eli Lilly	Phase 2	NCT04881760 (Coskun, Urva et al. 2022)
Efpeglenatide	Long acting GLP-1R agonist	Hamni Pharmaceutical	Phase 2	NCT02075281 (Pratley, Jacob et al. 2022)
Rybelsus (oral Semaglutide)	Long acting GLP-1R agonist	NovoNordisk	Phase 3	NCT05035095
HM15136	Glucagon analogue	Hamni Pharmaceutical	Phase 1	NCT04167553
GLPR-NPA	Long acting GLP-1R biased agonist	Eli Lilly	Phase 2	NCT05051579
NNC0165 ±-Semaglutide	PYY analogue	NovoNordisk	Phase 1	NCT03707990
NNC0165	PYY analogue	NovoNordisk	Phase 1	NCT02568306
NN9838/Cagrilintide	Amylin analogue	NovoNordisk	Phase 2	NCT03856047
LY3463251	GDF15 analogue	Eli Lilly	Phase 1	NCT03764774
GDF15	GDF15 analogue	NovoNordisk	Phase 1	NCT04010786

Table 1.1: Drugs currently in clinical trial phases for the treatment of overweight and obesity.

1.7 Glucagon as an anti-obesity agent - overview

Several important technological breakthroughs over the last 60 years have paved the way for a greater understanding of glucagon as a protein and its biology. A brief mention of these also help put important research efforts in the context of the technological advantages on which they are founded. A major technological breakthrough was the use of the radio-immunoassay procedure (RIA) by Roger Unger in 1959, to detect glucagon using glucagon antibodies (Unger, Eisentraut et al. 1959). Roger Unger had worked closely with the laboratory of Solomen Berson who initially developed this technique for

the detection of insulin (Yalow and Berson 1960). The technique led to experiments benefiting from the direct detection of glucagon itself rather than relying on the pharmacodynamic effects (ie. Hyperglycaemia or hepatic glycogenolytic activity) as measured outcomes. Furthermore, RIA opened possibilities for the entire research field with the ability to detect a wide variety of proteins present in very small concentrations. Other important technological breakthroughs include the discovery of somatostatin and its inhibitory role on pancreatic endocrine function (Koerker, Ruch et al. 1974). This allowed dissection of the physiological roles of insulin and glucagon by suppressing endogenous endocrine function whilst administering either hormone. Another important development was the generation of glucagon receptor knockout mice by Gelling and colleagues in 2003 allowing a mechanistic approach to studying glucagon biology (Gelling, Du et al. 2003).

1.8 Glucagon – discovery of the protein

Glucagon, a 29-amino acid pancreatic hormone is established as a key protein which plays a role in glucose homeostasis with its serendipitous discovery dating back to studies carried out in the early 20th Century. Following the observation that pancreatectomised dogs develop hyperglycaemia and administration of pancreatic extract reversed this, work intensified to isolate the substance implicated (von Mering J 1890, Murlin JR 1923). This resulted in the isolation of insulin which was subsequently used for the first time to treat a patient with type 1 diabetes (Banting, Best et al. 1922). In some of these studies to isolate pure pancreatic extracts, protein precipitation experiments were carried out by Dr Charles Kimball and Dr John Murlin at the University of Rochester. In their seminal study, in addition to various alcohol precipitation reactions, they demonstrated that with acetone precipitation, a substance could be isolated which had a 'distinctly hyperglycemic effect' (Kimball C 1923); They coined this substance 'glucagon' after its primary observed effect of being a **gluc**ose **agon**ist. These foundational observations were noted in early December 1922, and this centenary of this discovery has recently been marked at the well-attended Glucagon Meeting 2022 (Danish Diabetes Academy, Novo Nordic Foundation).

Further seminal studies in the 1940's demonstrated that glucagon stimulated glycogenolysis in *ex vivo* liver slices and was produced within the pancreatic islet independent to the presence of beta cells (Sutherland and De Duve 1948). Through efforts of the Lilly research laboratory, the crystal structure of glucagon was determined which allowed later successful determination of the protein sequence in 1957 (Staub, Sinn et al. 1953, Bromer, Sinn et al. 1957). The next major breakthrough in the glucagon research was the sequencing of the primary protein by Bell and colleagues. The sequence of mammalian (hamster) preproglucagon was successfully characterised, identifying the glucagon sequence in addition to two 'glucagon-related polypeptides', named GLP-1 and GLP-2 (Bell, Santerre et al. 1983). Not long before this important finding, the anglerfish variant of preproglucagon had been identified, facilitated by the advantageous separation of the exocrine and endocrine parenchyma (Lund, Goodman et al. 1983). The full sequence of mammalian preproglucagon identified in 1983 by Bell and colleagues was consistent with previous research efforts utilising RIA and 'glucagon-like-reactivity (GLI)' to identify the protein sequences for parts of the precursor protein in gut extracts, including Glicentin, Glicentin-related-polypeptide (GRPP) and glucagon with an 8-amino acid c-terminal extension (later to be called oxyntomodulin) (Sundby, Jacobsen et al. 1976, J.J 1980, Moody, Holst et al. 1981, Bataille, Tatemoto et al. 1982). These earlier efforts were fuelled by prior observations that glucagon-like reactivity was detected in response to an oral glucose load (Unger, Ohneda et al. 1968). Research in the early 1980's also revealed evidence for post-translational processing of preproglucagon to glucagon, glicentin and GRPP in the porcine pancreas (Moody, Holst et al. 1981). The processing of preproglucagon was later determined to be tissue specific with major divergence between pancreatic and gut tissues and the preproglucagon protein products are now well established (Figure 1.2); Preproglucagon processing in the pancreas is mediated by the prohormone convertase 2 (PC2) and in the gut by prohormone convertase 1/3 (PC1/3) (Holst, Bersani et al. 1994, Ugleholdt, Zhu et al. 2004).

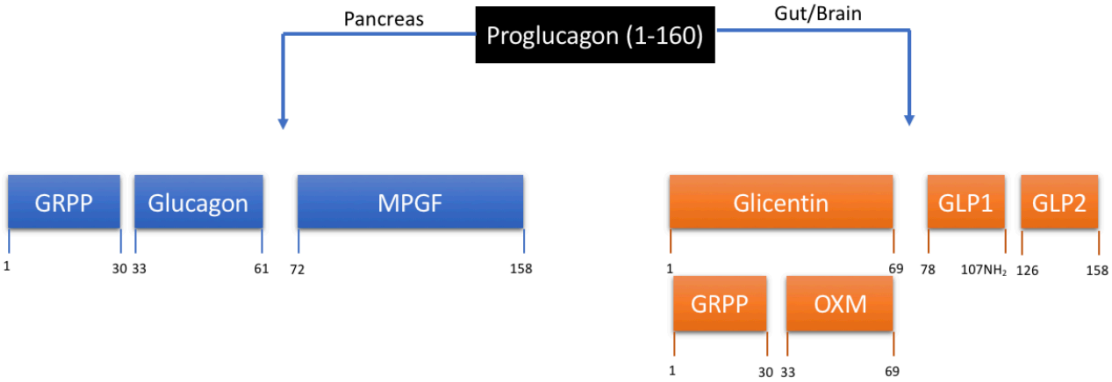


Figure 1.2: Proglucagon derives peptides. From Striking the balance: GLP-1/glucagon co-agonism as a treatment strategy for obesity. 2021. Frontiers in Endocrinology (Hope, Vincent et al. 2021).

1.9 Glucagon secretion

The cellular mechanisms of glucagon secretion are complex and governed by both intra- and extra-islet factors (Gaisano, Macdonald et al. 2012). As with the beta-cell, alpha-cells contain secretory granules under the control of voltage-dependent calcium channels (VDCC's). The main stimulus to glucagon secretion in the alpha cell is directly through plasma glucose availability. In contrast to the beta-cell, in response to low glucose the potassium ATP channel activity is low in the alpha-cell, paradoxically due to an elevated ATP:ADP ratio. Therefore, the alpha cell membrane is partially depolarised and action potential firing through sodium channels leading to calcium influx via the VDCC and exocytosis of glucagon-containing granules (Zhang, Ramracheya et al. 2013). In response to hyperglycaemia, the ATP:ADP ratio is elevated further and results in full inhibition of the potassium ATP channel and further membrane depolarisartion. In this case, enhanced membrane depolarisation leads to inactivation of the sodium channels and action potential firing. Therefore, VDCC's are inactivated with no calcium influx and lack of glucagon-granule exocytosis (Zhang, Ramracheya et al. 2013). In contrast, in response to low glucose, the beta-cell membrane is hyperpolarised and in response to hyperglycaemia, an increased ATP:ADP ratio leads to membrane depolarisation and calcium-dependent (via the VDCC) insulin-granule exocytosis (Gaisano, Macdonald et al. 2012). Therefore, full membrane depolarisation in response to hyperglycaemia appears to switch off voltage dependent ion channels in the alpha-cell membrane, which are regulated in the opposite manner in the beta-cell under the same conditions (Ramracheya, Ward et al. 2010). In addition to glucose, the alpha cell can directly respond to plasma nutrient availability. For example, amino acids are potent regulators of

glucagon secretion (Unger, Ohneda et al. 1969), whereas free fatty acids inhibit glucagon secretion (Gerich, Langlois et al. 1974). Arginine, alanine, glutamine and glycine are examples of amino acids which may potently stimulate glucagon secretion (Capozzi, D'Alessio et al. 2022).

In addition to the direct action of nutrients, the regulation of alpha-cell function is complicated by intra-islet (paracrine) and extra-islet hormone/metabolite signalling in addition to control by the central nervous system. The suppressive effect of intra-islet signalling metabolites secreted from the beta-cell include glutamate (Cabrera, Jacques-Silva et al. 2008), zinc (Hardy, Serino et al. 2011), ephrin (Hutchens and Piston 2015), insulin (Kawamori, Kurpad et al. 2009) and serotonin (5-HT) (Almaca, Molina et al. 2016). Paracrine control of alpha-cell function is also regulated mainly by the suppressive action of somatostatin from the delta-cell, which has been shown to be under the control of insulin and urocortin-3 secreted from the beta-cell, in response to hyperglycaemia (DiGruccio, Mawla et al. 2016, Vergari, Knudsen et al. 2019). Extra-islet hormones which stimulate alpha-cell glucagon secretion include GIP (Meier, Gallwitz et al. 2003), GLP-2 (Meier, Nauck et al. 2006) and Ghrelin (Chuang, Sakata et al. 2011). In contrast, GLP-1 has been shown to have a glucagonostatic effect through GLP-1R signalling on the alpha cell membrane (Ramracheya, Chapman et al. 2018). The multiple humoral suppressive signals described on alpha cell secretory function point towards a basal suppressive tone which, in times of hypoglycaemia is lost leading to glucagon secretion. Interestingly, there appears to be a synergistic effect of nutrient availability, in particular amino acids and alpha cell secretory function through GCPR activation (El, Gray et al. 2021). In addition to humoral control of alpha cell function, both the sympathetic and parasympathetic nervous system have been implicated in glucagon secretion (Faber, Deem et al. 2020).

1.10 Hepatic glucagon signalling

Numerous studies have investigated potential pathways of glucagon signalling and understandably these initially focused on hepatic carbohydrate metabolism, however important roles in hepatic lipid and amino acid metabolism have subsequently emerged. An early discovery of a signalling messenger regulated by glucagon was by Rall and Sutherland (Rall and Sutherland 1958). This was further confirmed by Robinson and colleagues who identified stimulation of cyclic AMP (cAMP) in a series of experiments

characterising a wide range of hormones, including glucagon (G A Robison 1968). The interaction between glucagon, insulin, cAMP and hepatic carbohydrate metabolism was further explored in a series of seminal experiments by Exton and Park in 1968, and defines our current knowledge of the potent acute and chronic glucose mobilising effect glucagon exerts through glycogenolysis and gluconeogenesis (Exton 1987).

The glucagon receptor (GCGR) is a G-protein coupled receptor expressed primarily in the liver, kidney and adrenal glands (Figure 1.3). Glucagon signals via the GCGR to activate a Gs protein which itself stimulates adenylate cyclase and cAMP accumulation within the cell. This in turn mediates the acute and chronic effects on glycogenolytic and gluconeogenic pathways (Exton 1987). Glucagon has also been shown to activate gluconeogenesis and glycogenolysis through cAMP-independent signalling molecules, including inositol phosphates (IP), calcium, mTORC2 and CREB (Wakelam, Murphy et al. 1986) (Jelinek, Lok et al. 1993) (Koo, Flechner et al. 2005). It is thought that glucagon generally signals through cAMP-dependent and cAMP-independent pathways with cAMP being able to acutely activate both post-translational and transcriptional signalling pathways, the latter through carbohydrate response binding element (CREB); In this way, glucagon is able to adapt acutely to fasting and also regulate these pathways chronically (Albrechtsen 2021). For example, chronic upregulation of rate limiting gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6P) through cAMP/PKA/CREB, enhances glucose output (Exton 1987). Acutely, glucagon-mediated induction of cAMP/PKA enhances phosphorylation of glycogen phosphorylase kinase and therefore subsequent phosphorylation and activation of glycogen phosphorylase, leading to hepatic glucose mobilisation (Albrechtsen 2021). Furthermore, PKA activity enhances phosphorylation targets within the glycolysis/gluconeogenesis pathway including FBPase2 to induce gluconeogenesis, and pyruvate kinase to suppress glycolysis. In addition to the effect on hepatic glucose metabolism, glucagon-mediated hepatic metabolism of amino acids also occurs through cAMP-dependent (cAMP/PKA-post-translational and cAMP/PKA/CREB-transcriptional) and cAMP-independent pathways, leading to enhanced amino acid catabolism and ureagenesis (Albrechtsen 2021). Glucagon-mediated hepatic metabolism of lipids is thought to occur through cAMP signalling both transcriptionally and post-translationally

via PKA, leading to both suppression of de novo lipogenesis and enhanced fatty acid beta-oxidation (Albrechtsen 2021).

Interestingly, more recently the state of hepatic 'glucagon resistance' in obesity and fatty liver disease has been characterised and gives further insight into hepatic signalling pathways regulated by glucagon. Glucagon resistance is characterised by a triad of hyperglucagonemia, hyperaminoacidemia and hyperglycemia (Winther-Sorensen, Galsgaard et al. 2020). The observation of hyperglycaemia in this setting suggests that the regulation of glycose and amino acid pathways downstream to the glucagon receptor may be independent to each other (Albrechtsen 2021). Based on the likely predominant signalling pathways involved, the divergent response to the metabolism of these nutrients in glucagon resistance states has been proposed to be due to the varying glucagon sensitivity on differential activation of transcriptional vs post-translational signalling pathways; For example, it has been proposed that in states of glucagon resistance, a reduction in post-translational signalling via cAMP may preferentially affect/reduce the post-translational signalling pathway of amino acid catabolism, whilst cAMP/PKA/CREB mediated stimulation of gluconeogenesis is sustained (Albrechtsen 2021). This presumably would rely on urea cycle activity being predominantly regulated by post-translational means and glucagon resistance affecting the ability of cAMP to activate these pathways rather than a lack of cAMP accumulation itself within the cell.

Based on the potent hepatic glucose mobilising effect of glucagon, it has been used as an emergency treatment for severe hypoglycaemia in the community for decades. However, broader biological functions of glucagon have subsequently emerged positioning itself as a potential breakthrough drug candidate for the treatment of obesity and metabolic disease; This is discussed further in section 1.7.7.

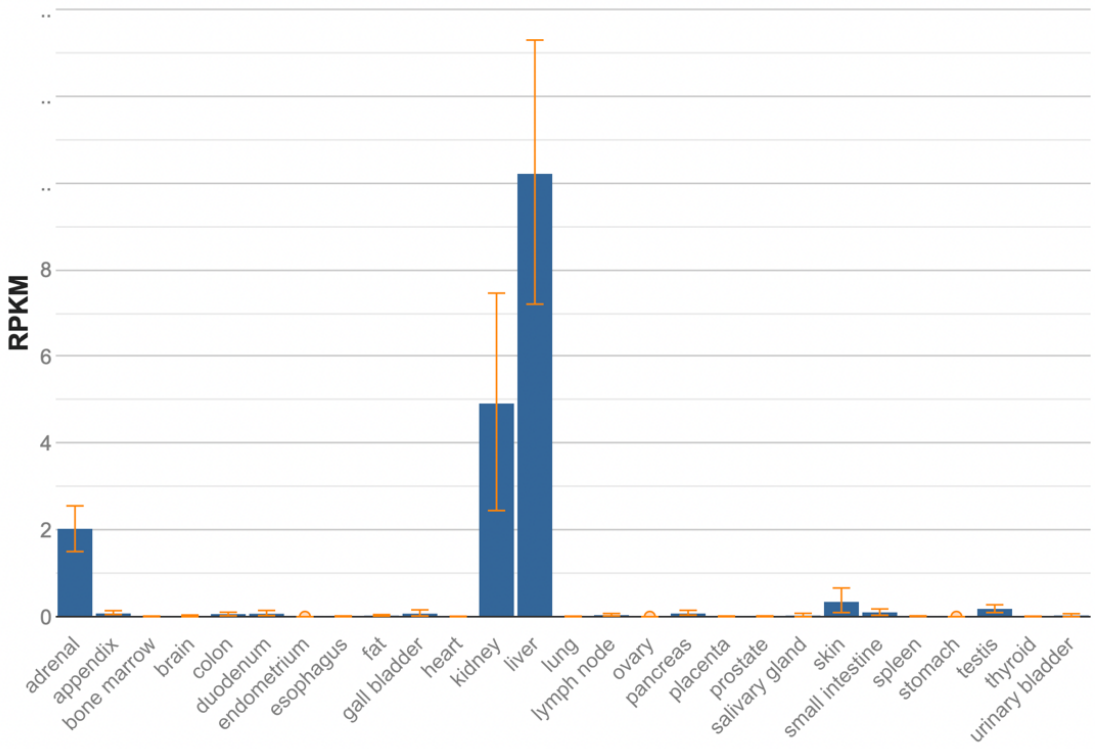


Figure 1.3: NCBI database search (October 2022) for pattern of tissue expression of the glucagon receptor. National Center for Biotechnology Information (NCBI). Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; Available from: https://www.ncbi.nlm.nih.gov/.

1.11 Glucagon in the pathophysiology of type 2 diabetes mellitus (T2DM)

The ''bihormonal theory'' of diabetes was proposed in 1975 by Roger Unger through three lines of evidence. Firstly, endogenous hyperglycaemia in various models had not been observed without the presence of glucagon (Unger and Orci 1975). Secondly, somatostatin suppression of both insulin and glucagon does not result in hyperglycaemia, which is restored with exogenous glucagon infusion (Mortimer, Tunbridge et al. 1974). Thirdly, somatostatin suppression of glucagon with exogenous insulin therapy improved glycaemia in individuals with diabetes (Gerich, Lorenzi et al. 1974). In patients with type 2 diabetes, studies have found that glucagon is elevated in both the fasting and postprandial state (Unger, Aguilar-Parada et al. 1970, Borghi, Wajchenberg et al. 1984). The primary cause of hyperglucagonemia in diabetes appears to be at partly due to defective insulin signalling within the islet and this was initially suggested in a study demonstrating whole body insulin resistance is independently associated with hyperglucagonemia (Ferrannini, Muscelli et al. 2007). The role for abnormal insulin signalling in the development of alpha cell hyperglucagonemia was also supported by

using alpha-cell specific insulin receptor knockout mice. Mice lacking the alpha cell IR exhibited a significantly higher glucagon and blood glucose levels than control mice (Kawamori, Kurpad et al. 2009). Other studies have demonstrated that other gut hormones in particular an exaggerated GIP secretion, play a role in enhancing glucagon secretion in response to an oral glucose load (Lund, Vilsboll et al. 2011). Several mechanisms have now been suggested to play a role in enhancing alpha cell function relative to beta cell function in the pathogenesis of type 2 diabetes (Martinez, Manzano et al. 2021). More recently the focus has shifted towards the role of altered amino acid metabolism in the hyperglucagonemia observed in type 2 diabetes, in particular dysfunction of the 'liver-alpha' axis (Holst, Wewer Albrechtsen et al. 2017) (further described in section 1.16.4). In a seminal study, it was discovered that increased plasma amino acids are associated with hyperglucagonemia independent to glycaemia/presence of diabetes (Wewer Albrechtsen, Junker et al. 2018). Therefore, resistance to amino acid metabolism in the liver may be the main cause of fasting hyperglucagonemia and therefore contributing factor to hyperglycaemia observed in those with type 2 diabetes and hepatic lipid accumulation. Earlier work has also shown that enhanced post-prandial hyperglucagonemia and hyperglycaemia following oral glucose load vs an intravenous load in type 2 diabetes may be a result of gut derived glucagon; Ongoing work is required to elucidate this mechanism (Knop 2018).

As a result of the glucagonocentric view of type 2 diabetes, glucagon receptor antagonism has been employed. Interestingly, glucagon receptor antagonists have been shown to modestly improve fasting glucose but not postprandial glucose levels. Furthermore, GRA's have been associated with side effects including hepatic lipid accumulation (Guzman, Zhang et al. 2017). As discussed in the next sections, paradoxically glucagon agonism has also been investigated for the treatment of type 2 diabetes and obesity.

1.12 The effect of glucagon on food intake

Glucagon has been exploited for two major effects on whole-body energy balance, food intake and energy expenditure. Indeed, it is precisely these actions on the two sides of the energy balance equation that has made a successful drug which targets the glucagon receptor so highly sought after (Hope, Vincent et al. 2021). The effect of glucagon on both food intake and energy expenditure were reported in the late 1950's. The first study to

show the effect on food intake was carried out in a small clinical study (Schulman, Carleton et al. 1957). 10 participants received a cross over intervention of 1mg glucagon intramuscularly prior to meals for 2 weeks and then a further 2 weeks receiving placebo. Glucagon was found to reduce food intake by an average of 440 calories per day which was also accompanied by a small amount of weight loss. This was further confirmed in human studies (Penick and Hinkle 1961). This finding has been replicated in rodent models which have also demonstrated that in rats, glucagon increases satiety via a hepatic mechanism, in particular by the hepatic branch of the vagus (Geary, Le Sauter et al. 1993). Glucagon also appears to signal directly to the CNS appetite centres as peripheral administration *in vivo* leads to enhanced activity in the hypothalamus and brainstem (Parker, McCullough et al. 2013). Specifically, glucagon can activate a GCGR/PKA/CaMKKbeta pathway in the hypothalamic ARC leading to enhanced AgRP neuron signalling and decreased food intake *in vivo* (Quinones, Al-Massadi et al. 2015). In addition to the role of glucagon during the fasting phase, these findings are consistent with a physiological role of glucagon as a post-prandial hormone to contribute towards satiety and meal termination.

1.13 The effect of glucagon on EE

The first report of glucagon's stimulatory effect on EE was in the late 1950's with acute dosing studies in Rats. In this study, it was shown that glucagon enhanced whole-body oxygen consumption within an hour of administration (Davidson, Salter et al. 1957). This confirmed a study by the same group in 1957 showing that glucagon-treated rats lost more weight than pair fed controls over 7 days with thrice daily administration (Salter, Davidson et al. 1957). To confirm glucagon-induced EE in a chronic setting, pair feeding studies demonstrated that daily dosing of glucagon (40 micrograms OD) in the male Wistar rat over 22 days, led to 75 % less body weight gain than a pair fed group (Davidson 1960, Salter 1960). Investigating the physiological role of glucagon in catabolic conditions, a subsequent glucagon-somatostatin co-infusion study demonstrated for the first time that glucagon acutely increases the resting metabolic rate in humans (Nair 1987). Several clinical studies have confirmed these observations in humans with doses of glucagon up to 50 ng/kg/min acutely (Calles-Escandon 1994, Tan, Field et al. 2013, Salem, Izzi-Engbeaya et al. 2016). However, not all human studies have demonstrated an

increase in EE, for example in one study where glucagon was administered at a lower dose (3 ng/kg/min) and following a meal (Bagger, Holst et al. 2015); This may suggest a possible link between prandial status and the ability of glucagon to increase EE. More recently, intranasal glucagon has also been demonstrated to increase resting energy expenditure in overweight/obese humans (Stahel, Lee et al. 2019). The ability of glucagon to increase EE in species other than rodents and humans was also demonstrated early on in pigs and dogs (Weiser and Grande 1974, Ingram and Kaciuba-Uscilko 1980).

1.14 Rationale for GLP-1/glucagon co-agonism as an anti-obesity strategy

As discussed in briefly in Section 1, the rationale for combining GLP-1 and glucagon was based on the individual action of these hormones on either side of the energy balance equation, food intake and energy expenditure, respectively. This rationale was supported by previous studies demonstrating that the endogenous GLP-1R-/GCGR co-agonist, oxyntomodulin (OXM) results in weight loss and reverses obesity (Dakin, Gunn et al. 2001, Dakin, Small et al. 2004, Pocai, Carrington et al. 2009). Indeed, glucagon receptor activation by OXM was shown to drive energy expenditure via the GCGR *in vivo* (Kosinski, Hubert et al. 2012). The combination approach was subsequently demonstrated in both humans with native hormone co-infusion studies (Tan, Field et al. 2013, Cegla, Troke et al. 2014), and with the development of unimolecular GLP-1R/GCGR co-agonists tested *in vivo* (Day, Gelfanov et al. 2012). Importantly, in both human co-infusion studies, combined administration of GLP-1 and glucagon did not adversely affect glycaemia due to the insulinotropic effect of GLP-1. Furthermore, in obese rodents, co-agonist treatment led to an improvement in glycaemia, in addition to other metabolic parameters including plasma lipids and hepatic steatosis (Day, Gelfanov et al. 2012). These also showed food-intake independent changes in body weight and fat mass in addition to increased EE (Day, Gelfanov et al. 2012). A raft of unimolecular co-agonists were subsequently developed and tested *in vivo*, with many progressing swiftly to clinical trial testing (Hope, Vincent et al. 2021). Despite the acceptance of the beneficial effect of glucagon on energy balance, the mechanism(s) of EE are less understood. However, to develop a safe and effective drug targeting the GCGR, an understanding of the EE mechanism is essential and prior studies contributing to this understanding will be discussed in the next section.

1.15 Glucagon mediated EE – Mechanisms

Since the initial finding by Davidson in 1957, several groups have studied various pathways implicated in glucagon-mediated EE. These initially focused on brown adipose tissue thermogenesis and more recently novel pathways including FGF21, FXR signalling using transgenic models (Al-Massadi, Ferno et al. 2019). However, despite numerous reports on the subject, a clear understanding of glucagon-mediated EE remains to be established. Furthermore, it remains possible that a combination of pathways is implicated in the EE response to glucagon, and these may be dependent for example on dose, timing, and chronicity of treatment. In the following sub-sections, previously proposed mechanisms of glucagon-mediated EE are discussed.

1.15.1 Glucagon mediated EE and BAT

Brown adipose tissue (BAT) is an important thermogenic organ in mammals, producing adaptive cold-induced non-shivering thermogenesis (R 1961, Donhoffer S 1964). Uncoupling protein 1 (UCP1) is the canonical protein in BAT which uncouples oxidative phosphorylation from ATP synthesis, with the resultant energy released as heat (Nicholls, Bernson et al. 1978, Golozoubova, Hohtola et al. 2001, Fedorenko, Lishko et al. 2012). The early observation of glucagon's stimulatory effect on BAT in rodents came from experiments on BAT tissue explants and use of supra-physiological doses of glucagon (Joel 1966, Kuroshima and Yahata 1979). *In vivo* studies showed indirect evidence for glucagon's effect on BAT for example interscapular temperature increases in rabbits and increased blood flow into BAT in rats (Cockburn, Hull et al. 1967, Yahata, Habara et al. 1983). Further *in vivo* studies also showed direct evidence for glucagon's thermogenic effect on BAT in rats (Doi and Kuroshima 1982). However, several other studies show that glucagon induced thermogenesis is not mediated by BAT. In species with minimal or no BAT, such as adult dogs or pigs, glucagon can still enhance thermogenesis (Weiser and Grande 1974, Ingram and Kaciuba-Uscilko 1980). Furthermore, studies in transgenic mice have shown that despite increased oxygen consumption in BAT tissue, whole-body *Ucp1* or BAT-specific *Gcgr* ablation does not prevent glucagon induced thermogenesis, indicating UCP-1 independent pathways (Beaudry, Kaur et al. 2019). Interestingly, glucagon-mediated EE was blunted in *Fgf21*$^{-/-}$ mice, suggesting it is an important

regulator. More recently, it has been shown that acute glucagon infusion (50 ng/kg/min) in humans increases energy expenditure independent of BAT-activity (Salem, Izzi-Engbeaya et al. 2016). Together these findings are generally conflicting and suggest that glucagon increases thermogenesis independent to BAT in humans.

1.15.2 Alternative possible mechanisms for glucagon-mediated EE

Glucagon's key catabolic actions include hepatic free fatty acid (FFA) and amino acid oxidation (Charlton, Adey et al. 1996, Longuet, Sinclair et al. 2008). Glucagon is also able to directly stimulate white adipose tissue lipolysis (Vaughan and Steinberg 1963), thereby liberating FFA's to the liver and other tissues. In addition to the stimulatory effect on glycogenolysis, glucagon is also able to stimulate gluconeogenesis, a pathway able to increase hepatic glucose output providing a whole-body energy substrate (Exton 1987). As gluconeogenesis is an energy-costly process (with a net consumption of ATP molecules), it is possible that this contributes to the energy effects observed with exogenous glucagon administration. Due to the challenges in measuring the energy expenditure of specific metabolic pathways, the contribution of these effects to the overall EE effects observed remains uncertain (Kleinert, Sachs et al. 2019). However, studies which have 'stressed' the gluconeogenesis pathway using a high protein/low carbohydrate diet, have demonstrated that hepatic gluconeogenesis contributes as much as 40% to the increased EE effects observed in humans (Veldhorst, Westerterp-Plantenga et al. 2009). More recently, it has been shown that in conditions of insulin resistance, gluconeogenesis may account for up to one third of resting energy expenditure (Quaye, Chacko et al. 2021). In addition to these energy consuming pathways, glucagon also stimulates another important catabolic hormone, cortisol (Waldhausl, Haydl et al. 1976) in addition to catecholamine release (Lawrence 1967), both with direct effects on the sympathetic nervous system. Consistent with the role of these adrenal hormones in glucagon-mediated EE, glucagon does not result in enhanced EE in adrenalectomized rats (Davidson 1960). Whole-body protein catabolism has also been implicated. Following early glucagon infusion studies demonstrating acute EE in humans (Nair 1987), subsequent studies showed that hyperglucagonemia was responsible for the enhanced resting metabolic rate and leucine oxidation (a marker of proteolysis) during insulin deprivation (Charlton and Nair 1998). The importance of protein catabolism for energy

metabolism in type 1 diabetes has therefore been highlighted previously (Hebert and Nair 2010).

In a seminal paper, Habbager *et al* demonstrated that hepatic derived FGF21 is an important mediator of the beneficial actions of glucagon on energy expenditure (Habegger, Stemmer et al. 2013). In a series of experiments, it was shown that the novel long-acting glucagon analogue, IUB288, resulted in food intake-independent body weight loss in DIO mice over 18 days. They demonstrated that the hepatic expression of FGF21 was increased by IUB288 and this mirrored increased circulating levels of FGF21. IUB288 treatment over 20 days did not result in body weight loss in global *Fgf21*$^{-/-}$ transgenic mice, compared to wild-type (WT) mice. Furthermore, using metabolic cages it was shown that the absence of *Fgf21* prevented IUB288-enhanced energy expenditure and associated locomotion. This research suggests that the EE effects of glucagon are dependent on the presence of FGF21. In keeping with these findings, FGF21 is known to increase energy expenditure acutely through central mechanisms targeting thermogenesis in brown and white adipose tissue (Owen, Ding et al. 2014, Douris, Stevanovic et al. 2015, BonDurant, Ameka et al. 2017). Supporting the central action of FGF21 on energy expenditure pathways, it has been demonstrated that the lack of FGF21 receptor in adipocytes does not prevent FGF21-induced body weight loss in DIO mice (BonDurant, Ameka et al. 2017). The effect of glucagon signalling on thermogenesis in adipose tissue has also been highlighted with studies in *Gcgr*$^{-/-}$ mice where a a decrease in thermogenic markers has been observed (Townsend, Medak et al. 2019). Collectively, these data support the glucagon's role as a regulator of thermogenesis in BAT and WAT *in vivo,* mediated through the action of FGF21 and CNS signalling to peripheral tissues. Indeed, more recently, glucagon mediated hepatic FGF21 induction has been shown to enhance EE through CNS signalling (Nason, Antipenko et al. 2021). However, it appears that hepatic FGF21 is not obligatory for glucagon induced EE. In another study using hepatic specific *Fgf21*$^{-/-}$ transgenic mice, it was shown that the lack of hepatic *Fgf21* only led to a partial blunting of body weight loss following chronic IUB288 treatment (Kim, Nason et al. 2018). Other contributory mediators of the glucagon-EE and body weight effect may also be partly due to hepatic farsenoid X receptor (FXR) signalling, mediating effects of various bile acid ligands (Kim, Nason et al. 2018). Specifically, loss of body weight in response to IUB288 was blunted in hepatic-specific *Fxr*$^{-/-}$ transgenic mice with

a prevention of IUB288 induced EE. Furthermore, in this study, it was noted that the thyroid hormone T4 also significantly increased with IUB288 treatment.

Further adding to the potential mechanisms by which glucagon enhances EE, it has been demonstrated that glucagon may also directly increase EE in rodents when administered intracerebroventricularly. This has been shown to be associated with BAT thermogenesis (Lockie, Heppner et al. 2012). Together these findings suggest that glucagon has the potential to enhance EE through several different mechanisms.

As glucagon is a protein catabolic hormone, the effects of chronic treatment with a long-acting glucagon analogue, as an anti-obesity strategy, on plasma amino acid availability, lean mass and energy expenditure is remains unexplored. These questions form the basis for the current thesis and therefore, in the next section, the background to the effect of glucagon on protein metabolism is further discussed.

1.16 Glucagon and protein metabolism

1.16.1 The urea cycle and amino acid catabolism

The hepatic Ornithine cycle (also known as the Urea cycle) comprises of five enzymes and is required for the detoxification of ammonia in mammals (Morris 2002). It was the first metabolic cycle to be discovered in 1932 by Hans Kreb and Kurt Henseleit in a series of biochemical studies described by Hans Kreb himself in 1973 (Kreb 1973). The five key enzymes of the urea cycle are now known to include Carbamyl phosphate synthetase-1 (CPS-1), Arginosuccinate synthetase (ASS), Arginosuccinate lyase (ASL), Ornithine transcarbamylase (OTC) and Arginase (Arg) (Morris 2002). The urea cycle enzymes are expressed in the periportal hepatocytes of the liver and therefore also readily available for presenting circulating amino acids. On a cellular level, the machinery of the urea cycle spans both the mitochondrial matrix and cytosol, with both CPS-1 and OTC located in the mitochondrial matrix. CPS-1 carries out the first step in the urea cycle pathway, transferring an ammonia molecule to phosphorylated bicarbonate, producing carbamoyl phosphate, which is subsequently condensed with ornithine (Figure 1.4). As CPS-1 has a requirement for the allosteric activator, N-acetyl-glutamate, N-acetyl-glutamate synthetase (NAGS) is also seen as the sixth urea cycle enzyme. The incoming ammonia

molecule is liberated from glutamate, interconverted from a variety of amino acids, and provides the nitrogen supply (in Carbomyl phosphate) to Ornithine (which contains two nitrogen atoms). A series of intermediate steps tightly regulate the transfer of nitrogen to the end-product urea, with the subsequent reformation of Ornithine (Figure 1.4). In addition to Carbamoyl phosphate supplying one nitrogen atom to Ornithine, through the action of ASS, aspartate also provides an additional nitrogen atom to the intermediate Citrulline generating the four nitrogen atom-containing Arginosuccinate. At the end of the cycle, Arginase facilitates the liberation of urea, containing two-nitrogen atoms, from arginine, reforming Ornithine (Figure 1.4).

Substrate availability to the urea cycle is an important factor in the acute regulation of cycle function. This is important as the urea cycle needs to have the capacity to respond to large fluxes in nitrogen load, for example during high levels of protein intake. Both short and longer-term control of the urea cycle is determined by the expression and activity of the individual urea cycle enzymes, influenced by insulin, glucagon and cortisol (Morris 2002). Increased activity of all five of the urea cycle enzymes has been demonstrated in response to glucagon *in vivo*, demonstrating its potent ability to increase activity of the cycle (Snodgrass, Lin et al. 1978). Subsequent studies have shown that glucagon is able to post-transcriptionally regulate urea cycle enzymes leading to enhanced activity within minutes (Janah, Kjeldsen et al. 2019). Other studies have shown that glucagon is also able to upregulate mRNA expression enzymes of the urea cycle in cultured rodent hepatocytes (Ulbright and Snodgrass 1993). As glucagon is secreted following a protein meal, it can therefore acutely facilitate metabolism of excess nitrogen load by increasing the activity of the urea cycle to produce the waste product, urea. Following a predominantly protein rich meal, insulin is also secreted from the pancreatic beta cell increasing the peripheral uptake of amino acids. Therefore, the hepatic glucose-mobilising action of glucagon may also serve to counter any potential hypoglycaemic effect of insulin in the presence of limited carbohydrate availability (Janah, Kjeldsen et al. 2019).

More recently, using glucagon receptor knockout models, glucagon analogues and glucagon receptor blocking antibodies, hepatic GCGR activity has been associated with divergent effects on both the transcriptional and post-translational regulation of the urea

cycle and hence its overall activity (Solloway, Madjidi et al. 2015, Kim, Okamoto et al. 2017, Emilie Elmelund 2022).

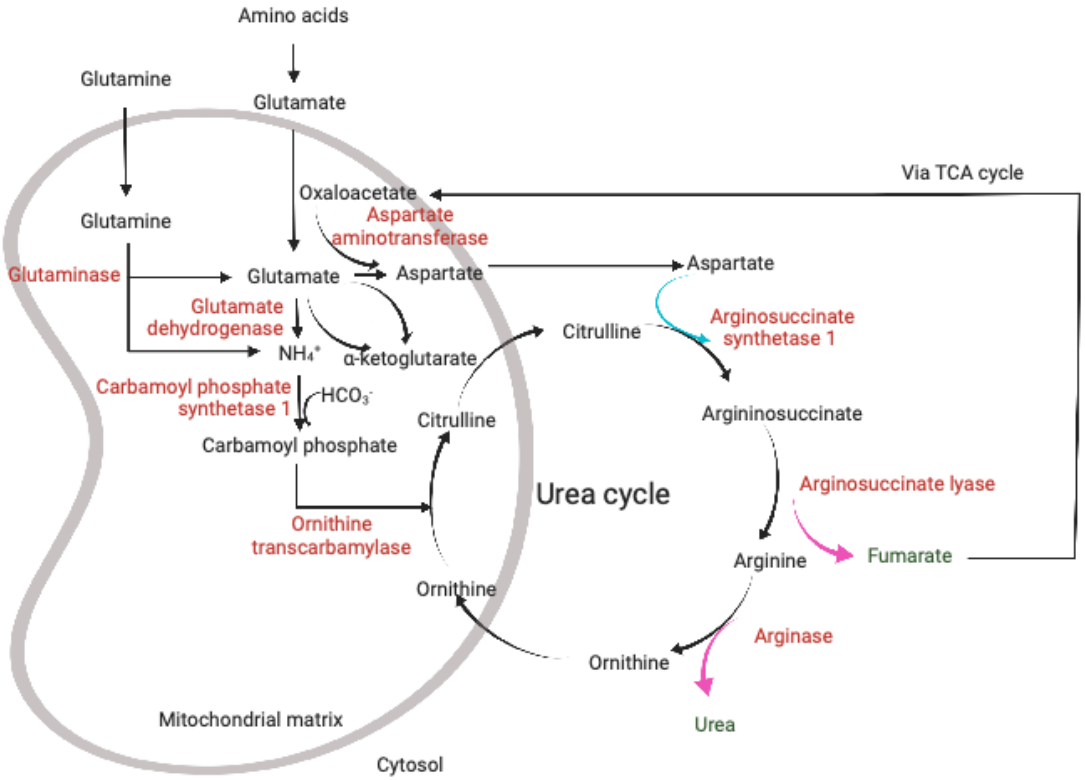


Figure 1.4: Mechanisms of amino acid entry to the urea cycle. Created using Biorender.com. Adapted from 'Exploring Metabolic Consequences of *CPS1* and *CAD* Dysregulation in Hepatocellular Carcinoma by Network Reconstruction' (Dumenci, U et al. 2020).

1.16.2 Early experiments exploring the interaction between native glucagon and nitrogen balance

The catabolic effect of glucagon on protein was discovered in the early 1950's not long after it was first purified and synthesised in at Eli Lilly and Co (Staub, Sinn et al. 1955, Bromer, Sinn et al. 1957). In these first *in vivo* experiments glucagon stimulated an increase in urinary nitrogen following administration in Rabbit (Tyberghein 1953). For the first time, the authors described the counter-regulatory action of insulin and glucagon, not only on carbohydrates but on protein metabolism. Urinary nitrogen was measured on the knowledge that increased amino acid catabolism results in nitrogen-containing urea (see Section 1.9.1). Using the measurement of urinary nitrogen content,

these observations were subsequently confirmed in other tissue and animal models (Salter, Davidson et al. 1957, Miller 1960, Salter 1960, Izzo and Glasser 1961). Due to a similar effect of steroids and glucagon on urine nitrogen production, early studies were quick to investigate the effect of glucagon in conditions of inflammation. Indeed, Salter suggested the potential use of glucagon for inflammatory conditions and when glucagon was administered to patients with rheumatoid arthritis, despite gastrointestinal side effects, a significant improvement in joint symptoms was observed (Ezrin, Salter et al. 1958).

With regards to the interaction between glucagon and lean mass, early studies indicated the role of reduced plasma amino acid availability on loss of muscle mass. For example, *ex vivo* studies demonstrated early on that nitrogen is released from skeletal muscle in glucagon treated rats (Peterson, Beatty et al. 1963). Furthermore, following a study in Rabbits whereby amino acids were measured in blood released from muscle, it was first suggested that glucagon stimulated reduction in amino acids may be the stimulus to release of amino acids from skeletal muscle, sustaining gluconeogenesis (Daniel, Pratt et al. 1977).

Through the application of more sophisticated methods, including the use of isotopes, studies in humans further described the whole-body changes in protein metabolism with administration of glucagon infusions. These studies sought to understand the whole-body change in nitrogen balance, beyond the primary catabolic action of glucagon in the liver. Indeed, these were important aspects to characterise as in the 1970's hyperglucagonemia was beginning to be associated with trauma (Meguid, Brennan et al. 1972), sepsis (Rocha, Santeusanio et al. 1973) and diabetes (Unger 1971). Isotope-labelled leucine was used in humans undergoing glucagon infusions to investigate the interaction between glucagon and protein balance. As leucine is an essential BCAA oxidised in the muscle, using this method, leucine oxidation, the rate of muscle proteolysis (leucine flux) and synthesis (non-oxidative leucine flux) could be estimated. These measures give an overall estimate of whole-body protein balance. Glucagon infusions achieving high physiological circulating levels lower plasma amino acids, enhance leucine oxidation and proteolysis in skeletal muscle (Nair, Halliday et al. 1987, Nair, Woolf et al. 1987, Hartl, Miyoshi et al. 1990, Pacy, Cheng et al. 1990, Charlton, Adey et al. 1996). As leucine is a positive

regulator of protein synthesis, these findings suggest that glucagon action, through stimulating hypoaminoacidemia and muscle leucine oxidation may result in reduced intramuscular leucine and a subsequent suppression of protein synthesis. Indeed, using leucine isotopes, infusions of glucagon in humans reduce markers of protein synthesis (Pacy, Cheng et al. 1990). Furthermore, in a human study, an infusion of glucagon attenuated skeletal muscle protein synthesis in response to an amino acid mixture (Charlton, Adey et al. 1996). In addition to the amino group released from muscle leucine oxidation, it has been suggested that the suppression of muscle protein synthesis in response to lowered intramuscular leucine allows valuable amino acids to be re-directed to the liver for glucose production (Adeva-Andany, Fernandez-Fernandez et al. 2021).

1.16.3 Protein metabolism in the glucagonoma syndrome

In parallel to studies characterising the physiological effects of glucagon on protein metabolism, the first reports of the glucagonoma syndrome were published, highlighting glucagon's effects in this pathological condition. In this report, Mallinson and colleagues first described the glucagonoma syndrome in a case series where patients exhibited low plasma amino acids and marked muscle wasting (Mallinson, Bloom et al. 1974). Further case reports have confirmed hypoaminoacidemia is a common finding in the glucagonoma syndrome (Barazzoni, Zanetti et al. 1999). However, the mechanism of muscle wasting in the glucagonoma syndrome has not been characterised.

1.16.4 Plasma amino acid availability and amino acid sensing; Recent insights from the liver-alpha axis

Plasma amino acids are tightly controlled and therefore any alteration of this would be expected to have systemic effects. The idea that amino acid sensing is important in healthy metabolism has recently been highlighted with the finding that glucagon-dependent clearance of plasma amino acids is coupled to alpha cell hyperplasia in a nutrient circuit (Longuet, Robledo et al. 2013, Solloway, Madjidi et al. 2015). In this circuit, disruption in glucagon action at the level of the liver leads to impaired amino acid catabolism and therefore increased plasma amino acid levels. Subsequently, plasma amino acids provide the signal to the pancreatic alpha cell for growth and differentiation (Dean, Li et al. 2017). This explained the previous finding that glucagon receptor

inactivating mutations in humans or transgenic GCGR knock-out mice exhibit pancreatic alpha-cell hyperplasia (Gelling, Du et al. 2003). This nutrient circuit was later termed the liver-alpha axis (Holst, Wewer Albrechtsen et al. 2017) with the ability of individual amino acids to enhance alpha-cell hyperplasia and glucagon secretion via mammalian target of rapamycin (mTOR) (Dean, Li et al. 2017).

Interestingly, increased fasting plasma glucagon was shown in patients with hepatic steatosis in the presence, or absence of type 2 diabetes (Junker, Gluud et al. 2016). In keeping with a disrupted liver-alpha axis, an important study demonstrated that hyperglucagonemia correlates with plasma amino acids in patients with hepatic steatosis without diabetes (Wewer Albrechtsen, Faerch et al. 2018, Wewer Albrechtsen, Junker et al. 2018), hence confirming glucagon resistance in this individuals (Janah, Kjeldsen et al. 2019). Using trans-jugular liver biopsies and gene expression analysis, hepatic glucagon resistance and disrupted hepatic amino acid catabolism has subsequently been shown directly in patients with hepatic steatosis (Suppli, Bagger et al. 2020); Interestingly, in this study by Suppli et al., the ability of glucagon to stimulate endogenous glucose production was not impaired in those with hepatic steatosis. Consistent with the data in humans, glucagon-stimulated amino acid catabolism is also blunted in a mouse model of hepatic steatosis (Winther-Sorensen, Galsgaard et al. 2020). Together, these important findings have heralded further clinical studies including one which is currently investigating the role of plasma amino acids as sensitive markers for hepatic steatosis when given a glucagon challenge (NCT04907721).

Amino acid sensing is not only applicable to the pancreatic alpha cell but occurs in multiple tissues including skeletal muscle (Moro, Ebert et al. 2016). Therefore, the wider potential effects of chronic hypoaminoacidemia and altered amino acid sensing will be discussed below. Interestingly, more recently a study has described alpha-cell hypoplasia in response to the use of a long-acting GCG analogue, suggesting a significant impact on the beta-cell in this context (Emilie Elmelund 2022). Furthermore, this study has also characterised the pattern of individual amino acids which is established when perturbation of the liver-alpha axis results in either hypoaminoacidemia or hyperaminoacidemia. It appears that certain amino acids foe example alanine, arginine,

asparagine, threonine, methionine with lesser effects on the branched-chain and aromatic amino acids (Emilie Elmelund 2022).

1.17 The effect of amino acid availability on amino acid sensing and lean mass; implications for long-term glucagon treatment?

The human body comprises of approximately 40-50% muscle mass and is essential for physical function and healthy metabolism. Muscle mass is determined by the coordinated regulation of protein synthetic and proteolysis pathways. The resulting protein turnover is therefore dynamic and allows for continued adaptation to metabolic demands. When the rate of proteolysis exceeds that of protein synthesis, muscle mass is lost, and for example, is a key element of anabolic resistance in sarcopenia (Moro, Ebert et al. 2016).

Proteolysis in muscle is controlled through several pathways, primarily the macroautophagy pathway (autophagy-lysosome) and the ubiquitin-proteosome pathway (UPP). Both pathways are distinct and unique in the way they operate. The autophagy-lysosome pathway is responsible for clearing protein aggregates and organelles which may present cell stress if not removed. Through the coordinated action of many 'atrogenes', cell constituents are targeted to autophagosomes which then fuse with lysosomes to form the autolysosome which can facilitate the enzymatic degradation of the vesicle components (Sandri 2010). Key regulators of the autophagy-lysosome pathway are mechanistic target of rapamycin complex 1 (mTORC1) and AMPK (Peris-Moreno, Cussonneau et al. 2021) (Figure 1.5). In contrast, the ubiquitin-proteosome pathway (UPP) targets proteins to the 26S proteosome for proteolysis through the coordinated action of ubiquitin ligases (Lecker, Goldberg et al. 2006). Two well-known and key E3 ligases implicated in muscle atrophy are Muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx/atrogin-1) (Bodine and Baehr 2014). The UPP is regulated by AKT/FOXO1 pathway signalling (Peris-Moreno, Cussonneau et al. 2021) (Figure 1.5).

The widest recognised regulator of protein synthesis is the serine/threonine kinase, mechanistic target of rapamycin complex 1 (mTORC1), a complex of proteins present in all mammalian cells (Takahara, Amemiya et al. 2020). A number of upstream signals converge on the mTORC1 pathway including amino acid availability, glucocorticoids, mechanical stimulation, Insulin/IGF1 and myostatin (Yoon 2017). Positive signals from

amino acids, mechanical stimulation and the Insulin/IGF1 pathway result in mTORC1 activation at the lysosomal surface leading to subsequent activation of S6 kinase 1 (S6K) and inhibition of 4E-binding protein 1 (4EBP1) (Ma and Blenis 2009). S6K facilitates translation initiation at the S6 ribosomal. In contrast, 4E-BP1 is a translation repressor through recruiting complexes to the mRNA at the ribosome. Therefore, any activation signal to mTORC1 leads to the coordinated increase in protein synthesis. It is increasingly recognised that mTORC1 is also able to negatively regulate various steps of autophagy, which helps ensure suppression of the autophagic flux during times of protein synthesis (Dossou and Basu 2019).

As amino acids are required for cellular processes, any reduction in supply would be detrimental to the cell. In the muscle fibres, important protein synthetic and proteolytic pathways need to be responsive to amino acid availability. One of the most well characterised amino acid sensors is indeed the mTORC1 complex, at the helm of orchestrating protein synthesis. Over the last decade, significant advances have been made in understanding the mechanisms of amino acid sensing within the mTORC1 pathway (Moro, Ebert et al. 2016). Amino acids promote activation of the so-called Rag proteins which recruit mTORC1 to the lysosomal surface where it interacts and is activated by Rheb. A heterodimer of Rag (GTPases) proteins is also activated by amino acids and change their nucleotide state. A lysosomal amino acid transporter SLC38A9 which senses arginine in the lysosome also activated mTORC1. Upstream to the activated Rag heterodimer, amino acids arginine and leucine are sensed by Sestrin2 and Castor 1 proteins, leading to activation of Gator2, inhibition of Gator1 which allows mTORC1 to be activated. Collectively, this shows that the mTORC1 pathway is highly sensitive to amino acid availability. Another amino acid sensing pathway is the GCN2/EIF2alpha/ATF4 pathway. GCN2 is a known amino acid sensor and in contrast to mTORC1 is activated by low plasma amino acid availability inhibiting protein synthesis (Moro, Ebert et al. 2016). Activated ATF4 regulates a number of downstream genes which serve to inhibit protein synthesis, including activation of 4E-BP1.

In addition to the role of amino acids in regulator the activity of the mTORC1 pathway, therefore affecting protein synthesis and autophagy, there is increasing evidence that the ubiquitin proteosome pathway is amino acid responsive. For example, it has been shown

that leucine attenuates skeletal muscle wasting through the inhibition of muRF1 and Atrogin 1 (Baptista, Leal et al. 2010).

Collectively, these findings support the possibility that chronic glucagon-stimulated hypoaminoacidemia, through local amino acid sensing pathways, may result in lean mass loss through altering the balance of protein synthesis and proteolysis. This would also be consistent with glucagon-infusion studies using isotope-labelled leucine (Section 1.9.2) and the findings of muscle wasting in the glucagonoma syndrome (Section 1.9.3). Although leucine is a key positive regulator of mTORC1 and altered leucine metabolism has been demonstrated in glucagon infusion studies, it is possible that the reduction in multiple individual amino acids is implicated in lean mass loss. If this is confirmed, then a strategy that could rescue of plasma amino acids may well serve to prevent any aberrancy within these amino acid sensing pathways.

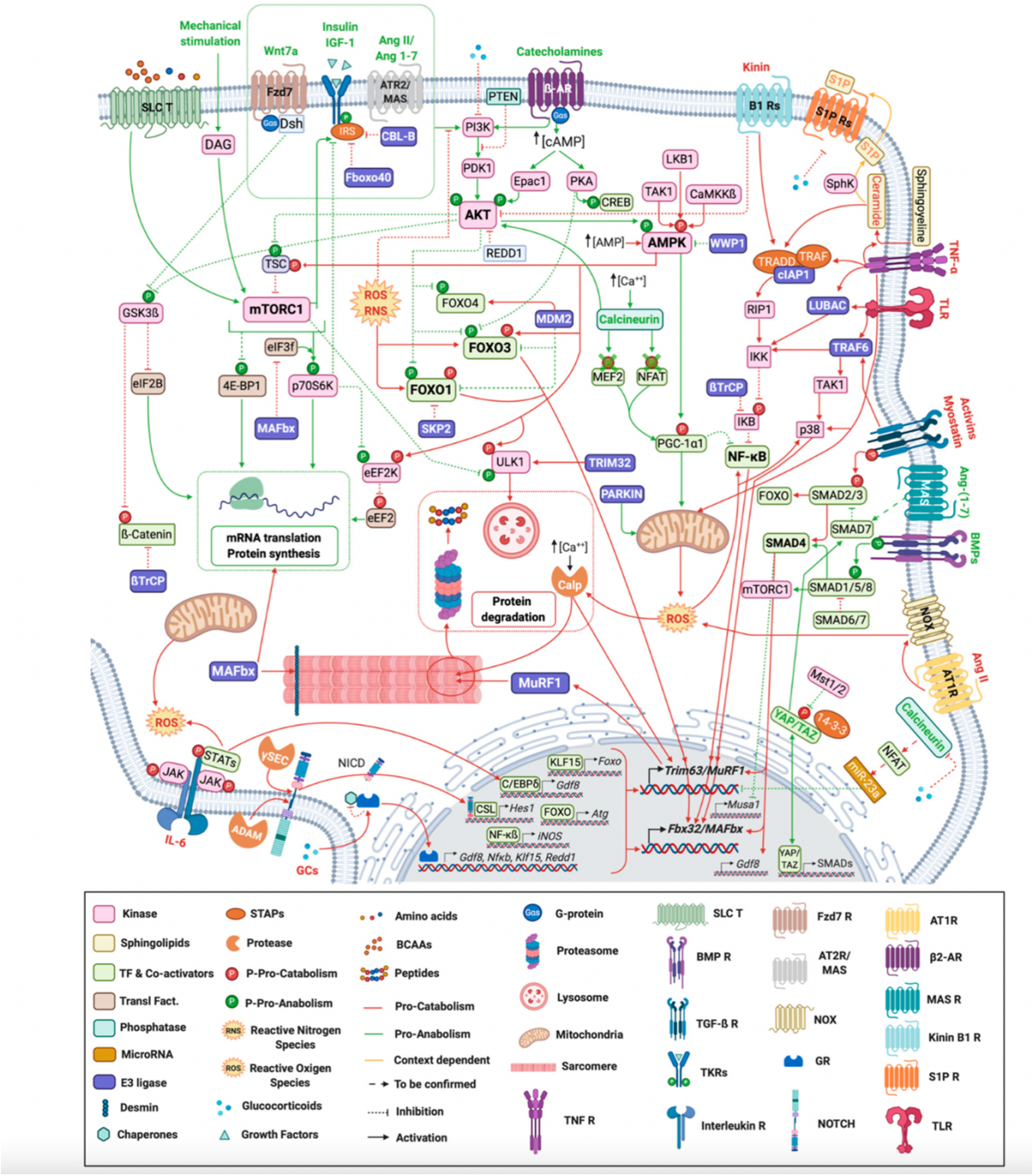


Figure 1.5: Pathways regulating muscle protein synthesis and proteolysis. Source of image: 'Ubiquitin Ligases at the Heart of Skeletal Muscle Atrophy Control (Peris-Moreno, Cussonneau et al. 2021).

1.18 The effect of amino acid availability on whole body energy balance – insight from models of dietary protein and amino acid restriction

The potential impact of glucagon-stimulated hypoaminoacidemia on energy balance is uncertain. However previous studies in models of dietary protein restriction provide insight into the possible effects of reproducing a state of low amino acid availability through a pharmacological intervention. Early studies first showed that dietary protein restriction led to enhanced longevity (Ross 1961, Miller and Payne 1968) and enhanced energy expenditure (Miller 1962, Miller and Mumford 1967, Stirling and Stock 1968) in rodents. These early studies used low protein diets to demonstrate reduced feed efficiency as a marker for energy expenditure. Further studies focused particularly on the effect of a low protein diet on the thermogenic brown adipose tissue (BAT), as a mechanism of energy dissipation (Rothwell and Stock 1979, Rothwell, Stock et al. 1982, Rothwell, Stock et al. 1983). The original definition of diet-induced thermogenesis (DIT) arose from these studies where it was thought that increased thermogenesis allowed disposal of excess non-essential energy consumed from protein-poor diets; In this way a strategy emerged to maintain protein and whole-body energy balance by overconsuming a low protein diet (Stock 1999). The increased food intake response to dietary protein restriction was subsequently termed 'protein leveraging' as a contributing factor to obesity in humans (Simpson and Raubenheimer 2005). Consistent with this and the early studies of protein restriction in rodents, more recent research has confirmed that a low protein diet in rodents may increase food intake through protein leveraging, and this can offset thermogenesis leading to increased adiposity (Sorensen, Mayntz et al. 2008, Huang, Hancock et al. 2013).

The early studies by Rothwell, Stock and others described the importance of brown adipose tissue in low protein diet stimulated thermogenesis. Since this time, numerous studies have led to further proposals of mechanisms leading to enhanced energy expenditure (Pezeshki and Chelikani 2021). FGF21 has received particular attention and therefore its potential role in low protein diet-thermogenesis is discussed below.

1.18.1 Hepatic FGF21, a key endocrine signal of protein restriction

FGF21, a hormone predominantly secreted in the liver was first identified as a factor enhancing glucose uptake in cultured adipocytes (Kharitonenkov, Shiyanova et al. 2005). FGF21 was subsequently shown to increase energy expenditure and insulin sensitivity *in vivo* (Coskun, Bina et al. 2008, Sarruf, Thaler et al. 2010, Owen, Ding et al. 2014). Using transgenic mouse models of FGF21 overexpression and hypothalamic KO of the co-receptor, FGF21 was found to signal through its cognate receptors in the hypothalamus to increase energy expenditure and body weight loss (Owen, Ding et al. 2014). The mechanism of body weight loss and energy expenditure appears to be through increased lipolysis and UCP1 thermogenesis in both BAT and WAT tissue, through upregulation of the sympathetic outflow to these thermogenic tissues (Owen, Ding et al. 2014). Previous work has suggested FGF21 also directly acts on adipose tissue increasing glucose uptake (BonDurant, Ameka et al. 2017). Therefore, it is proposed that FGF21 mediates both the 'fuel' and the 'fire' by increasing substrate supply to adipose tissue through direct glucose uptake and SNS-mediated lipolysis in addition to increasing thermogenic capacity with SNS-mediated upregulation of *Ucp1* in BAT and WAT (Owen, Mangelsdorf et al. 2015).

A seminal link between the metabolic effects of a low protein diet and fibroblast growth factor 21 (FGF21) was made in by Laeger et al., in 2014. This link changed the understanding of how low protein diets mediated their metabolic effects. Due to the similarity between the metabolic effects of a dietary protein restriction and FGF21 *in vivo*, Laeger et al., investigated whether FGF21 was responsible for mediating the long-observed metabolic effects of protein restriction (Laeger, Henagan et al. 2014). Not long before, a clue to this hypothesis emerged when it was demonstrated that amino acid restriction leads to increased FGF21 expression via ATF4, an integrated stress response pathway, in cultured murine hepatocytes and hepatocyte cell lines (De Sousa-Coelho, Marrero et al. 2012). Prior to this, the main physiological regulation of FGF21 was noted to be via PPARalpha, which in the fasting phase is upregulated by fatty acid supply from increased adipose tissue lipolysis (Badman, Pissios et al. 2007). Laeger and colleagues provided evidence of the additional regulation of FGF21 conferred by amino acid restriction and the ATF4 pathway. Indeed found that hepatic FGF21 is the key hepatic 'endocrine signal' of protein restriction, leading to enhanced energy expenditure and

body weight loss (Laeger, Henagan et al. 2014). Using GCN2 KO mice (GCN2 being upstream to Eif2alpha, ATF4 and FGF21, they demonstrated that hepatic FGF21 expression was prevented in response to a low protein diet. Furthermore, the EE and weight loss effects of a low protein diet were not observed in FGF21 KO mice (Laeger, Henagan et al. 2014).

To characterise these findings further, Maida et al., 2016 comprehensively demonstrated that dietary protein restriction leads to whole body metabolic remodelling with increased carbohydrate and fat oxidation and improved glucose homeostasis (Maida, Zota et al. 2016). These effects were not only dependent on hepatic FGF21 induction but also associated with the upregulation of an additional integrated stress response protein in the liver, NUPR. Interestingly, they demonstrated in murine hepatocytes that restriction of non-essential amino acids (NEAA) was necessary for these metabolic effects of a dietary protein restriction.

Similar to studies which demonstrated the central FGF21 co-receptor dependent effects of FGF21 on EE (Owen, Ding et al. 2014), using brain-specific FGF21 co-receptor KO mice, more recent work has confirmed that CNS FGF21 signalling mediates the metabolic effects of a low protein diet including EE, body weight loss and adipose tissue thermogenesis (Hill, Laeger et al. 2019).

Since the work by Laeger et al., research has focused on the likely amino acid suspects mediating the FGF21-dependent metabolic adaptations to dietary protein restriction. Conflicting reports have implicated both essential- and non-essential amino acids. For example, culturing murine hepatocytes in restricted NEAA media was able to recapitulate the effects of dietary protein dilution (Maida, Zota et al. 2016). In contrast other have shown that restriction of essential amino acids are necessary for these effects. Using a systematic experimental approach and a series of custom diets, Yap et al., demonstrated that the EE effect of dietary protein restriction was due to restriction of essential and not non-essential amino acids (Yap, Rusu et al. 2020). They further demonstrated that of the metabolically essential amino acids, lysine, threonine and tryptophan, dietary restriction of either threonine or tryptophan was able to reproduce the EE effect, with the add back of both being required to prevent it. The effects of

restriction of threonine and tryptophan on EE were also associated with a robust increase in hepatic FGF21 secretion and a measure of insulin sensitivity, the fasting insulin sensitivity index (fISI). Selective knockdown of hepatic FGF21 abolished the metabolic remodelling observed with Threonine restriction, confirming the EE effect of amino acid restriction is FGF21 dependent. Furthermore, enforcing hepatic threonine biosynthesis also prevented the EE effects observed with threonine restriction.

However, despite the strong evidence to suggest FGF21 mediates the EE effect of specific dietary amino acid restriction, others have shown FGF21 independent effects. For example, one study has shown that restriction of the branched-chain essential amino acids enhances body weight loss, however this appears to be independent to hepatic FGF21 induction (Fontana, Cummings et al. 2016). Furthermore, there is mounting evidence that individual amino acid restriction or depletion engages distinct pathways which lead to altered energy balance (Xiao and Guo 2022). Therefore, although hepatic FGF21 induction appears to be a key signal of protein restriction to metabolic adaptation, it may not be the only path to influencing energy balance. In the next section some studies are discussed which highlight the divergent effects of dietary amino acid restriction.

1.18.2 Restriction or depletion of individual amino acids can alter energy balance

As discussed above, exogenous glucagon administration may affect total and individual plasma amino acid levels in both rodents and humans. Is it possible that glucagon-stimulated hypoaminoacidemia may have direct or indirect effects on whole-body energy balance? Furthermore, is it possible that reduction in specific individual amino acids activate distinct pathways of energy balance? Early work demonstrated that diets deficient in single amino-acids, in particular tryptophan or histidine, led to weight loss in rats (Yokogoshi and Ashida 1975). Since then, several studies have attempted to characterise the effects of individual amino acids on body weight and energy balance. Interestingly, individual amino acids have been found to have a divergent effect on energy balance through specific signalling pathways and some of these will be discussed further below.

1.18.3 Methionine deprivation or restriction alters energy balance

In a seminal study investigating the effect of single amino acid restriction, it was shown that rats fed a 0.17% methionine diet had a 30% increase in life span and reduction in body weight despite increased food intake (Orentreich, Matias et al. 1993). These effects have been confirmed in subsequent studies (Hasek, Stewart et al. 2010). As much focus on energy expenditure in mammals has been on UCP1-dependent thermogenesis, studies of methionine restriction have been associated with an increased expression of *Ucp1* in rat BAT and WAT (Hasek, Stewart et al. 2010). Interestingly, methionine restriction also leads to direct action on both WAT lipogenesis (Hasek, Stewart et al. 2010) and lipolysis (Perrone, Mattocks et al. 2010), and it has been suggested this could lead to an adipose tissue-specific energy consuming futile cycle (Zhou, He et al. 2016). Furthermore, methionine restriction has been shown to increase physical activity in mice (Lees, Krol et al. 2014).

The mechanism by which methionine affects aspects of energy balance appears to be through hepatic FGF21 secretion and signalling. In diet-induced-obese mice, methionine restriction results in the increased hepatic expression and circulating levels of FGF21 (Ables, Perrone et al. 2012, Lees, Krol et al. 2014). Furthermore, the increase in energy intake and expenditure due to methionine restriction is not observed in *Fgf21*$^{-/-}$ mice (Wanders, Forney et al. 2017). Methionine is also not able to increase EE or expression levels of *Ucp1* in BAT or WAT, in mice with a CNS-specific deletion of the FGF21 co-receptor, β-klotho. (Forney, Fang et al. 2020). These data suggest that methionine increases the hepatic expression of FGF21 which then signals via the CNS to peripheral effector organs such as BAT and WAT. Human studies have also shed light on the role of methionine restriction and plasma FGF21 availability. As a vegetarian or vegan diet contain less methionine, it has also been shown that after short term vegetarian diet in humans increases plasma FGF21 levels (Castano-Martinez, Schumacher et al. 2019).

Studies have also demonstrated FGF21-independent effects of methionine restriction on energy balance. For example, mice with growth hormone deficiency are resistant to the effects of methionine restriction on energy balance (Brown-Borg, Rakoczy et al. 2014). Other FGF21-independent effects of methionine restriction on energy balance have also

been highlighted. Methionine can be converted to cysteine through a series of reactions to cysteine, levels of which are associated with obesity (Virtanen, Voutilainen et al. 2006). Studies have also demonstrated that cysteine supplementation can reverse the phenotype of methionine restriction in mice (Elshorbagy, Valdivia-Garcia et al. 2011). In addition to studies exploring effects of methionine restriction on energy balance, deprivation of dietary methionine is also associated with increased plasma norepinephrine levels in mice (Xiao, Du et al. 2016). Together, these findings suggest that methionine restriction can signal through a variety of pathways, both peripherally and centrally to affect overall body energy balance.

1.18.4 Threonine deprivation or restriction alters energy balance

A diet specifically restricted in threonine has been demonstrated to increase energy intake and expenditure in adult mice over 8-weeks (Yap, Rusu et al. 2020). This was associated with a reduced feed efficiency and loss of fat mass. An earlier study highlighted the different response to complete removal of threonine from the diet (Russell, Koehnle et al. 2003). In this study, it was demonstrated that threonine deficiency led to food aversion in rats, an effect reduced by direct injection of threonine into the anterior piriform complex (APC). Further studies have shown that threonine deficiency over 7 days in mice leads to reduced food intake in addition to increased energy expenditure (Xiao, Du et al. 2016).

Similar to other studies with methionine, the mechanism by which threonine restriction mediates energy balance also appears to partly depend on hepatic FGF21 secretion. In the study by Yap et al 2020, the EE effects of threonine restriction were not observed in adenoviral-mediated hepatic specific *Fgf21* knock out mice after 8-weeks of dietary intervention (Yap, Rusu et al. 2020). Activation of the sympathetic nervous system has also been implicated in the EE effects of altering threonine in the diet. In the study by Xiao et al, an increase in plasma norepinephrine levels were observed with dietary threonine deprivation in mice (Xiao, Du et al. 2016).

1.18.5 Lysine deprivation or restriction alters energy balance

In a study exploring the effect of altering individual EAA's in mice, it was shown that lysine deprivation led to a reduction in food intake and increased energy expenditure after 7 days (Xiao, Du et al. 2016). In contrast to these findings, in their single amino acid restriction study, Yap et al showed that individual reduction on lysine does not reproduce EE effects of low EAA diets (Yap, Rusu et al. 2020). Few studies have explored the specific effects of lysine deficiency on energy balance, however it appears that lysine deficiency results in the selective increase in the neurotransmitter, serotonin, regulating the anxiety and stress response (Smriga, Kameishi et al. 2002). In keeping with this, lysine is a known partial antagonist at the serotonin receptor (Smriga and Torii 2003). Similar to the effects of threonine or methionine deprivation, Xiao et al also demonstrated an increase in plasma norepinephrine levels with selective dietary lysine deprivation in mice, implicating the SNS in the EE effects observed (Xiao, Du et al. 2016).

1.18.6 Leucine deprivation or restriction alters energy balance

Individual dietary restriction of leucine in rodents has also shown to affect energy balance. For example, 80% restriction of leucine leads to increased food intake and energy expenditure in mice, with a similar however weaker response to 80% methionine restriction (Lees, Banks et al. 2017). This is supported by another study demonstrating the 85% restriction of leucine leads to enhanced energy intake and expenditure within 7 days in mice (Wanders, Stone et al. 2015). Interestingly, in contrast to these findings, it has recently been demonstrated that dietary leucine restriction in mice does not alter energy balance (Yu, Richardson et al. 2021). Several studies have investigated the possible mechanisms by which leucine restriction mediates its effects on whole body energy balance. In one report, leucine restriction was associated with increased hepatic FGF21 expression and circulating levels in addition to thermogenesis in BAT and WAT (Wanders, Stone et al. 2015). The involvement of SNS activation has also been suggested following reports of adrenergic stimulation to the WAT mice (Cheng, Zhang et al. 2011). Interest has been shown in general control nonderepressible 2 (Gcn2), a ubiquitously expressed amino acid sensor activated by uncharged tRNA's (Gallinetti, Harputlugil et al. 2013). Studies in mice have shown that GCN2 within amygdalar protein kinase C delta

neurons is activated when leucine is deficient from the diet. This subsequently triggers sympathetic nervous system mediated WAT browning (Yuan, Jiang et al. 2020). Furthermore, GCN2 activation in WAT macrophages upon dietary leucine deprivation has also been shown to enhance WAT browning (Wang, Xiao et al. 2021). The same pathway in the anterior piriform complex (APC) has been implicated in the food aversion response to EAA deprivation in rodents (Leung and Rogers 1971, Hao, Sharp et al. 2005) Another key amino acid sensor, mammalian target of rapamycin complex 1 (mTORC1) is also regulated by leucine. mTORC1 is a master coordinator of cellular protein synthesis (Gallinetti, Harputlugil et al. 2013). In the hypothalamus, leucine deprivation leads to increased activation of ribosomal protein S6 kinase beta 1 (S6K1), downstream, of mTOR, which subsequently leads to increased expression of corticotropin releasing hormone (CRH) in addition to thyrotropin releasing hormone (TRH), resulting in increased energy expenditure (Xia, Cheng et al. 2012, Xia, Zhang et al. 2015). To further add to the divergent mechanisms by which leucine deprivation alters energy balance, leptin receptor signalling has been shown to drive EE in response to dietary leucine deprivation in mice (Zhang, Liu et al. 2014).

1.18.7 Isoleucine or valine restriction alters energy balance

In a recent study it was demonstrated that that isoleucine or valine restriction, and not leucine restriction, led to increased energy intake and expenditure in rodents. A 67% reduction in dietary isoleucine or valine over 12 weeks in mice led to reduced gain of fat and lean mass despite a higher food intake (Yu, Richardson et al. 2021). Interesting, only dietary isoleucine restriction led to the induction of hepatic, iWAT and skeletal muscle FGF21 promoting energy expenditure. The energy expenditure effect was demonstrated to be due to the beiging process in iWAT due to a significant increase in a number of markers of thermogenic genes, including *Ucp1*, *Cidea* and *Elovl3*.

1.18.8 Histidine restriction alters energy balance

As histidine is an essential neurotransmitter which regulates energy intake and expenditure, it is possible that dietary restriction of histidine may affect energy balance (Tabarean 2016). Interestingly, more recently a study has found that individual

restriction of histidine increases energy expenditure, independent to FGF21 (Victoria Flores 2022). Further studies are required to further explore the effects of individual histidine restriction.

1.18.9 Phenylalanine deprivation or restriction alters energy balance

In addition to deprivation of other essential amino acids, Xiou et al demonstrated that individual restriction of dietary phenylalanine over 7 days in mice led to enhanced energy expenditure and reduced food intake (Xiao, Du et al. 2016). This was associated with increased markers of lipolysis and also plasma epinephrine levels suggesting activation of the sympathetic nervous system.

1.18.10 Tryptophan deprivation or restriction alters energy balance

Alterations in plasma levels of tryptophan, the precursor to serotonin has generated interest due to the ability of central serotonergic signalling to enhance energy expenditure and sympathetic nervous system activity (Yabut, Crane et al. 2019). However, a diet specifically deficient in tryptophan has been shown to increase energy expenditure and reduce food intake in mice over 7 days, and this was associated with an increase in plasma norepinephrine levels (Xiao, Du et al. 2016). Interestingly, in this study, tryptophan deficiency was the only EAA associated with a significant increase in physical activity.

More recently, it has been shown that tryptophan restriction can recreate the EE effects of a low EAA diet and this is also appears to be mediated by a hepatic FGF21 mechanism (Yap, Rusu et al. 2020).

1.19 Aims of thesis - overview

The initial aim of this thesis is to use a long-acting glucagon agonist to determine whether chronic GCGR agonism as a strategy for obesity and metabolic disease results in hypoaminoacidemia in a pre-clinical model. The project will then determine, in a pre-clinical model of obesity, whether glucagon-stimulated hypoaminoacidemia contributes

to lean mass loss through reduced plasma amino acid availability, over and above any effect of reduced calorie intake itself. This is particularly pertinent to the use of glucagon in anti-obesity drug strategies, as glucagon is known to enhance hepatic amino acid catabolism at both physiological and pathological levels (See section 1.9.2 and 1.9.3).

The project will then seek to determine whether 'rescue' of glucagon-stimulated hypoaminoacidemia will mitigate lean mass loss in a pre-clinical model of obesity, whilst preserving metabolic benefits of glucagon treatment, for example hepatic lipid metabolism and EE. Despite its progression in clinical trials (See section 1.6), our understanding of the impact of a pharmacotherapeutic targeting the GCGR on lean mass in a chronic setting, is unknown. To demonstrate translational relevance, a human study will be carried out to determine whether glucagon-stimulated hypoaminoacidemia is also observed in a Phase 1 trial of a GLP-1R/GCGR co-agonist in adults with overweight or obesity.

Finally, this thesis will also explore the wider whole-body physiological consequences of glucagon-stimulated hypoaminoacidemia, for example on energy balance. This is relevant as observations from models of dietary protein or amino acid restriction have consistently demonstrated enhanced energy expenditure in mouse models (See section 1.9.6). This therefore raises the prospect that hypoaminoacidemia is responsible at least in part, for enhanced EE associated with glucagon treatment. The translational relevance of these hypotheses remains high, as recent human studies have reported hypoaminoacidemia associated with pharmacotherapeutics targeting the GCGR (Bossart, Wagner et al. 2021, Martin Friedrichsen 2022).

2 Chapter 2– A long-acting glucagon analogue, G108, stimulates canonical glucagon signalling pathways causing chronic hypoaminoacidemia and lean mass loss.

2.1 Introduction

2.1.1 Early evidence that native glucagon enhances hepatic amino acid catabolism leading to reduced plasma amino acids

The effect of GCGR activation on hepatic signalling pathways of carbohydrate and protein metabolism is well established. Early studies demonstrated that native glucagon administration in rodents led to increased activity of the key enzymes of the urea cycle (McLean and Novello 1965, Snodgrass, Lin et al. 1978). Further work established that induction of the urea cycle is coordinated through various molecular mechanisms including transcriptional, post transcriptional and mRNA stabilisation (Ulbright and Snodgrass 1993) (See also section 1.7.3 and Section 1.9). These studies were informed by previous investigations aiming to characterise the whole-body metabolic effects of glucagon administration; In these, a consistent finding was of increased urinary nitrogen excretion in rodents and humans with exogenous native glucagon administration (Salter, Davidson et al. 1957, Salter, Ezrin et al. 1960). Confirming these findings, a longer-acting preparation of zinc-glucagon was shown to reduce individual amino acids following a single dose in rabbit (Bromer and Chance 1969). Concurrent to exogenous glucagon treatment paradigms in the 1960's and 1970's, glucagon-stimulated hepatic amino acid catabolism and the effect on plasma amino acids were highlighted in conditions of glucagon excess or depletion. For example, the effect of excess or depleted glucagon on plasma amino acids is evident from reports of hypoaminoacidemia in the glucagonoma syndrome (Mallinson, Bloom et al. 1974, Almdal, Heindorff et al. 1990, Barazzoni, Zanetti et al. 1999) and hyperaminoacidemia following total pancreatectomy (Boden, Master et al. 1980). Subsequent infusion studies in humans demonstrated the amino acid catabolism and lowered plasma amino acids with physiological infusions of glucagon (Boden, Tappy et al. 1990, Charlton, Adey et al. 1996). Collectively, these findings confirmed hepatic amino acid catabolism as a primary role of glucagon, which in various

settings may lead to enhanced hepatic amino acid catabolism and reduced plasma amino acids.

2.1.2 Mechanisms of hepatic GCGR-stimulated ureagenesis and hypoaminoacidemia

As the amino acid catabolic properties of glucagon have emerged as one of its primary biological activities, explanations for its physiological role have been suggested. As protein meals are a stimulus for not only insulin but also glucagon (Ang, Bruce et al. 2019), it is thought that the combined response of these 'counter-regulatory' hormones may be to serve as an optimum strategy for disposal of dietary protein and amino acids (Janah, Kjeldsen et al. 2019). In the post-prandial state following protein intake, insulin allows for peripheral uptake of glucose and amino acids, while glucagon enhances hepatic protein turnover and hepatic glucose output, therefore mitigating against the risk of insulin-induced hypoglycaemia in the setting of reduced carbohydrate availability (Ang, Bruce et al. 2019).

The mechanism of glucagon-stimulated hepatic amino acid catabolism appears to be primarily two-fold, with enhanced amino acid uptake in addition to catabolic processes (Janah, Kjeldsen et al. 2019). Although the urea cycle is substrate dependent, transcriptional induction by glucagon increases the activity of five key enzymes of the urea cycle is through a cAMP/PKA/CREB pathway (Snodgrass, Lin et al. 1978, Singer 2003). However, ureagenesis occurs within minutes of a glucagon infusion (Wolfe, Culebras et al. 1979) pointing towards mechanisms which regulate a swift increase in ureagenic activity when required. Of these, allosteric modifications of the urea cycle machinery through, for example, phosphorylation and acetylation have been suggested (Janah, Kjeldsen et al. 2019). Furthermore, the allosteric activator of Cabamoyl phosphate synthetase 1 (CPS1), N-acetyl glutamate (NAG) may serve as an important component for acute regulation of ureagenesis. As CPS1 mediates the initial step of the urea cycle condensing carbamoyl phosphate with ornithine, the increased transcription of N-acetyl glutamate synthetase (NAGS) by glucagon may potentiate these initial steps allowing for a quick increment in ureagenic activity (Heibel, Lopez et al. 2012).

As the urea cycle is substrate-dependent, a strategy to swiftly increase its activity is also through enhanced supply of the amino acid substrate. Although glucagon is known to regulate amino acid transporters in rodent hepatocytes (Kilberg, Barber et al. 1985), more recently several amino acid transporters targeted by glucagon have emerged. For example, in a study investigating amino acids as a biomarker for the GCGR activity of GLP-1R/GCGR co-agonists, GCGR activity and plasma amino acids correlate well with expression levels of several amino acid transporters in the liver (Li, Kirchner et al. 2020). These include the neutral and basic amino acid transporter (*Slc3a1*), high affinity cationic amino acid transporter 1 (*Slc7a1*), sodium coupled neutral amino acid transporters 2 and 3 (*Slc38a2* and *Slc38a3*, respectively). These findings are consistent with pre-clinical models of glucagon resistance induced by fatty liver in addition to hepatic specific *Gcgr* knockout mice, whereby several hepatic amino acid transporters are down-regulated (Winther-Sorensen, Galsgaard et al. 2020). In addition to the above, a mechanism by which amino acid transport is acutely enhanced has been suggested through glucagon-stimulated hyperpolarisation of the hepatocyte (Edmondson and Lumeng 1980). Together these findings suggest that glucagon orchestrates a multi-faceted response to enhance ureagenesis through both increasing amino acid uptake (substrate supply) and amino acid catabolic processes.

2.1.3 Evidence that native glucagon increases lean mass loss and potential mechanisms involved

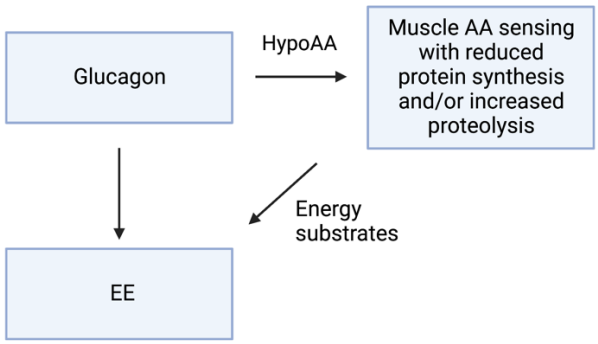
Given that glucagon enhances hepatic amino acid catabolism and lowers plasma amino acids, it is reasonable to hypothesise an effect on lean mass. One of the earliest studies to document the interaction between glucagon and lean mass demonstrated that nitrogen is released from skeletal muscle *ex vivo* in glucagon treated rats (Peterson, Beatty et al. 1963). Later on, isotopic infusion studies with native glucagon in humans allowed determination of the acute and sub-acute effects of physiological doses of glucagon on whole body protein balance. These studies gave important indications of the effect of glucagon on lean mass, for example skeletal muscle. Studies of acute physiological increases in glucagon (525 pg/kg/min achieving glucagon concentrations of 349 pmol/l) or even sustained increases over 5 days (1 mg/day infusions) in humans demonstrated no change in markers of muscle proteolysis (Pozefsky, Tancredi et al. 1976, Wolfe,

Culebras et al. 1979). In contrast, other studies in humans also using physiological doses of glucagon, show increased markers of muscle proteolysis and reduced markers of protein synthesis with short term glucagon infusions studies (Nair, Halliday et al. 1987, Charlton, Adey et al. 1996); In these studies, circulating levels of glucagon achieved were approximately 100 – 200 pmol/l. The inconsistent effect on muscle in these studies may be due to the dose-ranges used as all achieved high-physiological levels of circulating glucagon, where higher doses or longer-term exposure may be required. Indeed, in humans, the physiological range of circulating glucagon varies in the basal and fasted setting from below 20 pmol/l up to 3-4 times higher during hypoglycaemia (Knop, Vilsboll et al. 2007).

In excess however, the effect of chronic glucagon exposure on lean mass loss have been clearly observed in two main situations, firstly observations of the glucagonoma syndrome and secondly observations of critical illness. The glucagonoma syndrome is associated with marked reduction in plasma amino acid levels, lean mass loss and functional decline (Stevens, Flanagan et al. 1984, Almdal, Heindorff et al. 1990). Furthermore, the circulating levels of glucagon may reach approximately 1500 pmol/l (Almdal, Heindorff et al. 1990). In mouse models of critical illness, increased plasma glucagon is associated with increased hepatic amino acid catabolism, reduction in plasma amino acid levels and increased markers of muscle proteolysis, however other catabolic hormones likely play a contributing role (Thiessen, Derde et al. 2017).

Theoretically, glucagon-induced lean mass loss at high physiological or pathological circulating levels of glucagon may be due to direct sensing of low plasma amino acid availability (see Chapter 1). The amino acid substrates may also provide an energy source for glucagon-mediated EE (Schematic 1). To maintain energy balance, the liberation of energy substrates from loss of lean mass would be expected to be utilised by an energy consuming process. Therefore, it is possible that glucagon-stimulated hypoaminoacidemia facilitates amino acid supply from lean mass to fuel processes of glucagon-mediated energy expenditure.

Schematic 1: Potential mechanisms of glucagon-mediated lean mass loss. Created using Biorender.



Although amino acid availability may directly affect various steps in protein pathways in muscle (Chapter 1, Section 1.10), with regards to glucagon administration much focus has been given to leucine, an important positive regulator mTORC1 and protein synthesis (Adeva-Andany, Fernandez-Fernandez et al. 2021). Furthermore, using stable isotopes of leucine, the rate of muscle proteolysis (leucine flux) and synthesis (non-oxidative leucine flux) can be estimated. This is because leucine is an essential BCAA which may be oxidised in muscle tissue to liberate energy and amino groups for amino acid release into the circulation. Glucagon infusions to achieve physiological circulating levels have been shown to enhance leucine oxidation and leucine flux (marker of proteolysis) in skeletal muscle (Nair, Halliday et al. 1987, Nair, Woolf et al. 1987, Hartl, Miyoshi et al. 1990, Pacy, Cheng et al. 1990, Charlton, Adey et al. 1996). Leucine oxidation is also increased during fasting in humans (Fryburg, Barrett et al. 1990), suggesting that low amino acid availability enhances leucine oxidation in muscle. As leucine is a positive regulator of mTOR, these findings suggests that glucagon action, through stimulating hypoaminoacidemia and leucine oxidation may result in the suppression of protein synthesis. Indeed, infusions of glucagon in humans reduce markers of protein synthesis (Pacy, Cheng et al. 1990). Furthermore, in a human study, an infusion of glucagon lowered plasma amino acids and attenuated skeletal muscle protein synthesis in response to an amino acid mixture (Charlton, Adey et al. 1996). In addition to the amino group released from muscle leucine oxidation, it has been suggested that the suppression of muscle protein synthesis in response to glucagon allows valuable amino acids to be re-directed to the liver for glucose production (Adeva-Andany, Fernandez-Fernandez et al. 2021). Therefore, through studying the interaction between glucagon and leucine, a plausible

mechanisms of lean mass loss is through the combined effect of enhanced proteolysis and reduced protein synthesis.

Interestingly, following an early study in Rabbit where muscle blood amino acids were measured, it was first suggested that glucagon-stimulated reduction in amino acids may be the stimulus to release of amino acids from skeletal muscle, sustaining gluconeogenesis (Daniel, Pratt et al. 1977). Although leucine metabolism has been discussed, the mechanism by which glucagon may reduce lean/muscle mass is likely complex and may involve multiple individual amino acid sensing (Moro, Ebert et al. 2016). These aspects of glucagon-mediated lean mass loss remain unexplored.

