# The Effects of Protein on Gastrointestinal Responses, Associated with Energy Intake and Blood Glucose Regulation – a Management Strategy for Obesity and Type 2 Diabetes

A thesis submitted by
Rachel Anne Elovaris

For the degree of
Doctor of Philosophy

Adelaide Medical School
Faculty of Health and Medical Sciences
The University of Adelaide

July 2021

# Table of Contents

**Abstract** ...... VII

**Declaration of originality** ...... IX

**Dedication** ...... I

**Acknowledgements** ...... II

**Publications arising from this thesis** ...... III

**Abbreviations** ...... IV

**Chapter 1: Introduction** ...... 1

**1.1 An overview of obesity and type 2 diabetes** ...... 2

1.1.1 Obesity: prevalence, diagnosis, pathogenesis and metabolic consequences ...... 2

1.1.1.1 Prevalence ...... 2

1.1.1.2 Diagnosis ...... 3

1.1.1.3 Pathogenesis ...... 4

1.1.1.4 Metabolic consequences ...... 5

1.1.2 T2D: prevalence, diagnosis, pathogenesis and complications ...... 7

1.1.2.1 Prevalence ...... 7

1.1.2.2 Diagnosis of T2D ...... 7

1.1.2.3 Pathogenesis and complications of T2D ...... 8

**1.2 Treatment interventions for weight loss and management of blood glucose** ...... 9

1.2.1 Lifestyle interventions ...... 10

1.2.1.1 Hypocaloric diets ...... 11

1.2.1.1.1 Very low calorie diets and low calorie diets ...... 11

1.2.1.1.2 Hypocaloric diets with varying macronutrient composition ...... 12

1.2.1.2 High-protein diets ...... 13

1.2.1.2.1 Effects of high-protein diets on weight loss ...... 14

1.2.1.2.2 Effects of high-protein diets on metabolic parameters ...... 15

1.2.1.2.3 Safety of high-protein diets ...... 17

1.2.2 Pharmacotherapies for obesity ...... 18

Table 1.1: ...... 20

1.2.3 Bariatric surgery ...... 21

Figure 1.1: ...... 22

1.2.4 Pharmacotherapies for T2D ...... 23

Table 1.2: 26
**1.3 The effects of protein on GI function, energy intake and blood glucose 27**
1.3.1 Meal-related signals and effects on GI functions 28
1.3.1.1 Meal ingestion and related signals 28
1.3.1.2 GI motor function and gastric emptying of a meal 29
1.3.1.3 GI hormone responses 31
1.3.1.3.1 Ghrelin 31
1.3.1.3.2 Cholecystokinin 33
1.3.1.3.3 Peptide tyrosine tyrosine 34
1.3.1.3.4 Incretin hormones 36
1.3.1.3.5 Insulin 39
1.3.1.3.6 Glucagon 40
1.3.2 Acute effects of protein on GI function, energy intake and blood glucose 42
1.3.2.1 Effects of protein on energy intake and associated GI mechanisms 44
Figure 1.2: 47
1.3.2.2 Acute effects of protein on blood glucose and associated GI mechanisms 47
Figure 1.3: 48
Figure 1.4: 51
**1.4 The effects of amino acids on GI function, energy intake and blood glucose 52**
1.4.1 Effects of individual amino acids on GI function, energy intake and blood glucose 52
1.4.1.1 Effects of tryptophan on energy intake, blood glucose and GI responses 52
Figure 1.5: 54
1.4.1.2 Effects of phenylalanine on energy intake, blood glucose and GI responses 55
Figure 1.6: 56
1.4.1.3 Effects of lysine on energy intake, blood glucose and GI responses 57
Figure 1.7: 59
1.4.2 Effects of the BCAAs on body weight and glucose homeostasis 60
1.4.2.1 Effects of leucine on energy intake, blood glucose and GI responses 63
Figure 1.8: 67
1.4.2.2 Effects of isoleucine on energy intake, blood glucose and GI responses 68
Figure 1.9: 70
1.4.2.3 Effects of valine on energy intake, blood glucose and GI responses 71
**1.5 Aims and hypotheses 73**

**Chapter 2: Plasma free amino acid responses to whey protein and their relationships with gastric emptying, blood glucose- and appetite-regulatory hormones and energy intake in lean healthy men** ..... **75**

**2.1 Abstract** ..... **78**

**2.2 Introduction** ..... **78**

**2.3 Materials and methods** ..... **80**

2.3.1 Participants ..... 80

2.3.2 Study Outline ..... 81

2.3.3 Protein Drinks ..... 81

2.3.4 Protocol ..... 82

2.3.5 Measurements ..... 82

2.3.5.1 Gastric Emptying ..... 82

2.3.5.2 Plasma Ghrelin, CCK, GLP-1, Insulin, Glucagon, Free AA and Blood Glucose Concentrations ..... 83

2.3.5.3 Energy Intake ..... 85

2.3.6 Data and Statistical Analysis ..... 85

**2.4 Results** ..... **86**

2.4.1 Plasma AA Concentrations in Response to Increasing Protein Loads ..... 86

Table 2.1: ..... 87

Figure 2.1: ..... 88

Figure 2.2: ..... 89

Table 2.2: ..... 91

2.4.2 Relationships between Gastric Emptying, Ghrelin, CCK, GLP-1, Insulin, Glucagon, Blood Glucose and Energy Intake with Plasma AA concentrations ..... 92

Figure 2.3: ..... 93

**2.5 Discussion** ..... **94**

**Chapter 3: Intraduodenal administration of L-valine has no effect on antropyloroduodenal pressures, plasma cholecystokinin concentrations or energy intake in healthy, lean men** ..... **99**

**3.1 Abstract** ..... **102**

**3.2 Introduction** ..... **103**

**3.3 Materials and methods** ..... **105**

3.3.1 Participants ..... 105

3.3.2 Study Design ........ 106
3.3.3 Intraduodenal Infusions ........ 106
3.3.4 Study Protocol ........ 106
3.3.5 Measurements ........ 108
3.3.5.1 Energy Intake ........ 108
3.3.5.2 Appetite Perceptions and GI Symptoms ........ 108
3.3.5.3 Antropyloroduodenal Pressures ........ 109
3.3.5.4 Plasma CCK and Blood Glucose Concentrations ........ 109
3.3.6 Data and Statistical Analyses ........ 110
**3.4 Results ........ 111**
3.4.1 Energy Intake ........ 111
Figure 3.1: ........ 111
3.4.2 Appetite Perceptions and GI Symptoms ........ 112
Figure 3.2: ........ 112
3.4.3 Antropyloroduodenal Pressures ........ 113
Table 3.1: ........ 113
3.4.4 Plasma CCK and Blood Glucose Concentrations ........ 114
Figure 3.3: ........ 114
**3.5 Discussion ........ 114**

**Chapter 4: Comparative effects of the branched-chain amino acids, leucine, isoleucine and valine, on gastric emptying, plasma glucose, C-peptide and glucagon in healthy men ........ 118**
**4.1 Abstract ........ 121**
**4.2 Introduction ........ 121**
**4.3 Participants and methods ........ 123**
4.3.1 Study Participants ........ 123
4.3.2 Study Design ........ 124
Figure 4.1: ........ 125
4.3.3 Study Treatments ........ 125
4.3.4 Study Protocol ........ 126
4.3.5 Measurements ........ 127
4.3.5.1 Plasma Glucose and Hormone Analyses ........ 127
4.3.5.2 Gastric Emptying ........ 127

4.3.5.3 GI Symptoms ... 128
4.3.6 Data and Statistical Analysis ... 128
**4.4 Results ... 129**
4.4.1 Plasma Glucose Concentrations ... 129
4.4.2 Plasma C-Peptide Concentrations ... 130
4.4.3 Plasma Glucagon Concentrations ... 131
Figure 4.2: ... 132
Table 4.1: ... 133
4.4.4 Gastric Emptying ... 134
Figure 4.3: ... 134
4.4.5 Gastrointestinal Symptoms ... 134
4.4.6 Relationships Between Plasma Glucose with C-Peptide, Glucagon or Gastric Emptying ... 134
**4.5 Discussion ... 135**

**Chapter 5: Intragastric administration of leucine and isoleucine does not reduce the glycaemic response to, or slow gastric emptying of, a carbohydrate-containing drink in type 2 diabetes ... 139**
**5.1 Abstract ... 142**
**5.2 Introduction ... 142**
**5.3 Materials and methods ... 145**
5.3.1 Study participants ... 145
5.3.2 Study outline ... 146
5.3.3 Study treatments ... 146
5.3.4 Study protocol ... 147
5.3.5 Measurements ... 148
5.3.5.1 Plasma glucose and hormone analyses ... 148
5.3.5.2 Gastric emptying ... 149
5.3.5.3 Gastrointestinal symptoms ... 149
5.3.6 Data and statistical analysis ... 149
**5.4 Results ... 150**
5.4.1 Plasma glucose ... 150
5.4.2 Insulin ... 151
5.4.3 Glucagon ... 152

5.4.4 Gastric emptying....152
Table 5.1:....153
Figure 5.1:....154
Figure 5.2:....155
**5.5 Discussion....155**

**Chapter 6: Conclusions....159**

**Appendices....165**
**Appendix I:....165**
Table 2.A1:....165
Table 2.A2:....166
Table 2.A3:....167

**Chapter 7: References....168**

# Abstract

The research reported in this thesis investigated whether dietary AAs, including the BCAAs, had the potential to reduce energy intake and/or postprandial blood glucose in health and T2D, by stimulating gastrointestinal functions (gut hormone release and gastric emptying). If this proved to be the case it would support the goal of identifying nutrient-based treatment approaches for obesity and T2D.

The key findings of the studies are:

1. Following whey protein drinks (30 and 70 g), plasma concentrations of specific AAs, increased in a 'load-of-protein-dependent' manner, and there were strong relationships between plasma CCK, GLP-1, insulin and glucagon with the BCAAs, methionine, tryptophan, aspartic acid and tyrosine (**Chapter 2**). Ghrelin and energy intake correlated inversely, but only weakly, with AAs. Blood glucose did not correlate with any AAs, possibly because the drinks contained no carbohydrate, and any effect of insulin was counteracted by glucagon.

2. Intraduodenal infusion of valine (3.3 and 9.9 g), had no effect on antral, pyloric or duodenal pressures, plasma CCK, blood glucose, appetite perceptions or energy intake (**Chapter 3**). These results suggest that valine, unlike leucine, as shown in a previous study, does not have an energy intake-suppressant effect.

3. Leucine and isoleucine, but not valine, administered intragastrically (10 g), reduced the blood glucose response to a mixed-nutrient drink (**Chapter 4**). Leucine and isoleucine slowed early gastric emptying of the drink, while neither AA affected early (t = 0-30 min) postprandial C-peptide. Valine did not stimulate C-peptide, but stimulated glucagon markedly prior to drink

ingestion. The lack of effect of valine to reduce blood glucose despite slowing gastric emptying may have reflected the simulation of glucagon.

4. In contrast to previous findings in healthy individuals, leucine and isoleucine, administered intragastrically (10 g), did not reduce the blood glucose response to a mixed-nutrient drink, in T2D (**Chapter 5**). Both AAs stimulated insulin before and after the drink. Isoleucine simulated glucagon before and after the drink, while leucine tended to stimulate glucagon, before the drink. Neither AA slowed gastric emptying. It is uncertain why insulin stimulation by leucine and isoleucine was not associated with glucose lowering, but the stimulation of glucagon, presumably by circulating AAs, is likely to be important.

In conclusion, the research presented in this thesis has established that AAs vary widely in their effects on energy intake and postprandial blood glucose in health and T2D. Leucine and isoleucine, lower blood glucose, in part mediated by the slowing of gastric emptying, while valine does not have an effect on energy intake or blood glucose. Surprisingly, leucine and isoleucine did not lower the blood glucose response to a mixed-nutrient drink in T2D. Accordingly, these observations indicate that the underlying mechanisms, mediating the effects of leucine and isoleucine, when given in isolation, on blood glucose, are differential and complex, particularly in relation to comparative effects of leucine and isoleucine in health and T2D. The conditions under which the BCAAs may be more effective (i.e. in combination), to reduce energy intake and postprandial blood glucose, warrant further evaluation.

# Declaration of originality

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

12 July 2021

Rachel Anne Elovaris

BSc (Hons), MA NutDiet

# Dedication

***Dedicated to my late Father Captain Philip Hammond***

***and late Brother-in-law Matthew Start.***

***Fathers' both passionate about learning and who by their own example and encouragement inspired their children to seek knowledge through perseverance in study.***

***"The big challenge is to become all that you have the possibility of becoming. You cannot believe what it does to the human spirit to maximise and stretch yourself to the limit."***

***Jim Rohn***

# Acknowledgements

Professor Christine Feinle-Bisset and Professor Michael Horowitz have been tireless in their efforts to guide me through to the finalisation of my PhD. I wish to express my sincere gratitude to both of them. They have both spent many hours of reading and correcting the drafts I have presented to them. Their input to my studies has been invaluable because their knowledge of the subject is vast. Christine's perseverance and time that was put into my work will always be valued by me. Thank you.

Thanks to Ms Penelope Fitzgerald for her patience and excellent teaching methods which gave me skills to enable me to carry out laboratory work efficiently and successfully complete studies. Thank you to everyone from the CRE and the 'CFB-group' including Dr Sina Ullrich, Dr Christina McVeay, Dr Vida Bitarafan, Ms Maryam Hajishafiee, Mr Peyman Rezaie, Professor Shahram Agah and Mr Braden Rose, for your assistance with my laboratory studies, moral support, and intelligent discussions together. Many thanks also to Dr Natalie Luscombe-Marsh and Dr Amy Hutchison for their advice and encouragement, Ms Kylie Lange for her assistance regarding statistical issues, to Mr Scott Standfield and Ms Judith Wishart for their help with hormone analysis.

Thank you to my children, Nikolaos and Emerson, and partner, family and friends for their support. Without their encouragement and faith in me, this thesis would not have been made possible.

# Publications arising from this thesis

Elovaris RA*, Hutchison AT*, Lange K, Horowitz M, Feinle-Bisset C, Luscombe-Marsh ND. Plasma free amino acid responses to whey protein and their relationships with gastric emptying, blood glucose- and appetite-regulatory hormones and energy intake in lean healthy men. *Nutrients, 2019; 11(10): 2465.*

*Both authors contributed equally to this work

Elovaris RA, Fitzgerald PCE, Bitarafan V, Ullrich SS, Horowitz M, Feinle-Bisset C. Intraduodenal administration of L-valine has no effect on antropyloroduodenal pressures, plasma cholecystokinin concentrations or energy intake in healthy, lean men. *Nutrients, 2019;11(1): 99.*

Elovaris RA, Bitarafan V, Agah S, Ullrich SS, Lange K, Horowitz M, Feinle-Bisset C. Comparative effects of the branched-chain amino acids, leucine, isoleucine and valine, on gastric emptying, plasma glucose, C-peptide and glucagon in healthy men. *Nutrients, 2021; 13(5):1613.*

Elovaris RA, Hajishafiee M, Ullrich SS, Fitzgerald PCE, Lange K, Horowitz M, Feinle-Bisset C. Intragastric administration of leucine and isoleucine does not reduce the glycaemic response to, or slow gastric emptying of, a carbohydrate-containing drink in type 2 diabetes. *Diabetes Res Clin Pract, 2021;171:108618.*

# Abbreviations

| | |
|---|---|
| AA | Amino acid |
| ANOVA | Analysis of variance |
| AUC | Area under the curve |
| $AUC_{-31-1min}$ | Area under the curve from t=-31 to -1 |
| $AUC_{-1-30min}$ | Area under the curve from t=-1 to 30 min |
| $AUC_{0-30min}$ | Area under the curve from t=0 to 30 min |
| $AUC_{0-120min}$ | Area under the curve from t=0 to 120 min |
| $AUC_{15-120min}$ | Area under the curve from t=15 to 120 min |
| BCAAs | Branched-chain amino acids |
| BMI | Body mass index |
| C | Control with 0 g of protein |
| CCK | Cholecystokinin |
| CNS | Central nervous system |
| CVs | Coefficients of variation |
| DPP-IV | Dipeptidyl peptidase-IV |
| EECs | Enteroendocrine cells |
| ELISA | Enzyme-linked immunosorbent assay |
| GHS-R | Growth hormone secretagogue receptor |
| GI | Gastrointestinal |
| GIP | Glucose-dependent insulinotropic peptide |
| GLP-1 | Glucagon-like peptide-1 |
| GLUT4 | Glucose transporter-4 |
| H | 70 g pure whey protein isolate |
| HbA1c | Glycated haemoglobin |
| ID | Intraduodenally |
| IG | Intragastrically |
| IPPWs | Isolated pyloric pressure waves |
| i.v. | Intravenous |
| $iAUC_{-1-120min}$ | Incremental area under the curve from t=-1 to 120 min |
| $iAUC_{0-180min}$ | Incremental area under the curve from t=0 to 180 min |
| L | 30 g pure whey protein isolate |
| LCDs | Low calorie diets |

| | |
|---|---|
| L-Val-0.15 | Intraduodenal valine at the dose of 3.3 g |
| L-Val-0.45 | Intraduodenal valine at the dose of 9.9 g |
| MMC | Migrating motor complex |
| MI | Motility indices |
| NS | Not significant |
| OGTT | Oral glucose tolerance test |
| PYY | Peptide tyrosine tyrosine |
| PWs | Pressure waves |
| RYGB | Roux-en-Y gastric bypass |
| SEM | Standard error of the mean |
| SGLT-2 | Sodium-glucose co-transporter-2 |
| SUR | Sulphonylurea receptor |
| T2D | Type 2 diabetes |
| VAS | Visual analogue scale |
| VLCDs | Very low calorie diets |
| WHO | World Health Organization |
| Y2R | Y2 receptor |
| 3D | 3-dimensional |

# Chapter 1: Introduction

## 1.1 An overview of obesity and type 2 diabetes

Obesity and type 2 diabetes (T2D) have reached pandemic proportions. The prevalence of obesity is closely associated with the marked increase in T2D (1). According to the National Health Survey, conducted by the Australian Bureau of Statistics, in 2017-18, individuals who were obese (i.e. body mass index (BMI) 30-39.9 kg/m$^2$) were almost five times as likely to have T2D, compared with those who were normal-weight (9.8 % compared with 2 %) (2). Worldwide, there are more people who are obese than underweight (with the exception of sub-Saharan Africa and Asia). Overweight and obesity are associated with more deaths than underweight, especially in developing countries (3). Both obesity and T2D are, accordingly, major public health issues throughout the world, associated with serious co-morbidities, as well as substantial economic costs.

Management of overweight and obesity reduces the risk of developing co-morbidities, such as T2D. Additionally, treatment strategies for overweight and obesity also have beneficial effects in individuals with prediabetes or T2D. The following section will discuss the prevalence, pathogenesis and associated co-morbidities of obesity and T2D. Moreover, the current available treatments for obesity, including diet and lifestyle, bariatric surgery and pharmacotherapy options that also benefit T2D, as well as specific treatments for T2D, will also be discussed.

### 1.1.1 Obesity: prevalence, diagnosis, pathogenesis and metabolic consequences

#### *1.1.1.1* Prevalence

According to the most recent data from the World Health Organization (WHO), more than 1.9 billion adults are overweight (BMI 25-29.9 kg/m$^2$: 39 % of men and 40 % of women) (3). Between 1975 and 2016, the worldwide prevalence of obesity has almost tripled (3). In 2016,

650 million (13 %) adults were obese (11 % of men and 15 % of women), 340 million children and adolescents aged 5-19 were overweight or obese and 41 million children under the age of 5 were overweight (3). The most recent data from the National Health Survey, conducted by the Australian Bureau of Statistics, indicate that approximately two thirds (67 %) of the Australian adult population are overweight or obese (74.5 % of men and 59.7 % of women), a progressive increase from 56 % in 1995 (4). Alarmingly, the prevalence of severe obesity (BMI 35-40 kg/m$^2$) amongst Australian adults has almost doubled, from 5 % in 1995 to 9 % in 2014-15 (4).

#### ***1.1.1.2*** **Diagnosis**

A common method of measuring obesity is calculating an individual's BMI. BMI is defined as an individual's weight in kilograms divided by the square of their height in meters (kg/m$^2$), with a BMI between 18.5-24.9 kg/m$^2$ defined as normal weight, between 25-29.9 kg/m$^2$ as overweight, 30-39.9 kg/m$^2$ as obese and ≥ 40 kg/m$^2$ as morbidly obese (5). However, BMI only provides a crude estimate of body size, as it does not measure body fat directly, hence, it may not reflect the same body fat percentage or associated health risks in different individuals (6). In addition, it does not take into consideration age, sex, bone structure, fat distribution or muscle mass (7). For example, individuals who have a high amount of muscle mass, adding to their weight, but minimal fat, may be misclassified as overweight or obese. An alternative, and more precise tool, is measurement of waist circumference, as this closely reflects abdominal fat mass and total body fat. Abdominal obesity is defined as waist circumference > 94 cm in males and > 80 cm in females (8, 9). Changes in waist circumference also reflect changes in risk factors for chronic diseases, such as T2D and cardiovascular disease, more closely than BMI (10). For example, the risk of T2D is greatly increased when waist circumference is above

102 cm in males and 88 cm in females. Therefore, waist circumference, assessing abdominal obesity, is a more accurate indicator of chronic diseases than total obesity assessed by BMI.

***1.1.1.3* Pathogenesis**

In simple terms, obesity ensues from an energy imbalance, where energy intake exceeds energy expenditure (11, 12), resulting in a positive energy balance leading to an increase in body fat (11). This energy imbalance results from a complex interaction of genetic, environmental and social factors (13).

Genome-wide association studies have located obesity loci to numerous parts of the genome (14). For example, a common variant at the FTO (fat mass and obesity associated) loci is associated with changes in BMI and increased obesity in childhood through to adulthood (15). Twin and adoption studies have shown that genetic factors play a major role in the development of obesity (16, 17). In one study, 12 pairs of male twins were overfed 1000 kcal per day, 6 days per week, for a period of 100 days (17). Siblings gained a similar amount of weight; in contrast, there was substantial variability in the weight gain between twin pairs, somewhat questioning the importance of genetic factors (17). In an adoption study, genetic factors and the family environment were investigated and a strong relationship was found between the adoptees and the BMI of their biological parents, but no relationship between the BMI of adoptees and that of their adoptive parents, supporting the importance of genetics in obesity (16). Furthermore, gene mutations have been found to cause obesity in humans. For example, studies in morbidly obese individuals have shown mutations in the *LEP* and *LEPR* genes (18, 19). The *LEP* gene, a gene encoding leptin, was first discovered in mice (20) and is also known as the *Ob* gene due to its association with obesity (21). Leptin is an adipocyte-specific protein which plays a fundamental role in the regulation of food intake and energy expenditure and, therefore, body

weight. As both *LEP* and *LEPR* are pivotal to the regulation of energy expenditure, mutations in both, affect body metabolism, which in turn leads to weight gain and obesity (21). Congenital leptin deficiency, which is extremely rare, can be treated by exogenous administration of leptin, resulting in a sustained reduction in weight and BMI (22).

Genetic factors are also influenced by environmental and societal changes (23, 24). For example, factors contributing to this obesogenic environment, ranging from the ready availability of calorie-dense foods that are high in saturated fat and sugar (25), large portion sizes and increased soft drink (high sugar) consumption. Reductions in physical activity, sedentary occupations and increased screen time (25, 26) are also important. Taken together, societal changes in food intake, which have led to passive over-consumption, as well as a decrease in physical activity, has resulted in energy intake exceeding energy expenditure in everyday life to promote weight (fat mass) gain (25).

#### *1.1.1.4 Metabolic consequences*

During prolonged periods of energy overconsumption, the initial deposition of triglycerides occurs in subcutaneous adipose tissue (21). As the subcutaneous depot increases, limiting further lipid accumulation, triglycerides are increasingly diverted to visceral adipose tissue depots (21). In addition, adipose tissue secretes a number of adipocytokines, which are involved in inflammation and potentially contribute to insulin resistance and the development of T2D in obesity (27). This excess visceral adipose tissue, particularly around the abdomen, associated with obesity, is a risk factor for a range of major comorbidities, including metabolic syndrome, musculoskeletal conditions (e.g. osteoarthritis), particular cancers (e.g. endometrial, breast, ovarian, prostate, liver, gallbladder, kidney, and colon), cardiovascular disease (e.g. heart disease and stroke) and T2D (3, 28). Hence, weight loss and the maintenance of this

weight loss, are fundamental to prevent the current and alarming rise in obesity and co-related comorbidities, particularly T2D.

### 1.1.2 T2D: prevalence, diagnosis, pathogenesis and complications

#### *1.1.2.1 Prevalence*

Obesity substantially increases the risk of developing T2D. As obesity in the population continues to rise, so does the prevalence of T2D. According to the Australian Bureau of Statistics, in 2017-18, one in twenty Australians (1.2 million) had diabetes (2). T2D is the most rapidly growing, non-communicable, chronic disease in Australia and accounts for 85 % of all diabetes (2). In 2019, the International Diabetes Federation's Diabetes Atlas Report estimated that 9.3 % (463 million) of adults aged 20-79 years, had diabetes worldwide, and by 2040, one in ten individuals worldwide will have diabetes, and annual global health expenditure related to diabetes will reach USD$802 billion by 2045 (29).

#### *1.1.2.2 Diagnosis of T2D*

In undiagnosed individuals, early symptoms of diabetes that are often dismissed include tiredness and lethargy, weight gain, rashes and genital yeast infections. As the undiagnosed diabetes progresses and glucose levels gradually increase, thirst, polyuria, weight loss and blurred vision may be experienced (30). Thus, in the absence of biochemical testing, hyperglycaemia which is sufficient to cause pathological and functional changes, may be present for a long time before the diagnosis is made (30).

In order to avoid substantial progression of undiagnosed diabetes, individuals with symptoms, or who are at high risk for diabetes (i.e. due to their age, ethnicity, family history of diabetes, overweight/obesity, increasing waist circumference, unhealthy diet, sedentary behaviour) should be screened. There are a number of diagnostic tests for prediabetes and T2D. These include two acute measurements - a fasting blood glucose and/or a 2-hour blood glucose response to a 300 mL drink containing 75 g of glucose (i.e. the oral glucose tolerance test

(OGTT)), and a long-term marker, known as glycated haemoglobin A1c (HbA1c), which is an indicator of an individual's average circulating blood glucose over the past ~ 3 months. For diagnosis of prediabetes, impaired fasting glucose is defined by the WHO as a fasting blood glucose of 6.1-6.9 mmol/L, impaired glucose tolerance as a 2-hour blood glucose response of 7.8-11.1 mmol/L to an OGTT and a HbA1c up to 6.0-6.4 % [42-46 mmol/L]. The diagnostic criteria for T2D are a fasting blood glucose of ≥ 7 mmol/L, a 2-hour blood glucose response ≥ 11.1 mmol/L to an OGTT, or a HbA1c of ≥ 6.5 % [≥ 48 mmol/L]. Only the OGTT is recommended to detect prediabetes, and all three diagnostic tests can be used when detecting T2D (30, 31). However, according to the Australian Diabetes Society, a HbA1c level of 6.0-6.4 % [42-46 mmol/L] is indicative of a substantially increased risk of T2D. Hence, individuals with a HbA1c within this range should be managed accordingly (i.e. follow-up). Additionally, the fasting blood glucose and HbA1c are ongoing tests which may assist in assessing the management of T2D.

#### *1.1.2.3 Pathogenesis and complications of T2D*

T2D is a chronic progressive condition resulting from two major metabolic dysfunctions: impaired insulin secretion (i.e. due to β-cell dysfunction) and resistance to insulin action on peripheral tissues, skeletal muscle and liver, so that there is relative insulin deficiency (32). In order to maintain euglycaemia, insulin lowers blood glucose via two major mechanisms; 1) suppressing glucose production from the liver; and 2) promoting uptake of glucose by peripheral tissues, particularly skeletal muscle. However, under conditions of insulin resistance, insulin action is impaired in the liver and peripheral tissues, resulting in both, fasting and postprandial hyperglycaemia. Fasting hyperglycaemia reflects both increased hepatic glucose production (i.e. via increased gluconeogenesis and glycogenolysis) and decreased peripheral uptake of glucose. If T2D is not managed optimally, chronic hyperglycaemia can

lead to microvascular (e.g. retinopathy, nephropathy, and neuropathy) and macrovascular (e.g. cardiovascular) complications. Hence, weight loss and blood glucose lowering are fundamental in their management and prevention.

## 1.2 Treatment interventions for weight loss and management of blood glucose

There are a number of weight loss strategies primarily aimed at overweight and obese individuals. These also benefit T2D patients, as weight loss is associated with improvement in glycaemic control. Reducing weight in order to achieve the 'normal' or 'healthy' weight guidelines, defined by a BMI of 18.5-25 kg/$m^2$ (age ≥ 18-64) or BMI of 23-30 kg/$m^2$ (age ≥ 65 years), is not a realistic goal for the general population. Therefore, in most individuals, a common goal is to achieve a modest weight loss (i.e. 5-10 % of total body weight), that has clear benefits in improved glycaemia, blood pressure and cholesterol control (33-35). A sustained weight loss of as little as ~ 4 kg is associated in high-risk patients with a reduction in the progression to T2D (36).

Lifestyle modifications remain the first-line treatment for weight loss and are recommended by the WHO for both obesity and T2D, given their safety and accessibility (37). While weight loss from diet and lifestyle interventions can be achieved, the majority of individuals regain the weight that they have lost over the long-term (38). This is because weight loss is 'defended' by the body's 'set point' by counter-regulatory mechanisms to increase energy intake (39). When lifestyle modifications are ineffective for weight loss in the treatment of obesity, pharmacological strategies are used (11, 40). These can lead to 5-10 % weight loss, and some also have glucose-lowering effects. In addition, bariatric surgery, an option particularly for the morbidly obese, results in a more sustained weight loss and improvements in glycaemic

control. The following section provides an overview of current weight-loss strategies, including lifestyle, pharmacotherapy and surgical options for obesity, and blood glucose lowering medications for T2D.

### 1.2.1 Lifestyle interventions

Weight loss is fundamental for overweight and obese individuals who are at risk of developing co-morbidities such as T2D. Given that obesity reflects an imbalance between energy intake and energy expenditure, evidence-based guidelines for weight loss recommend reducing energy intake and increasing energy expenditure (41). Physical activity is desirable for the achievement, as well as the maintenance, of weight loss. Current guidelines, provided by the National Health and Medical Research Council of Australia, recommend 150 min of vigorous activity, or 300 min of moderate-intensity activity, per week (41). Importantly, physical activity maintains metabolically active lean tissue and improves metabolic and cardiovascular health outcomes. On its own, physical activity may be insufficient to achieve weight loss, although, in combination with a reduction in caloric intake, it creates a caloric deficit resulting in weight loss (41). However, suboptimal adherence, due to behaviour and/or environmental factors, impacts adversely on the long-term effectiveness of lifestyle interventions (41). Accordingly, many weight loss interventions that initially result in significant weight loss are followed by a plateau in weight and then progressive weight gain.

A reduction in caloric intake, facilitating weight loss, can be achieved by a range of dietary approaches. The following sections focus on hypocaloric diets for weight loss, including very low calorie diets (VLCDs) and low calorie diets (LCDs), as well as diets with varying macronutrient compositions, with a specific focus on high-protein diets. However, caution should be applied when interpreting the outcomes of these studies, as they are susceptible to

confounding by the other necessary changes to the diet (i.e. the proportion of other macronutrients or palatability).

#### *1.2.1.1 Hypocaloric diets*

Hypocaloric diets are well established weight loss strategies recommended for accelerating the weight loss process. Under the guidance of a nutrition expert, VLCDs and LCDs are an effective option to achieve, rapid, weight loss associated with improvements in glycaemic control and plasma lipid profiles. Furthermore, the longstanding belief that weight loss is more likely to be sustained if it is gradual as opposed to rapid, is incorrect.

##### *1.2.1.1.1 Very low calorie diets and low calorie diets*

VLCDs (i.e. ≤ 800 kcal/day), not surprisingly, lead to a greater and more rapid weight loss over the short-term compared with LCDs (i.e. ~ 1200-1600 kcal/day) (42). VLCDs are most commonly used to achieve initial rapid weight loss or as a preoperative weight loss strategy for morbidly obese patients scheduled for bariatric surgery. Weight loss using VLCDs averages between 1.5 to 2.5 kg per week, with a mean weight loss of 20 kg after 12 to 16 weeks (43, 44). In comparison, weight loss using LCDs averages between 0.4 to 0.5 kg per week, with a mean weight loss of 6 to 8 kg after 12 to 16 weeks (44). The Optifast® VLCD program is an example of a hypocaloric diet. Optifast® VLCD, consisting of the recommended daily requirements for vitamins, minerals, trace elements, fatty acids and protein, is used as a meal replacement. Regular dietetic support is needed to ensure that individuals are not at risk of macronutrient and/or micronutrient deficiencies. These diets are not recommended for long-term use, and they are not appropriate for all individuals, such as breastfeeding women and individuals with diabetes who are receiving hypoglycaemic medications, including insulin.

#### ***1.2.1.1.2 Hypocaloric diets with varying macronutrient composition***

Definition of the 'ideal' macronutrient composition of diets for the management of obesity, as well as associated metabolic conditions, has been the subject of many studies, and remains an area of substantial controversy. In the 1970s and 1980s, diets advocating low-fat, moderate-protein and high-carbohydrate intake (i.e. < 20 % fat, 20 % protein, > 60 % carbohydrate) were recommended widely based on the rationale that fat, which is of high caloric density (9 kcal/g), compared with carbohydrates or protein (~ 4 kcal/g), promotes passive overconsumption contributing to increased body weight (i.e. fat mass) and also increases the risk of cardiovascular disease. A meta-analysis of 19 controlled *ad libitum* low-fat studies, over 2-12 months, concluded that a reduction in dietary fat was associated with a weight loss of 1.9 – 4.5 kg (45). Low-fat diets have also shown to improve insulin sensitivity and cholesterol levels (46, 47). Low-carbohydrate diets ~ 20-30 g/day, with an increase in protein and unsaturated fats, may be more effective in inducing weight loss and improving glycaemia and cardiovascular risk factors over the first 6 months, when compared with low-fats diets (46, 48, 49). Low-carbohydrate diets lead to less insulin stimulation, potentially increasing insulin sensitivity, and also reduce glucose fluctuations (50). Samaha et al. (46) reported the effects of a low-carbohydrate diet (≤ 30 g carbohydrate/day) compared with a low-fat diet (deficit of 500 kcal/per day, with ≤ 30 % of less of energy from fat) in 132 morbidly obese participants. After 6 months, participants on the low-carbohydrate diet lost more weight (-5.8 ± 8.6 kg vs -1.9 ± 4.5 kg, respectively) and greater improvements in triglyceride levels and insulin sensitivity, compared with those on the low-fat diet (46). Taken together, while both low-fat and low-carbohydrate diets have beneficial effects on weight loss, low-carbohydrate diets may be more effective in promoting weight loss, as well as improving postprandial glycaemia.

### *1.2.1.2 High-protein diets*

Evidence generated during the past 20 years indicates that high-protein diets may be particularly effective for weight loss, maintenance of weight loss and improvement in co-morbidities associated with obesity (i.e. T2D and cardiovascular disease) (51). Furthermore, protein appears to have superior satiating effects compared with fat or carbohydrate (52), thereby facilitating weight loss by suppressing appetite and reducing energy intake (53-55). The WHO recommends a protein intake of 10-15 % of total daily energy or ~ 0.83 g/kg body weight (i.e. moderate or standard-protein intake) for healthy adults, and in high-protein diets protein intake is ≥ 20 % of total daily energy (i.e. ~ 0.8-1.6 g/kg body weight) (56, 57). One theory to account for the beneficial effects of a higher protein diet is the ‘protein leverage hypothesis’ (58) which suggests that the proportional energy from protein will decrease the amount of carbohydrate and fat consumed. Gosby et al. (59) tested the protein leverage theory in a short-term study (i.e. over a 4-day period) and found that lean individuals consumed ~ 10 % more energy on a low protein diet (10 % of energy derived from protein) when compared with a normal-protein diet (15 % of energy derived from protein) (59). The ‘protein leverage’ theory is likely to be of particular relevance in our current obesogenic environment, whereby the general population has access to a variety of processed foods that are likely to be high in fat and refined carbohydrate (i.e. increased caloric density), which are also highly palatable.

The following discussion examines current evidence on the effects of high-protein diets, on weight loss, as well as the effect on blood glucose control and cardiovascular risk factors. The potential adverse effects/risks of higher protein intakes will also be discussed.

***1.2.1.2.1 Effects of high-protein diets on weight loss***

The beneficial effects of high-protein diets for weight loss are now well-established as attested to by the outcomes of a meta-analysis of 24 randomised controlled trials that included studies with energy-matched, high and standard-protein arms (60). For example, when compared with a standard-protein diet (0.72 ± 0.09 g/kg/day; range: 0.55-0.88 g/kg/day), a high-protein diet (1.25 ± 0.17 g/kg/day; range: 1.07-1.60 g/kg/day) was more effective for weight loss (i.e. a 0.79 kg greater weight loss), body composition (i.e. inducing fat loss and preserving lean mass) and resting energy expenditure, over a duration of 12.1 ± 9.3 weeks (range: 4-52 week) (60). High-protein diets have also been reported to reduce energy intake and induce weight loss under *ad libitum* conditions. For example, in a landmark trial performed by Weigle et al. (61) the effects of both an isocaloric and *ad libitum* high-protein diet on appetite, weight loss and energy intake were investigated over a 4-month period in healthy individuals. Participants consumed a baseline diet consisting of 15 % protein, 35 % fat and 50 % carbohydrate for 2 weeks, followed by an isocaloric high-protein diet consisting of 30 % protein, 20 % fat and 50 % carbohydrate for a further two weeks. They then consumed the same high-protein diet *ad libitum for* 12 weeks. Satiety was markedly increased with the isocaloric high-protein diet, and during the *ad libitum* high-protein diet, participants decreased their daily intake by ~ 441 kcal, resulting in a weight loss of ~ 4.9 kg and decrease in fat mass of ~ 3.7 kg over 12 weeks.

Collectively these data suggest that high-protein diets, under both energy-restricted and *ad libitum* conditions, are beneficial for weight loss in obesity. At least in part, this is likely to reflect their greater satiating effect when compared with high-carbohydrate or high-fat meals. These satiety and appetite responses are modulated and maintained by both pre-absorptive and post-absorptive factors.

#### ***1.2.1.2.2 Effects of high-protein diets on metabolic parameters***

*Longer term effects of high-protein diets on glycaemia*

High-protein diets have beneficial effects on glycaemia, leading to reductions in blood glucose and HbA1c, as well as improvements in oral glucose tolerance and insulin sensitivity, over the longer term (62-64). For example McAuley et al. (65) compared an *ad libitum* high-protein diet (i.e. Zone diet – total energy provided by each meal and snack: 30 % from protein, 40 % from carbohydrate and 30 % from fat) with a high-fat diet or high-carbohydrate, high-fibre diet (control), in insulin-resistant obese women. At 8 weeks, insulin sensitivity was greater in the high-protein group compared with the high-fat and high-carbohydrate group (65). Piatti et al. (66) also reported that in women with normal glucose-tolerance, 21 days on a hypocaloric (800 kcal) high-protein diet (i.e. 45 % protein, 35 % carbohydrate and 20 % fat) improved glucose disposal, glucose oxidation and insulin sensitivity compared with a high-carbohydrate diet (i.e. 20 % protein, 60 % carbohydrate and 20 % fat). Furthermore, in a 5-week study in patients with T2D, a diet containing 30 % vs 15 % of total energy from protein, with a reduced carbohydrate content, was associated with reductions in HbA1c (~ 0.5 %) (67). Additionally, in obese patients with T2D, 7 days on their usual diet followed by 14 days on a low-carbohydrate/high-protein Atkins diet (i.e. carbohydrate ~ 20 g/day and protein ~ 151 g/day) was associated with an improvement in HbA1c (reduction from 7.3 to 6.8 %), decreased daily energy intake (3111 to 2164 kcal/day), weight loss (1.65 kg), as well as improved insulin sensitivity (68). Overall, this evidence strongly supports the benefits of high-protein diets to improve glycaemic control in both insulin resistant individuals and patients with T2D over the longer term.

*Cardiovascular benefits of high-protein diets*

High-protein diets have also been reported to exert beneficial effects on cardiovascular risk. Jenkins et al. (69) reported that in participants with hyperlipidaemia, over a one-month period, a high-wheat protein diet (27 % of energy derived from protein), compared with a control diet (16 % of energy derived from protein), was associated with reductions in triglyceride and LDL cholesterol concentrations. Samaha et al. (46) reported the effects of a high-protein Atkins diet (22 % of energy derived from protein), compared with a low-fat diet (16 % of energy derived from protein), in 79 obese and morbidly obese participants. After 6 months, participants on the high-protein diet had significantly lower triglycerides compared with the low-protein diet ( -20 % vs -4 %) (46). Farnsworth et al. (70) found in 57 overweight and obese hyperinsulinemic men and women that in those randomised to a high-protein diet (30 % of protein derived from energy), compared with a moderate-protein diet (15 % of protein derived from energy), there was a significant reduction in plasma triglyceride levels (-23 % vs -10 %) after 4 months. Furthermore, a number of studies have also reported that a high-protein diet, compared with a moderate-protein diet, over a 6-month period was associated with a significant decrease in plasma triglyceride levels (46, 48, 71).

Taken together, these beneficial effects of high-protein diets on weight loss, glycaemic control and cardiovascular risk factors support the use of high-protein diets as a nutrient-based therapy in the management of obesity and its associated co-morbidities, including T2D. These effects of protein on appetite, energy intake and blood glucose control may, at least in part, be mediated by underlying gastrointestinal (GI) mechanisms that are reviewed in **Section 1.3.2.**

***1.2.1.2.3 Safety of high-protein diets***

The long-term safety of high-protein diets is controversial. Specifically, higher protein intakes > 2.0 g/kg/day, compared with the recommended 'normal' protein intakes 0.8 – 1.2 g/kg/day), have been associated with increased risks of osteoporosis and renal dysfunction (72, 73).

The potential for higher protein intakes to be associated with osteoporosis is supported by the observation of increased urinary calcium excretion following high-protein meals, demonstrated in a number of short-term studies whereby dietary intakes of 2 g/kg/day of protein were associated with an increased renal calcium excretion and negative calcium balance compared with diets containing low-moderate protein intakes of 0.7 – 1.0 g/kg/day (72). It has been suggested that these effects may relate to the acid load generated by high protein-diets, specifically from animal protein, which represents a greater metabolic acid load compared with vegetable protein (72). This large acid load leads to bone resorption and hypercalciuria, unless buffered by the consumption of alkali-rich foods (i.e. fruits and vegetables) (74). However, a study of postmenopausal women over a 7-week period, a diet with moderate-high meat protein (1.6 g/kg/day), compared with a diet with moderate meat protein (0.8 g/kg/day), led to an increase in intestinal calcium absorption, which may, at least in part, compensate for an increase in urinary calcium excretion (75). Further supporting these findings, a review by Cuenca-Sanchez et al. (73) concluded that a higher protein intake could positively affect bone mineralisation and maintenance by increasing intestinal calcium absorption and insulin-like growth factor 1 concentrations. Accordingly, based on these findings and outcomes from other studies, protein intakes between ~ 1.0-1.5 g/kg/day, do not appear to be associated with adverse effects on net bone turnover or calcium balance (76, 77), or an increased risk of osteoporosis.

High-protein diets have also been associated with an increased renal acid load that may lead to kidney stones and/or an increase in the glomerular filtration rate, which may both be detrimental (72). While a number of short-term studies have shown that high-protein intake leads to hyperfiltration (a well-established phenomenon in early stages of chronic kidney disease) up to saturation point of ~ 125 g/day (78, 79), is likely to represent an adaptive mechanism of high-protein intakes that is not associated with a decline in kidney function (73). In overweight subjects, net filtration (i.e. filtration expressed as a function of renal mass), was not associated with protein intakes in the range 70–108 g/day, as higher protein intakes were linked to increased renal mass (80). Furthermore, in obese men and women, who consumed a calorie-restricted, high-protein diet (35 % of energy derived from protein or ~ 126 g/day) over a period of one year, no adverse effects on renal function were evident (81).

In conclusion, there is strong experimental support for the benefit of higher protein diets to reduce energy intake in both healthy weight and obese individuals to facilitate weight loss and over the longer-term subsequent weight maintenance. There is also a lack of strong evidence to suggest renal contraindications to high-protein intakes in either healthy or overweight/obese individuals, however, potentially susceptible groups, such as individuals living with T2D and those with existing chronic kidney disease, should seek professional advice regarding higher protein intakes.

### 1.2.2 Pharmacotherapies for obesity

There are a number of medications to treat obesity, however, only four drugs are currently approved by the Therapeutic Goods Administration for use in Australia, i.e. orlistat, phentermine, liraglutide and bupropion-naltrexone (Table 1.**1**). Orlistat is a gastric and pancreatic lipase inhibitor that reduces fat digestion, associated with a reduced absorption of

fat in the small intestine, resulting in lipids being transported through the GI tract and excreted via faeces (82). After one year, mean weight loss is 2.6-2.9 kg (83, 84), less than expected based on ~ 30 % lipase suppression, implying that food consumption increases. Adverse effects are frequent and include flatulence, faecal urgency and steatorrhea that is potentiated by high-fat foods (82, 85). Prolonged use may also lead to deficiencies in fat-soluble vitamins A, D, E and K (85). Phentermine is an amphetamine analogue acting centrally by increasing hypothalamic norepinephrine levels, which increases satiety and suppresses appetite (86). Phentermine is associated with a mean weight loss of 3.6 kg after a 13 week period (87). Adverse effects include paraesthesia, insomnia, dry mouth, unpleasant taste, constipation, vomiting, arrhythmia and tachycardia (85). This drug is only recommended for short-term use due to concerns regarding long-term cardiovascular, psychiatric and cognitive side effects. Liraglutide is a long-acting glucagon-like peptide-1 (GLP-1) agonist initially developed as a T2D medication (88), reducing postprandial glucose levels through an insulinotropic effect and inhibition of glucagon secretion and, possibly slowing of gastric emptying. Liraglutide is associated with a mean weight loss of 5.6 kg after one year (89). A disadvantage of liraglutide is that it requires subcutaneous injection (88). Furthermore, adverse effects, such as nausea, vomiting, diarrhoea and dyspepsia, occur frequently (88). Naltrexone is an opioid receptor agonist used to treat alcohol dependence and bupropion is a nonselective inhibitor of the dopamine transporter and norepinephrine transporter. In combination, bupropion-naltrexone increases satiety and reduces appetite, resulting in ~ 5 kg weight loss after one of year (83, 90). Common adverse effects include nausea, constipation, headache, vomiting, dizziness, insomnia, dry mouth, diarrhoea (82), as well as increasing heart rate and blood pressure. In relation to all pharmacotherapy, weight loss at one year is ~ 5 %, and less with orlistat, however, the response is variable. Pharmacotherapies for obesity are expensive and all have adverse effects. Additionally, therapy in responders should logically be prolonged, but this is not usually feasible.

**Table 1.1:** Pharmacotherapies for obesity approved by the Therapeutic Goods Administration in Australia.

| **Medication (trade names)** | **Route** | **Mechanism of action** | **Effect on weight** | **Main adverse effects** |
|---|---|---|---|---|
| Orlistat (Prolistat®, Xenical®) | Oral | Gastric and pancreatic lipase inhibitor; ↓ fat absorption (82) | After ~ 1 year mean weight loss in RCTs is 2.6-2.9 kg (83, 84) | ↑ fat in stools causing flatulence, faecal urgency, steatorrhea, fat-soluble vitamin deficiency, headache and fatigue (82, 85) |
| Phentermine (Duromine®, Metermine®) | Oral | Norepinephrine agonist; ↓ appetite (82) | After 13 weeks mean weight loss in RCTs is 3.6 kg (87) | Paraesthesia, insomnia, dry mouth, constipation, vomiting, arrhythmia, tachycardia and unpleasant taste (82, 85) |
| Liraglutide (Saxenda®) | Subcutaneous injection | GLP-1 receptor agonist; slows gastric emptying; ↑ satiety (82) | After ~ 1 year mean weight loss in RCTs is 5.6 kg (89) | Nausea, vomiting, diarrhoea and dyspepsia (88) |
| Naltrexone/Bupropion (Contrave ®) | Oral | Opioid receptor antagonist/ inhibitor of the dopamine and norepinephrine transporters; ↑ satiety, ↓ appetite (82) | After ~ 1 year mean weight loss in RCTs is 5 kg (83) | Constipation, headache, nausea, vomiting, dizziness, insomnia, dry mouth, diarrhoea (82), ↑ heart rate and ↑ blood pressure (90) |

RCTs, Randomised Control Trials

### 1.2.3 Bariatric surgery

Bariatric surgery is the most effective treatment for obesity (91). However, it is reserved for adults with a BMI ≥ 40 or a BMI ≥ 35 with at least one co-morbidity (92). In addition, it is only considered after other weight loss methods have been unsuccessful. The most commonly performed bariatric procedures are gastric banding, sleeve gastrectomy and Roux-en-Y Gastric Bypass (RYGB) (Figure **1.1**). Gastric banding is the least invasive of the three and is reversible. This procedure involves the placement of an inflatable ring around the gastro-oesophageal junction creating a small pouch. The band reduces the amount of food the stomach can accommodate leading to an increased feeling of postprandial fullness and reduction in hunger. For sleeve gastrectomy, the greater curvature and fundus of the stomach are resected, with the remaining stomach formed into a narrow tube with a volume of ~ 60-80 mL. RYGB is considered by many as the "gold-standard" procedure. It involves dividing the stomach into two parts forming a small gastric pouch < 30 mL volume (92). The pouch is connected to the mid-jejunum, thus bypassing the majority of the stomach, all of the duodenum, and part of the proximal jejunum (92). The duodenum is reconnected ~ 150 cm distally from the pylorus (92). All three procedures are associated with weight loss (i.e. 30-35 % total body weight range 1-3 years post-operation for sleeve gastrectomy and RYGB) (92-94), as well as a reduction in mortality (95). In 4047 obese patients, with a follow-up period ranging from 4 to 18 years, a greater long-term weight loss, in the bariatric surgical intervention group was associated with an overall reductions in mortality, compared with the control group (96). Improved co-morbidities, such as glycaemic control in T2D and hypertension have also been reported in both sleeve gastrectomy and RYGB (94). Following sleeve gastrectomy and RYGB surgery, up to 75-80 % of patients with T2D experience complete remission of diabetes, even before significant weight loss (97, 98). Sleeve gastrectomy and RYGB are superior to gastric banding, but more invasive. Common adverse effects of bariatric surgery include surgical complications,

vomiting, dumping syndrome and hypoglycaemia (i.e. usually in patients with diabetes) (99). Long-term complications can include micronutrient deficiencies (e.g. iron, thiamine, vitamin B12 and vitamin D deficiency, associated with the malabsorption of the fat-soluble vitamins A, D and K), as well as weight regain (99).

