THE EFFECTS OF AN ACUTE BOUT OF MODERATE-INTENSITY EXERCISE ON PLASMA AMINO ACID CONCENTRATIONS IN ADOLESCENT BOYS WITH PHENYLKETONURIA

By

Joanna Helm

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CERTIFICATE OF APPROVAL

This is to certify I have read the Master's Thesis of

Joanna Ruth Helm

and approve the research presented here

Melanie Gillingham, PhD, RD

Dr. Cary Harding, MD

Dr. Kerry Kuehl, MD, DrPH

Diane Stadler, PhD, RD, LD

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BCKDH……………………………….….. branched-chain alpha-keto acid dehydrogenase

BCOAD…………………….………………..…….branched-chain oxoacid dehydrogenase

BH_4 ……………….…………………………………….………….…….…….tetrahydrobiopterin

BMI…………………………………………….……………………….……body mass index

DXA……………………………………………….…….…dual energy x-ray absorptiometry

IAA………………………………………………………………….…….indispensable amino acids

IAAO……………………………………………………………..….indicator amino acid oxidation

IRMS…………………………………………………………. isotope ratio mass spectrometry

LNAA…..…………………………………………………….….……..large neutral amino acids

METS………………………………………………………..……….……metabolic equivalents

OCTRI………….……………………….…….… Oregon Clinical & Translational Research Institute

PAH……………………………………………………………….…..phenylalanine hydroxylase

PEG-PAL……………………….….….… pegylated recombinant phenylalanine ammonia lyase

PHE……………………………………………………….…………….………….phenylalanine

PKU…………………………………………………….……………….……….phenylketonuria

REE.....resting energy expenditure

TYR.....tyrosine

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ABSTRACT

Background: Phenylketonuria (PKU) is an autosomal recessive inherited metabolic disorder that can cause neurocognitive dysfunction and mental retardation if dietary treatment is not initiated within the first weeks of life. The restrictive nature of the phenylalanine-restricted diet makes compliance a challenge for many patients. Exercise promotes muscle protein synthesis and lean body mass. We hypothesized that an acute bout of moderate-intensity exercise would decrease amino acid oxidation, increase muscle protein synthesis, and promote tissue uptake of indispensable amino acids, thereby lowering plasma phenylalanine concentrations in patients with PKU.

Objectives: To assess the impact of an acute bout of moderate intensity exercise on plasma phenylalanine concentrations in adolescent males with PKU.

Design & Methods: A randomized cross-over clinical trial was completed on two adolescent males and the effects of an acute bout of moderate intensity exercise and sedentary activities on amino acid oxidation were compared using the indicator amino acid oxidation technique. Amino acid concentrations were measured in urine and plasma. The oxidation of the indicator amino acid, ^{13}C-lysine, was measured in expired breath as $^{13}CO_2$.

Results: Plasma phenylalanine concentrations decreased from baseline measurement for both participants during the sedentary visit (-28.7%, -66%). During the exercise visit, Participant 1 had a 23% increase in plasma phenylalanine concentrations, a 404% increase in urinary phenylalanine concentration and oxidized 22% of dosed ^{13}C-Lysine. Participant 2 had a 46%

decrease in plasma concentration, a 65% decrease in urinary phenylalanine concentration and oxidized 42% of dosed ^{13}C-Lysine.

Conclusions: This research did not support our hypothesis yet did not cause either participant to increase their plasma phenylalanine concentrations above baseline concentrations during the sedentary visit and only one subject experienced and increase in plasma phenylalanine concentration after exercise which plateaued for the duration of the study visit. Plasma phenylalanine concentrations decreased in one participant but no effect was seen in the other. Future studies involving the impact of exercise on acute and long term phenylalanine concentrations are needed to understand the impact of exercise on quality of life and neurocognitive function in patients with PKU.

Keywords: Phenylketonuria, PKU, Exercise, Stable Isotope, Oxidation, Muscle Protein Synthesis

CHAPTER 1

INTRODUCTION

Summary and Specific Aims

Phenylketonuria (PKU), also known as phenylalanine hydroxylase deficiency (PAH), is an autosomal recessive inherited metabolic disorder that can cause a wide spectrum of neurocognitive dysfunction if left untreated. The cornerstone of treatment is through nutritional management that requires adherence to a phenylalanine (phe) restricted diet. However, phenylalanine is an essential amino acid required for optimal growth, proper brain development, and cognitive function and cannot be completely eliminated from the diet. The restrictive nature of the diet for PKU makes compliance a challenge for many patients. Plasma phenylalanine concentrations can rise if patients consume too much phenylalanine in their daily food and beverage intake, or if they become ill and catabolic. Changes in the circulating total free amino acid pool are the result of the balance between gastrointestinal absorption of amino acids from the diet, protein oxidation to form ATP, and the uptake of amino acids into tissues to support protein synthesis. During negative energy balance, the body enters a catabolic state, oxidizing protein for energy, thereby causing the release of amino acids from muscle. Consequently, plasma amino acid concentrations, including phenylalanine, rise. Promoting anabolism, the uptake of free amino acids from the extracellular space for muscle protein synthesis, is a key component to maintaining plasma phenylalanine concentrations within treatment range among patients with PKU. Exercise promotes muscle protein synthesis and anabolism, but the effect on blood phenylalanine levels in patients with PKU has not been reported.

The promotion of muscle protein synthesis and accretion of lean body mass through exercise is well established in the literature. Our objective was to assess the impact of an acute bout of moderate-intensity exercise on amino acid oxidation and subsequent changes in plasma amino acid concentrations.

Hypothesis

We hypothesized that an acute bout of moderate-intensity exercise would decrease lysine oxidation, and promote tissue uptake of indispensable amino acids, thereby lowering plasma phenylalanine concentrations in patients with PKU.

Specific Aim

To determine if an acute bout of moderate-intensity exercise alters the oxidation of an indicator amino acid, ^{13}C-Lysine, in breath and changes the concentrations of plasma and urinary amino acids when compared to sedentary activity in adolescent boys with PKU.

Using exercise as a tool to manage plasma phenylalanine concentrations and maintain levels within treatment range is a novel approach in the therapy for patients with PKU. Exercise has a wide range of established health benefits that in turn help create a positive quality of life. We investigated the role of exercise as a potential adjunctive therapy in the management of plasma phenylalanine concentrations in patients with PKU.

Significance

Phenylalanine (phe) is an indispensable amino acid required for normal protein synthesis, cognitive development, and optimal growth. Phenylketonuria (PKU) is an autosomal recessive inherited metabolic disorder typically diagnosed at birth through newborn screening. If left untreated, PKU can result in severe neurocognitive dysfunction [1]. The prevalence of PKU in the United States is about 1:15,000 live births, with the highest prevalence in Turkey (1:4500) due to consanguinity in this population [2]. Phenylketonuria is caused by a mutation in the enzyme phenylalanine hydroxylase (PAH), which converts phenylalanine to tyrosine. Loss of PAH activity results in increased concentrations of plasma phenylalanine which can cross the blood brain barrier, causing neurotoxicity in the brain. Chronically elevated plasma phenylalanine concentrations are associated with intellectual impairment, declined executive function, ADHD symptoms, autistic tendencies, seizures, neurocognitive and motor deficits [1, 3].

Early diagnosis and immediate intervention allows most individuals with PKU to develop along normal growth trajectories, attain educational results similar to their unaffected peers, build normal relationships and obtain gainful employment. The cornerstone of dietary treatment requires compliance with a low phenylalanine diet, which usually begins immediately upon confirmation of a positive newborn screening result within the first few days of life. Phenylalanine-restricted diets must be individualized and regularly adjusted to maintain adequate nutrition for growth and development. The diet prescription consists of hydrolyzed amino acids, low or without phenylalanine, in the form of a medical formula and strict avoidance of foods high in phenylalanine, such as meat, fish, eggs, bread, cheese, and milk. The continuous development of alternative therapies has been researched since the discovery of

PKU in the 1930's. Currently, these therapies include: sapropterin dihydrochloride (Kuvan®), large neutral amino acids (LNAA), pegylated phenylalanine ammonia lyase (PEG-PAL), gene therapy, and potentially, liver transplantation. Recently, a novel whole protein source naturally low in phenylalanine, glycomacropeptide (GMP) has been developed as an option to include in the dietary regimen [4]. Although some patients have used these therapies with limited success, overall the outcomes have been unsatisfactory. Additional reasons for suboptimal compliance to dietary therapy include: poor adherence to the highly restrictive diet, psychosocial and emotional factors, poor family cohesion, lack of parental guidance and commitment, ethnic background, socio-economic status, and the quality of health care and health insurance [5-8]. Incorporating exercise and the health benefits that are associated with an active lifestyle as a potential adjunctive therapy could be a simple addition to the current treatment regimen for patients with PKU.

Poor dietary compliance and illness can lead to increased plasma phenylalanine concentrations. During times of negative energy balance, such as illness, the body enters a catabolic state, oxidizing protein for energy and causing the release of amino acids from protein tissues, primarily muscle. Consuming adequate energy, combined with a balance of indispensable (essential) amino acids, initiates a nutrient signaling cascade, stimulating the uptake of free amino acids from the extracellular pool, consequently stimulating protein synthesis and preventing protein breakdown. The indispensable or essential amino acids are: phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, histidine, leucine and lysine (Table 1).

Table 1. Indispensable, Dispensable, and Conditionally Indispensable Amino Acids in the Human Diet

Indispensable	Dispensable	Conditionally Indispensable
Histidine	Alanine	Arginine
Isoleucine	Aspartic Acid	Cysteine
Leucine	Asparagine	Glutamine
Lysine	Glutamic Acid	Glycine
Methionine	Serine	Proline
Phenylalanine		Tyrosine
Threonine		
Tryptophan		
Valine		

The human body cannot synthesize indispensable amino acids and they must be consumed in the diet. The effect of exercise on muscle protein synthesis and subsequent increases in lean body mass has been well established in the literature [9-11]. However, the effect of exercise on blood phenylalanine concentrations in patients with PKU has not been reported.

Our objective was to assess the impact of an acute bout of moderate intensity exercise on plasma phenylalanine concentrations in two adolescent males with PKU using a randomized crossover study where the participants served as their own controls. To assess changes in the indispensable amino acid pool, we used the indicator amino acid oxidation technique. This method utilizes a carbon-labeled isotope (L-[1-^{13}C]) tracer that is ingested orally and oxidation of this label is measured in expired breath as $^{13}CO_2$. This method is based on the assumption that if one indispensable amino acid is deficient, all other amino acids will be oxidized until that particular indispensable amino acid is available in adequate amounts, at which point oxidation

of the amino acid pool, including the tracer, will be the lowest [12]. If all the indispensable amino acids are available in adequate amounts, they will be oxidized at a similar rate. The long-term goal of this novel research is to determine if exercise could be used as an adjunctive therapy to improve the management of plasma phenylalanine concentrations and promote a normal, healthy quality of life among patients with PKU.

CHAPTER 2

A REVIEW OF PHENYLKETONURIA

Introduction

Phenylketonuria (PKU), (OMIM 261600), also referred to as phenylalanine hydroxylase deficiency (PAH)[1, 13], is an inherited autosomal recessive disorder characterized by mutations in the phenylalanine hydroxylase gene [14]. Phenylalanine hydroxylase converts phenylalanine to tyrosine in the presence of the cofactor tetrahydrobiopterin (BH_4), molecular oxygen, and iron. Loss of phenylalanine hydroxylase activity results in increased concentrations of phenylalanine in the blood which can lead to toxic concentrations of phenylalanine in the brain. If left untreated, phenylketonuria causes severe mental impairment, physical and neurodevelopmental complications, aberrant behavior, and psychiatric disorders.

Until the mid-1960's, most children born with PKU were permanently cognitively disabled by two years of age and typically lived in institutional care. The discovery of phenylketonuria can be credited to the persistence and perseverance of a mother, Borgny Egeland, in Sweden in the 1930's [15]. Her son and daughter were physically and mentally disabled, and she sought information about the cause of their disability and their unique odor. Initially reported by chemist Asbjorn Folling in 1933 [15], he named the disorder 'imbecillitis phenylpyruvica' due to the high concentration of phenylpyruvic acid (PPA) in the urine and the clinical manifestation of severe mental retardation in these children [16]. Lionel Penrose, an English geneticist suggested the term "Phenylketonuria" in 1937, now the traditionally accepted name for PKU [1, 17]. In 1953,

Horst Bickel described the effects of a low phenylalanine diet on a two-year old girl with PKU. He recommended a low-phenylalanine diet for her and began initial experiments with elemental formulas as the treatment for this disorder [18]. In the 1960's, Robert Guthrie developed the first diagnostic tool to identify phenylketonuria through the use of a simple blood sample taken after birth by pricking the heel of the infant [19]. Diagnosis was made utilizing this test until the more recent development of tandem mass spectrometry (MS/MS) in the early 1990's [20].

Outcomes of Untreated PKU

Phenylketonuria, if undiagnosed and untreated, results in devastating consequences for the affected individual. The two most apparent clinical findings in untreated PKU are severe neurocognitive dysfunction and a "mousey" smell due to high concentrations of phenylpyruvic acid in the urine. Other clinical signs often noted, even in well-controlled patients, include hyperactivity, light pigmentation of skin, delayed speech, skin rash, and seizures [14, 21, 22]. During the 1960's several prominent researchers suggested that the low phenylalanine diet could be discontinued around 6 years of age with no adverse effects [23-26], however more recent studies indicate that for the best outcomes, a "diet for life" is the optimal mode of treatment [3, 27-31].

Epidemiology

The prevalence of phenylketonuria varies widely between ethnic groups and geographical regions around the world. In the United States the prevalence is about one in 15,000 live births [32]. In Europe, the prevalence is about one in 10,000 live births [33], although in regions such as Northern Ireland and Turkey it is closer to 1:4,000 live births [2, 34]. This is due to the high

consanguinity within these populations. In Poland and Germany, approximately 1 in 8000 children are affected; in Denmark and Norway the prevalence is 1 in 13,000; and Sweden is 1 in 20,000 births [35]. In stark contrast, Finland has the lowest prevalence of PKU in Europe, with one birth in 100,000 affected [36]. The Czech Republic reports a rate of 1 in 8,015 births [37]. The prevalence in Latin America is highly variable, ranging from 1 in 25,000 to 1 in 50,000 births, with the highest rates closest to the equator [38]. As of 2010, Chile reported one birth in 18,916 as affected with PKU [39]. Prevalence rates in Asia vary widely from 1 in 11,572 to 1 in 25,000 on mainland China [40, 41] and less than one in 200,000 live births in Thailand [42]. Hyperphenylalanemia, or mild PKU, occurs at a high incidence throughout Spain [43], whereas Sub-Saharan Africa/Black British/Black populations have a very low prevalence of all types of this disorder [44].

Molecular Genetics and Classification

Phenylketonuria is an autosomal recessive condition. For a child to acquire PKU, each parent must be a carrier of a phenylalanine hydroxylase gene mutation and the child must inherit a defective gene from each parent. The majority (98%) of genetic mutations associated with phenylketonuria occur at the phenylalanine hydroxylase locus [45], on chromosome 12, in the region q22-q24.1. Little or no enzyme activity results in a diagnosis of classical PKU, whereas a mutation that only partially inhibits enzyme activity, typically leads to a diagnosis of mild PKU. There are currently over 600 known mutations of the phenylalanine hydroxylase gene [46].

Phenylketonuria is classified by the severity of mutations and its effect on phenylalanine hydroxylase activity, consequently affecting the plasma phenylalanine concentrations at diagnosis. Plasma phenylalanine concentration ranges for medical diagnosis are described in

Table 2 [47]. 1-2% of cases of phenylketonuria are due to mutations in the genes coding for the enzymes for tetrahydrobiopterin (BH_4) biosynthesis, a cofactor for phenylalanine hydroxylase [48].

Table 2. Classification of Diagnosis of Phenylketonuria

Classification of Phenylketonuria	Plasma Phenylalanine Concentrations
Unaffected	50-100 µmol/L (0.50-1.8 mg/dL)
Mild or Hyperphenylalanemia	120-600 µmol/L (2-6 mg/dL)
Moderate	600-1200 µmol/L (6-20 mg/dL)
Classic	>1200 µmol/L (>20 mg/dL)

Classification of PKU is not always straightforward when measured in neonates. There is the potential that children with PKU may not have reached their maximum plasma phenylalanine concentration when the diagnosis is made through newborn screening. The amount of phenylalanine consumed by an infant prior to the newborn screening test ranges from 350- 500 mg per day depending on the volume the infant is consuming and if they are breastfed or formula fed. Newborn screening tests occur between 24-48 hours after birth and in Oregon specifically, a second screening is conducted 2 weeks later to ensure proper diagnosis and eliminate differential diagnoses. The tolerance for dietary phenylalanine is typically around 250 mg/d (4 g of whole protein) among children with classical PKU. Children with mild and moderate phenylketonuria may tolerate from 250-400 mg/d of phenylalanine or higher [49].

CHAPTER 3

METABOLIC BIOCHEMISTRY

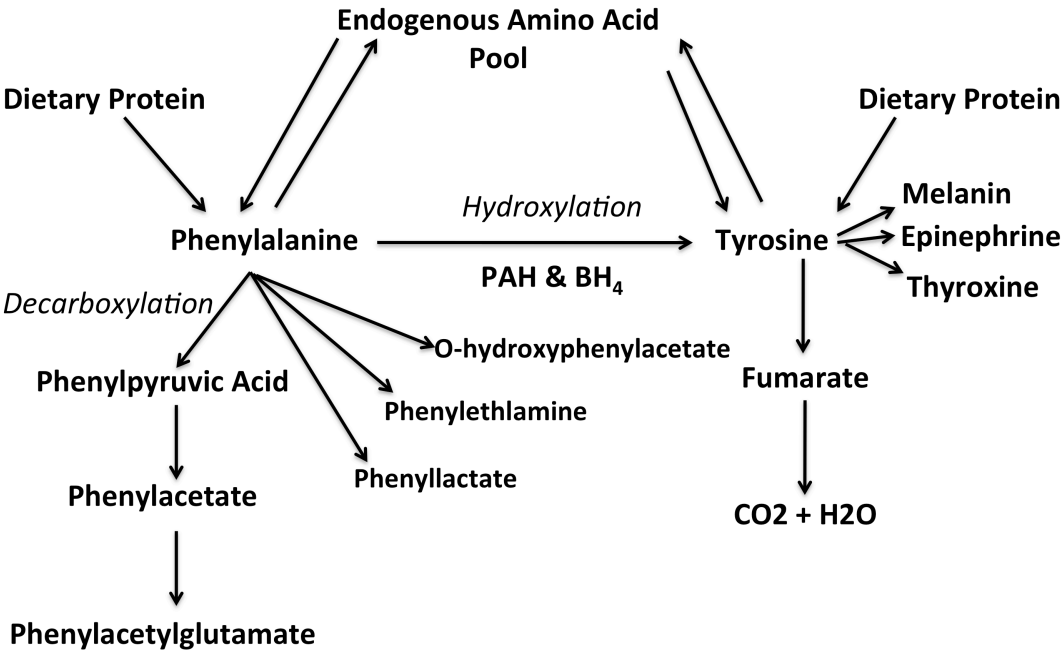
Biochemistry

Phenylalanine Metabolism

Phenylalanine (phe) is an essential amino acid that cannot be synthesized by the human body. Phenylalanine concentration in the blood is determined by a number of processes, including energy intake, endogenous protein turnover, catabolism, and incorporation of phenylalanine into newly synthesized protein. In patients with PKU, although they require a dietary restriction of phenylalanine, some amount of this indispensable amino acid is necessary for adequate growth and development. Phenylalanine is absorbed from the gastrointestinal tract via the portal vein. Phenylalanine may be hydroxylated into tyrosine via phenylalanine hydroxylase in the liver, or it may be incorporated into new proteins in tissues [45]. Due to the lack of phenylalanine hydroxylase activity, excessive intake of phenylalanine by patients with PKU will increase circulating blood phenylalanine concentrations that can ultimately cross the blood-brain barrier causing neurological damage. The etiology of this neurological damage is a current area of research with indeterminate results to date.

The phenylalanine hydroxylase reaction which occurs primarily in the liver, although some activity exists in the kidney, is the rate-limiting step in the catabolic pathway leading to the hydroxylation of phenylalanine [50]. Through this metabolic pathway the supply of the conditionally essential amino acid tyrosine, a precursor to the neurotransmitter dopamine, is

created [49]. Tyrosine is also supplied through the diet or daily protein turnover. The catabolic process begins with the presence of the cofactor tetrahydrobiopterin (BH_4), which phenylalanine hydroxylase (also called phenylalanine monooxygenase) oxidizes to dihydrobiopterin (BH_2), while phenylalanine is simultaneously combined with molecular oxygen and is hydroxylated to tyrosine (Figure 1) [14, 45].



Phenylalanine hydroxylase (PAH) and tetrahydrobiopterin (BH_4) in the presence of molecular O2 hydroxylates phenylalanine to tyrosine. The metabolites of phenylpyruvic acid are excreted in the urine.

Adapted from Scriver, Kaufman 2001 and Acosta 2010.

Figure 1. Catabolism of Phenylalanine to Tyrosine and Related Metabolites

Initially, prominent researchers believed that about 75% of dietary phenylalanine was catabolized to tyrosine and only 25% of phenylalanine was used for protein synthesis. Recent studies using the indicator amino acid oxidation method have shown the amount of dietary

phenylalanine used in protein synthesis is closer to 40-44% [51]. When phenylalanine hydroxylase is not functioning, phenylalanine is also converted to orthohydroxyphenylactic acid (OHPAA), phenylpyruvic acid (PPA), and phenylethylamine. Phenylpyruvic acid is catabolized to phenylacetate, and simultaneously, phenylacetate is converted to phenylacetylglutamate [14].

Pathophysiology

The effect of mutations in the gene encoding for phenylalanine hydroxylase and the resulting disruption of phenylalanine homeostasis continues to be investigated, but the major clinical effect of phenylalanine hydroxylase deficiency is the impact on brain development and executive function [52]. The primary cause of cognitive impairment is increased phenylalanine in the brain. A number of hypotheses have been proposed, with no definitive result, regarding the contributing factors of neurotoxicity in PKU that leads to severe cognitive and structural brain damage [53-55]. Nine amino acid transporters, each of which binds to a fairly specific set of amino acids, facilitate the transport of amino acids from the blood into the brain. Phenylalanine is transported into the brain by one of the large neutral amino acid (LNAA) carriers, the L-amino acid transporter 1 (LAT1) [55, 56]. This transporter also selectively transports valine, isoleucine, methionine, threonine, tryptophan, tyrosine, and histidine. The binding of the LNAA to the LAT-1 transporter is a competitive process; the rate of transport is proportionate to the blood concentration of all the transported amino acids [57]. When plasma phenylalanine concentrations are higher than the other LAT1 transported amino acids, more phenylalanine is transported into the brain.

Neurologically, patients with PKU have displayed white matter abnormalities, identified by structural and functional magnetic resonance imaging [27, 58, 59]. These abnormalities have been found adjacent to the ventricle trigones and occipital horns, and in the frontal and subcortical areas in the brain. The white matter abnormalities are generally regarded as a reduction in the myelin sheath of the axonal neurons [60]. Oligodendrocytes are responsible for myelin formation and maintenance. The myelin sheath is wrapped around the axons of the neurons, and increases the speed of the transmission of action potentials along the axon. If the axon becomes demyelinated, the speed of the transmission is reduced, ultimately affecting cognitive processing speed [61]. Encouragingly, there has been some recent evidence indicating potential reversibility of white matter abnormalities, although this finding has not been consistently replicated [62].

