THE ACUTE EFFECTS OF L-LEUCINE AND L-ISOLEUCINE ON GLYCEMIC RESPONSES IN HEALTHY AND INACTIVE ADULTS

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DEDICATION

I dedicate this dissertation to my family and the friends whom I consider family, who helped support me directly and indirectly through the four years of trying to capture my doctorate.

"*Take care of the ones who take care of you and you will be taken care of*."

- Unknown

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ABSTRACT

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The ingestion of whey protein or insulinogenic amino acids (AA) with a CHO drink has been shown to blunt the elevated post-prandial glucose response. It has been suggested that AA may facilitate secretion of hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) that are 50-70% responsible for regulating insulin secretion. The purpose of this study was to examine the "*priming*" effect of pre-ingested L-Isoleucine (ISO) and L-Leucine (LEU) on glucose metabolism and glycemic hormones in healthy, inactive adults. We hypothesized that preingested ISO and LEU would diminish the post prandial rise in glucose prior to a 75 g oral glucose tolerance test (OGTT) and have minimal effect on enteroendocrine hormone secretion. To test this, 12 healthy adults (Females: n = 6, males: n = 6, Age 27.39 ± 2.05 year; height 167.42 ± 2.23 cm; weight 77.77 ± 3.73 kg; BMI 26.30 ± 2.14 kg/m^2; lean body mass [LBM] 53.20 ± 4.67 kg; body fat 34.14 ± 2.96%; fasting blood glucose [FBG] 89.5 ± 4.67 mg/dl) completed four trials in a randomized, single-blinded fashion. Each trial consisted of ingestion of either ISO + LEU in combination (50:50), ISO, LEU, or placebo (PLA). Each treatment was ingested 30 min prior to a 2 hr 75 g (GLU) OGTT. The amino acid drink (200 mL) was prepared based on the participant's LBM at a standardized dose

(0.3g/kg), while the PLA dose was 3.54 g. Blood samples were collected at baseline (0), followed by AA or PLA drink, 6, 10, 30, followed by GLU drink, 36, 40, 60, 90, 120, and 150 min with appropriate inhibitors used for valid quantification. Results show that Δ area of glucose analysis ISO+LEU, ISO, and LEU reduced glucose response more than PLA (p = .005); ISO + LEU and ISO lowered blood glucose at 60 min and 90 min (p = < .05) compared to PLA. There was no difference between treatments in the AUC insulin concentration from baseline (p = .053); Δ change of C-peptide concentration was greater in ISO than PLA (p = .04), AUC differences showed ISO+LEU > PLA; the Δ change glucagon analysis showed no difference (p = .12); Δ change of $GLP\text{-}1_{Active}$ analysis showed no difference (P = .12); Δ change GIP_{Total} analysis ISO > LEU and PLA (p = .04). It appears that ISO and LEU combined or independently diminish glucose responses at peak time and ISO stimulates GIP and C-peptide concentrations more so than does LEU, and ISO and LEU have a negligible impact on GLP-1.

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CHAPTER I

INTRODUCTION

Background

Under conditions, ~75-85% of whole-body glucose is taken up into skeletal muscle by insulin (~70-100 mU/L). During hyperglycemia (220 mg/dl), this value increases to ~95% of insulin mediated glucose uptake into skeletal muscle (Baron, Brechtel, Wallace, & Edelman, 1988; Thiebaud et al., 1982). The process of insulin communication to uptake glucose is a complex system of intracellular interactions of numerous proteins. Glucose transport type 4 (GLUT-4) is a glucose transporter that is found predominantly in skeletal muscle and fat tissue and is in the 14-member family of transmembrane hexose transporters, each of which has a distinct affinity for particular hexose, and differ in tissue distribution. GLUT-4 cell transduction is mediated by the hormone insulin, which is a peptide hormone that is synthesized and secreted by the β-cells of the pancreas. Insulin responds to an ingestion of macronutrients carbohydrates, proteins, and lipids to differing degrees and influences cellular uptake of the macronutrient catabolized substrates glucose, lipids, and amino acids. Whole-body glucose uptake is known to be influenced by exercise, age, autoimmune responses (Type 1 diabetes), obesity (when combined with a lifestyle of chronic sedentary behavior), and high calorie intake.

Diabetes is classified as an impairment of the body's ability to modulate blood glucose concentrations. By definition, Type 2 diabetes (T2D) results from impaired insulin sensitivity combined with inadequate insulin secretion (Pfeiffer & Klein, 2014). T2D accounts for ~90–95% of those with diabetes, and has also been referred to as non–insulin-dependent diabetes (NIDDM) or adult-onset diabetes. T2D encompasses individuals who have insulin resistance with differing magnitude of insulin deficiencies. Chronic hyperglycemia, as a result of untreated or uncontrolled T2D, is associated with long term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels (Association, 2014a). Hyperglycemia is the result of glucose concentrations exceeding glucose disappearance from the serum compartment. In a fasting state, hyperglycemia may be directly related to increased hepatic glucose production and efflux into the circulation. In the postprandial state, hyperglycemia may result from a combination of increased plasma glucose concentrations, dysregulation of hepatic glucose output suppression, and defective insulin signaling in target tissues. This dysregulation is mainly found in skeletal muscle, which normally accounts for ~75% of whole body insulin-stimulated glucose uptake. The renal system assists in the removal of glucose from blood during chronic states of hyperglycemia. However, once the renal tubular transport of glucose has exceeded its maximum capacity, glycosuria is unable to reduce plasma glucose and does not prevent further hyperglycemic events (Inzucchi et al., 2012, 2015; Kramer et al., 2006). Furthermore, uncontrolled hyperglycemia may lead to macrovascular (i.e., ischemic heart

disease, stroke, and peripheral vascular disease) and microvasculature damage such as retinopathy, nephropathy, and neuropathy. These comorbidities may definitively reduce the quality of life. The current diagnostic criteria for T2D is a fasting plasma glucose of ≥ 7.0 mmol/l (126 mg/dl) or a 2 hr plasma glucose of ≥ 11.1 mmol/l (200 mg/dl) after consumption of a 75 g glucose drink. Impaired glucose tolerance (IGT) is defined by a fasting plasma glucose of < 7.0 mmol/l (126 mg/dl) and a 2 hr plasma glucose of ≥ 7.8 and < 11.1 mmol/l (140 mg/dl and 200 mg/dl) following consumption of a 75 g glucose drink. Lastly, an impaired fasting glucose (IFG) is defined by a fasting plasma glucose of 6.1 to 6.9 mmol/l (110 mg/dl to 125 mg/dl) and a 2 hr plasma glucose < 7.8 mmol/l (140 mg/dl) after consumption of a 75 g glucose drink (Organization, 2006).

Glycated hemoglobin (HbA_{1C}) is commonly used clinically to diagnose diabetes in individuals with risk factors, and reflects average blood glucose concentrations over a 2 to 3-month period. It is also used to identify those at higher risk of developing diabetes in the future. HbA_{1C} concentrations above the laboratory analyzed normal range (6.0%) but below the diagnostic cut point for diabetes (> 6.5%) indicate that an individual is at very high risk for developing diabetes. The incidence of developing diabetes in people with HbA_{1C} concentrations in this range (6.0 – 6.5%) are more than 10 times greater than people with lower concentrations (Association, 2014a; Organization, 2006). However, the 6.0 to < 6.5% range fails to identify a considerable number of patients who have IFG and IGT. Prospective studies indicate that people within the HbA_{1C} range of 5.5–6.0% have a 5-year cumulative incidence of the diagnosis of diabetes that ranges from 12% to 25%. It is feasible that a lower HbA_{1C} cut point may offer a greater practicality, a wider

application, and a more useful test that may increase the number of diagnoses made (Association, 2014a). The proposed and most appropriate level of HbA_{1C} to initiate preventive interventions is likely to be somewhere in the range of 5.5 – 6% (Association, 2014a).

Categorization for Pre-diabetes and Diabetes

- *Fasting plasma glucose*: 100 mg/dl (5.6 mmol/l) to 125 mg/dl (6.9 mmol/l) (IFG)
- *2 hr plasma glucose in the 75-g OGTT*: 140 mg/dl (7.8 mmol/l) to 199 mg/dl (11.0 mmol/l) (IGT)
- *HbA_{1C}*: 5.7–6.4%

Note: Impaired fasting glucose (IGF); oral glucose tolerance (OGTT); impaired glucose tolerance (IGT) (Standards of medical care in diabetes-2014, 2014).

Criteria for the diagnosis of diabetes

- $HbA_{1C} \geq 6.5\%$. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay (Little & Rohlfing, 2009)
- Fasting plasma glucose ≥ 126mg/dl (7.0mmol/l).
- Fasting is defined as no caloric intake for at ~ 8 hr.
- In a patient with classic symptoms of hyperglycemia or hyperglycemic event, a random plasma glucose ≥ 200 mg/dl (11.1 mmol/l)
- 2 hr plasma glucose ≥ 200 mg/dl (11.1 mmol/l) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose

load containing the equivalent of 75 g anhydrous glucose dissolved in water.

Note: National Glycohemoglobin Standardization Program (NGSP); Diabetes Control and Complications Trial (DCCT) (Standards of medical care in diabetes-2014, 2014).

The Impact of Diabetes

In the year 2000, the World Health Organization estimated that approximately 171 million people in the world had diabetes and that this number will increase to 366 million by 2030 (World Health Organization, 2006). The International Diabetes Foundation states that 387 million people in the world are currently living with diabetes (Aguiree et al., 2013), anticipating this figure to increase by 205 to 592 million by 2035. Additionally, a number of other strong figures point to grand scale of diabetes' impact: 1 out of every 12 individuals has diabetes, 46.3% (179 million people) of those people are living with the condition undiagnosed, in 2014, a total of 4.9 million died in relation to diabetic health issues, and in regards to healthcare expenditure $1 out of every $9 is spent on diabetic health related issues. (Aguiree et al., 2013; Beagley, Guariguata, Weil, & Motala, 2014).

Health Resources Strain Due to Diabetes

With the increasing diagnosis of this disease, there inevitably is a rise in health care costs. The American Diabetes Association (ADA) estimated the national costs of diabetes in the U.S. for 2002 to be $132 billion. By 2012, the financial stress in the U.S. from diabetes totaled $245 billion, which included $176 billion in direct medical cost and $69 billion in lost productivity (absenteeism). Additionally, 59% of direct medical costs are related to the population aged 65 years and over. However, roughly 88% of indirect

costs were associated with diabetes in individuals less than 65 years of age (American Diabetes Association, 2013; U.S. Department of Health and Human Services, 2014; World Health Organization, 2006). American health resources devoted to diabetes care in 2012 is projected to have been ~168 million hospital inpatient days, with 43.1 million of these days (25.7%) being accumulated by people with diabetes alone, and roughly 33% of all nursing and residential facility days occupied by people with diabetes as well. Moreover, half of all physician office visits, emergency department visits, outpatient hospital visits, and medication prescriptions (not including insulin and other antidiabetic agents) were related to people with diabetes (Association, 2013). More recent data from the International Diabetes Federations (IDF) has shown that in 2013, the majority of diabetes related health expenditures worldwide occurred in the U.S., which spent ~$239 billion on diabetes or 43.6% of the total global health expenditure. Internationally, in 2014, diabetes was responsible for a staggering ~$612 billion dollars in health spending, which equated to roughly 11% of total spending relative to adults. This equated to a cost-per-person in the U.S. that was roughly estimated at $11,000 compared to the average world estimated cost of $1,600 (Aguiree et al., 2013). The growing number of individuals affected by diabetes is staggering. This, combined with the overwhelming financial stress of treating this condition has motivated more research to discover innovative routes toward therapies and co-therapies that mediate hyperglycemia, and ultimately save lives.

Non-pharmacological Therapies for Diabetes

When lifestyle changes and efforts fall short of the desired goal, pharmacological

therapeutic approaches to treat T2D usually consists of 1) Metformin, depending on tolerance, is the preferred initial pharmacological agent, 2) insulin therapy for patients who have been recently diagnosed with T2D and have robustly elevated glucose levels or HbA_{1C} values, 3) if the non-insulin therapy at maximum tolerated dose is unable to achieve goal of HbA_{1C}, then additional pharmacological agents are added such as a GLP-1 receptor agonist or basal insulin analogs (Standards of Medical Care in Diabetes-2016 Abridged for Primary Care Providers, 2016).

Newer research has focused on less expensive non-pharmacological nutritional therapies such as whey protein or amino acid supplementation. Whey or its constituent amino acids independently, and in a mixture form, have shown to have a glucose lowering effect when consumed with, or previous to a glucose load or mixed meal (Frid, Nilsson, Holst, & Björck, 2005; Nilsson, Holst, & Bjorck, 2007; Nilsson, Stenberg, Frid, Holst, & Bjorck, 2004; Petersen et al., 2009). The research of the effects of protein and/or its constituent amino acids on glucose metabolism is not considered novel. However, recent research has shown that whey has an insulinotropic effect which is regulated by enteroendocrine hormones incretins glucose-dependent insulinotropic peptide (GIP) and glucagon like peptide-1 (GLP-1). Individuals with T2D may be prescribed medications that mediate incretin secretion by dipeptidyl peptidase IV(DPP-IV) inhibition, which inactivate these gut hormones and cancel the actions of these incretins. These medications are appropriately named DPP-IV inhibitors and have been shown to have a significant glucose lowering effect without inducing negative responses such as weight gain or increase the risk of hypoglycemia that is seen in persons with

metformin and sulfonylurea use (Avogaro & Fadini, 2014). Interestingly, whey protein may directly stimulate GLP-1 secretion, which slows gastric emptying and indirectly influence both incretins through the inhibition of the incretin degradation enzyme DPP-IV. Lastly, whey protein and its constituent amino acids may directly influence skeletal muscle uptake of glucose independent of GIP and GLP responses (Baggio & Drucker, 2007; Doi, Yamaoka, Fukunaga, & Nakayama, 2003; Liu et al., 2014; Nilsson et al., 2007; Standards of medical care in diabetes-2014, 2014).

The effects of amino acids on glucose and pancreatic hormones insulin and glucagon have been well documented. Gannon and Nuttall (2010) reviewed the effects of numerous amino acids and their individual relationships with glucose, glucagon, and insulin. They reported that the insulin area response of an individual amino acid ingested with glucose was relatively small. However, with the exception of leucine, when it was co-ingested at a lower glucose drink dose, a larger insulin response was observed. Additionally, they suggested that specific amino acids stimulate a differing magnitude of insulin responses when they were ingested with glucose compared to when ingested alone. Amino acid ingestion with glucose seemed to minimize the glucagon reduction that is normally seen with glucose ingestion (insulin inhibits glucagon secretion). Similar to insulin, they found that specific amino acids that stimulated glucagon secretion to a differing magnitude when ingested alone or with glucose. The GIP and GLP-1 responses were not investigated (Gannon & Nuttall, 2010).

There is an acute amount of research investigating amino acid mixtures that have shown to be insulin secretagogues and therefore increasing the concentration of insulin

synergistically with glucose to augment glucose uptake. This action is similar to whey protein. However, these same amino acid mixtures seem to have a negligible effect of stimulating both GIP and GLP-1 secretion compared to whey (Nilsson et al., 2007). Moreover, rodent model and myocellular culture research has shown how L-Isoleucine and or L-Leucine mechanistically influence glucose uptake into skeletal muscle cells through a process of intramuscular cell signaling that *mimics* insulin regulated signaling and induces GLUT-4 translocation similar to the actions of insulin (Doi et al., 2003; Doi et al., 2005; Doi, Yamaoka, Nakayama, Sugahara, & Yoshizawa, 2007; Liu et al., 2014; Nishitani, Takehana, Fujitani, & Sonaka, 2005). Nishitani et al., and Doi et al. (2003), observed the impact of ingested of L-Isoleucine and L-Leucine influencing glucose uptake in rodent muscle models. This action was regulated by the process of intramuscular cell signaling of phosphorylated proteins found in the IRS-$_{1/2}$ Akt, and GLUT-4 transporter that is normally governed by insulin. Furthermore, others have investigated the pancreatic hormones response to amino acids with little focus on incretin responses, yet it is suggested that incretins are 50-70% responsible for insulin-mediated glucose uptake (Baggio & Drucker, 2007). This creates questions with regard to how certain mixtures of amino acids or individual amino acids may have an effect on glucose uptake independent of incretin responses and normal insulin driven glucose uptake (Baggio & Drucker, 2007; Gannon & Nuttall, 2010; Wang et al., 2012), or more specifically, how BCAA's L-Leucine and L-Isoleucine may independently and synergistically affect glucose uptake in relation to an incretin response.

Hypothesis and Purpose Statement

Impaired glucose tolerance or diabetes is classified as an impairment of the body's ability to regulate blood glucose concentrations (hyperglycemia). Uncontrolled hyperglycemia can give rise to macrovascular (i.e., heart disease and stroke) and microvasculature damage such as retinopathy, nephropathy, and neuropathy. These comorbidities may definitively reduce the quality of life. The purpose of this research is to measure the effects of a dose of L-Leucine and L-Isoleucine on incretin responses of GLP-1 and GIP and glycemic responses to explore further the relationship of BCAA's and their influence on glucose uptake. Our *hypothesis* is that the ingestion of BCAA family members, L-Isoleucine and L-Leucine at a dose of 0.3g/kg/LBM (Doi et al., 2003; Doi et al., 2005; Doi et al., 2007) ingested prior to a glucose load (75 g) in 50:50 g combination and independently will improve glucose tolerance. The hypotheses will be supported if we observe a reduction in glucose concentrations with the ingestion of L-Isoleucine (ISO) and L-Leucine (LEU) separately or together with minimal incretin responses ($GIP_{Total,}$ $GLP\text{-}1_{Active}$).

- Hypothesis 1 – L-Leucine will have no effect ($p > .05$) on GIP and GLP-1 secretion.

- Hypothesis 2 – L-Isoleucine will have no effect ($p > .05$) on GIP and GLP-1 secretion.
- Hypothesis 3 – L-Leucine and L-Isoleucine in combination will have an effect ($p < .05$) on GIP and GLP-1 secretion.
- Hypothesis 4 – L-Leucine will have an effect ($p < .05$) on insulin secretion.
- Hypothesis 5 – L-Isoleucine will have no effect ($p < .05$) on insulin secretion.
- Hypothesis 6 – The combination of L-Leucine and L-Isoleucine will have a greater effect ($p < .05$) on glycemic responses compared to the amino acids ingested individually.
- Hypothesis 7 – All amino acid treatments will have a greater effect ($p < .05$) on glycemic responses compared to control.

Significance

This investigation addresses a unique and important issue of examining the relationships of BCAA family members L-Leucine and L-Isoleucine, pre-ingested before a glucose load on: blood glucose, enteroendocrine incretins GLP-1 and GIP, and pancreatic derived endocrine hormones C-peptide, insulin, and glucagon. Previous research has investigated how these amino acids have an insulinogenic effect by directly stimulating pancreatic insulin secretion. Others have examined how these amino acids may directly influence skeletal muscle uptake of glucose in either a rodent or myocellular culture model. Additionally, it has been reported that these BCAA's have a limited effect on incretins GLP-1 and GIP secretion, which are stated to be primarily responsible for

insulin release. To our knowledge, the effect of BCAA's L-Leucine and L-Isoleucine independently and synergistically influencing incretin secretion has not been assessed.

To address this question, we developed a research design to determine the effect of a standard dose of each amino acid, ingested individually or in combination, prior to a glucose load on plasma glucose, insulin, C-peptide, glucagon, GLP-1, and GIP responses in apparently healthy, inactive, adult men and women. Investigating these glycemic responses will be additive to current research and further isolate the relationship of these BCAA's on glucose concentrations and their influence on incretins GLP-1 and GIP. Moreover, for practical means, these study outcomes may lead to a possible supplemental co-therapy for persons with impaired glucose disposal; and lastly motivating further research to investigate the direct relationship between BCAAs and glucose uptake in human skeletal muscle.

Abbreviations

GLUT-4	Glucose transport type 4
LEU	L-Leucine
ISO	L-Isoleucine
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
DPP-IV	Dipeptidyl peptidase-IV
OGTT	Oral glucose tolerance test
OGAATT	Oral glucose and amino acid tolerance test

Definitions

Glucose Transport 4 Protein (GLUT-4): is a transporter protein that in humans is the insulin-regulated glucose transporter predominantly found in adipose tissues and skeletal muscle that is responsible for insulin-regulated glucose transport into the cell (Huang & Czech, 2007).

Glucose-dependent insulinotropic polypeptide (GIP): is a 42-amino acid peptide incretin hormone that is produced by enteroendocrine K-cells and released into the circulation in response to macronutrient stimulation The main actions of GIP are to stimulate insulin secretion in a glucose-dependent manner, promotion of growth and survival of the pancreatic ß-cell, and stimulation of adipogenesis (McIntosh, Widenmaier, & Kim, 2009).

Glucagon-like peptide-1(GLP-1): is a 30-amino acid peptide incretin hormone produced in the intestinal epithelial endocrine L-cells by differential processing of proglucagon (normally found in pancreatic synthesis of glucagon). The predominant actions of GLP-1 are to stimulate insulin secretion and to inhibit glucagon secretion, thereby indirectly regulating glucose deposition (Holst, 2007).

Phosphatidylinositol 3-kinase (PI3K): is a lipid kinase that phosphorylates the D-3 position on the inositol ring in phosphoinositides. It is a heterodimeric enzyme that is activated in response to a number of extracellular signals including insulin and insulin-

like growth factor-I. Insulin stimulation results in the recruitment of PI3K to the plasma membrane where it subsequently phosphorylates Akt. PI3K plays a primary role in the signaling of GLUT-4 to the surface and glucose and amino acid uptake (Tsakiridis et al., 1995)

Assumptions

- Participants are expected to maintain similar eating patterns, kilocalorie intake, and food choices during the three days before each treatment day.
- Participants are expected to maintain minimal exercise activity and or a sedentary lifestyle during the duration of the study and to refrain from any exercise or abnormal and excessive physical activity concentrations, compared to baseline three days prior to treatment.
- All laboratory equipment is functioning properly with validity and reliability measurements being established. Proper calibration and the use of qualified and trained research staff to minimize any potential for errors.
- All participants are to arrive at each testing session in a fasted state (≥ 8 hours)
- All participants are to arrive with adequate sleep (7-8 hr) before each of the testing sessions
- All participants are expected to follow the experimental protocol and are able to discontinue at any time.
- The treatment dose of either amino acid and control days are fully ingested, and prescribed dose is ingested for each person appropriately.

- Data collection for female participants is only during the follicular phase of menses, or menstruation cycle (1-10th day).
- Anthropometric measures of weight (kg), fat mass (FM), and lean body mass (LBM) will remain the same during the duration of the study.

Limitations

- The 24-hour food frequency questionnaire (FFQ) is considered the "gold standard" for dietary intake data for large cohort investigations. However, it has been scrutinized for its variability and reproducibility (Kristal, Peters, & Potter, 2005). It has been suggested that the use of personal cell phone dietary applications may reduce participant burden and have shown a higher correlation (r = 0.60) of energy intake and total energy expenditure compared to traditional paper format (r = 0.45); although, they show similar kilocalorie/day variability. It is suggested that mobile applications for dietary recording maintain a similar bias and are a more cost-effective, less laborious, and more acceptable route of data collection (Illner et al., 2012; McClung et al., 2009).

- The oral glucose tolerance test (OGTT) is not recognized as the "gold standard" for measuring insulin sensitivity compared to the euglycemic-hyperinsulinemic clamp technique (Sacks et al., 2011). However, a few studies have shown strong correlations between the results of an OGTT and the euglycemic-hyperglycemic clamp in estimating the metabolic clearance rate of glucose (r = .80); insulin

sensitivity index (r = .79 - .86) (Matsuda & DeFronzo, 1999; Soonthornpun et al., 2003; Stumvoll et al., 2000).

- It has been suggested that gastric emptying accounts for 34% of variance in peak glucose concentrations (Horowitz, Edelbroek, Wishart, & Straathof, 1993). To observe any possible peak concentration differences, we utilized acute time points (i.e. 6 min, 10 min, 36 min, 40 min) additional to the standard OGTT data collection time points (i.e. 0 min, 30 min, 60 min, 120 min) similar to the protocol Horowitz et al. (1993) used.

Delimitations

- Twelve apparently healthy males and females between the ages of 21-45 who are considered to live a sedentary lifestyle of exercising less than 2-3 days/week.
- Participants were recruited from Texas Woman's University and within the surrounding Denton, TX area by flyers, mass email, and online advertisements.
- Participants were excluded from the study if they had prior usage (≥ 6 months) of any protein based BCAA dietary supplement or medications that may alter glycemic and metabolic responses.
- Participants were informed to consume water before treatment days to maintain relative euhydrative state.
- All participants were tested at the Texas Woman's University Exercise Physiology and Biochemistry Laboratories and in accordance with Helsinki Code

signing and comprehending the University IRB-approved informed consent documents.

CHAPTER II

LITERATURE REVIEW

Normal Insulin Transduction Signaling

Insulin cell transduction is initiated by the tyrosine phosphorylation of the insulin receptor substrate (IRS-1), while IRS-2 is considered dispensable during the interaction. Once insulin binds to IRS-1 this activates a cascade of phosphorylation reactions beginning with activation of class I phosphatidylinositol-3-kinase (p85/p110; PI3K) to generate the phospholipid phosphatidylinositol-3,4,5-P_3 (PIP_3). The activation of both phosphatidylinositol-dependent kinase (PDK) and mechanistic target of rapamycin 2 ($mTORC_2$) phosphorylate the serine^{473}/threonine kinase^{308} of Akt and then the phosphorylation of the Akt substrate AS160/TBC1D4 (160 kDa) and TBC1D1. AS160/TBC1D1 are highly homologous Rab-GTPase activating proteins that promote hydrolysis of guanosine-5'-triphosphate (GTP) to guanosine diphosphate (GDP) on GLUT-4-containing vesicles. In the GDP bound form, the GLUT-4-containing vesicles are inhibited from translocating to the cell membrane surface (Klip, Sun, Chiu, & Foley, 2014; Middelbeek et al., 2013). Newer research has added to this interaction with molecular machinery that mobilizes GLUT-4 to the surface via insulin signaling. Ras-related C3 botulinum toxin substrate 1 (Rac1) is a small (~21 kDa) signaling G protein (GTPase) and is a member of the Rac subfamily of the family Rho family of GTPases.

Rac1 is activated in response to insulin to PI3K signaling in skeletal muscle fibers and leads to cortical actin branching. This branching is mediated by the cytoskeleton regulators Actin-Related Protein 2 and 3 (Arp2/3) complex that may be considered to be the actual '*mechanical hardware*' in the pathway leading to GLUT-4 mobilization. Additionally, Rab GTPases transmit their signals to interact with myosin's Va, Vb, and Vc (MyoV) molecules that bind to actin filaments. MyoV molecules belong to the class of progressive '*molecular motors'* based on their inherent ability to facilitate product transfer along actin filaments (Klip et al., 2014).

Impaired Insulin Transduction Signaling

Insulin resistance (IR) is defined as a reduction in the responsiveness of tissues to normal insulin concentrations, and is a principal dysregulated action found in the pathology of Type 2 diabetes (T2D). A failure in IRS signaling leads to a reduction in basal or normal cellular signaling of uptake of glucose, which leads to compensatory hyperinsulinemia, hyperglycemia, and dyslipidemia. The binding of insulin to the insulin receptor (IR) in skeletal muscle tissue, promotes the auto-phosphorylation of three regulatory loop tyrosine residues, which lessens the inhibition of IR tyrosine kinase activity toward insulin receptor substrate (IRS). Tyrosine phosphorylation of IRS by IR generates binding sites for Src homology 2 (SH2) domain proteins, including the regulatory subunits of class 1a PI3K with subsequent signaling downstream for the activation of GLUT-4 endocytosis. IRS-1 and IRS-2 are large proteins which have roughly > 1,200 amino acid residues that contain highly similar amino terminal pleckstrin

homology and phosphotyrosine binding (PTB) domains (~100 AA each), followed by long, unstructured carboxyl terminal tail regions. The PH domain is the essential module that links IRS-1 with the activated IR (Copps & White, 2012). These ~1,000 AA tails are enriched in serine and tyrosine (S/T) residues, including many within canonical kinase phosphorylation motifs within the IRS-1 tail. In cultured cells, it has been observed that IRS-1 phosphorylation has a positive effect on S/T residues of insulin signaling; however, adverse effects have been observed also from other S/T signaling. For example, insulin-stimulated phosphorylation of human S1223 reduces IRS-1 and SH2 domain-containing protein tyrosine phosphatase-2 (SHP2) interaction, sustaining IRS-1 tyrosine phosphorylation and PI3K association. This promotes insulin signaling output by decreasing tyrosine dephosphorylation and inhibitory S/T phosphorylation at other sites. TNFα activates pathways involving the S/T kinases inhibitor of nuclear factor kB kinase-β (IKKβ) and c-Jun N-terminal kinase (JNK) this may alter human S312 phosphorylation, which is frequently suggested as evidence for IR. However, the influence of insulin on human S312 phosphorylation may also be explained by insulin stimulus or contribution of other phosphorylated S/T combinations (Copps & White, 2012).

Additionally, it has been shown the over nutrition and the over stimulating effect of insulin signaling to form a feedback loop of S/T phosphorylation of IRS by $mTORC_1$, and downstream protein ribosomal protein S6 kinase beta-1 (S6K1) may be responsible for altered phosphorylation. Among the IRS-1 S/T phosphorylated directly by these kinases in vitro are human S307 and S527 by S6K1 and S636) by $mTORC_1$. Other S/T

phosphorylation in vivo by S6K1 in human model S270 and S1101 or by $mTORC_1$ other kinases S312 and S616 when $mTORC_1$ mediated S6K1 signaling is robustly activated (Copps & White, 2012; Rivas et al., 2009). Skeletal muscle samples and cultures from T2D individuals have observed deficits in IRS to PI3K mediated atypical protein kinase C (aPKC), and Akt/PKB signaling transduction, which is shown to be similar to those seen in persons with impaired glucose tolerance (IGT). Increased basal phosphorylation of IRS at serine sites (S) S312, S323, and S636 has been shown to be decreased in the muscle of T2D individuals. Whereas basal and stimulated phosphorylation of S330 is specifically reduced in muscle of obese insulin resistant individuals. Lastly, it has been observed in the skeletal muscle of healthy and lean individuals that insulin fails to stimulate the phosphorylation of S531 and S1142 or a decrease in phosphorylation of S1100. These serine phosphorylating actions may normally be seen in the skeletal muscle of T2D's (Copps & White, 2012).

With so many factors and phosphorylation mechanisms to induce positive or negative outcomes of insulin signaling, it becomes very difficult to isolate particular S/T phosphorylation. A complete understanding of the relationship between S/T phosphorylation and insulin resistance remains challenging due to the large number of unknown and potential phosphorylations of S/T on IRS makes it difficult to isolate and determine overall patterns in human and animal tissues related to insulin signaling (Copps & White, 2012). However, it seems as though tyrosine phosphorylation of IRS-1 results in positive activation of the p85 regulatory subunit of PI3K and activates the p110 catalytic subunit, leading to an increase in PIP_3. This activation of downstream protein

Akt/PKB and phosphorylation of substrate AS160 mediates the translocation of GLUT-4 to the sarcolemma and subsequent entry of glucose into the muscle fiber. Whereas, overall serine phosphorylation is associated with dysregulation of IRS-1 functions and standard cell transduction (DeFronzo & Tripathy, 2009).

Therapeutic Options for Type 2 Diabetes

The approach to managing hyperglycemia in persons diagnosed with T2D is multifactorial and dynamic. The preferential course prescribes dietary and exercise-based intervention concurrent with pharmacological agents and possible non-pharmacological therapies to assist in the modulation of hyperglycemic events. Similarly, a comprehensive program to attenuate cardiovascular risk factors which include smoking cessation, blood pressure control, and lipid management may reduce the progression of microvascular complications. Personalization of patient therapy becomes necessary, particularly when balancing the benefits of glycemic control with its potential risks, and accounting for the adverse effects of glucose-lowering medications (hypoglycemia), as well as a patient's age and health status. The patient and disease factors may influence the target for glucose control, as reflected by a patient's respective glycated hemoglobin (HbA_{1c}) control. HbA_{1c} measurements are a valid assessment of a person's glucose control. Relative to the person's diabetic disease diagnosis and progression, these measurements may identify what therapies are used, which is why HbA_{1c} tests are considered a particularly good determination of glucose control over longer periods of time. Other factors, such as age, life expectancy, comorbidities, and the

risks and consequences to the patient from an adverse drug event, are definitive factors that influence therapeutic directions for health professionals.

Lifestyle Modification of Weight Management

Weight management or weight loss has been suggested as an intricate factor to modulate the onset of T2D, and to therapeutically treat those diagnosed with T2D. A moderate weight loss of ~ 5% of total body weight can improve insulin sensitivity, which then leads to decrease fasting blood glucose concentrations, and additionally may lead to minimization of pharmacological medications related to T2D (Klein et al., 2004). Furthermore, the relationship between obesity via accumulation of fat mass and T2D has been well reported (Bjorntorp, 1988; Cassano, Rosner, Vokonas, & Weiss, 1992; Rush, Plank, Mitchelson, & Laulu, 2002).

The incidence of T2D diagnosis is 3 to 7 times higher in obese individuals, as compared with normal-weight adults. More specifically, those with a BMI > 35 kg/m^2 are 20 times more likely to develop diabetic conditions than those with a BMI between 18.5 and 24.9 kg/m2 (Klein et al., 2004). Additionally, weight gain in adulthood has been strongly associated with onset of T2D. An epidemiologic study by Colditz et al. (1990) observed a cohort of 113,861 US women, healthy without a history of diagnosed T2D, aged 30-55 year in 1976-1990. Among women with an average body mass index, 23-23.9 kg/m^2, the relative risk was 3.6 times that of women having a body mass index less than 22 kg/m^2. After adjusting for family history, there was an observation that an increase of 20-35 kg body weight was related to an increased risk (11.3%) of T2D, and with an increase of 35 kg or more, the risk increased even more (17.3%). However, in

contrast to weight gain, women who lost more than 5 kg reduced their risk for diabetes mellitus by 50% or more (Colditz et al., 1990). Similarly, in another prospective cohort study of 20,187 male alumni from Harvard University and the University of Pennsylvania (starting in the years 1962 and 1966 respectively, to the year 1998) were weighed and followed. Here, researchers observed 1,223 men develop T2D, with weight gain significantly increasing the risk of disease onset. Even among men with an initial BMI below 21 kg/m^2, weight gain significantly increased the risk of T2D, which suggests that a lower initial BMI does not attenuate the increased risk of T2D with weight gain (Oguma, Sesso, Paffenbarger, & Lee, 2005).

It has been reported that 80 to 90% of people with T2D are overweight or obese. Each kg of weight lost through health behavior modification in people with IGT is associated with a relative diabetes risk reduction of 16% (Lau & Teoh, 2013). The benefits of weight loss for individuals diagnosed with T2D has also been well supported. A moderate weight loss of ~ 5% may improve cellular insulin actions, thereby decreasing fasting blood glucose concentrations, and possibly reducing dependence on pharmacological agents prescribed. A 1990 prospective T2D study by the United Kingdom Prospective Diabetes Group observed on average, patients with a fasting plasma glucose of 10 to 12 mmol/l needed to lose 28% ideal body weight (18 kg) to attain a fasting plasma glucose level of less than 6 mmol/l after 3 months of dieting. However, during the second and third months of diet treatment, the patients' fasting blood glucose increased by a mean of 0.4 mmol/l independent of weight reduction (1.4 kg). It was suggested that the decrease in fasting plasma glucose is determined more by

the factor of energy intake restriction than by the body weight (UK Prospective Diabetes Study 7,1990).

It is possible that T2D patients may be less responsive to the therapy of dietary restriction; Watts et al. (1990) categorized T2D into "*responders*" and "*non-responders*" when fifty-five (41%) of 135 obese patients with basal blood glucose ~ 10 mmol/l experienced a ~9.1 kg weight loss and a reduction in plasma glucose level to ~ 7.0 mmol/l. Many of the deemed responders had improved plasma glucose concentrations after only a modest weight loss. In contrast, eighty (59%) of 135 patients with a ~ 9.1 kg weight loss, had plasma glucose levels that showed ~18.3 mmol/l (Watts et al., 1990).

In regards to patients who may not respond to traditional therapies of weight loss, whom are diagnosed morbidly obese, T2D, and may benefit from bariatric surgical procedures, a recent meta-analysis observed the long term effects of bariatric surgery. Yu et al. (2015) observed a decrease in BMI 13.4 kg/m^2, fasting blood glucose 59.7 mg/dl, and HbA_{1c} 1.8% with bariatric surgery. This group suggested that 89.2% of patients with T2D had improved their diabetic status to the point of remission (Yu et al., 2015), and appropriately expanded its criteria for those who needed metabolic surgery to patients with T2D and a BMI of 30-35 kg/m^2 (Fruhbeck, 2015). Further information may be found in these reviews since bariatric surgery is out of the scope of this research study (Reis, Alvarez-Leite, Bressan, & Alfenas, 2012; Ribaric, Buchwald, & McGlennon, 2014).

Weight loss has other health benefits other than reduction of plasma glucose. It has been suggested that weight loss also reduces the risk of cardiovascular disease (CVD)

mortality and macrovascular complications, (Staimez, Weber, & Gregg, 2014; Wing, 2010), blood pressure reduction (Bjorntorp, 1988; Holzgreve, 2008; Szabo, 1998; Wing, 2010), attenuating serum lipid composition and concentrations by way of decreasing serum triglycerides (TG), total cholesterol (TC), and LDL cholesterol while increasing serum HDL cholesterol (Krentz, 2003; Pascual et al., 2009; Pudar-Brankovic, Trpkovic, & Janosevic, 1995). Additionally, with weight loss, there has been an observation of a reduction in serum cytokine markers that are implicated in inflammatory responses associated with IR (Aldhahi & Hamdy, 2003; Calder et al., 2011; Chae et al., 2013; Cottam et al., 2004; Ryan & Nicklas, 2004). Lastly, weight loss may also be used as a prophylactic therapy to reduce the incidence or stave off the onset of T2D and its comorbidities. It has been shown that lifestyle interventions, which include diet and physical exercise modifications, may result in a reduction of around 50% in diabetes incidence that remains stable even after the individual lifestyle counseling has discontinued (Sanz, Gautier, & Hanaire, 2010). Other research directed at lifestyle modifications for T2D prevention has examined positive changes in the incidence of diabetes, and has shown risk reductions to such interventions when therapies of dietary, exercise, and lifestyle modifications are pooled together (G. N. Thomas et al., 2010).

Lifestyle Modifications of Dietary Alterations

The goal of modifying dietary or nutritional intake and choices of persons with T2D is to promote and support healthy eating patterns, choosing a variety of nutrient dense foods with appropriately portioned sizes, to improve the overall health of the individual and more specifically to mediate individualized glycemic, blood pressure, and

lipid goals. The American Diabetes Association (ADA) general recommended goals for this population are HbA_{1c} < 7%, (BP) blood pressure < 140/80 mmHg, LDL cholesterol < 100 mg/dl, triglycerides < 150 mg/dl, HDL cholesterol > 40 mg/dl for men and HDL cholesterol > 50 mg/dl for women to prevent further complications of diabetes. The success of reaching these aims is predicated on assisting and addressing individual nutritional needs based on personal and cultural preferences, health literacy, access to healthful food choices, willingness and ability to make behavioral changes, as well as acknowledging barriers to change. The attentional focus and direction of healthful food choices increases the pleasure of eating and it is maintained by providing positive feedback about food choices while limiting food choices only when indicated by the empirical evidence. Lastly, providing the individual with T2D with practical tools for regular meal planning rather than focusing on individual macronutrients, micronutrients, or singular foods, has shown to be effective (Evert et al., 2014).

Due to the relationship between body weight (i.e., fat mass accumulation) and insulin resistance, weight loss is a recommended strategy for overweight or obese adults with diabetes; however, the long term reduction of fat mass is difficult for most people to achieve and may be even harder for individuals with diabetes to achieve with diet alone. Lifestyle intervention weight loss research of 12 months (or longer) for T2D individuals noted a range of weight loss of 1.9 to 8.4 kg which was defined as moderate. In the 10 year Look AHEAD (The Action for Health in Diabetes) trial, the mean weight loss from baseline was 6% in the intervention group (ILI) and 3.5% in the control group (DSE). Additionally, during this trial they observed a mean decrease in HbA_{1c} from 7.3 to 6.6%

in ILI ($p < 0.001$) vs. 7.3 to 7.2% in DSE (Evert et al., 2014; Pi-Sunyer et al., 2007).

The current literature does not support a specific nutrition therapy approach to reduce excess weight (i.e. Mediterranean style, DASH, vegetarian or vegan, low carbohydrate, and low fat), but rather a range of eating patterns that result in reduced energy intake. A weight loss of > 6 kg (~7-8.5% loss of initial body weight), regular physical activity, and consistent interaction with registered dieticians (RDs) are of great importance for the beneficial effects of weight loss interventions. Successful strategies for weight loss in the Look AHEAD study were associated with lower BMI in overweight or obese T2D individuals that included weekly self-weighing, regular consumption of breakfast, and reduced intake of fast foods (Evert et al., 2014; Franz, Boucher, Rutten-Ramos, & VanWormer, 2015).

Table 1

Nutritional Strategies for All People with Diabetes

- Portion control is recommended for weight loss and maintenance.
- Carbohydrate based foods and beverages and endogenous insulin response are the greatest factors of the post meal blood glucose level. It is important to know what foods contain carbohydrates (i.e. starchy vegetables, whole grains, fruit, milk and milk products, vegetables, and sugar).
- When choosing carbohydrate-based foods, choose nutrient dense, high fiber foods opposed to processed foods with added sodium, fat, and

sugars. Nutrient dense foods and beverages provide essential vitamins, minerals, and other healthful substances with less calories.

- Avoid sugar-sweetened beverages.
- Unnecessary to subtract the amount of dietary fiber or sugar alcohols from total carbohydrates when counting carbohydrate.
- Substitute foods higher in unsaturated fat (liquid oils at room temperature) for foods higher in trans or saturated fat.
- Leaner protein selections and meat alternatives.
- Due to lack of empirical evidence, vitamin and mineral supplements, herbal products, or cinnamon to manage diabetes are not recommended.
- Moderate alcohol consumption (≤ 1/day for adult women and ≤ 2/day for adult men) has shown to have minimal acute or chronic effects on blood glucose in people with diabetes.
- Limit sodium intake to 2,300 mg/day.

Adapted from (Evert et al., 2014)

Successful dietary strategies for diabetes management and prevention include meals rich in wholegrains, fruits, vegetables, nuts, legumes, moderation in alcohol consumption, and lowered consumption of refined grains, red or processed meats, and sugar-sweetened beverages. To achieve long-term adherence to this diet plan, individuals can have flexibility in food choices without compromising overall diet quality. There is no standard meal plan or eating pattern that works universally for all people with diabetes. In order for an intervention to be effective the nutrition therapy should be

individualized for each patient/person based on his or her individual health goals, personal and cultural preferences, health literacy and comprehension of fundamental arithmetic, access to healthy lifestyle choices, and most importantly readiness, willingness, and ability to change current lifestyle for the better (Evert et al., 2014; Ley, Hamdy, Mohan, & Hu, 2014; Standards of medical care in diabetes-2014, 2014).

Pharmacological Therapeutic Agents

Sodium-glucose Cotransporter 2

Sodium–glucose cotransporter 2 (SGLT2) inhibitors are a newer class of antidiabetic drugs that reduce renal glucose reabsorption in the proximal convoluted tubule, leading to increased urinary glucose excretion (Canagliflozin, Dapagliflozin, Empagliflozin). The SGLT2 is a high capacity and low affinity transporter that is highly expressed and activated in patients with T2D and is responsible for 80-90% of renal glucose reabsorption and therefore reducing glucose reabsorption and increasing urinary glucose excretion by up to 80 g/day (Inzucchi et al., 2015; Vasilakou et al., 2013). SGLT2 showed associated with a 0.66% reduction in the HbA_{1c} level and had glycemic efficacy similar to that of other antidiabetic agents. Additionally, SGLT2 demonstrated to influence a modest weight loss (~2 kg) which showed to stabilize over 6–12 months and a consistent lowering of systolic and diastolic blood pressure (systolic ~ 2–4 and diastolic ~1–2 mmHg). However, the side effects such as include genital mycotic infections, vulvovaginal candidiasis in women and candida balanitis in men (Nyirjesy & Sobel, 2013). Women tended to have a higher rate of infection (11%), while men had a

lower rate (4%) of infection when compared with placebo with usage. Similarly, participants reported higher rates of urinary tract infections which were attributed to glycosuria. Lastly, SGLT2 seemingly had a diuresis effect which may become a more compounded issue with an older adult who may already be prescribed diuretic medications. Small increases in serum creatinine have been observed, which may be reversed; increased urine calcium excretion, and small elevations in LDL cholesterol (~5%) has also been observed in some trials, though these results have not been fully elucidated. Microvascular outcomes concerning SGLT2 are currently being evaluated for safety and risks (Inzucchi et al., 2015; Vasilakou et al., 2013).

Thiazolidinediones

The peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that play integral roles in the regulation of metabolic homeostasis and inflammation. There are three PPAR isoforms, PPARα, β/δ, and γ, and these vary in their expression and distributions in differing tissues. Their selectivity to differing ligands, which leads to the modulation of differing sets of genes. Thiazolidinediones (TZDs) also known as Glitazones (Pioglitazone and Rosiglitazone) have a high affinity to PPARγ ligands and are potent insulin-sensitizing agents that effectively improve glycemic control in T2D's. The mechanism of action of TZDs is bind to PPARγ, which leads to the formation of heterodimers with retinoid-X-receptors (RXRs). Followed by binding to specific DNA sequences termed peroxisome proliferator response elements (PPREs), found on the promoters of PPARγ target genes, subsequently stimulate their transcription, known as a transactivation, which is involved in regulating metabolic homeostasis. PPARγ plays a

key role in adipogenesis and it has been demonstrated it is crucial for adipocyte differentiation (Cariou, Charbonnel, & Staels, 2012). Ligand-activated PPARγ induces genes involved in lipogenesis and adipogenesis, including the CAAT/enhancer binding protein-α (C/EBPα). C/EBPα, which in turn promotes the transcription of PPARγ in a positive feedback regulatory loop. TZDs improve insulin sensitivity by enhance the storage of white adipocytes and indirectly reduce lipotoxicity by inhibiting intracellular lipid storage from liver and skeletal muscle which has directly shown that their accumulation impairs insulin signaling (Badin, Langin, & Moro, 2013; Van Steenbergen & Lanckmans, 1995). Additionally, PPARγ inhibits expression of inflammatory response genes via a mechanism termed ligand-dependent transrepression. This mechanism is independent of PPARγ ligand binding to its nuclear receptor (transactivation) and initiates antagonistic signal activation of its target genes by other classes of transcription factors, including NF-κB and AP-1 expressed proteins, which inhibits inflammatory signaling pathways. PPARγ also modulates the production and secretion of adipokines, including adiponectin, resistin, tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and monocyte chemoattractant protein (MCP-1). Adiponectin expression is increased by TZDs and has been shown to play an integral role in insulin sensitizing effect. WAT related macrophage-induced immune inflammatory response has shown pathogenic cause of insulin resistance. An accumulation of inflammatory M1-type macrophages in WAT increases the production of inflammatory cytokines TNFα and IL-6, which contribute to the deterioration of cellular insulin sensitivity. However, PPARγ acts as an anti-inflammatory factor in M1 macrophages and promotes the

polarization of human monocytes into contrasting anti-inflammatory M2 macrophages (Cariou et al., 2012; Gordon, 2003).

The primary outcome of PPARγ target gene activation in regards to adipocyte storage where it influences a lipid repartitioning toward adipocytes and thereby prevents skeletal muscle and liver lipid deposition and therefore lipotoxicity, and macrophage lipotoxicity and M1 polarization. Side effects associated with TZDs consist of fluid retention and edema, which may be related to both increased vascular permeability and vasodilatation and fluid retention by the kidney. Additionally, it has been shown that possibly in relation to the edema, the clinical use of TZDs was associated with a higher risk of further macrovascular events concerning congestive heart failure (CHF) with a 70% increased risk of CHF. Bladder cancer incidence was also increased, with a relationship between both the dose and the duration of TZD treatment and the risk of bladder cancer (Cariou et al., 2012; Charbonnel et al., 2004; Goldstein, 2006; Lee & Marcy, 2014). Lastly, in women predominantly, it was found that there was an increased incidence of bone fractures (OR = 1.94; 95% CI: 1.60-2.35; $p < 0.001$) while not in men (OR = 1.02; 95% CI: 0.83-1.27; $p = 0.83$) (Zhu, Jiang, & Ding, 2014).