2.1.4 Implications of using a long-acting GCGR agonist for metabolic disease, on plasma amino acids and lean mass

Glucagon action has emerged more recently as a potentially useful component in long-acting unimolecular multi-agonists, as a treatment for obesity and metabolic disease (Hope, Vincent et al. 2021). The benefits conferred by targeting the GCGR in pre-clinical models include increased energy expenditure, improved hepatic lipid catabolism, and reduced food intake (Al-Massadi, Ferno et al. 2019). Indeed, original seminal and recent reports of unimolecular multi-agonists highlight some of these metabolic benefits attributed to GCGR-signalling in rodents (Day, Ottaway et al. 2009, Knerr, Mowery et al. 2022). However, as glucagon promotes hepatic catabolism of amino acids, the chronic effect of glucagon on plasma amino acid availability and therefore lean mass becomes important, particularly in the context of the use of long-acting GCGR-targeted multi-agonists in a therapeutic setting. If these long-acting multi-agonists lead to reduced plasma amino acid availability through sustained GCGR activation, it is possible that lean mass may ensue through sensing pathways in lean tissue, for example skeletal muscle. As such, it may be possible to rescue a reduction in plasma amino acids to mitigate any effect on lean mass, whilst preserving metabolic benefits of GCGR agonism, for example energy expenditure and hepatic lipid metabolism. Answering the initial question of whether a long-acting glucagon analogue leads to hypoaminoacidemia is topical and important given the drive to develop GCGR-targeted multi-agonists, which leverage the thermogenic

and lipid catabolic properties of glucagon for the treatment of obesity and metabolic disease.

More recently, the first evidence emerged that GCGR-targeted multi-agonists lead to a reduction in plasma amino acids. In these pre-clinical studies, plasma amino acids have been shown to be a specific biomarker of GCGR activity when assessing the receptor balance in GLP-1R/GCGR co-agonists (Li, Kirchner et al. 2020). This therefore provides an *in vivo* 'read-out' or confirmation of GCGR activity during pre-clinical drug development. As a result, a handful of early clinical trials have now also reported hypoaminoacidemia associated with novel GCGR-targeted multi-agonists, primarily as a marker and confirmation of GCGR activity (Parker, Robertson et al. 2020, Bossart, Wagner et al. 2021). Interestingly, for these agonists, human GCGR receptor potencies vary from 2- to 10-fold less compared to native glucagon, highlighting wider drug characteristics on GCGR activity in these clinical studies.

Evidence for lean mass loss with glucagon analogues is scarce and comes from pre-clinical models. A previous study using a long-acting glucagon analogue, IUB288, demonstrated a reduction in lean mass and lack of fat mass gain in lean mice fed a high fat diet (Habegger, Stemmer et al. 2013). Using the same peptide, another group demonstrated that a three-week treatment with the long-acting glucagon analogue led to preferential loss of lean mass with no effect on fat mass (Okun, Rusu et al. 2021). In the latter study, using co-culture experiments with liver and muscle tissue *ex vivo*, enhanced hepatic alanine catabolism in a model of obesity/T2D leads to reduced muscle protein synthesis, suggested through reduction in the mTORC1 pathway. It is important to note that the long-acting glucagon analogue, IUB288 has a sizeably increased activity at the GCGR relative to native glucagon, and peak circulating levels achieved after a single dose reach approximately 5000 pmol/l.

2.1.5 Implications of the pharmacodynamic and pharmacokinetic properties of GCGR-targeted agonists on hepatic amino acid catabolism and plasma amino acids

To investigate a metabolic outcome following native hormone or analogue administration, an understanding of the pharmacodynamic (PD) and pharmacokinetic

(PK) properties are required. Whilst the PD properties of a substance pertain to the peptide-receptor interaction, the PK properties range from absorption, distribution, metabolism, and excretion. In Section 2.1.1, it was highlighted that native glucagon enhances amino acid uptake and catabolism. High doses of native glucagon were required due to its short half-life and clearance within minutes (Pontiroli, Calderara et al. 1993); in one study this is equivalent to approximately 300 nmol/kg in rodents (McLean and Novello 1965). To circumvent this issue, human studies used continuous intravenous glucagon infusions to investigate the glucagon-protein interaction and reach high physiological circulating levels (Section 2.1.2) (Wolfe, Culebras et al. 1979, Charlton, Adey et al. 1996). In other pre-clinical models, zinc-glucagon was administered to rabbits following single-dose administration demonstrating a prolonged effect on blood amino acids for up to 12 hours (Bromer and Chance 1969). The combination of zinc and glucagon was found to enhance the pharmacokinetics of glucagon by delaying absorption through depot formation. Since then, several strategies have been employed to increase the circulating half-life of glucagon and also receptor potency by modifying the primary sequence and peptide tail; These have allowed further characterisation of glucagon biology *in vivo* (Habegger, Stemmer et al. 2013). Due to the sequence similarity between glucagon, GLP-1, and oxyntomodulin, similar modifications have been made to either the GLP-1 or oxyntomodulin sequences to enhance GLP1R/GCGR receptor co-agonism with favourable PK profiles in a unimolecular agonist (Hope, Vincent et al. 2021). It is therefore not surprising that reports have emerged of hypoaminoaciemia associated with the use of long-acting GCGR targeted multi-agonists in rodents and humans (Li, Kirchner et al. 2020, Parker, Robertson et al. 2020, Bossart, Wagner et al. 2021). Indeed, the effect of long-acting glucagon agonists on plasma amino acids has been leveraged to provide a sensitive biomarker for GCGR activity (Li, Kirchner et al. 2020). The extent hypoaminoacidemia in the context of longer-acting GCGR-targeted multi-agonists will therefore depend on both variations in PD and PK properties.

2.1.6 G108 as a research tool to explore the effects of sustained GCGR agonism on hepatic amino acid catabolism, plasma amino acids and lean mass

To initially explore the chronic effects of a long-acting GCG analogue on hepatic amino acid catabolism, plasma amino acid levels and lean mass, peptide G108 was selected as a

research tool for these pre-clinical studies. In this Chapter, the effect of G108 on the murine GCGR and GLP-1R will first be determined. Single dose-administration studies in mice will also be carried out to confirm the effect on molecular markers of hepatic amino acid and carbohydrate metabolism, in addition to plasma amino acids. The dependency of the hepatic GCGR will be determined for the effects of G108 on hepatic amino acid metabolism and plasma amino acids.

Whether chronic treatment with a glucagon receptor agonist leads to hypoaminoacidemia associated with lean mass loss is unknown. In this Chapter, to further determine whether a GCGR agonism chronically leads to hepatic amino acid catabolism, hypoaminoacidemia and lean mass loss, the effects of chronic G108 administration were explored. To better understand the effect primarily on lean mass, a lean mouse model is used. To determine the food-intake independent effects of potential appetite suppressive peptides, a pair-fed paradigm can be used. However, due to the limitations associated with the practicality of pair feeding studies, a 'sub-anorectic' dose of G108 was first identified and then used to explore the specific effects of altered plasma amino acid levels on lean mass. This work will confirm whether G108 is an appropriate long-acting GCGR agonist to explore the chronic effect on plasma amino acid availability and lean mass in a pre-clinical model.

2.2 Chapter summary and aims

- Determine the murine GLP-1R and GCGR receptor potency of peptide G108.
- Determine the pharmacokinetic profile in plasma of G108 following single dose administration.
- Determine the acute *in vivo* effects of G108 on plasma amino acids.
- Determine the acute *in vivo* effects of G108 on hepatic amino acid metabolism.
- Determine the acute *in vivo* effects of G108 on glycaemia.
- Explore the effect of G108 on hepatic amino acid catabolism and plasma amino acids in mice lacking the hepatic glucagon receptor.

2.3 Methods

2.3.1 Peptide G108

Peptide G108 was synthetised using solid-phase synthesis, is based on the amino acid sequence of oxyntomodulin (OXM) and purchased from WuXi AppTec, China. 19 amino acid modifications were made, conferring a predicted increased potency at the GCGR whilst reducing GLP-1R potency. The pharmacokinetic profile was enhanced by the addition of a proline-linked N-terminal octadecanoic fatty acid tail. Following synthesis, the peptide was purified with reverse phase high performance liquid chromatography (HPLC). Prior to administration, the peptide was diluted in HPLC grade water.

2.3.2 Animal husbandry

All studies were carried out in accordance with the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012. 8-14-week-old lean male C57BL/6J mice and 8 week old male Wistar rats were purchased from Charles River, Margate, UK. Hepatic specific glucagon knockout mice were purchased from Jackson laboratories. To generate $Gcgr^{\Delta liver}$ mice, $Gcgr^{flox/flox}$ mice were crossed with hemizygous *Albumin-Cre* mice. This generated $Gcgr^{flox/flox}$*:Alb-Cre±* (*$Gcgr^{\Delta liver}$*) mice and $Gcgr^{flox/flox}$*:Alb*-Cre- (control) mice. The Cre-recombinase status of all mice was confirmed with genotyping studies. Animals were housed at 21^{o}C under standard 12-hour light cycle. Food and water were provided *ad libitum*.

2.3.3 Diets

Three chronic *in vivo* studies were conducted. For the chronic dose finding study and the chronic study in *Gcgr*$^{\Delta liver}$ mice, a standard RM1 diet was used (Special Diets Services). For the chronic single dose study in C57BL/6J lean mice, a custom diet with a 'standard' kcal composition of protein was used (Research Diets, Table 2.1).

Diet	Carbohydrate (kcal%)	Fat (kcal%)	Protein (kcal%)
RM1 chow diet	65	20	15
D11112201	65	15	20

Table 2.1: Diet composition. Approximate values.

2.3.4 Murine receptor potency experiments

Murine glucagon receptor (mGCGR) or GLP-1 receptor (mGLP-1R) plasmids (Sinobiological) were reconstituted in sterile-water at a concentration of 200 ng/μl. Lipofectamine 2000 (Invitrogen) was then used to encapsulate plasmids and stably transfect HEK293T cells. Cells were then incubated for 48 hours prior to cAMP accumulation assays. 12.5 μl of ascending dilutions from a serial dilution of either G108 or the native ligands GLP-1 and glucagon, were added to 12.5 μl of plasmid-transfected cells in a 96-well plate. The transfected cells were then stimulated for measurement of cAMP accumulation for 30 mins at 37^{o}C, using the cAMP-GS dynamic assay kit (Cisbio). The plate was then read using a SpectraMax i3x reader.

2.3.5 Pharmakokinetic profile study

The pharmacokinetic study was carried out in 10-week-old male Wistar rats (N=4). Each animal was administered subcutaneously with 0.5 mg of G108. Blood samples were obtained via tail venesection at baseline immediately prior to injection and at 1.5, 3, 6-, 24-, 48- and 72-hours following injection. Blood samples were collected on ice prior to centrifugation at 8000 g for 10 mins at 4^{o}C. The resulting plasma supernatant was stored at -80^{o}C prior to further radioimmunoassay analysis. Specific antiserum was produced in rabbits containing antibodies raised against the purified glucagon (1-16) fragment. This

was specifically able to cross-react with the N-terminal glucagon-like sequence of G108. Iodine-125 glucagon was used in the assay which was incubated for 3 nights at 4^{o}C prior to the separation phase (free vs antibody-bound label) with charcoal. A γ-counter was then used to quantify the ratio of free to bound label from the pellet and supernatant. The plasma peptide concentration was calculated using two phase exponential decay and comparing readings with the standard curve.

2.3.6 *In vivo* single dose administration studies

12-week-old lean C57BL/6J mice were administered with a single subcutaneous dose of 20 nmol/kg G108 (diluted in sterile HPLC water) or vehicle control (sterile HPLC water) (N=5-8/group). Tail venesection was carried out immediately prior to injection at 9 am and 2-hours after injection with 50 μl of blood collected on ice. On a separate occasion, 20 nmol/kg G108 was administered subcutaneously followed by collection of blood samples at 2, 24 and 48 hours. Blood samples were centrifuged at 2800 g for 10 minutes prior to storage of plasma at -80^{o}C. Plasma was subsequently used for measurement of total amino acids. For hepatic amino acid catabolism gene expression, a further cohort of 12-week-old lean C57Bl/6J mice (N=4/group) were administered with a single subcutaneous dose of 20 nmol/kg G108 or vehicle control, prior to dissection of liver tissue at 24 hours and storage at -80^{o}C. For analysis of plasma markers of amino acid catabolism in obese mice, 12-week-old lean C57BL/6J were provided *ab libitum* with a 60 kcal% high fat diet for 8-10 weeks, prior to single dose administration of 20 nmol/kg G108 or vehicle control (N=5-7/group). Blood was collected by tail vein venesection as described above with the addition of a 24-hour time point. Plasma samples were subsequently used to measure plasma total amino acids, alanine and urea. For studies in transgenic mice, a cohort of wild-type and *Gcgr*$^{\Delta liver}$ lean mice were used for hepatic gene expression studies and plasma amino acid measurements. Hepatic gene expression analysis and plasma amino acid measurement was carried out 24-hours following a single dose of 20 nmol/kg G108.

For the glucose profile study following G108 administration, 12-week-old lean C57BL/6J mice (N=6/group) were fasted for 2 hours prior to either an intraperitoneal injection of 10 nmol/kg G108 or vehicle control (N=6/group). A tail vein blood drop sample was

collected immediately prior to treatment injection and blood glucose was measured with a handheld GlucoRx meter. Subsequent tail vein blood samples were collected for glucose levels at 15, 30, 60 and 120 minutes after treatment injection. A subsequent longer glucose time-course study was carried out in 12-week-old lean C57BL/6J mice (N=8-10) over 24 hours following a single subcutaneous dose of 20 nmol/kg G108.

2.3.7 Chronic dose finding study

Twenty-four 14-week-old C57BL/6J mice were split into four groups based on treatment allocation: Vehicle control, 5 nmol/kg G108, 10 nmol/kg G108 and 20 nmol/kg G108. All mice were individually housed prior to commencing treatment and had *ad lib* access to RM1 diet and water. Daily subcutaneous administration of treatment at 9 am each day was tracked with daily food intake and body weight measurements over 18 days. Body composition measured by MRI was assessed at baseline prior to commencing treatment and day 4, 7, 12, 14 and 18 (Section 2.3.10). Grip strength measurements were taken at baseline and on days 7 and 18 (Section 2.3.12). A tail venesection was performed at baseline and on day 7 prior to measurement of total plasma amino acid levels. An intraperitoneal glucose tolerance test was also performed on day 14 of the study (Section 2.3.11).

2.3.8 Chronic 5 nmol/kg G108 administration in lean mice

Fourteen 14-week-old C57BL/6J mice were single housed and split into two groups based on treatment allocation: Vehicle control (HPLC water) or 5 nmol/kg G108. Mice were administered at 9 am with a daily subcutaneous dose of either Vehicle control or G108 for 21 days. Mice had *ad libitum* access to a standard diet consisting of 20 kcal% protein (diet D11112201, Table 2.1). Food intake and body weight were measured daily. Body composition measured by MRI was assessed at baseline prior to commencing treatment and day 7, 14 and 21. Blood samples were collected on day 7 of the study, prior to- and 4-hours following treatment administration. These were subsequently used to obtain plasma for measurement of total amino acid levels. An intraperitoneal glucose tolerance test was carried out on day 14 (Section 2.3.11). A forelimb grip strength test was carried out at baseline, day 14 and 21 (Section 2.3.12). At the end of the study on day 21, five

muscle groups were dissected and weighed: quadriceps, gastrocnemius, soleus, tibialis anterior and extensor digitorum longus.

2.3.9 Chronic 5 nmol/kg G108 administration in hepatic specific glucagon knockout mice (*Gcgr*$^{\Delta liver}$)

Thirty two 12-week old hepatic *Gcgr*$^{-/-}$ (Gcgr$^{flox/flox}$:Alb-Cre±) and control (Gcgr$^{flox/flox}$:Alb-Cre-) mice received a daily subcutaneously dose of either 5 nmol/kg G108 or vehicle control for 14 days. Body weight and food intake was measured every 48 hours. Body composition measured by MRI was assessed at baseline prior to commencing treatment and on day 14. Blood samples were also collected on day 14 of the study prior to treatment administration.

2.3.10 Food intake, body weight and body composition (EchoMRI)

Food intake and body weight was measured at 9am daily, 24 to 48-hourly in all studies using a calibrated weighing scale (Ohaus Traveller, Ohaus). Food was topped up as required. An EchoMRI analyser was used to measure body composition at various timepoints during the studies. Body composition was measured at the same time of day on each occasion. To measure body composition, each animal was placed into a Perspex cylinder which was then carefully inserted into the MRI analyser (EchoMRI – 100H). Following each individual scan, the animal was placed back into its respective cage. Data was obtained for both lean and fat mass.

2.3.11 Glucose tolerance tests

Intraperitoneal (IP) glucose tolerance tests were carried out following a 5 hour fast and subcutaneous administration of either vehicle control or 5 nmol/kg G108. Each animal was administered with IP glucose (2 g/kg body weight). A blood sample was collected via tail venesection at baseline prior to treatment administration and at 20, 40, 60 and 90-minutes following treatment administration. A handheld glucose monitor (GlucoRx, UK) was used to measure blood glucose at each time point.

2.3.12 Forelimb grip strength

Forelimb grip strength was measured in mice using a dedicated grip strength meter (Bioseb, France). Each animal was carefully lowered onto a grip strength bar and allowed to grip. Gentle retraction of the tail allowed the grip to release at which point the maximum grip force applied to the bar was measured and recorded (g). Three to four measurements were made with an interval period of 1 minute. An average of readings was then subsequently made.

2.3.13 Biochemical analysis of plasma

Total plasma amino acids were measured using commercially available fluorometric-based ELISA assays as directed (MAK002, Sigma, Ab65347, Abcam). Plasma alanine and urea levels were measured using an ELISA assay (MAK001, Sigma and MAK006, Sigma).

2.3.14 Quantitative polymerase chain reaction (qPCR) analysis.

1ml of Trizol (Sigma) was added to each Eppendorf containing a frozen piece of liver tissue. A small 5 mm steel bead was also added to each Eppendorf tube. All samples were then homogenised with a mechanical homogeniser for 2-3 minutes. 200 μl of chloroform was then added to each sample prior to further centrifugation at 12000 g for 15 minutes. 400 μl of supernatant was removed with a pipette into separately labelled Eppendorf tubes. 70% isopropanolol (2-propanol) was then added to the samples at an equal volume (400 μl). The mixture was gently mixed prior to centrifugation again at 16000 g for 15 minutes. The resulting RNA pellet was washed three times using 70% ethanol. After allowing the residual ethanol to precipitate, the RNA pellet was resuspended in 100-150 μl of sterile RNAse-free water, and stored at -80^{o}C. RNA was then quantified using a NanoDrop spectrophotometer (NanoDrop™). 1 ug of RNA was used to synthesise cDNA (iScript cDNA synthesis kit, Bio-Rad UK). qPCR analysis was carried out using the SYBR-green method using the following primers: m_nags_1, m_cps1_1, m_ass1_1, m_asl_1. m_arg_1, m_g6pc_1, m_pepck_1, m_pcg_1, m_sds_1, m_pygl_1, m_slc22a2_1, m_slc22a3_1, m_ Slc22a22_*1* (kickstart primers, Bio-Rad, UK).

2.4 Results

Key results for Chapter 2

- **Peptide G108 is a long-acting CGGR agonist.**
- **Single-dose administration of G108 in lean and obese mice increases gene expression of hepatic amino acid catabolism and reduces plasma amino acids.**
- **Hepatic GCGR is required for the acute effect of G108 on hepatic amino acid catabolism and plasma amino acids in lean mice.**
- **Following transient hyperglycaemia, a single dose of G108 leads to improved blood glucose levels for up to 24 hours in lean mice.**
- **A 'sub-anorectic' dose of G108 causes GCGR-dependent chronic hypoaminoacidemia and lean mass loss in lean mice.**

2.4.1 G108 is a selective GCGR agonist *in vitro*

Prior to *in vivo* testing, peptide G108 was designed and manufactured based on the primary sequences of the endogenous GLP-1R/GCGR co-agonist, oxyntomodulin (OXM) and glucagon. Several amino acid modifications were made with enhanced predicted potency to the GCGR and reduced potency to the GLP-1R. Furthermore, an octadecandioic fatty acid tail was also incorporated at the C-terminus, allowing reversible binding to albumin. The sequence modifications compared to OXM and glucagon are shown in Figure 2.4.1.



Figure 2.1: Amino acid sequence of peptide G108 compared to native glucagon and native oxyntomodulin (OXM). From Hope, Hinds et al. (In press). Underlined amino acids represent modifications made and red amino acids were conserved. B=aminoisobutyric acid.

Receptor potency studies measuring cyclic-AMP accumulation revealed four-fold increased potency at the GCGR and 32-fold less potency at the GLP-1R (Figure 2.2, Table 1).

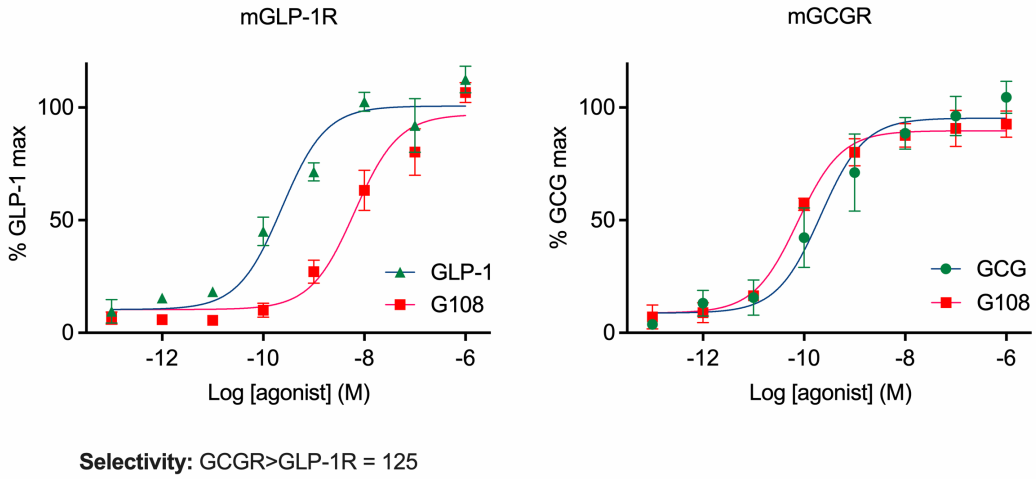


Figure 2.2: Receptor potency of G108 at the murine GLP-1R and GCGR receptors in HEK293T cells. From Hope, Hinds et al. (In press) n=4, data expressed as mean ± SEM.

	G108	**GCG**	**GLP-1**
mGCGR	10.1 ± 0.1	9.5 ± 0.5	n.d.
mGLP-1R	8.2 ± 0.3	n.d.	9.7 ± 0.1

Table 2.1: Receptor potency as measured by cAMP accumulation for G108 at both the GLP-1R and CGGR. From Hope, Hinds et al. (In press). Data expressed as log10-transformed pEC50 comparisons (M) for G108 and endogenous ligands.

2.4.2 G108 has an extended pharmacokinetic profile in plasma

Following a single 0.5 mg intraperitoneal dose of G108 in male Wistar rats, the plasma level of G108 increased rapidly to a peak mean of 12057 pmol/L (Figure 2.3). This gradually fell over 48 hours (2038 pmol/L) and retuned to baseline by 72 hours (126.4 pmol/L).

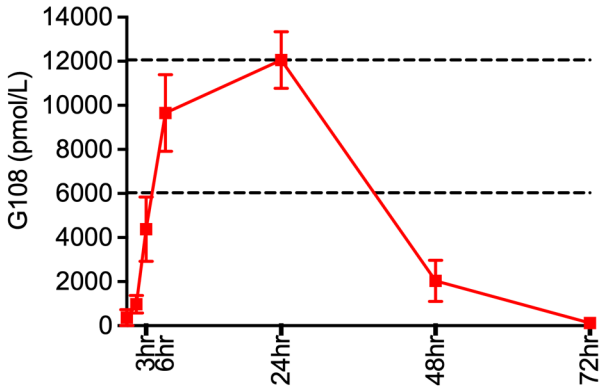


Figure 2.3: Pharmacokinetic profile of G108 in male Wistar rats. From Hope, Hinds et al. (In press). N=4, data expressed as mean ± SEM.

2.4.3 Single dose administration of G108 in lean and obese mice reduces total plasma amino acids

Following a single dose of 20 nmol/kg G108, total plasma amino acids were reduced significantly over 2 hours from baseline, compared to vehicle control (Figure 2.4A, mean ± SEM, baseline level: Vehicle control; 2.83 ± 0.24 nmol/µl, G108; 3.08 ± 0.17 nmol/µl, 2-hour level: Vehicle control; 2.96 ± 0.12 nmol/µl, G108; 2.49 ± 0.23 nmol/µl). To confirm these findings and further determine the longevity of G108-mediated hypoaminoacidemia, a time course was carried out with plasma analysis for total amino acids over 48 hours. This showed a significant reduction in total plasma amino acids over 24 hours which then returned to baseline at 48 hours (Figure 2.4B, mean ± SEM, baseline level; 2.97 ± 0.08 nmol/µl, 2-hour level 2.56 ± 0.21 nmol/µl, 24-hour level; 2.42 ± 0.21 nmol/µl, 48-hour level; 3.18 ± 0.22 nmol/µl).

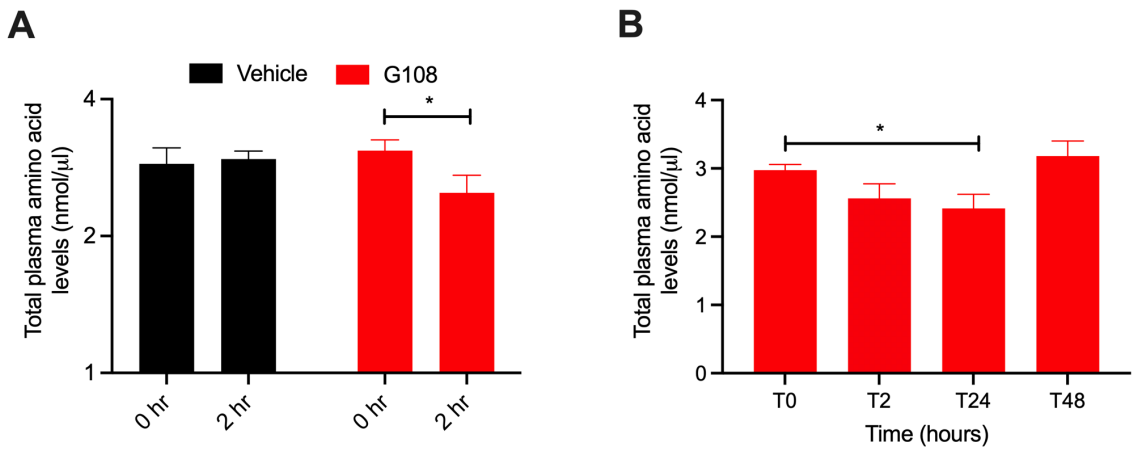


Figure 2.4: (A) Total plasma amino acids at baseline and 2 hr following single-dose subcutaneous administration of either 20 nmol/kg G108 or vehicle control. **(B)** Time-course profile of plasma amino acids following single dose subcutaneous admiration of 20 nmol/kg G108. Data expressed as mean ± SEM. *p<0.05. Analysed with a combination of one- and two-way ANOVA with post hoc Sidak and Dunnett tests.

'Glucagon resistance' in obesity associated hepatic steatosis has been demonstrated to affect hepatic amino acid catabolism in rodents and humans (Suppli, Bagger et al. 2020, Winther-Sorensen, Galsgaard et al. 2020). Therefore, the potential for a long-acting glucagon agonist, for example G108 to cause hypoaminoacidemia in an obese mouse model was explored. 20 nmol/kg G108 or vehicle control was subcutaneously administered to diet-induced-obese (DIO) mice. In addition to total plasma amino acid levels, a major glucogenic amino acid and sensitive marker of glucagon action (Wewer Albrechtsen, Faerch et al. 2018), alanine, was also measured. Plasma urea levels were also measured. A single dose of G108 led to a significant reduction in total plasma amino acids at 24 hours, with a trend towards a reduction in total plasma amino acids at 2 hours (Figure 2.5A, mean ± SEM, baseline total plasma amino acids: Vehicle; 6.19 ± 0.40 nmol/µl, G108; 6.40 ± 0.29 nmol/µl, 2-hour levels: Vehicle; 5.82 ± 0.80 nmol/µl, G108; 6.08 ± 0.22 nmol/µl, 24-hour levels: Vehicle 5.92 ± 0.18 nmol/µl, G108; 4.85 ± 0.30 nmol/µl). A significant reduction in plasma alanine was observed at both 2- and 24-hours following G108 administration, with a significant reduction also found following vehicle administration at 2 hours (Figure 2.5B, mean ±_SEM, baseline plasma alanine: Vehicle; 8.89 ± 0.30 nmol/µl, G108; 8.75 ± 0.60 nmol/µl, 24-hour levels: Vehicle; 9.40 ± 0.62 nmol/µl, G108; 4.54 ± 0.22 nmol/µl). Reflecting reduced plasma amino acid availability (substrate supply) at 24 hours (Figure 2.5A), plasma urea levels were also significantly reduced at the 24-hour time point following G108 administration (Figure 2.5C, mean ±

SEM, baseline plasma urea: Vehicle; 3.31 ± 0.16 ng/µl, G108; 3.25 ± 0.35 ng/µl, 24-hour levels: Vehicle 3.30 ± 0.23 ng/µl, G108 2.15 ± 0.16 ng/µl).

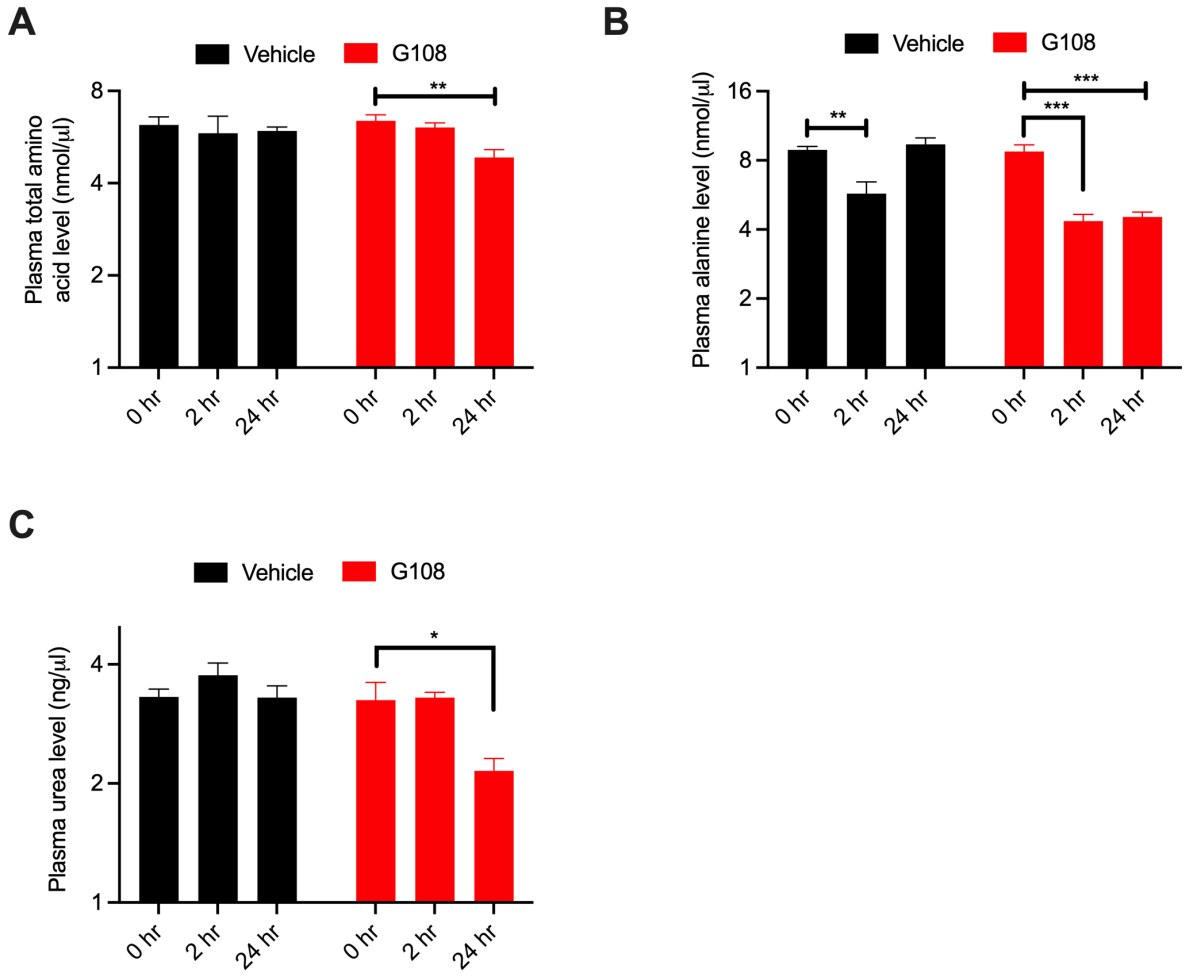


Figure 2.5: Total plasma amino acid, alanine and urea levels in diet-induced obese (DIO) mice following subcutaneous administration of either 20 nmol/kg G108 or vehicle control. Data expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. Analysed using two-way ANOVA with post hoc Sidak's test.

2.4.4 Single dose administration of G108 leads to upregulation of hepatic amino acid catabolism and gluconeogenesis.

The maximum reduction in total plasma amino acid levels were observed at 24 hours in lean (Figure 2.4B) and obese mice (Figure 2.5A) were consistent with the pharmacokinetic profile of G108 (Figure 2.3). Therefore, gene expression analysis of key hepatic gluconeogenic and urea cycle pathways were determined at 24 hours following a single dose of G108 in lean mice. At 24 hours, compared to vehicle control, there was a significant upregulation in the expression levels of multiple urea cycle enzymes (Figure

2.6A, Table 2.2). An increase in expression levels of the rate-limiting gluconeogenesis enzymes glucose-6-phosphatase (*G6p*) and phosphoenol pyruvate carboxykinase (*Pepck*) was observed in addition to glycogen phosphorylase (*Pygl*), serine dehydratase (*Sds*) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargc1a*) (Figure 2.6B, Table 2.2). Consistent with the actions of glucagon, these data demonstrate the upregulation of hepatic protein catabolic and carbohydrate glucose mobilising pathways in response to G108.

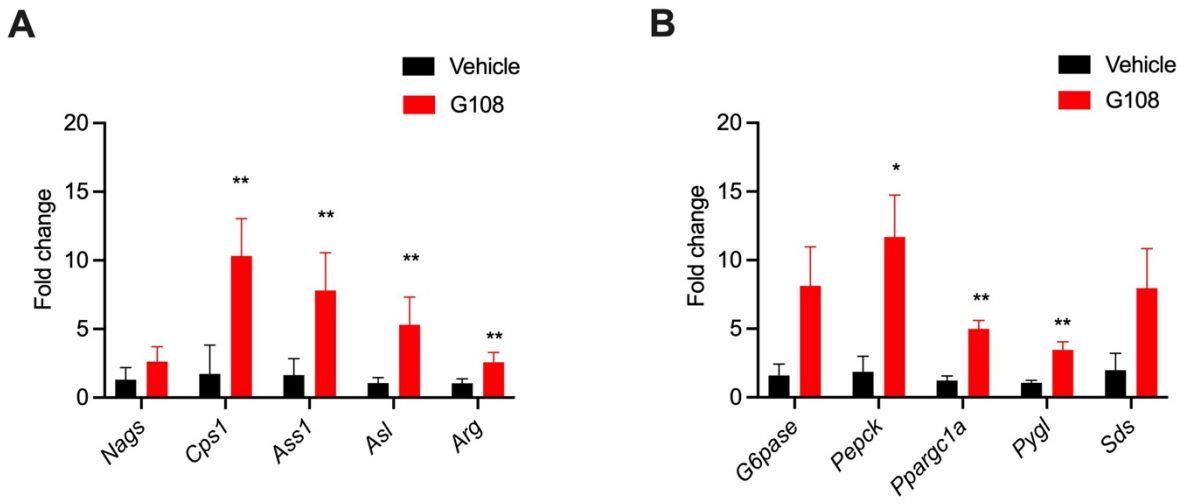


Figure 2.6 – Gene expression data 24 hours following administration of 20 nmol/kg G108. From Hope, Hinds et al. (In press). **(A)** Hepatic urea cycle enzymes. **(B)** Hepatic glucogenesis and carbohydrate metabolism enzymes. N-acetylglutamate synthetase deficiency (*Nags*), Carbamoyl phosphate synthase 1 (*Cps1*), Arginosuccinate synthase 1 (*Ass1*), Arginosuccinate lyase (*Asl*), Arginase (*Arg),* Glucose 6-phosphatase (*G6pase*), Phosphoenolpyruvate carboxykinase (*Pepck*), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α),* Glycogen phosphorylase (*Pygl*), Serine dehydratase (*Sds*). Data expressed as mean ± SEM, significance indicates change compared to vehicle control. *p<0.05, **p<0.01, analysed using unpaired t-test.

Target gene	Vehicle mean fold change (mean ± SEM)	20 nmol/kg G108 mean fold change (mean ± SEM)
N- acetylglutamate synthetase (*Nags*)	1.31 ± 0.44	2.63 ± 0.54
Carbamoyl phosphate synthase 1 (*Cps1*)	1.72 ± 1.05	10.34 ± 1.36 ***P*<0.01**
Arginosuccinate synthase 1 (*Ass1*)	1.65 ± 0.60	7.81 ± 1.38 ***P*<0.01**
Arginosuccinate lyase (*Asl*)	1.05 ± 0.20	5.31 ± 1.00 ***P*<0.01**
Arginase (*Arg*)	1.05 ± 0.17	258 ± 0.36 ***P*<0.01**
Glucose 6-phosphatase (*G6pase*)	1.60 ± 0.83	8.12 ± 2.86
Phosphoenolpyruvate carboxykinase (*Pepck*)	1.86 ± 1.14	11.70 ± 3.06 ***P*<0.05**
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1 alpha*)	1.23 ± 0.33	4.99 ± 0.61 ***P*<0.01**
Glycogen phosphorylase (*Pygl*)	1.06 ± 0.19	3.46 ± 0.59 ***P*<0.01**
Serine dehydratase (*Sds*)	1.98 ± 1.24	7.97 ± 2.89

Table 2.2: Tabulated gene expression data 24 hours following administration of 20 nmol/kg G108. From Hope, Hinds et al. (In press). Data expressed as mean ± SEM. *p<0.05, **p<0.01.

2.4.5 Single dose administration of G108 leads to acute dynamic changes in blood glucose.

Based on the increased expression of glycogenolytic and gluconeogenic enzymes following G108 dosing, to understand the acute drug effect on blood glucose, a glucose profile was performed. This included multiple timepoints within the first 2 hours and timepoints subsequently at 4- and 24-hours. A single 20 nmol/kg subcutaneous dose of G108 led to a hyperglycaemic excursion at 15 minutes which retuned to levels seen in the vehicle control group at 30 minutes (Figure 2.7A). The blood glucose levels then further reduced at 60 minutes and 2 hours compared to the vehicle control group (Figure 2.7A). A separate glucose profile for mice treated with G108 was consistent with this and demonstrated a reduced glucose level compared to baseline at 4- and 24-hours (Figure 2.7B).

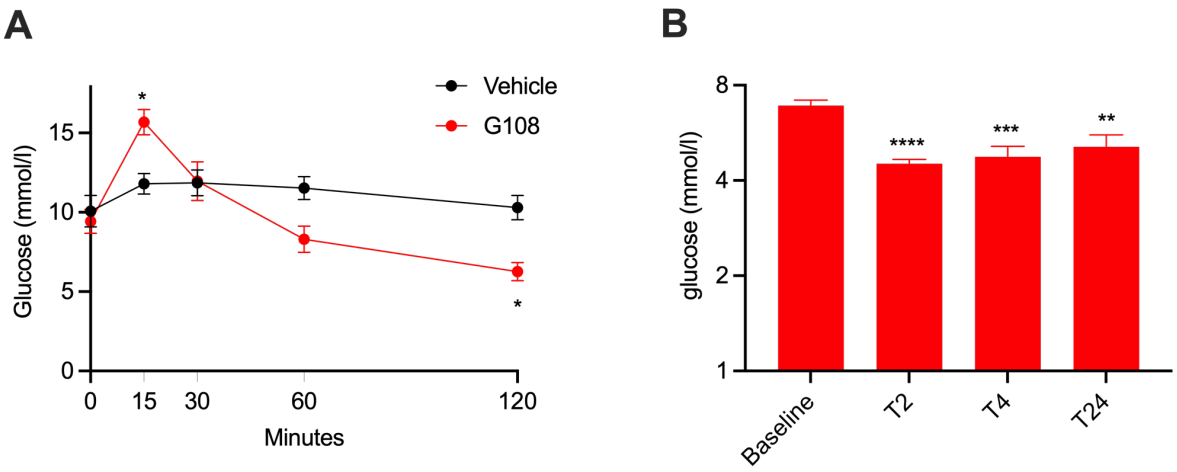


Figure 2.7 (A) Blood glucose profile over 2 hours following a single 10 nmol/kg subcutaneous dose of G108 or vehicle control in lean C57BL/6J mice (n=6/group). From Hope, Hinds et al. (In press). **(B)** Blood glucose profile over 24 hours following single 20 nmol/kg subcutaneous dose of G108 in lean C57BL/6J mice (n=8-10/group). Data expressed as mean ± SEM. Significance indicates change compared to vehicle control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Analysed using a combination of one- and two-way ANOVA with post hoc Sidak's or Dunnett's tests.

2.4.6 G108-mediated hepatic amino acid catabolism and hypoaminoacidemia is dependent on the presence of the hepatic glucagon receptor.

The acute effect of G108 on hepatic urea cycle upregulation was next determined in mice lacking the hepatic glucagon receptor. A single 20 nmol/kg dose of G108 or vehicle control was administered to both WT and hepatic specific *Gcgr*$^{\Delta liver}$ mice. After 4 hours, the mouse dissected, and liver collected for PCR analysis. Compared to wild-type mice receiving vehicle control, a significant increase in the expression of several genes central to hepatic amino acid uptake and catabolism was observed in wild-type mice receiving a single dose of G108 (Figure 2.8A, Table 2.3). A trend towards increased gene expression was also observed in *Gcgr*$^{\Delta liver}$ mice.

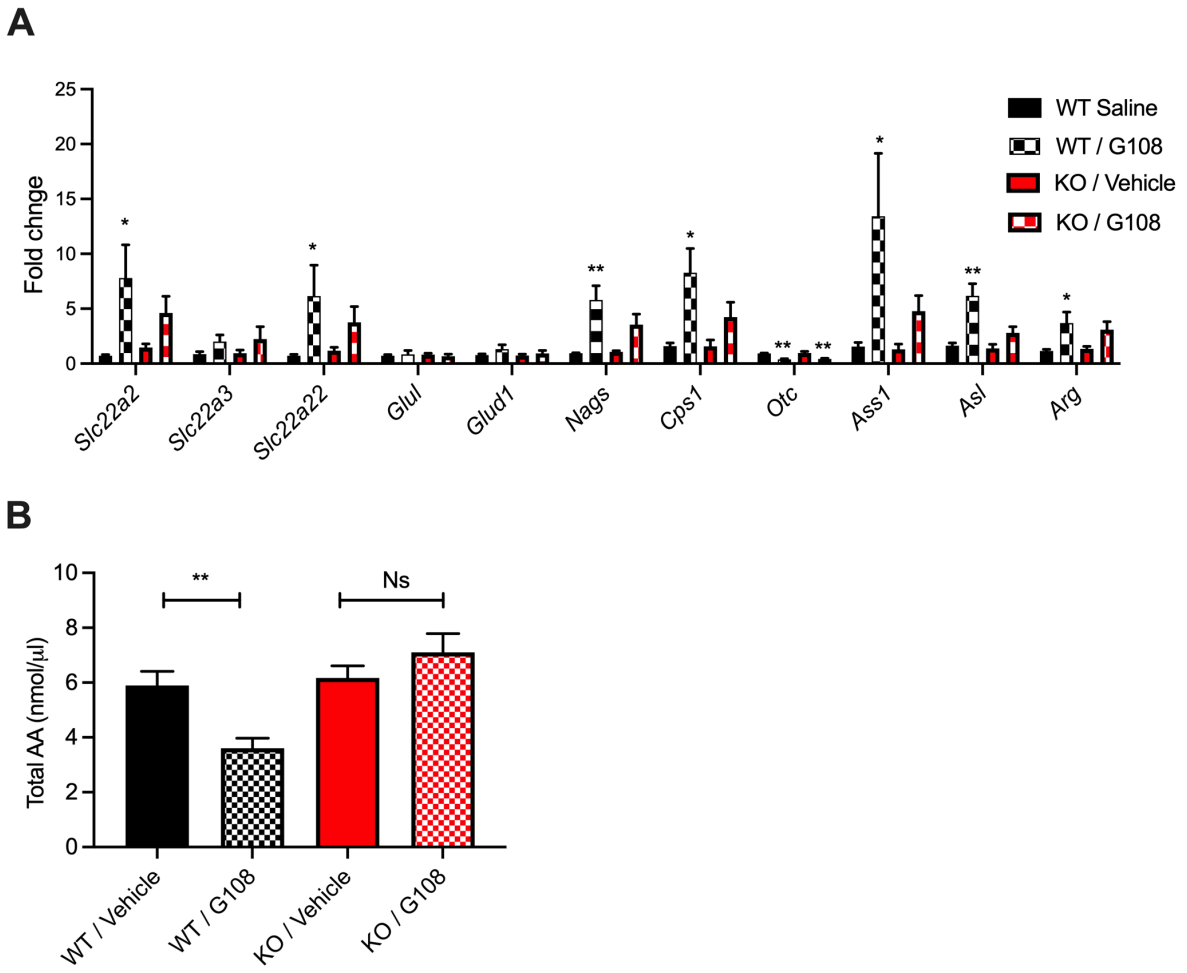


Figure 2.8: (A) Hepatic urea cycle enzyme expression 24-hours post administration of 20 nmol/kg G018. Solute Carrier Family 22 Member 2 (Slc22a2), Solute Carrier Family 22 Member 3 (Slc22a3), Solute Carrier family 22 member 22 (Slc22a22), Glutamine synthetase (*Glul*), Glutamate dehydrogenase 1 (*Glud1*), N-acetylglutamate synthetase (*Glul*), Glutamate dehydrogenase 1 (*Glud 1*), N-acetylglutamate suynthetase (*Nags*), Carbamoyl phosphate synthase 1 (*Cps1*), Ornithine transcarbamylase (*Otc*), Arginosuccinate Synthase 1 (*Ass1*), Arginosuccinate lyase (*Asl*), Arginase (*Arg*). **(B)** Plasma total amino acid levels 24-hours following a single dose of 20 nmol/kg G108. Data expressed as mean ± SEM, n=4/group. Significance indicates change compared to vehicle control. *p<0.05, **p<0.01, ***p<0.001. Data analysed using one-way ANOVA with post hoc Dunnett's test.

Target gene	WT / Vehicle mean fold change (mean ± SEM)	WT / 20 nmol/kg G108 mean fold change (mean ± SEM)	hKO / Vehicle mean fold change (mean ± SEM)	hKO / 20 nmol/kg G108 mean fold change (mean ± SEM)
Solute Carrier Family 22 Member 2 (*Slc22a2*)	0.71 ± 0.11	7.80 ± 3.02 ***P*<0.05**	1.46 ± 0.34	4.62 ± 1.51
Solute Carrier Family 22 Member 3 (*Slc22a3*)	0.87 ± 0.23	2.01 ± 0.61	0.95 ± 0.28	2.24 ± 1.12
Solute Carrier family 22 member 22 (*Slc22a22*)	0.72 ± 0.12	6.16 ± 2.82 ***P*<0.05**	1.17 ± 0.31	3.76 ± 1.43
Glutamine synthetase (*Glul*)	0.73 ± 0.10	0.85 ± 0.34	0.82 ± 0.14	0.68 ± 0.18
Glutamate dehydrogenase 1 (*Glud1*)	0.80 ± 0.09	1.12 ± 0.41	0.73 ± 0.13	0.92 ± 0.27
N- acetylglutamate synthetase (*Nags*)	0.95 ± 0.05	5.80 ± 1.29 ***P*<0.01**	1.07 ± 0.11	3.54 ± 0.97
Carbamoyl phosphate synthase 1 (*Cps1*)	1.60 ± 0.29	8.29 ± 2.21 ***P*<0.05**	1.58 ± 0.57	4.23 ± 1.37
Ornithine transcarbamylase (*Otc*)	0.92 ± 0.06	0.39 ± 0.06 ***P*<0.01**	0.97 ± 0.15	0.44 ± 0.06 ***P*<0.01**
Arginosuccinate synthase 1 (*Ass1*)	1.55 ± 0.38	13.43 ± 5.71 ***P*<0.05**	1.31 ± 0.47	4.70 ± 1.43
Arginosuccinate lyase (*Asl*)	1.63 ± 0.25	6.17 ± 1.10 ***P*<0.01**	1.38 ± 0.90	2.81 ± 0.55
Arginase (*Arg*)	1.16 ± 0.14	3.70 ±0.97 ***P*<0.05**	1.35 ± 0.23	3.09 ± 0.75

Table 2.3. Tabulated gene expression data 24 hours following administration of 20 nmol/kg G108. Data expressed as mean ± SEM. *p<0.05, **p<0.01.

As the significant G108-mediated upregulation of hepatic amino acid catabolic pathways was only observed in the presence of the hepatic GCGR, the effect on plasma amino acid levels was also determined. A single dose of 20 nmol/kg G108 or vehicle control was administered to lean WT and *Gcgr*$^{\Delta liver}$ mice. After 24 hours, total plasma amino acid levels were measured (Figure 2.8B). Compared to the WT group administered with vehicle control, a significant reduction in total plasma amino acid levels was observed in only the WT mice administered with G108 (Figure 2.8B, mean ± SEM, 24-hour cross sectional result: WT / Vehicle; 5.89 ± 0.51 nmol/μl, WT / G108; 3.60 ± 0.37 nmol/μl, KO / Vehicle; 6.20 ± 0.44 nmol/μl, KO / G108; 7.10 ± 0.68 nmol/μl).

2.4.7 Chronic dose finding with G108 reveals the sub-anorectic dose range

To determine the dose-dependent effects of peptide G108 on body weight and food intake in a chronic setting, various doses of G108 were subcutaneously administered daily to lean mice for 18 days. Three doses of G108 were chosen, 5 nmol/kg, 10 nmol/kg and 20 nmol/kg. Over 18 days the two higher peptide doses, 10 nmol/kg and 20 nmol/kg led to a significant reduction in body weight to a maximum body weight loss of -8.53 ± 0.39 g and -15.93 ± 3.54 g, respectively (Figure 2.9A and 2.9B). The two higher peptide doses also led to a reduced total food intake over 18 days compared to vehicle control (Figure 2.9C and 2.9D).

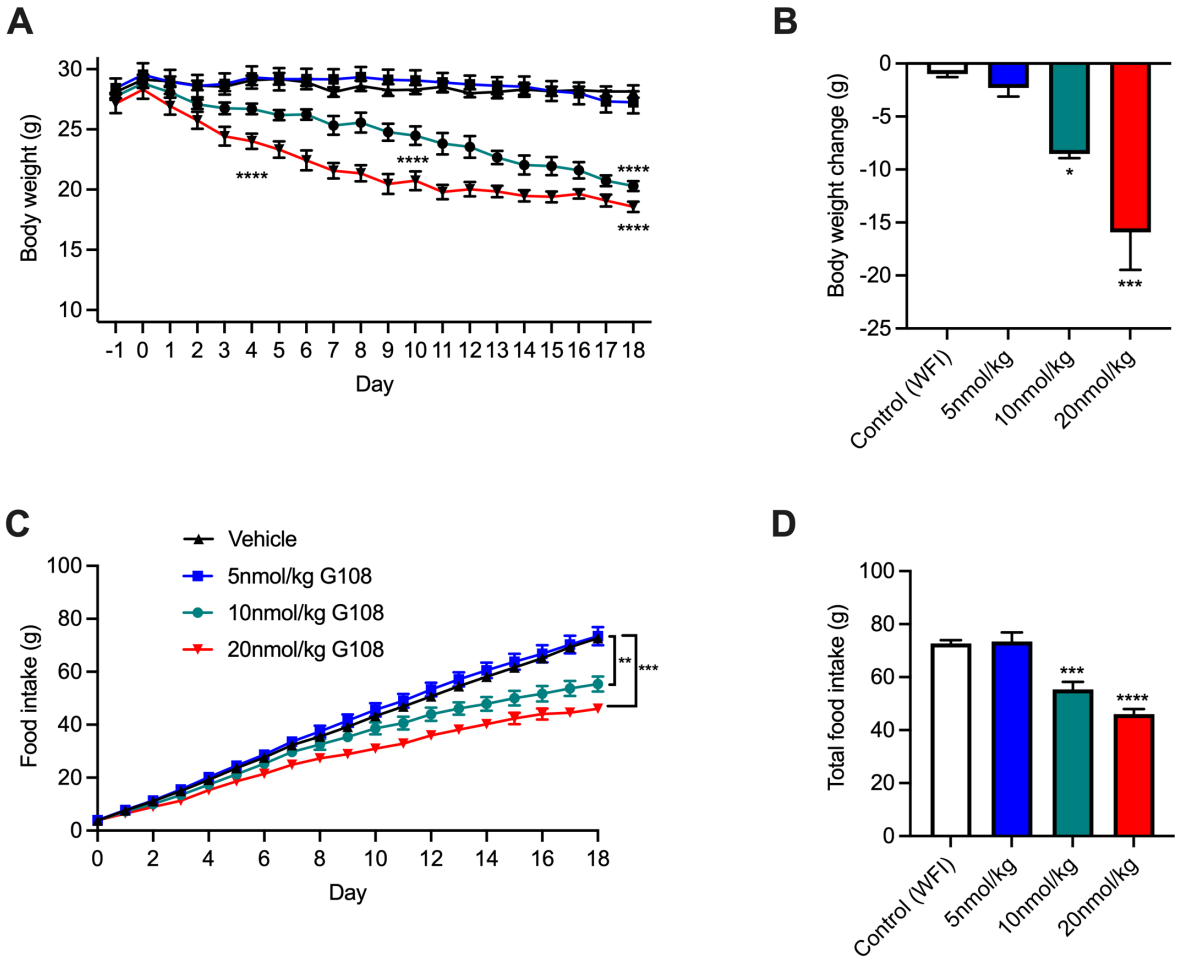


Figure 2.9: Body weight and food intake, n=6/group. **(A)** Daily body weight (g). **(B)** Total body weight loss (g) at study day 18. **(C)** Cumulative food intake (g). From Hope, Hinds et al. (In press). **(D)** Total food intake (g) at study day 18. Data expressed as mean ± SEM. Significance indicates change compared to vehicle control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data analysed using one-way ANOVA and post hoc Dunnett's test.

2.4.8 Chronic dose finding with G108 reveals dose-dependent effects on plasma amino acid levels, lean mass, and muscle strength

In addition to dose-dependent effects on food intake and body weight (Section 2.4.7), dose-dependent effects were observed on body composition including both lean and fat mass (Figure 2.10A and 2.10B change in lean mass from baseline to day 18: Control (WFI); -1.71 ± 0.30 g, 5 nmol/kg G108; -3.2 ± 0.65 g, 10 nmol/kg G108; -7.12 ± 0.21 g, 10 nmol/kg G108; -7.44 ± 0.65 g, Figure 2.10C and 2.10D change in fat mass from baseline to day 18: Control (WFI); 0.33 ± 0.20 g, 5 nmol/kg G108; 0.29 ± 0.46 g, 10 nmol/kg G108; -2.31 ± 0.37 g, 20 nmol/kg G108; -2.71 ± 0.31 g).

Consistent with the effects of G108 observed on lean mass, a dose-dependent effect of G108 was also seen in muscle strength, as measured by fore-limb grip strength. The highest peptide dose led to a significant reduction in fore-limb grip strength compared to baseline (Figure 2.10E grip strength change from baseline to day 18: Control (WFI); 7.02 ± 12.19 g, 5 nmol/kg G108; -28.22 ± 25.27 g, 10 nmol/kg G108; -41.29 ± 22.15 g, 20 nmol/kg G108; -91.96 ± 29.80 g).

To explore whether plasma total amino acid levels are associated with lean mass loss and functional muscle loss, plasma levels were measured after 7 days of daily treatment. A significant reduction from baseline in total plasma amino acids was observed in the two higher peptide dose groups, 10 nmol/kg and 20 nmol/kg G108 (Figure 3.2G). After 7 days, a trend toward reduced total plasma amino acids was observed at the lowest 5 nmol/kg dose of G108 (Figure 2.10G).

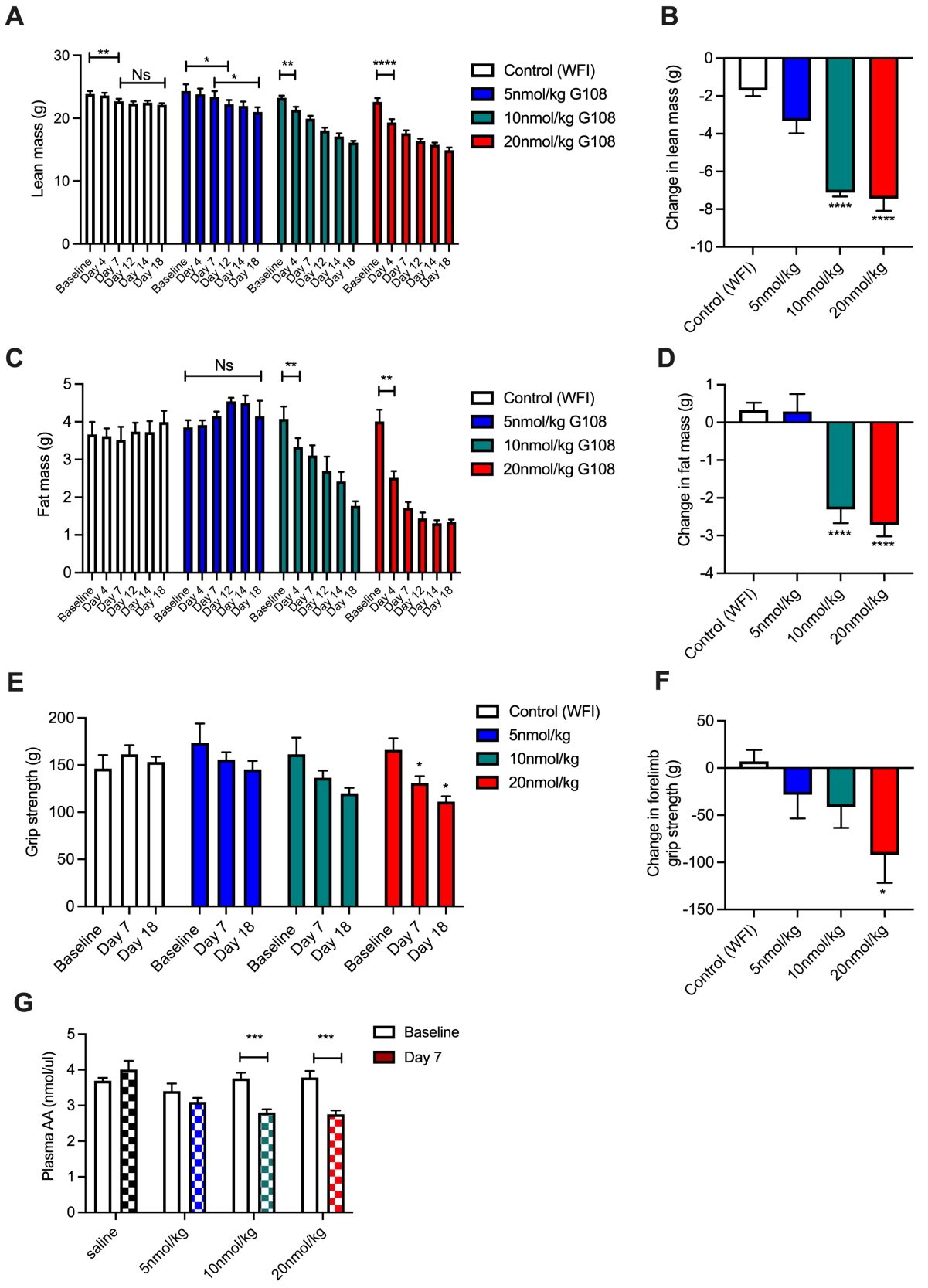


Figure 2.10: Body composition, forelimb grip strength and plasma amino acids, n=6/group. **(A)** Change in lean mass (g) at days 4, 7, 12, 14 and 18, compared to baseline. **(B)** Change in lean mass from baseline at

day 18 (g). **(C)** Change in fat mass (g) at days 4, 7, 12, 14 and 18, compared to baseline. **(D)** Change in fat mass from baseline at day 18 (g). **(E)** Change in grip strength (g) from baseline on day 7 and day 18 of study, significance indicates change from baseline. **(F)** Change in forelimb grip strength (g) from baseline at day 18, significance indicates change compared to vehicle control. **(G)** Change in total plasma amino acid levels from baseline after 7 days of treatment. Data expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data analysed using one-way or two-way ANOVA and post hoc Dunnett or Sidak test's.

2.4.9 Chronic dose finding with G108 reveals bidirectional changes in glucose tolerance.

To explore the effect of chronic glucagon agonist treatment on glycaemia, an intraperitoneal glucose tolerance test was performed on study day 14. Compared to vehicle control, a significant improvement in glucose tolerance was observed in the lowest 5 nmol/kg G108 dose after 30 minutes of a 2 g/kg glucose bolus (Figure 2.11A). No significant difference was observed in the higher dose peptide groups following the same glucose bolus and the highest 20 nmol/kg G108 dose led to a worsening of glucose tolerance. The change in glycaemia was also observed from incremental area under the curve (AUC) analysis of the intraperitoneal glucose tolerance test (Figure 2.11B).

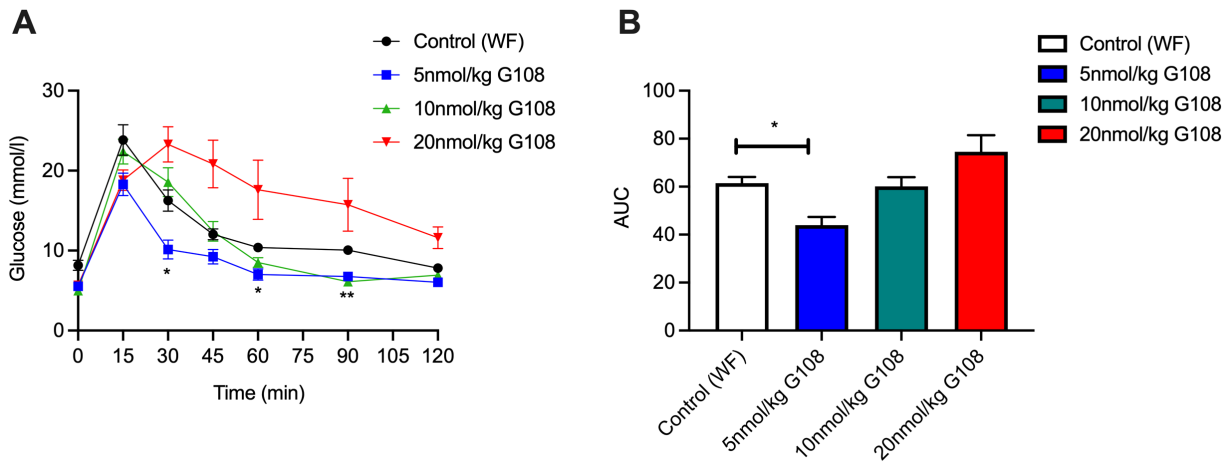


Figure 2.11: Glucose tolerance and AUC glucose, n=6/group. **(A)** Intraperitoneal glucose tolerance test (IPGTT), 2 g/kg. **(B)** Incremental area under the curve (AUC) glucose. Data expressed as mean ± SEM. Significance indicates change compared to vehicle control. *p<0.05, **p<0.01, Data analysed using one and two-way ANOVA and post hoc Dunnett's test.

2.4.10 Chronic sub-anorectic administration of G108 leads to hypoaminoacidemia and lean mass loss

To confirm and further explore the effect of chronically administered G108 on plasma amino acid levels and lean mass, a further chronic dosing study was carried out. In this study, two groups of lean mice were administered daily with either vehicle control or 5 nmol/kg G108, for 21 days (Figure 2.12). Primary outcomes for the study included plasma total amino acid levels, body composition, muscle function as measured by grip strength, and glucose tolerance.

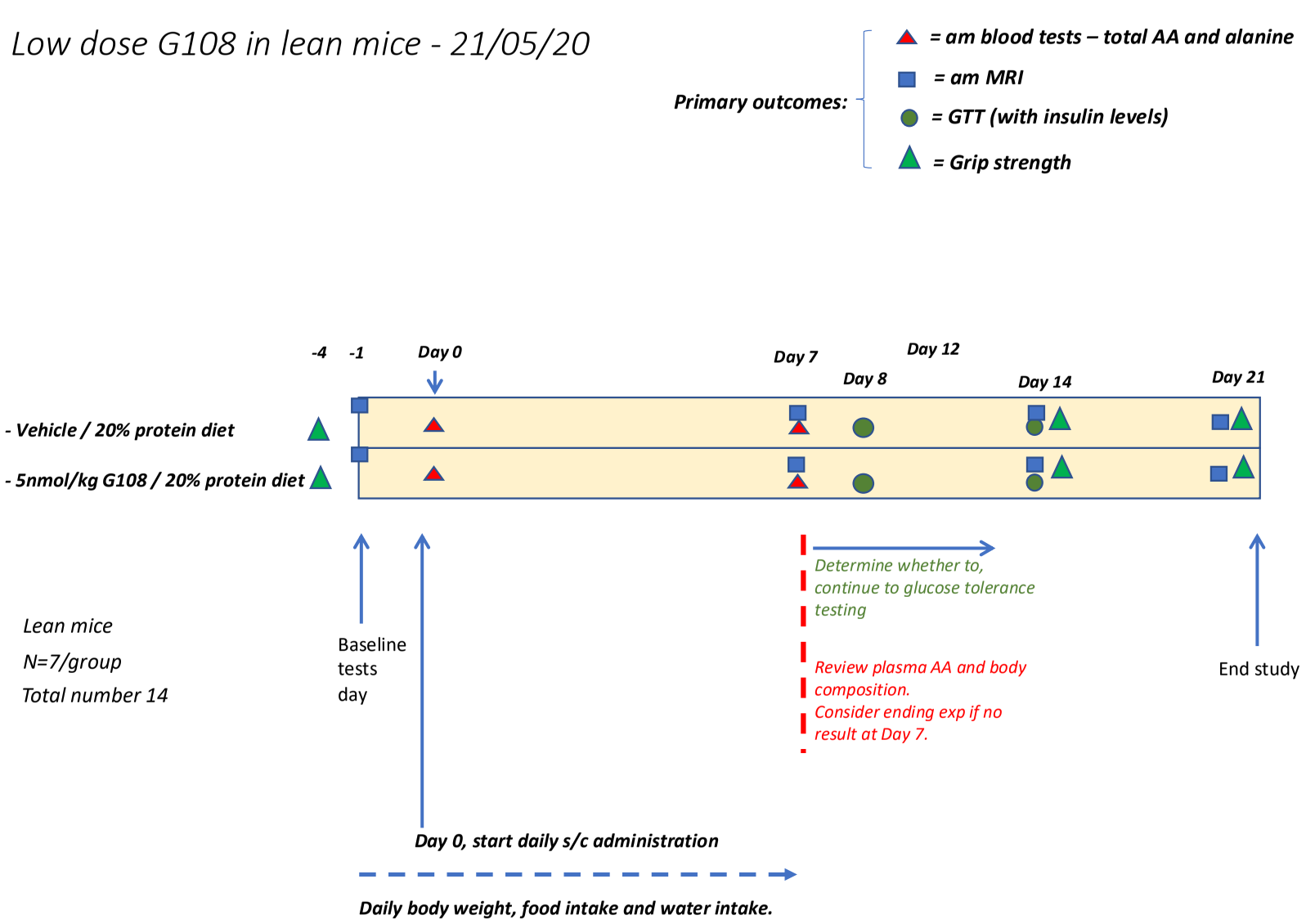


Figure 2.12: Schematic of 5 nmol/kg G108 chronic study plan.

Over 21 days there was no difference observed in the food intake consumed between the two groups (Figure 2.13A, total food consumed: Vehicle; 62.76 ± 2.33 g, 5 nmol/kg G108; 63.49 ± 1.34 g). A suppression of total body weight gain from baseline was observed in the 5 nmol/kg G108 group compared to vehicle control, which reached statistical significance from study day 8 (Figure 2.13B). Body composition analysis, as measured by MRI, demonstrated a significant reduction in lean mass from baseline in the G108 group, an effect not observed in the vehicle control group (Figure 2.13C and 2.13D, percentage lean mass loss from baseline in vehicle group: Day 7; 0.48 ± 0.95 %, Day 14; 0.53 ± 0.89 %, Day 20; -0.05 ± 0.87 %, percentage lean mass loss from baseline in 5 nmol/kg G108 group: Day 7; -4.33 ± 0.80 %, Day 14: -6.13 ± 0.89%, Day 20: -8.10 ± 1.51%). In contrast to these findings, fat mass increased throughout the study in both groups (Figure 2.13E and 2.13F, percentage fat mass loss from baseline in vehicle group: Day 7; 15.04 ± 2.74 %, Day 14; 26.12 ± 4.34 %, Day 20; 35.76 ± 7.38 %, percentage fat mass loss from baseline in 5 nmol/kg G108 group: Day 7; 26.82 ± 5.84 %, Day 14; 39.67 ± 6.65 %, Day 20; 47.21 ± 8.40 %). Consistent with the change in lean mass, a significant reduction in plasma total amino acid levels were observed in only the 5 nmol/kg G108 group on study day 7, 4 hours following treatment administration (Figure 2.13G, plasma total amino acid levels in 5 nmol/kg G108 group: baseline; 3.14 ± 0.22 nmol/μl, 4-hour post dose: 2.16 ± 0.15 nmol/μl).

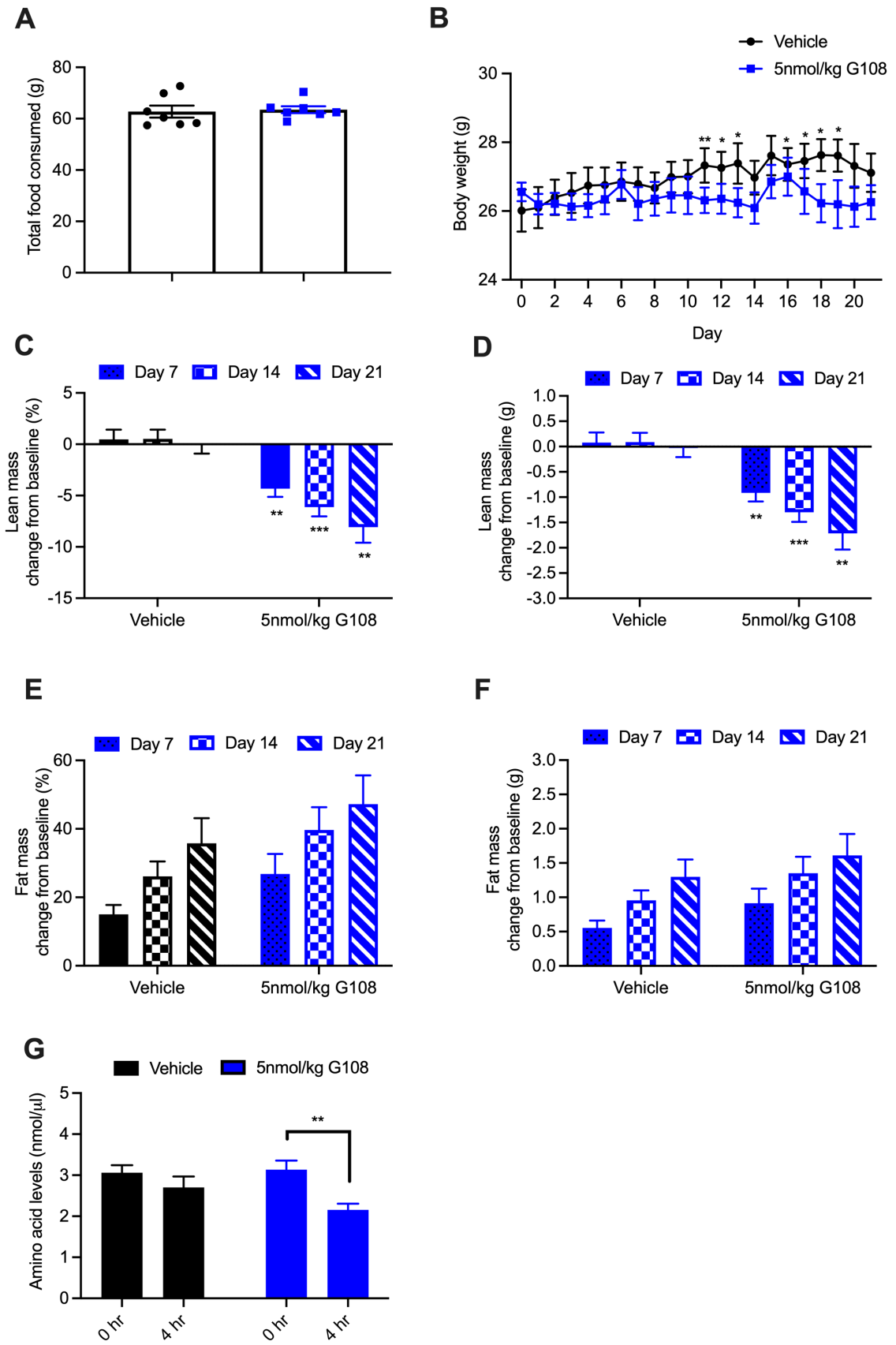

A
Total food consumed (g)
80
60
40
20
0
B
Vehicle
5nmol/kg G108
Body weight (g)
30
28
26
24
0 2 4 6 8 10 12 14 16 18 20
Day
C
Day 7
Day 14
Day 21
Lean mass change from baseline (%)
5
0
-5
-10
-15
Vehicle
5nmol/kg G108
D
Day 7
Day 14
Day 21
Lean mass change from baseline (g)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
Vehicle
5nmol/kg G108
E
Day 7
Day 14
Day 21
Fat mass change from baseline (%)
60
40
20
0
Vehicle
5nmol/kg G108
F
Day 7
Day 14
Day 21
Fat mass change from baseline (g)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
5nmol/kg G108
G
Vehicle
5nmol/kg G108
Amino acid levels (nmol/µl)
5
4
3
2
1
0
0 hr
4 hr
0 hr
4 hr

Figure 2.13: Food intake, body weight, body composition and total plasma amino acid levels in chronic 21-day study with administration of either vehicle control or 5 nmol/kg G108, n=7/group. From Hope, Hinds et al. (In Press). **(A)** Total food intake over 21 days. **(B)** Body weight over 21 days. **(C)** Change in lean mass from baseline (%) on day 7, 14 and 20. **(D)** Change in lean mass from baseline (g) on day 7, 14 and 20. **(E)** Change in fat mass from baseline (%) on day 7, 14 and 20. **(F)** Change in fat mass from baseline (g) on day 7, 14 and 20. **(G)** Plasma total amino acid levels at baseline and 4-hours post treatment on day 7 of study. Data expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. Significance indicates change compared to vehicle control. Data analysed using one and two-way ANOVA and post hoc Dunnett's or Sidak's tests.

2.4.11 Chronic sub-anorectic administration of G108 leads to reduced muscle weight and function

To determine the effect of G108-mediated lean mass loss on skeletal muscle, muscle function and weight were assessed. Forelimb grip strength tests revealed a significant reduction in grip strength in the 5 nmol/kg G108 group on day 15 and 21 compared to baseline (Figure 2.14A, forelimb grip strength (g) in 5 nmol/kg G108 group: Baseline grip strength; 145.71 ± 6.63 g, Day 15 grip strength; 115.90 ± 6.76 g, Day 21 grip strength; 87.60 ± 5.21 g). On study completion, muscle weights in the 5 nmol/kg G108 group were significantly lower in the largest hindlimb muscle groups including the quadriceps and gastrocnemius (Figure 2.14B, quadriceps muscle weight (g): vehicle; 0.22 ± 0.01 g, 5 nmol/kg G108; 0.18 ± 0.01 g, gastrocnemius muscle weight (g): vehicle; 0.22 ± 0.01 g, 0.17 ± 0.01 g)

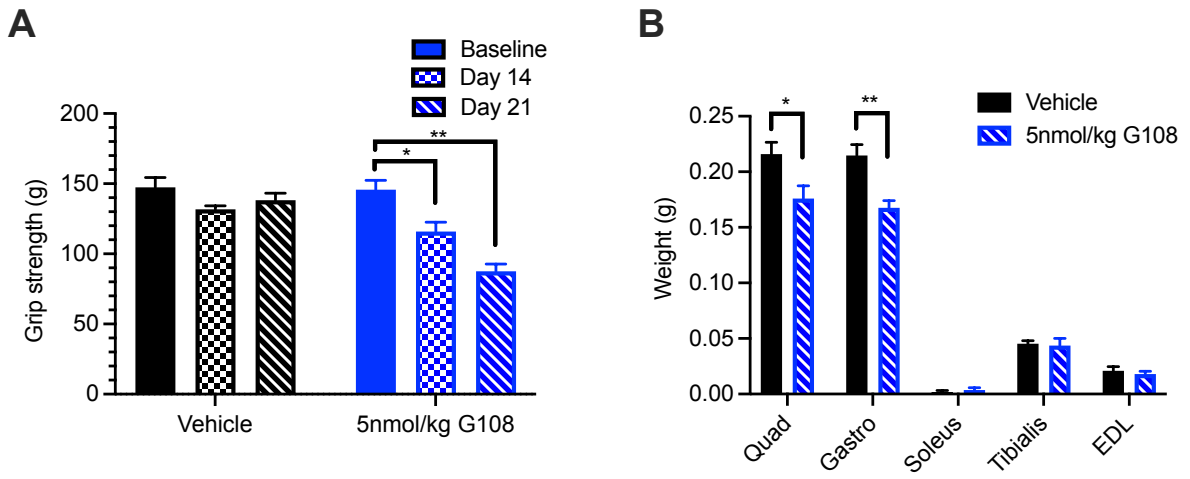


Figure 2.14: Forelimb grip strength and terminal muscle weights from 21-day daily treatment with either vehicle control or 5 nmol/kg G108, n=7/group. From Hope, Hinds et al. (In Press). **(A)** Forelimb grip strength (g) at baseline, day 14 and 21. **(B)** Muscle weights (g) from quadriceps (Quad), gastrocnemius

(Gastro), soleus, tibialis anterior (tibialis) and extensor digitorum longus (EDL). Data expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. Data analysed using a combination of unpaired t-tests and two-way ANOVA with post hoc Sidak's test.

2.4.12 Chronic sub-anorectic administration of G108 leads to improved glucose tolerance.

To determine whether daily treatment with 5 nmol/kg G108 leads to a chronic improvement in glycaemia, a glucose tolerance test was performed on day 14 of the 21-day study. G108 led to a significant reduction in blood glucose compared to vehicle control, 40 minutes following a glucose bolus (Figure 2.15A). Furthermore, the area under the curve for the glucose tolerance test revealed a significant reduction in AUC Figure 2.15B).

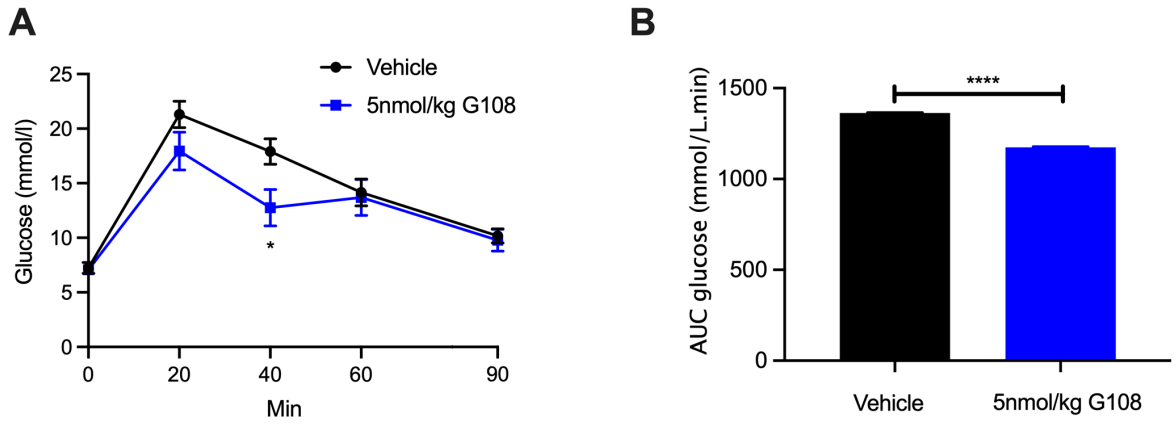


Figure 2.15: Intraperitoneal glucose tolerance test (iPGTT) on day 14 of 21-day daily treatment study with vehicle control or 5 nmol/kg G108, n=6-7/group. **(A)** iPGTT profile. **(B)** Incremental area under the curve (AUC) of iPGTT as in Figure 3.7A. Data expressed as mean ± SEM. Significance indicates change compared to vehicle control. *p<0.05, ***p<0.001. Glucose profile analysed using two-way ANOVA with post hoc Dunnett's test. AUC data analysed with paired t-test.

2.4.13 Loss of lean mass with chronic sub-anorectic administration of G108 is dependent on the presence of the hepatic glucagon receptor.

In section 2.4.10 and 2.4.11, the effect of G108 on lean mass and skeletal muscle was demonstrated. The effect of G108 on total plasma amino acid levels raise the possibility that G108-stimulated hepatic amino acid catabolism leads to indirect lean mass and muscle loss. To demonstrate the link between G108-stimulated hepatic amino acid catabolism, plasma hypoaminoacidemia and lean mass loss, the chronic effect of G108 in mice lacking the hepatic glucagon receptor was determined.

Daily administration of 5 nmol/kg G108 over 14 days to lean wild-type (WT) mice led to a significant reduction in total body weight compared to vehicle control, despite no change in total food intake (Figure 2.16A and 2.16B, total body weight loss at 14 days: vehicle / WT; 1.21 ± 0.14 g, 5 nmol/kg G108 / WT; -2.93 ± 1.00 g). In contrast, G108 had no effect on body weight loss in mice lacking the hepatic glucagon receptor (Figure 2.16B, total body weight loss at 14 days: 5 nmol/kg G108 / hepatic *Gcgr* KO; 1.59 ± 0.23 g).

Body composition analysis on day 14 revealed a significant loss of lean mass in WT mice treated with G108, not observed in either vehicle or *Gcgr* KO mice (Figure 2.16C, total loss of lean mass: 5 nmol/kg G108 / WT; -4.01 ± 0.61 g, 5 nmol/kg G108 / KO; 0.36 ± 0.35 g, vehicle / WT; -0.25 ± 0.16 g, vehicle / KO; 0.71 ± 0.20 g). Furthermore, the pattern of change in lean mass across treatment groups was associated with a similar pattern of change in plasma levels of total amino acids on study day 14 (Figure 2.16D, total plasma amino acid levels: 5 nmol/kg G108 / WT; 1.17 ± 0.14 nmol/μl, 5 nmol/kg G108 / KO; 2.82 ± 0.19 nmol/μl, vehicle / WT; 2.30 ± 0.25 nmol/μl, vehicle / KO; 3.14 ± 0.31 nmol/μl). The total plasma amino acid levels observed trended higher in the *Gcgr* KO mice than WT mice treated with vehicle control (Figure 2.16D).

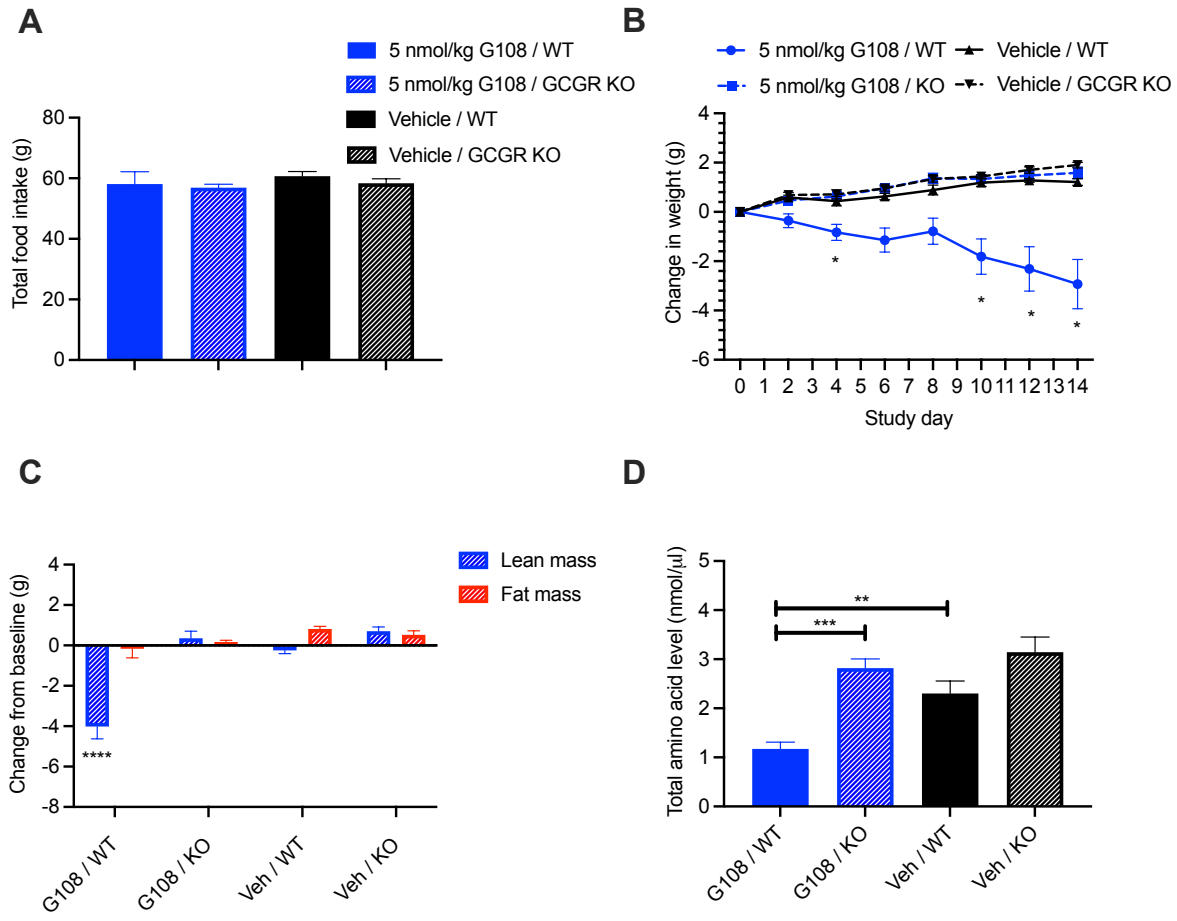


Figure 2.16: Food intake, body weight, body composition and total plasma amino acid levels in WT and hepatic specific Gcgr KO mice, treated once daily with either vehicle control or 5 nmol/kg G108 over 14 days, n=7-8/group. From Hope, Hinds et al. (In Press). **(A)** Total food intake (g) on day 14 of study. **(B)** Change in body weight from baseline (g). **(C)** Change in body composition (lean and fat mass) over 14 days from baseline (g). **(D)** Total plasma amino acid levels on day 14 of study. Data expressed as mean ± SEM. *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001. Analysed using a combination of one- and two-way ANOVA with post hoc Dunnett's and Sidak's test.

2.5 Discussion

2.5.1 G108 is a long-acting glucagon analogue which acutely enhances hepatic AA catabolism and reduces plasma amino acids

The aims of this Chapter are to determine whether a candidate peptide 'G108' is a suitable research tool to explore the effects of a long-acting GCGR agonist on plasma amino acids and lean mass in a pre-clinical model.

Since its discovery, studying the biological effects of glucagon with exogenous administration has been challenging due to its low aqueous solubility and formation of amyloid-like fibrils (Beaven, Gratzer et al. 1969). However, modifications to the primary sequence of native glucagon have emerged as a successful approach to generate long-acting GCGR agonists and mitigate these issues (Habegger, Stemmer et al. 2013). Furthermore, due to the primary sequence similarity between GLP1 and glucagon, unimolecular co-agonists with activity at both these receptors have been developed using the same strategy by modifying the primary sequence of GLP-1 (Day, Ottaway et al. 2009). G108 has also been modified from the primary sequence of glucagon (Figure 2.1), to confer similar potency at the GCGR to native glucagon and minimise GLP-1R activity (Figure 2.2, Table 2.1), whilst increasing its pharmacokinetic profile (Figure 2.3). The experiments in this Chapter demonstrate that peptide G108 is a long-acting GCGR agonist which, after single-dose administration, can cause a sustained upregulation of hepatic amino acid catabolism pathways leading to reduced plasma total amino acids.