CHAPTER 4

NUTRITION MANAGEMENT

Dietary Management

The cornerstone of the dietary management of PKU is to limit the consumption of the offending amino acid, phenylalanine. In general, the therapeutic diet is restricted in all high protein foods, such as meats, fish, eggs, dairy, breads, pasta and legumes. The amount of phenylalanine tolerated by an individual with PKU is highly individualized and currently determined by the resulting plasma phenylalanine concentration which is monitored on a regular basis through blood tests. The total amount of phenylalanine a patient can consume in one day depends on the residual activity of phenylalanine hydroxylase, the patient's age, rate of growth, and weight [63]. It is fairly common practice in most countries, but not all, to calculate the amount of phenylalanine consumed in all foods throughout each day to maintain adequate blood concentrations [64, 65]. The phenylalanine in fruits and vegetables has traditionally been accounted for to ensure the individual does not exceed their individual tolerated amount of phenylalanine. Recently, studies have shown minimal to nearly no effect on blood phenylalanine concentrations when fruits and certain types of vegetables are liberalized in the diet, and there is the beginning of a paradigm shift within the medical community to allow unlimited consumption of these foods [66-68]. For individuals with limited or no enzymatic activity, the dietary restriction is typically between 200-500 mg of phenylalanine per day, or equivalent to 4-10 grams of whole, natural protein. In comparison, the intake of phenylalanine in the normal pediatric population is approximately 3400 mg per day, or 68 g of protein [49]. Even though the diet to treat PKU is highly restrictive in naturally occurring whole protein,

adequate intake of protein is provided by a medical formula derived from hydrolyzed protein. PKU medical formulas contain all of the essential amino acids, except for phenylalanine, and age appropriate vitamins and minerals that allow children to develop normally along their growth trajectory. Current recommendations from Genetic Metabolic Dietitians International for phenylalanine, tyrosine and protein intakes for individuals with PKU are outlined in Table 3.

Table 3. Recommended intakes of phenylalanine, tyrosine, and protein for individuals with PKU [3]

Age	Phenylalanine (mg/day)	Tyrosine (mg/day)	Protein[a] (g/kg)
0 to < 3 months[b,c]	130-430	1100-1300	3-3.5
3 to < 6 months[b]	135-400	1400-2100	3-3.5
6 to < 9 months[b]	145-370	2500-3000	2.5-3
9 to < 12 months[b]	135-330	2500-3000	2.5-3
1 to < 4 years[b,d]	200-1100	4000-6000	≥ 30
>4 years to adults[e]	200-1100	4000-6000	120-140% RDA for age[f]

[a]Protein recommendations for individuals consuming phenylalanine-free amino acid-based medical foods as part of their protein source.
[b]Recommended intakes for infants and children <4 years of age are adapted from [14] and are for individuals with the classical form of phenylketonuria treated with a phenylalanine-restricted diet alone.
[c]Phenylalanine requirements for premature infants with phenylketonuria may be higher.
[d]Tolerance is usually stable by 2-5 years of age as phenylalanine requirements are based on a combination of size (increasing with age) and rate of growth (decreasing with age). For any individual, phenylalanine intake is adjusted based on frequent blood phenylalanine monitoring.
[e]Adapted from [69]. Range of phenylalanine intake is for the entire spectrum of phenylketonuria (mild to classical).
[f]Recommended protein intake from hydrolyzed-protein medical food is greater than the RDA and necessary to support growth in individuals with phenylketonuria.

Glycomacropeptide (GMP)

Glycomacropeptide is a naturally occurring whole protein that is produced during cheese processing. It is one of several proteins that make up whey protein and it is naturally low in phenylalanine. Current research suggests that patients with PKU report increased satiety, palatability, and expanded food choices when GMP is consumed [4]. Currently, clinical trials are underway studying the effect on satiety, plasma phenylalanine levels, dietary compliance, and cognitive function among individuals with PKU when GMP is provided as a protein source [70, 71].

Large Neutral Amino Acids (LNAA)

This treatment modality is based upon the theory that cognitive outcome and quality of life may improve in some patients with PKU by supplementing their protein intake with large neutral amino acids other than phenylalanine. The large neutral amino acids include histidine, isoleucine, leucine, methionine, tyrosine, tryptophan, valine, and phenylalanine. This treatment increases the plasma concentration of all other large neutral amino acids that compete with phenylalanine for uptake through the LAT1 transporter, thereby decreasing the rate and amount of phenylalanine transported across the blood brain barrier. Several researchers have postulated theories on how supplementation with large neutral amino acids may affect multiple targets such as: a reduction in brain phenylalanine concentration, a reduction in plasma phenylalanine concentration, an increase in brain neurotransmitter concentrations, and an increase in essential amino acid concentration in the brain [53, 55]. LNAA therapy is used as the medical food for some individuals with a more relaxed phenylalanine restriction or older adult patients who have struggled with current diet therapies [3].

Sapropterin dihydrochloride (Kuvan®)

Blood phenylalanine concentrations can be significantly reduced in patients with PKU who respond to the administration of the synthetic form of tetrahydrobiopterin that has been methylated, commonly known as Kuvan®. Kuvan® (BioMarin Pharmaceuticals Inc, Novato, California), is an oral tablet approved for use in the United States in 2007 and internationally in 2008. An individual's responsiveness to Kuvan® depends on the residual activity of the enzyme phenylalanine hydroxylase and milder mutations have been found to be more likely to produce better outcomes [72]. Although not clearly defined, the typical response to Kuvan® is a reduction in blood phenylalanine concentration by approximately 20% and as a result patients usually can consume a more liberalized diet [72, 73]. More subjective signs of responsiveness, including improved mood, attention and cognition, have been reported anecdotally.

Pegylated recombinant phenylalanine ammonia lyase

Pegylated recombinant phenylalanine ammonia lyase (PEG-PAL) is an investigational drug currently in clinical trials (BioMarin Corporation, Novato, California). Phenylalanine ammonia lyase is a potential option for patients with PKU as it is an injectable enzyme substitution for phenylalanine hydroxylase. Found in plants, fungi, and bacteria, phenylalanine ammonia lyase catabolizes the deamination of phenylalanine to ammonia and trans-cinnamic acid, which is safely excreted in urine [74]. Polyethylene glycol (PEG) is a water soluble, non-toxic compound that has been used pharmacologically in a process called "PEGylation" [75]. By encapsulating phenylalanine ammonia lyase in PEG, it increases the enzyme's resistance to degradation and decreases the natural immune response [72].

Although most patients have used one or more of the previously discussed therapies with limited success, overall, outcomes have been less than ideal. Additional treatment options that improve overall plasma phenylalanine control and quality of life for patients with PKU are needed.

CHAPTER 5

AMINO ACID METABOLISM AND EXERCISE

Indicator Amino Acid Technique

In the early 1980's a series of tracer studies was begun at Massachusetts Institute of Technology by Vernon Young and associates to revisit the determination and estimation of amino acid requirements in adults, using stable isotopic techniques to measure amino acid oxidation [76]. Amino acid oxidation studies are based on the principle that any amino acid, provided in excess of the needs of protein synthesis, is preferentially oxidized [77]. The tracer used is an indispensable amino acid labeled at its carboxyl carbon (typically C^{13} for humans and C^{14} for animals). The indicator amino acid oxidation method (IAAO) was applied initially in studies of young growing pigs and validated against traditional approaches based on criteria for growth, nitrogen balance, and body composition [78, 79]. The test amino acid(s) and the tracer are separate, and not linked to nutritionally significant quantities of the tracer. The IAAO technique is based on the concept that when one indispensable amino acid is deficient in the diet, all other indispensable amino acids are oxidized, including the labeled indicator amino acid. As the requirement concentration of the test amino acid is approached, the excess oxidation of the indicator amino acid declines until the test amino acid intake meets the physiological requirement. This is the inflection point and is seen at the plateau in the oxidation rate. The assumption is the indicator amino acid and all other essential amino acids are being supplied at a constant infusion rate at or above the requirement level of the individual. This method was first applied in adult humans by Zello et al. in 1993 to determine the lysine requirements in

healthy men [80]. It is critical that the intake of the indicator amino acid remains constant throughout the entire study period with either IV or oral infusion. Changes in the amino acid concentration in plasma or extra-cellular fluid can alter the rates of transportation into the cells and will change the intracellular, extracellular, and plasma enrichment [81]. For the amino acids phenylalanine and lysine, for which there are no surrogate measures of the intracellular enrichment, such as alpha-ketoisocaproate for leucine, the change in this ratio might confound results. The indicator amino acid oxidation method is mainly a measure of the intake of the test amino acid(s) as a proportion of its content in the amino acid mixture required to minimize oxidation and maximize protein synthesis.

When the intake of one indispensable amino acid is limited, it is expected that there will be an increased oxidation pattern of all other amino acids owing to inefficiency in protein synthesis. As the requirement for each indispensable amino acid is approached, the excess oxidation of the indicator amino acid declines, until it is at its lowest concentration when all the indispensable amino acids are provided in the required amounts. If the intake of the indicator amino acid exceeds the requirement, the low rate of oxidation of the indicator amino acid remains at a plateau. However, there are assumptions with this method regarding the changes in amino acid pool sizes and kinetics that might affect behavior of the tracer isotope and therefore interpretation of the data should be made cautiously.

The IAAO method has been used to estimate phenylalanine and tyrosine requirements in children with phenylketonuria [82] [51] and a healthy population [83, 84] [85]. In addition, IAAO has been used to determine the requirements for the following amino acids: methionine [86-89], threonine [90], tryptophan in adult women [91], branched chain amino acids [92, 93], lysine [80, 83, 94-96], and aromatic amino acids [97]. The oxidation of the indicator amino acid is inversely proportional

to whole body protein synthesis, and responds quickly to changes in the bioavailability of amino acids for metabolic processes, thereby reflecting the true metabolic availability of amino acids.

This method has provided results markedly different from previous nitrogen balance studies. The protein requirements for healthy, non-affected adults and children using the IAAO method were determined to be 0.93-1.2 g/kg/day, and 1.3-1.55 g/kg/day, respectively [98].

Metabolic Availability of Amino Acids

Metabolic availability is determined by measuring the slope of the response (ie: ^{13}C-indicator amino acid) when a test protein is consumed and by calculating the ratio of the test response to the slope of the reference protein or free amino acids. Excess amino acids cannot be stored, so their fate lies in being incorporated into muscle protein synthesis or oxidized (Figure 2) [99, 100].

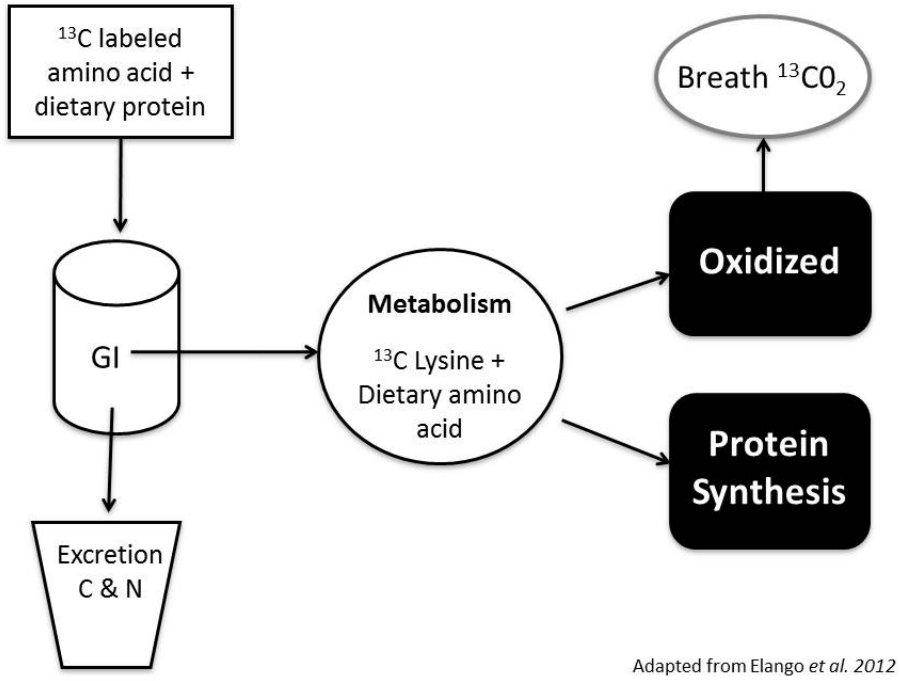


Figure 2. Indicator Amino Acid Oxidation Method & Fate of Indispensable Amino Acids

The labeled tracer amino acid + protein are consumed orally and digested. The majority of amino acids are absorbed into the body and enter the portal vein. Some trace amounts of amino acids are excreted in feces but this is considered negligible. Amino acids pass through the liver and are either taken up by the hepatocytes or pass through and enter the free amino acid pool in general circulation. Amino acids in the free amino acid pool can be transported into cells and oxidized for ATP synthesis or incorporated into body protein. Some amino acids will be degraded and the excess nitrogen excreted as urea in urine. If the tracer is oxidized, it will appear as labeled CO^2 in breath.

Based upon this principle, the change in the oxidation rate of the indicator amino acid is inversely proportional to the change in the rate of protein synthesis. As muscle protein synthesis incorporates amino acids from circulation, the oxidation rate decreases. The intersection between the indicator amino acid and indispensable amino acid pool is the estimated average requirement (EAR) (Figure 3).

Figure 3. Determining the indispensable amino acid requirement using the indicator amino acid oxidation method.

Indicator amino acid oxidation technique classically used to determine amino acid requirements in healthy individuals.

While the indicator amino acid oxidation method has classically been used to determine the amino acid requirements in healthy individuals, we used the basic principles to measure the effect of exercise on the change in indispensable amino acid concentrations by analyzing the oxidation of a tracer amino acid (^{13}C-Lysine).

Exercise

Exercise changes the blood flow, transit time of nutrients, and consequently, the uptake and release of amino acids from muscle tissue. Muscle mass is maintained through the regulated balance of muscle protein synthesis and muscle protein breakdown. A net gain of muscle mass is only possible when muscle protein synthesis exceeds muscle protein breakdown, causing a positive net balance in tissue formation [101]. In a resting, fasted state, protein net balance is negative and usually through feeding, positive balance is achieved. During negative energy balance, such as illness, the body enters a catabolic state, oxidizing protein for energy and causing the release of amino acids from muscle tissue. In contrast, consuming adequate energy and essential amino acids, stimulates muscle protein synthesis and prevents muscle protein breakdown.

It is well documented that exercise increases muscle protein synthesis and lean body mass [9-11]. Exercise programs have been used in spaceflight and during extended periods of bed-rest to promote muscle protein synthesis. Early mobility programs are used by rehabilitation therapy to encourage physical activity of intensive care unit patients [102]. A growing body of literature suggests this approach is safe and effective in selected patient populations to attenuate lean muscle loss [103-105]. During exercise, blood flow to working muscles increases, resulting in decreased blood flow to the liver and a decrease in hepatic amino acid synthesis, subsequently, the gut begins to supply amino acids to the extracellular pool [106, 107]. If the liver catabolizes the excessive amino acids, the result is a decrease in plasma amino acid concentrations whereby muscle protein may be lost through either the suppression of exercise-induced protein synthesis or the increase in muscle protein breakdown. During a bout of exercise at an intensity over 70%

VO_2max, there is a marked increase in the mobilization of amino acids into the free amino acid pool [108, 109].

Since 1975 when human myofibrillar and sarcoplasmic protein synthesis were first measured [110] advances in techniques and technology have enabled researchers to reliably measure the effects of physiological changes to muscle protein synthesis. Biolo et al. in 1997 created a state of amino acid excess by direct intravenous infusion of a complete mixture of amino acids and showed an increase in protein synthesis, a decrease in protein degradation, resulting in a net anabolic effect on muscle [111]. Increased net muscle protein balance indicates arterial blood flow is greater than venous blood flow, suggesting that muscle has increased its uptake of amino acids from the blood to supply the increased rate of protein synthesis.

Intramuscular amino acid concentrations show little change with short-term exercise or exercise at less than 70% VO_2max, but thereafter the concentration of glutamate falls sharply [112, 113]. In prolonged exercise or exercise at high intensity, the release of amino acids that are not metabolized in the muscle (e.g. phenylalanine, tyrosine, tryptophan, lysine, and threonine) increase in plasma [112]. The concentration of glutamine in plasma during short term exercise tends to increase [114, 115], whereas the intramuscular concentration of glutamine remains constant [112, 115, 116].

With regard to plasma amino acid concentrations, there is an increase in the uptake of glutamate during the initial period of moderate intensity exercise and an increase in both the release of alanine and glutamine [114-118]. Various studies have also demonstrated increased

plasma concentrations of lysine, histidine, and arginine during short-term bouts of exercise [114, 119]. There is considerable variation among studies in the magnitude of response, but the overall pattern is similar and appears to be related to the intensity of work. Small changes in the concentrations of other amino acids may occur in the plasma and muscle but, notably, the intramuscular and arterial plasma concentrations of the branch-chain amino acids remain unchanged during exercise, regardless of intensity [117, 118, 120]. Nonetheless, there appears to be an increased rate of branch-chain amino acid oxidation during prolonged exercise, as evidenced by a higher muscle uptake of branch-chain amino acids [117, 118]. Exercise activates the branched-chain alpha-keto acid dehydrogenase (BCKDH) complex in human and rat skeletal muscles [121, 122], an increase in the activation of the rate limiting enzyme, branched-chain oxoacid dehydrogenase (BCOAD) [123] and increased production of $^{13}CO_2$ in breath from infused L-[1-^{13}C] leucine [124]. Data from studies in both rodent muscle [125] and human muscle [11, 126] confirm that muscle protein synthesis is depressed during resistance-type exercise. It is generally agreed that resistance exercise results in increased muscle protein synthesis in the post-exercise recovery period [127].

Tryptophan, a precursor to serotonin, is theorized to suppress the feeling of pain in the body. Studies have attempted to determine plasma concentrations of tryptophan and the impact on aerobic endurance but data is limited and conflicting. Tryptophan does not appear to impact muscle protein synthesis in the current literature [106]. Cysteine supplementation has also been shown to be effective in suppressing loss of muscle mass in animal studies that limited loading and unloading of muscle groups [128].

These previously reported studies on healthy populations demonstrate the flux of amino acids within the body during pre- and post-exercise periods. Regardless of exercise mode, the principal conclusion from these studies is that muscle stimulation generated by exercise is a central physiological driver of muscle protein synthesis. Increased muscle protein synthesis will increase uptake of amino acids into tissues from the free amino acid pool and decrease the concentration of circulating amino acids, such as phenylalanine, in the blood. In combination, nutritional interventions that maintain amino acid availability and exercise therapies that stimulate protein synthesis seem to have a therapeutic potential for preserving mass and function of muscle. This research and feasibility study examined the effects of exercise on amino acid oxidation, flux in plasma amino acid concentrations over a 4-hour post-exercise period, and estimated protein synthesis in two participants with phenylketonuria.

CHAPTER 6

IMPACT OF AN ACUTE BOUT OF MODERATE-INTENSITY EXERCISE ON PLASMA AMINO ACIDS IN ADOLESCENT BOYS WITH PHENYLKETONURIA

METHODS

Patient Recruitment: Three participants diagnosed with classical phenylketonuria (PKU), ranging in age from 10 to 17 years and treated at Oregon Health & Science University, were recruited to participate in this feasibility study. Participants were recruited during their routine clinic visits at Doernbecher Children's Hospital Metabolic Clinic and through a letter in the mail along with a follow-up phone call.

Study Design: A randomized crossover clinical trial was conducted to compare the effects of an acute bout of moderate intensity exercise and sedentary activities on amino acid oxidation and change in plasma amino acid concentrations using the indicator amino acid oxidation technique. Each participant completed two separate study visits over a 30-day period and served as their own control.

Inclusion/Exclusion Criteria: To be eligible for this study, each potential participant: had a plasma phenylalanine concentration of >1200 μmol/L at diagnosis following a newborn screen, had been treated with a dietary phenylalanine restriction from early infancy, and was willing to participate in this study. Participants who had previously or were currently taking approved or experimental pharmacologic treatments (i.e.: Biopterin (BH_4), sapropterin dihydrochloride (Kuvan®), Large Neutral Amino Acids (LNAA), pegylated phenylalanine ammonia lyase (PEG-PAL)) were excluded from this study. Patients who had a recent history of weight

loss, endocrine disorder, were anemic, or involved in any other study or research protocol were excluded. Written consent was obtained from the participant and legal guardians with an explanation of the purpose, benefits and risks. The Institutional Review Board at Oregon Health & Science University approved all procedures used in this study.

Experimental Diet: Energy and protein was provided to the participant as eight iso-caloric and iso-nitrogenous hourly meals. Each meal provided one-eighth of the participant's daily energy needs and consisted of a macronutrient composition of approximately: 14% protein, 56% carbohydrate, and 30% fat [84]. The protein was provided by a phenylalanine-free amino acid medical food mixture at 1.5 g/kg/day for each participant to ensure adequate intake to support anabolism. The source of phenylalanine was a whey protein powder (Beneprotein®) and gluten-free flour base in a homemade lemon sugar cookie. The primary source of energy in the study diet was supplied from the formula and lemon sugar cookie to which the Camino Bar Pro® (Cambrooke Foods, Massachusetts) and Beneprotein® were added to complete the dietary requirements. The base of the liquid formula diet was an orange flavored phenylalanine free drink (LoPhlex LQ®, Nutricia North America) originally developed to supply energy, vitamins, and minerals to individuals with phenylketonuria. Tang® (Kraft Foods) was added to the formula as an additional source of phenylalanine-free energy and to enhance the palatability of the drink. Dietary composition, recipes and cooking procedures are provided in the Appendix.

Tyrosine was provided at 100 mg/kg/day and phenylalanine intake was 14 mg/kg/day [51, 82]. This provided a phenylalanine intake of 950 mg for Participant 1 and 770 mg for Participant 2. The diet was prepared and measured by Registered Dietitians in the Bionutrition Unit at Oregon Clinical & Translational Research Institute (OCTRI) at Oregon Health & Science University.

Metabolizable energy in the formula was calculated from analyses provided by suppliers. Gross energy content of each cookie was determined using the dietary analysis program, ProNutra™ (VioCare, Princeton, NJ). The phenylalanine-free liquid beverage and lemon sugar cookies provided 65% and 25% respectively of the total energy intake. The amino acid mixture provided the remaining 10% of energy.

Body composition measurements: Body composition was determined using dual energy x-ray absorptiometry (DEXA) during the baseline visit. Height was measured while the participant was barefoot using a wall-mounted stadiometer. Weight was taken on a digital standing scale, without shoes, after voiding, and in light-weight clothing.

Oral Isotope Infusions: Each study day required 8 hours (480 minutes) and was divided into 2 separate periods. The first period, hours 1 through 3, allowed for oral infusion equilibration and a baseline measurement; the second period, hours 4 through 9, was when the priming bolus lysine tracer isotope and subsequent half hour oral infusions were administered and the exercise test conducted. L-[1-^{13}C]lysine:HCL was the isotope used in this study, obtained from Cambridge Isotopes (Cambridge, MA). Lysine was used as the indicator amino acid as it does not produce metabolic intermediates and the carboxyl carbon is excreted as CO_2 between the conversion of α-ketoadipate and glutaryl-CoA (Figure 4).