Dipeptidyl Peptidase IV Inhibitors

Dipeptidyl peptidase IV inhibitors (DPP-IV) are a relatively new family of oral hypoglycemic pharmacological treatment. Sitagliptin, vildagliptin, and saxagliptin are a few that are currently approved by the US Food and Drug Administration (FDA) or the European Medicines Agency (EU), while others are awaiting approval or are currently in development. The DPP-IV gene family includes four enzymes—DPP-IV, DPP-VIII,

DPP-IX, and fibroblast activation protein (FAP)—and catalytically inactive proteins DPP-VI and DPP-X. DPP-IV has a ubiquitous organ distribution (hepatic, GI, endothelial capillaries; acinar cells of mucous and salivary glands of the pancreas, uterus, and immune organs such as thymus, spleen, and lymph node) with the highest concentrations found in the kidney (Gupta & Kalra, 2011). The therapeutic efficacy of DPP-IV inhibitors is related to their inherent ability to increase circulating concentrations of the intact, biologically active forms of the incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). In response to a meal, the flux in GLP-1 and GIP concentrations postprandial is followed by a rapid degradation by the enzyme dipeptidyl peptidase-IV (DPP-IV).

Both GLP-1 and GIP increase the sensitivity to glucose of the β-cells and thus enhances glucose-dependent insulin secretion. GLP-1 also improves the sensitivity to glucose of the α-cells under hyperglycemic conditions and modulates unneeded glucagon secretion resulting in a decrease insulin resistance. Similarly, GIP enhances the sensitivity of the α-cell to glucose under hypoglycemic conditions and stimulates the enhancement of glucagon counter-regulation to maintain glycemic homeostasis (Ahrén et al., 2011; Foley, 2014).

There are several peptides released (i.e. oxyntomodulin, peptide YY, cholecystokinin) by the intestine into the circulation in a nutrient-mediated manner, that stimulate postprandial insulin release by the pancreas, act as a signal for the regulate appetite, influence the gut absorption of nutrients, and the maintenance of energy homeostasis. GIP is predominantly synthesized and released by enteroendocrine K-cells

present in the proximal region of the small intestines (duodenum and jejunum) in response to mainly glucose or lipid ingestion and enhances glucose-stimulated insulin secretion (Figure 1) (Baggio & Drucker, 2007; Duez, Cariou, & Staels, 2012; Gutierrez-Aguilar & Woods, 2011). GLP-1 is synthesized in the gut from a precursor proglucagon peptide that can also be cleaved into GLP-2, oxyntomodulin and glicentin-related pancreatic polypeptide (GRPP). GLP-1 is synthesized and secreted by L-cells primarily of the distal small intestines (ileum) and colon. Basal incretin plasma concentrations of both peptides are low in the fasted state, though will rise postprandial within minutes and contribute to lower blood glucose by exerting potent glucose-dependent insulin stimulatory actions (Figure 1). It has been suggested, that the incretin effect is responsible for ~50–70% of the postprandial insulin responses in healthy individuals. However, the contribution to the overall insulin response in T2D's after oral glucose ingestion may amount to < 20% (Baggio & Drucker, 2007; Meier & Nauck, 2010; Nauck et al., 1986).

GLP-1 is secreted as GLP-1 (7-36)-amide (~80% as the major circulating and primary form of GLP-1) (Gupta & Kalra, 2011) and GLP-1 (7-37). GLP-1 is released within minutes of food ingestion and has a very short half-life (~2-3 min) because of its rapid degradation (Figure 3) through the action of DPP-IV and its renal clearance. Only ~25% of the secreted GLP-1 reaches the portal vein, and only 10-15% the systemic circulation. Comparatively, in humans, the basal circulating active form of GIP (1-42) and DPP-IV degraded form of the inactive metabolite GIP (3-42) concentrations range between 0.06-0.1 nmol/l (60-100 pmol/l), depending on the assay used to measure total

vs. intact GIP, and postprandial increase to 0.2–0.5 nmol/l (200-500 pmol/l) the time period of activity for GIP is 5-7 min in diabetic and non-diabetic subjects, respectively (Baggio & Drucker, 2007).

In comparison to GLP-1, GIP similarly promotes insulin secretion in response to glucose; however, it does not inhibit glucagon secretion nor does it mediate gastric emptying. Additionally, it does influence the increase of fatty acid uptake and lipogenesis by adipocytes and therefore promotes fat deposition shown in more depth in Figure 2 (Duez et al., 2012). The comparison of intact incretin hormones concentrations after pharmacological intravenous infusion in humans found that 40% of GIP remains intact and bioactive compared to only 20% of GLP-1, which proposes that GIP may be less susceptible to DPP-IV degradation in vivo, and may have a slightly longer plasma half-life when comparing GIP vs. GLP-1 (Baggio & Drucker, 2007). It has also been suggested that in diabetics that the GIP response to a glucose load may be compromised, ~36% compared to a normal healthy person of ~72% which would facilitate the use of pharmacological incretin agonists (Nauck, Stöckmann, Ebert, & Creutzfeldt, 1986).

DPP-IV inhibitors have differing pharmacokinetic and pharmacodynamic characteristics though are remarkably similar with regards mediating hyperglycemic responses. The current and future family of 'gliptins': *Sitagliptin* (Merck Sharp and Dohme Corp, approved as Januvia by US FDA in year 2006), *Vidagliptin* (Novartis, approved as Galvus by EU in year 2007), *Saxagliptin* (Bristol-Myers Squibb, approved as Onglyza by US FDA in 2010), *Linagliptin* (Boerhinger Ingelheim, approved as Tradjenta by US FDA in year 2011), *Alogliptin* (in process by Takeda Pharmaceutical Company

Limited, approved for use in Japan), *Dutogliptin* (in process by Phenomix Corporation), *Gemiglaptin* (in process by LG Life Sciences), *Sitagliptin, Vidagliptin, Saxagliptin* (approved for use in India) (Gupta & Kalra, 2011).

The family of DPP-IV inhibitors can be divided into mimetics of the DPP-IV molecule (*peptidomimetics, vildagliptin and saxagliptin*) and those that are not (*non-peptidomimetics, sitagliptin, alogliptin, linagliptin*) They are competitive reversible inhibitors of the DPP-IV substrate acting extracellularly. These molecules have varying affinities toward the DPP-IV substrate. Overall, the peptidomimetics have lesser selectivity toward DPP-IV compared to DPP-VIII/IX. The lower the relative selectivity toward DPP-IV, and greater the relative inhibition of DPP-VIII/IX, yields an increased possibility of side effects (Gupta & Kalra, 2011).

In general, DPP-IV inhibitors prolong the physiological meal influenced response of GLP-1 and GIP. The magnitude of prolongation depends on how complete the DPP-IV inhibition is. A ~50% inhibition would extend the half-life 2-fold, a 90% inhibition increases 10-fold, a 95% inhibition increases 20-fold. Other than the degradation rates, the half-life is primarily determined by the renal clearance and excretion of GLP-1 and GIP. However, it should be noted that the actual degree of DPP-IV enzyme activity, when it is inhibited by 90–95%, is essentially at the limit of detection of the enzyme assays. Consequently, it is not possible to truly distinguish between values above 90% inhibition of DPP-IV (Ahrén et al., 2011; Foley, 2014).

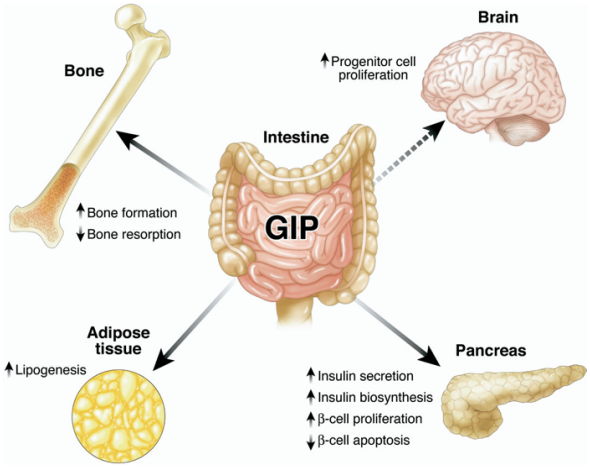

Figure 1: The effect of GIP on multiple tissues and physiological outcomes (Baggio & Drucker, 2007)

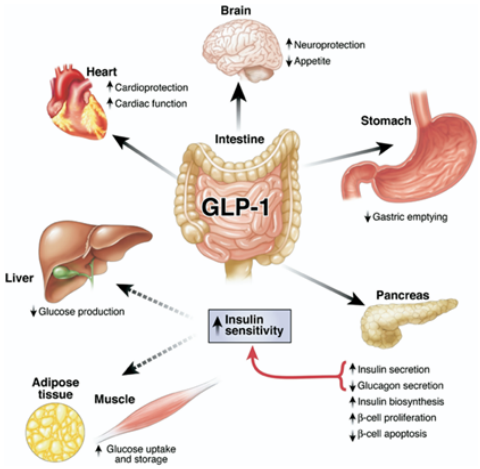

Figure 2: The effect of GLP-1 on multiple tissues and physiological outcomes (Baggio & Drucker, 2007).

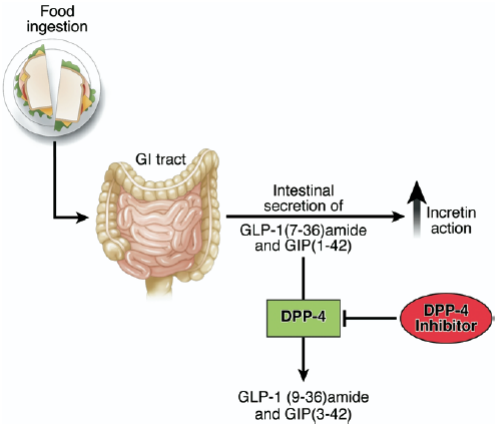

Figure 3: Enteroendocrine hormones GLP-1 and GIP secretion and subsequent degradation after nutrient ingestion. Diagram from (Baggio & Drucker, 2007).

Metformin

Metformin is currently the optimal drug for hyperglycemic therapy. However, it may be prescribed in conjunction with other pharmacological and non-pharmacological treatments. It has been in clinical use for over 50 years and based on its low cost,

efficacy, safety record reliability, weight neutral effects, and possible benefits of reduction in cardiovascular disease (CVD) and mortality (Paul, Klein, Majeed, & Khunti, 2015) when compared with less intensive treatments, and a possible reduction in cancer incidence (Vallianou, Evangelopoulos, & Kazazis, 2013; Yin, Zhou, Gorak, & Quddus, 2013). Other studies do not support these same positive outcomes (Mc Menamin, Murray, Hughes, & Cardwell, 2015). However, metformin is the recommended first-line oral therapy for the treatment of T2D (Inzucchi et al., 2015; Rena, Pearson, & Sakamoto, 2013).

The proposed mechanism of action of metformin is that it suppresses hepatic glucose production (HGP) through the protein-threonine kinase or liver kinase-B1 (LKB1) actuated adenosine monophosphate–activated protein kinase (AMPK) pathway. Metformin is transported into hepatocytes mainly via organic cation transporter-1 (OCT-1) this action influences an inhibition of the mitochondrial respiratory chain (complex I) through a mechanism currently not fully elucidated. This inhibition results in a decrease in energy production which is balanced by reducing the consumption of energy into the cell, namely, reduced gluconeogenesis in the liver. This anti-hyperglycemic action is suggested to be mediated in two main ways: 1) the increase in adenosine monophosphate (AMP) to adenosine triphosphate (ATP) ratio, which is proposed to stimulate gluconeogenesis directly due to low ATP concentrations; 2) increased AMP concentrations function as a key multi-signaling mediator that has been suggested to allosterically inhibit cyclic-AMP (cAMP) induced protein kinase A (PKA) signaling through the suppression of adenylate cyclase and allosterically inhibit fructose-1,6-

bisphosphatase (FBPase) (a vital gluconeogenic enzyme), and lastly activate AMPK (Rena et al., 2013).

AMPK is a conserved regulator of the cellular response to low cellular energy, and it is enabled when intracellular ATP concentrations decrease and its dephosphorylated counterpart AMP concentrations increase in response to nutrient deprivation (low energy status) and pathological stresses. AMPK maintains a critical role in many metabolic processes, including substrate glucose uptake and fatty acid oxidation in muscle, fatty acid synthesis and gluconeogenesis in the liver, and hypothalamic regulation of food intake. Structurally, AMPK is a heterotrimer, composed of the catalytic kinase α-subunit and two associated regulatory subunits, β and γ. During times of low energy status, AMP directly binds and partners with repeats of crystathionine-β synthase (CBS) domains in the AMPK γ-subunit causing a conformation change, which exposes the activation loop in the α-subunit that allows it to be phosphorylated by an upstream kinase. This cell transduction sequence is mandatory for activation loop of AMPKα, which is conserved across species, and its phosphorylation is requisite for AMPK activation (He, Meng, Germain-Lee, Radovick, & Wondisford, 2014; Shaw, 2005). It has been proposed that metformin stimulated LKB1–AMPK signaling modulates the expression of intricate gluconeogenic genes through the regulation of a transcription coactivator cAMP response element-binding protein-regulated transcription coactivator 2 ($CRTC_2$). $CRTC_2$ is in a dephosphorylated state in a fasting state and is localized in the nucleus where it enhances the transcriptional activation of gluconeogenic genes peroxisome proliferator-activated receptor-γ coactivator-1α (PPARGC1A) and its

ensuing gluconeogenic targets phosphoenolpyruvate carboxykinase (PCK1) and glucose-6 phosphatase (G6PC). Similar to an insulin secreted postprandial state, once these gluconeogenic genes are inhibited, the hepatic release of glucose is reduced (Rena et al., 2013).

The United Kingdom Prospective Diabetes Study (United Kingdom Prospective Diabetes Study 24, 1998) supported the safety and efficacy of metformin in obese patients with observing a greater effect than both sulphonylurea or insulin for any diabetes related all-cause mortality and stroke. Additionally, a significant reduction was also observed regarding myocardial infarction (MI) at the end of the trial in the metformin group compared with the conventional group [relative risk (RR):0.61; $p <$ .010] (United Kingdom Prospective Diabetes Study 24, 1998). In a 10-year follow up study they observed an early loss of glycemic differences (between-group differences in HbA_{1c}) after the first year, although a continued reduction in microvascular risk (24%, p = .001), risk reductions for MI (33%, p = .005), and death from any cause (27%, p = .002) [RR: 0.67; 95%, confidence interval (CI):0.51–0.89] (Holman, Paul, Bethel, Matthews, & Neil, 2008).

Although metformin has been considered the gold standard choice in the pharmacological modulation of hyperglycemia, it is not without contraindications and or side effects. Lactic acidosis (LA) has been associated with metformin treatment, although considered a rare conditional response, it is a critical adverse event because it can be fatal. Several conditions may increase the risk of LA associated with metformin, which can be categorized into: 1) promotion of the formation of lactate by the peripheral

tissues due to of hypoxia (circulatory failure, severe respiratory incapacity); 2) impairment of lactate metabolism through the pathway of gluconeogenesis due to primary or secondary hepatic failure; 3) robustly increased concentrations of metformin due to renal failure and inability to excrete active agent, leading to metformin accumulation and thereby compound the process of inhibiting liver gluconeogenesis (Scheen & Paquot, 2013). LA is defined as an arterial lactate of > 5 mmol/l and a blood pH ≤ 7.35. The two forms of LA associated with metformin use is Type A that is anaerobic LA caused by lactate production above normal ranges to resynthesize ATP in the absence of oxygen (O^2) and is usually associated with heart failure, sepsis, and shock. Type B, aerobic version, is caused by reduced usage of lactate due to impairment its oxidation or gluconeogenic conversion which is seen in liver disease, diabetes, cancer, and alcohol and metformin intoxication (combinations of Type A & B have been observed). The therapeutic serum level for metformin is 0.7 (0.3-1.0) mg/l (for μmol/l, multiply by 7.75). The sensible upper therapeutic limit is ~ 5 mg/l (Heaf, 2014).

Nutraceutical Therapeutic Agents

A multifaceted intervention is intricate for the improvement and control of hyperglycemia and may reduce the risk of comorbidities related to T2D. A nutrient dense, eucaloric dietary intake, in conjunction with physical activity, an appropriate pharmacological therapy is the gold standard for a therapy for persons with T2D. However, there may be additional improvements with the usage of certain efficacious nutraceutical products as a complementary therapy.

This section will elaborate on certain nutritional aides or supplements that have shown some efficacy to mediate hyperglycemia and other health issues associated with T2D.

Polyphenols

Polyphenols are phytochemical compounds found naturally in plant-based foods i.e. fruits, vegetables, whole grains, cereals, legumes, tea, coffee, wine, and cocoa. Currently, there > 8000 polyphenolic compounds known including phenolic acids, flavonoids, stilbenes, lignans, and polymeric lignans that have been identified in whole plant foods. These polyphenolic compounds are secondary metabolites of their native plants that are the first line of defense against ultraviolet radiation, oxidants and pathogenic organisms (Bahadoran, Mirmiran, & Azizi, 2013).

Polyphenols are classified into categories based on the number of phenol rings and structural elements that bind these rings to one another. Phenolic acids are approximately ⅓ of the polyphenolic compounds found in the diet which include two main classes of *hydroxybenzoic acid derivatives*: protocatechuic acid (PCA or 3,4-Dihydroxybenzoic acid), gallic acid (3,4,5-Trihydroxybenzoic acid), *p*-Hydroxybenzoic acid (4-Hydroxybenzoic acid); and *hydroxycinnamic acid derivatives*: caffeic acid (3,4-Dihydroxy-cinnamic acid trans-caffeate), chlorogenic acid (CGA or ((1*S*,3*R*,4*R*,5*R*)-3--1,4,5 trihydroxycyclohexane- carboxylic acid). Foods with a high density of phenolic acids are berry fruits, kiwis, cherries, apples, pears, chicory, and coffee (Bahadoran et al., 2013).

Flavonoids are considered the most abundant of the polyphenol family found in the human diet with > 4000 variations of identified compounds. There are six subclasses

of flavonoids including anthocyanins, flavonols, flavanols, flavanones, flavones, and isoflavones. Anthocyanin family of cyanidin, pelargonidin, delphinidin, and malvidin are predominantly found in the berry families of cherry, black grapes, and strawberries, and found in red wine and red cabbage. Flavonols, including quercetin, kampferol, and myricetin, have been mainly observed in onion, curly kale, leeks, broccoli, and blueberries. Isoflavones are also considered another class of highly important dietary flavonoids that include daidzein, genistein, and glycitein. Isoflavones are found in high concentrations in soybeans and soy products and are considered the richest sources of these phytoestrogenic structural compounds. Lignans are diphenoilc components with phytoestrogen activity and have been found in high concentrations in linseed and other grains and cereals. Stilbenes are found low quantities in the human diet. Lastly, resveratrol is one of the most readily researched compounds of these groups, and is mainly found in grapes and red wine (Bahadoran et al., 2013).

van Dam, et al. (2013) assessed the daily intake of polyphenol and flavonoid in a population of ~5 k French men and women (45–60 years). The intake of polyphenols and flavonoids were polyphenols were ~1.2 g/day and 506 mg/day for flavonoids. The main contributing food items to total polyphenols were coffee (44%), tea (9%), apples (6%), and red wine (6%), with coffee contributing to an intake of phenolic acids rather than flavonoids. Three cohort studies from USDA databases observed a mean total flavonoid intake that ranged from 358-414 mg/day. Tea was the main intake source for total flavonoids followed by apples, orange juice, and strawberries. (van Dam, Naidoo, & Landberg, 2013). The bioavailability is interdependent on food preparation processes,

gastrointestinal (GI) digestion and absorption, and cellular uptake and metabolism. During GI absorption, dietary polyphenols must be hydrolyzed by the intestinal enzymes or colon inhabited microflora, and then be consolidated in the intestinal cells and subsequent hepatic translocation for further methylation, sulfation or glucuronidation. Polyphenols accumulate in the target tissues and initiate a biological action. Lastly, polyphenol metabolites are predominantly excreted through bile and urine (Bahadoran et al., 2013).

The proposed antihyperglycemic benefits of polyphenols are mainly attributed to the reduction of intestinal absorption of dietary carbohydrate, modulation of the enzymes involved in glucose metabolism, improvement of β-cell function and therefore insulin action through the stimulation of insulin secretion, and anti-oxidative and anti-inflammatory properties. Polyphenols such as flavonoids, phenolic acids, and tannins, may mediate carbohydrate metabolism by inhibition of enzymes α-glucosidase and α-amylase that are intricate enzymes responsible for digestion and breakdown of dietary carbohydrates to glucose. Other notable polyphenols of green tea catechins, epicatechins, CGA, ferulic acids, caffeic and tannic acids, quercetin, and naringenin, have been suggested to influence the absorption of glucose from the intestine by inhibiting of (sodium) Na^+-dependent glucose transporters 1/2 (SGLT1/SGLT2). Other research has shown that polyphenolic compounds from coffee (CGA) may be also able to regulate postprandial glycemia through prevention of the development of impaired glucose tolerance by facilitating insulin responses, and resveratrol may amplify secretion of GIP and GLP-1 (Bahadoran et al., 2013; van Dam et al., 2013). A hydroxycinnamic acid

derivate, ferulic acid, has shown to blunt blood glucose elevations by elevating glucokinase activity, facilitating glycogen storage in the liver, and increase plasma insulin concentrations in diabetic rodent model. Similarly, in another diabetic rodent model the supplementation of hesperidin and naringin (two primary citrus bioflavonoids) influenced an increase in hepatic glucokinase activity and glycogen content, ameliorated hepatic gluconeogenesis by inhibiting glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPCK) (Bahadoran et al., 2013; Jung, Ran Kim, Hwang, & Youl Ha, 2007; Jung, Lee, Jeong, & Choi, 2004).

L-Carnitine

L-carnitine (β-hydroxy-γ-N-trimethylaminobutyric acid or IUPAC 3-Hydroxy-4-(trimethylazaniumyl) butanoate) is ubiquitous in food from animal sources but is found in limited availability in plants. In humans, ~75% of carnitine is obtained from the diet, the biologically active stereoisomer is absorbed from foods via both active and passive transport across GI enterocyte membranes. The bioavailability of L-carnitine varies due to dietary composition and adaptability; vegetarians who consume low carnitine diets have a higher uptake (66% to 86%) of available carnitine, comparatively to regular red meat eaters that have a lower adaptive uptake to higher carnitine diets (54% to 72%) of available carnitine. The rate of L-carnitine biosynthesis in vegetarians is suggested to be ~1.2 μmol/kg/day. Omnivorous humans ingest ~ 2-12 μmol/kg/day, which equates to the 75% reflected from dietary intake. The bioavailability of oral carnitine dietary supplements is low, the absorption of L-carnitine from dietary supplements (0.5-6 g) is primarily passive and its bioavailability is 14-18% of the dose. Unabsorbed L-carnitine is

mostly degraded by microorganisms in the large intestine. Alternatively, carnitine may be synthesized endogenously from two essential amino acids of lysine and methionine. This process is initiated by the enzymatic posttranslational modification and methylation of lysine into trimethyllysine. Trimethyllysine undergoes four enzymatic reactions in the course of endogenous L-carnitine biosynthesis; the last step in the biosynthesis of carnitine from lysine is modulated by the enzyme Gamma-butyrobetaine dioxygenase (γ-butyrobetaine hydroxylase) where it is expressed found, and synthesis mainly occurs in kidney, liver, and the brain. Ninety-nine percent of L-carnitine is found in intracellular compartments and primarily in cardiac and skeletal muscle tissues, which maintain the highest concentrations. However, these tissues are unable to endogenously synthesize carnitine and are dependent on a form of plasma transport facilitated by nutritional means. Unabsorbed L-carnitine is subjected to degradation by microorganisms in the large intestine (Derosa, Limas, Macías, Estrella, & Maffioli, 2014; Flanagan, Simmons, Vehige, Willcox, & Garrett, 2010; Rüetschi, Nordin, Odelhög, Jörnvall, & Lindstedt, 1993).

L-carnitine is actively transported via organic cation/carnitine transporter 2 (OCTN2) into the cytosol to regulate and participate in the shuttling of activated long-chain fatty acids into the mitochondria where β-oxidation takes place. Carnitine also regulates the Coenzyme-A (CoA)/acyl-CoA ratio within the mitochondria to mediate the accumulation of toxic acyl-CoA compounds and maintains energy production (Flanagan et al., 2010). It has shown to influence fatty acid uptake and lipid metabolism; however, it has also been proposed to indirectly mediate carbohydrate metabolism by modulating

the rate of acyl-CoA formation from pyruvate oxidation that is catalyzed by the pyruvate dehydrogenase complex (PDC). Due to the excess of its utilization by the TCA cycle through the condensation with oxaloacetate, which happens to be less than its rate of formation, leads to its subsequent accumulation. However, during times of increased PDC flux, carnitine buffers the excess acetyl groups formed, in a reaction catalyzed by carnitine acetyltransferase (CAT), ensuring an attainable and assessable pool of free CoA for the continuation of the PDC and TCA cycle reactions (Stephens, Constantin-Teodosiu, & Greenhaff, 2007).

Relative to T2D's, it has been suggested that there is an increase in pyruvate dehydrogenase kinase 4 (PDK4) mRNA expression in skeletal muscle that influences an increase in PDC activity. An upregulation of PDK4 occurs in humans with T2D and therefore an increase in PDC activity. Greater activity in PDC may exacerbate insulin resistance by increasing in malonyl-CoA, a robust inhibitor of carnitine palmitoyltransferase I (CPT1), which is the rate limiting enzyme in fatty acid oxidation. When long chain acyl-CoA esters are converted to carnitine esters by CPT1, inhibition of CPT1 by malonyl-CoA may increase long-chain acyl-CoA esters which in response may increase diacylglycerol and ceramide, which have been implicated in insulin resistance (Jeoung & Harris, 2010; McAinch et al., 2014).

L-carnitine as a therapy for T2D has shown some promise, Mingrone et al. (1999) investigated a 2 hr euglycemic hyperinsulinemic clamp with simultaneous constant infusion of L-carnitine (0.28 μmole/kg/min) or saline solution in a T2D and control population observed a significant increase in glucose oxidation only in the diabetic group

(17.61 ± 3.33 vs 16.45 ± 2.95 μmoles/kg of FFM/minute, $p < .001$), and a significant plasma lactate decreased during L-carnitine infusion compared to saline (0.028 ± 0.0191 without carnitine and 0.0759 ± 0.0329 with carnitine, $p < .0003$) (Mingrone et al., 1999). They suggested that the L-carnitine infusion increased pyruvate dehydrogenase activity.

Whey Protein

The investigation of the effects of protein and amino acids on glycemic responses was first observed by Dr. Graham Lusk (1866–1932). During this era, it was suggested that ~16% of protein consisted of nitrogen, and the deamination of the carbon skeleton of the amino acids in protein could be used for glucose synthesis. Using phlorhizin treated (a competitive inhibitor of renal glucose transport) rabbit and canine models and measuring protein intake and using dextrose to nitrogen excretion ratio (D/N), he proposed that roughly 60% of the protein was converted into glucose. However, this presumption of protein to glucose conversion did not provide information regarding the disposition of the ingested protein under normal physiological conditions, which could reduce the amount of glucose synthesized. The glucose produced could be stored directly as glycogen in the liver, or the amino acids resulting from the digestion of the protein could in part be used normal protein turnover and not influence the blood glucose concentration. The limitations of analysis and technology affected the ability to correctly analyze amino acids and other plasma hormones affecting glycemic responses (Gannon & Nuttall, 2010). Recently, research has investigated the effects of whey protein on glycemic responses with differing populations and have found conflicting evidence in contrast to Dr. Lusk's proposed glucose synthesis theory. Humans consume dairy protein

predominantly from bovine milk which consists of around 80% casein and 20% whey proteins. The casein in cow's milk comprises α-s1, α-s2, β- and κ-casein, while the whey consists of multiple globular proteins including β-lactoglobulin, α-lactalbumin, lactoferrin, lactoperoxidase, immunoglobulins, serum albumin, glycomacropeptide, enzymes, and growth factors (McGregor & Poppitt, 2013; Sousa et al., 2012).

The Glycemic Effect of Whey Protein

In relation to glycaemia, whey protein (WP) has been investigated in both healthy human and diseased populations, and more specifically T2D. WP has shown to reduce postprandial blood glucose perturbations by differing proposed mechanisms, which have not been entirely isolated or explained to date. Peterson et al. (2009) observed the glycemic response in healthy, obese subjects (44.4 ± 9.3 years and average BMI 33.6 ± 4.8 kg/m^2). They evaluated how differing doses of protein (5, 10, and 20 g) co-ingested with 50 g of anhydrous glucose influenced blood glucose responses. Glucose AUC concentrations were correlated inversely with protein doses, which indicated that higher doses of protein (20 g) resulted in minimized blood glucose concentrations ($r = -.48$, $p = .002$). The authors concluded that based on previous rodent model research that whey protein directly inhibited the actions of Dipeptidyl peptidase IV (DPP-IV), which is a peptidase enzyme which hydrolyzes incretin hormones like GLP-1 (Petersen et al., 2009). The authors did not measure serum DPP-IV, nor were any of the incretins measured to validate this suggested mechanism; so much is left to question. Additionally, the possibility of altered digestion rate with co-ingestion of both protein

and carbohydrate source may have influenced gastric emptying rate (Calbet & MacLean, 1997).

Claessens, Calame, Siemensma, van Baak, and Saris (2009) researched the effects of four protein hydrolysates from vegetable (pea, gluten, rice, and soy) and two protein hydrolysates from animal origin (whey and egg) on glucagon and insulin responses on healthy non-obese male participants (age 32 ± 13, weight 76.2 ± 4.7 kg, and BMI 23.7 ± 1.4 kg m^2). Each participant ingested ~250 ml of 0.2 g/kg protein hydrolysate and 0.2 g/kg maltodextrin except for the control drink that only contained 0.2 g/kg maltodextrin. They observed that the glucose area under the curve (AUC) for pea, soy, gluten, and whey protein hydrolysates differed significantly from the AUC for the control drink ($p < .006$) but AUCs did not differ between all six protein hydrolysates. Additionally, they found that the insulin response to the different protein hydrolysate and carbohydrate mixtures was significantly higher than the control carbohydrate drink. All protein hydrolysates seemed to synergistically stimulate insulin with carbohydrate intake and that resulted in lower plasma glucose concentration (Claessens, Calame, Siemensma, van Baak, & Saris, 2009).

The T2D population used in this investigation fell within the following metrics: seven males age 58 ± 3 years, BMI 28.6 ± 1.3 kg/m^2, and HbA_{1C} 6.5 ± 0.2%. Ma et al. (2009) investigated the effects of three separate meals (59.1 g carbohydrate, 4.3 g fat, 5.2 g protein; 1,276.5 kJ) where 2 of the 3 meals contained 55 g of whey protein; 1) preload (in soup), 2) in potato meal, or 3) control (no whey). They assessed gastric emptying, blood glucose, plasma insulin, glucagon-like peptide-1 ($GLP\text{-}1_{Total}$), gastric inhibitory

polypeptide also known as glucose-dependent insulinotropic peptide (GIP_{Total}), and cholecystokinin (CCK-8). Their results showed that gastric emptying was slowest on the whey preload day (87.3 ± 5.4 min; $p = .0001$) and was slower with whey in the meal (53.0 ± 8.3 min; $p < .01$) than with no whey (39.0 ± 6.2 min). Additionally, the incremental area under the curve (iAUC) for blood glucose was less after the whey preload (363.7 ± 64.5 mmol/min/l) and whey in meal (406.3 ± 85.9 mmol/min/l) compared with no whey (734.9 ± 98.9 mmol/min/l ($p < .005$). The iAUC for insulin, GLP-1, GIP, and CCK were greater with the whey preload treatment ($p < .05$ for all) or in the meal ($p < .005$ for all) compared with no whey treatment. However, the iAUC for insulin did not differ between whey preload and whey meal ($p = .50$). This study highlighted the multiple level effect that whey has on glycemic responses. A lengthened gastric emptying rate that may slow the glycemic response, an increased incretin effect which has been suggested to be responsible for 50-70% of insulin release (Edholm et al., 2010), and an overall reduction in the iAUC of glucose (Ma et al., 2009).

Other research has focused on the insulinogenic amino acids found in whey and how they influence glycemic responses compared to whey. Nilsson et al. (2007) compared four test drinks and a control drink (glucose in 250 mL water). The test drinks consisted of 1) lysine and threonine (AA2); 2) BCAA's leucine, isoleucine, and valine (AA3); 3) leucine, isoleucine, valine, lysine, and threonine (AA5); and 4) whey protein (adjusted to contain 18 g protein). All test drinks contained 25 g carbohydrates in the form of glucose. A healthy participant population (6 males, 6 females; age 20–30 years, BMI 22.4 ± 0.6 kg/m^2, fasting blood glucose concentrations 4.6 ± 0.04 mmol/l) was

recruited. The study assessed blood glucose, serum insulin, and plasma GIP and GLP-1 outcomes. At the conclusion of the study, they found that the blood glucose responses after ingestion of the whey (−56%) and the AA5 (−44%) drinks were significantly lower than those after ingestion of the reference glucose drink ($p < .05$). However, there were no significant differences found between the three amino acid drinks (AA2, AA3, and AA5) on postprandial glycemia. In regards to postprandial insulin responses, whey and AA3 (BCAA's) drinks were found to prompt a significantly higher AUC ($p < .05$) than did the control glucose drink. Whey and AA5 drink influenced a similar insulin response, though AA5 was not found to be significant. Whey induced a significantly higher insulin response than did the glucose drink or glucose drink with AA2. Moreover, whey influenced a greater GIP response than any of the other test drinks ($p < .05$). Lastly, GLP-1 responses showed to be similar between all test drinks. The authors concluded that whey indeed has a synergistic hyperinsulinemic effect, followed by AA5 and BCAA's. Similarly, AA5 (leucine, isoleucine, valine, lysine, and threonine) are likely to act as insulin secretagogues. They suggest that BCAA's are a significant factor in insulin release. Although the free amino acids drinks had an effect on insulin release, the authors proposed a direct stimulus of the amino acid mediated insulin release via pancreatic β-cell actuation due to minimal secretion of GIP and GLP-1 (Nilsson et al., 2007).

Whey protein (a primary source of both L-Leucine and L-Isoleucine) has been purported to have an inhibitory effect on dipeptidyl-peptidase-IV (DPP-IV). However, the potential inhibitor sequences of these peptides to become activated must be

hydrolyzed or digested from their designated proteins to influence inhibition of DPP-IV. Whey protein concentrate is rich in the protein β-lactoglobulin, and when hydrolyzed, these bioactive peptides have been observed as a moderate DPP-IV inhibitor (Jakubowicz & Froy, 2013; Jakubowicz et al., 2014; Silveira, Martinez-Maqueda, Recio, & Hernandez-Ledesma, 2013; Tulipano, Sibilia, Caroli, & Cocchi, 2011), which delays the degradation of GLP-1 and GIP and subsequent stimulation of insulin secretion by the pancreas. A few studies have used individual amino acids or a mixture form to observe the impact on incretins (Mansour, Hosseini, Larijani, Pajouhi, & Mohajeri-Tehrani, 2013) and only one study found leucine and isoleucine to increase incretin hormone in a human intestinal cell culture model (Chen & Reimer, 2009).

The Glycemic Effect of Amino Acid Mixtures

There is a body of research that shows amino acid mixtures seemingly have an effect on insulin release which indirectly may influence glucose uptake. These mixtures of amino acids have been appropriately named '*insulinogogues*' or '*secretogogues*'. It has been purported that combined mixtures of amino acids arginine with leucine, and arginine with phenylalanine has the largest synergistic increase in plasma insulin concentrations (van Loon, Saris, Verhagen, & Wagenmakers, 2000). Floyd et al. (1966) observed IV infusions of AA on insulin response and suggested that the following seven amino acids are ranked according to decreasing stimulus of the insulin responses: arginine, lysine, leucine, phenylalanine, valine, methionine, and histidine (Floyd Jr, Fajans, Conn, Knopf, & Rull, 1966). Moreover, other research has observed other amino

acids such as BCAA's (leucine, valine, and isoleucine), lysine, and threonine stimulate insulin secretion (Nilsson et al., 2004).

Bernard et al. (2011) used an amino acid mixture in a rodent model of isoleucine, leucine, cysteine, methionine, and valine, and found an improved blood glucose response to an oral glucose challenge without an increase in the plasma insulin response. They observed significantly lower blood glucose in carbohydrate + amino acid (CHO + AA) groups compared to CHO alone while insulin responses were similar. Muscle glucose uptake was higher in CHO + AA compared with CHO in both fast twitch red (8.36 ± 1.3 vs 5.27 ± 0.7 μmol/g/hr) and white muscle (1.85 ± 0.3 vs 1.11 ± 0.2 μmol/g/hr). Moreover, with assessing cell signaling mechanisms they found no difference in Akt/PKB phosphorylation between treatment groups; although, the amino acid mixture treatment resulted in increased AS160 phosphorylation in both muscle fiber types. Glycogen synthase (GS) phosphorylation was reduced in fast twitch red muscle of CHO + AA compared with CHO group; although, in contrast, $mTOR_1$ phosphorylation and activity was increased. The authors suggested that an amino acid mixture combined with glucose may improve glucose tolerance by increasing skeletal muscle glucose uptake and intracellular disposal which may be explained by enhanced associated intracellular signaling (Bernard et al., 2011).

This same amino acid treatment protocol was used later in a human model on 22 healthy overweight men and women (3 males:19 females) age of 25 ± 1.53 (M), 31.79 ± 1.75 (F); BMI (kg/m^2) 31.53 ± 2.15 (M), 35.04 ± 1.16 (F); fasting blood glucose (mmol/l) 4.92 ± 0.46 (M), 5.52 ± 0.13 (F) or 88.56 ± 8.28 mg/dl (M), 99.36 ± 2.34 mg/dl (F).

Wang et al. (2012) observed the effects of this same amino acid mixture (0.088 g cysteine 2HCl, 0.043 g methionine, 0.086 g valine, 12.094 g isoleucine, and 0.084 g leucine per 355 mL solution) with a carbohydrate beverage consisting of a 355-mL orange flavored glucose drink (100 g of dextrose) during an OGTT protocol. The amount of amino acid mixture provided was based on their previous rodent model research (154 mg/kg). They observed a significant treatment effect, with the blood glucose response to the CHO + AA treatment being significantly lower than that of the CHO treatment ($p < .01$). Additionally, there was no significant difference between mean fasting plasma insulin values between the two treatments (CHO + AA 161.34 ± 13.66 pmol/l, CHO 163.80 ± 14.53 pmol/l) nor was there a significant difference in the insulin AUC. Plasma glucagon concentrations were significantly higher in CHO + AA than in CHO at 60 min post treatment ($p < .05$). The authors concluded that the AA mixture of isoleucine, cysteine, methionine, valine, and leucine, improved the glucose response to an OGTT and they found that the blood glucose response was significantly reduced within 30 min of ingesting the CHO + AA mixture, and that blood glucose returned to the fasting or basal concentrations more rapidly during the CHO + AA treatment than during the CHO treatment. In regards to the elucidation of these responses, they felt that this could be explained by the previous reports of protein and individual amino acids such as leucine, isoleucine, arginine, and methionine, act as insulin secretagogues. Additionally, they stated that the hypoglycemic effect of these amino acids could be influenced by a reduced hepatic glucose output mediated by the inhibition of gluconeogenesis. However, the authors reported plasma insulin concentrations reaching ~1200 pmol/l in both the CHO +

AA and CHO treatment groups and they suggested gluconeogenic activity was not a confounding factor due to the robust insulin response. Lastly, in corroboration with previous work there is a suggestion that the hypoglycemic effect of amino acid mixtures is mediated by PI3K activity, which is a downstream target of IRS-1 and upstream signaling agent for GLUT-4 translocation (Wang et al., 2012).

Lindgren et al. (2015) compared the IV infusion of an amino acid mixture (AA) (7.0 g with ingestion of 100 ml of water) versus an oral group (intradoudenal) of ingested 100 ml AA mixture (6.5 g) amino acids in 12 apparently healthy males with BMI of 22.4 ± 1.4 kg/m^2, age 22.5 ± 1.4 years, who had no history of diabetes, HbA_{1c} 4.9 ± 0.3%, and a fasting glucose of 4.7 ± 0.2 mmol/l (84.6 ± 3.6 mg/dl). They observed no significant difference in the increase in amino acid concentrations between oral and IV treatments for all except three individual amino acids of aspartic acid, glutamic acid, and phenylalanine. All increased in value after IV compared to oral treatment ($p < .001$). They found no difference in glucose responses between oral or IV AA administration. However, insulin, C-peptide, and glucagon all rose in both treatments. C-peptide (AUC) increased, and total insulin secretion was 25% higher after oral than after IV AA administration ($p = .006$). Insulin clearance was also higher after oral amino acid administration (3.35 ± 0.45 L/min) compared to IV amino acid administration (2.60 ± 0.32 L/min). GIP (intact and total) increased after oral ingestion of AA, although no impact was seen during IV AA administration. Peak GIP concentrations were seen at 30 min after oral ingestion and a significant increase was observed in the AUC for both intact and total GIP after oral AA administration compared to IV amino acid

administration. Interestingly, GLP-1 (intact and total) AUC did not change significantly after either treatment; the authors suggested that the lower dose utilized was a possible factor in the lack of responsiveness of GLP-1, which has shown in previous research to be secreted after predominant protein meals. They concluded that based on this dose and amino acid composition, GIP is indeed a factor in stimulating insulin release. GIP would appear to be most sensitive to oral amino acid ingestion and confirms previous research that oral (intraduodenal) administration of an amino acid mixture augments insulin secretion compared to that when equally given through IV (Lindgren et al., 2015).

It seems as though the amino acid mixture protocols utilized in human and rodent models may have a positive effect on glucose uptake in an insulin dependent and independent manner. Less is known about the role specific amino acids play in the regulation of glucose uptake. Subsequent research has further isolated the glycemic responses from either individual amino acids or pairs of amino acids typically found in whey protein to further elaborate relationships of amino acid metabolism and glucose uptake.

The Metabolism of Branch Chain Amino Acids

The BCAA family consists of L-Leucine (LEU), L-Isoleucine (ISO), and L-Valine (VAL). The three BCAA's contain a 3 or 4 carbon side chain and are virtually the same molecular weight (LEU 131.17 g/mol; ISO 131.17 g/mol; VAL 117.15 g/mol. BCAA's comprise ~35% of the indispensable or essential amino acids (EAA's) in muscle proteins and ~33% of all amino acids in the body. Interestingly, BCAA's are ~50% of

the EAA's in the food supply, so deficiencies are not naturally observed (Harper, Miller, & Block, 1984). Mammals cannot de novo synthesize BCAA's, requiring intake from external food sources. A person must consume approximately 40 (LEU), 20 (ISO), and 19 (VAL) mg/kg/day. Collectively, this amounts in total to around ~5.5 g/day for a 70 kg adult. The best sources of these amino acids are red meat or dairy products. However, vegans are able to reach this recommended needs by consuming soy protein and other vegetarian sources (Rajendram, Preedy, & Patel, 2014).

The process of BCAA catabolism involves the first step of a reversible transamination to form a branched chain keto acid (BCKA): α-ketoisocaproate (KIC), α-keto-β-methylvalerate (KMV), or α-ketoisovalerate (KIV). This reaction is catalyzed by the enzyme branched-chain aminotransferase (BCAT). There are two known mammalian BCAT's a mitochondrial (BCATm) and a cytosolic (BCATc) isozyme. These aminotransferases are dependent on the active forms of vitamin B-6 coenzyme pyridoxal phosphate (PLP); in the first half reaction, the PLP form of the enzyme reacts with the α-amino group of a BCAA. Subsequently, the reaction proceeds to the pyroxamine monophosphate (PMP) form of the enzyme, releasing the respective BCKA. The PMP enzyme then transaminates a second α-keto acid, predominantly α-ketoglutarate (α-KG), later followed by a reversal of the first half reaction to reform the PLP enzyme and release glutamate. The mammalian BCAT isozymes are very specific for BCAA's and glutamate, with a substrate preferential sequence of isoleucine, leucine, valine, and lastly glutamate (Harper et al., 1984; Hutson, Sweatt, & LaNoue, 2005).

The second step is an irreversible oxidative decarboxylation of the branched-chain α-keto acids catalyzed by the mitochondrial branched-chain α-ketoacid dehydrogenase complex (BCKDC). This action commits the BCAA carbon skeleton to the degradative pathway. The mammalian BCKDC contains several copies of three distinct enzymes: branched-chain α-ketoacid decarboxylase (E1), dihydrolipoamide acyltransferase (E2), and dihydrolipoamide dehydrogenase (E3). The overall structure of the BCKDC resembles the structures of the mammalian pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase enzyme (α-KGDH) complexes. During the BCKDC reaction, BCKA substrate is oxidatively decarboxylated, forming their respective branched-chain acyl-CoA derivative and NADH. BCKD is highly regulated by both short term and long term mechanisms. Short term regulation is mediated by the highly specific 44 kDa BCKD kinase that can associate and dissociate from the BCKDC. The kinase phosphorylates and inactivates the E1 enzyme. Short-term regulation of the kinase is achieved by changes in concentrations of KIC, which inhibits the kinase. More specifically, in the presence of excess KIC, the kinase is inactivated, facilitating oxidation of the BCKA's and net nitrogen transfer (Harper et al., 1984; Hutson et al., 2005).

The Glycemic Effects of Branch Chain Amino Acids

The glycemic effects of BCAA's have been more examined in the last few decades of research. Some metabolomics research has implicated and associated plasma BCAA concentrations with insulin resistance and suggest a higher risk for T2D after finding a much higher concentration in the plasma of people with T2D (Xu et al., 2013).

The European Prospective Investigation into Cancer and Nutrition (*EPIC-Postdam*) study observed ~27,600 participants from the general population in Germany with the age range of 35–65 yrs. In conjunction with The Cooperative Health Research in the Region of Augsburg (*KORA*) study, consisting of population based surveys and follow up periods in the area of Augsburg in southern Germany with a total of 4,261 individuals between 25-74 years of age. Lastly, The Tübingen Family (*TüF*) study investigated the pathophysiology of T2D in southern Germany. In these combined prospective studies using a targeted metabolomics approach they found higher concentrations of phenylalanine, isoleucine, tyrosine, and valine to be associated with increased risk of T2D; although, glycine was associated with reduced risk of T2D. Along with other prospective research, it has been found that an increase of BCAA in plasma of subjects with insulin resistance may be the result of reduced activities of key BCAA catabolic enzymes in liver and adipose tissue. Furthermore, they suggest that amino acids may directly cause skeletal muscle insulin resistance by disrupting insulin signaling through $mTORC_1$ dependent activation and downstream phosphorylation of SK61 and subsequent IRS-1 inhibition (Floegel et al., 2013; Lu, Xie, Jia, & Jia, 2013). Amino acid activation of S6K1 through $mTORC_1$ is suggested to be comparable to insulin stimulated actions of the ingestion of amino acids and an insulin response, which appears to be an additive effect. The phosphorylation and activation of S6K1 following amino acid treatment results in inhibition of insulin mediated PI3K activity. Additionally, the inhibition of PI3K activity by amino acid is suggested to be the outcome of S6K1 serine

phosphorylation of IRS-1 which prevents the normal tyrosine phosphorylation (Rivas et al., 2009; Tremblay et al., 2005; Frédéric Tremblay & Marette, 2001).

In contrast to this relationship, other research has observed positive effects on glycemic responses with the use of BCAA's as a treatment. The aforementioned prospective studies that eluded to BCAA's factoring as a possible cause of T2D or even pre-diabetes have been met with scrutiny. Everman at al. (2015) acutely observed the IV infusion of dextrose and BCAA's to investigate how BCAA's influenced insulin sensitivity and glucose disposal in normal men. Participants in the BCAA group received BCAA (4%, concentration of 1.38 g ISO, 1.38 g LEU, and 1.24 g VAL g per 100 ml) at an infusion rate of 5 umol/kg/min to increase the concentration of plasma BCAA. The participants received either BCAA's or a saline infusion (control treatment). Additionally, the participants had insulin infused via hyperinsulinemic-euglycemic clamp method, which was started at a rate of either 40 mU/m^2/min or 80 mU/m^2/min to stimulate whole body glucose turnover.

Their results showed no differences in the plasma glucose concentration between groups at any of the study periods ($p > .05$) in the 40U (40 mU/m^2/min) experiment. Similarly, there were no differences in plasma glucose concentrations in either the control or the BCAA group over time in the 80U (80 mU/m^2/min) experiment, and no differences were observed between groups at any of the study periods ($p > .05$). During the hyperinsulinemic-euglycemic clamp, whole body glucose disposal rate (GDR) increased in both the control and BCAA groups in both the 40U and 80U experiments. Moreover, endogenous glucose production (EGP) decreased in the control group during insulin

infusion and similarly observed in the presence of increased plasma BCAA concentrations, and in both the 40U and 80U experiments. Furthermore, the increase in GDR between control and BCAA was not observed in either the 40U experiments ($p > .05$). Lastly, insulin infusion stimulated the glucose metabolic clearance in all experiments ($p < .05$) and they found no differences between control and BCAA in either of the experiments ($p > .05$).

The authors concluded that the study design and number and characteristics of participants (n = 10; 18–25 year; healthy) that the acute IV infused increase of BCAA concentrations do not induce insulin resistance (IR) nor does this increase have an additive effect on glucose uptake. The increased plasma BCAA concentrations may be a metabolic biomarker of the systemic environment that may be associated with obesity and insulin resistance rather than a cause of insulin resistance, which may suggest in persons with IGT that there may be an impaired ability to uptake and metabolize BCAA in skeletal muscle (Everman, Mandarino, Carroll, & Katsanos, 2015). This suggests that increase in BCAA concentration may possibly be a result of skeletal muscle proteolytic efflux of BCAA's for gluconeogenic needs (Wijekoon, Skinner, Brosnan, & Brosnan, 2004).