The receptor potency studies of G108 in murine cell lines demonstrate that G108 is approximately 4 times more potent at the mGCGR than native glucagon itself, with respect to cyclic-AMP (cAMP) accumulation (Table 2.1). They also confirm that G108 is approximately 30 times less potent at the mGLP1-R than native GLP1 itself (Table 2.1). This results in a selectivity factor towards the mGCGR of approximately 120, comparable to native glucagon (Svendsen, Larsen et al. 2018). Following single-dose administration, G108 was found to be long-acting with a peak plasma concentration at 24 hours, which was cleared by 72 hours (Figure 2.3). G108 achieved the same approximate Tmax concentration of a similar long-acting glucagon agonist previously described (Habegger, Stemmer et al. 2013).

A single dose of G108 led to a reduction in plasma total amino acids in both lean (Figure 2.4) and obese (Figure 2.5) mice. This is consistent with the actions of GCGR activation in the setting of physiological infusions of glucagon (Wolfe, Culebras et al. 1979, Boden, Tappy et al. 1990, Charlton, Adey et al. 1996) and pathological levels in humans (Mallinson, Bloom et al. 1974, Almdal, Heindorff et al. 1990). Recent reports have also emerged demonstrating that GCGR-targeted multi-agonists may result in hypoaminoacidemia in both rodents and humans. For example, studies exploring plasma amino acids as biomarkers for GCGR activity have demonstrated that a long-acting GLP1R/GCGR co-agonist, 'compound 2', reduces plasma total amino acids following a single dose in mice (Li, Kirchner et al. 2020). In contrast to G108, 'compound 2' is reported to have an approximately two-fold decreased GCGR potency compared to native glucagon. Evidence that the acute effect of GCGR activity on plasma amino acids translates to humans has recently emerged in human studies. For example, compound SAR441255, a lipidated long-acting triple agonist with a two-fold increased receptor potency at the GCGR compared to native glucagon, reported a broad reduction in amino acids for up to 12 hours following a single-dose in humans (Bossart, Wagner et al. 2021). As the receptor potency to the GCGR remains high in current multi-agonists being developed, these results suggest that chronic hypoaminoacidemia may be a common finding in a chronic setting. They also suggest that G108 is a valid research tool to explore the chronic effects of hypoaminoacidemia on lean mass.

2.5.2 G108 causes a chronic reduction in plasma amino acids and lean mass

G108 demonstrated a dose-dependent effect on lean mass (Figure 2.10A and B). At the lowest dose (5 nmol/kg) where food intake was not affected, G108 led to a modest reduction in lean mass which became significant between day 7 and day 18 of study (Figure 2.10A). This was associated with a reduction in amino acids compared to vehicle (Figure 2.10G). These findings demonstrate that in a chronic setting, G108 leads to lean mass loss and hypoaminoacidemia independent to a reduction in food intake, and therefore is a peripheral effect of the peptide. To confirm these findings, a further chronic study was carried out using only 5 nmol/kg G108 and demonstrated a significant reduction in plasma total amino acids associated with a reduction in lean mass at day 7, 14 and 21 (Figure 2.13C); At day 21 lean mass loss approached 2 g. The reduction in lean

mass in this study was also associated with a reduction in muscle weight and function, as measured by grip strength (Figure 2.14).

The first long-acting agonists targeting the GCGR incorporated modifications of the GLP-1, glucagon and oxyntomodulin peptide sequences to create GLP-1R/GCGR co-agonists. One of first of these did not report lean mass and only fat mass with total body weight loss in obese mice (Day, Ottaway et al. 2009). In a follow up study of various co-agonist peptides, the same group showed a significant reduction in fat-free mass, independent to food intake, was reported associated with one of the peptides used, 'Peptide 5' (Day, Gelfanov et al. 2012). Over 13 days this amounted to approximately 3 g of lean mass loss. Peptide 5 has enhanced selectivity to the GCGR, a five-fold-lower potency vs native glucagon, and was dosed every 4 days at 19 nmol/kg. In another study, daily dosing with a co-agonist 'DualAG' over 13 days in obese mice led to a reduction in lean mass of approximately 3g, however a reduction in food intake was also observed (Pocai, Carrington et al. 2009). The resulting lean mass loss in these studies is comparable to that seen with daily G108 over 21 days (Figure 2.13C). The lean mass lost in response to G108 is also similar to a study using a long-acting glucagon analogue 'IUB288', where a three-week treatment with this peptide led to preferential loss of lean mass with no effect on fat mass (Okun, Rusu et al. 2021). In this study, reduced muscle weights and grip strength was also noted comparable to the reductions observed with G108. Despite these findings in response to activating the GCGR, no data has been reported on plasma amino acids in these studies. However, collectively the findings from chronic G108-adminsitration and studies by others suggest that there is peripheral effect on lean mass of GCGR-targeted agonists, as food intake was generally not affected.

Although plasma amino acids have not been reported in the *in vivo* chronic studies described above, they are sensitive biomarkers for GCGR activity of GLP1R/GCGR co-agonists in the acute setting (Li, Kirchner et al. 2020). Due to the acute change in amino acids observed, understandably lean mass is not reported. In human pre-clinical studies, hypoaminoacidemia has been reported in the acute and chronic setting however not along-side data for lean mass, and the effect of food intake is not clear (Parker, Robertson et al. 2020, Bossart, Wagner et al. 2021). Interestingly, the human GCGR receptor potency for one of these (Cotadutide) is 10-fold less than native glucagon (Parker, Robertson et

al. 2020). Therefore, the findings in this Chapter highlight for the first time the association between chronic hypoaminoacidemia and lean mass loss in a pre-clinical model, in response to the peripheral effects of the long-acting glucagon analogue, G108.

2.5.3 The hepatic GCGR is required for G108-stimulated hypoaminoacidemia and lean mass loss

Studies have emerged suggesting that the hepatic GCGR action is required for the effect of long-acting GCGR-targeted agonists on lean mass. For example, the long-acting glucagon analogue, 'IUB288' enhances lean mass loss over 3 weeks *in vivo* and this effect is abolished with silencing of liver aminotransferases (Okun, Rusu et al. 2021). Furthermore, in this study using co-culture experiments with liver and muscle tissue *ex vivo*, enhanced hepatic amino acid catabolism in a model of hyperglucagonemia leads to reduced protein synthesis in muscle tissue (Okun, Rusu et al. 2021). This is suggestive of the direct effects of reduced amino acid availability on lean mass.

Our group has recently demonstrated that the lean mass loss associated with chronic administration of a long-acting glucagon analogue is dependent on the hepatic GCGR (Hinds, Owen et al. 2021). However, in this study, plasma amino acids were not reported. This Chapter extends these findings by demonstrating that both hypoaminoacidemia and lean mass loss in response to a long-acting glucagon analogue, G108, are dependent on the presence of the hepatic GCGR (Figure 2.16A-D). This points towards direct amino acid sensing as a mechanism of lean mass loss and is in keeping with the suggestion that native glucagon may exerts effects on muscle mass directly through plasma amino acid availability (Adeva-Andany, Fernandez-Fernandez et al. 2021).

2.5.4 Evidence of metabolic benefits with chronic G108 administration

Interestingly, the lowest dose G108 (5 nmol/kg) in the dose-finding study led to an improved glucose tolerance (Figure 2.11), and this was confirmed in a separate study (Figure 2.15). At higher doses, exceeding 10 nmol/kg, chronic G108 administration led to a deterioration in glucose tolerance (Figure 2.11). As glucagon mobilises glucose, particularly from hepatic glycogen stores, the chronic effect of glucagon therapy on glycaemia had been previously studied *in vivo* and clinical studies characterising the

effects of GCGR agonism. In human glucagon and GLP-1 infusion studies, it has previously been demonstrated that glucagon can acutely worsen glycaemia which is off-set by GLP-1 (Tan, Field et al. 2013). A recent early phase clinical trial of the GLP1/GCGR co-agonist 'NN1177' has highlighted the chronic effects of higher glucagon receptor preference and reported several safety issues including impaired glucose tolerance (Martin Friedrichsen 2022). Due to the marked differences in pre-clinical models using the same drug, it has been suggested that several factors lead to unpredictable effects when translating GCGR-targeted agonists, including species differences, study length and drug exposure (Simonsen, Lau et al. 2022). The findings from this Chapter demonstrate that G108 at higher doses can also lead to a chronic deterioration in glucose tolerance, however at low sub-anorectic doses, it leads to an improvement in glycaemia. Interestingly, it has previously been demonstrated that in the acute setting, a long-acting glucagon analogue 'IUB288' which also does not lead to a reduction in food intake, leads to improved insulin sensitivity and glycaemia for many hours following transient hyperglycaemia (Kim, Holleman et al. 2018). However, in contrast to G108, when administered chronically, IUB288 leads to worsened glycaemia (Habegger, Stemmer et al. 2013). This perhaps is not unsurprising as this compound had a sizeably increased potency at the GCGR compared to native glucagon (Habegger, Stemmer et al. 2013).

The effect of chronic GCGR activity on glycaemia is therefore complex, with several drug and experimental model factors playing a role. It is likely that with higher GCGR activity the detrimental effect on glucose needs to be offset with incretin (ie GLP-1 or GIP) action, however the precise balance of receptor activity remains uncertain. Elucidating the optimal characteristics for a GCGR-targeted agonist remains challenging as pre-clinical findings based on *in vivo* PD and PK data does not necessarily translate to humans. However, the effects of low-dose glucagon in a chronic setting remains unexplored and the findings presented in this Chapter indicate the potential metabolic benefits of this approach.

2.6 Conclusions

This Chapter presents findings from both acute and chronic studies with sub-anorectic doses of G108. The experiments carried out confirm that G108 is a selective glucagon agonist with comparable receptor profiles to GCGR-targeted agonists in development.

Furthermore, the effects on hepatic amino acid catabolism, plasma amino acid availability and lean mass are dependent on the presence of the hepatic GCGR. This work has therefore confirmed that G108 is an appropriate long-acting GCGR agonist to further explore the chronic effects of reduced plasma amino acid availability in a pre-clinical model of obesity.

3 Chapter 3 – A high protein diet protects lean mass in G108-treated obese mice and reveals whole-body effects of amino acid availability on energy expenditure and body weight loss.

3.1 Introduction

3.1.1 Hypoaminoacidemia and lean mass loss in response to a long-acting glucagon analogue may occur in both lean and obese rodents

The findings from chapter 2 demonstrated that chronic G108 treatment at a dose of 5 nmol/kg once daily causes food-intake independent weight loss over 21 days in lean mice. The weight loss observed was a result of selective lean mass loss and associated with a significant reduction in plasma amino acid levels, suggesting that low plasma amino acid availability stimulated by G108 may enhance lean mass loss. Whether preferential loss of lean over fat mass occurs in obesity due to chronic GCG-stimulated hypoaminoacidemia is unknown. Furthermore, whether dietary protein supplementation is sufficient to overcome hypoaminoacidemia and therefore lean mass loss in obesity, is also unknown; The effect of chronic glucagon treatment on body composition in an obese model may indeed be different than in a lean one. For example, recent reports of hepatic glucagon resistance have highlighted the reduced potential for GCG-stimulated amino acid catabolism in fatty liver disease (Suppli, Bagger et al. 2020). Therefore, it is possible that obesity-associated fatty liver disease may disturb GCG-stimulated hepatic amino acid catabolism (Winther-Sorensen, Galsgaard et al. 2020), and therefore the extent of lean mass loss. However, the chronic effect of long-acting glucagon analogues on plasma amino acids and lean mass in obese models, in addition to potential preventative strategies, remains an important question as multi-agonists with 'high' GCGR activity are currently being developed for the treatment of obesity and metabolic disease (Hope, Vincent et al. 2021).

3.1.2 Previous evidence for lean mass loss in obese rodents in response to glucagon receptor active analogues

Few studies have previously explored the effects of long-acting glucagon analogues on lean mass in obese rodents. The lack of evidence is partly due to the difficulty in using native glucagon as a research tool due to its very short half-life and low aqueous solubility

(Chabenne, DiMarchi et al. 2010). In addition, there has been a general hesitance to use glucagon analogues without the glucoregulatory counterbalance of GLP-1R activity, ie in the GLP1R/GCGR co-agonist. However, to understand the physiological actions of glucagon receptor signalling, select reports of long-acting glucagon analogues are available. For example, it has been demonstrated that daily dosing of the glucagon analogue, IUB288, over 16 days led to fat mass loss and reduced plasma cholesterol in obese mice, despite no change in food intake (Habegger, Stemmer et al. 2013). The effect of IUB288 on lean mass in obese mice were not reported however lean mass loss was noted in lean mice that after 19 days of treatment with IUB288. In a further study by the same team investigating the mechanisms of GCG-mediated EE, lean mass loss was observed in DIO mice after 17 days of daily treatment with IUB288 (Kim, Nason et al. 2018). However, in this study a reduction of food intake was also observed, therefore the contribution of any underlying GCG-stimulated hypoaminoacidemia to lean mass loss is unclear. Therefore, it remains uncertain whether chronic administration of a long-acting glucagon analogue can potentially cause hypoaminoacidemia and lean mass loss in an obese model.

Using GLP-1R/GCGR co-agonists, studies in obese rodents have also implicated chronic GCGR activation in lean mass loss. For example, in one study, several different GLP-1R/GCGR co-agonists were investigated in obese mice (Day, Gelfanov et al. 2012). Over 13 days the most selective GCGR agonist (Peptide 5) did not result in a significant reduction in food intake however did result in a significant reduction in fat and lean mass, comparable to co-agonist peptides which caused a greater decrease in food intake (Peptides 1, 2 and 3). Although plasma amino acids were not reported, these results point towards fat and lean mass loss associated with glucagon-driven energy expenditure when DIO mice were chronically administered with peptide 5. One of the first studies to investigate a GLP-1R/GCGR co-agonist in obese mice used a long-acting form of oxyntomodulin (OXM), the naturally occurring endogenous co-agonist to both GLP-1 and GCGR receptors (Pocai, Carrington et al. 2009). By conjugating cholesterol to the c-terminus of oxyntomodulin and incorporating an amino acid substitution at position two (preventing DPP-4 mediated degradation), a more glucagon selective GLP-1R/GCGR dual-agonist 'DualAG' was shown to enhance body weight loss by 10-12 g over 13 days, with both fat and lean mass loss over and above that of a GLP-1R monoagonist which

achieved weight loss of around 4-5 g. Food intake was significantly reduced in both groups therefore the effect on lean and fat mass loss may be partly attributed to this and partly attributed to enhanced energy expenditure.

3.1.3 Glucagon-stimulated hepatic amino acid catabolism resulting in hypoaminoacidemia is predicted to enhance lean mass loss in obese rodents

Collectively, the studies described in the previous section suggest that chronic therapy with long-acting GCGR-active analogues can have a catabolic effect on fat and lean mass in obese rodents, however the concurrent effects on food intake and lack of plasma amino acid data limit their interpretation on the role of GCGR-stimulated hypoaminoacidemia driving lean mass loss. However, a recent study has shown that plasma amino acids are a sensitive biomarker for GCGR activity for long-acting GLP1R/GCGR co-agonists in obese rodents (Li, Kirchner et al. 2020). Therefore, these data justify further work to determine whether GCG-stimulated hepatic amino acid catabolism, hypoaminoacidemia and lean mass loss occurs in response to long-acting glucagon analogues or GCGR-targeted co-agonists in obese rodents. Indeed, it is the triple combination of these findings which is required to answer this important question, and some studies have added further insight. For example, our group has previously demonstrated that a long-acting GLP-1R/GCGR co-agonist administered daily to rats for 22 days increased hepatic urea cycle activity, reduced plasma amino acids and increased molecular markers of muscle proteolysis (Rebecca Scott 2019). More recently, treatment with a long-acting glucagon agonist for three weeks in lean mice was shown to result in loss of lean mass with reduced muscle weight; These effects were prevented with silencing of hepatic alanine aminotransferase, implicating GCG-stimulated alanine catabolism in lean mass loss (Okun, Rusu et al. 2021).

3.1.4 Using G108 and a high protein diet intervention to explore effects on glucagon-stimulated amino acid catabolism and lean mass loss

The work in this chapter primarily sets out to explore the effect of chronic GCGR agonism on the triple combination of hepatic amino acid catabolism, plasma amino acids levels and lean mass in an obese model. It also aims to determine whether a high protein diet intervention can 'rescue' plasma amino acid levels and therefore lean mass. Secondary outcomes include exploring the effects of chronic glucagon receptor agonism on changes

in fat mass, glucose tolerance and hepatic lipid metabolism. By using low sub-anorectic doses of G108, the comparative effects on energy expenditure will also be assessed between groups.

It is hypothesised that chronic G108 treatment leads to enhanced hepatic amino acid catabolism resulting in a reduction in plasma amino acid availability and therefore lean mass loss. The perturbation in this liver-muscle axis may be mitigated with a high protein diet whilst maintaining the metabolic benefits of glucagon receptor agonism, for example energy expenditure and hepatic fat clearance. This finding would be of translational significance to individuals offered chronic glucagon therapy for obesity and metabolic disease.

3.2 Chapter summary and aims

- Determine the effect of chronic sub-anorectic doses of G108 treatment on total and individual plasma amino acid levels in obese mice.
- Determine the effect of chronic G108 treatment on lean mass in obese mice.
- Determine whether a high protein diet can rescue plasma amino acids and mitigate any loss of lean mass observed.
- Determine the effect of G108 and a high protein diet on molecular markers of hepatic amino acid catabolism, muscle protein synthesis and muscle proteolysis.
- Explore whether key beneficial effects of glucagon receptor agonism are maintained in the context of protein supplementation, for example hepatic steatosis, plasma lipids, glycaemia and energy expenditure.

3.3 Methods

3.3.1 *In vivo* studies

The following *in vivo* study was carried out according to the Scientific Procedures Act 1986.

3.3.2 Animal husbandry

Adult male C57Bl/6J mice (Charles River UK) were individually housed in temperature-controlled room (21oC) with a 12-hour light/dark cycle and acclimatised prior to study start. All mice had *ad libitum* access to chow diet and water until 2 weeks prior to study commencement, where the diet was switched to a custom diet (Research diets) which continued to be supplied *ad libitum* for the remainder of the study.

3.3.3 Diets

All diets were obtained from ResearchDiets.com, stored at 4^{o}C and used within 6 months of production. A standard DIO series diet (D12492) containing 60 kcal% fat was provided to all mice for 10 weeks to induce obesity prior to switching to a custom diet. Openstandard diet D18031206 was used as the 'standard protein' diet, consisting of 20 kcal% protein and 45 kcal% fat. Openstandard diet D08091801 was used as the 'high protein' diet. Both diets contained a higher fat content (45 kcal%) to maintain diet induced obesity.

Diet	Carbohydrate (kcal%)	Fat (kcal%)	Protein (kcal%)
D12492	20	60	20
D08091803	35	45	20
D08091801	15	45	40

3.3.4 Chronic DIO study

Twenty-seven lean 10-week-old C57BL/6J mice were provided *ad libitum* a 60 kcal% fat diet (diet D12492, supplementary table 1) for 12 weeks prior to the start of the study. The cohort of mice were individually housed and divided into six treatment groups, based on treatment and diet allocation; Vehicle / High fat, standard protein diet (N=9), 7.5 nmol/kg peptide G108 / High fat, standard protein diet (HFNP) (N=9) and 7.5 nmol/kg peptide G108 / High fat, high protein diet (N=9). The high fat standard protein diet, D08091803 and high fat high protein diet D08091801 are detailed in supplementary table 1. All mice had *ad libitum* access to water and their allocated diets. Mice were subcutaneously administered with their respective treatments at 9am each day for 27 days. Food intake, water intake and body weight were measured using the same weighing scale each morning prior to vehicle or peptide G108 administration. EchoMRI was carried out at baseline, day 14 and day 25. Tail venesection was carried out on day 13 of the study immediately pre-treatment. 40-50 μl of blood was collected and immediately transferred on ice, centrifuged at 5000 rpm for 10 minutes prior to analysis for total plasma amino acid levels. A glucose tolerance test was carried out on day 15 of the study following a 5-hour fast as described below. An insulin tolerance test was also carried out on day 18 of the study following a 5 hour-fast as described below. Forelimb grip strength testing was performed at baseline (day-3) and day 25 of the study. On day 22 of the study, all mice were placed into metabolic cages, single housed for 72 hours, as described below. After 72 hours, all mice were removed from the metabolic cages and returned to their previous cages. Following a 4-hour fast, on day 27 all mice were culled with the following tissues dissected and snap frozen in liquid nitrogen prior to storage at -80^{o}C; gastrocnemius, quadriceps, s/c WAT, BAT and liver. Using a sensitive weighing scale, five muscle groups (gastrocnemius, quadriceps, solues, tibialis anterior and extensor digitorum longus) were weighed and recorded. Terminal plasma was also collected and transferred on ice immediately for centrifugation as previous described and storage at -80^{o}C.

3.3.5 Food intake, body weight and body composition (EchoMRI)

Food intake was measured daily using a dedicated weighing scale and topped up accordingly. Body weight was recorded at 9 am daily using the same weighing scale. Body composition was measured using an EchoMRI analyser at various timepoints during

chronic studies. All body composition measurements were taken on the same time of day to reduce intra-animal variability. Animals were placed in a transparent cylindrical perspex tube and both ends secured with Perspex clips. The tubes containing the animal was then carefully placed into the MRI analyser (EchoMRI -100H). Each scan lasted 90 seconds where lean mass and fat mass were recorded in grams. Following the scan, the mice were removed from the Perspex tube and returned to their respective cages.

3.3.6 Glucose and insulin tolerance tests

For glucose tolerance tests, following a 5-hour fast and administration of peptide or vehicle treatment, mice were administered with glucose by intraperitoneal injection at a dose of 2 g/kg body weight. Tail vein blood samples were taken at baseline, 15, 20, 30, 40, 60, 90 and 120 minutes and glucose measured (mmol/l) using a handheld GlucoRx monitor. For insulin tolerance tests, following a 5-hour fast, mice were administered with actrapid insulin by intraperitoneal injection at a dose of 0.75 U/kg body weight. Tail vein blood samples were taken at baseline, 15, 30, 45, 60 and 90 minutes and glucose measured as described above.

3.3.7 Metabolic cages

For whole-body energy expenditure studies, The Comprehensive Lab Animal Monitoring System (CLAMS) was used (Columbus Instruments, Columbus, Ohio). This allowed simultaneous measurement of whole-body oxygen consumption (VO2), carbon dioxide production (VCO2) and movement. Open circuit calorimetry allows assessment of VO2 and VCO2 for 1-minute at 20-minute intervals, whilst infrared light beams are used to measure movement, locomotor (XTOT) and ambulatory (XAMB). The respiratory exchange ratio (RER) is the ratio of VCO2 to VO2 and indicates the energy source during aerobic respiration. Pure fat, protein or carbohydrate will result in an RER of 0.7, 0.8 or 1.0, respectively (Lusk 1924, Frayn 1983). Metabolic assessments were carried out over 72 hours. Following the assessment period, mice were placed back into their respective home cages.

3.3.8 Biochemical assessments

Total plasma amino acids were measured using a commercially available fluorometric-based ELISA assay (MAK002, Sigma, Ab65347, Abcam). Individual amino acids were measured using a gas-chromatography-mass spectrometry platform (Measurements carried out by Professor N Dalton and Dr C Turner at the WellChild Laboratory, Evelina Children's Hospital, London). Plasma FGF-21 (ab229382, Abcam), triglycerides (ab65336, Abcam) cholesterol (ab65390, Abcam), leptin (ab199082, Abcam), corticosterone (ab108821, Abcam), norepinephrine (ab287789, Abcam), free T4 (ab285258, Abcam) were measured using commercially available ELISA assay kits.

3.3.9 Western blots

The gastrocnemius muscle was dissected and snap frozen in liquid nitrogen on collection, prior to storage at -80°C. The frozen muscle was quickly thawed in ice cold buffer containing 10 mM Tris-HCl, 150 mM NaCl, 0.5 % NP40, 10% Glycerol, 5 mM EDTA, 50 mM Sodium fluoride, 10 mM beta-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 complete protease inhibitor cocktail tablet (Roche). Samples were homogenised and denatured at 95^{o}C, prior to determining protein concentration using the Pierce BCA protein assay kit (ThermoFisher Scientific). 30 μg of protein was loaded to individual wells and resolved by SDS-PAGE at 80 V for 30 min, followed by 150 V for 60 min. Protein was then transferred to a 0.45 nm nitrocellulose membrane for 1 hour at 200 mA, prior to blocking with 5% bovine serum albumin/TBST for 1 hour at room temperature. Membranes were incubated with rabbit anti-mouse polyclonal primary antibodies overnight at 4°C; 1:500 P-S6K (#9205), 1:1000 MAFbx (sc-166806), 1:2500 GAPDH (#2118). Membranes were then washed with TBST for 15 min prior to incubating with HRP-conjugated goat anti-rabbit secondary antibody (1:5000, Cell Signalling Technology #7404). A final wash was performed 15 min prior to incubating in horseradish peroxidase substrate. Membranes were then imaged using a Odyssey CLx Imager (LI-COR). For protein quantification analysis, protein blots were normalised using the loading control, GAPDH.

3.3.10 Histology

Frozen samples of liver were mounted in a cryostat and frozen sections were cut at 12 microns thickness, prior to storage at -80^{o}C. Prior to staining, sections were initially thawed. Sections were subsequently stained with Oil Red O solution for 5 minutes prior to a 30-minute wash in running tap water. They were then counter-stained with haematoxylin prior to a further wash step. Sections were then mounted and imaged using bright field light microscopy at a 10-20 x magnification.

3.3.11 Quantitative polymerase chain reaction (qPCR) analysis

qPCR analysis was carried out as described previously (Chapter 2, section 2.3.14). Specific qPCR primers used include: m_nags_1, m_cps1_1, m_ass1_1, m_asl_1, m_slc25a22, m_slc38a2, m_got_1, m_oat_1, m_g6p_1, m_pepck_1, m_pcg_1, m_fbxo32_1, m_maplc3b_1, m_bnip3_1, m_glul_1, m_4ebp1_1, m_fgf21_1, m_adrb2_1, m_serca1_1, m_ucp3_1, m_ucp1_1, m_cidea_1, m_dio_1, m_adrb3_1 (Kickstart primers, Bio-Rad, UK).

3.3.12 Forelimb grip strength tests

Forelimb grip strength testing was carried out as described previously (Chapter 2, section 2.3.12).

3.4 Results

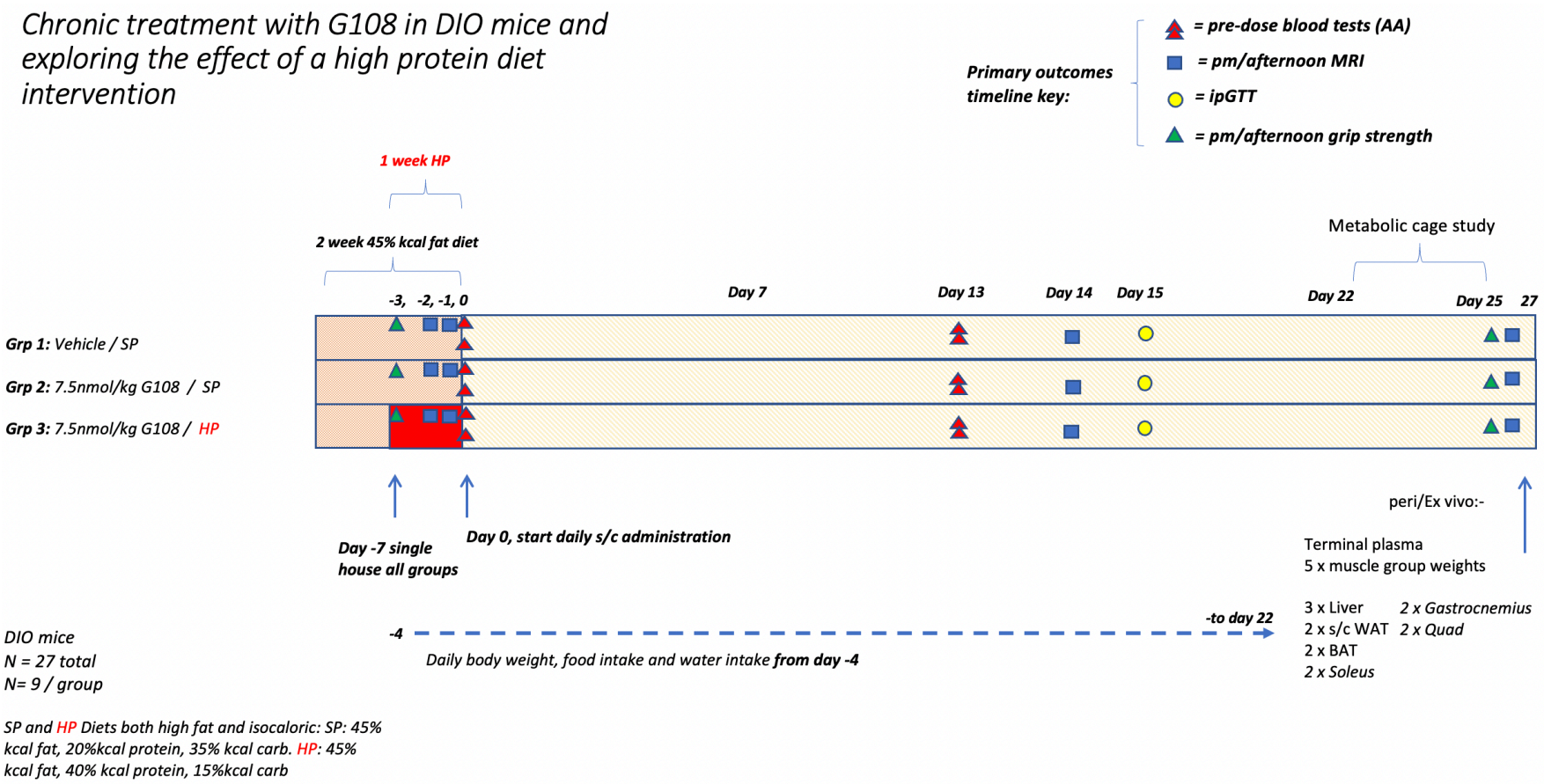
Key results for Chapter 3

- **Chronic daily G108 administration suppresses plasma amino acids and results in lean mass loss in diet-induced obese (DIO) mice.**
- **G108-stimulated hypoaminoacidemia results from a broad reduction in individual plasma amino acids in DIO mice.**
- **A high protein diet rescues chronic G108-stimulated hypoaminoacidemia and mitigates against lean mass loss in DIO mice.**
- **A high protein diet prevents G108-mediated EE and total body weight loss in DIO mice.**
- **Despite feeding with a high protein diet, key metabolic benefits of glucagon signalling remain include improved plasma lipids, hepatic lipid metabolism and glycaemia.**

3.4.1 Chronic daily G108 administration results in food-intake independent loss of body weight in diet-induced-obese mice

To determine whether G108 results in chronic hypoaminoacidemia and loss of lean mass in obesity, the effect of chronic peptide administration on food intake, body weight, plasma amino acids and body composition in diet-induced obese (DIO) mice was investigated. The effect of a high protein diet on these outcomes, in particular the ability to 'rescue' plasma amino acids was also investigated in mice receiving G108. To avoid a food intake-dependent effect on body weight and body composition, and to demonstrate the peripheral actions of the glucagon analogue, sub-anorectic doses of G108 were used. Due to recently described 'glucagon resistance' in states of fatty liver disease (Winther-Sorensen, Galsgaard et al. 2020), a slightly higher dose of G108 (7.5 nmol/kg) was chosen for the chronic studies in obese rodents. A 7.5 nmol/kg dose of G018 was administered subcutaneously, daily for 27 days in DIO mice. A high fat diet was provided to all mice with either standard protein (SP: 20 kcal%) or high protein (HP: 40 kcal%) content. Over 27 days, compared to the vehicle control group, mice in the 7.5 nmol/kg / SP diet group experienced a significant reduction in body weight (Figure 3.1A, average weight loss ± SEM (g), Vehicle: -1.89 ± 0.56 g, 7.5 nmol/kg G108 / SP: -7.73 ± 1.21 g, 7.5 nmol/kg G108 / HP: -2.42 ± 1.12 g). A reduction in food intake was not observed in any treatment group compared to vehicle control (Figure 3.1B, average total food intake ± SEM (g), Vehicle: 79.74 ± 2.03 g, 7.5 nmol/kg G108 / SP: 80.69 ± 2.74 g, 7.5 nmol/kg G108 / HP: 80.94 ± 1.97 g). Mice fed a high protein diet consumed significantly more water throughout the study than mice fed the standard protein diet (Figure 3D).

Schematic 3.1: Protocol for DIO study.



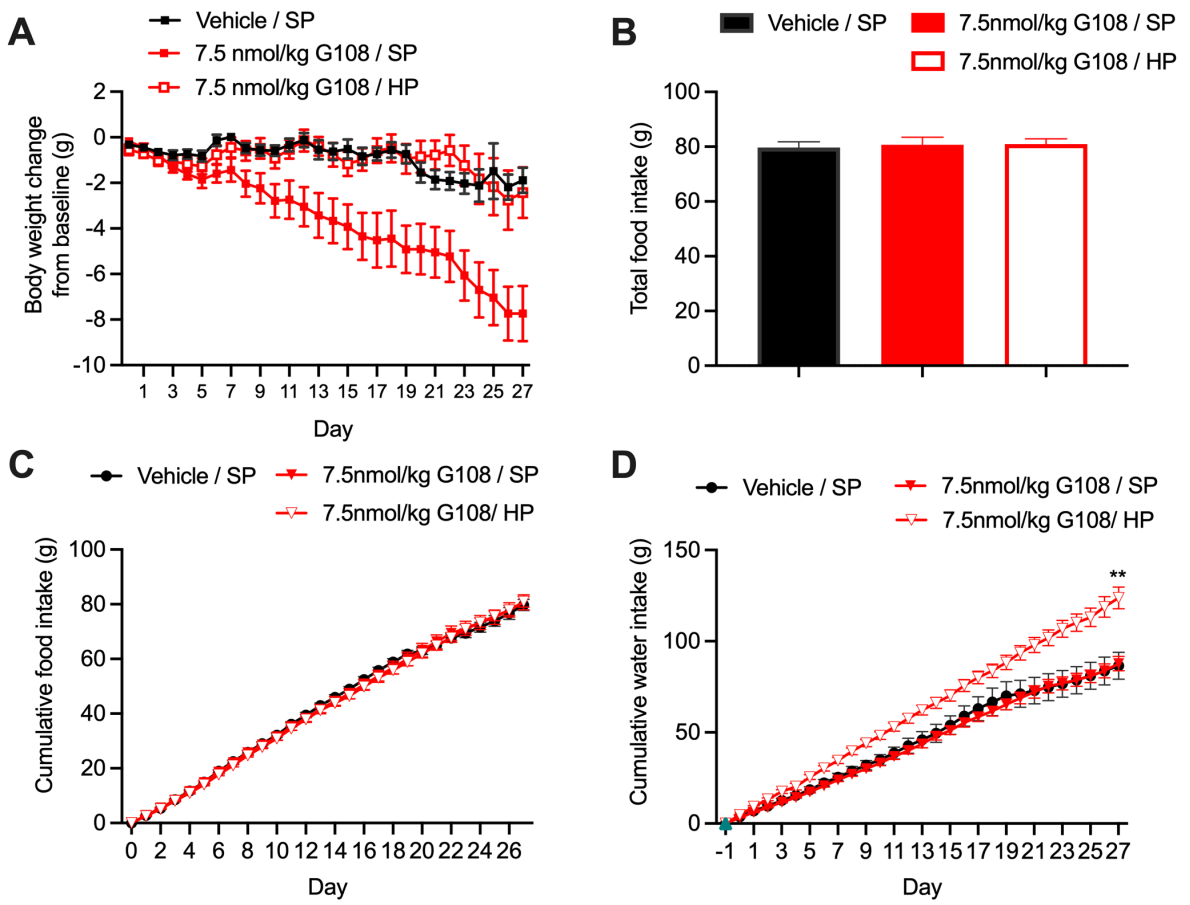


Figure 3.1: Body weight, food and water intake over 27 days in diet-induced-obese (DIO) mice receiving daily subcutaneous administration of either 7.5 nmol/kg G108 (red) on both a standard protein (SP) diet (solid symbols) or a high protein (HP) diet (open symbols), N=8-9/group. From Hope, Hinds et al. (In Press). **(A)** Body weight change (g). **(B)** Total food intake (g). **(C)** Cumulative food intake (g). **(D)** Cumulative water intake (g), significance only indicated at last timepoint. Data expressed as mean ±_SEM, significance indicates change compared to vehicle control. *p<0.05, **p<0.01, ***p<0.001, body weight and cumulative intake data analysed with linear mixed effects model, total food intake data analysed using one-way ANOVA with post hoc Dunnett's test.

3.4.2 Chronic daily G108 administration results in lean and fat mass loss in obese mice, prevented with a high protein diet

To determine whether G108 or the high protein diet intervention affected plasma amino acid levels and lean mass, body composition was measured at baseline, day 14 and day 25 using MRI (EchoMRI). A significant reduction in lean mass from baseline was observed in the G108-treated mice receiving a SP diet, compared to vehicle-treated mice after 14 days. However, a high protein diet mitigated lean mass loss after 14 days (Figure 3.2A,

average percentage change ± SEM (%), Vehicle: -3.78 ± 0.89 %, 7.5 nmol/kg G108 / SP: -9.87 ± 0.99 %, 7.5 nmol/kg G108 / HP: -7.03 ± 1.01 %). A further reduction in lean mass in G108-treated mice receiving a SP diet was observed at 25 days compared to vehicle treated mice. A high protein diet also mitigated lean mass loss after 25 days (Figure 3.2A, average percentage change ± SEM (%), Vehicle: -5.79 ± 1.02 %, 7.5 nmol/kg G108 / SP: -13.78 ± 0.80 %, 7.5 nmol/kg G108 / HP: -10.43 ± 2.20 %). Percentage changes in lean mass were reflected in absolute change (g), as indicated by change in lean mass (g) (Figure 3.2B).

A significant reduction in fat mass was only observed in mice receiving 7.5 nmol/kg G108 fed the SP diet, which became progressively evident after 25 days (Figure 3.2C, average percentage change ± SEM (%) at day 14, Vehicle: 0.73 ± 1.53 %, 7.5 nmol/kg G108 / SP: -6.52 ± 3.92 %, average percentage change ± SEM (%) at day 25, Vehicle: -3.88 ± 2.95 %, 7.5 nmol/kg G108 / SP: -19.79 ± 5.68 %). Percentage changes in fat mass were reflected in absolute change (g), as indicated by change in fat mass (g) (Figure 3.2D).

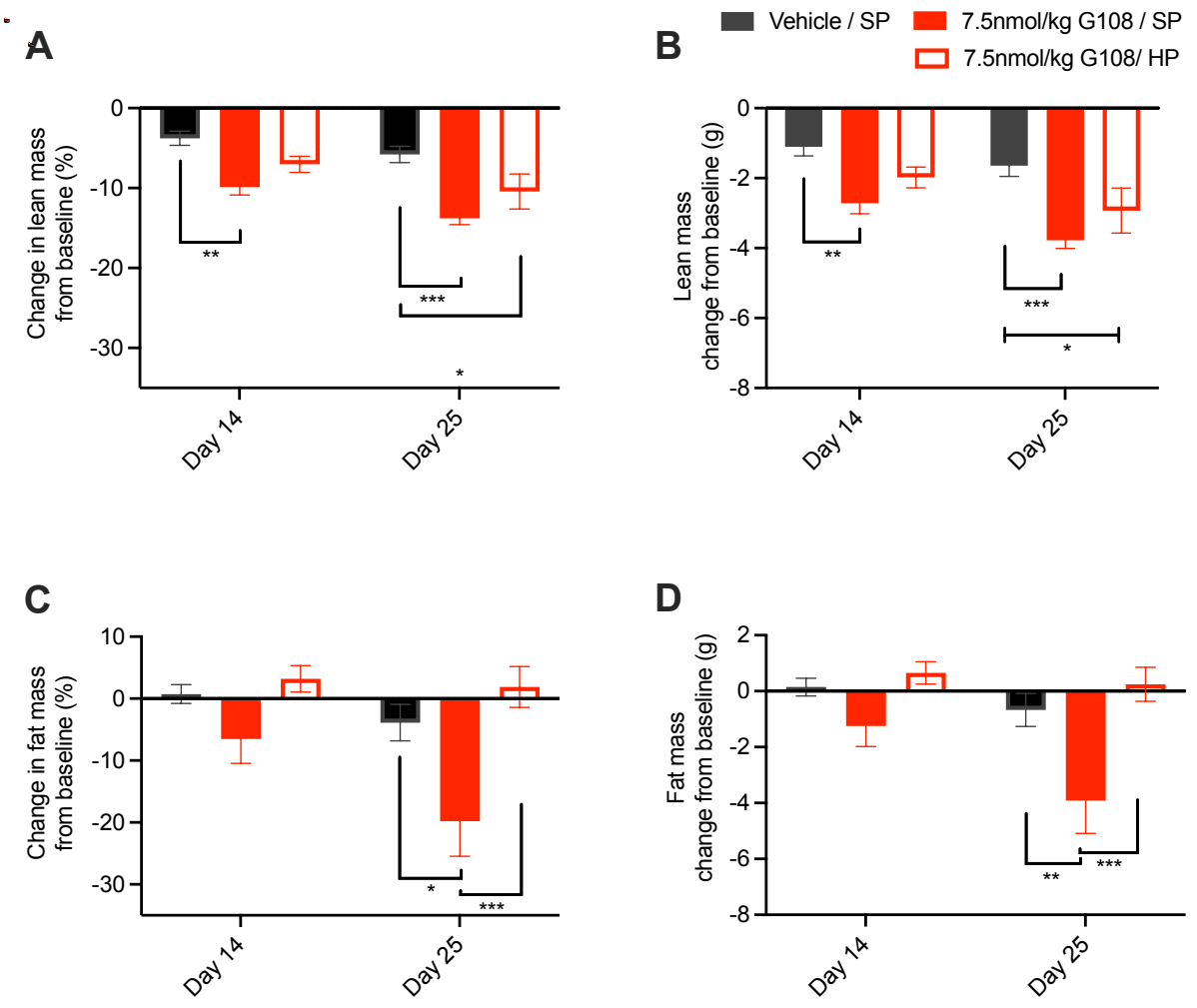


Figure 3.2: Body composition data during 27-day DIO study. N=9/group. From Hope, Hinds et al. (In Press). **(A)** Percentage change from baseline in lean mass at day 14 and day 25. **(B)** Absolute change (g) from baseline in lean mass. **(C)** Percentage change from baseline in fat mass at day 14 and day 25. **(D)** Absolute change (g) in fat mass. Data expressed as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, Analysed using two-way ANOVA with post hoc Tukey's test,

3.4.3 Chronic daily G108 administration results in a decline in muscle weight and function, mitigated with a high protein diet

Consistent with the body composition data in section 3.4.2, there was a reduction in muscle weights in the larger hindlimb muscle groups in mice treated with G108 receiving a standard protein diet (Figure 3.3A). A significant reduction in quadriceps muscle weight was prevented with a high protein diet. To determine the effects of chronic G108 dosing and the high protein diet intervention on muscle physical function, forelimb grip strength

testing was performed. A small but significant reduction in grip strength in mice administered with 7.5 nmol/kg G108 and a standard protein diet (116 g vs 131 g, P<0.05) (Figure 3.3B); Similar to the trends observed with muscle weight, the reduction in grip strength at the 7.5 nmol/kg G108 dose was also prevented in mice receiving a high protein diet.

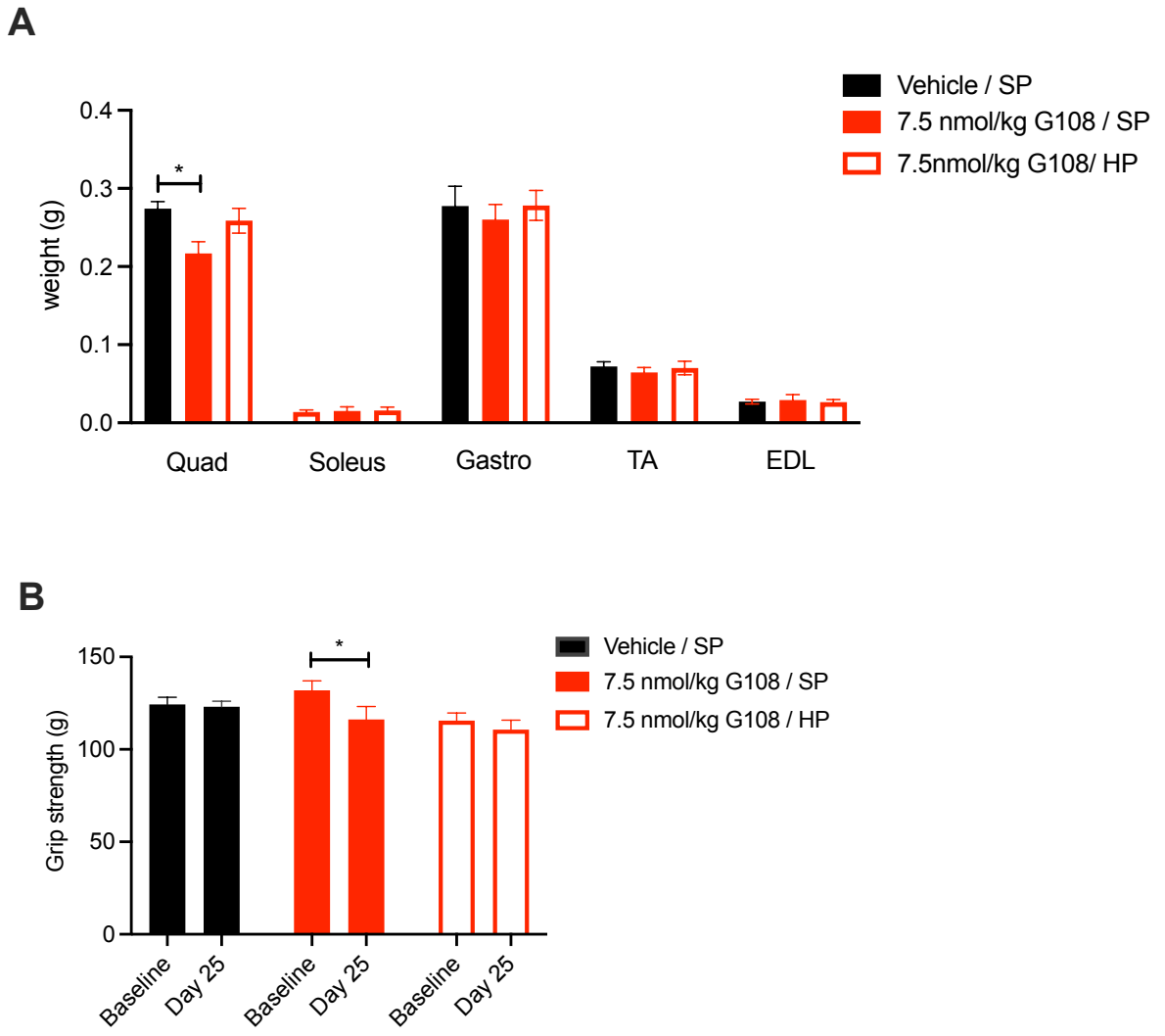


Figure 3.3: Muscle weight and function data following chronic dosing with G108 in DIO mice. From Hope, Hinds et al. (In Press). **(A)** *Ex vivo* muscle weighs after 27 days of daily subcutaneous administration of G108 treatment or vehicle control, Quad = quadriceps, Soleus, Gastro = gastrocnemius, TA = tibialis anterior, EDL = Extensor digitorum longus, N=9/group. **(B)** Forelimb grip strength test at baseline prior to commencing treatments, compared to grip strength on day 25 of daily treatments. N=9/group. Data expressed as mean ± SEM *p<0.05. Two-way ANOVA with post hoc Sidak's test.

3.4.4 Chronic daily G108 administration in obese mice increases hepatic catabolism of amino acids resulting in suppressed plasma amino acids, rescued by a high protein diet

At the end of the study, analysis of hepatic gene expression revealed a significant increase in the expression of key enzymes of the urea cycle and amino acid transporters including N-acetyglutamate synthetase (*Nags*), carbamoyl phosphate synthase (*Cps*), arginosuccinate synthase (*Ass*), arginosuccinate lyase (*Asl*), solute carrier family 25 member 22 (*Slc25a22*) and solute carrier family 38 member 2 (*Slc38a2*) (Figure 3.4A). In addition to this, the expression of catabolic intermediates, glutamic-oxaloacetic transaminase (*Got*) and ornithine aminotransferase (*Oat*) were also increased with G108 treatment. Changes in hepatic expression of catabolic enzymes and amino acid transporters were independent to a high protein diet intervention (Figure 3.4A). Following 13 days of daily dosing, total plasma amino acid levels were significantly lower immediately pre-dose in all G108 treatment groups and this was completely prevented in groups receiving a high protein diet intervention at the timepoint measured (Figure 3.4B). The hepatic expression of key rate-limiting gluconeogenic enzymes glucose-6-phosphatase (*G6p*) and phosphoenolpyruvate carboxykinase (*Pepck*), were also significantly increased with G108 treatment, independent to dietary protein enrichment (Figure 3.4C-D). In contrast to the plasma total amino acid levels on day 13 (Figure 3.4B), at the end of the study total plasma amino acids were noted to be significantly reduced in G108-treated mice receiving either the SP or HP diets (Figure 3.4E). As corticosterone is a known mediator of muscle proteolysis, terminal corticosterone plasma levels were measured with no difference across treatment groups (Figure 3.4F).

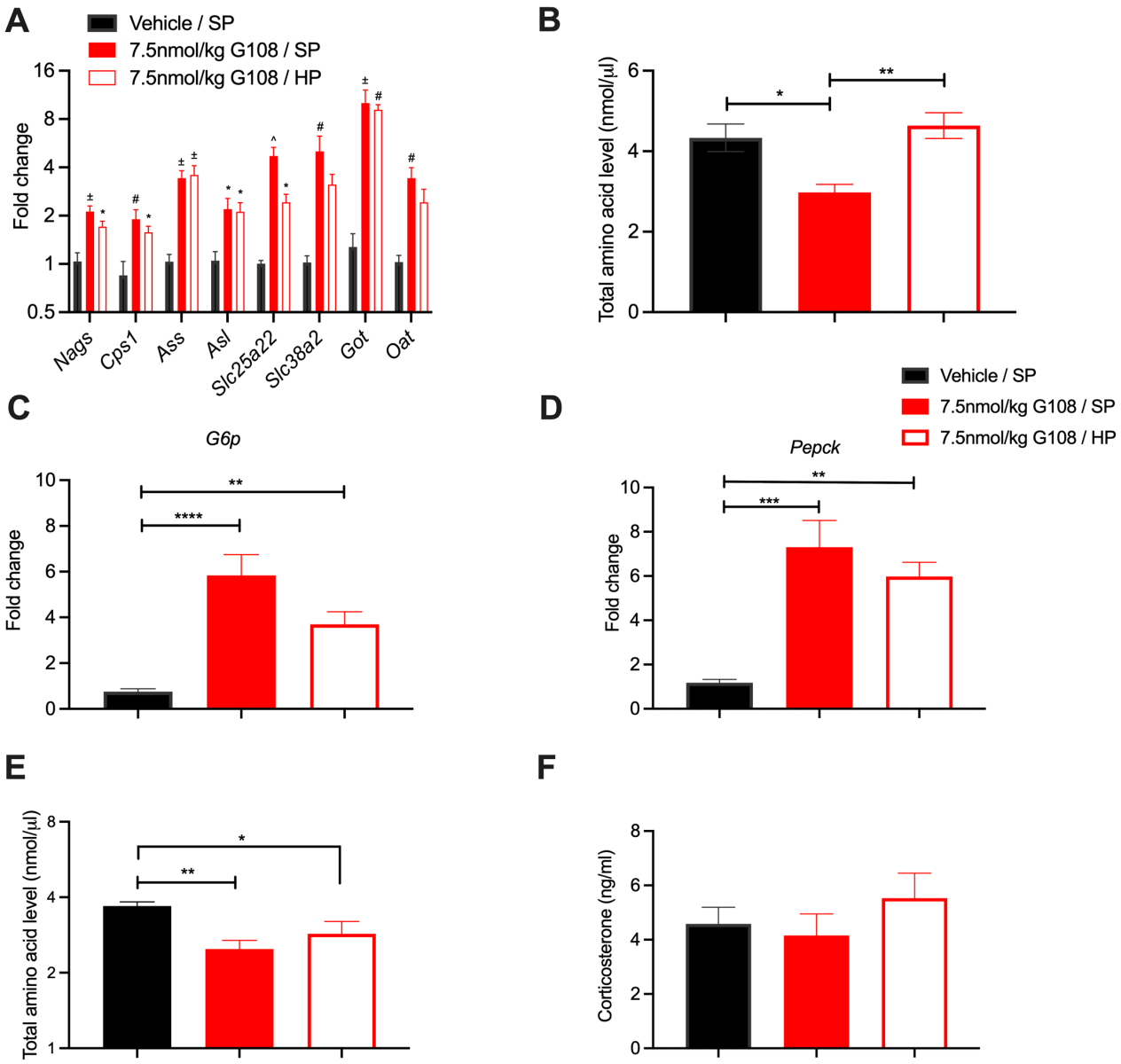


Figure 3.4: Hepatic gene expression of markers of protein catabolism and gluconeogenesis. From Hope, Hinds et al. (In Press). **(A)** Hepatic expression of urea cycle enzymes, catabolic intermediates and amino acid transporters, N-acetylglutamate synthetase deficiency (*Nags*), Carbamoyl phosphate synthase 1 (*Cps1*), Arginosuccinate synthase 1 (*Ass1*), Arginosuccinate lyase (*Asl*), inner mitochondrial membrane glutamate carrier Solute Carrier Family 25 Member 22 (*Slc25a22*), Solute Carrier Family 38 Member 2 (*Slc38a2*), Glutamic oxaloacetic transaminase 1 (*Got1*), Ornithine aminotransferase (*Oat*), N=6/group. **(B)** Total plasma amino acids on day 13 of study, N=9/group. Hepatic gene expression of **(C)** glucose-6-phosphatase (*G6p*) **(D)** Phosphoenolpyruvate carboxykinase (*Pepck*) and **(E)** Total plasma amino acids on day 27 of study, N=8-9/group. **(F)** Plasma corticosterone on day 27 on study, N=8-9/group. Data expressed as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, data analysed using one-way ANOVA with post hoc Dunnett's test.

3.4.5 Chronic daily G108 administration suppresses plasma individual amino acids in obese mice which are rescued by a high protein diet

Mass spectrometry confirmed a broad reduction in both essential and non-essential plasma amino acids associated with G108 treatment (Figure 3.5, Table 3.1). Of the nine essential amino acids, a significant reduction was observed with threonine, methionine and lysine, and to a lesser degree with histidine in G108-treated mice receiving a standard protein diet. A trend to reduction seen with leucine, isoleucine, valine. No change was observed in levels of other essential amino-acid, tryptophan or phenylalanine. Amino acid levels for all essential amino acids were rescued with a high protein diet, although in the case of lysine and threonine, the high protein diet only blunted the reduction in the level of this amino acid in mice treated with G108. A high protein diet not only rescued the branched chain amino acids, leucine, isoleucine and valine, but significantly increased plasma levels (Figure 3.5, Table 3.1).

Of the 11 non-essential amino acids analysed, G108 led to a significant reduction in plasma levels of serine, glutamine, glycine, alanine, citrulline, tyrosine and ornithine. A numerical reduction in levels of other non-essential amino acids was seen with proline, glutamate and arginine. No change was observed in levels of taurine. As with the essential amino acids, G108-stimulated reductions in non-essential amino acids was rescued in mice fed the high protein diet (Figure 3.5, Table 3.1).

Collectively, these results show the broad effect of G108 on individual plasma amino acids with specific amino acids being more prone to being suppressed. However, the reductions in amino acids were equally broadly rescued by increasing dietary protein content from 20 kcal% to 40 kcal%.

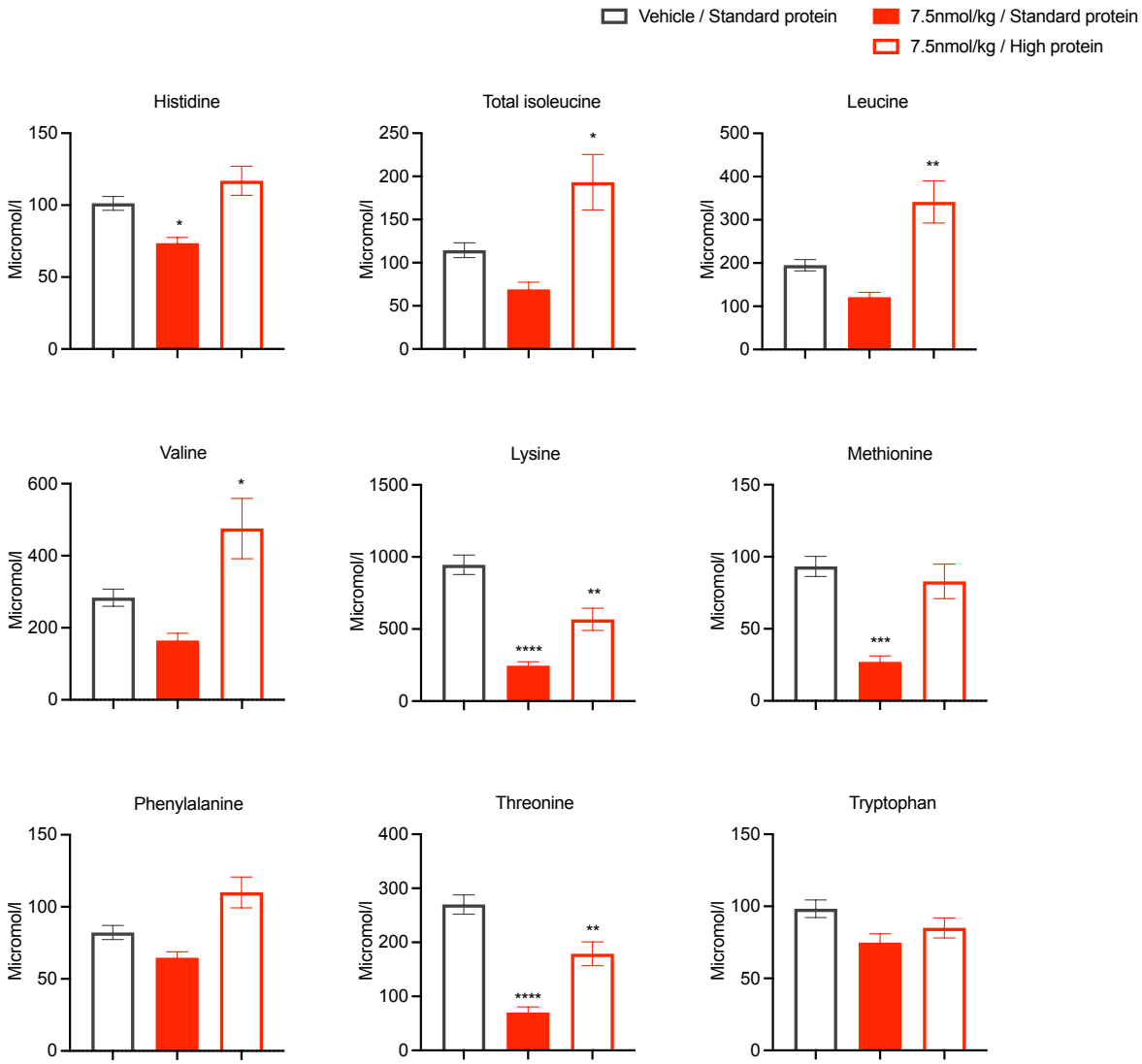


Figure 3.5: Plasma levels of individual essential amino acids (EAA's), as measured by gas-chromatography-mass-spectrometry (GCMS), following 13 days of treatment with vehicle and 7.5 nmol/kg G108 with and without a high protein diet intervention, N=6/group. From Hope, Hinds et al. (In Press). Data expressed as mean ± SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, difference compared to vehicle control group. Data analysed with one-way ANOVA with post hoc Dunnett's test.

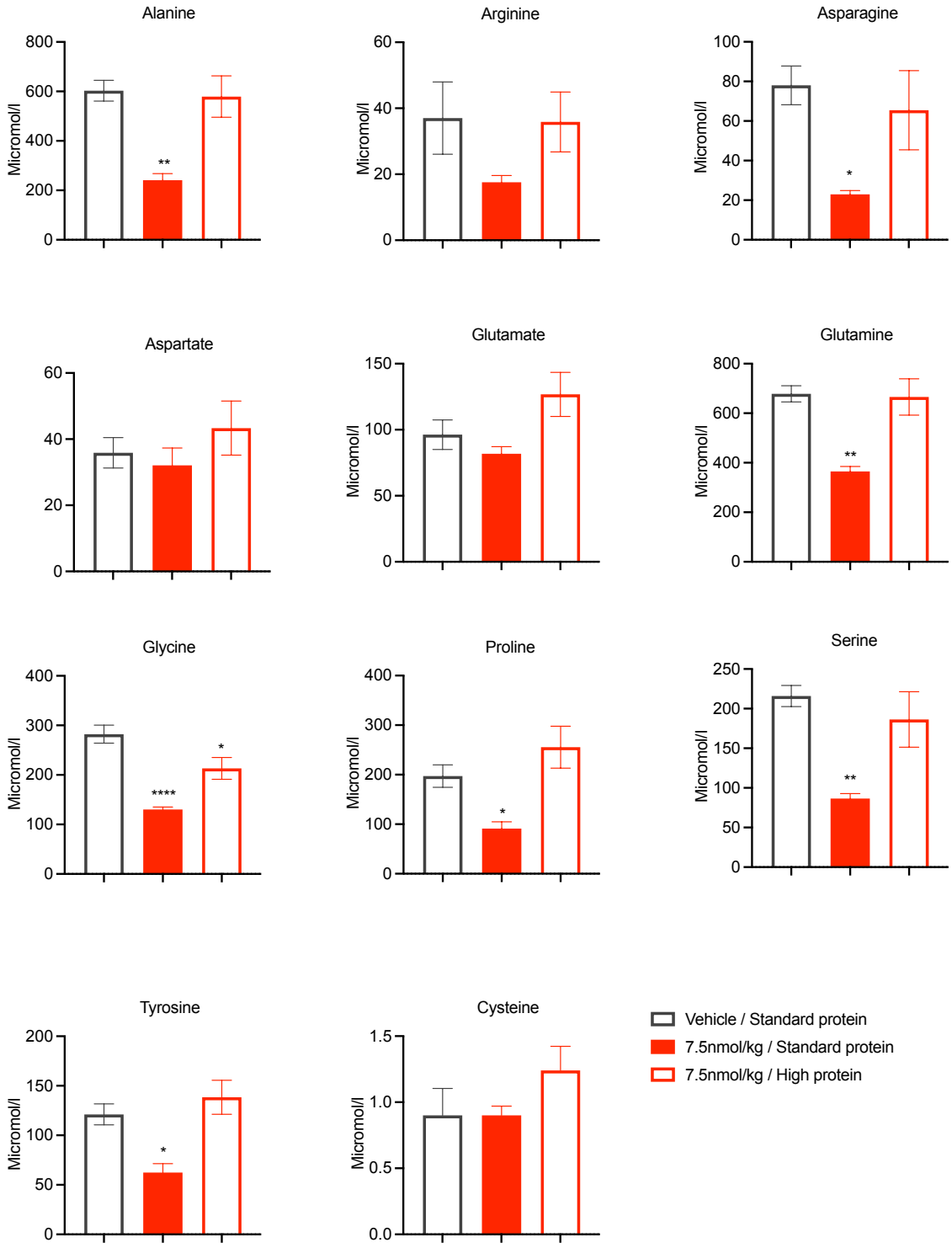


Figure 3.6: Plasma levels of individual non-essential amino acids (NEAA's), as measured by gas-chromatography-mass-spectrometry (GCMS), following 13 days of treatment with vehicle and 7.5 nmol/kg G108 with and without a high protein diet intervention, N=6/group. From Hope, Hinds et al. (In Press). Data expressed as mean ± SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, difference compared to vehicle control group. Data analysed with one-way ANOVA with post hoc Dunnett's test.

Amino Acid	Vehicle/SP	7.5 nmol/kg G108/SP	7.5 nmol/kg G108/HP
Histidine	101.3 ± 4.8	73.6 ± 4.0 ***P*<0.05**	116.9 ± 10.2
Total Isoleucine	114.5 ± 8.6	69.0 ± 8.3	193.2 ± 32.1 ***P*<0.05**
Leucine	195.0 ± 13.3	121.0 ± 11.1	341.6 ± 48.8 ***P*<0.01**
Valine	283.6 ± 23.6	164.6 ± 20.2	475.6 ± 83.9 ***P*<0.05**
Lysine	945.8 ± 67.5	245.1 ± 28.1 ***P*<0.0001**	567.5 ± 77.4 ***P*<0.01**
Methionine	93.4 ± 6.9	27.1 ± 4.2 ***P*<0.001**	82.9 ± 12.0
Phenylalanine	82.2 ± 4.9	64.7 ± 4.1	110.0 ± 10.7
Threonine	270.0 ± 17.9	70.12 ± 10.5 ***P*<0.0001**	178.9 ± 21.9 ***P*<0.01**
Tryptophan	98.4 ± 6.1	74.8 ± 6.1	85.0 ± 6.9

Table 3.1: Plasma levels of individual essential amino acids, as measured by gas-chromatography-mass-spectrometry (GCMS), following 13 days of treatment with vehicle and G108 with and without a high protein diet intervention, N=6/group. Data expressed as mean ± SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, difference compared to vehicle control group. Data analysed with one-way ANOVA with post hoc Dunnett's test.

Amino Acid	Vehicle/SP	7.5 nmol/kg G108/SP	7.5 nmol/kg G108/HP
Alanine	603.1 ± 42.0	240.9 ± 26.5 ***P*<0.01**	579.2 ± 83.7
Arginine	37 ± 10.9	17.6 ± 2.1	35.9 ± 9.1
Aspartate	35.9 ± 4.6	32.1 ± 5.3	43.3 ± 8.2
Asparagine	78 ± 9.8	22.9 ± 1.9 ***P*<0.05**	65.4 ± 19.9
Glutamate	96.3 ± 11.2	81.7 ± 5.3	126.8 ± 16.7
Glutamine	678.5 ± 32.8	364.9 ± 20.4 ***P*<0.01**	665.5 ± 73.3
Glycine	282.3 ± 18.1	130.0 ± 5.0 ***P*<0.0001**	212.8 ± 22.0 ***P*<0.05**
Proline	197.1 ± 22.8	91.2 ± 13.6 ***P*<0.05**	255.5 ± 42.3
Serine	216.0 ± 13.4	86.5 ± 6.3 ***P*<0.01**	186.4 ± 35.0
Tyrosine	121.1 ± 10.6	62.4 ± 9.0 ***P*<0.05**	138.4 ± 17.1
Cysteine	0.9 ± 0.2	0.9 ± 0.1	1.2 ± 0.2

Table 3.2: Plasma levels of individual non-essential amino acids, as measured by gas-chromatography-mass-spectrometry (GCMS), following 13 days of treatment with vehicle and G108 with and without a high protein diet intervention, N=6/group. Data expressed as mean ± SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, difference compared to vehicle control group. Data analysed with one-way ANOVA with post hoc Dunnett's test.

3.4.6 G108-stimulated hypoaminoacidemia in obese mice is associated with markers of proteolysis in skeletal muscle, mitigated with a high protein diet.

To explore whether increased hepatic catabolism of amino acids and subsequent reduction in plasma amino acid availability affected cellular pathways of muscle protein turnover, the expression of key regulators of muscle protein breakdown in skeletal muscle (gastrocnemius) were determined after 27 days of daily peptide dosing. An increase in the expression of the E3-ligase, *Fbxo32* was observed in mice receiving 7.5 nmol/kg G108 and a standard protein diet (Figure 3.7A). Upregulation of *Fbxo32* expression was not prevented in mice receiving 7.5 nmol/kg G108 and a high protein diet. A similar pattern of expression was observed for markers of the autophagy-lysosomal pathway, including the autophagy related genes (ATG) *Maplc3b* and *Bnip3* (Figure 3.7B and C). Furthermore, a similar pattern of gene expression was observed for glutamine synthetase (*Glul*), a key enzyme which transferring ammonia from the breakdown of the branched chain amino acids to glutamine (Figure 3.7D). In mice receiving 7.5 nmol/kg G108, a trend towards increased expression of the downstream mTORc1 target, *4ebp1* (a repressor of protein translation) was observed (Figure 3.7E). Upregulation of *4ebp1* was not observed in mice receiving G108 and a high protein diet (Figure 3.7E). To further characterise changes in key skeletal muscle enzyme levels involved in protein synthetic and proteolysis pathways, western blot analysis was carried out. Ribosomal protein S6 kinase beta-1 (S6K) is an important marker of mammalian target of rampamycin (mTORC1) activity, with mTORC1 established as a key amino acid sensing complex regulating both muscle protein synthesis and proteolysis. Compared to the vehicle control group, no significant change was identified in the phosphorylation status of S6K1 in G108-treated mice, however a trend towards increased activity was observed in mice receiving the high protein diet (Figure 3.8A). No change in total levels of the E3 ligase Atrogin-1 were observed (Figure 3.8B). To determine whether there were any detectable changes in markers of cellular energy status, the phosphorylation status of AMPK was also measured. No significant difference in the T-172 phosphorylated AMPK:Total AMPK ratio was identified in the G108 group on either normal protein diet or a high protein diet, compared to vehicle control (Figure 3.8C).

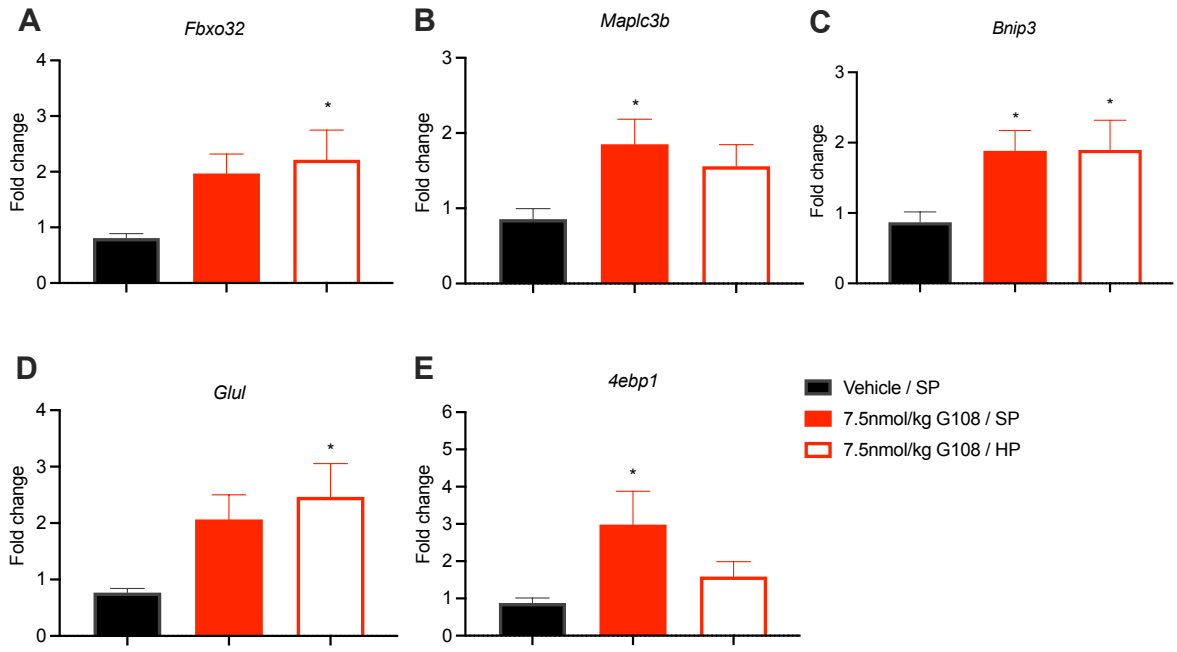


Figure 3.7: Gene expression of key ubiquitin-proteosome and proteolysosome autophagy pathways in skeletal muscle following 27 days of daily subcutaneous treatment with G108 peptide or vehicle control. N=5-6/group. From Hope, Hinds et al. (In Press). **(A)** F-box only protein 32 (*Fbxo32).* **(B)** Microtubule associated protein 1 light chain 3 beta (*Map1lc3b).* **(C)** BCL2 interacting protein 3 (*Bnip3).* **(D)** Glutamine synthetase (*Glul).* **(E)** Eukaryotic translation initiation factor 4E-binding protein 1 (*4ebp1).* Gene expression data analysed by the $2^{-\Delta\Delta CT}$ method, normalised to beta-actin endogenous control. Data expressed as mean ±- SEM. *p<0.05, ****p<0.0001, difference compared to vehicle control group.

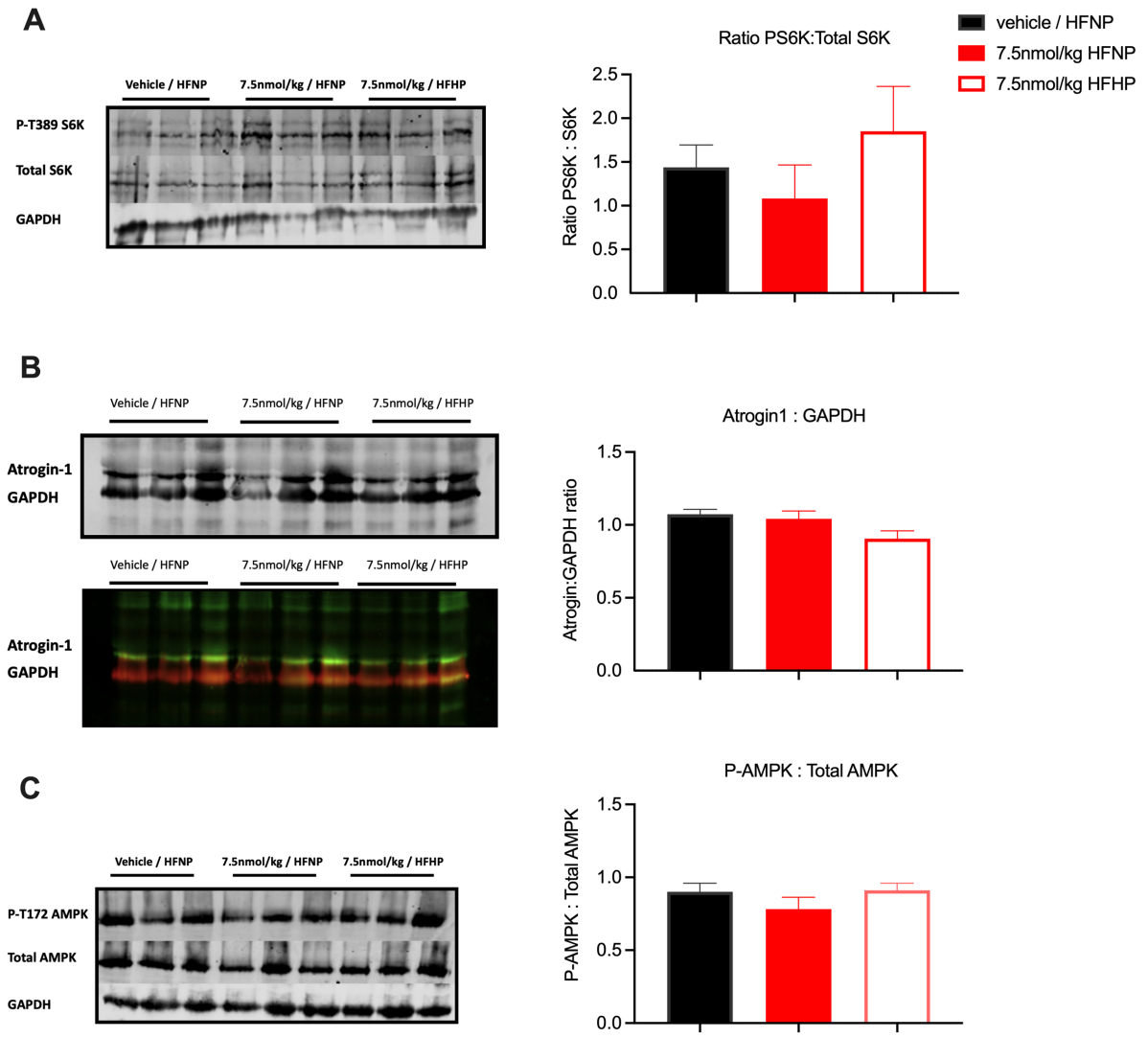


Figure 3.8: Protein quantification of key ubiquitin-proteosome and protein synthetic pathways in skeletal muscle following 27 days of daily subcutaneous treatment with G108 peptide or vehicle control. N=5-6/group. **(A)** Representative western blot image of p-S6k and total S6K comparing Vehicle / SP diet, 7.5 nmol/kg G108 / SP diet and 7.5 nmol/kg G108 / HP diet groups; Histogram of phosphorylated S6K:total S6K ratio, P-389 S6K and Total S6K normalised to endogenous control (GAPDH). **(B)** Representative multiplex western blot image of Atrogin-1 comparing Vehicle / SP diet, 7.5 nmol/kg G108 / SP diet and 7.5 nmol/kg G108 / HP diet groups; Histogram of Atrogin-1:GAPDH ratio normalised to endogenous control (GAPDH). **(C)** Representative western blot image of p-AMPK and total AMPK comparing Vehicle / SP diet, 7.5 nmol/kg G108 / SP diet and 7.5 nmol/kg G108 / HP diet groups; Histogram of p-AMPK:total AMPK ratio normalised to endogenous control (GAPOH). N=3-6/group. Western blot data analysed by one-way ANOVA. Data expressed as mean ± SEM. *p<0.05, ****p<0.0001.

3.4.7 G108-stimulated hypoaminoacidemia is associated with increased hepatic expression and circulating levels of fibroblast growth factor 21 (FGF21)

The hepatic expression of *Fgf21* was significantly upregulated in mice receiving 7.5 nmol/kg G108 and a standard protein diet (Figure 3.9A). This response was not observed in mice receiving the same dose level of G108 and a high protein diet (Figure 3.9A). The circulating plasma levels of FGF21 mirrored the pattern of hepatic gene expression in G108-treated mice receiving the standard protein diet and likewise a high protein diet prevented an increase in circulating FGF21 in G108-treated mice (Figure 3.9B).

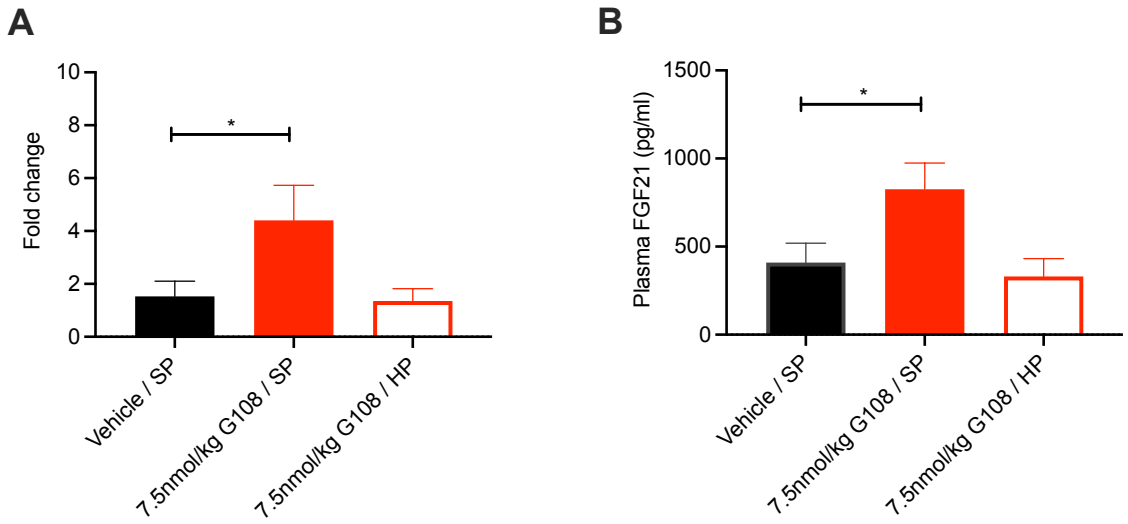


Figure 3.9: Changes in hepatic expression and circulating levels of fibroblast growth factor 21 (FGF21). From Hope, Hinds et al. (In Press). **(A)** Fold change in expression of hepatic Fgf21, n=6/group. **(B)** Circulating plasma levels of FGF21, n=9/group. Data expressed as mean ± SEM, *p<0.05, analysed using one-way ANOVA with post hoc Dunnett's test.

3.4.8 The effect of 7.5 nmol/kg G108 and a high protein diet on whole-body energy expenditure

Daily dosing of 7.5 nmol/kg G108 led to a sustained increase in average oxygen consumption (VO2) compared to mice receiving vehicle control (Figure 3.10A and B). The increase in oxygen consumption was also similar across time phases (Table 3.3, average oxygen consumption (ml/hr) in the light phase; Vehicle / SP : 118.32 ± 3.03 ml/hr, 7.5 nmol/kg / SP: 132.85 ± 3.02 ml/hr, average oxygen consumption in the dark phase Vehicle / SP: 134.39 ± 3.34 ml/hr, 7.5 nmol/kg / SP: 149.09 ± 3.33 ml/hr). Consistent

with the change in oxygen consumption, the average energy expenditure as measured by kcal expended per hour was also increased in mice receiving G108 (Figure 3.10C, Table 3.4), average energy expenditure (kcal/hr) in the light phase; Vehicle / SP : 0.57 ± 0.01 kcal/hr, 7.5 nmol/kg / SP: 0.64 ± 0.01 kcal/hr, average energy expenditure in the dark phase; Vehicle / SP : 0.63 ± 0.02 kcal/hr, 7.5 nmol/kg / SP: 0.69 ± 0.02 kcal/hr). A high protein diet prevented the increase in whole-body oxygen consumption and energy expenditure (kcal/hr) in both light and dark phases in mice treated with G108 (Figure 3.10B and C, Table 3.3 and 3.4).

G108 treatment led to an increase in carbon dioxide production (VCO2) however this did not reach statistical significance (Figure 3.10D, Table 3.5). A lower VCO2 to VO2 ratio were reflected in the lower respiratory exchange ratio (RER) between Vehicle / SP and 7.5 nmol/kg G108 / SP groups during the dark and full day time phases (Figure 3.10E). A significant reduction in RER was noted between Vehicle / SP and 7.5 nmol/kg HP groups, particularly in the dark and full day phases (Figure 3.10E, Table 3.5), consistent with the predicted increase in feeding of a high protein diet during the dark period.

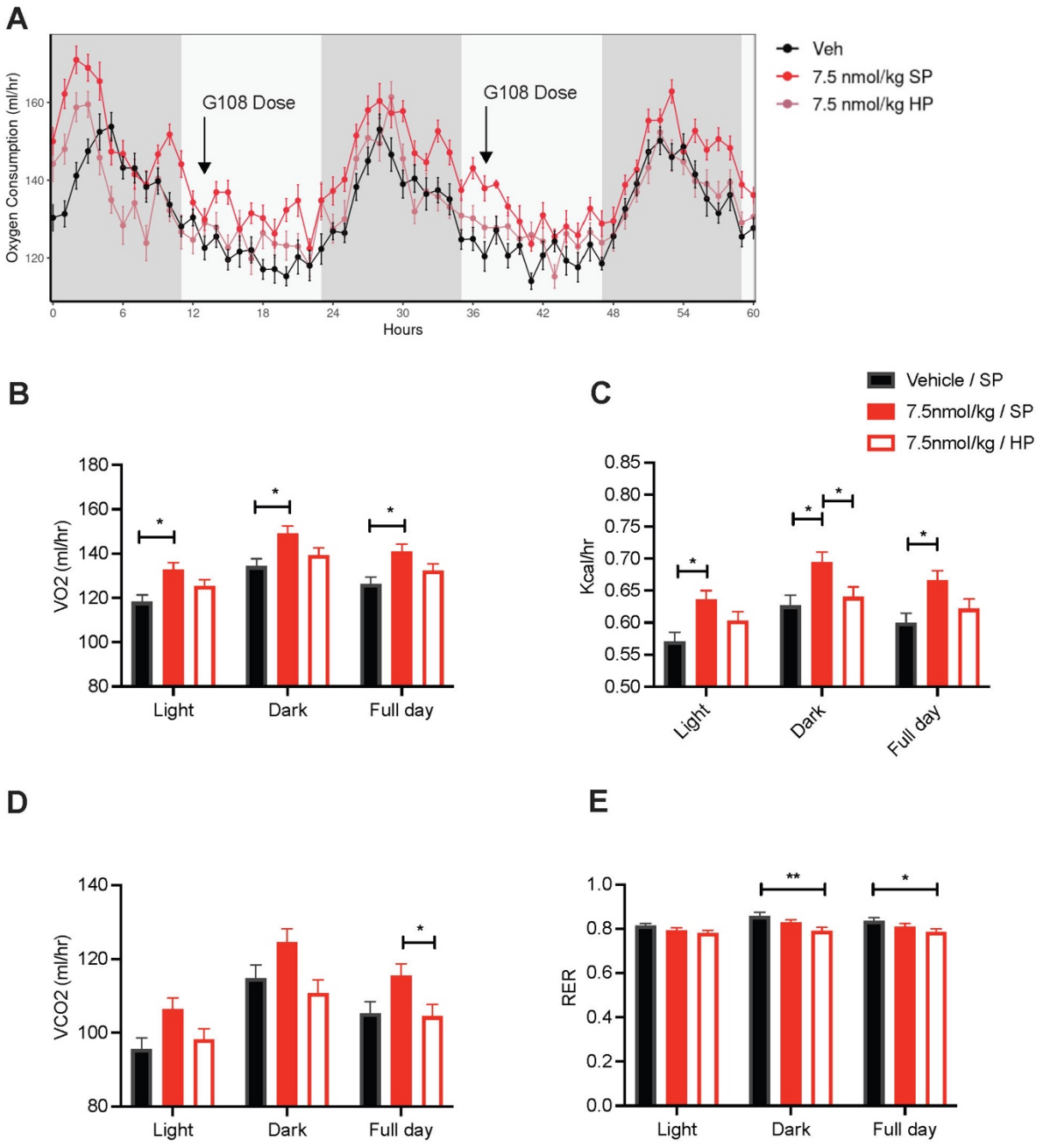


Figure 3.10: Whole-body oxygen consumption and carbon dioxide production in 7.5 nmol/kg G108-treated DIO mice. From Hope, Hinds et al. (In Press). **(A)** Oxygen consumption (ml/hr). **(B)** Average oxygen consumption (ml/hr) over the full day, dark or light phase. **(C)** Average energy expenditure (kcal/hr) over the full day, dark or light phase. **(D)** Average carbon dioxide production (ml/hr) over the full day, dark or light phase. **(E)** Respiratory exchange ratio (RER) over the full day, dark or light phase. Data expressed as mean ± SEM, *p<0.05. VO2, VCO2 and EE data analysed using regression-based ANOVA. RER data analysed with one-way ANOVA with post hoc Dunnett's test.

Average O2 consumption (ml/hr)	Light	Dark	Full day
Vehicle / SP	118.32 ± 3.03	134.39 ± 3.34	126.36 ± 3.13
7.5 nmol/kg / SP	132.85 ± 3.02 ***P*<0.05**	149.09 ± 3.30 ***P*<0.05**	140.97 ± 3.31 ***P*<0.05**
7.5 nmol/kg / HP	125.26 ± 2.99	139.27 ± 3.29	132.27 ± 3.08

Table 3.3: Average oxygen consumption (VO2) during metabolic cage study day 22-25. N=7-8/group. Significance indicates difference between treatment group and vehicle / SP group. Analysed by regression based ANCOVA with lean mass as co-variate.

Average kcal/hr	Light	Dark	Full day
Vehicle / SP	0.57 ± 0.01	0.63 ± 0.02	0.60 ± 0.01
7.5 nmol/kg / SP	0.64 ± 0.01 ***P*<0.05**	0.69 ± 0.02 ***P*<0.05**	0.67 ± 0.02 ***P*<0.05**
7.5 nmol/kg / HP	0.60 ± 0.02	0.64 ± 0.02	0.62 ± 0.02

Table 3.4: Average energy expenditure (kcal/hr) during metabolic cage study day 22-25. N=7-8/group. Significance indicates difference between treatment group and vehicle / SP group. Analysed by regression based ANCOVA with lean mass as co-variate.