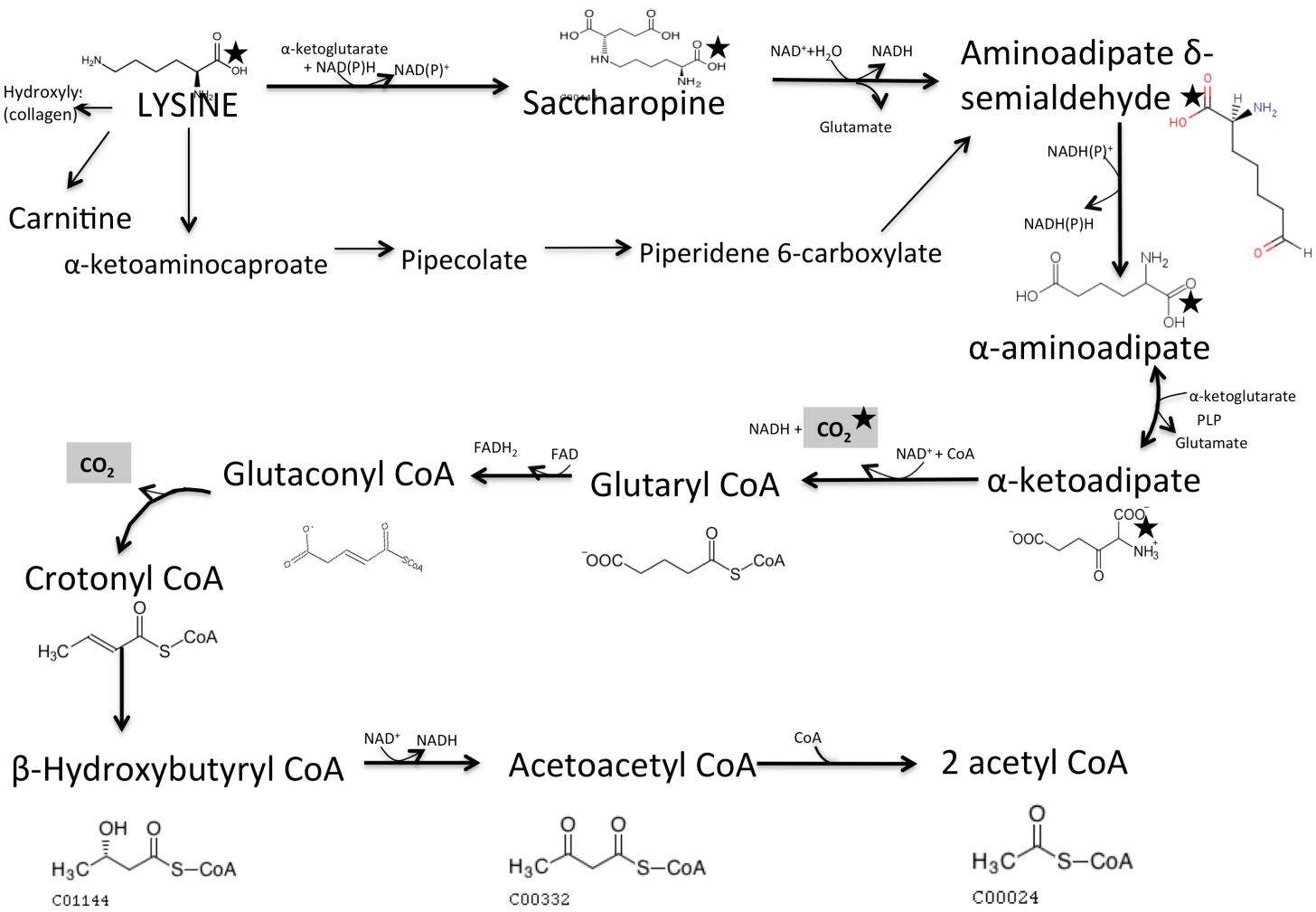


Figure 4. Metabolic Pathway of Lysine

Carboxyl carbon is noted with ★, indicating where it is excreted as CO_2 between α-ketoadipate and Glutaryl-CoA.

Each participant consumed an oral priming dose of 2.5 mg/kg of L-[1C^{13}]lysine:HCL in 150 mL of water. Every thirty minutes thereafter, the participant consumed an oral infusion bolus of 0.7 mg/kg for nine subsequent doses. The oral isotope infusion was followed by consumption of a water rinse of the isotope container to ensure 100% consumption of the tracer. Water (150 mL) was consumed with each meal to ensure a steady production of urine throughout the study day. Urine samples (~5mL) were taken at baseline (Hour 4) and hourly thereafter for the duration of the study for amino acid concentration analysis.

Isotope Oxidation Measurement:

The rate of oxidation of the ^{13}C-Lysine was measured in the expiration of $^{13}CO_2$. Calculation of the rate of oxidation required one fasting 30-minute measure of the rate of CO_2 production (VCO_2, ml/min) taken at the beginning of each study visit, prior to the first meal, through indirect calorimetry using a Sensormedics Metabolic Cart (Sensor Medics, Yorba Linda, CA). Two baseline breath samples were collected prior to administration of the oral isotope and hourly through the end of both study days. Breath samples were collected by having the participant exhale through a straw into a glass test tube. Breath samples were measured for $^{13}CO_2/^{12}CO_2$ enrichment using isotope ratio mass spectrometry (IRMS) by Dr. Jim Delaney at the University of Pittsburgh.

Exercise Testing:

Participants began treadmill testing ergometry immediately following Meal #4. Speed and grade of incline of the treadmill were increased until the participant reached his goal heart rate (75-

85% max heart rate as calculated using the equation 220-age in years). Treadmill testing was performed on an SMC 2000 treadmill (Sensor Medics, Yorba Linda, CA) with continuous ECG monitoring using a Sensor Medics Cardiosoft digital system. Respiratory gases were collected using a mouthpiece and Hans-Rudolph mask with gas exchange measured using a Sensor Medics VMAX 29 metabolic cart (Sensor Medics, Yorba Linda, CA).

Patient Schedule

Baseline visit: Participants arrived at the Clinical & Translational Research Center (CTRC) after an overnight fast of 10 hours. Height, weight and blood pressure were recorded. Baseline resting energy expenditure (REE) was measured by indirect calorimetry (IC). Total body composition was measured by DEXA. Total energy needs were calculated as REE X 1.5 activity factor for subsequent study visits. Participants were provided a sample of the hourly meals to ensure consumption on the study day and then discharged.

Study Visits: Each participant was studied on 2 non-consecutive days over a 1-month period with one sedentary and one exercise visit. The order of the study visits was randomly assigned. Participants arrived after an overnight fast of 10 hours. The participant's weight, height, vitals, REE and volume of CO_2 expired (VCO_2) at rest was measured by IC prior to the first meal. This took approximately 1 hour.

Exercise Study Visit: Procedures were the same as the baseline visit for hours 1-3. Between meal #3 and meal #4 an indwelling-catheter was placed for repeated venous blood sampling. Participants completed a moderate-intensity treadmill exercise test between meal #4 and #5 (Figure 5). Participants walked on a treadmill for 45 minutes at 75-85% of their estimated maximum heart rate. Predicted maximum heart rate was calculated using the formula: maximum heart rate = 220 - age in years. Blood samples were drawn prior to ^{13}C-Lysine bolus administration and every hour through the end of the study day. Blood was analyzed for amino acid concentrations. The exercise protocol was performed as follows: 3 minute warm-up phase with a slow walk at 1.5 miles per hour at 0% grade followed by increases in speed and incline every two minutes until the participant's heart rate achieved 75-85% of his predicted maximum heart rate. Participants continued at this rate for an additional 40 minutes. Heart rate monitors

were worn for continual monitoring of beats per minute during exercise and electrocardiography was conducted for the duration of the exercise. Blood pressure was taken at 10-minute intervals during the exercise test in order to determine consistency in cardio output as compared to the expiration rate of CO_2. Exercise was stopped if the participant developed any symptoms such as dizziness, respiratory distress, chest pains or muscle pain during exercise. A diagram of the hourly exercise/sedentary study procedures is given in Figure 5. Each of the two study visits required a 10-hour outpatient stay at the CTRC.

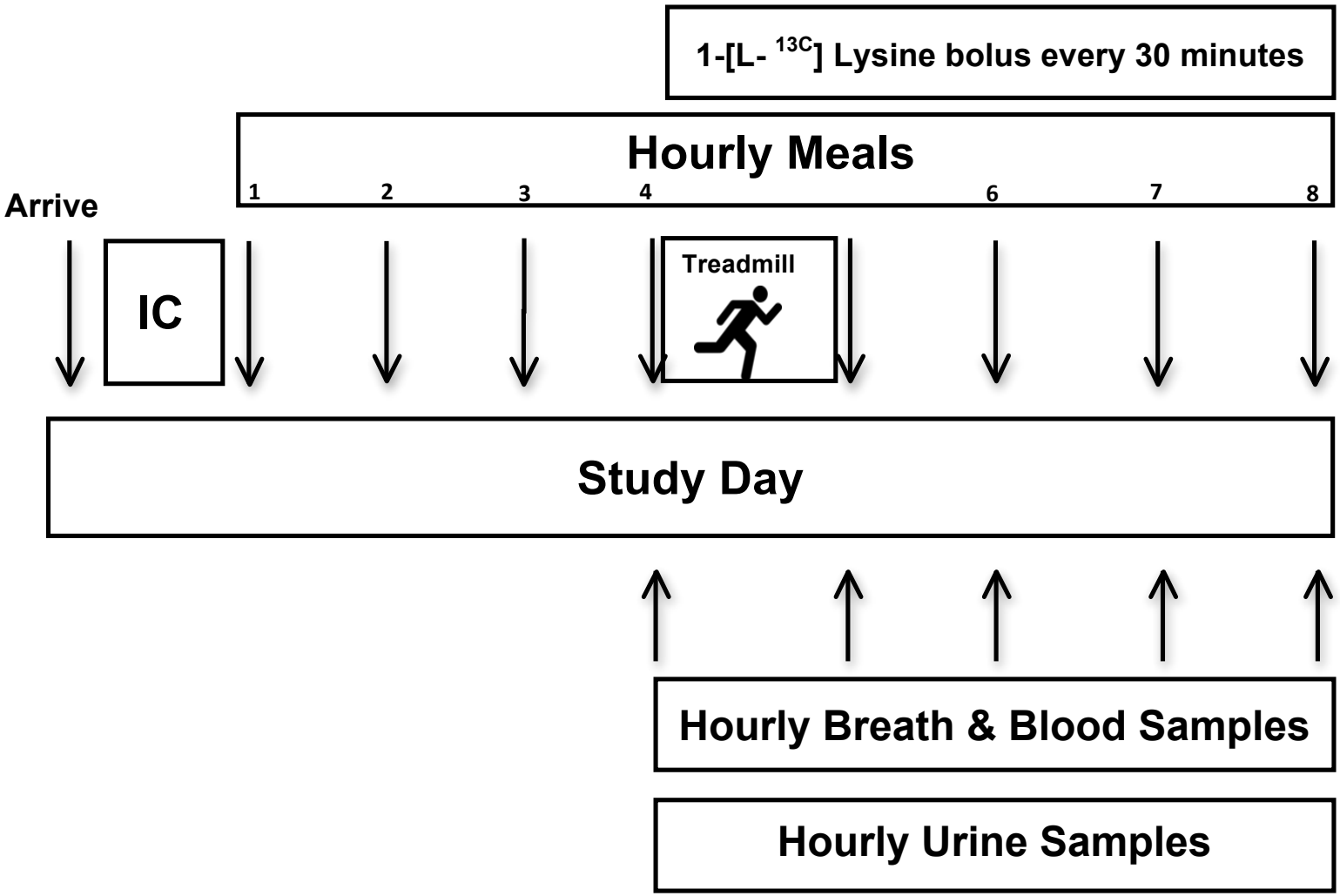


Figure 5: Hourly study procedures during the exercise and sedentary study visits.
Hourly breakdown of study procedures for each study day with arrival noted before indirect calorimetry. Hourly meal #1 through #3 provided for constant oral infusion of amino acids and energy. Hour #4 begins oral labeled isotope infusion bolus with meal and breath, blood and urine sampling.

Sedentary Study Visit: Participants were allowed to do non-physical activities such as watching TV or movies, playing on an iPad or reading for the duration of the entire 10-hour stay. IV placement and sampling occurred during the same time frame as the exercise study visit. Completion of the final sample collection, removal of the IV catheter, and discharge took about 1 hour for both study visits.

Outcome Measures: Breath samples collected every hour after the 4th meal through the end of the study were analyzed for $^{13}CO_2/^{12}CO_2$ enrichment. Increased enrichment of breath with $^{13}CO_2$ indicated the lysine tracer was oxidized. Blood samples were collected after meals 4, 5, 6, 7, and 8. Hourly urine samples were collected at the same time as breath and blood samples. Blood and urine samples were analyzed for amino acid concentrations. Influx of amino acids into the free amino acid pool was held constant with the defined hourly meals.

Whole blood was collected into sodium heparinized tubes and placed on ice. Blood was spun in a centrifuge at 2400 RPM, at 4C for 10 minutes. Plasma was removed, aliquoted into 1.8 mL freezer tubes and stored at -80C until analysis. Plasma amino acids were analyzed in the Biochemical Genetics Laboratory at Oregon Health & Science University. Plasma and urine amino acids were derivitized with 6-aminoquinoly-N-hydroxysuccinimidyl carbamate (AQC, Waters ACCQ Tag^{TM}derivitization system) followed by separation with ultra-high performance liquid chromatography and Tunable ultraviolet/visible (TUV) detector (Waters Acquity^{TM} UPLC, Milford, MA).

Expired $^{13}CO_2$ enrichment in breath samples was measured using an isotopic ratio mass spectrometer (Thermo Finnigan, Brahn, Germany) and expressed as atoms percent excess (APE) $^{13}CO_2$ over the baseline $^{13}CO_2$. The cumulative percentage of the ^{13}C-Lysine to the dose of $^{13}CO_2$ exhaled at each hour (L) was calculated using the following equations and constants:

Constants:

22.4 = molecular weight of CO_2

60 = minutes in 1 hour

146.19 = molecular weight of ^{13}C-Lysine

APE = atoms percent excess as reported from breath sample analyses

0.01109 = natural background of ^{13}C

VCO2 L/min during exercise= average of minutes 3 through 40 during exercise test

VCO2 L/min during rest = as measured by IC at the start of the study day

Where:

A = ^{13}C-Lysine dose given during hour 4 (priming and bolus dose)

B = ^{13}C-Lysine dose for each hour 5 through 9 (each hour is the same)

*C = CO_2 production in mmol/hr =(VCO_2 L/min at rest / 22.4) * 1000 ml/L * 60 minutes*

*D = CO_2 production mmol/hr = (VCO_2 L/min for exercise/ 22.4) * 1000 ml/L * 60 minutes*

E = Excess ^{13}C-Lysine dosed over exercise period =

=A/146.19

F = Excess 13C dosed at rest=

=B/146.19

G = APE in mmol of which is ^{13}C

=(APE /1000) * 0.01109

H = % of G that was excreted =

*=G/E *100*

I = area of the dose over a 1 hour time frame

*=(G + H/2) * 1*

J = cumulative area of the dose excreted

=I + H

L= % of the APE dose that was oxidized each hour in CO_2

*L = J * D*

We were not able to measure urine ^{13}C-lysine enrichment to calculate lysine flux. The analysis of urinary ^{13}C-lysine/^{12}C-lysine requires an HPLC connected to an IRMS which was not available. Future presentations of this data will incorporate flux calculations. Complete spreadsheets with calculations are attached in the Appendix.

Data Analysis: Descriptive statistics were computed to assess central tendency and variance at baseline and each hour post exercise. Statistical analysis was not possible because of the low number of participants (n=2). Differences in lysine oxidation (as measured in expired $^{13}CO_2$) and changes in plasma amino acid concentrations between sedentary and exercise study visits were compared as absolute and percent of baseline change over the study visit. Participant data that was inaccurate or missed during collection of set intervals was excluded.

RESULTS

Demographics. Three male participants were consented for this study and two completed both study days of the trial and contributed to the results reported (Table 4). The participants who completed this study were 14 and 17 years old, had a diagnosis of classical PKU, and were from the local Portland, Oregon area. The third participant withdrew prior to conducting his exercise study day. The nutrient composition of study hourly meals for each participant is provided in Table 5.

Plasma Amino Acid Concentrations

Figure 6 shows plasma phenylalanine concentration for the prior 18 months as recorded in each participants' electronic medical record. Plasma phenylalanine concentrations for both participants had been increasing and some values were noted to be above treatment range (120-360 µmol/L) during the time this study was conducted. Baseline, sedentary and exercise visits are noted in Figure 6 for reference.

Both participants' plasma phenylalanine concentrations at the beginning of the sedentary visit were within treatment range (316 µmol/L and 322 µmol/L, respectively) and concentrations steadily declined over the study day (Figure 7A & 7B). Participant 1 has plasma phenylalanine concentrations above treatment range at the beginning of the exercise visit (538 µmol/L), which remained elevated during and after exercise (Figure 7A). Conversely, participant 2 had well-controlled plasma phenylalanine concentrations at the start of the exercise visit (343 µmol/L) which declined over the course of the study day (Figure 7B). Plasma tyrosine concentrations remained relatively stable for both participants during the sedentary visit and increased in concentration and volatility during the exercise visit (Figure 7C & 7D).

Table 4. Participant Characteristics

	Participant 1	Participant 2
Sex	**M**	**M**
Age (yr)	**14**	**17**
Weight (kg)	**62.2**	**56.2**
Weight-for-age percentile (%)	**80**	**25**
Height (cm)	**155.5**	**165.7**
BMI (kg/height m^2)	**25.7**	**20**
BMI-for-age percentile (%)	**95**	**45**
Body Surface Area (m^2)	**1.8**	**1.9**
Phenylalanine intake (mg/kg/d)	**450**	**650**
Average Plasma Phenylalanine concentration (µmol/L)[¥]	**316**	**336**

** Weight and Height from baseline measurements; BMI, body mass index; and Body Surface Area determined by dual energy x-ray absorptiometry. Phenylalanine intakes represent total amounts prescribed in most recent chart note in EPIC. [¥]Average blood phenylalanine concentration per EPIC record since 1/2013.*

Table 5. Nutrient Composition of Hourly Study Meals

Dietary Constituents									
		Calculated energy (kcal/d)	Provided energy	Protein g	Fat g	CHO g	Phe mg	Tyr mg	Lysine mg
Participant 1	Total Intake	2716	2712 (44 kcal/kg)	97 (14%)	91 (30%)	383 (56%)	950 (15 mg/kg)	7200 (116 mg/kg)	7927.36 (127 mg/kg)
	Hourly Meal		339 (6 kcal/kg)	12	11.5	47.8	120	900	991
Participant 2	Total Intake	2100	2096 (37 kcal/kg)	78 (15%)	70 (30%)	295 (55%)	770 (14 mg/kg)	5740 (100 mg/kg)	6256 (109 mg/kg)
	Hourly Meal		262 (5 kcal/kg)	10	9	37	91	712	782

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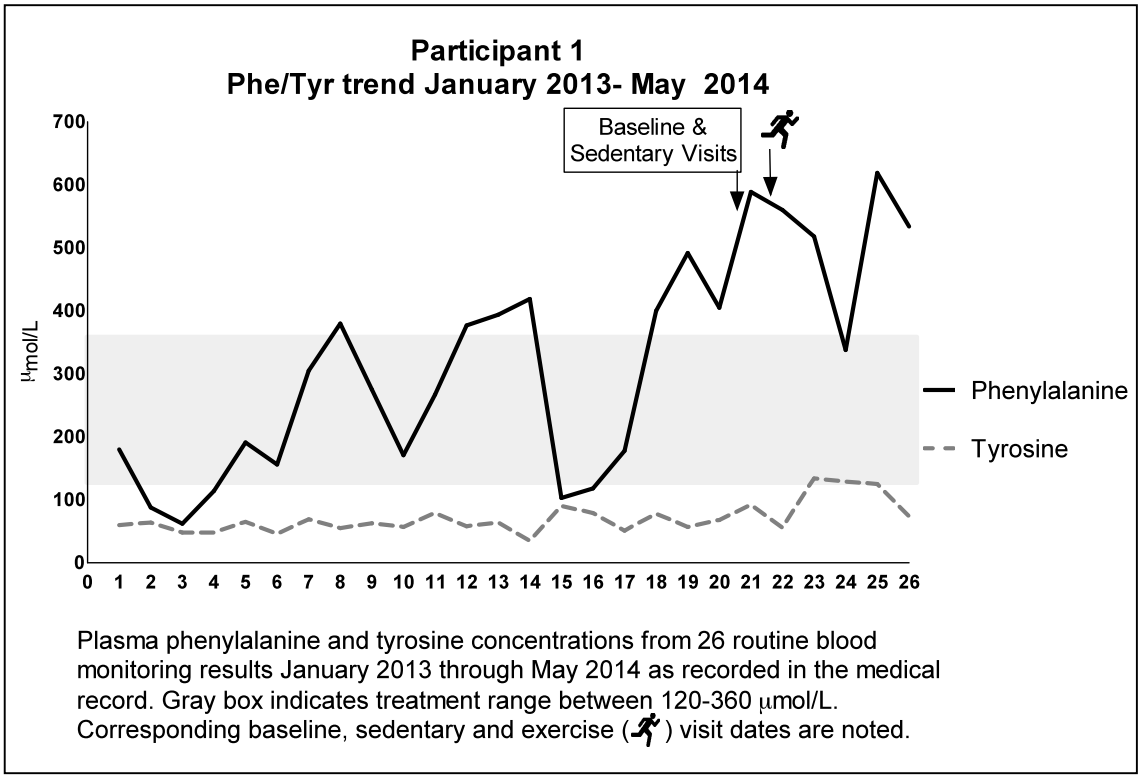


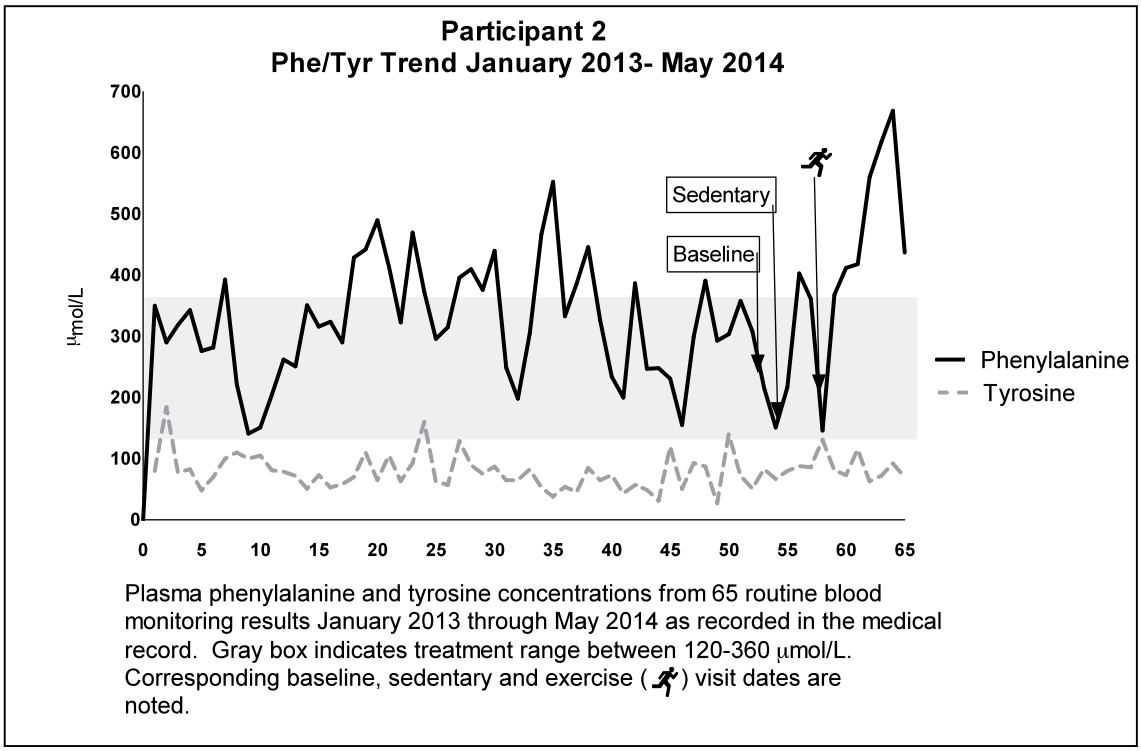


Figure 6. Plasma Phenylalanine and Tyrosine Concentrations from January 2013 - May 2014

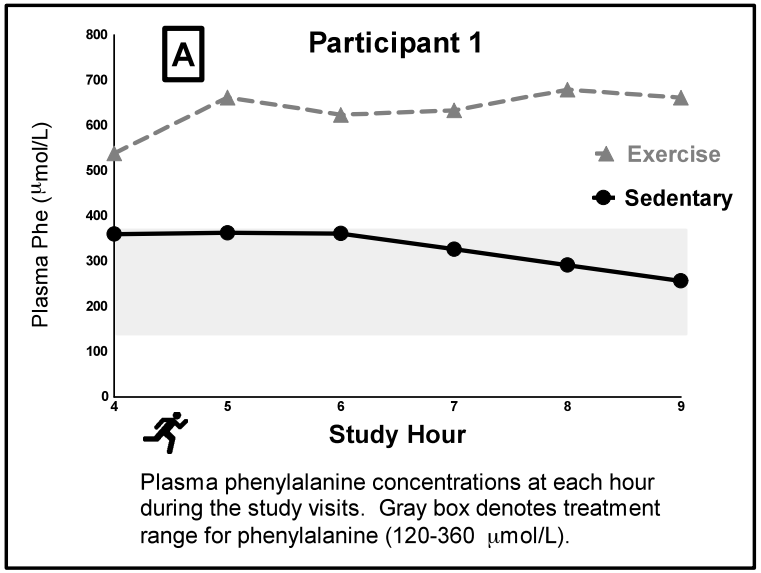
Exercise data.