Oral administration of a BCAA mixture has been shown to reduce hyperglycemic events in a virus-induced T2D mouse model. The BCAA treatment group was administered orally 0.9 g/kg/day of each BCAA from the day after viral inoculation. They found the blood glucose concentrations in the BCAA group were 54.5 ± 3.7 mg/dl at baseline, 145.2 ± 8.7 mg/dl at 30 min, and 128.7 ± 18.3 mg/dl at 60 min after the

glucose loading, which when compared to the control group were not significantly higher. Based on their data collection they suggested that the oral administration of BCAA is able to improve glucose intolerance induced by encephalomyocarditis virus (EMCV) (Utsugi et al., 2000).

Similarly, in a streptozotocin induced T2D rodent model fed a high protein diet or a control balanced diet, were injected intraperitoneally with mixtures of BCAA's (0.25 or 0.50 g/kg BW), or with each BCAA separately (0.50 g/kg) prior to streptozotocin administration. It was observed that 0.75-1.5 g/kg BW of BCAA's significantly decreased plasma glucose concentrations. However, it was unknown which amino acid (LEU, ISO, or VAL) or a possible combination that may have influenced glucose metabolism the most, or the mechanisms to explain the outcome (Eizirik, Kettelhut, & Migliorini, 1986; Rajendram et al., 2014).

Further relationship isolation and mechanistic work by Nishitani et al. (2005) investigated the BCAA's in a cirrhotic rodent model (CCl4). Cirrhosis has been associated with IGT. It has been observed in a large number of cirrhotic patients (~60–80%) that are diagnosed glucose intolerance that a percent of this population (~10–15%) may subsequently develop diabetes (Nishitani et al., 2005; Petrides & DeFronzo, 1989). They examined how BCAA's may regulate glucose metabolism in an IGT rodent model with cirrhosis by investigating the location of glucose transporters, the activity of glycogen synthesis in skeletal muscles, and related functions of glucose metabolism mediated by the liver. The CCl4 were administered either 4 g/kg of glucose and 1.5 g/kg BCAA's LEU, ISO, and VAL or saline for the control group.

They observed that LEU (30-120 min) and ISO (60-120 min) improved glucose tolerance in CCl4 model. Both ISO and LEU groups significantly decreased ($p < .05$) glucose concentrations compared to VAL. However, blood glucose concentrations in the LEU group remained unchanged from 30 to 120 min. Blood glucose concentrations in the ISO group decreased continuously beyond 60 min. Additionally, the AUC changes in plasma insulin concentrations were similar between all three groups. Their data suggest that glucose concentrations were reduced to a greater extent by ISO than by LEU. They suggested that the glucose uptake in soleus muscle of CCl4 model treated with LEU and ISO was not mediated by additional insulin secretion due to the similar responses in each group. Therefore, they alluded that LEU and ISO were able to equally influence greater glucose uptake ($p < .05$) either independent of insulin or act concurrently to directly stimulate glucose uptake.

To decipher the effects of BCAA's on GLUT-1/4 translocation they used immunoblotting to measure the total amount of transporters in soleus skeletal muscle membrane fractions, which was standardized against activity for Na^+-K^+-ATPase protein concentrations of the plasma membrane. The oral administration of LEU or ISO alone mediated translocation of the GLUT-4 protein to the plasma membrane, similar to the actions after the administration of glucose; however, VAL had no effect. GLUT-1 translocation was similarly promoted by the administration of LEU, ISO, or glucose alone. Moreover, the plasma insulin concentrations at the time of soleus muscle collection increased significantly after administration of glucose or LEU, whereas not observed with ISO treatment ($p < .05$).

The glycogen content and activity in the isolated soleus muscles and liver was investigated by assessing the effect of ISO treatment in CCI4 rodent model for glycogen storage capacity due to previous observations showing that cirrhosis directly reduces glucose uptake in both tissues, and therefore a reduction in glycogen content. They assessed glycogen synthase (GS) activity and found a difference in blood glucose concentration between the control group and the BCAA group and the GS activity of the soleus in the CCl4 was upregulated significantly by the administration of LEU ($p < .05$), whereas nothing found by the ISO group. Similarly, they also observed that glycogen content in soleus muscle tended to be greater in the LEU group than in the control group ($p < .05$).

Additionally, they investigated p70SK6 expressed protein content in soleus which is a downstream protein in $mTORC_1$ signaling. While using an $mTORC_1$ inhibitor rapamycin to reduce the function of the complex, they found that LEU augmented GS activity in soleus muscle, whereas ISO had no effect. They suggested LEU effect on GS was inhibited completely by pretreatment with rapamycin which is found to be in contrast to previous hepatocyte work (Krause, Bertrand, Maisin, Rosa, & Hue, 2002) that showed LEU had no effect on GS activity; suggesting $mTORC_1$ action has tissue specificity in regards to GS effects.

The authors concluded that the mechanism of signal transduction specifically in skeletal muscle stimulated by LEU and ISO mediate glucose transport independently of insulin. This suggests that LEU and ISO may partially '*mimic'* the actions of insulin in the regulation of glucose transport (GLUT-4 translocation promotion) and also improve

glucose metabolism by promoting glycogen synthesis (GS) and therefore facilitate glucose uptake and storage (Nishitani et al., 2005).

Glycemic Effects of L-Leucine

L-Leucine (LEU) is another indispensable or essential amino acid that has received much research attention due its potential as an ergogenic aid in exercise (resistance training), and a possible therapeutic role in some diseases such as cancer, muscle cachexia, obesity, and sarcopenia. Other than its role in energy supply as a nitrogen donor for muscle alanine synthesis, LEU is also an amino acid that is able to modulate some cellular pathways involved in skeletal muscle protein synthesis (MPS) and muscle protein breakdown or degradation (MPB), innate immune system (inflammation), skeletal muscle glucose uptake and glycogen synthesis, and direct β-cell stimulated insulin secretion (Rajendram et al., 2014).

The suggested or recommended LEU intakes in developed countries among healthy adults is ~200 mg/kg/day and currently there is no set safe upper tolerable limit (UL) defined for LEU intake. Research by Elango et al. (2012) and Pencharz et al. (2012) investigated the upper limit of LEU oxidization and metabolism. using stable isotope labeled L- [1- 13 C]-Leucine and graded test intakes of LEU (50–1,250 mg/kg/day) in young and adult men. They concluded that the oxidation of LEU (rate of tracer oxidation) plateaued with intakes above 550 mg/kg/day. Additionally, the increases in plasma ammonia concentrations, plasma LEU concentrations, and urinary LEU excretion with intakes > 500 mg/kg/day. Altogether, the data suggests that ~500

mg/kg/day as a rough estimate of the UL for leucine intake (Elango, Chapman, Rafii, Ball, & Pencharz, 2012; Pencharz, Elango, & Ball, 2012; Rajendram et al., 2014).

It has been proposed that LEU directly stimulates pancreatic β-cell generated insulin release by two proposed mechanisms: 1) It has been suggested that LEU or its metabolite KIC regulates K_{ATP} channel activity, and this results in an increase of free cytosolic calcium (Ca^{2+}), which then stimulates the exocytosis of insulin secretory granules by mechanisms involving activation of some protein kinases and protein acylation; 2) There are currently two known glutamate dehydrogenase (GDH) isoenzymes in human tissues. One is translated by GLUD1 gene with ubiquitous expression in differing tissues, while the other is encoded by GLUD2 gene with specific expression found in neural tissues. The GDH isotype in pancreatic β-cells is encoded by the GLUD1 gene. GDH is the key enzyme controlling amino acids and ammonia metabolism in pancreatic β-cells, liver, and brain. LEU and ADP have shown to activate GDH robustly, whereas VAL, ISO, and methionine activates GDH minimally. GDH is usually allosterically inhibited by GTP and ATP. It had been reported that a non metabolic analog of LEU, 2-aminobicyclo (2,2,1) heptane-2-carboxylic acid (BCH), significantly stimulates insulin secretion from pancreatic β-cells; although, in comparison LEU has been shown to be a more efficient as an insulin secretagogue. The selective activation of GDH is the primary or the only mechanism by which BCH stimulates insulin secretion from β-cells because it cannot be metabolized. It has been shown that LEU mediates glutaminolysis through GDH activation, which may play an intricate role in insulin release between meals when blood glucose falls below 90 mg/dl. This basal

insulin release accounts for ~50% of the daily required insulin secretion from β-cells. Additionally, the overexpression of GDH and increased activity significantly stimulates insulin secretion by glutamine action alone (2.7 fold) or glutamine plus BCH (~6 fold) in pancreatic β-cells (Liu et al., 2003; Mastorodemos et al., 2015; Yang, Chi, Burkhardt, Guan, & Wolf, 2010).

LEU also serves as a communicating agent with skeletal muscle. The amino acid transporter system L, Large Neutral AA Transporter (LAT1) transports LEU intracellular by way of amino acid sensing that currently is not fully understood. The kinetic actions of LEU in regards to glucose metabolism in skeletal muscle is dynamic due to is proposed interaction with numerous enzymes. Di Camillo et al. (2014) examined the effect of LEU on insulin signaling cascades in a human skeletal muscle cell culture line. The cell line was exposed to insulin, LEU, and LEU + insulin stimulus and total and phosphorylated AKT, Extracellular-signal-regulated kinases (ERK1/2), Glycogen synthase kinase 3 Beta (GSK3-β), Forkhead box protein-O1 (FOXO1), $mTORC_1$, Eukaryotic translation initiation factor 4E-binding protein1 (4EBP1), and p70S6K were investigated by western blot. The authors found no significant difference in time course phosphorylations by LEU, which the authors felt that was due to variability amongst the 'replicates' of the cell line donors. However, insulin had a major stimulatory effect on Akt, ERK, and GSK3-β. Similarly, and parallel with previous research, LEU + insulin-stimulated phosphorylation of these proteins, there was also an increase in 4EBP1 protein expression. The authors found a 2 fold upregulation of this protein by insulin and an even more magnified effect (18 fold) when combined with LEU. Interestingly, the

phosphorylated and total $mTORC_1$ showed no difference among any treatment, which has been seen in previous research. The authors again possibly attribute this to variability in the replicates. Lastly, in relation to glycogen synthesis, LEU independently had no effect on glycogen synthesis in comparison to insulin and LEU + insulin treatments, which resulted in a significant increase (p = .0157 and .006, respectively). The difference between the two conditions was found to be statistically significant (p = .008), which LEU amplified insulin-mediated stimulus of glycogen synthesis by 50% (Di Camillo, Eduati, Nair, Avogaro, & Toffolo, 2014).

Similarly, Peyrollier et al. (2000) utilized a rodent L6 myotubes culture model with a LEU as a treatment to investigate PI3K, $mTOR_1$, activity, p70S6 sequence and activity, and Akt/PKB activation. They also examined the effect of LEU on upregulation of amino acid system A transporter activity and lastly, associated inactivation of GSK-3. The L6 myotubes were incubated for 10 min with 2 mM LEU, this resulted in a modest, yet significant, stimulation increase of 40% of p70S6 kinase activity compared to basal activity ($18.8 \geq 0.8$ mU/mg of protein) and compared to amino acid deprivation they observed basal p70S6 kinase activity was significantly lower (7.1 ± 0.3 mU/mg of protein). Similarly, p70S6 kinase was activated by 2-fold upon addition of LEU to the incubation medium. When LEU was added to the culture, it influenced a very rapid transitory ~5-fold activation in PI3K activity assessed PIP_3. However, the increase in PI3K activity was greater (~10-12-fold) in muscle cells exposed to 100 nM insulin for 10 min. The authors suggested that insulin promoted tyrosine phosphorylation of IRS-1 and its subsequent downstream activation of PI3K. Comparatively, LEU did not stimulate the

activation of PI3K as insulin does and may likely be related to a recruitment of the kinase to some other tyrosine phosphorylated signaling complex. Lastly, the authors observed a significant LEU mediated inactivation of GSK-3 (therefore inhibit glycogen synthesis) that was comparable with insulin induced inhibition (Peyrollier, Hajduch, Blair, Hyde, & Hundal, 2000).

New mechanistic insights concerning LEU and glucose metabolism have focused on a new protein that is a counterpart to $mTORC_1$. The protein named the mechanistic target or rapamycin complex 2 ($mTORC_2$). Liu et al. (2014) investigated the effects of LEU on both mTOR complex 1 and 2 and its influences on glucose metabolism. Using a culture of primary rodent skeletal muscle cells and measured glucose uptake in these skeletal muscle cells by a non radioisotope, enzymatic fluorescence assay; this method enabled the detection of 2-deoxyglucose-6-phosphate (2DG6P). They observed that LEU independently did not alter the basal level of glucose uptake but did increase the insulin-mediated glucose uptake at 0.4, 2.0, 4.0 and 8.0 mmol/l ($p = .05$) with the peak effect happening at 2.0 mmol/l ($p < .01$). Moreover, LEU promotes the insulin stimulated glucose uptake despite induction of a PI3K inhibitor (wortmannin) in skeletal muscle cells, which contrasts with the aforementioned outcomes and suggestions from the previous paper. They observed that LEU further augmented the insulin stimulated glucose uptake by 15% ($p = .05$), and reversed inhibitory induced effect PI3K by wortmannin by 60% on the insulin-induced glucose uptake ($p = .05$). Interestingly, they additionally used an $mTORC_1$ inhibitor of rapamycin and found that independently it had no effect on the insulin stimulated glucose uptake; however, it eliminated the promoting

effect of LEU in the insulin induced glucose uptake (p = .05). Furthermore, the combination of both wortmannin and rapamycin significantly decreased the insulin promoting actions of glucose uptake with or without LEU treatment. The authors suggested by their further observations that the LEU promoting effects on insulin signaling are blunted by rapamycin, but not by wortmannin in skeletal muscle cells. The authors suggested this through the investigation the relationship of Akt and $mTORC_1$ and found that LEU alone did not stimulate Akt phosphorylation at residue 473 whereas insulin did. However, comparatively, LEU independently caused a small but significant increase in $mTORC_1$ phosphorylation at residue 2448 similarly as insulin alone. The synergistic effect of LEU enhanced the insulin mediated phosphorylations of Akt^{473} and $mTOR^{2448}$ significantly (p = .05). The insulin modulated phosphorylations of Akt^{473} and $mTOR^{2448}$ were inhibited by wortmannin, and the inhibition was counteracted by the administering of LEU. Moreover, the insulin mediated phosphorylations of AKT^{473} and $mTOR^{2448}$ were not affected by rapamycin; although, the promoting effect of LEU on the insulin mediated phosphorylations of Akt^{473} and $mTOR^{2448}$ were entirely inhibited by rapamycin (p = .05). The insulin induced phosphorylations of Akt^{473} and $mTOR^{2448}$ were significantly suppressed by the concurrent use of both wortmannin and rapamycin regardless of the presence of LEU.

Research in the last decade has focused on the further elaboration of the relationship of $mTORC_2$ with glucose metabolism in the pancreas, skeletal muscle, and other tissues. It is known that insulin signaling activates Akt by phosphorylation at two residues, threonine 308 (Thr^{308}) and serine473 (Ser^{473}), both of which are required for full

activation of Akt in vitro. The residue Thr^{308}, which resides in the activation loop of Akt, is phosphorylated by phosphoinositide-dependent kinase-1 (PDK1). It was previously investigated in a kinase similar to PDK1 (possibly PDK2) that mediated the phosphorylation of Ser^{473} in the COOH-terminal hydrophobic motif of Akt. However, the current research has established that $mTORC_2$ is responsible for the Serine 473 phosphorylation of Akt (Kumar et al., 2008).

In further investigation of the relationship between $mTORC_2$ and glucose metabolism, Liu et al. (2014) investigated the relationship of $mTORC_2$ and Akt at residue 473 relationship in their muscle cell culture model. They knocked out Rictor protein which is essential for $mTORC_2$ activity by a particular gene silencing small interfering RNA (siRNA). Inhibition of Rictor repressed the residue phosphorylation of Akt^{473}. Conversely, insulin independently and insulin with LEU stimulated phosphorylation of Akt^{473} when Rictor was present. Furthermore, LEU, insulin independently, and LEU with insulin all stimulated phosphorylation of $mTORC_1{}^{2448}$. In conclusion, the authors suggested that LEU amplified the insulin stimulated phosphorylations of Akt^{473} and $mTORC_1{}^{2448}$ and purported that $mTORC_2$ may be mediated by LEU independently or in conjunction with an insulin induced Akt phosphorylation at residue 473 in skeletal muscle cells, and therefore this mechanism may help explain how LEU is able to influence glucose uptake (H. Liu et al., 2014).

Beyond the mechanistic elaboration, the practicality of the treatment in the human model becomes very interesting. Kalogeropoulou et al. (2008) investigated the co-ingestion of 1 mmol/kg of LBM (a mean of ~7 g and range of 5-9 g) co-ingested with 25

g of glucose in thirteen healthy participants (6 men and 7 women), mean age ~24 year (range 18-33 year), BMI ~24 kg/m^2 (range 21-27 kg/m^2), body weight 70.9 kg (range 54-102 kg), LBM of 51 kg (range 40-75 kg) and mean percentage of LBM of 75.6% (range 68%-86%). Their results showed that LEU attenuated glucose responses; 50 min after glucose ingestion alone blood glucose peaked at ~131 mg/dl during the control treatment. However, when glucose was coingested with LEU blood glucose was lower ~114 mg/dl 50 min after coingestion. The glucose area response (iAUC) was ~50% less when LEU was ingested with glucose (p = .019). The insulin responses showed fasting serum insulin concentration was 32 ± 3 pmol/l (5.4 ± 0.5 μU/ml). Postprandial consumption of glucose influenced the mean serum insulin concentration which reached a peak of 186 pmol/l (31 μU/ml) at 50 min, followed by a gradual decrease down to fasting concentration at 150 min. Postprandial co-ingestion of glucose and LEU influenced the mean serum insulin concentration that was 72% greater 318 pmol/l (53 μU/ml) than that after the ingestion of glucose alone of and observed 10 (40 min) minutes earlier than glucose only treatment. Insulin, then rapidly returned to the fasting level by 150 minutes. Ingestion of LEU independently resulted in only a modest increase in insulin concentration. The insulin iAUC was 66% greater after ingestion of glucose plus LEU compared with when glucose consumed individually (p = .003). Additionally, LEU ingestion alone resulted in a modest but significantly higher insulin iAUC than water (control) intake (p = .0001). Mean fasting glucagon concentration was 68 ± 5 pg/ml, and after ingestion of glucose glucagon concentration decreased which is expected under normal conditions and remained below the initial fasting concentration value. With the

coingestion of glucose plus LEU, the observed glucagon concentration decreased similarly to the glucose treatment to its lowest point of ~56 pg/ml. It then increased back to a fasting concentration at 150 min. The iAUC of glucagon increased with LEU ingestion; comparatively to decreasing after the ingestion of the other treatments. The authors reported a wide amount of variance assessing glucagon. The glucagon iAUC after ingestion of LEU with glucose was significantly less negative when compared with the iAUC after glucose ingestion independently (p = .04). The glucagon iAUC after LEU ingestion was significantly higher when compared with the iAUC the control treatment of water ingestion (p = .006).

The authors conclusion from this study is that it corroborates with others studies from this same group (Gannon & Nuttall, 2010), that LEU has an independent and synergistic effect with glucose on insulin responses in normal healthy persons. It is suggested that their glucose response becomes blunted by the amplification of insulin through possible direct interactions with the pancreas β-cells. The authors did not measure incretin concentrations to observe any influences they may or may not have in these glycemic responses (Kalogeropoulou, Lafave, Schweim, Gannon, & Nuttall, 2008).

Glycemic Effects of L-Isoleucine

L-Isoleucine (ISO) is a fellow family member of the BCAA family and also an EAA required by diet and unable to be synthesized de novo. For a ~58-72 kg male, the recommended minimal ISO intake is suggested to be 0.70-0.65 g/day respectively, to a higher threshold intake of 1.40 g/day based on a nitrogen balance assessment (Rose,

Eades, & Coon, 1955), and there is currently not a set upper tolerable intake suggested for ISO. ISO has received far less attention compared to LEU in regards to its propensity to stimulate the pancreatic release of insulin, influence insulin sensitivity, and as a stimulator of MPS. However, ISO has received attention for its potential use as a glycemic modulator. Compared to LEU, ISO has shown to be less influential in stimulating the pancreatic release of insulin and its actions on glucose uptake has largely been regarded as insulin independent. Mechanistically, research has suggested a direct interaction with PI3K and PKC as a possible pathway to induce GLUT-4 translocation and glucose uptake (Nishitani et al., 2005).

Doi et al. (2003) investigated all of the BCAA's in a cell culture of C2C12 myotubes and rodent model. A dose of 0.3 g/10 mL/kg of body weight was delivered separately as a 3% solution by oral ingestion 30 min before the glucose bolus. The OGTT was performed by administration of a 2 g bolus of glucose/kg body weight by gavage as a 50% solution of glucose and water. VAL significantly increased the glucose level at 30 min after the glucose administration while LEU had a similar effect at the 120 min time point compared to the glucose only control. However, administration of ISO significantly decreased plasma glucose at 30 and 60 min time points after glucose ingestion. Additionally, in a dose-dependent manner, they investigated the ISO via oral administration at 0.05 or 0.10 g/kg BW caused no significant changes in plasma glucose concentrations. Plasma insulin concentrations at 30, 60, and 120 min post OGTT were not significantly different between any treatment. In regard to the C2C12 cell culture model, the differentiated myotubes cells were exposed to 1 mM LEU, ISO, and VAL for

4 hr. The glucose uptake was elevated by 16.8% in the presence of 1 mM isoleucine compared with the control. Comparatively, 1 mM LEU or VAL had no significant effect on glucose uptake in the myotubes. The assessment of 2-Deoxyglucose (2DG) uptake showed that 2 mM ISO caused the largest increase in glucose uptake (35.0%; $p < .05$). The uptake of 2-DG into C2C12 cells was significantly increased by exposure to 1–10 mM ISO and to 5–10 mM LEU. When myotube treatments of ISO, insulin alone or ISO plus insulin were assessed by 2DG uptake; insulin and ISO independently caused a significant increase in glucose uptake (1.1-fold; $p < .01$ and 1.3-fold; $p < .001$). The stimulatory effect of combined treatments (insulin + ISO) was significantly greater than that of insulin alone; however, this did not show to be significantly different from ISO alone. Lastly, the myotubes treated with 2 mM LEU or 2 mM VAL resulted in a significant increase in labeled D-[U-14C] glucose deposition into intracellular glycogen compared with control. Comparatively, 2 mM ISO showed no significant effect on glycogen synthesis.

The authors suggest through cell signaling pathway analysis by way of using several inhibitors that inhibited PI3K and PKC isolated their involvement and enhancement of glucose uptake by ISO. Furthermore, the amplified glucose uptake caused by ISO was not mediated by $mTORC_1$, which is in contrast to other research where the $mTORC_1$ inhibitor rapamycin showed an additive effect of glucose uptake when administered concurrently with ISO. This response suggests an alternate pathway of stimulus that ISO signaling involves PI3K and uses a differing route than IRS-1 mediated insulin signaling. In contrast, ISO showed no influence on glycogen synthesis

as it did for glucose uptake, and LEU and VAL despite having a weak effect on glucose uptake affected a higher labeled glucose deposition and level of glycogen synthesis (Doi et al., 2003).

To further explore the relationship of ISO and glucose uptake, Doi et al. (2005) using a similar study design, investigated the effects of LEU and ISO on glucose uptake in rodent skeletal muscle in vivo used labeled 2- [1,2-3 H]-deoxyglucose (2- [3 H] DG) and [U-14 C]-glucose. Additionally, they investigated whether the glucose uptake occurred via an AMPK mediated mechanism, by measuring the activity of AMPK isoforms α-1 and α-2, and the energy status contents of ATP, ADP, and AMP. Similarly, compared to data above, they observed that ISO decreased the plasma glucose concentration ($p < .05$) compared to control. The oral administration of LEU and ISO did not alter the plasma insulin concentrations at 1 h after administration. However, LEU transiently increased at 30 min after administration, though plasma glucose concentration was not changed. Comparatively, ISO administration in the rodent model had no effect on plasma glucose or insulin concentrations at 30 min. The rate of glucose uptake in the skeletal muscle of rats administered ISO was significantly greater than that in the control. Compared to LEU values the authors stated that the ISO treatment "tended" to be more significant ($p < .10$). The administration of LEU increased [U-14C]-glucose deposition into glycogen ($p < .05$) compared with the control group. On the other hand, the [U-14C]-glucose deposition into glycogen in the skeletal muscle did not differ between groups administrated ISO or the control. Furthermore, AMPK α-1 isoform was unchanged in

both LEU and ISO-administered groups compared to control. The activity of the AMPK α-2 isoform was also consistent during the administration of LEU compared with control.

In comparison, AMPK α-2 activity was decreased when administered ISO ($p < .05$) compared to the control group. Lastly, the administration of LEU or ISO did not alter the ADP or ATP contents in the skeletal muscle compared with control group. However, ISO influenced a 21% decrease in AMP content in skeletal muscle compared with the control ($p < .05$) and LEU ($p < .05$) groups. Additionally, the AMP concentration was not affected after administration of LEU compared with the control group. Furthermore, although LEU did not change the AMP to ATP ratio, ISO modulated a significant 20% decrease in this ratio ($p < .05$) in the skeletal muscle compared with control groups.

Based on their results, the authors concluded that ISO stimulated glucose uptake is increased independently of AMPK α-1 and α-2 activity based on its limited response to ISO in skeletal muscle. The decreases in the AMP concentration and the AMP to ATP ratio in the skeletal muscle in the group administered ISO was consistent and associated with the reduction in activity for AMPK α-2. In summary, the data suggests that skeletal muscle glycogen synthesis was significantly increased by administration of LEU; however not with ISO in vivo compared with the control group. LEU had less effect on glucose uptake in skeletal muscle compared to its influence on glycogen synthesis without affecting the AMP concentration, AMP to ATP ratio, nor the AMPK activity. However, in contrast, ISO showed a greater ability to stimulate glucose uptake and improve the cellular energy state via lowered AMP concentration and enhanced ATP to

AMP ratio in skeletal muscle than LEU; although it has less effect on protein synthesis signaling and glycogen synthesis (Doi et al., 2005). Both ISO and LEU seemingly had positive roles in glucose uptake or glucose deposition into skeletal muscle via glycogen synthesis, yet AMPK activity was unaffected and not a factor in mediating GLUT-4 translocation.

Continuing to elucidate the hypoglycemic effect of ISO, Doi et al. (2007) investigated the acute effects ISO in a IGT diabetic rodent model and the chronic effects of ISO on glucose metabolism with a high-fat/high-sucrose (HFD/HSD) diet. The mice eating The HFD and HSD diet freely drank water containing 20% sucrose without or with 1 or 2% of ISO for 6 weeks. The hypoglycemic activity of ISO was investigated in a normal rodent model as a control with a protocol of an oral dose of 30—300 mg/kg of ISO immediately followed with 2 g/kg of glucose. ISO significantly and in a dose dependent manner reduced the blood glucose level 16.2% and 24.6% of the glucose AUC in rodents given 100 and 300 mg/kg of ISO respectively. The glucose AUC was significantly increased by 15.7% in the HFD group with IGT compared with the control group ($p < .01$) confirming the dysregulation of glucose tolerance. The oral ISO dose of 300 mg/kg significantly reduced the height of the glucose tolerance curve in both control and HFD groups. The plasma insulin concentrations after the OGTT only showed a significant increase in the HFD group with ISO treatment, suggesting a different mechanism of hypoglycemic actions in the HFD group compared to the normal group. They next observed the hypoglycemic effect of ISO in db/db mice. The OGTT showed a decrease, in the blood glucose concentrations of db/db mice treated with ISO in a dose

dependent manner, and the difference was significant at an increased dose of 500 mg/kg. Moreover, the insulin AUC was not significantly different between ISO and insulin secretagogue (Nateglinide) comparison suggesting that ISO treatment in the T2D group similarly unaffected insulin secretion yet influenced glucose uptake. Lastly, the authors investigated a combined HFD and HSD (HFHS) with chronic ISO administration of either 1 or 2%. They observed that there was no significant effect of ISO on the blood glucose responses after the OGTT. However, the insulin AUC showed a significant decrease in a dose dependent effect (2% > 1% ISO) of ISO, that suggest that chronic feeding of 2% ISO possibly improved insulin sensitivity in this group.

In conclusion, the authors indicate that acute and chronic ISO supplementation improves glucose metabolism in normal animals, in IGT, HFD, and in T2D rodent models. Additionally, they observed that insulin sensitivity was improved in HFHS rodent model after 6 wk of 2% chronic ISO treatment. The ISO at the dose of 300 mg/kg (.03 g/kg) that had an acute hypoglycemic effect increased the ISO plasma concentrations from a basal 89.3 μmol/l up to a peak of 1220.6 μmol/l at 30 min after administration. Additionally, the amino acid drink was able to reach an ISO plasma level over 1300 μmol/l and was well tolerated without adverse effects and considered applicable in a human model. Lastly, the authors attribute these ISO mediated hypoglycemic effects through an enhanced glucose uptake by peripheral tissues and a possible synergistic stimulated insulin release from pancreatic β-cells. However, the data has shown the hypoglycemic effects of ISO mainly seem to be insulin secretion independent (Ikehara, Kawasaki, Maezono, Komatsu, & Konishi, 2008).

In more of a practical human model, Nuttall, Schweim, & Gannon (2008) investigated a dose of 1 mmol ISO/kg lean body mass (LBM) (a mean of ~7.4 g) coingested with 25 g of glucose (GLU) compared to ISO alone, GLU alone, and a water control trial. Observing 9 non diabetic participants (3 males; 6 females) with characteristics of: mean age ~26 year, height ~66 in, ~81 kg, BMI ~28 kg/m^2, LBM ~56.5 kg, ~83 mg/dl ~9.5 μU/ml. After completion of four trials, it was observed that when glucose was consumed alone, it resulted in a mean increase in serum glucose concentration from a basal of 84 mg/dl to a peak of 120 mg/dl at 40 min postprandial. After 150 min concentrations decreased back to the fasting basal concentrations. After co-ingestion of ISO and GLU, the glucose response was similar but reached a peak of only 114 mg/dl earlier at 30 min followed by a rapid decrease to fasting value by 65 min. Interestingly, blood glucose decreased further to 76 mg/dl and remained below the fasting value at the termination of treatment. The ISO only treatment resulted in a reduction in basal glucose concentration from 84 mg/dl to 77 mg/dl or an 8% reduction. Comparing the AUC of glucose, when ISO was coingested with GLU, it reduced the glucose AUC substantially when compared to that the ingestion of glucose alone (~61%) ($p < .03$). Similarly, ISO ingestion independently decreased the glucose AUC when compared to the intake of water alone ($p < .02$).

Insulin responses after glucose ingestion increased the mean basal concentration of insulin from 9 μU/ml to a peak of 48 μU/ml at 40 min, roughly a ~5-fold increase. After the coingestion of ISO and GLU stimulated an increase in mean insulin concentrations of roughly ~8-fold higher than basal conditions, which was ~49% higher

than the glucose treatment, suggesting a synergistic insulinogenic effect. Lastly, ISO independently did not augment the insulin concentration. However, after further examination using AUC; the insulin AUC was ~13% greater when ISO was coingested with GLU. Regardless, of the smaller insulin AUC response to glucose alone, when compared to the ISO and GLU AUC the difference was not significant ($p < .4$). Glucagon responses to ISO alone resulted in a minimal, transient increase in glucagon concentration; although, the coingestion of both GLU and ISO led to an expected decrease in glucagon. The glucagon AUC of postprandial ISO was not statistically different from water ingestion. Lastly, the reduction in glucagon resulting from GLU compared to coingestion of GLU with ISO similarly was not significantly different ($p = .35$).

The authors concluded that larger ingested physiological amounts of ISO is readily absorbed. When it is ingested independently, it did not influence insulin secretion and concentration but did decrease the glucose concentration. Additionally, it increased glucagon modestly. Moreover, when ISO was coingested with GLU it resulted in a smaller decrease in glucose concentration, though a similar increase in insulin concentration compared to glucose alone (Nuttall, Schweim, & Gannon, 2008)

CHAPTER III

RESEARCH METHODS

Participants

The inclusion criteria to participate in this study required an age range of 20 to 45 years of age. This range was selected because age is an independent factor that influences glucose uptake in healthy persons (Shimokata et al., 1991). Both genders were recruited equally and fairly. However, females admitted into the study were limited to testing during the follicular phase (days 1-10) for data collection (Figure 6). This is in consideration of the fact that insulin sensitivity may vary throughout the luteal and follicular phases of the menstrual cycle (Matsuda & DeFronzo, 1999; Naito, Kuma, & Mizushima, 2013; Tam et al., 2012), however, during the follicular phase, women respond to insulin in ways more similar to men when estrogen concentrations are low. Additionally, with prescribed usage of birth control, insulin sensitivity (IS) may be influenced during the luteal phase of birth control with medication (Blaak, 2008; Toth, Suthijumroon, Crockford, & Ryan, 1987; Yki-Järvinenf, 1984).

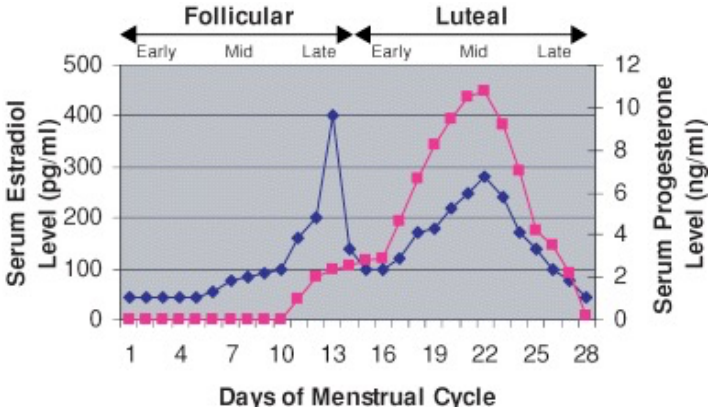

Figure 4: Women were investigated on days 1-10 to compare to men

Physical Activity and Exercise Training Status

Physical activity and exercise influence insulin sensitivity and therefore glucose uptake. A short bout of exercise or physical activity acutely influences a greater sensitivity to insulin by stimulating an increase in the actions of adenosine monophosphate kinase (AMPK) and other cell signaling actions to upregulate GLUT-4 translocation and glucose entrance into the cell (Borghouts & Keizer, 2000; Goodyear & Kahn, 1998). Though the definition of sedentary behavior is debated, it is best characterized as a sedentary or inactive lifestyle that includes most days spent sitting or reclining at a quantified intensity of 1.0-1.5 metabolic equivalents (METS), ≤ 1-3 days/week of exercise, and ≤ 20-150 min/week of activity or exercise, which is below the CDC/ACSM recommended guidelines for physical activity (Bennett, Winters-Stone, Nail, & Scherer, 2006; Garber et al.; Owen, Healy, Matthews, & Dunstan, 2010). Participants who did not meet the ACSM recommendation of weekly physical activity recommendations or self-identified as completely sedentary were admitted into the study.

Moreover, participants were also asked to refrain from activity during the three days prior to a treatment.

Study Design Summary

Participant entered lab, was informed of study details, and filled out informed consent. Additionally, basic anthropometric measures of height and weight were collected along with body composition via Dual-Energy X-ray Absorptiometry (DXA) (Lunar Prodigy GE Inc., Madison WI) three days prior to visit. Participants were told to abstain from physical activity, alcohol, and caffeine consumption 24 hours prior to experimental conditions. Nutritional self-recall mobile apps have shown to be somewhat resourceful. It has been reported similar variability when compared with conventional methods nutrient data collection such as the 3 day dietary recall (McClung et al., 2009). Dietary intake was recorded with the program MyFitnessPal (www.myfitnesspal.com), and participants were asked to record everything they ate and drank for three days prior to a treatment day. Additionally, all participants were coached to maintain similarity in their dietary choices during those three days to avoid confounding factors.

After a 12 hr overnight fast, participants were asked to arrive at the lab on the morning of a treatment day. An intravenous catheter was inserted either into a posterior forearm vein or antecubital vein and attached to a saline drip. A 4 ml baseline blood sample was taken at each of the following time points: baseline, 6 min, 10 min, 30 min, 36 min, 40 min, 60 min, 90 min, 120 min, and 150 min. For each of these visits, participants were randomized to for ingestion of one of the following 4 treatment drinks

after the baseline sample: control, 0.3 g/kg LBM L-isoleucine (ISO), 0.3 g/kg LBM L-leucine (LEU), 0.3 g/kg LBM of a combination of L-isoleucine and L-leucine drink (ISO+LEU). Thirty minutes after consuming the treatment drink, participants were asked to ingest a 75-g glucose drink. Participants were allowed to watch "non-stimulating" movies, documentaries, and/or TV shows during the treatment days and were placed in a semi-recumbent position for the duration of the visit. Written informed consent was obtained from all participants. Finally, this study was approved by the Department of Kinesiology and the Texas Woman's University Internal Review Board (*Protocol # 17757*), following The World Medical Association's Declaration of Helsinki, and was approved by clinicaltrials.gov (NCT02634164/).

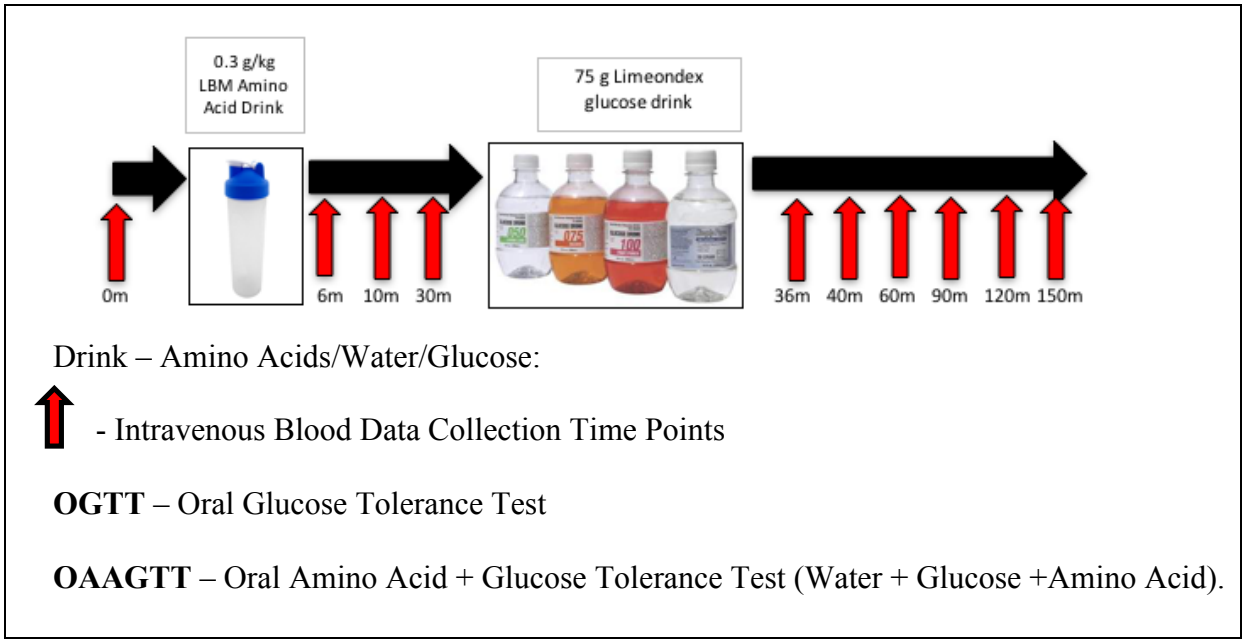

Figure 5: Overview of study design and sample collection time points

Preliminary Testing Session

This session served to screen participants and familiarize them with the procedures of the study. During this preliminary testing session, we obtained informed written consent, a medical health questionnaire, anthropometric measures (height and weight), DXA for whole-body soft-tissue (fat mass/fat-free mass) composition, and a blood sample taken for participant's complete blood panel for beneficence. During this testing session, a nutritional log (Myfitnesspal.com) was recorded by the participant for the three days prior to the day of the preliminary testing session to control for dietary intake and physical activity among experimental sessions.

Oral Glucose Tolerance Test (OGTT)

Blood glucose and insulin will be obtained following the guidelines and recommendations of the American Diabetes Association and the protocol similar to Eicher et al. (Association, 2014b; Eicher, Maresh, Tsongalis, Thompson, & Pescatello, 2010). Participants ingested amino acid with water (150 ml) with additional water (50 ml) to ensure the dose was fully consumed. Blood collections were completed at 0 min, 6 min, 10 min, 30 min prior to a standard 75 g glucose solution ingestion, followed by blood collections at 36 min, 40 min, 60 min, 90 min, 120 min, and 150 min post ingestion. A total of 16 blood collections were taken. Each 4 ml sample was then immediately placed on ice, and centrifuged at 3000 RPM (4°C) for 10 min. Plasma samples were transferred into storage cryotubes and frozen at -80°C until later analysis.

Finally, data analysis was completed at the Texas Woman's University Kinesiology Exercise Biochemistry Laboratory.

Oral Amino Acid and Glucose Tolerance Test (OAAGT)

L-Leucine

For this visit, participants ingested a powdered form of *L-Leucine* (Figure 6) standardized to of 0.3 g/kg of lean body mass weight with water (150 ml) with additional water (50 ml) to ensure full dose is consumed. The amino acid solution was ingested 30 min prior to 75 g glucose solution. Data collection time points were 0 min, 6 min, 10 min, 30 min, 36 min, 40 min, 60 min, 90 min, 120 min, and 150 min.

L-Isoleucine

For this visit, participants ingested a powdered form of *L-Isoleucine* (Figure 7) in the amount of 0.3 g/kg of lean body mass weight with water (150 ml) with additional water (50 ml) if needed to ensure the dose is fully consumed. The amino acid solution was ingested 30 min prior to a 75 g glucose solution. Data collection time points were 0 min, 6 min, 10 min, 30 min, 36 min, 40 min, 60 min, 90 min, 120 min, and 150 min.

L-Leucine and L-Isoleucine (50:50) Combination

For this visit, participants ingested a powdered form of both *L-Leucine and L-Isoleucine* in the amount of 0.3 g/kg of lean body mass weight each with water (150 ml) with additional water (50 ml) if needed to ensure the dose is fully consumed. The amino acid solution was ingested 30 min prior to 75 g glucose solution.

Data collection time points were 0 min, 6 min, 10 min, 30 min, 36 min, 40 min, 60 min, 90 min, 120 min, and 150 min.

Placebo or Control

For this visit, participants ingested a powdered form of 3.54 g of inert, lecithin, calorie free, and stevia sweetener (which is also found in the composition of the amino acids treatments). Lecithin was used to improve the dissolving of hydrophobic amino acids, LEU and ISO; the stevia sweetener was re used to increase palatability. Again, additional water (50 ml) was used if needed ensure the dose was fully consumed. The placebo solution was ingested 30 minutes prior to ingestion of a 75 g glucose solution. Data collection time points were 0 min, 6 min, 10 min, 30 min, 36 min, 40 min, 60 min, 90 min, 120 min, and 150 min.

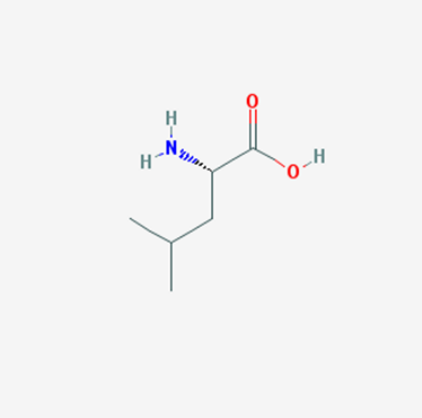

Figure 6: L-Leucine

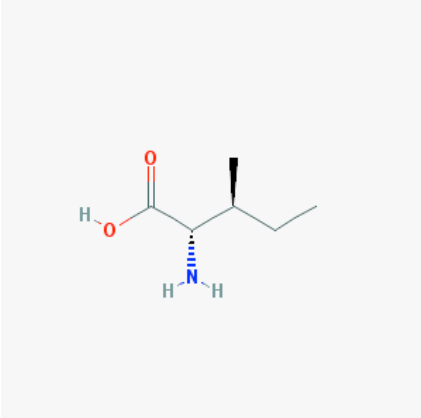

Figure 7: L-Isoleucine

Sampling and Analysis Protocol

All blood samples were drawn without stasis from the antecubital vein (or dorsal forearm vein if present) of the right or left arm using a 20 or 22 gauge indwelling Teflon catheter. All of the samples were collected in the morning (0800 hr) following overnight fast (12 hr) and at least three days of not participating in exercise. The cannula was kept patent with a solution of 0.9% saline. Baseline blood samples were taken prior to the treatment sessions for fasted samples of blood glucose and insulin, glucagon, C-peptide, $GLP\text{-}1_{Active,}$ and GIP_{Total}. The same metabolites were measured in all four treatments following the same time course data collection (listed in Study Design Summary) similar to (Nuttall et al., 2008)Additionally, a warming pad was wrapped around the sampling catheter for arterialization of the venous samples similar to the protocol that has been shown to be effective in previous glucose studies (Kjems, Holst, Vølund, & Madsbad, 2003; McGuire, Helderman, Tobin, Andres, & Berman, 1976; Nauck, Liess, Siegel, Niedmann, & Creutzfeldt, 1992).

Blood samples were drawn into BD Vacutainer K2 EDTA blood collection tubes in accordance with Millipore manufacturer recommended doses of serine protease inhibitor Pefabloc® SC (*#11429876001*), Sigma-Aldrich Protease Cocktail Inhibitor (*#P2714-1BTL*), and Sigma-Aldrich Dipeptidyl Peptidase IV (*#D4943 Sigma*). Samples were centrifuged at 3000 RPM at 4°C for 10 minutes. Plasma samples were then transferred into storage cryotubes and frozen at -80°C until later analysis.

The YSI 2900 Bioanalyzer (YSI Life Science, OH, USA) enzyme electrode technology was used to determine the concentration of glucose (mg/dl) in plasma,

according to manufacturer's maximum accuracy limits. Linearity curves were generated for glucose using a calibration solution, and the integrity of the system was confirmed by daily integrity tests. Finally, 25 ul of plasma was used per measurement.

Plasma hormone concentrations were analyzed using Luminex MagPix® System multiplex technology following manufacturers recommendations and similar to the protocol used by (McFarlin, Johnson, Moreno, & Foreyt, 2013). For plasma hormone analysis, 25 ul of the sample was analyzed in duplicate to determine the concentration of C-peptide, GIP_{Total}, $GLP\text{-}1_{Active}$, glucagon, and insulin. This was done using a Luminex Human Metabolic Hormone multiplex assay (HMHEMAG-34K, EMD Millipore, Billerica, MA). Manufacturer-supplied quality controls were included to measure assay variation and standards were set to a suggested 1:3 dilution. A minimum of ~100-400 beads (a minimum manufacture requirement of ≥32 beads) was collected for each analyte. The Magpix system was calibrated and verified before each sample analysis. The data was assessed and quantified using Milliplex Analyst Software 5.1 (EMD Millipore). Millipore manufactured and supplied controls were used to monitor coefficients of variation, which were <10%, and minimum detectable concentration for each analyte was within the normal limits reported by the manufacturer: insulin 14.97 pmol/l; C-peptide 0.031 nmol/l; GIP 0.12 pmol/l; GLP-1 0.36 pmol/l.

Statistical Approach and Power Analysis

The primary statistical analysis will be a two-way repeated measures ANOVA (time x treatment) design. More specifically, the effects of the treatments (LEU, ISO,

LEU + ISO, PLA) on the primary dependent variables are GIP_{Total} and $GLP\text{-}1_{Active,}$ and the secondary variables insulin, C-peptide, and glucose. Upon identification of a significant main effect from the ANOVA, paired differences were evaluated from multiple comparisons when appropriate, using Tukey post-hoc analysis. The statistical analysis Pearson's product correlation coefficient was used when appropriate for correlations between glucose, insulin, C-peptide, GIP_{Total}, GLP-1Active, and the treatments provided.

Data that was not normally distributed or otherwise violated analysis assumptions underwent transformation (e.g., log transformation) or non-parametric analysis if appropriate. Participant characteristics were expressed in means ± SD while data were expressed in mean ± SEM. Differences resulting in values of $p < .05$ were considered significant. Both relative incremental change from baseline (Δ) and incremental area under the curve (iAUC) for plasma glucose and hormone responses were assessed by Graphpad Prism, which computes the area under the curve using the trapezoidal rule. The area equation [ΔX*(Y1+Y2)/2] Prism uses this formula repeatedly for each adjacent pair of points defining the curve for each of the test drinks ingested by each participant (treatment x time). Incremental AUC expressing the 0–30-min time course for plasma glucose, insulin, C-peptide, glucagon, GIP, and GLP-1. The mean ± SEM incremental AUCs 0–150 min and 0-30 min for plasma glucose, insulin, C-peptide, glucagon, GIP, and GLP-1 were similarly calculated for each participant (treatment x time), using GraphPad PRISM software (version 7.0a; GraphPad Software Inc., San Diego). All

iAUC below the baseline were excluded from the calculations (Wolever, Jenkins, Jenkins, & Josse, 1991).