Average CO2 production (ml/hr)	**Light**	**Dark**	**Full day**
Vehicle / SP	95.60 ± 3.02	114.70 ± 3.61	105.20 ± 3.24
7.5 nmol/kg / SP	106.40 ± 3.01	124.57 ± 3.59	115.48 ± 3.22 ***P*<0.05**
7.5 nmol/kg / HP	98.16 ± 2.98	110.76 ±3.55	104.47 ± 3.19

Table 3.5: Average carbon dioxide production (VCO2) during metabolic cage study day 22-25. N=7-8/group. Significance indicates difference between 7.5 nmol/kg / SP group and 7.5 nmol/kg / HP group. Analysed by regression-based ANCOVA with lean mass as co-variate.

Respiratory exchange ratio	**Light**	**Dark**	**Full day**
Vehicle / SP	0.82 ± 0.01	0.86 ± 0.01	0.84 ± 0.01
7.5 nmol/kg / SP	0.79 ± 0.01	0.83 ± 0.01	0.81 ± 0.01
7.5 nmol/kg / HP	0.78 ± 0.01	0.80 ± 0.01 ***P* <0.01**	0.79 ± 0.01 ***P* <0.05**

Table 3.6: Average respiratory exchange ratio (RER) during metabolic cage study day 22-25. N=7-8/group. Significance indicates difference between treatment group and vehicle / SP group. Analysed using two-way ANOVA with post hoc Dunnett's test.

3.4.9 Chronic G108 administration leads to improved glucose tolerance independent to dietary protein content

To determine whether a chronic beneficial effect on glycaemia could be observed in obese mice using the sub-anorectic dose of a glucagon agonist (7.5 nmol/kg G108), the effects on fasting glucose and glucose tolerance were determined. A glucose tolerance test on day 15 revealed improvements in fasting glucose and glucose tolerance across all treatment groups compared to vehicle control (Figure 3.11A and B, Table 3.7). This was not associated with a difference in 20-minute glucose stimulated plasma insulin levels (Figure 3.11C). No differences were observed in insulin tolerance in any treatment group compared to vehicle control (Figure 3.11D, E and F).

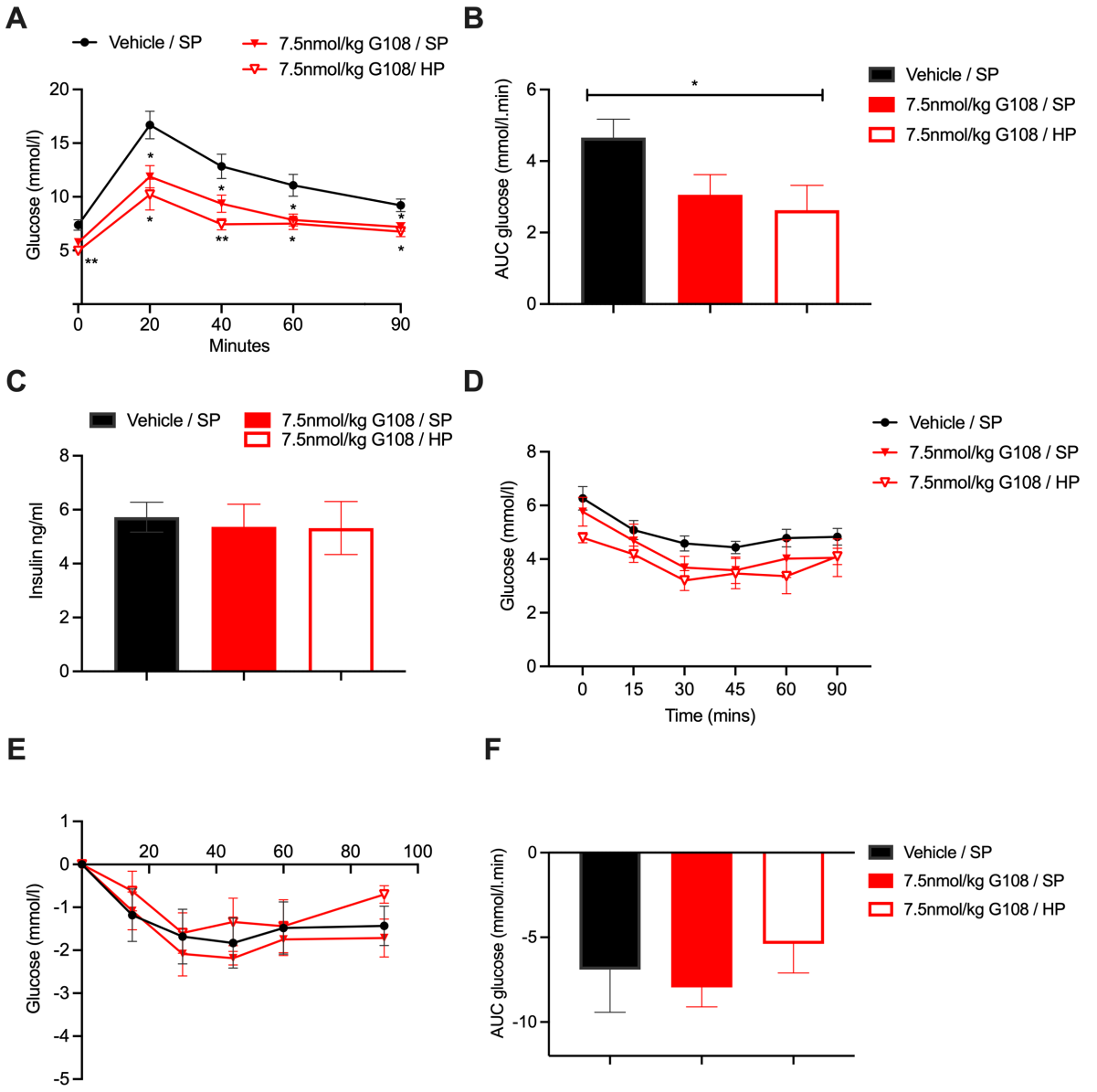


Figure 3.11: Glycaemic data. **(A)** 2 g/kg intraperitoneal glucose tolerance test on day 15 of study n=9/group. From Hope, Hinds et al. (In Press). **(B)** Incremental area under the curve (AUC) for glucose tolerance test (baseline to 20 min) **(C)** 20 minute-stimulated plasma insulin levels during ipGTT. **(D)** 0.75 U/kg intraperitoneal insulin tolerance test (IpGTT), n=6/group. **(E)** IpGTT results as in (D) corrected for baseline glucose **(F)** Incremental area under the curve (AUC) for glucose tolerance test. *p<0.05, **p<0.01, ***p<0.001, ***p<0.0001. Data expressed as mean ±- SEM. One- and two-way ANOVA with post hoc Dunnett's test.

Glucose (mmol/l)	Vehicle control	7.5 nmol/kg G108 / SP	7.5 nmol/kg G108 / HP
Baseline (0 mins)	7.38 ± 0.49	5.76 ± 0.38 ***P*<0.05**	4.96 ± 0.37 ***P*<0.01**
20 min	16.69 ± 1.28	11.87 ± 1.05 ***P*<0.05**	10.21 ± 1.44 ***P*<0.05**
40 min	12.84 ± 1.13	9.36 ± 0.81 ***P*<0.05**	7.44 ± 0.51 ***P*<0.01**
60 min	11.07 ± 1.03	7.84 ± 0.56 ***P*<0.05**	7.50 ± 0.56 ***P*<0.05**
90 min	9.20 ± 0.59	7.19 ± 0.41 ***P*<0.05**	6.76 ± 0.47 ***P*<0.05**

Table 3.7: Intraperitoneal glucose tolerance test (2g/kg) on day 15 of study, Vehicle control / SP diet, 7.5nmol/kg G108 / SP diet. 7.5 nmol/kg G108 / HP diet. Data represented as mean ± SEM, significance indicates difference between vehicle control group and treatment group, *p<0.05, **p<0.01. Analysed using two-way ANOVA with post hoc Dunnett's test.

3.4.10 Chronic G108 administration leads to a reduction in hepatic lipid accumulation independent to dietary protein content

To determine the effect of chronic G108 treatment on other key metabolic benefits attributed to GCGR signalling, plasma lipids and hepatic lipid metabolism were further explored. Although no change was observed in plasma total triglycerides across the groups (Figure 3.12A), a significant reduction in plasma total cholesterol was detected in mice administered with G108 and fed either the SP or HP diet (Figure 3.12B). Gene expression analysis revealed a reduction in the hepatic expression of *Fasn*, a key lipogenic marker and this reduction was also observed in mice fed a HP diet (3.12C). The expression of *Cpt1a*, a marker of fatty acid catabolism was increased only in G108-treated mice fed a SP diet. Consistent with the gene expression data, histological examination of hepatic slices revealed a marked reduction in hepatic lipid accumulation in G108-treated mice, irrespective of dietary protein content (Figure 3.12D).

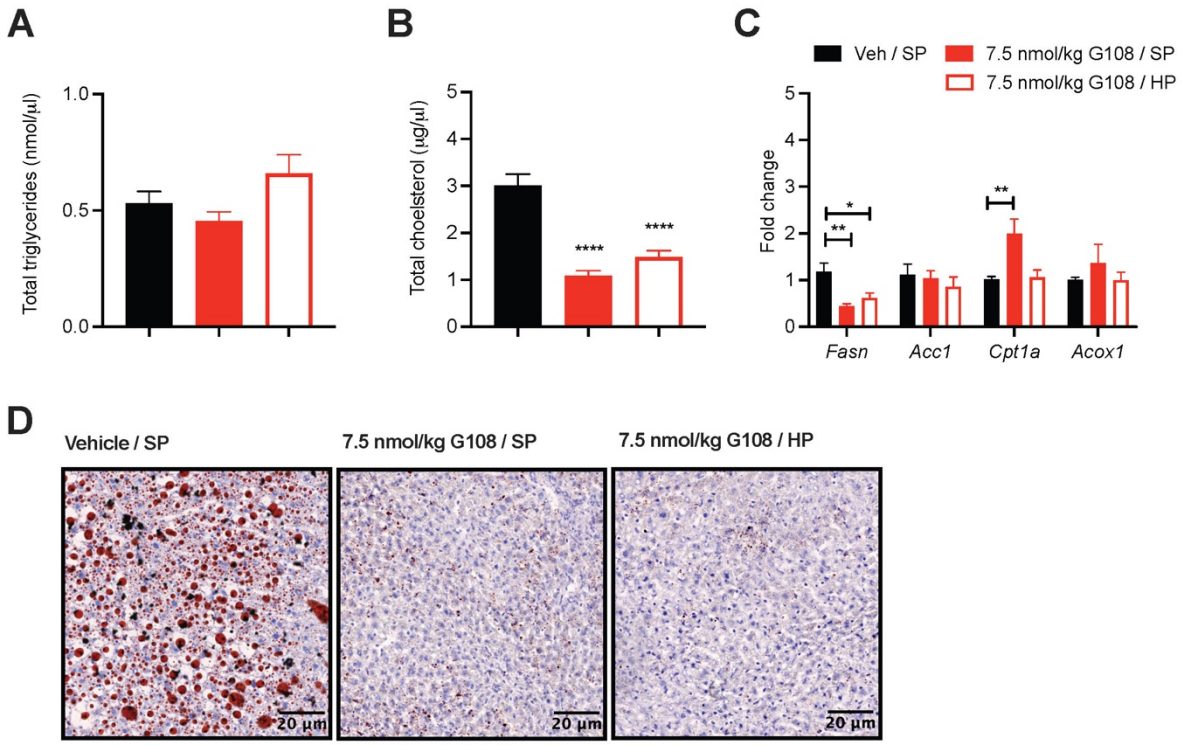


Figure 3.12: Lipid metabolism parameters from chronic 7.5 nmol/kg G108 daily dosing study in DIO mice. From Hope, Hinds et al. (In Press). **(A)** Plasma total triglyceride on day 14 of study, n=9/group. **(B)** Plasma total cholesterol on day 14 of study, n=9/group. **(C)** Expression of markers of hepatic lipid metabolism, n=8-9/group **(D)** Representative micrographs of terminal hepatic lipid content as indicated by Oil Red O staining. (F). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, indicates comparison between treatment and vehicle control group. Data plotted as mean ± SEM, analysed using one- and two-way ANOVA. Gene expression data analysed by the 2-DDCT method, normalised to cyclophilin endogenous control.

3.4.11 Chronic G108 administration activates a multi-organ energy consuming response associated with hypoaminoacidemia

Given that G108-stimulated energy expenditure and fat mass loss were prevented when feeding with a high protein diet, hypoaminoacidemia-mediated EE becomes a compelling mechanism explaining glucagon-mediated EE. Several early studies demonstrated the effect of dietary protein restriction on enhancing EE in rodents and humans (Miller and Payne 1962, Miller 1967, Stirling and stock 1968, McCraken 1976, Tulp 1979, Rothwell 1979, Rothwell and Stock 1982, Rothwell et al 1983). More recent research in rodents has demonstrated the divergent effects of dietary protein restriction on the EE mechanisms, including sympathetic activation, BAT and muscle thermogenesis and FGF21 action (Pezeshki, Zapata et al. 2016). Therefore, markers of energy expenditure

were measured in various tissues in the obese G108-treated mice receiving either the SP or HP diet.

During the assessment period in metabolic cages, a general trend towards increased locomotor activity across the light and dark phases was observed in G108-treated obese mice fed a standard protein diet (Figure 3.13A-C, Table 3.8). A significant increase in locomotor activity (XTOT) was observed in the G108 / SP group compared to vehicle control in the first dark phase (D1) of the assessment period (Figure 3.13C). G108-treated mice fed the high protein diet did not exhibit the same increase in activity throughout the light or dark phases with numerical reductions observed (Figure 3.13A-C, Table 3.8).

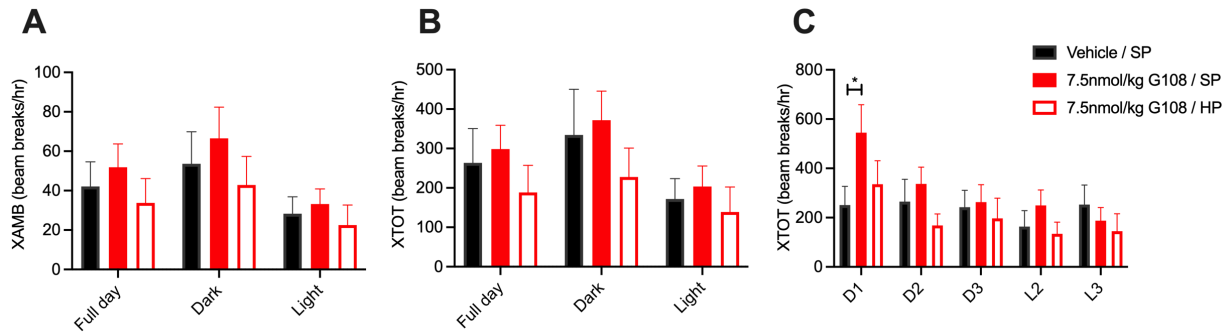


Figure 3.13: Activity levels in G108-treated obese mice. From Hope, Hinds et al. (In Press). **(A)** Ambulatory activity (XAMB) over a 60-hour period in 7.5 nmol/kg G108-treated mice. **(B)** Locomotor activity (XTOT) in 7.5 nmol/kg G108 treated mice. **(C)** Locomotor activity over individual dark and light periods in 7.5 nmol/kg G108-treated mice, n=7-8/group. *p<0.05, **p<0.01. Data expressed as mean ± SEM analysed using one- way ANOVA with post hoc Sidak's test, difference between treatment and vehicle group.

XTOT (Beam breaks in x-axis)	D1	D2	D3	L2	L3
Vehicle / SP	250.7 ± 76.11	265.2 ± 90.54	242.2 ± 68.46	163.7 ± 64.63	253.2 ± 79.37
7.5 nmol/kg / SP	545.1 ± 113.0 ***P*<0.05**	337.1 ± 68.23	234.1 ± 68.68	249.8 ± 62.65	187.6 ± 53.36
7.5 nmol/kg / HP	335.7 ± 95.54	168.3 ± 26.92	172.9 ± 75.13	134.4 ± 47.35	144.9 ± 71.07

Table 3.8: Activity levels in G108-treated obese mice across individual dark and light periods. As represented in Figure 3.12. *p<0.05, Data expressed as mean ± SEM analysed using one- way ANOVA with post hoc Sidak's test, difference between treatment and vehicle group.

The measurement of a selection of thermogenic markers in skeletal muscle (gastrocnemius) was made at the end of the 27-day daily dosing study in DIO mice. Increased expression of the beta 2 adrenergic receptor (*Adrb2*), Sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (*Serca1*) and uncoupling protein 3 (*Ucp3*) was found in G108-treated mice receiving a standard protein diet (Figure 3.14A-C, Table 3.9). This increase was not observed in mice receiving the high protein diet (Figure 3.14A-C). No significant change was observed in the skeletal muscle gene expression of either fibroblast growth factor-21 (*Fgf21)* or Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (*Pgc1-alpha)* (Figure 3.14A-C, Table 3.9).

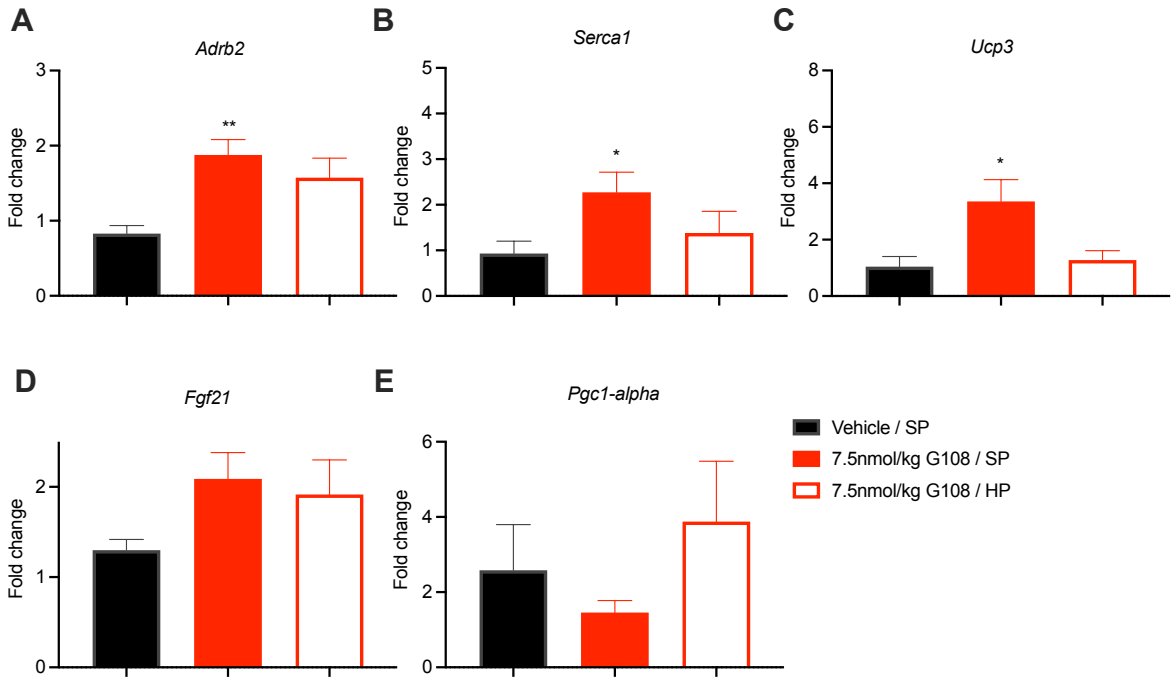


Figure 3.14: Markers of skeletal muscle EE from 27-day daily dosing study with G108 in diet induced obese (DIO) mice. From Hope, Hinds et al. (In Press). **(A)** Expression of skeletal muscle beta 2 adrenergic receptor (*Adrb2*) **(B)** sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (*Serca1*) and **(C)** uncoupling protein 3 (*Ucp3*) **(D)** Fibroblast growth factor 21 (*Fgf21*). **(E)** Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (*Pgc1-alpha).* *p<0.05, **p<0.01. Data expressed as mean ± SEM analysed using one-way ANOVA with post hoc Sidak's test, difference between treatment and vehicle group. Gene expression data analysed by the 2-DDCT method, normalised to beta-actin endogenous control.

Gene	Vehicle / SP	7.5 nmol/kg G108 / SP	7.5 nmol/kg G108 / HP
Adrb2	0.83 ± 0.11	1.88 ± 0.21 ***P*<0.01**	1.57 ± 0.26
Serca1	0.93 ± 0.27	2.27 ± 0.44 ***P*<0.05**	1.38 ± 0.47
Ucp3	1.05 ± 0.36	3.35 ± 0.77 ***P*<0.05**	1.27 ± 0.33
Fgf21	1.30 ± 0.12	2.09 ± 0.29	1.91 ± 0.38
Pgc1-alpha	2.58 ± 1.21	1.46 ± 0.32	3.89 ± 1.60

Table 3.9: Markers of skeletal muscle EE from 27-day daily dosing study with G108 in diet induced obese (DIO) mice. As represented in Figure 3.12.

In brown adipose tissue (BAT), chronic G108 administration did not result in a significant change in the expression of the key thermogenic uncoupling protein 1 (*Ucp1*) (Figure 3.15A, Table 3.10). No changes were observed in other regulators of Ucp1 or BAT thermogenesis including cell death-inducing DNA fragmentation factor alpha-like effector A (*Cidea*), Iodothyronine deiodinase 2 (*Dio2*) or peroxisome proliferator-activated receptor-gamma coactivator (*Pcg1-alpha*). A significant increase in fibroblast 21 (*Fgf21*) expression was observed in G108-treated mice fed the SP diet, and this was not observed in mice fed the HP diet.

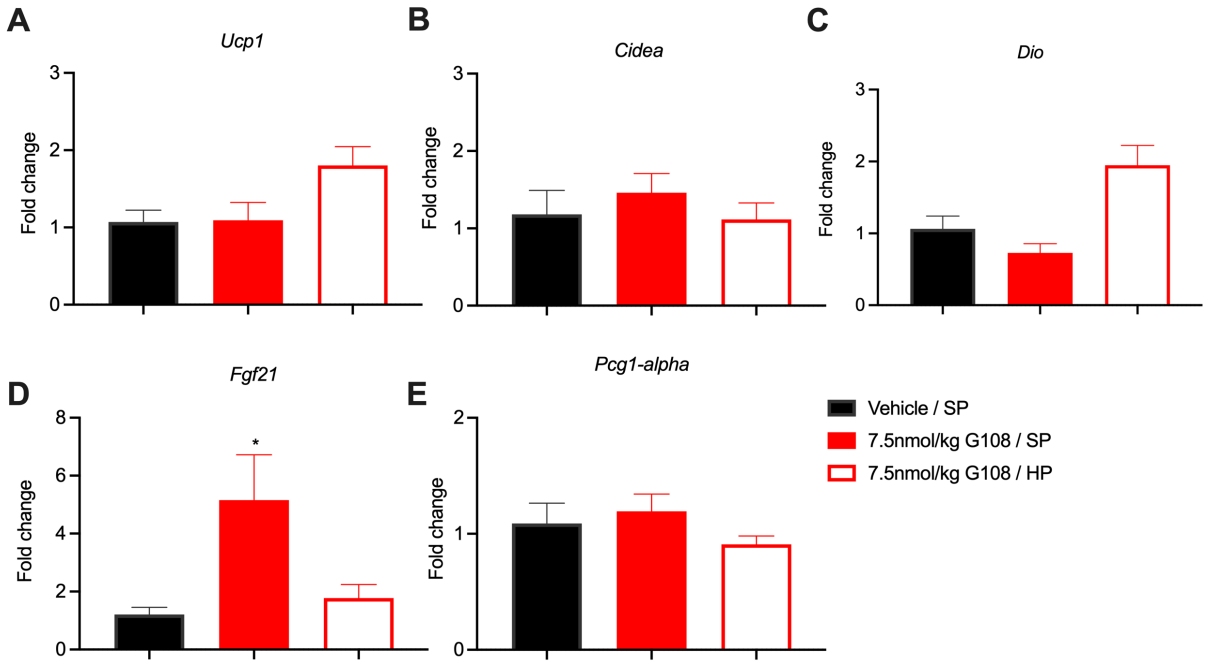


Figure 3.15: Markers of brown adipose tissue (BAT) markers of EE from 27-day daily dosing study with G108 in diet induced obese (DIO) mice. Adapted from Hope *et al*., 2022. **(A)** Uncoupling protein 1 (*Ucp1*) **(B)** Cell Death Inducing DFFA Like Effector A (*Cidea*). **(C)** Iodothyronine diodinase (*Dio*) **(D)** Fibroblast growth factor 21 (*Fgf21*). **(E)** Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (*Pgc1-alpha).* *p<0.05, **p<0.01. Data expressed as mean ± SEM analysed using one- way ANOVA with post hoc Sidak's test, difference between treatment and vehicle group. Gene expression data analysed by the 2-DDCT method, normalised to beta-actin endogenous control.

Gene	Vehicle / SP	7.5 nmol/kg G108 / SP	7.5 nmol/kg G108 / HP
Ucp1	1.07 ± 0.15	1.10 ± 0.24	1.80 ± 0.14
Cidea	1.18 ± 0.47	1.46 ± 0.21	1.12 ± 0.48
Dio	1.06 ± 0.18	0.723 ± 0.13	1.95 ± 0.28
Fgf21	1.21 ± 4.19	5.16 ± 0.47 ***P<0.05***	1.78 ± 4.19
Pgc1-alpha	1.089 ± 0.1758	1.20 ± 0.15	0.91 ± 0.07

Table 3.10: Markers of brown adipose tissue (BAT) markers of EE from 27-day daily dosing study with G108 in diet induced obese (DIO) mice. As represented in Figure 3.14.

Although a trend towards increased expression of *Ucp1* was observed in white adipose tissue (WAT) of G108-treated mice fed the SP diet, this did not reach statistical significance (Figure 3.16).

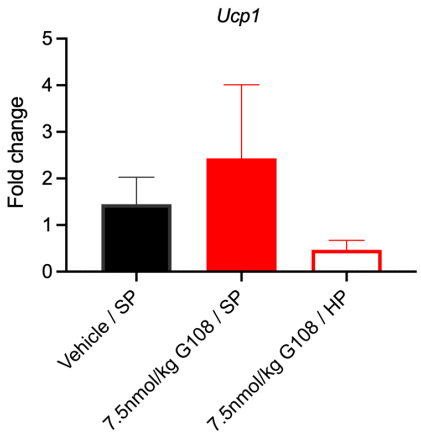


Figure 3.16: Gene expression of white adipose tissue (WAT) uncoupling protein 1 (*Ucp1*) from 27-day daily dosing study with G108 in diet induced obese (DIO) mice**.**

To determine whether any circulating markers of increased EE were detectable, both plasma norepinephrine and thyroxine were measured. G108-treated mice fed with either the SP or HP diet exhibited significantly higher circulating levels of norepipnephrine compared to vehicle control (Figure 3.17A, Table 3.11). No change was detected in circulating thyroxine (Figure 3.17B, Table 3.11).

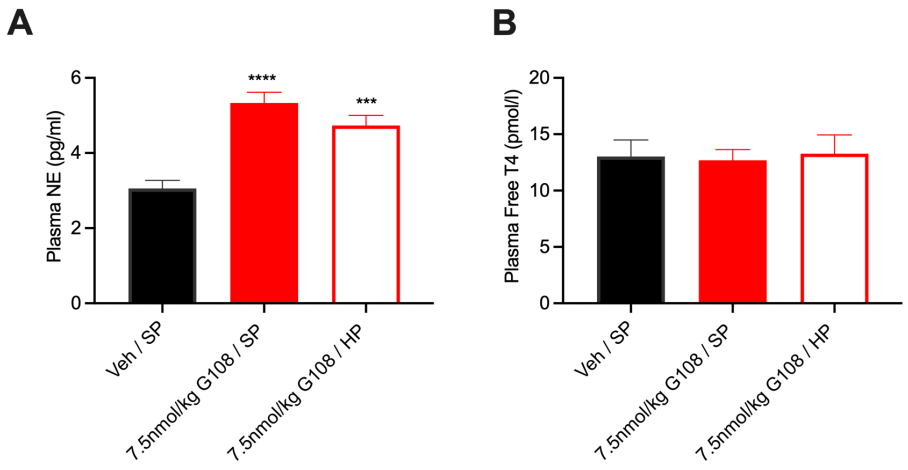


Figure 3.17: Terminal plasma norepinephrine and free thyroxine (T4) from 27-day daily dosing study with G108 in diet induced obese (DIO mice). ***p<0.001, ****p<0.0001. Data expressed as mean ± SEM analysed using one- way ANOVA with post hoc Dunnett's test, significance indicates difference between treatment and vehicle group.

Gene	Veh / SP	7.5 nmol/kg G108 / SP	7.5 nmol/kg G108 / HP
Plasma norepinephrine (pg/ml)	3.06 ± 0.22	5.33 ± 0.28 ***P*<0.0001**	4.73 ± 0.27 ***P*<0.001**
Plasma thyroxine (pmol/l)	13.03 ± 1.47	12.70 ± 0.94	13.27 ± 1.67

Table 3.11: Plasma norepinephrine and thyroxine levels from 27-day daily dosing study with G108 in diet induced obese mice.

3.5 Discussion

This Chapter investigated the chronic effect of G108, a long-acting glucagon analogue, on hepatic amino acid catabolism, plasma amino acids and lean mass in an obese model. The effects of G108 and a high protein diet on plasma amino acid levels and lean mass in obese mice were initially determined followed by a broader assessment of the metabolic benefits conferred by G108 on either the SP or HP diet. These will be discussed in more detail in the following sections.

3.5.1 G108 stimulates chronic hepatic amino acid catabolism leading to low plasma amino acids and lean mass loss in DIO mice

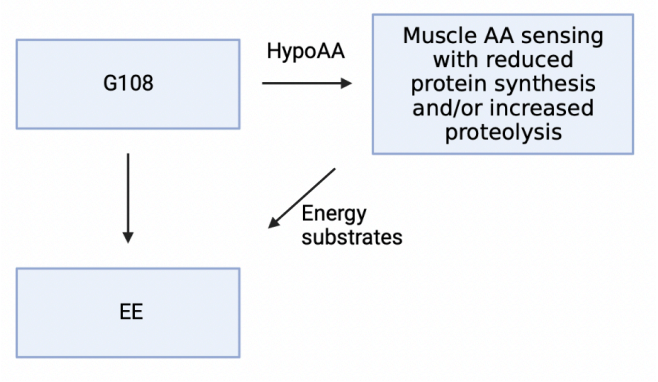
A chronic daily dosing study of G108 in DIO mice over 27 days was carried out using a sub-anorectic dose of G108 in mice receiving either a standard or high protein diet (SP and HP, respectively). G108 administration led to a striking reduction in lean mass compared to vehicle control as early as 14 days of treatment, which was further evident at day 25 (Figure 3.2). This was associated with a significant reduction in several individual essential- and non-essential amino acids (Figures 3.5 and 3.6). This is in keeping with the broad catabolic action of glucagon on amino acids in the clinical case of glucagon excess, for example the glucagonoma syndrome (Mallinson, Bloom et al. 1974, Almdal, Heindorff et al. 1990). A particular lowering in specific essential amino acids was observed, for example lysine, methionine, and threonine (Figure 3.5) the latter two also profoundly reduced in the glucagonoma syndrome (Almdal, Heindorff et al. 1990). The findings here are also consistent with previous glucagon infusion studies in man, in which a broad reduction in amino acids was observed over short term infusions of glucagon dose of 6 ng/kg/min (Boden, Rezvani et al. 1984, Charlton, Adey et al. 1996). Our group has previously demonstrated that a GLP-1R/GCGR co-agonist results in reduction in plasma total amino acids in rats over 22 days (Rebecca Scott 2019). The findings in this chapter extend these findings by demonstrating that a long-acting glucagon analogue results in a broad reduction in individual amino acids together with lean mass loss in an obese mouse model.

Consistent with G108-stimulated hypoaminoacidemia and the known actions of glucagon, an increase in key markers of hepatic amino acid catabolism were observed; G108 led to increased expression of urea cycle enzymes and amino acid transporters (Figure 3.4A). Glucagon induction of urea cycle enzyme activity is well established; In a 3-day study in rats, a particular increase in the activity of CPS, ASS and ASL was observed (McLean and Novello 1965). Another study demonstrated the increase the activity of all the urea cycle enzymes up to 7 days in rats with a continuous glucagon infusion (Snodgrass, Lin et al. 1978). Further studies *in* cultured rodent hepatocytes also support a general increase in mRNA expression of the urea cycle enzymes (Bobe, Velez et al. 2009) (Ulbright and Snodgrass 1993). Also consistent with the present findings of G108 in obese rodents, our group has previously demonstrated that a GLP-1R/GCGR co-agonist results in the increased expression of hepatic arginosuccinate synthetase (*Ass*) and carbamoyl phosphate Synthetase (*Cps*) after 22 days of daily injection in rats (Rebecca Scott 2019). The change in expression of hepatic amino acid transporters has also been demonstrated in a model of glucagon resistance, using hepatic-specific $Gcgr^{-/-}$ mice, which in addition to the urea cycle enzymes exhibit a marked reduction in the expression of amino acid transporters (Winther-Sorensen, Galsgaard et al. 2020). More recently, it has been shown that chronic administration of a long-acting GCG analogue in mice only increased the hepatic expression of *Ass* with no change in the expression of *Arg*, *Asl*, *Cps* or *Nags* (Emilie Elmelund 2022). Furthermore, interestingly the same study showed a specific increase in the enzyme activity of CPS1. This is consistent with previous studies demonstrating the post-transcriptional regulation of CPS1 by glucagon via Sirtuin proteins (Li, Zhang et al. 2016). Therefore, enhanced hepatic GCGR activity appears to have multiple paths to increase amino acid catabolism, and these may be context dependent for example on the dose, timing, and species. The findings in this chapter therefore are consistent with previous studies of native glucagon and demonstrate chronic upregulation of hepatic amino acid catabolism, including urea cycle and amino acid transporters in obese mice, in response to a long-acting glucagon analogue.

In keeping with observation from the glucagonoma syndrome (Stevens, Flanagan et al. 1984), chronic G108 exposure in the current DIO study led to a reduction in lean mass and decline in muscle function, in particular forelimb grip strength (Figure 3.3). A previous study exploring the effects of the glucagon agonist, IUB288, showed lean mass

loss in lean mice although plasma amino acid levels, skeletal muscle mass or function was not reported (Habegger, Stemmer et al. 2013). Another study using several custom GLP-1R/GCGR co-agonists has also demonstrated that a GCGR selective co-agonist, 'Peptide 5', may result in significant lean mass loss in obese mice (Day, Gelfanov et al. 2012); Again in this study, plasma amino acids, muscle weight or function were not reported. Interestingly, a recent study has demonstrated that hyperglucagonemia associated with type 2 diabetes leads to enhanced hepatic alanine catabolism (Okun, Rusu et al. 2021). In this study, a supporting experiment demonstrated that chronic glucagon analogue (IUB288) therapy over 3 weeks resulted in loss of lean mass and reduced grip strength in lean mice, restored with hepatic alanine aminotransferase silencing; Although plasma amino acids are not reported, this suggests that the loss of lean mass may be directly due to GCG-stimulated hepatic amino acid catabolism and a perturbation of this liver-muscle axis. Collectively, these previous studies are consistent with the findings presented in this Chapter where it has been demonstrated that in an obese mouse model, a long-acting glucagon analogue stimulates chronic hypoaminoacidemia which is associated with a reduction of lean mass and muscle function. These findings may have implications for the use of glucagon agonists as anti-obesity drugs, in particular as many drugs in development are multi-agonists and may target the GCGR (Hope, Vincent et al. 2021).

The findings presented in this chapter show that G108-stimulated hypoaminoacidemia is associated with lean mass loss however the precise mechanism leading to lean mass loss has not been established. A likely pathway to lean mass loss is direct amino acid sensing in lean mass. Local amino acid sensing in lean mass is a major regulator of mTOR and therefore this may be a prominent mechanism of lean mass loss (Kamei, Hatazawa et al. 2020). As glucagon enhances energy expenditure, a chronic net release of amino acids may contribute to energy requirements through provision of gluconeogenic substrates (Schematic 3.1). Supporting this, although the precise mechanism of lean mass loss in the glucagonoma syndrome remains unclear it is thought to be due to chronic hypoaminoacidemia (Almdal, Heindorff et al. 1990).



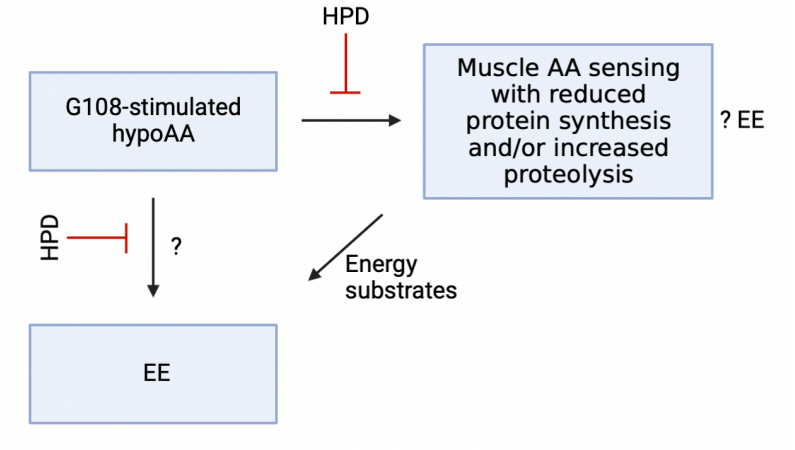
Schematic 3.1: Potential interaction between G108-stimulated hypoaminoacidemia, lean mass loss and EE

3.5.2 A high protein diet rescues G108-stimulated hypoaminoacidemia and limits lean mass loss

A high protein diet rescued pre-dose total plasma amino acid levels, as measured on day 13 of the study (Figure 3.4B) in G108-treated mice, and this was associated with blunting of lean mass loss by day 14 and day 25 (Figure 3.2). A high protein diet intervention has not been reported in models of exogenous native or glucagon analogue administration, however evidence for the effect of repleting amino acids in the context of hyperglucagonemia can be found in other situations. For example, previous studies have demonstrated the effects of excess glucagon and amino acid replacement on hepatic amino acid catabolism and muscle loss in models of critical illness (Thiessen, Derde et al. 2017). A key concern in critical illness has been sustained glucagon-driven hepatic nitrogen clearance despite amino acid replacement. Indeed, muscle loss is seen early on in critical illness and attempts to counteract the reduction in plasma amino acid availability with parenteral amino acid administration is limited by further stimulation of glucagon secretion and subsequent hepatic amino acid catabolism (Thiessen, Derde et al. 2017). Confounding the interpretation from this extreme clinical condition, in critical illness, other catabolic hormones also play a role in muscle proteolysis which are known to act together to enhance proteolysis (Gore, Jahoor et al. 1993). Given the role of other catabolic hormones in muscle proteolysis, plasma corticosterone was measured however no differences were observed between treatment groups (Figure 3.4F). In contrast to these models of critical illness, G108-driven hypoaminoacidemia in obese mice is rescued by dietary protein supplementation and this is associated with a mitigation of lean mass loss (Figure 3.2). Further studies would be required to determine whether a high protein

diet intervention can also overcome G108-stimulated hypoaminoacidemia when higher *anorectic* doses of peptide are used. At the sub-anorectic dose used of G108, although a 40 kcal% (high protein) diet broadly rescued plasma amino acids, specific amino acids were still reduced for example lysine, glycine and threonine. (Figure 3.5 and 3.6). Furthermore, at the end of the study, plasma total amino acids were also found to be reduced in G108-treated mice fed a HP diet (Figure 3.4E). Consistent with these findings, lean mass loss was not fully prevented in mice receiving the high protein diet (Figure 3.2). It is possible that the plasma amino acid measurements did not reflect any 'hidden' reductions in levels throughout the light/fasting period where the protective effect of the high protein diet on plasma amino acids and lean mass in G108-treated mice may have been attenuated.

The mechanism of a high protein diet protecting against lean mass through rescue of plasma amino acids from sub-normal to normal levels may be multi-faceted. However, based on established amino acid sensing pathways in lean tissue (Chapter 1, section 1.17), it is likely that direct amino acid sensing in lean mass is conferring the protective effects observed. From the body weight and food intake data (Figure 3.1), G108 enhances EE in obese mice receiving the SP diet. Therefore, it is possible that direct sensing of low amino acid availability in lean mass, acts as a mechanism to liberate glucogenic energy substrates fuelling energy consuming processes. However, as G108-mediated EE is *not* observed in obese mice fed a HP diet, G108-stimulated hypoaminoacidemia is itself implicated in the EE response. Therefore, by rescuing plasma amino acids, the high protein diet may act to prevent both the EE response and protect whole body protein stores (Schematic 3.2). It may also be possible that the protein catabolism/turnover in lean tissue may be source of the energy consuming response to G108-stimulated hypoaminoacidemia. Further mechanistic studies using amino acid diets are explored in Chapter 4.



Schematic 3.2: Potential interaction between G108-stimulated hypoaminoacidemia, lean mass loss and EE in the context of a high protein diet.

3.5.3 The effect of a high protein diet on molecular markers of muscle protein synthesis and proteolysis.

Amino acid availability to skeletal muscle directs the balance of major signalling pathways regulating muscle proteolysis and synthesis (Kamei, Hatazawa et al. 2020). Of these, the ubiquitin proteasome pathway and autophagy-lysosome pathways coordinate muscle proteolysis. In keeping with the loss of lean mass in the chronic DIO study, we observed changes in both pathways. Upregulation in expression of the E3-ligase, *Fbxo32* and other autophagy related genes including *Bnip3 and Maplc3b* was evident in G108-treated obese mice receiving a standard protein diet (Figure 3.7). Unexpectedly, despite mitigation of lean mass loss in mice fed a high protein diet, autophagy markers were also upregulated (Figure 3.7). The same pattern of expression was observed for glutamine synthetase (*Glul*) (Figure 3.7). Glutamine synthetase plays an important role in transferring ammonia from the breakdown products of branched chain amino acids in skeletal muscle (Holecek 2018). There are several explanations for the discrepancy between lean mass loss and skeletal muscle autophagy markers in G108-treated mice receiving a high protein diet. For example, it is possible that the high protein diet intervention was able to prevent upregulation of UPP and autophagy related genes and associated lean mass loss up to a certain time point in the DIO study. Following this, G108-stimulated hepatic amino acid catabolism may have overcome the effect of a high protein diet to maintain plasma amino acid availability therefore leading to upregulation of the muscle proteolytic markers. It is also possible that a high protein diet establishes a profile

of individual amino acids which confers a differential effect on proteolytic and synthetic pathways in skeletal muscle in this study. In addition, a terminal fast may have precluded the protective effect of the high protein diet on these markers of muscle proteolysis in peptide-treated mice, due to sustained action of glucagon on hepatic amino acid catabolism without dietary protein availability. As the UPP and autophagy pathways in muscle are regulated by amino acid availability, the changes in molecular markers of these key muscle pathways are supported by the observation of depleted terminal plasma amino acid levels across in both G108 groups irrespective of diet (Figure 3.4). Together, these findings may support a cautious use of long-acting glucagon containing multi-agonists during the fasting phase where a high protein diet intervention is not practical or sustainable to maintain plasma amino acid levels.

Despite a significant change in expression of autophagy genes, western blot analysis did not show a significant difference in the total levels of Atrogin-1 in gastrocnemius muscle with G108 treatment (Figure 3.8B). Furthermore, the phosphorylation status of Ribosomal protein S6 kinase beta-1 (S6K1) was unchanged a downstream effector of the amino acid sensor Mammalian target of rapamycin (mTORC1), regulating protein synthesis (Figure 3.8A). The absence of any measurable change in total protein levels and phosphorylation status of these enzymes may be due to the subtle chronic changes in lean mass in our model. In contrast to other models of lean mass loss e.g. cancer cachexia or starvation where higher degrees of muscle loss are observed, using a sub-anorectic dose of a glucagon agonist has revealed small but significant lean mass loss. Although the 'amino acid sensor' conferring plasma amino acid availability to lean mass in skeletal muscle has not been identified in the G108-DIO mouse model, possible candidates include MTORC1/S6K, AKT and AMPK (Bond 2016, Moro, Ebert et al. 2016, Kjobsted, Hingst et al. 2018). The absence of change in S6K1 suggests mTOR may not be the amino acid sensor here (Figure 3.8A). Furthermore, no change in AMPK status was observed in G108-treated mice (Figure 3.8C). To further identify changes in the amino acid sensing pathway at the level of protein activity in skeletal muscle, an experimental model with a higher extent of glucagon induced lean mass loss may be required; In this study, protein quantification may not be sensitive enough to detect this.

3.5.4 G108 leads to hepatic FGF21 induction and fat mass loss

Increased circulating plasma FGF21 is associated with enhanced white adipose tissue lipolysis in rodents (Hotta, Nakamura et al. 2009) and both transgenic and knockout FGF21 mice demonstrate the role of FGF21 in enhancing expression or protein levels of WAT lipases (Inagaki, Dutchak et al. 2007, Badman, Koester et al. 2009). Both direct and indirect mechanisms have been implicated. Indeed, evidence for the direct effect of FGF21 on WAT lipolysis has been demonstrated in vivo (Adams, Yang et al. 2012). However, the role for CNS signalling on the metabolic response to FGF21 (including adipose tissue lipolysis through sympathetic signalling) has also been demonstrated with central FGF21 administration (Owen, Ding et al. 2014).

Consistent with the role of FGF21 in WAT lipolysis, significant fat mass loss was observed in G018-treated mice and was associated with elevated plasma FGF21 (Figure 3.9). The ability of a glucagon analogue to enhance fat mass loss associated with FGF21 action has been shown previously with the acylated glucagon analogue, IUB288 (Habegger, Stemmer et al. 2013). In the study using IUB288, a 10 nmol/kg daily dose over 16 days resulted in approximately 10 g of fat mass loss independent to food intake in diet-induced obese mice, and this was associated with a significant increase in hepatic expression and circulating levels of FGF21. Furthermore, FGF21 KO mice were resistant to fat mass gain when provided with a high fat diet.

Could altered levels of total or individual amino acids play a role in hepatic FGF21 induction and the associated fat mass loss observed in the G108 DIO study? This is strongly suggested by the lack of FGF21 and fat mass loss with plasma amino acid rescue despite treatment with G108. Although glucagon has been shown to increase circulating FGF21 *in vivo* (Arafat, Kaczmarek et al. 2013, Habegger, Stemmer et al. 2013), the exact mechanism is uncertain and thought to be via both transcriptional and post translational mechanisms downstream to the GCGR/cAMP signalling pathway (Habegger, Stemmer et al. 2013, Cyphert, Alonge et al. 2014). Around the same time that the effect of glucagon on FGF21 was established, it also emerged that a low protein diet is a potent stimulator of hepatic *Fgf21* expression and circulating FGF21, associated with fat mass loss (Laeger, Henagan et al. 2014, Yap, Rusu et al. 2020). More recently, it has been shown the dietary

restriction of specific essential amino acids can recapitulate this effect (Yap, Rusu et al. 2020). In particular, dietary restriction of threonine or tryptophan have been implicated in a robust hepatic FGF21 response (Yap, Rusu et al. 2020), threonine also being dramatically reduced at the higher G108 peptide dose. The mechanism implicated in hepatic FGF21 induction to amino acid restriction is now established as the endoplasmic reticulum (ER) stress response, involving the eukaryotic translation initiation factor (eIF)2α-activating transcription factor (ATF4)-CHOP pathway (De Sousa-Coelho, Marrero et al. 2012). Therefore, the supressing effect of G108 on plasma amino acids could explain the effects on fat mass loss through hepatic induction of FGF21. Indeed, more recently, it has been shown that a low protein diet signals through central FGF21 signalling to enhance fat mass loss (Hill, Laeger et al. 2019).

It could be suggested that G108 directly leads to increased hepatic *Fgf21* expression and a HP diet 'switches' it off through an independent mechanism; Both amino acid availability and glucagon are known to be able to activate induce hepatic FGF21 through independent cascade signalling pathways. However, as a high protein diet is not resulting in above baseline plasma amino acid levels, excess plasma amino acid levels are not observed, therefore it is unlikely that a 'normal' plasma level of amino acids would provide a negative/brake signal on the increased hepatic expression of *Fgf21,* if mediated directly by glucagon signalling.

As a sub-anorectic dose of G108 was used, any dose-dependent effects on food intake could not be assessed. Low protein diets have been demonstrated to increase food intake (Laeger, Henagan et al. 2014, Maida, Zota et al. 2016). However, a previous study has demonstrated the divergent effects of low and very low protein diets on food intake with mild-moderately restricted diets increases intake, whereas severe restriction reduces intake (Pezeshki, Zapata et al. 2016). At the dose of G108 given (7.5 nmol/kg), as expected there was no effect on food intake (Figure 3.1B).

The findings from this Chapter therefore provide compelling evidence that plasma amino acid levels are the common denominator to the phenotypic effects of low protein diets and chronic glucagon agonist therapy *in vivo*. This data and that of others suggest that G108-stimulated hyopoaminoacidemia could be driving lipolysis and fat mass loss

through the action of FGF21. However, as discussed below, the findings in this chapter suggest that plasma amino acid levels are not only coordinating energy substrate supply (through lipolysis and proteolysis) but may also enhancing the unknown mechanism(s) of glucagon-mediated EE, due to the reversal of G108-mediated EE (Figure 3.10) with a HP diet-rescue of plasma amino acid levels. Therefore, suppression of plasma amino acids by G108 may mediate the weight loss and EE effects observed in our DIO study, both driving the 'fuel' and the 'fire' of glucagon-driven EE.

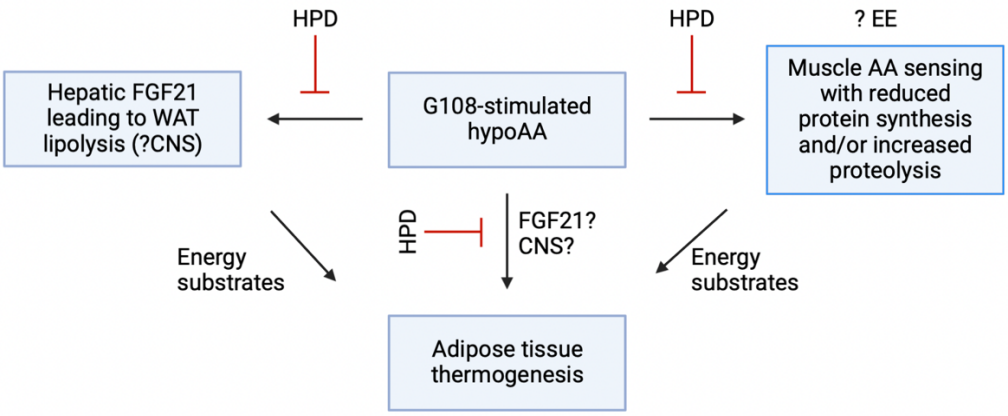
3.5.5 Plasma amino acid availability; Implications for understanding glucagon-stimulated EE

The mechanism by which glucagon increases energy expenditure is likely complex, multi-faceted with species-dependent variation (Kleinert, Sachs et al. 2019). Early studies demonstrated the ability of exogenous glucagon to enhance EE (Davidson, Salter et al. 1957, Davidson 1960). More recent studies have demonstrated the energy expenditure effect of a long-acting glucagon agonist, IUB288 in mice (Habegger, Stemmer et al. 2013) and that the EE effect of the GLP-1/glucagon co-agonist in rats is mediated through glucagon receptor signalling (Scott, Minnion et al. 2018).

In this chapter, the effect of the long-acting glucagon analogue G108 on energy expenditure was assessed directly in dedicated metabolic cages. The initial aim was to determine whether G108-mediated EE could be sustained whilst providing a high protein diet and mitigating lean mass loss. The effects of G108 on whole-body EE however became apparent before the mice were placed into metabolic cages, evidenced by food intake-independent fat mass loss in peptide treated mice (Figure 3.2). Indeed, a significant increase in oxygen consumption and energy expenditure was observed during both the light and dark phase in DIO mice receiving daily doses of G108 and a standard protein diet (Figure 3.10). The increased oxygen consumption observed in the metabolic cages are consistent with previous findings using a long-acting glucagon agonist *in vivo* (Habegger, Stemmer et al. 2013).

The effects of a high protein diet on whole body energy expenditure also became apparent before the mice were placed into metabolic cages, with a complete prevention

of food intake-independent fat mass loss in G108-treated mice (Figure 3.2). As discussed in section 3.5.4, fat mass loss may be associated with hepatic sensing of hypoaminoacidemia and subsequent FGF21 secretion, a known driver of white adipose tissue lipolysis (directly or indirectly). Therefore, restoring plasma amino acid levels may provide the 'brake' on fat mass loss in G108-treated mice. To support this, a recent study has demonstrated an indirect effect of FGF21 on WAT tissue through CNS FGF21 signalling, in response to dietary protein restriction (Hill, Laeger et al. 2019). Despite the clear link between FGF21 and WAT lipolysis, there is evidence that hepatic FGF21 also enhances EE. For example, FGF21 not only stimulates lipolysis, but activates thermogenesis in adipose tissue in response to low protein diets (Laeger, Henagan et al. 2014, Hill, Laeger et al. 2019). WAT thermogenesis is also observed with central administration of FGF21 (Owen, Ding et al. 2014). The long-acting glucagon analogue, IUB288, also enhances EE through the action of FGF21 (Habegger, Stemmer et al. 2013). Therefore, it is possible that G108-stimulated hypoaminoacidemia may enhance hepatic FGF21 induction leading to increased adipose tissue thermogenesis and WAT lipolysis (Schematic 3.3).



Schematic 3.3: Potential interactions between G108-stimulated hypoaminoacidemia, FGF21, lean mass loss and EE in the context of a high protein diet.

As energy supply and consumption (EE) are intrinsically linked, the ability of a high protein diet to 'switch off' G108-mediated fat mass loss and EE, suggest that plasma amino acid availability is responsible for both the 'fuel' and the 'fire' of the enhanced EE response observed. This hypothesis will be directly tested in Chapter 4 using amino acid-

based diets. The possible mechanisms of EE in response to G108-stimulated hypoaminoacidemia in obese mice are explored below.

3.5.6 Mechanisms of EE in response to G108-stimulated hypoaminoacidemia

Given that G108-mediated EE was prevented with a high protein diet, mechanisms of EE driven by hypoaminoacidemia are a compelling explanation for the energy consumption observed. Evidence for this comes from models of dietary protein or amino acid restriction dating back to the 1960's. Several early studies demonstrated the effect of dietary protein restriction on enhancing EE in rodents and humans (Miller and Payne 1962, Miller 1967, Stirling and stock 1968, McCraken 1976, Tulp 1979, Rothwell 1979, Rothwell and Stock 1982, Rothwell et al 1983). More recent research in rodents has demonstrated the divergent effects of dietary protein restriction on the EE mechanisms, including sympathetic and serotonergic activation, BAT and muscle thermogenesis and FGF21 action (Pezeshki, Zapata et al. 2016). Therefore, models of dietary protein or amino acid restriction can be used as a reference for exploring the potential mechanisms of G108-mediated EE.

3.5.7 G108 increases protein turnover

Based on the known physiology of glucagon, several potential energy expensive pathways are implicated following chronic G108 treatment. Although the gluconeogenesis and urea cycle pathways are both energy-dependent (Veldhorst, Westerterp-Plantenga et al. 2009), G108-treated mice did not exhibit increased EE when supplemented with a high protein diet, despite increased G108-mediated expression of these catabolic pathways and increased exogenous substrate (high protein diet) supply (Figure 3.4A). This suggests that G108-stimulated hepatic catabolism is not contributing to the EE effects observed. Another potential source of energy demand in G108-treated mice is muscle proteolysis which relies on ATP-dependent autophagy and the ubiquitin-proteolysis system. Indeed the reliance on a supply of ATP has been demonstrated for the individual pathways of autophagy including macroautophagy (Schellens, Vreeling-Sindelarova et al. 1988), microautophagy (Sahu, Kaushik et al. 2011) and maintenance of lysosomal pH (Van Dyke 1988). Furthermore, the ubiquitin-proteosome pathway is also ATP-

dependent at several steps (Peth, Uchiki et al. 2010). Mitigation of lean mass loss was observed in G108-treated mice receiving a high protein diet and therefore it is possible that sustained proteolysis in mice receiving a standard diet may contribute to energy consumption and therefore EE effects observed. Early reports of glucagon-infusions in humans were driven by the association of hyperglucagonemia, EE and protein catabolism in type 1 diabetes (Nair, Halliday et al. 1984). Indeed, glucagon-infusion in humans was later found to enhance resting metabolic rate and leucine-oxidation, a marker of whole-body proteolysis, during insulin deficiency (Charlton and Nair 1998). It has been suggested that protein catabolism plays an important role in the energy consumption observed in type 1 diabetes (Hebert and Nair 2010).

Other mechanisms of hypoaminoacidemia-mediated EE (besides proteolysis) are also possible. Recent research in rodents has also demonstrated the divergent effects of dietary protein restriction on EE mechanisms, including sympathetic activation, BAT and muscle thermogenesis and FGF21 action (Pezeshki, Zapata et al. 2016). Therefore, some of these potential mechanisms are explored below.

3.5.8 G108 increases markers of physical activity

Physical activity is an important contributor to total daily energy expenditure. In mice, up to 26% of total daily EE can be attributed to activity under thermoneutral conditions (Virtue, Even et al. 2012). However, in most laboratory studies, which are conducted below thermoneutral conditions, the contribution of activity to this total daily EE may be masked by other thermogenic processes (Virtue, Even et al. 2012). Indeed, a previous study demonstrated that at 24^{o}C, 20^{o}C or 5^{o}C, increases in physical activity in mice did not lead to an independent effect on total daily energy expenditure (Virtue, Even et al. 2012). Therefore, any changes in physical activity associated with EE between study groups in the DIO study presented in this chapter may need to be interpreted with caution when inferring a causal link.

The results in this chapter demonstrate that G108 can increase physical activity as measured by x-axis beam breaks (XTOT). This increased physical activity was observed throughout the metabolic cage assessment however only reached statistical significance

in specific light and dark phases. Although there are known limitations of physical activity assessment in metabolic cages (Speakman 2013), the appropriate control groups help to determine any given effect of the treatment.

Few reports have demonstrated a link between exogenous glucagon or glucagon-receptor activation and physical activity. In exploring the metabolic effects of glucagon-mediated FGF21 secretion in mice, Habagger et al demonstrated that daily treatment with their long-acting glucagon analogue (IUB288) over 72 hours in lean mice caused an increase in EE associated with increased beam breaks (Habegger, Stemmer et al. 2013). The increased activity was not observed in whole body FGF21 KO mice, suggesting a role for this hormone. A link between glucagon-receptor activation and physical activity has also been made in studies investigating other multi-agonists targeting the glucagon receptor. For example, twice daily treatment with a modified glucagon analogue in high fat fed mice resulted in increased EE which was associated with increased beam breaks in the x- and z-axis only during the dark phase (Lynch, Pathak et al. 2014). Increased activity has also been observed with twice daily administration of a triple-agonist in high fat fed mice, targeting GCGR, GLP-1 and GIP receptors (Gault, Bhat et al. 2013). However, in this study the triple agonist only increased z-axis activity in the light and dark phase and exenatide also resulted in increased z-axis activity in the light phase. Studies with the endogenous GLP-1R/GCGR co-agonist, oxyntomodulin have also revealed changes in physical activity in humans; One of the first studies of oxyntomodulin administration in humans demonstrated an increase in activity related energy expenditure by 26.2% (143 kcal/day) over 4 days, as measured by a movement sensor (Wynne, Park et al. 2006). Together, these results suggest that glucagon receptor activation may be implicated in the increased physical activity observed, however, to date no glucagon receptor knockout studies have been carried out to confirm a causal link.

The prevention of increased physical activity with a high protein diet and associated amino acid rescue in G108-treated mice, suggests a direct role for plasma amino acids. Previous studies have implicated individual amino acid restriction on physical activity in rodents, for example methionine (Lees, Krol et al. 2014). In a study investigating the effects of complete deprivation of individual essential amino acids, Xiao et al demonstrated that deprivation of tryptophan increased locomotion in mice (Xiao, Du et

al. 2016). These studies demonstrate the divergent effects of individual amino acids and degrees of alteration, whether this is by dietary protein restriction or depletion. However, other studies using total protein restriction, for example 5-6% casein restriction have shown inconsistent changes in physical activity (Laeger, Henagan et al. 2014, Blais, Chaumontet et al. 2018). Furthermore, in a more recent study, a similar 5% total protein dietary restriction did not lead to an increase in physical activity in mice (Yap, Rusu et al. 2020). The differences in study designs, total vs individual amino acid restriction and the degree of restriction may all play a role in the effect on physical activity.

3.5.9 G108 increases circulating FGF21 and thermogenic markers

Due to the similarity between the metabolic benefits conferred by a low protein diet, and the action of exogenous fibroblast growth factor 21 (FGF21) (Xu, Lloyd et al. 2009, Zhang, Xie et al. 2012), this led to Laeger and colleagues demonstrating in a seminal study that hepatic FGF21 is an endocrine signal of protein restriction mediating effects on whole body energy balance. (Laeger, Henagan et al. 2014). In this study, it was demonstrated that hepatic FGF21 is a signal of protein restriction in mice, through activation of the GCN/EIF2alpha pathway. They further showed that the observed increase in hepatic and circulating FGF21 levels in response to starvation or a ketogenic diet is due to protein restriction. Importantly, the low protein diet increased EE and this was prevented in global FGF21 KO mice suggesting an obligatory role for FGF21. A more recent study has demonstrated that central signalling of hepatic FGF21 is required for the metabolic adaptations, including EE and fat mass loss, in response to a 5% casein dietary protein restriction (Hill, Laeger et al. 2019).

The observation that hepatic FGF21 is expressed in response to a low protein diet is consistent with an earlier study which showed that AA restriction leads to hepatic FGF21 induction through the GCN/ATF4 mechanism in cell and tissue experiments (De Sousa-Coelho, Marrero et al. 2012). Justifying this investigation, De Souza and colleagues had linked observations of low amino acid availability and the GCN/ATF4 pathway and FGF21 upregulation with lipolysis, which helped them show that low amino acid availability upregulates FGF21 through GCN/ATF4.

Central FGF21 signalling is required for the metabolic adaptations to a low protein diet (Hill, Laeger et al. 2019); A low protein diet enhances EE and fat mass loss in mice with upregulation of thermogenic markers such as *Ucp1* in WAT. These effects are prevented in mice lacking the central FGF21 receptor. This supports previous reports of exogenous FGF21 administration, showing that FGF21 acts via central signalling to increase sympathetic outflow to adipose tissue enhancing lipolysis and adipose tissue thermogenesis in both BAT and WAT (Owen, Ding et al. 2014). Circulating FGF21 was increased in G108-treated mice and prevented in those fed a high protein diet (Figure 3.9). In addition, an increase in the expression of *Ucp1* was observed in WAT tissue, however this did not reach statistical significance (Figure 3.16). Although G108 did not affect the main BAT thermogenic markers, a significant increase in *Fgf21* expression was observed (Figure 3.15). This is noteworthy as it has been demonstrated previously that dietary protein restriction may enhance the expression of FGF21 in extra-hepatic tissues, for example skeletal muscle, small intestine and BAT (Pezeshki, Zapata et al. 2016, Chaumontet, Azzout-Marniche et al. 2019, Zapata, Singh et al. 2019, Dommerholt, Blankestijn et al. 2021). Indeed, a trend towards increased *Fgf21* expression as observed in muscle from G108-treated mice, in addition to a significant increase in other thermogenic markers (Figure 3.14). Consistent with these findings, it has been suggested that in response to dietary protein restriction, FGF21 secretion from multiple thermogenic tissue may be involved in a feed-forward mechanism with the sympathetic nervous system to enhance energy expenditure (Pezeshki, Zapata et al. 2016).

Although G108-stimulated hypoaminoacidemia appears to induce hepatic and circulating FGF21, its contribution to the EE effects observed in this model remain unclear. In previous studies of low protein diet interventions, plasma levels of FGF21 appear to be much higher (Hill, Laeger et al. 2019). It is likely that it is one contributing component of many. For example, the EE and weight loss effect of a low protein diet is only partially blunted in liver-specific FGF21 knockout transgenic mice (Perez-Marti, Garcia-Guasch et al. 2017). Furthermore, the glucagon analogue, IUB288, enhances EE in mice partially through FGF21 (Kim, Nason et al. 2018). Together this data suggests that G108-stimulated hypoaminocidemia enhances EE and the hepatic expression of FGF21, which is possible mediator of adipose tissue lipolysis and energy expenditure.

3.5.10 Other possible amino acid centric mechanisms of G108-mediated EE

Serotonergic signalling has also been implicated in the EE response to low-protein diets as administration of ondansetron to rodents reduces EE in rodents fed a 10 kcal% protein restricted diet (Pezeshki, Zapata et al. 2016). Furthermore, it has been shown that serotonergic signalling is important for thermogenesis in BAT and WAT tissue (Rothwell and Stock 1987, McGlashon, Gorecki et al. 2015). Several of these mechanisms have been recently reviewed (Pezeshki and Chelikani 2021).

3.5.11 Effects on glucose tolerance and hepatic fat

A significant improvement in in glucose tolerance on day 15 was observed in G108-treated mice (Figure 3.11A and B). Importantly, these effects were maintained when providing a high protein diet intervention. In obese mice, no change in glucose stimulated insulin levels were observed and no change in measured insulin sensitivity (Figure 3.11C-F). A previous study investigating the effects of the long-acting glucagon analogue, IUB288, showed improvements in glucose tolerance (Kim, Holleman et al. 2018). Using a euglycaemic clamp model IUB288 acutely enhances both glucose stimulated insulin secretion and insulin sensitivity; This was shown to be mediated augmentation of the insulin signalling pathway, in particular hepatic AKT phosphorylation, however the observed enhanced uptake of ^{14}C-labelled glucose into several skeletal muscle groups remains unexplained (Kim, Holleman et al. 2018). Further investigation is therefore required to understand the mechanisms of improved glucose tolerance secondary to chronic sub-anorectic dosing with G108 and whether this is mediated by insulin or insulin-independent glucose uptake pathways. It is becoming more evident that glucagon coordinated receptor activity at the hepatic GCGR and beta cell GCGR, particularly during the post-prandial phase may confer improved nutrient metabolism and not a hyperglycaemic response (Capozzi, D'Alessio et al. 2022).

A significant improvement in plasma cholesterol and hepatic lipid accumulation was also observed in G108-treated mice irrespective of dietary protein content (Figure 3.12). This was associated with a significant reduction in *Fasn* a key regulator of de novo lipogenesis. These findings are in keeping with the potent action of glucagon on circulating and

hepatic lipid metabolism (Galsgaard, Pedersen et al. 2019, Janah, Kjeldsen et al. 2019). Together, these findings suggest that G108 confers metabolic benefits in the chronic setting in obesity, independent to increasing dietary protein content which helps to mitigate lean mass loss.

3.6 Conclusions

A key aim of Chapter 3 was to determine whether chronic sub-anorectic dosing of a long-acting glucagon analogue in an obese model could suppress individual plasma amino acid levels and result in lean mass loss. This findings from this Chapter demonstrate that chronic dosing of G108 in diet-induced obese mice results in the increased hepatic catabolism of a broad range of individual amino acids resulting in total- and individual-hypoaminoacidemia. This is associated with a significant loss of lean mass. Supporting the reduction in lean mass, increased markers of skeletal muscle proteolysis were observed.

Importantly, a high protein diet was able to rescue plasma amino acid levels and reduce lean mass loss. A high protein diet was also able to rescue muscle weight and function. Interestingly, at the dose of G108 used, a high protein diet appeared to have a weakened effect on mitigating lean mass loss over time, and this was also reflected in the change in molecular markers of skeletal muscle proteolysis. This may be a result of the opposing effects on lean mass of high protein diet intake and G108-stimulated hypoaminoacidemia over time and/or being lost during 24-hour feed/fasting phases. Therefore, protein intake may become insufficient to overcome G108-stimulated hepatic amino acid catabolism and hypoaminoacidemia. This is also supported by the terminal plasma amino acid levels which show a broad reduction despite a high protein diet intervention. Although this hypothesis requires further investigation, if true this may have important implications for the use of long-acting glucagon agonists in humans, where prolonged fasting periods during sleep may increase susceptibility to lowered plasma amino acid availability and lean mass loss. The preservation of lean mass in individuals with obesity and type 2 diabetes is becoming more prominent. A recent study has demonstrated that hyperglucagonemia associated with these conditions contributes to lean mass loss through enhanced hepatic amino acid catabolism (Okun, Rusu et al. 2021).

The in vivo model described in this chapter highlights that the chronic G108-mediated fat mass loss and EE benefits are lost with repleting plasma total amino acids. The amino-acid centric responses observed in this chapter focused on whole-body energy expenditure, hepatic FGF21 and fat mass loss. These individual responses have been previously associated with paradigms of protein or amino acid restriction. Thus, the results in this chapter suggest that the mechanism of chronic glucagon-mediated EE in rodents may be due to chronic glucagon-stimulated hypoaminoacidemia. These findings may also provide a contributing mechanism by which glucagon confers significant weight loss effects in previously described GCGR-targeted multi-agonists in vivo (Pocai, Carrington et al. 2009, Day, Gelfanov et al. 2012). The observations in this Chapter will be further explored using amino acid-based diets in Chapter 4.

In contrast to the effects on fat mass and EE, other metabolic benefits of chronic G108 treatment including improved glucose tolerance and hepatic fat catabolism were maintained independent to a high protein diet intervention. This suggests the in vivo model presented in this chapter exhibits amino-acid centric effects of glucagon signalling in addition to effects of direct glucagon signalling *per se* (glycaemia and hepatic fat catabolism). Although the effects of chronic glucagon signalling on hepatic fat catabolism are well known, the effects on glycaemia are less defined and require further investigation. Emerging evidence suggests a glucose-regulatory benefit of hepatic GCGR-agonism (Kim, Holleman et al. 2018, Kim, Nason et al. 2022). It will therefore be important to determine whether these effects translate to humans. In the first instance it will be important to determine whether a broad reduction in individual plasma amino acids occurs in humans in response to chronic glucagon therapy (See Chapter 5). This will need to be investigated as part of a multi-agonist strategy, ie in combination with GLP-1, as many GCGR/GLP-1 co-agonists are currently in clinical development (Hope, Vincent et al. 2021).

In the next chapter the specific underpinning of EE and body weight loss by G108-stimulated hypoaminoacidemia is further explored.

4 Chapter 4 – Hypoaminoacidemia underpins chronic G108-mediated energy expenditure and body weight loss

4.1 Introduction

4.1.1 Dietary protein supplementation prevents G108-mediated EE and total body weight loss; Similarities to dietary protein or amino acid restriction paradigms

Chapter 3 described the effect of chronic G108 administration on EE and total body weight loss in DIO mice. In particular, the EE response observed in G108-treated mice was associated with hypoaminoacidemia, which would support historic and recent findings by others of EE in response to dietary protein or amino acid restriction (Rothwell, Stock et al. 1982, Maida, Zota et al. 2016, Pezeshki, Zapata et al. 2016, Zapata, Singh et al. 2019, Yap, Rusu et al. 2020). Indeed, a high protein diet rescued plasma amino acids, prevented EE and total body weight loss in G108-treated obese mice (Chapter 3, Sections 3.4.1, 3.4.5 and 3.4.8). Furthermore, similar changes in the hepatic amino acid response pathway (AAR) resulting in hepatic FGF21 induction, and components of EE are observed between these dietary restriction paradigms and in G108-treated mice fed a standard protein diet (Chapter 3, Sections 3.4.7 and 3.4.11). Although the plasma amino acid and EE findings in Chapter 3 are associative, the rescue of amino acids with dietary protein supplementation suggests that G108-stimulated hypoaminoacidemia may underpin the EE and body weight loss observed.

4.1.2 Hepatic amino acid sensing and FGF21 induction in response to dietary protein or amino acid restriction

A key amino acid sensing tissue is the liver, and this has been proposed to coordinate the systemic response to dietary protein or amino acid restriction to restore protein balance (Pezeshki and Chelikani 2021). A key pathway in which this occurs is through activation of the GCN2 (general control non-derepressible) pathway. In this pathway, low amino acid availability *in* vitro leads to accumulation of cytoplasmic tRNA molecules, activating GCN2 leading to an activation cascade involving eIF2alpha (eukaryotic translation initiation factor alpha), activating transcription factor 4 (ATF4) and CCAAT/Enhancer-

binding protein homologous protein (CHOP). This pathway ultimately results in the transcription and secretion of hepatic FGF21 and a reduction in hepatocyte protein synthesis (De Sousa-Coelho, Relat et al. 2013, Wan, Lu et al. 2014). By reducing protein synthesis through the activation of eIF2alpha, the local demand for amino acids is subsequently reduced. Hepatic FGF21 was identified as an end-transcriptional product of amino acid restriction *in* vitro after similar favourable metabolic outcomes, in particular hepatic fat oxidation, were made between protein restriction and starvation-induced FGF21 (Reitman 2007, De Sousa-Coelho, Relat et al. 2013). A seminal *in vivo* study later demonstrated that FGF21 is an endocrine signal of dietary protein restriction, and that low dietary protein-induced FGF21 was ameliorated in GCN2 knockout mice (Laeger, Henagan et al. 2014). In this study it was also demonstrated that the FGF21 deficient animals did not exhibit hyperphagia, EE or body weight loss when fed a low protein diet, suggesting the importance of FGF21 in this response (Laeger, Henagan et al. 2014). Indeed, in addition to the inhibition of hepatic protein synthesis described above, hepatic FGF21 has been implicated in the hyperphagic response to restore protein balance when fed a low protein diet (Hill, Laeger et al. 2019). Interestingly, another study confirmed the role of hepatic FGF21, however also found that in GCN2 knockout mice, plasma FGF21, hyperphagia, EE and body weight loss appeared after 14 days of a low protein diet, suggesting an GCN2-indpeendent route to hepatic FGF21 induction in response to chronic amino acid restriction (Laeger, Albarado et al. 2016).

4.1.3 Mechanisms of EE in response to dietary protein or amino acid restriction

When considering the EE response to low protein diets in rodents, because of diet-induced thermogenesis, it is important to distinguish the directionality of EE and food intake. It is also important to determine the effect on other dietary macronutrients when lowering protein content. As EE is a compensatory response to hyperphagia (Danforth 1981), the EE response to a dietary protein restriction may be secondary to increased food intake. However, dietary protein restriction in rodents can lead to EE without a hyperphagic response which suggests the EE response to low protein is independent to energy intake (Zapata, Singh et al. 2019). To determine the primary role of protein restriction rather than increased dietary carbohydrate content on EE, a study in rodents was carried out using fixed carbohydrate low protein diets. In this study, it was

demonstrated that despite fixed carbohydrate content, protein restriction still elicits an increased EE response (Zapata, Singh et al. 2019). Collectively, these studies demonstrated that the EE response observed in these studies is *specifically* due to protein restriction rather than increased carbohydrate content or energy intake.

Several mechanisms have been implicated in the EE response to low protein diets. Brown adipose tissue (BAT) received much early interest and was shown to play a role in low protein diet-induced thermogenesis (Rothwell and Stock 1979). More recently, it has been shown that transcripts of thermogenic markers, such as UCP1 are increased in BAT tissue when fed a low protein-diet (Pezeshki, Zapata et al. 2016). This is associated with increased expression of BAT adrenergic signalling markers, consistent with the role for sympathetic nervous system in BAT thermogenesis (Rothwell, Stock et al. 1983). As discussed in the previous section, FGF21 has been shown to be required for the EE response to low protein diets (Laeger, Albarado et al. 2016) and itself is implicated in adipose tissue thermogenesis through both central-sympathetic and endocrine mechanisms (Fisher, Kleiner et al. 2012, Hill, Laeger et al. 2019). Interestingly, in the latter study, using brain FGF21 co-receptor knockouts, WAT thermogenesis in response to a low-protein diet appears to occur exclusively through FGF21 signalling in the brain to increase sympathetic outflow (Hill, Laeger et al. 2019), in keeping with previous studies of exogenous FGF21 therapy (Owen, Ding et al. 2014). Adding complexity to this understanding, sympathetic stimulation of BAT increases BAT FGF21 transcription (Chartoumpekis, Habeos et al. 2011) and FGF21 transcription is increased in other thermogenic tissue for example skeletal muscle in response to a low protein diet (Pezeshki, Zapata et al. 2016). Therefore, it has been suggested that in response to dietary protein restriction, FGF21 secretion from thermogenic tissue may be involved in a feed-forward mechanism with the sympathetic nervous system to enhance energy expenditure (Pezeshki, Zapata et al. 2016). Interestingly, a low protein diet increases circulating FGF21 in humans which correlates with body weight loss (Vinales, Begaye et al. 2019).

Serotonergic signalling has also been implicated in the EE response to low-protein diets as administration of ondansetron to rodents reduces EE in rodents fed a 10 kcal% protein restricted diet (Pezeshki, Zapata et al. 2016). Furthermore, it has been shown that

serotonergic signalling is important for thermogenesis in BAT and WAT tissue (Rothwell and Stock 1987, McGlashon, Gorecki et al. 2015). To add to the multi-faceted EE response to dietary protein restriction, one study has also reported activity related EE accounting for the EE response in mice (Blais, Chaumontet et al. 2018).

In addition to balanced restriction/dilution of dietary protein, studies have also shown that reductions in individual dietary amino acids may recapitulate the EE response to dietary total protein. Methionine restriction is the most extensively studied model of individual amino acid restriction, and its EE effects have also been shown to be due to sympathetic signalling (Plaisance, Henagan et al. 2010, Spring, Singh et al. 2019) which appears to be dependent on hepatic FGF21 induction (Wanders, Forney et al. 2017). Other studies have demonstrated that dietary tryptophan restriction leads to sympathetic mediated EE response (Zapata, Singh et al. 2018). Using a systematic approach of exploring the effects of individual essential amino acid restriction, a recent study demonstrated that dietary restriction of threonine or lysine may increase EE, and this is dependent on hepatic FGF21 induction (Yap, Rusu et al. 2020). Also, lysine deprivation has been shown to increase serotonergic singling and energy expenditure (Smriga, Kameishi et al. 2002). Recently, dietary restriction of histidine in mice has been reported to increase energy expenditure independent to FGF21 (Victoria Flores 2022). Therefore, in addition to the role of hepatic FGF21, individual amino restriction may regulate distinct and divergent pathways of EE, further adding complexity to the mechanisms involved (Xiao and Guo 2021).

The mechanisms of dietary protein or amino acid restriction leading to EE and body weight loss are likely complex due to the divergent pathways involved and the distinct pathways activated by individually lowered amino acids (Xiao and Guo 2021). Therefore, it has been suggested that thermogenesis in response to dietary protein restriction may be mediated by parallel or interdependent pathways involving sympathetic, serotonergic and FGF21 signalling pathways (Pezeshki, Zapata et al. 2016).

4.1.4 Using custom amino acid-based diets to determine whether G108-stimulated hypoaminoacidemia underpins glucagon-mediated EE and body weight loss

This Chapter therefore sets out to determine whether G108-stimulated hypoaminoacidemia underpins chronic G108-mediated EE and body weight loss. Initially, single-dose administration studies of G108 will be carried out to determine whether plasma amino acid levels are implicated in the *acute* EE response to G108. In this acute study, a casein-based standard protein and high protein diet will be used. For the subsequent chronic studies, custom synthetic amino acid diets will be used whereby the additional casein component comprising the high protein diet, will be replaced by total individual amino acids. This diet therefore comprises of the standard protein (SP) diet supplemented with total individual amino acids (TAA), to match the protein content of the high protein (HP) diet. The standard diet supplemented with total amino acids (SP±TAA diet) can also be modified further to examine the contributions of specific amino acids. The SP±TAA diet specifically allows determination of whether dietary 'add-back' of total individual amino acids can rescue G108-stimulated hypoaminoacidemia, preventing EE and total body weight loss. In addition to dietary amino acid 'add back' studies, a custom low amino acid (LAA) diet will be made, where the reduction in individual amino acid constituents is matched to the reduction in plasma amino acids from obese mice chronically treated with G108 (Chapter 3, Section 3.4.5). The LAA diet will be provided *ad libitum* to obese mice to determine whether this will reproduce the EE and body weight loss effects mediated by chronic G108 administration. Collectively, these studies aim to provide a mechanistic explanation for G108-mediated EE and body weight loss, specifically through the action of G108-stimulated hypoaminoacidemia.

4.2 Chapter summary and aims

- Explore whether hypoaminoacidemia and EE occur in response to acute administration of G108, and whether this is prevented by feeding with a high protein diet.
- Determine whether supplementation of a standard protein diet with total individual amino acids rescues chronic G108-stimulated hypoaminoacidemia, in addition to preventing EE and total body weight loss.
- Determine whether supplementation of a standard protein diet with either essential or non-essential amino acids rescues chronic G108-stimulated hypoaminoacidemia, in addition to preventing EE and total body weight loss.
- Determine whether a custom low amino acid diet, matched to reductions in plasma amino acid levels from G108-treated mice, reproduces chronic G108-mediated EE and total body weight loss.

4.3 Methods

4.3.1 Animal husbandry

All studies were carried out in accordance with the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012. 8-14-week-old lean male C57BL/6J were purchased from Charles River, Margate, UK. *Fgf21*$^{\Delta liver}$ mice were obtained from Jackson laboratories. *Fgf21*$^{flox/flox}$ mice were crossed with hemizygous Albumin-Cre mice to generate *Fgf21*$^{flox/flox}$*:Alb-Cre±* (*Fgf21*$^{\Delta liver}$) and *Fgf21*$^{flox/flox}$*:Alb-Cre-* (control mice) as previous discussed Animals were housed at 21^{o}C under standard 12-hour light cycle. Food and water were provided *ad libitum* and all mice were provided with a 60 kcal% high fat diet on arrival to the animal unit.

4.3.2 Diets

All diets were obtained from ResearchDiets.com, (Table 4.1 and 4.2) stored at 4^{o}C. A standard DIO series diet (D12492) containing 60 kcal% fat was provided to all mice for 8-10 weeks to induce obesity prior to switching to a custom diet. Custom diets D08091803 and D08091801 were used as a standard protein high fat diet (SP diet) and high protein high fat diet (HP diet) similarly to that used in the previous chronic DIO study

(Chapter 3). Five new custom diets were obtained (Table 4.1 and 4.2). The first is D22011202 (SP±TAA diet, Formula 1), containing 20 kcal% casein (200 g) with the addition of total individual amino acids from a further 200g of casein; The SP±TAA diet contains 37 kcal% protein due to casein not being 100% amino acid based. The second new diet is D22011203 (SP±EAA diet, Formula 2), which contains 20 kcal% casein (200 g) with the addition of additional individual essential amino acids (EAA) only from 200 g of casein; The total protein content of Formula 2 is 27 kcal% protein. The third new diet is D22011204 (SP±NEAA diet, Formula 3) which contains additional synthetic non-essential amino acids (NEAA) only; The total protein content of this diet is 30 kcal% protein.

All new diets contain 45 kcal% fat content and to allow for different protein contents has varying degrees of carbohydrate content, based on the amount of corn starch added. The fourth new diet is A22012001 (SPAA diet, Formula 4), a completely amino acid-based version of the standard protein (SP) diet, D08091803. The SPAA diet contains 17 kcal% protein. The last new diet is A2212002 (LAA, Formula 5), which also contained individual amino acids however the amino acid content was reduced to match reductions in plasma amino acid levels in G108-treated DIO mice, based on mass spectrometry quantification of individual plasma amino acid levels (Chapter 3, Section 3.4.5).

Diet	Carbohydrate (kcal%)	Fat (kcal%)	Protein (kcal%)
D12492	20	60	20
D08091803	35	45	20
D08091801	15	45	40
D22011202 (Formula 1)	18	45	37
D22011203 (Formula 2)	28	45	27
D22011204 (Formula 3)	25	45	30
A22012001 (Formula 4)	38	45	17
A22012002 (Formula 5)	45	45	10

Table 4.1: Macronutrient composition of diets.

Constituent	Formula 1		Formula 2		Formula 3		Formula 4		Formula 5	
	200 g casein ± total amino acids from 200 g casein (SP±TAA diet)		200 g casein ± EAA from 200 g casein (SP±EAA diet)		200 g casein ± NEAA from 200 g casein (SP±NEAA diet)		Amino acid version of D08091803		'GCMS matched' low amino acid diet	
	g%	kcal %	g%	kcal%	g%	kcal%	g%	kcal %	g%	kcal %
Protein	44	37	32	27	35	30	20	17	11	10
Carbohydrate	21	18	33	28	29	25	44	38	53	45
Fat	23	45	23	45	23	45	23	45	23	45
Kcal/g		4.69		4.69		4.69		4.69		4.69
Casein	200	800	200	800	200	800	0	0	0	0
L-Histidine	4.5	18	4.5	18	0	0	4.5	18	3.27	13
L-Isoleucine	7.5	30	7.5	30	0	0	7.5	30	4.52	18
L-Leucine	15.7	63	15.7	63	0	0	15.7	63	9.74	39
L-Lysine	13	52	13	52	0	0	13	52	3.37	13
L-Methionine	5	20	5	20	0	0	5	20	1.45	6
L-Phenylalanine	8.3	33	8.3	33	0	0	8.3	33	6.53	26
L-Threonine	7.1	28	7.1	28	0	0	7.1	28	1.84	7
L-Tryptophan	3.2	8	2.1	8	0	0	2.1	8	1.60	6
L-Valine	9.2	37	9.2	37	0	0	9.2	37	5.34	21
L-Alanine	5	20	0	0	5	20	5	20	2.00	8
L-Arginine	5.9	24	0	0	5.9	24	5.9	24	2.80	11
L-Asparagine	7	28	0	0	7	28	7	28	2.06	8
L-Aspartic acid	5	20	0	0	5	30	5	20	4.47	18
L-Cystine	4.2	17	3	12	4.2	17	4.2	17	4.20	17
L-Glutamine	17	68	0	0	17	68	17	68	9.14	37
L-Glutamic acid	20.7	83	0	0	20.7	83	20.7	83	17.61	70
Glycine	3	12	0	0	3	12	3	12	1.38	6
L-Proline	17.6	70	0	0	17.6	70	27.6	70	8.14	33
L-Serine	9.9	40	0	0	9.9	40	9.9	40	3.96	16
L-Tyrosine	9	36	0	0	9	36	9	36	4.64	19
Corn Starch	3.1	12	104.4	418	75.5	302	201.3	805	279.94	1120
Maltodextrin 10	100	400	100	400	100	400	100	400	100	400
Sucrose	68.8	275	68.8	275	68.8	275	68.8	275	68.8	275
Cellulose	50	0	50	0	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225	25	225	25	225
Lard	177.5	1598	177.5	2598	177.5	1598	177.5	1598	177.5	1598
Mineral Mix	10	0	10	0	10	0	10	0	10	0
Dicalcium Phosphate	13	0	13	0	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0	5.5	0	5.5	0

Potassium Citrate, 1 H20	16.5	0	16.5	0	16.5	0	16.5	0	16.5	0
Sodium Bicarbonate	7.5	0	7.5	0	7.5	0	7.5	0	7.5	0
Vitamin Mix	10	40	10	40	10	40	10	40	10	40
Choline Bitrartrate	2	0	2	0	2	0	2	0	2	0
Yellow dye	0	0	0.05	0	0	0	0.025	0	0	0
Red dye	0.025	0	0	0	0.05	0	0	0	0	0
Blue dye	0.025	0	0	0	0	0	0.025	0	0.05	0
Total	865.65	4057	865.65	4057	865.65	4057	863.85	4050	863.85	4050

Table 4.2: Macronutrient composition of diets Formula 1-5.

4.3.3 Acute metabolic cage study

For the acute metabolic cage study (Figure 4.1), two groups (N=10/group) of lean male C57BL/6J mice were provided with a high fat diet for 10 days, prior to switching to either a standard protein (SP) diet or a high protein (HP) diet for 1 week (D08091803, D08091801, Table 4.1). All mice were individually housed in metabolic cages and acclimatised for 24 hours. On day 2, baseline readings were obtained for oxygen consumption and carbon dioxide production. On day 3, all mice were administered subcutaneously with G108 (7.5 nmol/kg) at 8 am in the morning. Subsequent metabolic readings were taken for 48 hours following peptide administration to the morning of day 5. On the morning of day 5, all mice were further administered with a higher dose of G108 (20 nmol/kg), with readings up to 72 hours later up until the morning of day 8. On the morning of day 8, all animals were returned to their home cages. A subsequent parallel study was conducted out of the metabolic cages after a wash-out period week where tail venesection and blood collection were taken at 9am prior to a single dose of G108 (7.5 nmol/kg). A repeat tail vein venesection and blood collection were carried out daily each morning for up to 48 hours. As in the metabolic cage study, a higher dose of G108 (20 nmol/kg) was then administered on day 5 with tail vein venesection daily each morning for up to 72 hours. After collection, the blood was stored on ice and immediately transferred for storage at -80^{o}C. These samples were subsequently used for plasma total amino acid analysis.