Participants completed a 45-minute moderate intensity treadmill test. Participant 1 was slightly below the goal range of 74%-85% of maximum heart rate, averaging 71%. Participant 2 maintained the goal for the entirety of the test, averaging 78%. Both participants maintained normal blood pressure throughout the test. Respiratory quotient (RQ) of 0.98 and 0.97 indicated both participants burned primarily carbohydrates for the duration of the exercise test. Participant 1 expired a greater volume of CO_2 but had similar metabolic equivalent (METS) and perceived exertion (BORG) as participant 2 (Table 6). Participant 2 exercised at a steeper grade and faster speed to reach his target heart rate suggesting a greater level of fitness as compared to Participant 1.

Table 6. Treadmill test data at 10 -minute intervals and total average

	Participant 1					Participant 2				
	Minute 1-10	Minute 11-20	Minute 21-30	Minute 31-42	Average	Minute 1-10	Minute 11-20	Minute 21-30	Minute 31-42	Average
Heart Rate % (BPM)	71% (146)	71% (147)	71% (147)	57% (121)	71% (147)	78% (158)	79% (161)	77% (157)	79% (160)	78% (159)
Blood Pressure	118/60	116/58	118/58	118/52	118/57	135/70	132/70	130/60	NA	132/67
RQ	1.00	0.97	0.98	0.94	.98	1.00	0.97	0.97	0.94	.97
VCO_2 (L/hr)	1489	1725	1571	1499	1567	1281	1452	1471	1309	1375
VO_2 (L/hr)	11.57	23.37	27.22	25.30	25.28	8.93	21.70	25.15	26.03	23.99
SPEED (mph)	2.07	3.30	3.40	3.99	4.00	2.03	4.10	4.26	4.06	3.70
GRADE (incline)	0	9.0	10	3.4	2.0	0	7.7	10	9.8	9.0
METS	6.7	7.8	7.2	7.2	7.2	6.2	7.2	7.4	6.8	6.9
BORG	3	4	4	1	3	3	3	3.5	3	3

Heart Rate is the percentage of maximum heart rate as calculated using the equation: 220-age
Blood Pressure is presented as systolic/diastolic
RQ is the respiratory quotient
VCO_2 is the volume of carbon dioxide excreted in breath over the hour of testing
VO_2 is the volume of oxygen excreted in breath over the hour of testing
METS is the metabolic equivalent used as a measure of exercise intensity
BORG is the perceived level of exertion on a scale of 1-10 with 1 being the least and 10 the highest



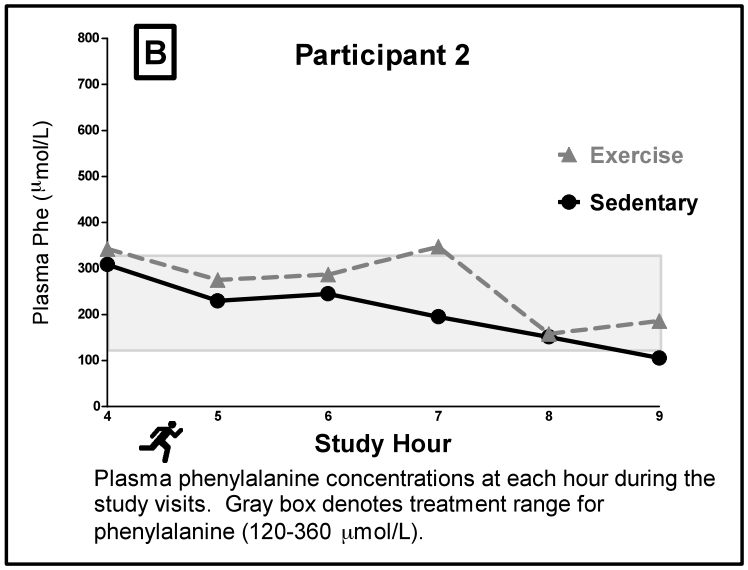
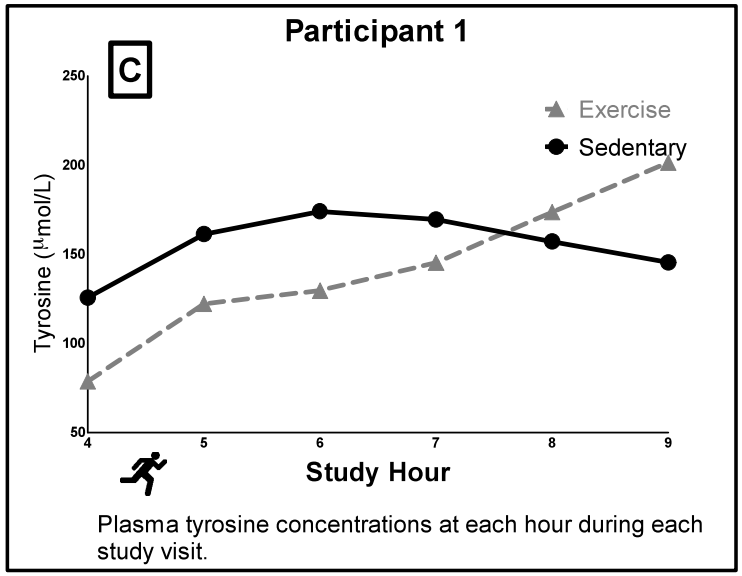


Figure 7A & 7B. Plasma Phenylalanine Concentrations during the last 5 hours on sedentary and exercise study days

Plasma phenylalanine concentrations in participant 1 (A) and participant 2 (B)are indicated by a dashed-gray line during the sedentary study day and a solid black line during the exercise study day beginning at hour 4 until hour 9.



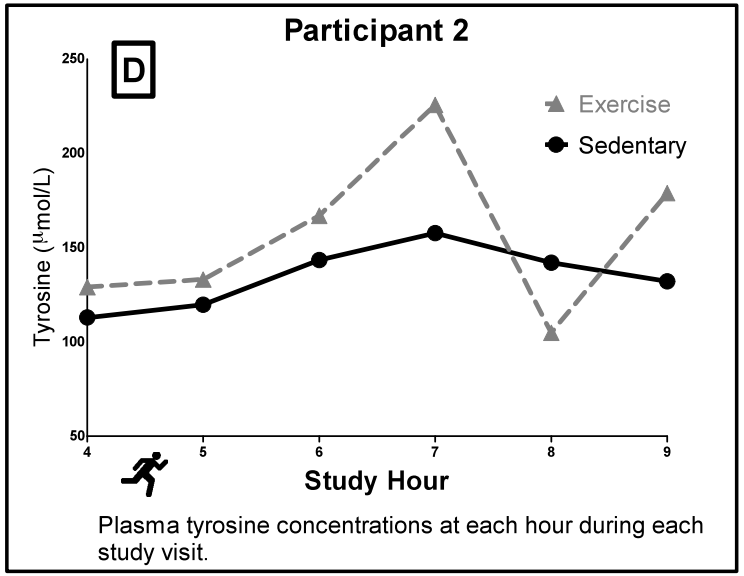


Figure 7C & 7D. Plasma Tyrosine Concentrations during the last 5 hours of sedentary and exercise study days

Plasma Tyrosine concentrations in participant 1 (C) and participant 2 (D) during the exercise study day beginning before exercise at hour 4 and post exercise until hour 9.

Indispensable amino acid concentrations at each hour are shown in Figure 8. Plasma indispensable amino acid concentrations remained relatively stable with a slight declining trend throughout the sedentary visit (Figure 8A & 8B) but tended to increase after exercise (Figure 8 C & 8D).

Both participants' indispensable amino acid pools were assumed to be at a steady state at baseline (Hour 4) during both study visits based on previous indicator amino acid oxidation studies [82, 83] . There was a slight decrease in the indispensable amino acid pool at hour 5 for both participants on the sedentary study day. Concentrations increased over hours 5 and 6 and then steadily declined at hour 7 during the sedentary visit.

During the exercise study day Participant 1 had plasma phenylalanine concentrations that exceed all other amino acid concentrations, particularly the branch-chain amino acids leucine, isoleucine and valine. Plasma indispensable amino acid concentrations increased immediately after exercise and reached a plateau. At the end of the study day, phenylalanine began to decrease slightly as all other indispensable amino acids increased slightly. Due to the restrictions in the protocol, we were not able to fully determine the effects of exercise and potential protein synthesis beyond 4 hours post exercise. In Participant 2, the plasma indispensable amino acid concentrations decreased during exercise and increased after exercise, spiking at hour 7 and declined again at hour 8. There was a net increase in plasma indispensable amino acid concentrations by the end of the exercise study visit.

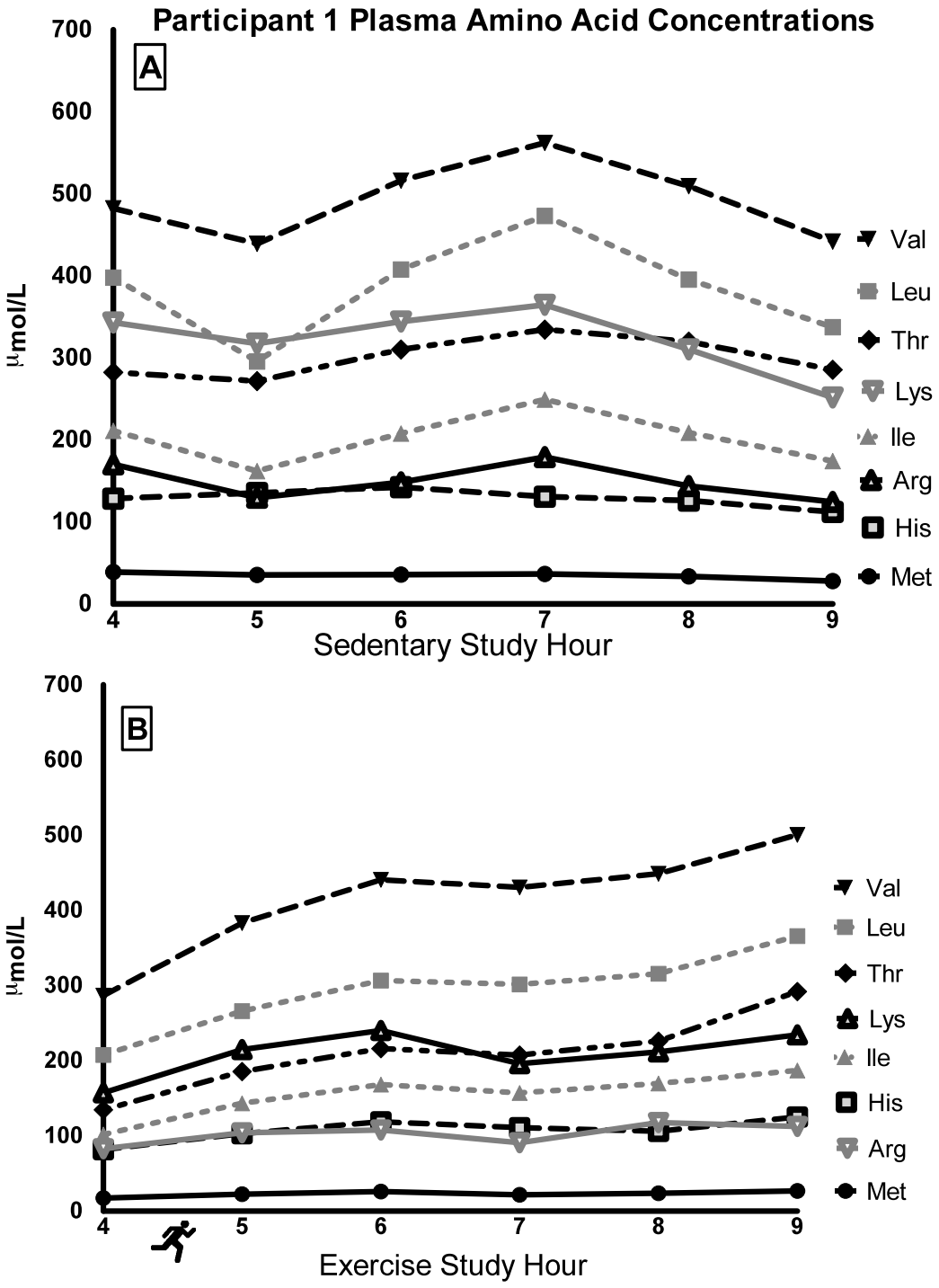


Figure 8A & 8B. Hourly indispensable amino acid concentrations for Participant 1 during the sedentary and exercise visits.

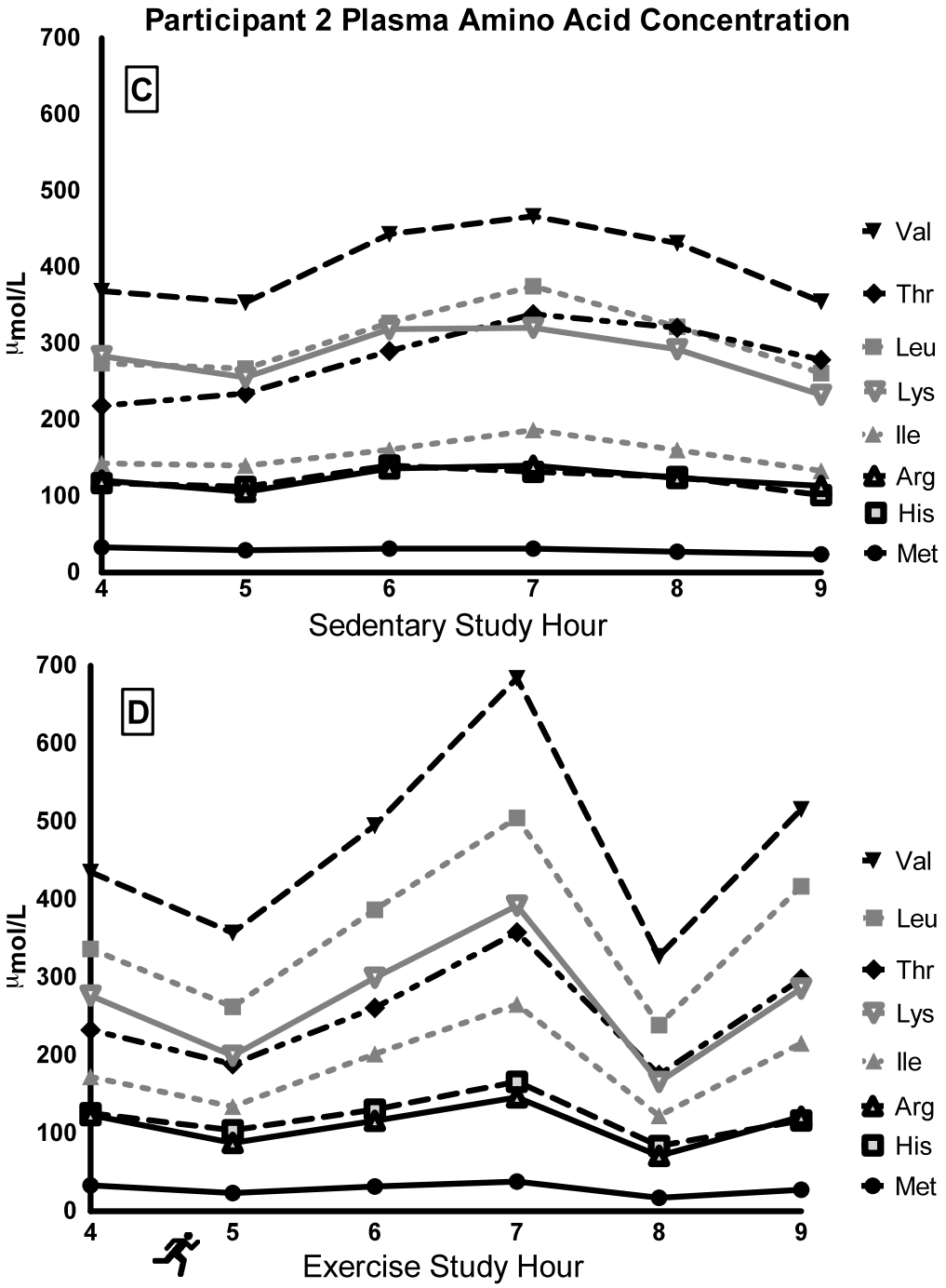


Figure 8C & 8D. Hourly indispensable amino acid concentrations for Participant 2 during the sedentary and exercise visits.

The percent change from baseline in phenylalanine and tyrosine concentrations between the sedentary and exercise study visits was calculated and is presented in Figure 9. Participant 1 had a 28.7% decrease in plasma phenylalanine concentration during the sedentary study day (Figure 9A, Hour 9). Conversely, on the exercise study day, phenylalanine concentrations were elevated prior to exercise, increased 23% immediately after exercise and remained at this level for the rest of the study. Participant 2 experienced a 66% decrease in phenylalanine concentration during the sedentary visit and a 46% decrease during the exercise visit (Figure 9B, Hour 9). Tyrosine concentrations increased in both study participants during both study days (Participant 1, 156%; Participant 2, 38%). The percent change in each indispensable amino acid between study hour 4 and hour 9 for both sedentary and exercise visits are illustrated in Figure 10. Almost all of the indispensable amino acids increased after the exercise visit compared to the sedentary visit.

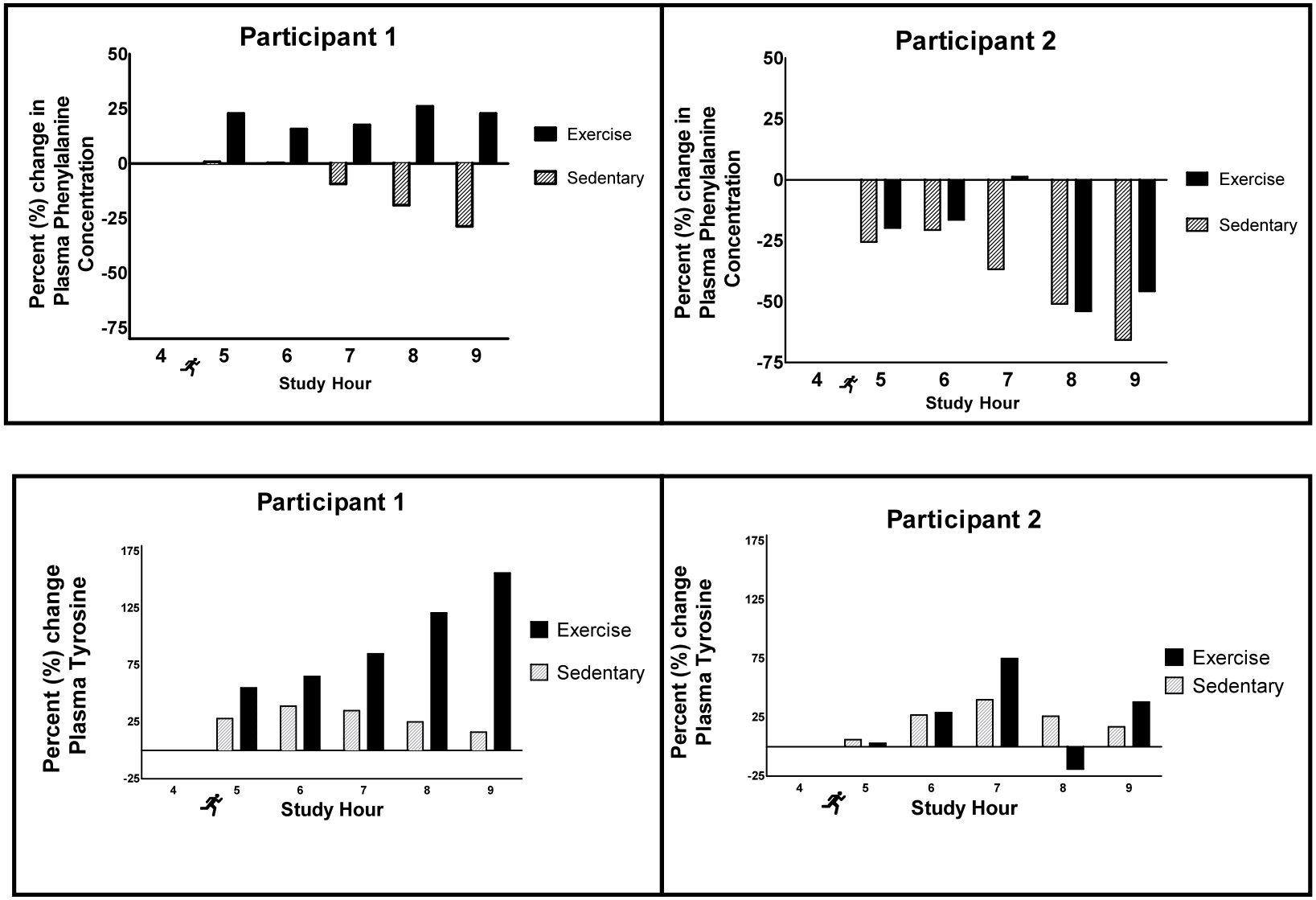


Figure 9. Hourly percent change from baseline in plasma phenylalanine and tyrosine between sedentary and exercise visits.