Interpretation of Data

The objective of the current study was to determine the effect of ISO+LEU, ISO, and, LEU treatments on lowering plasma glucose concentrations compared to PLA during a 2.5 hr OGTT. We hypothesized that the amino acid treatments would have no effect on increasing enteroendocrine incretin hormones GLP-1 and GIP concentrations, while in contrast, independently reduce the plasma glucose concentration. Additionally, we suggest that without an incretin effect, the insulin concentration response should be similar to PLA. Lastly, if there is no perceived incretin and insulin responses due to amino acid treatments we suggest glucose reduction may be possibly mediated by intramuscular skeletal muscle signaling, which has shown to influence glucose uptake.

CHAPTER IV

RESULTS

Presentation of Findings

Thirteen healthy and inactive adults were recruited for this study. Of the 13 participants, one did not complete the study due to compliance and scheduling issues. Twelve participants completed all four treatments. Table 2 shows the participant characteristics presented in mean and range where appropriate (n = 12; 6 females, 6 males). The average age of the group was 27 years (21-33 years); weight 82.5 kg (60-102 kg); lean body mass 48.6 kg (38.9-58.4 kg); fat mass 30.9 kg of (14-52.5 kg); BMI 30.1 kg/m^2 (21-41 kg/m^2); body fat percent 37.3% (20.3-52.7%); total cholesterol 148 mg/dl (108-180 mg/dl;); and fasting glucose 89.5 mg/dl or 4.97 mmol/l (79-102 mg/dl; 4.38-5.67 mmol/l). The majority of the participants selected for this study were from the Texas Woman's University campus in Denton, TX, and had self-reported themselves as inactive prior to and during data collection. Participants included a combination of both female and male subjects. As such, body composition values for LBM and FM were highly variable among these participants. However, fasting blood glucose concentration levels were considered healthy and normal throughout the group (< 126 mg/dl) (Standards of Medical Care in Diabetes-2016 Abridged for Primary Care Providers, 2016; Standards of medical care in diabetes-2014, 2014).

Table 2

Participant Characteristics	
n =12	(6M, 6F)
Age (yrs)	27.4 ± 7.1
Height (cm)	167.4 ± 7.7
Weight (kg)	77.8 ± 12.9
FM (kg)	26.5 ± 12.1
LBM (kg)	48.6 ± 6.3
BMI (kg/m2)	26.3 ± 7.4
BF %	34.1 ± 10.3
Cholesterol (mg/dL)	147.5 ± 26.6
HDL (mg/dL)	50.8 ± 18.2
LDL (mg/dL)	83 ± 25.4
TG (mg/dL)	88.8 ± 22.9
Glucose (mg/dL)	89.5 ± 6.0
Glucose (mmol/L)	4.97 ± 0.3
GIPTotal (pmol/L)	7.16 ± 2.1
GLP-1Active (pmol/L)	1.95 ± 1.3
Glucagon (ng/L)	54.2 ± 30.9
Insulin (pmol/L)	96.8 ± 44.1
C-peptide (nmol/L)	4.02 ± 1.97

Note: Values are expressed as means ± SD. Fat mass (FM); lean body mass (LBM); body fat (BF); triglycerides (TG); glucose-independent polypeptide (GIP); glucagon-like peptide-1 (GLP-1)

Data analysis of glucose samples (n = 12) was assessed with the YSI 2900 Biochemistry Analyzer, and all standards for calibration were within the manufacture recommendations of < 5% coefficient of variation (% CV). Typically, standard values were accepted if they were observed between 2-3% of listed manufacture standard concentration values. Luminex Magpix multiplexing data analysis results were maintained within manufacturer recommended intra- and inter-assay CV's (see Methods section). All data fit within manufacturer quality control ranges. One participant's plasma samples were omitted from final analysis due to errors induced by equipment malfunction (n = 11), and another participant's hormone glucagon analysis (n = 10) was also excluded due to the same reason. All data outside of recommended minimal assay

sensitivities were not used during final data analysis and interpretation. For treatments with 2-3 sample time points that were either corrupted or an outlier, GraphPad Prism's "Interpolate a standard curve" program was used to estimate best suitable time points.

Amino Acid Treatment Compared to Placebo

Each participant ingested either L-Isoleucine + L-Leucine (50:50) (ISO+LEU), L-Isoleucine (ISO), L-Leucine (LEU), and placebo (PLA) treatment. The average amino acid dose was 14.6 g, with a range of 11.67 to 17.5 g for each of the three amino acid treatments. The inert placebo dose was always 3.54 g for each participant.

Plasma Glucose

The incremental change (Δ) of plasma glucose from baseline (mmol/l) results (Mean ± SEM) (n = 12) showed a significant treatment main effect $F(3, 440) = 4.269$; $p = .005$) when comparing treatments. After Tukey post hoc multi comparison analysis found differences between ISO+LEU ($p = .02$), ISO ($p = .01$), and LEU ($p = .02$) compared to PLA treatment. Further interaction effect assessment found an interaction with lower glucose responses at 60 min in ISO+LEU vs. PLA ($p = .008$); at 90 min ISO+LEU ($p = .02$) and ISO ($p = .04$) vs. PLA (Figure 8). Assessment of incremental (Δ) changes of plasma glucose during the 30 min ingestion of amino acid prior to the glucose drink (Figure 9), showed a time x treatment interaction ($p = .001$) and a treatment effect $F(3, 33) = 4.03$; $p = .01$). ISO reduced plasma glucose (~2.5 mmol/l or 45 mg/dl) more so than the ISO+LEU treatment ($p = .01$). Additionally, interactions were observed at 6 min post amino acid ingestion, where ISO resulted in lower plasma glucose

than ISO+LEU (p = < .0001) and PLA (p = .01), and LEU lowered plasma glucose more so than ISO+LEU (p = .0001), with no differences found between ISO and LEU treatments.

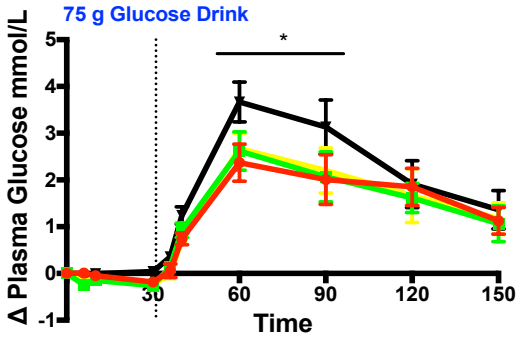

Figure 8: (Mean ± SEM) main effect (0-150 min) Δ Plasma glucose comparing ISO+LEU, ISO, LEU, and PLA. Main treatment effect (P = .005); (*) differences were observed between ISO+LEU (P = .02), ISO (P = .01), and LEU (P = .02) vs. PLA. Simple effect time differences at 60 min ISO+LEU vs. PLA (P = .008) and at 90 min ISO+LEU (P = .02) and ISO (P = .04) vs. PLA.

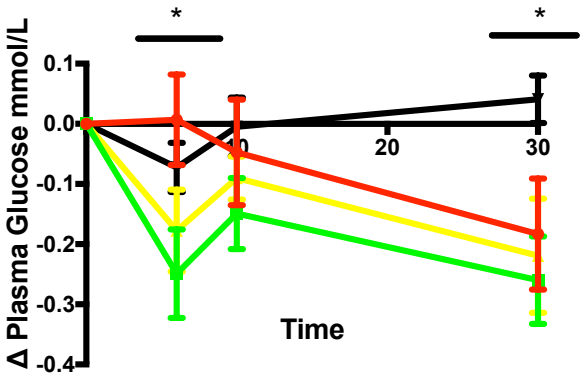

Figure 9: (Mean ± SEM) Δ Plasma glucose (mmol/l) comparing ISO+LEU, ISO, LEU, and PLA 0-30 min (amino acid); (*) time x treatment interaction (p= .001); main effect of treatment (P = .01), ISO vs. PLA (p = .01); simple effects 6 min ISO+LEU vs. ISO (<0.0001), ISO+LEU vs. LEU (p = .007), ISO vs. PLA (p = .01); 30 min ISO+LEU vs. PLA (p = .0007), ISO and LEU vs. PLA (p = <0.0001).

Plasma Insulin

The incremental plasma insulin change (n = 11) showed no main effect differences between amino acid treatments (p = .053; 0-150 min). Additionally, no differences were found between amino acid treatments and PLA, and no interactions were found between treatments at any time point (Figure 10). The incremental plasma insulin analysis of the 0-30 min phase of amino acid ingestion similarly showed no differences between treatments on insulin responses. An interaction was found between all amino acid

treatments vs. PLA at 30 min (p = .05). All amino acid treatments showed to stimulate an increase in insulin concentration (Figure 11).

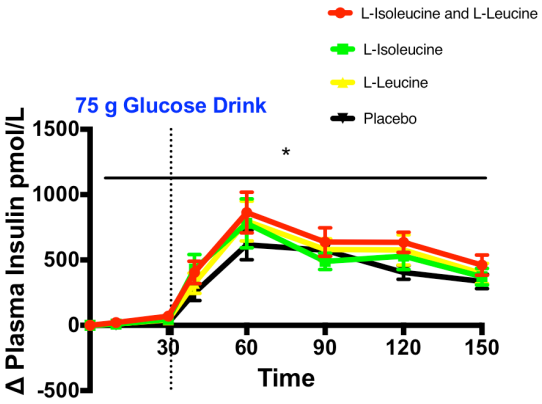

Figure 10: (Mean ± SEM) Δ Plasma insulin (pmol/l) comparing ISO+LEU, ISO, LEU, and PLA 0-150 min; no main effect differences were found between treatments (p = .0531). (*) When compared to PLA only ISO+LEU had a main effect (p = .03). No differences were found between amino acid treatments.

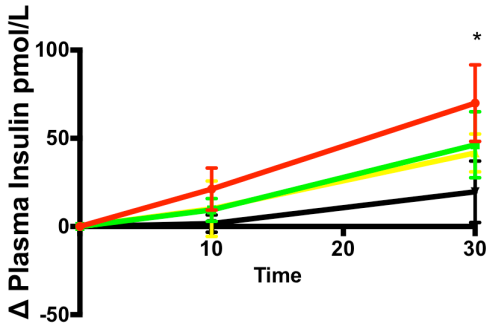

Figure 11: (Mean ± SEM) Δ Plasma insulin (pmol/l) (amino acid) comparing ISO+LEU, ISO, LEU, and PLA 0-30 min. No main effect was found between treatments; ISO+LEU vs. PLA (p = .01).

Plasma C-peptide

The incremental change in plasma (n = 11) C-peptide concentrations (0-150 min) showed a main effect of treatment between groups (F (3, 30) = 3.102; p = .04). ISO showed to have a greater effect on increasing C-peptide concentrations when compared to PLA (p = .04); however, no differences were found between the other amino acid treatments on C-peptide concentrations. Interactions were found at multiple time points for C-peptide concentrations: at 40 min ISO was greater than PLA (p = .01); 60 min ISO+LEU was greater than PLA (p = .04), and ISO was greater than PLA (p = .03); 120 min ISO+LEU was greater than PLA (p = .002), ISO greater than PLA (p = .02); 150

min ISO+LEU greater than PLA (p = .0005), ISO greater than PLA (p = .0003) (Figure 12).

The incremental change of plasma C-peptide concentrations during amino acid ingestion alone (0-30 min) showed no differences between treatments. An interaction was found showing a greater difference in C-peptide concentrations at 30 min: ISO+LEU greater than PLA (p = .004), ISO greater than PLA (p = .001), and LEU was greater than PLA (p = .01) (Figure 13).

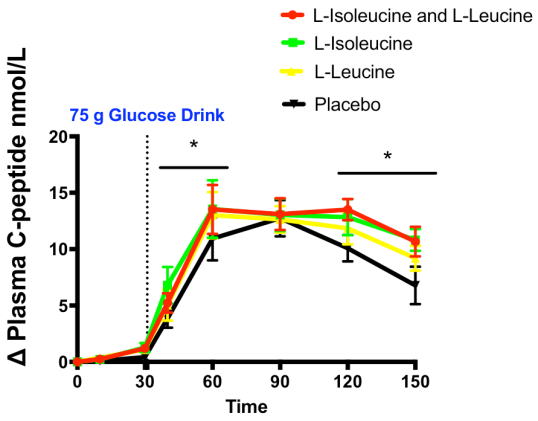

Figure 12: (Mean ± SEM) Δ Plasma C-peptide concentrations (nmol/l) comparing ISO+LEU, ISO, LEU, and PLA 0-150 min. (*) main effect treatment ISO > PLA (p = .04); differences on C-peptide concentrations: at 40 min ISO > PLA (p = .01); 60 min ISO+LEU > PLA (p = .04), and ISO > PLA (p = .03); 120 min ISO+LEU > PLA (p = .002), ISO > PLA (p = .02); 150 min ISO+LEU > PLA (p = .0005), ISO > PLA (p = .0003).

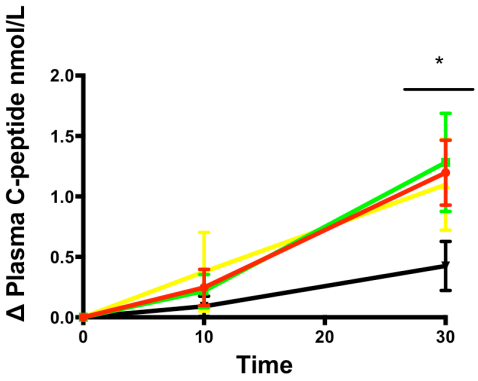

Figure 13: (Mean ± SEM) Δ Plasma C-peptide concentrations (nmol/l) (amino acid) comparing ISO+LEU, ISO, LEU, and PLA 0-30 min. (*) greater difference in C-peptide concentrations at 30 min: ISO+LEU > PLA (p = .004), ISO > PLA (p = .001), and LEU > PLA (p = .01).

Plasma Glucagon

The incremental change in plasma glucagon concentrations (0-150 min) showed no main effect differences between treatments (n = 10) (Figure 14). However, there were

interactions found between ISO+LEU a greater concentration than PLA (p = .003), ISO greater concentration than PLA (p = .01), which peaked at 30 min (~10 ng/l) followed by a robust decrease (~20 ng/l) after the subsequent glucose drink. The analysis of the incremental change of plasma glucagon concentration during amino acid ingestion (0-30 min) revealed a time x treatment interaction (F (6, 54) = 2.308; p = .04). Further Tukey post-hoc analysis showed interactions of greater glucagon concentrations at 30 min ISO+LEU greater than PLA (p = .002), ISO greater than PLA (p = .001), and LEU greater than PLA (p = .01) (Figure 15).

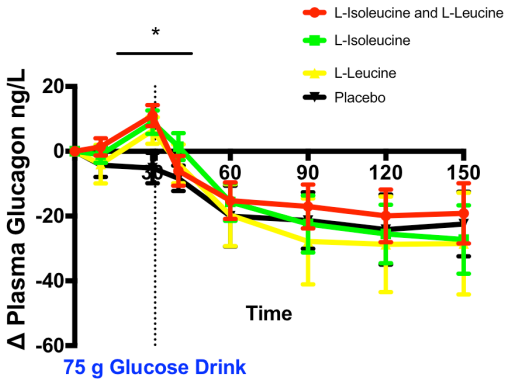

Figure 14: (Mean ± SEM) Δ Plasma glucagon concentrations (ng/l) comparing ISO+LEU, ISO, LEU, and PLA 0-150 min: (*) at 30 min, greater glucagon concentrations differences between ISO+LEU > PLA (p = .003), ISO > PLA (p = .01).

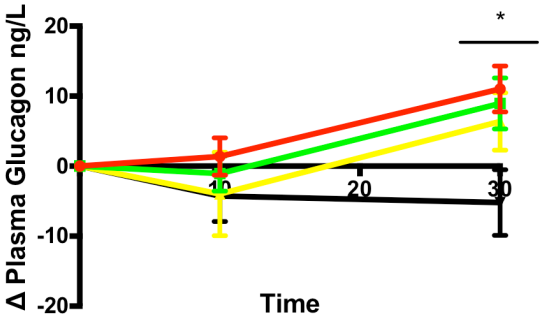

Figure 15: (Mean ± SEM) Δ Plasma glucagon concentrations (ng/l) comparing ISO+LEU, ISO, LEU, and PLA 0-30 min: (*) time x treatment interaction (p = .04), greater glucagon concentrations at 30 min ISO+LEU > PLA (p = .002), ISO > PLA (p = .001), and LEU > PLA (p = .01).

Plasma Glucagon-like peptide-1 (GLP-1)

Incremental change in plasma GLP-1_{Active} (pmol/l) concentrations (0-150 min) showed no main effect difference between treatments (n = 11). An interaction was found with greater GLP-1_{Active} concentrations: at 40 min ISO+LEU greater than PLA (p = .01) and ISO greater than PLA (p = .002); at 120 min ISO was greater than PLA (p = .01) (Figure 16).

The incremental change in plasma GLP-1_{Active} concentrations at the 0-30 min phase similarly showed no main treatment effect differences between groups on GLP-1_{Active} concentrations (Figure 17). However, there were interactions found where ISO+LEU was observed to be greater than PLA at 10 min (p = .03) and at 30 min (p = .02).

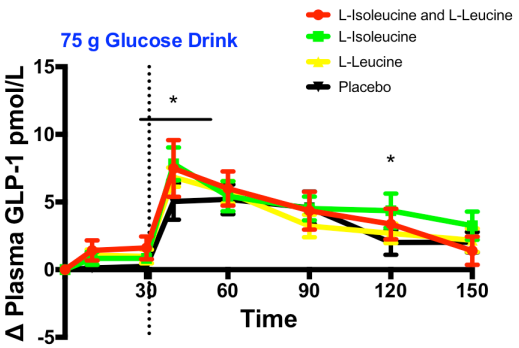

Figure 16: (Mean ± SEM) Δ Plasma GLP-1_{Active} concentrations (pmol/l) comparing ISO+LEU, ISO, LEU, and PLA 0-150 min: (*) at 40 min ISO+LEU > PLA (p = .01) and ISO > PLA (p = .002); at 120 min ISO > PLA (p = .01).

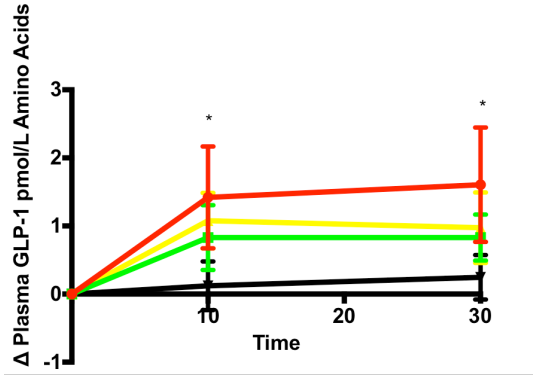

Figure 17: (Mean ± SEM) Δ Plasma GLP-1_{Active} concentrations (pmol/l) comparing ISO+LEU, ISO, LEU, and PLA 0-30 min: (*) differences between ISO+LEU > PLA observed at 10 min (p = .03) and 30 min (p = .02).

Plasma Glucose-dependent Insulinotropic peptide (GIP)

Analysis of the incremental change of plasma GIP_{Total} (pmol/l) concentrations (0-150 min) showed a main effect time x treatment interaction ($F(21, 210) = 1.993$; $p = .007$) and a treatment effect ($F(3, 30) = 4.062$; $p = .01$) (n = 11). A greater concentration of GIP_{Total} was observed during the ISO treatment as compared with LEU treatment ($p = .02$). Additionally, interactions were found at 40 min ISO greater than LEU ($p = .004$) and ISO greater than PLA ($p = .0001$); 60 min ISO greater than LEU ($p = .009$); 90 min ISO greater than LEU ($p = .009$); 120 min ISO+LEU greater than LEU ($p = < .0001$) and greater than PLA ($p = < .0001$), ISO greater than LEU ($p = .0008$) and greater than PLA ($p = .0003$); 150 min ISO+LEU greater than LEU ($p = .004$) and ISO greater than LEU ($p = .0005$) (Figure 18).

Incremental plasma GIP_{Total} concentrations (0-30 min) during amino acid ingestion resulted in a main effect time x treatment interaction ($F(6, 60) = 2.522$; $p = .03$) and a treatment effect ($F(3, 30) = 3.738$; $p = .02$). ISO showed to have a greater effect on stimulating GIP_{Total} concentrations than LEU ($p = .04$) and PLA ($p = .04$) (Figure 19). Moreover, interactions were found in GIP_{Total} concentrations at 10 min where ISO+LEU was greater than PLA ($p = .04$) and ISO greater than PLA ($p = .02$); at 30 min (~4 pmol/l) ISO greater than LEU ($p = .0002$) and (~2 pmol/l) ISO greater than PLA ($p = .0004$). Similar to the incremental change assessments, GIP_{Total} concentration iAUC analysis resulted in a treatment effect difference ($F(2.127, 21.27) = 3.473$; $p = .04$) and showed a higher concentration of GIP_{Total} in the treatment of ISO which was greater than LEU ($p = .03$) and greater than PLA ($p = .008$) (Figure 20).

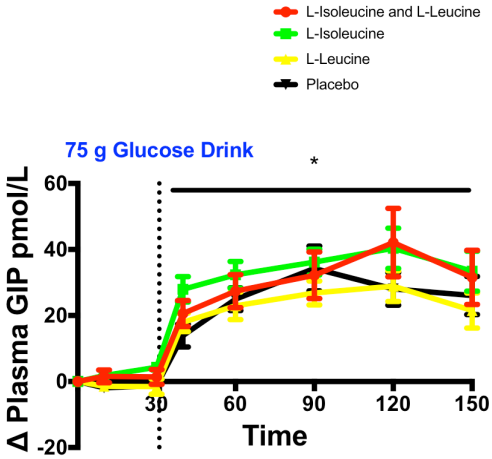

Figure 18: (Mean ± SEM) Δ Plasma GIP_{Total} concentrations (pmol/l) comparing ISO+LEU, ISO, LEU, and PLA 0-150 min (n = 11): (*) main effect differences of time x treatment (p = .007); treatment (p = .01); Tukey post hoc analysis ISO > LEU (p = .02); simple effect at 40 min ISO > LEU (p = .004) and ISO > PLA (p = .0001); 60 min ISO > LEU (p = .009); 90 min ISO > LEU (p = .009); 120 min ISO+LEU > LEU (p = <0.0001) and > PLA (p = <0.0001), ISO > LEU (p = .0008) and > PLA (p = .0003); 150 min ISO+LEU > LEU (p = .004) and ISO > LEU (p = .0005).

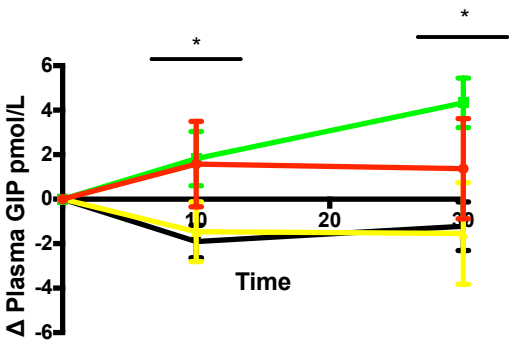

Figure 19: (Mean ± SEM) Δ Plasma GIP_{Total} concentrations (pmol/l) comparing ISO+LEU, ISO, LEU, and PLA 0-30 min: (*) main effect differences of treatment x time (p = .03) and treatment effect (p = .02, Tukey post hoc ISO > LEU (p = .04) and > PLA (p = .04). Simple effect differences at 10 min ISO+LEU > PLA (p = .04) and ISO > PLA (p = .02); at 30 min (~4 pmol/l) ISO > LEU (p = .0002) and (~2 pmol/l) ISO > PLA (p = .0004).

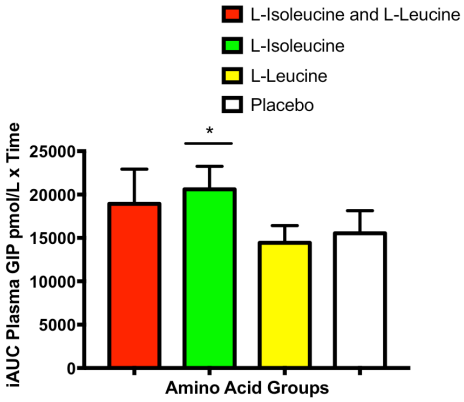

Figure 20: (Mean ± SEM) iAUC Plasma GIP_{Total} (pmol/l x time): (*) treatment effect (p = .04), Tukey post hoc ISO > LEU (p = .03) and ISO > PLA (p = .008).

MyFitnessPal Dietary Recall

Data collection and one-way ANOVA analysis of 3-day dietary intake (n = 10) showed no difference between treatments, macronutrient intake CHO (p = .30), FAT (p = .84), PRO (p = .37), and total Kcal intake (p = .42). The average kcal intake was found to be 1912 ± 319.4 kcal/day.

CHAPTER V

SUMMARY, DISCUSSION, CONCLUSION, AND RECOMMENDATION FOR FUTHER STUDIES

Statement of Problem

The purpose of this study was to determine the effects of a preingested therapeutic dose of L-Isoleucine (ISO) and L-Leucine (LEU) individually or equally combined, on the secretion of enteroendocrine hormone incretins GLP-1 and GIP, and subsequent C-peptide, and pancreatic hormones, insulin, and glucagon. Our overall aim was to elaborate further the relationship of these BCAA's and their influence on glucose uptake. Numerous studies have investigated both preingested and coingested whey protein with glucose or a mixed meal in both healthy and diseased populations and found a positive effect. With the addition of whey protein these investigations found a reduced post prandial blood glucose, increasing insulin concentrations, and a reduced glucagon secretions. Many of these investigators have eluded to whey protein directly and indirectly influencing GIP and GLP-1 which directly impact glycemic hormones insulin and glucagon. Whey protein indirectly assists glucose uptake by way of mediating insulin, GIP and GLP-1 secretion. It has been suggested that whey protein has a dual effect by stimulating greater enteroendocrine hormone GIP and GLP-1 secretion and a moderate inhibition of DPP-IV, which regulates the actions of GIP and GLP-1. The

augmentation of these incretins may indirectly reduce the blood glucose responses normally found from a glucose load or mixed meal (Gunnerud, Östman, & Björck, 2013; Jakubowicz & Froy, 2013; Jakubowicz et al., 2014; Marshall, 2004; Mignone, Wu, Horowitz, & Rayner, 2015; Nilsson et al., 2007; Nilsson et al., 2004; Salehi et al., 2012; Smithers, 2008). Additionally, other research has elected to observe a combination of amino acids found in whey (BCAA's) or specific mixture of amino acids that have been found to stimulate insulin secretion more so than others. These amino acids have been categorized as insulinogenic, and when consumed with glucose they synergistically stimulate a greater insulin response than when consumed alone. This amino acid mixture has shown to reduce blood glucose concentrations concurrent with a greater rise in insulin (Bernard et al., 2011; Iverson, Gannon, & Nuttall, 2013; Nilsson et al., 2007; Nilsson et al., 2004; Nishitani et al., 2005; van Loon et al., 2000; Wang et al., 2012; Yoshizawa, 2012; Zhang et al., 2011). Lastly, a few researchers have assessed the effect of an individual or pair of amino acids on glycemic hormones, as well as their related responses. These inquiries have found a similar glucose lowering effect as found in whey and amino acid mixtures (Gannon & Nuttall, 2010; Gannon, Nuttall, & Nuttall, 2002a, 2002b; Iverson et al., 2013; Iverson, Gannon, & Nuttall, 2014; Kalogeropoulou et al., 2008; Kalogeropoulou, Lave, Schweim, Gannon, & Nuttall, 2009; F. Q. Nuttall, Ngo, & Gannon, 2008; Nuttall, Schweim, & Gannon, 2008; Nuttall, Schweim, & Gannon, 2006). To our knowledge, our study is the first to observe the impact of a preingested model of BCAA family members L-Isoleucine and L-Leucine prior to a glucose drink on enteroendocrine hormones GLP-1 and GIP concentrations. Furthermore, investigating

the actions of these hormones and their subsequent effect on pancreatic derived hormones insulin, glucagon, which influence plasma glucose kinetics.

The Physiological Influence of L-Isoleucine, L-Leucine, and the Combination of L-Isoleucine and L-Leucine on Glycemic Responses

The purpose of this study was to investigate the effects of L-Leucine and L-Isoleucine on enterorendocrine derived incretin hormones GLP-1 and GIP, and the subsequent impact on C-peptide, and pancreatic derived insulin and glucagon hormones that regulate blood glucose. Our goal was to explore further the relationship of BCAA's and their influence on glucose metabolism. Our hypothesis was that the ingestion of BCAA family members, L-Isoleucine and L-Leucine at dose of 0.3 g/kg/LBM, in combination (50:50) and independently, prior to a glucose load (75 g) will improve glucose tolerance. Our hypotheses will be supported if we observe a reduction in the area of blood glucose compared to control with the ingestion of L-Isoleucine and L-Leucine. Additionally, we will see a change in blood glucose with no effect on incretin responses ($GIP_{Total,}$ $GLP\text{-}1_{Active}$).

Plasma Glucose

Due to differences in study designs between previous research and our protocol of amino acid and glucose administration (i.e. preingestion vs. coingestion; oral ingestion vs. IV), dose of amino acid, and use of an individual amino acid or a mixture of amino acids. It is difficult to compare our results fairly to the outcomes of other research studies. With this limitation, we separated our analysis into 0-30 min amino acid

ingestion only phase and 30-150 min post-glucose drink phase. Comparatively, our glucose response observations were slightly different than previous research on the L-Isoleucine in that we did not see a meaningful area reduction; although, disparate outcomes may be a result of the differing protocols. Nuttall, Schweim, & Gannon (2008) co-administered 25 g of glucose with 1 mmol/kg LBM (~7 g of ISO) of L-Isoleucine (n = 9). In the ISO ingestion group only, they showed an 8% reduction in blood glucose 84 to 77 mg/dl (4.66 to 4.28 mmol/l) at baseline to completion of treatment at 150 min. In the 0-30 min measurement (amino acid only) of our ISO treatment we showed a similar reduction response of glucose. However, the magnitude of reduction (-4.68 mg/dl; -0.26 mmol/l) was not as robust, which may be a factor of the time phase observed and possibly the differing sample of participants. Our participants were to maintain an inactive, and non exercising lifestyle to enter our study. It is unknown if physical activity or exercise was controlled for or assessed by Nuttall, Schweim, & Gannon (2008). It is unknown if physical activity or exercise was controlled for or assessed by Nuttall, Schweim, & Gannon (2008). It has been well documented that acute exercise has a positive effect on insulin sensitivity (Goodyear & Kahn, 1998), which may be a confounding factor influencing ISO mediated effect on glucose uptake (Nuttall, Schweim, & Gannon, 2008).

During ISO treatment, glucose concentration rose from a baseline 89.5 mg/dl (4.97 mmol/l) to ~104 mg/dl (5.8 mmol/l) at 40 min (10 min post glucose ingestion), and subsequently peaked at 60 min (30 min post glucose drink) at ~134 mg/dl (~7.5 mmol/l). Glucose then fell to ~106 mg/dl (5.9 mmol/l) at the 150 min end point of the

treatment. This final time point was ~18% higher than baseline levels at the beginning of the treatment before amino acid ingestion. Compared to PLA, the ISO treatment reduced plasma glucose at the 60 min (p = .05) and 90 min (p = .008) time point. This response suggests that the plasma glucose peak response was reduced at the 60 and 90 min time points, although this "*blunted effect*" may have influenced a longer duration of plasma glucose above baseline beyond that found at the end time point of 150 min. We may have observed blood glucose fall back to baseline levels if observation time was extended (180 min). However, the PLA group was found to be similar to the amino acid treatment groups in that it did not return to baseline after 150 min. This suggests that the 2-hr post-glucose drink observational period may not have been enough time to observe glucose return back to baseline with this sample population.

Similar to ISO, our observation of LEU is slightly different than previous research examining the effect of LEU on glucose metabolism. Kalogeropoulou et al. (2008) using the same protocol for LEU ingestion as Nuttall, Schweim, & Gannon (2008) administering 1 mmol/kg LBM of LEU with 25 g of glucose, found that a modest amount of glucose coingested with LEU had a synergistic effect of increasing insulin concentrations and therefore a reduction in blood glucose (Kalogeropoulou et al., 2008). Our observations of the LEU treatment showed to be similar to the ISO treatment by reducing plasma glucose and showed to be significantly different than PLA (p = .02). Still, we did not find a significant difference from the other treatments. Additionally, after further analysis we found that LEU showed no difference between

other treatments at any time point other than the near significant 60 min (p = .06) time point.

Comparatively, Kalogeropoulou et al. (2008) had similar sample population characteristics as we did (51 vs. 47 kg of LBM; fasting blood glucose was 84 ± 2.3 mg/dl [4.7 ± 0.1 mmol/l] vs. 89.5 ± 6.0 mg/dl [4.97 ± 0.3 mmol/l]). However, their activity level was not reported, nor if their participants were asked to refrain from exercise before each trial. In the 0-30 LEU phase, prior to the glucose drink, we saw a total reduction in glucose of 3.6 mg/dl (0.2 mmol/l) and a significant difference from PLA at the 30 min time point (p = .0001); however, a 3.6 mg/dl reduction in blood glucose may not be considered physiologically meaningful. Moreover, Kalogeropoulou et al. (2008) also reported no significant or meaningful glucose changes with LEU when ingested alone compared to their control treatment of water. In respect to the differences in the glucose and LEU dose and ingestion protocols between studies, there were comparable responses. Kalogeropoulou et al. (2008) observed a blunted peak at 50 min from LEU (114 mg/dl; 7.3 mmol/l) in comparison to their control (131 mg/dl; 6.3 mmol/l). This was similar to our observation that blood glucose was reduced at 60 min by the LEU treatment (134 mg/dl; 7.4 mmol/l) compared to PLA (150 mg/dl; 8.3 mmol/l). Still, we did not see a significant difference between the other amino acid treatments. In both Nuttall, Schweim, & Gannon (2008), and Kalogeropoulou et al. (2008), participants returned to baseline glucose values after 150 min while our population in all treatments (including PLA) returned to 106 mg/dl (5.8 mmol/l) after 150 min, which was 15.6% higher than original baseline values. Kalogeropoulou et al's

(2008) analysis of LEU and glucose iAUC showed a 50% reduction in the area of glucose (p = .019) in comparison to our data where we showed no iAUC differences between treatments (p = .08). Again, taking into consideration the differences in protocols and doses, it seems as though co-ingestion of LEU with a 25 g dose of glucose may elicit a stronger effect at blunting the blood glucose response than does pre-ingestion of LEU prior to a glucose drink (Kalogeropoulou et al., 2008).

In our analysis of the ISO+LEU treatment on glucose responses, we did find a treatment effect via reduction of blood glucose compared to PLA (p = .02), although this effect was similar to the other amino acid treatments. During the 0-30 min phase prior to glucose drink, the combination of ISO+LEU ingestion was not significantly different than the PLA. However, we did observe ISO+LEU treatment reduce blood glucose ~ 3 mg/dl (.18 mmol/l) more so than PLA (p = .0007) at 30 min similar to LEU. During the 30-150 min phase of this treatment, the response in glucose reduction was similar to ISO and LEU treatments; a reduction ~20 mg/dl (1.3 mmol/l) in glucose at 60 min (p = .008) and 90 min (p = .02) compared to PLA. Area analysis showed no differences between treatments of amino acids ISO, LEU, and ISO+LEU from PLA. We found time point interactions (60 and 90 min) where glucose was reduced by all amino acid treatments compared to PLA. However, the amino acid treatments at the chosen doses were not strong enough to elicit a reduction in glucose area compared to PLA.

Plasma Insulin

The insulin response during the ISO treatment in the 0-30 min prior to the glucose drink phase resulted in a change from a baseline mean of ~97 to a 30 min peak of 137 pmol/l (13.5 to 19.1 μU/ml), which was roughly a 41% increase from baseline. Conversely, Nuttall, Schweim, & Gannon (2008) showed no meaningful insulin responses from ISO compared to their control treatment of water ingestion. This insulin response may be a dose related due to the mean dose if ISO for their study was 7.4 g (1 mmol/kg LBM) compared to our study design with a mean dose of 14.6 g. In analyzing the baseline insulin concentration after glucose ingestion (30-150 min), at 30 min prior to the glucose drink it was ~136 pmol/l (18.9 μU/ml) with no difference found between treatments. However, 30 min after 75 g glucose ingestion (60 min time point) during the ISO treatment, the concentration of insulin rose to a peak value of ~869 pmol/l (~121 μU/ml); comparatively, Nuttall, Schweim, & Gannon (2008), showed a peak of ~400 pmol/l (70 μU/ml) at 45 min post coingestion.

Our data showed that a preingestion of ISO at 0.3g/kg/LBM induces more than a 2-fold difference in insulin concentration than a co-ingested 25 g glucose with ~7.4 g of ISO. This higher insulin response found in our data may again be explained by the differences in populations; our population self reported as inactive and our mean percent body fat was 34%. Higher levels of body fat have shown to be a large factor to influence insulin resistance (Boden, Chen, DeSantis, & Kendrick, 1993). Additionally, Nuttall, Schweim, & Gannon (2008) used a differing tool of body compositional assessment via bioelectrical impedance (BIA) for compositional measures. BIA is

known to have some limitations through error in its assessments; Kushner (1992) found a standard estimation of error (SEE) for fat free mass to range from ~2-4 kg (Kushner, 1992); this possible error in fat free mass assessments may then influence the amino acid dose, and therefore the response of the treatment. Nuttall, Schweim, & Gannon (2008) and Kalogeropoulou et al. (2008) investigated a population with greater LBM and lesser body fat percent than our sample population (56.5 kg vs. 48.6 kg; 30% vs. 34% respectively) (Nuttall, Schweim, & Gannon, 2008). Furthermore, it is unknown how physically active their population was, and compared to our inactive participants, they may have been more insulin sensitive if physically active. Further insulin multi-comparisons using area iAUC analysis of our data showed no difference between ISO and the other treatments (0-150) min.

During the 0-30 min (amino acid only) phase of LEU ingestion, we did not find a significant main effect of treatment compared to PLA (p = .13) on insulin response, nor any interactions at differing time points compared to other treatments. In contrast, Kalogeropoulou et al. (2008) found a significant difference in the insulin response area with the ingestion of LEU compared to their control treatment of water (p = .0001). Our observations of insulin values during the 30-150 min post glucose phase for LEU increased from ~42 pmol/l at 30 min and peaked at ~860 pmol/l at 60 min; comparatively, Kalogeropoulou et al. (2008) showed an insulin peak of 186 pmol/l (31 μU/ml) at 50 min after glucose drink (control) and when LEU was co-ingested with glucose they observed a peak of 318 pmol/l (53 μU/ml) at 40 min. This great discrepancy between our observed insulin concentration peaks and Kalogeropoulou et

al. (2008) may be explained by: (a) the dose of glucose (25 g vs. 75 g); (b) dose of LEU (7.4 g vs. 14.6 g); although, our assessment did not show a main effect difference between amino acid treatments, nor interactions at any time point in the 0-150 min on insulin concentrations; (c) day to day and between participant insulin concentration variations (Olefsky & Reaven, 1974).

The insulin action of the combination ISO+LEU treatment was similar to that of Nilsson et al. (2007), with the notable differences being: the variation of doses used, the ratio of ISO to LEU (1:1 vs. 1:2), and the possible additive factor of L-Valine. Participants showed a peak increase in insulin concentration of 400 pmol/l, 30 min after co-ingestion of 25 g of glucose and 4.4 g of BCAA's. Additionally, in comparison to ISO data presented by Nuttall, Schweim, & Gannon (2008), the ingestion of ISO and 25 g of glucose-stimulated a peak increase in insulin concentrations of ~400 pmol/l at ~40 min; Kalogeropoulou et al. (2008) showed a similar peak increase of insulin (~300 pmol/l at 40 min) when LEU was combined with 25 g of glucose (Kalogeropoulou et al., 2008; Nilsson et al., 2007; Nuttall, Schweim, & Gannon, 2008). Our combination of ISO+LEU at average intake of 14.6 g, without the third BCAA L-Valine, showed a meaningful increase in insulin without an additive effect of glucose during the 0-30 min phase, reflecting a mean increase of 70 pmol/l above baseline compared to Nuttall, Schweim, & Gannon (2008) and Kalogeropoulou et al. (2008), who both suggested these amino acids did not have an impact on insulin when consumed alone. Moreover, we observed a substantially higher concentration of insulin (~970 pmol/l) at 60 min (30 min post 75 g glucose drink). This figure is nearly 3 fold greater than both Nuttall,

Schweim, & Gannon (2008) and Kalogeropoulou, et al. (2008) reported. Based on our data, this may suggest a possible dose-effect of the ISO and LEU treatment due to our dose being ~50% greater (7.4 vs. 14.6 g), and the dose of glucose drink used in our study was 3-fold higher than the 25 g glucose drinks used in these study designs (Gannon & Nuttall, 2010; Kalogeropoulou et al., 2008; Nilsson et al., 2007; Nuttall, Schweim, & Gannon, 2008)

Plasma C-peptide

Our incremental C-peptide analysis showed during the 0-30 min prior to glucose phase, C-peptide concentrations in response to ISO treatment minimally increased ~1.3 nmol/l from a mean baseline of 4.0 nmol/l; however, no differences were found between groups. At 30 min post amino acid ingestion, ISO stimulated a greater (p = .001) C-peptide secretion compared to PLA, suggesting that ISO does indeed independently stimulate C-peptide and therefore insulin secretion. Following consumption of the glucose drink, our assessment of the 30-150 min post-glucose phase showed a peak C-peptide concentration of ~18 nmol/l at 60 min, with ISO having a greater impact on increasing C-peptide secretion than PLA (p = .04). Subsequent iAUC analysis of C-peptide comparing treatments found no differences (p = .1) between treatments. In summarization, our incremental change analysis demonstrates that ISO individually influences C-peptide more than LEU or PLA. However, based on our dual analysis to support differences between treatments we did not find a difference in iAUC to support a difference between treatments. This suggests that ISO may influence a greater insulin secretion via C-peptide than LEU or PLA. Furthermore, considering the

physiological relationship in which C-peptide is cosecreted from the ß-cells of the pancreas in an equimolar ratio with insulin (liver extracts 50% of insulin), this may suggest that ISO stimulates insulin secretion more so than LEU. It has been suggested that peripheral C-peptide levels may more accurately reflect pancreatic insulin secretion rates than measurements of peripheral insulin levels, and is now suggested to be a biomarker for insulin secretion when assessed during steady state time periods. However, due to the administration of both ISO and the glucose drink stimulation, it is more likely that a non steady state condition was observed in our participants. During non steady state conditions, the C-peptide to insulin molar ratio is influenced by hepatic insulin extraction, change of insulin secretion and the clearance rate of C-peptide (K Polonsky et al., 1986; Polonsky et al., 1988; KS Polonsky et al., 1986). Lastly, based on our data analysis and investigation, ISO appears to stimulate greater concentrations of C-peptide over the 150 min compared to PLA, and therefore possibly influence insulin secretion more so than LEU.

In our incremental analysis of LEU treatment on C-peptide concentration, we found LEU's effect on C-peptide to be secondary to its effect on the hormone insulin. The 0-30 min prior to glucose drink phase showed an increase at the 30 min time point, where it minimally climbed ~2 nmol/l from baseline (~4 nmol/l) with LEU ingestion, while PLA showed no real change. Additionally, there was no difference between amino acid treatments, in that they all influenced C-peptide secretion similarly in the 0-30 min phase. During the 30-150 min post glucose drink phase, LEU did not show any significant area differences compared to the PLA treatment. The lack of C-peptide

response difference compared to PLA implies that when LEU is preingested before a glucose drink, it seems to have a minimal effect on stimulating C-peptide and therefore insulin secretion.

The analysis of change of C-peptide concentration from baseline during the ISO+LEU treatment showed no difference when compared to PLA (p = .07). After assessing the 0-30 min prior to glucose drink phase, we again found no treatment differences between amino acid groups. Similar to the other amino acid treatments we found an interaction by the ISO+LEU treatment during the 0-30 min phase that increased C-peptide concentrations at the 30 min time point (p = .004) compared to PLA. During the 30-150 min phase of the treatment, we detected similar level changes as found in the ISO treatment. We observed an increase at 60 min (p = .04) and 90 min (p = .002), with a peak C-peptide concentration of ~18 nmol/l at 60 min. After our iAUC analysis, we found that ISO+LEU did not show a difference in C-peptide concentration compared to PLA. However, when all areas were compared we found no differences between treatments.

It appears that the ingestion of ISO seems to influence an insulin secretion action in the form of greater concentration of proinsulin C-peptide in comparison to LEU and PLA and in summary, our data suggest that ISO when consumed individually may stimulate C-peptide secretion and therefore insulin more so than LEU alone.

Plasma Glucagon

The glucagon response to ISO in the 0-30 min prior to glucose phase resulted in a small increase from baseline of 54 to 61.9 ng/l. Nuttall, Schweim, & Gannon (2008)

showed an identical response in their ISO, only ingestion in the 0-30 min phase showed a slight increase from baseline ~53 to 60 ng/l; followed by a return to baseline at 150 min. After the glucose drink, we observed glucagon concentrations fall 30 ng/l, which is considered a typical response when insulin levels rise (Maruyama, Hisatomi, Orci, Grodsky, & Unger, 1984). No other differences were found between ISO and other treatments during the 0-150 phase in glucagon concentrations. Interestingly, similar to our multiplexing analysis, Nuttall, Schweim, & Gannon (2008), showed robust variability in participant glucagon analysis via radioimmunoassay Nuttall, Schweim, & Gannon, 2008).

The effect LEU had on glucagon concentrations were similar to ISO. In the 0-30 min prior to glucose drink phase of LEU ingestion only, glucagon increased an average of ~6 ng/l (at 30 min) from a baseline value of 54 ng/l. This change was found to be significantly different from PLA (p = .01). However, it becomes debatable how physiologically meaningful and impactful this transient increase in glucagon is during this phase when we observed a slight reduction in blood glucose mixed with a slight rise in insulin. Our glucagon data seemed to be consistent with Kalogeropoulou et al. (2008) and Nuttall, Schweim, & Gannon (2008), which showed both LEU, ISO stimulated glucagon secretion in a differing magnitude. LEU seemed to have a greater stimulate greater glucagon secretion than did ISO when consumed individually. However, during our observed amino acid only treatment time period, we did not see a meaningful rise in glucagon, and we observed was no difference between amino acid

treatments at 30 min (p = .12) (Kalogeropoulou et al., 2008; Nuttall, Schweim, & Gannon, 2008)

It appears that LEU had a modest effect on glucagon secretion, which may be an effect of time, and suggesting that a greater concentration may have been observed if the treatment time included a longer duration prior to glucose drink. However, Kalogeropoulou et al. (2008) observed values in their normal healthy population peak at roughly ~70 ng/l before 30 min, and maintaining this for up to > 90 min. This suggests that LEU had no meaningful effect on stimulating glucagon in response to the dose used. The effect of LEU on glucagon in the 30-150 min phase was similar to other amino acid treatments, ISO+LEU and ISO, considering that after 30 min it reduced ~27 ng/l from 30 min time point prior to the glucose drink of 54 ng/l. This response, as previously explained, is considered normal in healthy, non diseased population in that insulin is an antagonistic hormone to the secretion of glucagon (Kalogeropoulou et al., 2008).

Our analysis of glucagon concentrations in relation to the ISO+LEU treatment showed the similar response to both ISO and LEU treatments in that no treatment differences were found. During the 0-30 min prior to glucose drink phase, ISO+LEU peaked (p = .0002) at 30 min with the peak concentration of glucagon increasing to 65 ng/l. The following 30-150 min measurements following the post-glucose drink phase were similar to all other treatments where glucagon fell ~20 ng/l below baseline values, with a concurrent rise in insulin concentration. The glucagon response to ISO+LEU was statistically similar to the other treatments, which is comparable to both Nuttall,

Schweim, & Gannon (2008) and Kalogeropoulou et al. (2008) who both observed similar glucagon responses with different doses of ISO or LEU with glucose (Gannon & Nuttall, 2010; Kalogeropoulou et al., 2008; Nuttall, Schweim, & Gannon, 2008).

Plasma GLP-1

GLP-1 concentration response to ISO in the 0-30 min prior to the glucose drink phase was found to increase minimally (1.34 to 2.17 pmol/l). No significant treatment effect was found between ISO and the other treatments (p = .19) on GLP-1 concentration. It has been suggested that roughly 50-60% of BCAA's may be directly extracted by splanchnic tissues for metabolic use while the other portion is delivered to the systemic system. However, amino acid absorption in the gut may be dependent on a) nutritional status, b) the essential amino acid composition of a meal, c) the high level of protein synthesis of the gut, and lastly d) transit time. These factors may influence the interaction with L-cells of the lower intestine and colon where GLP-1 is synthesized. The transit theory was also suggested by Lindgren et al. (2014) as an effect of interaction time more so than treatment effect. Lastly, a difference in study design ingestion protocols may partially explain why GLP-1 concentrations were unaffected; a synergistic effect of both amino acids ingested with glucose may have stimulated a differing response. (Lindgren et al., 2014; Ten Have, Engelen, Luiking, & Deutz, 2007).