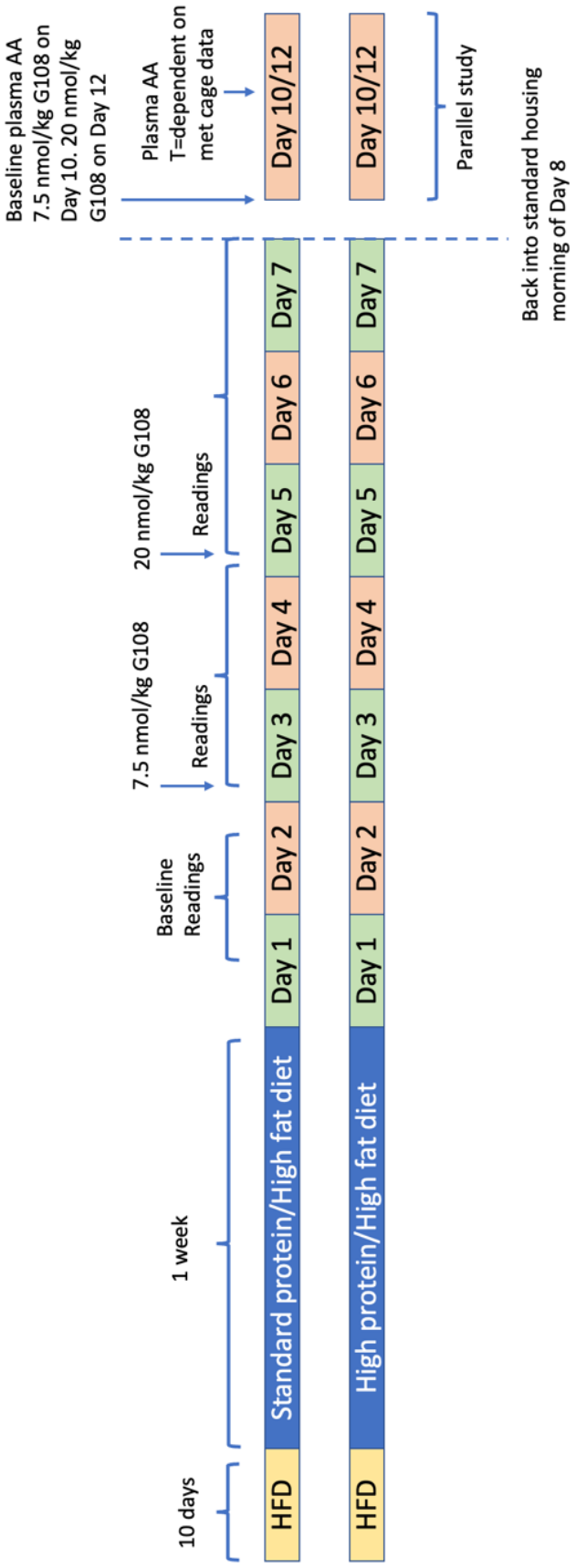


Figure 4.1. Schematic of acute metabolic cage study protocol.

4.3.4 Chronic G108 administration in DIO mice with total individual amino acid dietary 'add-back'

For the total individual amino acid dietary 'add-back' study, 32 diet-induced obese (DIO) male C57BL/6J mice were switched to custom diets for two weeks; Half of the cohort (N=16) were provided with a standard protein diet (D08091803, Table 4.1), and the other half (N=16) provided with standard protein diet supplemented with additional total individual amino acids (Formula 1, D22011202, Table 4.1 and 4.2). Food intake and body weight was recorded daily and mice from each diet group received daily subcutaneous administration of 7.5 nmol/kg G108 (N=8) or vehicle control (N=8). Therefore, the four treatment groups were as follows: Vehicle / SP diet, G108 / SP diet, Vehicle / SP ± total AA diet, G108 / SP ± total AA diet.

Prior to switching onto these custom diets on day 0, a baseline MRI analysis was carried out to measure body composition. A glucose multiple time course profile was taken prior to first treatment injection on day 0 and at 15, 30, 60 mins, 2, and 4 hours following this first injection. A glucose time course profile was also carried out on day 19. Body composition analysis was also carried out on day 18. Tail venesection was carried out on day 16 for plasma total amino acids and FGF21 quantification. All mice were placed into metabolic cages from day 20 of the study for analysis of metabolic variables and energy expenditure.

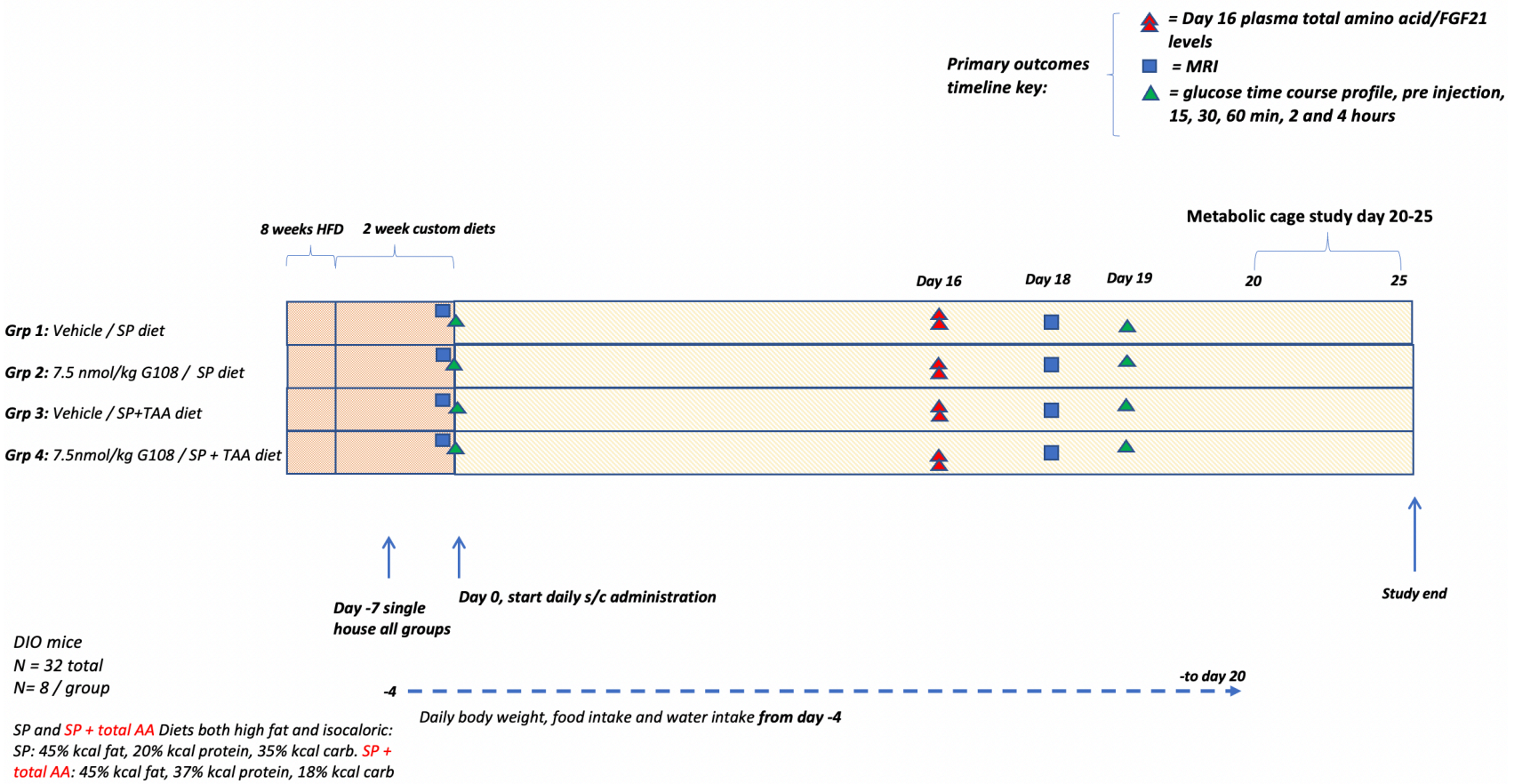


Figure 4.2. Schematic of total amino acid dietary 'add back' study protocol.

4.3.5 Chronic G108 administration in DIO mice with either EAA or NEAA 'add-back'

For the essential amino acid (EAA) and non-essential amino acid (NEAA) individual amino acid dietary 'add-back' study (Figure 4.3), 48 diet-induced obese male C57BL/6J mice were switched to custom diets for two weeks. A third of the cohort (N=16) were provided with a standard protein (SP) diet (D08091803, Table 4.1), Another third of the cohort (N=16), were provided with either provided with a standard protein diet supplemented with EAA's (Formula 2, D22011203, Table 4.1 and 4.2). Another third of the cohort were provided with a standard protein diet supplemented with NEAA's (Formula 3, D22011204, Table 4.1 and 4.2). Mice from each diet group received daily subcutaneous administration of 7.5 nmol/kg G108 (N=8) or vehicle control (N=8). The six treatment groups were as follows: Vehicle / SP diet, 7.5 nmol/kg G108 / SP diet, Vehicle / SP ± EAA diet, 7.5 nmol/kg G108 / SP ± EAA diet, Vehicle / SP ± NEAA diet, 7.5 nmol/kg G108 / SP ± NEAA diet.

Prior to switching onto these custom diets on day 0, a baseline MRI analysis was carried out to measure body composition. Body composition analysis was also carried out on day 14 in all groups. Tail venesection was carried out on day 13 for plasma FGF21 levels.

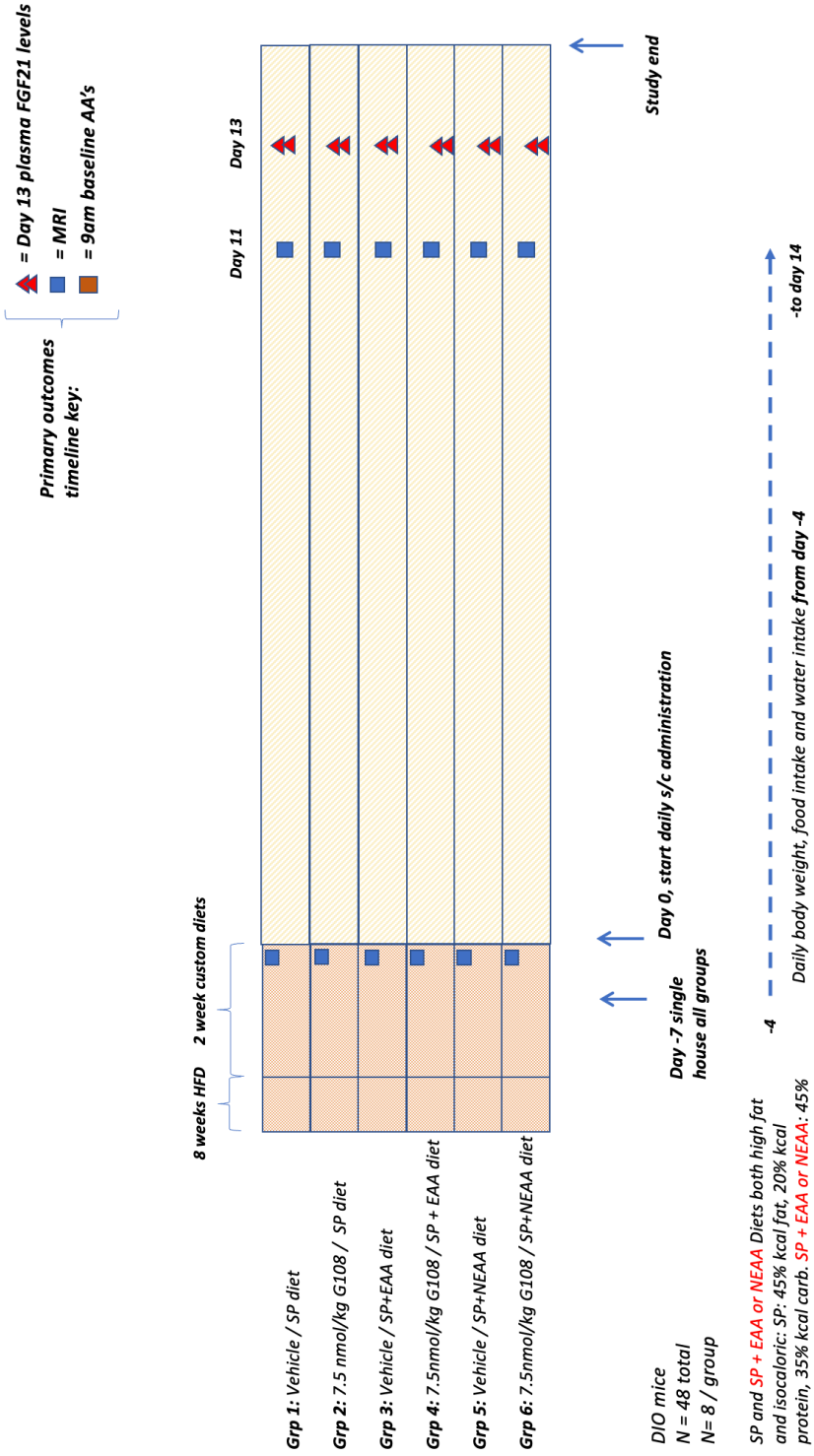


Figure 4.3. Schematic of EAA or NEAA dietary 'add back' study protocol.

4.3.6 Custom low amino acid profile diet study in obese mice

For the custom low amino acid profile diet study (Figure 4.4), two groups (N=7-8/group) of diet-induced-obese (DIO) male C57BL/6J mice were provided with either an amino acid-based standard protein (SP) diet (SPAA diet: A22012001, Table 4.1 and 4.2), containing 17 kcal% protein or a custom low amino acid profile diet, (LAA diet, Formula 5, A22012002, Table 4.1 and 4.2), consisting of 10 kcal% protein. Both diets were matched for fat content with variation in carbohydrate content to allow changes in protein content. The reduction in amino acid composition of the LAA diet relative to the SPAA diet was matched to reduction in plasma amino acid levels in 7.5 nmol/kg G108-treated obese mice, relative to vehicle-treated obese mice (Chapter 3, Section 3.4.5).

Prior to switching onto these custom diets on day 0, a baseline MRI analysis was carried out to measure body composition. Food intake and body weight was measured daily at 9 am in the morning and subsequent body composition analysis was carried out again on day 14. On day 26 of the study, all mice were placed into metabolic cages to measure energy expenditure. At the end of the study on day 34, plasma was collected and analysed for FGF21 and individual amino acid levels using gas-chromatography/mass-spectrometry (GC/MS).

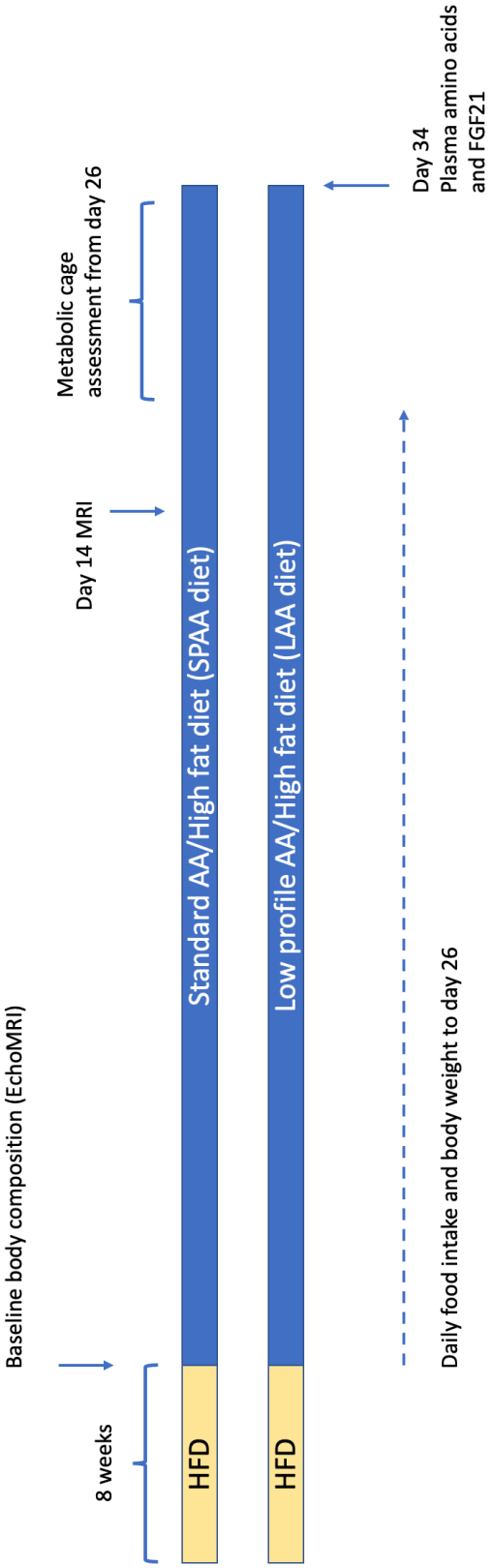


Figure 4.4. Schematic of dietary low amino acid study protocol.

4.3.7 Dietary restriction of amino acids in hepatic specific *Fgf21* (*Fgf21*$^{\Delta liver}$) knockout mice

Lean 10-week-old WT and *Fgf21*$^{\Delta liver}$ mice were provided with the low amino acid diet for two weeks, N=7-8/group (LAA diet, described in section 4.3.6 above). After two-weeks of *ad libitum* feeding with the LAA diet, a tail venesection was carried out for subsequent analysis of plasma FGF21. All mice were then placed into metabolic cages for assessment of EE over a 48-hour period.

4.3.8 Food intake, body weight and body composition (EchoMRI)

Food intake and body weight were measured at 9 am daily, 24- to 48-hourly in all studies using a calibrated weighing scale (Ohaus Traveller, Ohaus). Food was topped up as required. An EchoMRI analyser was used to measure body composition at various timepoints during the studies (technique described in Chapter 2, section 2.3.10). Body composition was measured at the same time of day on each occasion. Following each individual scan, the animal was placed back into its respective cage. Data was obtained for both lean and fat mass.

4.3.9 Metabolic cages

As described in Chapter 3, Section 3.3.7, whole-body energy expenditure studies were carried out using the Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, Ohio). Data was analysed using regression based-ANCOVA analysis in CalRapp and SPSS, using lean or total mass as a covariate.

4.3.10 Glucose time-course profile

For the glucose profile study as part of the total amino acid dietary 'add-back' study, mice were administered with 7.5 nmol/kg G108 or vehicle control on day 0 and day 19. A tail vein blood drop sample was collected immediately prior to treatment injection and blood glucose was measured with a handheld GlucoRx meter. Blood glucose was subsequently measured at 15, 30, 60, 120 and 240 minutes.

4.3.11 Biochemical analysis of plasma

Total plasma amino acids were measured using commercially available fluorometric-based ELISA assays as directed (MAK002, Sigma, Ab65347, Abcam). Plasma FGF21 was measured also used an ELISA based assay (ab229382, Abcam).

4.4 Results

Key results for Chapter 4

- **A high protein diet prevents acute hypoaminoacidemia and EE in response to a single high dose (20 nmol/kg) of G108 in obese mice.**
- **Dietary total amino acid supplementation prevents G108-stimulated hypoaminoacidemia, enhanced EE and body weight loss in obese mice.**
- **Dietary replacement with either EAA or NEAA components is not sufficient to prevent G108-stimulated EE and body weight loss in obese mice.**
- **A rationally-designed low amino acid diet mimics the chronic effect of G108 on EE and body weight loss in obese mice.**
- **Induction of hepatic FGF-21 does not appear to be required for the EE response to a low amino acid diet in obese mice.**

4.4.1 Acute metabolic cage study

To determine the acute effect of G108 on plasma amino acids and EE, single dose administration studies were carried out in mice receiving either a standard protein (SP) or high protein (HP diet). As in the chronic study described in Chapter 3, a sub-anorectic dose of G108 (7.5 nmol/kg) was used. To determine whether a dose-dependent effect could be observed, a higher dose of 20 nmol/kg was also used. These doses were chosen based on the potential outcome that the lower dose may not acutely result in either hypoaminoacidemia or EE.

Following a single dose of 7.5 nmol/kg G108, there was a reduction in plasma total amino acids in both the HP and SP groups after 24 hours (Figure 4.5A). This was associated with a trend to increased average energy expenditure (kcal/hr) in G108-treated mice fed the SP diet in the light phase between 24 and 36 hours (Figure 4.5B, Table 4.3). A trend towards increased energy expenditure was also observed in G108-treated mice fed the

SP diet, between 36 and 48 hours of the dark phase (Figure 4.5B, Table 4.3). No change in average energy expenditure was observed in the HP group (Figure 4.5B, Table 4.4).

Following a single dose of 20 nmol/kg G108, there was a significant and sustained reduction in plasma total amino acids for up to 48 hours in mice fed the SP diet (Figure 4.5C). This was associated with a significant increase in the average energy expenditure in G108-treated mice fed the SP diet between 0 and 12 hours of the light phase, 36 and 48 hours of the dark phase and 60 to 72 hours of the dark phase (Figure 4.5D, Table 4.5). Despite a reduction in total plasma amino acids in mice fed the HP diet following 20 nmol/kg G108 administration, the lowest level of plasma amino acids at 24 hours was comparable to the baseline level of plasma amino acids in the SP group (Figure 4.5C). In keeping with this, there was no detectable change in average energy expenditure in the HP group following administration of the higher dose of G108 (Figure 4.5D, Table 4.6).

Consistent with the changes in energy expenditure, the average oxygen consumption also increased in the same photoperiods (Tables 4.3-4.6). The respiratory exchange ratio (RER) as expected was lower in mice fed a high protein diet however a change in RER from the baseline photoperiod did not appear to associate consistently with the changes in EE or oxygen consumption (Tables 4.3-4.6).

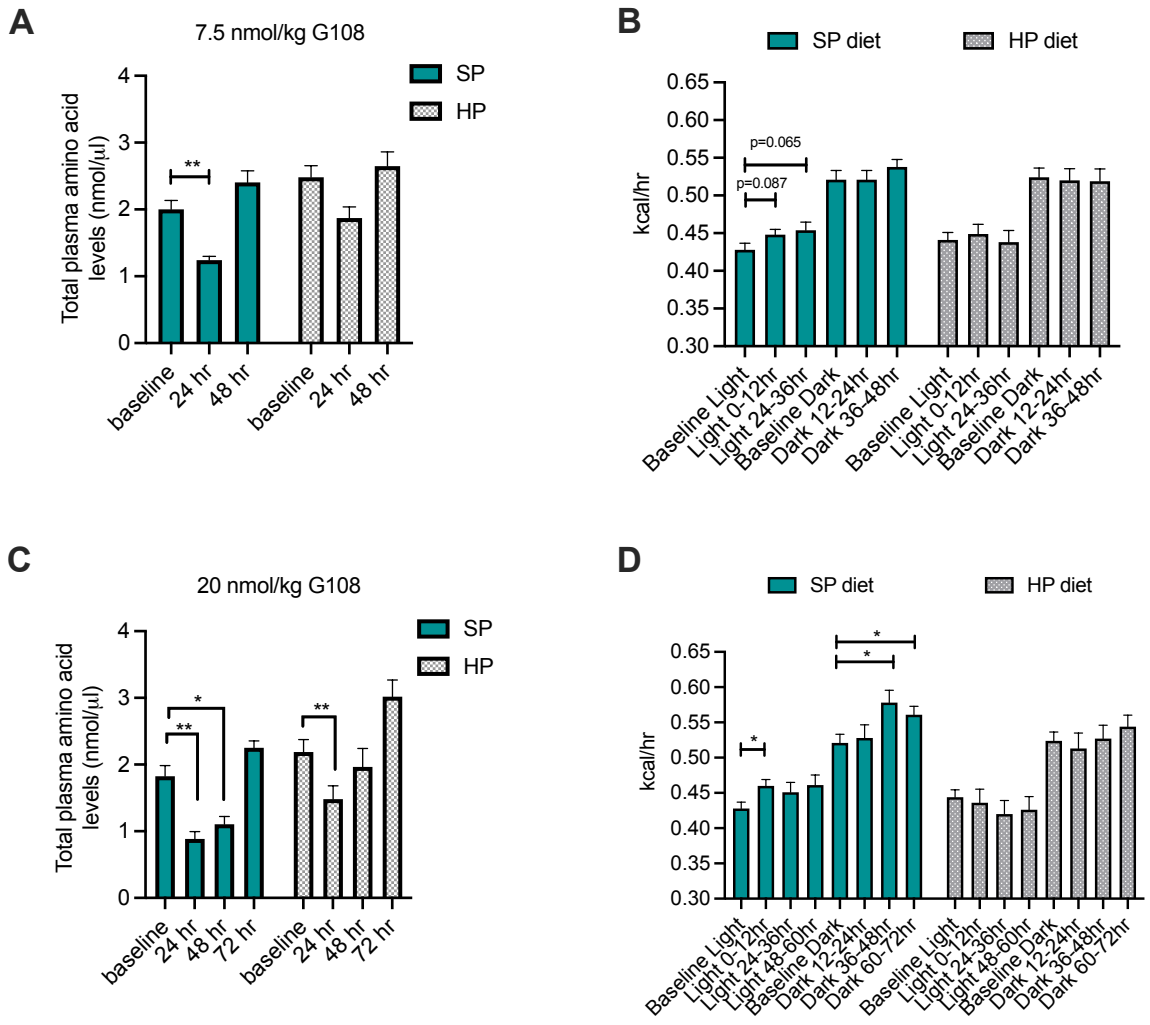


Figure 4.5: Plasma total amino acids and average energy expenditure (kcal/hr) in mice administered with a single dose of G108. SP – Standard protein diet, HP – High protein diet, n=10/group. From Hope, Hinds et al. (In Press). **(A)** Plasma total amino acids in mice administered with a single 7.5 nmol/kg dose of G108. **(B)** Average energy expenditure (kcal/hr) over 48 hours in mice administered with a single 7.5 nmol/kg dose of G108. (**C)** Plasma total amino acids in mice administered with a single 20 nmol/kg dose of G108. **(D)** Average energy expenditure (kcal/hr) over 72 hours in mice administered with a single 20 nmol/kg dose of G108. *p<0.05, **p<0.01, data plotted as mean ± SEM. Energy expenditure data analysed using regression based ANCOVA comparing baseline to subsequent photoperiods and using lean mass as a covariate. Total amino acid data analysed using two-way ANOVA with post hoc Sidak's test.

Time period	Vo2 (ml/hr)	Co2 (ml/hr)	RER	Kcal/hr
Baseline Light	89.18 ± 1.85	72.073 ± 1.313	0.81 ± 0.005	0.427 ± 0.008
Light 0-12 hr	92.62 ± 1.63	75.51 ± 1.31	0.81 ± 0.004	0.448 ± 0.008
Light 24-36 hr	95.59 ± 1.85 ***P*<0.05**	73.59 ± 1.80	0.77 ± 0.008 ***P*<0.01**	0.455 ± 0.010 ***P*=0.065**
Baseline Dark	106.83 ± 2.44	90.63 ±2.35	0.85 ± 0.005	0.520 ± 0.012
Dark 12-24 hr	108.12 ± 2.44	88.98 ± 2.35	0.82 ± 0.007 ***P*<0.05**	0.521 ± 0.012
Dark 36-48 hr	111.49 ± 2.13	92.34 ± 2.20	0.83 ± 0.004	0.538 ± 0.011

Table 4.3: Metabolic variables from the 7.5 nmol/kg G108 acute metabolic cage study in mice receiving a standard protein diet. Data expressed as mean ± SEM. Analysed using regression based ANCOVA comparing baseline to subsequent photoperiods and using lean mass as a covariate. Significance denotes change from light or dark photoperiod baseline.

Time period	Vo2 (ml/hr)	Co2 (ml/hr)	RER	Kcal/hr
Baseline Light	91.31 ± 2.68	72.86 ± 2.27	0.79 ± 0.004	0.444 ± 0.012
Light 0-12 hr	92.74 ± 2.68	73.65 ± 2.27	0.79 ± 0.003	0.449 ± 0.012
Light 24-36 hr	92.11 ± 2.81	69.74 ± 2.46	0.75 ± 0.007 **P<0.0001**	0.438 ± 0.013
Baseline Dark	108.83 ± 2.82	87.57 ± 2.72	0.80 ± 0.006	0.524 ± 0.014
Dark 12-24 hr	108.79 ± 2.82	85.58 ± 2.73	0.78 ± 0.007	0.520 ± 0.014
Dark 36-48 hr	108.92 ± 2.84	86.26 ± 2.75	0.79 ± 0.007	0.519 ± 0.017

Table 4.4: Metabolic variables from the 7.5nmol/kg G108 acute metabolic cage study in mice receiving a high protein diet. Data expressed as mean ± SEM. Analysed using regression based ANCOVA comparing baseline to subsequent photoperiods and using lean mass as a covariate. Significance denotes change from light or dark photoperiod baseline.

Time period	Vo2 (ml/hr)	Co2 (ml/hr)	RER	Kcal/hr
Baseline Light	89.18 ± 1.61	72.073 ± 1.49	0.81 ± 0.005	0.427 ± 0.009
Light 0-12 hr	95.60 ± 1.61 ***P*<0.05**	75.88 ± 1.49 ***P*<0.05**	0.79 ± 0.007	0.460 ± 0.009 *P*<0.05
Light 24-36 hr	95.88 ± 2.45	71.47 ± 2.16	0.74 ± 0.006 ***P*<0.0001**	0.452 ± 0.012
Light 48-60hr	96.29 ± 2.44	75.82 ± 2.40	0.78 ± 0.009	0.461 ± 0.012
Baseline Dark	106.83 ± 2.95	90.63 ± 3.10	0.85 ± 0.005	0.520 ± 0.015
Dark 12-24 hr	109.74 ± 2.95	88.25 ± 3.08	0.80 ± 0.009 ***P*<0.0001**	0.526 ± 0.016
Dark 36-48 hr	119.74 ± 3.10 ***P*<0.05**	100.10 ± 2.820 ***P*<0.05**	0.83 ± 0.004	0.578 ± 0.015 ***P*<0.05**
Dark 60-72 hr	115.33 ± 2.40 ***P*<0.05**	97.87 ± 2.24 ***P*<0.05**	0.85 ± 0.008	0.560 ± 0.012 ***P*<0.05**

Table 4.5: Metabolic variables from the 20nmol/kg G108 acute metabolic cage study in mice receiving a standard protein diet. Data expressed as mean ± SEM. Analysed using regression based ANCOVA comparing baseline to subsequent photoperiods and using lean mass as a covariate. Significance denotes change from light or dark photoperiod baseline.

Time period	Vo2 (ml/hr)	Co2 (ml/hr)	RER	Kcal/period
Baseline Light	91.31 ± 3.26	72.86 ± 2.86	0.79 ± 0.004	0.444 ± 0.016
Light 0-12 hr	91.59 ± 3.26	72.08 ± 2.86	0.78 ± 0.006	0.437 ± 0.016
Light 24-36 hr	88.48 ± 3.17	66.74 ± 2.58	0.75 ± 0.005 P**<0.0001**	0.420 ± 0.016
Light 48-60hr	88.74 ± 3.08	69.74 ± 2.59	0.78 ± 0.005	0.426 ± 0.015
Baseline Dark	108.83 ± 3.60	87.58 ± 3.10	0.80 ± 0.006	0.524 ± 0.018
Dark 12-24 hr	106.93 ± 3.60	85.66 ± 3.10	0.80 ± 0.003	0.513 ± 0.018
Dark 36-48 hr	110.43 ± 3.28	89.41 ± 2.81	0.81 ± 0.003	0.527 ± 0.017
Dark 60-72 hr	113.77 ± 3.13	88.67 ± 3.82	0.79 ± 0.005	0.546 ± 0.015

Table 4.6: Metabolic variables from the 20nmol/kg G108 acute metabolic cage study in mice receiving a high protein diet. Data expressed as mean ± SEM. Analysed using regression based ANCOVA comparing baseline to subsequent photoperiods and using lean mass as a covariate. Significance denotes change from light or dark photoperiod baseline.

4.4.2 Chronic G108 administration in DIO mice with dietary total amino acid 'add-back'

To demonstrate that chronic G108-stimulated hypoaminoacidemia increases energy expenditure and total body weight loss, a study was carried out using the sub-anorectic dose of G108 (7.5 nmol/kg), where total individual amino acids were supplemented in the diet. G108 was administered to mice receiving either a casein-based standard protein diet (SP) or the SP diet supplemented with individual total amino acids (SP ±TAA diet, Table 4.1 and Table 4.2). The total protein content of the SP±TAA diet was matched to that of the casein-based high protein diet used in Chapter 3. Therefore, this study aims to provide a mechanistic explanation for the findings in Chapter 3 and determine whether rescue of chronic G108-stimulated hypoaminoacidemia prevents EE and body weight loss.

As seen previously in Chapter 3, daily administration of 7.5 nmol/kg G108 in obese mice did not result in a chronic reduction in food intake (Figure 4.6A, Table 4.7). Despite this, G108-treated animals fed the SP diet lost a significant amount of total body weight compared to baseline (Figure 4.6B, Table 4.7). The loss of body weight reflected a reduction in both lean and fat mass (Figure 4.6C, Table 4.8). When individual total amino acids were supplemented in the diet, loss of total body weight in G108-treated mice was prevented (Figure 4.6B, Table 4.7) and this was also associated with preserved lean and fat mass in this group (Figure 4.6C, Table 4.8). Compared to mice receiving the SP diet, no independent effect of the dietary intervention on body weight or composition was observed in vehicle treated mice (Figure 4.6B and 4.6C, table 4.7 and 4.8). In keeping with the body weight loss independent to food intake, the average energy expenditure in the G108-treated mice fed the SP diet was increased (Figure 4.6D, Table 4.9). The increase in energy expenditure was blunted in mice receiving the SP±TAA diet (Figure 4.6D, Table 4.9).

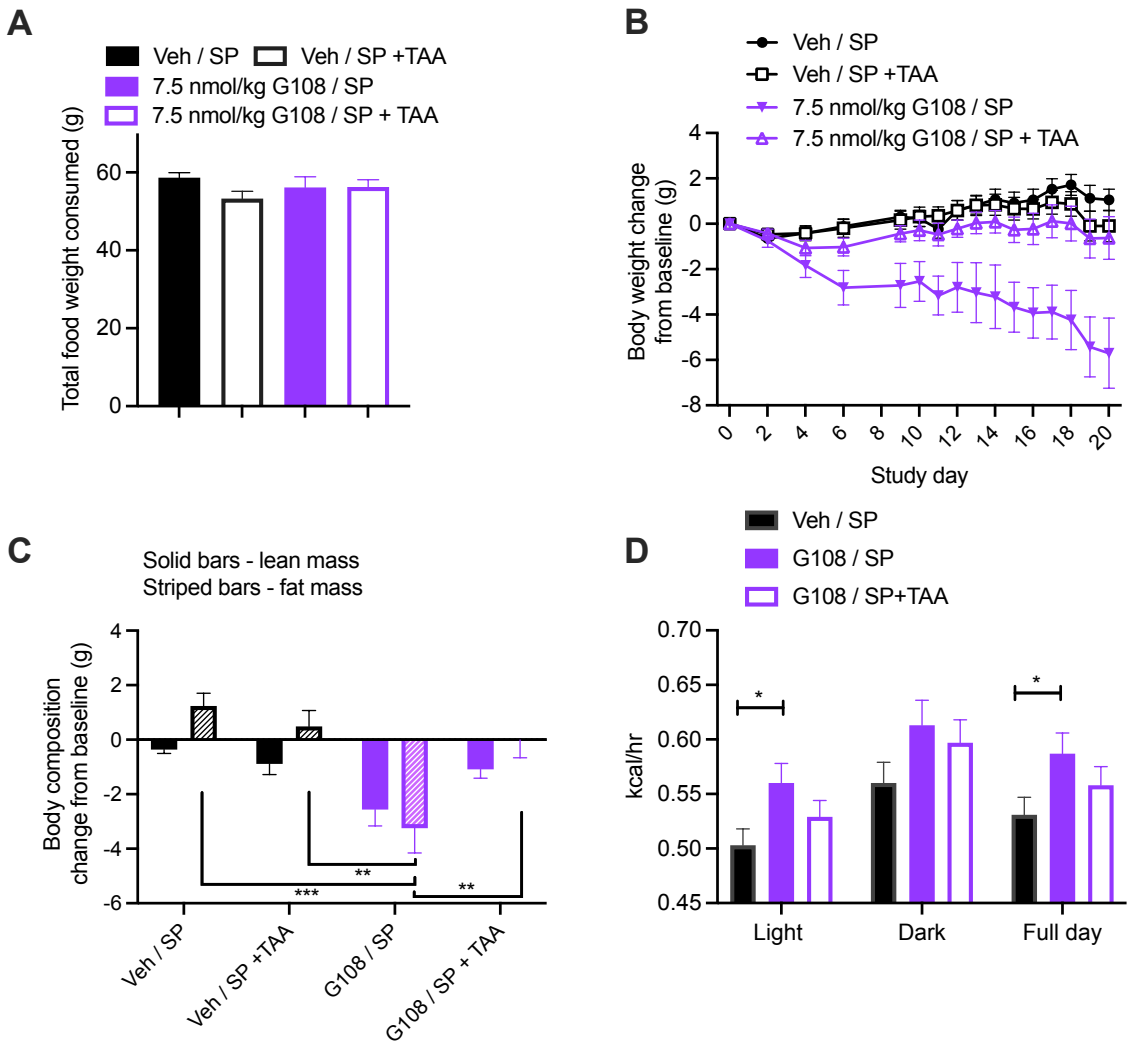


Figure 4.6: Effect of dietary total amino acid replacement in G108-treated DIO C57BL/6J mice, n=8/group. SP – standard protein diet, SP±TAA – SP diet with additional total amino acids. From Hope, Hinds et al. (In Press). **(A)** Total food weight consumed (g) in 7.5 nmol/kg G108-treated C57BL/6J DIO mice receiving dietary intervention. **(B)** Body weight change (g) from baseline over 20 days of daily administration of G108. **(C)** Change in body composition after 18 days of daily administration of G108 in mice receiving dietary intervention. **(D)** Average energy expenditure (kcal/hr) after 20 days of daily administration of G108 in mice receiving dietary intervention, n=6-8/group. *p<0.05, **p<0.01, ***p<0.001, data plotted as mean ± SEM. EE data analysed using regression based ANCOVA using lean mass as a covariate. All other data analysed using a combination of one- and two-way ANOVA with post hoc Sidak or Dunnett tests.

	Veh / SP	Veh / SP ±TAA	7.5 nmol/kg G108 / SP	7.5 nmol/kg G108 / SP ±TAA
Total food intake (g)	58.66 ± 1.27	53.26 ± 1.89	56.18 ± 2.72	56.25 ± 1.90
Change in total body weight from baseline (g)	1.05 ± 0.48	-0.10 ± 0.70	-5.70 ± 1.54 ***P*<0.001**	-0.63 ± 0.94

Table 4.7: Total food intake and change in body weight over 20 days. Data expressed as mean ± SEM. ***p<0.001, analysed with one-way ANOVA with post hoc Dunnett test.

	Veh / SP	Veh / SP ±TAA	7.5 nmol/kg G108 / SP	7.5 nmol/kg G108 / SP ±TAA
Change in fat mass from baseline (g)	1.24 ± 0.14	0.49 ± 0.58	-3.2 ± 0.91 ***P*<0.0001**	-0.01 ± 0.65
Change in lean mass from baseline (g)	-0.36 ± 0.14	-0.89 ± 0.39	-2.56 ± 0.60	-1.10 ± 0.33

Table 4.8: Change in body composition from baseline over 18 days. Data expressed as mean ± SEM. ****p<0.0001, significance denotes difference between treatment group and Veh / SP group. Analysed with two-way ANOVA with post hoc Dunnett's test.

	Veh / SP	7.5 nmol/kg G108 / SP	7.5 nmol/kg G108 / SP ±TAA
Vo2 (ml/hr)	113.14 ± 3.13	124.27 ± 3.92. ***P*=0.054**	119.59 ± 3.39
RER	0.79 ± 0.008	0.82 ± 0.02	0.76 ± 0.004
Kcal (kcal/hr)	0.531 ± 0.016	0.587 ± 0.019 ***P*<0.05**	0.558 ± 0.017

Table 4.9: Averaged metabolic variables for full-day photoperiod. Data expressed as mean ± SEM. ****p<0.0001, significance denotes difference between treatment group and Veh / SP group. Vo2 and kcal data analysed using ANCOVA with lean mass as a covariate. RER data analysed using one-way ANOVA.

The changes in body weight and EE observed with dietary 'add-back' of total individual (Figure 4.6) were associated with a rescue of plasma total amino acids (Figure 4.7A), confirming that G108-stimulated hypoaminoacidemia increases EE and body weight loss.

Plasma FGF21 was also increased in G108-treated mice fed a SP diet, a response which was prevented by dietary total amino acid supplementation (Figure 4.7B).

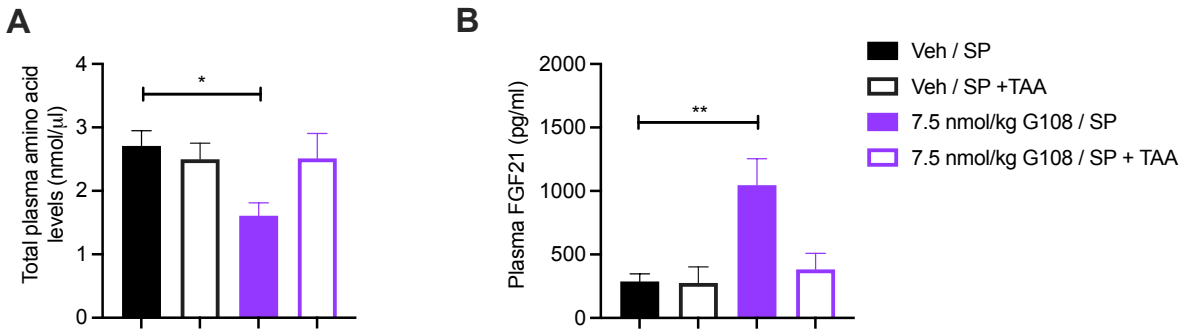


Figure 4.7: Effect of dietary total amino acid replacement on plasma total amino acids and FGF21, in G108-treated obese mice, n=8/group. From Hope, Hinds et al. (In Press). Plasma total amino acids **(A)** and plasma FGF21 **(B)** after 16 days of daily administration of G108. *p<0.05, **p<0.01, data plotted as mean ± SEM. Analysed using one-way ANOVA with post hoc Dunnett's test.

4.4.3 Chronic G108 administration in DIO mice with dietary 'add-back' of either EAA or NEAA

To determine whether G108-stimulated reduction in specific amino acid components of the SP±TAA diet could enhance EE and body weight loss, the SP±TAA diet was further modified. The broadest and most well-known classification of amino acids is into essential and non-essential amino acids (EAA and NEAA respectively). A further chronic daily dosing study was carried out with 7.5 nmol/kg G108, feeding with either SP, SP±EAA or SP±NEAA diets (diet compositions detailed in Tables 4.1 and 4.2). This study was designed based on the hypothesis that if a reduction in EAA mediates the EE response to G108, then this effect should be prevented when feeding with the SP±EAA diet. Conversely, if the NEAA's mediate the EE response to G108, then this effect should be prevented when feeding with the SP±NEAA diet. If lowering of either EAA's or NEAA's, in response to G108, is required for the EE effects observed, then G108-stimulated body weight loss and EE should be observed irrespective of the diet intervention.

Over 14 days of daily treatment, total food intake remained similar across all treatment groups except G108-treated mice fed with the SP±NEAA diet (Figure 4.8A, Table 4.10).

Despite no broad change in total food intake, all G108-treated mice lost total body weight to a similar degree (Figure 4.8B). The reduction in total body weight in all peptide groups reached approximately 8 grams over the 14-day study period (Table 4.10).

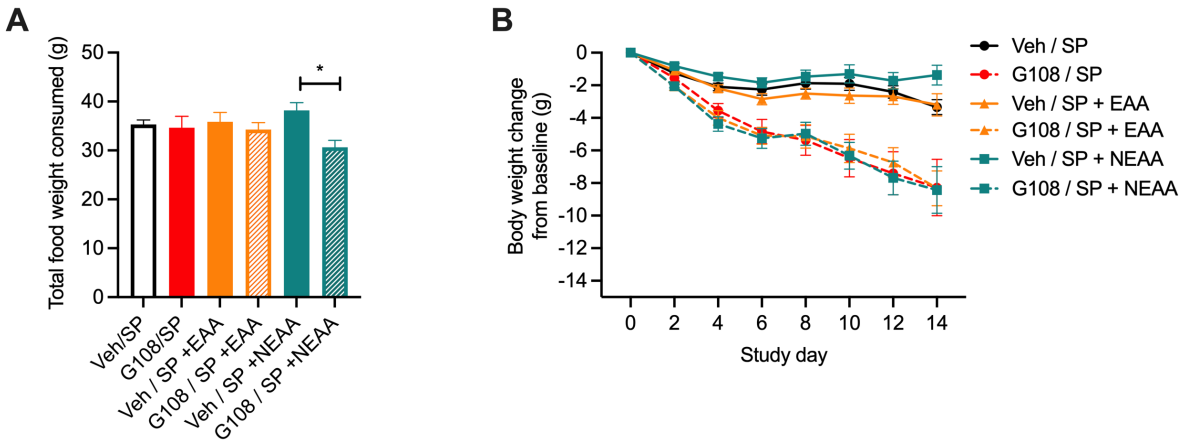


Figure 4.8: Effect of EAA or NEAA dietary 'add-back' in G108-treated obese male C57BL/6J mice, n=8/group. From Hope, Hinds et al. (In Press). **(A)** Total food weight consumed over 14 days. **(B)** Change in body weight from baseline over 14 days. Data plotted as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001. Data analysed with one- and two-way ANOVA with post hoc Sidak or Dunnett's tests.

	Veh / SP	**G108 / SP**	**Veh / SP ± EAA**	**G108 / SP ± EAA**	**Veh / SP ±NEAA**	**G108 / SP ±NEAA**
Total food intake (g)	35.30 ± 0.95	34.65 ± 2.34	35.85 ± 1.92	34.26 ± 1.44	38.20 ± 1.58	30.63 ± 1.44 ***P*<0.05**
Change in total body weight from baseline (g)	-3.34 ± 0.46	-8.28 ± 1.73 ***P*<0.05**	-3.2 ± 0.68	-8.33 ± 1.07 ***P*<0.05**	-1.38 ± 0.60	-8.43 ± 1.43 ***P*<0.05**

Table 4.10: Total food intake and change in body weight over 14 days. Data expressed as mean ± SEM. *p<0.05, analysed with one-way ANOVA with post hoc Tukeys test.

The total body weight loss constituted a loss of both fat and lean mass, which was again similar across all G108-treatment groups (Figure 4.9A, Table 4.11). In keeping with food intake-independent body weight loss, the feed efficiency was reduced to a similar extent in all G108-treated mice (Figure 4.9B).

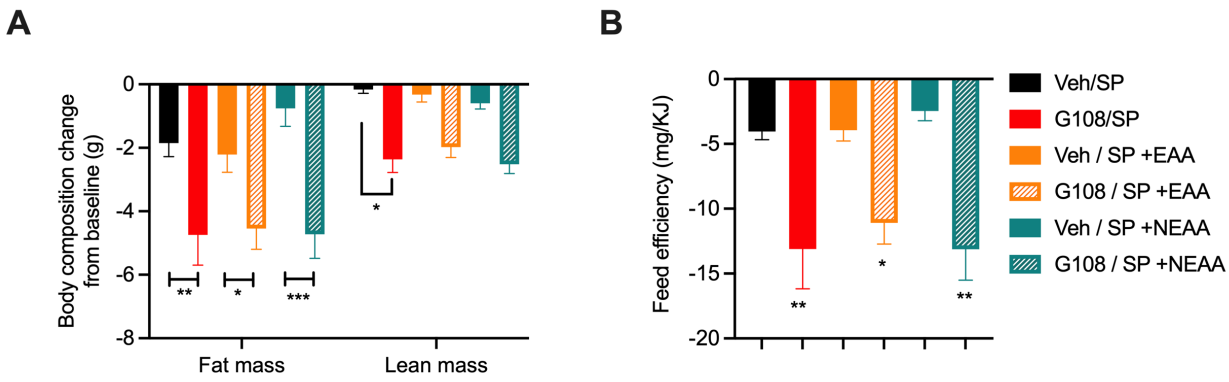


Figure 4.9: Effect of EAA or NEAA dietary 'add-back' in G108-treated obese male C57BL/6J mice, n=8/group. From Hope, Hinds et al. (In Press). **(A)** Change in body composition from baseline over 11 days. **(B)** Feed efficiency over 14 days. Data plotted as mean ± SEM, significance denotes change compared to vehicle control groups on the same dietary intervention, *p<0.05, **p<0.01, ***p<0.001. Data analysed with one- and two-way ANOVA with post hoc Sidak or Dunnett's tests.

	Veh / SP	**G108 / SP**	**Veh / SP ± EAA**	**G108 / SP ± EAA**	**Veh / SP ±NEAA**	**G108 / SP ±NEAA**
Change in fat mass from baseline (g)	-1.85 ± 0.43	-4.75 ± 0.95 ***P*<0.01**	-2.21 ± 0.56	-4.54 ± 0.65 ***P*<0.05**	-0.76 ± 0.57	-4.73 ± 0.76 ***P*<0.01**
Change in lean mass from baseline (g)	-0.16 ± 0.12	-2.37 ± 0.41 ***P*<0.01**	-0.32 ± 0.24	-1.97 ± 0.33 ***P*<0.05**	-0.60 ± 0.18	-2.52 ± 0.30 ***P*<0.01**

Table 4.11: Change in body composition from baseline over 14 days. Data expressed as mean ± SEM. *p<0.05, **p<0.01, significance denotes change compared to vehicle control groups on the same dietary intervention. Analysed with two-way ANOVA with post hoc Dunnett's test.

An increase in plasma FGF21 levels were associated with the increased body weight loss and feed efficiency in all G108-treated mice, irrespective of dietary intervention (Figure 4.10). The increase in plasma FGF21 in the EAA and NEAA add back groups was non-significantly lower to that observed in the G108-treated mice receiving the SP (Figure 4.10).

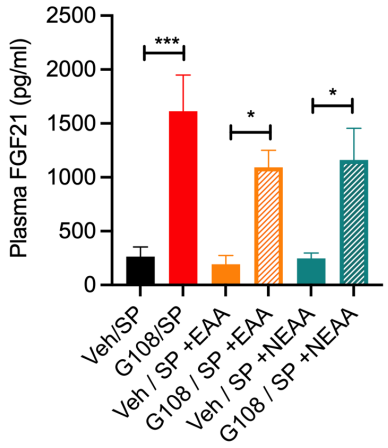


Figure 4.10: Plasma FGF21 on day 13 of study. From Hope, Hinds et al. (In Press). Data expressed as mean ± SEM. *p<0.05, analysed with one-way ANOVA with post hoc Tukey's test.

4.4.4 Custom low amino acid profile diet study in obese mice

To further support the finding that G108-stimulated hypoaminoacidemia increases EE, DIO mice were fed a custom low amino acid diet (LAA diet, Table 4.1 and Table 4.2). The individual amino acid concentrations in the LAA diet were reduced relative to the SP diet, matched to the degree of reduction of individual plasma amino acids in DIO mice chronically treated with 7.5 nmol/kg G108 (Chapter 3, Section 3.4.5). As a comparator, DIO mice were also fed with a standard protein amino acid diet (SPAA diet, Table 4.1 and Table 4.2), equivalent to the protein content of the previously used standard protein (SP) diet. Therefore, any chronic effect on EE observed in mice receiving the LAA diet was independent to G108 treatment *per se*. Increased EE, as measured by metabolic cages, was hypothesised to occur in mice fed with the LAA diet, relative to mice fed with the SPAA diet.

Mice were fed with the LAA or SPAA diet for 34 days, at which point terminal plasma samples were collected to confirm a reduction in individual plasma amino acid levels. As expected, mice fed with the LAA diet exhibited a broad reduction in plasma AA's (Figure 4.11), similar to that seen in G108-treated mice (Chapter 3, Section 3.4.5).

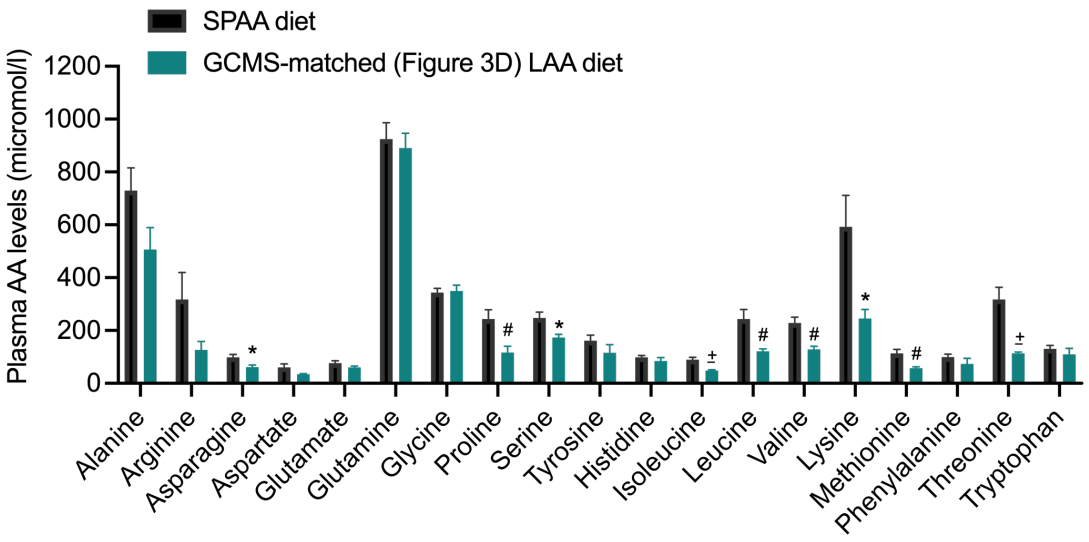


Figure 4.11: Individual plasma amino acid levels in DIO mice after 34 days of receiving either a low amino acid (LAA) diet or a standard protein amino acid (SPAA) diet, n=7-8/group. From Hope, Hinds et al. (In Press). Reduction in LAA diet AA concentrations matched to the reduction in plasma AA's in G108-trearted mice (Chapter 3, Section 3.4.5). Data plotted as mean ± SEM, *$p<0.05$, # $p<0.01$, ± $p<0.001$, analysed using unpaired t-tests.

Amino Acid	Vehicle/SP	7.5 nmol/kg G108/SP	Percentage change (7.5 nmol/kg G108/ SP vs Vehicle / SP)
Histidine	97.9 ± 6.7	84.5 ± 12.9	-13.7
Total Isoleucine	88.4 ± 8.7	48.5 ± 2.5 ***P*<0.001**	-45.1
Leucine	242.0 ± 36.4	121.71 ± 8.5 ***P*<0.01**	-49.7
Valine	227.8 ± 21.7	128.7 ± 11.5 ***P*<0.01**	-56.3
Lysine	591.2 ± 119.5	245.1 ± 34.0 ***P*<0.05**	-58.4
Methionine	112.9 ± 14.3	57.9 ± 4.1 ***P*<0.01**	-48.7
Phenylalanine	98.4 ± 10.7	73.3 ± 21.6	-25.5
Threonine	316.2 ± 46.1	113.1 ± 5.4 ***P*<0.001**	-64.2
Tryptophan	129.2 ± 13.1	109.9 ± 22.1	-14.9

Table 4.12: Individual essential plasma amino acid levels after 34 days of receiving either a low amino acid (LAA) diet or a standard protein amino acid (SPAA) diet, n=7-8/group. Measured using gas chromatography/mass spectrometry.

Amino Acid	Vehicle/SP	7.5 nmol/kg G108/SP	Percentage change (7.5 nmol/kg G108/ SP vs Vehicle / SP)
Alanine	728.4 ± 85.8	506.3 ± 83.1	-30.4
Arginine	316.2 ± 101.9	126.4 ± 32.1	-60.0
Aspartate	59.6 ± 12.7	34.9 ± 2.5	-41.4
Asparagine	91.1 ± 12.7	34.4 ± 2.5 ***P*<0.05**	-62.2
Glutamate	75.1 ± 9.1	60.9 ± 4.7	-18.9
Glutamine	923.4 ± 62.4	890.5 ± 55.7	-3.6
Glycine	342.5 ± 16.4	349.5 ± 21.9	±2.0
Proline	242.3 ± 35.2	116.7 ± 23.9 ***P*<0.01**	-51.8
Serine	246.0 ± 22.6	173.5 ± 11.9 ***P*<0.05**	-29.5
Tyrosine	160.9 ± 20.4	115.2 ± 30.9	-28.4
Cysteine	8.5 ± 1.4	8.9 ± 2.2	±4.7

Table 4.13: Individual non-essential plasma amino acid levels after 34 days of receiving either a low amino acid (LAA) diet or a standard protein amino acid (SPAA) diet, n=7-8/group. Measured using gas chromatography/mass spectrometry.

The LAA diet did not affect food intake (Figure 4.12A), however led to a reduction in both fat and lean mass (Figure 4.12B).

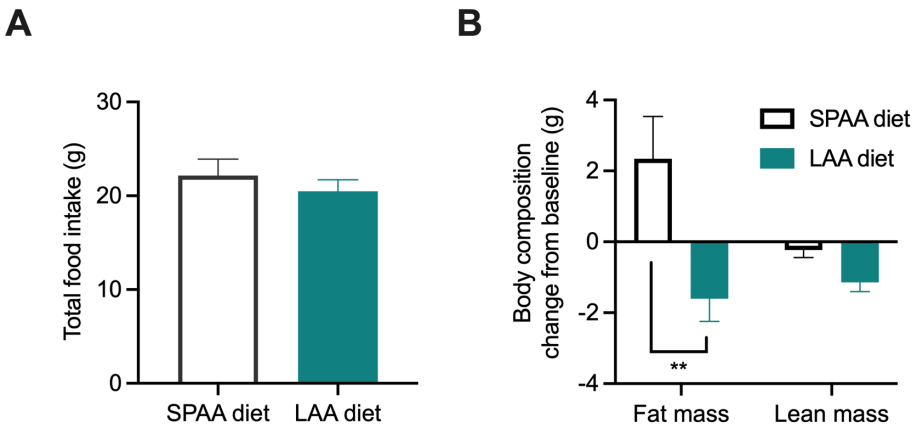


Figure 4.12: Total food intake and body composition in DIO mice fed a LAA or SPAA diet. From Hope, Hinds et al. (In Press)**. (A)** Total food intake over 7 days **(B)** Change in body composition from baseline over 14 days. n=7-8/group. Data plotted as mean ± SEM, ***p<0.001. Analysed using a combination of unpaired t-test and two-way ANOVA with post hoc Sidak's test.

In keeping with the food intake-independent effects of the LAA diet on body composition, both feed efficiency and EE were increased in mice fed the custom diet (Figure 4.13A and B).

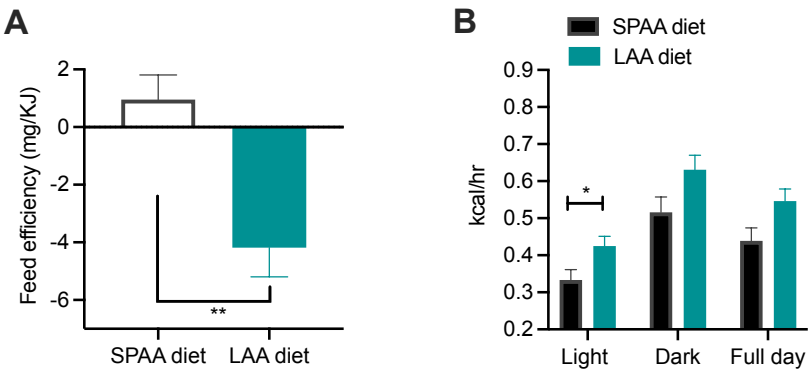


Figure 4.13: Feed efficiency and EE in DIO mice fed a LAA or SPAA diet. From Hope, Hinds et al. (In Press). **(A)** Feed efficiency over 10 days. **(B)** Average oxygen consumption over a 24-hour period. n=7-8/group. Data plotted as mean ± SEM, *p<0.05, **p<0.01. Feed efficiency data analysed using unpaired t-test. Oxygen consumption data analysed using ANCOVA using lean mass as a covariate.

Finally, to determine whether FGF21 induction is associated with reduced feed efficiency and enhanced EE, plasma FGF21 was measured. A significant increase in plasma FGF21 was detected in mice fed the LAA diet compared to those fed the SPAA diet (Figure 4.14)

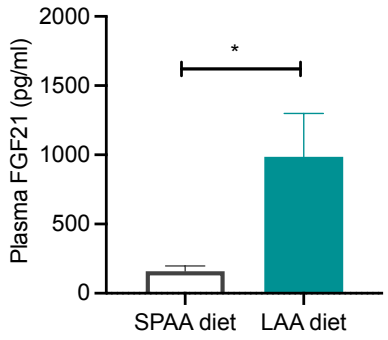


Figure 4.14: Terminal plasma FGF21 levels on day 34 of study. n=7-8/group. From Hope, Hinds et al. (In Press). Data plotted as mean ± SEM, *p<0.05. Analysed using unpaired test.

4.4.5 Increased circulating FGF21 is not required to enhance EE in response to a custom low amino acid diet

As hepatic FGF21 is activated in response to amino acid deprivation, the effect of G108-stimulated hypoaminoacidemia on EE was explored indirectly in mice lacking hepatic FGF21. For this study, the custom low amino acid diet (LAA diet, Table 4.1 and 4.2) was fed to both WT and *Fgf21*$^{\Delta liver}$ mice for two weeks prior to assessing plasma FGF21 and EE.

After two weeks of feeding with the LAA diet, plasma FGF21 levels were significantly elevated in the WT mice compared to the *Fgf21*$^{\Delta liver}$ (Figure 4.15A). Furthermore, plasma FGF21 was increased in the WT mice to a similar level as observed in previous chronic studies where either G108 was administered (Figure 4.7B, Figure 4.10) or the LAA diet was used (Figure 4.14). Following measurement of plasma FGF21, EE analysis was carried out. However, despite a difference in plasma FGF21 between the two groups, a difference in EE was not observed over a 48-hour period (Figure 4.15B).

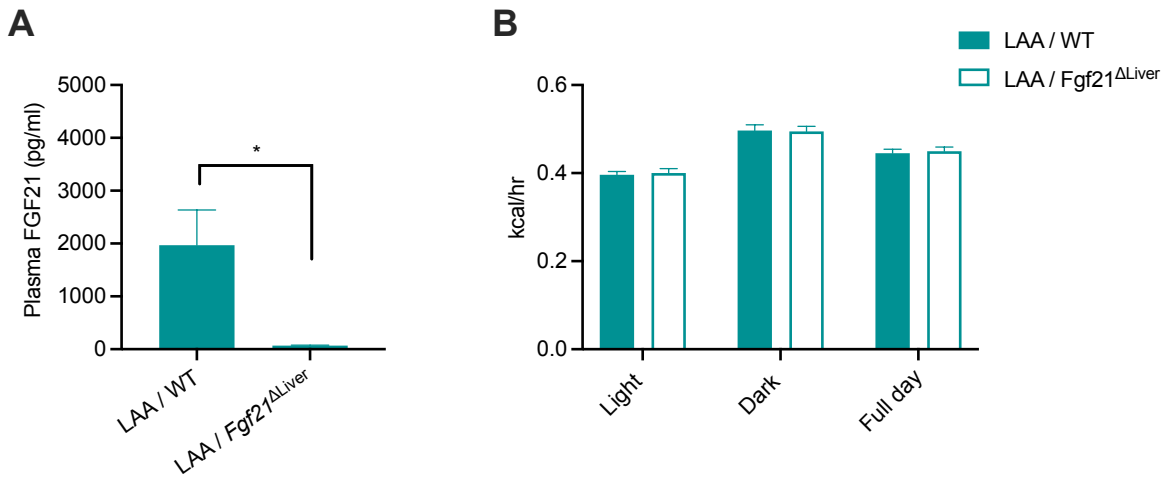


Figure 4.15: Plasma FGF21 and EE in WT and hepatic-specific *Fgf21* knockout mice fed a LAA diet, n=7-8/group. From Hope, Hinds et al. (In Press). **(A)** Plasma FGF21 on day 14. **(B)** Average energy expenditure (kcal/hr) over 48 hours after 14 days of a LAA diet. Data plotted as mean ± SEM, *p<0.05. Analysed using unpaired test. EE data analysed with ANCOVA using lean mass as a covariate.

4.5 Discussion

4.5.1 G108 enhances EE acutely and is prevented by a high protein diet

The results from Chapter 3, in the context of previously described models of dietary protein restriction (Pezeshki and Chelikani 2021), suggest that G108-stimulated hypoaminoacidemia may explain the body weight loss and EE responses observed. To validate this finding, further studies were carried out to determine whether single-dose administration of G108, also leads to acute hypoaminoacidemia and whether this is associated with enhanced EE, recapitulating the chronic effects previously observed in Chapter 3. Although a single 7.5 nmol/kg dose of G108 led to a reduction in plasma total amino acids at 24 hours (Figure 4.5A), this was only associated with a trend towards increased EE for up to 48 hours (Figure 4.5B). In mice fed a high protein diet who also received a single 7.5 nmol/kg dose of G108, plasma amino acids were not significantly reduced at 24 hours and the EE response was flat over the subsequent 48 hours (Figure 4.5B). These findings at the lower 7.5 nmol/kg dose of G108 suggests that a high protein diet may be preventing an EE response. As expected, following administration of the higher G108 dose (20 nmol/kg) there was a prolonged and significant reduction in plasma total amino acids (Figure 4.5C). This was associated with a significant increase in EE for up to 72 hours in mice fed the SP diet and was not observed in mice fed a HP diet (Figure 4.5D). Although a reduction in plasma total amino acids was observed at 24 hours in mice fed the HP diet and administered with 20 nmol/kg G108, this level did not fall significantly below that of the baseline plasma amino acid level in the SP fed mice (Figure 4.5C). Together these results confirm that the acute reduction in plasma total amino acid availability is associated with the acute EE response in G108-treated mice. This is consistent with the findings from Chapter 3, where a chronic reduction in total plasma amino acid availability is also associated with increased total body weight loss and EE (Chapter 3, section 3.4.1 and 3.4.8).

As previous studies have described an increased EE response to dietary protein or amino acid restriction (Rothwell, Stock et al. 1982, Maida, Zota et al. 2016, Pezeshki, Zapata et al. 2016, Zapata, Singh et al. 2019, Yap, Rusu et al. 2020), the initial findings from this Chapter and that of Chapter 3 suggest that G108-stimulated hypoaminoacidemia may mediate the EE and body weight loss observed. Previous paradigms of dietary protein

restriction in rodents have understandably measured EE in a chronic setting. The acute effect of a low protein diet has been investigated using over-feeding studies (Dulloo and Jacquet 1999). Interestingly, overfeeding with a low-protein (3 kcal%) diet over 24 hours results in increased energy expenditure measured in humans using whole-room metabolic chambers (Vinales, Begaye et al. 2019). Therefore, the findings from the acute studies carried out in this Chapter lay the foundation for further mechanistic studies to determine whether G108-stimulated hypoaminoacidemia underpins the EE and body weight responses observed.

4.5.2 Hypoaminoacidemia underpins chronic G108-mediated EE and total body weight loss

Further chronic studies were carried out to provide a mechanistic explanation for the EE and body weight loss effects observed in response to G108, specifically that these effects are mediated by G108-stimulated hypoaminoacidemia. Supplementing a 'standard protein' casein-based diet with total individual amino acids rescued hypoaminoacidemia and prevented the increase in EE and body weight loss in chronic G108-treated obese mice (Figure 4.6). As the individual amino acid supplementation *per se* did not alter body weight or food intake (Figure 4.6A and B), this suggests that the prevention of total body weight loss and EE in G108-treated mice is due to the rescue of hypoaminoacidemia. The effect of G108 treatment on chronic lean and fat mass is comparable to those previously observed in obese mice (Chapter 3, Section 3.4.2). Interestingly, a trend towards reduction in lean mass was observed in G108-treated mice receiving the amino acid supplementation (SP±TAA group) (Figure 4.6C). It is likely that plasma amino acid availability and therefore lean mass, is not protected throughout the 24-hour day/night clock cycle due to the long-acting nature of G108 which likely overcomes the effect of a dietary intervention during the fasting period. The loss of lean mass of lean mass in this group (SP ±TAA) despite no change in food intake, is also reflected in the trend to increases EE in all photoperiods (Figure 4.6A, C and D). Furthermore, the plasma amino acids were measured from blood samples collected early in the morning in the post-prandial period, so therefore any 'escape' from the amino acid supplementation resulting in mild hypoaminoacidemia during the fasting period may not have been captured (Figure 4.7A).

The effect chronic glucagon-stimulated hypoaminoacidemia on energy balance has not previously been reported. However, due to the parallels between the findings presented here and models of dietary protein restriction (leading to low plasma amino acid availability), these two treatment paradigms can be compared. Observations of the EE enhancing effects of low protein diets were noted in the 1960's (Miller 1962, Miller and Mumford 1967, Stirling and Stock 1968). Since then, several studies have reported that balanced dilution of dietary protein content in rodents enhances EE and body weight loss (Maida, Zota et al. 2016, Pezeshki, Zapata et al. 2016, Zapata, Singh et al. 2019). A consistent finding is that a low/moderate (5 kcal%) dietary protein restriction is required to enhance EE (Pezeshki, Zapata et al. 2016). Due to similarities in the EE and body weight responses, it is interesting to note that individual reduction in amino acids is largely similar in rodents fed a 5 kcal% protein diet (Maida, Zota et al. 2016) to that observed with chronic G108 administration (Chapter 3, Section 3.4.5).

The physiological response to dietary protein restriction is altered food intake. It has previously been found that dietary protein restriction in rodents leads to a 'divergent' response in energy balance. For example, a 'mild' (10 kcal%) reduction in total dietary protein content leads to increased food intake with no effect on EE, a low/'moderate' (5 kcal%) reduction leads to an increase in both food intake and EE, whereas a diet totally deficient in protein increases EE whilst severely reducing food intake (Pezeshki, Zapata et al. 2016). Therefore, studies in rodents using a diet with 5 kcal% protein and increased fat content have shown a variable response to obesity resistance due to concomitant hyperphagia (Maida, Zota et al. 2016, Hill, Laeger et al. 2019, Zapata, Singh et al. 2019). Despite this, however, reduced feed efficiency is a consistent finding demonstrating the increased energy expenditure effect of low protein diets on body weight. It is known that animals and humans change dietary behaviours to increase food intake (''protein leverage'') in response to reduced dietary protein and this may lead to weight gain (Simpson and Raubenheimer 2005). In the total 'add-back' study described in this Chapter, 7.5 nmol/kg G108 did not lead a change in food intake (Figure 4.6A), however this dose was specifically chosen within the sub-anorectic range. Therefore, it is expected that an established sub-anorectic dose of G108 may lead to hypoaminoacidemia and enhanced EE without an effect on energy intake. Consistent with this, the absence of

increased food intake despite increased EE in G108-treated mice fed a SP diet translated to total body weight loss in this groups during the study (Figure 4.6B and C).

Chronic G108-stimulated hypoaminoacidemia was associated with an increase in plasma FGF21 and this response was prevented with the total amino acid dietary supplement (Figure 4.7B). Hepatic FGF21 is secreted in response to low plasma amino acid availability and is required for the EE effects observed (Laeger, Henagan et al. 2014). Many studies have subsequently demonstrated that the EE response to moderate (5 kcal%) low protein diets is dependent on hepatic FGF21 (Laeger, Albarado et al. 2016, Maida, Zota et al. 2016, Hill, Laeger et al. 2017, Hill, Laeger et al. 2019). Interestingly, the hyperphagic response to low protein diets has also been found to be FGF21-dependent (Laeger, Albarado et al. 2016, Maida, Zota et al. 2016, Hill, Laeger et al. 2019). Therefore, the role of hepatic FGF21 induction in G108-treated mice is further explored in Section 4.5.4.

The findings from the total amino acid dietary 'add-back' study demonstrate that G108-stimulated hypoaminoacidemia drive the increase in EE observed, resulting in total body weight loss in this diet-induced obesity mouse model.

4.5.3 Replacing EAA or NEAA separately does not prevent EE effect

Since the discovery that low protein diets increase EE (Miller 1962, Miller and Mumford 1967, Stirling and Stock 1968), the research field has subsequently investigated the effects of dietary individual amino acid restriction. For example, restriction or depletion of individual essential amino acids (EAA's) has been shown to enhance EE in pre-clinical models (Xiao and Guo 2022). Recently, it has been demonstrated that a specific dietary restriction of either threonine or tryptophan is sufficient to recapitulate the *full* EE response to a 5 kcal% protein diet in mice (Yap, Rusu et al. 2020). In this study, silencing hepatic FGF-21 prevented the EE response to a low threonine diet. These findings demonstrate that restriction of individual amino acids in the diet can alter energy balance comparable to that of total protein diluted diets and that the mechanism by which it does so may also be through the induction of hepatic FGF21.

To determine whether G108-stimulated reductions in individual amino acid components mediate the EE and body weight loss response, custom amino-acid based diets were further modified and used in a follow up chronic study. G108-treated mice (7.5 nmol/kg) fed a diet supplemented with either EAA's or NEAA's were not resistant to body weight loss over two weeks (Figure 4.8B, 4.9A). Furthermore, G108-treated mice fed with either supplemented diet had an increased plasma FGF21 level after 2 weeks of study (Figure 4.10). Although individual amino acid data is not available to confirm the specific rescue of either component of the total amino acid profile, these findings suggest that G108-stimulated reduction in either EAA or NEAA may increase EE and body weight loss. Therefore, consistent with the total amino acid 'add-back' study (Section 4.4.2), the repletion of total amino acids is necessary to prevent G108-mediated EE and body weight loss. These findings contrast with a recent study where only dietary EAA restriction was found to enhance EE in rodents (Yap, Rusu et al. 2020). However, it is important to note that in other studies, a reduction in NEAA has also been shown to enhance EE and the hepatic FGF21 secretion in primary hepatocytes and *in vivo* (Shimizu, Maruyama et al. 2015, Wilson, Lennox et al. 2015, Maida, Zota et al. 2016).

Together, these findings suggest that rescue of total amino acids is required to prevent G108-mediated EE and body weight loss and therefore reduction of individual amino acids from both EAA and NEAA components are implicated in the effects observed.

4.5.4 The EE response to a rationally designed low amino acid diet suggests an FGF-21-independent effect.

Soon after it was recognised as a downstream target of the hepatic amino acid stress response pathway (De Sousa-Coelho, Relat et al. 2013), FGF21 was demonstrated to be a key 'endocrine signal' of dietary protein restriction (Laeger, Henagan et al. 2014). FGF21 leads to enhanced EE in response to a 5% casein diet, a response which is lost in *Fgf21*-knockout mice (Laeger, Henagan et al. 2014). Several studies have subsequently demonstrated that EE and body weight loss is dependent on hepatic FGF21 (Maida, Zota et al. 2016, Hill, Laeger et al. 2019). Furthermore, it has been shown that the metabolic programming of a low protein diet through hepatic FGF21 is mediated by a CNS sympathetic-WAT thermogenic pathway (Hill, Laeger et al. 2019). Interestingly, it has

recently been shown that dietary restriction of specific amino acids such as threonine or tryptophan increases hepatic FGF21 and EE, and in the case of threonine restriction these effects are prevented when hepatic FGF21 is silenced (Yap, Rusu et al. 2020). Dietary methionine restriction also leads to enhanced EE and induction of hepatic FGF21 (Lees, Krol et al. 2014) and the hepatic FGF21 is required for the thermogenic effects of restricting this amino acid (Wanders, Forney et al. 2017).

To explore whether G108-mediated EE and body weight loss in mice may be dependent on hepatic FGF21, the low amino acid diet (LAA diet) was fed to wild-type and hepatic-specific *Fgf21* knockout mice (*Fgf21*$^{\Delta liver}$). After two weeks of feeding with the LAA diet there was no difference in EE between genotypes (Figure 4.15B). Despite this, an increase in circulating FGF21 was detected (Figure 4.15A) which was comparable to the induction of FGF21 by G108 or the LAA diet in previous studies (Figures 4.7B, Figure 4.10 and Figure 4.14). Although the EE response to G108 does not appear to be FGF21-dependent based on these results, there are several lines of evidence which may support these findings. The maximum plasma FGF21 levels reached in response to either G108 administration or the rationally designed LAA diet, were in the range of approx. 1000 pg/ml (Figures 4.7B, Figure 4.10 and Figure 4.14). Studies by others which demonstrate FGF-21 dependent effects of dietary protein restriction (5 kcal% casein diet) have demonstrated levels of circulating FGF21 up to approximately 4000 pg/ml after 4 days (Laeger, Henagan et al. 2014), approaching approximately 8000 pg/ml after 27 weeks of a low protein diet (Laeger, Albarado et al. 2016). It is possible that the subtle induction of hepatic FGF21 by G108 or the custom LAA diet is a marker of the reduced amino acid availability however this is not sufficient to induce the thermogenic effects observed. In keeping with this, the proposed mechanisms of EE in response to a low protein diet are multi-faceted and may also involve FGF-21 independent pathways (Pezeshki and Chelikani 2021). Furthermore, due to the divergent effects on EE of individually restricting amino acids (Xiao and Guo 2022), the individual amino acid profile of G108-stimulated hypoaminoacidemia may result in the activation of different EE mechanisms than those observed with a balanced reduction in dietary protein (ie 5 kcal% protein diets). To support the distinct effects of restricting individual amino acids, dietary lysine restriction alone may result in increased serotonergic signalling and EE (Smriga and Torii

2003) and more recently, dietary histidine restriction was noted to increase EE in an FGF21-independent manner (Victoria Flores 2022).

A source of energy consumption from lean tissue protein turnover has also not been excluded. It is interesting that the lean mass loss appears to mirror the pattern of energy expenditure from the dietary total amino acid add back study (Figure 4.6). This was also observed with a high protein diet replacement (Chapter 3). However, there is evidence that dietary restriction of a single EAA, ie threonine may recapitulate the full EE effect of a low amino acid diet, without any effect on lean mass (Yap, Rusu et al. 2020). It may be that the EE response to G108-stimulated hypoaminoacidemia in obese mice is multi-faceted including direct effects of lowering individual amino acids, in addition to a whole-body increase in lean tissue protein turnover.

The findings from the *Fgf21*$^{-/-}$ study supports the fact that G108-stimulated hyopoaminoacidemia resulted in a subtle induction of hepatic FGF21 and therefore a subtle increase in circulating levels of the hormone. However, a limitation of this study was the short-duration of low amino acid diet feeding prior to metabolic assessments. Furthermore, in this study lean mice were used and not obese mice. It has previously been shown that the effects of FGF21 on EE are dependent on dietary fat content suggesting obesity status (Owen, Ding et al. 2014). Therefore, it would be appropriate to further investigate whether the custom low amino acid diet used in this Chapter confers the same EE effect in DIO WT and *Fgf21*$^{-/-}$ mice in addition to the EE response to a standard amino acid diet.

Together, these discussions may explain how G108-stimulated hypoaminoacidemia may mediate different mechanisms of EE than those seen in low protein diet paradigms and explanations for why the findings here may differ.

4.6 Conclusions

The results from this Chapter confirm that chronic G108-stimulated hypoaminoacidemia leads to increased energy expenditure and total body weight loss in obese mice. Therefore, these findings suggest that chronic hypoaminoacidemia underpins glucagon-mediated EE and body weight loss in this pre-clinical model. This effect does not appear

to be dependent on the induction of hepatic FGF21, as observed in other models of dietary protein restriction and energy expenditure, however key differences are highlighted between dietary protein restriction and G108 administration (Section 4.5.4). Interestingly, the acute EE response to G108 is associated with hypoaminoacidemia in mice fed with a standard protein diet, and this is abolished in mice fed with a high protein diet. This also strongly supports the notion that G108-stimulated hypoaminoacidemia underpins the EE responses observed. The mechanisms of EE activated by G108-stimulated hypoaminoacidemia remain uncertain. Experiments from Chapter 3 suggest that several EE components may be involved including muscle thermogenesis and locomotion (Chapter 3, Section 3.4.11). Using a more potent glucagon-analogue at a higher dose, it has previously been demonstrated that the EE response may be partially due to hepatic FGF21 and FXR signalling pathways (Kim, Nason et al. 2018). Therefore, it will be important to further explore the potential mechanisms of EE in response to glucagon-stimulated hypoaminoacidemia in addition to the extent to which it contributes to the overall EE effects observed, particularly in higher dose ranges.

5 Chapter 5– A novel long-acting GLP-1/glucagon co-agonist (G3215) results in markers of increased protein turnover in humans in a Phase 1 trial

5.1 Introduction

5.1.1 GCGR-targeted multi-agonists as a treatment for obesity and metabolic disease in humans

The metabolic benefits of exogenous glucagon administration on lipid metabolism and energy expenditure, were described early on in pre-clinical studies with native glucagon or zinc-glucagon (Davidson, Salter et al. 1957, Caren and Corbo 1960, Davidson 1960, Salter 1960, Eaton 1973). Since then, several studies in rodents have confirmed glucagon's effects on lipid metabolism for example by reducing hepatic lipid production (Guettet, Mathe et al. 1989), stimulating hepatic lipid oxidation (Lv, Qiu et al. 2017) and reducing plasma cholesterol (Guettet, Mathe et al. 1988). Human studies with short-term native glucagon infusions have also confirmed its role in stimulating energy expenditure (Nair 1987, Tan, Field et al. 2013, Salem, Izzi-Engbeaya et al. 2016).

Due to the hyperglycaemic nature of glucagon (Exton 1987), and in the context of the rising obesity levels over the last four decades, efforts have been made to develop a 'druggable' glucagon analogue with sustained metabolic benefits without deleterious side effects on glycaemia. As a proof-of-principle and based on earlier successful studies of the endogenous GLP1/GCGR co-agonist oxyntomodulin (Wynne, Park et al. 2006), co-infusion studies of GLP-1 and GCG demonstrated the benefits of this combination therapy in humans; In particular, co-infusion of these hormones led to enhanced EE whilst ameliorating glucagon-stimulated hyperglycaemia (Tan, Field et al. 2013). Long-acting analogues of glucagon have also been developed with the novel strategy of modification of 'sequence-similar' preproglucagon peptides, for example glucagon, GLP-1 or oxyntomodulin (Day, Ottaway et al. 2009, Pocai, Carrington et al. 2009, Day, Gelfanov et al. 2012). Using this strategy, long-acting glucagon or GCGR-targeted unimolecular multi-agonists have been developed which have had marked success in pre-clinical obesity models. (Hope, Vincent et al. 2021). For example, the first unimolecular GLP1/GCG co-agonists were shown to enhance EE and total body weight loss in DIO mice, in addition to improving whole body lipid profile and glucose tolerance (Day, Ottaway et al. 2009, Pocai,

Carrington et al. 2009). Since then, several successful pre-clinical reports have emerged of unimolecular GLP-1/GCG co-agonists which lead to weight loss in obese rodents (Henderson, Konkar et al. 2016, Evers, Haack et al. 2017). Through the addition of GIP activity, a GCGR-targeted triple agonist (SAR441255) has recently reported significant weight loss in obese mice of up to 60 percent (Bossart, Wagner et al. 2021, Knerr, Mowery et al. 2022). A key advantage of the addition of insulinotropic GIP has been to allow increases GCGR potency whilst avoiding hyperglycaemia, therefore maximising the energy expenditure and body weight loss response; This is exemplified with the balanced triple agonist 'Peptide 16' (Knerr, Mowery et al. 2022).

Following the initial stream of pre-clinical reports, some of the subsequent early phase clinical trials of GLP-1R/GCGR co-agonists have been underwhelming in terms of safety and efficacy (Hope, Vincent et al. 2021). For example, the forerunner Cotadutide (formerly MEDI0832), by Medimmune, is administered once daily and achieved a modest ~5% weight loss over 54 weeks, although striking beneficial effects were observed on plasma lipids and hepatic parameters of non-alcoholic hepatic steatosis and therefore this is being developed for the treatment of fatty liver disease (Nahra, Wang et al. 2021). Other co-agonist candidates for example NN1177 (Novo Nordisk) are associated with significant gastrointestinal adverse events and cardiovascular safety concerns (Martin Friedrichsen 2022). However, recently a phase 1 report of the once-weekly co-agonist IBI362 shows a body weight loss of up to 6.4 % over 12 weeks with a favourable safety profile (Ji, Gao et al. 2022). Furthermore, a recent report of the once weekly GLP-1R/GCGR/GIP triple agonist, LY3439743 (Lilly) has also shown a favourable safety profile in addition to weight loss of up to 8.96 kg over 12 weeks (Urva 2022). GCGR-targeted multi-agonists in development are summarised in Table 5.1.

The inconsistent results when translating to humans may be based on the various factors which may affect the behaviour of unimolecular multi-agonists, for example species and drug exposure differences; This has been highlighted in a pre-clinical report investigating these effects of NN1177 at a given GLP-1 to glucagon ratio, showing unexpected physiological differences between species and when compound exposure changes (Simonsen, Lau et al. 2022). This may explain why certain successful animal reports of drug candidates have not translated to similar success in humans (Tillner, Posch et al.

2019, Martin Friedrichsen 2022). Furthermore, Cotatutide may have benefited from not succumbing to similar safety concerns due to its notably weaker receptor potency to the GCGR, at 5-10-fold less than glucagon itself (Henderson, Konkar et al. 2016).

	GCGR-targeted multi-agonists in development				
	Class	**Developer**	**Receptor balance GLP-1R/GCGR/GIPR**	**Status**	**Reference**
Cotadutide	GLP1R/GCGR Co-agonist	AstraZeneca	5:1	Phase 2B Fatty liver disease	(Nahra, Wang et al. 2021)
ALT-801/Pemvidutide	GLP1R/GCGR Co-agonist	Altimmune	1:1	Phase 2 Obesity	NCT05295875
BI456906	GLP1R/GCGR Co-agonist	Boehringer Ingelheim	?	Phase 2 Obesity	NCT04667377
Efinopegutide	GLP1R/GCGR Co-agonist	Marck	1:1	Phase 2 Fatty liver disease	NCT04944992
IBI362 (LY3305677)	GLP1R/GCGR Co-agonist	Eli Lilly	1:1.6	Phase 2 Obesity	NCT04904913(Ji, Gao et al. 2022)
SAR441255	GLP1R/GIPR/GCGR Triple agonist	Sanofi	2:2:1	Phase 1 Obesity/T2DM	(Bossart, Wagner et al. 2021)
LY3437943	GLP1R/GIPR/GCGR Triple agonist	Eli Lilly	1:10:1	Phase 1 Obesity/T2DM	(Coskun, Urva et al. 2022)
NN1177	GLP1R/GCGR Co-agonist	Novo Nordisk	3:1	Discontinued	(Martin Friedrichsen 2022)
SAR425899	GLP1R/GCGR Co-agonist	Sanofi	5:1	Discontinued	(Tillner, Posch et al. 2019)
MOD-6031	GLP1R/GCGR Co-agonist	OPKO	?	Discontinued	NCT02692781
MK-8521	GLP1R/GCGR Co-agonist	Merck	?	Discontinued	NCT02492763
TT401	GLP1R/GCGR Co-agonist	Transition Therapeutics	?	Discontinued	NCT03406377
NN9204-1706	GLP1R/GIPR/GCGR Triple agonist	Novo Nordisk	?	Discontinued	NCT03661879

Table 5.1: GCGR-targeted multi-agonists in development. Individual references provided.

5.1.2 Emerging pre-clinical evidence for amino acid catabolism and lean mass loss in GCGR-targeted multi-agonists

In the pre-clinical reports of the first unimolecular GLP1-R/GCGR co-agonists, circulating plasma amino acids were not reported (Day, Ottaway et al. 2009, Pocai, Carrington et al. 2009, Day, Gelfanov et al. 2012, Henderson, Konkar et al. 2016). Evidence does exist however which may indicate lean mass loss through GCG-stimulated hypoaminoacidemia for previously reported GLP-1/GCG co-agonists and glucagon analogues. For example, in one study, a long-acting co-agonist 'Peptide 5' was administered over 13 days and led to approximately 3 g of lean mass loss, independent to food intake (Day, Gelfanov et al. 2012). In other studies, using a long-acting glucagon analogue, IUB288, a similar loss of lean mass was observed over 3 weeks (Habegger, Stemmer et al. 2013, Okun, Rusu et al. 2021).