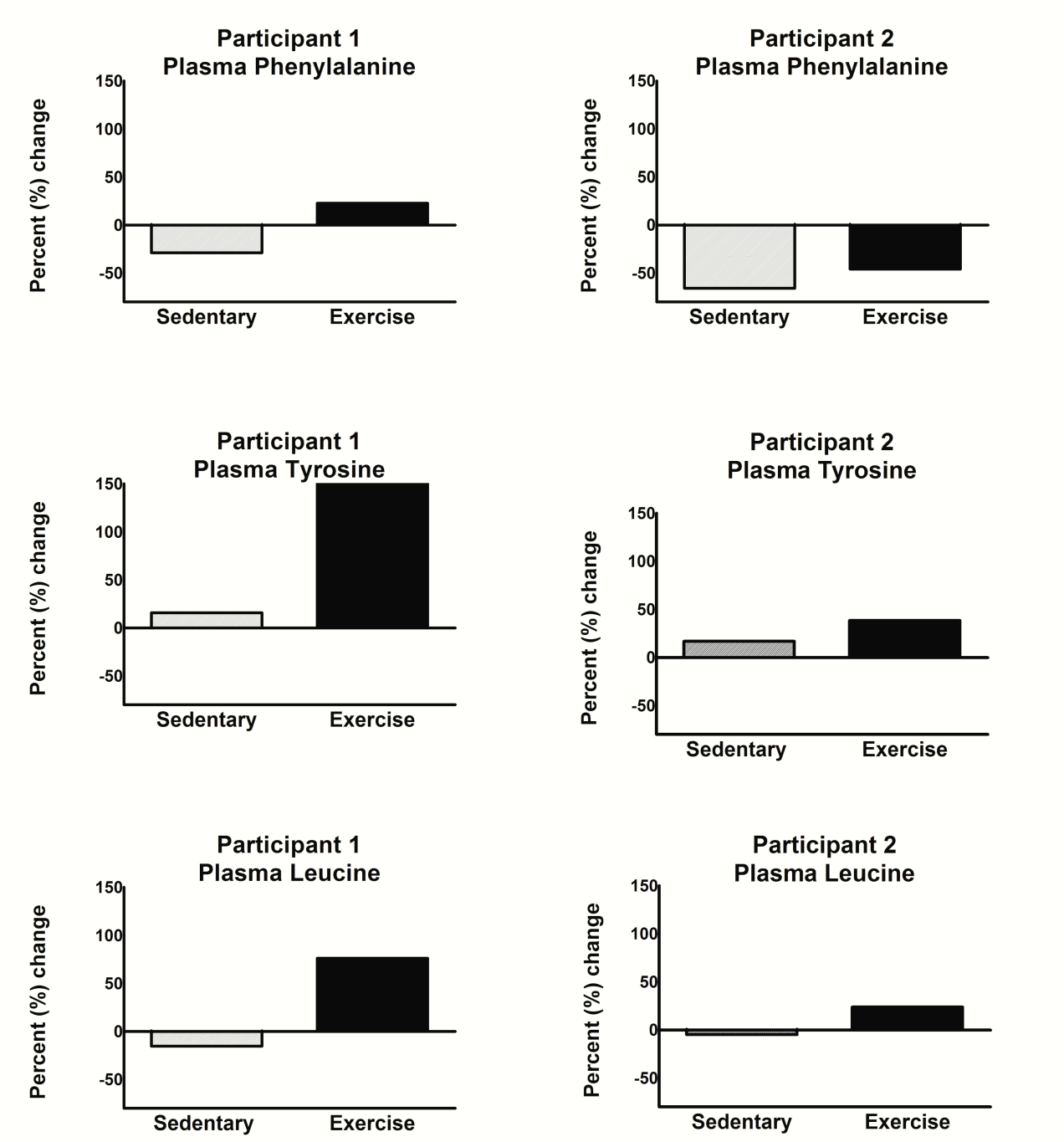


Figure 10. Percent change from baseline and end of study of plasma indispensable amino acid concentrations

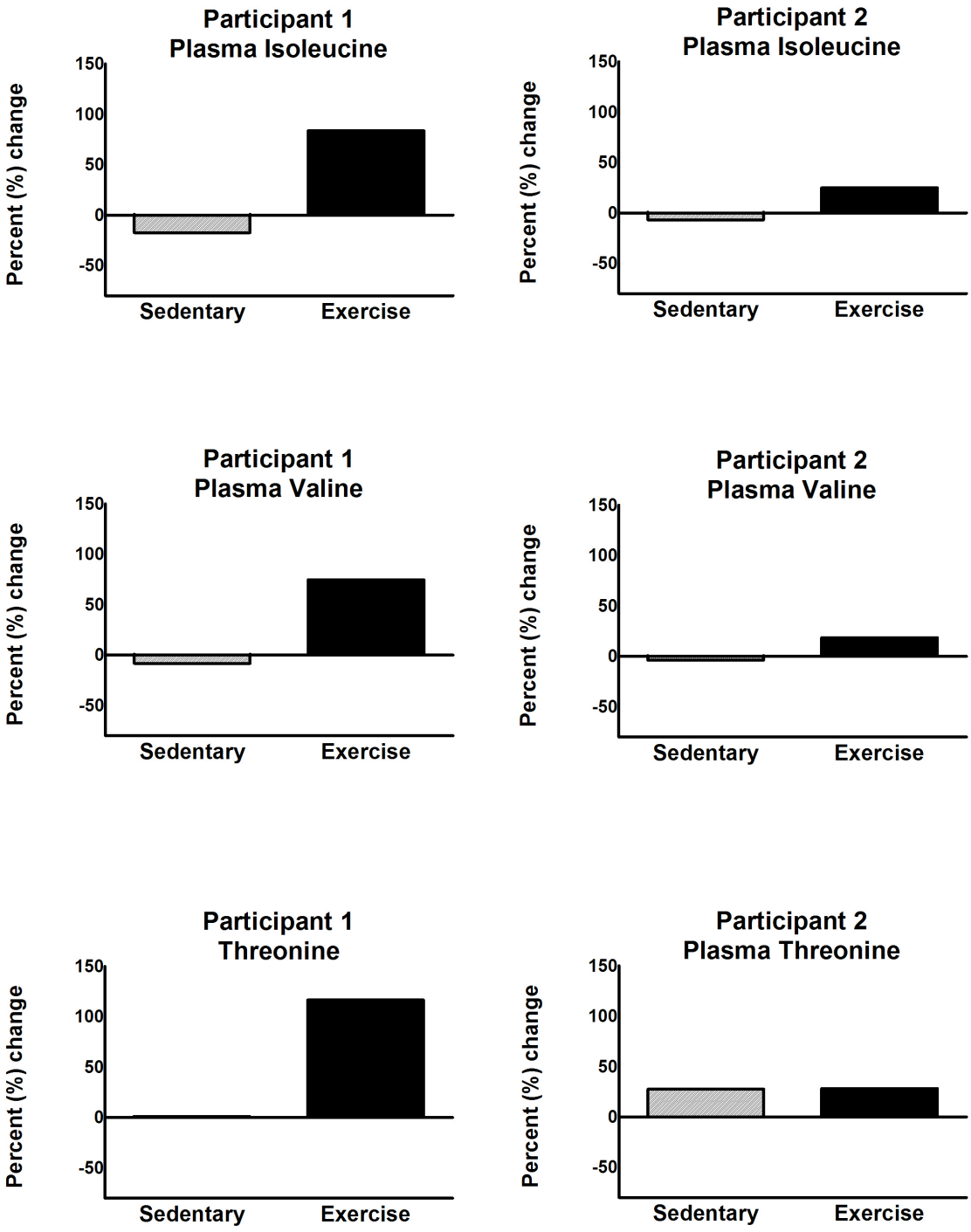


Figure 10. Percent change from baseline and end of study of plasma indispensable amino acid concentrations

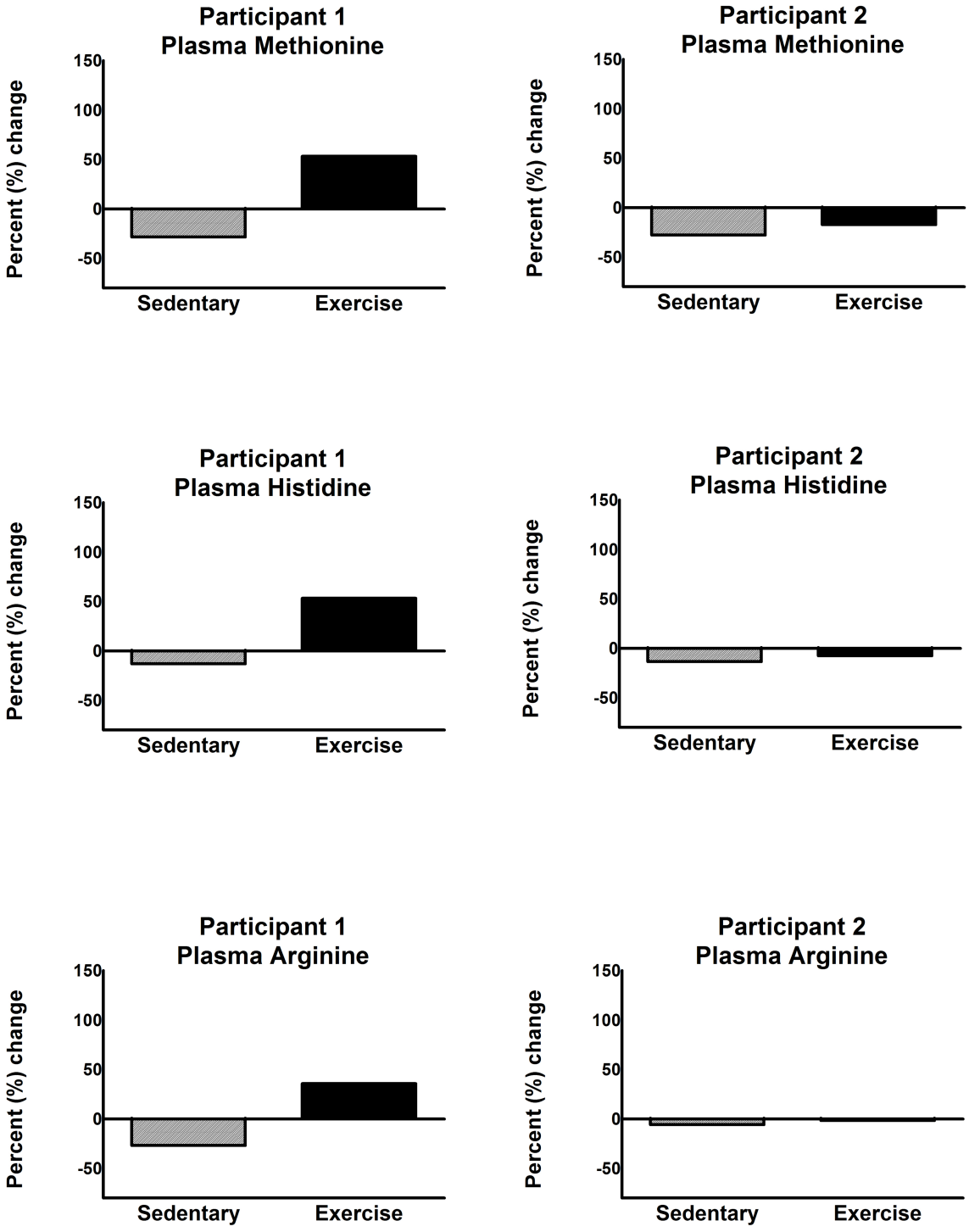


Figure 10. Percent change from baseline and end of study of plasma indispensable amino acid concentrations

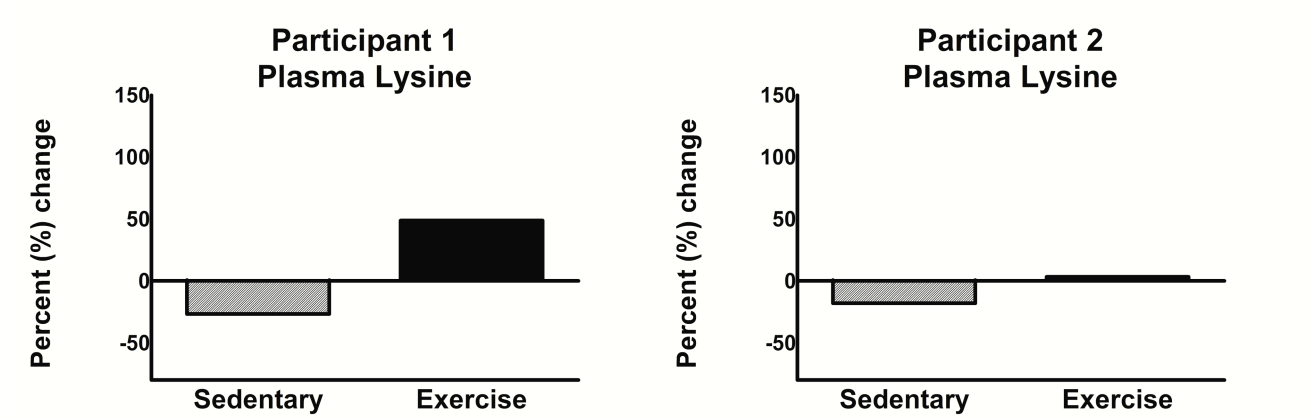


Figure 10. Percent change from baseline and end of study of plasma indispensable amino acid concentrations

Urinary Amino Acids.

Participant 1 had a urinary concentration of phenylalanine 404% higher during the exercise visit as compared to the sedentary visit. It should be noted that this participant had plasma phenylalanine concentrations above treatment range (538 -661 µmol/L) for the duration of this study visit. Participant 2 urinary phenylalanine concentrations were 65% lower during the exercise visit compared to his sedentary visit (Figure 11).

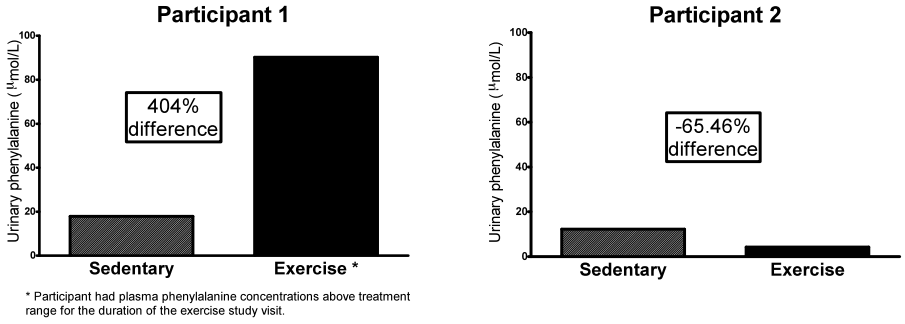


Figure 11. Percent change in urinary phenylalanine concentrations at the end of each study day between sedentary and exercise visits.

The percent difference in urinary indispensable amino acid concentrations between sedentary and exercise visits for Participant 1 ranged from 101% to 404% whereas Participant 2 excreted less indispensable amino acids with changes ranging from -65% to -100% (Figure 12). A comparison between the absolute change in urine indispensable amino acid concentrations is described in the Appendix.

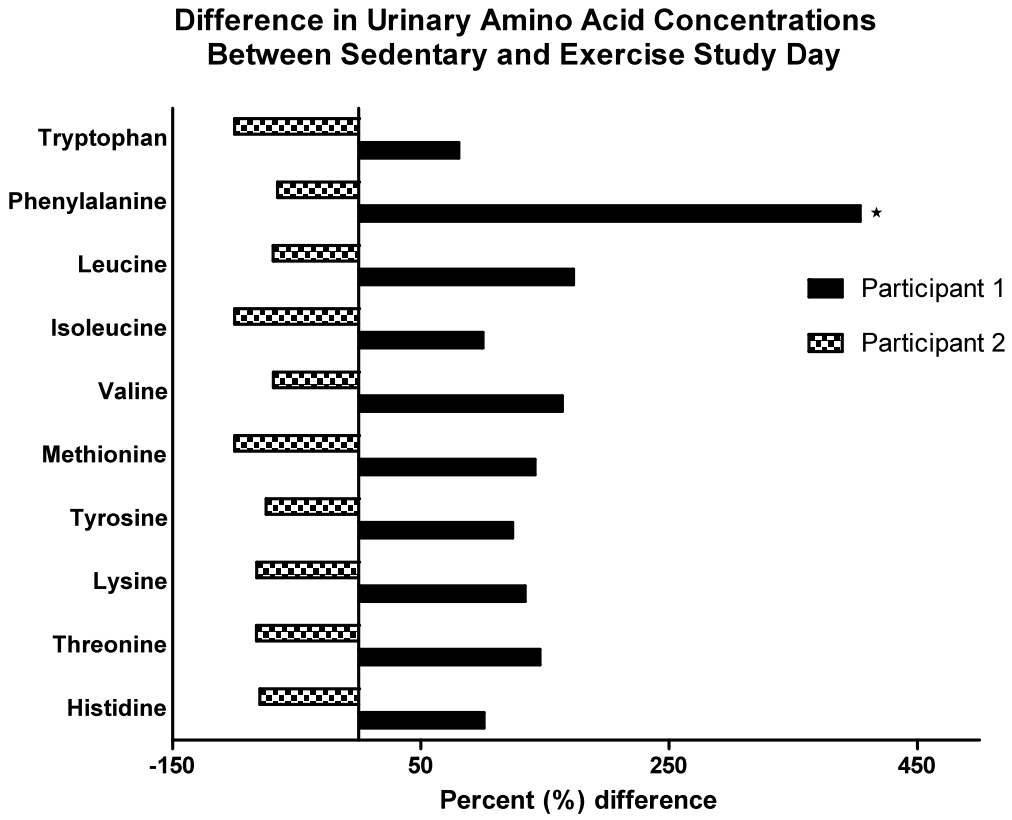


Figure 12. Percent difference in urinary indispensable amino acid concentration at the end of the study day between the sedentary and exercise visits. *Participant has plasma phenylalanine concentrations above treatment range

Lysine Oxidation.

The atoms percent excess of $^{13}CO_2$ released by oxidation of the lysine tracer at each hour of both study visits is shown in Table 7.

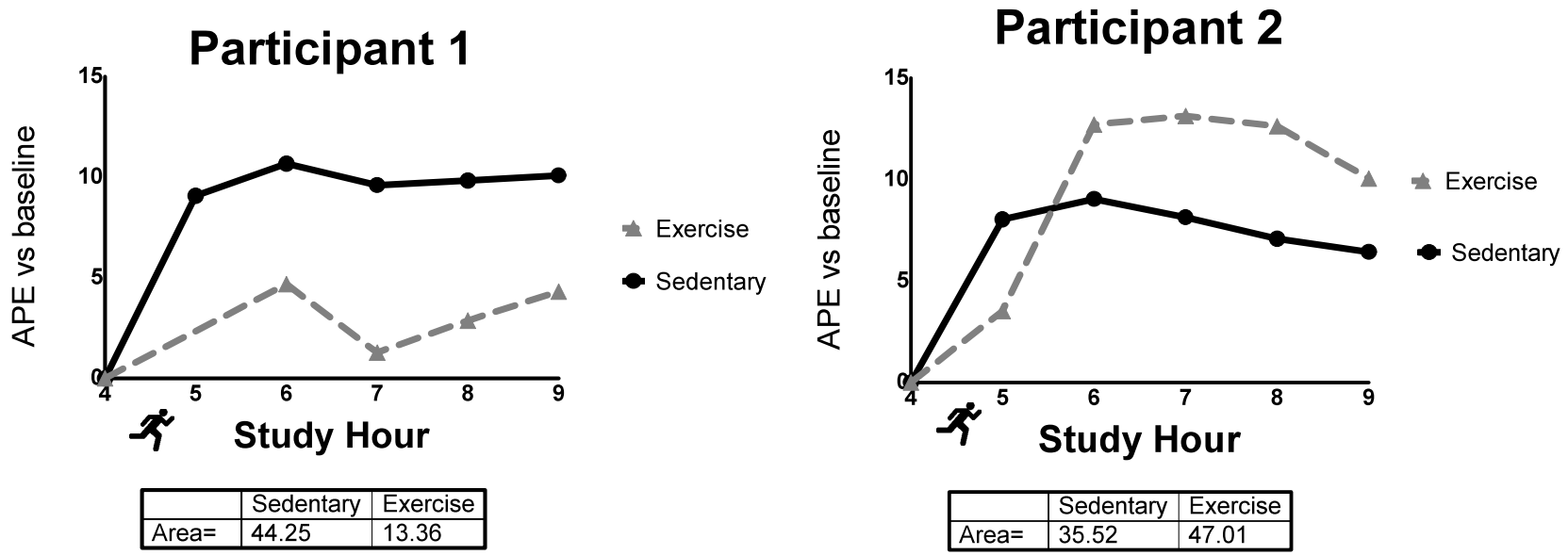
Table 7. Atoms percent excess (APE) of $^{13}CO_2$ expired in breath

	Participant 1		Participant 2	
	Sedentary	**Exercise**	**Sedentary**	**Exercise**
Hour 4	**0**	**0**	**0**	**0**
Hour 5	**9.09%**	**ND***	**8.04%**	**3.52%**
Hour 6	**10.68%**	**4.69%**	**9.04%**	**12.71%**
Hour 7	**9.61%**	**1.29%**	**8.14%**	**13.13%**
Hour 8	**9.81%**	**2.88%**	**7.08%**	**12.62%**
Hour 9	**10.10%**	**4.32%**	**6.44%**	**10.05%**

Numbers are expressed as Average Percent Excess (APE); this represents the percent of ^{13}C from the hourly labeled lysine dose that was excreted. Natural background of ^{13}C was accounted for with a molecular weight of 0.01109.

*Breath sample collection for Participant 1 at hour 5 during the exercise visit was missing.

Total area under the curve (TAUC) is the measureable effect of the "phenomenon" or how much $^{13}CO2$ was exhaled in breath when comparing the sedentary day to the exercise day. Participant 1 expired more $^{13}CO_2$ during the sedentary visit (44.25 APE/hour) compared to 11.02 APE/hour for the exercise visit. Participant 2 expired more $^{13}CO_2$ after exercise (47.005 APE/hour) compared to 35.52 APE/hour during his sedentary visit (Figure 13).



	Sedentary	Exercise
Area=	44.25	13.36

	Sedentary	Exercise
Area=	35.52	47.01

Figure 13. Total area under the curve of atoms percent excess $^{13}CO_2$ excreted above baseline over the last 5 hours of the exercise and sedentary study visits.

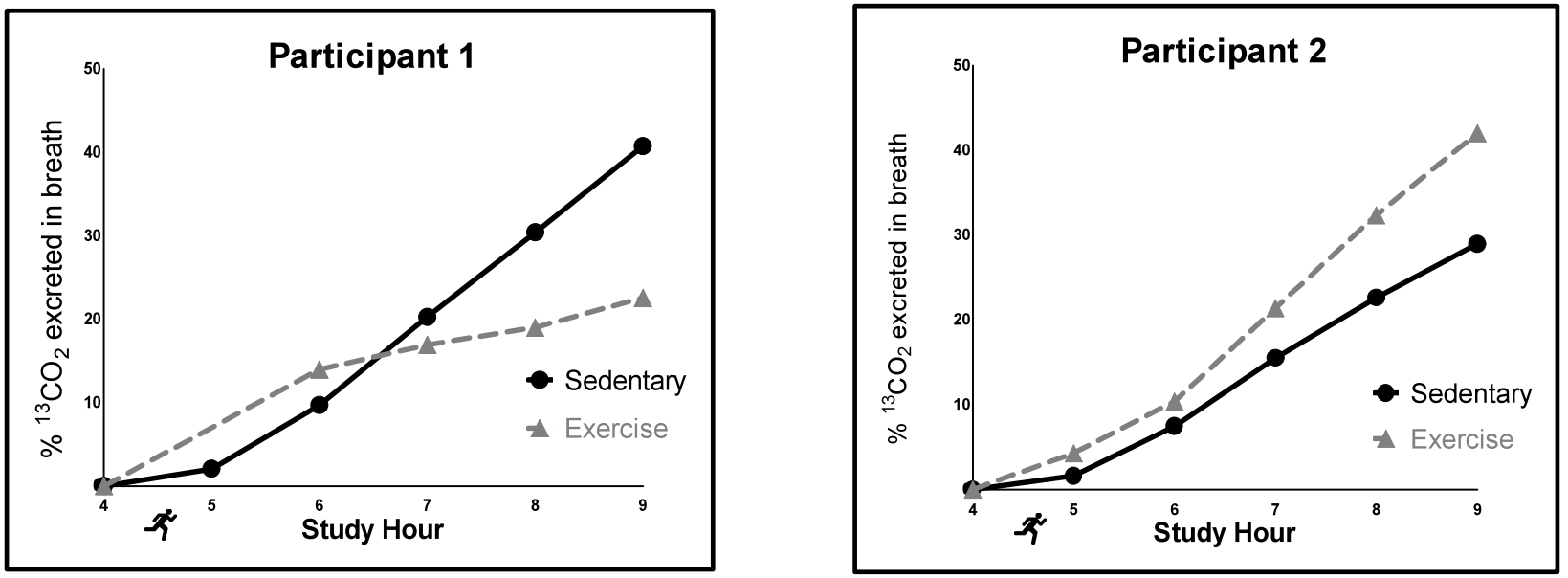


Figure 14. Cumulative atoms percent excess of $^{13}CO2$ excreted in breath samples over last the 5 hours of the exercise and sedentary study visits.