The impact of the LEU on GLP-1 showed no effect or meaningful change during the 0-30 min prior to glucose drink phase. To our knowledge, we have not come across a study that has looked at individual amino acid ingestion on incretin concentrations;

though some have observed the effect of multiple amino acids on incretin responses. Lindgren et al. (2015) observed the impact of oral (intradoudenal) and IV induced amino acid mixtures ~7 g on GIP, GLP-1, glucagon, insulin, and blood glucose. Similar to our outcomes, they did not see an effect of the amino acid mixture treatment on intact or total GLP-1 responses. Moreover, it does not seem plausible that the differences of dose and administration between previous studies and ours may have affected the outcomes. Lindgren et al. (2015) used a dose 6.5 g (our dose 14.6 g) of the full spectrum of 19 amino acids, via intradoudenal administration which maintained interaction of the amino acids with GI tract (O. Lindgren et al., 2015)

Interestingly, our data was found to be in contrast to previous research using an intestinal cell culture (NCI-H716 human) model, and showed a dramatic 474% increase in GLP-1 from a 3% solution of LEU (Chen & Reimer, 2009). It seems as though in a culture model, LEU is able to stimulate a robust GLP-1 secretion. Based on our data, the ingested form of LEU has no effect on GLP-1 compared to cell culture models. Furthermore, the process becomes more convoluted with the ingestion of whey, in that other factors such as dipeptides found in whey may inhibit DPP-IV (Nongonierma & FitzGerald, 2013), or may stimulate GLP-1 secretion (Matsumura, Miki, Jhomori, Gonoi, & Seino, 2005) compared to LEU individually.

Our data show that LEU had no effect on GLP-1 secretion when it is ingested in a higher dose, suggesting that it may be another amino acid, mixture of amino acids, dipeptides found in whey, or a substrate action not yet discovered that regulates GLP-1 secretion and therefore an increases the concentration. Furthermore, our GLP-1

analysis of the impact of LEU on the post glucose drink phase 30-150 min, showed no differences either; further supporting that the amino acid LEU is not responsible for the stimulus and secretion of GLP-1.

The assessment of GLP-1 concentration in response to the ISO+LEU treatment was similar to LEU and ISO treatments, and showed no significant effect. During the analysis of the 0-30 min prior to glucose drink phase we observed that ISO+LEU stimulated GLP-1 concentration more so than PLA at 10 min (p = .03) and 30 min (p = .02), while the other treatments ISO and LEU individually did not show a difference from PLA. The mean rise in the concentration of GLP-1 during the 0-30 min phase was roughly 1.5 pmol/l above the baseline value of 1.95 pmol/l, or roughly a 57% increase in concentration. However, it is unknown how physiologically meaningful a ~2 pmol/l rise in GLP-1 is above baseline if we did not observe a meaningful difference in insulin concentrations or a reduction in blood glucose area. During the 30-150 min post glucose phase, the GLP-1 concentration was found different than PLA at 30 min (p = .01) and peaked at ~10 pmol/l at 40 min (10 min after glucose intake). However, further iAUC analysis showed no concentration differences between treatments. This outcome was similar to Nilsson et al. (2007), who observed the effect of 4.4 g of BCAA's and 25 g of glucose on GLP-1 concentrations, showing no differences (Nilsson et al., 2007). With distinct differences in treatment protocols and doses between our study and Nilsson et al. (2007), based on our data analysis it appears that the physiological GLP-1 response to ISO+LEU in combination, increases only slightly. However, it is uncertain how meaningful an increase of GLP-1 of that magnitude might

be during the amino acid only phase, due to our observation that the incretin stimulated hormones, insulin and glucagon, were not found to be different than PLA. This further suggests that ISO and LEU ingestion in a higher combined dose may not explain the increased GLP-1 response found in trials using whey in vitro. Which further supports that either another amino acid, group of amino acids, or dipeptides found in whey may stimulate GLP-1 secretion and DPP-IV inhibition (Matsumura et al., 2005; Nongonierma & FitzGerald, 2013).

Plasma GIP

We observed an incremental change of GIP from baseline of 7.5 pmol/l to 11.9 pmol/l, or roughly 37%, during the ISO treatment in the 0-30 min prior to glucose drink. This was a significantly greater increase compared to both PLA (p = .04) and LEU (p = .04) treatments. Based on the 30-150 min phase, GIP did seem to be influenced by ISO ingestion; we detected a treatment difference between ISO and PLA (p = .02) with further iAUC analysis showing that ISO stimulated a greater area of GIP throughout the 0-150 min of the treatment, more so than LEU (p = .03) and PLA (p = .008).

The analysis of GIP concentrations during the LEU treatment's 0-30 min phase showed no effect compared to the other treatments. Additionally, LEU showed to have no effect on GIP during the 30-150 min period with glucose compared to the other treatments. Similarly, Lindgren et al. (2015) found that their amino acid ingestion did induce a GIP response, which peaked at ~20 pmol/l at 30 min into the trial. However, their protocol used a multiple amino acid mixture in the solution, which suggests that

other amino acids, independently or in combination, may have induced the GIP response (Lindgren et al., 2015)

Furthermore, we were unable to detect any differences between ISO+LEU on GIP secretion. Considering that LEU, when ingested alone, had no real impact on GIP secretion when compared to PLA, it is plausible that the combination treatment of ISO+LEU may have had no effect due to LEU in the mixture. Similarly, the ratio of ISO to LEU was 1:1 in the combined amino acid treatment mixture. This amount equates to half the dose of each amino acid compared to the other treatments in our study. It could be suggested that ISO does influence a GIP response more so than LEU regardless of dose; however, there was no effect observed by the treatments LEU, ISO+LEU.

The Incretin Effect

The incretin responses between GIP and GLP-1 in the ISO+LEU treatment and glucose ingestion were much different in that ISO had no meaningful effect on GLP-1 secretion. During the 0-30 min phase, GLP-1 concentrations rose from baseline by ~2 pmol/l at 30 min (pre-glucose), peaking at 40 min (10 min post glucose drink) at ~9 pmol/l, and were also found to be significantly different than PLA at this time point. However, the iAUC area of GLP-1 in response to ISO was not found to be different than any other treatment. This may suggest that ISO treatment alone lacks the ability to stimulate GLP-1 secretion in comparison to whey protein, which seemingly offers other factors that have shown to both stimulate GLP-1 secretion and inhibit DPP-IV activity.

For further analysis of the impact of ISO treatment on GIP and glucose, we used Pearson's "r" to assess correlative relationships between GIP and glucose concentration. We found a nonsignificant and weak relationship (r = .44; data not shown) between GIP and glucose. Consistent with previous reports and known to be a normal physiological response, we found a very strong relationship between insulin and C-peptide (Figure 21: r = .88, r^2 = .76, *p* = .0004). However, in the analysis of the ISO treatment, we found a nonsignificant and weak relationship between insulin and GIP concentrations (Figure 22: r = .14; r^2 = .02; *p* = ns). This outcome may possibly highlight a disconnect between ISO driven GIP and subsequent insulin secretion compared to glucose driven GIP and subsequent insulin secretion. Further research needs to examine the independent relationships between amino acids and glucose on GIP and their impact on insulinogenic responses.

This GIP response suggests that during the ISO treatment, as plasma glucose increased, the concentration of GIP seemed to be unaffected. Similarly, due to the poor stimulating effects of GIP on the pancreatic release of insulin and the nonsignificant differences found between insulin in all treatment groups, it could be suggested that other amino acid driven mechanisms may have influenced a greater uptake of glucose during the peak time points that we observed. In other research, the relationship between GIP and insulin concentrations has shown to have a strong relationship (r = .89) in a healthy population with administering an OGTT test with 50 g of glucose (Lauritsen & Moody, 1980). It has been widely accepted and acknowledged that glucose driven GIP secretion does indeed stimulate ß-cell receptor secretion of C-

peptide and therefore insulin release (Baggio & Drucker, 2007; Edholm et al., 2010; Gautier, Choukem, & Girard, 2008; Nauck et al., 1986).

Our data was contrary to the associative relationships found by Wachters-Hagedoorn et al. (2006), who noted a strong positive correlation between GIP and the rate of glucose appearance ($r = .73$; $p = .01$), as well as between insulin rate of appearance ($r = .68$, $p = .01$). However, this study reported that the treatment meals contained ~50 g of plant-based carbohydrates of differing digestion rates compared to the 75 g of dextrose we consistently used. Furthermore, they did not report a meaningful amount of protein or amino acids ingested during meal. Lastly, this same group reported a significant, yet weak, correlation between GLP-1 concentrations and the rate of appearance of glucose ($r = .32$, $p = .01$), whereas we did see a rise from glucose ingestion, yet no effect was found from the amino acid treatments used during each trial (Seino, Fukushima, & Yabe, 2010; Wachters-Hagedoorn et al., 2006). It appears that in conjunction with Nilsson et al., (2007), our analysis of the incretin response to ISO and LEU ingestion seemed to have a minimal effect on stimulating GLP-1 secretion; although, ISO individually showed to drive GIP concentrations similar to the response seen by Lindgren et al., (2015) who used an amino acid mixture (~7 g), which stimulated a similar GIP response (10 pmol/l) compared to our ISO treatment (~15 g) effect on GIP (4 pmol/l) (Lindgren et al., 2015; Nilsson et al., 2007).

At this time, we are unable to explain the peak rise of GIP concentrations at the 120 min time point in the ISO+LEU treatment. This response may be related to a delayed gastric emptying factor when the combination of these amino acids were ingested prior to the glucose drink. However, that seems less likely due to the

homogenous mass in all amino acid treatment trials. It may be plausible that there are K-cells located in the lower portion of the GI tract, and due to its distal location, which could explain the late GIP response seen in this treatment. Currently, we are also uncertain of a physiological explanation for the late peak in GIP, and how this may relate to glycemic responses and the population, or whether it may be beneficial or even consequential to glucose metabolism. Comparatively, the ISO+LEU treatment trial did not show an effect on the area of GIP concentrations compared to the other treatments. This further highlights the impact of ISO on GIP due to the lessened dose of amino acid (50:50) in the ISO+LEU treatment and the lack of effect that LEU alone had on GIP changes. This may suggest that LEU inhibited the impact of ISO on GIP responses when it was consumed with LEU.

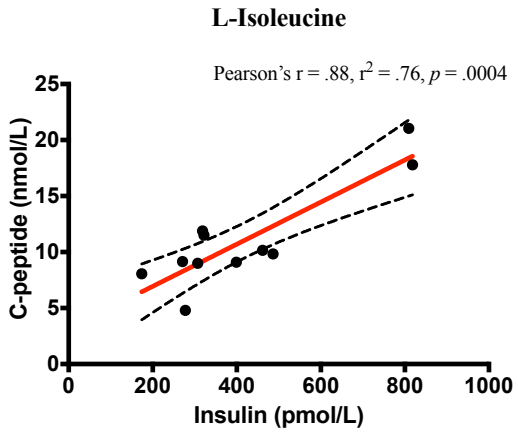

Figure 21: The association of the increase of plasma insulin and C-peptide in response to ISO treatment (r = .88).

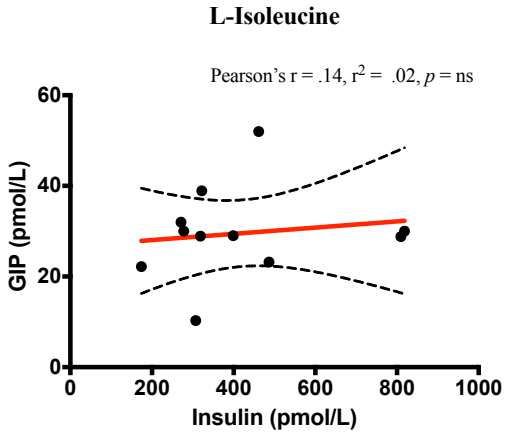

Figure 22: The association of the increase in plasma GIP and insulin in response to ISO treatment (r = .14).

Plasma Glucose Peak Reduction

It appears that the pre-ingestion of either ISO+LEU, ISO, or LEU before a glucose load does indeed reduce glucose peak responses. Overall, these amino acids impacted the 60 and 90 min time points, with the greatest impact seen at the 60 min time point compared to the peak point in the concentration of glucose (150 mg/dl; 8.3 mmol/l) in the PLA group found at 60 min. Interestingly, in our sample population, we observed glucose levels elevated above baseline levels until the cessation of the trial (~107 mg/dl; 5.94 mmol/l). Similarly, insulin levels at 150 min of the amino acid trials were elevated above baseline by ~370-460 pmol/l, and ~330 pmol/l above baseline in the PLA trial. This glucose response coincides with the decreased concentration of glucagon in each trial at 150 min of -20 ng/l below baseline levels. Due to the lowered glucagon concentrations, it could be assumed that the hepatic gluconeogenic properties were minimized, therefore the higher concentration levels of glucose and insulin found in plasma at the 150 min time point may be an effect of reduced glucose uptake through a reduced insulin sensitivity. It has been suggested that protein intake or amino acid intake will stimulate $mTORC_{1,}$ a major protein related to cell signaling for protein synthesis. During normal feeding when insulin is secreted it binds to the subsequent insulin receptor (IR) and then sets of a chain of signaling via IRS-1,2 to PI3K, and then regulating GLUT-4 translocation to uptake glucose into the cell. However, in a negative feedback manner during amino acid uptake into skeletal muscle, these amino acids may also directly or indirectly stimulate $mTORC_1$ phosphorylation and therefore downstream associated protein synthetic pathway enzyme S6K1. S6K1 plays an

impactful role in a negative feedback loop to inhibit IRS-1,2 activity. The inhibition of IRS-1,2 decreases insulin sensitivity and therefore a reduces glucose uptake through GLUT-4 translocation signaling restriction (Figure 23) (Rivas et al., 2009). It is plausible, and in respect to gastric emptying rates, an ingestion of a larger amounts of amino acid via ISO and LEU followed by 75 g glucose drink led to a subtle increase, which was followed by a substantial increase in insulin that remained elevated above baseline values 2-hours after ingestion. A response to this reduced insulin sensitivity action, may partially explain why the blood glucose levels remained elevated above basal values in conjunction with insulin at cessation of the treatment.

An additional suggestion to explain the increased glucose concentrations is that the amino acid ingestion blunted the peak portion of the area, yet it did not influence a greater disposal of glucose by increased tissue uptake. It merely blunted the peak response and "*stretched*" out the time portion of disposal. However, PLA experienced the same outcome, and insulin area levels were not different among the treatments, so this does not seem plausible. Additionally, it could be possible that the inert ingredients given to the participant via stevia had a similar action compared to the amino acid treatment intake, and may have decreased the glucose peak time points, thereby extending the time needed for values to fall back to baseline. There is work that shows that stevioside, which is found in the plant *Stevia rebaudiana Bertoni*, may influence the reduction of blood glucose with and without stimulating insulin secretion (Anton et al., 2010; Chen et al., 2005). However, the dose found in one study in a human model was 20g/day for three days. Our dose combined stevia and lecithin to a total amount of

3.54 g in each treatment. With this understanding, we are unable to fully explain the elevated glucose and insulin levels above baseline at the cessation of the treatment we observed.

Another observation made during this investigation was the influence that ISO had on GIP secretion and concentrations compared to LEU. In support of previous research that looked at the protein and or amino acids affecting GIP secretion, Lindgren et al. (2015) found that an intradoudenally absorbed amino acid mixture (6.5 g) induced a significant rise in GIP concentrations when compared to the same dose during IV infusion. In our protocol, we gave a much larger dose (14.6 g) of ISO+LEU, ISO, and LEU, and observed that either LEU nor the combination of ISO+LEU treatment was able to solicit the same increased area change outcome as ISO did alone; this identifies ISO as a specific amino acid that directly regulates GIP synthesis and secretion. Lindgren et al. (2015) saw a peak concentration of total GIP at ~20 pmol/l with the amino acid ingestion alone; while we saw a peak level of ~40 pmol/l after glucose treatment was implemented. In our 0-30 min prior to glucose drink phase of the amino acid only test, ISO independently influenced a 4 pmol/l increase from ~7 pmol/l at baseline at the 30 min time point. Currently, to our knowledge, there is no known threshold level of GIP to induce an insulin response, and therefore stimulate glucose uptake. So, while a slight increase in GIP may be statistically different in our case, it does not explain any greater insulin concentrations to facilitate glucose uptake since we did not show a difference between any group, in regards to insulin area, when compared.

The GIP response to amino acids and protein ingestion has been well reported (Fieseler et al., 1995; Lindgren et al., 2015; Thomas et al., 1978) by differing study designs. Another factor that may play a role in protein or amino acid regulation of GIP secretion is the dose. Nilsson et al. (2007), found that whey protein (18 g) with glucose had increased in GIP secretion 80% from baseline compared to three different amino acid groups (AA2: lysine and threonine; AA3: leucine, isoleucine, and valine; AA5: leucine, isoleucine, valine, lysine, and threonine with 3, 4.4, and 7.4 g respectively). They reported no differences in GIP area when comparing amino acid groups and the control drink of 25 g of glucose (Nilsson et al., 2007). GIP secretion from K-cells is enhanced in response to ingestion of meals or total glucose. GIP during fasting conditions are 5–20 pmol/l in healthy adults, and reach 50–100 pmol/l within 30 min in response to ingestion of 75 g glucose drink (OGTT). Whereas, total GIP may reach 100–150 pmol/l within 60 min in response to ingestion of a mixed meal (there may be ethnic variance). It has been observed that GIP secretion is enhanced by protein or fat intake; however, protein ingestion stimulates a more rapid and robust GIP secretion concentration compared to isocaloric fat ingestion (Seino et al., 2010).

The analysis of our GLP-1 data supports previous literature that amino acids do not stimulate a meaningful GLP-1 response compared to our control trial (PLA). Lindgren et al. (2015), showed no effect on GLP-1 area concentrations with ingested amino acid mixture. Similarly, Nilsson et al. (2007) showed no area concentration differences when comparing whey with amino acids. In contrast, other literature has shown that whey protein (Carr et al., 2008) and amino acids (Chen & Reimer, 2009;

Fieseler et al., 1995) augment GLP-1 concentrations in differing study designs and models. GLP-1 is secreted from L-cells and is also found to be synthesized and secreted in a co-localized manner in more superior located K-cells that are known to secrete GIP (Mortensen, Christensen, Holst, & Orskov, 2003). GLP-1 secretion has shown to be enhanced in response to ingestion of meals or glucose. Plasma levels of total GLP-1 in a normal healthy population while fasting range from 10–20 pmol/l, while 30 min after 75 g glucose drink or a mixed meal levels of total GLP-1 reach 30–60 pmol/l.

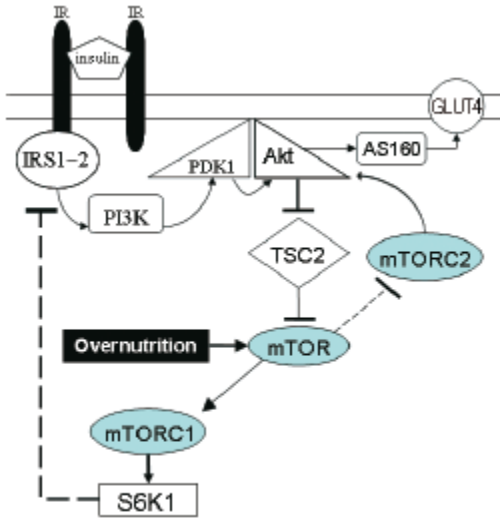

Figure 23: Over nutrition or amino acid induced stimulation of mTORC1 and downstream protein phosphorylation of S6K1 inducing negative feedback loop and inhibition of IRS-1,2 (Rivas, Lessard, & Coffey, 2009).

Mechanism of Action

Our data suggests that L-Isoleucine is an amino acid that plays a significant role in the amino acid driven GIP secretion, while L-Leucine does not seem to have the same effect. The mechanism of action of L-Isoleucine to stimulate GIP synthesis and secretion, is suggested to involve aliphatic L-amino acid and oligopeptide sensing sensitive G protein–coupled receptors (GPCRs). The GPCRs are more specifically named the calcium-sensing receptor (CaSR), metabotropic glutamate receptors (mGluRs), GPRC6A, the umami receptor (TAS1R1/R3), and LPAR5 (GPR92/93). The CaSR is associated with a variety of gut hormones such as gastrin, SST, CCK, GIP, and GLP-1, which have been strongly linked to incretin hormone secretion in vitro and in vivo, and is the principle Gαq-coupled receptor that is activated by calcium (Ca^{2+}), aromatic amino acids, and oligopeptides. The presumed model of incretin exocytosis is a sodium (Na^{2+}) and Ca^{2+} dependent action potential induced membrane depolarization, which is accompanied by a voltage dependent Ca^{2+} influx that occurs partially through Ca^{2+} channels (L-type cells). These cell actions are suggested to be essential for triggering the release of GIP and GLP-1 enteroendocrine hormone secretory granules (Gribble & Reimann, 2016; Mace, Tehan, & Marshall, 2015). Furthermore, intestinal aromatic amino acid transporters (TAT1 [SLC16A10]) are pH independent and electroneutral, showing that protons (H^{+}) not involved in the transport process are substantially expressed and observed in all sections of the small intestine. In the gut, the expression of TAT1 increases toward the tip of the villi and is observed in the basolateral membrane, which could contribute to a net efflux of other neutral amino acids, and in

turn allows for the efflux of aromatic amino acids, which may then influence the exchange for other neutral amino acids (i.e. L-Isoleucine), via system L-amino acid transporters (LAT2 [SLC7A8]) (Bröer, 2008; Verrey et al., 2004).

It is unknown how L-Isoleucine would compare to other individual amino acids, mixtures, or even differing doses to augment GIP concentrations. The whey protein regulated incretin hormone secretion has not fully been explained, and the factors that influence secretion are still being observed and debated. A prominent amino acid found in whey protein is L-Glutamine (GLN). GLN has shown to stimulate GLP-1 secretion when ingested independently, which may induce insulin driven postprandial glucose reductions (Cohen & Hall, 2009; Greenfield et al., 2009; Reimann, Williams, da Silva Xavier, Rutter, & Gribble, 2004; Samocha-Bonet et al., 2011; Tolhurst et al., 2011). GLN is ~16% of the total amino acid and ~50% of nonessential amino acid composition of 20 g serving of whey protein (Pennings et al., 2011), which may suggest that GLN plays a prominent role in the whey-driven GLP-1 secretion.

Due to our observations of L-Isoleucine driven GIP secretion, and its poor relationship with the pancreatic synthesis and release of C-peptide and insulin, the diminished glucose effect seen at the 60 and 90 min peak time points compared to our control treatment cannot be explained by insulin driven glucose uptake alone. The insulin area was found to be no different than the placebo control trial, so it could be suggested that the glucose blunting effect of L-Isoleucine and L-Leucine may be better explained by intramuscular amino acid insulin signaling mimicking these actions. This

cellular signaling action may be independent of insulin or the enteroendocrine incretin hormones GIP and GLP-1.

Doi et al. (2005) compared both L-Leucine and L-Isoleucine (1.35 g/kg body weight) in terms of glucose uptake. Using radiolabeled glucose (2-[1,2-3H]-deoxyglucose or 2-[3H] DG) they observed the glucose uptake in skeletal muscle while controlling for AMPK activity. L-Isoleucine showed to have a greater effect on gastrocnemius glucose uptake (2-[3H]DG) than did L-Leucine, independent of AMPK activity which is known to have an effect on GLUT-4 translocation (Doi et al., 2005). In another study with a similar study design by Doi et al. (2007), they compared the same dose of L-Isoleucine and L-Leucine on glucose uptake in both skeletal muscle and liver concurrent with hepatic enzyme measures associated with gluconeogenesis. Results were consistent with previous research that an effective dose of ISO (0.45 g/kg/bodyweight) influenced a hypoglycemic effect by way of a greater glucose uptake (2-[3H]DG) when compared to L-Leucine. This was accompanied by a reduction of gluconeogenic hepatic gene expression phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) mRNA (Doi et al., 2007).

Lastly, Nishitani et al. (2003) used a cirrhotic rodent model (CCl4) with similar glucose intolerant responses as a diabetic population. The rodents were given an OGTT with either 1.5 g/kg of L-Isoleucine, L-Leucine, or L-Valine. Results showed there was no difference between treatments and insulin responses, and L-Isoleucine and L-Leucine both lowered glucose regardless. They hypothesized that these two amino acids influenced GLUT-4 translocation, and L-Leucine independently affected $mTORC_1$

phosphorylation and activation, as well as stimulating glycogen synthase for the deposition of glucose (Figure 24). Additionally, it was suggested that L-Isoleucine may have interacted with PI3K and protein kinase-C (PKC), which are vital proteins in the intrinsic signaling pathway to stimulate GLUT-4 translocation (Nishitani et al., 2005).

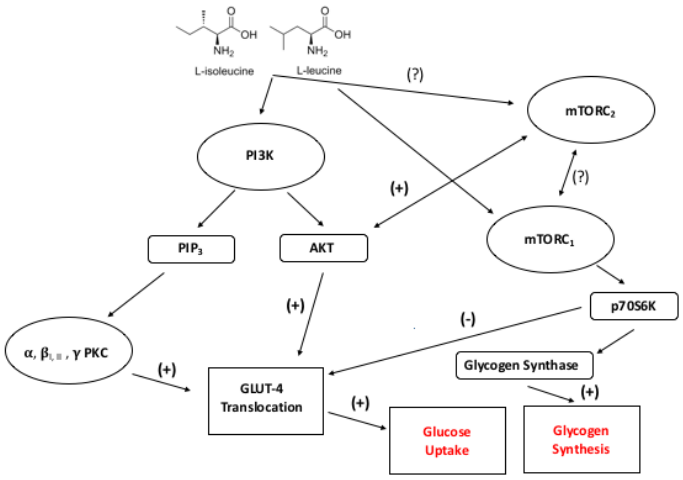

Figure 24: A proposed mechanism of action and cellular signaling pathways associated with L-Isoleucine and L-Leucine and their influence on glucose uptake and glycogen storage. Phosphatidylinositide 3-kinases (PI3K); Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3); protein kinase-B (PKB/AKT); **α**, $\boldsymbol{\beta}_{\text{I and II}}$, **γ** protein kinase-C (PKC); mechanistic target of rapamycin complex-1 ($mTORC_1$); mechanistic target of rapamycin complex-2 ($mTORC_2$); ribosomal protein S6 kinase beta-1(p70S6K/S6K1); glucose transporter type 4 (GLUT-4).

Future Research and Conclusion

Future research should observe the impact of BCAA's L-Isoleucine and L-Leucine on glucose uptake in a human model at the cellular skeletal muscle level. Our study was limited to the blood responses and hormones associated with glucose metabolism. Due to the interaction and the magnitude of interaction between BCAA's and skeletal muscle in regards to protein metabolism, observing the duality of intramuscular signaling proteins such as PI3K and $mTORC_{1/2}$ may give way to investigating possible nutritional therapies that are not typically associated with normal substrate metabolism, such as amino acid cell signaling and glucose uptake.

In conclusion, we observed that the pre-ingested effects of BCAA's L-Isoleucine and L-Leucine individually and in an equal mass on blood plasma glucose, C-peptide, pancreatic-derived hormones insulin, glucagon, and enteroendocrine synthesized hormones GIP and GLP-1. Our analysis showed that all amino acid treatments lowered blood glucose peak time points during treatment. Although we did not observe a successful blood glucose area reduction overall compared to the control, we did observe that L-Isoleucine seemed to have a greater impact on GIP concentrations than did L-Leucine, independently or when it's ingested in combination with L-Leucine. This amino acid driven outcome is similar to other research observing an amino acid mixture driving GIP secretion. Lastly, it appears that the blunted increase in glucose concentration during the regular peak period following a glucose drink response due to pre-amino acid ingestion cannot be fully explained by GIP augmentation and subsequent stimulation of pancreatic C-peptide and insulin release due to the similar insulin response found when

compared to the control treatment. This suggests that other amino acid driven insulin independent pathways may influence glucose uptake. More specifically, BCAA's in the majority bypass gut metabolism and reach the systemic system to be prominently uptaken into skeletal muscle. BCAA's have been known to regulate protein synthesis pathways and may also mimic glucose pathways that influence the uptake of glucose.

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APPENDIX A

Institutional Review Board Approval Letter

Institutional Review Board
Office of Research and Sponsored Programs
P.O. Box 425619, Denton, TX 76204-5619
940-898-3378
email: IRB@twu.edu
http://www.twu.edu/irb.html

DATE: October 13, 2014

TO: Mr. Dan Newmire
Department of Kinesiology

FROM: Institutional Review Board - Denton

Re: Approval for The Effects of Leucine and Isoleucine on Glucose Metabolism (Protocol #: 17757)

The above referenced study has been reviewed and approved at a fully convened meeting of the Denton Institutional Review Board (IRB) on 7/11/2014. This approval is valid for one year and expires on 7/11/2015. The IRB will send an email notification 45 days prior to the expiration date with instructions to extend or close the study. It is your responsibility to request an extension for the study if it is not yet complete, to close the protocol file when the study is complete, and to make certain that the study is not conducted beyond the expiration date.

If applicable, agency approval letters must be submitted to the IRB upon receipt prior to any data collection at that agency. A copy of the approved consent form with the IRB approval stamp is enclosed. Please use the consent form with the most recent approval date stamp when obtaining consent from your participants. A copy of the signed consent forms must be submitted with the request to close the study file at the completion of the study.

Any modifications to this study must be submitted for review to the IRB using the Modification Request Form. Additionally, the IRB must be notified immediately of any adverse events or unanticipated problems. All forms are located on the IRB website. If you have any questions, please contact the TWU IRB.

cc. Dr. Charlotte Sanborn, Department of Kinesiology
Dr. Vic Ben-Ezra, Department of Kinesiology
Graduate School

APPENDIX B

Informal Consent Form

TEXAS WOMAN'S UNIVERSITY
CONSENT TO PARTICIPATE IN RESEARCH

Title: *The Effects of L-Leucine and L-Isoleucine on Glycemic Responses in Apparently Healthy and Sedentary Adults*

Principal Investigator: Dan Newmire, MS ..... dnewmire@twu.edu 319-321-2144
Co-Investigator: Sarah Deemer, MS ..... sdeemer@twu.edu
Co-Investigator: Eric Rivas, MS..... erivas@twu.edu
Advisor: Vic Ben-Ezra, PhD ..... vbenezra@twu.edu 940-898-2597

Instructions:
Please read this consent form carefully and take your time making a decision about whether to participate. As the researchers discuss this consent form with you, please ask him/her to explain any words or information that you do not clearly understand. The purpose of the study, risks, inconveniences, discomforts, and other important information about the study are listed below. If you decide to participate, you will be given a copy of this form to keep.

Why is this study being done?
This study is being done to investigate the effect of amino acids L-Leucine and L-Isoleucine effects on how your body uptakes glucose from the blood. Normal blood glucose levels need to be maintained. Blood glucose levels that are too high or low can cause health problems such as diabetes, stroke, and cardiovascular disease. This study will investigate the effects of these amino acids on glucose metabolism.

Why am I being asked to take part in this research study?
You are being asked to take part in this study because you are either:

- Male or female
- Healthy or overweight
- Between the ages of 20 and 65 years
- Sedentary (exercise less than 3 days a week)
- Do not currently supplement your diet with branch chain amino acids (BCAAs) or supplemental proteins

You are not eligible in this study if you:

- Exercise 3+ days a week
- Use supplemental forms of BCAAs or Protein (Whey)
- Smoke

Do I have to take part in this research study?"
No. You have the right to choose whether you want to take part in this research study. If you decide to participate and later change your mind, you are free to stop participation at any time.

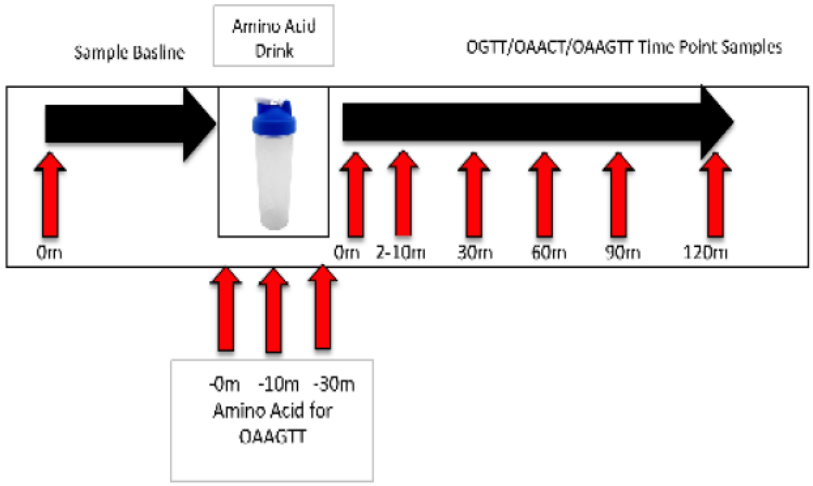
What is involved in the study?
If you volunteer to take part in this research study, you will be asked to sign this consent form and will have the following tests and procedures conducted. All tests and procedures are done solely for the purpose of the study and are not intended to diagnose or treat medical problems

Initial here:_____

To complete this project, information will be collected during 8 different days. If you agree to participate in the study, you will be required to visit the laboratory up to 8 times. The first day will involve screening procedures and preliminary testing. You will then complete an experimental oral glucose tolerance test (OGTT) as a control trial and then be randomly assigned into the following treatments: The oral amino acid concentration test (OAACT) where there will be three treatments where you will ingest a powdered amino acid with water solution of L-Leucine and/or L-Isoleucine amino acids with 150 mL water (with additional water if needed to completely consume dose), the oral amino acid combined with glucose tolerance test (OAAGTT), also three treatments; where you will also ingest the same amino acids L-Leucine and/or L-Isoleucine with water (150 mL water, with additional water if needed to completely consume dose) 30 minutes prior to a 75g standard glucose drink. This study duration will last 12 weeks.

Study Summary:

Blood samples for the oral glucose tolerance test (OGTT) and oral amino acid concentration test (OAACT) and oral amino acid and glucose tolerance test (OAAGTT) will be taken at 13 time points (-0,-10,-30,0,2,4,6,8,10,30,60,90,120min). Each sample will consist of 4mL amount of blood. Each participant will complete preliminary (visit 1) and OGTT (visit 2) for a control then be randomly assigned into the oral amino acid concentration test (OAACT) treatment groups (3x) and the oral amino acid combined with glucose tolerance test (OAAGT) treatment groups (3x) for the duration of this 12 week study



Drink – Amino Acids/Water/Glucose

↑ - Intravenous Blood Data Collection Time Points

OGTT – Oral Glucose Tolerance Test

Approved by the Texas Woman's University Institutional Review Board
Approved: June 5, 2016

Initial here:_____

OAACT – Oral Amino Acid Concentration Test (Water + Amino Acid

OAAGTT – Oral Amino Acid + Glucose Tolerance Test (Water + Amino Acid prior to Glucose

1. Procedure

Screening Procedures

To help decide if you qualify for this study, the researchers will ask you questions about your health, including medications you take and any surgical procedures you have had.

You will also complete these procedures:

- Medical History Questionnaire;
- Dual Energy X-Ray Absorptiometry (DXA)
- Body Mass Index: Measure of human body shape based on body mass divided by the square of your height;
- Fasting blood glucose: Blood sugar level measurement after 12 hours (overnight) of abstaining from food consumption;
- 2.5 h oral glucose tolerance test;
- Oral ingestion of powdered amino acids, L-Leucine and L-Isoleucine

If you decide to participate in the study, you are committing to approximately ~26 hours of testing and having less than <1 pint or 364mL (about ~25 tablespoons) of blood drawn. This study involves 8 total visits to the laboratory over 12 weeks. These visits include one preliminary and familiarization session (1), the OGTT (1), three experimental oral amino acid concentration sessions (OAACT)(3) and three oral amino acid and glucose tolerance sessions (OAAGTT) (3) to observe how your body uses glucose.

During the course of this study you will have the following tests and procedures:

Preliminary sessions: In this first session, you will provide informed written consent, complete a medical history questionnaire, and have the following measurements taken: height, weight, and waist circumference, and a small blood sample of 4mL (~1/4 tablespoon). This blood sample will a comprehensive metabolic test for the benefit of participant. You will then be familiarized with all experimental procedures. It will be important to keep diet the same before all experimental sessions. You will be given a food log (3-day dietary recall or myfitnesspal.com) and will record all food intake for 3 days prior to each treatment session. You will also have your body composition (bone mineral density, fat mass, and lean body mass) measured by a Dual-Energy X-ray Absorptiometry (DXA).

Oral Glucose Tolerance Test – Visit 2): You will then complete a 2.5-hr oral glucose tolerance test.

Peripheral intravenous catheter during oral glucose tolerance test:

Description of Procedure: A sterile catheter, which is a thin flexible plastic tube, will be inserted into an arm vein so that blood can be taken several times without having multiple sticks with a needle. A trained phlebotomist will draw blood samples for various chemicals in the body [glucose (sugar in the blood), insulin and C-peptide, (hormones produced by the pancreas), and lipids (Cholesterol: HDL/LDL).

Potential Risks: The risks of collecting a blood sample from you include the possibility of requiring more than one attempt to obtain the blood sample, local discomfort (pinch when the needle enters your skin), minor bruising or bleeding at the site (10%), or possible temporary lightheadedness, infection (<0.01%), or development of a blood clot (< 0.01%). These risks are slightly increased compared to a standard blood draw. The amount of blood being withdrawn for 8 visits over the course of the study is about ~25 tablespoons or ~364mL and will not affect your ability to participate in normal daily activities. One single donation of blood is roughly a pint (1 pint = 473mL). A trained and an experienced phlebotomist will perform the technique and your blood will be collected in a hygienic setting with sterile materials and biohazard protection measures to minimize these risks. On a rare occasion, a person may feel dizzy or faint. All testing will be concluded if any of these issues arise.
Duration of Procedure: The catheter will be in place for the entire experiment (approximately 3 hours).

Oral Glucose Tolerance Test

Description of Procedure: During this study, you will undergo an oral glucose tolerance test. This is a two-hour test that involves several blood samples. On the night before your test, you will eat your last meal and should not eat or drink anything other than water for at least 10 to 12 hours after. For this test, you will have a peripheral intravenous catheter (thin plastic tube described above) inserted into a large vein in your arm. The catheter will allow the phlebotomist to draw several blood samples from your vein without reinserting a needle each time. The phlebotomist will then give you a sugary substance to drink (75g glucose) over a short period. Blood samples will be drawn at -0, -10, -30, 0, 2, 4, 6, 8, 10, 30, 60, 90, 120 minutes, for a total of 150 minutes. The time at which you begin drinking the beverage will count as "0 minute." The phlebotomist will take a total of 13 small samples (4 mL each) of blood from your vein during the 2.5-hour test (4 times before you drink the beverage and 9 after you drink the beverage). The total amount of blood taken during each test will be 52 ml or ~4 tablespoons The results of the oral glucose tolerance test will allow the researchers to observe how your body reacts to a glucose load.
Potential Risks: You may experience hyperglycemia (high blood glucose) and possibly hypoglycemia (low blood glucose) as a result of the oral glucose tolerance test. You may find it difficult to drink the extremely sweet glucose (sugary) liquid. Some people feel sick after drinking the glucose liquid and may feel nauseated and vomit. Vomiting may prevent you from completing the test on that day.
Duration of Procedure: Blood samples will be drawn at -0, -10, -30, 0, 2, 4, 6, 8, 10, 30, 60, 90, 120 minutes for total of 150 minutes. The oral glucose tolerance test will take approximately 2.5 hours.

Oral Amino Acid Concentration Test

Description of Procedure: During this study, you will undergo an oral amino acid concentration test. This is a 2.5-hour test that involves 13 blood samples. On the night before your test, you will eat your last meal and should not eat or drink anything other than water for at least 10 to 12 hours after. For this test, you will have a peripheral intravenous catheter (thin plastic tube described above) inserted into a large vein in your arm. The catheter will allow the study phlebotomist to draw several blood samples from your vein without continual reinserting of a new needle each time. The phlebotomist will then give you an amino acid drink (0.3g/kg of LBM of either L-Isoleucine and/or L-Leucine) to consume. Blood samples will be drawn at -0, -10, 30, 0, 2, 4, 6, 8, 10, 30, 60, 90, 120 minutes. The time at which you begin drinking the beverage will count as "0 minute." The phlebotomist will take a total of 13 small samples (4 mL each or ~1/4

of a tablespoon) of blood from your vein during the 2.5-hour test (4 times before you drink the beverage and 9 after you drink the beverage). The total amount of blood taken during each test will be ~52mL or ~3.5 tablespoons. The results of the oral amino acid concentration test will allow the researchers to observe how your body reacts to amino acid intake.
Potential Risk: You may experience hyperglycemia (high blood glucose) and possibly hypoglycemia (low blood glucose) as a result of ingesting amino acids. You may find it difficult to swallow capsules. Some people may feel sick after ingesting the capsules with water and may feel nauseated and vomit. Vomiting may prevent you from completing the test on that day.
Duration of Procedure: Blood samples will be drawn at (-0, -10, -30, 2, 4, 6, 8, 10, 30, 60, 90, 120 min). The oral amino acid concentration test will take approximately 2.5 hours.

Oral Amino Acid + Glucose Tolerance Test

Description of Procedure: During this study, you will undergo an oral amino acid concentration test. This is a 2.5 hour test that involves 13 blood samples. On the night before your test, you will eat your last meal and should not eat or drink anything other than water for at least 10 to 12 hours after. When you come to the lab the morning for this test, you will have a peripheral intravenous catheter (thin plastic tube described above) inserted into a large vein in your arm. The catheter will allow the study phlebotomist to draw several blood samples from your vein without continual reinserting of a new needle each time. The researchers will then give you an amino acid drink of L-Leucine or L-Isoleucine or both pending which was randomly selected (0.3g/kg of LBM) 30 minutes prior to a standard 75g Glucose drink (total 150mL), which will be consumed together over a short period. Blood samples will be drawn at -0,-10,-30, 0, 2, 4, 6, 8, 10, 30, 60, 90, 120 minutes for a total of 150 minutes. The time at which you begin drinking the beverage will count as "0 minute." The phlebotomist will take a total of 13 small samples (4 mL each or ~1/4 tablespoon) of blood from your vein during the 2.5-hour test (4 samples before you drink the beverage and 9 after you drink the beverage). The total amount of blood taken during each test will be 52 ml or ~4 tablespoons. The results of the oral amino acid concentration test will allow the researchers to observe how your body reacts to amino acids and glucose intake.
Potential Risk: You may experience hyperglycemia (high blood glucose) and possibly hypoglycemia (low blood glucose) as a result ingesting amino acids with glucose drink. You may find it difficult to ingest the amino acid water mixture and you may find it difficult to drink the extremely sweet glucose (sugary) liquid. Some people may feel sick after ingesting the capsules with glucose and may feel nauseated and vomit. Vomiting may prevent you from completing the test on that day.
Duration of Procedure: Blood samples will be drawn at time points -0, -10, 30, 0, 2, 4, 6, 8, 10, 30, 60, 90, 120 minutes for a total of 150 minutes. The oral amino acid and glucose tolerance test will take approximately 2.5 hours.

Anthropometric Measurements:
During measurement of height, weight and waist circumference you may feel embarrassed. To minimize this embarrassment you will have the option to have measurements taken by a research team member of the same gender. Additionally to ensure privacy procedures will be performed in a small private room located in the exercise physiology lab (PH 114) and not shared with anyone except the principal investigators and faculty advisor.

Body Weight:

Description of Procedure: You will be asked to stand on a scale in a private room and provide a body weight wearing gym clothes.
Potential Risks: There are no risks associated with this procedure.
Duration of Procedure: Each weight measurement will take about 30 seconds.

Waist Circumference:

Description of Procedure: To determine your waist circumference, you will stand with your arms at your sides, feet together, and abdomen relaxed. A horizontal measure will be taken at the narrowest part of your torso above the bellybutton.
Potential Risks: There are no risks associated with this procedure.
Duration of Procedure: Each waist measurement will take about 30 seconds.

Body Height:

Description of Procedure: You will be asked to stand on a scale which will measure your height.
Potential Risks: There are no risks associated with this procedure.
Duration of Procedure: Each height measurement will take about 30 seconds.

Body Composition:

Description of Procedure: You will be asked to have your body composition of lean body mass (LBM), fat mass (FM), and bone mineral density (BMD) measured in a Dual Energy X-Ray Absorptiometry (DXA).
Potential Risks: DXA scanning utilizes radiation to obtain an image of your body. Everyone receives a small amount of unavoidable radiation from the environment each year. The DXA scan technique gives your body the equivalent of about 4 extra days' worth of this natural radiation. If you are pregnant or trying to get pregnant, you should not have this procedure. It is possible that having several of these tests may add to possible risk of injury or disease. To minimize this risk only 1 scan will take place at the preliminary visit for this research project.
Duration of Procedure: 1 DXA scan will take place during study and will take 20 minutes

Data Collection and Private Participant Information:

Description of Procedure: During research personal identity and biological measurements will be recorded and stored. This information will be pertinent to the study and needed.
Potential Risks: It is possible that there may be a potential risk of loss of confidentiality in all email, downloading, and internet transactions. To minimize this risk all data will be stored in password-protected computer and a locked cabinet at PI's residence. All data forms collected will be coded using alphabets and numbers. A single identification form linking names with their respective codes will be kept in a separate folder from the other data. Persons not associated with the study will have no access to the folders (soft or hard copies) confidentiality will be protected to the extent allowed by law. Also, during the collection of personal information (i.e., menstrual and menopausal history, dietary information) you may feel emotionally uneasy. To minimize emotional discomfort with the collection of this information, you will have the option to share this information with a research team member of the same gender. If you feel that you are unable to share this information you will be precluded from participation from the study.
Duration of Procedure: Data will be collected through the entirety of study.

Singular or Multiple Blood Collection:

Description of Procedure: During this study blood will be either drawn by way of singular venipuncture (preliminary visit 1) or by way of catheter or multiple blood collections. Blood will be drawn from the antecubital space (front side of arm, or opposite of elbow) this procedure is carried out by trained personnel and proper equipment is used, as it will be in this case.

Universal precautions will be used during all blood draw procedures. Sites for blood draws will be cleaned with alcohol immediately prior to each venipuncture blood will be collected in a hygienic setting with sterile materials and biohazard protection measures to minimize these risks. A registered nurse or a trained phlebotomist will obtain these blood samples.
Potential Risks: The risks of collecting a blood sample include the possibility of requiring more than one attempt to obtain the blood sample, local discomfort (pinch when the needle enters your skin), minor bruising or bleeding at the site (10%), possible temporary lightheadedness, infection (<0.01%), or development of a blood clot (<0.01%). There may also a small risk of the catheter perforating (going through) the vein or not being inserted into a blood vessel. Also, participants may experience discomfort, bleeding, and/or bruising. On a rare occasion, you may feel dizzy or faint. The likelihood of these complications is very remote (about 1 in 10,000)
Duration of Procedure: A singular blood collection will happen at the preliminary visit 1 (15min) and the catheter multiple blood draw will obtain 13 collections for the oral glucose tolerance test, oral amino acid concentration test, and oral amino acid and glucose tolerance test for 2.5hrs.

Glucose and Amino Acid drinks:

Description of Procedure: During this study you will consume a glucose drink (OGTT), a amino acid with water drink (OAACT), and amino acid drink followed by a glucose drink (OAAGTT).
Potential Risks: You may find it difficult to drink the extremely sweet glucose (sugary) liquid and the amino acid drink. Some people feel sick after drinking the glucose liquid and may vomit. In our lab this has occurred twice in 20 years (over 500 OGGTs). It is possible that blood glucose levels may drop very low toward the end of the test. Symptoms of low blood glucose include weakness, hunger, sweating, and feeling nervous or restless. If levels are very low, the test will be stopped. Hypoglycemia (low blood sugar, (<4mmol/L or <72mg/dL) may result from prolonged fasting. If you show any signs of hypoglycemia during the testing session, the test will be terminated. Signs of hypoglycemia include headache, confusion, hallucinations, bizarre behavior, tremors, cold sweat, low body temperature, blurry vision, shaking or trembling, fast heartbeat, sweating, tiredness/ weakness, convulsions, and coma. You will be given a glass of orange juice or carbohydrate rich food and monitored in the lab until signs of hypoglycemia subside. Also, to minimize the possibility of GI distress with administration independently and combined forms of glucose and amino acids all you will have immediate access to restroom facilities, additional water will be available and research will be stopped immediately if any signs or issues of nausea are witnessed by the research team and or by yourself. You will be notified of taste and possible GI distress during consent when administering oral drinks.
Duration of Procedure: Each drink will take less than 10-15 minutes to consume. 7 of the 8 visits will involve consumption of a drink.
How long can I expect to be in this study?
You will be asked to participate in 8 visits over 12 weeks. You will need to come into the lab for 1 preliminary visit, 1 oral glucose tolerance test, and 6 amino acid treatments with or without glucose. The study will last a total of 12 weeks. Each visit will take approximately 1.5 to 3.5 hours for a total of 26 hours for entirety of this study.

Dietary Considerations

You will be asked to consume a similar diet before each treatment following the preliminary visit. For three days before as well as after each session you will keep a dietary record to insure that food intake in terms of total calories and composition (percentages of fat, carbohydrate, and protein) are similar. You will receive counseling on how to record three day dietary record (3-Day Dietary Recall or http://www.myfitnesspal.com/). You will be asked to maintain the same evening meal consistently before each treatment throughout each trial, as well as information about keeping a similar diet.