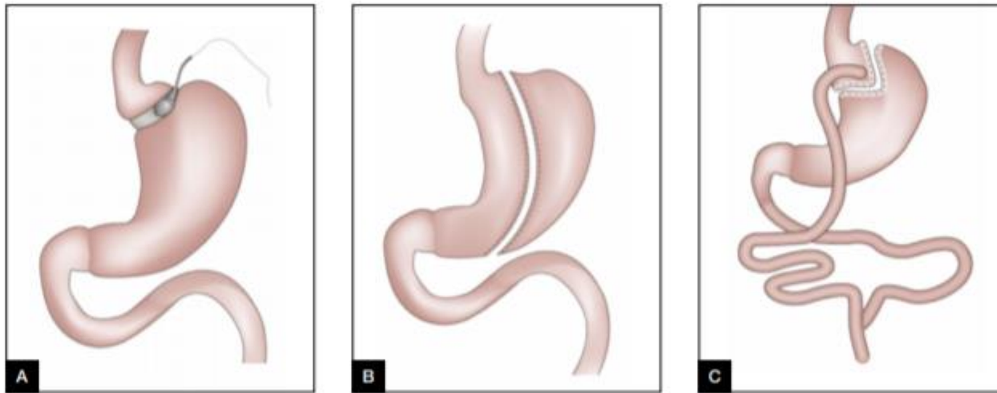


**Figure 1.1:** The three most common bariatric procedures for weight loss: A. Gastric banding; B. Sleeve gastrectomy; C. Roux-en-Y Gastric Bypass (RYGB).
Adapted from Lee and Dixon (100).

Caloric restriction, nutrient malabsorption and GI hormones, associated with appetite and energy intake, have been suggested to be the main mechanisms contributing to weight loss following sleeve gastrectomy and RYGB (92). The re-arrangement of the gut is associated with an acceleration of gastric emptying, as well as small intestinal transit. This is linked to a decrease in the hormone ghrelin, released from the stomach and an increase in the hormones peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) released, in particular, from the distal small intestine (97, 100). This more potent suppression of ghrelin and enhanced postprandial release of PYY and GLP-1 (and possibly also cholecystokinin (CCK)), induce satiety supporting weight loss (92, 101). Additionally, the increased stimulation of GLP-1 is likely to contribute to improved glycaemic control (102). The success of bariatric surgery in weight loss and the concomitant changes in GI hormones and glycaemic control, attests to the importance of GI hormones in the regulation of appetite, energy intake and blood glucose (i.e. discussed

in **Section 1.3.1.3**), and the potential for the upper GI tract as a target for novel, effective and adverse effect-free treatment approaches for obesity and T2D.

### 1.2.4 Pharmacotherapies for T2D

While management of T2D by diet and lifestyle, with the aim to achieve optimal blood glucose levels, is recommended as the first treatment option, most T2D patients fail to achieve the target range for blood glucose control (i.e. HbA1c level of ≤ 7 % [≤ 53 mmol/L]; fasting 6-8 mmol/L; 2 hours postprandial 6-10 mmol/L) (103, 104). Several pharmacotherapeutic options are available, acting via different mechanisms, in order to lower blood glucose levels (Table ). These options are commonly used under monotherapy and/or combination therapy, in order to achieve blood glucose levels within this target range or close to. Importantly, target ranges are often individualised, as they are dependent on an individual's background (i.e. age, medical or medication history).

Metformin is generally the first-line of oral hypoglycaemic medication. It was concluded that metformin acted primarily by reducing hepatic glucose output by inhibiting gluconeogenesis (105) and increasing insulin sensitivity in peripheral tissue and muscle by increasing the insulin receptor tyrosine kinase activity and glucose transporter (GLUT)-4 translocation to the cell membrane (106). The effects of metformin on glycaemia may be mediated primarily from the gut. Metformin enhances GLP-1 secretion, inhibits intestinal bile acid resorption and is associated with the modulation of gut microbiota (107). The main side-effects of metformin are gastrointestinal upset, including nausea and diarrhoea (105). Sulphonylureas (i.e. gliclazide and glipizide) are another form of oral hypoglycaemic medication. The effect is to stimulate the pancreas to produce more insulin (108). Insulin secretagogues bind to the sulphonylurea receptor (SUR)1 on the pancreatic β-cell membrane and close the associated ATP-sensitive $K^+$

channel (109). Inhibition of these channels leads to depolarization of the cell membrane which then release insulin into the blood (109). This effect is independent of glucose concentrations and there is an increased risk of hypoglycaemia (105). Another adverse effect of the release of insulin is weight gain (108). The incretin hormones, glucose-dependent insulinotropic peptide (GIP) and GLP-1 are rapidly inactivated by the enzyme dipeptidyl dipeptidase IV (DPP-IV) (110). Oral DPP-IV inhibitors (e.g. linagliptin, saxagliptin, sitagliptin) inhibit the enzymatic degradation of incretins, this, in turn, enhances the levels of active GLP-1 (the insulinotropic effect of GIP is markedly attenuated in T2D (111)), which act to lower blood glucose levels by stimulating insulin, and reducing glucagon, secretion (112). Adverse effects are rare. Another class of T2D medication, also targeting the incretin pathway, are the GLP-1 agonists (e.g. exenatide, liraglutide, lixisenatide, dulaglutide and semaglutide). These stimulate a rise in glucose-dependent insulin secretion, inhibit glucagon secretion, hence lowering hepatic glucose output (110) and they slow gastric emptying, which may promote early satiety to reduce food intake (108, 110). Adverse effects include nausea, vomiting and diarrhoea (113). A mean weight loss of ~ 2-5 kg has been reported over a 6 to 12 month period (113, 114). A disadvantage of GLP-1 analogues is that they involve subcutaneous injections (i.e. twice per day - once per week). Sodium-glucose co-transporter-2 (SGLT-2) inhibitors (e.g. dapagliflozin and empagliflozin) are another type of diabetes medication. SGLT-2 inhibitors block SGLT-2, which is responsible for 90 % of glucose reabsorption, and inhibit glucose reabsorption in the kidneys, independent of insulin concentrations. This results in increased glucose output associated with a mean weight loss of ~ 2 kg over one year (115). The adverse effects include urinary, fungal infections (108) and eugylcaemic ketoacidosis. Importantly, the use of GLP-1 receptor agonists or SGLT-2 inhibitors in high-risk individuals are associated with cardiovascular and renal protection (116), that is, reducing major cardiovascular events and/or the progression of chronic kidney disease (116). Insulin therapy is used when euglycaemia is

not achieved with the use of these hypoglycaemic therapies. Insulin is secreted from the beta cells in the pancreas with short-acting bursts of insulin at meal times (105). Due to this physiological pattern, the available preparation of action is classified into three groups basal, prandial or combination (premixed) (105). The use of insulin analogues increases the risk of hypoglycaemia, as well as its association with weight gain.

In summary, a wide variety of pharmacotherapy options is available for weight loss and/or to improve blood glucose control in T2D individuals. However, they are all costly and associated with adverse effects, and for some T2D medications, an increased risk of hypoglycaemia. While bariatric surgery is an effective intervention for long-term weight loss, it is currently only available for morbidly obese individuals where previous lifestyle interventions have failed. Bariatric surgery is also highly invasive and associated with risks. Additionally, all of these options impact quality of life.

**Table 1.2:** Pharmacotherapies for T2D (106, 107).

| **Medication (generic/trade name)** | **Route** | **Mechanism of action** | **A1c reduction** | **Main adverse effects** |
|---|---|---|---|---|
| Metformin | Oral | ↓ hepatic glucose production | 1.0 % – 1.3 % | GI upset: nausea, diarrhoea; vitamin $B_{12}$ deficiency |
| Sulphonylurea (Gliclazide, Glipizide) | Oral | ↑ insulin secretion | 0.4 % - 1.2 % | ↑ risk of hypoglycaemia;<br>↑ weight |
| DPP-IV inhibitors (Linagliptin, Saxagliptin, Sitagliptin) | Oral | ↑ insulin secretion;<br>↓ glucagon secretion | 0.5 % - 0.9 % | Well tolerated |
| GLP-1 receptor agonists (Exenatide, Liraglutide, Lixisenatide, Dulaglutide, Semaglutide) | SQ/oral | ↑ insulin secretion;<br>↓ glucagon secretion;<br>slows gastric emptying;<br>↑ satiety | 0.8 % - 2.0 % | Nausea, vomiting, diarrhoea;<br>↓ weight |
| SGLT-2 inhibitors (Dapagliflozin, Empagliflozin) | Oral | Inhibits glucose reabsorption via kidneys;<br>↑ glucosuria | 0.5 % - 0.9 % | Urinary and fungal infections;<br>↓ weight; eugylcaemic ketoacidosis |
| Insulin: Rapid-acting (NovoRapid®, Humalog®, Apidra®)<br>Short-acting (Actrapid®, Humulin®)<br>Immediate-acting (Humulin NPH®, Protaphane®)<br>Long-acting (Levemir®, Lantus®, Semglee®, Optisulin®, Toujeo®)<br>Premixed (several types) | SQ | ↑ glucose disposal,<br>↓ hepatic glucose production | 0.5 % - 2.0 % | ↑ risk of hypoglycaemia;<br>↑ weight |

SQ, subcutaneous; GI, gastrointestinal

## 1.3 The effects of protein on GI function, energy intake and blood glucose

As discussed in section 1.2.1.2, nutrient-based interventions, particularly those in which dietary protein content is increased, have been shown to be successful strategies in the management of obesity and co-morbidities, including T2D, by reducing weight and improving glycaemic control (53, 54, 117-119). A substantial amount of research has investigated the mechanisms underlying these effects of protein. Studies in which 'preloads' of protein have been administered, have demonstrated potent effects of protein to decrease energy intake, as well as postprandial glycaemia, with whey being particularly effective (120-122). The concept of a 'preload' is to administer a small amount of macronutrient at a fixed time before the main meal in order to stimulate the GI functions associated with the regulation of energy intake and/or blood glucose at that meal, including the slowing of gastric emptying and stimulation of the release of GI hormones. These effects of protein are likely to primarily reflect the action of peptides and amino acids - the digestion products of proteins. Amino acids, including branched-chain amino acids (BCAAs), which are abundant in whey, are of particular relevance, as they are potential mediators of these effects of protein, acting via both pre-absorptive and post-absorptive mechanisms. Pre-absorptive effects result from the 'sensing' of the digestive products of protein, including peptides and amino acids, by specialised receptors on enteroendocrine cells (EECs), located throughout the GI tract. This triggers the release of gut hormones, which initiate changes in GI motor function, underlying the slowing of gastric emptying (123). Post-absorptive effects include activation by hormones of vagal afferents that transmit signals to the brain, direct effects of amino acids on pancreatic β-cells to stimulate insulin (124, 125), and central mechanisms, including directs effect of amino acids on the brain, regulating food intake (125). The following sections briefly review GI functions, specifically gut hormones and gastric emptying, as well as underlying motor patterns, that play important roles in the GI regulation of energy intake and postprandial blood glucose, the acute effects of

protein, when consumed as a single meal or given intragastrically (IG) or intraduodenally (ID), on GI function, energy intake and postprandial blood glucose, as well as the role of amino acids, in particular the BCAAs, leucine, isoleucine and valine, in mediating these effects.

### 1.3.1 Meal-related signals and effects on GI functions

In order to appreciate the effects of protein on energy intake and postprandial blood glucose, which are discussed subsequently, an understanding of GI functions, specifically the motor patterns underlying gastric emptying and contributing to the digestion and transportation of ingested food, as well as key appetite- and blood glucose-regulatory hormones, released following meal ingestion, is important. These will be discussed in the following sections.

#### *1.3.1.1 Meal ingestion and related signals*

Digestion begins in the oral cavity whereby food is masticated with assistance of enzymes secreted by the salivary glands (i.e. salivary amylase). Components of food interact with taste buds which are located on the tongue and soft palate. A taste bud contains 50 - 100 taste cells and can detect sugars, amino acids, poisons, acids, and minerals (126). These tastants are signals for sweet, umami, bitter, sour, and salty tastes, respectively (126). Individual tastants have been proposed to be detected by different taste cells and gustatory nerves (126). Once food is swallowed, the ingested nutrients pass through the oesophagus (a muscular tube lined with stratified squamous epithelium) into the stomach via an involuntary process called peristalsis. As the bolus enters the oesophagus, voluntary control of the movement is lost. Alternating waves of contraction and relaxation assist the movement of the bolus through the oesophagus into the stomach. Between swallowing, the lower oesophageal sphincter closes to prevent reflux of gastric acid from the stomach. As the bolus enters the stomach, contractions mix the food with gastric secretions creating ‘chyme’. Chyme is then gradually emptied into

the small intestine, the major site of nutrient digestion and absorption, in a process referred to as 'gastric emptying'.

The presence of a meal in the upper GI tract initiates signals, including mechanical distension of the stomach, due to meal volume, and chemical components of a meal (e.g. macronutrients). These nutrients, are sensed in the GI tract by taste-like cells, primarily EECs (126), resulting in the modulation of postprandial GI functions, including GI motility and release of GI hormones, which in turn are associated with the regulation of energy intake and/or blood glucose (127).

***1.3.1.2 GI motor function and gastric emptying of a meal***

There are two distinct patterns of upper GI motility; these include i) a fasting pattern, characterised by the interdigestive migrating motor complex (MMC) and ii) a 'fed' pattern, which is initiated by meal ingestion (128). During fasting, the upper GI tract exhibits a cyclical motor pattern, known as the MMC. This motor pattern commences either in the antrum or the duodenum, and then takes ~ 90 - 120 min until it reaches the distal ileum. The main purpose of the MMC is to clear the stomach and small intestine of "debris", such as undigested food remains and bacteria. The MMC comprises four phases, consisting of distinct motor patterns. Phase I is a period of motor quiescence, characterised by an absence of contractions, with a duration of ~ 40 - 60 min; phase II consists of irregular, intermittent low-amplitude contractions, with a duration of ~ 20 - 40 min; phase III is characterised by a 5 - 10 min period of contractions, which occur at the maximum frequency of the electric pacemaker, i.e. ~ 3 contractions per minute in the stomach and ~ 12 contractions per minute in the duodenum; and phase IV constitutes a short period prior to the return to phase I. Ingestion of a meal converts the 'fasting motor pattern' to a so-called 'fed pattern'. This motor pattern is characterised by a

complex and coordinated interaction of motor activity in the proximal and distal stomach regions, the pylorus and the small intestine. The proximal stomach, serving as a reservoir, relaxes to accommodate food without a substantial increase in intragastric pressure. Subsequent, low-level, tonic contraction of the proximal stomach assists in gradually transferring gastric contents to the distal stomach, where it is mixed with gastric secretions and ground into particles, usually with a diameter of < 1-2 mm, through the forces of antral, and tonic and phasic pyloric, contractions. The 'liquefied' contents, known as chyme, enter the duodenum predominantly in a pulsatile manner, occurring only when the pylorus is open and the duodenum relaxed. In contrast, when the pylorus is closed, distal antral contractions move chyme towards the closed pylorus, facilitating the grinding of solids (129). This coordinated pattern of motor activity in the stomach, pylorus and duodenum underlies the process of gastric emptying.

Gastric emptying is influenced by the physical characteristics (e.g. solid, semi-solid, liquid, viscosity, volume) and chemical composition (e.g. nutrient and energy content, osmolarity, pH) of a meal. Gastric emptying of solids is characterised by an initial lag phase of 20-40 min in duration, which represents the time period before emptying of solid commences, followed by an overall linear phase (130). Nutrient liquids also empty from the stomach in a linear fashion, while nutrient-free liquids (e.g. water) empty in a volume-dependent, mono-exponential fashion without a measurable lag phase (130, 131). The presence of a meal in the stomach generates a mechanical distension, activating vagovagal reflexes. As gastric emptying progresses, the small intestine is increasingly exposed to nutrient components (e.g. glucose, amino acids, fatty acids), initiating feedback signals to the stomach and triggering the release of GI hormones which activate their receptors on vagal afferents, providing feedback to the central nervous system and stomach. This reinforces the relaxation of the stomach, stimulation

of pyloric pressures, and slowing of gastric emptying. In health, gastric emptying rates usually range from 1 - 4 kcal/min (132). Accordingly, there is modest variability in the rate of gastric emptying within an individual, the rate of gastric emptying varies substantially between individuals (133).

#### *1.3.1.3 GI hormone responses*

After meal ingestion, and as gastric emptying progresses, the effect of gastric distention decreases, and the contribution of intestinal exposure of the nutrients, sensed by specialised receptors on the intestinal mucosal EECs, becomes increasingly important. When nutrients enter the small intestine, they activate specialised receptors located on EECs throughout the small intestinal mucosa. Once activated, these receptors trigger the release of key GI hormones, including CCK, PYY and the incretin hormones (i.e. GLP-1 and GIP), which modulate the release of insulin and glucagon via the pancreas, and the suppression of ghrelin from the gastric mucosa. These GI hormones are associated with the regulation of GI motility, including GI motor function and gastric emptying, and energy intake and postprandial blood glucose. Key GI hormones, their release and physiological functions, are discussed in the following section.

##### *1.3.1.3.1 Ghrelin*

Ghrelin is the only known appetite stimulatory (orexigenic) GI hormone. It was originally characterised as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (134). Ghrelin is a 28 amino acid peptide with an octanoyl-group, essential for binding to the GHS-R and crossing the blood-brain barrier (134, 135). Ghrelin circulates in the blood stream in two different forms, acylated and des-acylated. It is predominantly produced in the X/A cells of the gastric mucosa (136). Ghrelin activates neuropeptide Y and agouti-related protein neurons in the hypothalamic arcuate nucleus (137). Plasma ghrelin concentrations are

elevated in the fasting state and decrease rapidly in response to food ingestion (138) due to cephalic- and intestinal-phase reflexes (123), suggesting that ghrelin is a potent signal for meal initiation. Ghrelin is suppressed postprandially by all three macronutrients. While some studies have suggested that carbohydrate ingestion suppressed ghrelin levels more than the other macronutrients (139), other studies have reported that high-protein meals are most effective, high-fat meals moderately effective, and high-carbohydrate meals least effective (140).

Ghrelin release is affected by metabolic dysfunction. For example, circulating levels of ghrelin are lower in obese, compared with lean, individuals so that plasma concentrations are inversely correlated with adiposity (141). Moreover, in anorexia nervosa ghrelin concentrations are higher, decreasing following weight gain (142).

Ghrelin has been shown to stimulate appetite and increase food intake when administered intravenously in humans, supporting its role as a meal initiation signal (143). Furthermore, intravenous administration of ghrelin in rats stimulates gut motility and gastric acid secretion (144), and in humans induces the MMC in the fasting state and, when administered postprandially, accelerates gastric emptying (145).

Ghrelin may also modulate glycaemic control. Ghrelin and the full length functional receptor GHS-R1α RNAs are expressed within the pancreas and β-cells (146). Acylated and des-acylated ghrelin have direct metabolic actions at the peripheral level to influence pancreatic function and affecting glucose metabolism (147, 148). Acylated ghrelin suppresses insulin secretion in vivo and in vitro (146) and, in humans, intravenous administration of acyl-ghrelin is associated with an increase in glucose levels in both healthy and obese individuals (149,

150). In contrast, des-acylated ghrelin, when given intravenously in humans, improves glucose metabolism, insulin sensitivity, as well as the inhibition of lipolysis (147, 148).

***1.3.1.3.2 Cholecystokinin***

CCK is secreted from enteroendocrine I-cells of the small intestine, primarily in the duodenum and proximal jejunum (151). CCK plays an important role in a number of metabolic and digestive processes (152), including the stimulation of gallbladder contraction, and exocrine pancreatic secretion (153). CCK circulates primarily in a 58-amino acid form (CCK-58) (154). CCK's actions are mediated by binding to two G protein-coupled receptors, CCK-A and CCK-B (154). The CCK-A receptor is expressed predominantly in the stomach, GI tract, liver, pancreas and on vagal afferents (155), and the CCK-B receptor in the central nervous system and stomach (156). CCK is released primarily by the digestive products of fat and protein (157, 158) and, to a lesser degree, carbohydrate (159).

Additional functions of CCK include the slowing of gastric emptying, as well as its appetite- and energy intake-suppressant effects (160, 161). For example, CCK-8 was shown to stimulate pyloric contractions, suppress antral and duodenal contractions, as well as increase plasma PYY and reduce energy intake, in a dose-dependent fashion (162). The effect of CCK on satiation may occur through actions in the pyloric region producing signals which are communicated back to the brain via vagal afferents or local actions on vagal afferents in the lamina propria, and the effect to slow gastric emptying may reflect both a direct endocrine effect and a vago-vagal reflex (123).

A role for endogenous CCK in these functions has been established using antagonists to CCK-A receptors. i.v. infusion of loxiglumide, a CCK-A receptor antagonist, inhibited the slowing

of gastric emptying of a mixed-nutrient or glucose drink, and reduced intragastric pressure, in response to a lipid solution (250 mL of 10 % Intralipid), consistent with an important role for endogenous CCK in the regulation of gastric emptying (163-165). In addition, in healthy men, i.v. infusion of loxiglumide before a meal consisting of orange juice, ham sandwich, chocolate mousse and coffee with cream, was associated with a small increase in food intake compared with control (166), indicating that endogenous CCK is involved in the regulation of acute energy intake, but it is not the only mechanism (166). Thus endogenous CCK appears to have a weak food intake-suppressant effect.

A direct role for CCK in the regulation of blood glucose remains to be established (123). However, CCK may indirectly contribute to the control of postprandial glycaemia via the slowing of gastric emptying (167). For example, after intravenous infusions of CCK at 24 pmol/kg/h, plasma CCK increased from 1 pmol/L to 8 pmol/L, and CCK slowed gastric emptying which was associated with lower postprandial blood glucose (167), the opposite effect to that observed when compared with an i.v infusion of loxiglumide (165).

***1.3.1.3.3 Peptide tyrosine tyrosine***

PYY is a 36 amino acid peptide, secreted by enteroendocrine L-cells, primarily in the ileum and large intestine (128). PYY is also expressed in the endocrine pancreas. There are two endogenous forms, $PYY_{1-36}$ and $PYY_{3-36}$. $PYY_{1-36}$ is secreted, then broken down by the enzyme, dipeptidyl peptidase DPP-IV to $PYY_{3-36}$. PYY is secreted in response to all three macronutrients, with fat probably being the most, and carbohydrate, the least potent stimulus (168-170). Following food ingestion, PYY levels increase within 15 - 30 min, peak at 60 - 90 min and remain elevated for several hours (123). This initial rise suggests the involvement of neuronal pathways, potentially activated by CCK, as only a small (if any), amount of nutrients

can reach the L-cells at that time (123). In obese individuals, plasma PYY levels are lower in the fasted, as well as the postprandial state, compared with healthy weight individuals (171). However, it is unclear whether this is a consequence, or contributing factor to the pathogenesis of obesity.

PYY is one of the hormones involved in the 'ileal brake' (171). This is a physiological mechanism to slow gastric emptying and small intestinal transit, activated by the presence of nutrients in the ileum, with fat probably being the most potent nutrient trigger. Earlier studies by Spiller et al. (172) and Read et al. (173) were the first to describe this mechanism. For example, Spiller et al. (172) reported that transit of a test meal was delayed, when small amounts of a lipid emulsion were infused into the ileum. Lin et al. (174) confirmed the role of PYY in the fat-induced ileal brake, by reporting that the slowing of intestinal transit by distal gut fat was abolished by i.v. administration of polyclonal PYY antibody in dogs. Furthermore, in humans, i.v. administration of $PYY_{3-36}$ at 0.18 or 0.51 pmol/kg/min has been shown to prolong mouth to caecum intestinal transit time of a 200 mL liquid meal and slow gastric emptying, in a dose-dependent manner (175). It is important to note, that an increase in PYY in response to lipid, is at least in part, mediated by CCK, as shown in studies using CCK-A receptor antagonists (176, 177).

PYY has been shown to reduce appetite and food intake in healthy and obese individuals, as well as in rodents (178, 179). Peripheral PYY may inhibit food intake by acting via the Y2 receptor (Y2R) in the hypothalamic arcuate nucleus (135). The effects of endogenous $PYY_{3-36}$ have been studied in rats in vivo using an antagonist to Y2 receptor, whereby the effect of $PYY_{3-36}$ on food intake is diminished by intravenous administration of the Y2 antagonist, BIIE0246 (180). In Y2R knockout mice, $PYY_{3-36}$ had no effect on food intake (178). Moreover,

PYY-knockout mice develop obesity which is reversed by the administration of exogenous PYY.

These findings suggest that PYY plays a role in the regulation of food intake via central and peripheral pathways, in humans and animals. Moreover, PYY is involved in the ileal brake mechanism, which underlies the slowing of gastric emptying.

#### ***1.3.1.3.4 Incretin hormones***

The currently known incretin hormones are GLP-1 and glucose-dependent insulinotropic peptide (GIP). They are released in response to oral, but not intravenous, glucose administration and induce a reduction in postprandial glucose levels, through an insulinotropic effect and in the case of GLP-1, inhibition of glucagon secretion. This increased insulin response to oral/enteral compared to i.v. glucose, is referred to as the 'incretin' effect. GLP-1 and GIP are rapidly degraded and inactivated by the enzyme DPP-IV.

*Glucagon-like peptide-1*

GLP-1 is a peptide hormone, secreted from enteroendocrine L-cells, located in both the small and large intestinal mucosa, although GLP-1 cells are more concentrated in the distal jejunum and ileum compared to the proximal small intestine, in humans (181). GLP-1 receptors are expressed in the GI tract, pancreas, abdominal vagal afferents, and various brain areas (123). GLP-1 is generated by the posttranslational processing of proglucagon (which also generates GLP-2, a trophic factor for the intestinal mucosa), and stimulates insulin, whilst inhibiting glucagon, secretion in a glucose-dependent manner (153). Carbohydrate, fat and protein all stimulate GLP-1 (182).

GLP-1 is involved in a wide range of physiological functions, including GI motility, blood glucose control and energy intake. GLP-1 is also one of the hormones involved in the 'ileal brake mechanism'. GLP-1 apparently reduces postprandial blood glucose by three mechanisms - the slowing of gastric emptying, possibly via a vagal-vagal reflex, and by increasing pancreatic β–cell insulin section and inhibiting pancreatic α–cell glucagon secretion (183). It is now thought that gastric emptying is probably the predominant mechanism how GLP-1 lowers postprandial blood glucose (111). The use of the GLP-1 receptor antagonist, exendin(9-39)amide, has allowed further investigation regarding the role of GLP-1 in blood glucose control. In healthy individuals, i.v. infusion of exendin(9-39)amide (500 pmol/kg/min), increased postprandial blood glucose, reduced the stimulation of insulin, as well as attenuating the suppression of glucagon, after consuming a 150 g oral glucose tolerance test (184). There is also evidence that GLP-1 has a major role to regulate motility to account for the slowing of gastric emptying. For example, i.v. infusion of exendin(9-39)amide, during a 120 min intraduodenal infusion of glucose at 1 kcal/min or 2.5 kcal/min, abolished the suppression of antral and duodenal pressures and the stimulation of pyloric pressures (183).

The outcomes of studies investigating the effects of GLP-1 on energy intake have been inconsistent. In healthy men, i.v. infusion of GLP-1 (50 pmol/kg/h) was reported to increase both satiety and fullness feelings after an energy fixed breakfast, and reduce energy intake at an *ad libitum* lunch 4.5 later (185). These effects were replicated in a study with T2D individuals, whereby reduced sensations of appetite were found in participants assigned continuous subcutaneous infusion of GLP-1 (4.8 pmol/kg/min) compared to those receiving the saline infusion over a six week period (186). However, in contrast, in obese individuals, i.v. infusion of GLP-1 (0.75 pmol/kg/min) administered at the beginning of the meal slowed gastric emptying and reduced postprandial hunger ratings, but did not affect the amount of food eaten

(187). Furthermore, i.v. infusion of exendin(9-39)amide, over 2 hr, slightly reduced appetite ratings, but did not affect food intake from an *ad libitum* meal 2 hr later, in response to nutrient induced GLP-1 release (oral mixed-nutrient drink, intraduodenal glucose or oleic acid) (188). Hence, the role of endogenous GLP-1 in energy intake remains unclear, warranting further investigation.

The physiological effects of GLP-1 on GI motility and glycaemia are of great interest due to its clinical relevance for the treatment of T2D and obesity. GLP-1 analogues/agonists (e.g. exenatide, liraglutide, dulaglutide, semaglutide) have been developed to mimic the effects of GLP-1. Moreover, an advantage of GLP-1 analogues/agonists is that they are not rapidly degraded by the enzyme DPP-IV. As a result, there is an increase in glucose-dependent insulin secretion, inhibition of glucagon secretion and slowing of gastric emptying, overall assisting with glycaemic control in T2D patients (108), without increasing the propensity to hyperglycaemia. The use of GLP-1 receptor agonists, particularly in higher dosage, is also associated with weight loss (114). Drugs which attenuate the breakdown of GLP-1, DPP-IV inhibitors, are also used widely in the management of T2D, but do not affect body weight (189).

*Glucose-dependent insulinotropic peptide*

GIP is produced by enteroendocrine K-cells in the duodenum and proximal jejunum, predominantly secreted in response to fat and carbohydrate. Like GLP-1, it enhances glucose-stimulated insulin secretion (182). Oral administration of glucose induces a biphasic GIP response peaking at 5 and 45 min (190). Like GLP-1, GIP exerts its insulinotropic affect via G-protein-coupled receptors which are expressed on the β cells (182). GIP is also involved in lipid metabolism (191) and stimulates lipoprotein activity, to increase fat disposition and storage (101). Unlike GLP-1, GIP does not slow gastric emptying (192), suggesting it lowers

postprandial blood glucose only by stimulation of insulin release. The later capacity is markedly impaired in individuals with T2D (111) so that clinical development for glucose-lowering has focused on 'GLP-1-related' approached.

There is limited information about the effects of GIP on energy intake. In obese and T2D mice, a daily injection of the GIP receptor antagonist (Pro3) GIP, had no effect on body weight or energy intake over a 14 day period (193). Drugs combining GLP-1 agonists with GIP antagonists are in development for the management of T2D and obesity (194).

#### ***1.3.1.3.5 Insulin***

Insulin is a polypeptide with 51-amino acids, synthesised in the pancreas by the β-cells of the islets of Langerhans. Insulin is a derivative of proinsulin, which is cleaved into insulin and C-peptide, a 31-amino acid peptide. When insulin is released, equal amounts of C-peptide are also released. C-peptide is a useful marker of insulin production, as it is not metabolised by the liver or other organs. Insulin secretion is stimulated by the ingestion of carbohydrates and by certain amino acids, free fatty acids, ketone bodies, glucagon and secretin (195). Insulin's main role relates to glucose homeostasis. Insulin mediates glucose uptake into adipose tissue and skeletal muscle via GLUT4 glucose transporters, and stimulates the storage of glycogen (i.e. glycogenesis), which enables the metabolism of glucose via the glycolytic pathway (195). The insulin response to the carbohydrate, is attenuated in patients with long-term T2D (196).

The effects of insulin on GI motility and the rate of gastric emptying have been investigated in a limited number of studies. For example, under euglycaemic conditions (insulin levels 46 ± 4mU/L), hyperinsulinemia eliminated antral MMC phase III and slowed gastric emptying in both post-absorptive and postprandial states, in healthy individuals (197). Notably, this study

was performed in only four participants. While Kong et al.(198), investigating the effect of euglycaemic hyperinsulinaemia, in healthy individuals, found that hyperinsulinemia delayed gastric emptying of both liquid and solid meals modestly, no effect was evident in a subsequent study in T1D and T2D (199).

The role of insulin in appetite regulation remains unclear. In a study performed by Speechly et al. (200), in lean individuals, fasting insulin and insulin concentrations after a pre-load meal and before a subsequent *ad libitum* test meal, were inversely correlated to energy intake. However, this was not the case in obese individuals. Similar observations were found in a study performed by Verdich et al. (201). In lean, but not in obese, individuals, energy intake at an *ad libitum* meal, was inversely related to both fasting insulin concentration prior to the fixed test-meal and insulin concentrations immediately before the *ad libitum* meal (201). While it appears that glucose is required for insulin to elicit a satiating effect in lean individuals, any potential effect of insulin on energy intake in obese individuals, is apparently interrupted.

***1.3.1.3.6 Glucagon***

Glucagon is a 29-amino acid peptide hormone, synthesised in the pancreas by the α-cells of the islets of Langerhans. The action of glucagon opposes that of insulin. In the fasting state, glucagon regulates blood glucose concentration, whereby hepatic glucose is obtained from the breakdown of stored glycogen (glycogenolysis) and the synthesis of glucose (gluconeogenesis). Following a meal, glucagon secretion is normally suppressed through the actions of endogenous insulin secretion (202), as well as the incretin hormones, GLP-1 and GIP (203). Glucose homeostasis is dependent on the balanced secretion of glucagon and insulin from pancreatic α-cells and β-cells, respectively. In healthy individuals, this is tightly regulated via a multi-loop feedback system. In the postprandial state, high plasma glucose levels

stimulate the pancreatic β-cells to release insulin. Insulin initiates glucose uptake and its use by insulin dependent tissues, stimulating the formation of glycogen from glucose (glycogenesis) in the liver and muscle, and suppressing glucagon secretion. If plasma glucose levels become low (hypoglycaemia), the pancreatic α-cells release glucagon. When glucagon reaches the liver, it initiates glycogenolysis, stimulates gluconeogenesis, and as a consequence distributes glucose into the circulation. In addition to glucose metabolism, glucagon plays a role in lipid metabolism, the regulation of energy intake, inhibition of gastric motility. It may also promote autophagy, and increase cardiac output (204). Glucagon also regulates hepatic amino acid catabolism, as well as serum amino acid levels (205). These functions are impaired in T2D, as is the postprandial suppression of glucagon (205).

A number of studies have described the effects of glucagon on motility. For example, glucagon given i.v. (0.25 to 2mg) was shown to slow gastric emptying of glucose or a standard meal (206, 207). Evidence relating to the effects of glucagon on appetite and energy intake is limited. In a study of lean and obese individuals and patients with type 1 diabetes who received intramuscular glucagon (1 mg), followed by a questionnaire, evaluating satiety and hunger (208), lean individuals and patients with T1D reported feeling more full after receiving glucagon compared to obese individuals (208). Geary et al. (209) reported that i.v. infusion of glucagon (0.86 pmol/kg/min) or CCK (1.75 pmol/kg/min) over a 4 hour period, reduced food intake in lean individuals. In contrast, simultaneous i.v. infusion of glucagon and CCK did not, the reason for this apparent inconsistency is unclear (209).

In conclusion, meal-related signals, in the upper GI tract, and their effects to modulate GI functions, including the slowing of gastric emptying and release of GI hormones, are critical to the regulation of energy intake and postprandial blood glucose. The acute effects of protein

and amino acids, in particular the BCAAs, and their relationship with GI functions on energy intake and blood glucose control, represents a focus of this thesis.

**1.3.2 Acute effects of protein on GI function, energy intake and blood glucose**

Dietary nutrients, particularly protein and fat, have potent effects to stimulate GI functions, associated with the suppression of energy intake and/or blood glucose. Protein may be most effective, when compared with fat or carbohydrate, at inducing satiety and reducing energy intake (52, 210). For example, in a study performed by Barkeling et al. (211), participants were fed either a high protein (43 % energy from protein) or a low protein (10 % energy from protein)/high-carbohydrate lunch. At the subsequent *ad libitum* evening meal, the high protein group ate 12 % less than the low protein group (211). Rolls et al. (210) conducted a study in which participants were given 5 different 'preloads', including a high protein (56 g of protein), high fat, high starch, high sucrose and mixed (fat and sucrose). At a subsequent *ad libitum* meal, the high protein preload resulted in increased satiety and consumption of fewer calories compared with all other preloads except for the high starch preload. Hence, both the high protein and high starch preloads were effective in suppressing appetite and reducing energy intake. Porini et al. (212) performed a study in 12 healthy males, who were fed lunches, consisting of either a low protein (baked macaroni, 41 g protein) or a high protein (meatballs with tomato sauce, 121 g protein) meals, and at two calorie levels, 2 hr before an *ad libitum* meal (212). Energy intake at the lunch was less after the high protein preload compared to the low protein preload (438 kcal vs 812 kcal, respectively) (212). Moreover, in a study by Stubbs et al. (213), 6 healthy males were fed breakfast consisting of 60 % of energy from either fat, protein, carbohydrate, or a mixture of all three macronutrients followed by an *ad libitum* lunch meal (13 % of energy from protein, 40 % of energy from fat and 47 % of energy from carbohydrate). Participants reported increased satiety levels from the high protein breakfast,

although no difference was found in subsequent energy intake after the *ad libitum* meal. It is important to appreciate that it was a very small study and the high content of macronutrients may have made the meals unpalatable, confounding measurement of energy intake. Poppitt et al. (52) performed a study in 12 healthy females, in which the macronutrient composition of a test meal was manipulated on 4 separate occasions. Each treatment comprised a 1MJ baseline meal and drink (40 % fat, 48 % carbohydrate, 12 % protein) to which an additional 1MJ of protein, alcohol, fat or carbohydrate was added. *Ad libitum* energy intake at a subsequent lunch meal and short-term hunger were less after the high protein preload compared with the other preloads, including high alcohol, high fat and high carbohydrate (522 ± 210 kcal, 660 ± 284 kcal, 596 ± 162 kcal, 609 ± 250 kcal, respectively) (52). It is important to note that some studies have failed to control for all dietary factors (i.e. factors that potentially influence satiety and hunger), including palatability. While the orally consumed meals were usually made to taste comparably, the high protein content would be likely to have affected palatability, thus, an effect of palatability on appetite and energy intake, as opposed to protein, cannot be excluded. Amongst proteins, whey protein, which is a major component of dairy and digested relatively rapidly, when compared with other sources of protein (i.e. casein, soy, tuna, turkey and egg albumin), has frequently been reported to be more satiating (214-217). The effects of whey protein, to suppress energy intake and/or postprandial blood glucose have been demonstrated in numerous studies (122, 124, 218-221). For instance, Akavan et al. (222) reported that in healthy individuals drinks containing whey protein loads of 20 g, 30 g or 40 g (consumed 30 min before a standardised carbohydrate-rich pizza meal) suppressed energy intake and reduced post-meal blood glucose (120).

Accordingly, the acute ingestion of dietary protein has been recognised to have potent effects to modulate GI functions, including the slowing of gastric emptying and GI hormone release,

which may mediate, at least in part, the energy intake-suppressant and blood glucose lowering effects of protein. The following sections will discuss acute effects of protein, particularly whey protein, on energy intake and blood glucose, as well as key GI mechanisms potentially underlying these effects.

#### *1.3.2.1 Effects of protein on energy intake and associated GI mechanisms*

Several studies have reported the acute effects of protein when ingested orally or given intraduodenally, on the suppression of appetite and subsequent *ad libitum* energy intake, and associated GI mechanisms (140, 215, 223-227). For example, Batterham et al. (225) reported that a high-protein mixed-nutrient meal (~ 65 % energy from protein, 17 % energy from carbohydrate, 17 % energy from fat) caused the greatest reduction in hunger when compared to a high-fat (65 % energy from fat, 17 % energy from protein, 17 % energy from carbohydrate) or high-carbohydrate (65 % energy from carbohydrate, 17 % energy from protein, 17 % of energy from fat) meal. Furthermore, the effect of the high protein meal correlated with the greatest increase in plasma PYY concentrations, in both lean and obese individuals. In support, Brennan et al. (140) investigated the acute effects of meals (i.e. conventional spaghetti bolognese and a vanilla yoghurt dessert), in lean and obese individuals. To vary macronutrient composition, whey protein isolate was added to increase the protein, pure cream to increase the fat, and corn flour and raw sugar to increase the carbohydrate content of the meals. On four separate occasions, following a standardised breakfast, participants received a lunch meal consisting of high protein (45 % energy from protein), adequate protein (30 % energy from protein), high-carbohydrate/low-protein (10 % energy from protein, 60 % energy from carbohydrate) or high-fat (55 % energy from fat, 15 % energy from protein). In lean individuals, the high-protein and high-fat meals reduced energy intake compared with the adequate protein meal by ~ 14 % and 9 % respectively. In obese individuals, the high-protein and high-fat meals

reduced energy intake compared with high-carbohydrate/low-protein (~ 23 %) and high-fat meals (~ 18 %), the adequate protein meal also reduced energy intake compared with the high-carbohydrate meal (~ 10 %). Additionally, the CCK and ghrelin responses, to the high-protein and adequate-protein meal, were sustained in both lean and obese individuals, supporting the concept, that the effect of protein on appetite, is attributable to the release and/or suppression of GI hormones. In obese individuals, the sensitivity to the satiating effects of protein, appears to be maintained, further supporting the potential for protein to have beneficial effects on weight loss (140).

Hall et al. (215) reported that a preload of whey protein, decreased eating, increased plasma concentrations of CCK and GLP-1, and slowed gastric emptying, when compared with a preload of casein protein. As discussed, CCK and GLP-1 slow gastric emptying and this in turn may be relevant to the increased satiety response to whey (215). Similar to Akhavan et al. (222), Hutchison et al. (224) compared the effect of oral whey protein drinks consisting of 30 and 70 g, and found that both protein loads diminished hunger, desire to eat, and prospective food consumption, and suppressed subsequent energy intake, at a buffet meal 240 min later, by 147 kcal and 177 kcal respectively (**Figure 1.2**). The amount of calories emptied from the stomach at 60 min was related to the magnitude of the changes over the first 60 min in plasma CCK and GLP-1, and these hormones were each modestly associated with the suppression of energy intake by both protein drinks (224). This study also established that the rate of gastric emptying of the increasing loads of protein was independent of load. Even though these GI responses were attributable to a more sustained GI response, following the 70 g of protein compared with the 30 g of protein after 60 min, the rates of gastric emptying of both protein loads, were related, and the GI hormone responses were related to the rate of emptying rather than the protein load. Additionally, administration of whey directly into the duodenum (in order to exclude the

potential influences of orosensory factors), at rates of 0.5 - 3 kcal/min over 60 min, stimulated pyloric pressures, and the release of the GI hormones, CCK, GLP-1 and PYY and, at the highest rate, also suppressed energy intake (226). In this study, the stimulation of pyloric pressures, a key determinant of the slowing of gastric emptying, was found to be the main determinant for a reduction in energy intake. Soenen et al. (227) also investigated the effects of ID whey protein loads (0.5 - 3 kcal/min over 60 min) on energy intake, GI motility and appetite, in healthy young and older men. At the buffet meal, the suppression of energy intake was inversely related to the change in isolated pyloric pressures and positively related to the number of antral pressure waves. GI hormone concentrations were not analysed. These findings further support the relationship between GI motor function and feeding behaviour. Moreover, it is important to note that administration of whey, directly into the duodenum, indicates that these effects of protein occur in the absence of any potentially aversive influences due to palatability. Accordingly, there is compelling evidence confirming that higher protein intakes, are associated with major changes in GI functions, involving interrelated GI mechanisms (i.e. GI motility patterns and GI hormones), which contribute to reductions in appetite and subsequent energy intake.

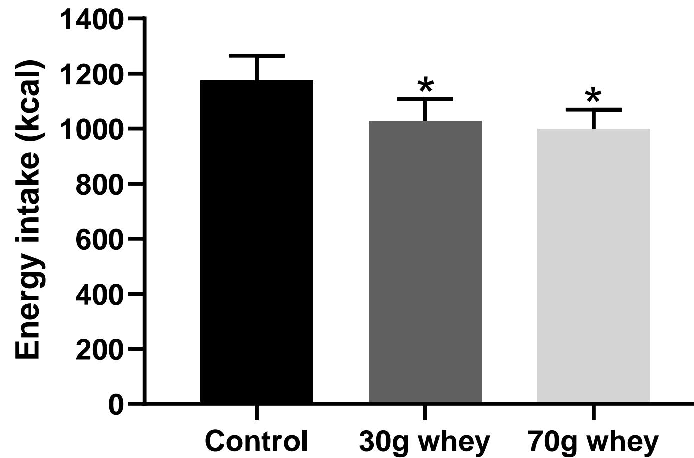


**Figure 1.2:** Energy intake (kcal) from the buffet meal at an ad libitum lunch 3 h after oral test drinks with 30 g or 70 g of whey protein, or control. *Significantly less than control, $P < 0.05$. Adapted from Hutchison et al. (224).

***1.3.2.2 Acute effects of protein on blood glucose and associated GI mechanisms***

The effect of protein on the postprandial glycaemic response, in healthy individuals and those with T2D, is well-established (121, 222). For example, in healthy individuals, the addition of protein (30 g gelatin) to an oral glucose load (50 g), reduced the blood glucose response to that load, primarily by slowing of gastric emptying, but also by stimulating the incretin hormones (228). Furthermore, in healthy individuals, protein in loads of 4.5 – 18 g (in a drink containing 25 g glucose), dose-dependently lowered postprandial glycaemia, with a minimum of 9 g required for an effect (229). Similarly, drinks containing whey protein isolate loads of 10 g, 20 g, 30 g or 40 g, consumed 30 min before a standardised carbohydrate-rich pizza meal, reduced postprandial blood glucose in a dose-dependent fashion (120). It appears that this effect is maintained in T2D. For example a study performed by Gannon et al. (230) reported that ingestion of 25 g of protein from different sources (e.g. beef, turkey, gelatine, cottage cheese, fish and soy) with 50 g of glucose, attenuated the blood glucose response in patients with T2D, compared with the response to glucose alone. Moreover, in diet-controlled patients with T2D, ingestion of 55 g whey protein, given as a preload 30 min before a carbohydrate meal, slowed gastric emptying (**Figure 1.3A**), associated with a substantial reduction in postprandial

glycaemia (**Figure 1.3B**) (121). The effect of whey protein, on the lowering of postprandial blood glucose, therefore appears to be related in part to the slowing of gastric emptying.

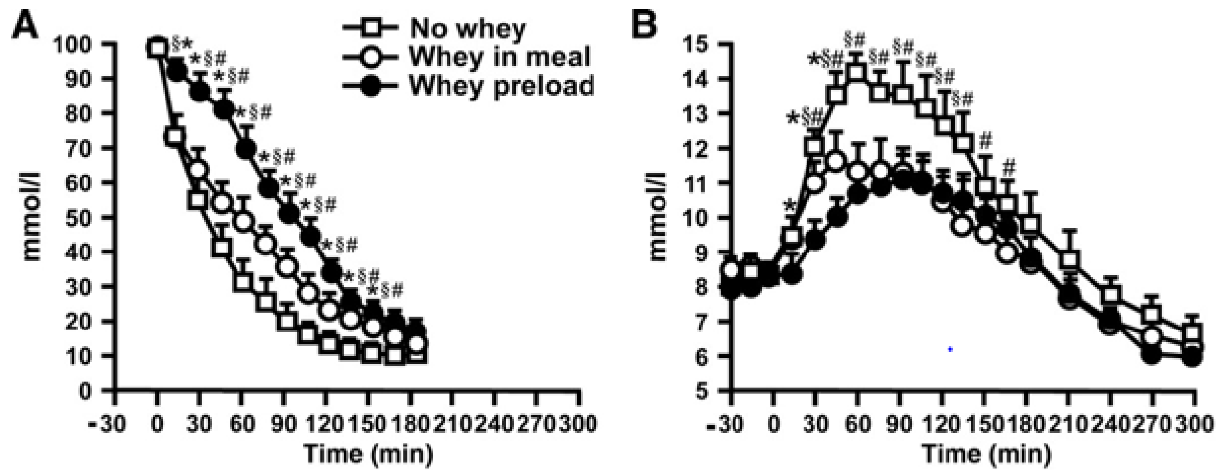


**Figure 1.3:** Gastric emptying (A) and blood glucose concentrations (B) after ingestion of beef-flavoured soup followed by a mashed potato meal 30 min later, in T2D patients. 55g whey protein was added into the soup (whey preload) or mixed in to the potato meal (whey in meal). *P < 0.05, whey preload vs. whey in meal; #P < 0.05, whey in meal vs. no whey; §P < 0.05, whey preload vs. no whey. Adapted from Ma et al. (121).

The effect of whey protein to stimulate insulin, may occur via the incretin hormones, in a glucose-dependent fashion (124, 196, 231) or by direct effect on the pancreatic β-cells (232-234). A number of studies have provided important insights into the effects of protein, on gastric emptying and/or GI hormone release, including GLP-1 and GIP, associated with the lowering of blood glucose. Veldhorst et al. (216) compared the effects of custards with either a normal content (10 % of energy from protein) or high content (25 % of energy from protein) of protein, from different sources of protein (whey/soy/casein) on plasma insulin and GLP-1 responses. Whey triggered the greatest responses in insulin and GLP-1 concentrations compared with casein and/or soy (216). Bowen et al. (235) reported in obese individuals that plasma GLP-1 concentrations were greater after ingestion of a drink containing 50 g whey protein compared to drinks containing 50 g fructose or 50 g glucose. However, the stimulation of GLP-1 was not evident in all studies. In healthy individuals, oral ingestion of 18 g of intact

whey protein compared with 25 g glucose alone, was associated with greater plasma GIP and insulin responses and reduced blood glucose compared to 25 g glucose alone, however no increase in GLP-1 (221). The GIP response is possibly key to the higher insulin response and lowering of postprandial blood glucose after whey protein in healthy participants. As discussed, the insulinotropic effect of GIP is markedly attenuated in T2D.