The cumulative percent of the labeled lysine dose expired as $^{13}CO_2$ steadily increased in both participants during both trial arms (sedentary and exercise). During the exercise visit Participant 2 expired 42% of the labeled lysine dose, whereas Participant 1 expired 22%. The converse was seen during the sedentary visit where Participant 1 expired 41% of the labeled lysine dose and Participant 2 was lower, expiring 29% of the dose (Figure 14). The cumulative amount of L-[1C^{13}] lysine oxidized in milligrams as measured by breath $^{13}CO_2$ was calculated and is expressed in Table 8.

Table 8. Amount of L-[1-C13] Lysine administered within each hour and total L-[1C^{13}] lysine oxidized over the course of the study.

	Total L-[1-C^{13}] Lysine Dosed (mg)	Hourly L-[1-C^{13}] Lysine Dose (mg)					Total L-[1-C^{13}] Lysine Oxidized (mg)
		4	5	6	7	8	End of Study Visit
Participant 1							
Sedentary	**551.5**	**199.5**	**88**	**88**	**88**	**88**	**224.96**
Exercise	**551.5**	**199.5**	**88**	**88**	**88**	**88**	**124.5**
Participant 2							
Sedentary	**491.5**	**179.5**	**78**	**78**	**78**	**78**	**142.39**
Exercise	**516**	**188**	**82**	**82**	**82**	**82**	**216.84**

The relationship between plasma phenylalanine concentrations to $^{13}CO_2$ in expired breath samples is described in Figure 15. Participant 1 had a steady concentration of plasma phenylalanine (~600 µmol/L) even in the presence of fluctuating $^{13}CO_2$ enrichment where

participant 2 had a dramatic increase in $^{13}CO_2$ enrichment with a steady concentration of plasma phenylalanine within treatment range (~300 µmol/L).

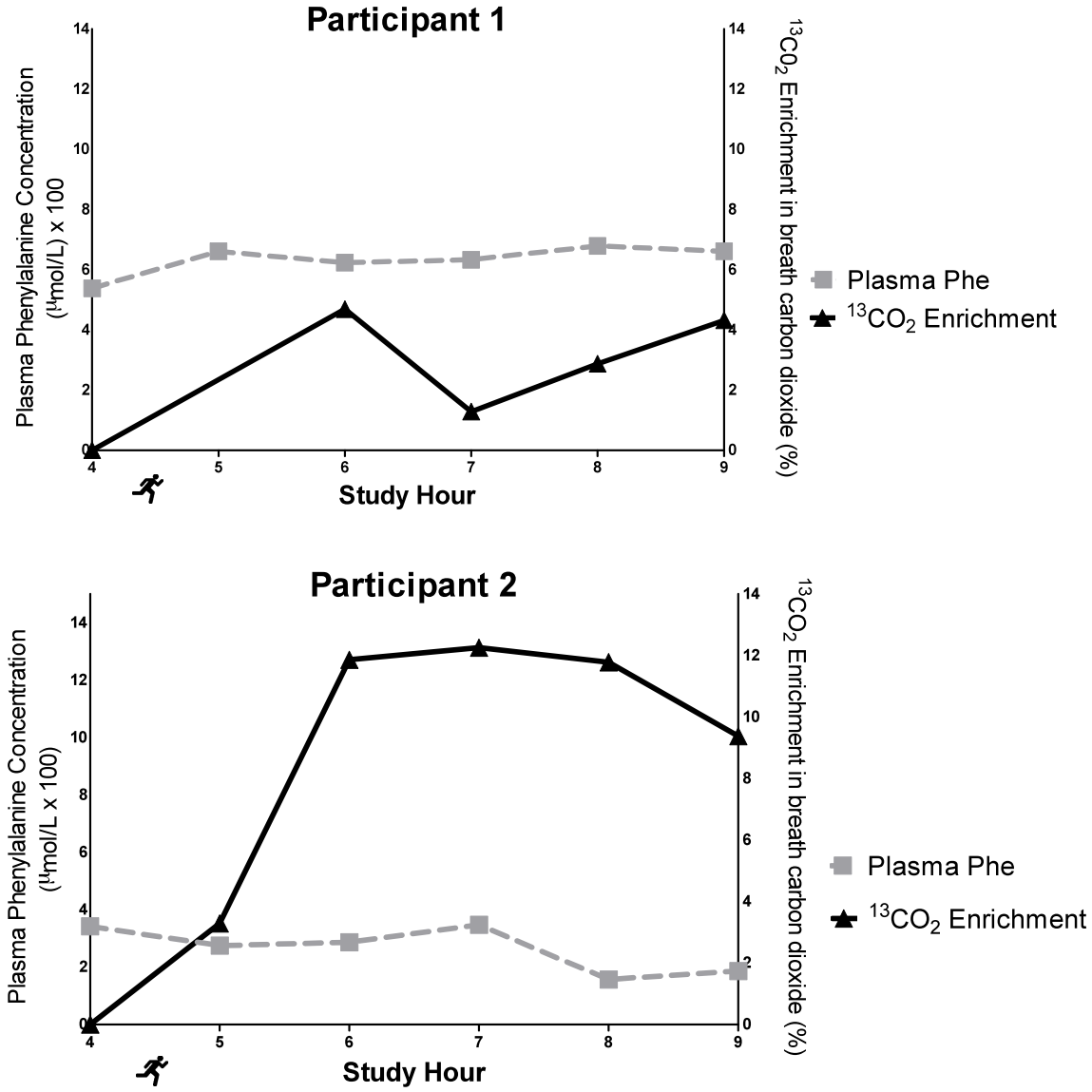


Figure 15. The relationship between plasma phenylalanine concentrations and $^{13}CO_2$ enrichment in breath samples each hour during the exercise study visit.

DISCUSSION

The most important finding of this feasibility study was that both participants decreased their plasma phenylalanine concentrations or maintained their baseline plasma phenylalanine concentrations during this study even when provided a diet of 14 mg/kg phenylalanine and an acute bout of exercise. Participants' dietary phenylalanine intake for each study visit was 111% and 19% higher than their respective current diet prescriptions of phenylalanine (participant 1: 450 mg phe/day; participant 2: 650 mg phe/day). It has been demonstrated that blood phenylalanine concentration is not greatly impacted by incidental additional intake of phenylalanine at 100% of an individual's typical daily phenylalanine intake, and our data supports this literature [129]. Phenylalanine intake of over 200% of normal is tolerated in some patients when blood phenylalanine concentrations are stable and below 360 µmol/L (2-6 mg/dL), the upper limit of the treatment range. The participants in this study were well-matched to each other based on age, activity level, and fat-free mass (77% and 78%). This is important because the percentage of metabolically active tissue mobilizing and incorporating amino acids from the amino acid pool into proteins was similar between participants despite their difference in BMI.

Plasma Amino Acid Concentrations

Participants' plasma phenylalanine concentrations decreased from baseline over the course of the sedentary visits (-29%, -66%) and remained within treatment range. During the exercise arm, Participant 1 has a plasma phenylalanine concentration that was above treatment range (538 µmol/L) at baseline and immediately after his bout of exercise, his phenylalanine concentration increased by 23% (from 538 to 662 µmol/L) which was a larger increase in

concentration than all other essential amino acids, and his phenylalanine concentration remained elevated over the course of the study day. Upon completion of this study a review of plasma phenylalanine concentrations for both participants since January 1, 2013 was conducted. Both participants' plasma phenylalanine concentrations steadily increased over the past 18 months (Figure 3). Noteworthy is the 282 µmol/L increase in plasma phenylalanine concentrations between October 1, 2013 and November 19, 2013 for Participant 1. His average phenylalanine concentration was 449 µmol/L in December 2013. Participant 1 was randomized to the sedentary visit on December 17 and at the end of the study day, he had a 28% decrease in plasma phenylalanine concentration from 360 µmol/L to 257 µmol/L. The next phenylalanine concentration reported in the medical record was on January 4, 2014 at 589 µmol/L. His exercise visit was conducted 7 days later and at the end of his exercise study day he had a 23% increase in plasma phenylalanine concentration from 538 µmol/L to 662 µmol/L. Participant 1 entered the exercise study day with plasma phenylalanine concentrations above treatment range, which may have compromised his ability to synthesize protein given the altered amino acid ratios in his indispensable amino acid pool. His plasma phenylalanine concentration reported almost one month after his exercise study visit was 560 µmol/L. The plasma phenylalanine concentration that was above treatment range in this participant during the exercise study day may have compromised our ability to make conclusions about protein synthesis in this participant.

Participant 2 had a decreased plasma phenylalanine concentration by 46% (350 µmol/L to ~200 µmol/L) by the end of the exercise study day. It is noted that at hour 7 there was a sharp increase in all indispensable amino acid concentrations followed by a dramatic drop by hour 8. We do not have an explanation for this occurrence, as all blood processing, procedures and

activity levels were kept constant and no confounding factors were noted. Both participants had fluctuating tyrosine concentrations throughout the exercise visit. This was expected as tyrosine is not metabolized in the muscle and during higher intensity exercise it has been shown to increase in plasma until taken up by the liver [112].

Urinary Amino Acid Concentrations

Participant 1 has a urinary concentration that was 404% higher at the end of the study day during the exercise visit that the sedentary visit. This is not surprising given his plasma phenylalanine concentrations were above treatment range. Participant 2 excreted 65% less phenylalanine in urine after exercise and also had a 46% decrease in plasma phenylalanine concentration. Although we were unable to obtain a measure of the ^{13}C-Lysine concentration in urine, these changes suggest that phenylalanine was being resorbed in the proximal tubules, possibly to be recycled and incorporated into muscle protein.

$^{13}CO_2$ Enrichment of Breath

The rate of $^{13}CO_2$ excretion in breath (2.8% APE) for Participant 1 was lower after exercise than during the sedentary visit (9.8% APE). This in an unexplained phenomenon suggesting decreased protein oxidation when plasma phenylalanine levels were consistently above treatment range. It is possible protein oxidation is inhibited during periods of plasma phenylalanine concentrations above treatment range but the current body of literature is lacking in evidence. Our goal was to provide a constant infusion of energy, carbohydrate and

protein, and a stimulus to induce muscle protein synthesis (exercise) with the hypothesis that phenylalanine concentrations would drop after exercise but the study protocol may not have been long enough to notice a significant drop in phenylalanine concentrations. Hoeska et al analyzed cerebral protein synthesis via PET scans in patients with PKU and determined that protein synthesis ceases when plasma phenylalanine concentration exceeds 600 μmol/L. It is unknown if this occurs in muscle tissue but may provide an explanation as to why protein synthesis may be inhibited when plasma phenylalanine concentration is significantly higher than all other indispensable amino acid concentrations.

In contrast, changes in plasma amino acid concentrations and rate of lysine oxidation, as measured by the rate of production of $^{13}CO_2$ for Participant 2 who had plasma phenylalanine levels within treatment range, suggests exercise may have increased protein synthesis which supports our hypothesis showing a decrease in plasma phenylalanine concentrations after exercise. In Participant 2 the total rate of $^{13}CO_2$ production was similar between the two visits and urinary phenylalanine excretion was lower following exercise, which may suggest the decrease in plasma phenylalanine was due to phenylalanine being taken up into muscle for protein synthesis. The major promoter of protein synthesis in skeletal muscle is plasma leucine concentrations and insulin, via the regulation of the mammalian target of rapamycin (mTOR) signaling pathway [130-132]. Our protocol with hourly meals would maintain plasma leucine and increase insulin concentrations above fasting concentrations, potentially promoting anabolism and protein synthesis.

One caveat to interpretation of the data is that the percent change in almost all of the indispensable amino acid concentrations over the course of the study day was higher following exercise compared to the sedentary visit (Figure 7A & 8B). Based on the changes in concentration of all amino acids, it appears that exercise stimulated mobilization of indispensable amino acids from muscle, at least in the immediate post-exercise period. It is possible there is a period of mild catabolism and amino acid mobilization immediately after moderate exercise followed by a period of anabolism but we did not measure changes in tracer oxidation, amino acids in plasma or urine beyond 4 hours after exercise.

Clinical Implications

Based upon the results of this study there are several points to consider from a clinical perspective. First, recent changes in a patient's activity level, and the time of day and the day of week the routine blood samples are collected is critical to obtain a representative phenylalanine concentration from which to make dietary changes. It is noted in this study that Participant 2 started spring break three days prior to his exercise visit. He reported being "tired and sore from playing tag football" every day during his break. We did not control for diet or activity level prior to both visits, and this could be a confounding factor in our results. The increase in physical activity before the exercise visit, along with similar or lower energy intake, may have induced catabolism in Participant 2. Upon administration of adequate dietary energy and phenylalanine, the decrease in plasma phenylalanine concentration may be related to the change in weekly activity and not the acute bout of exercise during the study. A blood draw obtained during a week when the patient's schedule has drastically changed or new regimen has

been implemented, may skew the blood phenylalanine concentrations and result in an unnecessary or inadequate diet change. Based on our results, if a blood sample is drawn between 1 and 4 hours after a moderately intense activity, phenylalanine and tyrosine levels may be higher due to the mobilization of the indispensable amino acid pool. Additionally if the patient has phenylalanine concentrations above the treatment range, consideration needs to be given as to how well this person is synthesizing proteins [133].

Secondly, the reduction in plasma phenylalanine concentrations in both participants over the course of the sedentary visit is noteworthy and may be due to either unique diurnal patterns observed in patients with PKU or increased anabolism. In children without PKU on a normal diet, plasma phenylalanine concentrations are typically higher in the evening than in the morning, the converse of which is found in children affected by PKU. Patients with PKU have been shown to have inverse diurnal fluctuation [134]. Sixty-three percent of patients in a recent study experienced the highest phenylalanine concentrations of the day between 6:00 and 9:00 in the morning [134-136]. Concentrations decreased throughout the day and were lower in the evening. It is possible the decline observed in both participants reflects their normal diurnal pattern for plasma phenylalanine and tyrosine. Alternatively, our study protocol provided adequate energy and was aimed at promoting anabolism, which may have been reflected in the decreasing phenylalanine concentrations over the sedentary study period. This suggests that protein catabolism predominates over protein anabolism during fasting periods. Supporting this, prolonged fasting results in a small rise in phenylalanine concentrations [137, 138]. A significant correlation between the timing of formula consumption and percent change in plasma phenylalanine concentrations has been reported. The larger the quantity of formula consumed

after 4:00 pm, the larger the decrease in daytime phenylalanine concentrations [135]. The less formula consumed after 4 pm, the larger the change in plasma phenylalanine concentrations between 4 pm and 6 am. When formula was given every 4 hours, blood phenylalanine concentration had a median difference of 40 µmol/L, versus 3 equal servings during a 10-hour period resulted in a 140 µmol/L difference. We provided an "oral infusion" every hour over the course of the study visit. The gradual decrease in plasma phenylalanine concentration with the constant intake of phenylalanine may reflect anabolism and improved protein synthesis during periods of frequent and adequate energy and protein.

The third clinical consideration is with regards to daily fluctuation of plasma phenylalanine concentrations. Current literature reports more variation in plasma phenylalanine concentrations than was observed in this study. Adult patients with PKU who had been continuously treated since diagnosis at birth were found to have day-to-day plasma phenylalanine concentrations that varied between 55 and 275 µmol/L with an individual standard deviation of 6-99 µmol/L [134]. In this study we observed variations at baseline between 308 and 538 µmol/L. In patients with PKU, the day to day plasma phenylalanine concentration may vary up to 400%. In unaffected individuals, plasma phenylalanine concentrations fluctuate no more than 50% over a 24 hour period [139, 140]. Participant 2 experienced the greatest percent and absolute change in plasma phenylalanine concentration (-66%, -203 µmol/L) during the sedentary visit. This may be attributed to good control at baseline as Participant 1 has a similar decrease in phenylalanine (-28%, -103 µmol/L) when levels were within treatment range. Phenylalanine values vary throughout the day according to dietary intake, with less fluctuation associated with more frequent meals [139, 140]. During our study protocol, the participants were

given a continuous infusion and we did not observe highly variable phenylalanine concentrations, although controlling for intake on the day prior to a study visit should be noted as a key addition for future study design.

Finally, further research and consideration with regard to patients in a state of chronic plasma phenylalanine concentrations above treatment range is critical. Based on the results from this study, protein synthesis may be inhibited if phenylalanine concentrations exceed all other indispensable amino acids, particularly the branched-chain amino acids (Leucine, Isoleucine, and Valine), thereby limiting protein synthesis and rate of muscle accretion.

This is noteworthy, as an imbalance in the indispensable amino acid pool may also have negative consequences on whole body protein synthesis, further adding to the body of evidence supporting a "diet for life" in these patients. Calculation of the protein-energy ratio may be useful in assessing protein balance in the PKU diet. Currently there are no specific diet recommendations for the PKU population with regards to increased activity levels or exercise regimen. Further studies exploring the effects of exercise on plasma phenylalanine concentrations and corresponding dietary recommendations would be beneficial.

Clinical Considerations

1.) Chronically elevated phenylalanine concentrations may suppress protein synthesis and negatively impact lean body mass accretion.

2.) Recent changes in activity level may affect plasma phenylalanine concentrations.

3.) Time of day and day of week impact plasma amino acid concentrations.

4.) Careful analysis of the "whole picture" is recommended prior to initiating a diet change based on plasma phenylalanine concentrations.

Limitations:

This study had many limitations and possible confounding factors. This research was conducted as a feasibility study on a limited budget with a small number of participants (n=2). Analysis of only plasma and urinary amino acid concentrations is not an optimal indicator of protein synthesis or turnover in the body due to compartmentalization, intermediate metabolite production, and flux within the amino acid pool. We did not control for baseline plasma phenylalanine concentrations and this may have skewed our results. Limitations also existed in our methods. The indirect amino acid oxidation technique is not a direct measure of the maintenance requirement of indispensable amino acids in the same way that the 24-hour carbon balance method is, but rather is a measure of the intake of the tracer amino acid as a proportion of its content in the amino acid mixture required for postprandial protein deposition. There is also a limitation as to whether measurements in the fed state only, with no prior period of adaptation to experimental diets, can accurately reflect the dietary requirement needed for protein synthesis.

Although we recruited participants with similar reported activity levels, we did not design the study to control for diet or activity level prior to each study visit. This created a limitation in the actual level of fitness for each subject and may have skewed the exercise results. There was no diet analysis completed assessing phenylalanine and energy intakes prior to the study. Challenges were also present in conduction of the treadmill exercise. We found difficulty in keeping the leads attached to the chest of Participant 2 during his test resulting in variations in his ECG. One breath sample was missing for Participant 1 during the exercise test which altered

his $^{13}CO_2$ oxidation data. The third participant recruited (no data is reported) had an adverse event prior to beginning the treadmill tests and passed out as leads were placed on his chest prior to exercise. This participant elected to withdraw from the rest of the study.

A final limiting factor surrounding this type of study is cost. The ^{13}C-Lysine is expensive ($1200 per gram) and use of the research kitchen, sports medicine treadmill and equipment and lab processing of blood, breath and urine samples is costly to run and have analyzed. Future studies will require adequate funding and careful protocol development to achieve optimal results.

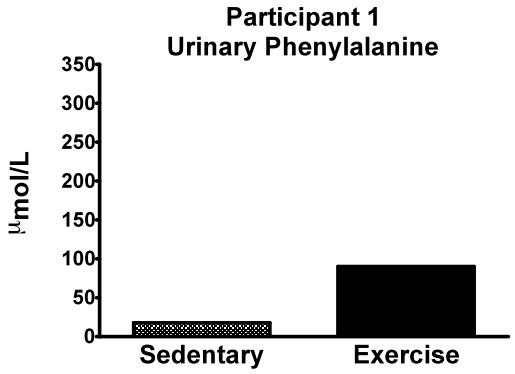
Future Considerations:

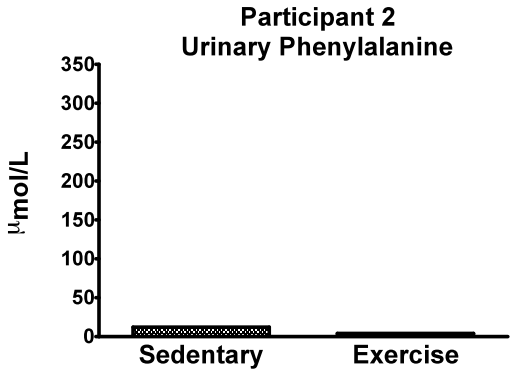
Future considerations for expanding this feasibility study include: 1.) Increase the duration of the study day to a minimum of 24 hours, to allow for complete assessment of indispensable amino acid dynamics to determine the level of protein synthesis occurring and diurnal variation; 2.) Increase the exercise intervention to several months or a year for analysis of long-term muscle accretion that could be non-invasively assessed using a DEXA scan or ultrasound; 3.) Collect 3-day diet records prior to each study visit; 4.) Add a blood draw after a 10-hour fast for amino acid analysis; 4.) Control for baseline hyperphenylalanemia prior to each study visit to decrease the variability and improve interpretation of results; 5.) Add a resistance exercise arm to determine differences between type of exercise (aerobic vs resistance) and which stimulates the greatest uptake of phenylalanine.

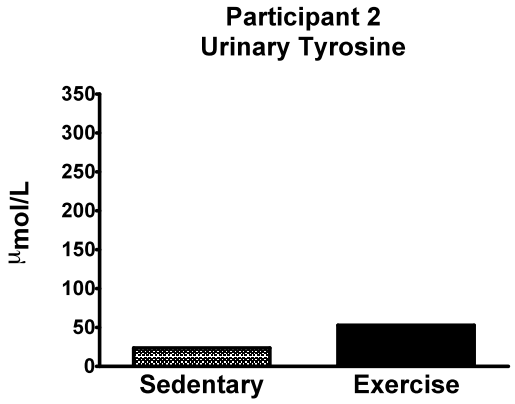
Conclusion:

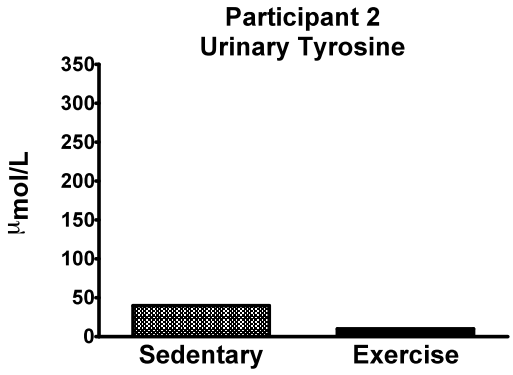
In conclusion, we did not support our hypothesis that moderate intensity exercise would decrease post-exercise amino acid oxidation, and increase muscle protein synthesis. Moderate-intensity exercise transiently increased plasma amino acid concentrations but did not cause either participant to dramatically increase plasma phenylalanine concentrations even with the administration of 14 mg/kg of phenylalanine each day and in the presence of mobilizing indispensable amino acids after exercise. Plasma phenylalanine concentrations decreased over the 4-hour post exercise period in one participant but no effect was seen in the other. The 2014 American College of Medical Genetics Practice Guidelines for PKU states "current and future therapies should be evaluated not only for their ability to lower phenylalanine but also for effects on enhancing quality of life for affected individuals and their families"[1]. Future studies involving the impact of exercise on acute and long term phenylalanine concentrations are needed to substantiate the positive impact of exercise on quality of life and neurocognitive function in patients with phenylketonuria.