2. Benefits

Participation in the study will be of no monetary cost to you. Following completion of the study, you will have the results of the study and your individual results. For participating in this study all participants will be financially compensated for their time. For each visit you will be compensated $25 with the ability of a combined total of $200 (8x$25). Secondly, you will receive $150 worth of supplemental products from Dymatize (http://www.dymatize.com/). Lastly, you will also receive information regarding your complete blood panel, glucose tolerance, body composition, and nutritional intake. This information is supplied free of charge and will increase your awareness of your personal health.

In case of a medical emergency, the fire department's Emergency Medical Team will be alerted. Telephones are available in the testing laboratories.

We will try to prevent any problem that could result from this research. Please let us know at once if there is a problem and we will help you. You should understand, however, that TWU does not provide medical services or financial assistance for injuries that might happen because you are taking part in this research. The investigators are prepared to advise you in case of adverse effects, which you should report to them promptly. Phone numbers where the investigators may be reached are provided in this form.

To reduce the possibility of improper disclosure, your name will be kept confidential and will not be associated with the data in any presentation of results. All subjects will be given a code number. Your data will be kept on file in a locked cabinet for a maximum of 5 years after the data are published. All data will be destroyed (shredded) after the five-year period. All data will be discarded in the recycle bin or regular trash, making sure that your name is completely obliterated on any documents or data files.

You will be given a copy of this signed and dated consent form to keep. If you have any questions about the research study you should ask the researchers; their phone numbers are at the top of this form. If you have questions about your rights as a participant in this research or the way this study has been conducted, you may contact the Texas Woman's University Office of Research and Sponsored Programs at 940-898-3378 or via e-mail at IRB@twu.edu.

_____ _____

Signature of Participant Date

*If you would like to know the results of this study tell us where you want them to be sent:

Email: _____

Or

Address:

_____

_____

_____

_____

Initial here:_____

APPENDIX C

Lifestyle Survey

LIFESTYLE SURVEY

The questions in this survey are designed to help us understand your health. Try to answer each question truthfully. There are no right or wrong answers. If you are not sure, try your best guess. If you have any questions please ask the research assistant. You do not have to answer any of the questions if they make you uncomfortable. YOUR ANSWERS WILL BE KEPT STRICTLY CONFIDENTIAL.

HEALTH HABITS

1. Now thinking about your physical health, which includes physical illness and injury, for how many days during the past 30 days did you have poor physical health?	Number of days	
2. Now thinking about your mental health, which includes stress, depression, and emotional problems, for how many days during the past 30 days did you have poor mental health?	Number of days	
3. During the past 30 days, for about how many days did poor physical or mental health keep you from doing your usual activities, such as self-care, work or recreation?	Number of days	
4. Have you smoked at least 100 cigarettes (5 packs) in your whole life? **(IF NO, GO TO QUESTION #7)**	YES	NO
5. About how many cigarettes do you now smoke each day?	Cigarettes /day	
a. If 0, how long has it been since you quite smoking?	years /months	
6. About how old were you when you started smoking cigarettes regularly? (at least one cigarette per week)	years old	
7. A drink is 1 can or bottle of beer, 1 glass of wine, 1 can or bottle of wine cooler, 1 cocktail or 1 shot of liquor. About how many days a week do you have an alcoholic drink?	days /week	
8. On the days that you drink, about how many drinks do you have (on average)?	drinks each time I drink	

9. How would you consider your health? (Please mark one only)				
VERY GOOD	GOOD	AVERAGE	POOR	VERY POOR
1	2	3	4	5

10. Have you ever had or do you have the following? If you check YES, please provide an additional explanation.			
YES	NO	DON'T KNOW	(CHECK ALL THAT APPLY)
			Free or easy bleeding (hemophilia) Please Explain:
			Low blood iron (anemia) Please Explain:
			Sickle cell anemia Please Explain:
			Rheumatic fever Please Explain:
			Heart murmur Please Explain:
			Irregular heart beat Please Explain:
			Mitral valve prolapse Please Explain:
			Any heart problem Please Explain:
			Pacemaker or implanted defibrillator Please Explain:
			High blood pressure Please Explain:
			High blood cholesterol Please Explain:
			Varicose veins Please Explain:
			Cancer Please Explain:
			Emphysema Please Explain:
			Chronic bronchitis Please Explain:
			Asthma Please Explain:

YES	NO	DON'T KNOW	(CHECK ALL THAT APPLY)
			Lung disease Please Explain:
			Seizures Please Explain:
			Stroke Please Explain:
			Low blood sugar Please Explain:
			Diabetes Please Explain:
			Kidney Disease Please Explain:
			Hepatitis Please Explain:
			Liver Disease Please Explain:
			Eye problems Please Explain:
			Hearing problems Please Explain:
			Thyroid problems Please Explain:
			Orthopedic problems Please Explain:
			Back problems Please Explain:
			Joint problems Please Explain:
			Arthritis Please Explain:
			AIDS Please Explain:
			Alcoholism Please Explain:
			Other medical problems Please Explain:

11. Have you recently (within the last year) had any of the following symptoms?
If you check YES, please provide an additional explanation.

YES	NO	DON'T KNOW	(CHECK ALL THAT APPLY)
			Chest pain Please Explain:
			Shortness of breath Please Explain:
			Heart palpations or fast heartbeat Please Explain:
			Arm or shoulder pain Please Explain:
			Burning sensations Please Explain:
			Unusual fatigue with slight exertion Please Explain:
			Severe headache Please Explain:
			Blurred vision Please Explain:
			Low or high blood sugar Please Explain:
			Frequent urination Please Explain:
			Blood in urine Please Explain:
			Coughing of blood Please Explain:
			Feeling faint or dizzy Please Explain:
			Difficulty walking Please Explain:
			Low bone density (osteoporosis) Please Explain:
			Leg or ankle swelling Please Explain:

YES	NO	DON'T KNOW	(CHECK ALL THAT APPLY)
			Swelling in your joints Please Explain:
			Low-back pain Please Explain:
			Weakness in arm Please Explain:
			Leg numbness Please Explain:
			Significant emotional problem Please Explain:
			Other medical problems Please Explain:

12. Are you taking any medications or supplements? If yes, please list and describe below.	**YES**	**NO**	**DON'T KNOW**

13. Of the following members of your family, describe any cardiovascular disease, heart disease, stroke, or diabetes each has (or had).

YES	NO	DON'T KNOW	
			Mother Please Explain:
			Father Please Explain:
			Mother's Mother Please Explain:
			Mother's Father Please Explain:
			Father's Mother Please Explain:
			Father's Father Please Explain:

MENSTRUAL HISTORY			
14. What is your date of birth?	MM	DD	YY
15. At approximately what age (year and months) did you begin menstruating?	AGE		MM
16. Do you currently have regular menstrual cycles (i.e., regularly spaced periods of menstrual bleeding)?	YES		NO
If you answered "**YES**" to question #16:			
a. Approximately how many days separate your periods?	Less than 25 days	25-32 days	More than 32 days
b. Approximately how many days does your bleeding last?	Less than 3 days	3-7 days	More than 7 days
c. When did your most recent period begin?	MM	DD	YY
d. Is there any reason to believe that you are pregnant?	YES		NO
If you answered "**NO**" to question #16:			
a. Have your menstrual periods stopped completely?	YES		NO
b. When did your most recent period begin?	MM	DD	YY
c. If you still occasionally have menstrual bleeding, describe the pattern.			

17. How would you consider your eating habits? (PLEASE MARK ONE ONLY)				
VERY GOOD	GOOD	AVERAGE	POOR	VERY POOR
1	2	3	4	5

18. How often do you eat the following foods? (CIRCLE ONE NUMBER FOR EACH ITEM)							
	never or few times a year	**about once a month**	**several times a month**	**few times a week**	**once a day**	**3 or 4 times a day**	**5 times a day or more**
FRESH FRUITS AND VEGETABLES	1	2	3	4	5	6	7
PASTRIES (pie, cake, cookies, brownies, sweet rolls, donuts)	1	2	3	4	5	6	7
BREAD (bread, pasta, tortilla)	1	2	3	4	5	6	7
POULTRY	1	2	3	4	5	6	7
FISH	1	2	3	4	5	6	7
PORK	1	2	3	4	5	6	7
RED MEATS (beef, lamb, lunch meats)	1	2	3	4	5	6	7
WHOLE MILK	1	2	3	4	5	6	7
FRIED FOOD	1	2	3	4	5	6	7
HOW OFTEN DO YOU EAT AT RESTAURANTS	1	2	3	4	5	6	7

These next few questions are about exercise, recreation and physical activities other than your regular job duties. For each of the following activities, please tell us how many times you did them in the past MONTH. For each activity that you did in the last MONTH, please tell us how much time on average in hours or minutes you spent doing the activity EACH TIME YOU DID IT.

19. First we will start with typical sport activities like basketball, volleyball and soccer and fitness related activities like jogging, running, riding a bike, dancing, and aerobics. These are NOT activities you do during work. These are NOT activities you do with your children either; only with other adults or by yourself.

a. In the past MONTH, have you done any typical sport or fitness related activities? (if "NO", go to Question #20)	**YES**	**NO**
b. If Yes, please tell us how many times in the past MONTH you have done the sport or fitness related activities listed below. If you did the activity every day then you would say you did it 30 times in the last MONTH. Then tell us how much time ON AVERAGE in minutes or hours you spent doing each sport or fitness related activity each time you did it.	**Times in last MONTH**	**Average Hours/ Minutes Each Time**
Aerobics/Aerobic Dance (using a video during a class in a gym)		
Other Dancing (for fun with friends and family)		
Jogging or Running		
Golf		
Bowling		
Tennis		
Basketball		
Baseball or Softball		
Soccer		
Weight lifting/training		
Riding a bike outside or a stationary bike inside		
Swimming		
Yoga		
Roller skating or blading		
Other1:		
Other2:		
Other3:		

20. Now we would like to know about recreational activities that you do when you have free time like watching television, reading, and spending time with your family. These are NOT activities you do during work.		
a. In the past MONTH, have you participated in any recreational activities? (IF "NO" go to Question #21)	**YES**	**NO**
b. If Yes, please tell us how many times in the past MONTH you have done the following recreational activities. If you did the activity once every day then you would say you did it 30 times in the last MONTH. Then tell us how much time ON AVERAGE in minutes or hours you spent doing each recreational activity each time you did it.	**Times in past MONTH**	**Avg Hours/ Minutes Each Time**
Using a computer		
Going to church		
Quiet play with children (board games, drawing, coloring, reading stories)		
Active play with children (playing tag, soccer, baseball, hide and seek)		
Talking on the telephone		
Listening to music		
Watching television or videos		
Watching a movie in a theater		
Visiting with friends (in person, not on the phone)		
Playing games (dominos, checkers, boardgames, cards)		
Reading		
Other 1:		
Other 2:		
Other 3:		
Other 4:		

21. Now we would like to know about household activities that you do such as cleaning, gardening, and taking care of children. These are NOT activities you do during work.		
a. In the past MONTH, have you done any household activities? (if "NO", go to Question 22)	**YES**	**NO**
b. If Yes, please tell us how many times in the past MONTH you have done the following household activities. If you did the activity once every day then you would say you did it 30 times in the last MONTH. Then tell us how much time ON AVERAGE in minutes or hours you spent doing each household activity each time you did it.	**Times in past MONTH**	**Average Hours/ Minutes Each Time**
Light Cleaning (picking up the house, dusting, sweeping, ironing, dishes)		
Hard Cleaning (scrub floors, move objects, carry loads up stairs)		
Childcare (feeding, bathing, dressing)		
Gardening or Yardwork		
Shopping (grocery, clothes)		
Light home repair/maintenance (changing light bulbs, fixing loose fixtures)		
Heavy home repair/maintenance (carpentry, lifting large objects)		
Laundry (time loading, unloading, hanging, folding only)		
Food Preparation (cooking, serving)		
Other 1:		
Other 2:		
Other 3:		
Other 4:		

22. ON AVERAGE , how would you describe your walking pace when you walk? (PLEASE CHECK ONLY ONE)	
	Slow, like taking a leisurely stroll.
	Moderate, like you had somewhere to go.
	Fast, like walking (without jogging) to catch a bus or make an appointment.

23. ON AVERAGE , how would you describe the intensity with which you clean the house or do other work around the house? (PLEASE CHECK ONLY ONE)	
	Light, like taking a leisurely stroll.
	Moderate, like walking to get somewhere.
	Hard, like fast walking (without jogging) to catch a bus or make an appointment.

24. Compared to others of your age and gender, would you say you are: (PLEASE CHECK ONE ONLY)	
	MUCH LESS ACTIVE
	SOMEWHAT LESS ACTIVE
	ABOUT AS ACTIVE
	SOMEWHAT MORE ACTIVE
	MUCH MORE ACTIVE

25. For each of the following statements, please indicate how often they prevent you from exercising on a scale of one to five. One represents Never, three represents Sometimes and five represents Always. Two is in between Never and Sometimes. Four is in between Sometimes and Always.

	Never		Sometimes		Always
a. I do not have a safe place to exercise.	1	2	3	4	5
b. I feel self-conscious about exercising because I am too overweight.	1	2	3	4	5
c. My health prevents me from exercising. Please Explain:	1	2	3	4	5
d. I do not enjoy exercising.	1	2	3	4	5
e. I cannot motivate myself to exercise.	1	2	3	4	5
f. I do not have anyone to exercise with.	1	2	3	4	5
g. I do not have time to exercise.	1	2	3	4	5
h. I do not have the energy to exercise.	1	2	3	4	5
i. I am afraid I will hurt myself when I exercise.	1	2	3	4	5
j. It is too hot or too cold to exercise.	1	2	3	4	5
k. My spouse/significant other does not want me to exercise.	1	2	3	4	5
l. I cannot find anyone to watch my children while I exercise.	1	2	3	4	5
m. I cannot walk/jog/run in my neighborhood. Please Explain:	1	2	3	4	5
n. I do not have clothes and/or shoes to exercise in.	1	2	3	4	5
o. Other. Please Explain:	1	2	3	4	5

PERSONAL INFORMATION

1. Please check the ethnic group that you most identify with:

If you are Hispanic, please check one of the following:

☐ Mexican National ☐ Mexican American ☐ Chicana/o

☐ Latina/o ☐ Other, please detail: _____

If you are **NOT** Hispanic, please check one of the following:

☐ American Indian ☐ Alaskan Native ☐ Asian or Pacific Islander

☐ African American, not of Hispanic Origin ☐ Caucasian, not of Hispanic Origin

☐ Other, please detail: _____

2. When were you born? _____/_____/_____
Month Day Year

3. What is your gender? ☐ Female ☐ Male

4. How tall are you? _____ Feet **AND** _____ inches **OR** _____ meters

5. How much do you weigh? _____ pounds **OR** _____ kilograms

6. Please check marital status: ☐ SINGLE ☐ MARRIED ☐ DIVORCED

☐ WIDOWED ☐ COMMON LAW ☐ OTHER: _____

7. Please check highest level of education COMPLETED:

☐ Less than 7th Grade
☐ Junior High/Secondary School (9th Grade)
☐ Some High School (10th or 11th Grade)
☐ Completed High School
☐ Some College or Vocational Training
☐ Completed Associate Degree
☐ Completed Bachelor Degree
☐ Completed Graduate Degree

8. Status of employment: ☐ Full Time ☐ Part Time ☐ Unemployed

9. If student, give status of schooling: ☐ Full Time ☐ Part Time _____

APPENDIX D

Myfitnesspal Food Diary Instructions

Food Diary and Food Database

How do I add a food to my food diary?

To add a food to your diary, just take the following steps:

A. **On the Web**
   - Click on the "Food" tab, then click "Add Food" under the meal you'd like to log.

B. **In our iPhone App**
   - Tap the large blue "+" button in the navigation bar, then tap "Food"
   - Or, on the Diary page, tap "+ Add Food" under a meal category in your diary.

C. **In our other Mobile Apps**
   - Tap the "Add to Diary" button on your Home screen **or** on the Food Diary page tap the "+" (or "Add") button
   - Tap the meal to which you would like to add your food.
   - To search for a food, type its name in the search field. You can enter brand names, or just key words. Then, tap the "Search" button. For greater convenience, some of our apps support searching by scanning an item's barcode.
   - Tap on a matching search result, then adjust the Number of Servings or Serving Size to reflect how much of that item you've eaten.
   - That's it. Your food should be logged in your diary.

D. **Pro Tip**: Our database has an enormous number of food items. You may improve your search results by adding terms like "uncooked," "cooked," "raw" or "generic" (for instance "apple generic") if your initial search does not provide the specific item you are seeking.

Exercise Diary and Exercise Database

How do I add an exercise to my diary?

Adding an exercise to your diary is similar to adding a food. Just take the following steps:

A. **On the Web**
   - Click on the "Exercise" tab, then click "Add Exercise" under the type of exercise you'd like to log.

B. **In the iPad App**
   - Tap the "Add to Diary" button on the "Home" screen OR tap the "Exercise Diary" tab, and then tap the "+" button in the upper right hand corner.

C. In our iPhone App

- Tap the large blue "+" button in the navigation bar, then tap "Exercise"

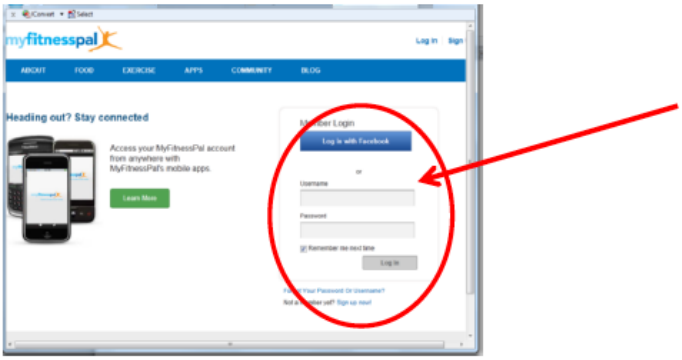
D. In our other Mobile Apps

- Tap the “Add to Diary” button on your “Home” screen OR on the “Diary” page tap the "+" (or "Add") button
- Choose either “Cardiovascular” or “Strength."
- Type the name of an exercise in the search box and press the “Search” button -- (on software keyboards, you'll find it in place of the "Return" key). You'll be shown a list of exercises in our database that match your search.
- Select the item you'd like to add to your diary, then enter the number of minutes for a cardio exercise, or the number of sets, reps and weight for a strength exercise. Finally, select "Add" or " " (apps) or "Add Exercise to Diary" (site).
- That’s it. Your exercise should be logged in your diary.

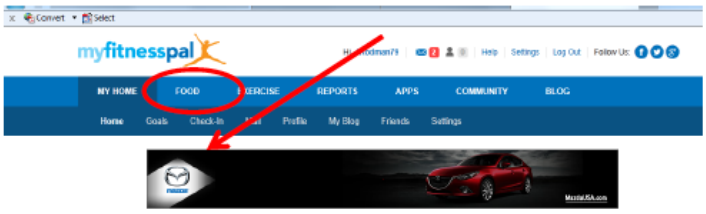
Printing your MyFitnessPal Diaries

You can view a printable version of your daily diary by logging into your MFP account at http://www.myfitnesspal.com (Mobile app users should log in using the same username and password they use in the app.)

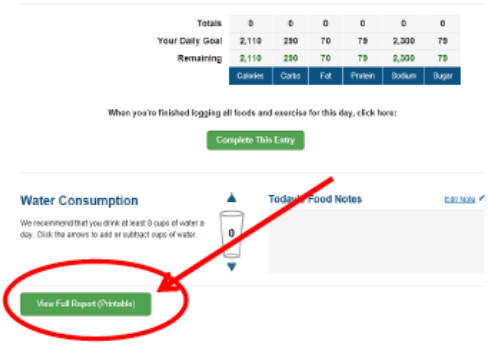
1. Log in to MFP



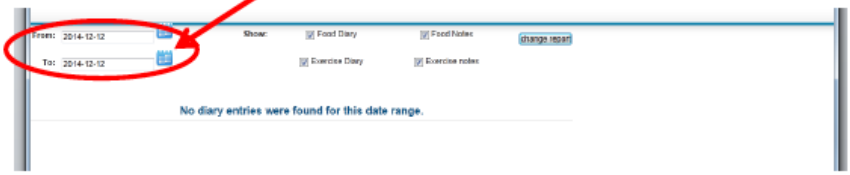
2. Click the "Food" tab.



3. Scroll to the bottom of the page and click "View Printable Report."



4. Choose a date range and click “Change Report.”



5. Select the dates to print then print as PDF.

APPENDIX E

Data Collection Sheets

The Effects of Leucine and Isoleucine on Glucose Metabolism
Data Collection Sheet

File Name: _____ Amino Acid: _____

Name: _____ Time of Arrival: _____

Date: _____

Height (in): _____ Weight (kg): _____ Waist Circumference (in): _____

LBM (kg): _____ x 0.3g/kg/LBM = _____

Glucometer Samples: **Amino Acid Drink**

Time (min)	0*	2	4	6	8	10	30
Sample #1							
Sample #2							

Glucometer Samples: **Glucose Drink (75g)**

Time (min)	0	2	4	6	8	10	30	60	90	120
Sample #1										
Sample #2										

Restroom Usage (Time): _____

Subjective Participant Responses:

Important Protocol Information:

1. **Amino Acid** (Leucine and/or Isoleucine) 0.3g/kg of LBM, when combined, ½ of each to equal same mass (g) as individually weighed Amino Acid.

2. **Amino Acid drink given** after 1st blood sample taken (0*), and 2, 4, 6, 8, 10, 30 min post-drink. Saline flushing only if needed (clotting) during these samples. Followed by 30 min sample.

3. **Glucose drink** given 30 min post Amino acid drink (whole content must be consumed), followed by 2, 4, 6, 8, 10 min blood samples (Saline flushing only if needed [clotting] during these samples), followed by 30, 60, 90, 120 min.

4. **Saline flushing** pre and post sample (1 mL), and then draw 4 mL for throw away to ensure sample is not diluted by saline.

5. **Restroom** may be used due to fluid consumption and saline drip used, record time and may use tagaderm to cover catheter to protect from movement, tape should be used to anchor catheter down and tagaderm only used if needed.

6. **Protein inhibitors** should be premixed into microcentrifuge prior to blood samples. 40 uL will be placed into the 4 mL samples (AA: 0, 10, 30) (GLU:0, 10, 30, 60, 90, 120). 20 uL will be placed in the 2 mL vials (AA: 2, 4, 6, 8) (GLU: 2, 4, 6, 8). **Pipette into vial before centrifuge.**

7. **Ice** (concession stand or lab freezer ice) will be used to slow metabolism of blood of time points 0-10 min, being they will be all be centrifuged as once due to time points so close together.

8. **Centrifuging** of all vials will be at ~2500 RPMs for 15 min after inhibitor has been placed in vial.

YSI 2900 Samples: **Amino Acid Drink**

Time (min)	0*	2	4	6	8	10	30
Sample #1							
Sample #2							

YSI 2900 Samples: **Glucose Drink (75g)**

Time (min)	0	2	4	6	8	10	30	60	90	120
Sample #1										
Sample #2										

Notes:

APPENDIX F

Research Flyer

TWU
TEXAS WOMAN'S UNIVERSITY
DENTON DALLAS HOUSTON

Exercise Physiology and Biochemistry Laboratories

Your Help is Needed for a Research Study

The Effects of Leucine and Isoleucine on Glucose Metabolism

The purpose of the research is to observe the effects of the BCAA's L-Leucine and L-Isoleucine on glucose metabolism. This study requires 8 visits for a total of 26hrs. *Participation is voluntary*

You are Eligible if: Non-smoker, exercise less than 3-days a week, normal healthy weight, and do not currently use whey protein or BCAAs

Participants will receive a free blood health screening analysis (CBC, HDL, LDL, TC, etc), body composition (DEXA)

For participating in this study all participants will be financially compensated for their time. Each visit will be compensated $25 with the ability of a combined total of $200 (8)

To learn more about this research, email Dan Newmire or stop by my office (PH211), Exercise Physiology Labs (PH 116 or PH112), Exercise Biochemistry Lab (PH114)

This research is conducted by PI Dan Newmire in the Exercise Physiology Department located in Pioneer Hall.

There is a potential risk of loss of confidentiality in all email, downloading and internet transactions

TWU IRB Validation Stamp

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APPENDIX G

Data Analysis and Raw Data

Table 12: 2-Way RMANOVA Δ Delta Glucose mg/dL 0-150 min

Two-way RM ANOVA	Matching: Both factors				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Treatment	53.64	<0.0001	****	Yes	
Time	1.268	0.0308	*	Yes	
Interaction: Treatment x Time	1.465	0.028	*	Yes	
Interaction: Treatment x Subjects	18.94				
Interaction: Time x Subjects	4.172				
Subjects	10.63				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment	173746	9	19305	F (9, 99) = 31.16	P<0.0001
Time	4109	3	1370	F (3, 33) = 3.345	P=0.0308
Interaction: Treatment x Time	4746	27	175.8	F (27, 297) = 1.629	P=0.0280
Interaction: Treatment x Subjects	61337	99	619.6		
Interaction: Time x Subjects	13514	33	409.5		
Subjects	34429	11	3130		
Residual	32043	297	107.9		

Table 13: 2-Wa
Two-way RM AI
Number of fam
Number of com
Alpha
Tukey's multipl

0
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

6
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

10
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

30
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

36
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

40
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

60
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

90
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

120
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

150
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

Test details

0
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

6
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

10
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

30
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

36
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

40
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

60
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

90
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

120
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

150
L-Isoleucine a
L-Isoleucine a
L-Isoleucine a
L-Isoleucine v
L-Isoleucine v
L-Leucine vs.

Table 14: 2-Way RMANOVA Δ Delta Glucose mg/dL 0-150 min

Two-way RM ANOVA	Compare column means (main column effect)							
Number of families	1							
Number of comparisons per family	3							
Alpha	0.05							
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
Placebo vs. L-Isoleucine and L-Leucine	6.674	0.2417 to 13.11	Yes	*	0.0406			
Placebo vs. L-Isoleucine	6.935	0.503 to 13.37	Yes	*	0.0322			
Placebo vs. L-Leucine	6.646	0.214 to 13.08	Yes	*	0.0415			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
Placebo vs. L-Isoleucine and L-Leucine	20.95	14.27	6.674	2.613	120	120	2.555	33
Placebo vs. L-Isoleucine	20.95	14.01	6.935	2.613	120	120	2.655	33
Placebo vs. L-Leucine	20.95	14.3	6.646	2.613	120	120	2.544	33

Table 15: 2-Way RMANOVA Δ Delta Glucose from Amino Acid mg/dL 0-30 min

Two-way RM ANOVA	Matching: Both factors				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	4.951	0.2464	ns	No	
Time	7.005	0.0044	**	Yes	
Treatment	6.391	0.0187	*	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	83.85	6	13.97	(6, 132) = 1.3	P=0.2464
Time	118.6	2	59.31	(2, 132) = 5.6	P=0.0044
Treatment	108.2	3	36.07	(3, 132) = 3.4	P=0.0187
Residual	1383	132	10.47		
Number of missing values	0				

Table 16: 2-Way RMANOVA Δ Delta Glucose from Amino Acid mg/dL 0-30 min

Two-way RM ANOVA	Compare column means (main column effect)
Number of families	1
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
L-Isoleucine and L-Leucine vs. L-Isoleucine	2.14	0.1547 to 4.125	Yes	*	0.0292
L-Isoleucine and L-Leucine vs. L-Leucine	1.363	-0.6217 to 3.348	No	ns	0.2841
L-Isoleucine and L-Leucine vs. Placebo	0.23	-1.755 to 2.215	No	ns	0.9904
L-Isoleucine vs. L-Leucine	-0.7764	-2.761 to 1.209	No	ns	0.7394
L-Isoleucine vs. Placebo	-1.91	-3.895 to 0.07526	No	ns	0.064
L-Leucine vs. Placebo	-1.133	-3.118 to 0.8517	No	ns	0.449

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.2408	-2.381	2.14	0.7629	36	36	3.967	132
L-Isoleucine and L-Leucine vs. L-Leucine	-0.2408	-1.604	1.363	0.7629	36	36	2.527	132
L-Isoleucine and L-Leucine vs. Placebo	-0.2408	-0.4708	0.23	0.7629	36	36	0.4264	132
L-Isoleucine vs. L-Leucine	-2.381	-1.604	-0.7764	0.7629	36	36	1.439	132
L-Isoleucine vs. Placebo	-2.381	-0.4708	-1.91	0.7629	36	36	3.54	132
L-Leucine vs. Placebo	-1.604	-0.4708	-1.133	0.7629	36	36	2.101	132

Table 17: 2-Way RMANOVA Δ Delta Glucose mmol/L 0-150 min

Two-way RM ANOVA	Matching: Both factors				
Alpha	0.05				
Source of Variation	% of total variation	P value	value summa	Significant?	
Time	54.76	<0.0001	****	Yes	
Treatment	0.4332	0.3314	ns	No	
Interaction: Time x Treatment	1.498	0.0277	*	Yes	
Interaction: Time x Subjects	19.32				
Interaction: Treatment x Subjects	4.029				
Subjects	9.851				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Time	535.7	9	59.52	F (9, 99) = 31.18	P<0.0001
Treatment	4.237	3	1.412	F (3, 33) = 1.183	P=0.3314
Interaction: Time x Treatment	14.65	27	0.5425	F (27, 297) = 1.631	P=0.0277
Interaction: Time x Subjects	189	99	1.909		
Interaction: Treatment x Subjects	39.41	33	1.194		
Subjects	96.36	11	8.76		
Residual	98.82	297	0.3327		

Table 18: 2-Way RMANOVA Δ Delta Glucose mmol/L 0-150 min

Two-way RM ANOVA Within each row, compare columns (simple effects within rows)

Number of families	10
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.06083	-0.6692 to 0.5476	No	ns	0.9939
L-Isoleucine and L-Leucine vs. L-Leucine	-0.035	-0.6434 to 0.5734	No	ns	0.9988
L-Isoleucine and L-Leucine vs. Placebo	0.1292	-0.4792 to 0.7376	No	ns	0.9469
L-Isoleucine vs. L-Leucine	0.02583	-0.5826 to 0.6342	No	ns	0.9995
L-Isoleucine vs. Placebo	0.19	-0.4184 to 0.7984	No	ns	0.8512
L-Leucine vs. Placebo	0.1642	-0.4442 to 0.7726	No	ns	0.8981
6					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.1917	-0.4167 to 0.8001	No	ns	0.8479
L-Isoleucine and L-Leucine vs. L-Leucine	0.1475	-0.4609 to 0.7559	No	ns	0.9233
L-Isoleucine and L-Leucine vs. Placebo	0.2083	-0.4001 to 0.8167	No	ns	0.8128
L-Isoleucine vs. L-Leucine	-0.04417	-0.6526 to 0.5642	No	ns	0.9977
L-Isoleucine vs. Placebo	0.01667	-0.5917 to 0.6251	No	ns	0.9999
L-Leucine vs. Placebo	0.06083	-0.5476 to 0.6692	No	ns	0.9939
10					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.03917	-0.5692 to 0.6476	No	ns	0.9984
L-Isoleucine and L-Leucine vs. L-Leucine	0.006667	-0.6017 to 0.6151	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. Placebo	0.08667	-0.5217 to 0.6951	No	ns	0.9829
L-Isoleucine vs. L-Leucine	-0.0325	-0.6409 to 0.5759	No	ns	0.9991
L-Isoleucine vs. Placebo	0.0475	-0.5609 to 0.6559	No	ns	0.9971
L-Leucine vs. Placebo	0.08	-0.5284 to 0.6884	No	ns	0.9865
30					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.01417	-0.5942 to 0.6226	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	-0.0025	-0.6109 to 0.6059	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. Placebo	-0.09417	-0.7026 to 0.5142	No	ns	0.9783
L-Isoleucine vs. L-Leucine	-0.01667	-0.6251 to 0.5917	No	ns	0.9999
L-Isoleucine vs. Placebo	-0.1083	-0.7167 to 0.5001	No	ns	0.9676
L-Leucine vs. Placebo	-0.09167	-0.7001 to 0.5167	No	ns	0.9799
36					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.1467	-0.7551 to 0.4617	No	ns	0.9247
L-Isoleucine and L-Leucine vs. L-Leucine	0.05833	-0.5501 to 0.6667	No	ns	0.9947
L-Isoleucine and L-Leucine vs. Placebo	-0.1658	-0.7742 to 0.4426	No	ns	0.8954
L-Isoleucine vs. L-Leucine	0.205	-0.4034 to 0.8134	No	ns	0.8201
L-Isoleucine vs. Placebo	-0.01917	-0.6276 to 0.5892	No	ns	0.9998
L-Leucine vs. Placebo	-0.2242	-0.8326 to 0.3842	No	ns	0.7767
40					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.245	-0.8534 to 0.3634	No	ns	0.7258
L-Isoleucine and L-Leucine vs. L-Leucine	-0.05083	-0.6592 to 0.5576	No	ns	0.9964
L-Isoleucine and L-Leucine vs. Placebo	-0.375	-0.9834 to 0.2334	No	ns	0.3845
L-Isoleucine vs. L-Leucine	0.1942	-0.4142 to 0.8026	No	ns	0.8428
L-Isoleucine vs. Placebo	-0.13	-0.7384 to 0.4784	No	ns	0.9459
L-Leucine vs. Placebo	-0.3242	-0.9326 to 0.2842	No	ns	0.515
60					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.315	-0.9234 to 0.2934	No	ns	0.5397
L-Isoleucine and L-Leucine vs. L-Leucine	-0.3392	-0.9476 to 0.2692	No	ns	0.4752
L-Isoleucine and L-Leucine vs. Placebo	-1.169	-1.778 to -0.5608	Yes	****	<0.0001
L-Isoleucine vs. L-Leucine	-0.02417	-0.6326 to 0.5842	No	ns	0.9996
L-Isoleucine vs. Placebo	-0.8542	-1.463 to -0.2458	Yes	**	0.0019
L-Leucine vs. Placebo	-0.83	-1.438 to -0.2216	Yes	**	0.0028
90					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.1242	-0.7326 to 0.4842	No	ns	0.9524
L-Isoleucine and L-Leucine vs. L-Leucine	-0.2325	-0.8409 to 0.3759	No	ns	0.7568
L-Isoleucine and L-Leucine vs. Placebo	-0.9992	-1.608 to -0.3908	Yes	***	0.0002
L-Isoleucine vs. L-Leucine	-0.1083	-0.7167 to 0.5001	No	ns	0.9676
L-Isoleucine vs. Placebo	-0.875	-1.483 to -0.2666	Yes	**	0.0014
L-Leucine vs. Placebo	-0.7667	-1.375 to -0.1583	Yes	**	0.0069
120					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.1733	-0.4351 to 0.7817	No	ns	0.8825
L-Isoleucine and L-Leucine vs. L-Leucine	0.1508	-0.4576 to 0.7592	No	ns	0.9188
L-Isoleucine and L-Leucine vs. Placebo	0.07083	-0.5376 to 0.6792	No	ns	0.9905
L-Isoleucine vs. L-Leucine	-0.0225	-0.6309 to 0.5859	No	ns	0.9997
L-Isoleucine vs. Placebo	-0.1025	-0.7109 to 0.5059	No	ns	0.9723
L-Leucine vs. Placebo	-0.08	-0.6884 to 0.5284	No	ns	0.9865
150					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.0025	-0.6059 to 0.6109	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	-0.08167	-0.6901 to 0.5267	No	ns	0.9856
L-Isoleucine and L-Leucine vs. Placebo	-0.1058	-0.7142 to 0.5026	No	ns	0.9697
L-Isoleucine vs. L-Leucine	-0.08417	-0.6926 to 0.5242	No	ns	0.9843
L-Isoleucine vs. Placebo	-0.1083	-0.7167 to 0.5001	No	ns	0.9676
L-Leucine vs. Placebo	-0.02417	-0.6326 to 0.5842	No	ns	0.9996

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
0								
L-Isoleucine and L-Leucine vs. L-Isoleucine	4.783	4.844	-0.06083	0.2355	12	12	0.3653	297
L-Isoleucine and L-Leucine vs. L-Leucine	4.783	4.818	-0.035	0.2355	12	12	0.2102	297
L-Isoleucine and L-Leucine vs. Placebo	4.783	4.654	0.1292	0.2355	12	12	0.7757	297
L-Isoleucine vs. L-Leucine	4.844	4.818	0.02583	0.2355	12	12	0.1551	297
L-Isoleucine vs. Placebo	4.844	4.654	0.19	0.2355	12	12	1.141	297
L-Leucine vs. Placebo	4.818	4.654	0.1642	0.2355	12	12	0.9859	297
6								
L-Isoleucine and L-Leucine vs. L-Isoleucine	4.79	4.598	0.1917	0.2355	12	12	1.151	297
L-Isoleucine and L-Leucine vs. L-Leucine	4.79	4.643	0.1475	0.2355	12	12	0.8858	297
L-Isoleucine and L-Leucine vs. Placebo	4.79	4.582	0.2083	0.2355	12	12	1.251	297
L-Isoleucine vs. L-Leucine	4.598	4.643	-0.04417	0.2355	12	12	0.2652	297
L-Isoleucine vs. Placebo	4.598	4.582	0.01667	0.2355	12	12	0.1001	297
L-Leucine vs. Placebo	4.643	4.582	0.06083	0.2355	12	12	0.3653	297
10								
L-Isoleucine and L-Leucine vs. L-Isoleucine	4.736	4.697	0.03917	0.2355	12	12	0.2352	297
L-Isoleucine and L-Leucine vs. L-Leucine	4.736	4.729	0.006667	0.2355	12	12	0.04004	297
L-Isoleucine and L-Leucine vs. Placebo	4.736	4.649	0.08667	0.2355	12	12	0.5205	297
L-Isoleucine vs. L-Leucine	4.697	4.729	-0.0325	0.2355	12	12	0.1952	297
L-Isoleucine vs. Placebo	4.697	4.649	0.0475	0.2355	12	12	0.2853	297
L-Leucine vs. Placebo	4.729	4.649	0.08	0.2355	12	12	0.4804	297
30								
L-Isoleucine and L-Leucine vs. L-Isoleucine	4.6	4.586	0.01417	0.2355	12	12	0.08508	297
L-Isoleucine and L-Leucine vs. L-Leucine	4.6	4.603	-0.0025	0.2355	12	12	0.01501	297
L-Isoleucine and L-Leucine vs. Placebo	4.6	4.694	-0.09417	0.2355	12	12	0.5655	297
L-Isoleucine vs. L-Leucine	4.586	4.603	-0.01667	0.2355	12	12	0.1001	297
L-Isoleucine vs. Placebo	4.586	4.694	-0.1083	0.2355	12	12	0.6506	297
L-Leucine vs. Placebo	4.603	4.694	-0.09167	0.2355	12	12	0.5505	297
36								
L-Isoleucine and L-Leucine vs. L-Isoleucine	4.844	4.991	-0.1467	0.2355	12	12	0.8808	297
L-Isoleucine and L-Leucine vs. L-Leucine	4.844	4.786	0.05833	0.2355	12	12	0.3503	297
L-Isoleucine and L-Leucine vs. Placebo	4.844	5.01	-0.1658	0.2355	12	12	0.9959	297
L-Isoleucine vs. L-Leucine	4.991	4.786	0.205	0.2355	12	12	1.231	297
L-Isoleucine vs. Placebo	4.991	5.01	-0.01917	0.2355	12	12	0.1151	297
L-Leucine vs. Placebo	4.786	5.01	-0.2242	0.2355	12	12	1.346	297
40								
L-Isoleucine and L-Leucine vs. L-Isoleucine	5.522	5.767	-0.245	0.2355	12	12	1.471	297
L-Isoleucine and L-Leucine vs. L-Leucine	5.522	5.573	-0.05083	0.2355	12	12	0.3053	297
L-Isoleucine and L-Leucine vs. Placebo	5.522	5.897	-0.375	0.2355	12	12	2.252	297
L-Isoleucine vs. L-Leucine	5.767	5.573	0.1942	0.2355	12	12	1.166	297
L-Isoleucine vs. Placebo	5.767	5.897	-0.13	0.2355	12	12	0.7807	297
L-Leucine vs. Placebo	5.573	5.897	-0.3242	0.2355	12	12	1.947	297
60								
L-Isoleucine and L-Leucine vs. L-Isoleucine	7.151	7.466	-0.315	0.2355	12	12	1.892	297
L-Isoleucine and L-Leucine vs. L-Leucine	7.151	7.49	-0.3392	0.2355	12	12	2.037	297
L-Isoleucine and L-Leucine vs. Placebo	7.151	8.32	-1.169	0.2355	12	12	7.021	297
L-Isoleucine vs. L-Leucine	7.466	7.49	-0.02417	0.2355	12	12	0.1451	297
L-Isoleucine vs. Placebo	7.466	8.32	-0.8542	0.2355	12	12	5.13	297
L-Leucine vs. Placebo	7.49	8.32	-0.83	0.2355	12	12	4.985	297
90								
L-Isoleucine and L-Leucine vs. L-Isoleucine	6.789	6.913	-0.1242	0.2355	12	12	0.7457	297
L-Isoleucine and L-Leucine vs. L-Leucine	6.789	7.022	-0.2325	0.2355	12	12	1.396	297
L-Isoleucine and L-Leucine vs. Placebo	6.789	7.788	-0.9992	0.2355	12	12	6.001	297
L-Isoleucine vs. L-Leucine	6.913	7.022	-0.1083	0.2355	12	12	0.6506	297
L-Isoleucine vs. Placebo	6.913	7.788	-0.875	0.2355	12	12	5.255	297
L-Leucine vs. Placebo	7.022	7.788	-0.7667	0.2355	12	12	4.604	297
120								
L-Isoleucine and L-Leucine vs. L-Isoleucine	6.634	6.461	0.1733	0.2355	12	12	1.041	297
L-Isoleucine and L-Leucine vs. L-Leucine	6.634	6.483	0.1508	0.2355	12	12	0.9058	297
L-Isoleucine and L-Leucine vs. Placebo	6.634	6.563	0.07083	0.2355	12	12	0.4254	297
L-Isoleucine vs. L-Leucine	6.461	6.483	-0.0225	0.2355	12	12	0.1351	297
L-Isoleucine vs. Placebo	6.461	6.563	-0.1025	0.2355	12	12	0.6156	297
L-Leucine vs. Placebo	6.483	6.563	-0.08	0.2355	12	12	0.4804	297
150								
L-Isoleucine and L-Leucine vs. L-Isoleucine	5.91	5.908	0.0025	0.2355	12	12	0.01501	297
L-Isoleucine and L-Leucine vs. L-Leucine	5.91	5.992	-0.08167	0.2355	12	12	0.4905	297
L-Isoleucine and L-Leucine vs. Placebo	5.91	6.016	-0.1058	0.2355	12	12	0.6356	297
L-Isoleucine vs. L-Leucine	5.908	5.992	-0.08417	0.2355	12	12	0.5055	297
L-Isoleucine vs. Placebo	5.908	6.016	-0.1083	0.2355	12	12	0.6506	297
L-Leucine vs. Placebo	5.992	6.016	-0.02417	0.2355	12	12	0.1451	297

Table 19: 2-Way RMANOVA Δ Delta Glucose mmol/L 0-150 min

Two-way RM ANOVA	Compare column means (main column effect)				
Number of families	1				
Number of comparisons per family	6				
Alpha	0.05				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.01533	-0.316 to 0.3466	No	ns	0.9994
L-Isoleucine and L-Leucine vs. L-Leucine	-0.001583	-0.3329 to 0.3297	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. Placebo	-0.3705	-0.7018 to -0.0392	Yes	*	0.0214
L-Isoleucine vs. L-Leucine	-0.01692	-0.3482 to 0.3144	No	ns	0.9992
L-Isoleucine vs. Placebo	-0.3858	-0.7171 to -0.05454	Yes	*	0.0149
L-Leucine vs. Placebo	-0.3689	-0.7002 to -0.03762	Yes	*	0.0222

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.7928	0.7775	0.01533	0.1285	120	120	0.1688	440
L-Isoleucine and L-Leucine vs. L-Leucine	0.7928	0.7944	-0.001583	0.1285	120	120	0.01743	440
L-Isoleucine and L-Leucine vs. Placebo	0.7928	1.163	-0.3705	0.1285	120	120	4.079	440
L-Isoleucine vs. L-Leucine	0.7775	0.7944	-0.01692	0.1285	120	120	0.1862	440
L-Isoleucine vs. Placebo	0.7775	1.163	-0.3858	0.1285	120	120	4.247	440
L-Leucine vs. Placebo	0.7944	1.163	-0.3689	0.1285	120	120	4.061	440

Table 20: 2-Way RMANOVA Δ Delta Glucose from Amino Acid mmol/L 0-30 min

Two-way RM ANOVA	Matching: Both factors			
Alpha	0.05			
Source of Variation	% of total variation	P value	P value summary	Significant?
Time	7.035	0.0104	*	Yes
Treatment	7.283	0.0151	*	Yes
Interaction: Time x Treatment	6.009	0.0013	**	Yes
Interaction: Time x Subjects	17.62			
Interaction: Treatment x Subjects	19.88			
Subjects	22.42			

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Time	0.66	3	0.22	F (3, 33) = 4.393	P=0.0104
Treatment	0.6833	3	0.2278	F (3, 33) = 4.03	P=0.0151
Interaction: Time x Treatment	0.5637	9	0.06263	F (9, 99) = 3.345	P=0.0013
Interaction: Time x Subjects	1.653	33	0.05008		
Interaction: Treatment x Subjects	1.865	33	0.05651		
Subjects	2.103	11	0.1912		
Residual	1.853	99	0.01872		

Table 21: 2-Way RMANOVA Δ Delta Glucose from Amino Acid mmol/L 0-30 min

Two-way RM ANOVA Compare column means (main column effect)					
Number of families	1				
Number of comparisons per family	6				
Alpha	0.05				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	djusted P Value
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.1085	-0.02272 to 0.2398	No	ns	0.1344
L-Isoleucine and L-Leucine vs. L-Leucine	0.06563	-0.06563 to 0.1969	No	ns	0.5372
L-Isoleucine and L-Leucine vs. Placebo	-0.04688	-0.1781 to 0.08438	No	ns	0.7695
L-Isoleucine vs. L-Leucine	-0.04292	-0.1742 to 0.08834	No	ns	0.8129
L-Isoleucine vs. Placebo	-0.1554	-0.2867 to -0.02416	Yes	*	0.0152
L-Leucine vs. Placebo	-0.1125	-0.2438 to 0.01876	No	ns	0.1144

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.05604	-0.1646	0.1085	0.04853	48	48	3.163	33
L-Isoleucine and L-Leucine vs. L-Leucine	-0.05604	-0.1217	0.06563	0.04853	48	48	1.913	33
L-Isoleucine and L-Leucine vs. Placebo	-0.05604	-0.009167	-0.04688	0.04853	48	48	1.366	33
L-Isoleucine vs. L-Leucine	-0.1646	-0.1217	-0.04292	0.04853	48	48	1.251	33
L-Isoleucine vs. Placebo	-0.1646	-0.009167	-0.1554	0.04853	48	48	4.529	33
L-Leucine vs. Placebo	-0.1217	-0.009167	-0.1125	0.04853	48	48	3.279	33

Table 22: 2-Way RMANOVA Δ Delta Glucose from Amino Acid mmol/L 0-30 min

Two-way RM ANOVA Within each row, compare columns (simple effects within rows)					
Number of families	4				
Number of comparisons per family	6				
Alpha	0.05				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	-0.146 to 0.146	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	0	-0.146 to 0.146	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. Placebo	0	-0.146 to 0.146	No	ns	>0.9999
L-Isoleucine vs. L-Leucine	0	-0.146 to 0.146	No	ns	>0.9999
L-Isoleucine vs. Placebo	0	-0.146 to 0.146	No	ns	>0.9999
L-Leucine vs. Placebo	0	-0.146 to 0.146	No	ns	>0.9999
6					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.2558	0.1099 to 0.4018	Yes	****	<0.0001
L-Isoleucine and L-Leucine vs. L-Leucine	0.1842	0.03819 to 0.3301	Yes	**	0.0073
L-Isoleucine and L-Leucine vs. Placebo	0.07917	-0.06681 to 0.2251	No	ns	0.4917
L-Isoleucine vs. L-Leucine	-0.07167	-0.2176 to 0.07431	No	ns	0.5759
L-Isoleucine vs. Placebo	-0.1767	-0.3226 to -0.03069	Yes	*	0.011
L-Leucine vs. Placebo	-0.105	-0.251 to 0.04097	No	ns	0.2434
10					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.1017	-0.04431 to 0.2476	No	ns	0.2701
L-Isoleucine and L-Leucine vs. L-Leucine	0.0425	-0.1035 to 0.1885	No	ns	0.8718
L-Isoleucine and L-Leucine vs. Placebo	-0.0425	-0.1885 to 0.1035	No	ns	0.8718
L-Isoleucine vs. L-Leucine	-0.05917	-0.2051 to 0.08681	No	ns	0.715
L-Isoleucine vs. Placebo	-0.1442	-0.2901 to 0.001807	No	ns	0.0542
L-Leucine vs. Placebo	-0.085	-0.231 to 0.06097	No	ns	0.4285
30					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.07667	-0.06931 to 0.2226	No	ns	0.5195
L-Isoleucine and L-Leucine vs. L-Leucine	0.03583	-0.1101 to 0.1818	No	ns	0.9183
L-Isoleucine and L-Leucine vs. Placebo	-0.2242	-0.3701 to -0.07819	Yes	***	0.0007
L-Isoleucine vs. L-Leucine	-0.04083	-0.1868 to 0.1051	No	ns	0.8844
L-Isoleucine vs. Placebo	-0.3008	-0.4468 to -0.1549	Yes	****	<0.0001
L-Leucine vs. Placebo	-0.26	-0.406 to -0.114	Yes	****	<0.0001