In healthy individuals, the addition of protein (30 g gelatin) to an oral glucose load (50 g glucose), reduced the postprandial blood glucose response substantially, associated with the slowing of gastric emptying (228). The latter is apparently the dominant factor to account for the reduction in postprandial glycaemia, by whey. As discussed, in patients with T2D, 55 g whey protein, when given as a pre-load before a potato meal, slowed gastric emptying, and reduced postprandial blood glucose excursions by ~ 3 mmol/L, as well as stimulating GLP-1 (**Figure 1.4D**), GIP (**Figure 1.4E**) and insulin (**Figure 1.4C**) (121). The slowing of gastric emptying and reduction in blood glucose were less marked when the 55 g of whey was ingested with the meal, suggesting that the timing of protein ingestion relative to a meal containing carbohydrate, is potentially important, particularly in T2D. Similarly, a 150-mL flavoured drink containing 17 g whey and 5 g guar, when consumed for 12 weeks before meals, reduced postprandial glycaemia, slowed gastric emptying and led to a modest reduction in glycated haemoglobin in T2D (236). A strong inverse relationship between the magnitude of the postprandial increase in blood glucose and rate of gastric emptying. There was a postprandial increase in glucagon, a decrease in plasma GLP-1 and no effect on insulin. The reduction in GLP-1 may reflect the capacity of guar to slow gastric emptying, modifying nutrient absorption in the small intestine, and the rise in glucagon probably reflects secondary effects of whey (i.e. amino acids, on pancreatic α-cells) (237). Overall, these observations are consistent with the

concept that the rate of gastric emptying, as well as the incretin responses to a meal, are key determinants of postprandial blood glucose excursions (238, 239).

Importantly, whey protein has been found to inhibit the activity of DPP-IV (240). For example, in rodents *in vivo*, acute administration of whey protein has been shown to significantly reduce DPP-IV activity in the proximal small intestine thus increasing GLP-1 and GIP concentrations (240). Thus, whey protein may enhance incretin action, with the potential to affect incretin degradation through inhibition of intestinal DPP-IV.

In summary, the potent effects of protein, in particular whey, to reduce appetite and subsequent energy intake (241) and lower postprandial glycaemia (238, 239), involve major effects to modulate GI motor function associated with the slowing of gastric emptying (120, 121), as well as the release of key GI hormones, including CCK, PYY, GLP-1 and GIP.

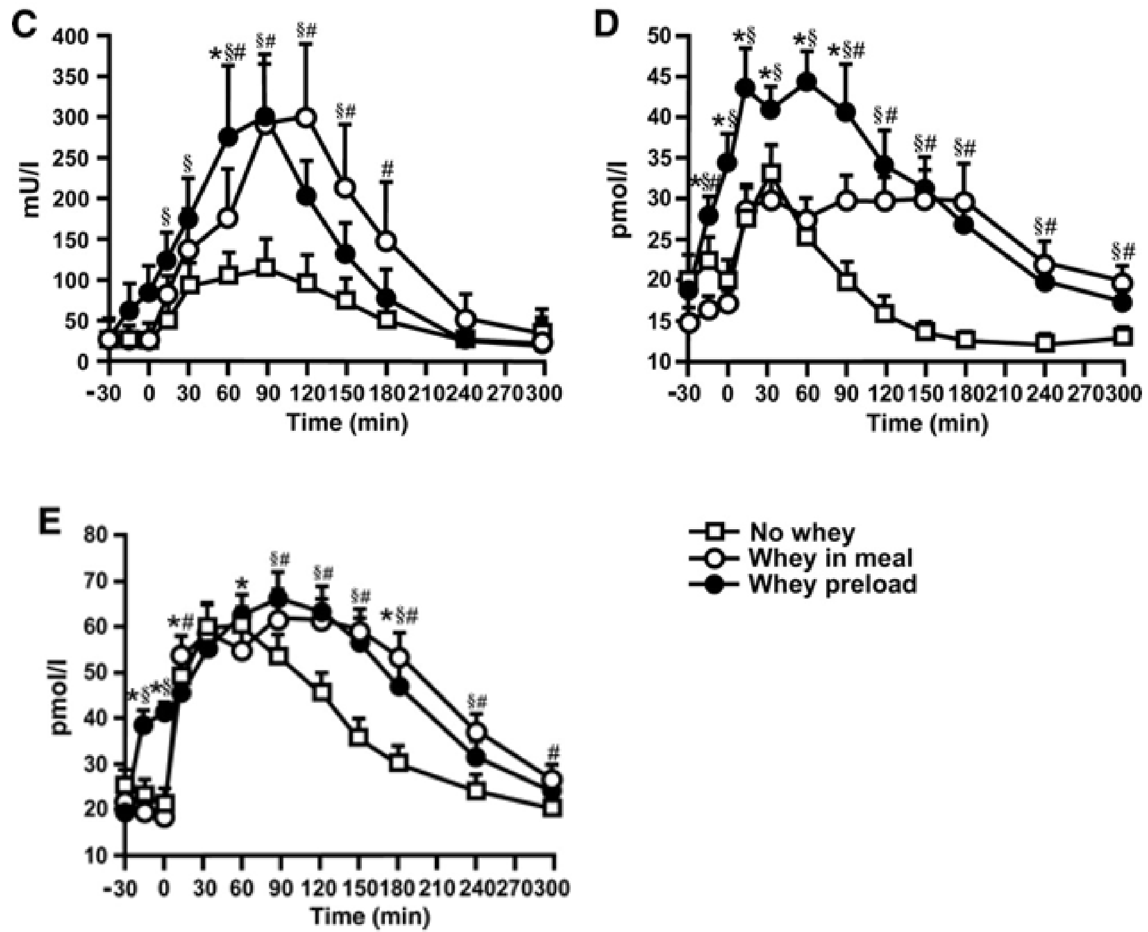


**Figure 1.4:** Plasma insulin (C), GLP-1 (D) and GIP (E) after ingestion of beef-flavoured soup followed by a mashed potato meal 30 min later, in T2D patients. 55g whey protein was added into the soup (whey preload) or mixed in to the potato meal (whey in meal). *P < 0.05, whey preload vs. whey in meal; #P < 0.05, whey in meal vs. no whey; §P < 0.05, whey preload vs. no whey. Adapted from Ma et al. (121).

## 1.4 The effects of amino acids on GI function, energy intake and blood glucose

There is evidence that the effects of protein on energy intake and blood glucose are mediated, at least in part, by the effects of individual amino acids. A number of amino acids, when consumed orally or administered into the GI lumen, have effects to stimulate GI hormones, slow gastric emptying, reduce energy intake and/or lower postprandial blood glucose. Interestingly, there is considerable variation in the effects of individual amino acids on these parameters. Thus, further clarification is needed, particularly in relation to the effects of individual amino acids and their roles in the regulation of energy intake and/or blood glucose. The following section discuss the effects of specific amino acids (i.e. tryptophan, phenylalanine and lysine), that have been reported to effect energy intake and/or blood glucose, and subsequently the focus is on the effects of the BCAAs, leucine, isoleucine and valine, given that they are abundant in whey and, therefore, likely to contribute to the effects of whey to reduce energy intake and lower blood glucose.

### 1.4.1 Effects of individual amino acids on GI function, energy intake and blood glucose

A number of studies have investigated the effects of tryptophan, phenylalanine and lysine, on energy intake and/or blood glucose, as well as potential underlying GI functions, including gastric emptying or gut hormone release.

#### *1.4.1.1 Effects of tryptophan on energy intake, blood glucose and GI responses*

Tryptophan, an aromatic amino acid, is one of the essential amino acids. It is known for its key role as a precursor for the neurotransmitter serotonin, which influences appetite (242). Tryptophan has potent effects on GI motor and hormone functions (243-245). For example, it stimulates pyloric pressures, and increases CCK and GLP-1 concentrations (243-245), all key

inhibitory regulators of gastric emptying. Intraduodenal infusion of tryptophan, at a dose of 0.15 kcal/min (3.3 g over 90 min), was shown to stimulate pyloric pressures (243, 246). Moreover, tryptophan ingested orally in capsules (~ 3 g), or when administered intragastrically (1.56 g in an aqueous solution) before a meal, slowed gastric emptying (247, 248). When tryptophan was infused intraduodenally at a dose of 0.15 kcal/min for 90 min, or when given intragastrically (1.56 g), it stimulated the release of CCK and GLP-1 (243, 248). The enhancement of these GI hormones, potentially underlie the effects of tryptophan on GI motility and gastric emptying.

Effects of tryptophan to suppress energy intake have been reported in a number of studies (243-245). When ingested orally in capsules (~ 3 g) 45 min before a meal, or infused intraduodenally (3.3 g over 90 min), energy intake was reduced, in healthy individuals (243, 247). At a meal, provided immediately after an intraduodenal infusion of tryptophan, healthy participants consumed ~ 200 kcal less, when compared with the control (i.e. saline), an amount which is in clear excess of its own energy content (energy content of tryptophan: 13.5 kcal) (243). Intraduodenal infusion of tryptophan also stimulated plasma CCK; moreover, there was a ~ 12-fold increase in plasma tryptophan (**Figure 1.5A**), compared with the control, and a strong inverse correlation was found between energy intake and plasma tryptophan (**Figure 1.5B**) (243). This suggests that both GI (pre-absorptive effects) and central factors (post-absorptive effects), acting in the periphery and/or brain, contribute to the appetite-suppressant effects of tryptophan.

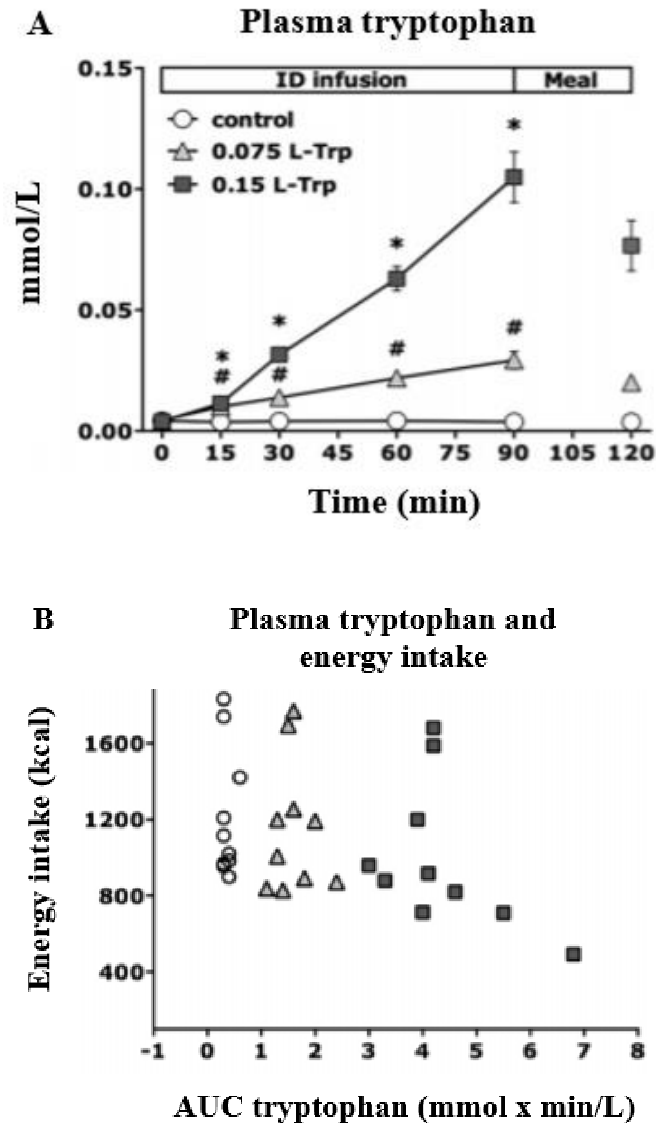


**Figure 1.5:** Plasma tryptophan concentrations after intraduodenal infusion of tryptophan at 0.075 kcal/min, 0.15 kcal/min, or control (A); correlation between plasma tryptophan concentrations and energy intake (B). # $P < 0.05$ tryptophan at 0.075 kcal/min vs control; * $P < 0.05$ tryptophan at 0.15 kcal/min vs control. Adapted from Steinert et al. (243).

The effects of tryptophan on postprandial blood glucose have been evaluated in lean, obese and T2D individuals, but the outcomes of these studies are inconclusive (243, 248, 249). In groups of healthy individuals and those with obesity, who did not have T2D, intragastric administration of 3 g of tryptophan slowed gastric emptying and attenuated the blood glucose response to a mixed-nutrient drink in the first 30 min modestly (248). The rise in plasma C-peptide (a measure of insulin secretion) was attenuated in lean individuals, whereas plasma glucagon was increased in both groups. The findings suggest that the lowering of postprandial blood glucose response primarily reflected the slowing of gastric emptying. In individuals with T2D, intragastric administration of tryptophan, at a dose of 3 g, slowed gastric emptying of a mixed-nutrient drink, associated with a delayed rise, but no overall reduction, in postprandial blood glucose (249). Tryptophan also simulated plasma C-peptide (although only modestly), as well as glucagon (249). The modest effect of tryptophan on blood glucose, observed in response to intragastric administration, may reflect a small insulinotropic effect and/or the stimulation of glucagon - possibly a direct response to circulating amino acids, which counteract glucose-lowering by stimulating glycogenolysis and gluconeogenesis (243, 248).

#### *1.4.1.2 Effects of phenylalanine on energy intake, blood glucose and GI responses*

The effects of the essential and aromatic amino acid, phenylalanine, on GI function, energy intake and blood glucose have been evaluated in a number of studies. In healthy individuals, phenylalanine, when given intraduodenally, at 0.45 kcal/min for 90 min (total amount 10 g), decreased antral motility and stimulated pyloric pressures (250). Furthermore, phenylalanine stimulated plasma CCK within 15 min, an effect which was sustained over the 90 min infusion (250). Similarly, both oral and intragastric ingestion of 10 g phenylalanine stimulated CCK at 20 or 30 min, respectively, before a meal, in healthy individuals (251, 252).

A small number of studies have evaluated the effect of phenylalanine on energy intake. Oral ingestion of 10 g phenylalanine, 20 min before a meal (mince meat, rice and cake), was associated with a reduction in energy intake by ~ 498 kcal, in healthy individuals (251). Similarly, intragastric administration of phenylalanine, at 10 g, reduced energy intake from a buffet meal, consumed 30 min later by ~ 184 kcal (**Figure 1.6**) (252). This energy intake-suppressant effect of phenylalanine was correlated with the stimulation of CCK and tended to be associated with stimulation of PYY (252). Accordingly, the reduction in energy intake, at least in part, is likely to be mediated by an increase in CCK and PYY. In contrast, there was no effect on energy intake in response to intraduodenal phenylalanine, despite the increase in CCK 256. This may be due to the low infusion rate providing a weaker, although constant, stimulus over the 90 min period.

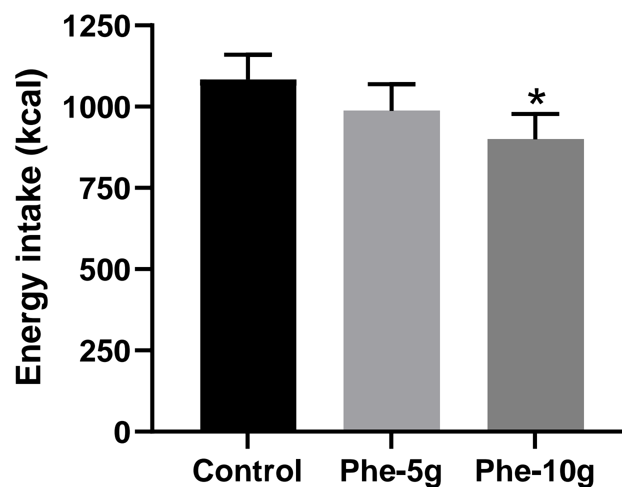


**Figure 1.6:** Energy intake (kcal) from the buffet meal at an ad libitum lunch 30 min after intragastric infusion of phenylalanine, at doses of 5 g (Phe-5 g), 10 g (Phe-10 g), or control. *Significantly less than control, $P < 0.05$. Adapted from Fitzgerald et al. (252).

There is limited information about the effects of phenylalanine on postprandial blood glucose. In healthy individuals, co-ingestion of phenylalanine (1 mmol/kg lean body mass) with 25 g glucose stimulated insulin and attenuated the blood glucose response to the glucose load, when compared to the control (i.e. 25 g glucose alone) (253). Intragastric administration of

phenylalanine, at a dose of 10 g, reduced plasma glucose by 0.6 mmol/L, in response to a mixed-nutrient drink consumed 30 min later, in healthy individuals (252). Phenylalanine also stimulated insulin, as well as glucagon, before and after the mixed-nutrient drink without any effect on gastric emptying or GLP-1 (252). This suggests that the glucoregulatory effect of phenylalanine may reflect the stimulation of insulin (possibly due to a direct effect of circulating phenylalanine on the pancreatic β-cells), independent of the slowing of gastric emptying or stimulation of plasma GLP-1, while the glucagon response may have minimised the glucose-lowering effect.

#### *1.4.1.3 Effects of lysine on energy intake, blood glucose and GI responses*

The effects of lysine, also an essential amino acid, on GI function, energy intake and blood glucose have been investigated in both animals and humans (254-257). In rats, intragastric administration of lysine, at very high loads of 6.7 mmo/L lysine/kg, equivalent to ~ 68 g in a 70 kg human, was reported to slow gastric emptying (256). Oral ingestion of a lysine solution in rats (in doses ranging 0-800 mg) and in humans (in doses ranging from 0.5 g to 7.5 g) slowed gastric emptying, measured via magnetic resonance imaging, in a dose-dependent fashion (257). In contrast, in healthy individuals, intragastric administration of lysine (in doses of 5 and 10 g), had no effect on gastric emptying, of a mixed-nutrient drink (254).

The outcomes of studies evaluating the effects of lysine on blood glucose are inconclusive. In healthy individuals, intragastric preloads of lysine, at doses of 5 g and 10 g, reduced the blood glucose response to a mixed-nutrient drink slightly (**Figure 1.7A**) (254). There was no effect on plasma insulin (**Figure 1.7B**), while glucagon concentrations were greater after lysine compared with control (254). Similarly, in lean and obese individuals, co-ingestion of lysine of ~ 11 g (1 mmol/kg lean body mass; dose range between 6 g to 13 g) with 25 g glucose, reduced

the blood glucose response, while there was no effect on plasma insulin concentrations (255). Thus, the effect of lysine to lower postprandial blood glucose appeared to be independent of insulin or slowing of gastric emptying. In contrast, in older, overweight individuals, lower doses of lysine (e.g. 2 g and 5 g) did not attenuate the blood glucose nor effect the plasma insulin responses, to a higher load of glucose (e.g. 75 g) (258), although the doses used may have been too low. Gastric emptying was not evaluated in the latter two studies. Accordingly, information on the effects of lysine on blood glucose is limited, there is evidence that in healthy, young individuals, the decrease in blood glucose by lysine is mediated by mechanisms other than the stimulation of insulin or slowing of gastric emptying, potentially including enhanced glucose uptake into peripheral tissues (e.g. skeletal muscle) and/or inhibition of glucose absorption in the small intestine.

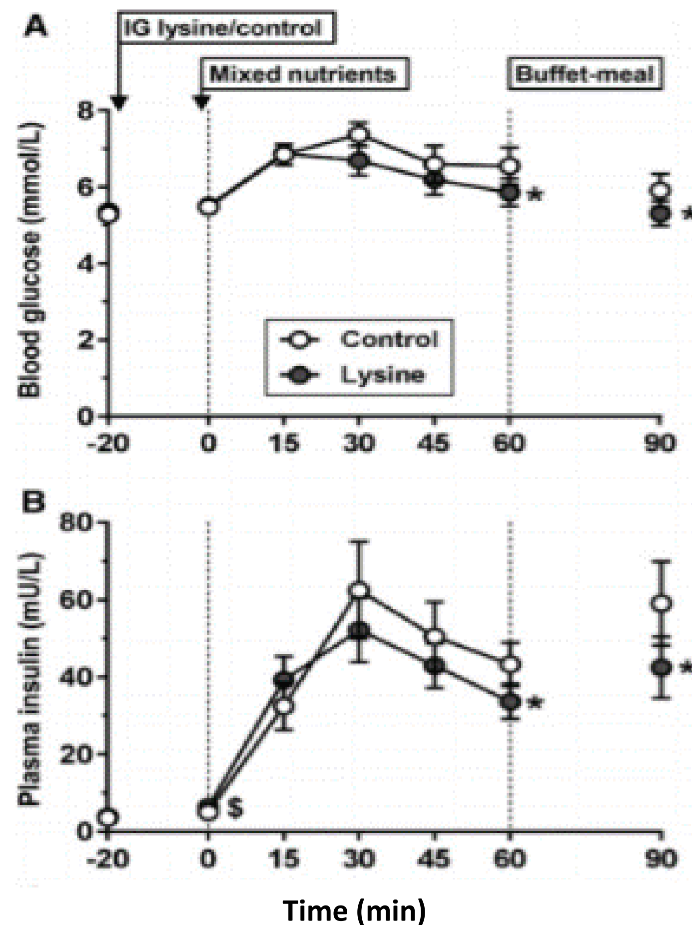


**Figure 1.7:** Blood glucose (A) and plasma insulin (B) in response to intragastric administration of lysine (pooled data from the 2 lysine loads), or control solution before consumption of a mixed-nutrient drink. * $P < 0.05$ (different from the control solution at that time point). Adapted from Ullrich et al. (254).

Information about the effects of lysine on energy intake is also limited. In rats, intragastric administration of lysine at the high load of 6.7 mmol/L, was reported to decrease energy intake over a 48 h period (256). The outcomes indicated that the anorectic effect of lysine was dependent on intact vagal afferents, since lysine did not inhibit food intake in vagus nerve-lesioned animals. In healthy individuals, the effect of intraduodenal administration of whey protein to suppress energy intake has been reported to correlate directly with plasma concentrations of lysine (as well as other essential amino acids, including the BCAAs, methionine and tyrosine) (259). In contrast, energy intake, 150 min after oral ingestion of lysine ~ 11 g (1 mmol/kg lean body mass; dose range 6 - 13 g) was not affected, although desire to

eat was reduced (255). Moreover, intragastric administration of lysine (in doses of 5 and 10 g), had no effect on subsequent energy intake after an *ad libitum* meal, despite a shorter time interval between administration and the meal (i.e. 90 min) (254). These observations suggest that in physiological amounts lysine does not reduce energy intake in humans, and the suppressive effect of lysine reported in rats (256) is most likely reflective of the very high doses used.

### *1.4.2 Effects of the BCAAs on body weight and glucose homeostasis*

The BCAAs leucine, isoleucine and valine, which are abundant in whey protein, have gained increasing interest as potential mediators of the effects of protein on energy intake, body weight and blood glucose (214, 217, 260). The BCAAs are among the most abundant, as well as hydrophobic, of amino acids. The side chains of amino acids are what differentiates them from each other and, in the case of leucine, isoleucine and valine, the side chains are referred to as aliphatic (i.e. contain 3 or 4 carbon side chains). BCAAs make up ~ 30 % of all the amino acids in the body. For optimal levels and/or benefits of these essential amino acids, recommended dietary consumption for humans per day is ~ 39 mg/kg of leucine, ~ 20 mg/kg of isoleucine and ~ 26 mg/kg of valine per day (57). Altogether, this is equivalent to ~ 7 g of BCAAs per day for a 70 kg adult and the best sources of BCAAs are red meat and dairy products.

There is evidence that dietary intake of BCAAs plays a role in body weight and blood glucose regulation (261-264). In a large cohort study, known as the INTERMAP study, including 4429 men and women, higher dietary intake of BCAAs was associated with a reduced prevalence of overweight or obesity (265), a finding which is consistent with outcomes of other population studies on BCAA intake and overweight and obesity (266, 267). While these cross-sectional studies limit inferences as to potential causal relationships between BCAA intake and risk of

overweight and/or obesity, studies on BCAA intake, using very high amounts of up to 135 g/day (range 10 - 135 g/day), in combination with caloric restriction, reported a reduction in body weight due to a decrease in fat mass, while maintaining muscle mass, as well as metabolic rate (261-264). For example, one short-term study in 25 elite wrestlers found that BCAA supplementation (~ 0.35 g/kg body weight/day or 24.5 g/day for a 70 kg adult) combined with a hypocaloric diet (1960 kcal/day for a 70 kg adult) for 19 days, was associated with a greater reduction in body weight (~ 4 kg) and body fat (- 17.3 %), compared with a hypocaloric low-protein or a high-protein diet, over 19 days (263). A similar study in 17 resistant-trained individuals, compared BCAA supplementation (14 g pre and post workout + 112 kcal dietary supplement) with a carbohydrate supplementation (28 g pre and post work out + 112 kcal dietary supplement) in combination with a hypocaloric diet (for workout days: 30 % of energy from carbohydrates, 35 % from protein, and 35 % from fat and caloric intake based on the Harris Benedict Formula, over an 8 week period (262). The individuals receiving BCAAs maintained lean mass and preserved skeletal muscle mass performance, while losing fat mass (-0.05 ± 0.08 kg) compared with the low-protein group (262). These latter studies suggest that weight loss induced by a high protein/low-carbohydrate diet may be mediated by BCAAs. However, these study findings are limited to high performing athletes, combining a high BCAA intake with a hypocaloric diet. Further evaluation is needed to investigate this association between BCAA intake and overweight and/or obesity in non-athletes, in particular association/s between the individual BCAAs, using smaller amounts with little additional calories, and any potential beneficial effects on energy intake regulation.

Evidence to suggest that dietary intake of BCAAs plays a beneficial role in glucose homeostasis is inconclusive (261). In a study performed in healthy rats consuming a low-protein diet, insulin secretion was reduced when leucine, valine or the three BCAAs were

deficient. Insulin secretion was normalised by supplementation of the low-protein diet with the three BCAAs in sham-operated, but not in vagotomised, rats (268). This suggests that the BCAAs play an important role in insulin release via a direct effect on β-cells, and that BCAA supplementation is sufficient to compensate for a reduced insulinotropic activity of the low-protein diet via the vagus nerve (268). Moreover, another study reported that the diabetogenic action of streptozotocin is diminished in rats adapted to a high-protein diet (70 % protein). Additionally, plasma concentrations of BCAAs are raised in rats adapted to a high-protein diet (269). To test the possibility that BCAAs play a role in the beneficial effects of high-protein diets, a BCAA mixture (0.75 – 1.5 g/kg body weight) was given orally to streptozotocin-induced rats, and was shown to lower plasma glucose levels (270). In healthy individuals, Nilsson et al. (221) investigated the effects of acute ingestion of a test drink containing a mixture of leucine, isoleucine and valine (i.e. ratio being 2:1:1, 4.4 g in total) + 25 g of glucose, compared with the control drink (i.e. 25 g glucose + 250 mL water). The test drink containing the BCAAs increased plasma insulin concentrations, but there was minimal effects on the glycaemic response to the glucose load, perhaps reflecting the release of glucagon, which was not a primary outcome. Similarly, in healthy males, ingestion of a 5 g BCAA mixture (i.e. weight ratio of 1 : 2.3 : 1.2 for isoleucine: leucine: valine), increased plasma insulin levels, but this was not associated with a reduction in blood glucose (271), perhaps because the BCAA mixture contained no carbohydrate. Since the doses (i.e. ≤ 5 g) used in the latter two studies for the BCAAs had no effect on blood glucose, the effects of higher doses of BCAAs, or individual BCAAs (i.e. > 5 g), given before a ‘pre-load’ (i.e. carbohydrate drink/meal with a similar carbohydrate content to that in an average main meal), warrant evaluation.

A potential additional mechanism for the beneficial effect of dietary intake of BCAAs on body weight/glucose tolerance is by effects in skeletal muscle (272). Because the largest site of

glucose disposal, loss of muscle mass, whether due to a reduced physical activity level or the aging process, may contribute to metabolic syndrome and/or T2D. The BCAAs, in particular leucine, are well known to promote an insulinogenic effect, which in turn influences skeletal muscle uptake of glucose potentially by directly and indirectly stimulating insulin signalling pathways and, possibly, via an incretin effect (124, 182, 273, 274). Interestingly, athletic populations often consume excess protein and/or BCAAs, but do not develop insulin resistance or other metabolic diseases (275). For example, in individuals that were resistance trained for 9 months and supplemented with whey protein, there was an increase fasting circulating leucine, which correlated positively with an increase in lean body mass (which is associated with improved insulin sensitivity) (276). However, it is important to note that athletes per se have higher energy and protein requirements.

Accordingly, while there is evidence that BCAAs may reduce energy intake and improve postprandial blood glucose, the potential differential effects of individual BCAAs on energy intake and blood glucose regulation is uncertain. Therefore, to better understand the relative effects of leucine, isoleucine and valine, into the potential mechanisms involved in energy intake and blood glucose regulation, the following section provides a summary of what is currently known about these BCAAs when used in isolation.

#### *1.4.2.1 Effects of leucine on energy intake, blood glucose and GI responses*

Amongst the BCAAs, leucine is the most comprehensively studied. Leucine is involved in regulating several cellular processes, including the stimulation of protein synthesis in skeletal muscle, as well as in adipose tissue (277, 278). The initiation of mRNA translation is the major mechanism by which leucine stimulates protein synthesis (279). Regulation of mRNA translation by leucine is dependent on the hypothalamic mammalian target of rapamycin (279).

Moreover, leucine is well known to acutely stimulate insulin release from pancreatic β-cells, reducing blood glucose levels (280). Studies in animal models have also shown leucine to modulate eating and energy intake (281). However, information relating to the effects of leucine on energy intake and blood glucose, as well as the potential involvement of changes in gut hormone function and upper gut motor function, in humans is limited.

The effects of leucine on energy intake in humans are inconsistent, while some studies have reported a reduction in energy intake, others have found no effect. For example, in a study performed in healthy individuals, leucine (~ 7 g) ingested alone or co-ingested with 25 g of glucose, did not affect energy intake, 150 min later (282) . Similarly, leucine (in doses of 5 g and 10 g) administered intragastrically, 15 min before a mixed-nutrient drink, did not affect energy at a subsequent meal, 90 min later (283). In contrast, in healthy individuals, when leucine was infused intraduodenally, at a rate of 0.45 kcal/min, but not 0.15 kcal/min, for 90 min, energy intake at a subsequent meal was reduced by a substantial 156 ± 57 kcal, furthermore, leucine stimulated the release of CCK which may have contributed to the suppression of intake (284). Interestingly, intragastric administration of leucine, did not enhance the stimulation of CCK and, as discussed, there was no reduction in energy intake (283). Similarly, intraduodenal administration of leucine (1.56 g) did not increase CCK concentrations, in lean and obese individuals (244), however the dose used in this study was comparatively lower. It is also important to note that the time intervals between leucine administration and energy-intake assessment, in these studies (244, 283), may have been too long. Thus, the effects of a shorter time interval to a meal (~ 30 min), resulting in higher plasma leucine concentrations during the meal, warrants evaluation.

The capacity of leucine to regulate food intake in animals also remains controversial. A review by Pedroso et al. (279) reported that a number of supplementation approaches, including leucine in the drinking water, in the diet through gavage, or peripherally via subcutaneous and intraperitoneal injections, have no effects on food intake in rodents. In contrast, acute administration of leucine, via intracerebroventricular injections, has been reported to reduce energy intake in rodents (281, 285), suggesting that high plasma leucine concentrations within the brain may be required for a reduction in energy intake. The latter may not be achievable via oral ingestion.

The effect of leucine to lower blood glucose is well-established and associated with underlying GI functions, including glucoregulatory hormones. In contrast, studies evaluating the effects of leucine on blood glucose, and the relationship with gastric emptying are limited. In healthy individuals, 5 g and 10 g of leucine administered intragastrically, 15 min before a mixed-nutrient drink, reduced blood glucose levels, however, there was no effect on the slowing of gastric emptying (286). Similarly, in a study performed in lean and obese individuals, leucine infused intraduodenally at a small dose of 1.56 g, had no effect on gastric emptying (244). These studies suggest that gastric emptying does not play a role in blood glucose regulation by leucine.

It is well-known that leucine stimulates insulin, contributing to the lowering of blood glucose levels. For example, co-ingestion of leucine (~ 7 g) with 25 g of glucose, stimulated insulin (**Figure 1.8B**) and attenuated the postprandial rise in blood glucose by ~ 1 mmol/L (**Figure 1.8A**), compared with glucose alone (282). Moreover, in healthy individuals, leucine (10 g) 15 min before a mixed-nutrient drink, reduced blood glucose and increased plasma insulin, with no effect on GLP-1 or GIP (283), suggesting that the effect of leucine to lower blood glucose

was most likely due to a direct effect of leucine on pancreatic β-cells. The mechanism/s underlying the effect of leucine to enhance insulin secretion from the pancreatic β-cells remains to be elucidated. However, it has been suggested, like glucose-mediated insulin secretion, that when leucine is metabolised in the pancreatic β-cell, it opens up the $Ca^{2+}$ channels resulting in insulin release (287), which in turn activates glutamate dehydrogenase, a key enzyme for insulin secretion (288). The effects of leucine on blood glucose, when consumed/administered as a 'pre-load', 30 min before a mixed nutrient drink, containing a significant amount of glucose-based carbohydrates, (as opposed to predominantly fructose-based carbohydrate in the previous study (283)), and whether these are associated with changes in gut and pancreatic hormones, or gastric emptying, is currently unknown. Thus, these effects of leucine on the response to a mixed-nutrient drink, and the association with gastric emptying and glucoregulatory hormones, in healthy individuals, have been investigated in **Study 3 in Chapter 4**.

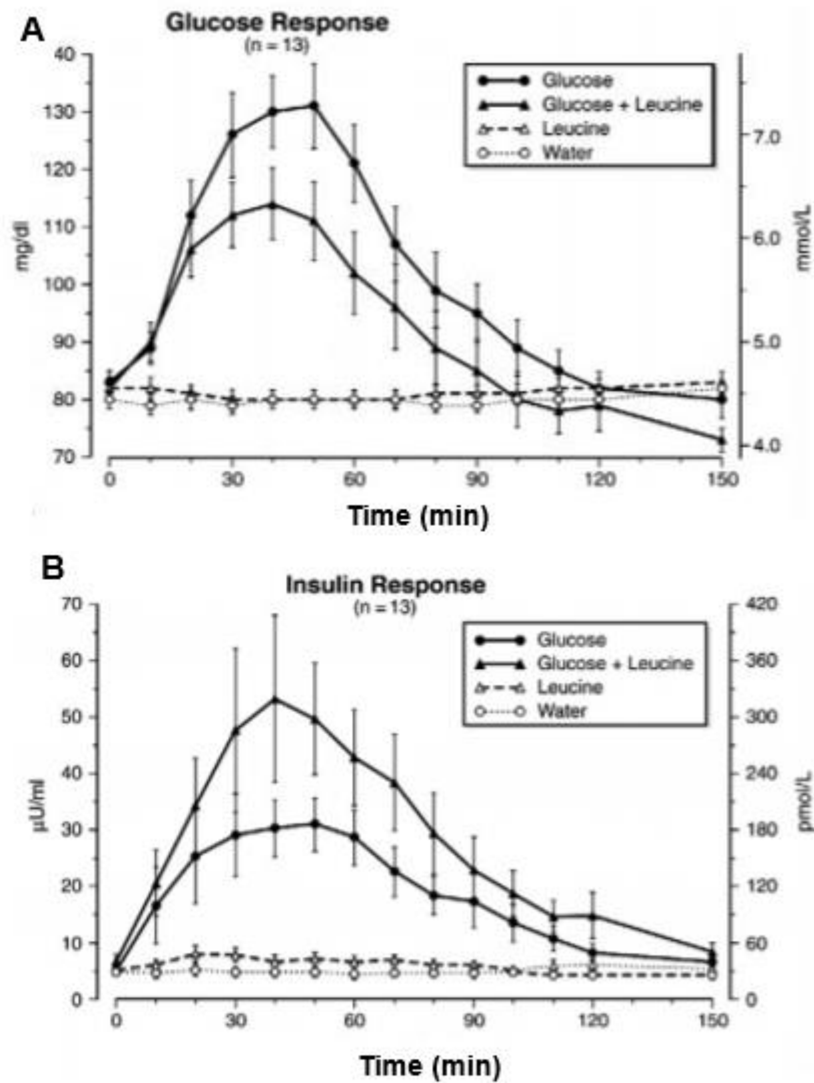


**Figure 1.8:** Blood glucose (A) and plasma insulin (B) in response to ingestion of 25 g of glucose, or leucine ~ 7 g alone, or leucine with glucose, or water, in healthy individuals. Blood glucose was reduced, while plasma insulin was increased after co-ingestion of leucine with glucose, compared with glucose alone ($P < 0.05$ for both). Adapted from Kalogeropoulou et al. (282).

#### ***1.4.2.2 Effects of isoleucine on energy intake, blood glucose and GI responses***

A limited number of studies have evaluated the effects of isoleucine on energy intake and blood glucose in both animals and humans. The available evidence, suggests that isoleucine does not affect energy intake. In mice, chronic supplementation of the diet with isoleucine (2.5 % supplemented in the drinking water) in combination with a high-fat diet (45 % energy) over a 6 week period (289), was associated with ~ 6 % lower body weight gain, however, food intake was not affected (289). It was suggested that weight loss was mediated by other factors, such as an increase in energy expenditure.

In healthy individuals, oral ingestion of isoleucine, in a dose of 1 mmol/kg lean body mass (corresponding to a mean amount of 7.4 g; range 5-9.2 g), ingested alone or co-ingested with 25 g of glucose, had no effect on energy intake, when assessed 150 min after isoleucine ingestion (290). Consistent with these findings, in healthy individuals, when isoleucine was administered intragastrically in 5 g and 10 g doses, 15 min before a mixed-nutrient drink, it did not reduce energy from a subsequent meal, 90 min later (283).

Information about the effects of isoleucine on blood glucose concentrations is derived mainly from rodents and showing a blood glucose lowering effect. In rats, oral administration of 30 and 60 min after glucose administration (273). The reduction in glucose was not associated with an increase in insulin, thus, isoleucine potentially stimulates glucose uptake in an insulin-independent manner (273). Consistent with this concept, in lean C57BL/6J mice, oral administration of isoleucine (30-300 mg/kg, equivalent to ~ 2-21 g for a 70 kg human), dose-dependently reduced blood glucose after glucose administration, in normal, in glucose-intolerant high-fat diet and in T2D, mice (291). A suggested underlying mechanism by which isoleucine may affect blood glucose relates to the enhancement of glucose uptake in skeletal

muscle, potentially by increased translocation of the glucose transporter GLUT-4 to the muscle cell membrane - observed in the skeletal muscle of Wistar rats, 30 min after ingestion of a 30 % glucose solution with 0.55 g/kg isoleucine (equivalent to ~ 38.5g in a 70 kg human) (292).

Findings in humans are consistent with those in animals. For example, in healthy individuals, oral ingestion of isoleucine, in a dose 1 mmol/kg lean body mass (corresponding to a mean amount to a mean amount of 7.4 g; range 5-9.2 g), co-ingested with 25 g of glucose, decreased the blood response (**Figure 1.9A**), without an increase in plasma insulin (**Figure 1.9B**) (290), and intragastric administration of 10 g, but not 5 g, of isoleucine acutely lowered blood glucose levels by a modest ~ 1.1 mmol/L, 15 min before a mixed-nutrient drink, containing fructose-based carbohydrates (283). Isoleucine had no effect on insulin, but was shown to slow gastric emptying of the mixed nutrient drink (283). Thus, isoleucine diminished the blood glucose response most likely by the slowing of gastric emptying, while insulin does not appear to play a role. Whether a greater effect of isoleucine on blood glucose, when administered as a 'pre-load', e.g. 30 min before a mixed nutrient drink, containing a significant amount of glucose-based carbohydrates, (as opposed to predominantly fructose-based carbohydrate in the previous study (283)), will be observed is not known. The effects of isoleucine on the response to a mixed-nutrient drink, and the association with gastric emptying and glucoregulatory hormones, in healthy individuals, have been investigated in **Study 3 in Chapter 4**.

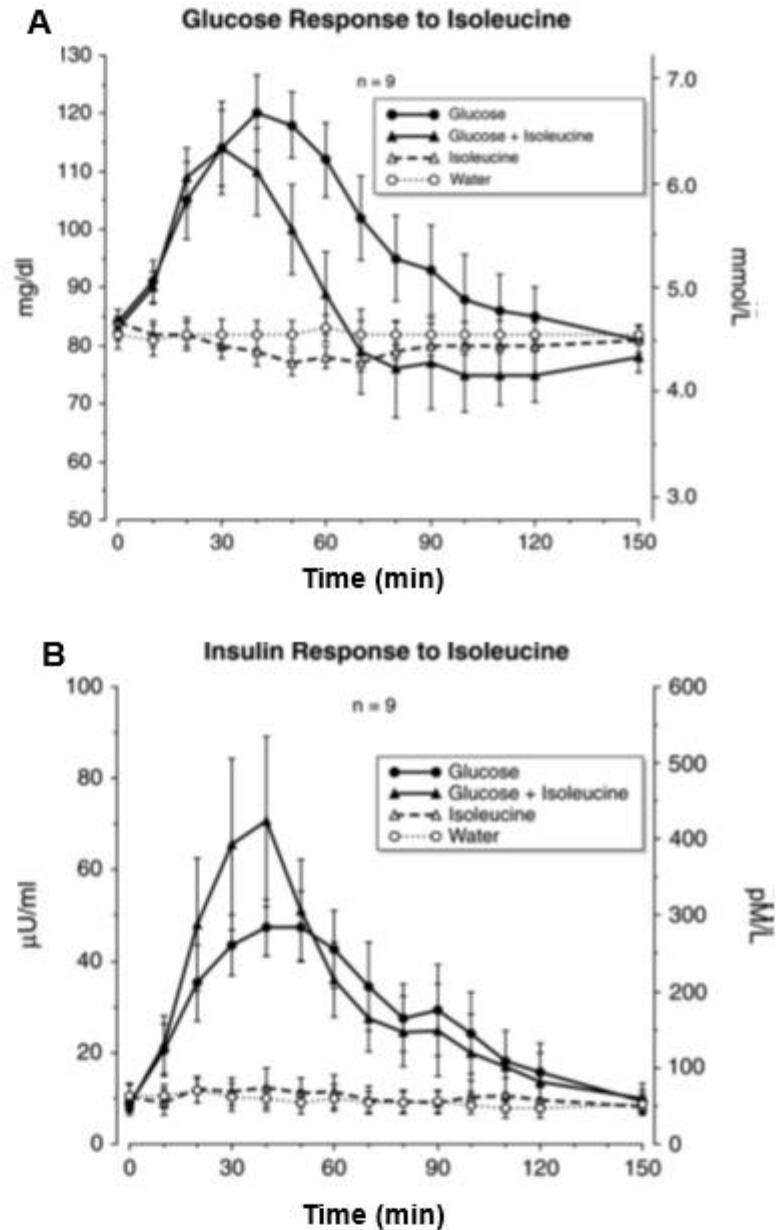


**Figure 1.9:** Blood glucose (A) and plasma insulin (B) in response to ingestion of 25 g of glucose, or isoleucine ~ 7.4 g alone, or isoleucine with glucose, or water, in healthy individuals. Blood glucose was reduced, after co-ingestion of isoleucine with glucose, compared with glucose alone ($P < 0.05$). There were no differences in plasma insulin concentrations after ingestion of glucose alone or co-ingested with isoleucine. Adapted from Nuttall et al. (290).

Altogether, the blood glucose lowering effects of both leucine and isoleucine support the potential for them to improve blood glucose control in individuals with impaired glucose tolerance and/or T2D. No studies have evaluated the acute effects of leucine or isoleucine, given as a as a 'pre-load', 30 min before a carbohydrate-rich meal, on the blood glucose-response in T2D. Given that postprandial blood glucose is higher in T2D than health, and pharmacologic strategies that stimulate insulin and slow gastric emptying are effective in the management of T2D, it may be predicted that the postprandial glucose-lowering effects of leucine and isoleucine would be greater. The effects of leucine and isoleucine on blood glucose, and the association with gastric emptying and glucoregulatory hormones, in T2D patients**,** have been investigated in **Study 4 in Chapter 5.**

#### *1.4.2.3 Effects of valine on energy intake, blood glucose and GI responses*

In contrast to leucine and isoleucine, much less information is available about the effects of valine on blood glucose regulation, in both animals and humans, and to the author's knowledge, there is no information about the effects of valine on energy intake.

Valine is a glucogenic amino acid; that is, it can be converted into glucose via gluconeogenesis. This glucogenic property of valine means that it has the capacity to increase blood glucose. In rats, acute oral administration of valine (1 g/kg, equivalent to 70 g in a 70 kg human), 30 min prior to exercise, prevented the reduction in liver glycogen and blood glucose (293). Also in rats, acute oral administration of valine (0.3 g/kg, equivalent to ~ 21g in a 70 kg human), increased the blood glucose response, 30 min after glucose administration (273). These findings are consistent with an effect of valine to maintain blood glucose. In contrast, in a study performed in fasted healthy humans, i.v. administration of 30 g valine slightly decreased blood glucose and stimulated insulin (196). It is important to note, however, that the doses of valine

used in these studies (196, 273, 293), were extremely high, and the data, as presented were difficult to interpret. Furthermore, these discrepant effects on the blood glucose response may reflect species differences (rats vs humans), as well as different routes of administration used (oral vs. i.v.).

There are no studies that have evaluated the effects of valine when consumed orally, given intragastrically or intraduodenally, on fasting or postprandial blood glucose in humans. The effects of valine, when given intragastrically, on the blood glucose response to a mixed-nutrient drink, and the relationship with gastric emptying and glucoregulatory hormones, in healthy individuals, has been investigated in **Study 3 in Chapter 4**. Because it is not known whether valine affects gut functions, including GI motility or GI hormone release, in association with effects on energy intake, in humans, the effects of valine, when infused intraduodenally, on antropyloroduodenal motility and plasma CCK concentrations, in the regulation of energy intake, in healthy individuals, have been investigated in **Study 2 in Chapter 3**.

## 1.5 Aims and hypotheses

To address the inconsistencies in the literature and limited information about the effects of the BCAAs, in isolation, on energy intake and blood glucose regulation, and the relevance of gastric emptying and gut hormones, in humans, the studies in this thesis aimed to:

1. Investigate the effects of drinks containing 30 g and 70 g pure whey protein isolate on the temporal release of all 20 AAs, and relationships with gastric emptying, GI hormone release, plasma insulin, glucagon, blood glucose, appetite and energy intake, in healthy individuals. It was hypothesised that oral whey protein loads of 30 and 70 g, would lead to load-dependent rises in specific AAs, and that the effects of protein on gastric emptying, blood glucose- and appetite-regulatory hormones and energy intake would be related to circulating concentrations of specific BCAAs and other essential AAs (**Chapter 2**).

2. Investigate the effects of intraduodenal infusion of valine, on upper GI motor functions, gut hormones, appetite perceptions, blood glucose and energy intake, in healthy individuals. It was hypothesised that intraduodenal infusion of valine would dose-dependently stimulate plasma CCK, modulate antropyloroduodenal motility and suppress of energy intake (**Chapter 3**).

3. Investigate the comparative effects of intragastric administration of leucine, isoleucine and valine on the blood glucose, C-peptide (as a measure of insulin secretion) and glucagon responses to, and gastric emptying of, a mixed-nutrient drink, in healthy individuals (**Chapter 4**).

4. Investigate the effects of intragastric administration of leucine and isoleucine on the blood glucose, insulin and glucagon responses to, and gastric emptying of, a mixed-nutrient drink, in individuals with T2D. Based on the outcomes of the study reported in Chapter 4, and because T2D is associated with abnormally elevated postprandial blood glucose concentrations, it was hypothesised, that leucine and isoleucine would attenuate the blood glucose response to a mixed-nutrient drink, containing glucose-based carbohydrates (**Chapter 5**).

The proposed studies will increase current knowledge on the individual effects of the BCAAs on energy intake, blood glucose, and relevant GI functions, including gastric emptying and gut hormone release and will also provide evidence as to whether BCAAs have the potential to be utilised for nutrient-based approaches, in the prevention and/or management of obesity and T2D.