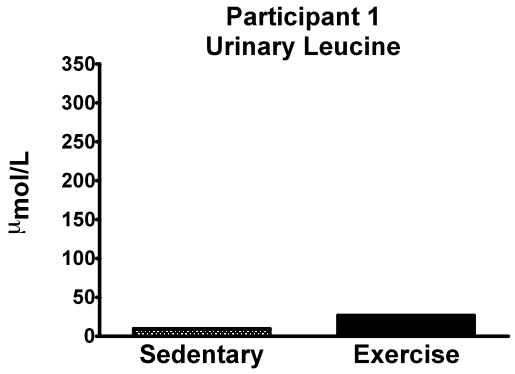
APPENDIX A: URINARY INDISPENSABLE AMINO ACID CONCENTRATIONS AFTER THE 9TH HOUR OF THE SEDENTARY AND EXERCISE VISITS.

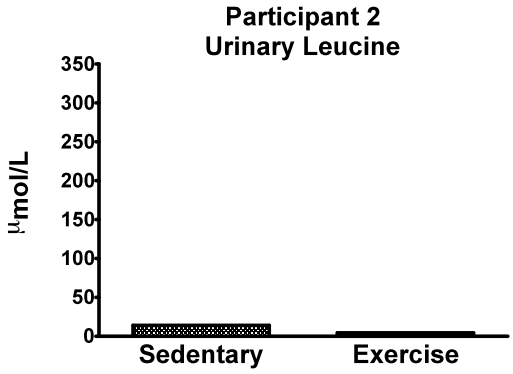


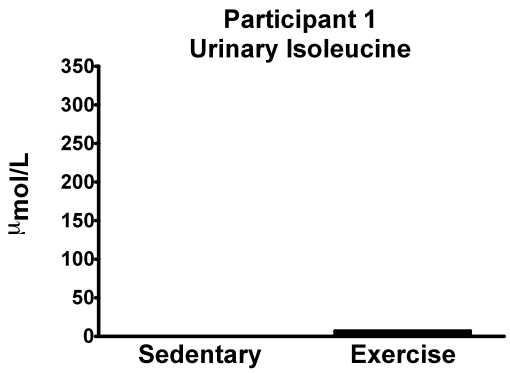


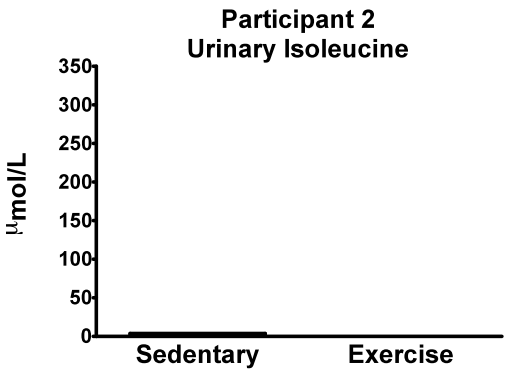









Participant 1
Urinary Isoleucine
µmol/L
350
300
250
200
150
100
50
0
Sedentary
Exercise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Urinary Isoleucine
µmol/L
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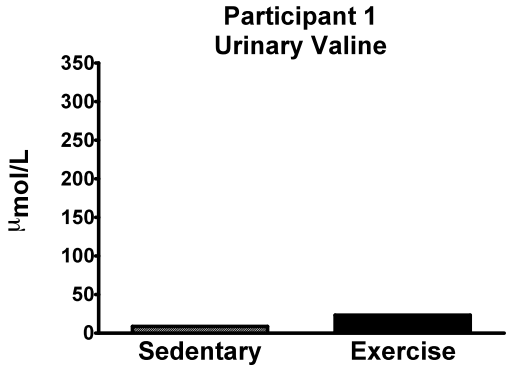
300
250
200
150
100
50
0
Sedentary
Exercise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Urinary Valine
µmol/L
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200
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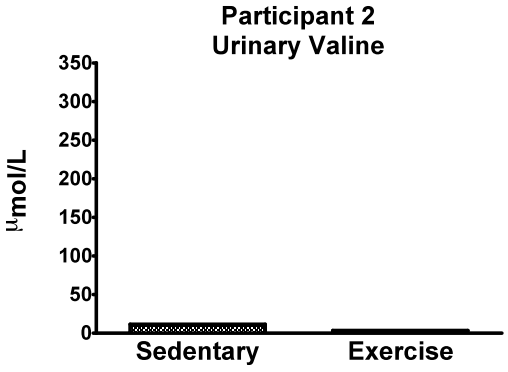
0
Sedentary
Exercise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Urinary Valine
µmol/L
350
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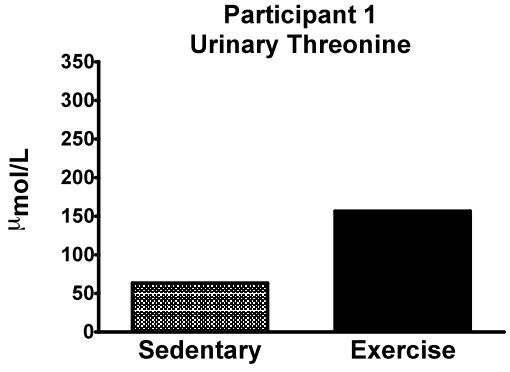
100
50
0
Sedentary
Exercise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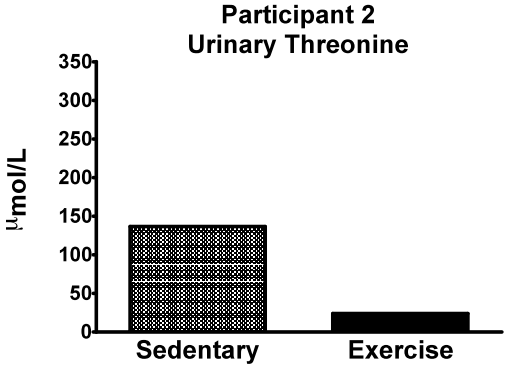
Urinary Threonine
µmol/L
350
300
250
200
150
100
50
0
Sedentary
Exercise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Urinary Threonine
µmol/L
350
300
250
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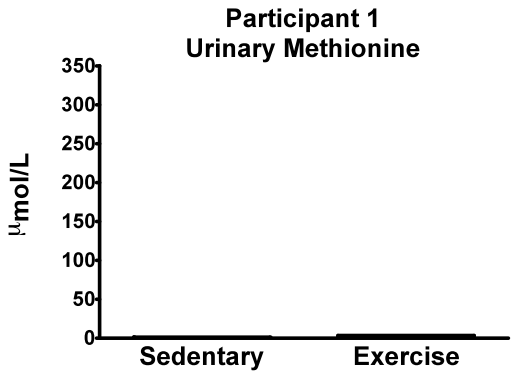
0
Sedentary
Exercise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Urinary Methionine
μmol/L
350
300
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200
150
100
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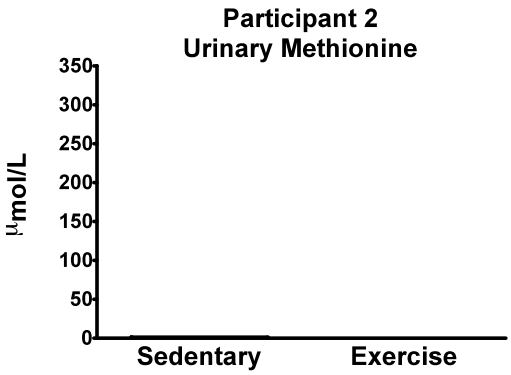
0
Sedentary
Exercise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Urinary Methionine
μmol/L
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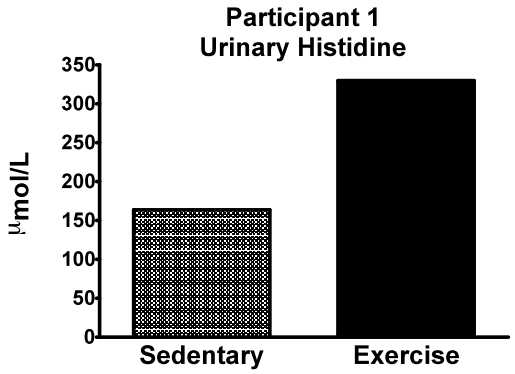
0
Sedentary
Exercise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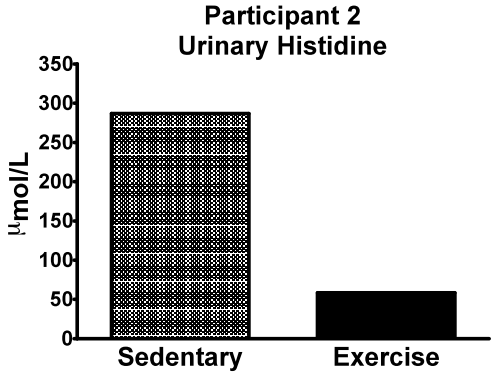
Urinary Histidine
μmol/L
350
300
250
200
150
100
50
0
Sedentary
Exercise


Participant 2
Urinary Histidine
μmol/L
350
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200
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100
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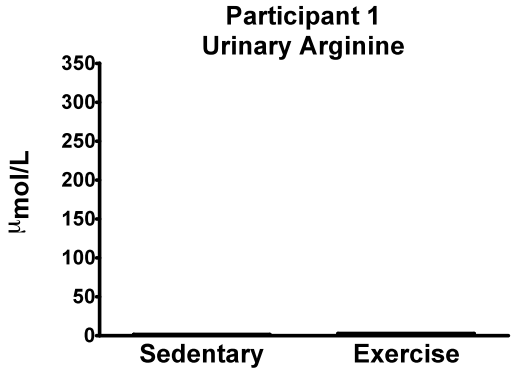
Sedentary
Exercise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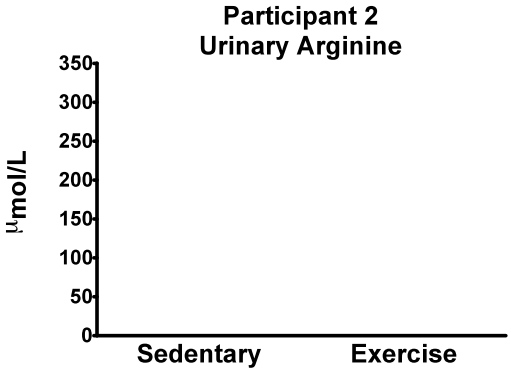
Urinary Arginine
μmol/L
350
300
250
200
150
100
50
0
Sedentary
Exercise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Urinary Arginine
μmol/L
350
300
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150
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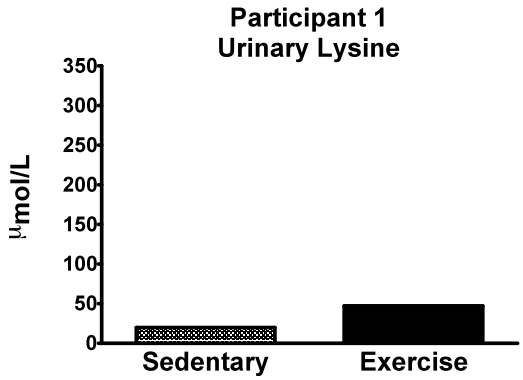
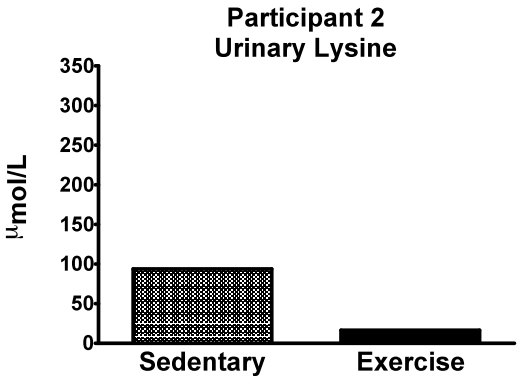
0
Sedentary
Exercise


Participant 1
Urinary Lysine
μmol/L
350
300
250
200
150
100
50
0
Sedentary
Exercise


Participant 2
Urinary Lysine
μmol/L
350
300
250
200
150
100
50
0
Sedentary
Exercise

APPENDIX B.

APPROVED: Aug. 08 2013

Research opportunity:

Patients with Phenylketonuria (PKU)

A new study looking at the effects of exercise in patients with PKU is being conducted at OHSU. If you have PKU and are a male between the ages of14-17, you may be eligible to participate. Participants must come to OHSU on 3 different occasions. Participants will be asked to drink a medical formula used to supplement the diet of people with PKU. Participants will also walk on a treadmill for 40 minutes at one visit.

For more information, please contact Melanie Gillingham, PhD at (503) 494-1682 or email gillingm@ohsu.edu

OHSU

OHSU is an equal opportunity, affirmative action institution. eIRB# 9202

OREGON HEALTH & SCIENCE UNIVERSITY

Child Assent Form

IRB# 9202

Protocol Approval Date: 3/28/2013

TITLE: The Effects of Moderate Exercise on Plasma Amino Acids in Subjects with Phenylketonuria

PRINCIPAL INVESTIGATOR: Melanie B. Gillingham, PhD, RD (503) 494-1682

CO-INVESTIGATORS: Cary O. Harding, MD (503) 494-2783

This research study was explained to me. I know how it may or may not help me. I also know that this study will help doctors learn more about Phenylketonuria (PKU). To be sure that I know what is going to happen, the investigator will ask me the following:

1. To explain what I will do and what will happen in this study.

2. If I have any questions or want to know anything else about this study or PKU.

3. To explain some of the good and bad things that might happen to me if I enter this study.

I have thought about being a part of this study. I have asked and received answers to my questions. I agree to be in this study. I know that I don't have to agree to be in the study. Even though I agree to be in it now, I know I may feel differently later on and can ask to stop being in

the study. I know that I may talk with my parents and/or doctor about not being in this study at any time.

OREGON HEALTH & SCIENCE UNIVERSITY

INSTITUTIONAL REVIEW BOARD

PHONE NUMBER (503) 494-7887
CONSENT/AUTHORIZATION FORM APPROVAL DATE

Apr. 24, 2013

Do not sign this form after the
Expiration date of: 3/27/2014

Name/signature:_____ Date:_____

OREGON HEALTH & SCIENCE UNIVERSITY

MED. REC. NO. _____

NAME _____

BIRTHDATE _____

IRB#: 9202

Protocol Approval Date: 03/28/2013

Clinical Research Consent Summary

You are being asked to join a research study. You do not have to join the study. Even if you decide to join now, you can change your mind later.

If you decide to join, you will be asked to sign a consent form, which shows you give permission to be in the study, and an authorization form, which shows you give permission for us to use your health information for the study.

1. The purpose of this study is to learn more about Phenylketonuria (PKU).
2. In this study, we will learn about PKU and how muscles utilize individual components of dietary protein when exercising. These components are called "amino acids." Phenylalanine is an amino acid. We want to learn
    a. Whether moderate exercise can have an effect on phenylalanine control in PKU subjects
3. This study is being funded by an internal grant.
4. We do not know if exercise will have an effect on phenylalanine control in PKU subjects.
5. You will be asked to consume hourly medical formulas and a cookie, equal to 1/8th your daily nutrient needs, for 8 hours. You will also be asked to consume a labeled isotope of lysine, which is also an amino acid, every 30 minutes starting after hour 4 until the end of your visit. How your body uses protein from the medical formula, and the labeled isotope of lysine, will be measured by collecting hourly breath, blood, and urine samples.
6. If you join the study, you will come for a total of 3 visits, 1 baseline visit and 2 study visits, over the course of 1-2 months. The baseline visit will take approximately 3 hours. The study visits will require approximately a 10-hour stay for each visit. You will be contacted after study visits to ask how you are doing.
7. There are risks involved in participating in the study, some of which may be very serious.

8. If you agree, samples and information collected during the study may be saved for future research.

Subject #: _____

Protocol #: _____

OREGON HEALTH & SCIENCE UNIVERSITY

MED. REC. NO. _____

NAME _____

BIRTHDATE _____

IRB#: 9202

Clinical Research Consent Form

TITLE: The Effects of Moderate Exercise on Plasma Amino Acids in Subjects with Phenylketonuria

PRINCIPAL INVESTIGATOR:	Melanie B. Gillingham, PhD, RD (503) 494-1682
CO-INVESTIGATOR:	Cary O. Harding, MD (503) 494-7608

PURPOSE:

"You" means you or your child in this consent form.

You have been invited to be in this research study because you have Phenylketonuria. The purpose of this study is to learn about how exercise affects phenylketonuria, specifically blood levels of phenylalanine.

The study may help us to understand how the body uses specific components of protein called amino acids in subjects with PKU. Phenylalanine is an amino acid.

Right now, we do not know how exercise effects blood phenylalanine levels in subjects with PKU.

This study requires 3 visits to the clinic and will take approximately 1-2 months to complete.

We are asking you to provide samples for a blood and urine bank, also called a repository. These samples will be stored indefinitely and may be used in the future for research. You may choose whether to have your samples included in this repository or not.

5 male subjects will be enrolled into this study at OHSU.

PROCEDURES:

This study will compare how your body uses amino acids, small components of protein, at two different times:

1. When you are exercising
2. When you are at rest

If you agree to be in this study, you will complete 3 separate study visits.

At your baseline visit we will collect a drop of blood from your finger to check for anemia. In the unlikely event that you are anemic, you will be withdrawn from the study.

If you can continue in the study, a series of tests will be completed. Each test is explained below. All of the tests listed will be completed at the Clinical and Translational Research Center (CTRC) but the order of the tests may be different than listed in this consent form.

Day 1:

You will arrive at the CTRC in the morning for a baseline visit that will last about 3 hours. You will provide a urine sample and a blood sample. You will undergo a simple test to measure how many calories you burn at rest.

Energy Expenditure Test: Energy expenditure is how many calories you use during the day. A clear, colorless, Plexiglass canopy (bubble) will be placed over your head and chest while you rest on a bed. Samples of the air that you breathe out will be collected for about 30 minutes. A

trained research assistant will perform this test in a private room to help you feel comfortable and relaxed.

Dual Energy X-Ray Absorptiometry (DEXA): We will measure how much muscle and fat you have by giving you a DEXA scan. DEXA is a low power x-ray machine. This test is painless and involves a very small amount of radiation exposure. To perform this test, you must lie still on a bed while the DEXA machine scans your body. This procedure takes up to 10 minutes. To complete this test you must remove all metal and change into a hospital gown or wear clothes that have no metal in them.

Your next two visits will take place at the Clinical and Translational Research Center and will last about 10 hours each and will be scheduled for non-consecutive days. Your exercise and rest visits will be randomly assigned.

Day 2:

At your next visit you will be randomly assigned to either exercise or rest for the day. You will arrive at the CTRC after an overnight fast. You will undergo the same test as before in which the amount of calories your body uses at rest is measured. Once this test is finished you will be given $1/8^{th}$ of your daily calorie and nutrient needs in the form of a medical formula. You will be given this same meal every hour for the next 8. You will also provide urine samples every hour for the next 8 hours.

Between your 3^{rd} and 4^{th} hourly meal, an IV will be placed to draw blood from your arm. At this time you will start giving hourly breath samples where you blow through a straw into an empty test tube. You will then be given a stable isotope of Lysine, an amino acid. This will be mixed in water for you to drink. You will then take a smaller dose of this every 30 minutes for the duration of this study visit. After you have received your first dose of the Lysine, you will then begin either the treadmill exercise testing or continue to remain seated and calm, depending on which you are randomized to do during your first visit. At the next visit you will complete the other task.

Exercise Test: During the exercise testing you will walk on a treadmill for 40 minutes. Your blood pressure and heart activity will be measured in both a standing and sitting position. Blood pressure and heart activity will also be measured during the exercise test. To measure your heart activity, small wires are taped to your chest and both arms. The electrocardiogram instrument will record the electrical signals produced by your beating heart. The equipment does not give an electrical shock.

The study investigators will monitor you during the exercise session. Two teaspoons of blood will be drawn from the IV catheter right before you begin the exercise. You will be asked to

breathe into a mask during the exercise so that samples of the air you breathe out can be collected. You will begin walking slowly on the treadmill and the pace of walking will gradually increase to a brisk walk. At the end of the exercise two more teaspoons of blood will be drawn from the IV catheter. A total of 8 teaspoons of blood will be drawn during this and the following visit. If for any reason you cannot complete the exercise testing you may ask to stop and the test will be stopped immediately.

After the exercise test you will continue to receive the labeled isotope of Lysine as well as your hourly meals. You will continue to give breath, blood, and urine samples for the next 3 hours until the end of this study visit.

Day 3:

Your third study visit will be a non-consecutive day. You will again be asked to fast overnight before coming to the CTRC. All study procedures will remain the same except, if you completed the exercise testing at the second visit you will not complete it again. You will instead be asked to do quiet sedentary activities like reading or watching movies. Or, if you completed the resting portion of the study on your second visit, you will be asked to complete the treadmill exercise at this visit.

Other information:

Meals: At your baseline visit you will be given breakfast at the end of the morning testing before you are sent home. This will be the same meal you will eat every hour during your 2^{nd} and 3^{rd} study visits. We will make sure this medical formula is tolerable to you. At your next two study visits you will be asked to eat this meal hourly, which will provide $1/8^{th}$ of your calorie and nutrient needs for the day. This will be in the form of a medical formula specifically made for people with PKU. These are commercially available and readily used for the treatment of people with PKU. If you currently use a medical formula, this may or may not be the same one you use. You will also consume a small amount of food which will naturally provide all amino acids, including some phenylalanine.

All subjects will receive the same treatment however, the order of their visits may vary due to randomization.

Your medical records will be reviewed. We will collect information about when you were diagnosed with PKU and what medical formula or medications you are currently taking.

In the future, your blood and urine samples may be given to researchers for other research studies. The samples will be stored in a secure freezer indefinitely for future analysis. The blood samples will be coded with a number and only the investigators will be able to identify the

sample. You may choose to have your samples stored for future use. If you choose not to have your blood and urine stored, the samples will be destroyed at the conclusion of the study. You can indicate your choice at the end of this form.

If you have any questions regarding this study now or in the future, contact Melanie Gillingham, PhD, (503) 494-1682.

RISKS AND DISCOMFORTS:

Blood Samples/IV Catheter: We will draw blood from your arm. You may feel some pain when your blood is drawn. There is a small chance the needle will cause bleeding, a bruise, or an infection. There is a small risk of developing anemia from the blood sampling in this study, but this is an unlikely risk. You will have a catheter (tube) placed in your vein. You may get an infection where the tube is placed. This would cause swelling, redness, and pain. You may bleed or get a bruise. There is a small chance your blood stream or heart valves may get a serious infection. You may get a blood clot that could go to your lungs. These problems are very rare. If you have these problems, you will need hospital care. You will have an IV catheter in your arm for about 5 hours twice during the study.

DEXA scans: In this study, you will be exposed to a small dose of radiation (x-rays). This test will measure the total amount of fat, muscle, and bone in your body. While we cannot be sure any dose of radiation is entirely safe, the amount you will be exposed to in this study is not known to cause health problems.

Confidentiality: Efforts will be made to keep your personal information confidential as described in the CONFIDENTIALITY section, but we cannot guarantee total privacy. There is a small chance that your information could be accidentally released.

BENEFITS: You may or may not personally benefit from participating in this study. However, by serving as a subject, you may contribute to new information that may benefits patients in the future.

ALTERNATIVES:

1. You may choose not to be in this study. If you choose not to participate, you should continue to follow your current phenylalanine restricted diet and take your medical formulas as prescribed by your physician or dietitian to treat your disorder.
2. You may choose to participate. If you choose to participate:
    a. You may choose to participate and allow your blood and urine samples to be stored for future research.
    b. You may choose to participate and request your blood samples to be destroyed at the end of the study.