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
0								
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	0	0	0.05586	12	12	0	99
L-Isoleucine and L-Leucine vs. L-Leucine	0	0	0	0.05586	12	12	0	99
L-Isoleucine and L-Leucine vs. Placebo	0	0	0	0.05586	12	12	0	99
L-Isoleucine vs. L-Leucine	0	0	0	0.05586	12	12	0	99
L-Isoleucine vs. Placebo	0	0	0	0.05586	12	12	0	99
L-Leucine vs. Placebo	0	0	0	0.05586	12	12	0	99
6								
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.006667	-0.2492	0.2558	0.05586	12	12	6.477	99
L-Isoleucine and L-Leucine vs. L-Leucine	0.006667	-0.1775	0.1842	0.05586	12	12	4.663	99
L-Isoleucine and L-Leucine vs. Placebo	0.006667	-0.0725	0.07917	0.05586	12	12	2.004	99
L-Isoleucine vs. L-Leucine	-0.2492	-0.1775	-0.07167	0.05586	12	12	1.814	99
L-Isoleucine vs. Placebo	-0.2492	-0.0725	-0.1767	0.05586	12	12	4.473	99
L-Leucine vs. Placebo	-0.1775	-0.0725	-0.105	0.05586	12	12	2.658	99
10								
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.0475	-0.1492	0.1017	0.05586	12	12	2.574	99
L-Isoleucine and L-Leucine vs. L-Leucine	-0.0475	-0.09	0.0425	0.05586	12	12	1.076	99
L-Isoleucine and L-Leucine vs. Placebo	-0.0475	-0.005	-0.0425	0.05586	12	12	1.076	99
L-Isoleucine vs. L-Leucine	-0.1492	-0.09	-0.05917	0.05586	12	12	1.498	99
L-Isoleucine vs. Placebo	-0.1492	-0.005	-0.1442	0.05586	12	12	3.65	99
L-Leucine vs. Placebo	-0.09	-0.005	-0.085	0.05586	12	12	2.152	99
30								
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.1833	-0.26	0.07667	0.05586	12	12	1.941	99
L-Isoleucine and L-Leucine vs. L-Leucine	-0.1833	-0.2192	0.03583	0.05586	12	12	0.9072	99
L-Isoleucine and L-Leucine vs. Placebo	-0.1833	0.04083	-0.2242	0.05586	12	12	5.675	99
L-Isoleucine vs. L-Leucine	-0.26	-0.2192	-0.04083	0.05586	12	12	1.034	99
L-Isoleucine vs. Placebo	-0.26	0.04083	-0.3008	0.05586	12	12	7.616	99
L-Leucine vs. Placebo	-0.2192	0.04083	-0.26	0.05586	12	12	6.582	99

Table 23: 2-Way RMANOVA Δ Delta Glucose from Amino Acid mmol/L 0-30 min

Two-way RM ANOVA	Compare column means (main column effect)							
Compare column means (main column effect)								
Number of families	1							
Number of comparisons per family	3							
Alpha	0.05							
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
Placebo vs. L-Isoleucine and L-Leucine	-0.23	-2.259 to 1.799	No	ns	0.985			
Placebo vs. L-Isoleucine	1.91	-0.1192 to 3.939	No	ns	0.0687			
Placebo vs. L-Leucine	1.133	-0.8956 to 3.162	No	ns	0.3883			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
Placebo vs. L-Isoleucine and L-Leucine	-0.4708	-0.2408	-0.23	0.8241	36	36	0.2791	33
Placebo vs. L-Isoleucine	-0.4708	-2.381	1.91	0.8241	36	36	2.317	33
Placebo vs. L-Leucine	-0.4708	-1.604	1.133	0.8241	36	36	1.375	33

Table 24: 2-Way RMANOVA Δ Delta Insulin pmol/L 0-150 min

Two-way RM ANOVA	Matching: Both factors				
Alpha	0.05				
Source of Variation	% of total variation	P value	value summa	Significant?	
Interaction	1.178	0.9934	ns	No	
Time	51.88	<0.0001	****	Yes	
Treatment	1.112	0.0531	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	584661	21	27841	F (21, 320) = 0.3916	P=0.9934
Time	25751346	7	3678764	F (7, 320) = 51.75	P<0.0001
Treatment	551770	3	183923	F (3, 320) = 2.587	P=0.0531
Residual	22747947	320	71087		
Number of missing values	0				

Table 26: 2-Way RMANOVA Δ Delta Insulin pmol/L 0-150 min

Two-way RM ANOVA Within each row, compare columns (simple effects within rows)

Number of comparisons per	6				
Alpha	0.05				
Tukey's multiple comparisor	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
ISO and LEU vs. ISO	0	-293.6 to 293.6	No	ns	>0.9999
ISO and LEU vs. LEU	0	-293.6 to 293.6	No	ns	>0.9999
ISO and LEU vs. PLA	0	-293.6 to 293.6	No	ns	>0.9999
ISO vs. LEU	0	-293.6 to 293.6	No	ns	>0.9999
ISO vs. PLA	0	-293.6 to 293.6	No	ns	>0.9999
LEU vs. PLA	0	-293.6 to 293.6	No	ns	>0.9999
10					
ISO and LEU vs. ISO	11.84	-281.8 to 305.5	No	ns	0.9996
ISO and LEU vs. LEU	11.1	-282.5 to 304.7	No	ns	0.9997
ISO and LEU vs. PLA	19.49	-274.1 to 313.1	No	ns	0.9982
ISO vs. LEU	-0.7386	-294.3 to 292.9	No	ns	>0.9999
ISO vs. PLA	7.65	-286 to 301.3	No	ns	0.9999
LEU vs. PLA	8.389	-285.2 to 302	No	ns	0.9999
30					
ISO and LEU vs. ISO	23.53	-270.1 to 317.1	No	ns	0.9969
ISO and LEU vs. LEU	28.2	-265.4 to 321.8	No	ns	0.9946
ISO and LEU vs. PLA	50.18	-243.4 to 343.8	No	ns	0.9712
ISO vs. LEU	4.674	-288.9 to 298.3	No	ns	>0.9999
ISO vs. PLA	26.65	-267 to 320.3	No	ns	0.9955
LEU vs. PLA	21.98	-271.6 to 315.6	No	ns	0.9974
40					
ISO and LEU vs. ISO	-24.99	-318.6 to 268.6	No	ns	0.9962
ISO and LEU vs. LEU	83.84	-209.8 to 377.4	No	ns	0.8819
ISO and LEU vs. PLA	157.3	-136.3 to 451	No	ns	0.5103
ISO vs. LEU	108.8	-184.8 to 402.4	No	ns	0.7738
ISO vs. PLA	182.3	-111.3 to 475.9	No	ns	0.3779
LEU vs. PLA	73.51	-220.1 to 367.1	No	ns	0.9167
60					
ISO and LEU vs. ISO	83.44	-210.2 to 377	No	ns	0.8834
ISO and LEU vs. LEU	66.04	-227.6 to 359.6	No	ns	0.9378
ISO and LEU vs. PLA	247	-46.56 to 540.7	No	ns	0.133
ISO vs. LEU	-17.4	-311 to 276.2	No	ns	0.9987
ISO vs. PLA	163.6	-130 to 457.2	No	ns	0.4759
LEU vs. PLA	181	-112.6 to 474.6	No	ns	0.3846
90					
ISO and LEU vs. ISO	148.4	-145.3 to 442	No	ns	0.5604
ISO and LEU vs. LEU	56.47	-237.1 to 350.1	No	ns	0.9598
ISO and LEU vs. PLA	62.93	-230.7 to 356.5	No	ns	0.9456
ISO vs. LEU	-91.88	-385.5 to 201.7	No	ns	0.8506
ISO vs. PLA	-85.43	-379 to 208.2	No	ns	0.876
LEU vs. PLA	6.452	-287.2 to 300.1	No	ns	>0.9999
120					
ISO and LEU vs. ISO	104.1	-189.5 to 397.7	No	ns	0.7964
ISO and LEU vs. LEU	58.11	-235.5 to 351.7	No	ns	0.9564
ISO and LEU vs. PLA	229.8	-63.84 to 523.4	No	ns	0.1824
ISO vs. LEU	-46.02	-339.6 to 247.6	No	ns	0.9775
ISO vs. PLA	125.6	-168 to 419.2	No	ns	0.6866
LEU vs. PLA	171.7	-121.9 to 465.3	No	ns	0.4327
150					
ISO and LEU vs. ISO	88.73	-204.9 to 382.3	No	ns	0.8633
ISO and LEU vs. LEU	64.36	-229.3 to 358	No	ns	0.9421
ISO and LEU vs. PLA	124.4	-169.2 to 418	No	ns	0.6932
ISO vs. LEU	-24.37	-318 to 269.2	No	ns	0.9965
ISO vs. PLA	35.7	-257.9 to 329.3	No	ns	0.9893
LEU vs. PLA	60.07	-233.5 to 353.7	No	ns	0.9522

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
0								
ISO and LEU vs. ISO	0	0	0	113.7	11	11	0	320
ISO and LEU vs. LEU	0	0	0	113.7	11	11	0	320
ISO and LEU vs. PLA	0	0	0	113.7	11	11	0	320
ISO vs. LEU	0	0	0	113.7	11	11	0	320
ISO vs. PLA	0	0	0	113.7	11	11	0	320
LEU vs. PLA	0	0	0	113.7	11	11	0	320
10								
ISO and LEU vs. ISO	21.2	9.354	11.84	113.7	11	11	0.1473	320
ISO and LEU vs. LEU	21.2	10.09	11.1	113.7	11	11	0.1381	320
ISO and LEU vs. PLA	21.2	1.703	19.49	113.7	11	11	0.2425	320
ISO vs. LEU	9.354	10.09	-0.7386	113.7	11	11	0.009188	320
ISO vs. PLA	9.354	1.703	7.65	113.7	11	11	0.09517	320
LEU vs. PLA	10.09	1.703	8.389	113.7	11	11	0.1044	320
30								
ISO and LEU vs. ISO	69.9	46.37	23.53	113.7	11	11	0.2927	320
ISO and LEU vs. LEU	69.9	41.69	28.2	113.7	11	11	0.3508	320
ISO and LEU vs. PLA	69.9	19.71	50.18	113.7	11	11	0.6243	320
ISO vs. LEU	46.37	41.69	4.674	113.7	11	11	0.05815	320
ISO vs. PLA	46.37	19.71	26.65	113.7	11	11	0.3316	320
LEU vs. PLA	41.69	19.71	21.98	113.7	11	11	0.2734	320
40								
ISO and LEU vs. ISO	405	430	-24.99	113.7	11	11	0.3109	320
ISO and LEU vs. LEU	405	321.2	83.84	113.7	11	11	1.043	320
ISO and LEU vs. PLA	405	247.7	157.3	113.7	11	11	1.957	320
ISO vs. LEU	430	321.2	108.8	113.7	11	11	1.354	320
ISO vs. PLA	430	247.7	182.3	113.7	11	11	2.268	320
LEU vs. PLA	321.2	247.7	73.51	113.7	11	11	0.9144	320
60								
ISO and LEU vs. ISO	863.2	779.7	83.44	113.7	11	11	1.038	320
ISO and LEU vs. LEU	863.2	797.1	66.04	113.7	11	11	0.8215	320
ISO and LEU vs. PLA	863.2	616.1	247	113.7	11	11	3.073	320
ISO vs. LEU	779.7	797.1	-17.4	113.7	11	11	0.2164	320
ISO vs. PLA	779.7	616.1	163.6	113.7	11	11	2.035	320
LEU vs. PLA	797.1	616.1	181	113.7	11	11	2.252	320
90								
ISO and LEU vs. ISO	637.1	488.7	148.4	113.7	11	11	1.845	320
ISO and LEU vs. LEU	637.1	580.6	56.47	113.7	11	11	0.7025	320
ISO and LEU vs. PLA	637.1	574.1	62.93	113.7	11	11	0.7828	320
ISO vs. LEU	488.7	580.6	-91.88	113.7	11	11	1.143	320
ISO vs. PLA	488.7	574.1	-85.43	113.7	11	11	1.063	320
LEU vs. PLA	580.6	574.1	6.452	113.7	11	11	0.08026	320
120								
ISO and LEU vs. ISO	634.5	530.4	104.1	113.7	11	11	1.295	320
ISO and LEU vs. LEU	634.5	576.4	58.11	113.7	11	11	0.7229	320
ISO and LEU vs. PLA	634.5	404.7	229.8	113.7	11	11	2.858	320
ISO vs. LEU	530.4	576.4	-46.02	113.7	11	11	0.5725	320
ISO vs. PLA	530.4	404.7	125.6	113.7	11	11	1.563	320
LEU vs. PLA	576.4	404.7	171.7	113.7	11	11	2.135	320
150								
ISO and LEU vs. ISO	460.5	371.8	88.73	113.7	11	11	1.104	320
ISO and LEU vs. LEU	460.5	396.2	64.36	113.7	11	11	0.8005	320
ISO and LEU vs. PLA	460.5	336.1	124.4	113.7	11	11	1.548	320
ISO vs. LEU	371.8	396.2	-24.37	113.7	11	11	0.3032	320
ISO vs. PLA	371.8	336.1	35.7	113.7	11	11	0.444	320
LEU vs. PLA	396.2	336.1	60.07	113.7	11	11	0.7472	320

Table 27: 2-Way RMANOVA Δ Delta Insulin pmol/L from Amino Acid 0-30 min

Two-way RM ANOVA	Matching: Both factors				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	2.859	0.5978	ns	No	
Time	18.94	<0.0001	****	Yes	
Treatment	3.579	0.1302	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	7152	6	1192	F (6, 120) = 0.7663	P=0.5978
Time	47370	2	23685	F (2, 120) = 15.23	P<0.0001
Treatment	8952	3	2984	F (3, 120) = 1.918	P=0.1302
Residual	186656	120	1555		
Number of missing values	0				

Table 28: 2-Way RMANOVA Δ Insulin pmol/L from Amino Acid 0-30 min

Two-way RM ANOVA Compare column means (main column effect)					
Number of families	1				
Number of comparisons pe	6				
Alpha	0.05				
Tukey's multiple comparisc	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
ISO and LEU vs. ISO	11.79	-13.51 to 37.09	No	ns	0.619
ISO and LEU vs. LEU	13.1	-12.19 to 38.4	No	ns	0.5335
ISO and LEU vs. PLA	23.23	-2.071 to 48.52	No	ns	0.0841
ISO vs. LEU	1.312	-23.98 to 26.61	No	ns	0.9991
ISO vs. PLA	11.43	-13.86 to 36.73	No	ns	0.642
LEU vs. PLA	10.12	-15.17 to 35.42	No	ns	0.7248

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
ISO and LEU vs. ISO	30.37	18.57	11.79	9.709	33	33	1.717	120
ISO and LEU vs. LEU	30.37	17.26	13.1	9.709	33	33	1.908	120
ISO and LEU vs. PLA	30.37	7.139	23.23	9.709	33	33	3.383	120
ISO vs. LEU	18.57	17.26	1.312	9.709	33	33	0.1911	120
ISO vs. PLA	18.57	7.139	11.43	9.709	33	33	1.666	120
LEU vs. PLA	17.26	7.139	10.12	9.709	33	33	1.474	120

Table 29: 2-Way RMANOVA Δ Delta Insulin pmol/L from Amino Acid 0-30 min

Two-way RM ANOVA Within each row, compare columns (simple effects within rows)

Number of families	3
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
ISO and LEU vs. ISO	0	-43.81 to 43.81	No	ns	>0.9999
ISO and LEU vs. LEU	0	-43.81 to 43.81	No	ns	>0.9999
ISO and LEU vs. PLA	0	-43.81 to 43.81	No	ns	>0.9999
ISO vs. LEU	0	-43.81 to 43.81	No	ns	>0.9999
ISO vs. PLA	0	-43.81 to 43.81	No	ns	>0.9999
LEU vs. PLA	0	-43.81 to 43.81	No	ns	>0.9999
10					
ISO and LEU vs. ISO	11.84	-31.97 to 55.66	No	ns	0.8952
ISO and LEU vs. LEU	11.1	-32.71 to 54.92	No	ns	0.9117
ISO and LEU vs. PLA	19.49	-24.32 to 63.31	No	ns	0.6537
ISO vs. LEU	-0.7386	-44.55 to 43.08	No	ns	>0.9999
ISO vs. PLA	7.65	-36.16 to 51.47	No	ns	0.9686
LEU vs. PLA	8.389	-35.43 to 52.2	No	ns	0.9592
30					
ISO and LEU vs. ISO	23.53	-20.29 to 67.34	No	ns	0.5024
ISO and LEU vs. LEU	28.2	-15.61 to 72.02	No	ns	0.3403
ISO and LEU vs. PLA	50.18	6.369 to 94	Yes	*	0.0179
ISO vs. LEU	4.674	-39.14 to 48.49	No	ns	0.9925
ISO vs. PLA	26.65	-17.16 to 70.47	No	ns	0.391
LEU vs. PLA	21.98	-21.83 to 65.8	No	ns	0.5603

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
0								
ISO and LEU vs. ISO	0	0	0	16.82	11	11	0	120
ISO and LEU vs. LEU	0	0	0	16.82	11	11	0	120
ISO and LEU vs. PLA	0	0	0	16.82	11	11	0	120
ISO vs. LEU	0	0	0	16.82	11	11	0	120
ISO vs. PLA	0	0	0	16.82	11	11	0	120
LEU vs. PLA	0	0	0	16.82	11	11	0	120
10								
ISO and LEU vs. ISO	21.2	9.354	11.84	16.82	11	11	0.9959	120
ISO and LEU vs. LEU	21.2	10.09	11.1	16.82	11	11	0.9338	120
ISO and LEU vs. PLA	21.2	1.703	19.49	16.82	11	11	1.639	120
ISO vs. LEU	9.354	10.09	-0.7386	16.82	11	11	0.06211	120
ISO vs. PLA	9.354	1.703	7.65	16.82	11	11	0.6433	120
LEU vs. PLA	10.09	1.703	8.389	16.82	11	11	0.7055	120
30								
ISO and LEU vs. ISO	69.9	46.37	23.53	16.82	11	11	1.979	120
ISO and LEU vs. LEU	69.9	41.69	28.2	16.82	11	11	2.372	120
ISO and LEU vs. PLA	69.9	19.71	50.18	16.82	11	11	4.22	120
ISO vs. LEU	46.37	41.69	4.674	16.82	11	11	0.3931	120
ISO vs. PLA	46.37	19.71	26.65	16.82	11	11	2.241	120
LEU vs. PLA	41.69	19.71	21.98	16.82	11	11	1.848	120

Table 30: 2-Way RMANOVA Δ Delta C-peptide nmol/L 0-150 min

Two-way RM ANOVA	Matching: Both factors				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Time	65.09	<0.0001	****	Yes	
Treatment	1.01	0.0414	*	Yes	
Interaction: Time x Treatment	0.8716	0.2124	ns	No	
Interaction: Time x Subjects	15				
Interaction: Treatment x Subjects	3.256				
Subjects	7.801				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Time	10220	7	1460	F (7, 70) = 43.38	P<0.0001
Treatment	158.6	3	52.86	F (3, 30) = 3.102	P=0.0414
Interaction: Time x Treatment	136.9	21	6.517	F (21, 210) = 1.251	P=0.2124
Interaction: Time x Subjects	2356	70	33.65		
Interaction: Treatment x Subjects	511.2	30	17.04		
Subjects	1225	10	122.5		
Residual	1094	210	5.211		

Table 31: 2-Way RMANOVA Δ C-peptide nmol/L 0-150 min

Two-way RM ANOVA Compare column means (main column effect)								
Number of families	1							
Number of comparisons per family	6							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.1367	-1.829 to 1.555	No	ns	0.9962			
L-Isoleucine and L-Leucine vs. L-Leucine	0.5319	-1.16 to 2.224	No	ns	0.8278			
L-Isoleucine and L-Leucine vs. Placebo	1.57	-0.1217 to 3.263	No	ns	0.0764			
L-Isoleucine vs. L-Leucine	0.6686	-1.023 to 2.361	No	ns	0.7074			
L-Isoleucine vs. Placebo	1.707	0.01503 to 3.399	Yes	*	0.0474			
L-Leucine vs. Placebo	1.039	-0.6536 to 2.731	No	ns	0.3573			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
L-Isoleucine and L-Leucine vs. L-Isoleucine	7.184	7.321	-0.1367	0.6223	88	88	0.3107	30
L-Isoleucine and L-Leucine vs. L-Leucine	7.184	6.652	0.5319	0.6223	88	88	1.209	30
L-Isoleucine and L-Leucine vs. Placebo	7.184	5.614	1.57	0.6223	88	88	3.569	30
L-Isoleucine vs. L-Leucine	7.321	6.652	0.6686	0.6223	88	88	1.519	30
L-Isoleucine vs. Placebo	7.321	5.614	1.707	0.6223	88	88	3.88	30
L-Leucine vs. Placebo	6.652	5.614	1.039	0.6223	88	88	2.36	30

Table 32: 2-Way RMANOVA Δ Delta C-peptide nmol/L 0-150 min

Two-way RM ANOVA Within each row, compare columns (simple effects within rows)

Number of families	8
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	-2.521 to 2.521	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	0	-2.521 to 2.521	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. Placebo	0	-2.521 to 2.521	No	ns	>0.9999
L-Isoleucine vs. L-Leucine	0	-2.521 to 2.521	No	ns	>0.9999
L-Isoleucine vs. Placebo	0	-2.521 to 2.521	No	ns	>0.9999
L-Leucine vs. Placebo	0	-2.521 to 2.521	No	ns	>0.9999
10					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.03	-2.491 to 2.551	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	-0.1291	-2.65 to 2.392	No	ns	0.9992
L-Isoleucine and L-Leucine vs. Placebo	0.1564	-2.364 to 2.677	No	ns	0.9985
L-Isoleucine vs. L-Leucine	-0.1591	-2.68 to 2.362	No	ns	0.9984
L-Isoleucine vs. Placebo	0.1264	-2.394 to 2.647	No	ns	0.9992
L-Leucine vs. Placebo	0.2855	-2.235 to 2.806	No	ns	0.9912
30					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.08545	-2.606 to 2.435	No	ns	0.9998
L-Isoleucine and L-Leucine vs. L-Leucine	0.09818	-2.423 to 2.619	No	ns	0.9996
L-Isoleucine and L-Leucine vs. Placebo	0.7709	-1.75 to 3.292	No	ns	0.858
L-Isoleucine vs. L-Leucine	0.1836	-2.337 to 2.704	No	ns	0.9976
L-Isoleucine vs. Placebo	0.8564	-1.664 to 3.377	No	ns	0.8153
L-Leucine vs. Placebo	0.6727	-1.848 to 3.193	No	ns	0.9004
40					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-1.571	-4.092 to 0.9498	No	ns	0.373
L-Isoleucine and L-Leucine vs. L-Leucine	0.1918	-2.329 to 2.713	No	ns	0.9973
L-Isoleucine and L-Leucine vs. Placebo	1.373	-1.148 to 3.893	No	ns	0.4943
L-Isoleucine vs. L-Leucine	1.763	-0.758 to 4.283	No	ns	0.271
L-Isoleucine vs. Placebo	2.944	0.4229 to 5.464	Yes	*	0.0148
L-Leucine vs. Placebo	1.181	-1.34 to 3.702	No	ns	0.6191
60					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.04545	-2.566 to 2.475	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	0.4991	-2.022 to 3.02	No	ns	0.956
L-Isoleucine and L-Leucine vs. Placebo	2.582	0.06109 to 5.103	Yes	*	0.0424
L-Isoleucine vs. L-Leucine	0.5445	-1.976 to 3.065	No	ns	0.9439
L-Isoleucine vs. Placebo	2.627	0.1065 to 5.148	Yes	*	0.0374
L-Leucine vs. Placebo	2.083	-0.438 to 4.603	No	ns	0.144
90					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.05727	-2.463 to 2.578	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	0.4627	-2.058 to 2.983	No	ns	0.9644
L-Isoleucine and L-Leucine vs. Placebo	0.3591	-2.162 to 2.88	No	ns	0.9828
L-Isoleucine vs. L-Leucine	0.4055	-2.115 to 2.926	No	ns	0.9756
L-Isoleucine vs. Placebo	0.3018	-2.219 to 2.823	No	ns	0.9896
L-Leucine vs. Placebo	-0.1036	-2.624 to 2.417	No	ns	0.9996
120					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.6645	-1.856 to 3.185	No	ns	0.9036
L-Isoleucine and L-Leucine vs. L-Leucine	1.661	-0.8598 to 4.182	No	ns	0.3229
L-Isoleucine and L-Leucine vs. Placebo	3.435	0.9147 to 5.956	Yes	**	0.0029
L-Isoleucine vs. L-Leucine	0.9964	-1.524 to 3.517	No	ns	0.7358
L-Isoleucine vs. Placebo	2.771	0.2502 to 5.292	Yes	*	0.0249
L-Leucine vs. Placebo	1.775	-0.7462 to 4.295	No	ns	0.2654
150					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.1436	-2.664 to 2.377	No	ns	0.9989
L-Isoleucine and L-Leucine vs. L-Leucine	1.472	-1.049 to 3.993	No	ns	0.4321
L-Isoleucine and L-Leucine vs. Placebo	3.887	1.367 to 6.408	Yes	***	0.0005
L-Isoleucine vs. L-Leucine	1.615	-0.9053 to 4.136	No	ns	0.3478
L-Isoleucine vs. Placebo	4.031	1.51 to 6.552	Yes	***	0.0003
L-Leucine vs. Placebo	2.415	-0.1053 to 4.936	No	ns	0.0658

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
0								
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	0	0	0.9734	11	11	0	210
L-Isoleucine and L-Leucine vs. L-Leucine	0	0	0	0.9734	11	11	0	210
L-Isoleucine and L-Leucine vs. Placebo	0	0	0	0.9734	11	11	0	210
L-Isoleucine vs. L-Leucine	0	0	0	0.9734	11	11	0	210
L-Isoleucine vs. Placebo	0	0	0	0.9734	11	11	0	210
L-Leucine vs. Placebo	0	0	0	0.9734	11	11	0	210
10								
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.2473	0.2173	0.03	0.9734	11	11	0.04359	210
L-Isoleucine and L-Leucine vs. L-Leucine	0.2473	0.3764	-0.1291	0.9734	11	11	0.1876	210
L-Isoleucine and L-Leucine vs. Placebo	0.2473	0.09091	0.1564	0.9734	11	11	0.2272	210
L-Isoleucine vs. L-Leucine	0.2173	0.3764	-0.1591	0.9734	11	11	0.2311	210
L-Isoleucine vs. Placebo	0.2173	0.09091	0.1264	0.9734	11	11	0.1836	210
L-Leucine vs. Placebo	0.3764	0.09091	0.2855	0.9734	11	11	0.4147	210
30								
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.196	1.282	-0.08545	0.9734	11	11	0.1242	210
L-Isoleucine and L-Leucine vs. L-Leucine	1.196	1.098	0.09818	0.9734	11	11	0.1427	210
L-Isoleucine and L-Leucine vs. Placebo	1.196	0.4255	0.7709	0.9734	11	11	1.12	210
L-Isoleucine vs. L-Leucine	1.282	1.098	0.1836	0.9734	11	11	0.2668	210
L-Isoleucine vs. Placebo	1.282	0.4255	0.8564	0.9734	11	11	1.244	210
L-Leucine vs. Placebo	1.098	0.4255	0.6727	0.9734	11	11	0.9774	210
40								
L-Isoleucine and L-Leucine vs. L-Isoleucine	5.228	6.799	-1.571	0.9734	11	11	2.282	210
L-Isoleucine and L-Leucine vs. L-Leucine	5.228	5.036	0.1918	0.9734	11	11	0.2787	210
L-Isoleucine and L-Leucine vs. Placebo	5.228	3.855	1.373	0.9734	11	11	1.994	210
L-Isoleucine vs. L-Leucine	6.799	5.036	1.763	0.9734	11	11	2.561	210
L-Isoleucine vs. Placebo	6.799	3.855	2.944	0.9734	11	11	4.277	210
L-Leucine vs. Placebo	5.036	3.855	1.181	0.9734	11	11	1.716	210
60								
L-Isoleucine and L-Leucine vs. L-Isoleucine	13.52	13.56	-0.04545	0.9734	11	11	0.06604	210
L-Isoleucine and L-Leucine vs. L-Leucine	13.52	13.02	0.4991	0.9734	11	11	0.7251	210
L-Isoleucine and L-Leucine vs. Placebo	13.52	10.94	2.582	0.9734	11	11	3.751	210
L-Isoleucine vs. L-Leucine	13.56	13.02	0.5445	0.9734	11	11	0.7912	210
L-Isoleucine vs. Placebo	13.56	10.94	2.627	0.9734	11	11	3.817	210
L-Leucine vs. Placebo	13.02	10.94	2.083	0.9734	11	11	3.026	210
90								
L-Isoleucine and L-Leucine vs. L-Isoleucine	13.1	13.05	0.05727	0.9734	11	11	0.08321	210
L-Isoleucine and L-Leucine vs. L-Leucine	13.1	12.64	0.4627	0.9734	11	11	0.6723	210
L-Isoleucine and L-Leucine vs. Placebo	13.1	12.75	0.3591	0.9734	11	11	0.5217	210
L-Isoleucine vs. L-Leucine	13.05	12.64	0.4055	0.9734	11	11	0.5891	210
L-Isoleucine vs. Placebo	13.05	12.75	0.3018	0.9734	11	11	0.4385	210
L-Leucine vs. Placebo	12.64	12.75	-0.1036	0.9734	11	11	0.1506	210
120								
L-Isoleucine and L-Leucine vs. L-Isoleucine	13.51	12.85	0.6645	0.9734	11	11	0.9655	210
L-Isoleucine and L-Leucine vs. L-Leucine	13.51	11.85	1.661	0.9734	11	11	2.413	210
L-Isoleucine and L-Leucine vs. Placebo	13.51	10.07	3.435	0.9734	11	11	4.991	210
L-Isoleucine vs. L-Leucine	12.85	11.85	0.9964	0.9734	11	11	1.448	210
L-Isoleucine vs. Placebo	12.85	10.07	2.771	0.9734	11	11	4.026	210
L-Leucine vs. Placebo	11.85	10.07	1.775	0.9734	11	11	2.578	210
150								
L-Isoleucine and L-Leucine vs. L-Isoleucine	10.67	10.81	-0.1436	0.9734	11	11	0.2087	210
L-Isoleucine and L-Leucine vs. L-Leucine	10.67	9.195	1.472	0.9734	11	11	2.138	210
L-Isoleucine and L-Leucine vs. Placebo	10.67	6.78	3.887	0.9734	11	11	5.648	210
L-Isoleucine vs. L-Leucine	10.81	9.195	1.615	0.9734	11	11	2.347	210
L-Isoleucine vs. Placebo	10.81	6.78	4.031	0.9734	11	11	5.857	210
L-Leucine vs. Placebo	9.195	6.78	2.415	0.9734	11	11	3.509	210

Table 33: 2-Way RMANOVA Δ Delta C-peptide nmol/L from Amino Acid 0-30 min

Two-way RM ANOVA	Matching: Both factors				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Time	25.99	<0.0001	****	Yes	
Treatment	2.715	0.2576	ns	No	
Interaction: Time x Treatment	3.199	0.0921	ns	No	
Interaction: Time x Subjects	15.12				
Interaction: Treatment x Subjects	19.18				
Subjects	17.14				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Time	24.12	2	12.06	F (2, 20) = 17.19	P<0.0001
Treatment	2.519	3	0.8398	F (3, 30) = 1.416	P=0.2576
Interaction: Time x Treatment	2.969	6	0.4948	F (6, 60) = 1.92	P=0.0921
Interaction: Time x Subjects	14.03	20	0.7015		
Interaction: Treatment x Subjects	17.8	30	0.5932		
Subjects	15.91	10	1.591		
Residual	15.46	60	0.2577		

Table 34: 2-Way RMANOVA Δ C-peptide nmol/L from Amino Acid 0-30 min

Two-way RM ANOVA Compare column means (main column effect)								
Number of families	1							
Number of comparisons per family	6							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.01848	-0.5341 to 0.4971	No	ns	0.9997			
L-Isoleucine and L-Leucine vs. L-Leucine	-0.0103	-0.5259 to 0.5053	No	ns	>0.9999			
L-Isoleucine and L-Leucine vs. Placebo	0.3091	-0.2065 to 0.8247	No	ns	0.3776			
L-Isoleucine vs. L-Leucine	0.008182	-0.5074 to 0.5238	No	ns	>0.9999			
L-Isoleucine vs. Placebo	0.3276	-0.188 to 0.8432	No	ns	0.3276			
L-Leucine vs. Placebo	0.3194	-0.1962 to 0.835	No	ns	0.3493			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.4812	0.4997	-0.01848	0.1896	33	33	0.1379	30
L-Isoleucine and L-Leucine vs. L-Leucine	0.4812	0.4915	-0.0103	0.1896	33	33	0.07684	30
L-Isoleucine and L-Leucine vs. Placebo	0.4812	0.1721	0.3091	0.1896	33	33	2.305	30
L-Isoleucine vs. L-Leucine	0.4997	0.4915	0.008182	0.1896	33	33	0.06102	30
L-Isoleucine vs. Placebo	0.4997	0.1721	0.3276	0.1896	33	33	2.443	30
L-Leucine vs. Placebo	0.4915	0.1721	0.3194	0.1896	33	33	2.382	30

Table 35: 2-Way RMANOVA Δ Delta C-peptide nmol/L from Amino Acid 0-30 min

Two-way RM ANOVA Within each row, compare columns (simple effects within rows)					
Number of families	3				
Number of comparisons per family	6				
Alpha	0.05				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	-0.572 to 0.572	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	0	-0.572 to 0.572	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. Placebo	0	-0.572 to 0.572	No	ns	>0.9999
L-Isoleucine vs. L-Leucine	0	-0.572 to 0.572	No	ns	>0.9999
L-Isoleucine vs. Placebo	0	-0.572 to 0.572	No	ns	>0.9999
L-Leucine vs. Placebo	0	-0.572 to 0.572	No	ns	>0.9999
10					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.03	-0.542 to 0.602	No	ns	0.999
L-Isoleucine and L-Leucine vs. L-Leucine	-0.1291	-0.7011 to 0.4429	No	ns	0.9328
L-Isoleucine and L-Leucine vs. Placebo	0.1564	-0.4156 to 0.7284	No	ns	0.8878
L-Isoleucine vs. L-Leucine	-0.1591	-0.7311 to 0.4129	No	ns	0.8826
L-Isoleucine vs. Placebo	0.1264	-0.4456 to 0.6984	No	ns	0.9366
L-Leucine vs. Placebo	0.2855	-0.2865 to 0.8574	No	ns	0.5548
30					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.08545	-0.6574 to 0.4865	No	ns	0.9789
L-Isoleucine and L-Leucine vs. L-Leucine	0.09818	-0.4738 to 0.6702	No	ns	0.9687
L-Isoleucine and L-Leucine vs. Placebo	0.7709	0.1989 to 1.343	Yes	**	0.004
L-Isoleucine vs. L-Leucine	0.1836	-0.3884 to 0.7556	No	ns	0.8311
L-Isoleucine vs. Placebo	0.8564	0.2844 to 1.428	Yes	**	0.0011
L-Leucine vs. Placebo	0.6727	0.1007 to 1.245	Yes	*	0.0149

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
0								
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	0	0	0.2165	11	11	0	60
L-Isoleucine and L-Leucine vs. L-Leucine	0	0	0	0.2165	11	11	0	60
L-Isoleucine and L-Leucine vs. Placebo	0	0	0	0.2165	11	11	0	60
L-Isoleucine vs. L-Leucine	0	0	0	0.2165	11	11	0	60
L-Isoleucine vs. Placebo	0	0	0	0.2165	11	11	0	60
L-Leucine vs. Placebo	0	0	0	0.2165	11	11	0	60
10								
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.2473	0.2173	0.03	0.2165	11	11	0.196	60
L-Isoleucine and L-Leucine vs. L-Leucine	0.2473	0.3764	-0.1291	0.2165	11	11	0.8434	60
L-Isoleucine and L-Leucine vs. Placebo	0.2473	0.09091	0.1564	0.2165	11	11	1.022	60
L-Isoleucine vs. L-Leucine	0.2173	0.3764	-0.1591	0.2165	11	11	1.039	60
L-Isoleucine vs. Placebo	0.2173	0.09091	0.1264	0.2165	11	11	0.8256	60
L-Leucine vs. Placebo	0.3764	0.09091	0.2855	0.2165	11	11	1.865	60
30								
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.196	1.282	-0.08545	0.2165	11	11	0.5583	60
L-Isoleucine and L-Leucine vs. L-Leucine	1.196	1.098	0.09818	0.2165	11	11	0.6415	60
L-Isoleucine and L-Leucine vs. Placebo	1.196	0.4255	0.7709	0.2165	11	11	5.037	60
L-Isoleucine vs. L-Leucine	1.282	1.098	0.1836	0.2165	11	11	1.2	60
L-Isoleucine vs. Placebo	1.282	0.4255	0.8564	0.2165	11	11	5.595	60
L-Leucine vs. Placebo	1.098	0.4255	0.6727	0.2165	11	11	4.395	60

Table 36: 2-Way RMANOVA C-peptide nmol/L 0-150 min

RM ANOVA Within each row C-peptide nmol/L x time iAUC					
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	2.323				
P value	0.11				
P value summary	ns				
Statistically significant (P < 0.05)?	No				
Geisser-Greenhouse's epsilon	0.8158				
R square	0.1885				
Was the matching effective?					
F	7.387				
P value	<0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.6665				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	536730	3	178910	(2.447, 24.47) = 2.32	P=0.1100
Individual (between rows)	5689255	10	568926	F (10, 30) = 7.387	P<0.0001
Residual (random)	2310380	30	77013		
Total	8536365	43			
Data summary					
Number of treatments (columns)	4				
Number of subjects (rows)	11				

Table 37: 2-Way RMANOVA C-peptide nmol/L 0-150 min

RM ANOVA Within each row C-peptide nmol/L x time iAUC								
Number of families	1							
Number of comparisons per family	6							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	ɑdjusted P Value			
L-Isoleucine and L-Leucine vs. L-Isoleucine	-7.591	-423.9 to 408.7	No	ns	>0.9999	A-B		
L-Isoleucine and L-Leucine vs. L-Leucine	99.75	-251.4 to 450.9	No	ns	0.8206	A-C		
L-Isoleucine and L-Leucine vs. Placebo	266.3	-23.16 to 555.7	No	ns	0.0737	A-D		
L-Isoleucine vs. L-Leucine	107.3	-333.6 to 548.3	No	ns	0.8769	B-C		
L-Isoleucine vs. Placebo	273.9	-96.97 to 644.7	No	ns	0.1727	B-D		
L-Leucine vs. Placebo	166.5	-105.5 to 438.5	No	ns	0.2979	C-D		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
L-Isoleucine and L-Leucine vs. L-Isoleucine	1397	1405	-7.591	136.1	11	11	0.07889	10
L-Isoleucine and L-Leucine vs. L-Leucine	1397	1297	99.75	114.8	11	11	1.229	10
L-Isoleucine and L-Leucine vs. Placebo	1397	1131	266.3	94.61	11	11	3.98	10
L-Isoleucine vs. L-Leucine	1405	1297	107.3	144.1	11	11	1.053	10
L-Isoleucine vs. Placebo	1405	1131	273.9	121.2	11	11	3.195	10
L-Leucine vs. Placebo	1297	1131	166.5	88.91	11	11	2.649	10

Table 38: 2-Way RMANOVA C-peptide nmol/L 0-150 min

RM ANOVA Within each row C-peptide nmol/L x time iAUC

Number of families	1
Number of comparisons per family	3
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	D-?	
Placebo vs. L-Isoleucine and L-Leucin	-266.3	-527.3 to -5.25	Yes	*	0.0456	A	L-Isoleucine and L-Leucine
Placebo vs. L-Isoleucine	-273.9	-608.3 to 60.57	No	ns	0.113	B	L-Isoleucine
Placebo vs. L-Leucine	-166.5	-411.8 to 78.78	No	ns	0.2064	C	L-Leucine

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Placebo vs. L-Isoleucine and L-Leucin	1131	1397	-266.3	94.61	11	11	2.815	10
Placebo vs. L-Isoleucine	1131	1405	-273.9	121.2	11	11	2.259	10
Placebo vs. L-Leucine	1131	1297	-166.5	88.91	11	11	1.873	10

Table 38: 2-Way RMANOVA Δ Delta Glucagon ng/L 0-150 min

Two-way RM ANOVA Matching: Both factors

Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	1.191	>0.9999	ns	No
Time	19.56	<0.0001	****	Yes
Treatment	0.7064	0.4605	ns	No

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2547	21	121.3	F (21, 288) = 0.208	P>0.9999
Time	41825	7	5975	F (7, 288) = 10.25	P<0.0001
Treatment	1510	3	503.4	F (3, 288) = 0.8634	P=0.4605
Residual	167930	288	583.1		

Number of missing values	0

Table 39: 2-Way RMANOVA Δ Glucagon ng/L 0-150 min

Two-way RM ANOVA Compare column means (main column effect)

Number of families	1
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
L-Isoleucine and L-Leucine vs. L-Isoleucine	2.062	-7.805 to 11.93	No	ns	0.9492
L-Isoleucine and L-Leucine vs. L-Leucine	5.148	-4.718 to 15.01	No	ns	0.5329
L-Isoleucine and L-Leucine vs. Placebo	5.099	-4.767 to 14.97	No	ns	0.541
L-Isoleucine vs. L-Leucine	3.087	-6.779 to 12.95	No	ns	0.8505
L-Isoleucine vs. Placebo	3.038	-6.828 to 12.9	No	ns	0.8564
L-Leucine vs. Placebo	-0.04875	-9.915 to 9.817	No	ns	>0.9999

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
L-Isoleucine and L-Leucine vs. L-Isoleucine	-8.133	-10.19	2.062	3.818	80	80	0.7636	288
L-Isoleucine and L-Leucine vs. L-Leucine	-8.133	-13.28	5.148	3.818	80	80	1.907	288
L-Isoleucine and L-Leucine vs. Placebo	-8.133	-13.23	5.099	3.818	80	80	1.889	288
L-Isoleucine vs. L-Leucine	-10.19	-13.28	3.087	3.818	80	80	1.143	288
L-Isoleucine vs. Placebo	-10.19	-13.23	3.038	3.818	80	80	1.125	288
L-Leucine vs. Placebo	-13.28	-13.23	-0.04875	3.818	80	80	0.01806	288

Table 40: 2-Way RMANOVA Δ Delta Glucagon ng/L 0-150 min

Two-way RM ANOVA Within each row, compare columns (simple effects within rows)

Number of families	8
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	-11.95 to 11.95	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	0	-11.95 to 11.95	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. Placebo	0	-11.95 to 11.95	No	ns	>0.9999
L-Isoleucine vs. L-Leucine	0	-11.95 to 11.95	No	ns	>0.9999
L-Isoleucine vs. Placebo	0	-11.95 to 11.95	No	ns	>0.9999
L-Leucine vs. Placebo	0	-11.95 to 11.95	No	ns	>0.9999
10					
L-Isoleucine and L-Leucine vs. L-Isoleucine	2.433	-9.515 to 14.38	No	ns	0.9523
L-Isoleucine and L-Leucine vs. L-Leucine	5.343	-6.605 to 17.29	No	ns	0.6533
L-Isoleucine and L-Leucine vs. Placebo	5.643	-6.305 to 17.59	No	ns	0.6123
L-Isoleucine vs. L-Leucine	2.91	-9.038 to 14.86	No	ns	0.9219
L-Isoleucine vs. Placebo	3.21	-8.738 to 15.16	No	ns	0.8984
L-Leucine vs. Placebo	0.3	-11.65 to 12.25	No	ns	>0.9999
30					
L-Isoleucine and L-Leucine vs. L-Isoleucine	2.07	-9.878 to 14.02	No	ns	0.9697
L-Isoleucine and L-Leucine vs. L-Leucine	4.638	-7.31 to 16.59	No	ns	0.746
L-Isoleucine and L-Leucine vs. Placebo	16.22	4.271 to 28.17	Yes	**	0.003
L-Isoleucine vs. L-Leucine	2.568	-9.38 to 14.52	No	ns	0.9445
L-Isoleucine vs. Placebo	14.15	2.201 to 26.1	Yes	*	0.013
L-Leucine vs. Placebo	11.58	-0.3671 to 23.53	No	ns	0.0612
40					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-7.481	-19.43 to 4.467	No	ns	0.3682
L-Isoleucine and L-Leucine vs. L-Leucine	-2.189	-14.14 to 9.759	No	ns	0.9645
L-Isoleucine and L-Leucine vs. Placebo	2.277	-9.671 to 14.23	No	ns	0.9604
L-Isoleucine vs. L-Leucine	5.292	-6.656 to 17.24	No	ns	0.6602
L-Isoleucine vs. Placebo	9.758	-2.19 to 21.71	No	ns	0.1515
L-Leucine vs. Placebo	4.466	-7.482 to 16.41	No	ns	0.7673
60					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.408	-11.54 to 12.36	No	ns	0.9998
L-Isoleucine and L-Leucine vs. L-Leucine	4.471	-7.477 to 16.42	No	ns	0.7667
L-Isoleucine and L-Leucine vs. Placebo	4.795	-7.153 to 16.74	No	ns	0.726
L-Isoleucine vs. L-Leucine	4.063	-7.885 to 16.01	No	ns	0.8145
L-Isoleucine vs. Placebo	4.387	-7.561 to 16.34	No	ns	0.7769
L-Leucine vs. Placebo	0.324	-11.62 to 12.27	No	ns	0.9999
90					
L-Isoleucine and L-Leucine vs. L-Isoleucine	5.412	-6.536 to 17.36	No	ns	0.6439
L-Isoleucine and L-Leucine vs. L-Leucine	10.78	-1.167 to 22.73	No	ns	0.093
L-Isoleucine and L-Leucine vs. Placebo	4.34	-7.608 to 16.29	No	ns	0.7825
L-Isoleucine vs. L-Leucine	5.369	-6.579 to 17.32	No	ns	0.6498
L-Isoleucine vs. Placebo	-1.072	-13.02 to 10.88	No	ns	0.9956
L-Leucine vs. Placebo	-6.441	-18.39 to 5.507	No	ns	0.5026
120					
L-Isoleucine and L-Leucine vs. L-Isoleucine	5.564	-6.384 to 17.51	No	ns	0.6231
L-Isoleucine and L-Leucine vs. L-Leucine	8.792	-3.156 to 20.74	No	ns	0.2284
L-Isoleucine and L-Leucine vs. Placebo	4.239	-7.709 to 16.19	No	ns	0.7944
L-Isoleucine vs. L-Leucine	3.228	-8.72 to 15.18	No	ns	0.8969
L-Isoleucine vs. Placebo	-1.325	-13.27 to 10.62	No	ns	0.9917
L-Leucine vs. Placebo	-4.553	-16.5 to 7.395	No	ns	0.7566
150					
L-Isoleucine and L-Leucine vs. L-Isoleucine	8.086	-3.862 to 20.03	No	ns	0.2989
L-Isoleucine and L-Leucine vs. L-Leucine	9.349	-2.599 to 21.3	No	ns	0.1813
L-Isoleucine and L-Leucine vs. Placebo	3.282	-8.666 to 15.23	No	ns	0.8922
L-Isoleucine vs. L-Leucine	1.263	-10.69 to 13.21	No	ns	0.9928
L-Isoleucine vs. Placebo	-4.804	-16.75 to 7.144	No	ns	0.7249
L-Leucine vs. Placebo	-6.067	-18.02 to 5.881	No	ns	0.5538