# Chapter 2: Plasma free amino acid responses to whey protein and their relationships with gastric emptying, blood glucose- and appetite-regulatory hormones and energy intake in lean healthy men

**Elovaris RA*, Hutchison AT*, Lange K, Horowitz M, Feinle-Bisset C, Luscombe-Marsh ND**

*both authors contributed equally to this work

**Published in**

***Nutrients 2019; 11(10): 2465.***

# Statement of Authorship

| | |
|---|---|
| Title of Paper | Plasma free amino acid responses to whey protein and their relationships with gastric emptying, blood glucose- and appetite-regulatory hormones and energy intake in lean healthy men. |
| Publication Status | ☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | Elovaris RA*, Hutchison AT*, Lange K, Horowitz M, Feinle-Bisset C, Luscombe-Marsh ND. Nutrients, 2019 Oct 15, 11(10): 2465. *These authors contributed equally to this work. |

## Principal Author

| | | | |
|---|---|---|---|
| Name of Principal Author (Candidate) | Rachel Elovaris | | |
| Contribution to the Paper | Analysed the data, conducted the statistical analysis, interpreted the data, prepared the manuscript, and contributed to the revision of the manuscript. | | |
| Overall percentage (%) | 40% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. | | |
| Signature | | Date | July 6, 2021 |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

| | | | |
|---|---|---|---|
| Name of Co-Author | Amy Hutchison | | |
| Contribution to the Paper | Conducted the experiments, interpreted the data, prepared the manuscript, edited and revised the manuscript, and approved the final version of the manuscript. | | |
| Signature | | Date | June 23, 2021 |

| Name of Co-Author | Kylie Lange | | |
|---|---|---|---|
| Contribution to the Paper | Contributed to the statistical analysis, interpreted the data, edited and revised the manuscript, and approved the final version of the manuscript. | | |
| Signature | | Date | 7 July 2021 |

| Name of Co-Author | Michael Horowitz | | |
|---|---|---|---|
| Contribution to the Paper | Conceived and designed the research, interpreted results of experiments, edited and revised the manuscript, and approved the final version of manuscript. | | |
| Signature | | Date | 7 July 2021 |

| Name of Co-Author | Christine Feinle-Bisset | | |
|---|---|---|---|
| Contribution to the Paper | Conceived and designed the research, interpreted results of experiments, edited and revised the manuscript, obtained funding, and approved the final version of manuscript. | | |
| Signature | | Date | June 15, 2021 |

| Name of Co-Author | Natalie Luscombe-Marsh | | |
|---|---|---|---|
| Contribution to the Paper | Conceived and designed the research, interpreted the data, prepared the manuscript, contributed to the revision of the manuscript, approved the final version of manuscript, obtained funding, and had overall responsibility for the project. | | |
| Signature | | Date | 1/7/2021 |

## 2.1 Abstract

This study determined the effects of increasing loads of whey protein on plasma amino acid (AA) concentrations, and their relationships with gastric emptying, blood glucose- and appetite-regulatory hormones, blood glucose and energy intake. Eighteen healthy lean men participated in a double-blinded study, in which they consumed, on 3 separate occasions, in randomised order, 450-mL drinks containing either 30 g (L) or 70 g (H) of pure whey protein isolate, or control with 0 g of protein (C). Gastric emptying, serum concentrations of AAs, ghrelin, cholecystokinin (CCK), glucagon-like-peptide 1 (GLP-1), insulin, glucagon and blood glucose were measured before and after the drinks over 180 min. Then energy intake was quantified. All AAs were increased, and 7/20 AAs were increased more by H than L. Incremental areas under the curve ($iAUC_{0–180\ min}$) for CCK, GLP-1, insulin and glucagon were correlated positively with iAUCs of 19/20 AAs ($p < 0.05$). The strongest correlations were with the branched-chain AAs as well as lysine, tyrosine, methionine, tryptophan, and aspartic acid (all $R^2 > 0.52$, $p < 0.05$). Blood glucose did not correlate with any AA (all $p > 0.05$). Ghrelin and energy intake correlated inversely, but only weakly, with 15/20 AAs (all $R^2 < 0.34$, $p < 0.05$). There is a strong relationship between glucoregulatory hormones with a number of (predominantly essential) AAs. However, the factors mediating the effects of protein on blood glucose and energy intake are likely to be multifactorial.

## 2.2 Introduction

High-protein diets, including those that incorporate about 2–3 serves of dairy protein, are effective in the management of obesity and associated cardio-metabolic conditions (70, 121, 140, 260, 294). As one of the main components of dairy, whey is common in the diet and, when compared with other sources of protein, has been shown to be more satiating and more effective in facilitating weight loss (214, 217, 260).

The importance of gastrointestinal (GI) mechanisms to the beneficial effects of foods and beverages rich in dietary protein, particularly whey, on weight loss and cardio-metabolic functions has been well established over the last decade (120, 121, 229, 260). Several studies have reported dose-dependent effects of whey protein on concentrations of blood glucose- and appetite-regulatory hormones, including cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), insulin and glucagon (122, 124, 218-221). In healthy individuals, whey protein in loads of 4.5–18 g (in a drink containing 25 g glucose) dose-dependently lowered postprandial glycaemia and increased insulin, with a minimum of 9 g required for a significant effect (229). Furthermore, in healthy young adults drinks containing whey protein isolate loads of 20 g, 30 g or 40 g (consumed 30 min before a standardised carbohydrate-rich pizza meal) suppressed energy intake and reduced post-meal blood glucose more than 0 or 10 g loads (120).

Specific amino acids (AAs) that reach the peripheral circulation are purported to be integral to the modulation of blood glucose- and appetite-regulatory hormones by protein-rich foods/beverages (220, 221). In healthy individuals, intraduodenal (ID) administration of whey protein in loads of 2.1, 6.3, and 12.5 kcal/min (to mimic the range of normal gastric emptying rates of protein of 1–4 kcal/min) over 60 min increased plasma concentrations of 19 out of 20 AAs (the exception being cysteine) in a protein load-dependent manner (259). Moreover, the increases in the branched-chain and essential (methionine, lysine and tyrosine) AAs were strongly, and positively, related with GLP-1 and insulin, and moderately, and inversely, with energy intake (259). In another study, in which healthy men consumed drinks with 30 or 70 g of whey protein, the amount of calories emptied from the stomach by 60 min was associated significantly, albeit modestly, with the magnitude of change over the first 60 min in ghrelin, CCK, GLP-1 and glucagon concentrations (all r values $> 0.5$, $p < 0.05$), and these gut hormones were each modestly associated (inversely in the case of ghrelin) with the concordant

suppression of energy intake (~ 11 % suppression) by both protein drinks [17]. There is no information about the relationships between the release of specific AAs with glucose- and appetite-regulatory hormones, gastric emptying and energy intake responses following the consumption of protein-enriched drinks.

We have now analysed remaining plasma samples from our previous study (224) to evaluate the hypothesis that oral whey protein loads of 30 and 70 g would lead to load-dependent rises in specific AAs, and that the effects of protein on gastric emptying, blood glucose- and appetite-regulatory hormones and energy intake would be related to circulating concentrations of specific branched-chain and other essential AAs.

## 2.3 Materials and methods

### 2.3.1 Participants

Twenty lean, healthy men (mean age 24.7 ± 1.2 years [range 18–37 years]; mean BMI 22.0 ± 0.5 kg/m$^2$ [range 18.6–25.0 kg/m$^2$]) were recruited into the study as described (224). All participants provided written, informed consent to participate in the study, which was approved by the Research Ethics Committee of the Central Adelaide Local Health Network. The number of participants was determined from power calculations on the basis of our previous work, indicating that $n = 16$ participants would allow detection of a mean difference of 20.5 min in gastric 50 % emptying time ($T_{50}$), while $n = 20$ participants would allow detection of a mean difference in energy intake between treatments of 215 kcal, with $\beta = 0.8$ and $\alpha = 0.05$ (226, 295). Only males were studied, due to known variations in energy intake across the menstrual cycle in females (296). Participants who were identified as restrained eaters (score ≥ 12 on the eating restraint component of the Three-Factor Eating Questionnaire) (297), had low ferritin (< 30 ug/L) or iron (< 8 umol/L) concentrations, were lactose-intolerant, vegetarians, or were high-performance athletes, were excluded from participating. The Royal Adelaide Hospital

Research Ethics Committee approved the study protocol, and the study was registered as a clinical trial with the Australia and New Zealand Clinical Trial Registry (www.anzctr.org.au, registration number 12611000706976).

### 2.3.2 Study Outline

The aims of the original study were to evaluate the effects of 450-mL drinks containing 30 g pure whey protein isolate (L), 70 g pure whey protein isolate (H), or 0 g (control) on gastric emptying, GI hormone release, plasma insulin, glucagon, total AAs, blood glucose, appetite and energy intake (224). Accordingly, the evaluation of the effects of the drinks on the temporal release of all 20 AAs, and relationships with the previously reported outcomes, represents an exploratory secondary analysis.

### 2.3.3 Protein Drinks

As described (224), the pure protein drinks (i.e., they did not contain any other macronutrients) were prepared in the morning of each study visit by a member of the research staff, who had no involvement in either the analysis or interpretation of the data. Whey protein isolate powder (8855 ClearPro, Fonterra Co-Operative Group Ltd., Auckland, New Zealand) was dissolved in distilled water and diet cordial (Bickford's Diet Lime Cordial, Bickford's Australia) to achieve the desired loads (i.e., L-30 g whey protein (total energy content = 126 kcal) or H-70 g whey protein (total energy content = 283 kcal). The control drink consisted of 90 mL cordial and 359 mL distilled water (total energy content = 11.5 kcal). Sodium chloride was added to the L and C drinks in amounts of 0.3 g and 1.2 g, respectively, to match the osmolarity with H (i.e., 88 mOsmol/L) because it is well-established that osmolarity of liquid solutions affects gastric emptying (298, 299). The drink was provided to each participant in an opaque cup, covered at all times, so that both the primary investigator and the participant were blinded to the treatment,

and consumed within 2 min. **Table 2.A1** (**Appendix I**) outlines the AA composition of the whey protein isolate, and the amounts present in each drink.

### 2.3.4 Protocol

Each participant was studied on three occasions, separated by 7–11 days, in a randomised, double-blind, cross-over design (300). Randomisation and preparation of the solutions were performed by an investigator who had no involvement in the studies or data analysis. Participants were provided with a standardised meal, consumed on the evening before each study day, and instructed to abstain from all food, drinks and vigorous exercise until attending the laboratory at the Discipline of Medicine at 0830 h. At t = −10 min, a 14-mL blood sample was collected, a visual analogue scale questionnaire (VAS) administered and a 3-dimensional (3D) image of the stomach, obtained using 3D ultrasound, was recorded. At t = −2 min, participants then ingested one of the test drinks. Immediately afterwards, at t = 0 min, and subsequently, at 15-min intervals, until t = 180 min, further 3D ultrasound images, blood samples and VASs were obtained. At t = 180 min, each participant was presented with a standardised, cold, buffet-style test meal, as described (226), and instructed to consume as much food until they felt comfortably full, for up to 30 min (t = 180–210 min).

### 2.3.5 Measurements

#### *2.3.5.1 Gastric Emptying*

Gastric emptying was measured by 3D ultrasonography with the use of a Logiq 9 ultrasound system (GE Health Care Technologies, Milwaukee, WI, USA) with TruScan Architecture (a built-in magnetic sensor for 3D image acquisitions), as described (224).

***2.3.5.2 Plasma Ghrelin, CCK, GLP-1, Insulin, Glucagon, Free AA and Blood Glucose Concentrations***

10-mL blood samples were collected into ice-chilled ethylenediaminetetraacetic acid-coated tubes. Blood samples were centrifuged immediately (3200 rpm for 15 min at 4 °C) to obtain plasma. Plasma samples were stored at −70 °C for subsequent analysis.

Plasma total ghrelin concentrations (pmol/L) were analysed by radioimmunoassay, as described (301), without peptide extraction (Phoenix Pharmaceuticals, Burlingame, CA, USA). No cross-reactions with relevant molecules have been evident. Detection limit was 1.0 mU/L, intra-assay and inter-assay coefficients of variation (CVs) were 7.0 % and 13.4 %, respectively.

Plasma CCK-8 concentrations (pmol/L) were analysed by radioimmunoassay after ethanol extraction, using an adaption of a previous method, as described (302). Standards were prepared with the use of a synthetic sulfated CCK-8 antibody (Sigma Chemical, St. Louis, MO, USA) which binds all CCK peptides containing a sulfated tyrosine residue in position 7, shows a 26 % cross-reactivity with unsulfated CCK-8, < 2 % cross-reactivity with human gastrin I, and does not bind to structurally unrelated peptides. Sulfated CCK-8 $^{125}$I-labeled with Bolton and Hunter reagent (Perkin Elmer) was used as a tracer, and samples were incubated for 7 days at 4 °C. The antibody-bound fraction was separated by the addition of dextran-coated charcoal containing gelatin and the radioactivity determined in the supernatants after centrifugation. The detection limit was 1 pmol/L, and intra-assay and inter-assay CVs were 8.4 % and 16.5 %, respectively.

Plasma GLP-1 concentrations (pmol/L) were analysed by radioimmunoassay (GLPIT-36HK; Millipore, Billerica, MA, USA) (303). There are no cross-reactions with glucagon, gastric

inhibitory polypeptide or other gut or pancreatic peptides, and it measures both GLP-1$_{(7–36)}$ and GLP-1$_{(9–36)}$ amide. The detection limit was 3 pmol/L, and intra- and inter-assay CVs were 7.1 % and 7.8 %, respectively.

Plasma insulin concentrations (mU/L) were analysed by ELISA (10-1113; Mercodia, Uppsala, Sweden) (303). The detection limit was 1.0 mU/L, and intra- and inter-assay CVs were 2.8 % and 8.2 %, respectively.

Plasma glucagon concentrations (pmol/L) were analysed by radioimmunoassay (GL-32K; Millipore, Burlington, MA, USA). The antibody used does not cross-react with insulin, proinsulin, C-peptide, somatostatin, or pancreatic polypeptide, and has < 0.1 % cross-reactivity with oxyntomodulin. The detection limit was 6 pmol/L, and intra-assay and inter-assay CVs were 4.2 % and 9.3 %, respectively.

Plasma free AA concentrations (mmol/L) for aspartic acid, alanine, arginine, asparagine, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine were measured, as described (259). The analysis was performed at the Australian Proteome Analysis Facility established under the Australian Government's National Collaborative Research Infrastructure Strategy.

Blood glucose concentrations (mmol/L) were measured immediately after collection, by the glucose oxidase method using a portable glucometer (FreeStyle Optimum H; Abbott Laboratories, Chicago, IL, USA).

#### *2.3.5.3 Energy Intake*

Each food item in the buffet meal (295) was weighed before and after consumption to quantify the amounts of food and beverages consumed (g). Energy intake (kcal) was then calculated using commercially available software (Foodworks 3.01, Xyris Software, Highgate Hill, QLD, Australia) (295).

### 2.3.6 Data and Statistical Analysis

Statistical analysis was performed using SPSS software (version 24; IBM, Armonk, NY, USA), in consultation with a biostatistician. Baseline plasma AA concentrations (i.e., $t = -2$ min) between study days were analysed using one-way repeated measures ANOVA with protein load as the factor. The effects of protein load on the net incremental area under the curve ($iAUC_{0\text{–}180\ min}$) for each AA were analysed by general linear model mixed model ANOVA. Two-way repeated measures ANOVA (treatment-by-time model) was conducted, with post-hoc pairwise comparisons at individual time points. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni correction, were performed when significant treatment, or treatment-by-time, effects, were found. To visually present the plasma AAs that had the "smallest" (bottom 5) and "greatest" (top 5) responses to the test drinks, we defined the response based on the magnitude of increase in each plasma AA concentration over the 180-min period following the L and H drink, respectively, and relative to C. The formula below was used to calculate the magnitude of increase in each plasma AA:

Magnitude increase (expressed as a %) = $iAUC_{0\text{–}180\ min}$ for each specific AA following H, divided by $iAUC_{0\text{–}180\ min}$ for each specific AA following C, multiplied by 100.

Relationships between the iAUC for each AA and protein load, total AA concentration within the protein drinks, gastric emptying (half-emptying time, $T_{50}$) (224), energy intake and iAUCs for ghrelin, CCK, GLP-1, insulin, glucagon and blood glucose, respectively, were evaluated

using linear within-subject correlations (*r*) with fixed slopes and subject-varying intercepts (304). Relationships of each AA with gastric emptying, energy intake and iAUCs for ghrelin, CCK, GLP-1, insulin, glucagon and blood glucose, respectively, were ranked in order of strongest to weakest response.

Statistical significance was accepted at $p < 0.05$. All data are reported as means ± SEMs.

## 2.4 Results

Twenty participants were recruited for the study. Data from 16 participants who completed all study days, who tolerated the drink and for whom no data were missing, was analysed. Reasons for excluding the data from 4 participants were failure to fast overnight ($n = 1$), completion of only 2 of the 3 study days before withdrawing due to time constraints ($n = 1$), and exclusion of gastric emptying data due to suboptimal image quality (i.e., presence of air in the stomach) ($n = 2$). There were no differences in the characteristics of the 16 completers compared with the 4 excluded participants, and no exclusion was related to the protein drinks.

### 2.4.1 Plasma AA Concentrations in Response to Increasing Protein Loads

Baseline concentrations of individual and total AAs did not differ between test days with the exception of tryptophan ($p < 0.05$) (**Table 2.1**).

There was a significant treatment-by-time interaction for plasma concentrations of 20/20 AAs ($p < 0.05$ for all). The greatest increases in plasma concentrations over the 180-min period following L and H, relative to C, were for leucine, lysine, valine, isoleucine and alanine (**Figure 2.1**), all of which diminished after 90 min, although more slowly after H than L. Conversely, smallest increases were evident for tryptophan, cysteine, histidine, glycine and aspartic acid (**Figure 2.2**).

**Table 2.1:** Baseline (fasting) plasma amino acid (AA) concentrations prior to consumption of the test drinks consisting of either 0 g (C), 30 g (L) or 70 g (H) of pure whey protein dissolved in varying amounts of distilled water, diet cordial, and sodium chloride (all 450 mL and 88 mOsm/L) [a].

| | Treatment | | | | |
|---|---|---|---|---|---|
| **AA** | **C** | **L** | **H** | **$F_{2,30}$** | ***P Value*** [b] |
| **mmol/L** | | | | | |
| Gln (C) | 0.80 ± 0.6 | 0.74 ± 0.07 | 0.81 ± 0.08 | 0.78 | 0.468 |
| Ala (NE) | 0.41 ± 0.03 | 0.38 ± 0.04 | 0.43 ± 0.04 | 1.41 | 0.261 |
| Gly (C) | 0.30 ± 0.02 | 0.28 ± 0.03 | 0.31 ± 0.03 | 1.15 | 0.329 |
| Val (E) | 0.28 ± 0.02 | 0.26 ± 0.02 | 0.29 ± 0.03 | 0.71 | 0.499 |
| Pro (C) | 0.26 ± 0.02 | 0.27 ± 0.04 | 0.27 ± 0.03 | 0.07 | 0.930 |
| Lys (E) | 0.20 ± 0.02 | 0.19 ± 0.02 | 0.21 ± 0.02 | 0.89 | 0.420 |
| Leu (E) | 0.14 ± 0.01 | 0.14 ± 0.01 | 0.15 ± 0.02 | 0.74 | 0.488 |
| Thr (E) | 0.14 ± 0.01 | 0.13 ± 0.01 | 0.14 ± 0.01 | 1.54 | 0.232 |
| Ser (C) | 0.12 ± 0.01 | 0.11 ± 0.01 | 0.12 ± 0.01 | 1.06 | 0.359 |
| Arg (C) | 0.10 ± 0.01 | 0.09 ± 0.01 | 0.10 ± 0.01 | 2.03 | 0.148 |
| His (E) | 0.10 ± 0.01 | 0.09 ± 0.01 | 0.10 ± 0.01 | 0.45 | 0.639 |
| Ile (E) | 0.08 ± 0.01 | 0.07 ± 0.01 | 0.08 ± 0.01 | 2.16 | 0.132 |
| Tyr (C) | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.08 ± 0.01 | 1.53 | 0.234 |
| Asn (NE) | 0.07 ± 0.01 | 0.06 ± 0.01 | 0.07 ± 0.01 | 0.56 | 0.580 |
| Phe (E) | 0.06 ± 0.01 | 0.06 ± 0.01 | 0.06 ± 0.01 | 0.65 | 0.527 |
| Glu (NE) | 0.06 ± 0.01 | 0.05 ± 0.01 | 0.05 ± 0.01 | 1.12 | 0.341 |
| Cys (C) | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.16 | 0.850 |
| Met (E) | 0.03 ± 0.003 | 0.03 ± 0.003 | 0.03 ± 0.003 | 0.62 | 0.543 |
| Trp (E) | 0.005 ± 0.00 | 0.005 ± 0.00 | 0.006 ± 0.00 | 3.88 | 0.032 |
| Asp (NE) | 0.004 ± 0.00 | 0.004 ± 0.00 | 0.004 ± 0.00 | 1.94 | 0.162 |
| Total | 3.27 ± 0.21 | 3.06 ± 0.26 | 3.35 ± 0.28 | 0.94 | 0.404 |

[a] Data are means ± SEMs, $n$ = 16; amino acids (AAs) have been presented in order of highest to lowest concentration. [b] Main effect of protein load was determined by one-way repeated measures ANOVA; statistical significance was accepted at $p < 0.05$. Abbreviations for AAs: Alanine: Ala; Arginine: Arg; Asparagine: Asn; Aspartic Acid: Asp; Cysteine: Cys; Glutamine: Gln; Glutamic acid: Glu; Glycine: Gly; Histidine: His; Isoleucine: Ile; Leucine: Leu; Lysine: Lys; Methionine: Met; Phenylalanine: Phe; Serine: Ser; Threonine: Thr; Tryptophan: Trp; Tyrosine: Tyr Valine: Val. Essential AAs (E); Non-essential AAs (NE); Conditional AAs (C).

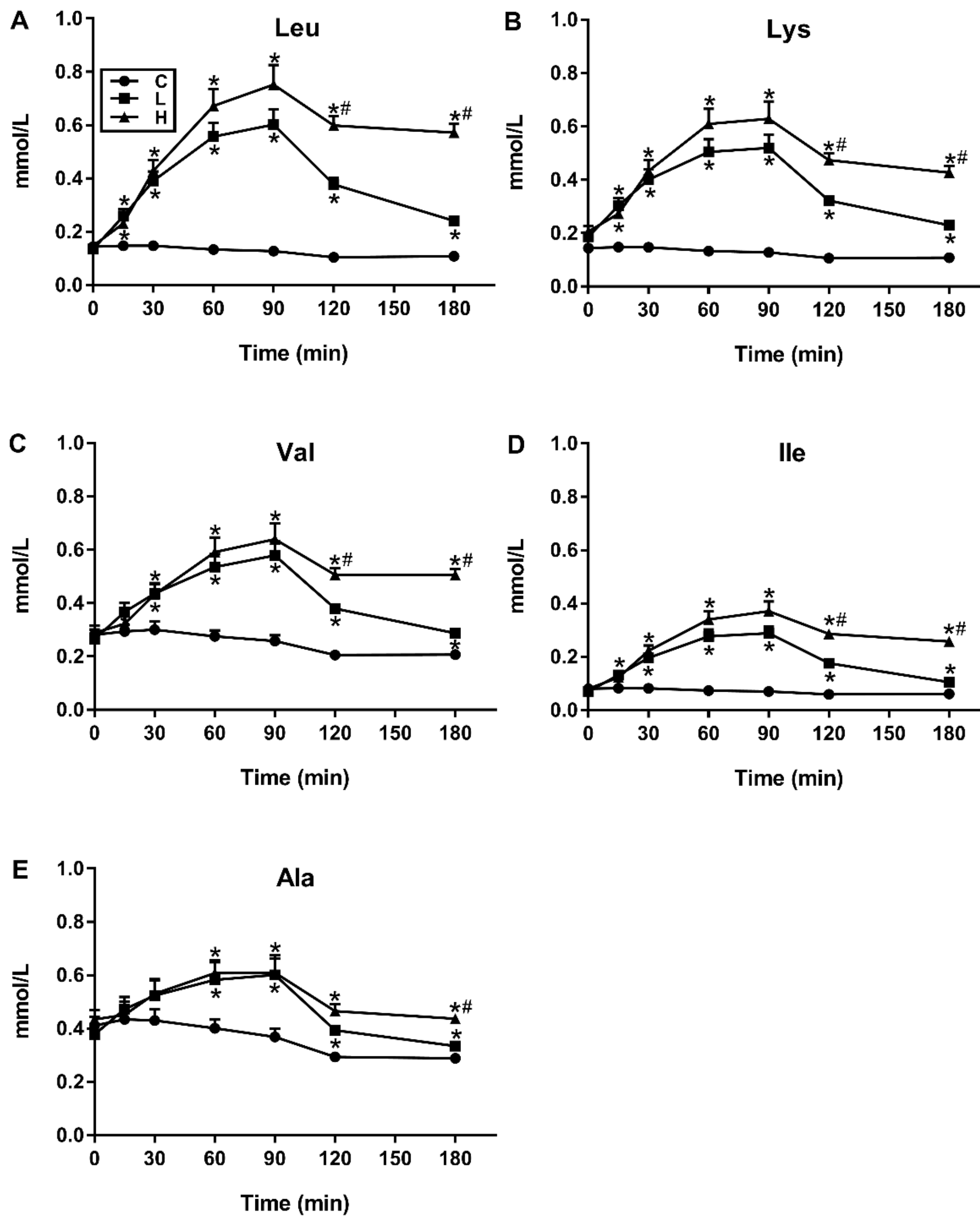


**Figure 2.1:** Temporal profiles of (A) Leucine (Leu), (B) Lysine (Lys), (C) Valine (Val), (D) Isoleucine (Ile) and (E) Alanine (Ala), the five amino acids whose plasma concentrations increased the most in response to test drinks containing either 0 g (C), 30 g (L) or 70 g (H) of pure whey protein dissolved in varying amounts of distilled water, diet cordial, and sodium chloride so they were matched for volume and osmolarity (all 450 mL and 88 mOsm/L). These responses were defined as being the strongest based on the "magnitude of increase" in each plasma concentration over the 180-min period following the L and H drink, respectively, and relative to C. Data are means ± SEMs, $n = 16$. Effects of protein load and time on individual AAs were determined by a two-way ANOVA, and post-hoc comparisons between two loads were determined using Bonferroni's correction; statistical significance was accepted at $p < 0.05$. * Significantly different from C; # Significantly different from L ($p < 0.05$).

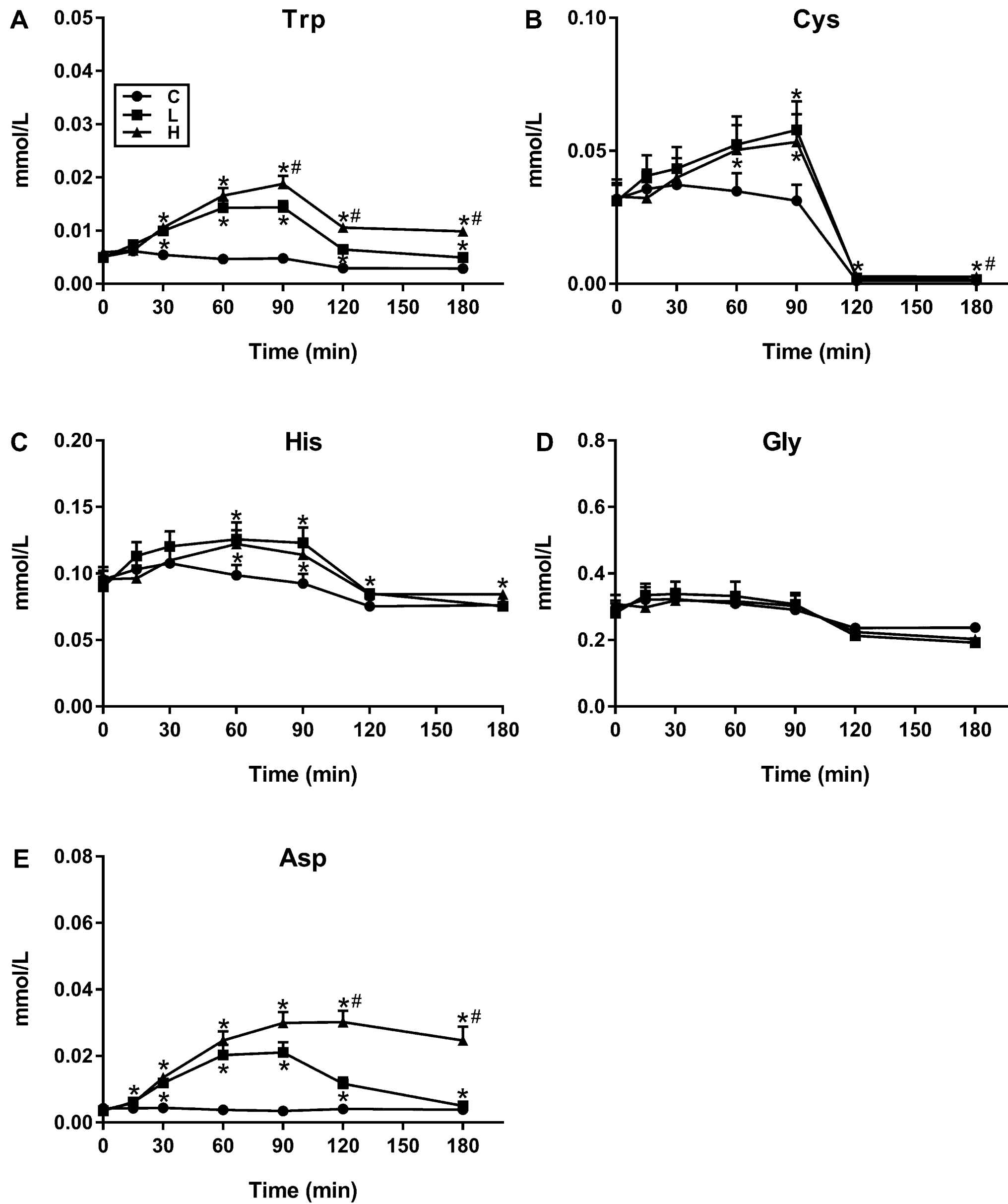


**Figure 2.2:** Temporal profiles of (A) Tryptophan (Trp), (B) Cysteine (Cys), (C) Histidine (His), (D) Glycine (Gly) and (E) Aspartic Acid (Asp), the five amino acids with the weakest response to test drinks containing either 0 g (C), 30 g of whey protein (L) or 70 g of pure whey protein (H) dissolved in varying amounts of distilled water, diet cordial and sodium chloride so they were matched for volume and osmolarity (all 450 mL and 88 mOsm/L). These responses were defined as being the weakest based on the "magnitude of increase" in each plasma concentration over the 180-min period following the L and H drink, respectively, and relative to C. Data are means ± SEMs, n = 16. Effects of protein load and time on individual AAs were determined by a two-way ANOVA, and post-hoc comparisons between two loads were determined using Bonferroni's correction; statistical significance was accepted at $p < 0.05$. * Significantly different from C; # Significantly different from L ($p < 0.05$).

The $iAUC_{0–180\ min}$ of each of the 20 AA profiles are depicted in **Table 2.2**. There was a significant main effect of protein load on the $iAUC_{0–180\ min}$ of plasma concentrations of 19 out of 20 AAs (exception: glycine) ($p < 0.05$ for all). Post-hoc analyses revealed that the $iAUC_{0–180\ min}$ of plasma concentrations of 19 out of 20 AAs ($p < 0.05$ for all) (exception: glycine) were greater in response to L compared with C. Furthermore, for 18 out of 20 AAs ($p < 0.05$ for all) (exceptions: glycine and glutamine) the responses to H were greater than to C. While plasma concentrations of 13 out of 20 AAs did not differ between L and H protein drinks, the $iAUC_{0–180\ min}$ of leucine, lysine, isoleucine, tyrosine, glutamic acid, methionine and aspartic acid were greater following H than L ($p < 0.05$ for all). Relationships between the $iAUCs_{0–180\ min}$ of each of the 20 AAs (displayed in order of abundance of the AAs within the whey protein drinks) with the load of protein are presented in **Table 2.A3** (**Appendix I**). The magnitude of increase in the plasma concentrations of 8 out of 9 essential AAs (exception: histidine) was moderately to strongly associated with the concentration of the protein in the drinks (i.e., range of $R^2$ values was 0.54–0.84, $p < 0.05$ for all). In contrast, the relationships for the non-essential AAs (i.e., range of $R^2$ values was 0.26–0.83, $p < 0.05$ for all) and 5 out of 6 conditional AAs (exception: glycine for which there was no relationship) (i.e., range of $R^2$ values was 0.14–0.72, $p < 0.05$ for all) were more variable.

**Table 2.2:** Plasma amino acid (AA) responses (incremental areas under the curve $(iAUC)_{0–180\ min}$) (displayed in order of abundance of the amino acids within the whey protein drinks) following consumption of the test drinks consisting of either 0 g (C), 30 g (L) or 70 g (H) of whey protein dissolved in varying amounts of distilled water, diet cordial, and sodium chloride (all 450 mL and 88 mOsm/L) [a].

| | Treatment | | | | |
|---|---|---|---|---|---|
| **AA** | **C** | **L** | **H** | **$F_{2,30}$** | ***P Value*** [b] |
| **mmol·180 min·L$^{-1}$** | | | | | |
| Glu (NE) | 1.9 ± 0.4 | 8.1 ± 1.2 [c] | 11.2 ± 1.0 [c,d] | 36.8 | <0.001 |
| Leu (E) | 0.6 ± 0.2 | 48.4 ± 3.5 [c] | 74.4 ± 5.2 [c,d] | 133 | <0.001 |
| Asp (NE) | 0.1 ± 0.02 | 1.7 ± 0.2 [c] | 3.5 ± 0.3 [c,d] | 74.0 | <0.001 |
| Lys (E) | 1.7 ± 0.4 | 33.7 ± 3.1 [c] | 49.6 ± 4.4 [c,d] | 67.6 | <0.001 |
| Ile (E) | 0.3 ± 0.1 | 22.4 ± 1.7 [c] | 35.0 ± 2.5 [c,d] | 123 | <0.001 |
| Val (E) | 1.5 ± 0.5 | 30.1 ± 3.3 [c] | 40.3 ± 4.1 [c] | 45.1 | <0.001 |
| Ala (NE) | 2.3 ± 0.7 | 22.1 ± 3.0 [c] | 18.7 ± 3.0 [c] | 15.0 | 0.001 |
| Thr (E) | 1.1 ± 0.3 | 14.1 ± 1.5 [c] | 18.2 ± 2.0 [c] | 35.7 | <0.001 |
| Pro (C) | 2.1 ± 0.6 | 15.3 ± 1.8 [c] | 14.1 ± 2.0 [c] | 16.1 | <0.001 |
| Tyr (C) | 0.3 ± 0.1 | 8.4 ± 0.9 [c] | 12.7 ± 1.3 [c,d] | 51.6 | <0.001 |
| Ser (C) | 0.9 ± 0.2 | 6.9 ± 1.0 [c] | 7.1 ± 1.0 [c] | 14.6 | <0.001 |
| Phe (E) | 0.3 ± 0.1 | 3.9 ± 0.6 [c] | 5.3 ± 0.8 [c] | 22.6 | <0.001 |
| Cys (C) | 0.3 ± 0.1 | 1.7 ± 0.4 [c] | 1.3 ± 0.3 [c] | 7.43 | 0.002 |
| Arg (C) | 0.5 ± 0.1 | 6.9 ± 0.9 [c] | 8.2 ± 1.0 [c] | 24.4 | 0.001 |
| Met (E) | 0.2 ± 0.05 | 4.2 ± 0.4 [c] | 6.6 ± 0.7 [c,d] | 52.7 | <0.001 |
| Trp (E) | 0.04 ± 0.01 | 0.82 ± 0.07 [c] | 1.15 ± 0.1 [c] | 66.2 | <0.001 |
| His (E) | 0.8 ± 0.2 | 3.9 ± 0.7 [c] | 2.8 ± 0.4 [c] | 8.13 | 0.006 |
| Gly (C) | 2.6 ± 0.6 | 6.3 ± 1.4 | 3.2 ± 0.8 | 3.50 | 0.066 |
| Gln (C) | 8.5 ± 1.9 | 25.4 ± 4.8 [c] | 14.9 ± 3.1 | 5.30 | 0.011 |
| Asn (NE) | 0.6 ± 0.1 | 5.9 ± 0.7 [c] | 6.9 ± 0.9 [c] | 23.9 | <0.001 |
| Total | 23.2 ± 6.0 | 255.25 ± 29.0[c] | 302.9 ± 34.0 [c] | 27.9 | <0.001 |

[a] Data are means ± SEMs, $n = 16$. [b] Main effect of treatment on the $iAUC_{0–180\ min}$ for individual AAs was determined by one-way repeated measures ANOVA and post-hoc comparisons between two loads were determined using Bonferroni's correction; statistical significance was accepted at $p < 0.05$; [c] Significantly different from C ($p < 0.05$); [d] Significantly different from L ($p < 0.05$). Abbreviations for AAs: Alanine: Ala; Arginine: Arg; Asparagine: Asn; Aspartic Acid: Asp; Cysteine: Cys; Glutamine: Gln; Glutamic acid: Glu; Glycine: Gly; Histidine: His; Isoleucine: Ile; Leucine: Leu; Lysine: Lys; Methionine: Met; Phenylalanine: Phe; Serine: Ser; Threonine: Thr; Tryptophan: Trp; Tyrosine: Tyr; Valine: Val. Essential AAs (E); Non-essential AAs (NE); Conditional AAs (C).

### 2.4.2 Relationships between Gastric Emptying, Ghrelin, CCK, GLP-1, Insulin, Glucagon, Blood Glucose and Energy Intake with Plasma AA concentrations

For the purpose of the current exploratory relationships analysis, the temporal profiles of gastric emptying, and plasma ghrelin, CCK, GLP-1, insulin, glucagon, and blood glucose reported previously [17] have been expressed in **Table 2.A2** (**Appendix I**).

The strength of the relationships between the $iAUCs_{0–180\ min}$ for each of the 20 amino acids with gastric emptying, ghrelin, CCK, GLP-1, insulin, glucagon, glucose and energy intake following consumption of the three test drinks is illustrated in **Figure 2.3**. There were positive correlations for gastric emptying with the $iAUCs_{0–180\ min}$ of 16 out of 20 AAs ($R^2$ range 0.16–0.71, $p < 0.05$ for all). There were also positive correlations between: (1) the $iAUC_{0–180\ min}$ of ghrelin with the $iAUCs_{0–180\ min}$ of 15 out of 20 AAs ($R^2$ range 0.12–0.34, $p < 0.05$ for all); (2) the $iAUCs_{0–180\ min}$ of both CCK and GLP-1 with the $iAUCs_{0–180\ min}$ of 18 out of 20 AAs (CCK: $R^2$ range 0.22–0.76 and GLP-1: $R^2$ range 0.13–0.68, $p < 0.05$ for all); (3) the $iAUC_{0–180\ min}$ of insulin with the $iAUCs_{0–180\ min}$ of 16 out of 20 AAs ($R^2$ range 0.22–0.65, $p < 0.05$ for all); and, (4) the $iAUC_{0–180\ min}$ of glucagon with the $iAUCs_{0–180\ min}$ of 18 out of 20 AAs ($R^2$ range 0.15–0.85, $p < 0.05$ for all). There was no significant relationship between any AA with blood glucose concentrations (all $p > 0.05$). Energy intake was correlated inversely with the $iAUCs_{0–180\ min}$ for 15 out of 20 AAs ($R^2$ range 0.12–0.21, $p < 0.05$). The $iAUCs_{0–180\ min}$ of the branched chain AAs, the essential AAs (particularly lysine, methionine and tryptophan), and the non-essential or conditional AAs (particularly aspartic acid and tyrosine) were most strongly related with gastric emptying and all glucose- and appetite-regulatory hormones, and weakly correlated with energy intake.

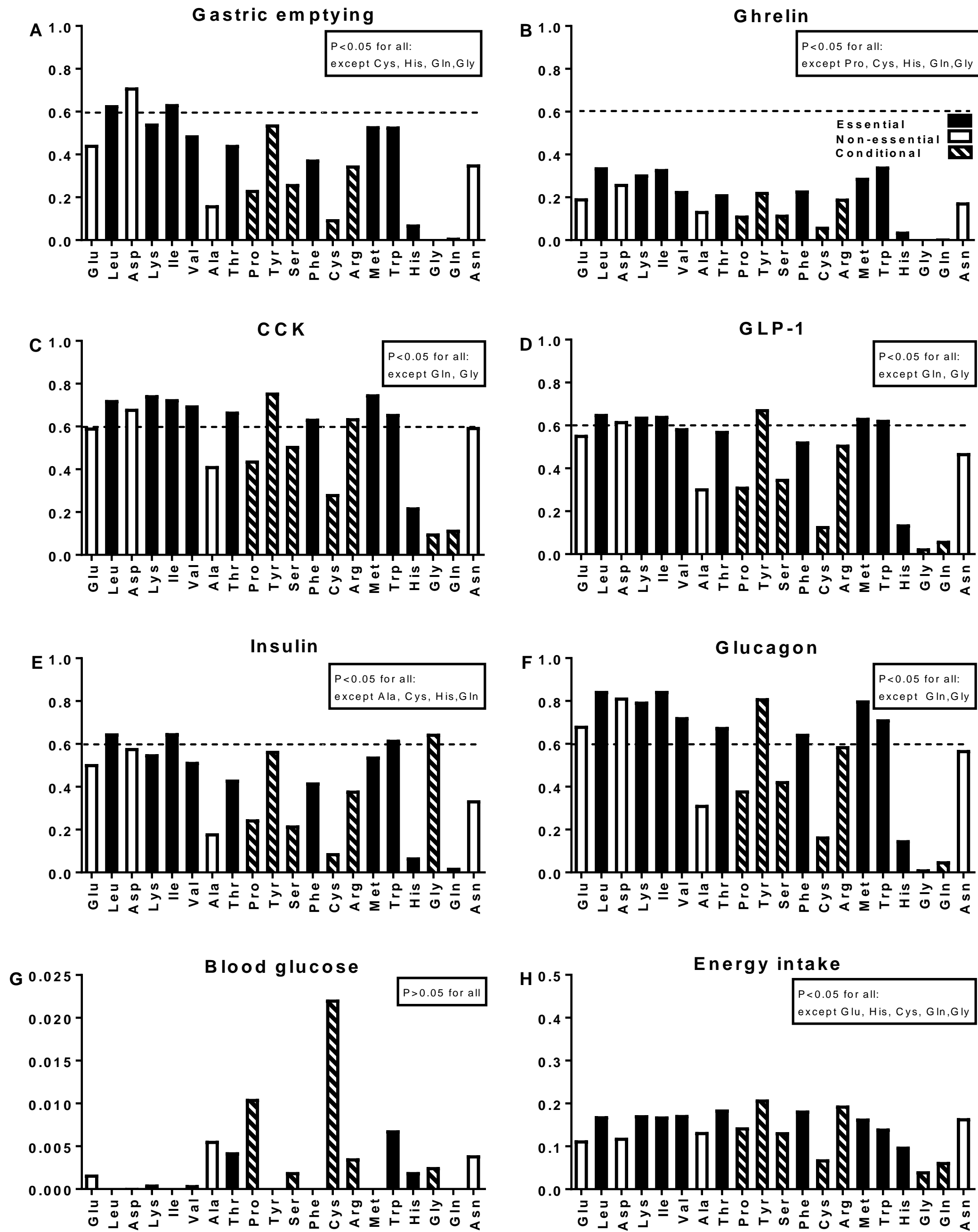


**Figure 2.3:** Within-subject relationships between the iAUCs$_{0–180\ min}$ of each of the 20 amino acids (displayed in order of abundance of the amino acids in the whey protein drinks) with (A) gastric emptying, as well as the iAUCs$_{0–180\ min}$ of (B) ghrelin, (C) CCK, (D) GLP-1, (E) insulin, (F) glucagon and (G) blood glucose and (H) energy intake, following consumption of the three test drinks which contained 0 g (C), 30 g (L), and 70 g (H) of whey protein. Data are within-subject $R^2$ values for $n$ = 16; Statistical significance was accepted at $p < 0.05$. The hatched horizontal line indicates the specific AAs which were consistently amongst the top ~10 amino acids strongly associated (defined as having an $R^2$ of $\geq 0.6$) with the majority of study outcomes presented in A to F except ghrelin. Abbreviations for AAs: Alanine: Ala; Arginine: Arg; Asparagine: Asn; Aspartic Acid: Asp; Cysteine: Cys; Glutamine: Gln; Glutamic acid: Glu; Glycine: Gly; Histidine: His; Isoleucine: Ile; Leucine: Leu; Lysine: Lys; Methionine: Met; Phenylalanine: Phe; Serine: Ser; Threonine: Thr; Tryptophan: Trp; Tyrosine: Tyr; Valine: Val.

## 2.5 Discussion

This study in healthy men expands existing knowledge relating to the interplay between specific AAs within foods containing an intact or hydrolysed protein and how they collectively influence the modulation of glucose- and appetite-regulatory hormones, as well as energy intake. We have established for the first time that 450 mL, isoosmolar test drinks containing either 30 or 70 g of pure protein (i.e., contained no other macronutrients) substantially increased the plasma AA responses ($iAUC_{0–180\ min}$) of only 7 (i.e., leucine, isoleucine, lysine, methionine, tyrosine, glutamic acid and aspartic acid) of the 20 AAs in a load-of-protein dependent manner. Confirming previous studies by others (305, 306) and our group [16], we found that the magnitude of increase in postprandial plasma concentrations for most of the essential AAs, reflected their abundance within the protein drinks, whereas the relationships for the non-essential and conditionally AAs were more variable. We have also established that plasma CCK, GLP-1, insulin and glucagon responses to the protein drinks were all strongly and positively correlated with specific AAs, particularly the essential AAs, while ghrelin and energy intake were only weakly, and inversely, correlated with 16 and 15 out of 20 AAs, respectively. Conversely, the blood glucose response was not correlated with any AA.

Previously we have reported that the postprandial plasma concentrations of 19 out of 20 AAs were increased in a load-of-protein dependent manner when increasing loads of whey protein (i.e., 8, 24 and 48 g) were delivered into the duodenum at rates mimicking the normal range of gastric emptying (i.e., 1–4 kcal/min) [16]. In contrast, despite the protein loads within the current study being substantially greater (i.e., 30 and 70 g) than used in our previous ID study, we observed that only 4 of the 9 essential AAs (leucine, lysine, isoleucine, methionine), 2 of the 7 conditionally essential AAs (tyrosine and glutamine), and 2 of the 4 non-essential AAs (glutamic and aspartic acids), were increased in a load-of-protein dependent manner.

Furthermore, in the current study we observed positive correlations, albeit variable in strength, between the concentration of AAs in the plasma, with the concentration of each AA in the protein drinks ($R^2$ range 0.11–0.84) and also between each AA with gastric emptying ($R^2$ range 0.16–0.71). Taken together, the current study extends these insights by demonstrating that factors other than the protein load and rate of gastric emptying of the protein test drinks influence the concentrations of specific AAs in the peripheral circulation. Such factors likely to account for the differences in concentrations of specific AAs reaching the peripheral circulation between studies may include the concentration of free AAs, as well as di- and tri-peptides within the source of protein (i.e., whether protein is intact, isolated, highly hydrolysed), differential digestion, metabolic transformation (which is particularly extensive for the dicarboxylic AAs, glutamic and aspartic acids) and absorption, of the exogenous and endogenous small intestinal proteins, and the rates of uptake and release of AAs from the liver and other tissues (307).

Evidence for differential roles of specific AAs in the regulation of glucose- and appetite-regulatory hormones, and the suppression of energy intake, is derived from studies in both animals (256, 308) and humans (243, 248, 254, 309). In healthy men, we have reported previously that leucine (284), but not valine (309), each infused ID at a rate of 0.45 kcal/min for 90 min, modulated gut motor and hormone functions, blood glucose and/or energy intake. We also found that intragastric administration of lysine (at loads of 5 and 10 g) reduced the glycaemic response to a mixed-nutrient drink moderately (254). Moreover, in lean and obese individuals, intragastric administration of 3 g of tryptophan slowed gastric emptying and increased glucagon, but did not suppress subsequent energy intake substantially (248), whereas a solution containing 3.3 g tryptophan infused ID at a rate of 0.15 kcal/min for 90 min, stimulated pyloric pressures, the release of CCK, and to a lesser extent, GLP-1 and PYY, and

significantly reduced energy intake by ~ 200 kcal (243) . In contrast to the aforementioned studies, a strength of the current design was that we measured all 20 AAs and, thereby, could evaluate the interplay between these AAs and how they collectively influence glucose- and appetite-regulatory hormones, as well as energy intake. As such, the current study extends current knowledge (124, 220, 221, 243, 248, 254, 259, 284, 309) by demonstrating that leucine, valine, isoleucine, methionine and lysine (all essential AAs) and tyrosine (conditionally-essential AA) are each associated strongly with postprandial concentrations of CCK, GLP-1, insulin and glucagon, to a lesser extent with ghrelin, and weakly with protein-induced suppression of energy intake.

Notably, the blood glucose response was not related to the release of any AA. This was not surprising since the protein drinks contained no carbohydrate, and the almost immediate release of insulin (i.e., from ~ 15 min) was presumably counteracted by the release of glucagon from ~ 60 min following the drinks so that euglycaemia was maintained. These findings again highlight the strong association between AAs with the release of insulin, which is likely to reflect direct stimulation of pancreatic β-cells (310). While plasma AA responses were also strongly, and positively, correlated with the glucagon response, whether its release is attributable to direct stimulation of the pancreatic α-cells by specific AAs remains uncertain. Interestingly, a study in rodents in which a high-protein, carbohydrate-free solution was perfused through pancreatic islet cells, found that arginine, glutamine and α-aminobutyrate stimulated glucagon secretion (311).

Several aspects of our study design should be considered when interpreting our results. Only healthy males were included, hence, our results may not reflect responses in women, overweight/obese or older individuals, although this is unlikely (312). Although the 30 and 70

g loads of whey protein isolate used in this study contained different amounts of energy, our hypothesis was to investigate the effects of increasing protein loads that are typically consumed within drinks, snacks or main meals. As such, it cannot be assumed that our observations would extend to drinks that are isocaloric, or that contain fully hydrolysed form of proteins. Had we used a fully hydrolysed form of whey protein, the AAs may have been rapidly absorbed from the GI tract and may have differentially affected the responses we assessed (313). Moreover, it cannot be assumed that our observations would extend to drinks containing other sources of protein, or a solid protein. For example, it has been reported that casein compared to whey, and solid compared to liquefied protein, are more slowly digested and absorbed (216, 314). However, evidence from a 2013 systemic review indicates that findings remain inconsistent regarding the protein kinetics from intact compared to hydrolysed forms of whey or casein, or of various protein sources (315). Finally, we recognise that the relationships explored in this secondary analysis do not establish causality, and with no adjustment to $P$-values for multiple comparisons, our results are hypothesis-generating in nature. Hence, our findings dictate the need for further investigation to elucidate the role of these specific AAs in the regulation of glucose homeostasis and energy intake. A carefully designed, prospective study is clearly warranted to disentangle the effects of protein, different AAs and energy on correlations between postprandial responses of individual AAs and other study outcomes.

In conclusion, this study provided new insights into the concentrations of 20 AAs reaching the peripheral circulation following drinks containing loads of whey protein isolate that are representative of loads commonly consumed by humans. Our observations indicate that, in healthy, lean men, plasma concentrations of specific AAs (particularly the essential AAs) increase in a load-of-protein-dependent manner, and there are strong relationships between CCK, GLP-1, insulin and glucagon with leucine, isoleucine, valine, lysine, methionine,

tryptophan, aspartic acid and tyrosine. In contrast, there was no relationship between blood glucose concentrations with plasma AAs, and relationships between ghrelin and energy intake with AAs were weak. Accordingly, our observations demonstrate that factors mediating the effects of dietary protein on blood glucose and energy intake are multifactorial and inter-related.