Over the last 5 years, the role of glucagon in amino acid catabolism has become increasingly reported for at least two reasons. Firstly, an important physiological liver-alpha axis has been described whereby glucagon and amino acids regulate a feedback cycle in which the extent of GCGR signalling determines circulating amino acids as messengers to alpha cell glucagon secretion (Holst, Wewer Albrechtsen et al. 2017). Secondly, circulating amino acids have been shown to be a sensitive biomarker of GCGR activity when using GCGR-targeted agonists in pre-clinical models (Li, Kirchner et al. 2020). Indeed, in humans, individual plasma amino acids have been reported with the use of a GCGR-targeted triple agonist (GLP-1R/GCGR/GIP) (SAR441255) as a 'biomarker' of GCGR activity (Bossart, Wagner et al. 2021). Recently, a GLP1R/GCGR/GIP triple agonist (Peptide 16) has been reported to cause a significant loss of lean mass in obese rodents and this was not consistent with a reduction in energy intake, but associated with a higher GCGR receptor activity (Knerr, Mowery et al. 2022); Although plasma amino acids were not reported in this study, it was noted that this indicated a potential for the triple-agonist to cause amino acid catabolism and muscle wasting.

Together these studies have highlighted the increasing interest in reporting plasma amino acids when using GCGR-targeted multi-agonists in pre-clinical models.

Furthermore, they also highlight the important link between GCGR-stimulated hypoaminoacidemia and lean mass loss, investigated in Chapters 2 and 3 of this thesis.

5.1.3 Evidence for hypoaminoacidemia in humans treated with GCGR-targeted agonists

Increased circulating glucagon at higher physiological and pathological circulating levels results in increased hepatic amino acid catabolism and hypoaminoacidemia. This has been shown with exogenous glucagon infusion studies in humans (Wolfe, Culebras et al. 1979, Nair, Halliday et al. 1987, Boden, Tappy et al. 1990) or with excess endogenous glucagon production in the glucagonoma syndrome (Mallinson, Bloom et al. 1974, Almdal, Heindorff et al. 1990). Despite these findings, the effect of GCGR-targeted multi-agonists on circulating amino acids in humans has only recently been reported. This is due to the relatively recent advancement of these agonists into clinical phase testing (Hope, Vincent et al. 2021) in addition to the recent validation of plasma amino acids as a sensitive biomarker for GCGR activity when using these agonists (Li, Kirchner et al. 2020).

A phase 2a study of Cotadutide was the first to report changes in circulating amino acids with chronic treatment of the GLP-1R/GCGR co-agonist (Parker, Robertson et al. 2020). Over 43 days of daily dosing with Cotadutide, there was a significant reduction in plasma alanine, in addition to numerical reductions in cysteine, tyrosine, isoleucine, tryptophan, and glutamate. More recently, in a phase 1 trial, the chronic effect of a GLP-1R/GCGR co-agonist on circulating amino acids has been demonstrated. In this study, in addition to cardiovascular safety concerns being raised, weekly treatment with NN1177 (Novo Nordisk) led to a broad reduction in individual amino acids after 12 weeks (Martin Friedrichsen 2022). GCGR-stimulated hypoaminoacidemia has also been observed in higher combination multi-agonists. For example, in a single-ascending dose study in humans, a GLP-1R/GCGR/GIPR triple-agonist (SAR441255) led to a broad reduction in individual amino acids at least up to 12 hours following a single-dose (Bossart, Wagner et al. 2021). Although lean mass was not measured due to the single-dose study design, it was also noted that the chronic implications of reduced amino acids require investigation (Bossart, Wagner et al. 2021). More recently, in a single ascending first-in-human trial,

another GLP1R/GCGR/GIPR triple agonist (LY3437943) reported a chronic reduction in selected glucogenic amino acids, including alanine, serine and asparagine (Coskun, Urva et al. 2022); However a complete amino acid profile has not been reported. In a follow up phase 1b study, plasma amino acids are not reported (Urva 2022). Interestingly, the length of duration and receptor potency varies between drug candidates, and this may have implications for the effect on circulating amino acids. For example, Cotadutide necessitates once daily treatment and has weaker GCGR potency relative to native glucagon, whereas SAR441255, LY3437943 and NN1177 have enhanced GCGR receptor potency and/or greater half-life; This may explain why Cotadutide has a modest effect on individual plasma amino acids in which the most affected amino acid is alanine, a major glucogenic substrate (Parker, Robertson et al. 2020, Bossart, Wagner et al. 2021, Martin Friedrichsen 2022).

5.1.4 Infusion of a novel GLP1/GCGR co-agonist, G3215, in humans to determine the effect on plasma amino acids and lean mass

G3215 is a novel GLP-1R/GCGR co-agonist developed at Imperial College London, based on the endogenous co-agonist, oxyntomodulin. Through several modifications, receptor potency to both cognate receptors relative to native ligands, GLP-1 and GCG are enhanced. In a Phase 1 double-blind randomised control trial, the safety, tolerability, pharmacokinetics and pharmacodynamics was assessed in overweight or obese adults. Using an infusion strategy, these outcomes were assessed over 14 days of a continuous subcutaneous infusion using an mini-pump, worn by the participant. As an exploratory outcome, individual plasma amino acids were measured at multiple time points during the infusion period. To determine any effect on lean mass, body composition and functional strength were also measured. In this Chapter, the trial methods are detailed fully with results of exploratory outcomes highlighted pertinent to the primary focus of this thesis, including the effects of G3215 on plasma amino acid metabolism, body composition and grip strength.

5.2 Chapter summary and aims

- Determine whether a rationally designed GLP1/GCGR co-agonist, G3215 results in reduced plasma alanine in a Phase 1 clinical trial.
- Determine whether administration of G3215 results in reduction of individual plasma amino acids.
- Determine whether administration of G3215 in the short term leads to increased lean mass loss.

5.3 Methods

5.3.1 Study design and participants

This study was carried out at the NIHR Wellcome Trust Clinical Research Facility at Hammersmith Hospital, London, from October 2021 to August 2022. It was a double-blind, randomised-control trial and compared the effect of a 14-day continuous subcutaneous infusion of a novel GLP1R/GCGR co-agonist, 'G3215' versus placebo, on the primary outcomes of safety, tolerability, pharmacokinetics and pharmacodynamics in overweight or obese adults.

23 subjects were recruited to three cohorts (8 subjects per cohort for cohort 1 and cohort 2, 7 subjects in cohort 3), in which 6 subjects in each cohort received G3215 infusion and the remaining receiving placebo infusion. Cohort 1 included those with normoglycaemia and later cohorts also included those with prediabetes or type 2 diabetes (as per WHO 2006 and 2011 diagnostic criteria).

The starting dose for Cohort 1 was 0.6 mg/24 hours and the dose G3215 was titrated up through multiple-stepwise increments to 2.4 mg/24 hours over 14 days by increasing the infusion rate. The dose increments were applied to all participants within the cohort. Therefore, the placebo infusion rate was also increased in line with subjects receiving G3215. The final dose achieved in cohort 2 and 3 will be increased and this will depend on the safety and tolerability assessed in cohort 1. Between cohorts, a safety monitoring committee will determine whether G3215 is safe to proceed with in further cohorts.

Primary outcomes included safety and tolerability assessments through recording of adverse events (AE's), clinical examination, ECG monitoring, vital signs, and organ-specific safety blood tests. Secondary outcomes included pharmacokinetic assessments. Exploratory outcomes included food intake, change in body weight, muscle strength, body composition, markers of nitrogen balance, and glucose tolerance.

Sentinel dosing was carried out in each cohort with randomisation to ensure that 1 was received G3215 and 1 received placebo.

The study was carried out according to the guidance set by the International Conference on Harmonisation (ICH) and Good Clinical Practice (GCP). The Investigational medicinal product (IMP) (G3215), was developed and administered according to the requirements set by the European Union (EU) Good Manufacturing practice (GMP).

5.3.2 Peptide G2315

Peptide G3215 is an analogue based on the primary peptide sequence of the endogenous GLP1R/GCGR co-agonist, oxyntomodulin (OXM). Modifications were made based on the primary peptide sequence of OXM including the deletion of 2-residues and substitution of 14 residues. Through assessment of cAMP activity in human cell lines, G3215 has previously been demonstrated to have superior receptor activity at both the GLP1R and GCGR, compared to native OXM (data not shown). Receptor binding studies have demonstrated that G3215 binds with high affinity (within 3-fold of native ligands) to both GLP-1R and GCGR (data not shown). G3215 is therefore predicted to combine the GLP-1 effect on appetite suppression and glucose-stimulated insulin release, with glucagon's effect on energy expenditure and lipid metabolism.

Following pre-clinical toxicology studies, first in human clinical trial (FITH) were carried out between January 2015 and February 2019. This comprised of a single-ascending dose study (Part A), a multiple ascending dose study (Part B) and a subcutaneous infusion study (Part C). Parts A and B were carried out using G3215 reconstituted in a zinc formulation to prolong its duration after subcutaneous administration. In part A, the maximum single-dose of G3215 administered was 48 mg. In part B, weekly doses of

G3215 of up to 24 mg were administered subcutaneously for 5 weeks, with the maximum cumulative dose of 108 mg over the 5-week period. In part C, the highest dose administered through subcutaneous infusion was 2.2 mg/24 hours. Data for Part A-C not shown.

The starting dose of G3215 in the planned 14-day subcutaneous infusion study was therefore based on the doses administered and tolerability of the IMP in the FITH studies described above. Therefore, in Cohort 1, G3215 was titrated up over 14 days to a maximum dose of 2.4 mg/24 hours, with a potential maximum cumulative dose of 33.6 mg. In subsequent cohorts, this dose will be increased however not exceed the maximum cumulative doses in Part A of the FITH (48 mg) or Part B of the FITH (108 mg).

5.3.3 Inclusion and exclusion criteria

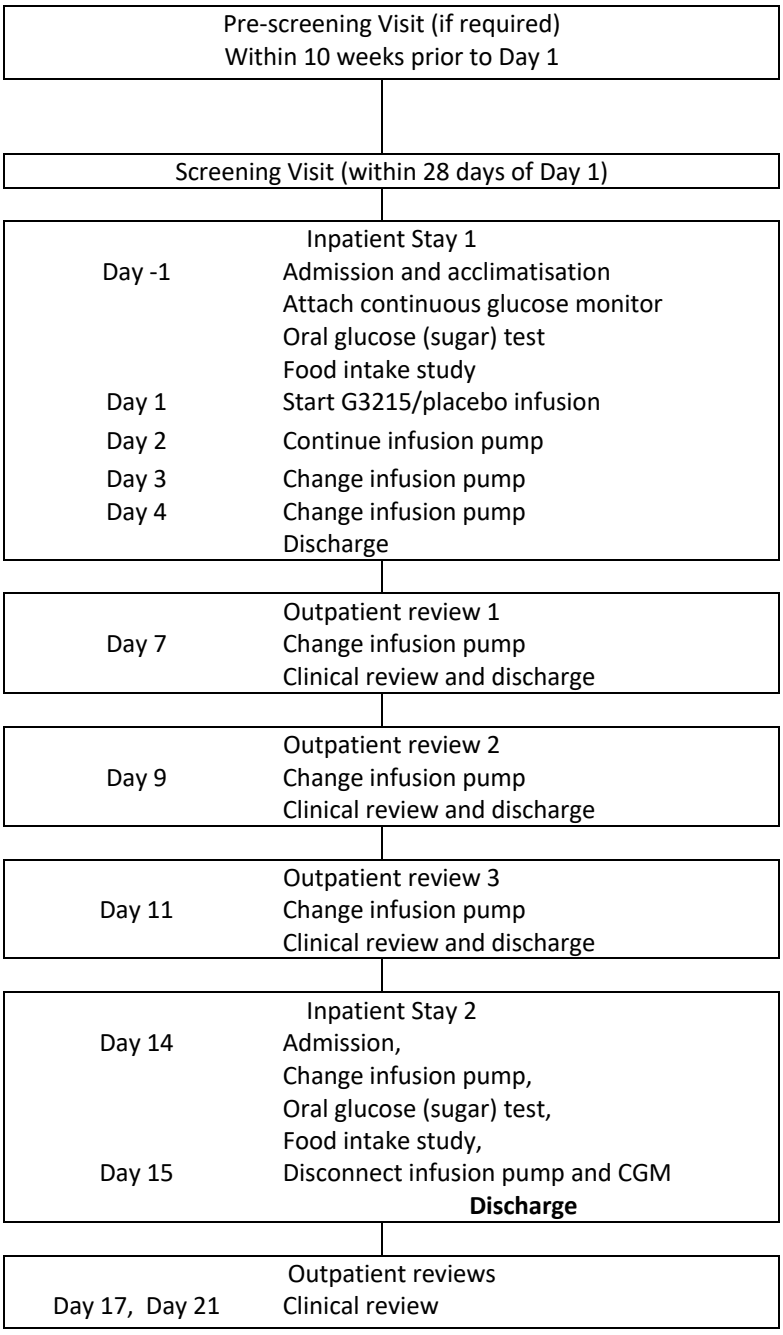
Inclusion criteria were adult males aged 18-65 years old, a BMI between 25 and 45 kg/m^2, normal glucose tolerance, impaired glucose tolerance (IGT), impaired fasting glucose (IFG), type 2 diabetes (according to WHO 2006 and 2011 diagnostic criteria). Participants with Type 2 diabetes, IFG or IGT were controlled with diet/lifestyle, monotherapy (sulphonylurea, metformin or an SGLT2i) or dual-therapy (sulphonylurea/metformin, or sulphonylurea/SGLT2i). A HbA1c of between 6.0-8.5%. Other criteria include a negative screen for hepatitis B surface antigen, hepatitis C antibody and human immunodeficiency virus 1 and 2 antibody. A negative screen was also required for drugs of abuse and nicotine. Other key exclusion criteria included, a history of atopy, drug hypersensitivity, a history of alcohol abuse, substance dependence, a history of clinically significant migraines, pancreatitis or pancreatic cancer, infections, known inflammatory conditions, a history of gastrointestinal symptoms, recent donation of blood products, previous use of an IMP within 3 months or engaged in extended follow up of another IMP, or previously received G3215.

5.3.4 Intervention

Participants were admitted to the Clinical Research Facility on Day -1, prior to the day of infusion commencing. In the morning, they underwent baseline safety blood tests and a baseline oral glucose tolerance test. This was followed by an *ad libitum* food intake study

and further acclimatisation to the facility throughout the day. A grip strength assessment was also carried out in the afternoon. On day 1, following baseline blood tests, a subcutaneous infusion pump was connected to the lower abdominal area and was subsequently changed every 24-48 hours throughout the 14-day infusion period. During days 1-4, all participants remained in the Clinical Research Facility where the infusion rate of the pump (and therefore dose of G3215) was titrated up until day 3 and multiple blood sample for PK analysis were taken throughout the day. During this time, safety assessments were made with blood tests, clinical examination, ECG analysis and vital sign recordings. On day 4, participants were discharged from the facility and returned for an outpatient visit on day 7 for PK and safety blood tests, ECG analysis, vital signs and clinical examination. Participants also returned on day 9 and 11 for clinical examination and vital signs. At each outpatient visit (Day 7, 9 and 11) to the facility, the infusion set, and syringe were changed. On day 14, all participants attended the facility and stayed for 2 days (study day 14 and day 15). Following vital signs, PK and safety blood tests on day 14, the infusion set and syringe were changed and then the participants underwent an oral glucose tolerance test. A food intake study was then carried out for lunch and dinner meals. A grip strength assessment was also carried out in the afternoon on Day 14. On day 15, vital signs, PK and safety blood tests were collected in the morning. All participants then underwent a food intake study for breakfast followed by permanent removal of the subcutaneous infusion pump. All participants returned for a final visit on Day 17 and Day 21 for safety assessments and PK analysis. During the two inpatient periods (day -1-4 and day 14-15), visual analogue scales were provided to participants pre- and post-meals and served to indicate the effect of the respective treatments on subjective levels of appetite. Local (infusion site) tolerability assessments were made as part of the clinical examination. Body weight was measured at each visit and body composition measured using bioimpedance was measured on days -1, 7, 11, 14 and day 21.

Summary of visits: Source (Informed Consent Form, G3215 study)



5.3.5 Randomisation process and IMP preparation

Participants were randomised to their respective treatment of G3215 or placebo using the Oracle Inform data capture system. The randomisation codes were provided to the pharmacist and 'code break' enveloped were maintained in the clinical unit and by designated individuals not working in the clinical team. The pharmacy within Hammersmith Hospital used the randomisation codes to prepare the infusion pump syringes throughout the study. The infusion pump syringes, blinded to treatment, were collected by the clinical team on each day the infusion set, and syringe were changed. A code-break protocol was in place if required.

5.3.6 Subcutaneous pump and dose-adjustments

A Dana Rs pump was used to administer G3215 or placebo continuously through the subcutaneous route over the 14-day infusion period. The infusion rate of the Dana Rs pump was changed to achieve a specific dose over 24 hours. This varied from 0.6 mg/day to 3.4 mg/day according to the cohort and final dose achieved for each individual participant.

5.3.7 Body weight and body composition

Body weight was recorded at the same time of day on each study day. Body composition measurements were recorded on study days -1, 7, 11, 14 and day 21, using Tanita bioelectrical impedance technology. Measurements for total body weight, fat and lean mass is provided.

5.3.8 Grip strength test

A hand grip strength test was recorded on day -1 and day 14 of the study, using a digital grip strength meter (Jamar® dynamometer, Patterson Medical). Participants held the grip strength meter in their hand with the arm at a right angle, prior to squeezing the device to maximum force over 5 seconds. Three recordings were taken from both the left and right hands.

5.3.9 Oral glucose tolerance test

An oral glucose tolerance test (75g glucose drink) was carried out during the two inpatient visits, specifically on the morning of day -1 and on day 14. Samples for insulin and glucose were collected at time points: 0, 15, 30, 60, 90, 120, 180 and 240 minutes following the glucose drink.

5.3.10 Food intake studies

Food intake studies were carried out during the two inpatient visits. A standardised lunch and dinner were provided on study day -1 and day 14, with a standardised breakfast provided on study day 1 and day 15. Participants were provided in excess with each meal and asked to eat these meals until they felt 'comfortably full' within a period of 20 minutes. The meal weight was recorded pre- and post- presentation to the participant and based on the fixed kcal/g content of each meal this allowed the total energy value consumed (kcal) to be calculated.

5.3.11 Safety blood tests, ECG, vital signs, and visual analogue scales

Safety blood tests and were collected on each inpatient study day and each study visit, excluding outpatient visit 9 and outpatient visit 11. Blood samples were collected through a peripherally sited cannula.

A 12-lead ECG was carried out twice daily during the inpatient study days (Day -1, 1, 2, 3, 4, 14, 15) and once during the outpatient visits (Day 7, 9, 11, 17, 21). After lying in the supine position for at least 5 minutes, the ECG was performed. The heart rate PR interval, QRS duration, QT and QTc values were recorded.

Blood pressure, pulse rate and temperature were recorded at multiple set time-points during the inpatient days and once during each outpatient visit.

Visual analogue scales were provided to participants pre- and post- all meals including the food intake studies. This comprised of a 100mm linear scale where the participant

was asked to mark where they subjectively rated appetite related questions. The mark along the 100mm scale was then recorded (ie 74mm = 74).

5.3.12 Physical examination

A physical examination was performed on each study day and included a comprehensive assessment of all the major body systems.

5.3.13 PK and Biochemical analysis

Safety blood tests were analysed by the NHS Clinical Laboratory at Hammersmith Hospital. PK samples were collected and transferred on wet ice to the research facility laboratory, where they were centrifuged and aliquoted into dedicated PK tubes for analysis. These were delivered to the Clinical Research Organisation, LCG for further analysis. Blood samples for amino acid analysis were centrifuged at 4000 rpm for 10 minutes and plasma stored at -80^{o}C prior to analysis. An alanine kit (MAK001, Sigma) was used to analyse plasma alanine levels. 16 samples (10 G3215 infusion and 6 placebo) were sent for individual plasma amino acid analysis using gas-chromatography-mass-spectrometry (GCMS) at the WellChild Lab, St Thomas' Hospital, London, UK.

5.4 Results

Key results for Chapter 5

- **Over 14-days, G3215 infusion causes a reduction in the glucogenic amino acid alanine.**
- **Over 14-days G3215 infusion also causes a broad reduction in individual amino acids**
- **A comparable reduction in lean and fat mass is observed over 14 days with G3215 infusion.**

5.4.1 G3215 infusion reduces plasma alanine over 14 days

23 participants completed the study and plasma samples from study days -1, 1, 7, 14 and 21 were used to measure amino acid levels. Alanine was initially measured using a commercially available ELISA kit, to determine whether thorough individual amino acid measurement was indicated. Plasma alanine levels in participants receiving placebo remained stable for the duration of the infusion period (study day 1 to study day 14) and during the follow up visit on study day 21 (Figure 5.1A). In contrast, plasma alanine was significantly reduced during the infusion period in participants receiving G3215 infusion (Figure 5.1B, mean ± SEM, G3215 infusion baseline level on study day 1: 359.1 ± 37.6 micromol/l, study day 7; 205.0 ± 44.8 micromol/l, study day 14; 217.6 ± 32.3 micromol/l). This then increased above baseline when measured during the follow up visit on day 21 (Figure 5.1B, study day 21; 526.3 ± 86.9 micromol/l).

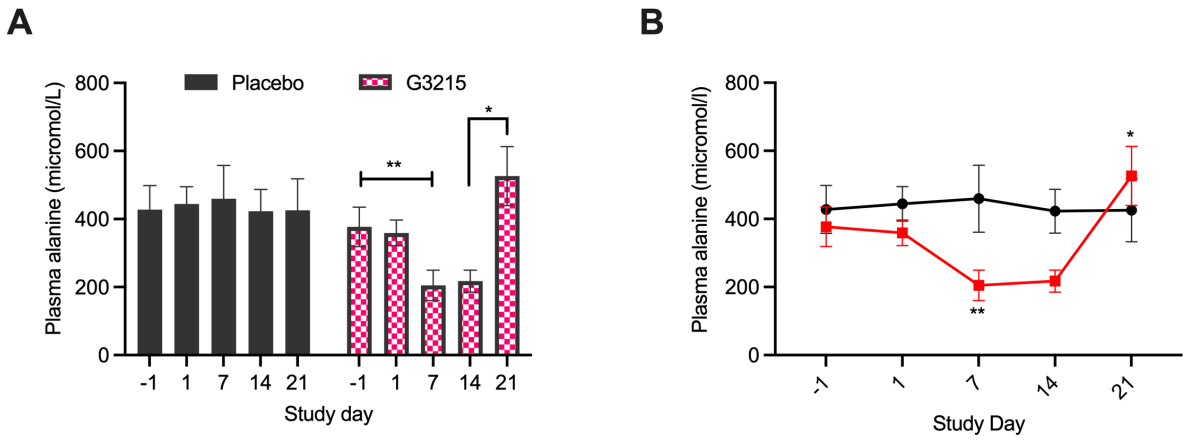


Figure 5.1: Plasma alanine levels over 21-day study period combined cohort 1-3. **(A)** Histogram and **(B)** overall plot of plasma alanine levels over 21-day study period with day -1 to day 14 infusion period. *p<0.05, **p<0.01, data plotted as mean ± SEM. Data analysed using two-way ANOVA with post hoc Tukey's test.

5.4.2 G3215 infusion reduces individual plasma amino acids over 14 days

Due to the significant reduction in plasma alanine in response to G3215 infusion, individual plasma amino acids were subsequently measured. For analysis, these were split into essential (EAA's, Figure 4.2, Table 5.2) and non-essential (NEAA's, Figure 5.3, Table 5.3) amino acids. Of the EAA's, compared to baseline (Day 1), there was a reduction in plasma histidine, leucine, valine, lysine, mehionine and threonine, at both measured timepoints during the G3215 infusion period (study day 7 and study day 14) (Figure 5.2, Table 5.2). Compared to baseline, there was also a significant reduction in plasma isoleucine at study day 14 and plasma tryptophan at study day 7, in participants receiving G3215 infusion. There was no significant reduction, compared to baseline, in any of the EAA's in the placebo group (Figure 5.2, Table 5.2). Interestingly, there was a rebound increase in plasma threonine at day 21, 7 days after the G3215 infusion was discontinued (Figure 5.2, Table 5.2). In participants receiving G3215 infusion, the EAA's with the most pronounced reduction were threonine (Day 14: -43.9% compared to baseline Day 1) and lysine (Day 14: -28.5% compared to baseline Day 1) (Table 5.2).

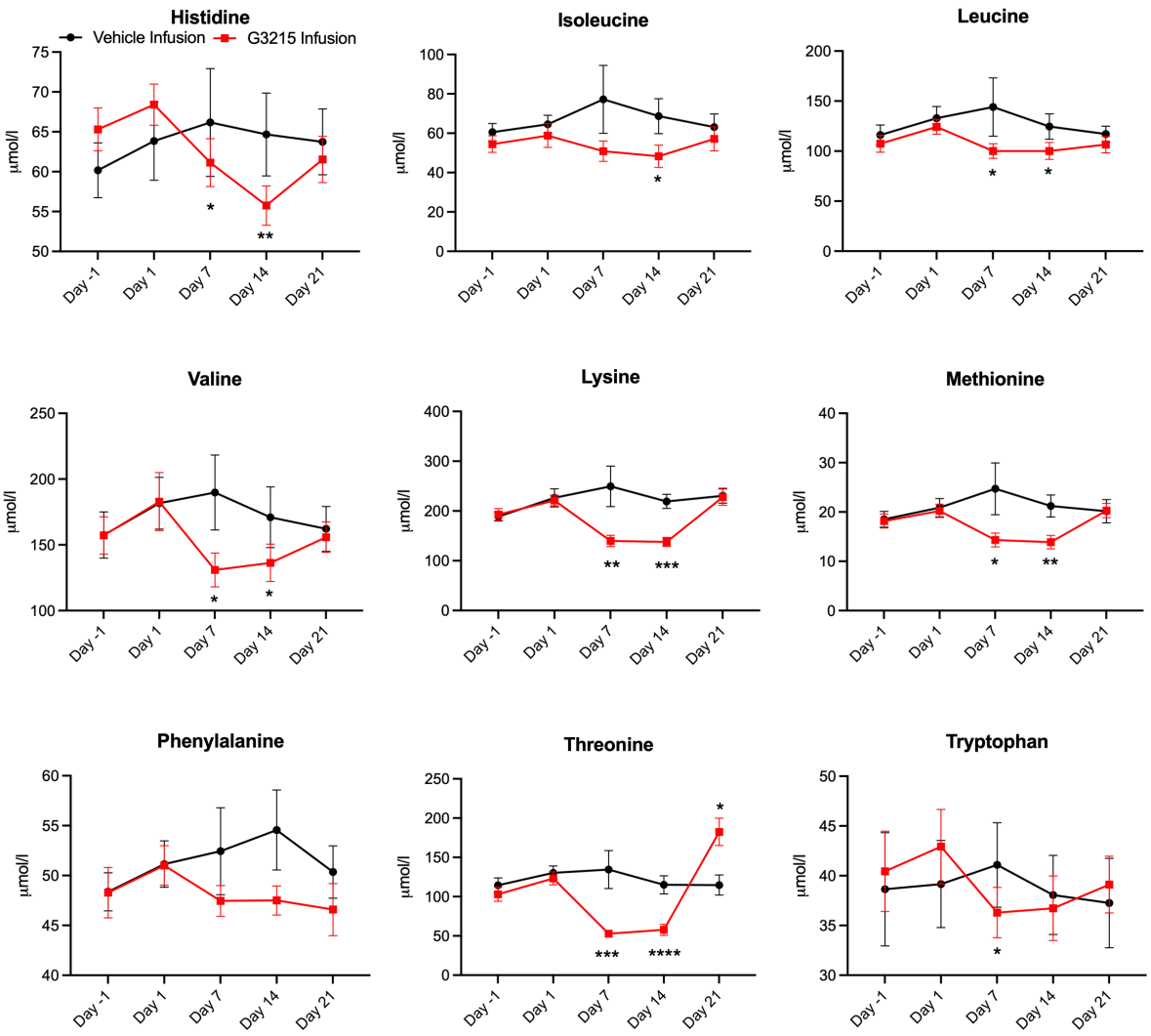


Figure 5.2 Plasma individual essential amino acids. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, plotted as mean ± SEM, significance indicates change from baseline (Day 1).. Data analysed using two-way ANOVA with post hoc Dunnett's test.

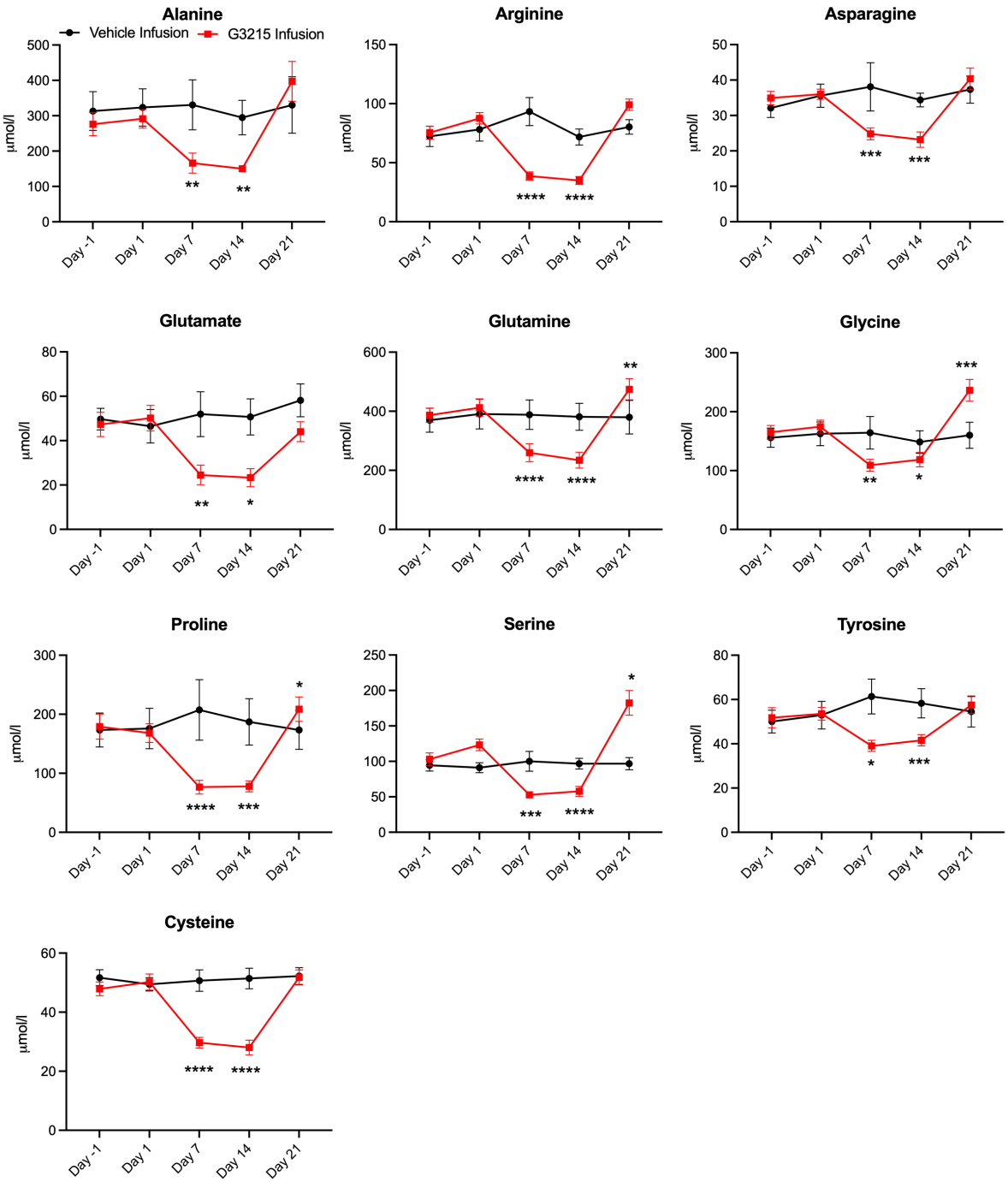


Figure 5.3 Plasma individual non-essential amino acids. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, plotted as mean ± SEM, significance indicates change from baseline (Day 1).. Data analysed using two-way ANOVA with post hoc Dunnett's test.

	Plasma individual essential amino acid level (micromol/l ± SEM)					
	Day-1	Day 1	Day 7	Day 14	Day 21	Change from baseline to day 14 (%)
Histidine Placebo	60.18 ± 3.44	63.83 ± 4.91	66.17 ± 6.76	64.67 ± 5.20	63.73 ± 4.13	7.46
Histidine G3215	65.31 ± 2.67	68.41 ± 2.59	61.14 ± 3.00 ***P*<0.05**	55.75 ± 2.48 ***P*<0.01**	61.54 ± 2.90	-14.64
Isoleucine Placebo	60.52 ± 4.43	64.55 ± 4.60	77.18 ± 17.24	68.67 ± 8.89	63.08 ± 6.71	13.47
Isoleucine G3215	54.49 ± 4.21	58.82 ± 6.05	50.91 ± 5.17 ***P*<0.05**	48.30 ± 5.67	57.12 ± 6.11	-11.36
Leucine Placebo	116.08 ± 9.99	132.88 ± 11.65	144.03 ± 29.17	124.62 ± 12.67	117.02 ± 7.95	7.36
Leucine G3215	107.43 ± 8.49	124.33 ± 7.40	100.09 ± 7.36 ***P*<0.05**	100.13 ± 8.40 ***P*<0.05**	106.54 ± 8.27	-6.80
Valine Placebo	157.48 ± 17.59	181.67 ± 19.69	189.77 ± 28.52	170.97 ± 23.06	162.17 ± 17.04	8.57
Valine G3215	157.14 ± 14.21	182.83 ± 22.06	130.92 ± 12.83 ***P*<0.05**	136.37 ± 14.18 ***P*<0.05**	155.87 ± 11.51	-13.22
Lysine Placebo	188.70 ± 8.92	226.33 ± 10.08	249.57 ± 40.61	219.25 ± 14.11	230.58 ± 14.86	16.19
Lysine G3215	192.84 ± 12.04	221.18 ± 10.66	139.82 ± 11.41 ***P*<0.01**	137.83 ± 9.07 ***P*<0.001**	227.78 ± 16.41	-28.53
Methionine Placebo	18.52 ± 1.60	20.87 ± 1.86	24.70 ± 5.27	21.22 ± 2.23	20.15 ± 2.38	14.58

Methionine G3215	18.16 ± 1.41	20.23 ± 1.34	14.31 ± 1.41 ***P*<0.05**	13.88 ± 1.38 ***P*<0.01**	20.29 ± 1.45	-23.57
Phenylalanine Placebo	48.37 ± 1.90	51.15 ± 2.33	52.43 ± 4.35	54.58 ± 4.00	50.35 ± 2.62	12.84
Phenylalanine G3215	48.29 ± 2.53	51.01 ± 1.98	47.45 ± 1.54	47.50 ± 1.46	46.59 ± 2.61	-1.64
Threonine Placebo	114.43 ± 9.45	130.33 ± 8.75	135.50 ± 24.16	115.07 ± 11.54	114.75 ± 12.87	0.56
Threonine G3215	103.08 ± 8.95	123.29 ± 8.23	52.74 ± 3.42 ***P*<0.001**	57.79 ± 6.98 ***P*<0.0001**	182.48 ± 17.43	-43.94
Tryptophan Placebo	38.63 ± 5.69	39.17 ± 4.37	41.08 ± 4.24	38.07 ± 3.97	37.25 ± 4.50	-1.45
Tryptophan G3215	40.44 ± 4.04	42.92 ± 3.74	36.30 ± 2.52 ***P*<0.05**	36.73 ± 3.24	39.11 ± 2.85	-9.17

Table 5.2: Individual plasma essential-amino acids (EAA's). White fill represents G3215 infusion, blue fill represents placebo infusion. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, mean ± SEM, significance indicates change from baseline (Day 1). Data analysed using two-way ANOVA with post hoc Dunnett's test.

	Plasma individual non-essential amino acid level (micromol/l ± SEM)					
	Day-1	Day 1	Day 7	Day 14	Day 21	Change from baseline to day 14 (%)
Alanine Placebo	313.30 ± 54.67	323.47 ± 52.69	330.78 ± 70.77	294.67 ± 48.70	330.27 ± 79.83	-5.95
Alanine G3215	276.17 ± 32.68	291.45 ± 26.10	165.90 ± 28.28 ***P*<0.01**	150.10 ± 9.34 ***P*<0.01**	397.38 ± 56.14	-45.65
Arginine Placebo	72.33 ± 8.61	78.23 ± 9.95	93.39 ± 11.83	71.80 ± 6.82	80.32 ± 6.14	-0.73
Arginine G3215	75.40 ± 5.47	87.62 ± 4.79	38.70 ± 3.47 ***P*<0.0001**	34.93 ± 3.39 ***P*<0.0001**	99.12 ± 4.86	-53.67
Asparagine Placebo	32.10 ± 2.66	35.58 ± 3.29	38.08 ± 6.82	34.38 ± 1.94	37.33 ± 3.86	7.10
Asparagine G3215	34.93 ± 1.90	36.01 ± 1.43	24.80 ± 1.67 ***P*<0.001**	23.18 ± 2.18 ***P*<0.001**	40.37 ± 3.02	-33.64
Glutamate Placebo	49.72 ± 4.87	46.52 ± 7.52	51.95 ± 10.11	50.68 ± 8.14	58.18 ± 7.41	1.93
Glutamate G3215	47.30 ± 5.46	50.19 ± 5.73	24.52 ± 4.45 ***P*<0.01**	23.28 ± 4.08 ***P*<0.05**	44.01 ± 4.46	-50.78
Glutamine Placebo	369.75 ± 40.65	390.95 ± 50.60	388.47 ± 49.66	381.17 ± 45.40	379.95 ± 56.84	3.08
Glutamine G3215	386.77 ± 24.10	412.06 ± 28.80	259.77 ± 30.49 ***P*<0.0001**	234.49 ± 26.98 ***P*<0.0001**	474.45 ± 35.70 ***P*<0.01**	-39.37

Glycine Placebo	155.88 ± 16.34	162.50 ± 20.13	164.28 ± 27.66	148.55 ± 19.06	160.08 ± 22.12	-4.70
Glycine G3215	165.04 11.60	174.65 ± 11.06	109.15 ± 10.07 ***P*<0.01**	118.63 ± 12.23 ***P*<0.05**	236.42 ± 18.52 ***P*<0.001**	-28.12
Proline Placebo	173.43 ± 28.73	175.80 ± 34.29	207.42 ± 51.30	187.12 ± 39.31	173.48 ± 32.79	7.89
Proline G3215	179.12 ± 21.35	168.08 ± 15.71	76.61 ± 11.61 ***P*<0.0001**	77.82 ± 9.22 ***P*<0.001**	208.64 ± 20.65 ***P*<0.05**	-56.55
Serine Placebo	94.42 ± 8.08	91.10 ± 7.00	100.13 ± 13.94	96.78 ± 7.46	96.73 ± 8.60	2.50
Serine G3215	103.08 ± 8.95	123.29 ± 8.23	52.74 ± 3.42 ***P*<0.001**	57.70 ± 6.98 ***P*<0.0001**	182.48 ± 17.43 ***P*<0.05**	-44.02
Tyrosine Placebo	50.03 ± 5.12	52.93 ± 6.21	61.37 ± 7.86	58.28 ± 6.62	54.57 ± 7.00	16.49
Tyrosine G3215	51.76 ± 4.55	53.55 ± 2.87	39.06 ± 2.51 ***P*<0.05**	41.59 ± 2.58 ***P*<0.001**	57.51 ± 3.81	-19.65
Cysteine Placebo	51.70 ± 2.69	49.43 ± 2.25	50.70 ± 3.64	51.40 ± 3.48	52.22 ± 2.86	-0.58
Cysteine G3215	47.89 ± 2.32	50.21 ± 2.71	29.69 ± 1.80 ***P*<0.0001**	28.01 ± 2.51 ***P*<0.05**	51.78 ± 2.56	-41.51

Table 5.3: Individual plasma non-essential-amino acids (NEAA's). White fill represents G3215 infusion, blue fill represents placebo infusion. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, mean ± SEM, significance indicates change from baseline (Day 1). Data analysed using two-way ANOVA with post hoc Dunnett's test.

Of the NEAA's, there was also a broad reduction in all individual amino acids in response to infusion of G3215 at both day 7 and 14. (Figure 5.3, Table 5.3). The largest reductions were seen for alanine, arginine, glutamate, proline, serine and cysteine; These were noted to have reduced by 40-60 % compared to the day -1 baseline measurement (Table 5.3). Similar to what was observed with the EAA's, following discontinuation of the G3215 infusion there was a rebound increase in certain amino acids, for example glutamine, glycine, proline and serine (Figure 5.3, Table 5.3).

5.4.3 Effects of G3215 infusion on body composition and functional strength

To determine the effect of G3215 infusion on body composition over the study period, an electrical bioimpedance method was utilised. This has the advantage of being non-invasive and quick to perform. The bioimpedance measurement was performed using a Tanita (TANITA, EU) weighing scale within the Clinical Research Facility, with measurements at study day -1 (study day prior to infusion commencement), day 11 and day 14 (study day prior to infusion discontinuation). A small but significant reduction in lean mass from baseline (-2.22 ± 0.99 % or -1.54 ± 0.69 kg) was observed in participants receiving G3215 infusion at day 11 (Figure 5.4, Table 5.4). A similar reduction in fat mass (-3.98 ± 1.82 % or -1.22 ± 0.55 kg) from baseline was also seen in participants receiving G3215 infusion at day 11. There were no significant changes from baseline in body composition when measured at day 11 in participants receiving the placebo infusion (Figure 5.4, Table 5.4). At day 14, the weight loss from lean mass in participants receiving G3215 infusion was comparable to that of the placebo infusion and therefore did not reach statistical significance; The lean mass loss from baseline at day 14 reached -1.08 ± 0.63 kg versus -0.50 ± 0.41 kg in those receiving placebo infusion (Figure 5.4, Table 5.4). However, at day 14, the fat mass loss from baseline progressed in participants receiving G3215 infusion (-6.56 ±1.76 % or -1.86 ± 0.58 kg), compared to participants receiving placebo infusion (0.10 ± 1.30 % or -0.08 ± 0.30 kg) (Figure 5.4, Table 5.4).

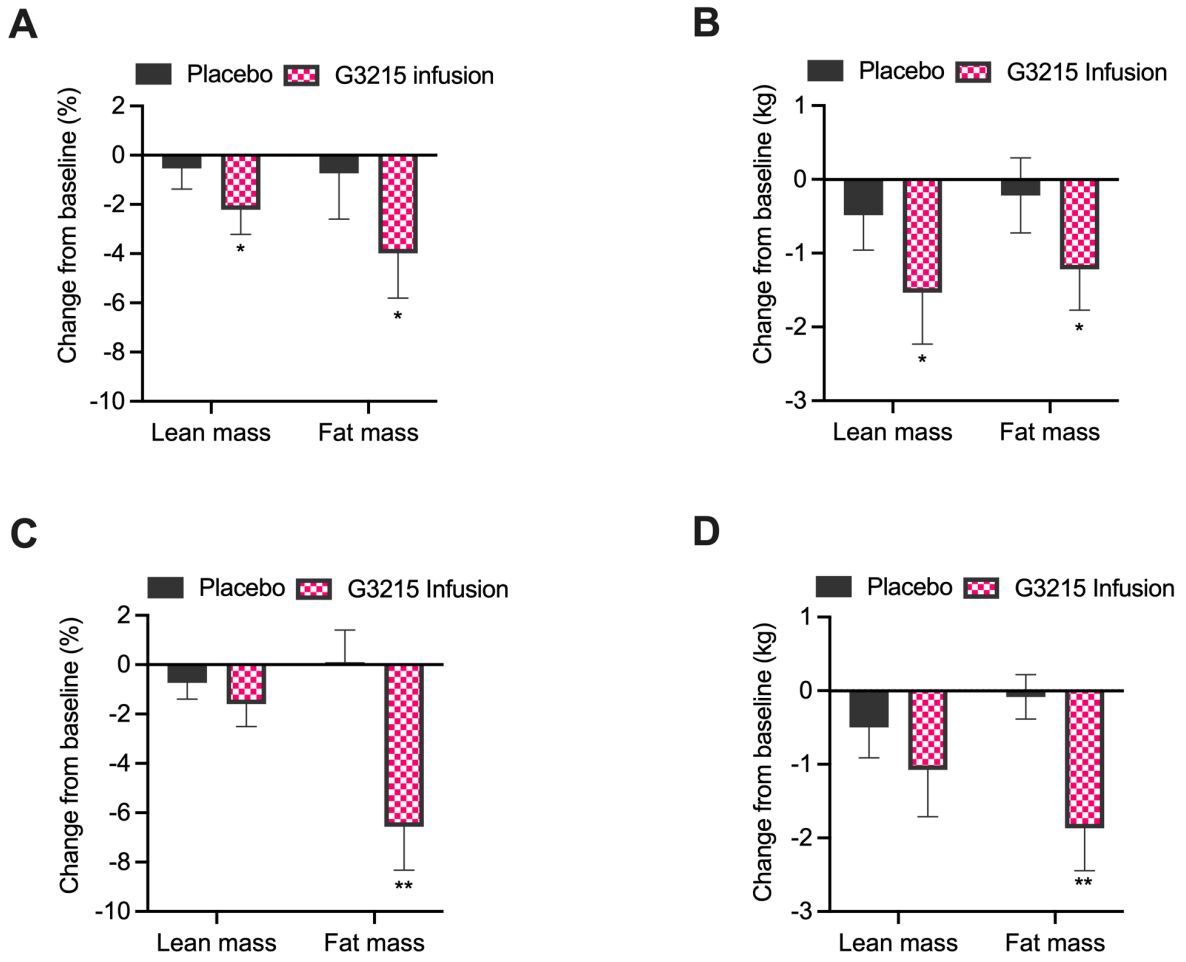


Figure 5.4: Body composition data, n=6 placebo group, n=17 G3215 group. **(A)** Body composition on day 11 percentage change from baseline **(B)** Body composition on day 11 absolute (g) change from baseline. **(C)** Body composition on day 14 percentage change from baseline **(D)** Body composition on day 14 absolute (g) change from baseline. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, data plotted as mean ± SEM. Solid bars = placebo infusion, Checked bars = G3215 infusion. Analysed from baseline using paired t-tests.

Body composition data, change from baseline (day -1)	Day 11 Placebo Mean ± SEM	Day 11 G3215 Mean ± SEM	Day 14 Placebo Mean ± SEM	Day 14 G3215 Mean ± SEM
Lean mass (%)	-0.54 ± 0.84	-2.22 ± 0.99 ***P*<0.05**	-0.733 ± 0.66	-1.59 ± 0.90
Lean mass (g)	-0.48 ± 0.47	-1.54 ± 0.69 ***P*<0.05**	-0.50 ± 0.41	-1.08 ± 0.63
Fat mass (%)	-0.74 ± 1.85	-3.98 ± 1.82 ***P*<0.05**	0.10 ± 1.30	-6.56 ± 1.76 ***P*<0.01**
Fat mass (g)	-0.22 ± 0.51	-1.22 ± 0.55 ***P*<0.05**	-0.08 ± 0.30	-1.86 ± 0.58 ***P*<0.01**

Table 5.4: Body composition change from baseline (day -1). White fill represents G3215 infusion, blue fill represents placebo infusion. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, mean ± SEM. Data analysed from baseline using paired t-tests.

To assess any observable effect of G3215 infusion on muscle strength, two functional strength tests were carried out at baseline (Day -1) and on study day 14. These included hand-grip-strength and the five time sit-to-stand test. There was a significant increase in hand grip strength in the G3215 infusion group, as measured on day 14 as change from baseline; This difference was not observed in participants receiving the placebo infusion (Figure 5.5A, Table 5.5, change from baseline in grip strength, mean ± SEM, placebo left hand: -0.28 ± 1.62 s, placebo right hand: -0.66 ± 1.25 s, G3215 left hand: 4.40 ± 1.19 s, G3215 right hand: 3.02 ±1.25 s). There was a greater reduction in the five time sit-to-stand time from baseline in participants receiving placebo infusion than in participants receiving G3215 infusion, however this reduction did not reach statistical significance (Figure 5.4B, Table 5.5, change in time from baseline, mean ± SEM, placebo infusion: -2.28 ± 1.30 s, G3215 infusion: -0.45 ± 1.25 s).

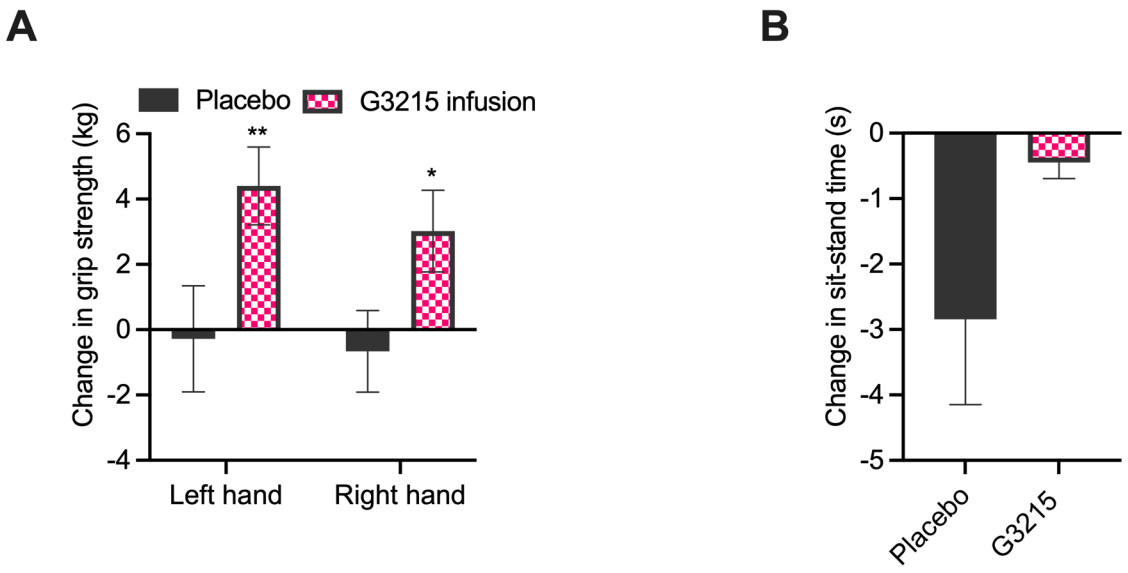


Figure 5.5: Functional assessments, n=6 placebo group, n=17 G3215 group. **(A)** Change in hand grip strength (kg) from baseline measured on study day 14. **(B)** Change in five sit-to-stand time (s) from baseline measured on study day 14. *p<0.05, **p<0.01, mean ± SEM. Data analysed from baseline using paired t-tests.

Functional assessments change from baseline (day -1)	Day 14 Placebo Mean ± SEM	Day 14 G3215 Mean ± SEM
Left hand grip strength (kg)	-0.28 ± 1.62	4.40 ± 1.19 ***P*<0.01**
Right hand grip strength (kg)	-0.66 ± 1.25	3.02 ± 1.25 ***P*<0.05**
Change in sit-to-stand time (s)	-2.28 ± 1.30	-0.45 ± 0.24

Table 5.5: Functional assessments. n=6 placebo group, n=17 G3215 group. Change in hand grip strength (kg) from baseline measured on study day 14. Change in five sit-to-stand time (s) from baseline measured on study day 14. White fill represents G3215 infusion, blue fill represents placebo infusion. *p<0.05, **p<0.01, mean ± SEM. Data analysed from baseline using paired t-tests.

5.5 Discussion

5.5.1 Infusion of a novel GLP1/GCGR co-agonist, G3215, over 14-days in humans causes a broad reduction in plasma amino acids

Plasma amino acids were measured at two time points during the 14-day infusion of either placebo of G3215 (Day 7 and Day 14). Using a mass-spectrometry approach, individual amino acids were measured and demonstrated a broad reduction in the majority of these at both timepoints. Of the nine EAA's and eleven NEAA's measured, all of these were supressed at day 7 and day 14 compared to baseline (Figure 5.2 and Figure 5.3). In contrast, there was no change in plasma amino acids across the EAA and NEAA components, in the placebo infusion group. The reduction in individual plasma amino acids in response to G3215 can be compared to reductions observed with other GCGR-targeted analogues recently reported in humans (Table 5.6). For example, the once-daily administered cotadutide also resulted in a broad reduction in amino acids over 49 days (esc to 300 μg daily), however to a lesser extent (Table 5.6) (Parker, Robertson et al. 2020). Although plasma several amino acids were reduced from baseline, only alanine reached statistical significance. In contrast, the once weekly NN1177, Novo Nordisk (esc to 4,200 μg weekly) caused a more marked reduction in individual plasma amino acids over 78 days, comparable to that seen in response to G3215 infusion (Table 5.6) (Martin Friedrichsen 2022). Interestingly, certain individual amino acids appeared to be less affected in response to either G3215 or NN1177 including phenylalanine, tryptophan, leucine and isoleucine (Table 5.6). More recently in a Phase 1 single dose administration study, the GLP-1R/GCGR/GIPR triple agonist, LY3437943 (Lilly) led to a reduction in three measured glucogenic amino acids up to 8 days following drug administration (Coskun, Urva et al. 2022) (Table 5.6). However, in a more recent report of a multiple-ascending dose study of LY3437943, plasma amino acids are not reported (Urva 2022). Another GLP-1R/GCGR/GIPR triple agonist (SAR441255, Sanofi) which has been tested in a phase 1 trial led to a broad reduction in amino acids for up to 12 hours after a single 80 μg or 150 μg dose (Bossart, Wagner et al. 2021).

It is challenging to infer specific causal factors when directly comparing between amino acid responses to different drug candidates. This is due to several differences between these compounds including dose administered and pharmacodynamic/pharmacokinetic

properties, which may have a divergent effect on hepatic GCGR activation underpinning amino acid uptake and catabolism. The latter may include drug potency, efficacy, circulating longevity therefore drug exposure, and any specific ligand-receptor interactions which may affect downstream signalling activity and therefore extent of amino acid uptake and catabolic activity. For example, cotadutide has a weak potency to the hGCGR relative to native glucagon (5-10-fold weaker relative to native glucagon) and is administered daily due to its shorter half-life (~9-12 hr) (Henderson, Konkar et al. 2016). In comparison, NN1177 has a high potency to the hGCGR (within 2-fold relative to native glucagon) and an extended half-life (~77-103 hr)(Simonsen, Lau et al. 2022). G3215 is administered continuously via a subcutaneous infusion and has a high potency to the hGCGR (within 3-fold relative to native glucagon, see section 5.3.2).

Beyond direct GCGR-dependent effects on plasma amino acid availability, the effect of insulin on peripheral amino acid uptake in response to varying GLP-1R activities of these compounds may also need to be considered. Furthermore, the extent of satiety and therefore caloric intake from protein sources may further affect the available circulating essential amino acids.

Plasma essential amino acids (EAA's) Percentage reduction from baseline (%)				
	G3215 (day 14)	Cotadutide (Day 49)	NN1177 (Day 78)	LY3437943 (Day 8)
Histidine	-14.64 % ***P*<0.01**	-9.99 %	-32 %	-
Isoleucine	-11.36 %	-3.21 %	-21 %	-
Leucine	-6.80 % ***P*<0.05**	14.07 %	-25%	-
Valine	-13.22 % ***P*<0.05**	-13.67 %	-37 %	-
Lysine	-28.53 % ***P*<0.001**	-20.06 % ***P*=0.084**	-46 %	-
Methionine	-23.57 % ***P*<0.01**	-8.72 %	-43 %	-
Phenylalanine	-1.64 %	-6.48 %	-8 %	-
Threonine	-43.94 % ***P*<0.0001**	-10.74 %	-61 %	-
Tryptophan	-9.17 %	-7.09 %	-23 %	-
Plasma non-essential amino acids (NEAA's) **Percentage reduction from baseline (%)**				
	G3215 (day 14)	Cotadutide (Day 43)	NN1177 (Day 78)	LY3437943 (Day 8)
Alanine	-45.65 % ***P*<0.01**	-13.81 % ***P*<0.05**	-56 %	-38.13 %
Arginine	-53.67 % ***P*<0.0001**	-5.40 %	-68 %	-
Asparagine	-33.64 % ***P*<0.001**	1.89 %	-57 %	-11.05 %
Glutamate	-50.78 % ***P*<0.01**	-5.74 %	-2 %	-
Glutamine	-39.37 % ***P*<0.0001**	4.72 %	-60 %	-
Glycine	-28.12 % ***P*<0.01**	-7.49 %	-33 %	-
Proline	-56.55 % ***P*<0.0001**	3.45 %	-66 %	-
Serine	-44.02 % ***P*<0.001**	11.45 %	-35 %	1.16 %
Tyrosine	-19.65 % ***P*<0.05**	-14.43 % ***P*=0.068**	-39 %	-
Cysteine	-41.51 % ***P*<0.0001**	-3.52 % ***P*=0.057**	-	-
Aspartate	-	16.93 %	24 %	-

Table 5.6: Percentage reductions of individual amino acids across comparator GCGR-targeted multi-agonists including G3215, Cotadutide, NN1177, and LY3437943 *p<0.05, **p<0.01, ***p<0.001,

****p<0.0001, mean ± SEM. Data from G3215 study analysed using two-way ANOVA with post hoc Dunnett's test. Data for Cotadutide, NN1177 and LY3437943 from respective clinical trial reports (Parker, Robertson et al. 2020, Coskun, Urva et al. 2022, Martin Friedrichsen 2022). Data for NN1177 analysed from ratio of geometric means. Data from SAR441255 not presented as represented in heat map (Bossart, Wagner et al. 2021).

The trial of G3215 reports normalisation of individual amino acids 7 days after drug discontinuation. This is important as it demonstrates that G3215-stimulated hypoaminoacidemia is transient and recovers following discontinuation. Interestingly, specific amino acids were noted to 'rebound' above baseline values, for example threonine, glutamine, glycine and serine (Figure 5.2 and 5.3). It is also important to determine whether a steady state of hypoaminoacidemia is achieved or whether a continual depression of amino acids will occur with chronic treatment. Plasma amino acids were measured at two timepoints during the infusion period and the results suggest a steady state of hypoaminoacidemia is achieved, which normalises upon drug discontinuation (Figure 5.3 and 5.3). Interestingly, of the three plasma amino acids reported from the phase 1 trial of LY3437943, following an initial reduction over 8 days, plasma amino acids recover by the end of the study (Day 43 of weekly dosing) (Coskun, Urva et al. 2022). The follow up plasma amino acid levels were not reported for Cotadutide or NN1177 (Parker, Robertson et al. 2020, Martin Friedrichsen 2022). It therefore will be important to determine whether plasma amino acids remain suppressed when G3215 is administered for longer-time periods.

The consequences of disturbed circulating amino acid availability is most recognised from 'Inborn errors of metabolism' which is itself a defined field of medicine established following the identification of the defect in tyrosine metabolism leading to Alkaptonuria (Garrod 1996). The subsequent discovery of a number of amino acid disorders are defined by excess of specific amino acids with distinct clinical characteristics (Aliu, Kanungo et al. 2018). Whereas the inborn errors of metabolic follow a one-gene-one enzyme defect pattern, the aetiology of protein deficiency is in the mainstay environmental, through dietary deficiency. Indeed, dietary protein deficiency is observed both in a severe form as kwashiorkor or marasmus, mainly in developing nations and less severe forms in the elderly (Aliu, Kanungo et al. 2018). Dietary protein is important to supply essential amino acids, which in turn are precursors for important components of

normal physiology for example protein synthesis, enzymes, neurotransmitters, nucleotides and other low weight substances (Wu 2016). Non-essential amino acids are also required for normal physiological function, for example glutamine provides a significant energy source to immune cells (Li, Yin et al. 2007). Due to the pleotropic function of individual amino acids, it is not unsurprising that symptoms of protein deficiency in humans are diverse and extensive (Table 5.7).

Consequences of protein deficiency in humans
Reduction in muscle protein synthesis and increased proteolysis
Skeletal muscle wasting, physical fatigue, impaired mobility
Impaired immune response
Reduction in hormones including insulin, growth hormone, IGF-1, thyroid hormones
Impaired growth and cognitive development
Impaired anti-oxidative reactions leading to increased oxidative stress and aging
Impaired absorption and transport of nutrients including vitamins, minerals, amino acids, glucose and fatty acids
Anaemia, reduced transport of oxygen
Cardiac failure, hypertension
Tissue fluid retention
Impaired synthesis of neurotransmitters leading to anxiety and depression
Reduced fertility
Intrauterine growth restriction in maternal protein deficiency

Table 5.7: Consequences of protein deficiency in humans. Adapted from the review article entitled 'dietary protein intake and human health' (Wu 2016)

Due to the diverse negative effects of protein deficiency on whole-body physiology, It therefore remains to be established whether G3215 or similar GCGR-targeted multi-agonists lead to a chronic suppression of plasma amino acids and if so, the implication this may have on whole-body physiology, beyond that of lean mass and muscle function.

This will further inform whether any degree of chronic GCGR-stimulated hypoaminoacidemia is an acceptable risk and whether preventative strategies (discussed in section 5.6) are required to mitigate this detrimental effect mediated by enhanced hepatic catabolism of amino acids.

5.5.2 A 14-day infusion of G3215 is associated with changes in body composition including lean mass loss

Increased loss of fat mass was observed in participants receiving G3215 over 14-days of the infusion period. As G3215 was effective in enhancing satiety, a degree of fat mass loss is expected however this was not extensive due to the short-term infusion. In comparison, later phase clinical trials would be designed to power for efficacy outcomes such as body weight loss and changes in body composition. Due to the potential effects of G3215 on plasma amino acids (see section 5.5.1), body composition data also aimed to capture any potential effects on lean mass during the infusion period. Interestingly, there was a small but detectable reduction in lean mass from baseline following 11 days of infusion with G3215, an effect not observed in participants receiving the placebo infusion (Figure 5.4, Table 5.4). The reduction in lean mass in the G3215 group was also observed following 14 days of infusion, however at this time-point did not reach statistical significance.

The change in lean mass from baseline between day 11 and day 14 does not follow the sustained reduction in individual plasma amino acids (Figure 5.2 and 5.3). Therefore, it is possible that after initial lean mass loss, there is an element of compensation within lean tissue over time to maintain lean mass in a low amino acid environment. It is also possible that G3215-stimulated insulin action may mitigate any detrimental effect on lean mass as insulin may enhance protein synthesis and/or reduce proteolysis (Everman, Meyer et al. 2016). Alternatively, it is possible that non-significant difference in lean mass between the two groups at day 14 are underpowered due to the short-term infusion period and/or lack of placebo participants number, leading to high variation in the body composition data. It is also possible that the detrimental effect on lean mass at day 11 in the G3215 group is instead coupled to calorific intake and due to heightened effects of satiety during the initial infusion period, which then subsides later during the infusion period.

It will therefore be important to power the outcome of body composition sufficiency in later phase clinical trials of G3215. If a reduction in lean mass is identified as a concern in future trials, then it will also be important to identify the likely contributing mechanisms to this. For example, this thesis has demonstrated that glucagon analogue-stimulated hypoaminoacidemia may contribute directly to enhanced lean mass loss (Chapter 2 and 3); This may be due to enhanced muscle proteolysis and/or reduced synthesis through defined amino acid sensing pathways in muscle (highlighted in Chapter 1). Furthermore, as G3215 increases satiety leading to reduced caloric intake, the effect of reduced dietary protein intake may compound any detrimental effect of GCGR-stimulated hypoaminoacidemia on lean mass. It is also possible that the insulinotropic action of GLP-1 may prevent lean mass loss and so therefore GCGR/GLP1R co-agonism may inherently offer this protective effect. Indeed, there is evidence that GLP-1's has a positive effect on skeletal muscle metabolism and perfusion (Abdulla, Phillips et al. 2022).

Recent reports of GCGR-targeted multi-agonists in early phase human trials have not reported changes in body composition. Some of these however have administered their candidate drugs in a chronic setting, for example in the case of cotadutide (Medimmune) over 41 days (Ambery, Parker et al. 2018) or 54 weeks (Nahra, Wang et al. 2021), NN1177 (Novo Nordisk) over 78 days (Martin Friedrichsen 2022) and LY3437943 (Eli Lilly) over 43 days or 12 weeks (Coskun, Urva et al. 2022). The latter drug from Eli Lilly has recently been tested in a multiple-ascending dose trial over 12 weeks, however despite significant weight loss, body-composition is not reported (Urva 2022). The phase 1 trial of G3215 (ICL) in this Chapter reports for the first time the effect of a GCGR/GLP-1R agonist on body composition together with the effects on individual plasma amino acids. Given that the multi-agonists described above all have a suppressive effect on plasma amino acids, it is likely that reporting of both these outcomes will be required in further clinical trials of GCGR-targeted multi-agonists. This is further supported by the work in Chapter 2 and 3 which demonstrate that hypoaminoacidemia stimulated by the GCGR/GLP-1 co-agonist G108 causes lean mass loss *in vivo* (Chapters 2 and 3).

5.5.3 A 14-day infusion of G3215 is associated with positive changes in functional strength tests

Functional strength tests were also carried out as an exploratory outcome. Participants in the G3215 group exhibited an increased hand grip strength from baseline after 14 days of infusion. This was associated with a sustained five-time-sit to stand time (Figure 5.5, Table 5.5). In contrast, participants in the placebo group did not exhibit and increased hand grip strength from baseline at day 14 and a reduction in the sit-to-stand time was observed. It is possible that the functional strength tests were underpowered particularly in the placebo group and so therefore this research question could not be answered. However, as a significant increase in hand grip strength was observed in the G3215 group (which contained a higher number of participants), this at least suggests that G3215 itself does not lead to a decline in function as assessed by muscle strength. If G3215-stimulated hypoaminoacidemia has a chronic effect on lean mass and muscle function, these results are perhaps not unexpected due to the short-term infusion protocol. Indeed, to firmly establish any effect on muscle function, longer-term studies with higher participant numbers are likely required.

5.6 Conclusions

The results from this Chapter show that a 14-day subcutaneous infusion of the GCGR/GLP1R agonist G3215, leads to a broad sustained suppression in plasma amino acids. This is consistent with previous *in vitro* characterisation of G3215 demonstrating its high receptor potency to the hGCGR relative to native glucagon. Furthermore, by administering G3215 as a continuous infusion, the drug exposure is prolonged in a similar way to a long-acting depot administration. Both these factors are likely to enhance the effect of G3215 on hepatic amino acid catabolism and therefore plasma availability.

It will be important to determine the physiological effects of chronically supressed plasma amino acids in response to GCGR-targeted multi-agonists such as G3215. These physiological effects may be wider than just the effect on the lean mass compartment. Furthermore, it will be important to understand the drug-characteristics, for example the pharmacodynamic and pharmacokinetic properties modulating hepatic amino acid catabolism (Section 5.5.1). If hypoaminoacidemia is identified as a concern in human

trials of GCGR-targeted multi-agonists, it will be important to determine effective preventative strategies. The results from this thesis demonstrate that dietary protein supplementation may be a viable strategy (*In vivo* studies, Chapter 3) however optimisation at the level of the drug/receptor interaction may also be required to mitigate effects on GCGR-stimulated hepatic amino acid catabolism. For example, the latter strategy may leverage engineered 'biased' unimolecular GCGR-targeted multi-agonists or 'decouple' GCGR/GLP-1 co-agonists to deliver differentially timed co-agonism throughout the 24-hour day/night cycle. A GCGR biased approach could be employed for unimolecular GCGR-targeted multi-agonists to minimise hepatic amino acid catabolism whilst maintaining other metabolic benefits of GCGR receptor signalling. Furthermore, by administering glucagon and GLP-1 analogues as separate entities, it may be possible to time glucagon analogue exposure to minimise excess hepatic amino acid catabolism, for example in the fasting periods.

The importance of mitigating against GCGR-stimulated hypoaminoacidemia is currently uncertain. The extreme 'end of the scale' of low plasma amino acid availability is highlighted by the multiple detrimental effects of protein deficiency in humans (section 5.5.1). Therefore, any further programme of work in this area will contribute towards the currently uncharacterised understanding of the implications of chronic hypoaminoacidemia in humans treated with GCGR-targeted multi-agonists. Furthermore, further research may offer potential translatable strategies to prevent this effect in humans receiving these agonists for the treatment of obesity and metabolic disease.

6 Chapter 6– Future treatment strategies to mitigate against glucagon-stimulated hypoaminoacidemia and lean mass loss

6.1 Introduction

6.1.1 A high protein diet prevents lean mass loss *in vivo* during chronic G108 administration

In Chapter 3, it was demonstrated that chronic G108-stimulated hypoaminoacidemia is associated with a significant loss of lean mass in obese mice. A high protein diet intervention was also found to rescue low plasma amino acids and in turn, lean mass loss was mitigated. However, it was noted that that a high protein diet intervention did not completely prevent lean mass loss over the course of a 27-day daily dosing study in obese mice (Chapter 3, section 3.4.2). As alluded to, this may be due to incomplete rescue of amino acids throughout the entire 24-hour time cycle, in particular during the fasting/sleeping phase. The lack of complete protection on lean mass afforded by the high protein diet may also be due to the balance of hepatic GCGR and dietary protein availability. For example, 40 kcal% dietary protein content may not be sufficient to halt lean mass loss in the presence of sustained hepatic GCGR activation by daily administration of G108 at a dose of 7.5 nmol/kg.

Therefore, strategies to further mitigate or fully prevent lean mass loss in response to chronic glucagon analogue therapy may be required. These include but may not limited to; altering the exposure time to the analogue, utilising biased agonism at the GCGR to supress amino acid catabolic pathways or increasing the dietary availability of protein. Indeed, it is possible that a combination of strategies is required to optimise efficacy of GCGR-targeted multi-agonists whilst minimising hypoaminoacidemia and lean mass loss. In any strategy to avoid GCG-stimulated hypoaminoacidemia and lean mass loss, it will be important to ensure that the key metabolic benefits of GCGR activation are maintained, as otherwise the value of targeting the GCGR may be minimal.

6.1.2 Alternative strategies to prevent lean mass loss during chronic glucagon analogue administration - Timing of glucagon administration

A more obvious solution to mitigate lean mass loss in response to chronic glucagon analogue therapy, is to limit the drug exposure time and therefore pharmacological effects. If prolonged hepatic GCGR activation leads to a sustained suppression of plasma amino acids, it is reasonable to assume that the extent of lean mass loss will be higher. Indeed, in the case of the glucagonoma syndrome, chronic hyperglucagonemia is present and associated with chronically suppressed plasma amino acids and severe muscle wasting (Mallinson, Bloom et al. 1974, Almdal, Heindorff et al. 1990, Barazzoni, Zanetti et al. 1999).

A key question which needs to be answered is whether shortening the drug exposure time, i.e., by using a short acting glucagon analogue, would also reduce other metabolic effects of glucagon, for example hepatic lipid and plasma profiles and glycaemia. From the studies in Chapter 3 and 4, it is predicted that if shorter-acting analogues reduce amino acid suppression, then the EE effects observed will also be attenuated or prevented; This is based on the findings in Chapter 3 and 4 that G108-stimulated hypoaminoacidemia explain the EE and body weight loss effects in obese mice. However, as GCG-dependent effects on hepatic amino acid, lipid metabolism and glycaemia may exhibit a divergent response to short-acting (SA) and long-acting (LA) GCG analogues, and this could be based on the different regulation of these pathways (Albrechtsen 2021). Therefore, an important question will be to determine whether SA GCG analogues can provide any key benefits of GCG signalling whilst mitigating against hepatic amino acid catabolism and lean mass loss.

6.1.3 Alternative strategies to prevent lean mass loss during chronic glucagon analogue administration - Partial agonism of a GLP1R/GCGR co-agonist

Biased agonism is a promising pharmacological strategy whereby receptor ligands differentially activate downstream signalling pathways to a single receptor. This has the advantage of potentially enhancing the therapeutic window by enhancing beneficial intracellular signalling pathways and reducing deleterious ones (Schmid, Kennedy et al. 2017). G-protein coupled receptors (GPCR's) are linked to proximal signalling

intermediates g-proteins or beta-arrestin, the latter regulating receptor endocytosis. Therefore, biased agonism at GCPR's may allow for a sustained signalling through g-proteins, i.e., Gs. Indeed, it has been demonstrated that through the selective reduction in beta-arrestin recruitment at the GLP-1R, biased GLP-1 analogues may enhance GLP-1 action leading to improved efficacy for glucose regulation (Jones, Buenaventura et al. 2018). G-proteins and beta-arrestins also regulate the intracellular pathways downstream to the GCGR. Whether a biased strategy is possible at the GCGR to selectively reduce intracellular signalling pathways regulating hepatic amino acid catabolism, is unknown. A previous study has demonstrated that biased GCG analogues do not affect hepatic glucose output in isolated hepatocytes (Jones, McGlone et al. 2021). Interestingly, it has recently been shown that partial agonism to obtain bias at the GLP1R leads to improved efficacy of a GLP1R/GCGR co-agonist (Pickford, Lucey et al. 2021); In this study, partial agonism at the GLP1R for only beta-arrestin recruitment by the peptide 'SRB103Q' led to reduced GLP-1R recycling and sustained improvements in glucose tolerance compared to a full GLP-1R agonist 'SRB103H'. Interestingly, SRB103Q is also a partial agonist at the GCGR for *both* Gs and beta-arrestin recruitment. Although plasma amino acids were not reported, in a chronic study there was no chronic effect on lean mass between SRB103Q and the full agonist, SRB103H. Therefore, it is possible that divergent partial agonism of SRB103Q at the GLP1R and GCGR lead to sustained improvements in glucose regulation without further increase in hepatic amino acid catabolism. Therefore, biased-partial agonism in GLP1R/GCGR co-agonists may offer a promising strategy to potentially selectively enhance GLP1R activity whilst maintaining GCGR activity.

6.1.4 Alternative strategies to prevent lean mass loss during chronic glucagon analogue administration - Increasing dietary protein availability to prevent lean mass loss during chronic glucagon analogue administration

In Chapter 3, increased dietary protein content to 40 kcal% rescued plasma amino acids which was associated with attenuated lean mass loss in obese mice receiving G108 in a chronic setting (Chapter 3, section 3.5.2). It is possible that plasma amino acids 'escaped' the protection of a high protein diet, during times of fasting or resting. In the case of mice, most of the feeding occurs during the initial phases of the dark phase. Therefore, a long-period of minimal food intake may result in an increased ratio of G108-GCGR activity:

dietary protein supply. As the plasma amino acids were measured in the morning (post-prandial phase), it is possible that there is a higher chance of amino acid repletion at this time from the high protein diet. If this is true, then sustained intake of protein at this level of dietary protein content (40 kcal%), i.e., little and often would be a possible strategy to more effectively prevent lean mass loss. An alternative strategy is to increase the dietary protein content further. In mice, this would represent an increase to 50 or 60 kcal% dietary protein. However, there is also the risk of adverse effects directly from excess dietary protein which include metabolic burden on the liver and kidneys, cancer and cardiovascular risk (Delimaris 2013). Therefore, increasing dietary protein content to extreme levels may not an appropriate strategy and is unlikely to be effective in isolation.

6.1.5 Investigating drug duration of GCG analogues and biased agonism in a GLP1/GCGR co-agonist on plasma amino acids and metabolic outcomes

From the possible strategies described, in this Chapter, a SA GCG analogue (G395) will be used to determine the effect on plasma amino acids in both the acute and chronic setting. The chronic effect of G395 on lean mass and other metabolic benefits of GCGR signalling will also be determined. The effects of G395 will also be compared to those of G108, the long-acting GCG analogue used in previous chapters. Furthermore, a Zinc-G395 preparation (Zn-G395/Zn-SA) will also be used to provide information from an intermediate acting analogue. Assuming no effect on food intake and maintained dietary protein intake, it is hypothesised that the SA G395 will not result in a marked degree of hypoaminoacidemia or lean mass loss, as observed with the LA G108. Furthermore, it is hypothesised that in the chronic setting, cumulative metabolic benefits of GCGR signalling, for example improved hepatic lipid metabolism may still be observed with G395 administration. Therefore, the SA GCG analogue G395 may confer a net benefit over the LA GCG analogue G108.

As biased signalling is a potential strategy to differentially activate downstream receptor signals, the known partial agonist properties of the GLP1/GCGR co-agonist peptides SRB103Q and SRB103H (Pickford, Lucey et al. 2021) will be used to explore the effects on both blood glucose and plasma amino acids. It is hypothesised that with the selective partial agonism at the GLP1R to reduce beta-arrestin recruitment, whilst remaining a

balanced partial agonist at the GCGR for both beta-arrestin and Gs recruitment, that SRB103Q may confer sustained improvements in blood glucose whilst not affecting hepatic amino acid catabolism.

6.2 Chapter summary and aims

- Determine an appropriate dose of short-acting (SA) GCG analogue, G395, which does not alter food intake.
- Determine whether the SA GCG analogue, G395, acutely reduces plasma amino acids compared to the long-acting (LA) GCG G108.
- In a chronic study, compared to G108, determine whether daily administration of G395 leads to a chronic suppression in amino acids and/or loss of lean mass.
- In a chronic study, compared to G108, determine whether daily administration of G395 leads to metabolic benefits attributed to GCGR signalling.
- Determine whether partial agonism in a GLP1/GCGR co-agonist can optimise metabolic benefits whilst not affecting hepatic amino acid catabolism.

6.3 Methods

6.3.1 Peptide selection

G395 was screened from a library of peptides with accompanying receptor potency data. G395 demonstrated similar receptor potency to G108 in human hepatocyte cell lines and therefore its GCGR selectivity was similar to native glucagon. G395 differed from G108 mainly by the lack of a long fatty acid tail at the c-terminal end. It therefore did not contain any peptide sequence modifications which would be expected to enhance its half-life. 7.5 nmol/kg G108 was used as a head-to-head comparator in the following studies due to its established effect on plasma amino acids and lean mass in obese mice (Chapter 3 and 4). Furthermore, a Zinc preparation of G395 was made by reconstituting G395 in a 0.5 mM ZnCl solution. This aimed to produce an intermediate acting preparation of the SA G395 analogue, and therefore provide further insight into the effect of drug exposure to the outcomes measured.

For the acute study investigating the effect of partial agonism, the peptides 'SRB103Q' and 'SRB103H' were used (Pickford, Lucey et al. 2021).

6.3.2 Acute dose finding study of SA GCG (G395) in DIO mice

Thirty-five 14-week-old lean male C57BL/6J mice were provided ad libitum with a 60 kcal% high fat diet and free access to water. 1 week prior to study commencement, all mice were individually housed. Mice were equally split into five groups and received a single subcutaneous dose of their respective treatment at 9 am: Vehicle control (N=6), 20 nmol/kg G395 (N=5), 50 nmol/kg G395 (N=6), 100 nmol/kg G395 (N=6), 7.5 nmol/kg G108 (N=5). Food intake was recorded at baseline and 24 hours later. A glucose time course profile was carried out including at baseline and following dose administration at 1, 2, 4 and 8 hours later. Blood glucose was measured using a handheld glucometer (GlucoRx). A tail venesection was performed at 4 hours and 24 hours following dose administration and plasma was analysed for total amino acids using a commercially available kit (MAK002, Sigma).

6.3.3 Chronic daily dosing study of SA GCG (G395) vs LA GCG (G108) in DIO mice

Following a 7-day wash-out period, twenty-eight 14-week-old lean C57BL/6J mice used in the dose finding study were then used in a chronic daily dosing study. In this study the 50 nmol/kg G395 dose was taken forward for a head-to-head comparison with 7.5 nmol/kg G108 with a vehicle control group. Furthermore, a Zinc-G395 preparation was made where the G395 dose was equivalent to the non-zinc preparation at 50 nmol/kg. Mice received a single subcutaneous dose of their respective treatment at 9am: Vehicle control (N=7), 50 nmol/kg G395 (N=7), 50 nmol/kg Zn-G395 (N=7) and 7.5 nmol/kg G108 (N=7). Food weight and body weight was measured daily, and all mice had ad libitum access to a 60 kcal% high fat diet and water. Lean mass was measured at baseline and on day 11 using MRI as previously described (Chapter 2, section 2.3.10). A tail venesection was performed on day 7 of the study 4-hours post dose and plasma was analysed for total amino acids as described in the previous section. An intraperitoneal tolerance test (2 g/kg) was carried out on day 11 of the study and described previously (Chapter 3, section 3.3.6). At the end of the study, liver was dissected and snap frozen in liquid nitrogen before storage at -80^{o}C. Frozen liver sections were then produced (as

previously described in Chapter 3, section 3.3.10), prior to imaging under bright field microscopy at x 10 magnification.

6.3.4 Biased-partial agonism by a GLP1/GCGR co-agonist at both the GLP1R and GCGR to optimise metabolic benefits whilst minimising amino acid catabolism

Twenty-four 14-week-old C57BL/6J mice were group housed (N=5/cage). Three groups were established: Vehicle control (N=8), 20 nmol/kg SRB103Q (N=8) and 20 nmol/kg SRB103H (N=8). All animals underwent tail venesection to collect blood at baseline, and 2, 4 and 8 hours following a single dose of vehicle of 20 nmol/kg peptide. Blood glucose was also measured at all time-points using a handheld glucometer (GlucoRx). The blood samples collected were processed and plasma alanine levels measured using a commercially available it (MAK001, Sigma).

6.4 Results

Key results for Chapter 6

- **An acute dose-finding study of a short-acting glucagon analogue demonstrates acute improvements in glycaemia with no effect on plasma total amino acids.**
- **Chronic administration of a short-acting glucagon analogue confers metabolic benefits with no detectable effect on plasma total amino acids and lean mass.**
- **Partial agonism to produce bias at the GLP-1R within a GLP-1R/GCGR co-agonist is a potential strategy to optimise receptor signalling.**

6.4.1 Acute dose-finding study of the SA GCG analogue G395

Following a single ascending dose of SA GCG (20-100 nmol/kg G395) or LA GCG (7.5 nmol/kg G108), there was no significant difference in food intake (Figure 6.1). A trend towards increased food intake was observed in the 100 nmol/kg SA GCG dose group (Figure 6.1A). A time course-profile following the single-dose administration revealed a dose-dependent improvement in blood glucose levels at 2- and 4-hours following SA GCG administration (Figure 6.1B and Table 6.1, 2-hour post dose glucose levels, mean ± SEM (mmol/l); Vehicle control: 9.85 ± 0.65, 20 nmol/kg G395: 8.86 ± 0.52, 50 nmol/kg G395: 7.55 ± 0.42, 100 nmol/kg G395: 6.32 ± 0.28, 4-hour post dose; Vehicle control: 8.88 ± 0.24, 20 nmol/kg G395: 7.66 ± 0.08, 50 nmol/kg G395: 7.28 ± 0.61, 100 nmol/kg G395: 5.97 ± 0.25. The LA GCG analogue G108 also improved blood glucose levels following a single-dose however its maximum effect observed was at the 8-hour time point (6.38 ± 0.47 mmol/l), by which time the glucose levels from all SA GCG doses had converged with the average glucose level from the vehicle control group (Figure 6.1B, Table 6.1). In keeping with these results an incremental area under the curve also demonstrated an improvement in glycaemia following a single dose of either treatment dose (Figure 6.1C).

To determine whether total plasma amino acids are differentially affected by SA and LA glucagon, these were also measured at both 4- and 24- hours following single-dose administration of G395 or G108. No change was observed in total plasma amino acids at either time point following either 20, 50 or 100 nmol/kg of G395 (Figure 6.1D, Table 6.2). In contrast, compared to the vehicle control group, a reduction in total plasma amino acids was observed following a single-dose of 7.5 nmol/kg G108 at the 4-hour time-point, which reached statistical difference at 24-hours (Figure 6.1D and Table 6.2, 4-hour post dose total plasma amino acids, mean ± SEM, (nmol/μl); Vehicle control: 1.57 ± 0.15, 7.5 nmol/kg G108: 1.12 ± 0.03, 24-hour post dose; Vehicle control: 1.81 ± 0.33, 7.5 nmol/kg G108: 1.07 ± 0.25.

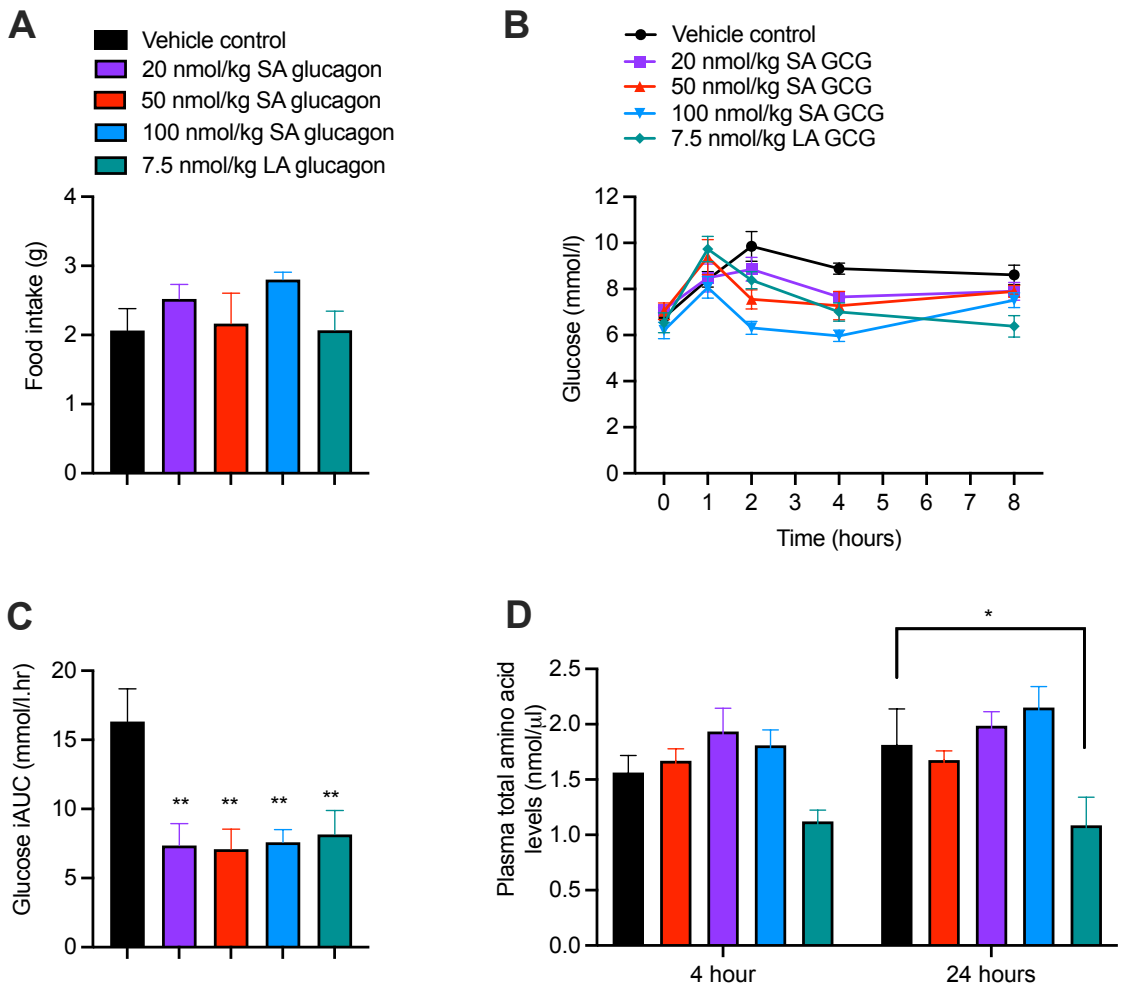


Figure 6.1: Differential effects of a single dose of a short-acting (SA) GCG analogue (G395) and the long-acting (LA) GCG analogue (G108) in C57BL/6J DIO mice. N=5-6/group. **(A)** Food intake (g) over 24 hours. **(B)** Glucose time-course profile over 8 hours. **(C)** Incremental area-under the curve (iAUC) for glucose time course-profile as in B. **(D)** Plasma total amino acids at 4- and 24- hours following dose administration. Data plotted as mean ± SEM. *p<0.05, **p<0.01. Data analysed using one-way ANOVA with post hoc Dunnett's test. Glucose time course profile and plasma amino acids data analysed using two-way ANOVA with post hoc Dunnett's test.