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
0								
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	0	0	4.61	10	10	0	189
L-Isoleucine and L-Leucine vs. L-Leucine	0	0	0	4.61	10	10	0	189
L-Isoleucine and L-Leucine vs. Placebo	0	0	0	4.61	10	10	0	189
L-Isoleucine vs. L-Leucine	0	0	0	4.61	10	10	0	189
L-Isoleucine vs. Placebo	0	0	0	4.61	10	10	0	189
L-Leucine vs. Placebo	0	0	0	4.61	10	10	0	189
10								
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.367	-1.066	2.433	4.61	10	10	0.7464	189
L-Isoleucine and L-Leucine vs. L-Leucine	1.367	-3.976	5.343	4.61	10	10	1.639	189
L-Isoleucine and L-Leucine vs. Placebo	1.367	-4.276	5.643	4.61	10	10	1.731	189
L-Isoleucine vs. L-Leucine	-1.066	-3.976	2.91	4.61	10	10	0.8928	189
L-Isoleucine vs. Placebo	-1.066	-4.276	3.21	4.61	10	10	0.9848	189
L-Leucine vs. Placebo	-3.976	-4.276	0.3	4.61	10	10	0.09204	189
30								
L-Isoleucine and L-Leucine vs. L-Isoleucine	11.02	8.952	2.07	4.61	10	10	0.6351	189
L-Isoleucine and L-Leucine vs. L-Leucine	11.02	6.384	4.638	4.61	10	10	1.423	189
L-Isoleucine and L-Leucine vs. Placebo	11.02	-5.197	16.22	4.61	10	10	4.976	189
L-Isoleucine vs. L-Leucine	8.952	6.384	2.568	4.61	10	10	0.7879	189
L-Isoleucine vs. Placebo	8.952	-5.197	14.15	4.61	10	10	4.341	189
L-Leucine vs. Placebo	6.384	-5.197	11.58	4.61	10	10	3.553	189
40								
L-Isoleucine and L-Leucine vs. L-Isoleucine	-6.06	1.421	-7.481	4.61	10	10	2.295	189
L-Isoleucine and L-Leucine vs. L-Leucine	-6.06	-3.871	-2.189	4.61	10	10	0.6716	189
L-Isoleucine and L-Leucine vs. Placebo	-6.06	-8.337	2.277	4.61	10	10	0.6986	189
L-Isoleucine vs. L-Leucine	1.421	-3.871	5.292	4.61	10	10	1.624	189
L-Isoleucine vs. Placebo	1.421	-8.337	9.758	4.61	10	10	2.994	189
L-Leucine vs. Placebo	-3.871	-8.337	4.466	4.61	10	10	1.37	189
60								
L-Isoleucine and L-Leucine vs. L-Isoleucine	-15.23	-15.64	0.408	4.61	10	10	0.1252	189
L-Isoleucine and L-Leucine vs. L-Leucine	-15.23	-19.7	4.471	4.61	10	10	1.372	189
L-Isoleucine and L-Leucine vs. Placebo	-15.23	-20.02	4.795	4.61	10	10	1.471	189
L-Isoleucine vs. L-Leucine	-15.64	-19.7	4.063	4.61	10	10	1.247	189
L-Isoleucine vs. Placebo	-15.64	-20.02	4.387	4.61	10	10	1.346	189
L-Leucine vs. Placebo	-19.7	-20.02	0.324	4.61	10	10	0.0994	189
90								
L-Isoleucine and L-Leucine vs. L-Isoleucine	-17.05	-22.47	5.412	4.61	10	10	1.66	189
L-Isoleucine and L-Leucine vs. L-Leucine	-17.05	-27.83	10.78	4.61	10	10	3.308	189
L-Isoleucine and L-Leucine vs. Placebo	-17.05	-21.39	4.34	4.61	10	10	1.332	189
L-Isoleucine vs. L-Leucine	-22.47	-27.83	5.369	4.61	10	10	1.647	189
L-Isoleucine vs. Placebo	-22.47	-21.39	-1.072	4.61	10	10	0.3289	189
L-Leucine vs. Placebo	-27.83	-21.39	-6.441	4.61	10	10	1.976	189
120								
L-Isoleucine and L-Leucine vs. L-Isoleucine	-19.95	-25.51	5.564	4.61	10	10	1.707	189
L-Isoleucine and L-Leucine vs. L-Leucine	-19.95	-28.74	8.792	4.61	10	10	2.697	189
L-Isoleucine and L-Leucine vs. Placebo	-19.95	-24.19	4.239	4.61	10	10	1.301	189
L-Isoleucine vs. L-Leucine	-25.51	-28.74	3.228	4.61	10	10	0.9904	189
L-Isoleucine vs. Placebo	-25.51	-24.19	-1.325	4.61	10	10	0.4065	189
L-Leucine vs. Placebo	-28.74	-24.19	-4.553	4.61	10	10	1.397	189
150								
L-Isoleucine and L-Leucine vs. L-Isoleucine	-19.17	-27.26	8.086	4.61	10	10	2.481	189
L-Isoleucine and L-Leucine vs. L-Leucine	-19.17	-28.52	9.349	4.61	10	10	2.868	189
L-Isoleucine and L-Leucine vs. Placebo	-19.17	-22.45	3.282	4.61	10	10	1.007	189
L-Isoleucine vs. L-Leucine	-27.26	-28.52	1.263	4.61	10	10	0.3875	189
L-Isoleucine vs. Placebo	-27.26	-22.45	-4.804	4.61	10	10	1.474	189
L-Leucine vs. Placebo	-28.52	-22.45	-6.067	4.61	10	10	1.861	189

Table 41: 2-Way RMANOVA Δ Delta Glucagon ng/L from Amino Acid 0-30 min

Two-way RM ANOVA	Matching: Both factors			
Alpha	0.05			
Source of Variation	% of total variation	P value	P value summary	Significant?
Time	7.988	0.0112	*	Yes
Treatment	6.293	0.1277	ns	No
Interaction: Time x Treatment	6.318	0.0469	*	Yes
Interaction: Time x Subjects	12.36			
Interaction: Treatment x Subjects	27.36			
Subjects	15.05			

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Time	1132	2	566	F (2, 18) = 5.818	P=0.0112
Treatment	892	3	297.3	F (3, 27) = 2.07	P=0.1277
Interaction: Time x Treatment	895.4	6	149.2	F (6, 54) = 2.308	P=0.0469
Interaction: Time x Subjects	1751	18	97.29		
Interaction: Treatment x Subjects	3878	27	143.6		
Subjects	2133	9	237		
Residual	3492	54	64.66		

Table 42: 2-Way RMANOVA Δ Glucagon ng/L from Amino Acid 0-30 min

Two-way RM ANOVA Compare column means (main column effect)					
Number of families	1				
Number of comparisons per family	6				
Alpha	0.05				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.501	-6.967 to 9.969	No	ns	0.9618
L-Isoleucine and L-Leucine vs. L-Leucine	3.327	-5.141 to 11.79	No	ns	0.7073
L-Isoleucine and L-Leucine vs. Placebo	7.287	-1.181 to 15.76	No	ns	0.1106
L-Isoleucine vs. L-Leucine	1.826	-6.642 to 10.29	No	ns	0.9342
L-Isoleucine vs. Placebo	5.786	-2.682 to 14.25	No	ns	0.2644
L-Leucine vs. Placebo	3.96	-4.508 to 12.43	No	ns	0.5832

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
L-Isoleucine and L-Leucine vs. L-Isoleucine	4.13	2.629	1.501	3.094	30	30	0.686	27
L-Isoleucine and L-Leucine vs. L-Leucine	4.13	0.8027	3.327	3.094	30	30	1.521	27
L-Isoleucine and L-Leucine vs. Placebo	4.13	-3.158	7.287	3.094	30	30	3.33	27
L-Isoleucine vs. L-Leucine	2.629	0.8027	1.826	3.094	30	30	0.8345	27
L-Isoleuci								
L-Leucine								

Table 43: 2-Way RMANOVA Δ Delta Glucagon ng/L from Amino Acid 0-30 min

Two-way RM ANOVA Within each row, compare columns (simple effects within rows)					
Number of families	3				
Number of comparisons per family	6				
Alpha	0.05				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	-9.533 to 9.533	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	0	-9.533 to 9.533	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. Placebo	0	-9.533 to 9.533	No	ns	>0.9999
L-Isoleucine vs. L-Leucine	0	-9.533 to 9.533	No	ns	>0.9999
L-Isoleucine vs. Placebo	0	-9.533 to 9.533	No	ns	>0.9999
L-Leucine vs. Placebo	0	-9.533 to 9.533	No	ns	>0.9999
10					
L-Isoleucine and L-Leucine vs. L-Isoleucine	2.433	-7.1 to 11.97	No	ns	0.9055
L-Isoleucine and L-Leucine vs. L-Leucine	5.343	-4.19 to 14.88	No	ns	0.453
L-Isoleucine and L-Leucine vs. Placebo	5.643	-3.89 to 15.18	No	ns	0.4046
L-Isoleucine vs. L-Leucine	2.91	-6.623 to 12.44	No	ns	0.8498
L-Isoleucine vs. Placebo	3.21	-6.323 to 12.74	No	ns	0.8087
L-Leucine vs. Placebo	0.3	-9.233 to 9.833	No	ns	0.9998
30					
L-Isoleucine and L-Leucine vs. L-Isoleucine	2.07	-7.463 to 11.6	No	ns	0.939
L-Isoleucine and L-Leucine vs. L-Leucine	4.638	-4.895 to 14.17	No	ns	0.5734
L-Isoleucine and L-Leucine vs. Placebo	16.22	6.686 to 25.75	Yes	***	0.0002
L-Isoleucine vs. L-Leucine	2.568	-6.965 to 12.1	No	ns	0.8911
L-Isoleucine vs. Placebo	14.15	4.616 to 23.68	Yes	**	0.0013
L-Leucine vs. Placebo	11.58	2.048 to 21.11	Yes	*	0.0113

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
0								
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	0	0	3.596	10	10	0	54
L-Isoleucine and L-Leucine vs. L-Leucine	0	0	0	3.596	10	10	0	54
L-Isoleucine and L-Leucine vs. Placebo	0	0	0	3.596	10	10	0	54
L-Isoleucine vs. L-Leucine	0	0	0	3.596	10	10	0	54
L-Isoleucine vs. Placebo	0	0	0	3.596	10	10	0	54
L-Leucine vs. Placebo	0	0	0	3.596	10	10	0	54
10								
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.367	-1.066	2.433	3.596	10	10	0.9568	54
L-Isoleucine and L-Leucine vs. L-Leucine	1.367	-3.976	5.343	3.596	10	10	2.101	54
L-Isoleucine and L-Leucine vs. Placebo	1.367	-4.276	5.643	3.596	10	10	2.219	54
L-Isoleucine vs. L-Leucine	-1.066	-3.976	2.91	3.596	10	10	1.144	54
L-Isoleucine vs. Placebo	-1.066	-4.276	3.21	3.596	10	10	1.262	54
L-Leucine vs. Placebo	-3.976	-4.276	0.3	3.596	10	10	0.118	54
30								
L-Isoleucine and L-Leucine vs. L-Isoleucine	11.02	8.952	2.07	3.596	10	10	0.814	54
L-Isoleucine and L-Leucine vs. L-Leucine	11.02	6.384	4.638	3.596	10	10	1.824	54
L-Isoleucine and L-Leucine vs. Placebo	11.02	-5.197	16.22	3.596	10	10	6.378	54
L-Isoleucine vs. L-Leucine	8.952	6.384	2.568	3.596	10	10	1.01	54
L-Isoleucine vs. Placebo	8.952	-5.197	14.15	3.596	10	10	5.564	54

Table 43: 2-Way RMANOVA Δ Delta GLP-1_{Active} pmol/L 0-150 min

Two-way RM ANOVA Matching: Both factors					
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Time	31.95	<0.0001	****	Yes	
Treatment	2.991	0.0181	*	Yes	
Interaction: Time x Treatment	1.622	0.1782	ns	No	
Interaction: Time x Subjects	13.21				
Interaction: Treatment x Subjects	7.654				
Subjects	30.09				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Time	1804	7	257.7	F (7, 70) = 24.2	P<0.0001
Treatment	168.9	3	56.3	F (3, 30) = 3.908	P=0.0181
Interaction: Time x Treatment	91.57	21	4.361	F (21, 210) = 1.299	P=0.1782
Interaction: Time x Subjects	745.6	70	10.65		
Interaction: Treatment x Subjects	432.2	30	14.41		
Subjects	1699	10	169.9		
Residual	705.1	210	3.358		

Table 44: 2-Way RMANOVA Δ Delta GLP-1_{Active} pmol 0-150 min

Two-way RM ANOVA Compare column means (main column effect)	
Number of families	1
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.258	-0.2976 to 2.814	No	ns	0.1466
L-Isoleucine and L-Leucine vs. L-Leucine	1.132	-0.4236 to 2.688	No	ns	0.2184
L-Isoleucine and L-Leucine vs. Placebo	1.928	0.3717 to 3.483	Yes	*	0.0106
L-Isoleucine vs. L-Leucine	-0.126	-1.682 to 1.43	No	ns	0.9961
L-Isoleucine vs. Placebo	0.6692	-0.8866 to 2.225	No	ns	0.6502
L-Leucine vs. Placebo	0.7952	-0.7606 to 2.351	No	ns	0.5153

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
L-Isoleucine and L-Leucine vs. L-Isoleucine	5.98	4.721	1.258	0.5722	88	88	3.11	30
L-Isoleucine and L-Leucine vs. L-Leucine	5.98	4.847	1.132	0.5722	88	88	2.799	30
L-Isoleucine and L-Leucine vs. Placebo	5.98	4.052	1.928	0.5722	88	88	4.764	30
L-Isoleucine vs. L-Leucine	4.721	4.847	-0.126	0.5722	88	88	0.3115	30
L-Isoleucine vs. Placebo	4.721	4.052	0.6692	0.5722	88	88	1.654	30
L-Leucine vs. Placebo	4.847	4.052	0.7952	0.5722	88	88	1.965	30

Table 45: 2-Way RMANOVA Δ Delta Glucagon ng/L 0-150 min

Two-way RM ANOVA Within each row, compare columns (simple effects within rows)

Number of families	8
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	-2.023 to 2.023	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	0	-2.023 to 2.023	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. Placebo	0	-2.023 to 2.023	No	ns	>0.9999
L-Isoleucine vs. L-Leucine	0	-2.023 to 2.023	No	ns	>0.9999
L-Isoleucine vs. Placebo	0	-2.023 to 2.023	No	ns	>0.9999
L-Leucine vs. Placebo	0	-2.023 to 2.023	No	ns	>0.9999
10					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.5882	-1.435 to 2.612	No	ns	0.8754
L-Isoleucine and L-Leucine vs. L-Leucine	0.3418	-1.682 to 2.365	No	ns	0.9719
L-Isoleucine and L-Leucine vs. Placebo	1.298	-0.7253 to 3.322	No	ns	0.3468
L-Isoleucine vs. L-Leucine	-0.2464	-2.27 to 1.777	No	ns	0.9891
L-Isoleucine vs. Placebo	0.71	-1.313 to 2.733	No	ns	0.8002
L-Leucine vs. Placebo	0.9564	-1.067 to 2.98	No	ns	0.6123
30					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.7773	-1.246 to 2.801	No	ns	0.7526
L-Isoleucine and L-Leucine vs. L-Leucine	0.6327	-1.391 to 2.656	No	ns	0.8498
L-Isoleucine and L-Leucine vs. Placebo	1.36	-0.6635 to 3.383	No	ns	0.3054
L-Isoleucine vs. L-Leucine	-0.1445	-2.168 to 1.879	No	ns	0.9977
L-Isoleucine vs. Placebo	0.5827	-1.441 to 2.606	No	ns	0.8784
L-Leucine vs. Placebo	0.7273	-1.296 to 2.751	No	ns	0.7884
40					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.3345	-2.358 to 1.689	No	ns	0.9736
L-Isoleucine and L-Leucine vs. L-Leucine	0.6509	-1.373 to 2.674	No	ns	0.8387
L-Isoleucine and L-Leucine vs. Placebo	2.444	0.4202 to 4.467	Yes	*	0.0108
L-Isoleucine vs. L-Leucine	0.9855	-1.038 to 3.009	No	ns	0.5886
L-Isoleucine vs. Placebo	2.778	0.7547 to 4.802	Yes	**	0.0026
L-Leucine vs. Placebo	1.793	-0.2307 to 3.816	No	ns	0.1026
60					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.56	-1.463 to 2.583	No	ns	0.8904
L-Isoleucine and L-Leucine vs. L-Leucine	0.2718	-1.752 to 2.295	No	ns	0.9855
L-Isoleucine and L-Leucine vs. Placebo	0.7927	-1.231 to 2.816	No	ns	0.7411
L-Isoleucine vs. L-Leucine	-0.2882	-2.312 to 1.735	No	ns	0.9828
L-Isoleucine vs. Placebo	0.2327	-1.791 to 2.256	No	ns	0.9908
L-Leucine vs. Placebo	0.5209	-1.503 to 2.544	No	ns	0.9095
90					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.1645	-2.188 to 1.859	No	ns	0.9967
L-Isoleucine and L-Leucine vs. L-Leucine	1.134	-0.8898 to 3.157	No	ns	0.4692
L-Isoleucine and L-Leucine vs. Placebo	-0.22	-2.243 to 1.803	No	ns	0.9922
L-Isoleucine vs. L-Leucine	1.298	-0.7253 to 3.322	No	ns	0.3468
L-Isoleucine vs. Placebo	-0.05545	-2.079 to 1.968	No	ns	0.9999
L-Leucine vs. Placebo	-1.354	-3.377 to 0.6698	No	ns	0.3095
120					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.9936	-3.017 to 1.03	No	ns	0.582
L-Isoleucine and L-Leucine vs. L-Leucine	0.6827	-1.341 to 2.706	No	ns	0.8184
L-Isoleucine and L-Leucine vs. Placebo	1.361	-0.6626 to 3.384	No	ns	0.3048
L-Isoleucine vs. L-Leucine	1.676	-0.3471 to 3.7	No	ns	0.1423
L-Isoleucine vs. Placebo	2.355	0.3311 to 4.378	Yes	*	0.0153
L-Leucine vs. Placebo	0.6782	-1.345 to 2.702	No	ns	0.8214
150					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-1.854	-3.877 to 0.1698	No	ns	0.0856
L-Isoleucine and L-Leucine vs. L-Leucine	-0.78	-2.803 to 1.243	No	ns	0.7505
L-Isoleucine and L-Leucine vs. Placebo	-0.6318	-2.655 to 1.392	No	ns	0.8503
L-Isoleucine vs. L-Leucine	1.074	-0.9498 to 3.097	No	ns	0.517
L-Isoleucine vs. Placebo	1.222	-0.8016 to 3.245	No	ns	0.4016
L-Leucine vs. Placebo	0.1482	-1.875 to 2.172	No	ns	0.9976

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
0								
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	0	0	0.7813	11	11	0	210
L-Isoleucine and L-Leucine vs. L-Leucine	0	0	0	0.7813	11	11	0	210
L-Isoleucine and L-Leucine vs. Placebo	0	0	0	0.7813	11	11	0	210
L-Isoleucine vs. L-Leucine	0	0	0	0.7813	11	11	0	210
L-Isoleucine vs. Placebo	0	0	0	0.7813	11	11	0	210
L-Leucine vs. Placebo	0	0	0	0.7813	11	11	0	210
10								
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.419	0.8309	0.5882	0.7813	11	11	1.065	210
L-Isoleucine and L-Leucine vs. L-Leucine	1.419	1.077	0.3418	0.7813	11	11	0.6187	210
L-Isoleucine and L-Leucine vs. Placebo	1.419	0.1209	1.298	0.7813	11	11	2.35	210
L-Isoleucine vs. L-Leucine	0.8309	1.077	-0.2464	0.7813	11	11	0.4459	210
L-Isoleucine vs. Placebo	0.8309	0.1209	0.71	0.7813	11	11	1.285	210
L-Leucine vs. Placebo	1.077	0.1209	0.9564	0.7813	11	11	1.731	210
30								
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.607	0.83	0.7773	0.7813	11	11	1.407	210
L-Isoleucine and L-Leucine vs. L-Leucine	1.607	0.9745	0.6327	0.7813	11	11	1.145	210
L-Isoleucine and L-Leucine vs. Placebo	1.607	0.2473	1.36	0.7813	11	11	2.462	210
L-Isoleucine vs. L-Leucine	0.83	0.9745	-0.1445	0.7813	11	11	0.2616	210
L-Isoleucine vs. Placebo	0.83	0.2473	0.5827	0.7813	11	11	1.055	210
L-Leucine vs. Placebo	0.9745	0.2473	0.7273	0.7813	11	11	1.316	210
40								
L-Isoleucine and L-Leucine vs. L-Isoleucine	7.486	7.821	-0.3345	0.7813	11	11	0.6055	210
L-Isoleucine and L-Leucine vs. L-Leucine	7.486	6.835	0.6509	0.7813	11	11	1.178	210
L-Isoleucine and L-Leucine vs. Placebo	7.486	5.043	2.444	0.7813	11	11	4.423	210
L-Isoleucine vs. L-Leucine	7.821	6.835	0.9855	0.7813	11	11	1.784	210
L-Isoleucine vs. Placebo	7.821	5.043	2.778	0.7813	11	11	5.028	210
L-Leucine vs. Placebo	6.835	5.043	1.793	0.7813	11	11	3.245	210
60								
L-Isoleucine and L-Leucine vs. L-Isoleucine	5.994	5.434	0.56	0.7813	11	11	1.014	210
L-Isoleucine and L-Leucine vs. L-Leucine	5.994	5.722	0.2718	0.7813	11	11	0.492	210
L-Isoleucine and L-Leucine vs. Placebo	5.994	5.201	0.7927	0.7813	11	11	1.435	210
L-Isoleucine vs. L-Leucine	5.434	5.722	-0.2882	0.7813	11	11	0.5216	210
L-Isoleucine vs. Placebo	5.434	5.201	0.2327	0.7813	11	11	0.4212	210
L-Leucine vs. Placebo	5.722	5.201	0.5209	0.7813	11	11	0.9428	210
90								
L-Isoleucine and L-Leucine vs. L-Isoleucine	4.357	4.522	-0.1645	0.7813	11	11	0.2978	210
L-Isoleucine and L-Leucine vs. L-Leucine	4.357	3.224	1.134	0.7813	11	11	2.052	210
L-Isoleucine and L-Leucine vs. Placebo	4.357	4.577	-0.22	0.7813	11	11	0.3982	210
L-Isoleucine vs. L-Leucine	4.522	3.224	1.298	0.7813	11	11	2.35	210
L-Isoleucine vs. Placebo	4.522	4.577	-0.05545	0.7813	11	11	0.1004	210
L-Leucine vs. Placebo	3.224	4.577	-1.354	0.7813	11	11	2.45	210
120								
L-Isoleucine and L-Leucine vs. L-Isoleucine	3.368	4.362	-0.9936	0.7813	11	11	1.798	210
L-Isoleucine and L-Leucine vs. L-Leucine	3.368	2.685	0.6827	0.7813	11	11	1.236	210
L-Isoleucine and L-Leucine vs. Placebo	3.368	2.007	1.361	0.7813	11	11	2.463	210
L-Isoleucine vs. L-Leucine	4.362	2.685	1.676	0.7813	11	11	3.034	210
L-Isoleucine vs. Placebo	4.362	2.007	2.355	0.7813	11	11	4.262	210
L-Leucine vs. Placebo	2.685	2.007	0.6782	0.7813	11	11	1.227	210
150								
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.398	3.252	-1.854	0.7813	11	11	3.355	210
L-Isoleucine and L-Leucine vs. L-Leucine	1.398	2.178	-0.78	0.7813	11	11	1.412	210
L-Isoleucine and L-Leucine vs. Placebo	1.398	2.03	-0.6318	0.7813	11	11	1.144	210
L-Isoleucine vs. L-Leucine	3.252	2.178	1.074	0.7813	11	11	1.943	210
L-Isoleucine vs. Placebo	3.252	2.03	1.222	0.7813	11	11	2.211	210
L-Leucine vs. Placebo	2.178	2.03	0.1482	0.7813	11	11	0.2682	210

Table 46: 2-Way RMANOVA Δ Delta GLP-1_{Active} pmol/L from Amino Acid 0-30 min

Two-way RM ANOVA	Matching: Both factors				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	1.189	0.9376	ns	No	
Time	3.978	0.0549	ns	No	
Treatment	14.59	0.0002	***	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	6.939	6	1.157	F (6, 120) = 0.2964	P=0.9376
Time	23.21	2	11.61	F (2, 120) = 2.975	P=0.0549
Treatment	85.17	3	28.39	F (3, 120) = 7.276	P=0.0002
Residual	468.2	120	3.902		
Number of missing values	0				

Table 47: 2-Way RMANOVA Δ GLP-1_{Active} pmol/L from Amino Acid 0-30 min

Two-way RM ANOVA Compare column means (main column effect)								
Number of families	1							
Number of comparisons per family	6							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.89	0.6233 to 3.157	Yes	***	0.0009			
L-Isoleucine and L-Leucine vs. L-Leucine	1.09	-0.1773 to 2.357	No	ns	0.1182			
L-Isoleucine and L-Leucine vs. Placebo	2.013	0.7463 to 3.28	Yes	***	0.0004			
L-Isoleucine vs. L-Leucine	-0.8006	-2.068 to 0.4664	No	ns	0.3568			
L-Isoleucine vs. Placebo	0.123	-1.144 to 1.39	No	ns	0.9943			
L-Leucine vs. Placebo	0.9236	-0.3434 to 2.191	No	ns	0.2338			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
L-Isoleucine and L-Leucine vs. L-Isoleucine	3.784	1.894	1.89	0.4863	33	33	5.497	120
L-Isoleuci								
L-Isoleuci								
L-Isoleuci								
L-Isoleuci								
L-Leucine								

Table 48: 2-Way RMANOVA Δ Delta GLP-1_{Active} pmol/L 0-30 min

Two-way RM ANOVA Within each row, compare columns (simple effects within rows)					
Number of families	3				
Number of comparisons per family	6				
Alpha	0.05				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	-5.3 to 5.3	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	0	-5.3 to 5.3	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. Placebo	0	-5.3 to 5.3	No	ns	>0.9999
L-Isoleucine vs. L-Leucine	0	-5.3 to 5.3	No	ns	>0.9999
L-Isoleucine vs. Placebo	0	-5.3 to 5.3	No	ns	>0.9999
L-Leucine vs. Placebo	0	-5.3 to 5.3	No	ns	>0.9999
10					
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.935	-3.364 to 7.235	No	ns	0.7771
L-Isoleucine and L-Leucine vs. L-Leucine	1.13	-4.17 to 6.43	No	ns	0.9449
L-Isoleucine and L-Leucine vs. Placebo	4.284	-1.016 to 9.583	No	ns	0.1571
L-Isoleucine vs. L-Leucine	-0.8055	-6.105 to 4.494	No	ns	0.9789
L-Isoleucine vs. Placebo	2.348	-2.951 to 7.648	No	ns	0.6566
L-Leucine vs. Placebo	3.154	-2.146 to 8.453	No	ns	0.411
30					
L-Isoleucine and L-Leucine vs. L-Isoleucine	2.557	-2.742 to 7.857	No	ns	0.5919
L-Isoleucine and L-Leucine vs. L-Leucine	2.087	-3.212 to 7.387	No	ns	0.7345
L-Isoleucine and L-Leucine vs. Placebo	4.487	-0.8124 to 9.787	No	ns	0.1275
L-Isoleucine vs. L-Leucine	-0.47	-5.77 to 4.83	No	ns	0.9956
L-Isoleucine vs. Placebo	1.93	-3.37 to 7.23	No	ns	0.7785
L-Leucine vs. Placebo	2.4	-2.9 to 7.7	No	ns	0.6407
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
0								
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	0	0	2.034	11	11	0	120
L-Isoleucine and L-Leucine vs. L-Leucine	0	0	0	2.034	11	11	0	120
L-Isoleucine and L-Leucine vs. Placebo	0	0	0	2.034	11	11	0	120
L-Isoleucine vs. L-Leucine	0	0	0	2.034	11	11	0	120
L-Isoleucine vs. Placebo	0	0	0	2.034	11	11	0	120
L-Leucine vs. Placebo	0	0	0	2.034	11	11	0	120
10								
L-Isoleucine and L-Leucine vs. L-Isoleucine	4.679	2.744	1.935	2.034	11	11	1.346	120
L-Isoleucine and L-Leucine vs. L-Leucine	4.679	3.549	1.13	2.034	11	11	0.7856	120
L-Isoleucine and L-Leucine vs. Placebo	4.679	0.3955	4.284	2.034	11	11	2.978	120
L-Isoleucine vs. L-Leucine	2.744	3.549	-0.8055	2.034	11	11	0.56	120
L-Isoleucine vs. Placebo	2.744	0.3955	2.348	2.034	11	11	1.633	120
L-Leucine vs. Placebo	3.549	0.3955	3.154	2.034	11	11	2.193	120
30								
L-Isoleucine and L-Leucine vs. L-Isoleucine	5.301	2.744	2.557	2.034	11	11	1.778	120
L-Isoleucine and L-Leucine vs. L-Leucine	5.301	3.214	2.087	2.034	11	11	1.451	120
L-Isoleucine and L-Leucine vs. Placebo	5.301	0.8136	4.487	2.034	11	11	3.12	120
L-Isoleucine vs. L-Leucine	2.744	3.214	-0.47	2.034	11	11	0.3268	120
L-Isoleucine vs. Placebo	2.744	0.8136	1.93	2.034	11	11	1.342	120

Table 49: 2-Way RMANOVA Δ Delta GIP_{Total} pmol/L 0-150 min

Two-way RM ANOVA Matching: Both factors					
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Time	49.16	<0.0001	****	Yes	
Treatment	2.311	0.0155	*	Yes	
Interaction: Time x Treatment	1.391	0.0077	**	Yes	
Interaction: Time x Subjects	15.61				
Interaction: Treatment x Subjects	5.69				
Subjects	18.86				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Time	71385	7	10198	F (7, 70) = 31.5	P<0.0001
Treatment	3356	3	1119	F (3, 30) = 4.062	P=0.0155
Interaction: Time x Treatment	2020	21	96.2	F (21, 210) = 1.993	P=0.0077
Interaction: Time x Subjects	22663	70	323.8		
Interaction: Treatment x Subjects	8263	30	275.4		
Subjects	27388	10	2739		
Residual	10136	210	48.27		

Table 50: 2-Way RMANOVA Δ Delta GIP_{Total} pmol/L 0-150 min

Two-way RM ANOVA Compare column means (main column effect)								
Number of families	1							
Number of comparisons per family	6							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
L-Isoleucine and L-Leucine vs. L-Isoleucine	-2.448	-9.251 to 4.355	No	ns	0.7627			
L-Isoleucine and L-Leucine vs. L-Leucine	5.213	-1.591 to 12.02	No	ns	0.1816			
L-Isoleucine and L-Leucine vs. Placebo	4.09	-2.713 to 10.89	No	ns	0.3752			
L-Isoleucine vs. L-Leucine	7.66	0.8572 to 14.46	Yes	*	0.0226			
L-Isoleucine vs. Placebo	6.538	-0.2656 to 13.34	No	ns	0.0631			
L-Leucine vs. Placebo	-1.123	-7.926 to 5.68	No	ns	0.9694			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
L-Isoleucine and L-Leucine vs. L-Isoleucine	19.6	22.05	-2.448	2.502	88	88	1.384	30
L-Isoleucine and L-Leucine vs. L-Leucine	19.6	14.39	5.213	2.502	88	88	2.946	30
L-Isoleucine and L-Leucine vs. Placebo	19.6	15.51	4.09	2.502	88	88	2.312	30
L-Isoleucine vs. L-Leucine	22.05	14.39	7.66	2.502	88	88	4.33	30
L-Isoleucine vs. Placebo	22.05	15.51	6.538	2.502	88	88	3.695	30
L-Leucine vs. Placebo	14.39	15.51	-1.123	2.502	88	88	0.6346	30

Table 51: 2-Way RMANOVA Δ Delta GIP_{Total} pmol/L 0-150 min

Two-way RM ANOVA Within each row, compare columns (simple effects within rows)

Within each row, compare columns (simple effects within rows)

Number of families	8
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	-7.672 to 7.672	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	0	-7.672 to 7.672	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. Placebo	0	-7.672 to 7.672	No	ns	>0.9999
L-Isoleucine vs. L-Leucine	0	-7.672 to 7.672	No	ns	>0.9999
L-Isoleucine vs. Placebo	0	-7.672 to 7.672	No	ns	>0.9999
L-Leucine vs. Placebo	0	-7.672 to 7.672	No	ns	>0.9999
10					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.2455	-7.917 to 7.426	No	ns	0.9998
L-Isoleucine and L-Leucine vs. L-Leucine	3.036	-4.635 to 10.71	No	ns	0.735
L-Isoleucine and L-Leucine vs. Placebo	3.482	-4.19 to 11.15	No	ns	0.6431
L-Isoleucine vs. L-Leucine	3.282	-4.39 to 10.95	No	ns	0.6851
L-Isoleucine vs. Placebo	3.727	-3.944 to 11.4	No	ns	0.5906
L-Leucine vs. Placebo	0.4455	-7.226 to 8.117	No	ns	0.9988
30					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-2.955	-10.63 to 4.717	No	ns	0.7511
L-Isoleucine and L-Leucine vs. L-Leucine	2.918	-4.754 to 10.59	No	ns	0.7581
L-Isoleucine and L-Leucine vs. Placebo	2.591	-5.081 to 10.26	No	ns	0.818
L-Isoleucine vs. L-Leucine	5.873	-1.799 to 13.54	No	ns	0.1979
L-Isoleucine vs. Placebo	5.545	-2.126 to 13.22	No	ns	0.2433
L-Leucine vs. Placebo	-0.3273	-7.999 to 7.344	No	ns	0.9995
40					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-7.3	-14.97 to 0.3717	No	ns	0.0686
L-Isoleucine and L-Leucine vs. L-Leucine	2.682	-4.99 to 10.35	No	ns	0.802
L-Isoleucine and L-Leucine vs. Placebo	6.709	-0.9627 to 14.38	No	ns	0.1097
L-Isoleucine vs. L-Leucine	9.982	2.31 to 17.65	Yes	**	0.0049
L-Isoleucine vs. Placebo	14.01	6.337 to 21.68	Yes	****	<0.0001
L-Leucine vs. Placebo	4.027	-3.644 to 11.7	No	ns	0.5262
60					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-4.927	-12.6 to 2.744	No	ns	0.3458
L-Isoleucine and L-Leucine vs. L-Leucine	4.464	-3.208 to 12.14	No	ns	0.4353
L-Isoleucine and L-Leucine vs. Placebo	2.445	-5.226 to 10.12	No	ns	0.8423
L-Isoleucine vs. L-Leucine	9.391	1.719 to 17.06	Yes	**	0.0094
L-Isoleucine vs. Placebo	7.373	-0.299 to 15.04	No	ns	0.0646
L-Leucine vs. Placebo	-2.018	-9.69 to 5.654	No	ns	0.9041
90					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-3.991	-11.66 to 3.681	No	ns	0.534
L-Isoleucine and L-Leucine vs. L-Leucine	5.355	-2.317 to 13.03	No	ns	0.2727
L-Isoleucine and L-Leucine vs. Placebo	-2.045	-9.717 to 5.626	No	ns	0.9007
L-Isoleucine vs. L-Leucine	9.345	1.674 to 17.02	Yes	**	0.0099
L-Isoleucine vs. Placebo	1.945	-5.726 to 9.617	No	ns	0.9131
L-Leucine vs. Placebo	-7.4	-15.07 to 0.2717	No	ns	0.0631
120					
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.727	-5.944 to 9.399	No	ns	0.9371
L-Isoleucine and L-Leucine vs. L-Leucine	13.21	5.537 to 20.88	Yes	****	<0.0001
L-Isoleucine and L-Leucine vs. Placebo	14.04	6.365 to 21.71	Yes	****	<0.0001
L-Isoleucine vs. L-Leucine	11.48	3.81 to 19.15	Yes	***	0.0008
L-Isoleucine vs. Placebo	12.31	4.637 to 19.98	Yes	***	0.0003
L-Leucine vs. Placebo	0.8273	-6.844 to 8.499	No	ns	0.9924
150					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-1.891	-9.563 to 5.781	No	ns	0.9195
L-Isoleucine and L-Leucine vs. L-Leucine	10.04	2.365 to 17.71	Yes	**	0.0046
L-Isoleucine and L-Leucine vs. Placebo	5.5	-2.172 to 13.17	No	ns	0.2501
L-Isoleucine vs. L-Leucine	11.93	4.256 to 19.6	Yes	***	0.0005
L-Isoleucine vs. Placebo	7.391	-0.2808 to 15.06	No	ns	0.0636
L-Leucine vs. Placebo	-4.536	-12.21 to 3.135	No	ns	0.4206

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
0								
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	0	0	2.962	11	11	0	210
L-Isoleucine and L-Leucine vs. L-Leucine	0	0	0	2.962	11	11	0	210
L-Isoleucine and L-Leucine vs. Placebo	0	0	0	2.962	11	11	0	210
L-Isoleucine vs. L-Leucine	0	0	0	2.962	11	11	0	210
L-Isoleucine vs. Placebo	0	0	0	2.962	11	11	0	210
L-Leucine vs. Placebo	0	0	0	2.962	11	11	0	210
10								
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.573	1.818	-0.2455	2.962	11	11	0.1172	210
L-Isoleucine and L-Leucine vs. L-Leucine	1.573	-1.464	3.036	2.962	11	11	1.45	210
L-Isoleucine and L-Leucine vs. Placebo	1.573	-1.909	3.482	2.962	11	11	1.662	210
L-Isoleucine vs. L-Leucine	1.818	-1.464	3.282	2.962	11	11	1.567	210
L-Isoleucine vs. Placebo	1.818	-1.909	3.727	2.962	11	11	1.779	210
L-Leucine vs. Placebo	-1.464	-1.909	0.4455	2.962	11	11	0.2127	210
30								
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.373	4.327	-2.955	2.962	11	11	1.41	210
L-Isoleucine and L-Leucine vs. L-Leucine	1.373	-1.545	2.918	2.962	11	11	1.393	210
L-Isoleucine and L-Leucine vs. Placebo	1.373	-1.218	2.591	2.962	11	11	1.237	210
L-Isoleucine vs. L-Leucine	4.327	-1.545	5.873	2.962	11	11	2.804	210
L-Isoleucine vs. Placebo	4.327	-1.218	5.545	2.962	11	11	2.647	210
L-Leucine vs. Placebo	-1.545	-1.218	-0.3273	2.962	11	11	0.1562	210
40								
L-Isoleucine and L-Leucine vs. L-Isoleucine	20.61	27.91	-7.3	2.962	11	11	3.485	210
L-Isoleucine and L-Leucine vs. L-Leucine	20.61	17.93	2.682	2.962	11	11	1.28	210
L-Isoleucine and L-Leucine vs. Placebo	20.61	13.9	6.709	2.962	11	11	3.203	210
L-Isoleucine vs. L-Leucine	27.91	17.93	9.982	2.962	11	11	4.765	210
L-Isoleucine vs. Placebo	27.91	13.9	14.01	2.962	11	11	6.688	210
L-Leucine vs. Placebo	17.93	13.9	4.027	2.962	11	11	1.923	210
60								
L-Isoleucine and L-Leucine vs. L-Isoleucine	27.39	32.32	-4.927	2.962	11	11	2.352	210
L-Isoleucine and L-Leucine vs. L-Leucine	27.39	22.93	4.464	2.962	11	11	2.131	210
L-Isoleucine and L-Leucine vs. Placebo	27.39	24.95	2.445	2.962	11	11	1.167	210
L-Isoleucine vs. L-Leucine	32.32	22.93	9.391	2.962	11	11	4.483	210
L-Isoleucine vs. Placebo	32.32	24.95	7.373	2.962	11	11	3.52	210
L-Leucine vs. Placebo	22.93	24.95	-2.018	2.962	11	11	0.9635	210
90								
L-Isoleucine and L-Leucine vs. L-Isoleucine	32.2	36.19	-3.991	2.962	11	11	1.905	210
L-Isoleucine and L-Leucine vs. L-Leucine	32.2	26.85	5.355	2.962	11	11	2.556	210
L-Isoleucine and L-Leucine vs. Placebo	32.2	34.25	-2.045	2.962	11	11	0.9765	210
L-Isoleucine vs. L-Leucine	36.19	26.85	9.345	2.962	11	11	4.461	210
L-Isoleucine vs. Placebo	36.19	34.25	1.945	2.962	11	11	0.9287	210
L-Leucine vs. Placebo	26.85	34.25	-7.4	2.962	11	11	3.533	210
120								
L-Isoleucine and L-Leucine vs. L-Isoleucine	42.09	40.36	1.727	2.962	11	11	0.8246	210
L-Isoleucine and L-Leucine vs. L-Leucine	42.09	28.88	13.21	2.962	11	11	6.306	210
L-Isoleucine and L-Leucine vs. Placebo	42.09	28.05	14.04	2.962	11	11	6.701	210
L-Isoleucine vs. L-Leucine	40.36	28.88	11.48	2.962	11	11	5.481	210
L-Isoleucine vs. Placebo	40.36	28.05	12.31	2.962	11	11	5.876	210
L-Leucine vs. Placebo	28.88	28.05	0.8273	2.962	11	11	0.3949	210
150								
L-Isoleucine and L-Leucine vs. L-Isoleucine	31.55	33.44	-1.891	2.962	11	11	0.9027	210
L-Isoleucine and L-Leucine vs. L-Leucine	31.55	21.51	10.04	2.962	11	11	4.791	210
L-Isoleucine and L-Leucine vs. Placebo	31.55	26.05	5.5	2.962	11	11	2.626	210
L-Isoleucine vs. L-Leucine	33.44	21.51	11.93	2.962	11	11	5.694	210
L-Isoleucine vs. Placebo	33.44	26.05	7.391	2.962	11	11	3.528	210
L-Leucine vs. Placebo	21.51	26.05	-4.536	2.962	11	11	2.166	210

Table 52: 2-Way RMANOVA Δ Delta GIP_{Total} pmol/L from Amino Acid 0-30 min

Two-way RM ANOVA	Matching: Both factors				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Time	0.6004	0.722	ns	No	
Treatment	8.842	0.0215	*	Yes	
Interaction: Time x Treatment	5.407	0.0304	*	Yes	
Interaction: Time x Subjects	18.13				
Interaction: Treatment x Subjects	23.65				
Subjects	21.93				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Time	15.71	2	7.855	F (2, 20) = 0.3311	P=0.7220
Treatment	231.3	3	77.12	F (3, 30) = 3.738	P=0.0215
Interaction: Time x Treatment	141.5	6	23.58	F (6, 60) = 2.522	P=0.0304
Interaction: Time x Subjects	474.5	20	23.72		
Interaction: Treatment x Subjects	618.8	30	20.63		
Subjects	573.8	10	57.38		
Residual	560.9	60	9.349		

Table 53: 2-Way RMANOVA Δ GIP_{Total} pmol/L from Amino Acid 0-30 min

Two-way RM ANOVA Compare column means (main column effect)								
Number of families	1							
Number of comparisons per family	6							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
L-Isoleucine and L-Leucine vs. L-Isoleucine	-1.067	-4.107 to 1.974	No	ns	0.7761			
L-Isoleucine and L-Leucine vs. L-Leucine	1.985	-1.055 to 5.025	No	ns	0.3047			
L-Isoleucine and L-Leucine vs. Placebo	2.024	-1.016 to 5.065	No	ns	0.2884			
L-Isoleucine vs. L-Leucine	3.052	0.01125 to 6.092	Yes	*	0.0489			
L-Isoleucine vs. Placebo	3.091	0.05064 to 6.131	Yes	*	0.0452			
L-Leucine vs. Placebo	0.03939	-3.001 to 3.08	No	ns	>0.9999			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
L-Isoleucine and L-Leucine vs. L-Isoleucine	0.9818	2.048	-1.067	1.118	33	33	1.349	30
L-Isoleucine and L-Leucine vs. L-Leucine	0.9818	-1.003	1.985	1.118	33	33	2.51	30
L-Isoleucine and L-Leucine vs. Placebo	0.9818	-1.042	2.024	1.118	33	33	2.56	30
L-Isoleucine vs. L-Leucine	2.048	-1.003	3.052	1.118	33	33	3.86	30
L-Isoleucine vs. Placebo	2.048	-1.042	3.091	1.118	33	33	3.909	30
L-Leucine vs. Placebo	-1.003	-1.042	0.03939	1.118	33	33	0.04983	30

Table 54: 2-Way RMANOVA Δ Delta GIP_{total} pmol/L 0-30 min

Two-way RM ANOVA Within each row, compare columns (simple effects within rows)					
Number of families	3				
Number of comparisons per family	6				
Alpha	0.05				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0					
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	-3.445 to 3.445	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. L-Leucine	0	-3.445 to 3.445	No	ns	>0.9999
L-Isoleucine and L-Leucine vs. Placebo	0	-3.445 to 3.445	No	ns	>0.9999
L-Isoleucine vs. L-Leucine	0	-3.445 to 3.445	No	ns	>0.9999
L-Isoleucine vs. Placebo	0	-3.445 to 3.445	No	ns	>0.9999
L-Leucine vs. Placebo	0	-3.445 to 3.445	No	ns	>0.9999
10					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-0.2455	-3.691 to 3.2	No	ns	0.9976
L-Isoleucine and L-Leucine vs. L-Leucine	3.036	-0.4088 to 6.482	No	ns	0.1028
L-Isoleucine and L-Leucine vs. Placebo	3.482	0.03661 to 6.927	Yes	*	0.0467
L-Isoleucine vs. L-Leucine	3.282	-0.1634 to 6.727	No	ns	0.0674
L-Isoleucine vs. Placebo	3.727	0.2821 to 7.172	Yes	*	0.029
L-Leucine vs. Placebo	0.4455	-3 to 3.891	No	ns	0.9861
30					
L-Isoleucine and L-Leucine vs. L-Isoleucine	-2.955	-6.4 to 0.4907	No	ns	0.1176
L-Isoleucine and L-Leucine vs. L-Leucine	2.918	-0.527 to 6.363	No	ns	0.1247
L-Isoleucine and L-Leucine vs. Placebo	2.591	-0.8543 to 6.036	No	ns	0.2042
L-Isoleucine vs. L-Leucine	5.873	2.428 to 9.318	Yes	***	0.0002
L-Isoleucine vs. Placebo	5.545	2.1 to 8.991	Yes	***	0.0004
L-Leucine vs. Placebo	-0.3273	-3.772 to 3.118	No	ns	0.9944

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
0								
L-Isoleucine and L-Leucine vs. L-Isoleucine	0	0	0	1.304	11	11	0	60
L-Isoleucine and L-Leucine vs. L-Leucine	0	0	0	1.304	11	11	0	60
L-Isoleucine and L-Leucine vs. Placebo	0	0	0	1.304	11	11	0	60
L-Isoleucine vs. L-Leucine	0	0	0	1.304	11	11	0	60
L-Isoleucine vs. Placebo	0	0	0	1.304	11	11	0	60
L-Leucine vs. Placebo	0	0	0	1.304	11	11	0	60
10								
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.573	1.818	-0.2455	1.304	11	11	0.2662	60
L-Isoleucine and L-Leucine vs. L-Leucine	1.573	-1.464	3.036	1.304	11	11	3.294	60
L-Isoleucine and L-Leucine vs. Placebo	1.573	-1.909	3.482	1.304	11	11	3.777	60
L-Isoleucine vs. L-Leucine	1.818	-1.464	3.282	1.304	11	11	3.56	60
L-Isoleucine vs. Placebo	1.818	-1.909	3.727	1.304	11	11	4.043	60
L-Leucine vs. Placebo	-1.464	-1.909	0.4455	1.304	11	11	0.4832	60
30								
L-Isoleucine and L-Leucine vs. L-Isoleucine	1.373	4.327	-2.955	1.304	11	11	3.205	60
L-Isoleucine and L-Leucine vs. L-Leucine	1.373	-1.545	2.918	1.304	11	11	3.165	60
L-Isoleucine and L-Leucine vs. Placebo	1.373	-1.218	2.591	1.304	11	11	2.81	60
L-Isoleucine vs. L-Leucine	4.327	-1.545	5.873	1.304	11	11	6.37	60
L-Isoleucine vs. Placebo	4.327	-1.218	5.545	1.304	11	11	6.015	60
L-Leucine vs. Placebo	-1.545	-1.218	-0.3273	1.304	11	11	0.355	60

Table 55: RMANOVA GIP_{Total} nmol/L 0-150 min

RM ANOVA Within each row GIP_{Total} pmol/L x time iAUC

Repeated measures ANOVA summary

Table 56: 2-Way RMANOVA GIPTotal pmol/L 0-150 min

RM ANOVA Within each row C-peptide nmol/L x time iAUC

Number of families	1
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
L-Isoleucine and L-Leucine vs. L-Isoleucine	-1680	-9476 to 6116	No	ns	0.9098	A-B
L-Isoleucine and L-Leucine vs. L-Leucine	4485	-4187 to 13158	No	ns	0.4298	A-C
L-Isoleucine and L-Leucine vs. Placebo	3389	-3632 to 10410	No	ns	0.4849	A-D
L-Isoleucine vs. L-Leucine	6165	422.7 to 11908	Yes	*	0.0349	B-C
L-Isoleucine vs. Placebo	5069	1400 to 8738	Yes	**	0.008	B-D
L-Leucine vs. Placebo	-1096	-7093 to 4901	No	ns	0.9419	C-D

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
L-Isoleucine and L-Leucine vs. L-Isoleucine	18926	20606	-1680	2548	11	11	0.9325	10
L-Isoleucine and L-Leucine vs. L-Leucine	18926	14441	4485	2835	11	11	2.238	10
L-Isoleucine and L-Leucine vs. Placebo	18926	15537	3389	2295	11	11	2.088	10
L-Isoleucine vs. L-Leucine	20606	14441	6165	1877	11	11	4.645	10
L-Isoleucine vs. Placebo	20606	15537	5069	1199	11	11	5.978	10
L-Leucine vs. Placebo	14441	15537	-1096	1960	11	11	0.791	10

Individual (between rows)	2918062726	10	291806273	F (10, 30) = 11.13	P<0.0001
Residual (random)	786484310	30	26216144		
Total	3977719908	43			

Data summary

Number of treatments (columns)	4
Number of subjects (rows)	11