# Chapter 3: Intraduodenal administration of L-valine has no effect on antropyloroduodenal pressures, plasma cholecystokinin concentrations or energy intake in healthy, lean men

Elovaris RA, Fitzgerald PCE, Bitarafan V, Ullrich SS,

Horowitz M, Feinle-Bisset C

**Published in**

***Nutrients 2019; 11(1): 99.***

# Statement of Authorship

| Title of Paper | Intraduodenal administration of L-valine has no effect on antropyloroduodenal pressures, plasma cholecystokinin concentrations or energy intake in healthy, lean men. |
|---|---|
| Publication Status | ☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | Elovaris RA, Fitzgerald PCE, Bitarafan V, Ullrich SS, Horowitz M, Feinle-Bisset C. Nutrients, 2019 Jan 5;11(1): 99. |

## Principal Author

| Name of Principal Author (Candidate) | Rachel Elovaris | | |
|---|---|---|---|
| Contribution to the Paper | Conducted the experiments, analysed the data, conducted the statistical analysis, interpreted the data, prepared the manuscript, and contributed to the revision of the manuscript. | | |
| Overall percentage (%) | 50% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. | | |
| Signature | | Date | 21/6/21 |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

| Name of Co-Author | Penelope Fitzgerald | | |
|---|---|---|---|
| Contribution to the Paper | Conducted the experiments, edited and revised the manuscript, and approved the final version of manuscript. | | |
| Signature | | Date | 21.6.21 |

| Name of Co-Author | Vida Bitarafan | | |
|---|---|---|---|
| Contribution to the Paper | Edited and revised the manuscript, and approved the final version of manuscript. | | |
| Signature | | Date | 21.6.21 |

| Name of Co-Author | Sina Ullrich | | |
|---|---|---|---|
| Contribution to the Paper | Conceived and designed the research, edited and revised the manuscript, and approved the final version of manuscript. | | |
| Signature | | Date | 15.06.2021 |

| Name of Co-Author | Michael Horowitz | | |
|---|---|---|---|
| Contribution to the Paper | Contributed to the design of the research, interpreted results of experiments, edited and revised the manuscript, and approved the final version of manuscript. | | |
| Signature | | Date | 21/06/21 |

| Name of Co-Author | Christine Feinle-Bisset | | |
|---|---|---|---|
| Contribution to the Paper | Conceived and designed the research, interpreted the data, prepared the manuscript, contributed to the revision of the manuscript, approved the final version of manuscript, obtained funding and had overall responsibility for the project. | | |
| Signature | | Date | June 15, 2021 |

## 3.1 Abstract

Whey protein is rich in the branched-chain amino acids, L-leucine, L-isoleucine and L-valine. Thus, branched-chain amino acids may, at least in part, mediate the effects of whey to reduce energy intake and/or blood glucose. Notably, 10 g of either L-leucine or L-isoleucine, administered intragastrically before a mixed-nutrient drink, lowered postprandial blood glucose, and intraduodenal infusion of L-leucine (at a rate of 0.45 kcal/min, total: 9.9 g) lowered fasting blood glucose and reduced energy intake from a subsequent meal. Whether L-valine affects energy intake, and the gastrointestinal functions involved in the regulation of energy intake, as well as blood glucose, in humans, is currently unknown. We investigated the effects of intraduodenally administered L-valine on antropyloroduodenal pressures, plasma cholecystokinin, blood glucose and energy intake. Twelve healthy lean men (age: 29±2 years, BMI: 22.5±0.7 kg/m$^2$) were studied on 3 separate occasions in randomised, double-blind order. Antropyloroduodenal pressures, plasma cholecystokinin, blood glucose, appetite perceptions and gastrointestinal symptoms were measured during 90-min intraduodenal infusions of L-valine at 0.15 kcal/min (total: 3.3g) or 0.45 kcal/min (total: 9.9g), or 0.9 % saline (control). Energy intake from a buffet-meal immediately after the infusions was quantified. L-valine did not affect antral, pyloric (number; control: 14 ± 5; L-Val-0.15: 21 ± 9; L-Val-0.45: 11 ± 4), or duodenal pressures, plasma cholecystokinin (mean concentration, pmol/L; control: 3.1 ± 0.3; L-Val-0.15: 3.2 ± 0.3; L-Val-0.45: 3.0 ± 0.3), blood glucose, appetite perceptions, symptoms or energy intake (kcal; control: 1040 ± 73; L-Val-0.15: 1040 ± 81; L-Val-0.45: 1056 ± 100), at either load. In conclusion, intraduodenal infusion of L-valine, at loads that are moderately (3.3 g) or substantially (9.9 g) above World Health Organization valine requirement recommendations, does not appear to have energy intake- or blood glucose-lowering effects.

## 3.2 Introduction

High-protein diets, when compared with diets high in carbohydrate or fat, appear to have more potent effects to decrease appetite and body weight and improve glycaemic control in obese people with and without type 2 diabetes (70, 242, 316, 317). Whey protein appears to be particularly effective and has been shown to decrease energy intake as well as the blood glucose response to a meal, associated with changes in gastrointestinal (GI) motor and hormone functions (224, 226, 318, 319). For example, administration of whey directly into the duodenum (designed to avoid the potentially confounding effects of orosensory influences and differences in gastric emptying), at rates of 0.5 - 3 kcal/min over 60 min (equivalent to ~ 8 - 48 g protein), stimulated pyloric pressures, a key determinant of the slowing of gastric emptying, and the release of the gut hormones, cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1), in a dose-dependent fashion, and, at the highest rate, also suppressed energy intake (226). In people with type 2 diabetes, managed by diet, ingestion of 55 g whey protein, 30 min before a carbohydrate meal, was shown to stimulate insulin, GLP-1 and glucose-dependent insulinotrophic peptide (GIP) release and slow gastric emptying, associated with a marked reduction in postprandial glucose (121). These effects were more marked than when the same amount of whey was co-ingested with the carbohydrate meal (121).

There is increasing evidence that, in analogy to triglycerides and fatty acids (157, 320), amino acids may mediate the effects of protein, including whey, on appetite and glycaemia. Whey protein is rich in the branched-chain amino acids (BCAAs), L-leucine, L-isoleucine and L-valine. In response to intraduodenal administration of whey protein, we have observed direct correlations between plasma concentrations of these amino acids with plasma concentrations of GLP-1 and insulin, and an inverse correlation with energy intake (259), supporting the concept that these amino acids, at least in part, mediate the effects of whey to reduce energy

intake and/or blood glucose. Indeed, both L-leucine and L-isoleucine have been shown in studies by ourselves and others to modulate gut motor and hormone functions, energy intake and/or blood glucose [15-19]; in contrast, there is very little information about the effects of L-valine.

There is evidence that L-leucine decreases energy intake in both animals and humans (281, 284). In rats, acute injection of L-leucine, directly into the brain, inhibited food intake apparently by stimulating L-leucine-sensitive neurons in the nucleus of the solitary tract, suggesting central effects of L-leucine in the regulation of energy intake (281). In humans, we have reported that intraduodenal infusion of L-leucine, at a rate of 0.45 kcal/min for 90 min, stimulated plasma CCK and reduced energy intake from a subsequent meal by a substantial 156 ± 57 kcal (284). Both L-leucine and L-isoleucine also decrease fasting and postprandial blood glucose. In the study referred to above (284), intraduodenal L-leucine stimulated insulin and reduced blood glucose, in the absence of carbohydrate. Moreover, 10 g L-leucine or L-isoleucine, ingested orally with 25 g of glucose (282, 290), or administered intragastrically 15 min before a carbohydrate-containing mixed-nutrient drink (283), attenuated the postprandial rise in blood glucose significantly.

The outcomes of studies conducted by ourselves (254, 283) and others (196, 321) indicate that the effects (and mechanisms) of amino acids to decrease appetite and glycaemia vary substantially. In fasted healthy humans, intravenous infusion of 30 g L-valine slightly decreased blood glucose and stimulated insulin (196). Whether L-valine, when given at doses that are modestly (3.3 g) or substantially (9.9 g) above World Health Organization valine requirement recommendations for L-valine (~ 26 mg/kg body weight, equivalent to ~ 2 g for a 70 kg person (57)), but comparable to doses that have previously been shown to be effective

for L-leucine and L-isoleucine (282, 283, 290), affects energy intake, or the gut functions associated with the regulation of energy intake or blood glucose, is currently unknown.

The aim of the present study was to evaluate the hypothesis that intraduodenal infusion of L-valine would modulate plasma hormone concentrations and upper GI motility, in a dose-dependent manner, associated with reduced energy intake and fasting glucose in healthy males.

## 3.3 Materials and methods

### 3.3.1 Participants

Twelve healthy males, aged 29 ± 2 years (range: 22-43 years) and of normal body weight (body mass index 22.5 ± 0.7 kg/m$^2$, range: 20-25 kg/m$^2$) were recruited from an existing pool of volunteers and by flyers placed around the Royal Adelaide Hospital, University of Adelaide, University of South Australia and Flinders University campuses, and through advertisements in local newspapers and on online sites (University of Adelaide and Gumtree). Subjects who smoked, consumed > 20 g of alcohol/day, had low ferritin (< 30 ug/L) or iron (< 8 umol/L) concentrations, were lactose-intolerant, vegetarians, or high-performance athletes, had significant GI symptoms, disease or surgery, or used medications known to affect GI functions and/or appetite, were excluded. All subjects were unrestrained eaters (score ≤ 12 on the eating restraint component of the Three-Factor Eating Questionnaire (297)) and had been weight-stable (< 5 % fluctuation) in the 3 months preceding the study. The study protocol was approved by the Human Research Ethics Committee of the Central Adelaide Local Health Network, and the study performed in accordance with the Declaration of Helsinki and the NHMRC Statement on Ethical Conduct in Human Research. Each subject provided written, informed consent prior to their enrolment. The study was registered as a clinical trial with the Australian New Zealand Clinical Trials Registry (www.anctr.org.au, ACTRN12617000715370).

### 3.3.2 Study Design

The study evaluated the dose-related effects of 90-min intraduodenal infusions of L-valine at loads of (i) 0.15 kcal/min (3.3 g over 90 min, "L-Val-0.15") or (ii) 0.45 kcal/min (9.9 g over 90 min, "L-Val-0.45"), or (iii) saline ("control"), on antropyloroduodenal pressures, plasma CCK and blood glucose concentrations, appetite perceptions and energy intake in healthy male subjects.

### 3.3.3 Intraduodenal Infusions

L-valine solutions were prepared by dissolving 4.6 g or 13.8 g crystalline L-valine (PureBulk, Roseburg, Oregon, USA), NaCl (2.2 g and 0.8 g, respectively) and 146 mg $CaCl_2$ x $2H_2O$, in distilled water to a final volume of 500 mL. The control solution contained 4.8 g NaCl and 146 mg $CaCl_2$ x $2H_2O$ dissolved in distilled water to a volume of 500 mL. All solutions were isotonic (300 mosmol) and had a pH of 7. Intraduodenal infusions were delivered at a rate of 4 mL/min, thus, delivering loads of L-valine at 0.15 kcal/min (total: 3.3 g) or 0.45 kcal/min (total: 9.9 g), in a total volume over 90 min of 360 ml. The loads were based on our previous study, which found significant effects of intraduodenal L-leucine on upper GI functions, blood glucose and energy intake (284). Solutions were prepared on the morning of each study day, and were administered in a randomised, double-blind fashion. Both the preparation of the solutions and the randomisation (using the online tool available at www.randomization.com) were performed by a research officer, who was not involved in the performance of the studies or data analysis.

### 3.3.4 Study Protocol

Each subject was studied on three occasions, separated by 3-7 days. Subjects were instructed to abstain from vigorous exercise and alcohol intake for 24 h before each study visit. They were

provided with a standardised evening meal (Beef Lasagne; McCain Food; energy content: 602 kcal) to be consumed by 7:00 p.m. on the night before each study, after which time they fasted overnight from solids and liquids. Subjects then attended the Clinical Research Facility at the University of Adelaide at 8:00 a.m. the following morning. They were intubated with a small-diameter (external diameter: 3.5 mm), 17-channel manometric catheter (length: 100 cm; Dentsleeve International, Mui Scientific, Mississauga, On, Canada), which was inserted into the stomach through an anaesthetised nostril and allowed to pass through the pylorus into the duodenum by peristalsis (319, 322). The manometric catheter contained 16 side-holes spaced at 1.5 cm intervals and was positioned with six side-holes in the antrum (*channels 1-6*), a 4.5-cm sleeve sensor (*channel 7*), with two side-holes (*channels 8, 9*) on the back of the sleeve, across the pylorus, and seven side-holes in the duodenum (*channels 10-16*). The correct positioning of the catheter, with the sleeve sensor straddling the pylorus, was maintained by continuous measurement of the transmucosal potential difference between the most distal antral, and the most proximal duodenal, channels (319, 323). An additional channel (with the side-hole positioned ~ 14.5 cm distal to the pylorus when the catheter was in position) was used for intraduodenal infusion of L-valine or control solutions.

Once the catheter was positioned correctly, fasting motility was observed until the occurrence of a phase III of the migrating motor complex. Immediately after phase III passed the antropyloroduodenal region, and during a subsequent period of motor quiescence (phase I), an intravenous cannula was placed in a forearm vein for blood sampling. At $t$ = -10 min (approximately 1030 h) and $t$ = 0 min, fasting blood samples were taken, and the subject completed visual analogue scale (VAS) questionnaires to assess appetite perceptions and GI symptoms. The intraduodenal infusion of L-valine or control was then commenced and continued for 90 min ($t$ = 0 - 90 min). Antropyloroduodenal motility was recorded continuously

during the pre-infusion period and throughout the infusion, and blood samples for measurements of plasma CCK and blood glucose concentrations were collected, and VAS questionnaires completed, at 15-min intervals. At *t* = 90 min, the infusion was terminated and the catheter removed. The subject was then presented with a standardised, cold, buffet-style test meal (295). Subjects were instructed to consume as much food as they wished until they felt comfortably full over up to 30 min (*t* = 90 - 120 min). The meal consisted of 4 slices (~ 120 g) each of whole-meal and white breads, 100 g sliced ham, 100 g sliced chicken, 85 g sliced cheddar cheese, 100 g sliced tomato, 100 g sliced cucumber, 100 g iceberg lettuce, 20 g margarine, 22 g mayonnaise, 120 g fruit salad, 175 g strawberry yoghurt, 100 g chocolate custard, 1 apple (~ 170 g), 1 banana (~ 190 g), 600 mL water, 350 mL orange juice and 375 mL iced coffee total energy content: ~ 2300 kcal; total weight: ~ 2924 g; macronutrient distribution: (~ 27 % fat, ~ 52 % carbohydrate and ~ 21 % protein). Immediately after the meal (i.e. at *t* = 120 min), the intravenous catheter was removed, and the subject was then allowed to leave the laboratory.

### 3.3.5 Measurements

#### *3.3.5.1 Energy Intake*

Each food item in the buffet meal was weighed before and after consumption to quantify the amount of food eaten (g). Energy intake (kcal) was then calculated using commercially available software (Foodworks 8.0, Xyris Software, Highgate Hill, QLD, Australia).

#### *3.3.5.2 Appetite Perceptions and GI Symptoms*

Perceptions of appetite (hunger, fullness, desire to eat and prospective food consumption) were assessed using validated 100-mm VAS questionnaires (324). GI symptoms (nausea and bloating) were also assessed. VAS scales consisted of 100-mm horizontal lines, where 0 mm

represented “not felt at all”, and 100 mm “felt the strongest possible”. Subjects were asked to place a vertical mark on each horizontal line to rate the strength of each sensation felt at that point in time.

### *3.3.5.3 Antropyloroduodenal Pressures*

Antropyloroduodenal pressures were digitised and recorded via a computer-based system running commercially available software (MMS DATAbase software, version 8.17; Solar GI). Antropyloroduodenal pressures were analysed for: (i) numbers and amplitudes of antral and duodenal pressure waves (PWs); (ii) numbers and amplitudes of isolated pyloric pressure waves (IPPWs); and (iii) basal pyloric pressure. Pressure waves were defined by an amplitude ≥10 mmHg, and a minimum interval of 15 seconds between peaks for antral and pyloric waves and 3 seconds for duodenal waves. Basal pyloric pressure was calculated by subtracting the mean basal pressure (with the exclusion of phasic pressures) recorded at the most distal antral side hole from the mean basal pressure recorded at the sleeve (323). This analysis was performed using custom-written software (by Prof. A. Smout, University Medical Centre Amsterdam, The Netherlands).

### *3.3.5.4 Plasma CCK and Blood Glucose Concentrations*

Blood samples were collected into ice-chilled ethylenediaminetetraacetic acid-coated tubes and centrifuged (3200 rpm at 4°C for 15 min) within 15 min of collection to obtain plasma. Plasma samples were stored at -80°C until analysed. Plasma cholecystokinin-8 (CCK-8) concentrations (pmol/L) were analysed by radioimmunoassay after ethanol extraction using an adaption of the method by Santangelo et al (241). The antibody used recognises sulfated CCK-8 and does not bind to structurally unrelated peptides. Cross-reactivity with unsulfated CCK-8

was ~ 15 % and with human gastrin I 0.2 %. The detection limit was 1 pmol/L, and intra-assay and inter-assay coefficients of variation were 9.2 % and 13.7 %, respectively.

Blood glucose concentrations (mmol/L) were determined immediately after collection by the glucose oxidase method using a portable glucometer (FreeStyle OptimumH; Abbott Laboratories, Chicago, IL, USA).

### 3.3.6 Data and Statistical Analyses

The number of subjects was based on power calculations derived from previous work (284). We calculated that $n = 12$ subjects would be required to detect a 15 % decrease in energy intake at $\alpha = 0.05$, with a power of 80 %.

For all data baseline values were calculated as means of values acquired between $t = -10$ and $t = 0$ min. During the 90-min infusion period, VAS scores, plasma CCK and blood glucose data were expressed as means at each time point, while the number of antral, isolated pyloric and duodenal PWs were expressed as total numbers, and basal pyloric pressures and the amplitude of antral, isolated pyloric and duodenal PWs, were expressed as mean values over the 90-min period. The numbers and amplitudes of antral and duodenal PWs were used to calculate antral and duodenal motility indices (MIs), as previously described (325).

Statistical analysis was performed with the use of SPSS software (version 24; IBM). VAS scores, plasma CCK and blood glucose data were analysed using repeated-measures two-factor analysis of variance (ANOVA), with time (0 – 90 min), and treatment (L-valine-0.15, L-valine-0.45, control) as factors. MIs for antral and duodenal PWs, basal pyloric pressure, number and amplitude of IPPWs and energy intake were analysed using one-factor ANOVA. Sphericity of

the time effect for all models was evaluated by Mauchly's test, and, if violated, the adjusted Greenhouse-Geisser $p$ value was reported. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni correction, were performed where ANOVAs revealed significant effects. All data are reported as means ± SEMs. All tests were two-tailed and statistical significance was accepted at $p < 0.05$.

## 3.4 Results

All subjects completed the 3 study visits and tolerated the study treatments well.

### 3.4.1 Energy Intake

There was no effect of treatment on energy intake (**Figure 3.1**), or the amount eaten (gram; control: 1198 ± 94; L-Val-0.15: 1156 ± 83; L-Val-0.45: 1156 ± 97), from the buffet-meal ($p > 0.05$ for both).

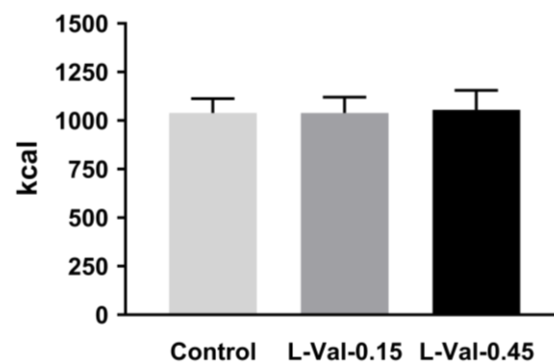


**Figure 2.1:** Energy intake from a buffet meal after 90-min intraduodenal infusions of control, or L-valine at 0.15 kcal/min ("L-Val-0.15") or 0.45 kcal/min ("L-Val-0.45"). One-way ANOVA was used to analyse the data. Statistical significance was accepted at $p < 0.05$. Data are means ± SEMs, $n = 12$.

### 3.4.2 Appetite Perceptions and GI Symptoms

There were no differences in baseline ratings of hunger, fullness, desire to eat, prospective consumption, bloating or nausea between study days. There were also no effects of treatment or time on ratings of these sensations and symptoms ($p > 0.05$ for all; **Figure 3.2A-F**).

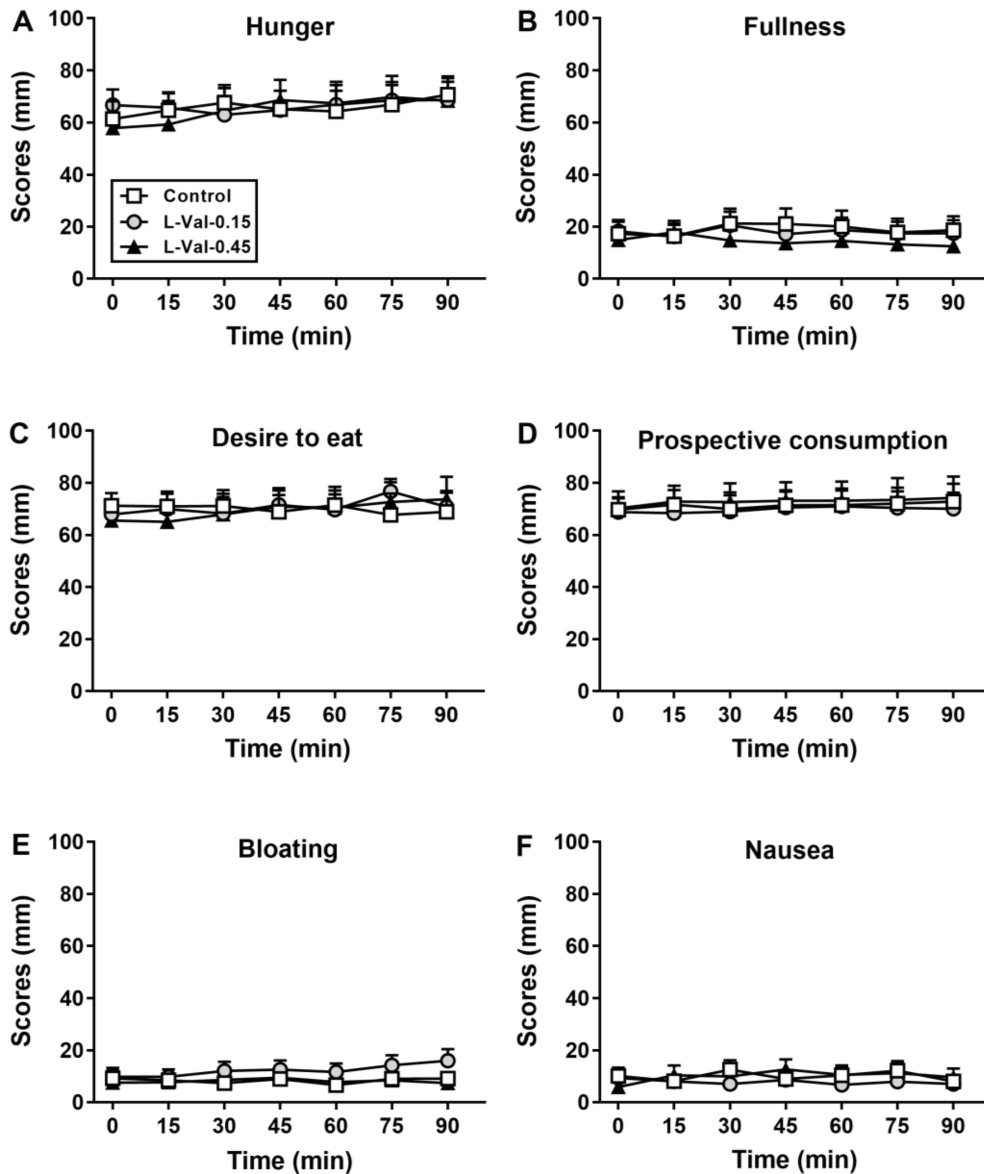


**Figure 3.2:** Scores for hunger (A), fullness (B), desire to eat (C), prospective consumption (D), bloating (E) and nausea (F) during 90-min intraduodenal infusions of L-valine at 0.15 kcal/min ("L-Val-0.15") or 0.45 kcal/min ("L-Val-0.45"), or control. Repeated-measures two-factor ANOVA, with treatment and time as factors, was used to analyse the data. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni correction, were used to determine significant differences between treatments if ANOVAs were significant. Statistical significance was accepted at $P < 0.05$. Data are means ± SEMs, $n = 12$.

### 3.4.3 Antropyloroduodenal Pressures

Baseline values for antral, pyloric and duodenal pressures did not differ between study days and were 0 ($p > 0.05$ for all).

There was no effect of treatment on the total number, mean amplitude or MI of antral PWs; mean basal pyloric pressures**;** total number or mean amplitude of IPPWs, or the total number, mean amplitude or MI of duodenal PWs ($p > 0.05$ for all; **Table 3.1**).

**Table 3.1:** Number, amplitude and motility indices of antral and duodenal pressure waves, basal pyloric pressure, and number and amplitude of isolated pyloric pressures waves during 90-min intraduodenal infusions of L-valine at 0.15 kcal/min ("L-Val-0.15"), L-valine at 0.45 kcal/min ("L-Val-0.45"), or control.

| | **Control** | **L-Val-0.15** | **L-Val-0.45** | ***P* value** |
|---|---|---|---|---|
| **Antral pressure waves** | | | | |
| *Number* | 51 ± 18 | 46 ± 11 | 35 ± 11 | > 0.05 |
| *Amplitude, mmHg* | 37 ± 8 | 46 ± 8 | 34 ± 7 | > 0.05 |
| *Motility index, mmHg*min* | 9 ± 1 | 10 ± 1 | 9 ± 1 | > 0.05 |
| **Basal pyloric pressure, mmHg** | -0.3 ± 0.9 | 0.2 ± 1.0 | 0.4 ± 0.4 | > 0.05 |
| **Isolated pyloric pressure waves** | | | | |
| *Number* | 14 ± 5 | 21 ± 9 | 11 ± 4 | > 0.05 |
| *Amplitude, mmHg* | 9 ± 3 | 16 ± 4 | 14 ± 4 | > 0.05 |
| **Duodenal pressure waves** | | | | |
| *Number* | 445 ± 66 | 495 ±77 | 361 ± 65 | > 0.05 |
| *Amplitude, mmHg* | 29 ± 2 | 29 ± 3 | 27 ± 2 | > 0.05 |
| *Motilityindex, mmH g*min* | 15 ± 0.4 | 15 ± 0.4 | 14 ± 0.5 | > 0.05 |

One-factor ANOVA was used to test for differences in number, amplitude and motility indices of antral and duodenal pressure waves, basal pyloric pressure, and number and amplitude of isolated pyloric pressures waves. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were used to determine significant differences between treatments if ANOVAs were significant. Data are means ± SEMs, $n = 12$.

### 3.4.4 Plasma CCK and Blood Glucose Concentrations

There were no differences in baseline values between study days for plasma CCK or blood glucose concentrations ($p > 0.05$ for all).

There was no effect of treatment ($p > 0.05$), but an effect of time ($p < 0.05$), on plasma CCK concentrations (**Figure 3.3A**). All 3 infusions slightly increased plasma CCK at $t = 15$ min ($p < 0.05$), after which time no further changes occurred.

There was no effect of treatment, or time, on blood glucose concentrations ($p > 0.05$; **Figure 3.3B**).

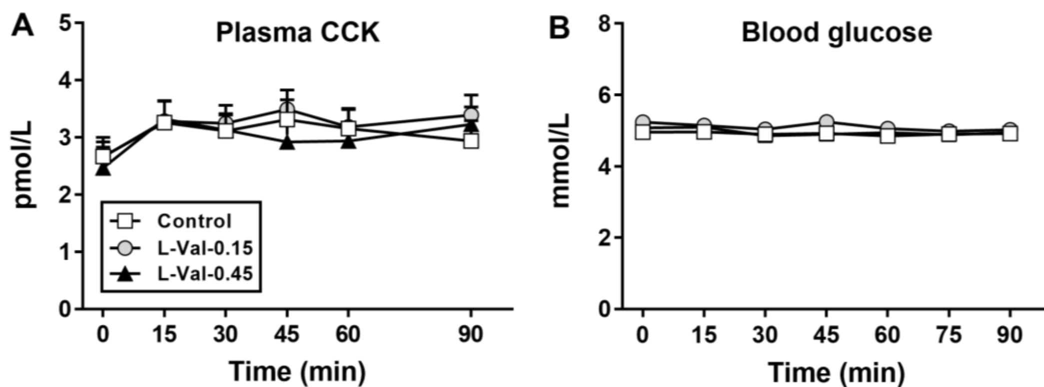


**Figure 3.3:** Plasma CCK (A) and blood glucose (B) concentrations during 90-min intraduodenal infusions of L-valine at 0.15 kcal/min ("L-Val-0.15") or 0.45 kcal/min ("L-Val-0.45"), or control. Repeated-measures two-factor ANOVA, with treatment and time as factors, was used to analyse the data. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni correction, were used to determine significant differences between treatments if ANOVAs were significant. Statistical significance was accepted at $p < 0.05$. Data are means ± SEMs, $n = 11$ for CCK (due to technical difficulties with blood sampling), $n = 12$ for glucose.

## 3.5 Discussion

We have investigated the effects of the BCAA, L-valine, which is abundant in whey protein, to elucidate whether L-valine, like L-leucine and L-isoleucine, has potent effects to reduce

energy intake and/or blood glucose and to modulate key underlying GI functions. Our study indicates that L-valine, when administered intraduodenally at loads of 0.15 kcal/min or 0.45 kcal/min for 90 min, has no effect on antropyloroduodenal motility, plasma CCK, energy intake or blood glucose, suggesting that L-valine, unlike L-leucine at these loads, does not have GI, energy intake-suppressant or glucoregulatory effects.

There has been substantial interest in BCAAs, particularly L-leucine and L-isoleucine, in relation to their potential role in mediating the effects of protein, particularly whey, on energy intake, blood glucose and other metabolic outcomes (317, 321). We have reported recently that intraduodenal infusion of L-leucine at the load of 0.45 kcal/min, but not 0.15 kcal/min (loads identical to those used in the current study), significantly reduced subsequent energy intake, associated with stimulation of plasma CCK concentrations, in healthy males (284). We have also reported that, in response to intraduodenal whey protein, subsequent energy intake is correlated inversely with circulating concentrations of L-leucine, L-isoleucine and L-valine (259). Based on these findings, we hypothesised that intraduodenal infusion of L-valine would also reduce energy intake. In contrast to our expectation, L-valine was ineffective – it did not affect subsequent energy intake, nor GI functions of relevance to the regulation of energy intake, specifically the stimulation of plasma CCK and pyloric pressures (326, 327). While it is possible that L-valine may be an amino acid that does not play a role in appetite regulation, it is worth noting the important contributory roles of orosensory and gastric influences (which we bypassed in the current study) with gut hormone release and the modulation of energy intake (328, 329). In support, while oral administration of L-phenylalanine has been shown to reduce subsequent energy intake in healthy humans (251), we reported recently that intraduodenal L-phenylalanine was ineffective (300). Thus, the effects of orally administered L-valine, and, if effective, potential underlying mechanisms, warrant further investigation.

Studies in healthy individuals have established the capacity of both L-leucine and L-isoleucine to reduce elevated blood glucose levels. For example, intragastric administration of 10 g L-leucine or 10 g L-isoleucine, 15 min before consumption of a carbohydrate-containing mixed-nutrient drink, reduced the blood glucose response to the drink by ~ 1.1 mmol/L (283). Furthermore, intraduodenal infusion of leucine at 0.45 kcal/min (9.9 g over 90 min), slightly stimulated insulin and reduced fasting blood glucose modestly (284). In contrast to L-leucine and L-isoleucine, the few studies on the effects of valine on blood glucose have yielded inconsistent findings (321). In fasted rats, L-valine (0.3 g/kg body weight, equivalent to ~ 22.5 g in a 75 kg human), given orally 30 min before an oral glucose tolerance test, increased plasma glucose levels, 60 min later by ~ 20 mg/dl (273), and, at a dose of 1 g/kg body weight, ameliorated the fall in blood glucose induced by exercise (293), while, in contrast, intrahypothalamic infusion of L-valine (12 *n*mol), given with a continuous intravenous glucose infusion, was reported to lower blood glucose, compared with control rats. In fasted healthy humans, intravenous administration of 30 g L-valine resulted in a small stimulation of insulin secretion and decrease in blood glucose concentrations (196). The L-valine loads used in these studies were extremely high. Moreover, the discrepant effects may be due to species differences (rats vs. humans), as well as the different routes of administration (oral vs. intravenous vs. intrahypothalamic). Our data, using doses spanning physiological and supraphysiological ranges, found no effects on blood glucose concentrations. It should be appreciated that our study design did not include a carbohydrate-containing meal or oral administration of L-valine, and, thus, did not evaluate effects on postprandial blood glucose. This also warrants evaluation.

Some limitations of our study should be recognised. We did not measure all of the key gut and pancreatic hormones involved in energy intake or blood glucose regulation, including ghrelin, peptide tyrosine tyrosine, GLP-1, GIP, insulin and glucagon, because of the lack of effect of

L-valine on energy intake, blood glucose and plasma CCK concentrations. Intraduodenal infusion of L-valine was used to exclude influences of orosensory factors and to standardise nutrient delivery to the small intestine, bypassing any inter-individual variations in gastric emptying. Thus, we cannot exclude the possibility that L-valine affects energy intake and/or blood glucose when ingested orally or administered intragastrically, thus, activating multiple afferent pathways concurrently (330, 331). We administered L-valine in isolation, thus, the apparent discrepancy in our findings, i.e. a lack of effect of intraduodenally administered L-valine on energy intake, versus the inverse correlation between plasma L-valine concentrations and energy intake after intraduodenal whey protein infusion in our previous study (259), may indicate that L-valine requires the presence of other amino acids to exert an effect on energy intake and/or blood glucose; this hypothesis may warrant investigation. Finally, we only studied healthy males, because they have been reported to be most sensitive to dietary manipulations (312), hence, we cannot generalise our findings to outcomes in women, overweight/obese or age, however, we believe a different outcome would be unlikely.

In conclusion, intraduodenal infusions of L-valine, at loads that are moderately (3.3 g) or substantially (9.9 g) above recommended daily intakes, do not affect antropyloroduodenal motility, plasma CCK or fasting blood glucose concentrations, or energy intake. These observations indicate that, unlike L-leucine or L-isoleucine, L-valine, does not play a critical role in the regulation of energy intake and/or blood glucose control, providing evidence that different amino acids, even though of similar chemical structure, can have distinct physiological effects.

# Chapter 4: Comparative effects of the branched-chain amino acids, leucine, isoleucine and valine, on gastric emptying, plasma glucose, C-peptide and glucagon in healthy men

**Elovaris RA, Bitarafan V, Agah S, Ullrich SS, Lange K, Horowitz M, Feinle-Bisset C**

**Published in**

***Nutrients 2021;13(5):1613.***

# Statement of Authorship

| | |
|---|---|
| Title of Paper | Comparative effects of the branched-chain amino acids, leucine, isoleucine and valine, on gastric emptying, plasma glucose, C-peptide and glucagon in healthy men. |
| Publication Status | ☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | Elovaris RA, Bitarafan V, Agah S, Ullrich SS, Lange K, Horowitz M, Feinle-Bisset C. Nutrients, 2021 May 11;13(5):1613. |

## Principal Author

| | | | |
|---|---|---|---|
| Name of Principal Author (Candidate) | Rachel Elovaris | | |
| Contribution to the Paper | Conducted the experiments, analysed the data and conducted the statistical analysis, interpreted the data, prepared the manuscript, and contributed to the revision of the manuscript. | | |
| Overall percentage (%) | 50% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. | | |
| Signature | | Date | 21/6/21. |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

| | | | |
|---|---|---|---|
| Name of Co-Author | Vida Bitarafan | | |
| Contribution to the Paper | Analysed the data and conducted the statistical analysis, interpreted the data, edited and revised the manuscript, and approved the final version of manuscript. | | |
| Signature | | Date | 21.6.21 |

| | | | |
|---|---|---|---|
| Name of Co-Author | Shahram Agah | | |
| Contribution to the Paper | Contributed to the experiments, edited and revised the manuscript, and approved the final version of manuscript. | | |
| Signature | | Date | 20/06/21 |

| Name of Co-Author | Sina Ullrich | | |
|---|---|---|---|
| Contribution to the Paper | Conceived and designed the research, edited and revised the manuscript, and approved the final version of manuscript. | | |
| Signature | | Date | 15.06.2021 |

| Name of Co-Author | Kylie Lange | | |
|---|---|---|---|
| Contribution to the Paper | Contributed to the statistical analysis, interpreted the data, edited and revised the manuscript, and approved the final version of the manuscript. | | |
| Signature | | Date | 21-06-21 |

| Name of Co-Author | Michael Horowitz | | |
|---|---|---|---|
| Contribution to the Paper | Contributed to the design of the research, interpreted results of experiments, edited and revised the manuscript, and approved the final version of manuscript. | | |
| Signature | | Date | 21/06/21 |

| Name of Co-Author | Christine Feinle-Bisset | | |
|---|---|---|---|
| Contribution to the Paper | Conceived and designed the research, interpreted the data, prepared the manuscript, contributed to the revision of the manuscript, approved the final version of manuscript, obtained funding and had overall responsibility for the project. | | |
| Signature | | Date | June 15, 2021 |

## 4.1 Abstract

**Background:** Whey protein lowers postprandial blood glucose in health and type 2 diabetes, by stimulating insulin and incretin hormone secretion and slowing gastric emptying. The branched-chain amino acids, leucine, isoleucine and valine, abundant in whey, may mediate the glucoregulatory effects of whey. We investigated the comparative effects of intragastric administration of leucine, isoleucine and valine on the plasma glucose, C-peptide and glucagon responses to, and gastric emptying of, a mixed-nutrient drink, in healthy men.

**Methods**: 15 healthy men (27 ± 3 yr) received, on 4 separate occasions, in double-blind, randomised fashion, either 10 g leucine, 10 g isoleucine, 10 g valine or control, intragastrically, 30 min before a mixed-nutrient drink. Plasma glucose, C-peptide and glucagon concentrations were measured before, and for 2 hours following, the drink. Gastric emptying of the drink was quantified using $^{13}$C-acetate breath-testing.

**Results:** Amino acids alone did not affect plasma glucose or C-peptide, while isoleucine and valine, but not leucine, stimulated glucagon ($p < 0.05$), compared with control. After the drink, isoleucine and leucine reduced peak plasma glucose compared with both control and valine (all $p < 0.05$). Neither amino acid affected early (t=0–30 min) postprandial C-peptide or glucagon. While there was no effect on overall gastric emptying, plasma glucose at t=30 min correlated with early gastric emptying ($p < 0.05$).

**Conclusion**: In healthy individuals, leucine and isoleucine lower postprandial blood glucose, at least in part by slowing gastric emptying, while valine does not appear to have an effect, possibly due to glucagon stimulation.

## 4.2 Introduction

Ingestion of protein, particularly whey, lowers postprandial plasma glucose in healthy individuals and in people with type 2 diabetes (T2D) (120, 121). This beneficial effect of whey

protein is associated with slowing of gastric emptying and the stimulation of insulin and the incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) (121, 122, 214, 219, 223, 229). Gastric emptying is a key determinant of postprandial blood glucose concentrations, particularly the initial rise in blood glucose, and slowing of gastric emptying reduces postprandial glucose excursions in both health and T2D (332-334). These GI functions can be stimulated in a targeted fashion by providing nutrients in isolated form, i.e. not as part of a meal, as a so-called 'preload', approximately 30 min before a meal. For example, in diet-controlled T2D, acute ingestion of whey protein (55 g), 30 min prior to a carbohydrate meal, leads to a marked reduction in postprandial glucose, particularly within the first 30 min, associated with stimulation of insulin and slowing of gastric emptying (121).

The branched-chain amino acids, leucine, isoleucine and valine, which are abundant in whey protein, are potential mediators of the effects of protein on glucose homeostasis. Preclinical studies suggest that leucine and isoleucine regulate glycaemia by different mechanisms - leucine by stimulating insulin release via enhancing glutaminolysis, regulating gene transcription and protein synthesis (335), and isoleucine by increasing glucose uptake in skeletal muscle (280) and decreasing hepatic gluconeogenesis (273, 336). In humans, both leucine and isoleucine (in amounts of ~ 8-10 g), co-ingested with 25 g glucose, attenuated the glycaemic response to the glucose load, although only leucine, but not isoleucine, stimulated insulin (282, 290). Moreover, intragastric administration of leucine and isoleucine (each at 10 g), 15 min before a mixed-nutrient drink (containing 56 g carbohydrates), reduced blood glucose modestly (~ 1.1 mmol/L) (283). Leucine stimulated insulin, while isoleucine slowed gastric emptying (283), indicating that the mechanisms underlying the glucose-lowering effects of the two amino acids differ. However, because the drink used in the latter study consisted

primarily of fructose-based carbohydrates (283), it is not known whether the glucoregulatory responses to a drink containing glucose-based carbohydrates would differ.

There is limited, and inconsistent, evidence regarding the glycaemic effects of valine in both animals and humans (293, 309, 337). In rats, acute oral administration of valine (in a dose of 1 g/kg body weight), 30 min prior to exercise, prevented the reduction in liver glycogen and blood glucose (293), consistent with an effect of valine to maintain blood glucose. In contrast, in rats, intrahypothalamic infusion of valine lowered circulating blood glucose comparably to leucine and isoleucine and, during a euglycaemic pancreatic clamp, reduced plasma glucose, apparently by decreasing liver glucose production (337). Finally, in humans, 90-min intraduodenal infusion of valine, at loads of 0.15 and 0.45 kcal/min (administering a total of ~ 3.3 g and 9.9 g, respectively), had no effect on fasting blood glucose (309), in contrast to intraduodenal leucine (284). Accordingly, the effects of valine on postprandial blood glucose remain uncertain.

The aim of the current study was to determine the comparative effects of leucine, isoleucine and valine on the blood glucose, C-peptide (as a measure of insulin secretion) and glucagon responses to, and gastric emptying of, a mixed-nutrient drink, containing maltodextrin and sucrose, in healthy men. Amino acids were administered intragastrically to avoid potential confounding effects due to their unpleasant taste.

## 4.3 Participants and methods

### 4.3.1 Study Participants

Fifteen healthy males, aged 27 ± 3 years (range: 20 – 51 years) and of normal body weight (body mass index 23 ± 0.5 kg/m$^2$ (range: 20 – 25 kg/m$^2$), participated in the study. The number

of participants was determined by power calculations derived from our previous study (283). We calculated that $n$ = 15 participants would allow detection of a 1.2 mmol/L reduction in plasma glucose, assuming a within-subjects standard deviation of 1.1 mmol/L, at $\alpha = 0.05$, with a power of 80 %. Participants were recruited through advertisements on online sites (University of Adelaide and Gumtree) and at the University of Adelaide, University of South Australia, Flinders University and Royal Adelaide Hospital, and from an existing pool of volunteers. All participants were unrestrained eaters (score ≤ 12 on the eating restraint component of the Three-Factor Eating Questionnaire (297)) and had been weight-stable (< 5 % fluctuation) in the 3 months preceding the study. Individuals who smoked, consumed > 20 g alcohol/day, had low ferritin (< 30 ug/L) or iron (< 8 umol/L) concentrations (a requirement of our Ethics Committee), were lactose-intolerant, vegetarians or high-performance athletes, had significant gastrointestinal (GI) symptoms, disease or surgery, or used medications known to affect GI functions and/or appetite, were excluded. Once included, participants were allocated a treatment order generated using an online tool (www.randomization.com) with balanced permutations by a researcher who was not involved in the data analysis. The study protocol was approved by the Human Research Ethics Committee of the Central Adelaide Local Health Network, and the study performed in accordance with the Declaration of Helsinki and the NHMRC Statement on Ethical Conduct in Human Research. All participants provided written, informed consent prior to their enrolment. The study was registered as a clinical trial with the Australian New Zealand Clinical Trials Registry (www.anzctr.org.au, number: ACTRN12619000718145).

### 4.3.2 Study Design

The study evaluated the effects of intragastric administration of 10 g leucine, 10 g isoleucine or 10 g valine, or control, on the plasma glucose, C-peptide and glucagon responses to, and the

gastric emptying of, a mixed-nutrient, carbohydrate-containing drink consumed 30 min later (**Figure 4.1**). The dose of the amino acids, and the 30 min between treatments and the drink, were based on our previous studies, in which nutrients stimulated glucoregulatory hormones within this time (283, 284).

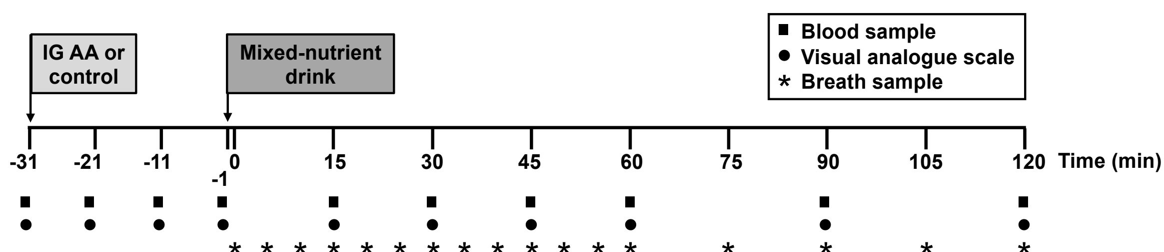


**Figure 4.1:** Schematic representation of the study design. At t = −31 min, a baseline blood sample (for measurement of plasma glucose and glucoregulatory hormones) was collected and a visual analogue scale questionnaire (to assess GI symptoms) completed, then study treatments (either an amino acid (AA; leucine, isoleucine or valine) or control) were administered intragastrically within 1 min. After 30 min, at t = −1 min, each participant consumed, within 1 min, 350 mL of a mixed-nutrient drink, containing 100 mg of $^{13}C$-acetate for measurement of gastric emptying by $^{13}CO_2$ breath test. Blood samples and visual analogue ratings were collected at the indicated time points throughout the study, and breath samples after the mixed-nutrient drink.

### 4.3.3 Study Treatments

Due to the low water solubility (particularly of leucine), amino acids were administered as suspensions at room temperature. 10 g crystalline leucine (Bulk Nutrients, Tasmania, Australia), isoleucine or valine (both from PureBulk Inc., Roseburg, Oregon, USA) and 29.2 mg $CaCl_2 \times 2H_2O$ were incorporated in 10 mL of a suspending agent (ORA-Plus®, Perrigo®, Minneapolis, USA), and isotonic saline was used to adjust to a final volume of 100 mL. The control treatment consisted of 10 mL suspending agent, 29.2 mg $CaCl_2 \times 2H_2O$ and 90 mL isotonic saline. Suspensions were prepared on the morning of each study visit by a research officer who was not involved in the performance of the study or analysis of the data. Syringes were covered so that both the study participant and primary investigator (RAE) were blinded to the treatments.

### 4.3.4 Study Protocol

Each participant was studied on 4 occasions, separated by at least 3, and up to 7, days. Participants were instructed to abstain from vigorous exercise and alcohol intake for 24 h before each study visit and provided with a standardised evening meal (Beef Lasagne; energy content: 602 kcal; McCain Food, Wendouree, Victoria, Australia) to be consumed by 7 p.m. on the night before each study. Participants were instructed not to consume any other solid foods or liquids (with the exception of water, which was allowed until 6.30 a.m.) until they arrived at the Clinical Research Facility at the University of Adelaide at 8.30 a.m. the following morning. Upon arrival they were seated in an upright position, and an intravenous cannula was placed into a forearm vein for regular blood sampling. At baseline (t = -31 min), a blood sample to assess plasma glucose and hormone concentrations, and a breath sample to measure gastric emptying, were collected, and the participant completed a visual analogue scale (VAS) questionnaire to assess GI symptoms. Participants were then intubated with a nasogastric, soft-silicone feeding tube (outer diameter: 4 mm; Dentsleeve International, Mississauga, Ontario, Canada), which was inserted through an anaesthetised nostril into the stomach. The correct positioning of the tube was checked by auscultating the stomach with the use of a stethoscope while pushing a 5-mL air bolus through the tube. Immediately thereafter, participants received, into the stomach, one of the study treatments, within 1 min. The tube was then removed, and blood samples and VAS questionnaires were collected every 10 min for the next 30 min (t = -31 to -1 min). At t = -1 min, each participant consumed, within 1 min, 350 mL of a mixed-nutrient drink (Resource Plus®; Nestle, Tongala, Victoria, Australia (325 ml); 500 kcal; 74 g carbohydrates, including maltodextrin and sucrose, 18 g protein, 15 g fat; plus 25 mL water to make up the final volume) containing 100 mg $^{13}$C-acetate for measurement of gastric emptying by breath-test (338). After consumption of the drink, VAS questionnaires and blood samples were collected at 15-min intervals for the next hour (t = 15 – 60 min). For the second hour (t =

60 – 120 min), VAS questionnaires and blood samples were collected every 30 min. Breath samples were collected every 5 min for the first hour and every 15 min subsequently. At t = 120 min, the cannula was removed, and participants were presented with a light lunch, after which they were free to leave the laboratory.

### 4.3.5 Measurements

#### *4.3.5.1 Plasma Glucose and Hormone Analyses*

Blood samples were collected into ice-chilled ethylenediaminetetraacetic acid-coated tubes. Plasma was obtained by centrifugation at 3200 × *g* (1832 × *g*-force) for 15 min at 4 °C within 15 min of collection and then stored at −80 °C for subsequent analysis.

Plasma glucose (mmol/L) was measured using the glucose oxidase technique (2300 STAT Plus; YSI, Yellow Springs, OH, USA).