	Vehicle control	20 nmol/kg SA	50 nmol/kg SA	100 nmol/kg SA	7.5 nmol/kg LA
Baseline	6.77 ± 0.16	7.10 ± 0.12	7.03 ± 0.36	6.20 ± 0.35	6.54 ± 0.43
1-hour	8.42 ± 0.33	8.48 ± 0.60	9.38 ± 0.76	8.07 ± 0.46	9.73 ± 0.55
2-hour	9.85 ± 0.65	8.86 ± 0.52	7.55 ± 0.42 ***P*<0.05**	6.32 ± 0.28. ***P*<0.01**	8.38 ± 0.37
4-hour	8.88 ± 0.24	7.66 ± 0.08 ***P*<0.001**	7.28 ± 0.61	5.97 ± 0.25 ***P*<0.0001**	7.00 ± 0.40
8-hour	8.62 ± 0.42	7.90 ± 0.38	7.90 ± 0.25	7.52 ± 0.32	6.38 ± 0.47 ***P*<0.05**

Table 6.1: Glucose time-course profile following single dose administration of vehicle control, 20 nmol/kg G395, 50 nmol/kg G395, 100 nmol/kg G395 and 7.5 nmol/kg G108. Data represented as mean ± SEM, significance indicates difference between vehicle control group and treatment group, *p<0.05, **p<0.01, ****p<0.0001. Analysed using two-way ANOVA with post hoc Dunnett's test.

	Vehicle control	20 nmol/kg SA	50 nmol/kg SA	100 nmol/kg SA	7.5 nmol/kg LA
4-hour	1.57 ± 0.15	1.67 ± 0.11	1.93 ± 0.21	1.81 ± 0.14	1.12 ± 0.03
24-hour	1.81 ± 0.33	1.68 ± 0.09	1.99 ± 0.13	2.15 ± 0.18	1.07 ± 0.25 ***P*<0.05**

Table 6.2: Plasma total amino acids at 4- and 24-hour timepoints following single dose administration of vehicle control, 20 nmol/kg G395, 50 nmol/kg G395, 100 nmol/kg G395 and 7.5 nmol/kg G108. Data represented as mean ± SEM, significance indicates difference between vehicle control group and treatment group, *p<0.05. Analysed using two-way ANOVA with post hoc Dunnett's test.

6.4.2 Chronic daily dosing of the short acting glucagon analogue G395 does not cause plasma amino acid suppression or lean mass loss

G395 and G108 were then used to determine whether chronic treatment with a SA GCG analogue (G395) delivers a net pharmacological benefit over a LA GCG (G108) analogue, by minimising hepatic amino acid catabolism and lean mass loss whilst sustaining other metabolic benefits. The metabolic benefits measured in this daily dosing study included glucose tolerance as measured by an intraperitoneal glucose tolerance test (ipGTT) on day 11, plasma cholesterol on day 7 and hepatic lipid accumulation at the end of the study.

Over 11 days of daily dosing with G395, Zn-G395 or G108, there was no significant change in food intake compared to vehicle control, confirming sub-anorectic doses used (Figure 6.2A). Over 11 days, mice in the G108 group lost a significant amount of body weight

compared to vehicle control group (Figure 6.2, Table 6.3). Consistent with enhanced energy expenditure (Chapters 3 and 4), the long-acting peptide (G108) led to a reduced feed efficiency compared to vehicle control (Figure 6.2D, Table 6.3). In keeping with the findings from Chapter 4, enhanced energy expenditure was associated with reduced total plasma amino acids in mice receiving G108 (Figure 6.2E, Table 6.3). A trend towards reduced total amino acids was observed in the Zn-G395 group (Figure 6.2E, Table 6.3) and this was consistent with a similar pattern of lean mass loss over the course of the study (Figure 6.2F, Table 6.3) and terminal weights of the quadriceps muscle (Figure 6.2G, Table 6.3). In keeping with findings from Chapter 4, the fat mass reduction in the G108 group (Figure 6.2H, Table 6.3) was associated with hypoaminoacidemia (Figure 6.2E) and markers of enhanced energy expenditure (Figure 6.2D).

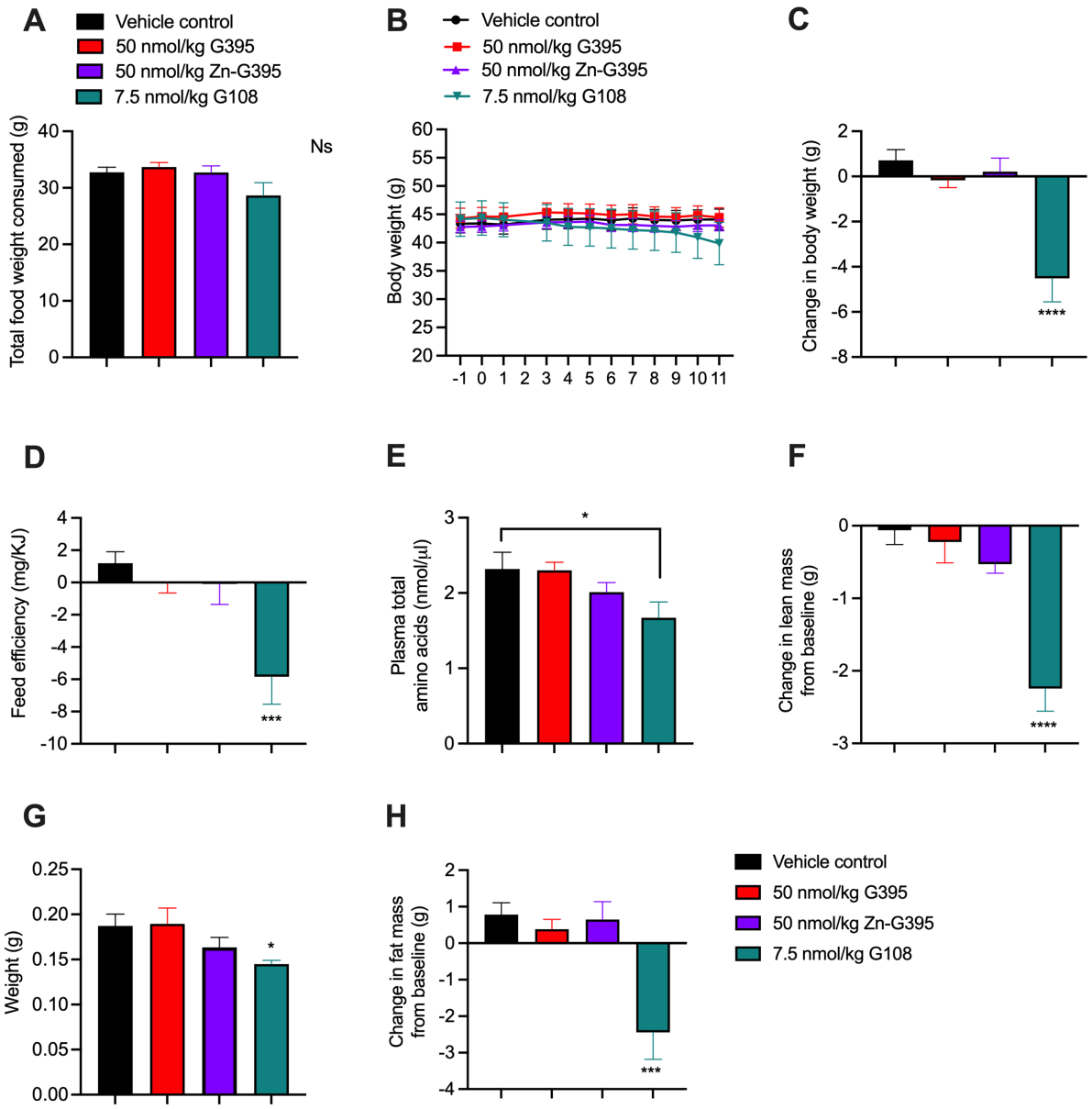


Figure 6.2: Chronic daily dosing study of 50 nmol/kg G395 (short acting), 50 nmol/kg Zn-G395 (intermediate acting) and 7.5 nmol/kg G108 (long-acting) in C57BL/6J DIO mice, N=7/group. **(A)** Total food weight (g) consumed over 11 days. **(B)** Daily change in body weight (g) over the study. **(C)** Total change in lean mass from baseline (%). **(D)** Feed efficiency (mg/KJ) **(E)** Plasma total amino acids on day 7 of study 4-hour post dose. **(F)** Change in lean mass (g) from baseline. **(G)** Quadriceps weight (g) at the end of the study. **(H)** Change in fat mass (g) from baseline. Data plotted as mean ± SEM, *p<0.05, ****p<0.0001. Data analysed using one-way ANOVA with post hoc Dunnett's test.

	Vehicle control	50 nmol/kg G395	50 nmol/kg Zn-G395	7.5 nmol/kg G108
Plasma total amino acids (nmol/μl)	2.32 ± 0.22	2.30 ± 0.10	2.011 ± 0.13	1.67 ± 0.21 ***P*<0.05**
Total body weight loss from baseline (g)	0.71 ± 0.48	-0.17 ± 0.32	0.21 ± 0.60	-4.51 ± 1.04 ***P*<0.0001**
Feed efficiency (mg/KJ)	1.19 ± 0.71	-0.04 ± 0.60	-0.08 ± 1.28	-5.84 ± 1.71 ***P*<0.001**
Change in fat mass from baseline (g)	0.78 ± 0.33	0.38 ± 0.27	0.65 ± 0.49	-2.44 ± 0.74 ***P*<0.001**
Change in lean mass from baseline (g)	-0.06 ± 0.19	-0.22 ± 0.29	-0.53 ± 0.12	-2.24 ± 0.31 ***P*<0.0001**
Quadriceps weight (g)	0.19 ± 0.01	0.19 ± 0.02	0.16 ± 0.01	0.14 ± 0.01 ***P*<0.05**

Table 6.3: Plasma total amino acids (nmol/μl), change in lean mass from baseline (%) and quadriceps weight (g). Data plotted as mean ± SEM, *p<0.05, ****p<0.0001. Data analysed using one-way ANOVA with post hoc Dunnett's test.

6.4.3 Chronic daily dosing of the short-acting glucagon analogue G395 confers similar metabolic benefits to the long-acting glucagon analogue G108

After confirming that the short-acting GCG analogue G395 does not cause chronic suppression of plasma amino acids and subsequent lean mass loss relative to the longer acting G108, beneficial metabolic effects were further explored. These included an intraperitoneal glucose tolerance test (ipGTT), plasma cholesterol and an assessment of hepatic lipid accumulation.

An ipGTT on day 11 demonstrated a significant improvement in glucose tolerance across all treatment groups (Figure 6.3A, 6.4.3B Table 6.4). The degree of improvement

observed in blood glucose at both baseline (fasting glucose) and throughout the 2-hour period was comparable for G395 and G108. After 7 days of daily dosing there was a marked reduction in plasma total cholesterol in the group receiving G108 (Figure 6.4C, Table 6.4). There was a trend towards reduction in total cholesterol in mice receiving G395 and a significant reduction was observed in mice receiving Zn-G395 (Figure 6.4C, Table 6.4). To assess the degree of hepatic lipid accumulation across treatment groups directly, liver lobes were imaged and then collected to image frozen liver sections. At the end of the study, there was macroscopic evidence of greater lipid accumulation in the livers of mice receiving vehicle control (Figure 6.3D). In contrast, livers from mice receiving either G395 or G108 appeared to have a reduction in lipid accumulation. This was confirmed by imaging frozen liver sections under bright field microscopy, demonstrating reduced lipid droplets in the G395 and G108 groups (Figure 6.3E)

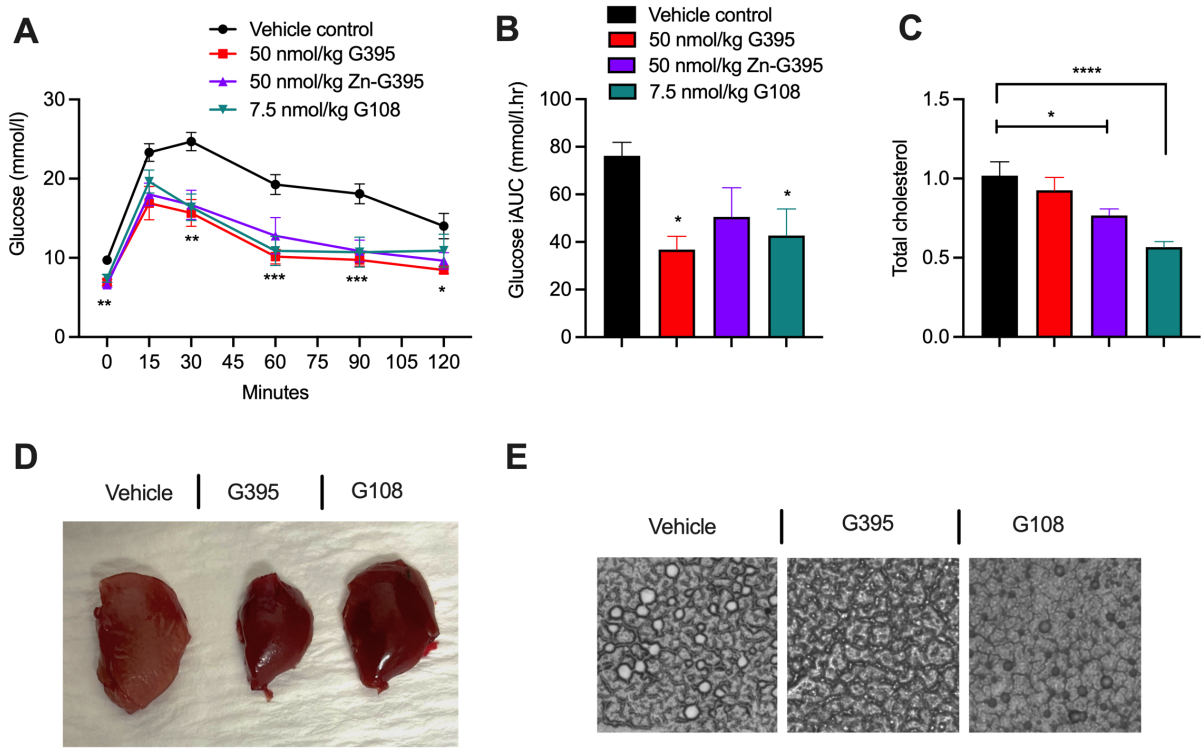


Figure 6.3: Effects of chronic administration of 50nmol/kg G395. 50 nmol/kg Zn-G395 and 7.5 nmol/kg G108 on glucose tolerance, plasma cholesterol and hepatic lipid accumulation. **(A)** Day 11 intraperitoneal glucose tolerance test (2g/kg), significance indicates glucose levels compared to vehicle control. **(B)** Area under the curve (iAUC) for ipGTT in 'A'. **(C)** Total plasma cholesterol levels 4-hours post dose on day 7 of study. **(D)** Representative ex vivo images of liver lobes from each treatment group. **(E)** Representative images of liver sections imaged under bright-field microscopy at x10 magnification, white hepatic lipid

droplets indicate lipid accumulation. Data plotted as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001. Data analysed with one- or two-way ANOVA with post hoc Dunnett's tests.

	Vehicle control	50 nmol/kg G395	50 nmol/kg Zn-G395	7.5 nmol/kg G108
Baseline (0 mins)	9.71 ± 0.45	6.92 ± 0.40 ***P*<0.01**	6.60 ± 0.31 ***P*<0.001**	7.41 ± 0.47 ***P*<0.01**
15 min	23.30 ± 1.11	16.92 ± 2.10	18.01 ± 1.42 ***P*<0.05**	19.64 ± 1.44
30 min	24.69 ± 1.56	15.67 ± 1.70 ***P*<0.01**	16.70 ± 1.84 ***P*<0.05**	16.39 ± 1.69 ***P*<0.01**
60 min	19.27 ± 1.25	10.15 ± 0.90 ***P*<0.001**	12.80 ± 2.30	10.87 ± 1.83 ***P*<0.01**
90 min	18.09 ± 1.25	9.73 ± 0.77 ***P*<0.001**	10.86 ± 1.38 ***P*<0.01**	10.73 ± 1.89 ***P*<0.05**
120 min	14.01 ± 1.60	8.47 ± 0.47 ***P*<0.05**	9.63 ± 1.04	10.91 ± 2.08

Table 6.4: Intraperitoneal glucose tolerance test (2g/kg) on day 11 of study, vehicle control, 50nmol/kg G395. 50 nmol/kg Zn-G395 and 7.5 nmol/kg G108. Data represented as mean ± SEM, significance indicates difference between vehicle control group and treatment group, *p < 0.05, **p <0.01, ***p<0.001. ****p < 0.0001. Analysed using two-way ANOVA with post hoc Dunnett's test.

	Vehicle control	50 nmol/kg G395	50 nmol/kg Zn-G395	7.5 nmol/kg G108
Glucose iAUC (mmol/l.hr)	76.20 ± 5.65	36.78 ± 5.63 ***P*<0.05**	50.51 ± 12.31	42.73 ± 11.13. ***P*<0.05**
Total plasma cholesterol (μg/μl)	1.02 ± 0.09	0.93 ± 0.08	0.77 ± 0.04 ***P*<0.05**	0.56 ± 0.04 ***P*<0.001**

Table 6.5: Glucose iAUC from iPGTT on day 11 and total plasma cholesterol on day 7 of study, Vehicle control. 50nmol/kg G395. 50 nmol/kg Zn-G395 and 7.5 nmol/kg G108. Data represented as mean ± SEM, significance indicates difference between vehicle control group and treatment group, *p<0.05, **p<0.01, ***p<0.001. ****p<0.0001. Analysed using two-way ANOVA with post hoc Dunnett's test.

6.4.4 Biased-partial agonism by a GLP1/GCGR co-agonist at both the GLP1R and GCGR to optimise metabolic benefits whilst minimising amino acid catabolism

To determine whether it is possible for biased-partial agonism within a GLP1R/GCGR co-agonist, to optimise metabolic effects, the peptides SRB103Q and SRB103H were used (Pickford, Lucey et al. 2021). As discussed previously (section 6.1.3), SRB103H is a full agonist at both the GLP1R and GCGR, whereas SRB103Q is a partial agonist at both receptors, showing bias for reduced beta-arrestin recruitment specifically at the GLP1R. Therefore, it is possible that SRB103Q may confer sustained efficacy on improved glucose regulation, with no change in hepatic amino acid catabolism.

Following a single dose of either peptide, there was an acute reduction in glucose levels at 2 hours, after which point, they diverged with SRB103Q showing a lower and more sustained improvement in glucose levels compared to vehicle control (Figure 6.4A). At the 8-hour timepoint after dose administration, only the SRB103Q group demonstrated a significantly reduced blood glucose level. Plasma alanine also reduced acutely following the single dose of either peptide (Figure 6.4B), however the effect size was less marked as the effect on blood glucose. In contrast to the divergent effect of the peptides on blood glucose levels, both SRB103Q and SRB103H led to a sustained moderate reduction in plasma alanine levels compared to vehicle control (Figure 6.4B).

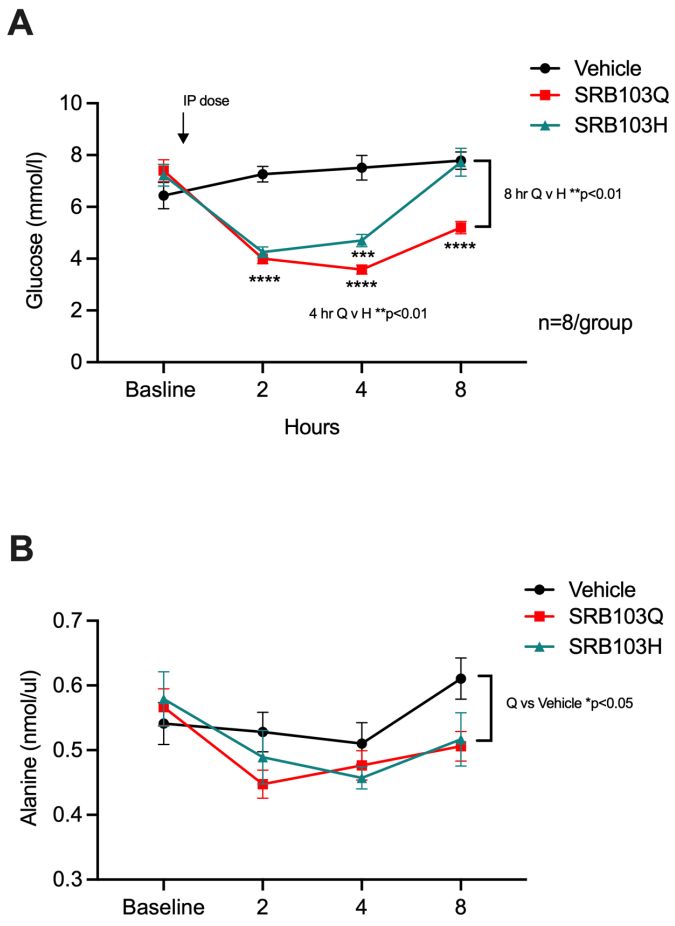


Figure 6.4: (A) Glucose and **(B)** plasma alanine levels following a single dose of either vehicle control, 20 nmol/kg SRB103Q or 20 nmol/kg SRB103H, N=8/group. Measurements taken at baseline, 2, 4 and 8 hours following dose administration. Data plotted as mean ± SEM, *p<0.05, ***p<0.001, ****p<0.0001, significance indicates difference between treatment group compared to vehicle control. Analysed using two-way ANOVA with post hoc Dunnett's test.

6.5 Discussion

6.5.1 The short-acting GCG analogue, G395, does not stimulate hypoaminoacidemia or lean mass loss

The findings from Chapter 2 and 3 demonstrated that the long-acting GCG analogue, G108, stimulated hepatic amino acid catabolism, hypoaminoacidemia and lean mass loss. To establish whether this occurred with administration of a short-acting GCG analogue, G395 was first administered in an acute dose-ranging study to determine effects on food intake and plasma amino acids. As with the previously established sub-anorectic dose of G108 (7.5nmol/kg), doses of G395 ranging from 20 nmol/kg to 100 nmol/kg did not have a notable effect on food intake (Figure 6.1A). Furthermore, G395 did not affect plasma amino acids at 4- or 24-hours following a single dose in contrast to G108 (Figure 6.1D). As all doses of G395 led to improved blood glucose acutely (Figure 6.1B and C), this provides a positive sign that G395 may offer a net metabolic benefit (ie glycaemic benefits without lean mass loss) in the context of chronic daily dosing.

A dose of 50 nmol/kg G395 was tested head-to-head with 7.5 nmol/kg G108 in a chronic daily dosing study in DIO mice. This confirmed a negligible effect of the SA GCG analogue on supressing plasma amino acids and lean mass loss (6.2E, F and G). In contrast, and in keeping with findings from Chapter 2 and 3, G108 led to a marked suppression in plasma amino acids on day 7 and lean mass loss over the course of the study (Figure 6.2E and F). The zinc preparation of G395 led to a trend in reduction in plasma amino acids on day 7 and this was also associated with a trend in lean mass loss over the course of the study (Figure 6.2E and F). This is in keeping with the mode of action of zinc when combined with glucagon, to enhance depot release from subcutaneous administration and lengthen the drug release into the circulation (F.Trading 1969). Although no circulating peptide concentrations are available, it is possible that compared to the non-zinc preparation, a sustained release of the Zn-G395 preparation led to prolonged hepatic GCGR activity and subsequent effects on amino acid catabolism. In keeping with this a similar pattern in muscle weight was observed whereby the average quadriceps weight from the G108 group was significantly rescued compared to vehicle control (Figure 6.2G). A trend to reduction in quadriceps weight was observed in the Zn-G395 group whereas no change was observed in the G395 group.

Only G108 led to both a chronic suppression in total amino acids and a reduction in fat mass (Figure 6.2E and H). This is in keeping with the results from Chapter 4 which demonstrate that hypoaminoacidemia underpins G108-stimulated energy expenditure and body weight loss. As no reduction in total plasma amino acid levels were observed in mice receiving the SA GCG preparations, energy expenditure was not expected to be increased and therefore no accompanying fat mass loss.

6.5.2 The short-acting GCG analogue, G395, provides metabolic benefits also attributed to the long-acting GCG analogue G108

It could be reasoned that the absence of effect on plasma amino acids or lean mass observed with G395 is due to its potential short half-life and immediate clearance from the body. To explore whether other metabolic effects of GCGR signalling were maintained, glucose tolerance, plasma cholesterol and hepatic lipid accumulation were measured in this daily dosing study. Interestingly, a significant improvement in glucose tolerance in all treatment groups was observed on day 11 of the study (Figure 6.3A and B). Glucagon is well known for its hyperglycaemic properties through enhanced gluconeogenesis and glycogenolysis (Exton 1987). However, a re-emerging field exploring the glucocentric side of glucagon physiology is realising the potential glucose-lowering properties of glucagon. For example, following a single dose, the long-acting glucagon analogue IUB288 enhances hepatic insulin sensitivity through AKT phosphorylation as demonstrated with hyperinsulinaemic euglycaemic clamps (Kim, Holleman et al. 2018). More recently, using the same analogue, it has been shown that the acute GCGR enhancement of hepatic insulin sensitivity through AKT is mediated by the upstream activation of mTORC2 (Kim, Nason et al. 2022). As G395 improved glucose tolerance without any effect on energy expenditure, this would be consistent with a mechanism such as improved hepatic insulin sensitivity which may be mediating these effects, rather than a general increased demand from a negative energy balance.

G108 led to a chronic reduction in plasma cholesterol (Figure 6.3C), in keeping with findings from Chapter 3. Although this was not observed with G395, a reduction in plasma cholesterol was noted in mice treated with the zinc preparation of G395. As glucagon increases hepatic fatty acid oxidation and suppresses de novo lipogenesis (Galsgaard,

Pedersen et al. 2019), the results are in keeping with these actions. Imaging of liver sections demonstrated that G395 reduced hepatic lipid accumulation to a similar degree as seen in the liver of mice treated with G108, compared to the vehicle control group (Figure 6.3D and E). Therefore, these findings suggest that daily administration of SA GCG analogue G395 has an enhanced cumulative effect on chronic hepatic lipid metabolism than on plasma total cholesterol.

How can the differential effects of SA vs LA GCG on lean mass, glucose tolerance and hepatic lipid accumulation be explained? It is possible that G395 has divergent effects on macronutrient metabolic pathways at a given dose. For example, at a given dose of G395, there may be a more pronounced effect on glucose regulation and hepatic lipid accumulation, compared to hepatic amino acid catabolism. For example, the dose-response curve for the former outcomes may be 'leftward shifted'. In contrast, sustained GCGR activity may be required for hepatic amino acid catabolism to effect plasma levels and so therefore may be precluded with a short acting analogue with a shorter plasma half-life. This divergent activity of a fixed dose of G395 could also be explained by varying duration of activation of signalling pathways downstream to the hepatic GCGR. This may be explained by the fact that the metabolic pathways for protein, lipid and glucose regulation in the liver are governed by divergent transcriptional and post-transcriptional mechanisms (Albrechtsen 2021). Together, the findings from the chronic study in this Chapter suggest that short-acting glucagon can be leveraged to provide metabolic benefits whilst minimising side effects, in particular amino acid catabolism and lean mass loss. Therefore, further work is required to fully characterise the changes in GCGR-dependent signalling pathways which, when SA GCG is administered, confers a net metabolic benefit and importantly mitigates against lean mass loss.

6.5.3 Biased-partial agonism by a GLP1/GCGR to optimise metabolic benefits whilst minimising amino acid catabolism

Biased agonism at the GLP1 receptor has been shown as a strategy to improve efficacy of GLP-1 analogues, in particular by enhancing their insulinotopic effects at the beta-cell (Jones 2022). GLP1/GCGR co-agonists have been employed in various drug development programmes for obesity and metabolic disease, to leverage multiple benefits of glucagon

signalling (Hope, Vincent et al. 2021). Recently, it was demonstrated that the primary peptide sequence of a co-agonist could be modified to confer bias through partial agonism at the GLP1R (Pickford, Lucey et al. 2021); The peptide SRB103Q was demonstrated to be biased at the GLP1R leading to reduced beta-arrestin recruitment through partial agonism. As beta-arrestin recruitment is key for receptor endocytosis, the reduced recruitment translated into sustained improvements in glucose tolerance in vivo compared to the full GLP1 unbiased agonist, SRB103H (Figure 6.4A). Interestingly, previous in vitro analysis demonstrated that SRB103Q was also a balanced partial agonist at the GCGR for Gs and beta-arrestin, whereas SRB103H was again a full agonist at GCGR (Pickford, Lucey et al. 2021). In keeping with this both peptides led to a sustained moderate reduction in plasma alanine levels (Figure 6.4B) and this is consistent with a similar degree of lean mass loss observed previously (Pickford, Lucey et al. 2021).

Based on the findings of the SA GCG analogue (G395), it is not expected that the short-acting SRB compounds would lead to sustained amino acid catabolism, such as seen with the LA GCG analogue, G108. Indeed, using the SRB compounds, plasma alanine was measured, a more sensitive marker of glucagon-stimulated amino acid catabolism. The findings from this chapter demonstrate that although the glucose lowering properties of SRB103Q are prolonged after a single dose, there is no change in plasma alanine between SRB103Q and the full agonist SRB103H. This suggests that the balanced partial agonism for Gs and beta-arrestin recruitment at the GCGR by SRB103Q does not lead to sustained or prolonged GCGR activation, compared to SRB103H. Whether this strategy would be beneficial to optimise long-acting GLP-1R/GCGR co-agonists by minimising hypoaminoacidemia requires further investigation.

6.6 Conclusions

The findings from this Chapter suggest that SA GCG therapy may be a promising approach for the treatment of metabolic disease, and indeed may be required to circumvent excess amino acid catabolism and lean mass loss. Currently, most GCGR-targeted multi-agonists in development are long-acting preparations. However, a number of these have not succeeded due to adverse events reported from early phase clinical trials (Hope, Vincent et al. 2021). Indeed, recently, the long-acting GLP1R/GCGR co-agonist NN1177 was shown to worsen glucose tolerance, lower plasma amino acids and present

cardiovascular risk, highlighting the deleterious effects of sustained GCGR activity (Martin Friedrichsen 2022). In contrast, the co-agonist which has progressed the furthest through clinical trials is the short-acting, once daily Cotadutide with no cardiovascular concerns and reduced effects on plasma amino acids (Parker, Robertson et al. 2020, Nahra, Wang et al. 2021). Although the improved safety profile of Cotadutide may be linked to its reduced receptor potency to both the GLP1R and GCGR, the short-acting nature of the drug may also contribute to its success by reducing drug exposure time and therefore the average time of GCGR activation.

From a drug development perspective, it may be possible to decouple GLP1 from glucagon and obtain metabolic benefits from SA glucagon whilst still administering LA GLP1. This approach would have the benefit of reducing plasma amino acid suppression and lean mass loss, whilst still offering metabolic benefits of glucagon for example hepatic lipid clearance and improved hepatic insulin sensitivity. However, the ideal ratio of GCGR to GLP1 potency and doses administered will need to be established to optimise GLP-1/GCGR co-agonism. Furthermore, through engineering bias agonism in a unimolecular co-agonist, it may be possible to prolong the insulinotropic effects of GLP1R signalling, whilst not prolonging GCGR activity and the subsequent excess amino acid catabolism. Based on recent updates in understanding of glucagon biology detailing the dynamic and adaptive roles of glucagon in physiology (Capozzi, D'Alessio et al. 2022), altering GCGR activity as described within a multi-agonist strategy may be a promising approach.

7 Chapter 7 – General discussions

7.1 100 years of glucagon research highlight the diverse actions of this intriguing hormone

It is 100 years since the discovery of glucagon (Kimball C 1923), and since then research on this intriguing hormone has paved the way to a greater understanding of its diverse physiological effects (Capozzi, D'Alessio et al. 2022). More recently (over the last two decades), glucagon research has intensified due to characterisation of favourable metabolic actions which may be targeted towards obesity and metabolic disease therapy. Of these, the description of enhanced energy expenditure and hepatic lipid catabolism in pre-clinical models testing GLP1R/GCGR co-agonists (Pocai, Carrington et al. 2009, Day, Gelfanov et al. 2012) have led to the positioning of GCGR-targeted multi-agonists in many drug development pipelines (Hope, Vincent et al. 2021).

Emerging evidence highlights a primary physiological action of glucagon is amino acid metabolism, and is perhaps of equal importance to glucose regulation (Emilie Elmelund 2022). Indeed, the recent 'Glucagon Meeting' (Copenhagen, Danish Diabetes Academy/Novo Nordisk Foundation) highlighted the amino-acid centric nature of important aspects of glucagon signalling. This thesis focuses on glucagon-stimulated amino acid metabolism, an underexplored area of chronic GCGR activity in a therapeutic context. A long-acting GCG analogue was used as a research tool to explore the chronic effects on hepatic amino acid catabolism and plasma amino acid availability which may have wider physiological implications on lean mass, total body weight and energy expenditure. This project therefore has relevance to the development of GCGR-targeted multi-agonists, currently positioned in a variety of drug development programmes for the treatment of obesity and metabolic disease. Indeed, part of this thesis also included a phase 1 trial of a GLP-1R/GCGR co-agonist which explored the effect on amino acid metabolism. The project therefore aimed to provide further understanding of the biology of glucagon in the context of a chronic treatment setting, whilst also demonstrating translational relevance.

7.2 The actions of G108, a long-acting GCG analogue, are consistent with the key amino acid catabolic action of glucagon

The activity of glucagon on whole-body nitrogen balance was noted early on with studies in humans and animals noting enhanced urinary nitrogen excretion, suggesting a protein catabolic action (Tyberghein 1953, Salter, Davidson et al. 1957, Ezrin, Salter et al. 1958, Salter 1960, Salter, Ezrin et al. 1960). Subsequent biochemical and molecular characterisation at the tissue level further confirmed increased activity and upregulation of key enzymes of the urea/ornithine cycle (Snodgrass, Lin et al. 1978, Ulbright and Snodgrass 1993). Altered amino acid catabolism due to glucagon receptor signalling is also evidenced from different clinical conditions where there is excess or a lack of the hormone. For example, in the case of the glucagonoma syndrome, a broad reduction in plasma amino acids is observed (Mallinson, Bloom et al. 1974). In contrast, in patients lacking pancreatic glucagon secretion, ie., pancreatectomised patients, display marked increase in circulating plasma amino acids (hyperaminoacidemia).

As a rationally designed glucagon analogue, it is therefore perhaps not surprising that G108 leads to an increased upregulation of key components of the urea cycle in both the acute and chronic setting. Furthermore, G108 increased the upregulation of hepatic amino acid transporters, also consistent with findings by others using either glucagon-receptor knockout models or models of fatty liver disease (Winther-Sorensen, Galsgaard et al. 2020, Emilie Elmelund 2022). The upregulation of amino acid catabolic pathways was associated with a significant reduction in plasma *total* amino acids, and in the chronic setting, a unique profile of hypoaminoacidemia was observed in diet-induced-obese (DIO) mice (Chapter 3, Section 3.4.5). In particular, the largest reductions in plasma essential amino acids were for lysine, methionine and threonine, whereas the largest reductions in non-essential amino acids were for alanine, arginine, asparagine, glutamine, glycine, proline, serine and tyrosine. A lesser effect, albeit still a reductive effect was observed for the following plasma amino acids: histidine, valine, isoleucine, leucine, phenylalanine, tryptophan, aspartate, glutamate and cysteine.

Evidence from multiple sources have previously provided insight into the specific individual amino acids which may be suppressed the most in response to enhanced hepatic GCGR activity. Early infusion studies of glucagon in humans have characterised

the plasma changes in individual plasma amino acids, for example one where 60 ng/kg/hr glucagon infusion over 8 hours led to largest reductions in for example proline, tyrosine, glycine, threonine, and alanine (Boden, Rezvani et al. 1984). In the case of pathological excess, for example the glucagonoma syndrome, similarly the largest reductions in plasma amino acid concentrations were in alanine, methionine, lysine and cysteine, with least affected the aromatic amino acids phenylalanine and tyrosine (Mallinson, Bloom et al. 1974). In the case of pancreatectomised patients, amino acids increased the most are for example serine, alanine, arginine, glycine, threonine and tyrosine (Boden, Master et al. 1980). These studies are from different clinical contexts however describe a similar pattern of amino acid perturbation to G108-stimulated hypoaminoacidemia. However, the effect of glucagon exposure time, circulating glucagon levels and other factors such as dietary nutrition may also play a role in determining these amino acid profiles.

More recent work has sought to also characterise the changes in individual amino acids due to glucagon action in the context of the growing prevalence of metabolic disease (Wewer Albrechtsen, Pedersen et al. 2019). In work which established the relationship between hepatic amino acid catabolism and regulation of alpha-cell mass in *Gcgr*$^{-/-}$ mice, elevated circulating concentrations of methionine, threonine and asparagine were observed with lesser effects on the branched chain amino acids and aromatic amino acids (Solloway, Madjidi et al. 2015). This amino acid and hepatic-alpha cell relationship, the liver-alpha axis has recently emerged as an important feedback system involving amino acids and glucagon (Holst, Wewer Albrechtsen et al. 2017). Other work to characterise the liver-alpha axis in rodents demonstrate a prominent role for alanine, arginine, cysteine and proline (Galsgaard, Jepsen et al. 2020). This is consistent with the use of glucagon receptor agonists and blocking antibodies in rodents (Emilie Elmelund 2022) which highlight a prominent role for these amino acids in addition to asparagine, threonine and methionine which may direct glucagon secretion from the alpha cell and whose clearance is more sensitive to the action of glucagon itself. The amino acids changed the least in the latter study were leucine, isoleucine, glutamate, tryptophan, and phenylalanine (Emilie Elmelund 2022), consistent with the findings of previous work of the liver-alpha relationship (Solloway, Madjidi et al. 2015). Furthermore, when this axis is disrupted directly, for example in the setting of fatty liver disease, a similar

perturbation in amino acids is observed (Wewer Albrechtsen, Junker et al. 2018). Together, these findings are also broadly consistent with the individual amino acids which change the most with chronic G108 administration in DIO mice, suggesting that chronic glucagon analogue therapy suppresses highly glucogenic amino acids the most, with branched chain and aromatic amino acids more resistant to this suppressive effect. Interestingly, it has been suggested that specific amino acids play more important roles in the liver-alpha axis, for example for glucagon secretion and alpha cell proliferation (Emilie Elmelund 2022). If the liver-alpha axis plays a role in restoring homeostasis, then it seems logical that in a physiological feedback loop, these amino acid candidates are most affected by altered glucagon activity at the level of the hepatic glucagon receptor. It will therefore be important to establish the effect of chronic glucagon-stimulated hypoaminoacidemia on elements of this axis, for example on the alpha cell. Indeed, very recent evidence has emerged demonstrating alpha-cell hypoplasia with chronic GCG analogue therapy in mice (Emilie Elmelund 2022).

Acute and chronic administration of G108 in lean and obese mice, respectively, led to upregulation of several enzymes of the urea cycle. In the acute setting, increases were observed in *Cps1*, *Ass1*, *Asl* and *Arg*, however this was a relatively large dose (20 nmol/kg) (Chapter 2, Section 2.4.4). An increase in these in response to the same single-high dose of G108 was blunted in hepatic specific $Gcgr^{-/-}$ mice (Chapter 2, Section 2.4.6). After 27 days of dosing with a lower, sub-anorectic dose (7.5 nmol/kg) of G018 in obese mice, upregulation was observed for *Nags*, *Cps1*, *Ass* and *Asl* with *Ass* showing the highest increase (Chapter 3, Section 3.4.4). Interestingly, recent evidence suggests a divergent effect of altered hepatic GCGR activity on transcriptional and post-transcriptional mechanisms of amino acid catabolic pathways. One of the first studies to investigate glucagon regulation of the urea cycle showed an increased enzyme activity of all 5 enzymes (Snodgrass, Lin et al. 1978). Further studies have shown that GCGR activity may regulate the gene expression of all the urea cycle enzymes (Solloway, Madjidi et al. 2015, Kim, Okamoto et al. 2017, Winther-Sorensen, Galsgaard et al. 2020). In a recent study, chronic therapy with a long-acting GCG analogue in mice leads to increased transcriptional upregulation of *Ass1* but not *Arg*, *Asl* or *Otc* (Emilie Elmelund 2022). Furthermore, whilst the *Cps1* was not upregulated, the enzymatic activity of CPS1 was significantly increased. This is consistent with the ability of glucagon to enhance CPS1

activity through deacetylation by Sirtuin proteins (Li, Zhang et al. 2016). Other mechanisms of glucagon-mediated increase in urea cycle activity have been suggested including increased glutaminase activity, which provides glutamate to NAGS to produce the co-enzyme NAG to CPS1 (Emilie Elmelund 2022). Therefore, further work is required to understand the variables which may affect this divergent response to hepatic GCGR activation.

Further research is required to understand the physiological effects of glucagon-stimulated suppression of individual amino acids in the context of chronic therapy with CGGR-targeted multi-agonists. In contrast, it may also be important to understand the effect of increases in individual amino acids in the case of hepatic glucagon resistance, ie fatty liver disease. As amino acids have a diverse range of functions, many research questions can be asked. For example, it will be interesting to investigate the physiological effects in various amino-acid sensing tissues, regulated by changes in individual amino acids in response to either chronic GCGR activation (hypoaminoacidemia in the context of therapy) or glucagon resistance (hyperaminoacidemia in the context of metabolic disease). It will also be important to explore the interplay between GCGR-agonist therapy and the concept of glucagon resistance in fatty liver disease.

7.3 Implications of G108-stimulated chronic hypoaminoacidemia on lean mass and a preventative strategy

The advances in peptide biochemistry combined with the inherent similarities between the preproglucagon derived products, glucagon, GLP-1 and oxyntomodulin, paved the way to the description of the first unimolecular co-agonists targeting both the GLP-1R and GCGR (Day, Ottaway et al. 2009, Pocai, Carrington et al. 2009, Day, Gelfanov et al. 2012). These studies immediately highlighted the additional metabolic benefits conferred by GCGR activity with improved lipid catabolism, enhanced energy expenditure and body weight loss *in vivo*. Fat mass loss attributed to enhanced energy expenditure and reduced food intake is clearly a beneficial synergistic action between GLP-1R activity and GCGR activity. However, the consequence of amino acid catabolism attributed to GCGR activity is that lean mass is at risk in a chronic setting and it is thought that this is due to reduced circulating levels of amino acids (Mallinson, Bloom et al. 1974); By extension therefore, an unforeseen consequence of chronic GCGR activation in a

therapeutic setting may also be loss of lean mass. As reduced calorie intake will affect not only fat mass, but also lean mass, it is challenging to identify whether lean mass loss reported in early GLP-1R/GCGR co-agonist studies in rodents are due to GCGR-dependent amino acid catabolism, or due to the GLP1 mediated suppressive effects on appetite. These early studies indeed reported variable degrees of lean mass loss however did not report plasma levels of amino acids and therefore interpretation of the cause of lean mass loss is not possible (Pocai, Carrington et al. 2009, Day, Gelfanov et al. 2012). However, in one study exploring the effects of various co-agonist peptides on body weight and body composition, 'peptide 5' resulted in a greater reduction in lean mass relative to its effect on food intake, suggesting a food-intake independent effect on lean mass (Day, Gelfanov et al. 2012). However, multiple pre-clinical studies (Henderson, Konkar et al. 2016, Evers, Haack et al. 2017, Elvert, Herling et al. 2018) which laid the foundation for progression of candidate GCGR-targeted co-agonists have not reported plasma amino acids or lean mass therefore and therefore may have (unintentionally) taken the focus away from this potential problem. Furthermore, as many GCGR-targeted multi-agonists have only reached early (Phase 1 or 2) trial testing, this further may explain why lean mass data is not available. Therefore, it is still unclear whether GCGR-mediated lean mass loss occurs in those treated with CGGR-targeted multi-agonists.

In this project, a sub-anorectic dosing strategy was used to explore the food-independent effects of G108 on amino acid metabolism and lean mass. G108 led to a marked reduction in lean mass in both lean and obese mice (Chapter 2, Section 2.4.10, Section 2.4.13, Chapter 3, Section 3.4.2). This was independent to food intake, associated with upregulation of the hepatic urea cycle and a broad suppression of plasma individual amino acids (Chapter 3, Section 3.4.4). The most practical and translational approach to try to prevent lean mass loss is through dietary supplementation, as amino acid availability is a known regulator of lean mass (Kamei, Hatazawa et al. 2020). Doubling the dietary protein content (to 40 kcal% protein), broadly rescued total plasma amino acids levels and mitigated lean mass loss in G108-treated obese mice (Chapter 3, Section 3.4.2 and Section 3.4.4). It is likely that a balance of dietary protein intake and G108-stimulated amino acid catabolism ultimately sets the extent of hypoaminoacidemia and lean mass loss; This is perhaps why lean mass loss occurred even in the high protein diet groups,

due to variable protein intakes during the 24-hour time cycle, where G108 is expected to have continued GCGR activity throughout.

Furthermore, the ability of a high protein diet to rescue plasma amino acids and lean mass may be dependent on the dose of glucagon administered. It is notable that since the first characterisation of the glucagonoma syndrome, (Mallinson, Bloom et al. 1974) in which very high circulating levels of glucagon are detected, high protein diets were advised due to the concern that the typical skin rash (necrolytic migratory erythema) is a result of reduced amino acid availability to the dermis. In this project, low doses of G108 were used and dietary protein supplementation was sufficient to broadly rescue G108-stimulated hypoaminoacidemia however whether this is the case at higher doses of G108 is unclear. Furthermore, it will be important to determine which specific individual amino acids regulate lean mass metabolism in the context of chronic GCGR activity and hypoaminoacidemia. For example, recently a meta-analysis has shown that the branched chain amino acids and tryptophan were significantly reduced in humans with sarcopenia (Dai, Lin et al. 2021). Therefore, further understanding of the roles for individual amino acids may allow for targeted amino acid supplementation to prevent lean mass loss in those treated with CGGR-targeted multi-agonists.

If lean mass loss becomes evident in human studies of GCGR-targeted multi-agonists, alternative strategies to prevent this may be required. For example, it will be important to investigate the effect of another key muscle stimulant – exercise, in particular mechanical stimulus through resistance training. Furthermore, the combination of increased dietary protein intake and mechanical stimulus may provide a compelling adjunct to the use of these multi-agonists as they are easily accessible and affordable.

Together the results of this work demonstrate that a long-acting CGG analogue, in the chronic setting, leads to marked hypoaminoacidemia through increased hepatic amino acid catabolism and this is associated with functional lean mass loss (Chapter 2, Section 2.4.10, Section 2.4.13, Chapter 3, Section 3.4.2, Section 3.4.4). However, a simple strategy to rescue glucagon-stimulated hypoaminoacidemia, dietary protein supplementation, is enough to broadly rescue plasma amino acids and mitigate lean mass loss (Chapter 3, Section 3.4.2, Section 3.4.4.).

7.4 Implications of G108-stimulated chronic hypoaminoacidemia on EE and total body weight loss in a pre-clinical model of obesity

Following the initial reports of increased EE in response to glucagon in Rats (Davidson, Salter et al. 1957, Salter, Davidson et al. 1957) the same group quickly confirmed these findings in both rats and humans in further studies (Davidson 1960, Salter 1960, Salter, Ezrin et al. 1960). A key clue to the EE effect of glucagon was in the initial discovery that glucagon-treated Rats (300μg three times daily-high dose) lost more weight than pair-fed controls (Salter, Davidson et al. 1957). Despite initial focus suggesting the role of brown adipose tissue (BAT) in rodents (Joel 1966, Cockburn, Hull et al. 1967, Yahata, Habara et al. 1983), further studies have provided conflicting results (Beaudry, Kaur et al. 2019) and more recently suggest that glucagon-mediated EE in humans is independent to BAT thermogenesis (Salem, Izzi-Engbeaya et al. 2016). However, despite an unclear mechanism, unimolecular co-agonists of GLP-1R/GCGR have shown successful weight loss in obese rodents through GCGR-dependent EE (Day, Ottaway et al. 2009, Pocai, Carrington et al. 2009), and enhanced EE has been confirmed in human glucagon-infusion studies with doses of glucagon of up to 50 ng/kg/min (Tan, Field et al. 2013, Salem, Izzi-Engbeaya et al. 2016). In 2013, Habbager *et al*., reported that hepatic derived FGF21 is a main regulator of glucagon-mediated EE in obese mice, using a long-acting acylated glucagon IUB288 (Habegger, Stemmer et al. 2013). In keeping with these findings, other had previously shown that FGF21 acts centrally to enhance sympathetic activity to adipose tissue and thermogenic activity via UCP1 (Owen, Ding et al. 2014). Further studies followed on from this work by demonstrating a partial FGF21 dependent effect on EE in response to IUB288, which is also contributed to by the activation of hepatic farsenoid X receptor (FXR) (Kim, Nason et al. 2018). Other hormones involved in glucagon-mediated EE have been proposed, including secretion of thyroxine, cortisol and nor-adrenaline, enhancing sympathetic nervous system activity (Lawrence 1967, Waldhausl, Haydl et al. 1976, Kim, Nason et al. 2018). Furthermore, other mechanisms including futile cycling and enhanced costs of hepatic metabolism have been suggested (Kleinert, Sachs et al. 2019). Therefore, multiple potential mechanisms driving glucagon-mediated EE appear to be possible.

Consistent with the known effects of glucagon, sub-anorectic doses of G108 in both lean and obese mice resulted in loss of body weight and so therefore indicated enhanced EE

(Chapter 2, Section 2.4.10, Chapter 3, Section 3.4.1). In obese mice, G108 led to a marked reduction in both lean and fat mass (Chapter 3, Section 3.4.2) and enhanced EE was confirmed using direct measurement of oxygen consumption in dedicated metabolic cages (Chapter 3, Section 3.4.8). Interestingly, these effects were prevented with dietary protein supplementation, an intervention aimed to rescue G108-stimulated hypoaminoacidemia and lean mass loss (Chapter 3, Section 3.4.2, Section 3.4.8). These findings suggest that reduced plasma amino acid availability in response to chronic G108 administration, is a key mediator of the EE responses observed. To rationalise these findings, in this thesis parallels have been made between models of dietary protein restriction, an intervention which also reduces plasma amino acid availability. Indeed, consistent with the interaction between G108, EE and a high protein diet, studies dating back to the 1960's demonstrate that restricting dietary protein enhanced EE in rodents (Miller 1962, Miller and Mumford 1967, Stirling and Stock 1968). In a striking similarity in research progress since the 1960's, as with glucagon, initial studies of low protein diet induced EE focused on BAT tissue as a main regulator of EE (Rothwell and Stock 1979, Rothwell, Stock et al. 1982, Rothwell, Stock et al. 1983) and then hepatic FGF21 (Laeger, Henagan et al. 2014). Indeed, the seminal study by Laeger *et al*., confirmed that hepatic FGF21 is an endocrine signal of dietary protein restriction *in vivo*, through an amino acid stress response signalling pathway, previously characterised by others in isolated hepatocytes or leucine deficient diets (De Sousa-Coelho, Marrero et al. 2012, De Sousa-Coelho, Relat et al. 2013). The involvement of hepatic FGF21 induction in the EE response to dietary protein restriction has been shown in subsequent studies (Maida, Zota et al. 2016, Yap, Rusu et al. 2020). The mechanism by which FGF21 enhances EE in response to dietary protein restriction is thought to be due to central FGF21 signalling, enhancing sympathetic activity and thermogenesis in adipose tissue (Hill, Laeger et al. 2019).

To further confirm whether G108-stimulated hypoaminoacidemia explains the EE and body weight loss responses observed, several additional studies were carried out. Single-dose administration studies with a higher (20 nmol/kg) dose of G108 led to marked and prolonged suppression of total plasma amino acids and this was associated with enhanced EE (Chapter 4, Section 4.4.1). A high protein diet not only rescued total plasma amino acids but also the coincident rise in EE (Chapter 4, Section 4.4.1). To provide mechanistic insight into the effects observed, supplementing the diet with individual

amino acids (rather than a casein-based high protein diet) also rescued chronic G108-stimulated hypoaminoacidemia and prevented EE and total body weight loss (Chapter 4, Section 4.4.2). Furthermore, a rationally designed low amino acid diet with reductions in amino acids matched to plasma reductions of G108-treated mice, resulted in enhanced EE and body weight loss (Chapter 4, Section 4.4.4). Interestingly, despite plasma FGF-21 consistently being elevated in G108-treated mice, mice lacking hepatic *Fgf21* appeared to exhibit a similar EE response to the low amino acid diet as wild-type mice (Chapter 4, Section 4.4.5). This indicates that hepatic FGF21 induction may not explain the EE in response to G108-stimualted hypoaminoacidemia and further studies are warranted to confirm this. Interestingly, studies of dietary protein restriction (typically using 5% casein diets) have shown a far greater elevation in circulating FGF21 levels in rodents (Maida, Zota et al. 2016, Hill, Laeger et al. 2019), therefore suggesting that hepatic induction of FGF21 by G108 may be a marker of hypoaminoacidemia but not be directly mediating the EE responses observed. In keeping with this hypothesis, reviews in the field of dietary protein or amino acid restriction highlight multi-faceted mechanisms of enhanced EE, including sympathetic and serotonergic signalling, in addition to induction of hepatic FGF21 (Pezeshki and Chelikani 2021, Xiao and Guo 2022). Furthermore, suppression of individual amino acids may elicit specific responses (Xiao and Guo 2022). The precise mechanism(s) of EE activated by G108-stimualated hypoaminoacidemia therefore warrant further investigation and may well reveal effects attributed to suppression of individual amino acids. It will also be important to determine whether general protein turnover in lean mass is also contributing to the EE responses observed, as early glucagon infusion studies suggested the intrinsic link between hyperglucagonemia, proteolysis and resting metabolic rate during insulin deficiency (Nair, Halliday et al. 1984, Nair 1987, Charlton and Nair 1998).

Together, the EE findings from this project reveal a paradigm shift on the mechanism of glucagon-mediated EE. Rather than a mediated by a cascade of endocrine signals downstream to the hepatic GCGR, this work suggests that the EE response may also be due to a whole-body response to reduced plasma amino acid availability, a consequence of GCGR-dependent amino acid catabolism. It will be important to determine the extent to which hypoaminoacidemia contributes to glucagon-mediated EE at higher doses of GCGR-targeted multi-agonists, and whether this mechanism also translates to humans.

7.5 Implications of G108-stimulated chronic hypoaminoacidemia for the use of GCGR-targeted multi-agonists in humans?

To further understand glucagon biology in humans, several glucagon-infusion studies were carried out in the 1980's and 1990's. With regards to protein metabolism, these consistently noted marked nitrogen loss and suppressed plasma amino acids (Boden, Rezvani et al. 1984, Nair, Halliday et al. 1987, Charlton, Adey et al. 1996). However, in the last decade, glucagon therapy in humans has garnered much attention due to the possibility of co-agonism with other anti-obesity agents, such as GLP-1, to offset glucagon-stimulated hyperglycaemia (Tan, Field et al. 2013). Combined with the success of the unimolecular GLP-1R/GCGR co-agonist in pre-clinical trials, this has led to a number of GCGR-targeted multi-agonists progressing to early phase clinical trials within the last 5 years (Hope, Vincent et al. 2021). More recently, the first GLP-1R/GCGR/GIPR triple agonists have been tested in phase 1 single ascending dose trials (Bossart, Wagner et al. 2021, Coskun, Urva et al. 2022). Interestingly, and perhaps not surprisingly, is that suppressed plasma amino acids are a common feature to the long-acting GCGR-targeted multi-agonists including both the co-agonists (GLP-1R/GCGR) and triple agonists (GLP-1R/GCGR/GIPR). The once-weekly long-acting GLP-1R/GCGR co-agonist NN1177 (Novo Nordisk) has been discontinued following a phase 1 multiple ascending dose (MAD) trial due to cardiovascular and glycaemic safety concerns over the course of 12 weeks (Martin Friedrichsen 2022). As part of the phase 1 trial, plasma amino acids were measured during a drug-drug interaction study over 12 weeks noting a broad reduction in all plasma amino acids at the last study visit. Of the GLP-1R/GCGR co-agonists which have reported data on plasma amino acids, Cotadutide (Medimmune) has progressed well to phase 2 trials with no safety concerns (Nahra, Wang et al. 2021). A recent Phase 2a study providing mechanistic insights into the drug has reported reductions in several amino acids after 49 days of daily dosing (esc to 300 μg/day) (Parker, Robertson et al. 2020); However, only a significant reduction in alanine was observed with numerical reductions for cysteine, glutamate, isoleucine, tryptophan and tyrosine. It is possible that due to the relatively week activity at the GCGR (10-fold less than glucagon) combined with the shorter half-life, that broad hypoaminoacidemia is not observed. In the last year, single-ascending dose protocols in phase 1 studies have been reported for two GLP-1R/GCGR/GIPR triple agonists, SAR441255 (Sanofi) and LY3437943 (Eli Lilly). After single administration of the highest doses of SAR441255 (80 μg or 150 μg), there was a

broad reduction in plasma amino acids which lasted up to the final measurement point of 12 hours post-dose (Bossart, Wagner et al. 2021). After a single dose of LY3437943, there was a reduction in three of the measured plasma amino acids, alanine, asparagine and serine, detected *eight days* post-dose administration (Coskun, Urva et al. 2022); However plasma amino acids are not reported in a follow up Phase 1b study (Urva 2022). A noticeable difference between the two unimolecular triple-agonists is that LY3437943 has an extended pharmacokinetic profile of LY3437943, with a half-life of six days, whereas SAR441255 has a shorter half-life of approximately 5 hours. Therefore, it is important to determine amino acid measurements in line with the length of peptide bioactivity. Chapter 5 presents plasma amino acid data from a phase 1 trial of a GLP-1R/GCGR co-agonist, G3215, being developed at Imperial College London. Uniquely, G3215 is delivered through a continuous subcutaneous infusion allowing for easy dose-titration and flexible dosing if required. Plasma amino acids were measured at various timepoints over the 14-day infusion period, and consistent with findings described by others above, also show a broad but stable suppression of amino acids which return to baseline upon discontinuation (Chapter 5, Section 5.2).

Despite plasma amino acids being more frequently reported in early phase clinical trials as a marker of GCGR activity, there are recent examples of this not being reported (Ji, Gao et al. 2022, Jiang, Pang et al. 2022). Once, routinely reported, it will be important to determine whether chronic suppression of plasma amino acids will be problematic or not, and if so which amino-acid sensitive pathways/tissue could be affected. A main reason that plasma amino acids are being reported in human trials to 'test for GCGR target engagement' (Bossart, Wagner et al. 2021). The consequence of chronic hypoaminoacidemia in response to a CGGR-targeted multi-agonist is currently unknown. It is likely that any physiological effect depends on the extent of plasma amino acid suppression by the agonist and this may be harmful or even beneficial. In this thesis, a clear negative consequence on lean mass is evident from the pre-clinical studies of G108. However, this was mitigated with a high protein diet suggesting its translational potential as a treatment adjuvant (Chapter 3, Section 3.4.2, Section 3.4.4). To determine the extent of amino acid suppression and, for example lean mass in humans, clinical trials will need to routinely report data on individual plasma amino acids and body composition. Furthermore, there may indeed be consequences of chronic hypoaminoacidemia in

amino acid sensitive tissues independent to lean mass, for example alpha-cell hypoplasia as recently noted in pre-clinical studies of a long-acting GCG analogue (Emilie Elmelund 2022). As amino acid-sensitive tissues are widespread, further investigation is warranted to determine the whole-body effects of reduced amino acid availability in response to GCGR-targeted multi-agonists.

Findings from this thesis demonstrate that G108-stimulated hypoaminoacidemia enhances EE and total body weight loss in a mouse model of obesity. Whether this phenomenon occurs in humans is uncertain however warrants further investigation. The reported weight loss effects of GCGR-targeted multi-agonists is variable and comparisons to the striking weight loss achieved by the latest GLP-1 mono-agonists is difficult due to the different stage of development of this class of drug. Therefore, the extent of weight loss which can be achieved with GCGR-targeted multi-agonists in a chronic setting is yet to be confirmed. Cotadutide, weakly potent at the GCGR, results in modest weight reduction over 54 weeks (Nahra, Wang et al. 2021), however recent Phase 1 reports of IBI362 (Eli Lilly) and LY3437943 suggest that much higher degrees of weight loss may occur when administered for longer time frames (Coskun, Urva et al. 2022, Ji, Gao et al. 2022, Urva 2022). The contribution of GCGR-mediated EE to the weight loss effects observed in pre-clinical models of these agonists is reported using metabolic cages with direct measurement of metabolic variables. However, in humans this is more difficult due to practicalities of measuring energy expenditure (being in a closed room/system). Therefore, it will be important to determine whether long-acting GCGR agonists achieve measurable increases in EE and the mechanism in which this occurs. Recent mechanistic clinical studies using native glucagon infusions in humans have shown conflicting evidence of glucagon-stimulated EE and this may be due to the dose of glucagon infused (Salem, Izzi-Engbeaya et al. 2016, Whytock, Carnero et al. 2021). Whether hypoaminoacidemia is a mechanism of EE in response to GCGR-targeted multi-agonists is uncertain. However, as hypoaminoacidemia is a common feature of long-acting preparations of these agonists in early phase trials, this does warrant further investigation. Interestingly, recently studies of low-protein overfeeding in humans have demonstrated that FGF-21 mediates EE in response to low dietary protein (Vinales, Begaye et al. 2019). Therefore, it *may* be possible that FGF21 mediates the EE response to higher doses of glucagon in response to suppressed plasma amino acids. Interestingly,

studies of native glucagon infusions have shown that the EE response depends on whether participants were fasted or not (Kleinert, Sachs et al. 2019), which also suggests that plasma amino acid availability could be mediating this effect.

Together these findings, along with those of others, suggest that hypoaminoacidemia is a common of prolonged GCGR activity with unimolecular agonists in humans. The extent of hypoaminoacidemia is likely largely dependent on drug factors and the long term-consequences need to be established.

7.6 Future directions to harness the metabolic benefits of chronic glucagon therapy whilst mitigating potential side effects of excess amino acid catabolism

Based on the discussions in the previous sections, if hypoaminoacidemia in humans occurs in response to GCGR-targeted multi-agonists, this may not be a favourable outcome in a chronic therapeutic setting. Therefore, options to rescue plasma amino acids and mitigate, for example lean mass loss, may be required. One potential solution, demonstrated in Chapter 3 is dietary protein supplementation, which rescued G108-stimulated hypoaminoacidemia and prevented lean mass loss in obese mice (Chapter 3, Section 3.4.2, Section 3.4.4). A balance of GCGR-dependent hepatic amino acid catabolism and dietary protein intake was achieved, which resulted in a broad rescue of amino acids. However, the requirement of protein intake is likely dependent on the extent of GCGR-dependent hepatic amino acid catabolism, which itself will depend on several drug factors such as receptor potency and drug exposure time. Therefore, the extent of dietary protein supplementation required to offset GCGR-mediated suppression of plasma amino acids remains to be answered. For example, what level of increased dietary protein intake would be required to rescue G3215-stimulated hypoaminoaciemia during a 14-day continuous infusion? As GCGR-targeted multi-agonists have unique pharmacodynamic and pharmacokinetic profiles, it is likely that the need for dietary protein supplementation will be equally as unique. This does however warrant further investigation as it is still unclear whether chronic hypoaminoacidemia in response to these agonists is harmful and whether replacement through dietary supplementation is required.

As hepatic glucagon signalling regulates a diverse set of metabolic pathways across all three major nutrient groups, protein, carbohydrate and lipids, the mechanisms controlling these at both the transcriptional and post-translational levels is complex (Albrechtsen 2021). Emerging evidence suggests that long-acting preparations of GCGR-targeted multi-agonists invariably leads to hypoaminoacidemia in both the acute and chronic setting (see section 7.5). In Chapter 6, the effects of a short-acting GCG analogue were compared with G108, a long-acting GCG analogue in a chronic daily dosing study in obese mice. Intriguingly, despite the lack of effect on plasma amino acids, the findings suggest that the short-acting GCG analogue, G395 was able to improve glucose tolerance and hepatic lipid accumulation. Furthermore, these beneficial effects appeared to comparable to those achieved by the long-acting analogue, G108 (Chapter 6, Section 6.4.2, Section 6.4.3). It is possible that the short-acting analogue, G395, has divergent effects on macronutrient metabolic pathways at a given dose. For example, sustained hepatic GCGR activity may be required to suppress plasma amino acids whereas shorter duration of GCGR activity may have a sizable impact on carbohydrate and lipid metabolic pathways. Furthermore, the much higher dose of the short-acting glucagon analogue may have resulted in biological activity across protein, carbohydrate, and lipid pathways, however after the short-acting drug has lost biological activity, effects on hepatic amino acid catabolism may have attenuated. The differences in the responses observed may be due to the underlying differences in how lipid, carbohydrate and amino acids are regulated to homeostasis. Together, the divergent effects of the short-acting GCG analogue on plasma amino acids, glucose tolerance and hepatic lipids, suggest that temporal effects of glucagon biology could be harnessed to optimise GCGR-targeted multi-agonist therapy. By 'uncoupling' glucagon from GLP-1 in a unimolecular co-agonist, this will allow for timed dosing of individual components. Therefore, these findings warrant further investigation.

Another strategy which is gaining attention in multi-agonist research is the concept of receptor bias. This is highlighted by the marked success of Tirzepetide in controlling glycaemia, a GLP-1/GIP co-agonist which demonstrates bias at the beta cell GLP-1R (Willard, Douros et al. 2020). In Chapter 6, the effect on plasma amino acids and glycaemia of two GLP-1R/GCGR co-agonists, one which exhibits partial agonism for beta-arrestin recruitment was explored. SRB103Q exhibits selective partial agonism for beta-

arrestin recruitment at the GLP-1R whereas it exhibits partial agonism for both beta-arrestin and Gs recruitment at the GCGR (Pickford, Lucey et al. 2021). In contrast, SRB103H exhibits full agonism for beta-arrestin and Gs proteins at both receptors. As expected, and consistent with previous findings (Pickford, Lucey et al. 2021), after a single dose SRB103Q led to a sustained improvement in blood glucose over 8 hours, superior to that of SRB103H. However, the reduction in plasma alanine at 8 hours was comparable between both co-agonists. The latter finding may be due to the fact these are short-acting analogues which have a greater effect on carbohydrate metabolic pathways than amino acid catabolic pathways (See above sections on SA GCG vs LA GCG). Alternatively, the equal partial agonism for beta-arrestin recrtuiment and Gs recrtuiment to the GCGR may have translated to a comparable biological effect on amino acid catabolic pathways as the full agonist (SRB103H). In this context, if partial agonism has resulted in an outcome of enhancing glycaemia through GLP-1R activity without further enhancement of hepatic amino acid catabolism via the GCGR, this strategy would also uncouple GLP1-1R activity from GCGR activity within the unimolecular co-agonist. This may provide a strategy to further optimise drug design by determining the extent by which receptors are activated using unimolecular agonists targeting multiple receptors. This may well be further complicated by differential sensitivity of various signalling pathways downstream to a single receptor, for example the GCGR (Albrechtsen 2021). Therefore, further work is warranted to further explore the potential effects of receptor bias on the action of unimolecular GCGR multi-agonists.

7.7 Conclusions

In summary, this project set out to determine whether a long-acting GCG analogue, G108, resulted in hepatic amino acid catabolism and the chronic suppression of plasma amino acids (Figure 7.1). G108 was used as a research tool due to its similar activity at the GCGR as multi-agonists in development targeting this receptor. Using a sub-anorectic dosing strategy, G108 was found to enhance hepatic amino acid catabolism in a pre-clinical model of obesity, leading to hypoaminoacidemia and lean mass loss in the chronic setting. However, a dietary protein supplementation broadly rescued plasma amino acids and mitigating lean mass loss. Furthermore, despite rescuing plasma amino acids and mitigating lean mass loss, the high protein diet did not affect the ability of G108 to improve glycaemia and hepatic lipid metabolism. Using the paradigm of dietary protein restriction in rodents as a lead, the EE response to the long-acting GCGR agonist G108 was found to be due to reduced plasma amino acid availability in response to enhanced hepatic amino acid catabolism in obese mice; This was subsequently confirmed *in vivo* with further mechanistic studies. A phase 1 trial of a GLP-1R/GCGR demonstrated a broad reduction in plasma amino acids, providing further opportunities to explore the longer-term effects of these findings. Finally, and briefly, strategies to optimise glucagon therapy were explored. The findings from this thesis lead to further intriguing questions, which if answered, will contribute to our understanding of the fascinating world of glucagon biology.

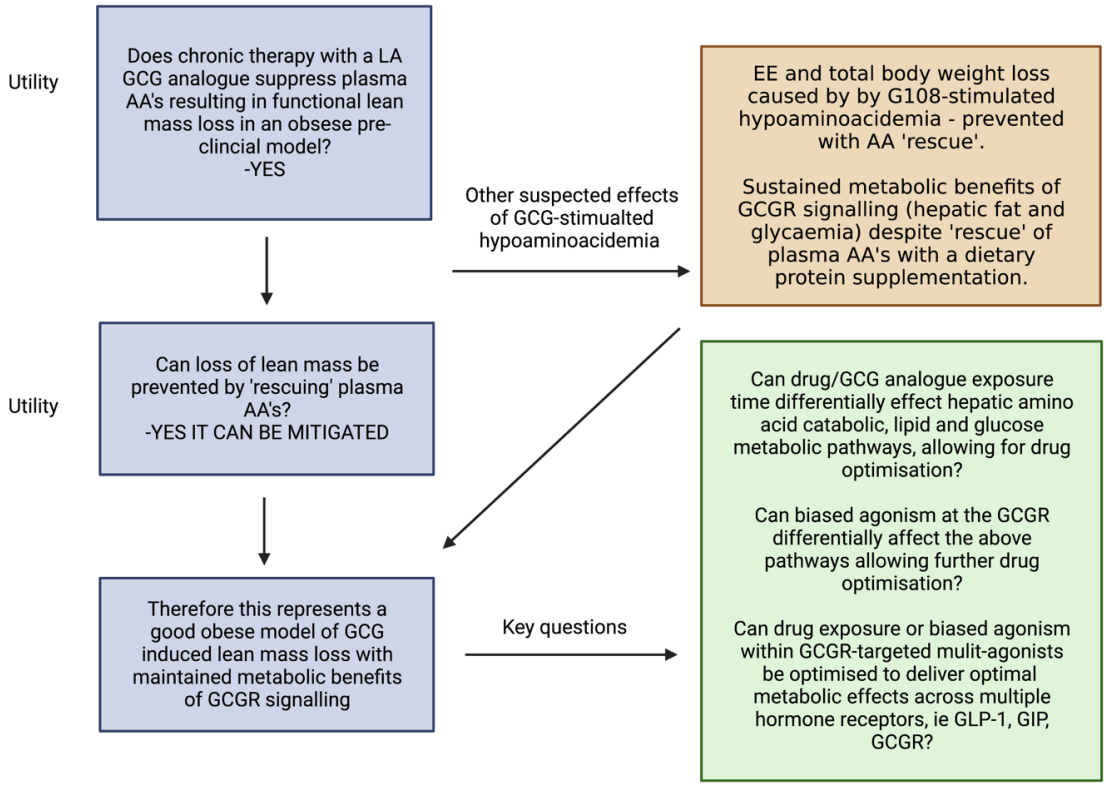


Figure 7.1: Schematic overview of project findings and future directions

8 REFERENCES

Abdulla, H., B. Phillips, D. Wilkinson, A. Gates, M. Limb, T. Jandova, J. Bass, J. Lewis, J. Williams, K. Smith, I. Idris and P. Atherton (2022). "Effects of GLP-1 infusion upon whole-body glucose uptake and skeletal muscle perfusion during fed-state in older men." J Clin Endocrinol Metab.

Ables, G. P., C. E. Perrone, D. Orentreich and N. Orentreich (2012). "Methionine-restricted C57BL/6J mice are resistant to diet-induced obesity and insulin resistance but have low bone density." PLoS One **7**(12): e51357.

Adams, A. C., C. Yang, T. Coskun, C. C. Cheng, R. E. Gimeno, Y. Luo and A. Kharitonenkov (2012). "The breadth of FGF21's metabolic actions are governed by FGFR1 in adipose tissue." Mol Metab **2**(1): 31-37.

Adeva-Andany, M. M., C. Fernandez-Fernandez, Y. Lopez-Pereiro, I. Castro-Calvo and N. Carneiro-Freire (2021). "The effects of glucagon and the target of rapamycin (TOR) on skeletal muscle protein synthesis and age-dependent sarcopenia in humans." Clin Nutr ESPEN **44**: 15-25.

Al-Massadi, O., J. Ferno, C. Dieguez, R. Nogueiras and M. Quinones (2019). "Glucagon Control on Food Intake and Energy Balance." Int J Mol Sci **20**(16).

Albrechtsen, N. J. W. (2021). "The glucose-mobilizing effect of glucagon at fasting is mediated by cyclic AMP." Am J Physiol Endocrinol Metab **321**: E571–E574.

Aliu, E., S. Kanungo and G. L. Arnold (2018). "Amino acid disorders." Ann Transl Med **6**(24): 471.

Almaca, J., J. Molina, D. Menegaz, A. N. Pronin, A. Tamayo, V. Slepak, P. O. Berggren and A. Caicedo (2016). "Human Beta Cells Produce and Release Serotonin to Inhibit Glucagon Secretion from Alpha Cells." Cell Rep **17**(12): 3281-3291.

Almdal, T. P., H. Heindorff, L. Bardram and H. Vilstrup (1990). "Increased amino acid clearance and urea synthesis in a patient with glucagonoma." Gut **31**(8): 946-948.

Ambery, P., V. E. Parker, M. Stumvoll, M. G. Posch, T. Heise, L. Plum-Moerschel, L. F. Tsai, D. Robertson, M. Jain, M. Petrone, C. Rondinone, B. Hirshberg and L. Jermutus (2018). "MEDI0382, a GLP-1 and glucagon receptor dual agonist, in obese or overweight patients with type 2 diabetes: a randomised, controlled, double-blind, ascending dose and phase 2a study." Lancet **391**(10140): 2607-2618.

Ang, T., C. R. Bruce and G. M. Kowalski (2019). "Postprandial Aminogenic Insulin and Glucagon Secretion Can Stimulate Glucose Flux in Humans." Diabetes **68**(5): 939-946.

Arafat, A. M., P. Kaczmarek, M. Skrzypski, E. Pruszynska-Oszmalek, P. Kolodziejski, D. Szczepankiewicz, M. Sassek, T. Wojciechowicz, B. Wiedenmann, A. F. Pfeiffer, K. W. Nowak and M. Z. Strowski (2013). "Glucagon increases circulating fibroblast growth factor 21 independently of endogenous insulin levels: a novel mechanism of glucagon-stimulated lipolysis?" Diabetologia **56**(3): 588-597.

Badman, M. K., A. Koester, J. S. Flier, A. Kharitonenkov and E. Maratos-Flier (2009). "Fibroblast growth factor 21-deficient mice demonstrate impaired adaptation to ketosis." Endocrinology **150**(11): 4931-4940.

Badman, M. K., P. Pissios, A. R. Kennedy, G. Koukos, J. S. Flier and E. Maratos-Flier (2007). "Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states." Cell Metab **5**(6): 426-437.

Bagger, J. I., J. J. Holst, B. Hartmann, B. Andersen, F. K. Knop and T. Vilsboll (2015). "Effect of Oxyntomodulin, Glucagon, GLP-1, and Combined Glucagon +GLP-

1 Infusion on Food Intake, Appetite, and Resting Energy Expenditure." J Clin Endocrinol Metab **100**(12): 4541-4552.
Banting, F. G., C. H. Best, J. B. Collip, W. R. Campbell and A. A. Fletcher (1922). "Pancreatic Extracts in the Treatment of Diabetes Mellitus." Can Med Assoc J **12**(3): 141-146.
Baptista, I. L., M. L. Leal, G. G. Artioli, M. S. Aoki, J. Fiamoncini, A. O. Turri, R. Curi, E. H. Miyabara and A. S. Moriscot (2010). "Leucine attenuates skeletal muscle wasting via inhibition of ubiquitin ligases." Muscle Nerve **41**(6): 800-808.
Barazzoni, R., M. Zanetti, A. Tiengo and P. Tessari (1999). "Protein metabolism in glucagonoma." Diabetologia **42**(3): 326-329.
Bataille, D., K. Tatemoto, C. Gespach, H. Jornvall, G. Rosselin and V. Mutt (1982). "Isolation of glucagon-37 (bioactive enteroglucagon/oxyntomodulin) from porcine jejuno-ileum. Characterization of the peptide." FEBS Lett **146**(1): 79-86.
Batterham, R. L., M. A. Cowley, C. J. Small, H. Herzog, M. A. Cohen, C. L. Dakin, A. M. Wren, A. E. Brynes, M. J. Low, M. A. Ghatei, R. D. Cone and S. R. Bloom (2002). "Gut hormone PYY(3-36) physiologically inhibits food intake." Nature **418**(6898): 650-654.
Beaudry, J. L., K. D. Kaur, E. M. Varin, L. L. Baggio, X. Cao, E. E. Mulvihill, J. H. Stern, J. E. Campbell, P. E. Scherer and D. J. Drucker (2019). "The brown adipose tissue glucagon receptor is functional but not essential for control of energy homeostasis in mice." Mol Metab **22**: 37-48.
Beaven, G. H., W. B. Gratzer and H. G. Davies (1969). "Formation and structure of gels and fibrils from glucagon." Eur J Biochem **11**(1): 37-42.
Bell, G. I., R. F. Santerre and G. T. Mullenbach (1983). "Hamster preproglucagon contains the sequence of glucagon and two related peptides." Nature **302**(5910): 716-718.
Blais, A., C. Chaumontet, D. Azzout-Marniche, J. Piedcoq, G. Fromentin, C. Gaudichon, D. Tome and P. C. Even (2018). "Low-protein diet-induced hyperphagia and adiposity are modulated through interactions involving thermoregulation, motor activity, and protein quality in mice." Am J Physiol Endocrinol Metab **314**(2): E139-E151.
Bobe, G., J. C. Velez, D. C. Beitz and S. S. Donkin (2009). "Glucagon increases hepatic mRNA concentrations of ureagenic and gluconeogenic enzymes in early-lactation dairy cows." J Dairy Sci **92**(10): 5092-5099.
Boden, G., R. W. Master, I. Rezvani, J. P. Palmer, T. E. Lobe and O. E. Owen (1980). "Glucagon deficiency and hyperaminoacidemia after total pancreatectomy." J Clin Invest **65**(3): 706-716.
Boden, G., I. Rezvani and O. E. Owen (1984). "Effects of glucagon on plasma amino acids." J Clin Invest **73**(3): 785-793.
Boden, G., L. Tappy, F. Jadali, R. D. Hoeldtke, I. Rezvani and O. E. Owen (1990). "Role of glucagon in disposal of an amino acid load." Am J Physiol **259**(2 Pt 1): E225-232.
Bodine, S. C. and L. M. Baehr (2014). "Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and MAFbx/atrogin-1." Am J Physiol Endocrinol Metab **307**(6): E469-484.
Bond, P. (2016). "Regulation of mTORC1 by growth factors, energy status, amino acids and mechanical stimuli at a glance." J Int Soc Sports Nutr **13**: 8.
BonDurant, L. D., M. Ameka, M. C. Naber, K. R. Markan, S. O. Idiga, M. R. Acevedo, S. A. Walsh, D. M. Ornitz and M. J. Potthoff (2017). "FGF21 Regulates Metabolism

Through Adipose-Dependent and -Independent Mechanisms." Cell Metab **25**(4): 935-944 e934.
Borghi, V. C., B. L. Wajchenberg and F. P. Cesar (1984). "Plasma glucagon suppressibility after oral glucose in obese subjects with normal and impaired glucose tolerance." Metabolism **33**(12): 1068-1074.
Bossart, M., M. Wagner, R. Elvert, A. Evers, T. Hubschle, T. Kloeckener, K. Lorenz, C. Moessinger, O. Eriksson, I. Velikyan, S. Pierrou, L. Johansson, G. Dietert, Y. Dietz-Baum, T. Kissner, I. Nowotny, C. Einig, C. Jan, F. Rharbaoui, J. Gassenhuber, H. P. Prochnow, I. Agueusop, N. Porksen, W. B. Smith, A. Nitsche and A. Konkar (2021). "Effects on weight loss and glycemic control with SAR441255, a potent unimolecular peptide GLP-1/GIP/GCG receptor triagonist." Cell Metab.
Bromer, W. W. and R. E. Chance (1969). "Zinc glucagon depression of blood amino acids in rabbits." Diabetes **18**(11): 748-754.
Bromer, W. W., L. G. Sinn, A. Staub and O. K. Behrens (1957). "The amino acid sequence of glucagon." Diabetes **6**(3): 234-238.
Brown-Borg, H. M., S. G. Rakoczy, J. A. Wonderlich, L. Rojanathammanee, J. J. Kopchick, V. Armstrong and D. Raasakka (2014). "Growth hormone signaling is necessary for lifespan extension by dietary methionine." Aging Cell **13**(6): 1019-1027.
Cabrera, O., M. C. Jacques-Silva, S. Speier, S. N. Yang, M. Kohler, A. Fachado, E. Vieira, J. R. Zierath, R. Kibbey, D. M. Berman, N. S. Kenyon, C. Ricordi, A. Caicedo and P. O. Berggren (2008). "Glutamate is a positive autocrine signal for glucagon release." Cell Metab **7**(6): 545-554.
Calles-Escandon, J. (1994). "Insulin dissociates hepatic glucose cycling and glucagon-induced thermogenesis in man." Metabolism **43**(8): 1000-1005.
Capozzi, M. E., D. A. D'Alessio and J. E. Campbell (2022). "The past, present, and future physiology and pharmacology of glucagon." Cell Metab **34**(11): 1654-1674.
Caren, R. and L. Corbo (1960). "Glucagon and cholesterol metabolism." Metabolism **9**: 938-945.
Carlsson, L. M. S., K. Sjoholm, P. Jacobson, J. C. Andersson-Assarsson, P. A. Svensson, M. Taube, B. Carlsson and M. Peltonen (2020). "Life Expectancy after Bariatric Surgery in the Swedish Obese Subjects Study." N Engl J Med **383**(16): 1535-1543.
Cassidy, S., J. Y. Chau, M. Catt, A. Bauman and M. I. Trenell (2017). "Low physical activity, high television viewing and poor sleep duration cluster in overweight and obese adults; a cross-sectional study of 398,984 participants from the UK Biobank." Int J Behav Nutr Phys Act **14**(1): 57.
Castano-Martinez, T., F. Schumacher, S. Schumacher, B. Kochlik, D. Weber, T. Grune, R. Biemann, A. McCann, K. Abraham, C. Weikert, B. Kleuser, A. Schurmann and T. Laeger (2019). "Methionine restriction prevents onset of type 2 diabetes in NZO mice." FASEB J **33**(6): 7092-7102.
Cegla, J., R. C. Troke, B. Jones, G. Tharakan, J. Kenkre, K. A. McCullough, C. T. Lim, N. Parvizi, M. Hussein, E. S. Chambers, J. Minnion, J. Cuenco, M. A. Ghatei, K. Meeran, T. M. Tan and S. R. Bloom (2014). "Coinfusion of low-dose GLP-1 and glucagon in man results in a reduction in food intake." Diabetes **63**(11): 3711-3720.
Chabenne, J. R., M. A. DiMarchi, V. M. Gelfanov and R. D. DiMarchi (2010). "Optimization of the native glucagon sequence for medicinal purposes." J Diabetes Sci Technol **4**(6): 1322-1331.
Charlton, M. R., D. B. Adey and K. S. Nair (1996). "Evidence for a catabolic role of glucagon during an amino acid load." J Clin Invest **98**(1): 90-99.

Charlton, M. R. and K. S. Nair (1998). "Role of hyperglucagonemia in catabolism associated with type 1 diabetes: effects on leucine metabolism and the resting metabolic rate." Diabetes **47**(11): 1748-1756.
Chartoumpekis, D. V., I. G. Habeos, P. G. Ziros, A. I. Psyrogiannis, V. E. Kyriazopoulou and A. G. Papavassiliou (2011). "Brown adipose tissue responds to cold and adrenergic stimulation by induction of FGF21." Mol Med **17**(7-8): 736-740.
Chaumontet, C., D. Azzout-Marniche, A. Blais, J. Piedcoq, D. Tome, C. Gaudichon and P. C. Even (2019). "Low-protein and methionine, high-starch diets increase energy intake and expenditure, increase FGF21, decrease IGF-1, and have little effect on adiposity in mice." Am J Physiol Regul Integr Comp Physiol **316**(5): R486-R501.
Chen, H. Y., M. E. Trumbauer, A. S. Chen, D. T. Weingarth, J. R. Adams, E. G. Frazier, Z. Shen, D. J. Marsh, S. D. Feighner, X. M. Guan, Z. Ye, R. P. Nargund, R. G. Smith, L. H. Van der Ploeg, A. D. Howard, D. J. MacNeil and S. Qian (2004). "Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein." Endocrinology **145**(6): 2607-2612.
Chen, X. Y., L. Chen, W. Yang and A. M. Xie (2021). "GLP-1 Suppresses Feeding Behaviors and Modulates Neuronal Electrophysiological Properties in Multiple Brain Regions." Front Mol Neurosci **14**: 793004.
Cheng, Y., Q. Zhang, Q. Meng, T. Xia, Z. Huang, C. Wang, B. Liu, S. Chen, F. Xiao, Y. Du and F. Guo (2011). "Leucine deprivation stimulates fat loss via increasing CRH expression in the hypothalamus and activating the sympathetic nervous system." Mol Endocrinol **25**(9): 1624-1635.
Chuang, J. C., I. Sakata, D. Kohno, M. Perello, S. Osborne-Lawrence, J. J. Repa and J. M. Zigman (2011). "Ghrelin directly stimulates glucagon secretion from pancreatic alpha-cells." Mol Endocrinol **25**(9): 1600-1611.
Cockburn, F., D. Hull and I. Walton (1967). "The effect of lipolytic hormones and theophylline on heat production in brown adipose tissue in vivo." Br J Pharmacol Chemother **31**(3): 568-577.
Connolly, H. M., J. L. Crary, M. D. McGoon, D. D. Hensrud, B. S. Edwards, W. D. Edwards and H. V. Schaff (1997). "Valvular heart disease associated with fenfluramine-phentermine." N Engl J Med **337**(9): 581-588.
Coskun, T., H. A. Bina, M. A. Schneider, J. D. Dunbar, C. C. Hu, Y. Chen, D. E. Moller and A. Kharitonenkov (2008). "Fibroblast growth factor 21 corrects obesity in mice." Endocrinology **149**(12): 6018-6027.
Coskun, T., S. Urva, W. C. Roell, H. Qu, C. Loghin, J. S. Moyers, L. S. O'Farrell, D. A. Briere, K. W. Sloop, M. K. Thomas, V. Pirro, D. B. Wainscott, F. S. Willard, M. Abernathy, L. Morford, Y. Du, C. Benson, R. E. Gimeno, A. Haupt and Z. Milicevic (2022). "LY3437943, a novel triple glucagon, GIP, and GLP-1 receptor agonist for glycemic control and weight loss: From discovery to clinical proof of concept." Cell Metab.
Courcoulas, A. P., W. C. King, S. H. Belle, P. Berk, D. R. Flum, L. Garcia, W. Gourash, M. Horlick, J. E. Mitchell, A. Pomp, W. J. Pories, J. Q. Purnell, A. Singh, K. Spaniolas, R. Thirlby, B. M. Wolfe and S. Z. Yanovski (2018). "Seven-Year Weight Trajectories and Health Outcomes in the Longitudinal Assessment of Bariatric Surgery (LABS) Study." JAMA Surg **153**(5): 427-434.
Cyphert, H. A., K. M. Alonge, S. M. Ippagunta and F. B. Hillgartner (2014). "Glucagon stimulates hepatic FGF21 secretion through a PKA- and EPAC-dependent posttranscriptional mechanism." PLoS One **9**(4): e94996.