Plasma C-peptide concentrations (pmol/L) were measured by ELISA (10-1113; Mercodia, Uppsala, Sweden). The minimal detectable limit was 15 pmol/L, and intra-assay and inter-assay CVs were 2.4 % and 4.9 %, respectively.

Plasma glucagon (pg/ml) was measured by radioimmunoassay (GL-32K; Millipore, Billerica, MA, USA). The minimum detectable limit was 15 pg/mL, and intra-assay and inter-assay CVs were 6.9 % and 4.2 %, respectively.

#### *4.3.5.2 Gastric Emptying*

Gastric emptying was measured by breath-test using $^{13}C$-acetate (338). $^{13}CO_2$ concentrations in exhaled air of end-expiratory breath samples were measured using an isotope ratio mass

spectrometer (FANci2 breath test analyser, Fischer Analysen Instrumente, Leipzig, Germany) with an online gas chromatographic purification system. Breath sample data were expressed as the percentage of recovery of $^{13}CO_2$ in the breath per hour.

#### *4.3.5.3 GI Symptoms*

Nausea and bloating were assessed using validated 100-mm VAS questionnaires (324). VAS scales consisted of 100-mm horizontal lines, where 0 mm represented “not felt at all”, and 100 mm “felt the strongest possible”. Participants were asked to place a vertical mark on each horizontal line to rate the strength of each sensation felt at that point in time.

### 4.3.6 Data and Statistical Analysis

Statistical analysis was performed with SPSS software (version 26.0; IBM, Chicago, IL, USA). Plasma glucose, C-peptide and glucagon concentrations and gastric emptying were expressed as absolute values, and VAS data as changes from baseline (i.e., t = −31 min), to account for variations in baseline values.

Effects of amino acids alone (i.e., prior to ingestion of the mixed-nutrient drink) on plasma glucose, C-peptide and glucagon concentrations and VAS symptom ratings were evaluated by repeated-measures two-way analysis of variance (ANOVAs) with treatment (leucine, isoleucine, valine and control) and time (t = −31–−1 min) as factors. Plasma glucose and hormone data were also analysed as total area under the curve (AUC) (expressed as $AUC_{-31\text{–}-1\ min}$), using repeated-measure one-way ANOVA with treatment as a factor. To evaluate responses to the mixed-nutrient drink, plasma glucose, C-peptide and glucagon concentrations and symptom ratings were analysed using repeated-measures two-way ANOVAs with treatment and time (t = 15–120 min), as factors. The data were also summarised as total AUC (expressed

as $AUC_{15–120\ min}$) and analysed using repeated-measures one-way ANOVA with treatment as a factor. Peak plasma glucose was analysed using repeated-measures one-way ANOVA with treatment as a factor. Gastric emptying was analysed as $AUC_{0–120\ min}$. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed where ANOVAs revealed significant effects. Sphericity of the repeated effects was evaluated by Mauchly's test and, when violated, the adjusted Greenhouse–Geisser *p*-value was reported. Following examination of the data, post-hoc analyses were also conducted over the first 30 min post-meal for C-peptide, glucagon (both expressed as $AUC_{−1–30\ min}$) and gastric emptying (expressed as $AUC_{0–30\ min}$), representing 'early changes'. Correlations between plasma glucose at t = 30 min with early changes in plasma C-peptide and glucagon and gastric emptying were evaluated, using data across all study days, with the use of linear within-subject correlations (339). All data are reported as means ± SEMs. All tests were two-tailed, and differences were considered statistically significant at $P \leq 0.05$.

## 4.4 Results

All participants tolerated the study treatments well and completed all study visits without reporting any adverse effects.

### 4.4.1 Plasma Glucose Concentrations

There were no differences in baseline (fasting) concentrations of plasma glucose between study days (**Figure 4.2A**).

Response to amino acids alone: There was a treatment × time interaction for plasma glucose ($p < 0.05$); isoleucine reduced plasma glucose at t = −1 min, compared with valine ($p < 0.05$),

while there was no difference between amino acids and control (**Figure 4.2A**). There was a trend for an effect of treatment on plasma glucose $AUC_{-31--1\ min}$ ($p = 0.098$; **Table 4.1**).

Response to the mixed-nutrient drink: Plasma glucose increased on all study days, with a maximum concentration of 7 mmol/L on the control day, and < 7 mmol/L on amino acid days. There was a treatment × time interaction for plasma glucose ($p < 0.05$); isoleucine reduced ($p < 0.05$), and leucine tended to reduce ($p = 0.055$), plasma glucose at t = 30 min, compared with control, while there was no difference between valine and control. Moreover, isoleucine at t = 15–60 min and leucine at t =15 min reduced blood glucose compared with valine (all $p < 0.05$) (**Figure 4.2A**). There was also an effect of treatment on peak plasma glucose ($p < 0.05$); leucine and isoleucine reduced peak glucose, compared with both control and valine ($p < 0.05$, control: 7.0 ± 0.2 mmol/L, leucine: 6.3 ± 0.2 mmol/L, isoleucine: 6.0 ± 0.1 mmol/L, valine: 6.8 ± 0.2 mmol/L). Finally, there was an effect of treatment on plasma glucose $AUC_{15–120\ min}$ ($p = 0.001$); isoleucine reduced $AUC_{15–120\ min}$ compared with control and valine ($p < 0.05$) (**Table 4.1**).

### 4.4.2 Plasma C-Peptide Concentrations

There were no differences in baseline C-peptide concentrations between study days (**Figure 4.2B**).

Response to amino acids alone: There was no effect of treatment or time on plasma C-peptide (**Figure 4.2B**) or on $AUC_{-31--1\ min}$ (**Table 4.1**).

Response to the mixed-nutrient drink*:* There were effects of treatment and time, but no interaction, on overall plasma C-peptide ($p < 0.05$). Plasma C-peptide increased markedly on all study days. Leucine, but not isoleucine or valine, increased C-peptide, compared with

control ($p < 0.05$). There were no effects of treatment or time, or an interaction, on plasma C-peptide over the first 30 min post-drink (**Figure 4.2B**). There was an effect of treatment on plasma C-peptide $AUC_{15–120\ min}$ ($p = 0.001$), but not on $AUC_{−1–30\ min}$; $AUC_{15–120\ min}$ was greater after leucine compared with control, isoleucine and valine (**Table 4.1**).

### 4.4.3 Plasma Glucagon Concentrations

There were no differences in baseline glucagon concentrations between study days (**Figure 4.2C**).

Response to amino acids alone: There was a treatment × time interaction for plasma glucagon ($p < 0.05$); valine increased glucagon at t = −10 and −1 min, and isoleucine at t = −1 min (all $p < 0.05$), compared with control, while there was no difference between leucine and control (**Figure 4.2C**). There was also an effect of treatment on plasma glucagon $AUC_{−31–−1\ min}$ ($p = 0.006$); there was a trend for plasma glucagon to be greater after valine compared with control ($p = 0.061$; **Table 4.1**).

Response to the mixed-nutrient drink: There were no effects of treatment or time, or an interaction, on overall plasma glucagon or during the first 30 min post-drink, or on $AUC_{−1–30\ min}$ or $AUC_{15–120\ min}$ (**Figure 4.2C, Table 4.1**).

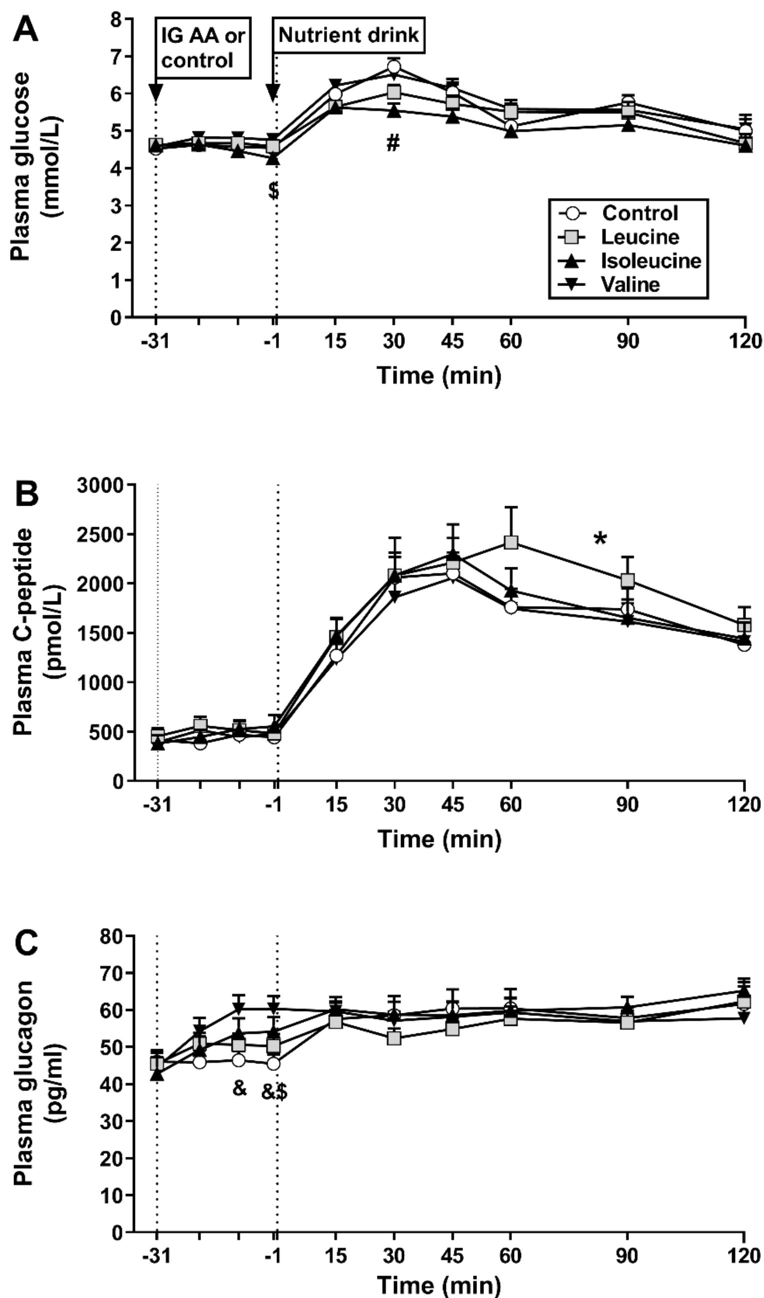


**Figure 4.2:** Plasma glucose (A), C-peptide (B) and glucagon (C) concentrations at baseline (t = −31 min), in response to intragastric (IG) administration of either 10 g of leucine, 10 g of isoleucine, 10 g of valine or control (t = −20, −10 and −1 min), and after a mixed-nutrient drink (t = 15–120 min). Data were analysed using repeated-measures two-way ANOVA with treatment and time as factors. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were conducted where ANOVAs revealed significant effects. (A) $ isoleucine significantly different from valine at t = −1 min, $p < 0.05$; # leucine and isoleucine significantly different from both control and valine, both $p < 0.05$. (B) * leucine, but not isoleucine or valine, significantly different from control, $p < 0.05$. (C) & valine significantly different at t = −10 and −1 min, and $ isoleucine at t = −1 min, from control, all $p < 0.05$. Data are means ± SEMs; $n$ = 15; AA, amino acid.

**Table 4.1:** Effects of intragastric administration of the branched-chain amino acids, leucine, isoleucine or valine, or control on plasma glucose, C-peptide and glucagon concentrations and gastric emptying [1].

| Unit | Control | Leucine | Isoleucine | Valine | ANOVA *p*-Value |
|---|---|---|---|---|---|
| **Plasma glucose** | | | | | |
| $AUC_{-31--1\ min}$, mmol/L × min | 137 ± 3 | 135 ± 1 | 139 ± 2 | 143 ± 2 | 0.098 |
| $AUC_{15-120\ min}$, mmol/L × min | 678 ± 14 | 654 ± 20 | 616 ± 17* | 687 ± 24 # | 0.001 |
| **Plasma C-peptide** | | | | | |
| $AUC_{-31--1\ min}$, pmol/L × min | 12,806 ± 1783 | 15,406 ± 2339 | 14,397 ± 2281 | 13,926 ± 1818 | 0.302 |
| $AUC_{-1-30\ min}$, pmol/L × min | 37,840 ± 4266 | 41,092 ± 4903 | 41,803 ± 5821 | 35,996 ± 4471 | 0.333 |
| $AUC_{15-120\ min}$, pmol/L × min | 184,431 ± 17,969 | 214,318 ± 23,924 *#$ | 191,239 ± 22,908 | 176,874 ± 16504 | 0.001 |
| **Plasma glucagon** | | | | | |
| $AUC_{-31--1\ min}$, pg/ml × min | 0 ± 87 | 98 ± 73 | 231 ± 53 | 333 ± 104 @ | 0.006 |
| $AUC_{-1-30\ min}$, pg/ml × min | 180 ± 75 | 116 ± 39 | 250 ± 46 | 208 ± 50 | 0.332 |
| $AUC_{15-120\ min}$, pg/ml × min | 1400 ± 587 | 1053 ± 301 | 1864 ± 351 | 1413 ± 355 | 0.537 |
| **Gastric emptying** | | | | | |
| $AUC_{0-30\ min}$, % recovery of $^{13}CO_2$ × min | 327 ± 49 | 249 ± 26 | 290 ± 23 | 261 ± 24 | 0.096 |
| $AUC_{0-120\ min}$, % recovery of $^{13}CO_2$ × min | 2555 ± 116 | 2169 ± 118 * | 2335 ± 100 | 2229 ± 90% | 0.004 |

[1] Data are means ± SEMs. AUC, area under the curve. All parameters are expressed as absolute data and were analysed using repeated-measures one-way ANOVA with treatment as a factor. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were conducted where ANOVAs revealed significant effects. * Significantly different from control ($p < 0.05$); # significantly different from isoleucine ($p < 0.05$); $ significantly different from valine ($p < 0.05$); @ trend for a difference from control ($p = 0.061$); % trend for a difference from control ($p = 0.051$).

### 4.4.4 Gastric Emptying

There was an effect of treatment on overall gastric emptying (i.e., $AUC_{0–120\ min}$) of the drink ($p < 0.05$). Leucine, but not isoleucine, slowed ($p < 0.05$), and valine tended to slow ($p = 0.051$), gastric emptying, compared with control (**Figure 4.3**). There was a trend for an effect of treatment on early gastric emptying (i.e., t = 0–30 min post-drink; $AUC_{0–30\ min}$) ($p = 0.096$).

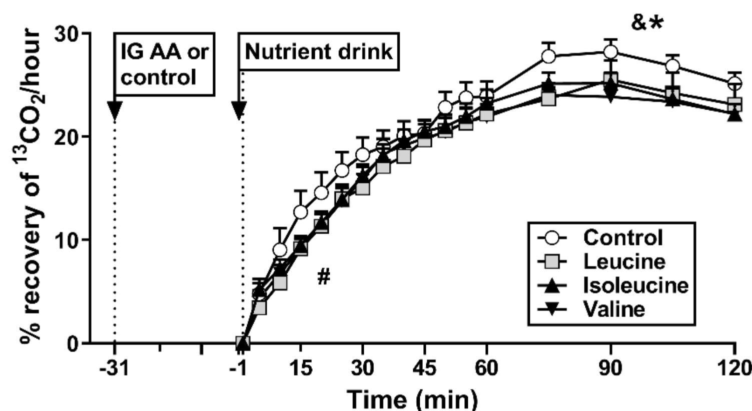


**Figure 4.3:** Recovery of $^{13}CO_2$ in breath samples as a measure of gastric emptying of a mixed-nutrient drink, containing 100 mg $^{13}$C-acetate, consumed at t = −1 min, 30 min after intragastric (IG) administration of either 10 g of leucine, 10 g of isoleucine, 10 g of valine or control. Gastric emptying was expressed as $AUC_{0–120\ min}$ and $AUC_{0–30\ min}$. * Leucine $AUC_{0–120\ min}$ significantly different from control, $p < 0.05$, & trend for valine to differ from control, $p = 0.051$. # Trend for an effect of treatment on early gastric emptying (i.e., t = 0–30 min post-drink), $p = 0.096$. Data are means ± SEMs; $n = 15$; AA, amino acid.

### 4.4.5 Gastrointestinal Symptoms

There were no differences in baseline ratings, or any effects of treatment, on nausea or bloating (data not shown).

### 4.4.6 Relationships Between Plasma Glucose with C-Peptide, Glucagon or Gastric Emptying

There were no relationships between plasma glucose at t = 30 min with early C-peptide $AUC_{−1–30\ min}$ (r = 0.1, $p = 0.3$) or glucagon $AUC_{−1–30\ min}$ (r = 0.08, $p = 0.5$), but a positive correlation with early gastric emptying $AUC_{0–30\ min}$ (r = 0.35, $p < 0.01$).

## 4.5 Discussion

Our study shows that both leucine and isoleucine when administered intragastrically, in a dose of 10 g, in healthy individuals, modestly reduced peak glucose in response to a mixed-nutrient drink, while valine had no effect. None of the amino acids affected early plasma C-peptide, a marker of insulin secretion. Valine and, to a lesser extent, isoleucine, but not leucine, stimulated glucagon before the drink. While all three amino acids appeared to have a small, albeit non-significant, effect to slow early gastric emptying, this was only associated with postprandial glucose lowering after leucine and isoleucine. The absence of an effect of valine to lower postprandial blood glucose may reflect its effect to stimulate glucagon.

That both leucine and isoleucine, at the dose of 10 g, modestly lowered blood glucose after a carbohydrate-containing drink, is consistent with previous findings by ourselves (283, 284) and others (282, 290). The effects of the branched-chain amino acid, valine, on the postprandial glucose response to a mixed-nutrient drink have, to our knowledge, not been investigated previously in humans.

While the magnitude of the effects of leucine and isoleucine to lower the blood glucose response to a drink containing primarily glucose-based carbohydrates is of the same order as their glucose-lowering effect in response to a predominantly fructose-based drink in our previous study (283), it appears that the underlying mechanisms may differ between the two test drinks. In our previous study, leucine stimulated C-peptide within the first 60 postprandial minutes and did not slow gastric emptying, while isoleucine slowed gastric emptying, albeit only after the first 30 postprandial minutes, but did not stimulate C-peptide. In the current study, while leucine stimulated C-peptide, this effect was apparently due to an increase in the second postprandial hour, indicating that the effect of leucine to lower blood glucose, which

was maximal at ~ 30 min post-drink, was unrelated to C-peptide stimulation. In contrast to our previous study (283), leucine slowed gastric emptying of the drink, including a trend for slowing of early gastric emptying (i.e. within the first 30 min post-drink), which probably contributed to blood glucose-lowering, as supported by the observed correlation between blood glucose concentrations at 30 min and early gastric emptying. It is not clear why leucine would slow gastric emptying of a drink containing predominantly glucose-, but not fructose-, based carbohydrates, but the observed differences may potentially reflect differential interactions with other macronutrients, which warrants further investigation. The effect of leucine to modestly slow early gastric emptying, even though it was not statistically significant, may account for the absence of a stimulatory effect on early C-peptide secretion.

In line with previous observations by ourselves (283) and others (290), isoleucine did not stimulate C-peptide. In contrast, there was a trend for an effect to slow early gastric emptying. We have demonstrated that even small changes in the rate of glucose delivery to the small intestine can have a major impact on blood glucose concentrations (340). Thus, the observed effect of isoleucine (and leucine) to lower postprandial blood glucose concentrations may be related to their effect to slow early gastric emptying.

To our knowledge, this is the first study to evaluate the postprandial glycaemic effects of valine in humans. Unlike leucine and isoleucine, valine does not appear to have glucose-lowering effects. Valine did not stimulate C-peptide, may have a modest (although statistically non-significant) effect to slow early gastric emptying and potently stimulated glucagon prior to drink ingestion. While it is unknown whether valine may selectively stimulate pancreatic alpha-cells (341), valine-induced glucagon stimulation would counteract any potential glucose-lowering due to slowing of gastric emptying.

Given that both leucine and isoleucine have been shown to have modest effects to lower postprandial blood glucose in both the current and previous (282, 290) studies, it is important to consider the potential clinical implications of these findings for the management, treatment or prevention of type 2 diabetes. It is tempting to hypothesise that if these amino acids lower blood glucose in healthy people with good blood glucose control, it is likely that these effects may even be more pronounced in patients with type 2 diabetes that have elevated blood glucose concentrations (283). However, we have reported recently [28], using an identical study design, that leucine and isoleucine, in the same doses, did not lower the blood glucose response to a mixed-nutrient drink in people with T2D. Thus, the glucose-lowering effect of these amino acids appears not to be maintained, arguing against the use of leucine or isoleucine as a nutrient-based strategy in the management of T2D.

A number of limitations for this study should be recognised. We studied only healthy participants, thus, our results cannot be extended to people with impaired glucose tolerance or T2D. Whether the effect of leucine and isoleucine may be greater in individuals with impaired glucose tolerance or T2D, due to elevated blood glucose levels, warrants further investigation. While the 10 g dose of each amino acid used is relatively high, it is representative of what may be consumed in a meal, given that there are relatively large amounts of leucine and isoleucine, and somewhat less valine, in foods, including beef, chicken and tuna (e.g. approximately 10 g leucine is contained in a 350-g steak). We were unable to evaluate the effect of oral ingestion of the amino acids, due to their unpleasant taste. Finally, we did not assess plasma amino acid concentrations, however, it is known from studies by ourselves (284) and others (282, 290) that plasma concentrations of these amino acids rise within 15 - 30 min of administration of preloads of whey (342, 343) or individual amino acids (282, 284, 290).

In conclusion, among the branched-chain amino acids, leucine and isoleucine, but not valine, modestly diminish the blood glucose response after a mixed-nutrient drink containing glucose-based carbohydrates in healthy people, most likely due to the slowing of early gastric emptying.

# Chapter 5: Intragastric administration of leucine and isoleucine does not reduce the glycaemic response to, or slow gastric emptying of, a carbohydrate-containing drink in type 2 diabetes

**Elovaris RA, Hajishafiee M, Ullrich SS, Fitzgerald PCE, Lange K, Horowitz M, Feinle-Bisset C**

**Published in**

***Diabetes Research and Clinical Practice 2021; 171: 108618.***

# Statement of Authorship

| | |
|---|---|
| Title of Paper | Intragastric administration of leucine and isoleucine does not reduce the glycaemic response to, or slow gastric emptying of, a carbohydrate-containing drink in type 2 diabetes. |
| Publication Status | ☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | Elovaris RA, Hajishafiee M, Ullrich SS, Fitzgerald PCE, Lange K, Horowitz M, Feinle-Bisset C. Diabetes Res Clin Pract. 2021 Jan.;171:108618. |

## Principal Author

| | | | |
|---|---|---|---|
| Name of Principal Author (Candidate) | Rachel Elovaris | | |
| Contribution to the Paper | Conducted the experiments, analysed the data and conducted the statistical analysis, interpreted the data, prepared the manuscript, and contributed to the revision of the manuscript. | | |
| Overall percentage (%) | 50% | | |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. | | |
| Signature | | Date | 21/6/21 |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

| | | | |
|---|---|---|---|
| Name of Co-Author | Maryam Hajishafiee | | |
| Contribution to the Paper | Interpreted results of experiments, edited and revised the manuscript, and approved the final version of manuscript. | | |
| Signature | | Date | 21.06.2021 |

| | | | |
|---|---|---|---|
| Name of Co-Author | Sina Ullrich | | |
| Contribution to the Paper | Conceived and designed the research, edited and revised the manuscript, and approved the final version of the manuscript. | | |
| Signature | | Date | 15.06.2021 |

| Name of Co-Author | Penelope CE Fitzgerald | | |
|---|---|---|---|
| Contribution to the Paper | Contributed to the experiments, edited and revised the manuscript, and approved the final version of the manuscript. | | |
| Signature | | Date | 21. 6. 21 |

| Name of Co-Author | Kylie Lange | | |
|---|---|---|---|
| Contribution to the Paper | Contributed to the statistical analysis, interpreted the data, edited and revised the manuscript, and approved the final version of the manuscript. | | |
| Signature | | Date | 21.6.21 |

| Name of Co-Author | Michael Horowitz | | |
|---|---|---|---|
| Contribution to the Paper | Contributed to the design of the research, interpreted results of experiments, edited and revised the manuscript, and approved the final version of manuscript. | | |
| Signature | | Date | 21/06/21 |

| Name of Co-Author | Christine Feinle-Bisset | | |
|---|---|---|---|
| Contribution to the Paper | Conceived and designed the research, interpreted the data, prepared the manuscript, contributed to the revision of the manuscript, approved the final version of manuscript, obtained funding and had overall responsibility for the project. | | |
| Signature | | Date | June 15, 2021 |

## 5.1 Abstract

**Aims:** In healthy individuals, intragastric administration of the branched-chain amino acids, leucine and isoleucine, diminishes the glycaemic response to a mixed-nutrient drink, apparently by stimulating insulin and slowing gastric emptying, respectively. This study aimed to evaluate the effects of leucine and isoleucine on postprandial glycaemia and gastric emptying in type-2 diabetes mellitus (T2D).

**Methods:** 14 males with T2D received, on 3 separate occasions, in double-blind, randomised fashion, either 10g leucine, 10g isoleucine or control, intragastrically 30 min before a mixed-nutrient drink (500 kcal; 74g carbohydrates, 18g protein, 15g fat). Plasma glucose, insulin and glucagon were measured from 30 min pre- until 120 min post-drink. Gastric emptying of the drink was also measured.

**Results:** Leucine and isoleucine stimulated insulin, both before and after the drink (all $P < 0.05$; peak (mU/L): control: 70 ± 15; leucine: 88 ± 17; isoleucine: 74 ± 15). Isoleucine stimulated ($P < 0.05$), and leucine tended to stimulate ($P$=0.078), glucagon before the drink, and isoleucine stimulated glucagon post-drink ($P$=0.031; peak (pg/mL): control: 62 ± 5; leucine: 70±9; isoleucine: 69 ± 6). Neither amino acid affected gastric emptying or plasma glucose (peak (mmol/L): control: 12.0 ± 0.5; leucine: 12.5 ± 0.7; isoleucine: 12.0 ± 0.6).

**Conclusions:** In contrast to health, in T2D, leucine and isoleucine, administered intragastrically in a dose of 10 g, do not lower the glycaemic response to a mixed-nutrient drink. This finding argues against a role for ‘preloads’ of either leucine or isoleucine in the management of T2D.

## 5.2 Introduction

Reducing postprandial glucose excursions, by dietary and/or pharmacological strategies, is now appreciated as an important therapeutic target in the management of type 2 diabetes

mellitus (T2D). Gastric emptying plays a critical role in the regulation of postprandial glucose; it accounts for ~ 35 % of the variance in the initial (0-30 min) rise in glucose after a meal in health and diabetes (332, 333). Ingestion of protein, particularly whey, as a ‘preload’ before a meal, lowers postprandial glycaemia, apparently by slowing gastric emptying and stimulating glucagon-like peptide-1 (GLP-1) and insulin, in both health and T2D (121, 122, 214, 219, 224, 226, 229). For example, in diet-controlled T2D, ingestion of 55 g whey protein, incorporated in 350 ml soup, 30 min before a carbohydrate meal, stimulated insulin and slowed gastric emptying, associated with a marked reduction in postprandial glycaemia (121). Moreover, ingestion of a 150-ml flavoured drink containing 17 g whey and 5 g guar, for 12 weeks before meals, reduced postprandial glycaemia, slowed gastric emptying and led to a modest reduction in glycated haemoglobin in T2D (236). Thus, preload strategies that reduce the glycaemic response to a meal may also improve longer-term blood glucose control in T2D. However, such preloads represent a substantial additional caloric load (up to 220 kcal for 55 g whey), which is undesirable.

Whey protein is rich in branched-chain amino acids, particularly leucine and isoleucine, which have both been reported to lower postprandial glucose in healthy individuals (283). Moreover, the effects of whey to stimulate GLP-1 and insulin were related to circulating levels of leucine and isoleucine (343). Thus, these amino acids may, at least in part, mediate the effects of whey protein to lower postprandial blood glucose. Supporting this concept, oral administration of 1 mmol/kg body weight (approximating ~ 9 g in a 70 kg person) isoleucine reduced the glycaemic response to ingestion of 25 g glucose (290). We recently reported that in healthy participants intragastric administration of leucine and isoleucine, each at a dose of 10 g (containing ~ 40 kcal), 15 min before a mixed-nutrient drink, reduced peak postprandial blood glucose modestly (by ~ 1.1 mmol/L) (283). Interestingly, leucine increased insulin (as observed

previously (282)), while only isoleucine slowed gastric emptying of the drink significantly and did not increase insulin, suggesting that the two amino acids may lower blood glucose via different mechanisms (283). It is not known whether leucine and isoleucine, at the dose of 10 g used in healthy people, reduce postprandial glucose excursions in T2D. Given that postprandial blood glucose is higher in T2D than health, and pharmacologic strategies that stimulate insulin and slow gastric emptying are effective in the management of T2D, it may be predicted that postprandial glucose-lowering effects of leucine and isoleucine would be greater. In db/db mice (a model of T2D), acute and chronic administration of isoleucine reduced the glycaemic response to an oral glucose challenge (291), although this finding contrasts with that of another study in lean and obese mice, which found no effect of isoleucine on postprandial blood glucose (344). On the other hand, the ingestion of protein and specific amino acids, including leucine, may stimulate glucagon (282, 345), which may counteract glucose lowering, particularly as the postprandial suppression of glucagon is attenuated in T2D (346). Valine is also a branched-chain amino acid, and therefore potentially of interest. However, intraduodenal infusion of valine, unlike leucine (284), had no effect on upper gut functions (including stimulation of plasma cholecystokinin which slows gastric emptying), fasting glucose or subsequent energy intake (309).

The aim of this study was, therefore, to evaluate the effects of leucine and isoleucine on blood glucose and plasma insulin and glucagon responses to, and the gastric emptying of, a mixed-nutrient drink in individuals with T2D. We hypothesised that intragastric administration of leucine or isoleucine before a mixed-nutrient drink containing maltodextrin and sucrose, would lower the glycaemic response substantially, leucine primarily by stimulating insulin, and isoleucine by slowing gastric emptying. Both amino acids were administered intragastrically to avoid any confounding effects due to their unpleasant taste.

## 5.3 Materials and methods

### 5.3.1 Study participants

Fourteen males, aged 62 ± 3 years (range: 41–70 years), with obesity (BMI 31 ± 1 kg/m$^2$ (range: 23–37 kg/m$^2$)) and reasonably controlled T2D (HbA1c ≤ 7.4), with a duration of known diabetes of 8 ± 2 years, participated in the study. Participants were recruited in consecutive order from an existing pool of volunteers, by flyers placed around the Royal Adelaide Hospital, Queen Elizabeth Hospital and University of Adelaide, and at diabetes seminars (Diabetes SA, Adelaide, Australia). Glycated haemoglobin, which was measured from a fasting sample (SA Pathology, Adelaide, Australia), was 6.6 ± 0.2 % (48 ± 2 mmol/mol) at the time of screening. All participants met WHO criteria for the diagnosis of diabetes (347) and were managed by metformin (1000–2000 mg/day) alone ($n$ = 9) or in combination with a DPP-IV inhibitor ($n$ = 2) or sodium-glucose transport inhibitor ($n$ = 2) alone, or a DPP-IV inhibitor in combination with a sodium-glucose transport inhibitor ($n$ = 1). None had impaired renal function or evidence of diabetic microvascular complications (including autonomic nerve dysfunction, as assessed using standardised cardiovascular reflex tests) (348). In addition, those who smoked, consumed > 20 g of alcohol/day, had significant gastrointestinal symptoms, disease or surgery, or used medications known to affect gastrointestinal function and/or appetite, were excluded. All participants were also unrestrained eaters (score ≤12 on the eating restraint component of the Three-Factor Eating Questionnaire (297)) and had been weight-stable (< 5 % fluctuation) in the 3 months preceding the study. Once participants were included they were assigned to a treatment order of balanced randomisation (using the online tool available at www.randomization.com) by a researcher who was not involved in either the performance of the studies or data analysis. The study protocol was approved by the Human Research Ethics Committee of the Central Adelaide Local Health Network (Protocol number: 140626), and the study performed in accordance with the Declaration of Helsinki and the NHMRC Statement

on Ethical Conduct in Human Research. Each participant provided written, informed consent prior to their enrolment. The study was registered as a clinical trial with the Australian New Zealand Clinical Trials Registry (www.anzctr.org.au, number: ACTRN12619000413123).

### 5.3.2 Study outline

This study evaluated the effects of intragastric administration of 10 g leucine, 10 g isoleucine or control, on the plasma glucose, insulin and glucagon responses to, and gastric emptying of, a mixed-nutrient, carbohydrate-containing drink in T2D. The loads of leucine and isoleucine were based on our previous study, which found significant effects of these amino acids, at 10 g each, to lower blood glucose in healthy individuals (283).

### 5.3.3 Study treatments

Due to the low water solubility (particularly of leucine), amino acids were administered as suspensions. 10 g crystalline leucine (Bulk Nutrients, Tasmania, Australia), or isoleucine (PureBulk Inc., Roseburg, Oregon, USA) and 29.2 mg $CaCl_2$ x $2H_2O$ were incorporated in 10 mL of a suspending agent (ORA-Plus®, Perrigo®, Minneapolis, USA), and isotonic saline used to adjust to a final volume of 100 mL. The control treatment consisted of 10 mL suspending agent, 29.2 mg $CaCl_2$ x $2H_2O$, and 90 mL isotonic saline. The treatments were prepared by a researcher who was not involved in either the performance of the study or analysis of the data on the morning of each study visit and administered at room temperature. Syringes were covered so that the study participant and primary investigator (RAE) were both blinded to the treatments.

### 5.3.4 Study protocol

Each participant was studied on 3 occasions in a randomised, double-blind, cross-over design, separated by at least 3, and up to 7, days. Participants were instructed to discontinue their glucose-lowering medication for 48 h, and abstain from vigorous exercise and alcohol intake for 24 h, before each study visit. They were provided with a standardised evening meal (beef lasagne; energy content: 603 kcal; McCain Foods, Wendouree, Victoria, Australia) to be consumed by 1900 h on the night before each study, after which time they fasted overnight from solids and liquids, except water which was allowed until 0700 h. Participants then attended the Clinical Research Facility at the University of Adelaide at 0830 h the following morning. Upon arrival they were seated in an upright position, and an intravenous cannula was placed into a forearm vein for regular blood sampling, and the arm was kept warm with a heat pad for regular sampling of 'arterialised' blood to minimise capillary-venous differences in glucose and hormone concentrations (349). At baseline ($t$ = -31 min), a blood sample to measure plasma glucose and hormone concentrations and a fasting/baseline breath sample, as a reference prior to the measurement of gastric emptying by breath test, were collected. Participants also completed a visual analogue scale (VAS) questionnaire to assess gastrointestinal symptoms. Each participant was then intubated with a nasogastric, soft-silicone feeding tube (outer diameter: 4 mm; Dentsleeve International, Mississauga, Ontario, Canada), which was inserted through an anaesthetised nostril into the stomach. The correct positioning of the tube was checked by auscultating the stomach with a stethoscope while pushing a 5-mL air bolus through the tube. Immediately thereafter ($t$ = -31 min, ~ 0845 h), one of the study treatments, i.e. either i) 10 g leucine, ii) 10 g isoleucine or iii) control, all in a volume of 100 mL, was administered intragastrically within 1 min. The tube was then removed and over the next 30 min (i.e. $t$ = -31 to -1 min), blood samples were collected every 10 min. At $t$ = -1 min, participants consumed, within 1 min, 350 mL of a mixed-nutrient drink (Resource Plus®

vanilla; Nestle Healthcare Nutrition, Tongala, Victoria, Australia (325 ml); 500 kcal; 74 g carbohydrates including maltodextrin and sucrose, 18 g protein (including milk protein caseinates and soy protein isolate), 15 g fat, plus 25 mL water to make up the final volume) labelled with 100 mg $^{13}$C-acetate (Cambridge Isotope Laboratories, Inc., Andover, Massachusetts, USA) for measurement of gastric emptying by breath test (338). Immediately after consumption of the drink ($t$ = 0 min) and for the next hour, blood samples were collected every 15 min and breath samples every 5 min. For the second hour ($t$ = 60 - 120 min), blood samples were collected every 30 min and breath samples every 15 min. Throughout the study, at the same time points as blood sample collections, participants rated gastrointestinal symptoms, including nausea and bloating. At $t$ = 120 min, the cannula was removed, and participants provided with a light lunch, after which they were free to leave the laboratory.

### 5.3.5 Measurements

#### *5.3.5.1 Plasma glucose and hormone analyses*

Blood samples were collected into ice-chilled ethylenediaminetetraacetic acid-coated tubes and centrifuged (3200 rpm at 4°C for 15 min) within 15 min of collection to obtain plasma. Plasma samples were stored at -80°C until further analysed.

Plasma glucose (mmol/L) was measured using the glucose oxidase technique (2300 STAT Plus; YSI, Yellow Springs, OH, USA).

Plasma insulin (mU/L) was measured by ELISA (10-1113; Mercodia, Uppsala, Sweden). The minimum detectable limit was 1.0 mU/L, and intra- and inter-assay CVs were 2.4 % and 4.9 %, respectively.

Plasma glucagon (pmol/L) was measured by radioimmunoassay (GL-32K; Millipore, Billerica, MA). The minimum detectable limit was 15 pg/mL, and intra-assay and inter-assay CVs were 6.9 % and 4.2 %, respectively.

#### *5.3.5.2 Gastric emptying*

$^{13}CO_2$ concentrations in exhaled air of end-expiratory breath samples were measured using an isotope ratio mass spectrometer (FANci2 breath test analyser, Fischer Analysen Instrumente, Leipzig, Germany) with an online gas chromatographic purification system. Data were expressed as the percentage of recovery of $^{13}CO_2$ in the breath per hour (338).

#### *5.3.5.3 Gastrointestinal symptoms*

Nausea and bloating were assessed using 100-mm VAS scales (324). The scales consisted of 100-mm horizontal lines, where 0 mm represented "not felt at all", and 100 mm "felt the strongest possible". Participants were asked to place a vertical mark on each horizontal line to rate the strength of each sensation felt at that point in time.

### 5.3.6 Data and statistical analysis

The number of participants was based on power calculations derived from previous work (283), indicating that $n$ = 14 participants would allow detection of a 1.2 mmol/L difference in peak plasma glucose (which was considered clinically meaningful), with a standard deviation of 1.1 mmol/L, at $\alpha = 0.05$, with a power of 80 %.

Effects of amino acids alone were evaluated by calculating total areas under the curve (AUC) (using the trapezoidal rule) for plasma glucose, insulin, glucagon and VAS data from $t$ = -31 to -1 min ($AUC_{-31 \text{ to } -1 \text{ min}}$). To evaluate the responses to the mixed-nutrient drink, plasma

glucose, insulin, glucagon and VAS were expressed as AUCs from $t$ = -1 to 120 min ($AUC_{-1\ to\ 120\ min}$) and gastric emptying data as AUCs from $t$ = 0 to 120 min ($AUC_{0\ to\ 120\ min}$). The insulin-secretory response was calculated using the incremental AUC from $t$ = -1 to 120 min ($iAUC_{-1\ to\ 120\ min}$) as: Insulin secretory response = ($iAUC_{-1\ to\ 120\ min}$ for insulin)/($iAUC_{-1\ to\ 120\ min}$ for glucose) (350). AUCs of plasma glucose, insulin, glucagon, gastric emptying and the insulin-secretory response to the drink were analysed using linear mixed effects models, including treatment and baseline values (i.e. at $t$ = -31 min) as fixed effects, and an unstructured covariance matrix to account for the repeated measurements per participant. Peak plasma glucose concentrations were evaluated using one-factor repeated-measures ANOVA. Plasma concentrations of glucose, insulin and glucagon at $t$ = 120 min were compared with baseline concentrations using a one-sample $t$-test to assess whether concentrations had returned to fasting levels at that time. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni correction, were performed where analyses revealed significant treatment effects. Statistical analysis was performed using SPSS software (version 25, IBM, Chicago, IL, USA). All data are reported as means ± SEMs. Differences were considered statistically significant at $P < 0.05$.

## 5.4 Results

All participants completed all 3 study visits and tolerated the study treatments well. No participant experienced any gastrointestinal symptoms, including nausea or bloating, in response to any treatment (data not shown). Because of technical problems, breath test data (for the measurement of gastric emptying) were unavailable in one participant.

### 5.4.1 Plasma glucose

There were no differences in fasting glucose concentrations between study days (**Figure 5.1A**).

*Response to amino acids alone*. There was no effect of treatment on plasma glucose $AUC_{-31\ to\ -1\ min}$ (**Figure 5.1A, Table 5.1**).

*Response to the drink*. There was a substantial rise in plasma glucose in response to all study treatments. However, there was no effect of treatment on plasma glucose $AUC_{-1\ to\ 120\ min}$ or peak plasma glucose concentrations (**Figure 5.1A, Table 5.1**). On all study days, plasma glucose concentrations remained higher at $t = 120$ min compared with baseline (all $P < 0.05$).

### 5.4.2 Insulin

There were no differences in fasting insulin concentrations between study days (**Figure 5.1B**). *Response to amino acids alone.* There was an effect of treatment on insulin $AUC_{-31\ to\ -1\ min}$ ($P < 0.001$) (**Figure 5.1B, Table 5.1**). Leucine ($P < 0.001$) and isoleucine ($P=0.008$) increased insulin $AUC_{-31\ to\ -1\ min}$, modestly compared with control.

*Response to the drink*. There was an effect of treatment on insulin $AUC_{-1\ to\ 120\ min}$ ($P=0.022$) (**Figure 5.1B, Table 5.1**). Leucine ($P=0.021$) and isoleucine ($P=0.034$) increased insulin $AUC_{-1\ to\ 120\ min}$, compared with control. There was also an effect of treatment on peak plasma insulin concentrations ($P=0.005$), which was higher after leucine, but not isoleucine, compared with control (all $P< 0.05$) (**Table 5.1**). On all study days, insulin concentrations remained higher at $t = 120$ min compared with baseline (all $P < 0.05$).

*Insulin-secretory response*. There was a trend for an effect of treatment on the insulin-secretory response ($P = 0.089$) (**Table 5.1**).

### 5.4.3 Glucagon

There were no differences in fasting glucagon concentrations between study days (**Figure 5.1C**).

*Response to amino acids alone*. There was an effect of treatment on glucagon $AUC_{-31\ to\ -1\ min}$ ($P$=0.049) (**Figure 5.1C, Table 5.1**). Leucine tended to increase ($P$=0.078), and isoleucine increased ($P$=0.048), glucagon $AUC_{-31\ to\ -1\ min}$, compared with control.

*Response to the drink.* There was an effect of treatment on glucagon $AUC_{-1\ to\ 120\ min}$ ($P$=0.030) (**Figure 5.1C, Table 5.1**). Isoleucine increased glucagon $AUC_{-1\ to\ 120\ min}$, compared with control ($P$=0.031). While mean glucagon concentrations were higher after leucine than control, this difference was not significant ($P$=0.225). There was no significant effect of treatment on peak glucagon concentrations (**Table 5.1**). On all study days, glucagon concentrations remained higher at $t$ = 120 min compared with baseline (all $P < 0.05$).

### 5.4.4 Gastric emptying

There was no effect of treatment on gastric emptying $AUC_{0\ to\ 120\ min}$ of the drink (**Figure 5.2, Table 5.1**).

**Table 5.1:** Plasma glucose, insulin and glucagon concentrations in response to amino acids alone ($t$ = -31 to -1 min) and a mixed-nutrient drink ($t$ = -1 to 120 min), and gastric emptying (measured with the use of a $^{13}$C-acetate breath test, $t$ = 0 to 120 min)) of the drink.

| | **Treatments** | | | ***P*** |
|---|---|---|---|---|
| | **Control** | **Leucine** | **Isoleucine** | ***(Treatment)*** |
| **Plasma glucose** | | | | |
| Peak concentration, mmol/L | 12.0 ± 0.5 | 12.5 ± 0.7 | 12.0 ± 0.6 | $P$=0.264 |
| $AUC_{-31\ to\ -1\ min}$, mmol/L×min | 219 ± 2 | 222 ± 4 | 223 ± 4 | $P$=0.454 |
| $AUC_{-1\ to\ 120min}$, mmol/L×min | 1242 ± 31 | 1280 ± 49 | 1261 ± 48 | $P$=0.413 |
| **Plasma insulin** | | | | |
| Peak concentration, mU/L | 70 ± 15 | 88 ± 17** | 74 ± 15 | $P$=0.005 |
| $AUC_{-31\ to\ -1\ min}$, mU/L×min | 243 ± 9 | 394 ± 27* | 335 ± 20* | $P$<0.001 |
| $AUC_{-1\ to\ 120min}$, mU/L×min | 5535 ± 115 | 7282 ± 1457 | 7036 ± 151 | $P$=0.033 |
| **Insulin secretory response** | | | | |
| mU/mmol | 13 ± 3 | 16 ± 4 | 16 ± 5 | $P$=0.089 |
| **Plasma glucagon** | | | | |
| Peak concentration, pg/mL | 62 ± 5 | 70 ± 9 | 69 ± 6 | $P$=0.105 |
| $AUC_{-31\ to\ -1\ min,}$ pg/mL×min | 1285 ± 28 | 1472 ± 64 | 1383 ± 27* | $P$=0.049 |
| $AUC_{-1\ to\ 120min,}$ pg/mL×min | 6144 ± 555 | 6875 ± 829 | 6878 ± 609* | $P$=0.030 |
| **Gastric emptying** | | | | |
| $AUC_{0\ to\ 120min}$, %×min/h | 2168 ± 120 | 2219 ± 148 | 2118 ± 181 | $P$=0.706 |

Data were analysed using linear mixed effects models, including treatment and baseline value as fixed effects, and an unstructured covariance matrix to account for repeated measurements. The insulin-secretory response (calculated as $iAUC_{-1\ to\ 120\ min}$ for insulin)/($iAUC_{-1\ to\ 120\ min}$ for glucose) was analysed using one-factor repeated-measures ANOVA. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni correction, were performed where analyses revealed significant effects. *Significantly different from control, $P < 0.01$. **Significantly different from control and isoleucine, $P < 0.05$. Data are means ± SEM, $n$ = 14 for plasma glucose, insulin and glucagon and $n$ = 13 for gastric emptying.

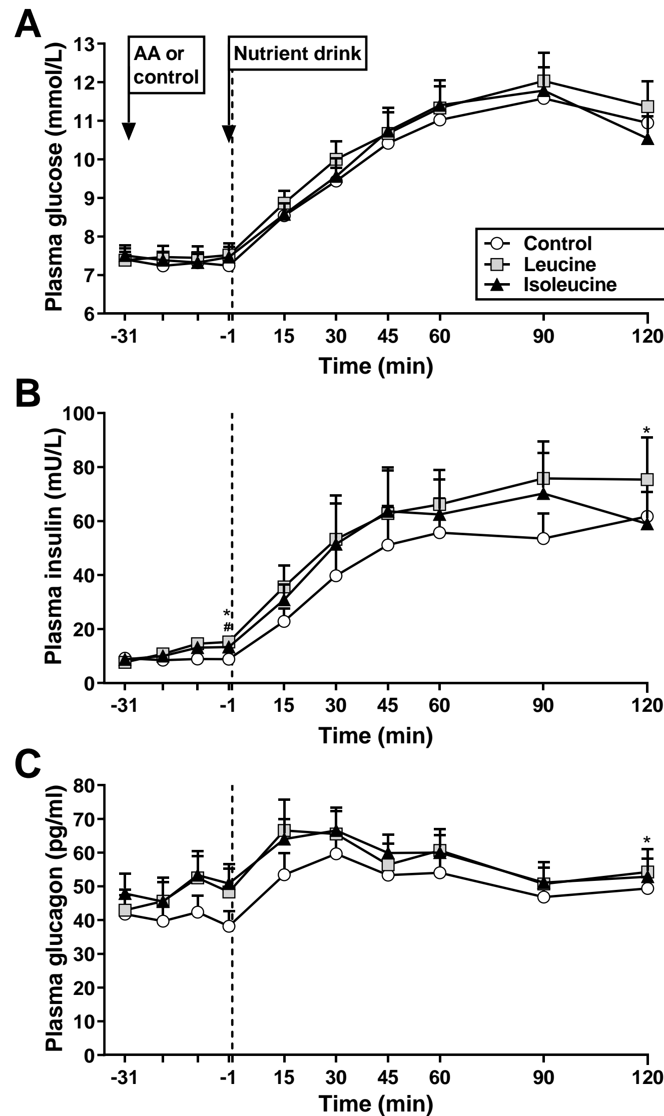


**Figure 5.1:** Plasma glucose (A), insulin (B) and glucagon (C) concentrations in response to intragastric administration of leucine or isoleucine alone (t = -31 to -1 min) and a mixed-nutrient drink (t = -1 to 120 min). (A) There was no effect of treatment on plasma glucose concentrations either in response to leucine and isoleucine alone or the drink. (B) There were effects of treatment on plasma insulin concentrations, which were greater in response to both leucine and isoleucine alone and the drink (*P < 0.05 for all). (C) There was an effect of treatment on plasma glucagon concentrations, which were greater in response to isoleucine (*P = 0.048), and tended to be greater in response to leucine alone (P = 0.078), compared with control. There was also an effect of treatment on plasma glucagon concentrations following the drink, which were greater in response to isoleucine (*P = 0.031), but not leucine (P = 0.225), compared with control. Data are means ± SEM, *n* = 14.