Dai, M., T. Lin, J. Yue and L. Dai (2021). "Signatures and Clinical Significance of Amino Acid Flux in Sarcopenia: A Systematic Review and Meta-Analysis." Front Endocrinol (Lausanne) **12**: 725518.
Dakin, C. L., I. Gunn, C. J. Small, C. M. Edwards, D. L. Hay, D. M. Smith, M. A. Ghatei and S. R. Bloom (2001). "Oxyntomodulin inhibits food intake in the rat." Endocrinology **142**(10): 4244-4250.
Dakin, C. L., C. J. Small, R. L. Batterham, N. M. Neary, M. A. Cohen, M. Patterson, M. A. Ghatei and S. R. Bloom (2004). "Peripheral oxyntomodulin reduces food intake and body weight gain in rats." Endocrinology **145**(6): 2687-2695.
Danforth, E., Jr. (1981). "Dietary-induced thermogenesis: control of energy expenditure." Life Sci **28**(15-16): 1821-1827.
Daniel, P. M., O. E. Pratt and E. Spargo (1977). "The mechanism by which glucagon induces the release of amino acids from muscle and its relevance to fasting." Proc R Soc Lond B Biol Sci **196**(1124): 347-365.
Davidson, I. W., J. M. Salter and C. H. Best (1957). "Calorigenic action of glucagon." Nature **180**(4595): 1124.
Davidson, I. W. F., Salter J.M, Best C.H (1960). "The effect of glucagon on the metabolic rate of rats." The American Journal of Clinical Nutrition **8**.
Day, J. W., V. Gelfanov, D. Smiley, P. E. Carrington, G. Eiermann, G. Chicchi, M. D. Erion, J. Gidda, N. A. Thornberry, M. H. Tschop, D. J. Marsh, R. SinhaRoy, R. DiMarchi and A. Pocai (2012). "Optimization of co-agonism at GLP-1 and glucagon receptors to safely maximize weight reduction in DIO-rodents." Biopolymers **98**(5): 443-450.
Day, J. W., N. Ottaway, J. T. Patterson, V. Gelfanov, D. Smiley, J. Gidda, H. Findeisen, D. Bruemmer, D. J. Drucker, N. Chaudhary, J. Holland, J. Hembree, W. Abplanalp, E. Grant, J. Ruehl, H. Wilson, H. Kirchner, S. H. Lockie, S. Hofmann, S. C. Woods, R. Nogueiras, P. T. Pfluger, D. Perez-Tilve, R. DiMarchi and M. H. Tschop (2009). "A new glucagon and GLP-1 co-agonist eliminates obesity in rodents." Nat Chem Biol **5**(10): 749-757.
De Sousa-Coelho, A. L., P. F. Marrero and D. Haro (2012). "Activating transcription factor 4-dependent induction of FGF21 during amino acid deprivation." Biochem J **443**(1): 165-171.
De Sousa-Coelho, A. L., J. Relat, E. Hondares, A. Perez-Marti, F. Ribas, F. Villarroya, P. F. Marrero and D. Haro (2013). "FGF21 mediates the lipid metabolism response to amino acid starvation." J Lipid Res **54**(7): 1786-1797.
Dean, E. D., M. Li, N. Prasad, S. N. Wisniewski, A. Von Deylen, J. Spaeth, L. Maddison, A. Botros, L. R. Sedgeman, N. Bozadjieva, O. Ilkayeva, A. Coldren, G. Poffenberger, A. Shostak, M. C. Semich, K. I. Aamodt, N. Phillips, H. Yan, E. Bernal-Mizrachi, J. D. Corbin, K. C. Vickers, S. E. Levy, C. Dai, C. Newgard, W. Gu, R. Stein, W. Chen and A. C. Powers (2017). "Interrupted Glucagon Signaling Reveals Hepatic alpha Cell Axis and Role for L-Glutamine in alpha Cell Proliferation." Cell Metab **25**(6): 1362-1373 e1365.
Delimaris, I. (2013). "Adverse Effects Associated with Protein Intake above the Recommended Dietary Allowance for Adults." ISRN Nutr **2013**: 126929.
DiGruccio, M. R., A. M. Mawla, C. J. Donaldson, G. M. Noguchi, J. Vaughan, C. Cowing-Zitron, T. van der Meulen and M. O. Huising (2016). "Comprehensive alpha, beta and delta cell transcriptomes reveal that ghrelin selectively activates delta cells and promotes somatostatin release from pancreatic islets." Mol Metab **5**(7): 449-458.

Dodd, G. T., S. J. Kim, M. Mequinion, C. E. Xirouchaki, J. C. Bruning, Z. B. Andrews and T. Tiganis (2021). "Insulin signaling in AgRP neurons regulates meal size to limit glucose excursions and insulin resistance." Sci Adv **7**(9).
Doi, K. and A. Kuroshima (1982). "Modified metabolic responsiveness to glucagon in cold-acclimated and heat-acclimated rats." Life Sci **30**(9): 785-791.
Dommerholt, M. B., M. Blankestijn, M. A. Vieira-Lara, T. H. van Dijk, H. Wolters, M. H. Koster, A. Gerding, R. P. van Os, V. W. Bloks, B. M. Bakker, J. K. Kruit and J. W. Jonker (2021). "Short-term protein restriction at advanced age stimulates FGF21 signalling, energy expenditure and browning of white adipose tissue." FEBS J **288**(7): 2257-2277.
Donhoffer S, S. F., Szegvari G (1964). "BROWN ADIPOSE TISSUE AND THERMOREGULATORY HEAT PRODUCTION IN THE RAT." Nature **15**(203): 765-766.
Dossou, A. S. and A. Basu (2019). "The Emerging Roles of mTORC1 in Macromanaging Autophagy." Cancers (Basel) **11**(10).
Douris, N., D. M. Stevanovic, F. M. Fisher, T. I. Cisu, M. J. Chee, N. L. Nguyen, E. Zarebidaki, A. C. Adams, A. Kharitonenkov, J. S. Flier, T. J. Bartness and E. Maratos-Flier (2015). "Central Fibroblast Growth Factor 21 Browns White Fat via Sympathetic Action in Male Mice." Endocrinology **156**(7): 2470-2481.
Dulloo, A. G. and J. Jacquet (1999). "Low-protein overfeeding: a tool to unmask susceptibility to obesity in humans." Int J Obes Relat Metab Disord **23**(11): 1118-1121.
Dumenci, O. E., A. M. U, S. A. Khan, E. Holmes and S. D. Taylor-Robinson (2020). "Exploring Metabolic Consequences of CPS1 and CAD Dysregulation in Hepatocellular Carcinoma by Network Reconstruction." J Hepatocell Carcinoma **7**: 1-9.
Eaton, R. P. (1973). "Hypolipemic action of glucagon in experimental endogenous lipemia in the rat." J Lipid Res **14**(3): 312-318.
Edmondson, J. W. and L. Lumeng (1980). "Biphasic stimulation of amino acid uptake by glucagon in hepatocytes." Biochem Biophys Res Commun **96**(1): 61-68.
El, K., S. M. Gray, M. E. Capozzi, E. R. Knuth, E. Jin, B. Svendsen, A. Clifford, J. L. Brown, S. E. Encisco, B. M. Chazotte, K. W. Sloop, D. J. Nunez, M. J. Merrins, D. A. D'Alessio and J. E. Campbell (2021). "GIP mediates the incretin effect and glucose tolerance by dual actions on alpha cells and beta cells." Sci Adv **7**(11).
Elias, C. F., C. Aschkenasi, C. Lee, J. Kelly, R. S. Ahima, C. Bjorbaek, J. S. Flier, C. B. Saper and J. K. Elmquist (1999). "Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area." Neuron **23**(4): 775-786.
Elshorbagy, A. K., M. Valdivia-Garcia, D. A. Mattocks, J. D. Plummer, A. D. Smith, C. A. Drevon, H. Refsum and C. E. Perrone (2011). "Cysteine supplementation reverses methionine restriction effects on rat adiposity: significance of stearoyl-coenzyme A desaturase." J Lipid Res **52**(1): 104-112.
Elvert, R., A. W. Herling, M. Bossart, T. Weiss, B. Zhang, P. Wenski, J. Wandschneider, S. Kleutsch, U. Butty, A. Kannt, M. Wagner, T. Haack, A. Evers, A. Dudda, M. Lorenz, S. Keil and P. J. Larsen (2018). "Running on mixed fuel-dual agonistic approach of GLP-1 and GCG receptors leads to beneficial impact on body weight and blood glucose control: A comparative study between mice and non-human primates." Diabetes Obes Metab **20**(8): 1836-1851.
Emilie Elmelund, K. D. G., Christian D. Johansen, Samuel A.J. Trammell, Anna B. Bomholt, Marie Winther-Sørensen, Jenna E. Hunt, Charlotte M. Sørensen, Thomas Kruse, Jesper F. Lau, Trisha J. Grevengoed, Jens J. Holst, Nicolai J. Wewer

Albrechtsen (2022). "Opposing effects of chronic glucagon receptor agonism and antagonism on amino acids, hepatic gene expression, and alpha cells." iScience.
England, P. H. (2017). "Health matters: obesity and the food environment."
Everman, S., C. Meyer, L. Tran, N. Hoffman, C. C. Carroll, W. L. Dedmon and C. S. Katsanos (2016). "Insulin does not stimulate muscle protein synthesis during increased plasma branched-chain amino acids alone but still decreases whole body proteolysis in humans." Am J Physiol Endocrinol Metab **311**(4): E671-E677.
Evers, A., T. Haack, M. Lorenz, M. Bossart, R. Elvert, B. Henkel, S. Stengelin, M. Kurz, M. Glien, A. Dudda, K. Lorenz, D. Kadereit and M. Wagner (2017). "Correction to Design of Novel Exendin-Based Dual Glucagon-like Peptide 1 (GLP-1)/Glucagon Receptor Agonist." J Med Chem **60**(13): 5941.
Exton, J. H. (1987). "Mechanisms of hormonal regulation of hepatic glucose metabolism." Diabetes Metab Rev **3**(1): 163-183.
Ezrin, C., J. M. Salter, M. A. Ogryzlo and C. H. Best (1958). "The clinical and metabolic effects of glucagon." Can Med Assoc J **78**(2): 96-98.
F.Trading, P. N., M.Pingel Aa.Vølund (1969). "Biological and chemical properties of two glucagon preparations with prolonged action." European Journal of Pharmacology.
Faber, C. L., J. D. Deem, C. A. Campos, G. J. Taborsky, Jr. and G. J. Morton (2020). "CNS control of the endocrine pancreas." Diabetologia **63**(10): 2086-2094.
Fedorenko, A., P. V. Lishko and Y. Kirichok (2012). "Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria." Cell **151**(2): 400-413.
Ferrannini, E., E. Muscelli, A. Natali, R. Gabriel, A. Mitrakou, A. Flyvbjerg, A. Golay, K. Hojlund, S. Relationship between Insulin and I. Cardiovascular Disease Risk Project (2007). "Association of fasting glucagon and proinsulin concentrations with insulin resistance." Diabetologia **50**(11): 2342-2347.
Fisher, F. M., S. Kleiner, N. Douris, E. C. Fox, R. J. Mepani, F. Verdeguer, J. Wu, A. Kharitonenkov, J. S. Flier, E. Maratos-Flier and B. M. Spiegelman (2012). "FGF21 regulates PGC-1alpha and browning of white adipose tissues in adaptive thermogenesis." Genes Dev **26**(3): 271-281.
Fontana, L., N. E. Cummings, S. I. Arriola Apelo, J. C. Neuman, I. Kasza, B. A. Schmidt, E. Cava, F. Spelta, V. Tosti, F. A. Syed, E. L. Baar, N. Veronese, S. E. Cottrell, R. J. Fenske, B. Bertozzi, H. K. Brar, T. Pietka, A. D. Bullock, R. S. Figenshau, G. L. Andriole, M. J. Merrins, C. M. Alexander, M. E. Kimple and D. W. Lamming (2016). "Decreased Consumption of Branched-Chain Amino Acids Improves Metabolic Health." Cell Rep **16**(2): 520-530.
Forney, L. A., H. Fang, L. C. Sims, K. P. Stone, L. Y. Vincik, A. M. Vick, A. N. Gibson, D. H. Burk and T. W. Gettys (2020). "Dietary Methionine Restriction Signals to the Brain Through Fibroblast Growth Factor 21 to Regulate Energy Balance and Remodeling of Adipose Tissue." Obesity (Silver Spring) **28**(10): 1912-1921.
Frayn, K. N. (1983). "Calculation of substrate oxidation rates in vivo from gaseous exchange." J Appl Physiol Respir Environ Exerc Physiol **55**(2): 628-634.
Fryburg, D. A., E. J. Barrett, R. J. Louard and R. A. Gelfand (1990). "Effect of starvation on human muscle protein metabolism and its response to insulin." Am J Physiol **259**(4 Pt 1): E477-482.
G A Robison, R. W. B., E W Sutherland (1968). "Cyclic AMP." Annu Rev Biochem **37**: 149-174.
Gaisano, H. Y., P. E. Macdonald and M. Vranic (2012). "Glucagon secretion and signaling in the development of diabetes." Front Physiol **3**: 349.

Gallinetti, J., E. Harputlugil and J. R. Mitchell (2013). "Amino acid sensing in dietary-restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR." Biochem J **449**(1): 1-10.
Galsgaard, K. D., S. L. Jepsen, S. A. S. Kjeldsen, J. Pedersen, N. J. Wewer Albrechtsen and J. J. Holst (2020). "Alanine, arginine, cysteine, and proline, but not glutamine, are substrates for, and acute mediators of, the liver-alpha-cell axis in female mice." Am J Physiol Endocrinol Metab **318**(6): E920-E929.
Galsgaard, K. D., J. Pedersen, F. K. Knop, J. J. Holst and N. J. Wewer Albrechtsen (2019). "Glucagon Receptor Signaling and Lipid Metabolism." Front Physiol **10**: 413.
Gao, M., C. Piernas, N. M. Astbury, J. Hippisley-Cox, S. O'Rahilly, P. Aveyard and S. A. Jebb (2021). "Associations between body-mass index and COVID-19 severity in 6.9 million people in England: a prospective, community-based, cohort study." Lancet Diabetes Endocrinol **9**(6): 350-359.
Garrod, A. E. (1996). "The incidence of alkaptonuria: a study in chemical individuality. 1902." Mol Med **2**(3): 274-282.
Gault, V. A., V. K. Bhat, N. Irwin and P. R. Flatt (2013). "A novel glucagon-like peptide-1 (GLP-1)/glucagon hybrid peptide with triple-acting agonist activity at glucose-dependent insulinotropic polypeptide, GLP-1, and glucagon receptors and therapeutic potential in high fat-fed mice." J Biol Chem **288**(49): 35581-35591.
Geary, N., J. Le Sauter and U. Noh (1993). "Glucagon acts in the liver to control spontaneous meal size in rats." Am J Physiol **264**(1 Pt 2): R116-122.
Gelling, R. W., X. Q. Du, D. S. Dichmann, J. Romer, H. Huang, L. Cui, S. Obici, B. Tang, J. J. Holst, C. Fledelius, P. B. Johansen, L. Rossetti, L. A. Jelicks, P. Serup, E. Nishimura and M. J. Charron (2003). "Lower blood glucose, hyperglucagonemia, and pancreatic alpha cell hyperplasia in glucagon receptor knockout mice." Proc Natl Acad Sci U S A **100**(3): 1438-1443.
Gerich, J. E., M. Langlois, V. Schneider, J. H. Karam and C. Noacco (1974). "Effects of alternations of plasma free fatty acid levels on pancreatic glucagon secretion in man." J Clin Invest **53**(5): 1284-1289.
Gerich, J. E., M. Lorenzi, V. Schneider, J. H. Karam, J. Rivier, R. Guillemin and P. H. Forsham (1974). "Effects of somatostatin on plasma glucose and glucagon levels in human diabetes mellitus. Pathophysiologic and therapeutic implications." N Engl J Med **291**(11): 544-547.
Golozoubova, V., E. Hohtola, A. Matthias, A. Jacobsson, B. Cannon and J. Nedergaard (2001). "Only UCP1 can mediate adaptive nonshivering thermogenesis in the cold." FASEB J **15**(11): 2048-2050.
Gore, D. C., F. Jahoor, R. R. Wolfe and D. N. Herndon (1993). "Acute response of human muscle protein to catabolic hormones." Ann Surg **218**(5): 679-684.
Guettet, C., D. Mathe, N. Navarro and B. Lecuyer (1989). "Effects of chronic glucagon administration on rat lipoprotein composition." Biochim Biophys Acta **1005**(3): 233-238.
Guettet, C., D. Mathe, M. Riottot and C. Lutton (1988). "Effects of chronic glucagon administration on cholesterol and bile acid metabolism." Biochim Biophys Acta **963**(2): 215-223.
Guh, D. P., W. Zhang, N. Bansback, Z. Amarsi, C. L. Birmingham and A. H. Anis (2009). "The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis." BMC Public Health **9**: 88.
Gutzwiller, J. P., B. Goke, J. Drewe, P. Hildebrand, S. Ketterer, D. Handschin, R. Winterhalder, D. Conen and C. Beglinger (1999). "Glucagon-like peptide-1: a potent regulator of food intake in humans." Gut **44**(1): 81-86.

Guzman, C. B., X. M. Zhang, R. Liu, A. Regev, S. Shankar, P. Garhyan, S. G. Pillai, C. Kazda, N. Chalasani and T. A. Hardy (2017). "Treatment with LY2409021, a glucagon receptor antagonist, increases liver fat in patients with type 2 diabetes." Diabetes Obes Metab **19**(11): 1521-1528.
Habegger, K. M., K. Stemmer, C. Cheng, T. D. Muller, K. M. Heppner, N. Ottaway, J. Holland, J. L. Hembree, D. Smiley, V. Gelfanov, R. Krishna, A. M. Arafat, A. Konkar, S. Belli, M. Kapps, S. C. Woods, S. M. Hofmann, D. D'Alessio, P. T. Pfluger, D. Perez-Tilve, R. J. Seeley, M. Konishi, N. Itoh, A. Kharitonenkov, J. Spranger, R. D. DiMarchi and M. H. Tschop (2013). "Fibroblast growth factor 21 mediates specific glucagon actions." Diabetes **62**(5): 1453-1463.
Hao, S., J. W. Sharp, C. M. Ross-Inta, B. J. McDaniel, T. G. Anthony, R. C. Wek, D. R. Cavener, B. C. McGrath, J. B. Rudell, T. J. Koehnle and D. W. Gietzen (2005). "Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex." Science **307**(5716): 1776-1778.
Hardy, A. B., A. S. Serino, N. Wijesekara, F. Chimienti and M. B. Wheeler (2011). "Regulation of glucagon secretion by zinc: lessons from the beta cell-specific Znt8 knockout mouse model." Diabetes Obes Metab **13 Suppl 1**: 112-117.
Hartl, W. H., H. Miyoshi, F. Jahoor, S. Klein, D. Elahi and R. R. Wolfe (1990). "Bradykinin attenuates glucagon-induced leucine oxidation in humans." Am J Physiol **259**(2 Pt 1): E239-245.
Hasek, B. E., L. K. Stewart, T. M. Henagan, A. Boudreau, N. R. Lenard, C. Black, J. Shin, P. Huypens, V. L. Malloy, E. P. Plaisance, R. A. Krajcik, N. Orentreich and T. W. Gettys (2010). "Dietary methionine restriction enhances metabolic flexibility and increases uncoupled respiration in both fed and fasted states." Am J Physiol Regul Integr Comp Physiol **299**(3): R728-739.
Hebert, S. L. and K. S. Nair (2010). "Protein and energy metabolism in type 1 diabetes." Clin Nutr **29**(1): 13-17.
Heibel, S. K., G. Y. Lopez, M. Panglao, S. Sodha, L. Marino-Ramirez, M. Tuchman and L. Caldovic (2012). "Transcriptional regulation of N-acetylglutamate synthase." PLoS One **7**(2): e29527.
Henderson, S. J., A. Konkar, D. C. Hornigold, J. L. Trevaskis, R. Jackson, M. Fritsch Fredin, R. Jansson-Lofmark, J. Naylor, A. Rossi, M. A. Bednarek, N. Bhagroo, H. Salari, S. Will, S. Oldham, G. Hansen, M. Feigh, T. Klein, J. Grimsby, S. Maguire, L. Jermutus, C. M. Rondinone and M. P. Coghlan (2016). "Robust anti-obesity and metabolic effects of a dual GLP-1/glucagon receptor peptide agonist in rodents and non-human primates." Diabetes Obes Metab **18**(12): 1176-1190.
Hill, C. M., T. Laeger, D. C. Albarado, D. H. McDougal, H. R. Berthoud, H. Munzberg and C. D. Morrison (2017). "Low protein-induced increases in FGF21 drive UCP1-dependent metabolic but not thermoregulatory endpoints." Sci Rep **7**(1): 8209.
Hill, C. M., T. Laeger, M. Dehner, D. C. Albarado, B. Clarke, D. Wanders, S. J. Burke, J. J. Collier, E. Qualls-Creekmore, S. M. Solon-Biet, S. J. Simpson, H. R. Berthoud, H. Munzberg and C. D. Morrison (2019). "FGF21 Signals Protein Status to the Brain and Adaptively Regulates Food Choice and Metabolism." Cell Rep **27**(10): 2934-2947 e2933.
Hinds, C. E., B. M. Owen, D. C. D. Hope, P. Pickford, B. Jones, T. M. Tan, J. S. Minnion and S. R. Bloom (2021). "A glucagon analogue decreases body weight in mice via signalling in the liver." Sci Rep **11**(1): 22577.
Holecek, M. (2018). "Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements." Nutr Metab (Lond) **15**: 33.

Holst, J. J., M. Bersani, A. H. Johnsen, H. Kofod, B. Hartmann and C. Orskov (1994). "Proglucagon processing in porcine and human pancreas." J Biol Chem **269**(29): 18827-18833.
Holst, J. J., N. J. Wewer Albrechtsen, J. Pedersen and F. K. Knop (2017). "Glucagon and Amino Acids Are Linked in a Mutual Feedback Cycle: The Liver-alpha-Cell Axis." Diabetes **66**(2): 235-240.
Hope, D. C. D., C. Hinds, T. Lopes, M. Vincent, J. V. Shrewsbury, A. T. C. Yu, I. Davies, R. Scott, B. Jones, K. G. Murphy, J. Minnion, A. Sardini, D. Carling, T. A. Lutz, S. R. Bloom, T. M. M. Tan and B. Owen (In press). "Hypoaminoacidemia Underpins Glucagon-mediated Energy Expenditure and Weight Loss."
Hope, D. C. D., T. M. M. Tan and S. R. Bloom (2018). "No Guts, No Loss: Toward the Ideal Treatment for Obesity in the Twenty-First Century." Front Endocrinol (Lausanne) **9**: 442.
Hope, D. C. D., M. L. Vincent and T. M. M. Tan (2021). "Striking the Balance: GLP-1/Glucagon Co-Agonism as a Treatment Strategy for Obesity." Front Endocrinol (Lausanne) **12**: 735019.
Hotta, Y., H. Nakamura, M. Konishi, Y. Murata, H. Takagi, S. Matsumura, K. Inoue, T. Fushiki and N. Itoh (2009). "Fibroblast growth factor 21 regulates lipolysis in white adipose tissue but is not required for ketogenesis and triglyceride clearance in liver." Endocrinology **150**(10): 4625-4633.
Huang, X., D. P. Hancock, A. K. Gosby, A. C. McMahon, S. M. Solon, D. G. Le Couteur, A. D. Conigrave, D. Raubenheimer and S. J. Simpson (2013). "Effects of dietary protein to carbohydrate balance on energy intake, fat storage, and heat production in mice." Obesity (Silver Spring) **21**(1): 85-92.
Hutchens, T. and D. W. Piston (2015). "EphA4 Receptor Forward Signaling Inhibits Glucagon Secretion From alpha-Cells." Diabetes **64**(11): 3839-3851.
Inagaki, T., P. Dutchak, G. Zhao, X. Ding, L. Gautron, V. Parameswara, Y. Li, R. Goetz, M. Mohammadi, V. Esser, J. K. Elmquist, R. D. Gerard, S. C. Burgess, R. E. Hammer, D. J. Mangelsdorf and S. A. Kliewer (2007). "Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21." Cell Metab **5**(6): 415-425.
Ingram, D. L. and H. Kaciuba-Uscilko (1980). "Metabolic effects of glucagon in the young pig." Horm Metab Res **12**(9): 430-433.
Izzo, J. L. and S. R. Glasser (1961). "Comparative effects of glucagon, hydrocortisone and epinephrine on the protein metabolism of the fasting rat." Endocrinology **68**: 189-198.
J.J, H. (1980). "Evidence that glicentin contains the entire sequence of glucagon." Biochem J **187**: 337-343.
Jackson, S. E., C. Kirschbaum and A. Steptoe (2017). "Hair cortisol and adiposity in a population-based sample of 2,527 men and women aged 54 to 87 years." Obesity (Silver Spring) **25**(3): 539-544.
James, W. P., I. D. Caterson, W. Coutinho, N. Finer, L. F. Van Gaal, A. P. Maggioni, C. Torp-Pedersen, A. M. Sharma, G. M. Shepherd, R. A. Rode, C. L. Renz and S. Investigators (2010). "Effect of sibutramine on cardiovascular outcomes in overweight and obese subjects." N Engl J Med **363**(10): 905-917.
Janah, L., S. Kjeldsen, K. D. Galsgaard, M. Winther-Sorensen, E. Stojanovska, J. Pedersen, F. K. Knop, J. J. Holst and N. J. Wewer Albrechtsen (2019). "Glucagon Receptor Signaling and Glucagon Resistance." Int J Mol Sci **20**(13).

Jastreboff, A. M., L. J. Aronne, N. N. Ahmad, S. Wharton, L. Connery, B. Alves, A. Kiyosue, S. Zhang, B. Liu, M. C. Bunck, A. Stefanski and S.-. Investigators (2022). "Tirzepatide Once Weekly for the Treatment of Obesity." N Engl J Med.
Jelinek, L. J., S. Lok, G. B. Rosenberg, R. A. Smith, F. J. Grant, S. Biggs, P. A. Bensch, J. L. Kuijper, P. O. Sheppard, C. A. Sprecher and et al. (1993). "Expression cloning and signaling properties of the rat glucagon receptor." Science **259**(5101): 1614-1616.
Ji, L., L. Gao, H. Jiang, J. Yang, L. Yu, J. Wen, C. Cai, H. Deng, L. Feng, B. Song, Q. Ma and L. Qian (2022). "Safety and efficacy of a GLP-1 and glucagon receptor dual agonist mazdutide (IBI362) 9 mg and 10 mg in Chinese adults with overweight or obesity: A randomised, placebo-controlled, multiple-ascending-dose phase 1b trial." EClinicalMedicine **54**: 101691.
Jiang, H., S. Pang, Y. Zhang, T. Yu, M. Liu, H. Deng, L. Li, L. Feng, B. Song, H. Han-Zhang, Q. Ma, L. Qian and W. Yang (2022). "A phase 1b randomised controlled trial of a glucagon-like peptide-1 and glucagon receptor dual agonist IBI362 (LY3305677) in Chinese patients with type 2 diabetes." Nat Commun **13**(1): 3613.
Joel, C. D. (1966). "Stimulation of metabolism of rat brown adipose tissue by addition of lipolytic hormones in vitro." J Biol Chem **241**(4): 814-821.
Jones, B. (2022). "The therapeutic potential of GLP-1 receptor biased agonism." Br J Pharmacol **179**(4): 492-510.
Jones, B., T. Buenaventura, N. Kanda, P. Chabosseau, B. M. Owen, R. Scott, R. Goldin, N. Angkathunyakul, I. R. Correa, Jr., D. Bosco, P. R. Johnson, L. Piemonti, P. Marchetti, A. M. J. Shapiro, B. J. Cochran, A. C. Hanyaloglu, A. Inoue, T. Tan, G. A. Rutter, A. Tomas and S. R. Bloom (2018). "Targeting GLP-1 receptor trafficking to improve agonist efficacy." Nat Commun **9**(1): 1602.
Jones, B., E. R. McGlone, Z. Fang, P. Pickford, I. R. Correa, Jr., A. Oishi, R. Jockers, A. Inoue, S. Kumar, F. Gorlitz, C. Dunsby, P. M. W. French, G. A. Rutter, T. Tan, A. Tomas and S. R. Bloom (2021). "Genetic and biased agonist-mediated reductions in beta-arrestin recruitment prolong cAMP signaling at glucagon family receptors." J Biol Chem **296**: 100133.
Junker, A. E., L. Gluud, J. J. Holst, F. K. Knop and T. Vilsboll (2016). "Diabetic and nondiabetic patients with nonalcoholic fatty liver disease have an impaired incretin effect and fasting hyperglucagonaemia." J Intern Med **279**(5): 485-493.
Kamei, Y., Y. Hatazawa, R. Uchitomi, R. Yoshimura and S. Miura (2020). "Regulation of Skeletal Muscle Function by Amino Acids." Nutrients **12**(1).
Kawamori, D., A. J. Kurpad, J. Hu, C. W. Liew, J. L. Shih, E. L. Ford, P. L. Herrera, K. S. Polonsky, O. P. McGuinness and R. N. Kulkarni (2009). "Insulin signaling in alpha cells modulates glucagon secretion in vivo." Cell Metab **9**(4): 350-361.
Kharitonenkov, A., T. L. Shiyanova, A. Koester, A. M. Ford, R. Micanovic, E. J. Galbreath, G. E. Sandusky, L. J. Hammond, J. S. Moyers, R. A. Owens, J. Gromada, J. T. Brozinick, E. D. Hawkins, V. J. Wroblewski, D. S. Li, F. Mehrbod, S. R. Jaskunas and A. B. Shanafelt (2005). "FGF-21 as a novel metabolic regulator." J Clin Invest **115**(6): 1627-1635.
Kilberg, M. S., E. F. Barber and M. E. Handlogten (1985). "Characteristics and hormonal regulation of amino acid transport system A in isolated rat hepatocytes." Curr Top Cell Regul **25**: 133-163.
Kim, J., H. Okamoto, Z. Huang, G. Anguiano, S. Chen, Q. Liu, K. Cavino, Y. Xin, E. Na, R. Hamid, J. Lee, B. Zambrowicz, R. Unger, A. J. Murphy, Y. Xu, G. D. Yancopoulos, W. H. Li and J. Gromada (2017). "Amino Acid Transporter Slc38a5

Controls Glucagon Receptor Inhibition-Induced Pancreatic alpha Cell Hyperplasia in Mice." Cell Metab **25**(6): 1348-1361 e1348.
Kim, T., C. L. Holleman, S. Nason, D. M. Arble, N. Ottaway, J. Chabenne, C. Loyd, J. A. Kim, D. Sandoval, D. J. Drucker, R. DiMarchi, D. Perez-Tilve and K. M. Habegger (2018). "Hepatic Glucagon Receptor Signaling Enhances Insulin-Stimulated Glucose Disposal in Rodents." Diabetes **67**(11): 2157-2166.
Kim, T., S. Nason, J. Antipenko, B. Finan, A. Shalev, R. DiMarchi and K. M. Habegger (2022). "Hepatic TORC2 Signaling Facilitates Acute Glucagon-Receptor Enhancement of Insulin-Stimulated Glucose Homeostasis in Mice." Diabetes.
Kim, T., S. Nason, C. Holleman, M. Pepin, L. Wilson, T. F. Berryhill, A. R. Wende, C. Steele, M. E. Young, S. Barnes, D. J. Drucker, B. Finan, R. DiMarchi, D. Perez-Tilve, M. Tschop and K. M. Habegger (2018). "Glucagon Receptor Signaling Regulates Energy Metabolism via Hepatic Farnesoid X Receptor and Fibroblast Growth Factor 21." Diabetes **67**(9): 1773-1782.
Kimball C, M. J. (1923). ""Aqueous extracts of pancreas III. Some precipitation reactions of insulin"." J Biol Chem **58**(1): 337-348.
King, A. (2010). "Prevention: Neuropsychiatric adverse effects signal the end of the line for rimonabant." Nat Rev Cardiol **7**(11): 602.
Kjobsted, R., J. R. Hingst, J. Fentz, M. Foretz, M. N. Sanz, C. Pehmoller, M. Shum, A. Marette, R. Mounier, J. T. Treebak, J. F. P. Wojtaszewski, B. Viollet and L. Lantier (2018). "AMPK in skeletal muscle function and metabolism." FASEB J **32**(4): 1741-1777.
Kleinert, M., S. Sachs, K. M. Habegger, S. M. Hofmann and T. D. Muller (2019). "Glucagon Regulation of Energy Expenditure." Int J Mol Sci **20**(21).
Knerr, P. J., S. A. Mowery, J. D. Douros, B. Premdjee, K. R. Hjollund, Y. He, A. M. Kruse Hansen, A. K. Olsen, D. Perez-Tilve, R. D. DiMarchi and B. Finan (2022). "Next generation GLP-1/GIP/glucagon triple agonists normalize body weight in obese mice." Mol Metab: 101533.
Knop, F. K. (2018). "EJE PRIZE 2018: A gut feeling about glucagon." Eur J Endocrinol **178**(6): R267-R280.
Knop, F. K., T. Vilsboll, S. Madsbad, J. J. Holst and T. Krarup (2007). "Inappropriate suppression of glucagon during OGTT but not during isoglycaemic i.v. glucose infusion contributes to the reduced incretin effect in type 2 diabetes mellitus." Diabetologia **50**(4): 797-805.
Koda, S., Y. Date, N. Murakami, T. Shimbara, T. Hanada, K. Toshinai, A. Niijima, M. Furuya, N. Inomata, K. Osuye and M. Nakazato (2005). "The role of the vagal nerve in peripheral PYY3-36-induced feeding reduction in rats." Endocrinology **146**(5): 2369-2375.
Koerker, D. J., W. Ruch, E. Chideckel, J. Palmer, C. J. Goodner, J. Ensinck and C. C. Gale (1974). "Somatostatin: hypothalamic inhibitor of the endocrine pancreas." Science **184**(4135): 482-484.
Koo, S. H., L. Flechner, L. Qi, X. Zhang, R. A. Screaton, S. Jeffries, S. Hedrick, W. Xu, F. Boussouar, P. Brindle, H. Takemori and M. Montminy (2005). "The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism." Nature **437**(7062): 1109-1111.
Kosinski, J. R., J. Hubert, P. E. Carrington, G. G. Chicchi, J. Mu, C. Miller, J. Cao, E. Bianchi, A. Pessi, R. Sinharoy, D. J. Marsh and A. Pocai (2012). "The glucagon receptor is involved in mediating the body weight-lowering effects of oxyntomodulin." Obesity (Silver Spring) **20**(8): 1566-1571.

Kreb, H. (1973). "THE DISCOVERY OF THE ORNITHINE CYCLE OF UREA SYNTHESIS." Biochemical Education **1**.
Kreymann, B., G. Williams, M. A. Ghatei and S. R. Bloom (1987). "Glucagon-like peptide-1 7-36: a physiological incretin in man." Lancet **2**(8571): 1300-1304.
Kuroshima, A. and T. Yahata (1979). "Thermogenic responses of brown adipocytes to noradrenaline and glucagon in heat-acclimated and cold-acclimated rats." Jpn J Physiol **29**(6): 683-690.
Laeger, T., D. C. Albarado, S. J. Burke, L. Trosclair, J. W. Hedgepeth, H. R. Berthoud, T. W. Gettys, J. J. Collier, H. Munzberg and C. D. Morrison (2016). "Metabolic Responses to Dietary Protein Restriction Require an Increase in FGF21 that Is Delayed by the Absence of GCN2." Cell Rep **16**(3): 707-716.
Laeger, T., T. M. Henagan, D. C. Albarado, L. M. Redman, G. A. Bray, R. C. Noland, H. Munzberg, S. M. Hutson, T. W. Gettys, M. W. Schwartz and C. D. Morrison (2014). "FGF21 is an endocrine signal of protein restriction." J Clin Invest **124**(9): 3913-3922.
Lam, T. M., I. Vaartjes, D. E. Grobbee, D. Karssenberg and J. Lakerveld (2021). "Associations between the built environment and obesity: an umbrella review." Int J Health Geogr **20**(1): 7.
Lawrence, A. M. (1967). "Glucagon provocative test for pheochromocytoma." Ann Intern Med **66**(6): 1091-1096.
Lecker, S. H., A. L. Goldberg and W. E. Mitch (2006). "Protein degradation by the ubiquitin-proteasome pathway in normal and disease states." J Am Soc Nephrol **17**(7): 1807-1819.
Lees, E. K., R. Banks, C. Cook, S. Hill, N. Morrice, L. Grant, N. Mody and M. Delibegovic (2017). "Direct comparison of methionine restriction with leucine restriction on the metabolic health of C57BL/6J mice." Sci Rep **7**(1): 9977.
Lees, E. K., E. Krol, L. Grant, K. Shearer, C. Wyse, E. Moncur, A. S. Bykowska, N. Mody, T. W. Gettys and M. Delibegovic (2014). "Methionine restriction restores a younger metabolic phenotype in adult mice with alterations in fibroblast growth factor 21." Aging Cell **13**(5): 817-827.
Leung, P. M. and Q. R. Rogers (1971). "Importance of prepyriform cortex in food-intake response of rats to amino acids." Am J Physiol **221**(3): 929-935.
Li, L., P. Zhang, Z. Bao, T. Wang, S. Liu and F. Huang (2016). "PGC-1alpha Promotes Ureagenesis in Mouse Periportal Hepatocytes through SIRT3 and SIRT5 in Response to Glucagon." Sci Rep **6**: 24156.
Li, P., Y. L. Yin, D. Li, S. W. Kim and G. Wu (2007). "Amino acids and immune function." Br J Nutr **98**(2): 237-252.
Li, W., T. Kirchner, G. Ho, F. Bonilla, K. D'Aquino, J. Littrell, R. Zhang, W. Jian, X. Qiu, S. Zheng, B. Gao, P. Wong, J. N. Leonard and R. C. Camacho (2020). "Amino acids are sensitive glucagon receptor-specific biomarkers for glucagon-like peptide-1 receptor/glucagon receptor dual agonists." Diabetes Obes Metab **22**(12): 2437-2450.
Lockie, S. H., K. M. Heppner, N. Chaudhary, J. R. Chabenne, D. A. Morgan, C. Veyrat-Durebex, G. Ananthakrishnan, F. Rohner-Jeanrenaud, D. J. Drucker, R. DiMarchi, K. Rahmouni, B. J. Oldfield, M. H. Tschop and D. Perez-Tilve (2012). "Direct control of brown adipose tissue thermogenesis by central nervous system glucagon-like peptide-1 receptor signaling." Diabetes **61**(11): 2753-2762.
Longuet, C., A. M. Robledo, E. D. Dean, C. Dai, S. Ali, I. McGuinness, V. de Chavez, P. M. Vuguin, M. J. Charron, A. C. Powers and D. J. Drucker (2013). "Liver-specific disruption of the murine glucagon receptor produces alpha-cell hyperplasia: evidence for a circulating alpha-cell growth factor." Diabetes **62**(4): 1196-1205.

Longuet, C., E. M. Sinclair, A. Maida, L. L. Baggio, M. Maziarz, M. J. Charron and D. J. Drucker (2008). "The glucagon receptor is required for the adaptive metabolic response to fasting." Cell Metab **8**(5): 359-371.
Loos, R. J. F. and G. S. H. Yeo (2022). "The genetics of obesity: from discovery to biology." Nat Rev Genet **23**(2): 120-133.
Ludvik, B., F. Giorgino, E. Jodar, J. P. Frias, L. Fernandez Lando, K. Brown, R. Bray and A. Rodriguez (2021). "Once-weekly tirzepatide versus once-daily insulin degludec as add-on to metformin with or without SGLT2 inhibitors in patients with type 2 diabetes (SURPASS-3): a randomised, open-label, parallel-group, phase 3 trial." Lancet **398**(10300): 583-598.
Lund, A., T. Vilsboll, J. I. Bagger, J. J. Holst and F. K. Knop (2011). "The separate and combined impact of the intestinal hormones, GIP, GLP-1, and GLP-2, on glucagon secretion in type 2 diabetes." Am J Physiol Endocrinol Metab **300**(6): E1038-1046.
Lund, P. K., R. H. Goodman, M. R. Montminy, P. C. Dee and J. F. Habener (1983). "Anglerfish islet pre-proglucagon II. Nucleotide and corresponding amino acid sequence of the cDNA." J Biol Chem **258**(5): 3280-3284.
Lusk, G. (1924). "Animal calorimetry: twenty-fourth paper. Analysis of the oxidation of mixtures of carbohydrate and fat." J. Biol. Chem. **59**: 41-42.
Lutz, T. A., B. Coester, L. Whiting, A. A. Dunn-Meynell, C. N. Boyle, S. G. Bouret, B. E. Levin and C. Le Foll (2018). "Amylin Selectively Signals Onto POMC Neurons in the Arcuate Nucleus of the Hypothalamus." Diabetes **67**(5): 805-817.
Lv, S., X. Qiu, J. Li, J. Liang, W. Li, C. Zhang, Z. N. Zhang and B. Luan (2017). "Glucagon-induced extracellular cAMP regulates hepatic lipid metabolism." J Endocrinol **234**(2): 73-87.
Lynch, A. M., N. Pathak, V. Pathak, F. P. O'Harte, P. R. Flatt, N. Irwin and V. A. Gault (2014). "A novel DPP IV-resistant C-terminally extended glucagon analogue exhibits weight-lowering and diabetes-protective effects in high-fat-fed mice mediated through glucagon and GLP-1 receptor activation." Diabetologia **57**(9): 1927-1936.
Ma, X. M. and J. Blenis (2009). "Molecular mechanisms of mTOR-mediated translational control." Nat Rev Mol Cell Biol **10**(5): 307-318.
Maes, H. H., M. C. Neale and L. J. Eaves (1997). "Genetic and environmental factors in relative body weight and human adiposity." Behav Genet **27**(4): 325-351.
Maida, A., A. Zota, K. A. Sjoberg, J. Schumacher, T. P. Sijmonsma, A. Pfenninger, M. M. Christensen, T. Gantert, J. Fuhrmeister, U. Rothermel, D. Schmoll, M. Heikenwalder, J. L. Iovanna, K. Stemmer, B. Kiens, S. Herzig and A. J. Rose (2016). "A liver stress-endocrine nexus promotes metabolic integrity during dietary protein dilution." J Clin Invest **126**(9): 3263-3278.
Mallinson, C. N., S. R. Bloom, A. P. Warin, P. R. Salmon and B. Cox (1974). "A glucagonoma syndrome." Lancet **2**(7871): 1-5.
Marso, S. P., G. H. Daniels, K. Brown-Frandsen, P. Kristensen, J. F. Mann, M. A. Nauck, S. E. Nissen, S. Pocock, N. R. Poulter, L. S. Ravn, W. M. Steinberg, M. Stockner, B. Zinman, R. M. Bergenstal, J. B. Buse, L. S. Committee and L. T. Investigators (2016). "Liraglutide and Cardiovascular Outcomes in Type 2 Diabetes." N Engl J Med **375**(4): 311-322.
Martin Friedrichsen, L. E., Frederik Flindt Kreiner, Ronald Goldwater, Martin Kankam, Søren Toubro, Sune Boris Nygård (2022). "Glucagon/GLP-1 receptor co-agonist NNC9204-1177 reduced body weight in adults with overweight or obesity but was associated with safety issue." MedRxiv.

Martin Friedrichsen, L. E., Frederik Flindt Kreiner, Ronald Goldwater, Martin Kankam, Søren Toubro, Sune Boris Nygård (2022). "Glucagon/GLP-1 receptor co-agonist NNC9204-1177 reduced body weight in adults with overweight or obesity but was associated with safety issues." Medrxiv.
Martin Friedrichsen, L. E., Frederik Flindt Kreiner, Ronald Goldwater, Martin Kankam, Søren Toubro, Sune Boris Nygård (2022). "Glucagon/GLP-1 receptor co-agonist NNC9204-1177 reduced body weight in adults with overweight or obesity but was associated with safety issues."
Martinez, M. S., A. Manzano, L. C. Olivar, M. Nava, J. Salazar, L. D'Marco, R. Ortiz, M. Chacin, M. Guerrero-Wyss, M. Cabrera de Bravo, C. Cano, V. Bermudez and L. Angarita (2021). "The Role of the alpha Cell in the Pathogenesis of Diabetes: A World beyond the Mirror." Int J Mol Sci **22**(17).
McAllister, E. J., N. V. Dhurandhar, S. W. Keith, L. J. Aronne, J. Barger, M. Baskin, R. M. Benca, J. Biggio, M. M. Boggiano, J. C. Eisenmann, M. Elobeid, K. R. Fontaine, P. Gluckman, E. C. Hanlon, P. Katzmarzyk, A. Pietrobelli, D. T. Redden, D. M. Ruden, C. Wang, R. A. Waterland, S. M. Wright and D. B. Allison (2009). "Ten putative contributors to the obesity epidemic." Crit Rev Food Sci Nutr **49**(10): 868-913.
McGlashon, J. M., M. C. Gorecki, A. E. Kozlowski, C. K. Thirnbeck, K. R. Markan, K. L. Leslie, M. E. Kotas, M. J. Potthoff, G. B. Richerson and M. P. Gillum (2015). "Central serotonergic neurons activate and recruit thermogenic brown and beige fat and regulate glucose and lipid homeostasis." Cell Metab **21**(5): 692-705.
McLean, P. and F. Novello (1965). "Influence of Pancreatic Hormones on Enzymes Concerned with Urea Synthesis in Rat Liver." Biochem J **94**: 410-422.
Meguid, M. M., M. F. Brennan, W. A. Muller and T. T. Aoki (1972). "Glucagon and trauma." Lancet **2**(7787): 1145.
Meier, J. J., B. Gallwitz, N. Siepmann, J. J. Holst, C. F. Deacon, W. E. Schmidt and M. A. Nauck (2003). "Gastric inhibitory polypeptide (GIP) dose-dependently stimulates glucagon secretion in healthy human subjects at euglycaemia." Diabetologia **46**(6): 798-801.
Meier, J. J., M. A. Nauck, A. Pott, K. Heinze, O. Goetze, K. Bulut, W. E. Schmidt, B. Gallwitz and J. J. Holst (2006). "Glucagon-like peptide 2 stimulates glucagon secretion, enhances lipid absorption, and inhibits gastric acid secretion in humans." Gastroenterology **130**(1): 44-54.
Miller, D. S. and P. Mumford (1967). "Gluttony. 1. An experimental study of overeating low- or high-protein diets." Am J Clin Nutr **20**(11): 1212-1222.
Miller, D. S. and P. R. Payne (1968). "Longevity and protein intake." Exp Gerontol **3**(3): 231-234.
Miller, D. S. P., P.R (1962). "Weight maintenance and food intake." Journal of Nutrition **78**: 255-262.
Miller, L. L. (1960). "Glucagon: a protein catabolic hormone in the isolated perfused rat liver." Nature **185**: 248.
Moody, A. J., J. J. Holst, L. Thim and S. L. Jensen (1981). "Relationship of glicentin to proglucagon and glucagon in the porcine pancreas." Nature **289**(5797): 514-516.
Moro, T., S. M. Ebert, C. M. Adams and B. B. Rasmussen (2016). "Amino Acid Sensing in Skeletal Muscle." Trends Endocrinol Metab **27**(11): 796-806.
Morris, S. M., Jr. (2002). "Regulation of enzymes of the urea cycle and arginine metabolism." Annu Rev Nutr **22**: 87-105.
Mortimer, C. H., W. M. Tunbridge, D. Carr, L. Yeomans, T. Lind, D. H. Coy, S. R. Bloom, A. Kastin, C. N. Mallinson, G. M. Besser, A. V. Schally and R. Hall (1974).

"Effects of growth-hormone release-inhibiting hormone on circulating glucagon, insulin, and growth hormone in normal, diabetic, acromegalic, and hypopituitary patients." Lancet **1**(7860): 697-701.
Muller, T. D., M. Bluher, M. H. Tschop and R. D. DiMarchi (2022). "Anti-obesity drug discovery: advances and challenges." Nat Rev Drug Discov **21**(3): 201-223.
Murlin JR, C. H., Gibbs CBF,Stokes AM (1923). "Aqueous extracts of the pancreas. I. Influence on the carbohydrate metabolism of depancreatized animals." J Biol Chem **56**: 253-296.
Nahra, R., T. Wang, K. M. Gadde, J. Oscarsson, M. Stumvoll, L. Jermutus, B. Hirshberg and P. Ambery (2021). "Effects of Cotadutide on Metabolic and Hepatic Parameters in Adults With Overweight or Obesity and Type 2 Diabetes: A 54-Week Randomized Phase 2b Study." Diabetes Care **44**(6): 1433-1442.
Nair, K. S. (1987). "Hyperglucagonemia increases resting metabolic rate in man during insulin deficiency." J Clin Endocrinol Metab **64**(5): 896-901.
Nair, K. S., D. Halliday and J. S. Garrow (1984). "Increased energy expenditure in poorly controlled Type 1 (insulin-dependent) diabetic patients." Diabetologia **27**(1): 13-16.
Nair, K. S., D. Halliday, D. E. Matthews and S. L. Welle (1987). "Hyperglucagonemia during insulin deficiency accelerates protein catabolism." Am J Physiol **253**(2 Pt 1): E208-213.
Nair, K. S., P. D. Woolf, S. L. Welle and D. E. Matthews (1987). "Leucine, glucose, and energy metabolism after 3 days of fasting in healthy human subjects." Am J Clin Nutr **46**(4): 557-562.
Nason, S. R., J. Antipenko, N. Presedo, S. E. Cunningham, T. H. Pierre, T. Kim, J. R. Paul, C. Holleman, M. E. Young, K. L. Gamble, B. Finan, R. DiMarchi, C. S. Hunter, A. Kharitonenkov and K. M. Habegger (2021). "Glucagon receptor signaling regulates weight loss via central KLB receptor complexes." JCI Insight **6**(4).
Ness-Abramof, R. and C. M. Apovian (2005). "Drug-induced weight gain." Timely Top Med Cardiovasc Dis **9**: E31.
Nicholls, D. G., V. S. Bernson and G. M. Heaton (1978). "The identification of the component in the inner membrane of brown adipose tissue mitochondria responsible for regulating energy dissipation." Experientia Suppl **32**: 89-93.
O'Brien, P. E., A. Hindle, L. Brennan, S. Skinner, P. Burton, A. Smith, G. Crosthwaite and W. Brown (2019). "Long-Term Outcomes After Bariatric Surgery: a Systematic Review and Meta-analysis of Weight Loss at 10 or More Years for All Bariatric Procedures and a Single-Centre Review of 20-Year Outcomes After Adjustable Gastric Banding." Obes Surg **29**(1): 3-14.
Okun, J. G., P. M. Rusu, A. Y. Chan, Y. Wu, Y. W. Yap, T. Sharkie, J. Schumacher, K. V. Schmidt, K. M. Roberts-Thomson, R. D. Russell, A. Zota, S. Hille, A. Jungmann, L. Maggi, Y. Lee, M. Bluher, S. Herzig, M. A. Keske, M. Heikenwalder, O. J. Muller and A. J. Rose (2021). "Liver alanine catabolism promotes skeletal muscle atrophy and hyperglycaemia in type 2 diabetes." Nat Metab **3**(3): 394-409.
Orentreich, N., J. R. Matias, A. DeFelice and J. A. Zimmerman (1993). "Low methionine ingestion by rats extends life span." J Nutr **123**(2): 269-274.
Organisation, W. H. "Fact Sheet “Obesity and overweight”."
Owen, B. M., X. Ding, D. A. Morgan, K. C. Coate, A. L. Bookout, K. Rahmouni, S. A. Kliewer and D. J. Mangelsdorf (2014). "FGF21 acts centrally to induce sympathetic nerve activity, energy expenditure, and weight loss." Cell Metab **20**(4): 670-677.

Owen, B. M., D. J. Mangelsdorf and S. A. Kliewer (2015). "Tissue-specific actions of the metabolic hormones FGF15/19 and FGF21." Trends Endocrinol Metab **26**(1): 22-29.
Pacy, P. J., K. N. Cheng, G. C. Ford and D. Halliday (1990). "Influence of glucagon on protein and leucine metabolism: a study in fasting man with induced insulin resistance." Br J Surg **77**(7): 791-794.
Parker, J. A., K. A. McCullough, B. C. Field, J. S. Minnion, N. M. Martin, M. A. Ghatei and S. R. Bloom (2013). "Glucagon and GLP-1 inhibit food intake and increase c-fos expression in similar appetite regulating centres in the brainstem and amygdala." Int J Obes (Lond) **37**(10): 1391-1398.
Parker, V. E. R., D. Robertson, T. Wang, D. C. Hornigold, M. Petrone, A. T. Cooper, M. G. Posch, T. Heise, L. Plum-Moerschel, H. Schlichthaar, B. Klaus, P. D. Ambery, J. J. Meier and B. Hirshberg (2020). "Efficacy, Safety, and Mechanistic Insights of Cotadutide, a Dual Receptor Glucagon-Like Peptide-1 and Glucagon Agonist." J Clin Endocrinol Metab **105**(3).
Penick, S. B. and L. E. Hinkle, Jr. (1961). "Depression of food intake induced in healthy subjects by glucagon." N Engl J Med **264**: 893-897.
Perez-Marti, A., M. Garcia-Guasch, A. Tresserra-Rimbau, A. Carrilho-Do-Rosario, R. Estruch, J. Salas-Salvado, M. A. Martinez-Gonzalez, R. Lamuela-Raventos, P. F. Marrero, D. Haro and J. Relat (2017). "A low-protein diet induces body weight loss and browning of subcutaneous white adipose tissue through enhanced expression of hepatic fibroblast growth factor 21 (FGF21)." Mol Nutr Food Res **61**(8).
Peris-Moreno, D., L. Cussonneau, L. Combaret, C. Polge and D. Taillandier (2021). "Ubiquitin Ligases at the Heart of Skeletal Muscle Atrophy Control." Molecules **26**(2).
Perrone, C. E., D. A. Mattocks, M. Jarvis-Morar, J. D. Plummer and N. Orentreich (2010). "Methionine restriction effects on mitochondrial biogenesis and aerobic capacity in white adipose tissue, liver, and skeletal muscle of F344 rats." Metabolism **59**(7): 1000-1011.
Peterson, R. D., C. H. Beatty and R. M. Bocek (1963). "Effects of insulin and glucagon on carbohydrate and protein metabolism of adductor muscle and diaphragm." Endocrinology **72**: 71-77.
Peth, A., T. Uchiki and A. L. Goldberg (2010). "ATP-dependent steps in the binding of ubiquitin conjugates to the 26S proteasome that commit to degradation." Mol Cell **40**(4): 671-681.
Pezeshki, A. and P. K. Chelikani (2021). "Low Protein Diets and Energy Balance: Mechanisms of Action on Energy Intake and Expenditure." Front Nutr **8**: 655833.
Pezeshki, A., R. C. Zapata, A. Singh, N. J. Yee and P. K. Chelikani (2016). "Low protein diets produce divergent effects on energy balance." Sci Rep **6**: 25145.
Pi-Sunyer, X., A. Astrup, K. Fujioka, F. Greenway, A. Halpern, M. Krempf, D. C. Lau, C. W. le Roux, R. Violante Ortiz, C. B. Jensen, J. P. Wilding, S. Obesity and N. N. S. G. Prediabetes (2015). "A Randomized, Controlled Trial of 3.0 mg of Liraglutide in Weight Management." N Engl J Med **373**(1): 11-22.
Pickford, P., M. Lucey, R. M. Rujan, E. R. McGlone, S. Bitsi, F. B. Ashford, I. R. Correa, D. J. Hodson, A. Tomas, G. Deganutti, C. A. Reynolds, B. M. Owen, T. M. Tan, J. Minnion, B. Jones and S. R. Bloom (2021). "Partial agonism improves the anti-hyperglycaemic efficacy of an oxyntomodulin-derived GLP-1R/GCGR co-agonist." Mol Metab **51**: 101242.
Plaisance, E. P., T. M. Henagan, H. Echlin, A. Boudreau, K. L. Hill, N. R. Lenard, B. E. Hasek, N. Orentreich and T. W. Gettys (2010). "Role of beta-adrenergic receptors

in the hyperphagic and hypermetabolic responses to dietary methionine restriction." Am J Physiol Regul Integr Comp Physiol **299**(3): R740-750.
Pocai, A., P. E. Carrington, J. R. Adams, M. Wright, G. Eiermann, L. Zhu, X. Du, A. Petrov, M. E. Lassman, G. Jiang, F. Liu, C. Miller, L. M. Tota, G. Zhou, X. Zhang, M. M. Sountis, A. Santoprete, E. Capito, G. G. Chicchi, N. Thornberry, E. Bianchi, A. Pessi, D. J. Marsh and R. SinhaRoy (2009). "Glucagon-like peptide 1/glucagon receptor dual agonism reverses obesity in mice." Diabetes **58**(10): 2258-2266.
Pontiroli, A. E., A. Calderara, M. G. Perfetti and S. R. Bareggi (1993). "Pharmacokinetics of intranasal, intramuscular and intravenous glucagon in healthy subjects and diabetic patients." Eur J Clin Pharmacol **45**(6): 555-558.
Pozefsky, T., R. G. Tancredi, R. T. Moxley, J. Dupre and J. D. Tobin (1976). "Metabolism of forearm tissues in man. Studies with glucagon." Diabetes **25**(2): 128-135.
Pratley, R. E., S. Jacob, S. Baek, M. E. Trautmann, M. Hompesch, O. Han, J. Stewart, C. H. Sorli, A. Shaunik and K. H. Yoon (2022). "Efficacy and safety of efpeglenatide in key patient subgroups from the BALANCE randomized trial, stratified by pre-diabetes status, BMI, and age at baseline." BMJ Open Diabetes Res Care **10**(1).
Quaye, E., S. Chacko, S. T. Chung, R. J. Brychta, K. Y. Chen and R. J. Brown (2021). "Energy expenditure due to gluconeogenesis in pathological conditions of insulin resistance." Am J Physiol Endocrinol Metab **321**(6): E795-E801.
Quinones, M., O. Al-Massadi, R. Gallego, J. Ferno, C. Dieguez, M. Lopez and R. Nogueiras (2015). "Hypothalamic CaMKKbeta mediates glucagon anorectic effect and its diet-induced resistance." Mol Metab **4**(12): 961-970.
R, S. (1961). "Thermogenic activity of the hibernating gland in the cold-acclimated rat." Physiologist **4**(113).
Rall, T. W. and E. W. Sutherland (1958). "Formation of a cyclic adenine ribonucleotide by tissue particles." J Biol Chem **232**(2): 1065-1076.
Ramracheya, R., C. Chapman, M. Chibalina, H. Dou, C. Miranda, A. Gonzalez, Y. Moritoh, M. Shigeto, Q. Zhang, M. Braun, A. Clark, P. R. Johnson, P. Rorsman and L. J. B. Briant (2018). "GLP-1 suppresses glucagon secretion in human pancreatic alpha-cells by inhibition of P/Q-type Ca(2+) channels." Physiol Rep **6**(17): e13852.
Ramracheya, R., C. Ward, M. Shigeto, J. N. Walker, S. Amisten, Q. Zhang, P. R. Johnson, P. Rorsman and M. Braun (2010). "Membrane potential-dependent inactivation of voltage-gated ion channels in alpha-cells inhibits glucagon secretion from human islets." Diabetes **59**(9): 2198-2208.
Raybould, H. E. (2007). "Mechanisms of CCK signaling from gut to brain." Curr Opin Pharmacol **7**(6): 570-574.
Rebecca Scott, D. H., Bryn Owen, Steve Bloom & Tricia Tan (2019). "GLP-1/Glucagon dual agonist affects amino acid metabolism." Endocrine Abstracts.
Reitman, M. L. (2007). "FGF21: a missing link in the biology of fasting." Cell Metab **5**(6): 405-407.
Rocha, D. M., F. Santeusanio, G. R. Faloona and R. H. Unger (1973). "Abnormal pancreatic alpha-cell function in bacterial infections." N Engl J Med **288**(14): 700-703.
Ross, M. H. (1961). "Length of life and nutrition in the rat." J Nutr **75**: 197-210.
Rothwell, N. J. and M. J. Stock (1979). "A role for brown adipose tissue in diet-induced thermogenesis." Nature **281**(5726): 31-35.

Rothwell, N. J. and M. J. Stock (1987). "Effect of diet and fenfluramine on thermogenesis in the rat: possible involvement of serotonergic mechanisms." Int J Obes **11**(4): 319-324.
Rothwell, N. J., M. J. Stock and R. S. Tyzbir (1982). "Energy balance and mitochondrial function in liver and brown fat of rats fed "cafeteria" diets of varying protein content." J Nutr **112**(9): 1663-1672.
Rothwell, N. J., M. J. Stock and R. S. Tyzbir (1983). "Mechanisms of thermogenesis induced by low protein diets." Metabolism **32**(3): 257-261.
Russell, M. C., T. J. Koehnle, J. A. Barrett, J. E. Blevins and D. W. Gietzen (2003). "The rapid anorectic response to a threonine imbalanced diet is decreased by injection of threonine into the anterior piriform cortex of rats." Nutr Neurosci **6**(4): 247-251.
Sahu, R., S. Kaushik, C. C. Clement, E. S. Cannizzo, B. Scharf, A. Follenzi, I. Potolicchio, E. Nieves, A. M. Cuervo and L. Santambrogio (2011). "Microautophagy of cytosolic proteins by late endosomes." Dev Cell **20**(1): 131-139.
Salem, V., C. Izzi-Engbeaya, C. Coello, D. B. Thomas, E. S. Chambers, A. N. Comninos, A. Buckley, Z. Win, A. Al-Nahhas, E. A. Rabiner, R. N. Gunn, H. Budge, M. E. Symonds, S. R. Bloom, T. M. Tan and W. S. Dhillo (2016). "Glucagon increases energy expenditure independently of brown adipose tissue activation in humans." Diabetes Obes Metab **18**(1): 72-81.
Salter, J. M. (1960). "Metabolic effects of glucagon in the Wistar Rat." The American Journal of Clinical Nutrition **8**(5): 535-539.
Salter, J. M. (1960). "Metabolic Effects of Glucagon in the Wistar Rat." The American Journal of Clinical Nutrition **8**(5).
Salter, J. M., I. W. Davidson and C. H. Best (1957). "The pathologic effects of large amounts of glucagon." Diabetes **6**(3): 248-252; discussion, 252-245.
Salter, J. M., C. Ezrin, J. C. Laidlaw and A. G. Gornall (1960). "Metabolic effects of glucagon in human subjects." Metabolism **9**: 753-768.
Sandri, M. (2010). "Autophagy in skeletal muscle." FEBS Lett **584**(7): 1411-1416.
Sarruf, D. A., J. P. Thaler, G. J. Morton, J. German, J. D. Fischer, K. Ogimoto and M. W. Schwartz (2010). "Fibroblast growth factor 21 action in the brain increases energy expenditure and insulin sensitivity in obese rats." Diabetes **59**(7): 1817-1824.
Scarpace, P. J. and Y. Zhang (2009). "Leptin resistance: a prediposing factor for diet-induced obesity." Am J Physiol Regul Integr Comp Physiol **296**(3): R493-500.
Schellens, J. P., H. Vreeling-Sindelarova, P. J. Plomp and A. J. Meijer (1988). "Hepatic autophagy and intracellular ATP. A morphometric study." Exp Cell Res **177**(1): 103-108.
Schmid, C. L., N. M. Kennedy, N. C. Ross, K. M. Lovell, Z. Yue, J. Morgenweck, M. D. Cameron, T. D. Bannister and L. M. Bohn (2017). "Bias Factor and Therapeutic Window Correlate to Predict Safer Opioid Analgesics." Cell **171**(5): 1165-1175 e1113.
Schulman, J. L., J. L. Carleton, G. Whitney and J. C. Whitehorn (1957). "Effect of glucagon on food intake and body weight in man." J Appl Physiol **11**(3): 419-421.
Scott, R., J. Minnion, T. Tan and S. R. Bloom (2018). "Oxyntomodulin analogue increases energy expenditure via the glucagon receptor." Peptides **104**: 70-77.
Sharretts, J., O. Galescu, S. Gomatam, E. Andraca-Carrera, C. Hampp and L. Yanoff (2020). "Cancer Risk Associated with Lorcaserin - The FDA's Review of the CAMELLIA-TIMI 61 Trial." N Engl J Med **383**(11): 1000-1002.
Shimizu, N., T. Maruyama, N. Yoshikawa, R. Matsumiya, Y. Ma, N. Ito, Y. Tasaka, A. Kuribara-Souta, K. Miyata, Y. Oike, S. Berger, G. Schutz, S. Takeda and H. Tanaka

(2015). "A muscle-liver-fat signalling axis is essential for central control of adaptive adipose remodelling." Nat Commun **6**: 6693.
Simonsen, L., J. Lau, T. Kruse, T. Guo, J. McGuire, J. F. Jeppesen, K. Niss, P. Sauerberg, K. Raun and C. Dornonville de la Cour (2022). "Preclinical evaluation of a protracted GLP-1/glucagon receptor co-agonist: Translational difficulties and pitfalls." PLoS One **17**(3): e0264974.
Simpson, S. J. and D. Raubenheimer (2005). "Obesity: the protein leverage hypothesis." Obes Rev **6**(2): 133-142.
Singer, M. A. (2003). "Dietary protein-induced changes in excretory function: a general animal design feature." Comp Biochem Physiol B Biochem Mol Biol **136**(4): 785-801.
Smriga, M., M. Kameishi, H. Uneyama and K. Torii (2002). "Dietary L-lysine deficiency increases stress-induced anxiety and fecal excretion in rats." J Nutr **132**(12): 3744-3746.
Smriga, M. and K. Torii (2003). "L-Lysine acts like a partial serotonin receptor 4 antagonist and inhibits serotonin-mediated intestinal pathologies and anxiety in rats." Proc Natl Acad Sci U S A **100**(26): 15370-15375.
Snodgrass, P. J., R. C. Lin, W. A. Muller and T. T. Aoki (1978). "Induction of urea cycle enzymes of rat liver by glucagon." J Biol Chem **253**(8): 2748-2753.
Solloway, M. J., A. Madjidi, C. Gu, J. Eastham-Anderson, H. J. Clarke, N. Kljavin, J. Zavala-Solorio, L. Kates, B. Friedman, M. Brauer, J. Wang, O. Fiehn, G. Kolumam, H. Stern, J. B. Lowe, A. S. Peterson and B. B. Allan (2015). "Glucagon Couples Hepatic Amino Acid Catabolism to mTOR-Dependent Regulation of alpha-Cell Mass." Cell Rep **12**(3): 495-510.
Sorensen, A., D. Mayntz, D. Raubenheimer and S. J. Simpson (2008). "Protein-leverage in mice: the geometry of macronutrient balancing and consequences for fat deposition." Obesity (Silver Spring) **16**(3): 566-571.
Speakman, J. R. (2013). "Measuring energy metabolism in the mouse - theoretical, practical, and analytical considerations." Front Physiol **4**: 34.
Spring, S., A. Singh, R. C. Zapata, P. K. Chelikani and A. Pezeshki (2019). "Methionine Restriction Partly Recapitulates the Sympathetically Mediated Enhanced Energy Expenditure Induced by Total Amino Acid Restriction in Rats." Nutrients **11**(3).
Stahel, P., S. J. Lee, S. K. Sud, A. Floh and S. Dash (2019). "Intranasal glucagon acutely increases energy expenditure without inducing hyperglycaemia in overweight/obese adults." Diabetes Obes Metab **21**(6): 1357-1364.
Staub, A., L. Sinn and O. K. Behrens (1953). "Purification and crystallization of hyperglycemic glycogenolytic factor (HGF)." Science **117**(3049): 628-629.
Staub, A., L. Sinn and O. K. Behrens (1955). "Purification and crystallization of glucagon." J Biol Chem **214**(2): 619-632.
Stevens, F. M., R. W. Flanagan, D. O'Gorman and K. D. Buchanan (1984). "Glucagonoma syndrome demonstrating giant duodenal villi." Gut **25**(7): 784-791.
Stirling, J. L. and M. J. Stock (1968). "Metabolic origins of thermogenesis induced by diet." Nature **220**(5169): 801-802.
Stock, M. J. (1999). "Gluttony and thermogenesis revisited." Int J Obes Relat Metab Disord **23**(11): 1105-1117.
Sundby, F., H. Jacobsen and A. J. Moody (1976). "Purification and characterization of a protein from porcine gut with glucagon-like immunoreactivity." Horm Metab Res **8**(5): 366-371.

Suppli, M. P., J. I. Bagger, A. Lund, M. Demant, G. van Hall, C. Strandberg, M. J. Konig, K. Rigbolt, J. L. Langhoff, N. J. Wewer Albrechtsen, J. J. Holst, T. Vilsboll and F. K. Knop (2020). "Glucagon Resistance at the Level of Amino Acid Turnover in Obese Subjects With Hepatic Steatosis." Diabetes **69**(6): 1090-1099.
Sutherland, E. W. and C. De Duve (1948). "Origin and distribution of the hyperglycemic-glycogenolytic factor of the pancreas." J Biol Chem **175**(2): 663-674.
Svendsen, B., O. Larsen, M. B. N. Gabe, C. B. Christiansen, M. M. Rosenkilde, D. J. Drucker and J. J. Holst (2018). "Insulin Secretion Depends on Intra-islet Glucagon Signaling." Cell Rep **25**(5): 1127-1134 e1122.
Tabarean, I. V. (2016). "Histamine receptor signaling in energy homeostasis." Neuropharmacology **106**: 13-19.
Takahara, T., Y. Amemiya, R. Sugiyama, M. Maki and H. Shibata (2020). "Amino acid-dependent control of mTORC1 signaling: a variety of regulatory modes." J Biomed Sci **27**(1): 87.
Tan, T. M., B. C. Field, K. A. McCullough, R. C. Troke, E. S. Chambers, V. Salem, J. Gonzalez Maffe, K. C. Baynes, A. De Silva, A. Viardot, A. Alsafi, G. S. Frost, M. A. Ghatei and S. R. Bloom (2013). "Coadministration of glucagon-like peptide-1 during glucagon infusion in humans results in increased energy expenditure and amelioration of hyperglycemia." Diabetes **62**(4): 1131-1138.
Thiessen, S. E., S. Derde, I. Derese, T. Dufour, C. A. Vega, L. Langouche, C. Goossens, N. Peersman, P. Vermeersch, S. Vander Perre, J. J. Holst, P. J. Wouters, I. Vanhorebeek and G. Van den Berghe (2017). "Role of Glucagon in Catabolism and Muscle Wasting of Critical Illness and Modulation by Nutrition." Am J Respir Crit Care Med **196**(9): 1131-1143.
Tillner, J., M. G. Posch, F. Wagner, L. Teichert, Y. Hijazi, C. Einig, S. Keil, T. Haack, M. Wagner, M. Bossart and P. J. Larsen (2019). "A novel dual glucagon-like peptide and glucagon receptor agonist SAR425899: Results of randomized, placebo-controlled first-in-human and first-in-patient trials." Diabetes Obes Metab **21**(1): 120-128.
Townsend, L. K., K. D. Medak, C. M. Knuth, W. T. Peppler, M. J. Charron and D. C. Wright (2019). "Loss of glucagon signaling alters white adipose tissue browning." FASEB J **33**(4): 4824-4835.
Turton, M. D., D. O'Shea, I. Gunn, S. A. Beak, C. M. Edwards, K. Meeran, S. J. Choi, G. M. Taylor, M. M. Heath, P. D. Lambert, J. P. Wilding, D. M. Smith, M. A. Ghatei, J. Herbert and S. R. Bloom (1996). "A role for glucagon-like peptide-1 in the central regulation of feeding." Nature **379**(6560): 69-72.
Tyberghein, J. (1953). "[Action of glucagon on the metabolism of proteins]." Arch Int Physiol **61**(1): 104-107.
Ugleholdt, R., X. Zhu, C. F. Deacon, C. Orskov, D. F. Steiner and J. J. Holst (2004). "Impaired intestinal proglucagon processing in mice lacking prohormone convertase 1." Endocrinology **145**(3): 1349-1355.
Ulbright, C. and P. J. Snodgrass (1993). "Coordinate induction of the urea cycle enzymes by glucagon and dexamethasone is accomplished by three different mechanisms." Arch Biochem Biophys **301**(2): 237-243.
Unger, R. H. (1971). "Glucagon and the insulin: glucagon ratio in diabetes and other catabolic illnesses." Diabetes **20**(12): 834-838.
Unger, R. H., E. Aguilar-Parada, W. A. Muller and A. M. Eisentraut (1970). "Studies of pancreatic alpha cell function in normal and diabetic subjects." J Clin Invest **49**(4): 837-848.

Unger, R. H., A. M. Eisentraut, C. M. Mc, S. Keller, H. C. Lanz and L. L. Madison (1959). "Glucagon antibodies and their use for immunoassay for glucagon." Proc Soc Exp Biol Med **102**: 621-623.
Unger, R. H., A. Ohneda, E. Aguilar-Parada and A. M. Eisentraut (1969). "The role of aminogenic glucagon secretion in blood glucose homeostasis." J Clin Invest **48**(5): 810-822.
Unger, R. H., A. Ohneda, I. Valverde, A. M. Eisentraut and J. Exton (1968). "Characterization of the responses of circulating glucagon-like immunoreactivity to intraduodenal and intravenous administration of glucose." J Clin Invest **47**(1): 48-65.
Unger, R. H. and L. Orci (1975). "The essential role of glucagon in the pathogenesis of diabetes mellitus." Lancet **1**(7897): 14-16.
Urva, S. (2022). "LY3437943, a novel triple GIP, GLP-1, and glucagon receptor agonist in people with type 2 diabetes: a phase 1b, multicentre, double-blind, placebo-controlled, randomised, multiple-ascending dose trial." The Lancet.
Van Dyke, R. W. (1988). "Proton pump-generated electrochemical gradients in rat liver multivesicular bodies. Quantitation and effects of chloride." J Biol Chem **263**(6): 2603-2611.
Vaughan, M. and D. Steinberg (1963). "Effect of Hormones on Lipolysis and Esterification of Free Fatty Acids during Incubation of Adipose Tissue in Vitro." J Lipid Res **4**: 193-199.
Veldhorst, M. A., M. S. Westerterp-Plantenga and K. R. Westerterp (2009). "Gluconeogenesis and energy expenditure after a high-protein, carbohydrate-free diet." Am J Clin Nutr **90**(3): 519-526.
Vergari, E., J. G. Knudsen, R. Ramracheya, A. Salehi, Q. Zhang, J. Adam, I. W. Asterholm, A. Benrick, L. J. B. Briant, M. V. Chibalina, F. M. Gribble, A. Hamilton, B. Hastoy, F. Reimann, N. J. G. Rorsman, Spiliotis, II, A. Tarasov, Y. Wu, F. M. Ashcroft and P. Rorsman (2019). "Insulin inhibits glucagon release by SGLT2-induced stimulation of somatostatin secretion." Nat Commun **10**(1): 139.
Victoria Flores, A. B. S., Michelle Sonsalla, Nicole E. Richardson, Deyang Yu, Grace E. Sheridan, Michaela E. Trautman, Reji Babygirija, Eunhae P. Cheng, Jennifer M. Rojas, Shany E. Yang, Matthew H. Wakai, Ryan Hubbell, Ildiko Kasza, Jay L. Tomasiewicz, Cara L. Green, Claudia Dantoin, Caroline M. Alexander, Joseph A. Baur, Kristen C. Malecki, View ORCID ProfileDudley W. Lamming (2022). "Regulation of metabolic health by dietary histidine in mice."
Vinales, K. L., B. Begaye, C. Bogardus, M. Walter, J. Krakoff and P. Piaggi (2019). "FGF21 Is a Hormonal Mediator of the Human "Thrifty" Metabolic Phenotype." Diabetes **68**(2): 318-323.
Virtanen, J. K., S. Voutilainen, T. H. Rissanen, P. Happonen, J. Mursu, J. A. Laukkanen, H. Poulsen, T. A. Lakka and J. T. Salonen (2006). "High dietary methionine intake increases the risk of acute coronary events in middle-aged men." Nutr Metab Cardiovasc Dis **16**(2): 113-120.
Virtue, S., P. Even and A. Vidal-Puig (2012). "Below thermoneutrality, changes in activity do not drive changes in total daily energy expenditure between groups of mice." Cell Metab **16**(5): 665-671.
von Mering J, M. O. (1890). "Diabetes mellitus nach Pankreasextirpation." Exp Pathol Pharmakol **26**: 371-378.
Wakelam, M. J., G. J. Murphy, V. J. Hruby and M. D. Houslay (1986). "Activation of two signal-transduction systems in hepatocytes by glucagon." Nature **323**(6083): 68-71.

Waldhausl, W., H. Haydl and P. Nowotny (1976). "ACTH and cortisol responses to glucagon stimulation." J Clin Endocrinol Metab **43**(3): 675-678.
Wan, X. S., X. H. Lu, Y. C. Xiao, Y. Lin, H. Zhu, T. Ding, Y. Yang, Y. Huang, Y. Zhang, Y. L. Liu, Z. M. Xu, J. Xiao and X. K. Li (2014). "ATF4- and CHOP-dependent induction of FGF21 through endoplasmic reticulum stress." Biomed Res Int **2014**: 807874.
Wanders, D., L. A. Forney, K. P. Stone, D. H. Burk, A. Pierse and T. W. Gettys (2017). "FGF21 Mediates the Thermogenic and Insulin-Sensitizing Effects of Dietary Methionine Restriction but Not Its Effects on Hepatic Lipid Metabolism." Diabetes **66**(4): 858-867.
Wanders, D., K. P. Stone, K. Dille, J. Simon, A. Pierse and T. W. Gettys (2015). "Metabolic responses to dietary leucine restriction involve remodeling of adipose tissue and enhanced hepatic insulin signaling." Biofactors **41**(6): 391-402.
Wang, F., F. Xiao, L. Du, Y. Niu, H. Yin, Z. Zhou, X. Jiang, H. Jiang, F. Yuan, K. Liu, S. Chen, S. Duan and F. Guo (2021). "Activation of GCN2 in macrophages promotes white adipose tissue browning and lipolysis under leucine deprivation." FASEB J **35**(6): e21652.
Weatherford, S. C. and S. Ritter (1988). "Lesion of vagal afferent terminals impairs glucagon-induced suppression of food intake." Physiol Behav **43**(5): 645-650.
Weiser, P. C. and F. Grande (1974). "Calorigenic effects of glucagon and epinephrine in anesthetized dogs." Proc Soc Exp Biol Med **145**(3): 912-917.
Wewer Albrechtsen, N. J., K. Faerch, T. M. Jensen, D. R. Witte, J. Pedersen, Y. Mahendran, A. E. Jonsson, K. D. Galsgaard, M. Winther-Sorensen, S. S. Torekov, T. Lauritzen, O. Pedersen, F. K. Knop, T. Hansen, M. E. Jorgensen, D. Vistisen and J. J. Holst (2018). "Evidence of a liver-alpha cell axis in humans: hepatic insulin resistance attenuates relationship between fasting plasma glucagon and glucagonotropic amino acids." Diabetologia **61**(3): 671-680.
Wewer Albrechtsen, N. J., A. E. Junker, M. Christensen, S. Haedersdal, F. Wibrand, A. M. Lund, K. D. Galsgaard, J. J. Holst, F. K. Knop and T. Vilsboll (2018). "Hyperglucagonemia correlates with plasma levels of non-branched-chain amino acids in patients with liver disease independent of type 2 diabetes." Am J Physiol Gastrointest Liver Physiol **314**(1): G91-G96.
Wewer Albrechtsen, N. J., J. Pedersen, K. D. Galsgaard, M. Winther-Sorensen, M. P. Suppli, L. Janah, J. Gromada, H. Vilstrup, F. K. Knop and J. J. Holst (2019). "The Liver-alpha-Cell Axis and Type 2 Diabetes." Endocr Rev **40**(5): 1353-1366.
Whytock, K. L., E. A. Carnero, R. B. Vega, J. Tillner, C. Bock, K. Chivukula, F. Yi, C. Meyer, S. R. Smith and L. M. Sparks (2021). "Prolonged Glucagon Infusion Does Not Affect Energy Expenditure in Individuals with Overweight/Obesity: A Randomized Trial." Obesity (Silver Spring) **29**(6): 1003-1013.
Wilding, J. P. H., R. L. Batterham, S. Calanna, M. Davies, L. F. Van Gaal, I. Lingvay, B. M. McGowan, J. Rosenstock, M. T. D. Tran, T. A. Wadden, S. Wharton, K. Yokote, N. Zeuthen, R. F. Kushner and S. S. Group (2021). "Once-Weekly Semaglutide in Adults with Overweight or Obesity." N Engl J Med **384**(11): 989.
Willard, F. S., J. D. Douros, M. B. Gabe, A. D. Showalter, D. B. Wainscott, T. M. Suter, M. E. Capozzi, W. J. van der Velden, C. Stutsman, G. R. Cardona, S. Urva, P. J. Emmerson, J. J. Holst, D. A. D'Alessio, M. P. Coghlan, M. M. Rosenkilde, J. E. Campbell and K. W. Sloop (2020). "Tirzepatide is an imbalanced and biased dual GIP and GLP-1 receptor agonist." JCI Insight **5**(17).
Wilson, G. J., B. A. Lennox, P. She, E. T. Mirek, R. J. Al Baghdadi, M. E. Fusakio, J. L. Dixon, G. C. Henderson, R. C. Wek and T. G. Anthony (2015). "GCN2 is required

to increase fibroblast growth factor 21 and maintain hepatic triglyceride homeostasis during asparaginase treatment." Am J Physiol Endocrinol Metab **308**(4): E283-293.
Winther-Sorensen, M., K. D. Galsgaard, A. Santos, S. A. J. Trammell, K. Sulek, R. E. Kuhre, J. Pedersen, D. B. Andersen, A. S. Hassing, M. Dall, J. T. Treebak, M. P. Gillum, S. S. Torekov, J. A. Windelov, J. E. Hunt, S. A. S. Kjeldsen, S. L. Jepsen, C. G. Vasilopoulou, F. K. Knop, C. Orskov, M. P. Werge, H. C. Bisgaard, P. L. Eriksen, H. Vilstrup, L. L. Gluud, J. J. Holst and N. J. Wewer Albrechtsen (2020). "Glucagon acutely regulates hepatic amino acid catabolism and the effect may be disturbed by steatosis." Mol Metab **42**: 101080.
Wolfe, B. M., J. M. Culebras, T. T. Aoki, N. E. O'Connor, R. J. Finley, A. Kaczowka and F. D. Moore (1979). "The effects of glucagon on protein metabolism in normal man." Surgery **86**(2): 248-257.
Wu, G. (2016). "Dietary protein intake and human health." Food Funct **7**(3): 1251-1265.
Wynne, K., A. J. Park, C. J. Small, K. Meeran, M. A. Ghatei, G. S. Frost and S. R. Bloom (2006). "Oxyntomodulin increases energy expenditure in addition to decreasing energy intake in overweight and obese humans: a randomised controlled trial." Int J Obes (Lond) **30**(12): 1729-1736.
Xia, T., Y. Cheng, Q. Zhang, F. Xiao, B. Liu, S. Chen and F. Guo (2012). "S6K1 in the central nervous system regulates energy expenditure via MC4R/CRH pathways in response to deprivation of an essential amino acid." Diabetes **61**(10): 2461-2471.
Xia, T., Q. Zhang, Y. Xiao, C. Wang, J. Yu, H. Liu, B. Liu, Y. Zhang, S. Chen, Y. Liu, Y. Chen and F. Guo (2015). "CREB/TRH pathway in the central nervous system regulates energy expenditure in response to deprivation of an essential amino acid." Int J Obes (Lond) **39**(1): 105-113.
Xiao, F., Y. Du, Z. Lv, S. Chen, J. Zhu, H. Sheng and F. Guo (2016). "Effects of essential amino acids on lipid metabolism in mice and humans." J Mol Endocrinol **57**(4): 223-231.
Xiao, F. and F. Guo (2021). "Impacts of essential amino acids on energy balance." Mol Metab: 101393.
Xiao, F. and F. Guo (2022). "Impacts of essential amino acids on energy balance." Mol Metab **57**: 101393.
Xu, J., D. J. Lloyd, C. Hale, S. Stanislaus, M. Chen, G. Sivits, S. Vonderfecht, R. Hecht, Y. S. Li, R. A. Lindberg, J. L. Chen, D. Y. Jung, Z. Zhang, H. J. Ko, J. K. Kim and M. M. Veniant (2009). "Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice." Diabetes **58**(1): 250-259.
Yabut, J. M., J. D. Crane, A. E. Green, D. J. Keating, W. I. Khan and G. R. Steinberg (2019). "Emerging Roles for Serotonin in Regulating Metabolism: New Implications for an Ancient Molecule." Endocr Rev **40**(4): 1092-1107.
Yahata, T., Y. Habara and A. Kuroshima (1983). "Effects of glucagon and noradrenaline on the blood flow through brown adipose tissue in temperature-acclimated rats." Jpn J Physiol **33**(3): 367-376.
Yalow, R. S. and S. A. Berson (1960). "Immunoassay of endogenous plasma insulin in man." J Clin Invest **39**: 1157-1175.
Yap, Y. W., P. M. Rusu, A. Y. Chan, B. C. Fam, A. Jungmann, S. M. Solon-Biet, C. K. Barlow, D. J. Creek, C. Huang, R. B. Schittenhelm, B. Morgan, D. Schmoll, B. Kiens, M. D. W. Piper, M. Heikenwalder, S. J. Simpson, S. Broer, S. Andrikopoulos, O. J. Muller and A. J. Rose (2020). "Restriction of essential amino acids dictates the systemic metabolic response to dietary protein dilution." Nat Commun **11**(1): 2894.

Yokogoshi, H. and K. Ashida (1975). "Comparison between the metabolic effects of tryptophan and histidine deficiencies in the rat." J Nutr **105**(5): 550-556.
Yoon, M. S. (2017). "mTOR as a Key Regulator in Maintaining Skeletal Muscle Mass." Front Physiol **8**: 788.
Yu, D., N. E. Richardson, C. L. Green, A. B. Spicer, M. E. Murphy, V. Flores, C. Jang, I. Kasza, M. Nikodemova, M. H. Wakai, J. L. Tomasiewicz, S. E. Yang, B. R. Miller, H. H. Pak, J. A. Brinkman, J. M. Rojas, W. J. Quinn, 3rd, E. P. Cheng, E. N. Konon, L. R. Haider, M. Finke, M. Sonsalla, C. M. Alexander, J. D. Rabinowitz, J. A. Baur, K. C. Malecki and D. W. Lamming (2021). "The adverse metabolic effects of branched-chain amino acids are mediated by isoleucine and valine." Cell Metab **33**(5): 905-922 e906.
Yu, Y., T. South, Q. Wang and X. F. Huang (2008). "Differential expression of hypothalamic CART mRNA in response to body weight change following different dietary interventions." Neurochem Int **52**(8): 1422-1430.
Yuan, F., H. Jiang, H. Yin, X. Jiang, F. Jiao, S. Chen, H. Ying, Y. Chen, Q. Zhai and F. Guo (2020). "Activation of GCN2/ATF4 signals in amygdalar PKC-delta neurons promotes WAT browning under leucine deprivation." Nat Commun **11**(1): 2847.
Zapata, R. C., A. Singh, N. M. Ajdari and P. K. Chelikani (2018). "Dietary Tryptophan Restriction Dose-Dependently Modulates Energy Balance, Gut Hormones, and Microbiota in Obesity-Prone Rats." Obesity (Silver Spring) **26**(4): 730-739.
Zapata, R. C., A. Singh, A. Pezeshki, B. S. Avirineni, S. Patra and P. K. Chelikani (2019). "Low-Protein Diets with Fixed Carbohydrate Content Promote Hyperphagia and Sympathetically Mediated Increase in Energy Expenditure." Mol Nutr Food Res **63**(21): e1900088.
Zhang, Q., C. T. Delessa, R. Augustin, M. Bakhti, G. Collden, D. J. Drucker, A. Feuchtinger, C. G. Caceres, G. Grandl, A. Harger, S. Herzig, S. Hofmann, C. L. Holleman, M. Jastroch, S. Keipert, M. Kleinert, P. J. Knerr, K. Kulaj, B. Legutko, H. Lickert, X. Liu, G. Luippold, D. Lutter, E. Malogajski, M. T. Medina, S. A. Mowery, A. Blutke, D. Perez-Tilve, C. Salinno, L. Sehrer, R. D. DiMarchi, M. H. Tschop, K. Stemmer, B. Finan, C. Wolfrum and T. D. Muller (2021). "The glucose-dependent insulinotropic polypeptide (GIP) regulates body weight and food intake via CNS-GIPR signaling." Cell Metab **33**(4): 833-844 e835.
Zhang, Q., B. Liu, Y. Cheng, Q. Meng, T. Xia, L. Jiang, S. Chen, Y. Liu and F. Guo (2014). "Leptin signaling is required for leucine deprivation-enhanced energy expenditure." J Biol Chem **289**(3): 1779-1787.
Zhang, Q., R. Ramracheya, C. Lahmann, A. Tarasov, M. Bengtsson, O. Braha, M. Braun, M. Brereton, S. Collins, J. Galvanovskis, A. Gonzalez, L. N. Groschner, N. J. Rorsman, A. Salehi, M. E. Travers, J. N. Walker, A. L. Gloyn, F. Gribble, P. R. Johnson, F. Reimann, F. M. Ashcroft and P. Rorsman (2013). "Role of KATP channels in glucose-regulated glucagon secretion and impaired counterregulation in type 2 diabetes." Cell Metab **18**(6): 871-882.
Zhang, Y., Y. Xie, E. D. Berglund, K. C. Coate, T. T. He, T. Katafuchi, G. Xiao, M. J. Potthoff, W. Wei, Y. Wan, R. T. Yu, R. M. Evans, S. A. Kliewer and D. J. Mangelsdorf (2012). "The starvation hormone, fibroblast growth factor-21, extends lifespan in mice." Elife **1**: e00065.
Zhou, X., L. He, D. Wan, H. Yang, K. Yao, G. Wu, X. Wu and Y. Yin (2016). "Methionine restriction on lipid metabolism and its possible mechanisms." Amino Acids **48**(7): 1533-1540.