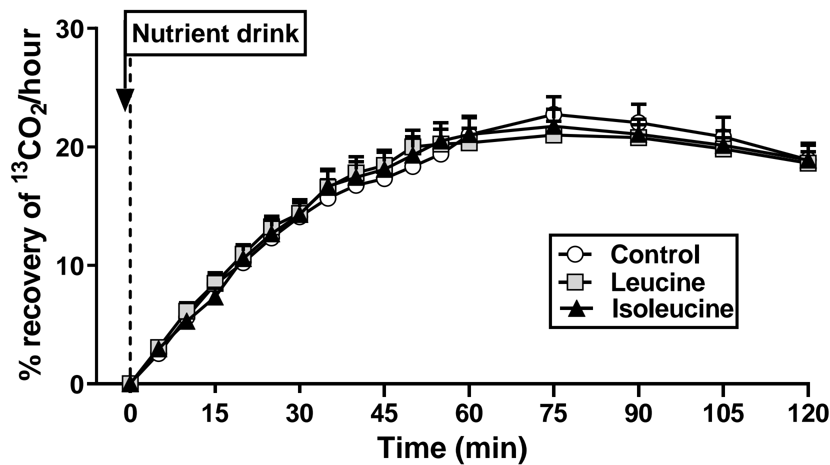


**Figure 5.2:** Gastric emptying (expressed as the percentage recovery of $^{13}CO_2$ in the breath per hour) of a mixed-nutrient drink containing 100 mg $^{13}$C-acetate, consumed at $t$ = -1 min, after the intragastric administration of 10 g leucine or isoleucine or control at $t$ = -31 min. Neither leucine nor isoleucine had any effect on gastric emptying. Data are means ± SEM, $n$ = 13.

## 5.5 Discussion

Our study indicates that in individuals with uncomplicated T2D (i.e. without overt microvascular complications, including cardiovascular autonomic neuropathy) and reasonable glycaemic control, neither leucine nor isoleucine, when administered intragastrically in a dose of 10 g before a mixed-nutrient drink (containing maltodextrin and sucrose), diminish the glycaemic response – we had reported previously that both leucine and isoleucine reduced the glycaemic response to a carbohydrate drink in health (283). Accordingly, this observation argues against the concept of using leucine or isoleucine as a nutrient-based strategy in the management of T2D.

The rationale for our study was underpinned by previous observations by ourselves and others that both of these branched-chain amino acids, when given at a relatively high dose of ~ 10 g, reduce the glycaemic response to both glucose (282, 290) and a mixed-nutrient drink containing mainly fructose-based carbohydrates (283). Although the observed reductions in glycaemia were modest (i.e. reduction in plasma glucose of ~ 1.1-1.2 mmol/L), these studies

were performed in healthy individuals where the maximum blood glucose after the drink was ~ 7.5 mmol/L. We hypothesised that the magnitude of blood glucose-lowering induced by these amino acids would be greater in individuals with T2D, simply because the glycaemic response to the drink would predictably be greater. However, in contrast to our hypothesis, neither amino acid attenuated the blood glucose response to the nutrient drink, despite a much greater elevation in postprandial glucose (to ~ 12 mmol/L) in the control condition, when compared with the response in healthy controls in the previous study, suggesting that the glucoregulatory responses to these amino acids are altered in T2D.

Our previous study in healthy participants suggested that leucine and isoleucine lower the glycaemic response to carbohydrate by different mechanisms – leucine by stimulating insulin, and isoleucine by slowing gastric emptying (283). In the current study, both leucine and isoleucine stimulated insulin, which may reflect an insulinotropic effect of circulating amino acids (218, 351). We did not measure plasma concentrations of the incretin hormones, GLP-1 and GIP, which stimulate insulin in a glucose-dependent manner, given the absence of changes in glycaemia (182). A major role for these hormones is unlikely; a recent study demonstrated in healthy participants that orally ingested leucine and isoleucine, at the even higher dose of ~ 15g, did not stimulate GLP-1, and only isoleucine stimulated GIP minimally (352). Furthermore, a significant insulinotropic effect of GIP would be most unlikely, given that GIP is known to be markedly diminished in T2D (111). It remains uncertain why insulin stimulation by leucine and isoleucine was not associated with glucose lowering, but the observed stimulation of glucagon, presumably by circulating amino acids, is likely to be pivotal. Defective postprandial suppression of glucagon is well characterised as a feature of T2D (346), and in our previous study in healthy participants neither leucine nor isoleucine stimulated

glucagon (283). It is also possible that in health the insulinotropic effect of leucine is greater because of the effect of GIP (111).

The rate of gastric emptying is a major determinant of postprandial glycaemic excursions, particularly during the first 30-60 min, and in the population studied i.e. well-controlled, uncomplicated individuals with T2D, gastric emptying is usually normal or slightly more rapid (353). We reported that isoleucine slowed gastric emptying in healthy participants (283), which may account for the absence of an effect to increase insulin, while in the current study neither amino acid slowed gastric emptying. This may explain the lack of glucose-lowering in response to these amino acids. Slowing of gastric emptying appears central to the effect of whey protein preloads, when given in isolation (121) or combined with guar (236), to diminish postprandial glycaemia in T2D. We do not have a clear explanation for the absence of an effect of isoleucine to slow gastric emptying in the current study. Acute elevations in blood glucose, even within the normal postprandial range (130), may slow gastric emptying, and this remains a potential confounder, i.e. blood glucose levels were predictably higher in the current study in T2D than in health.

Some limitations of our study should be recognised. Leucine and isoleucine were given in isolation, and it is possible that their capacity to lower blood glucose is influenced by the interaction with other amino acids or peptides (354). The study was performed in males, hence, our findings cannot be generalised to females, but we would not anticipate substantial differences. We only evaluated the effects of one dose of leucine and isoleucine (10 g), but, if anything, this could be regarded as supraphysiological (i.e. higher than contained in an average meal (284) or recommended by WHO guidelines (57)). We also did not evaluate the complete glycaemic response to the drink, i.e. blood glucose levels remained somewhat higher than

baseline at 120 min, however, the effect of 'preload' interventions to lower blood glucose are evident within the first 120 min, and usually within in the first 30-60 min (121, 236). Finally, we did not measure circulating amino acid concentrations, thus, cannot exclude that the absence of an effect to lower blood glucose was due to a reduced absorption of amino acids, associated with a lack of postabsorptive effects (e.g. on insulin).

In conclusion, in individuals with T2D, while both leucine and isoleucine stimulated insulin, in contrast to health, only isoleucine stimulated glucagon significantly, and neither amino acid slowed gastric emptying or diminished the glycaemic response to the carbohydrate-containing drink, attesting to the importance of gastric emptying to the regulation of postprandial blood glucose. Based on these observations, leucine or isoleucine 'preloads' as a nutrient-based strategy to optimise postprandial blood glucose control in T2D are unlikely to prove useful. Moreover, oral consumption of leucine or isoleucine, at the dose of 10 g, would most likely be challenging due to the unpleasant taste and/or low solubility of these amino acids.

# Chapter 6: Conclusions

The studies reported in this thesis examined whether dietary amino acids, particularly the BCAAs, leucine, isoleucine and valine, had the potential to reduce postprandial blood glucose and/or energy intake by modulating GI functions (including gastric emptying and gut hormone release), in both healthy and/or T2D. The studies investigated: (1) the effects of drinks containing 30 g and 70 g pure whey protein isolate on the temporal release of all 20 amino acids, and relationships with gastric emptying, GI hormone release, plasma insulin, glucagon, blood glucose, appetite and energy intake, in healthy individuals; (2) the effects of valine, when infused intraduodenally, on plasma CCK, antropyloroduodenal motility, appetite and energy intake, in healthy individuals; (3) the comparative effects of leucine, isoleucine and valine, when administered intragastrically, on the blood glucose and plasma hormone responses to, and gastric emptying of, a mixed-nutrient drink, in healthy individuals; (4) and whether leucine or isoleucine, when administered intragastrically, before a mixed-nutrient drink, have the potential to reduce postprandial blood glucose in T2D.

As discussed, obesity and T2D are major public health issues throughout the world, associated with serious co-morbidities, as well as substantial economic costs. Therapies, other than pharmacological or surgical, including nutrient-based treatments, that are effective, inexpensive and free of adverse effects, as a management strategy for obesity and/or T2D, by reducing weight and/or improving glycaemic control, are urgently needed. The work presented in this thesis investigated the broad hypothesis that amino acids, particularly the BCAAs, when consumed orally or administered into the stomach or GI lumen, have the potential to stimulate GI functions (including gastric emptying and gut hormone release), known to be associated with the regulation of energy intake and blood glucose. Existing information about the individual, as well as comparative effects, of leucine, isoleucine and valine on GI functions

associated with potential effects on blood glucose and/or energy intake, in both health and T2D, is limited.

The first study, presented in **Chapter 2**, represents an exploratory secondary data analysis. The original study characterised the effects of oral whey protein drinks (30 g or 70 g), on gastric emptying, GI hormone release, appetite and energy intake, in healthy individuals. Consequently, the effects of these drinks on the temporal release of all 20 amino acids, and relationships with the previously reported outcomes were analysed. The study demonstrated that following ingestion of oral whey protein drinks (30 g or 70 g), the effects of whey to simulate GLP-1 and insulin, and to reduce energy intake, correlated with circulating levels of amino acids, including the BCAAs. Thus, the BCAAs may, at least in part, mediate the effects of whey protein to lower postprandial blood glucose and reduce energy intake. The mechanisms underlying these effects of the BCAAs, associated with GI functions, blood glucose and energy intake, warrant further investigation.

The study presented in **Chapter 3** was designed based on the outcomes from a previous studies investigated in our laboratory, whereby the effect of intraduodenal administration of whey protein or leucine to suppress energy intake was directly correlated with plasma concentrations of the BCAAs or leucine, respectively, and also motivated by the lack of information about the effects of valine on blood glucose and/or energy intake in humans. The study investigated, in healthy individuals, the effects of valine, infused intraduodenally, to standardise the rate of delivery into the small intestine and to avoid the potentially confounding effects of orosensory influences and differences in gastric emptying. It was demonstrated that a 90-min infusion of valine (delivering rates of 0.15 kcal/min (total: 3.3 g) or 0.45 kcal/min (total: 9.9 g)), did not affect antral, pyloric or duodenal pressures, plasma CCK, appetite perceptions or energy intake.

Thus, it appears that valine, even when administered in moderate to relatively high doses, does not have GI or energy intake-suppressant effects. These observations indicate that, unlike leucine or isoleucine, valine in isolation, is not critical to the regulation of energy intake. Accordingly, different amino acids, even though of similar chemical structure, can have distinct physiological effects.

The study presented in **Chapter 4** was designed based on outcomes from previous studies, by ourselves and others, suggesting that leucine and isoleucine regulate blood glucose, while observations relating to the effect of valine on blood glucose were inconclusive. In this study, in healthy individuals, leucine and isoleucine (in doses of 10 g), but not valine, lowered the blood glucose response to a mixed-nutrient drink (containing a significant amount of carbohydrate; 74 g). Both leucine and isoleucine slowed gastric emptying, while neither amino acid affected postprandial C-peptide (a measure of insulin secretion). Valine did not stimulate C-peptide, but, instead, potently stimulated glucagon prior to drink ingestion. Any potential effect of valine on blood glucose was likely to have been counteracted by increased glucagon stimulation pre-drink. These findings are of relevance to the development of nutrient-based interventions for blood glucose control, in that leucine and isoleucine have beneficial effects. This study, as well as the study in **Chapter 5**, incorporated a design whereby the amino acids were administered intragastrically to avoid any confounding effects due to their unpleasant taste, and a mixed-nutrient test drink with a similar macronutrient composition to that of a ‘normal’ meal.

The study presented in **Chapter 5**, aimed to translate the findings in healthy individuals (described in **Chapter 4**) to individuals with T2D in order to establish whether the effects of leucine and isoleucine, on postprandial blood glucose, are evident in the target population.

Accordingly, the study in **Chapter 5**, was designed to determine whether intragastric administration of leucine and/or isoleucine (in doses of 10 g) would attenuate the blood glucose response to a mixed-nutrient drink, in individuals with T2D. Leucine and isoleucine did not lower the blood glucose response to the drink, although both stimulated insulin before and after the drink. Isoleucine stimulated glucagon both before and after the drink, and leucine tended to stimulate glucagon before the drink. It is uncertain as to why insulin stimulation by leucine and isoleucine was not associated with glucose lowering, but the stimulation of glucagon, presumably by circulating amino acids (i.e. directly stimulating pancreatic α-cells), is likely to be important. For example, defective postprandial suppression of glucagon is well characterised as a feature of T2D, and in our previous study in healthy participants neither leucine nor isoleucine stimulated glucagon.

Overall, the studies presented in this thesis have established that the effects of the BCAAs, leucine, isoleucine and valine, on energy intake and blood glucose, GI functions, including gastric emptying and gut hormone release, involved in the regulation of blood glucose and energy intake, vary substantially. In healthy individuals, leucine and isoleucine appear to be the most promising, reflecting their beneficial effects to lower postprandial blood glucose, in part by the slowing of gastric emptying, while valine does not appear to have an effect on either energy intake or blood glucose. In contrast to their effects in healthy individuals, leucine and isoleucine, surprisingly, did not lower the blood glucose response to a mixed-nutrient drink in T2D.

Some limitations should be taken into account when interpreting the results. Only healthy or T2D men were included, therefore, the findings may not reflect responses in women or obese men without T2D or apply to patients with less well-controlled T2D. In the studies presented

in **Chapters 3**, **4** and **5**, we did not measure all of the key gut and pancreatic hormones involved in energy intake or blood glucose regulation, including ghrelin, PYY, GLP-1, GIP, because of the lack of effect of leucine, isoleucine or valine on energy intake and/or blood glucose and/or CCK concentrations. The high dose we used (10 g) for individual amino acids is higher than the usual intakes within a meal, thus, the dose could be considered to be supraphysiological. Furthermore, doses were standardised, rather than individualised, thus, not taking into account a participant's height, weight and physical activity level. Finally, circulating amino acid concentrations were not measured, which would provide additional information on glucose control and aminostatic controls of eating.

In conclusion, the conditions under which the BCAAs may be effective clearly warrant further evaluation. Thus, studies using orally consumed amino acids, as a mixture/combination, to examine the interplay and/or synergistic effects of nutrients on absorption, as well as targeting other population groups (e.g. women or obese individuals without T2D or individuals with type 1 diabetes) to examine the intra-individual variability, may help to clarify the role of amino acids in the regulation of energy intake and/or blood glucose. Furthermore, by combining leucine or isoleucine, with other amino acids, such as tryptophan or phenylalanine or lysine (previously shown to effect energy intake and/or blood glucose), may enhance their beneficial effects by triggering similar/different GI mechanisms, to have more potent effects to reduce energy intake and/or postprandial blood glucose. This will also help to confirm and, at a minimum, better understand, the effects leucine and isoleucine, as well as their underlying GI mechanisms, associated with modulation of energy intake and/or blood glucose. Ultimately, this would represent important contributions to knowledge gain in this area, as well as facilitating in the search for effective and adverse-effect free, nutrient-based approaches for the prevention/management of overweight/obesity and impaired glucose tolerance/T2D.

# Appendices

## Appendix I:

**Table 2.A1:** Amino acid (AA) composition of test drinks containing 30 g (L) or 70 g (H) of pure whey protein isolate [a,b] (**Chapter 2**).

| | **Treatment** | | |
|---|---|---|---|
| **AA** | **L** | **H** | **Per 100 g** |
| | **g [mmol/L]** | **g [mmol/L]** | **g** |
| Glu | 5.7 [88] | 13.2 [211] | 18.9 |
| Leu | 4.3 [77] | 10.1 [181] | 14.4 |
| Asp | 4.0 [69] | 9.2 [163] | 13.2 |
| Lys | 3.8 [59] | 8.8 [142] | 12.5 |
| Ile | 2.0 [35] | 4.6 [83] | 6.6 |
| Val | 1.8 [35] | 4.1 [82] | 5.9 |
| Ala | 1.8 [46] | 4.1 [108] | 5.9 |
| Thr | 1.7 [33] | 3.9 [77] | 5.5 |
| Pro | 1.4 [28] | 3.4 [70] | 4.8 |
| Tyr | 1.3 [16] | 3.0 [39] | 4.3 |
| Ser | 1.2 [26] | 2.9 [65] | 4.1 |
| Phe | 1.2 [17] | 2.8 [40] | 4.0 |
| Cys | 1.2 [23] | 2.8 [54] | 4.0 |
| Arg | 0.9 [12] | 2.2 [30] | 3.1 |
| Met | 0.8 [12] | 1.9 [30] | 2.7 |
| Trp | 0.8 [9] | 1.9 [22] | 2.7 |
| His | 0.7 [10] | 1.5 [23] | 2.2 |
| Gly | 0.6 [18] | 1.4 [44] | 2.0 |
| Gln | | Not reported | |
| Asn | | Not reported | |
| Total | 35.04 | 81.76 | 116.8 |

[a] The amino acid (AA) composition of 'Whey Protein Isolate 8855' (Fonterra Co-Operative Group Ltd., Auckland, New Zealand) was provided by Fonterra, and glutamine and asparagine were not reported in the product specifications because they contributed to concentrations of glutamic acid and aspartic acid, respectively. [b] AAs (g) are presented in order of most to least abundant within the test drink. Abbreviations for AAs: Alanine: Ala; Arginine: Arg; Asparagine: Asn; Aspartic Acid: Asp; Cysteine: Cys; Glutamine: Gln; Glutamic acid: Glu; Glycine: Gly; Histidine: His; Isoleucine: Ile; Leucine: Leu; Lysine: Lys; Methionine: Met; Phenylalanine: Phe; Serine: Ser; Threonine: Thr; Tryptophan: Trp; Tyrosine: Tyr; Valine: Val.

**Table 2.A2:** Plasma gut hormone responses ($AUCs_{0–180\ min}$), gastric emptying and the amount and total energy consumed at a buffet meal, in response to the test drinks containing either 0 g (C), 30 g (L) or 70 g (H) of pure whey protein dissolved in varying amounts of distilled water, diet cordial, and sodium chloride (all 450 mL and 88 mOsm/L) [a] (**Chapter 2**).

| | **C** | **L** | **H** | ***P Value*** [b] |
|---|---|---|---|---|
| Ghrelin (pmol/L 180 min·$L^{-1}$) | 80,372 ± 10,256 | 66,690 ± 8536 | 59,053 ± 7854 | < 0.01 |
| CCK (pmol/L 180 min·$L^{-1}$) | 729 ± 102 | 1016 ± 131 | 1215 ± 119 | < 0.001 |
| GLP-1 (pmol/L 180 min·$L^{-1}$) | 4564 ± 439 | 6018 ± 408 | 7247 ± 470 | < 0.001 |
| Insulin (mU/L 180 min·$L^{-1}$) | 503 ± 63 | 1463 ± 175 | 2275 ± 300 | < 0.01 |
| Glucagon (pmol/L 180 min·$L^{-1}$) | 3187 ± 158 | 5973 ± 308 | 8219 ± 376 | < 0.001 |
| $T_{50}$ (min) | 12 ± 0.5 | 26 ± 3 [c] | 65 ± 9 [c,d] | < 0.001 |
| Gastric emptying rate (kcal/min) | NA | 2.6 ± 0.3 | 2.9 ± 0.3 | > 0.05 |
| Blood glucose (mmol/L 180 min·$L^{-1}$) | 932 ± 16 | 921 ± 19 | 900 ± 13 | > 0.05 |
| Energy intake (kcal) | 1174 ± 91 | 1027 ± 81 [c] | 998 ± 71 [c] | < 0.01 |
| Amount eaten (g) | 1097 ± 78 | 1114 ± 112 | 1068 ± 69 | > 0.05 |

[a] Data are means ± SEMs; $n = 16$ for gut hormones, blood glucose, energy intake, amount consumed, and gastric emptying. [b] Main effect of protein load determined by one-way repeated measures ANOVA and post-hoc comparisons between two loads were determined using Bonferroni's correction; statistical significance was accepted at $p < 0.05$; [c] Significantly different from C ($p < 0.05$); [d] Significantly different from L ($p < 0.05$). Abbreviations: CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; $T_{50}$, the time at which 50% of the drink had emptied from the stomach (T50) and which was used to calculate gastric emptying rate of each protein drink.

**Table 2.A3:** Within-subject relationships between the $iAUCs_{0–180\ min}$ of each of the 20 amino acids (displayed in order of abundance of the amino acids within the whey protein drinks) with the load of protein, following consumption of the test the drinks containing either 0 g (C), 30 g (L) or 70 g (H) of pure whey protein dissolved in varying amounts of distilled water, diet cordial and sodium chloride (all 450 mL and 88 mOsm/L) (**Chapter 2**).

| AA | $R^2$ *Value* | *P Value* |
|---|---|---|
| mmol/L | | |
| Glu (NE) | 0.66 | < 0.001 |
| Leu (E) | 0.84 | < 0.001 |
| Asp (NE) | 0.83 | < 0.001 |
| Lys (E) | 0.76 | < 0.001 |
| Ile (E) | 0.84 | < 0.001 |
| Val (E) | 0.66 | < 0.001 |
| Ala (NE) | 0.26 | 0.003 |
| Thr (E) | 0.61 | < 0.001 |
| Pro (C) | 0.31 | 0.001 |
| Tyr (C) | 0.72 | < 0.001 |
| Ser (C) | 0.35 | < 0.001 |
| Phe (E) | 0.54 | < 0.001 |
| Cys (C) | 0.14 | 0.034 |
| Arg (C) | 0.51 | < 0.001 |
| Met (E) | 0.74 | < 0.001 |
| Trp (E) | 0.74 | < 0.001 |
| His (E) | 0.11 | 0.062 |
| Gly (C) | 0 | 0.874 |
| Gln (C) | Not reported | |
| Asn (NE) | Not reported | |

Data are within-subject $R^2$ values for $n = 16$; Statistical significance was accepted at $p < 0.05$. Abbreviations for AAs: Alanine: Ala; Arginine: Arg; Asparagine: Asn; Aspartic Acid: Asp; Cysteine: Cys; Glutamine: Gln; Glutamic acid: Glu; Glycine: Gly; Histidine: His; Isoleucine: Ile; Leucine: Leu; Lysine: Lys; Methionine: Met; Phenylalanine: Phe; Serine: Ser; Threonine: Thr; Tryptophan: Trp; Tyrosine: Tyr; Valine: Val. Essential AAs (E); Non-essential AAs (NE); Conditional AAs (C).

# Chapter 7: References

1. Ogden CL, Flegal KM, Carroll MD, Johnson CL. Prevalence and trends in overweight among US children and adolescents, 1999-2000. JAMA. 2002;288(14):1728-32.
2. Australian Bureau of Statistics. National Health Survey: diabetes melllitus 2017-2018. Internet: https://www.abs.gov.au/statistics/health/health-conditions-and-risks/diabetes/latest-release (accessed 25 November 2019).
3. World Health Organization. Obesity and overweight - fact sheet 2019. Internet: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight (accessed 28 November 2019).
4. Australian Bureau of Statistics. National Health Survey: overweight and obesity 2017-2018. Internet: https://www.abs.gov.au/statistics/health/health-conditions-and-risks/national-health-survey-first-results/latest-release (accessed 25 November 2019).
5. World Health Organization. Body Mass Index (BMI). Internet: https://www.who.int/data/gho/data/themes/topics/topic-details/GHO/body-mass-index (acessed 8 July 2021).
6. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ Tech Rep Ser. 2000;894:i-xii, 1-253.
7. Rothman KJ. BMI-related errors in the measurement of obesity. Int J Obes. 2008;32(3):S56-S9.
8. Han TS, Seidell JC, Currall JE, Morrison CE, Deurenberg P, Lean ME. The influences of height and age on waist circumference as an index of adiposity in adults. Int J Obes Relat Metab Disord. 1997;21(1):83-9.
9. Pouliot MC, Despres JP, Lemieux S, Moorjani S, Bouchard C, Tremblay A, et al. Waist circumference and abdominal sagittal diameter: best simple anthropometric indexes of abdominal visceral adipose tissue accumulation and related cardiovascular risk in men and women. Am J Cardiol. 1994;73(7):460-8.
10. Han TS, Richmond P, Avenell A, Lean ME. Waist circumference reduction and cardiovascular benefits during weight loss in women. Int J Obes Relat Metab Disord. 1997;21(2):127-34.
11. Hill JO, Wyatt HR, Peters JC. Energy balance and obesity. Circulation. 2012;126(1):126-32.
12. Australian Institute of Health and Welfare 2016. Australia's health 2016. Australia's health series no. 15. Cat. no. AUS 199. Canberra: AIHW.
13. Carr RD, Larsen MO, Winzell MS, Jelic K, Lindgren O, Deacon CF, et al. Incretin and islet hormonal responses to fat and protein ingestion in healthy men. Am J Physiol Endocrinol Metab. 2008;295(4):E779-84.
14. Rankinen T, Zuberi A, Chagnon YC, Weisnagel SJ, Argyropoulos G, Walts B, et al. The human obesity gene map: the 2005 update. Obesity (Silver Spring). 2006;14(4):529-644.
15. Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, et al. A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science. 2007;316(5826):889-94.
16. Stunkard AJ, Sørensen TI, Hanis C, Teasdale TW, Chakraborty R, Schull WJ, et al. An adoption study of human obesity. N Engl J Med. 1986;314(4):193-8.
17. Bouchard C, Tremblay A, Després JP, Nadeau A, Lupien PJ, Thériault G, et al. The response to long-term overfeeding in identical twins. N Engl J Med. 1990;322(21):1477-82.
18. Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, et al. Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature. 1997;387(6636):903-8.
19. Saeed S, Bonnefond A, Manzoor J, Philippe J, Durand E, Arshad M, et al. Novel LEPR mutations in obese Pakistani children identified by PCR-based enrichment and next generation sequencing. Obesity (Silver Spring). 2014;22(4):1112-7.

20. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature. 1994;372(6505):425-32.
21. Wasim M, Awan FR, Najam SS, Khan AR, Khan HN. Role of leptin deficiency, inefficiency, and leptin receptors in obesity. Biochem Genet. 2016;54(5):565-72.
22. Gibson WT, Farooqi IS, Moreau M, DePaoli AM, Lawrence E, O'Rahilly S, et al. Congenital leptin deficiency due to homozygosity for the Delta133G mutation: report of another case and evaluation of response to four years of leptin therapy. J Clin Endocrinol Metab. 2004;89(10):4821-6.
23. Little TJ, Horowitz M, Feinle-Bisset C. Modulation by high-fat diets of gastrointestinal function and hormones associated with the regulation of energy intake: implications for the pathophysiology of obesity. Am J Clin Nutr. 2007;86(3):531-41.
24. Haslam DW, James WP. Obesity. Lancet. 2005;366(9492):1197-209.
25. Prentice AM, Jebb SA. Fast foods, energy density and obesity: a possible mechanistic link. Obes Rev. 2003;4(4):187-94.
26. Richard D. Cognitive and autonomic determinants of energy homeostasis in obesity. Nat Rev Endocrinol. 2015;11(8):489-501.
27. Vettor R, Milan G, Rossato M, Federspil G. Review article: adipocytokines and insulin resistance. Aliment Pharmacol Ther. 2005;22 Suppl 2:3-10.
28. Australian Institute of Health and Welfare. Overweight and obesity 2017. Internet: https://www.aihw.gov.au/reports/overweight-obesity/overweight-and-obesity-an-interactive-insight/contents/what-is-overweight-and-obesity (accessed 29 November 2019).
29. IDF Diabetes Atlas 9th edition 2019. Internet: https://www.diabetesatlas.org/en/ (accessed 5 August 2020).
30. Classification of diabetes mellitus. Geneva: World Health Organisarion; 2019.
31. The Royal Australian College of General Practioners. Management of type 2 diabetes: A handbook for general practice. East Melbounre, Vic: RACGP, 2020.
32. The Royal Australian College of General Practitioners. General practice management of type 2 diabetes: 2016–18. East Melbourne, Vic: RACGP, 2016.
33. Wilding JP. The importance of weight management in type 2 diabetes mellitus. Int J Clin Pract. 2014;68(6):682-91.
34. Van Gaal LF, Wauters MA, De Leeuw IH. The beneficial effects of modest weight loss on cardiovascular risk factors. Int J Obes Relat Metab Disord. 1997;21 Suppl 1:S5-9.
35. Vidal J. Updated review on the benefits of weight loss. Int J Obes Relat Metab Disord. 2002;26 Suppl 4:S25-8.
36. Van Gaal LF, Mertens IL, Ballaux D. What is the relationship between risk factor reduction and degree of weight loss? Eur Heart J Suppl. 2005;7(suppl_L):L21-L6.
37. World Health Organization. Redefining obesity and its treatment. Internet: http://www.wpro.who.int/nutrition/documents/docs/Redefiningobesity.pdf?ua=1 (accessed 28 November 2019).
38. Sumithran P, Proietto J. The defence of body weight: A physiological basis: For weight regain after weight loss. Clinical science (London, England : 1979). 2013;124:231-41.
39. Müller MJ, Bosy-Westphal A, Heymsfield SB. Is there evidence for a set point that regulates human body weight? F1000 Med Rep. 2010;2:59.
40. Arciero PJ, Edmonds R, He F, Ward E, Gumpricht E, Mohr A, et al. Protein-pacing caloric-restriction enhances body composition similarly in obese men and women during weight loss and sustains efficacy during long-term weight maintenance. Nutrients. 2016;8(8):476.
41. National Health and Medical Research Council (2013) Clinical practice guidelines for the management of overweight and obesity in adults, adolescents and children in Australia. Melbourne: National Health and Medical Research Council.

42. Doucet E, Imbeault P, St-Pierre S, Almeras N, Mauriege P, Richard D, et al. Appetite after weight loss by energy restriction and a low-fat diet-exercise follow-up. Int J Obes Relat Metab Disord. 2000;24(7):906-14.
43. Wadden TA, Sternberg JA, Letizia KA, Stunkard AJ, Foster GD. Treatment of obesity by very low calorie diet, behavior therapy, and their combination: a five-year perspective. Int J Obes. 1989;13 Suppl 2:39-46.
44. Wadden TA. Treatment of obesity by moderate and severe caloric restriction. Results of clinical research trials. Ann Intern Med. 1993;119(7 Pt 2):688-93.
45. Astrup A, Grunwald GK, Melanson EL, Saris WHM, Hill JO. The role of low-fat diets in body weight control: a meta-analysis of ad libitum dietary intervention studies. Int J Obes. 2000;24(12):1545-52.
46. Samaha FF, Iqbal N, Seshadri P, Chicano KL, Daily DA, McGrory J, et al. A low-carbohydrate as compared with a low-fat diet in severe obesity. N Engl J Med 2003;348(21):2074-81.
47. Brehm BJ, Seeley RJ, Daniels SR, D'Alessio DA. A randomized trial comparing a very low carbohydrate diet and a calorie-restricted low fat diet on body weight and cardiovascular risk factors in healthy women. J Clin Endocrinol Metab. 2003;88(4):1617-23.
48. Skov AR, Toubro S, Ronn B, Holm L, Astrup A. Randomized trial on protein vs carbohydrate in ad libitum fat reduced diet for the treatment of obesity. Int J Obes Relat Metab Disord. 1999;23(5):528-36.
49. Noakes M, Keogh JB, Foster PR, Clifton PM. Effect of an energy-restricted, high-protein, low-fat diet relative to a conventional high-carbohydrate, low-fat diet on weight loss, body composition, nutritional status, and markers of cardiovascular health in obese women. Am J Clin Nutr. 2005;81(6):1298-306.
50. Hall KD. A review of the carbohydrate-insulin model of obesity. Eur J Clin Nutr. 2017;71(3):323-6.
51. Abete I, Astrup A, Martínez JA, Thorsdottir I, Zulet MA. Obesity and the metabolic syndrome: role of different dietary macronutrient distribution patterns and specific nutritional components on weight loss and maintenance. Nutr Rev. 2010;68(4):214-31.
52. Poppitt SD, McCormack D, Buffenstein R. Short-term effects of macronutrient preloads on appetite and energy intake in lean women. Physiol Behav. 1998;64(3):279-85.
53. Leidy HJ, Clifton PM, Astrup A, Wycherley TP, Westerterp-Plantenga MS, Luscombe-Marsh ND, et al. The role of protein in weight loss and maintenance. Am J Nutr. 2015;101(6):1320S-9S.
54. Westerterp-Plantenga MS, Lemmens SG, Westerterp KR. Dietary protein – its role in satiety, energetics, weight loss and health. Br J Nutr. 2012;108(S2):S105-S12.
55. Westerterp-Plantenga MS, MJ IJ, Wijckmans-Duijsens NE. The role of macronutrient selection in determining patterns of food intake in obese and non-obese women. Eur J Clin Nutr. 1996;50(9):580-91.
56. Layman DK. Dietary Guidelines should reflect new understandings about adult protein needs. Nutr Metab. 2009;6(1):12.
57. Joint FAO/WHO/UNU Expert consultation on protein and amino acid requirements in human nutrition: report of a joint FAO/WHO/UNU expert consultation 2002; Geneva: World Health Organization. Internet: http://www.who.int/iris/handle/10665/43411 (accessed 29 November 2019).
58. Simpson SJ, Raubenheimer D. Obesity: the protein leverage hypothesis. Obes Rev. 2005;6(2):133-42.
59. Gosby AK, Conigrave AD, Lau NS, Iglesias MA, Hall RM, Jebb SA, et al. Testing protein leverage in lean humans: a randomised controlled experimental study. PloS one. 2011;6(10):e25929-e.

60. Wycherley TP, Moran LJ, Clifton PM, Noakes M, Brinkworth GD. Effects of energy-restricted high-protein, low-fat compared with standard-protein, low-fat diets: a meta-analysis of randomized controlled trials. Am J Clin Nutr. 2012;96(6):1281-98.
61. Weigle DS, Breen PA, Matthys CC, Callahan HS, Meeuws KE, Burden VR, et al. A high-protein diet induces sustained reductions in appetite, ad libitum caloric intake, and body weight despite compensatory changes in diurnal plasma leptin and ghrelin concentrations. Am J Clin Nutr. 2005;82(1):41-8.
62. Nuttall FQ, Gannon MC. The metabolic response to a high-protein, low-carbohydrate diet in men with type 2 diabetes mellitus. Metabolism. 2006;55(2):243-51.
63. Nuttall FQ, Gannon MC. Effect of a LoBAG30 diet on protein metabolism in men with type 2 diabetes. A Randomized Controlled Trial. Nutr Metab (Lond). 2012;9(1):43.
64. Gannon MC, Nuttall FQ. Effect of a high-protein, low-carbohydrate diet on blood glucose control in people with type 2 diabetes. Diabetes. 2004;53(9):2375-82.
65. McAuley KA, Hopkins CM, Smith KJ, McLay RT, Williams SM, Taylor RW, et al. Comparison of high-fat and high-protein diets with a high-carbohydrate diet in insulin-resistant obese women. Diabetologia. 2005;48(1):8-16.
66. Piatti PM, Monti LD, Magni F, Fermo I, Baruffaldi L, Nasser R, et al. Hypocaloric high-protein diet improves glucose oxidation and spares lean body mass: Comparison to hypocaloric high-carbohydrate diet. Metabolism. 1994;43(12):1481-7.
67. Gannon MC, Nuttall FQ, Saeed A, Jordan K, Hoover H. An increase in dietary protein improves the blood glucose response in persons with type 2 diabetes. Am J Clin Nutr. 2003;78(4):734-41.
68. Boden G, Sargrad K, Homko C, Mozzoli M, Stein TP. Effect of a low-carbohydrate diet on appetite, blood glucose levels, and insulin resistance in obese patients with type 2 diabetes. Ann Intern Med. 2005;142(6):403-11.
69. Jenkins DJ, Wolever TM, Taylor RH, Barker H, Fielden H, Baldwin JM, et al. Glycemic index of foods: a physiological basis for carbohydrate exchange. Am J Clin Nutr. 1981;34(3):362-6.
70. Farnsworth E, Luscombe ND, Noakes M, Wittert G, Argyiou E, Clifton PM. Effect of a high-protein, energy-restricted diet on body composition, glycemic control, and lipid concentrations in overweight and obese hyperinsulinemic men and women. Am J Clin Nutr. 2003;78(1):31-9.
71. Yancy WS, Jr., Olsen MK, Guyton JR, Bakst RP, Westman EC. A low-carbohydrate, ketogenic diet versus a low-fat diet to treat obesity and hyperlipidemia: a randomized, controlled trial. Ann Intern Med. 2004;140(10):769-77.
72. Eisenstein J, Roberts SB, Dallal G, Saltzman E. High-protein weight-loss diets: are they safe and do they work? A review of the experimental and epidemiologic data. Nutr Rev. 2002;60(7 Pt 1):189-200.
73. Cuenca-Sánchez M, Navas-Carrillo D, Orenes-Piñero E. Controversies surrounding high-protein diet intake: satiating effect and kidney and bone health. Adv Nutr. 2015;6(3):260-6.
74. Barzel US, Massey LK. Excess dietary protein can adversely affect bone. J Nutr. 1998;128(6):1051-3.
75. Cao JJ, Johnson LK, Hunt JR. A diet high in meat protein and potential renal acid load increases fractional calcium absorption and urinary calcium excretion without affecting markers of bone resorption or formation in postmenopausal women. J Nutr. 2011;141(3):391-7.
76. Kerstetter JE, O'Brien KO, Insogna KL. Dietary protein, calcium metabolism, and skeletal homeostasis revisited. Am J Clin Nutr. 2003;78(3 Suppl):584s-92s.

77. Spencer H, Kramer L, DeBartolo M, Norris C, Osis D. Further studies of the effect of a high protein diet as meat on calcium metabolism. Am J Clin Nutr. 1983;37(6):924-9.
78. Rodríguez-Iturbe B, Herrera J, García R. Relationship between glomerular filtration rate and renal blood flow at different levels of protein-induced hyperfiltration in man. Clin Sci. 1988;74(1):11-5.
79. Brändle E, Sieberth HG, Hautmann RE. Effect of chronic dietary protein intake on the renal function in healthy subjects. Eur J Clin Nutr. 1996;50(11):734-40.
80. Skov AR, Toubro S, Bülow J, Krabbe K, Parving HH, Astrup A. Changes in renal function during weight loss induced by high vs low-protein low-fat diets in overweight subjects. Int J Obes Relat Metab Disord. 1999;23(11):1170-7.
81. Brinkworth GD, Buckley JD, Noakes M, Clifton PM. Renal function following long-term weight loss in individuals with abdominal obesity on a very-low-carbohydrate diet vs high-carbohydrate diet. J Am Diet Assoc. 2010;110(4):633-8.
82. Pilitsi E, Farr OM, Polyzos SA, Perakakis N, Nolen-Doerr E, Papathanasiou AE, et al. Pharmacotherapy of obesity: Available medications and drugs under investigation. Metabolism. 2019;92:170-92.
83. Khera R, Murad MH, Chandar AK, Dulai PS, Wang Z, Prokop LJ, et al. Association of pharmacological treatments for obesity with weight loss and adverse events: a systematic review and meta-analysis. JAMA. 2016;315(22):2424-34.
84. Padwal R, Li SK, Lau DC. Long-term pharmacotherapy for obesity and overweight. Cochrane Database Syst Rev. 2004(3):Cd004094.
85. Australian Medicines Handbook 2019. Rossi S, Editor. Adelaide, Australia: Australian Medicines Handbook Ltd; 2019.
86. Rueda-Clausen CF, Padwal RS. Pharmacotherapy for weight loss. BMJ. 2014;348:g3526.
87. Haddock CK, Poston WS, Dill PL, Foreyt JP, Ericsson M. Pharmacotherapy for obesity: a quantitative analysis of four decades of published randomized clinical trials. Int J Obes Relat Metab Disord. 2002;26(2):262-73.
88. Black RL, Higbea AM, Duval C, Chastain LM. Pharmacological management of obesity in patients with type 2 diabetes. Expert Rev Endocrinol Metab. 2016;11(5):415-23.
89. Pi-Sunyer X, Astrup A, Fujioka K, Greenway F, Halpern A, Krempf M, et al. A Randomized, Controlled Trial of 3.0 mg of Liraglutide in Weight Management. N Engl J Med. 2015;373(1):11-22.
90. Bello NT. Update on drug safety evaluation of naltrexone/bupropion for the treatment of obesity. Expert Opin Drug Saf. 2019;18(7):549-52.
91. Neary MT, Batterham RL. Gut hormones: Implications for the treatment of obesity. Pharmacol Ther. 2009;124(1):44-56.
92. Malin SK, Kashyap SR. Differences in weight loss and gut hormones: rouen-y gastric bypass and sleeve gastrectomy surgery. Current obesity reports. 2015;4(2):279-86.
93. Wolfe BM, Kvach E, Eckel RH. Treatment of obesity: weight loss and bariatric surgery. Circ Res. 2016;118(11):1844-55.
94. Sjöström CD, Lissner L, Wedel H, Sjöström L. Reduction in incidence of diabetes, hypertension and lipid disturbances after intentional weight loss induced by bariatric surgery: the SOS Intervention Study. Obes Res. 1999;7(5):477-84.
95. Sjöström L, Narbro K, Sjöström CD, Karason K, Larsson B, Wedel H, et al. Effects of bariatric surgery on mortality in Swedish obese subjects. N Engl J Med. 2007;357(8):741-52.
96. Biobaku F, Ghanim H, Monte SV, Caruana JA, Dandona P. Bariatric surgery: remission of inflammation, cardiometabolic benefits, and common adverse effects. J Endocr Soc. 2020;4(9):bvaa049.

97. Pories WJ, Swanson MS, MacDonald KG, Long SB, Morris PG, Brown BM, et al. Who would have thought it? An operation proves to be the most effective therapy for adult-onset diabetes mellitus. Ann Surg. 1995;222(3):339-50; discussion 50-2.
98. Jiménez A, Casamitjana R, Flores L, Viaplana J, Corcelles R, Lacy A, et al. Long-term effects of sleeve gastrectomy and Roux-en-Y gastric bypass surgery on type 2 diabetes mellitus in morbidly obese subjects. Ann Surg. 2012;256(6):1023-9.
99. Lee PC, Dixon J. Bariatric-metabolic surgery: A guide for the primary care physician. Aust Fam Physician. 2017;46(7):465-71.
100. Ramón JM, Salvans S, Crous X, Puig S, Goday A, Benaiges D, et al. Effect of Roux-en-Y gastric bypass vs sleeve gastrectomy on glucose and gut hormones: a prospective randomised trial. J Gastrointest Surg. 2012;16(6):1116-22.
101. Flatt PR. Dorothy Hodgkin Lecture 2008. Gastric inhibitory polypeptide (GIP) revisited: a new therapeutic target for obesity-diabetes? Diabetes Medicine. 2008;25(7):759-64.
102. le Roux CW, Aylwin SJ, Batterham RL, Borg CM, Coyle F, Prasad V, et al. Gut hormone profiles following bariatric surgery favor an anorectic state, facilitate weight loss, and improve metabolic parameters. Ann Surg. 2006;243(1):108-14.
103. Yurgin N, Secnik K, Lage MJ. Antidiabetic prescriptions and glycemic control in German patients with type 2 diabetes mellitus: a retrospective database study. Clin Ther. 2007;29(2):316-25.
104. Mata-Cases M, Franch-Nadal J, Real J, Mauricio D. Glycaemic control and antidiabetic treatment trends in primary care centres in patients with type 2 diabetes mellitus during 2007-2013 in Catalonia: a population-based study. BMJ Open. 2016;6(10):e012463.
105. Upadhyay J, Polyzos SA, Perakakis N, Thakkar B, Paschou SA, Katsiki N, et al. Pharmacotherapy of type 2 diabetes: An update. Metabolism. 2018;78:13-42.
106. Goodarzi MO, Bryer-Ash M. Metformin revisited: re-evaluation of its properties and role in the pharmacopoeia of modern antidiabetic agents. Diabetes Obes Metab. 2005;7(6):654-65.
107. Wu T, Horowitz M, Rayner C. New insights into the anti-diabetic actions of metformin: from the liver to the gut. Expert Rev Gastroenterol Hepatol. 2016;11.
108. Davoren P. Glucose-lowering medicines for type 2 diabetes. Aust Fam Physician. 2015;44(5):176-9.
109. Proks P, Reimann F, Green N, Gribble F, Ashcroft F. Sulfonylurea stimulation of insulin secretion. Diabetes. 2002;51 Suppl 3:S368-76.
110. Nyenwe EA, Jerkins TW, Umpierrez GE, Kitabchi AE. Management of type 2 diabetes: evolving strategies for the treatment of patients with type 2 diabetes. Metabolism. 2011;60(1):1-23.
111. Nauck MA, Heimesaat MM, Orskov C, Holst JJ, Ebert R, Creutzfeldt W. Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. J Clin Invest. 1993;91(1):301-7.
112. Charbonnel B, Karasik A, Liu J, Wu M, Meininger G. Efficacy and safety of the dipeptidyl peptidase-4 inhibitor sitagliptin added to ongoing metformin therapy in patients with type 2 diabetes inadequately controlled with metformin alone. Diabetes Care. 2006;29(12):2638-43.
113. Fala L. Trulicity (Dulaglutide): A new GLP-1 receptor agonist once-weekly subcutaneous injection approved for the treatment of patients with type 2 diabetes. Am Health Drug Benefits. 2015;8(Spec Feature):131-4.

114. Apovian CM, Bergenstal RM, Cuddihy RM, Qu Y, Lenox S, Lewis MS, et al. Effects of exenatide combined with lifestyle modification in patients with type 2 diabetes. Am J Med. 2010;123(5):468 e9-17.
115. Vasilakou D, Karagiannis T, Athanasiadou E, Mainou M, Liakos A, Bekiari E, et al. Sodium-glucose cotransporter 2 inhibitors for type 2 diabetes: a systematic review and meta-analysis. Ann Intern Med. 2013;159(4):262-74.
116. Buse JB, Wexler DJ, Tsapas A, Rossing P, Mingrone G, Mathieu C, et al. 2019 update to: Management of hyperglycaemia in type 2 diabetes, 2018. A consensus report by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). Diabetologia. 2020;63(2):221-8.
117. Clifton P. Effects of a high protein diet on body weight and comorbidities associated with obesity. Br J Nutr. 2012;108(S2):S122-S9.
118. Dong JY, Zhang ZL, Wang PY, Qin LQ. Effects of high-protein diets on body weight, glycaemic control, blood lipids and blood pressure in type 2 diabetes: meta-analysis of randomised controlled trials. Br J Nutr. 2013;110(5):781-9.
119. Larsen TM, Dalskov SM, van Baak M, Jebb SA, Papadaki A, Pfeiffer AF, et al. Diets with high or low protein content and glycemic index for weight-loss maintenance. N Engl J Med. 2010;363(22):2102-13.
120. Akhavan T, Luhovyy BL, Brown PH, Cho CE, Anderson GH. Effect of premeal consumption of whey protein and its hydrolysate on food intake and postmeal glycemia and insulin responses in young adults. Am J Clin Nutr. 2010;91(4):966-75.
121. Ma J, Stevens JE, Cukier K, Maddox AF, Wishart JM, Jones KL, et al. Effects of a protein preload on gastric emptying, glycemia, and gut hormones after a carbohydrate meal in diet-controlled type 2 diabetes. Diabetes Care. 2009;32(9):1600-2.
122. Frid AH, Nilsson M, Holst JJ, Bjorck IM. Effect of whey on blood glucose and insulin responses to composite breakfast and lunch meals in type 2 diabetic subjects. Am J Clin Nutr. 2005;82(1):69-75.
123. Steinert RE, Feinle-Bisset C, Asarian L, Horowitz M, Beglinger C, Geary N. Ghrelin, CCK, GLP-1, and PYY(3-36): secretory controls and physiological roles in eating and glycemia in health, obesity, and after RYGB. Physiol Rev. 2017;97(1):411-63.
124. Lindgren O, Pacini G, Tura A, Holst JJ, Deacon CF, Ahren B. Incretin effect after oral amino acid ingestion in humans. J Clin Endocrinol Metab. 2015;100(3):1172-6.
125. Potier M, Darcel N, Tomé D. Protein, amino acids and the control of food intake. Curr Opin Clin Nutr Metab Care. 2009;12(1):54-8.
126. Iwatsuki K, Uneyama H. Sense of taste in the gastrointestinal tract. J Pharmacol Sci. 2012;118(2):123-8.
127. Hajishafiee M, Bitarafan V, Feinle-Bisset C. Gastrointestinal sensing of meal-related signals in humans, and dysregulations in eating-related disorders. Nutrients. 2019;11(6):1298.
128. Camilleri M. Integrated upper gastrointestinal response to food intake. Gastroenterology. 2006;131(2):640-58.
129. Azpiroz F, Malagelada JR. Intestinal control of gastric tone. Am J Physiol 1985;249(4 Pt 1):G501-9.
130. Phillips LK, Deane AM, Jones KL, Rayner CK, Horowitz M. Gastric emptying and glycaemia in health and diabetes mellitus. Nat Rev Endocrinol. 2015;11(2):112-28.
131. Horowitz M, Dent J, Fraser R, Sun W, Hebbard G. Role and integration of mechanisms controlling gastric emptying. Dig Dis Sci. 1994;39(12 Suppl):7S-13S.
132. Stevens JE, Jones KL, Rayner CK, Horowitz M. Pathophysiology and pharmacotherapy of gastroparesis: current and future perspectives. Expert Opin Pharmacother. 2013;14(9):1171-86.

133. Collins PJ, Horowitz M, Cook DJ, Harding PE, Shearman DJ. Gastric emptying in normal subjects--a reproducible technique using a single scintillation camera and computer system. Gut. 1983;24(12):1117-25.
134. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 1999;402(6762):656-60.
135. McGowan BM, Bloom SR. Gut hormones regulating appetite and metabolism. Drug Discov Today Ther Strateg. 2007;4(2):147-51.
136. Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, et al. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology. 2000;141(11):4255-61.
137. Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, et al. Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. Diabetes. 2001;50(2):227-32.
138. Näslund E, Hellström PM. Appetite signaling: from gut peptides and enteric nerves to brain. Physiol Behav. 2007;92(1):256-62.
139. Foster-Schubert KE, Overduin J, Prudom CE, Liu J, Callahan HS, Gaylinn BD, et al. Acyl and total ghrelin are suppressed strongly by ingested proteins, weakly by lipids, and biphasically by carbohydrates. J Clin Endocrinol Metab. 2008;93(5):1971-9.
140. Brennan IM, Luscombe-Marsh ND, Seimon RV, Otto B, Horowitz M, Wishart JM, et al. Effects of fat, protein, and carbohydrate and protein load on appetite, plasma cholecystokinin, peptide YY, and ghrelin, and energy intake in lean and obese men. Am J Physiol Gastrointest Liver Physiol. 2012;303(1):G129-40.
141. Wren AM, Bloom SR. Gut hormones and appetite control. Gastroenterology. 2007;132(6):2116-30.
142. Otto B, Cuntz U, Fruehauf E, Wawarta R, Folwaczny C, Riepl RL, et al. Weight gain decreases elevated plasma ghrelin concentrations of patients with anorexia nervosa. Eur J Endocrinol. 2001;145(5):669-73.
143. Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, et al. Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab. 2001;86(12):5992.
144. Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, et al. Ghrelin stimulates gastric acid secretion and motility in rats. Biochem Biophys Res Commun. 2000;276(3):905-8.
145. Tack J, Depoortere I, Bisschops R, Delporte C, Coulie B, Meulemans A, et al. Influence of ghrelin on interdigestive gastrointestinal motility in humans. Gut. 2006;55(3):327-33.
146. Churm R, Davies JS, Stephens JW, Prior SL. Ghrelin function in human obesity and type 2 diabetes: a concise review. Obes Rev. 2017;18(2):140-8.
147. Benso A, St-Pierre DH, Prodam F, Gramaglia E, Granata R, van der Lely AJ, et al. Metabolic effects of overnight continuous infusion of unacylated ghrelin in humans. Eur J Endocrinol. 2012;166(5):911-6.
148. Gasco V, Beccuti G, Marotta F, Benso A, Granata R, Broglio F, et al. Endocrine and metabolic actions of ghrelin. Endocr Dev. 2010;17:86-95.
149. Broglio F, Gottero C, Benso A, Prodam F, Destefanis S, Gauna C, et al. Effects of ghrelin on the insulin and glycemic responses to glucose, arginine, or free fatty acids load in humans. J Clin Endocrinol Metab. 2003;88(9):4268-72.
150. Guido M, Romualdi D, De Marinis L, Porcelli T, Giuliani M, Costantini B, et al. Administration of exogenous ghrelin in obese patients with polycystic ovary syndrome: effects on plasma levels of growth hormone, glucose, and insulin. Fertil Steril. 2007;88(1):125-30.

151. Small CJ, Bloom SR. Gut hormones and the control of appetite. Trends Endocrinol Metab. 2004;15(6):259-63.
152. Little TJ, Horowitz M, Feinle-Bisset C. Role of cholecystokinin in appetite control and body weight regulation. Obes Rev. 2005;6(4):297-306.
153. Vella A, Camilleri M. The Gastrointestinal Tract as an Integrator of Mechanical and Hormonal Response to Nutrient Ingestion. Diabetes. 2017;66(11):2729-37.
154. Prinz P, Stengel A. Control of Food Intake by Gastrointestinal Peptides: Mechanisms of Action and Possible Modulation in the Treatment of Obesity. J Neurogastroenterol Motil. 2017;23(2):180-96.
155. Sayegh AI. The role of cholecystokinin receptors in the short-term control of food intake. Prog Mol Biol Transl Sci. 2013;114:277-316.
156. Hill DR, Campbell NJ, Shaw TM, Woodruff GN. Autoradiographic localization and biochemical characterization of peripheral type CCK receptors in rat CNS using highly selective nonpeptide CCK antagonists. J Neurosci. 1987;7(9):2967-76.
157. Feinle C, O'Donovan D, Doran S, Andrews JM, Wishart J, Chapman I, et al. Effects of fat digestion on appetite, APD motility, and gut hormones in response to duodenal fat infusion in humans. Am J Physiol Gastrointest Liver Physiol. 2003;284(5):G798-G807.
158. Lieverse RJ, Jansen JB, Masclee AA, Rovati LC, Lamers CB. Effect of a low dose of intraduodenal fat on satiety in humans: studies using the type A cholecystokinin receptor antagonist loxiglumide. Gut. 1994;35(4):501-5.
159. Little TJ, Doran S, Meyer JH, Smout AJ, O'Donovan DG, Wu KL, et al. The release of GLP-1 and ghrelin, but not GIP and CCK, by glucose is dependent upon the length of small intestine exposed. Am J Physiol Endocrinol Metab. 2006;291(3):E647-55.
160. Moran TH. Cholecystokinin and satiety: current perspectives. Nutrition. 2000;16(10):858-65.
161. Muurahainen N, Kissileff HR, Derogatis AJ, Xavier Pi-Sunyer F. Effects of cholecystokinin-octapeptide (CCK-8) on food intake and gastric emptying in man. Physiol Behav. 1988;44(4):645-9.
162. Brennan IM, Little TJ, Feltrin KL, Smout AJ, Wishart JM, Horowitz M, et al. Dose-dependent effects of cholecystokinin-8 on antropyloroduodenal motility, gastrointestinal hormones, appetite, and energy intake in healthy men. Am J Physiol Endocrinol Metab. 2008;295(6):E1487-94.
163. Fried M, Erlacher U, Schwizer W, Lochner C, Koerfer J, Beglinger C, et al. Role of cholecystokinin in the regulation of gastric emptying and pancreatic enzyme secretion in humans. Studies with the cholecystokinin-receptor antagonist loxiglumide. Gastroenterology. 1991;101(2):503-11.
164. Mesquita MA, Thompson DG, Troncon LE, D'Amato M, Rovati LC, Barlow J. Effect of cholecystokinin-A receptor blockade on lipid-induced gastric relaxation in humans. Am J Clin Nutr. 1997;273(1 Pt 1):G118-23.
165. Fried M, Schwizer W, Beglinger C, Keller U, Jansen JB, Lamers CB. Physiological role of cholecystokinin on postprandial insulin secretion and gastric meal emptying in man. Studies with the cholecystokinin receptor antagonist loxiglumide. Diabetologia. 1991;34(10):721-6.
166. Beglinger C, Degen L, Matzinger D, D'Amato M, Drewe J. Loxiglumide, a CCK-A receptor antagonist, stimulates calorie intake and hunger feelings in humans. Am J Physiol Regul Integr Comp Physiol. 2001;280(4):R1149-54.
167. Liddle RA, Rushakoff RJ, Morita ET, Beccaria L, Carter JD, Goldfine ID. Physiological role for cholecystokinin in reducing postprandial hyperglycemia in humans. J Clin Invest. 1988;81(6):1675-81.
168. Onaga T, Zabielski R, Kato S. Multiple regulation of peptide YY secretion in the digestive tract. Peptides. 2002;23(2):279-90.

169. Miazza B, Palma R, Lachance JR, Chayvialle JA, Jonard PP, Modigliani R. Jejunal secretory effect of intraduodenal food in humans. A comparison of mixed nutrients, proteins, lipids, and carbohydrates. Gastroenterology. 1985;88(5 Pt 1):1215-22.
170. Groger G, Unger A, Holst JJ, Goebell H, Layer P. Ileal carbohydrates inhibit cholinergically stimulated exocrine pancreatic secretion in humans. Int J Pancreatol. 1997;22(1):23-9.
171. Chaudhri OB, Small CJ, Bloom SR. The gastrointestinal tract and the regulation of appetite. Drug Discovery Today: Disease Mechanisms. 2005;2(3):289-94.
172. Spiller RC, Trotman IF, Adrian TE, Bloom SR, Misiewicz JJ, Silk DB. Further characterisation of the 'ileal brake' reflex in man--effect of ileal infusion of partial digests of fat, protein, and starch on jejunal motility and release of neurotensin, enteroglucagon, and peptide YY. Gut. 1988;29(8):1042-51.
173. Read NW, McFarlane A, Kinsman RI, Bates TE, Blackhall NW, Farrar GB, et al. Effect of infusion of nutrient solutions into the ileum on gastrointestinal transit and plasma levels of neurotensin and enteroglucagon. Gastroenterology. 1984;86(2):274-80.
174. Lin HC, Zhao XT, Wang L, Wong H. Fat-induced ileal brake in the dog depends on peptide YY. Gastroenterology. 1996;110(5):1491-5.
175. Savage AP, Adrian TE, Carolan G, Chatterjee VK, Bloom SR. Effects of peptide YY (PYY) on mouth to caecum intestinal transit time and on the rate of gastric emptying in healthy volunteers. Gut. 1987;28(2):166-70.
176. Lin HC, Chey WY, Zhao X. Release of distal gut peptide YY (PYY) by fat in proximal gut depends on CCK. Peptides. 2000;21(10):1561-3.
177. Degen L, Drewe J, Piccoli F, Gräni K, Oesch S, Bunea R, et al. Effect of CCK-1 receptor blockade on ghrelin and PYY secretion in men. Am J Physiol Regul Integr Comp Physiol. 2007;292(4):R1391-9.
178. Batterham RL, Bloom SR. The gut hormone peptide YY regulates appetite. Ann N Y Acad Sci. 2003;994:162-8.
179. Batterham RL, Cohen MA, Ellis SM, Le Roux CW, Withers DJ, Frost GS, et al. Inhibition of food intake in obese subjects by peptide YY3-36. N Engl J Med. 2003;349(10):941-8.
180. Abbott CR, Small CJ, Kennedy AR, Neary NM, Sajedi A, Ghatei MA, et al. Blockade of the neuropeptide Y Y2 receptor with the specific antagonist BIIE0246 attenuates the effect of endogenous and exogenous peptide YY(3-36) on food intake. Brain Res. 2005;1043(1-2):139-44.
181. Eissele R, Goke R, Willemer S, Harthus HP, Vermeer H, Arnold R, et al. Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. Eur J Clin Invest. 1992;22(4):283-91.
182. Baggio LL, Drucker DJ. Biology of incretins: GLP-1 and GIP. Gastroenterology. 2007;132(6):2131-57.
183. Schirra J, Nicolaus M, Roggel R, Katschinski M, Storr M, Woerle HJ, et al. Endogenous glucagon-like peptide 1 controls endocrine pancreatic secretion and antro-pyloro-duodenal motility in humans. Gut. 2006;55(2):243-51.
184. Edwards CM, Todd JF, Mahmoudi M, Wang Z, Wang RM, Ghatei MA, et al. Glucagon-like peptide 1 has a physiological role in the control of postprandial glucose in humans: studies with the antagonist exendin 9-39. Diabetes. 1999;48(1):86-93.
185. Flint A, Raben A, Astrup A, Holst JJ. Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans. J Clin Invest. 1998;101(3):515-20.
186. Zander M, Madsbad S, Madsen JL, Holst JJ. Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and beta-cell function in type 2 diabetes: a parallel-group study. Lancet. 2002;359(9309):824-30.

187. Näslund E, Gutniak M, Skogar S, Rössner S, Hellström PM. Glucagon-like peptide 1 increases the period of postprandial satiety and slows gastric emptying in obese men. Am J Clin Nutr. 1998;68(3):525-30.
188. Steinert RE, Schirra J, Meyer-Gerspach AC, Kienle P, Fischer H, Schulte F, et al. Effect of glucagon-like peptide-1 receptor antagonism on appetite and food intake in healthy men. Am J Clin Nutr. 2014;100(2):514-23.
189. Gilbert MP, Pratley RE. GLP-1 Analogs and DPP-4 Inhibitors in Type 2 Diabetes Therapy: Review of Head-to-Head Clinical Trials. Front Endocrinol (Lausanne). 2020;11:178.
190. Herrmann C, Göke R, Richter G, Fehmann HC, Arnold R, Göke B. Glucagon-like peptide-1 and glucose-dependent insulin-releasing polypeptide plasma levels in response to nutrients. Digestion. 1995;56(2):117-26.
191. Elliott RM, Morgan LM, Tredger JA, Deacon S, Wright J, Marks V. Glucagon-like peptide-1 (7-36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns. J Endocrinol 1993;138(1):159-66.
192. Wu T, Rayner CK, Young RL, Horowitz M. Gut motility and enteroendocrine secretion. Curr Opin Pharmacol. 2013;13(6):928-34.
193. Parker JC, Irwin N, Lavery KS, Green BD, O'Harte FP, Gault VA, et al. Metabolic effects of sub-chronic ablation of the incretin receptors by daily administration of (Pro3)GIP and exendin(9-39)amide in obese diabetic (ob/ob) mice. Biol Chem. 2007;388(2):221-6.
194. Willard FS, Douros JD, Gabe MB, Showalter AD, Wainscott DB, Suter TM, et al. Tirzepatide is an imbalanced and biased dual GIP and GLP-1 receptor agonist. JCI Insight. 2020;5(17).
195. Mann JTS. Essentials of Human Nutrition. 2007;Third Edition.
196. Floyd JC, Jr., Fajans SS, Conn JW, Knopf RF, Rull J. Stimulation of insulin secretion by amino acids. J Clin Invest. 1966;45(9):1487-502.
197. Eliasson B, Björnsson E, Urbanavicius V, Andersson H, Fowelin J, Attvall S, et al. Hyperinsulinaemia impairs gastrointestinal motility and slows carbohydrate absorption. Diabetologia. 1995;38(1):79-85.
198. Kong MF, King P, Macdonald IA, Blackshaw PE, Perkins AC, Armstrong E, et al. Effect of euglycaemic hyperinsulinaemia on gastric emptying and gastrointestinal hormone responses in normal subjects. Diabetologia. 1998;41(4):474-81.
199. Kong MF, King P, Macdonald IA, Blackshaw PE, Horowitz M, Perkins AC, et al. Euglycaemic hyperinsulinaemia does not affect gastric emptying in type I and type II diabetes mellitus. Diabetologia. 1999;42(3):365-72.
200. Speechly DP, Buffenstein R. Appetite dysfunction in obese males: evidence for role of hyperinsulinaemia in passive overconsumption with a high fat diet. Eur J Clin Nutr. 2000;54(3):225-33.
201. Verdich C, Toubro S, Buemann B, Lysgård Madsen J, Juul Holst J, Astrup A. The role of postprandial releases of insulin and incretin hormones in meal-induced satiety—effect of obesity and weight reduction. Int J Obes. 2001;25(8):1206-14.
202. Aronoff SL, Berkowitz K, Shreiner B, Want L. Glucose metabolism and regulation: beyond insulin and glucagon. Diabetes Spectr. 2004;17(3):183-90.
203. Komatsu R, Matsuyama T, Namba M, Watanabe N, Itoh H, Kono N, et al. Glucagonostatic and Insulinotropic Action of Glucagonlike Peptide I-(7–36)-Amide. Diabetes. 1989;38(7):902-5.
204. Muller TD, Finan B, Clemmensen C, DiMarchi RD, Tschop MH. The new biology and pharmacology of glucagon. Physiol Rev. 2017;97(2):721-66.

205. Solloway MJ, Madjidi A, Gu C, Eastham-Anderson J, Clarke HJ, Kljavin N, et al. Glucagon couples hepatic amino acid catabolism to mTOR-dependent regulation of alpha-cell mass. Cell Rep. 2015;12(3):495-510.
206. Chernish SM, Brunelle RR, Rosenak BD, Ahmadzai S. Comparison of the effects of glucagon and atropine sulfate on gastric emptying. Am J Gastroenterol. 1978;70(6):581-6.
207. Dotevall G, Kock NG. The effect of glucagon on intestinal motility in man. Gastroenterology. 1963;45(3):364-7.
208. Arafat AM, Weickert MO, Adamidou A, Otto B, Perschel FH, Spranger J, et al. The impact of insulin-independent, glucagon-induced suppression of total ghrelin on satiety in obesity and type 1 diabetes mellitus. J Clin Endocrinol Metab. 2013;98(10):4133-42.
209. Geary N, Kissileff HR, Pi-Sunyer FX, Hinton V. Individual, but not simultaneous, glucagon and cholecystokinin infusions inhibit feeding in men. Am J Physiol Regul Integr Comp Physiol. 1992;262(6):R975-R80.
210. Rolls BJ, Hetherington M, Burley VJ. The specificity of satiety: The influence of foods of different macronutrient content on the development of satiety. Physiol Behav. 1988;43(2):145-53.
211. Barkeling B, Rössner S, Björvell H. Effects of a high-protein meal (meat) and a high-carbohydrate meal (vegetarian) on satiety measured by automated computerized monitoring of subsequent food intake, motivation to eat and food preferences. Int J Obes. 1990;14(9):743-51.
212. Porrini M, Crovetti R, Testolin G, Silva S. Evaluation of satiety sensations and food intake after different preloads. Appetite. 1995;25(1):17-30.
213. Stubbs RJ, van Wyk MC, Johnstone AM, Harbron CG. Breakfasts high in protein, fat or carbohydrate: effect on within-day appetite and energy balance. Eur J Clin Nutr. 1996;50(7):409-17.
214. Pal S, Ellis V. The acute effects of four protein meals on insulin, glucose, appetite and energy intake in lean men. Br J Nutr. 2010;104(8):1241-8.
215. Hall WL, Millward DJ, Long SJ, Morgan LM. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. Br J Nutr. 2003;89(2):239-48.
216. Veldhorst MA, Nieuwenhuizen AG, Hochstenbach-Waelen A, van Vught AJ, Westerterp KR, Engelen MP, et al. Dose-dependent satiating effect of whey relative to casein or soy. Physiol Behav. 2009;96(4-5):675-82.
217. Anderson GH, Tecimer SN, Shah D, Zafar TA. Protein source, quantity, and time of consumption determine the effect of proteins on short-term food intake in young men. J Nutr. 2004;134(11):3011-5.
218. Trico D, Frascerra S, Baldi S, Mengozzi A, Nesti L, Mari A, et al. The insulinotropic effect of a high-protein nutrient preload is mediated by the increase of plasma amino acids in type 2 diabetes. Eur J Nutr. 2018; 258:2253-61.
219. King DG, Walker M, Campbell MD, Breen L, Stevenson EJ, West DJ. A small dose of whey protein co-ingested with mixed-macronutrient breakfast and lunch meals improves postprandial glycemia and suppresses appetite in men with type 2 diabetes: a randomized controlled trial. Am J Clin Nutr. 2018;107(4):550-7.
220. Calbet JA, MacLean DA. Plasma glucagon and insulin responses depend on the rate of appearance of amino acids after ingestion of different protein solutions in humans. J Nutr. 2002;132(8):2174-82.
221. Nilsson M, Holst JJ, Bjorck IM. Metabolic effects of amino acid mixtures and whey protein in healthy subjects: studies using glucose-equivalent drinks. Am J Clin Nutr. 2007;85(4):996-1004.

222. Akhavan T, Luhovyy BL, Panahi S, Kubant R, Brown PH, Anderson GH. Mechanism of action of pre-meal consumption of whey protein on glycemic control in young adults. J Nutr Biochem. 2014;25(1):36-43.
223. Hutchison AT, Feinle-Bisset C, Fitzgerald PC, Standfield S, Horowitz M, Clifton PM, et al. Comparative effects of intraduodenal whey protein hydrolysate on antropyloroduodenal motility, gut hormones, glycemia, appetite, and energy intake in lean and obese men. Am J Clin Nutr. 2015;102(6):1323-31.
224. Hutchison AT, Piscitelli D, Horowitz M, Jones KL, Clifton PM, Standfield S, et al. Acute load-dependent effects of oral whey protein on gastric emptying, gut hormone release, glycemia, appetite, and energy intake in healthy men. Am J Clin Nutr. 2015;102(6):1574-84.
225. Batterham RL, Heffron H, Kapoor S, Chivers JE, Chandarana K, Herzog H, et al. Critical role for peptide YY in protein-mediated satiation and body-weight regulation. Cell Metab. 2006;4(3):223-33.
226. Ryan AT, Feinle-Bisset C, Kallas A, Wishart JM, Clifton PM, Horowitz M, et al. Intraduodenal protein modulates antropyloroduodenal motility, hormone release, glycemia, appetite, and energy intake in lean men. Am J Clin Nutr. 2012;96(3):474-82.
227. Soenen S, Giezenaar C, Hutchison AT, Horowitz M, Chapman I, Luscombe-Marsh ND. Effects of intraduodenal protein on appetite, energy intake, and antropyloroduodenal motility in healthy older compared with young men in a randomized trial. Am J Clin Nutr. 2014;100(4):1108-15.
228. Karamanlis A, Chaikomin R, Doran S, Bellon M, Bartholomeusz FD, Wishart JM, et al. Effects of protein on glycemic and incretin responses and gastric emptying after oral glucose in healthy subjects. Am J Clin Nutr. 2007;86(5):1364-8.
229. Gunnerud UJ, Ostman EM, Bjorck IM. Effects of whey proteins on glycaemia and insulinaemia to an oral glucose load in healthy adults; a dose-response study. Eur J Clin Nutr. 2013;67(7):749-53.
230. Gannon MC, Nuttall FQ, Neil BJ, Westphal SA. The insulin and glucose responses to meals of glucose plus various proteins in type II diabetic subjects. Metabolism. 1988;37(11):1081-8.
231. Floyd JC, Jr., Fajans SS, Conn JW, Knopf RF, Rull J. Insulin secretion in response to protein ingestion. J Clin Invest. 1966;45(9):1479-86.
232. Hutton JC, Sener A, Malaisse WJ. Interaction of branched chain amino acids and keto acids upon pancreatic islet metabolism and insulin secretion. J Biol Chem. 1980;255(15):7340-6.
233. Blachier F, Mourtada A, Sener A, Malaisse WJ. Stimulus-secretion coupling of arginine-induced insulin release. Uptake of metabolized and nonmetabolized cationic amino acids by pancreatic islets. Endocrinology. 1989;124(1):134-41.
234. Sener A, Malaisse WJ. The stimulus-secretion coupling of amino acid-induced insulin release. Insulinotropic action of L-alanine. Biochim Biophys Acta. 2002;1573(1):100-4.
235. Bowen J, Noakes M, Clifton PM. Appetite hormones and energy intake in obese men after consumption of fructose, glucose and whey protein beverages. Int J Obes (Lond). 2007;31(11):1696-703.
236. Watson LE, Phillips LK, Wu T, Bound MJ, Checklin HL, Grivell J, et al. A whey/guar “preload” improves postprandial glycaemia and glycated haemoglobin levels in type 2 diabetes: A 12-week, single-blind, randomized, placebo-controlled trial. Diabetes Obes Metab. 2019;21(4):930-8.
237. Trinick TR, Laker MF, Johnston DG, Keir M, Buchanan KD, Alberti KG. Effect of guar on second-meal glucose tolerance in normal man. Clin Sci (Lond). 1986;71(1):49-55.
238. Chaikomin R, Rayner CK, Jones KL, Horowitz M. Upper gastrointestinal function and glycemic control in diabetes mellitus. World J Gastroenterol. 2006;12(35):5611-21.

239. Rayner CK, Samsom M, Jones KL, Horowitz M. Relationships of upper gastrointestinal motor and sensory function with glycemic control. Diabetes Care. 2001;24(2):371-81.
240. Tulipano G, Sibilia V, Caroli AM, Cocchi D. Whey proteins as source of dipeptidyl dipeptidase IV (dipeptidyl peptidase-4) inhibitors. Peptides. 2011;32(4):835-8.
241. Santangelo A, Peracchi M, Conte D, Fraquelli M, Porrini M. Physical state of meal affects gastric emptying, cholecystokinin release and satiety. Br J Nutr. 1998;80(6):521-7.
242. Veldhorst M, Smeets A, Soenen S, Hochstenbach-Waelen A, Hursel R, Diepvens K, et al. Protein-induced satiety: effects and mechanisms of different proteins. Physiol Behav. 2008;94(2):300-7.
243. Steinert RE, Luscombe-Marsh ND, Little TJ, Standfield S, Otto B, Horowitz M, et al. Effects of intraduodenal infusion of L-tryptophan on ad libitum eating, antropyloroduodenal motility, glycemia, insulinemia, and gut peptide secretion in healthy men. J Clin Endocrinol Metab. 2014;99(9):3275-84.
244. Meyer-Gerspach AC, Häfliger S, Meili J, Doody A, Rehfeld JF, Drewe J, et al. Effect of L-tryptophan and L-leucine on gut hormone secretion, appetite feelings and gastric emptying rates in lean and non-diabetic obese participants: A randomized, double-blind, parallel-group Trial. PLoS One. 2016;11(11):e0166758.
245. Carney BI, Jones KL, Horowitz M, Sun WM, Hebbard G, Edelbroek MA. Stereospecific effects of tryptophan on gastric emptying and hunger in humans. J Gastroenterol Hepatol. 1994;9(6):557-63.
246. Edelbroek M, Sun WM, Horowitz M, Dent J, Smout A, Akkermans L. Stereospecific effects of intraduodenal tryptophan on pyloric and duodenal motility in humans. Scand J Gastroenterol. 1994;29(12):1088-95.
247. Hrboticky N, Leiter LA, Anderson GH. Effects of L-tryptophan on short term food intake in lean men. Nutr Res. 1985;5(6):595-607.
248. Ullrich SS, Fitzgerald PCE, Giesbertz P, Steinert RE, Horowitz M, Feinle-Bisset C. Effects of intragastric administration of tryptophan on the blood glucose response to a nutrient drink and energy intake, in lean and obese men. Nutrients. 2018;10(4).
249. Hajishafiee M, Elovaris RA, Jones KL, Heilbronn LK, Horowitz M, Poppitt SD, et al. Effects of intragastric administration of L-tryptophan on the glycaemic response to a nutrient drink in men with type 2 diabetes - impacts on gastric emptying, glucoregulatory hormones and glucose absorption. Nutr Diabetes. 2021;11(1):3.
250. Steinert RE, Landrock MF, Horowitz M, Feinle-Bisset C. Effects of Intraduodenal Infusions of L-phenylalanine and L-glutamine on antropyloroduodenal motility and plasma cholecystokinin in healthy men. J Neurogastroenterol Motil. 2015;21(3):404-13.
251. Ballinger AB, Clark ML. L-phenylalanine releases cholecystokinin (CCK) and is associated with reduced food intake in humans: evidence for a physiological role of CCK in control of eating. Metabolism. 1994;43(6):735-8.
252. Fitzgerald PCE, Manoliu B, Herbillon B, Steinert RE, Horowitz M, Feinle-Bisset C. Effects of L-phenylalanine on energy intake and glycaemia-impacts on appetite perceptions, gastrointestinal hormones and gastric emptying in healthy males. Nutrients. 2020;12(6):1788.
253. Nuttall FQ, Schweim KJ, Gannon MC. Effect of orally administered phenylalanine with and without glucose on insulin, glucagon and glucose concentrations. Horm Metab Res. 2006;38(8):518-23.
254. Ullrich SS, Fitzgerald PC, Nkamba I, Steinert RE, Horowitz M, Feinle-Bisset C. Intragastric lysine lowers the circulating glucose and insulin responses to a mixed-nutrient drink without slowing gastric emptying in healthy adults. J Nutr. 2017;147(7):1275-81.
255. Kalogeropoulou D, LaFave L, Schweim K, Gannon MC, Nuttall FQ. Lysine ingestion markedly attenuates the glucose response to ingested glucose without a change in insulin response. Am J Clin Nutr. 2009;90(2):314-20.

256. Jordi J, Herzog B, Camargo SM, Boyle CN, Lutz TA, Verrey F. Specific amino acids inhibit food intake via the area postrema or vagal afferents. J Physiol. 2013;591(22):5611-21.
257. Baruffol C, Jordi J, Camargo S, Radovic T, Herzog B, Fried M, et al. L-lysine dose dependently delays gastric emptying and increases intestinal fluid volume in humans and rats. Neurogastroenterol Motil. 2014;26(7):999-1009.
258. Kim IY, Williams RH, Schutzler SE, Lasley CJ, Bodenner DL, Wolfe RR, et al. Acute lysine supplementation does not improve hepatic or peripheral insulin sensitivity in older, overweight individuals. Nutr Metab (Lond). 2014;11(1):49.
259. Luscombe-Marsh ND, Hutchison AT, Soenen S, Steinert RE, Clifton PM, Horowitz M, et al. Plasma free amino acid responses to intraduodenal whey protein, and relationships with insulin, glucagon-like peptide-1 and energy intake in lean healthy men. Nutrients. 2016;8(1).
260. Stonehouse W, Wycherley T, Luscombe-Marsh N, Taylor P, Brinkworth G, Riley M. Dairy intake enhances body weight and composition changes during energy restriction in 18-50-year-old adults-a meta-analysis of randomized controlled trials. Nutrients. 2016;8(7).
261. Layman DK. The role of leucine in weight loss diets and glucose homeostasis. J Nutr. 2003;133(1):261s-7s.
262. Dudgeon WD, Kelley EP, Scheett TP. In a single-blind, matched group design: branched-chain amino acid supplementation and resistance training maintains lean body mass during a caloric restricted diet. J Int Soc Sports Nutr. 2016;13:1.
263. Mourier A, Bigard AX, de Kerviler E, Roger B, Legrand H, Guezennec CY. Combined effects of caloric restriction and branched-chain amino acid supplementation on body composition and exercise performance in elite wrestlers. Int J Sports Med. 1997;18(1):47-55.
264. Gordon-Elliott JS, Margolese HC. Weight loss during prolonged branched-chain amino acid treatment for tardive dyskinesia in a patient with schizophrenia. Aust N Z J Psychiatry. 2006;40(2):195.
265. Qin LQ, Xun P, Bujnowski D, Daviglus ML, Van Horn L, Stamler J, et al. Higher branched-chain amino acid intake is associated with a lower prevalence of being overweight or obese in middle-aged East Asian and Western adults. J Nutr. 2011;141(2):249-54.
266. Cogate PG, Natali AJ, de Oliveira A, Alfenas RC, Hermsdorff HH. Consumption of branched-chain amino acids is inversely associated with central obesity and cardiometabolic features in a population of Brazilian middle-aged men: potential role of leucine intake. J Nutr Health Aging. 2015;19(7):771-7.
267. Li YC, Li Y, Liu LY, Chen Y, Zi TQ, Du SS, et al. The ratio of dietary branched-chain amino acids is associated with a lower prevalence of obesity in young Northern Chinese adults: An internet-based cross-sectional study. Nutrients. 2015;7(11):9573-89.
268. Horiuchi M, Takeda T, Takanashi H, Ozaki-Masuzawa Y, Taguchi Y, Toyoshima Y, et al. Branched-chain amino acid supplementation restores reduced insulinotropic activity of a low-protein diet through the vagus nerve in rats. Nutr Metab (Lond). 2017;14:59.
269. Eizirik DL, Migliorini RH. Reduced diabetogenic effect of streptozotocin in rats previously adapted to a high-protein, carbohydrate-free diet. Diabetes. 1984;33(4):383-8.
270. Eizirik DL, Kettelhut IC, Migliorini RH. Administration of branched-chain amino acids reduces the diabetogenic effect of streptozotocin in rats. Braz J Med Biol Res. 1987;20(1):137-44.
271. Zhang Y, Kobayashi H, Mawatari K, Sato J, Bajotto G, Kitaura Y, et al. Effects of branched-chain amino acid supplementation on plasma concentrations of free amino acids, insulin, and energy substrates in young men. J Nutr Sci Vitaminol (Tokyo). 2011;57(1):114-7.
272. Mobbs CV, Isoda F, Makimura H, Mastaitis J, Mizuno T, Shu IW, et al. Impaired glucose signaling as a cause of obesity and the metabolic syndrome: the glucoadipostatic hypothesis. Physiol Behav. 2005;85(1):3-23.

273. Doi M, Yamaoka I, Fukunaga T, Nakayama M. Isoleucine, a potent plasma glucose-lowering amino acid, stimulates glucose uptake in C2C12 myotubes. Biochem Biophys Res Commun. 2003;312(4):1111-7.
274. Newsholme P, Cruzat V, Arfuso F, Keane K. Nutrient regulation of insulin secretion and action. J Endocrinol. 2014;221(3):R105-20.
275. Gannon NP, Schnuck JK, Vaughan RA. BCAA metabolism and insulin sensitivity - dysregulated by metabolic status? Mol Nutr Food Res. 2018;62(6):e1700756.
276. Volek JS, Volk BM, Gómez AL, Kunces LJ, Kupchak BR, Freidenreich DJ, et al. Whey protein supplementation during resistance training augments lean body mass. J Am Coll Nutr. 2013;32(2):122-35.
277. Columbus DA, Fiorotto ML, Davis TA. Leucine is a major regulator of muscle protein synthesis in neonates. Amino Acids. 2015;47(2):259-70.
278. López N, Sánchez J, Palou A, Serra F. Gender-associated impact of early leucine supplementation on adult predisposition to obesity in rats. Nutrients. 2018;10(1).
279. Pedroso JA, Zampieri TT, Donato J, Jr. Reviewing the effects of L-leucine supplementation in the regulation of food Intake, energy balance, and glucose homeostasis. Nutrients. 2015;7(5):3914-37.
280. Layman DK, Walker DA. Potential importance of leucine in treatment of obesity and the metabolic syndrome. J Nutr. 2006;136(1 Suppl):319s-23s.
281. Cota D, Proulx K, Smith KA, Kozma SC, Thomas G, Woods SC, et al. Hypothalamic mTOR signaling regulates food intake. Science. 2006;312(5775):927-30.
282. Kalogeropoulou D, LaFave L, Schweim K, Gannon MC, Nuttall FQ. Leucine, when ingested with glucose, synergistically stimulates insulin secretion and lowers blood glucose. Metabolism. 2008;57(12):1747-52.
283. Ullrich SS, Fitzgerald PC, Schober G, Steinert RE, Horowitz M, Feinle-Bisset C. Intragastric administration of leucine or isoleucine lowers the blood glucose response to a mixed-nutrient drink by different mechanisms in healthy, lean volunteers. Am J Clin Nutr. 2016;104(5):1274-84.
284. Steinert RE, Landrock MF, Ullrich SS, Standfield S, Otto B, Horowitz M, et al. Effects of intraduodenal infusion of the branched-chain amino acid leucine on ad libitum eating, gut motor and hormone functions, and glycemia in healthy men. Am J Clin Nutr. 2015;102(4):820-7.
285. Laeger T, Reed SD, Henagan TM, Fernandez DH, Taghavi M, Addington A, et al. Leucine acts in the brain to suppress food intake but does not function as a physiological signal of low dietary protein. Am J Physiol Regul Integr Comp Physiol. 2014;307(3):R310-20.
286. Ullrich SS, Otto B, Hutchison AT, Luscombe-Marsh ND, Horowitz M, Feinle-Bisset C. Comparative effects of intraduodenal protein and lipid on ghrelin, peptide YY, and leptin release in healthy men. Am J Physiol Regul Integr Comp Physiol. 2015;308(4):R300-4.
287. van Loon LJ. Leucine as a pharmaconutrient in health and disease. Curr Opin Clin Nutr Metab Care. 2012;15(1):71-7.
288. Fahien LA, Macdonald MJ. The complex mechanism of glutamate dehydrogenase in insulin secretion. Diabetes. 2011;60(10):2450-4.
289. Nishimura J, Masaki T, Arakawa M, Seike M, Yoshimatsu H. Isoleucine prevents the accumulation of tissue triglycerides and upregulates the expression of PPARalpha and uncoupling protein in diet-induced obese mice. J Nutr. 2010;140(3):496-500.
290. Nuttall FQ, Schweim K, Gannon MC. Effect of orally administered isoleucine with and without glucose on insulin, glucagon and glucose concentrations in non-diabetic subjects. e-SPEN. 2008;3(4):e152-e8.

291. Ikehara O, Kawasaki N, Maezono K, Komatsu M, Konishi A. Acute and chronic treatment of L-isoleucine ameliorates glucose metabolism in glucose-intolerant and diabetic mice. Biol Pharm Bull. 2008;31(3):469-72.
292. Morato PN, Lollo PC, Moura CS, Batista TM, Carneiro EM, Amaya-Farfan J. A dipeptide and an amino acid present in whey protein hydrolysate increase translocation of GLUT-4 to the plasma membrane in Wistar rats. Food Chem. 2013;139(1-4):853-9.
293. Tsuda Y, Iwasawa K, Yamaguchi M. Acute supplementation of valine reduces fatigue during swimming exercise in rats. Biosci Biotechnol Biochem. 2018;82(5):856-61.
294. Parker B, Noakes M, Luscombe N, Clifton P. Effect of a high-protein, high-monounsaturated fat weight loss diet on glycemic control and lipid levels in type 2 diabetes. Diabetes care. 2002;25(3):425-30.
295. Nair NS, Brennan IM, Little TJ, Gentilcore D, Hausken T, Jones KL, et al. Reproducibility of energy intake, gastric emptying, blood glucose, plasma insulin and cholecystokinin responses in healthy young males. Br J Nutr. 2009;101(7):1094-102.
296. Brennan IM, Feltrin KL, Nair NS, Hausken T, Little TJ, Gentilcore D, et al. Effects of the phases of the menstrual cycle on gastric emptying, glycemia, plasma GLP-1 and insulin, and energy intake in healthy lean women. Am J Physiol 2009;297(3):G602-10.
297. Stunkard AJ, Messick S. The three-factor eating questionnaire to measure dietary restraint, disinhibition and hunger. J Psychosom Res. 1985;29(1):71-83.
298. Maddern GJ, Chatterton BE, Collins PJ, Horowitz M, Shearman DJ, Jamieson GG. Solid and liquid gastric emptying in patients with gastro-oesophageal reflux. Br J Surg. 1985;72(5):344-7.
299. Vist GE, Maughan RJ. The effect of osmolality and carbohydrate content on the rate of gastric emptying of liquids in man. J Physiol. 1995;486 (Pt 2):523-31.
300. Steinert RE, Ullrich SS, Geary N, Asarian L, Bueter M, Horowitz M, et al. Comparative effects of intraduodenal amino acid infusions on food intake and gut hormone release in healthy males. Physiol Rep. 2017;5(21).
301. Parker BA, Doran S, Wishart J, Horowitz M, Chapman IM. Effects of small intestinal and gastric glucose administration on the suppression of plasma ghrelin concentrations in healthy older men and women. Clin Endocrinol (Oxf). 2005;62(5):539-46.
302. MacIntosh CG, Morley JE, Wishart J, Morris H, Jansen JB, Horowitz M, et al. Effect of exogenous cholecystokinin (CCK)-8 on food intake and plasma CCK, leptin, and insulin concentrations in older and young adults: evidence for increased CCK activity as a cause of the anorexia of aging. J Clin Endocrinol Metab. 2001;86(12):5830-7.
303. Pilichiewicz AN, Chaikomin R, Brennan IM, Wishart JM, Rayner CK, Jones KL, et al. Load-dependent effects of duodenal glucose on glycemia, gastrointestinal hormones, antropyloroduodenal motility, and energy intake in healthy men. Am J Physiol 2007;293(3):E743-53.
304. Bland JM, Altman DG. Calculating correlation coefficients with repeated observations: Part 2--Correlation between subjects. BMJ. 1995;310(6980):633.
305. Hegarty JE, Fairclough PD, Moriarty KJ, Clark ML, Kelly MJ, Dawson AM. Comparison of plasma and intraluminal amino acid profiles in man after meals containing a protein hydrolysate and equivalent amino acid mixture. Gut. 1982;23(8):670-4.
306. Adibi SA, Gray SJ. Intestinal absorption of essential amino acids in man. Gastroenterology. 1967;52(5):837-45.
307. Matthews DM. Intestinal absorption of amino acids and peptides. Z Ernahrungswiss Suppl. 1977;20(20):1-7.
308. Zhang Y, Guo K, LeBlanc RE, Loh D, Schwartz GJ, Yu YH. Increasing dietary leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism in mice via multimechanisms. Diabetes. 2007;56(6):1647-54.

309. Elovaris RA, Fitzgerald PCE, Bitarafan V, Ullrich SS, Horowitz M, Feinle-Bisset C. Intraduodenal administration of L-valine has no effect on antropyloroduodenal pressures, plasma cholecystokinin concentrations or energy intake in healthy, lean men. Nutrients. 2019;11(1).
310. Salehi A, Gunnerud U, Muhammed SJ, Ostman E, Holst JJ, Bjorck I, et al. The insulinogenic effect of whey protein is partially mediated by a direct effect of amino acids and GIP on beta-cells. Nutr Metab (Lond). 2012;9(1):48.
311. Eisenstein AB, Strack I. Amino acid stimulation of glucagon secretion by perifused islets of high-protein-fed rats. Diabetes. 1978;27(4):370-6.
312. Rolls BJ, Kim-Harris S, Fischman MW, Foltin RW, Moran TH, Stoner SA. Satiety after preloads with different amounts of fat and carbohydrate: implications for obesity. Am J Clin Nutr. 1994;60(4):476-87.
313. Little TJ, Russo A, Meyer JH, Horowitz M, Smyth DR, Bellon M, et al. Free fatty acids have more potent effects on gastric emptying, gut hormones, and appetite than triacylglycerides. Gastroenterology. 2007;133(4):1124-31.
314. Martens MJ, Lemmens SG, Born JM, Westerterp-Plantenga MS. A solid high-protein meal evokes stronger hunger suppression than a liquefied high-protein meal. Obesity (Silver Spring). 2011;19(3):522-7.
315. Bendtsen LQ, Lorenzen JK, Bendsen NT, Rasmussen C, Astrup A. Effect of dairy proteins on appetite, energy expenditure, body weight, and composition: a review of the evidence from controlled clinical trials. Adv Nutr. 2013;4(4):418-38.
316. Aller EE, Larsen TM, Claus H, Lindroos AK, Kafatos A, Pfeiffer A, et al. Weight loss maintenance in overweight subjects on ad libitum diets with high or low protein content and glycemic index: the DIOGENES trial 12-month results. Int J Obes (Lond). 2014;38(12):1511-7.
317. Layman DK, Baum JI. Dietary protein impact on glycemic control during weight loss. J Nutr. 2004;134(4):968S-73S.
318. Lejeune MP, Westerterp KR, Adam TC, Luscombe-Marsh ND, Westerterp-Plantenga MS. Ghrelin and glucagon-like peptide 1 concentrations, 24-h satiety, and energy and substrate metabolism during a high-protein diet and measured in a respiration chamber. Am J Clin Nutr. 2006;83(1):89-94.
319. Ryan AT, Luscombe-Marsh ND, Saies AA, Little TJ, Standfield S, Horowitz M, et al. Effects of intraduodenal lipid and protein on gut motility and hormone release, glycemia, appetite, and energy intake in lean men. Am J Clin Nutr. 2013;98(2):300-11.
320. Pilichiewicz A, O'Donovan D, Feinle C, Lei Y, Wishart JM, Bryant L, et al. Effect of lipase inhibition on gastric emptying of, and the glycemic and incretin responses to, an oil/aqueous drink in type 2 diabetes mellitus. J Clin Endocrinol Metab. 2003;88(8):3829-34.
321. Chartrand D, Da Silva MS, Julien P, Rudkowska I. Influence of amino acids in dairy products on glucose homeostasis: the clinical evidence. Can J Diabetes. 2017;41(3):329-37.
322. Feltrin KL, Little TJ, Meyer JH, Horowitz M, Smout AJ, Wishart J, et al. Effects of intraduodenal fatty acids on appetite, antropyloroduodenal motility, and plasma CCK and GLP-1 in humans vary with their chain length. Am J Physiol Regul Integr Comp Physiol. 2004;287(3):R524-33.
323. Heddle R, Dent J, Toouli J, Read NW. Topography and measurement of pyloric pressure waves and tone in humans. Am J Clin Nutr. 1988;255(4 Pt 1):G490-7.
324. Parker B, Sturm K, MacIntosh C, Feinle C, Horowitz M, Chapman I. Relation between food intake and visual analogue scale ratings of appetite and other sensations in healthy older and young subjects. Eur J Clin Nutr. 2004;58(2):212.
325. Camilleri M, Malagelada JR. Abnormal intestinal motility in diabetics with the gastroparesis syndrome. Eur J Clin Invest. 1984;14(6):420-7.

326. Seimon RV, Lange K, Little TJ, Brennan IM, Pilichiewicz AN, Feltrin KL, et al. Pooled-data analysis identifies pyloric pressures and plasma cholecystokinin concentrations as major determinants of acute energy intake in healthy, lean men. Am J Clin Nutr. 2010;92(1):61-8.
327. Schober G, Lange K, Steinert RE, Hutchison AT, Luscombe-Marsh ND, Landrock MF, et al. Contributions of upper gut hormones and motility to the energy intake-suppressant effects of intraduodenal nutrients in healthy, lean men - a pooled-data analysis. Physiol Rep. 2016;4(17).
328. Kissileff HR, Carretta JC, Geliebter A, Pi-Sunyer FX. Cholecystokinin and stomach distension combine to reduce food intake in humans. Am J Physiol Regul Integr Comp Physiol. 2003;285(5):R992-8.
329. Koop I, Ruppert-Seipp G, Koop H, Schafmayer A, Arnold R. Cholecystokinin release by gastric distension--an atropine-sensitive mechanism. Digestion. 1990;46(4):220-7.
330. Depoortere I. Taste receptors of the gut: emerging roles in health and disease. Gut. 2014;63(1):179-90.
331. Latorre R, Sternini C, De Giorgio R, Greenwood-Van Meerveld B. Enteroendocrine cells: a review of their role in brain-gut communication. Neurogastroenterol Motil. 2016;28(5):620-30.
332. Jones KL, Horowitz M, Carney BI, Wishart JM, Guha S, Green L. Gastric emptying in early noninsulin-dependent diabetes mellitus. J Nucl Med. 1996;37(10):1643-8.
333. Horowitz M, Edelbroek MA, Wishart JM, Straathof JW. Relationship between oral glucose tolerance and gastric emptying in normal healthy subjects. Diabetologia. 1993;36(9):857-62.
334. Gonlachanvit S, Hsu CW, Boden GH, Knight LC, Maurer AH, Fisher RS, et al. Effect of altering gastric emptying on postprandial plasma glucose concentrations following a physiologic meal in type-II diabetic patients. Dig Dis Sci. 2003;48(3):488-97.
335. Yang J, Chi Y, Burkhardt BR, Guan Y, Wolf BA. Leucine metabolism in regulation of insulin secretion from pancreatic beta cells. Nutr Rev. 2010;68(5):270-9.
336. Doi M, Yamaoka I, Nakayama M, Sugahara K, Yoshizawa F. Hypoglycemic effect of isoleucine involves increased muscle glucose uptake and whole body glucose oxidation and decreased hepatic gluconeogenesis. Am J Physiol Endocrinol Metab. 2007;292(6):E1683-E93.
337. Arrieta-Cruz I, Su Y, Gutierrez-Juarez R. Suppression of endogenous glucose production by isoleucine and valine and impact of diet composition. Nutrients. 2016;8(2):79.
338. Mossi S, Meyer-Wyss B, Beglinger C, Schwizer W, Fried M, Ajami A, et al. Gastric emptying of liquid meals measured noninvasively in humans with [13C]acetate breath test. Dig Dis Sci. 1994;39(12 Suppl):107S-9S.
339. Bland JM, Altman DG. Calculating correlation coefficients with repeated observations: Part 1--Correlation within subjects. BMJ. 1995;310(6977):446.
340. Ma J, Pilichiewicz AN, Feinle-Bisset C, Wishart JM, Jones KL, Horowitz M, et al. Effects of variations in duodenal glucose load on glycaemic, insulin, and incretin responses in type 2 diabetes. Diabet Med. 2012;29(5):604-8.
341. Dean ED. A primary role for α-cells as amino acid sensors. Diabetes. 2020;69(4):542-9.
342. Giezenaar C, Luscombe-Marsh ND, Hutchison AT, Standfield S, Feinle-Bisset C, Horowitz M, et al. Dose-dependent effects of randomized intraduodenal whey-protein loads on glucose, gut hormone, and amino acid concentrations in healthy older and younger men. Nutrients. 2018;10(1):78.
343. Elovaris RA, Hutchison AT, Lange K, Horowitz M, Feinle-Bisset C, Luscombe-Marsh ND. Plasma free amino acid responses to whey protein and their relationships with gastric emptying, blood glucose- and appetite-regulatory hormones and energy intake in lean healthy men. Nutrients. 2019;11(10):2465.

344. O'Rielly R, Li H, Lim SM, Yazbeck R, Kritas S, Ullrich SS, et al. The effect of isoleucine supplementation on body weight gain and blood glucose response in lean and obese mice. Nutrients. 2020;12(8):2446.
345. Claessens M, Saris WH, van Baak MA. Glucagon and insulin responses after ingestion of different amounts of intact and hydrolysed proteins. Br J Nutr. 2008;100(1):61-9.
346. Haedersdal S, Lund A, Knop FK, Vilsboll T. The Role of Glucagon in the Pathophysiology and Treatment of Type 2 Diabetes. Mayo Clin Proc. 2018;93(2):217-39.
347. Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia. Report of a WHO/IDF consultation. World Health Organization 2006, International Diabetes Federation. Geneva: Switzerland. Internet: https://www.who.int/diabetes/publications/Definition%20and%20diagnosis%20of%20diabetes_new.pdf (accessed 25 November 2019).
348. Ewing DJ, Clarke BF. Diagnosis and management of diabetic autonomic neuropathy. Br Med J (Clin Res Ed). 1982;285(6346):916-8.
349. Nauck MA, Liess H, Siegel EG, Niedmann PD, Creutzfeldt W. Critical evaluation of the 'heated-hand-technique' for obtaining 'arterialized' venous blood: incomplete arterialization and alterations in glucagon responses. Clin Physiol. 1992;12(5):537-52.
350. Tura A, Kautzky-Willer A, Pacini G. Insulinogenic indices from insulin and C-peptide: Comparison of beta-cell function from OGTT and IVGTT. Diabetes Res Clin Pract. 2006;72(3):298-301.
351. Gannon MC, Nuttall FQ. Amino acid ingestion and glucose metabolism--a review. IUBMB Life. 2010;62(9):660-8.
352. Newmire DE, Rivas E, Deemer SE, Willoughby DS, Ben-Ezra V. The impact of a large bolus dose of l-leucine and l-isoleucine on enteroendocrine and pancreatic hormones, and glycemia in healthy, inactive adults. Nutrients. 2019;11(11).
353. Watson LE, Xie C, Wang X, Li Z, Phillips LK, Sun Z, et al. Gastric emptying in patients with well-controlled type 2 diabetes compared with young and older control subjects without diabetes. J Clin Endocrinol Metab. 2019;104(8):3311-9.
354. Holwerda AM, Paulussen KJM, Overkamp M, Goessens JPB, Kramer IF, Wodzig W, et al. Leucine coingestion augments the muscle protein synthetic response to the ingestion of 15 g of protein following resistance exercise in older men. Am J Physiol Endocrinol Metab. 2019;317(3):E473-e82.