CONFIDENTIALITY:

We will not use your name or your identity for publication or publicity purposes.

A code number will be assigned to you, your cells and genetic information, as well as to information about you. Only the investigators named on this consent form will be authorized to link the code number to you. Other investigators who may receive your blood, urine, or breath samples for research will be given only the code number which will not identify you. Your breath samples will be sent to an investigator at the University of Pittsburgh, Dr. James Delany, for analysis.

Research records may be reviewed and copied by people involved in conducting or overseeing research including the OHSU Institutional Review Board, the Office for Human Research Protections, the Oregon Clinical and Translational Research Center, and the National Center for Research Resources.

All other parties including employers, insurance companies, and relatives will be refused access to your information unless you provide written permission or unless we are required by law to release it.

Under Oregon Law, suspected child or elder abuse must be reported to appropriate authorities.

COMMERCIAL DEVELOPMENT:

Samples obtained from you in this research may be used for commercial purposes, such as making a discovery that could be patented or licensed to a company. There are no plans to pay you if this happens. You will not have any property rights or ownership or financial interest in or arising from products or data that may result from your participation in this study. Further, you will have no responsibility or liability for any use that may be made of your samples or information.

COSTS: There will be no cost to you or your insurance company to participate in this study.

LIABILITY: If you believe you have been injured or harmed while participating in this research and require immediate treatment, contact Dr. Melanie Gillingham at 503-319-2404.

You have not waived your legal rights by signing this form. If you are harmed by the study procedures, you will be treated. Oregon Health & Science University does not offer to pay for the cost of the treatment. Any claim you make against Oregon Health & Science University may be limited by the Oregon Tort Claims Act (ORS 30.260 through 30.300). If you have questions on this subject, please call the OHSU Research Integrity Office at (503) 494-7887.

This federally-funded study does not have the ability to provide compensation for research-related injury. If you are injured or become ill from taking part in this study, it is important to tell your study doctor. Emergency treatment may be available but you or your insurance company will be charged for this treatment.

PARTICIPATION:

If you have any questions regarding your rights as a research subject, you may contact the OHSU Research Integrity Office at (503) 494-7887.
You do not have to join this or any research study. If you do join, and later change your mind, you may quit at any time. If you refuse to join or withdraw early from the study, there will be no penalty or loss of any benefits to which you are otherwise entitled.

You may be removed from the study prior to its conclusion if the study is stopped by the sponsor or if you do not follow study instructions.

You will not be given the results of the tests from this research.

If in the future you decide you no longer want to participate in this research, we will destroy all your blood, urine, and breath samples. However, if your samples are already being used in an ongoing research project and if their withdrawal jeopardizes the success of the entire project, we may ask to continue to use them until the project is completed.

We will give you any new information during the course of this research study that might change the way you feel about being in the study.

Signatures:

OPTIONAL STUDY PROCEDURES

The optional portions of this study are described in detail throughout this consent form and listed here as a summary. Please read the options and place your initials next to one of the choices below. You can still participate in the main part of the study if you choose not to participate in the optional parts.

_____ I give my consent to participate in the main part of this study, but I do not give my consent for the storage and future use of my blood/tissue samples.

Subject #: _____

Protocol #: _____

4OREGON HEALTH & SCIENCE UNIVERSITY

INSTITUTIONAL REVIEW BOARD

PHONE NUMBER (503) 494-7887

CONSENT/AUTHORIZATION FORM APPROVAL DATE

Aug. 8, 2013

Do not sign this form after

the expiration date of: **03-27-2014**

or

_____ I give my consent for my blood/tissue samples and information to be used for this and future research studies, which may include genetic research.

Your signature below indicates that you have read this entire form and that you agree to be in this study.

We will give you a copy of this form.

Subject #: _____

Protocol #: _____

_____

Name of Subject, Printed

Signature of Subject/Legal Guardian Date

_____

Name of Legal Guardian, Printed

Signature of Second Legal Guardian
Date_____ Name of Legal Guardian, Printed

Signature of Person Obtaining Consent Date

OREGON HEALTH & SCIENCE UNIVERSITY

IRB#: 9202

MED. REC. NO. _____

NAME _____

BIRTHDATE _____

HIPAA CLINICAL RESEARCH AUTHORIZATION

Subject #: _____

Protocol #: _____

Title of Study: with Phenylketonuria

Name of Principal Investigator: Melanie B. Gillingham, PhD, RD

Phone Number: (503) 494-1682

We have already asked you for your consent to be in the research study. We are also required to seek separate permission to use your health information for the study. The Health Insurance Portability and Accountability Act (HIPAA) is a federal law designed to help protect the privacy of health information.

During this study, OHSU will use and disclose (release) health information about you. Under federal law, we may not use or disclose it unless you authorize us to do so by signing this form. This authorization is voluntary. You do not have to sign this form. If you choose not to sign it, you cannot join this study.

The health information we will collect, use, and disclose is described in the attached consent form. The consent form also describes why we will use the health information. Investigators, study staff, and others at OHSU who are involved in the research or overseeing the research may use and disclose your health information.

We may send your health information to others outside OHSU who are involved in the research or overseeing the research, including:

- The Office for Human Research Protections, which oversees research involving humans
- The National Center for Research Resources
- The University of Pittsburgh

When we send information outside of OHSU, it may no longer be protected under federal law. In this case, your information could be used and re-released without your authorization.

We may continue to use and disclose your health information indefinitely.

Some of the information collected and created in this study may be useful for your future health care and will be placed in your OHSU medical record. While the research is in progress, you may not have access to this information. After the study is complete, you will be able to access any study information that was added to your OHSU medical record.

You have the right to withdraw this authorization at any time. To withdraw your permission for us to use information that identifies you, please send a written request or email to:

Melanie B. Gillingham

Subject #: _____

Protocol #: _____

Department of Molecular & Medical Genetics

Mail Code L103

Oregon Health & Science University

3181 SW Sam Jackson Park Rd

Portland, OR 97239

Telephone: 503-494-1682

Email: gillingm@ohsu.edu

The use and disclosure of your health information for this research will stop as of the date the principal investigator receives your request. However, the use and disclosure of information collected in good faith before your request arrives will continue. Withdrawing this authorization will not affect your health care or your relationship with OHSU.

Please ask the investigator or study staff if you have any questions about this HIPAA authorization.

We will give you a copy of this signed form.

_____

Name of Subject, Printed

Signature of Subject/ Legal Guardian Date

_____

Name of Legal Guardian, Printed

Signature of Second Legal Guardian Date

_____ Name of Legal Guardian,
Printed_____
__

Signature of Person Obtaining Authorization Date

MED. REC. NO. _____

NAME _____

BIRTHDATE _____

IRB#: 9202

HIPAA CLINICAL RESEARCH AUTHORIZATION

Subject #: _____

Protocol #: _____

OPTIONAL PROCEDURES

Title of Study:	The Effects of Moderate Exercise on Plasma Amino Acids in Subjects with Phenylketonuria
Name of Principal Investigator:	Melanie B. Gillingham, PhD, RD
Phone Number:	503-494-1682

You indicated in the consent form that you would like to participate in the optional banking of your samples for future research. The Health Insurance Portability and Accountability Act (HIPAA) requires that we seek separate permission from you to use your health information for the optional study procedures that you selected.

During the optional parts of the study, OHSU will use and disclose (release) health information about you. Under federal law, we may not use or disclose it unless you authorize us to do so by signing this form. This authorization is voluntary. You do not have to sign this form. If you choose not to sign it, you cannot participate in the optional parts of the study. However, you may still participate in the main part of the study.

The health information we will collect, use, and disclose is described in the attached consent form. The consent form also describes why we will use the health information. Investigators, study staff, and others at OHSU who are involved in the research or overseeing the research may use and disclose your health information.

We may send your health information to others outside OHSU who are involved in the research or overseeing the research, including:

- The Office for Human Research Protections, which oversees research involving humans
- The National Center for Research Resources
- The University of Pittsburgh

When we send information outside of OHSU, it may no longer be protected under federal law. In this case, your information could be used and re-released without your authorization. We may continue to use and disclose your health information indefinitely.

Some of the information collected and created in this study may be useful for your future health care and will be placed in your OHSU medical record. While the research is in progress, you may

not have access to this information. After the study is complete, you will be able to access any study information that was added to your OHSU medical record.

You have the right to withdraw this authorization at any time. To withdraw your permission for us to use information that identifies you, please send a written request or email to:

Melanie B. Gillingham
Department of Molecular & Medical Genetics
Mail Code L103
Oregon Health & Science University
3181 SW Sam Jackson Park Rd
Portland, OR 97239
Telephone: 503-494-1682 Email: gillingm@ohsu.edu

The use and disclosure of your health information for this research will stop as of the date the principal investigator receives your request. However, the use and disclosure of information collected in good faith before your request arrives will continue. Withdrawing this authorization will not affect your health care or your relationship with OHSU.

Please ask the investigator or study staff if you have any questions about this HIPAA authorization.

We will give you a copy of this signed form.

4OREGON HEALTH & SCIENCE UNIVERSITY

INSTITUTIONAL REVIEW BOARD

PHONE NUMBER (503) 494-7887

CONSENT/AUTHORIZATION FORM APPROVAL DATE

Aug. 8, 2013

Do not sign this form after

the expiration date of: **03-27-2014**

_____

Name of Subject, Printed

Signature of Subject/Legal Guardian Date

_____

Subject #: _____

Protocol #: _____

Name of Legal Guardian, Printed

Signature of Second Legal Guardian Date

_____

Name of Legal Guardian, Printed

Signature of Person Obtaining Authorization Date

The Effects of Moderate Exercise on Plasma Amino Acids in Subjects with Phenylketonuria

Initials: _____

DOB: _____

Gender _____

Date Consent signed: _____

Age: _____ Ht: _____ Wt: _____ BMI: _____

Arrival Time: _____ Fasting for 10 Hrs: Y___ N____

Diagnosis Confirmation:

Diagnosed on Newborn Screening: Y___ N___
Date of Diagnosis: _____

Currently on Medical Formula: ☐Yes ☐No

Type & Amount per day:

_____
_____

Subject #: _____

Protocol #: _____

Hgb/Hct

Result:

Date:

Subject eligible to participate:

- ☐ Yes
- ☐ No

Indirect Calorimetry:

Date: _____ Time: _____

IC	L/min	L/min	Kcals/day	Notes:
RQ	**VO2**	**VCO2**	**REE**	

Total Kcal/d: _____

DEXA:

Date: _____ Time: _____

DEXA					
BMD (g/cm^2)	**BMC** (g)	**Fat** (g)	**Lean** (g)	**Lean+BMC** (g)	**Total Mass** (g)

Metabolic Panel:

	Meal test				**Notes:**
	0	**1**	**2**	**3**	

Subject #: _____

Protocol #: _____

Time of draw:					
Tube:	**Aliquot**	**Samples collected (check)**			
RED	insulin				
	ketones, lactate, pyruvate				
	extra				
Grey	glucose				
EDTA	extra				
	total fatty acids				
	Free fatty acids				
Li Hep	extra				
	Lipid panel	Local lab			

Notes:

Study Day 1:

Date: _____ Age: _____ Ht:_____ Wt: _____

Arrival Time:_____ Fasting: Y____ N____

Indirect Calorimetry:

Date: _____ Time: _____

Subject #: _____

Protocol #: _____

IC	L/min	L/min	Kcals/day	Notes:
RQ	**VO2**	**VCO2**	**REE**	

Total Kcal/d**:** _____ Kcal/hourly meal**:** _____

Hourly Meals:

Description: _____

_____

Meal	**Time:**	**% Consumed**	**Notes:**
1			
2			
3			
4			

Subject #: _____

Protocol #: _____

5			
6			
7			
8			

Labeled Lysine:

Labeled Lysine Oral Priming dose: _____

Lysine Bolus dose: _____

Dose #	**Time:**	**Amount**	**Notes:**
1 (primer)			
2			

3			
4			
5			
6			
7			
8			
9			
10			

Indicator Amino Acid Oxidation

		Meal test				Notes:
		0	**1**	**2**	**3**	
Time of draw:						
Tube:	**Aliquot**	**Samples collected (check)**				
RED	insulin					

	ketones, lactate, pyruvate					
	extra					
Grey	glucose					
EDTA	extra					
	total fatty acids					
	Free fatty acids					
Li Hep	extra					
	Lipid panel	Local lab				

Breath Samples:

Test	**Time:**	**Notes:**
0 (2 times)		
1		
2		
3		
4		

Hourly Urine Samples:

Test	**Time:**	**Notes:**
0		
1		
2		

3		
4		
5		
6		
7		
8		
9		
10		

Exercise test (if done)

Date: _____ Time: _____

Notes:

<u>Study Day 2:</u>

Date: _____ Age: _____ Ht:_____ Wt: _____

Subject #: _____

Protocol #: _____

Arrival Time:_____ Fasting: Y____ N____

Indirect Calorimetry:

Date: _____ Time: _____

IC	L/min	L/min	Kcals/day	Notes:
RQ	**VO2**	**VCO2**	**REE**	

Total Kcal/d**:** _____ Kcal/hourly meal**:** _____

Hourly Meals:

Description: _____

_____

Meal	**Time:**	**% Consumed**	**Notes:**
1			
2			
3			
4			
5			
6			
7			
8			

Labeled Lysine:

Labeled Lysine Oral Priming dose: _____

Subject #: _____

Protocol #: _____

Lysine Bolus dose: _____

Dose #	Time:	Amount	Notes:
1 (primer)			
2			
3			
4			
5			
6			
7			
8			
9			
10			

Indicator Amino Acid Oxidation:

Subject #: _____

Protocol #: _____

		Meal test				Notes:
		0	**1**	**2**	**3**	
Time of draw:						
Tube:	**Aliquot**	**Samples collected (check)**				
RED	insulin					
	ketones, lactate, pyruvate					
	extra					
Grey	glucose					
EDTA	extra					
	total fatty acids					
	Free fatty acids					
Li Hep	extra					
	Lipid panel	Local lab				

Breath Samples:

Subject #: _____

Protocol #: _____

Test	Time:	Notes:
0 (2 times)		
1		
2		
3		
4		

Hourly Urine Samples:

Test	Time:	Notes:
0		
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

Exercise test (if done)

Date: _____ Time: _____

Notes

Exercise Treadmill Test

Est. Max HR: _____ HR range (70-80% est max) : _____ BP (sitting): _____

Labeled Lysine Primer (time) _____:_____ Medical Formula Consumed (Meal 4) _____:_____

Pre-Ex Blood (time) _____:_____ Pre-Ex Urine (time): _____:_____ Pre-Ex Breath (time):_____:_____ Time of Exercise: _____:_____

Labeled Lysine Bolus (Primer+30 mins) _____:_____

Time (min)	Speed (mph)	Grade (%)	HR (bpm)	BP	Comments/Borg
1	1.8	0			
2	1.8	0			
3	2.0	0			
4	2.0	0			
5	2.5				
6					
7					
8					
9					
10					
11					

12					
13					
14					
15					
16					
17					
18					
19					
20					
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42					
43					
44					
45					

Post Exercise:

Post-Ex Blood (time) _____:_____ Post-Ex Urine (time): _____:_____ Post-Ex Breath (time):_____:_____

Medical Formula Consumed (Meal 5) _____:_____

Adverse Events (Use this information for tracking then complete the AE Log):

Event	**Expected?** (Yes/N	**SAE?**	**Severity**	**Start Date/Time**	**End Date/ Time**	**Comments:** (Include relationship to study intervention, what action taken,

Concomitant Medications (List new medications or changes to previous medications)

Medication	**Indication**	**Dose, Route, Frequency**	**Start Date**	**Stop Date**

Recommendations/Discussion

OREGON HEALTH & SCIENCE UNIVERSITY

School of Medicine

Department of Molecular and Medical Genetics
Mail code: L103
3181 S.W. Sam Jackson Park Rd.
Portland, Oregon 97239-3098
tel 503 494-1682 | fax 503 494-6886

Melanie B. Gillingham, Ph.D.
Assistant Professor
gillingm@ohsu.edu

Date:

Dear _____,

We are recruiting subjects for a study entitled *"The Effects of Exercise on Plasma Amino Acids in Subjects with Phenylketonuria"* evaluating the effect of a short bout of exercise on phenylalanine levels in teenage males with phenylketonuria (PKU) which will be conducted at Oregon Health & Science University. People with phenylketonuria must consume a diet low in protein and often supplement with medical formulas. The goal of this study is to investigate whether a short bout of exercise can lower phenylalanine levels in the blood. We invite your child to join this study. This is a small pilot project which is approved by the OHSU Institutional Review Board.

Participation in this study is voluntary. A copy of the informed consent for the study is enclosed. The study coordinator will contact you sometime in the next week to ask if you would like to participate in the study and to go over the consent form with you. To participate in this study, you will complete 3 study visits. At 1 visit you will complete an exercise test on a treadmill. Additionally, you will provide blood, breath, and urine samples, and consume a specific hourly defined meal. If you have any questions, or concerns, we are happy to discuss them with you. Our contact information is listed below. Thank you for considering participating in this research study.

Sincerely,

School of Medicine

Department of Molecular and Medical Genetics
Mail code: L103
3181 S.W. Sam Jackson Park Rd.
Portland, Oregon 97239-3098
tel 503 494-1682 | fax 503 494-6886

Melanie B. Gillingham, Ph.D
Assistant Professor
gillingm@ohsu.edu

Melanie Gillingham PhD, RD
Assistant Professor
Phone: 503-494-1682
Oregon Health & Science University
3181 SW Sam Jackson Park Rd
Portland, OR 972

Cary O. Harding, MD
Associate Professor

4093-01

Meal	Food Description	Amount	Measure	Gram Weight	Water	Energy	Protein
Breakfast	4093 Beneprotein Powder	24	gram	24	0	85.71	20.57
Breakfast	4093 GF Lemon Sugar Cookie	316	gram	316	26.55	1556.5	6.35
Breakfast	4093 Lophlex LQ Orange	352	gram	352		294.82	50.83
Breakfast	Orange-flavor drink, KRAFT, TANG Drink	104	gram	104	0.31	380.64	0
Breakfast	Water	1560	gram	1560	1558.44	0	0
Breakfast	4093 Camino Pro Complete Bar 12+ PB	100	gram	100		395	19
Breakfast Total				2456	1585.31	2712.67	96.76
Diet Total :				2456	1585.31	2712.67	96.76

Meal	Food Description	Amount	Measure	Gram Weight	Phenylalanine	Tyrosine	Valine
Breakfast	4093 Beneprotein Powder	24	gram	24	0.58	0.58	1.15
Breakfast	4093 GF Lemon Sugar Cookie	316	gram	316	0.33	0.24	0.37
Breakfast	4093 Lophlex LQ Orange	352	gram	352	0	4.78	3.51
Breakfast	Orange-flavor drink, KRAFT, TANG Drink	104	gram	104			
Breakfast	Water	1560	gram	1560			
Breakfast	4093 Camino Pro Complete Bar 12+ PB	100	gram	100	0.05	1.6	1.11
Breakfast Total				2456	0.95	7.2	6.14
Diet Total :				2456	0.95	7.2	6.14

4093-01

Meal	Food Description	Amount	Measure	Total lipid (fat)	Ash	Carbohydrate, by difference	Fiber, total dietary
Breakfast	4093 Beneprotein Powder	24	gram	0		0	0
Breakfast	4093 GF Lemon Sugar Cookie	316	gram	81.36	2.9	200.79	4.56
Breakfast	4093 Lophlex LQ Orange	352	gram	0		23.64	1.27
Breakfast	Orange-flavor drink, KRAFT, TANG Drink	104	gram	0	1.35	102.34	0.31
Breakfast	Water	1560	gram	0	1.56	0	0
Breakfast	4093 Camino Pro Complete Bar 12+ PB	100	gram	9.9		56	4
Breakfast Total				91.26	5.81	382.76	10.14
Diet Total :				91.26	5.81	382.76	10.14

Meal	Food Description	Amount	Measure	Arginine	Histidine	Alanine	Aspartic acid
Breakfast	4093 Beneprotein Powder	24	gram	0.43	0.33	1.05	2.16
Breakfast	4093 GF Lemon Sugar Cookie	316	gram	0.48	0.16	0.37	0.59
Breakfast	4093 Lophlex LQ Orange	352	gram	5.08	2.01	2.95	4.45
Breakfast	Orange-flavor drink, KRAFT, TANG Drink	104	gram				
Breakfast	Water	1560	gram				
Breakfast	4093 Camino Pro Complete Bar 12+ PB	100	gram	1.98	0.47	0.72	1.09
Breakfast Total				7.97	2.97	5.08	8.29
Diet Total :				7.97	2.97	5.08	8.29

Meal	Food Description	Amount	Measure	Tryptophan	Threonine	Isoleucine	Leucine
Breakfast	4093 Beneprotein Powder	24	gram	0.35	1.34	1.28	2.2
Breakfast	4093 GF Lemon Sugar Cookie	316	gram	0.08	0.23	0.27	0.53
Breakfast	4093 Lophlex LQ Orange	352	gram	1.04	2.64	3.15	5.41
Breakfast	Orange-flavor drink, KRAFT, TANG Drink	104	gram				
Breakfast	Water	1560	gram				
Breakfast	4093 Camino Pro Complete Bar 12+ PB	100	gram	0.22	2	1.28	3.75
Breakfast Total				1.69	6.21	5.98	11.9
Diet Total :				1.69	6.21	5.98	11.9

Meal	Food Description	Amount	Measure	Glutamic acid	Glycine	Proline	Serine
Breakfast	4093 Beneprotein Powder	24	gram	3.52	0.33	1.13	0.91
Breakfast	4093 GF Lemon Sugar Cookie	316	gram	1.29	0.31	0.3	0.33
Breakfast	4093 Lophlex LQ Orange	352	gram	0	4.78	5.08	2.77
Breakfast	Orange-flavor drink, KRAFT, TANG Drink	104	gram				
Breakfast	Water	1560	gram				
Breakfast	4093 Camino Pro Complete Bar 12+ PB	100	gram	2.35	0.15	1.6	0.81
Breakfast Total				7.16	5.57	8.12	4.82
Diet Total :				7.16	5.57	8.12	4.82

Meal	Food Description	Amount	Measure	Lysine	Methionine	Cystine
Breakfast	4093 Beneprotein Powder	24	gram	2.28	0.45	0.49
Breakfast	4093 GF Lemon Sugar Cookie	316	gram	0.24	0.14	0.08
Breakfast	4093 Lophlex LQ Orange	352	gram	4.14	0.86	1.3
Breakfast	Orange-flavor drink, KRAFT, TANG Drink	104	gram			
Breakfast	Water	1560	gram			
Breakfast	4093 Camino Pro Complete Bar 12+ PB	100	gram	0.72	0.19	0.02
Breakfast Total				7.39	1.64	1.89
Diet Total :				7.39	1.64	1.89

Meal	Food Description	Amount	Measure	Alcohol, ethyl	Protein (%)	Fat (%)	Carbohydrate (%)
Breakfast	4093 Beneprotein Powder	24	gram	0	100	0	0
Breakfast	4093 GF Lemon Sugar Cookie	316	gram	2.42	1.61	46.41	50.91
Breakfast	4093 Lophlex LQ Orange	352	gram		68.26	0	31.74
Breakfast	Orange-flavor drink, KRAFT, TANG Drink	104	gram		0	0	100
Breakfast	Water	1560	gram	0	0	0	0
Breakfast	4093 Camino Pro Complete Bar 12+ PB	100	gram	0	19.53	22.9	57.57
Breakfast Total				2.42	14.04	29.8	55.55
Diet Total :				2.42	14.04	29.8	55.55

4093-02

Meal	Food Description	Amount	Measure	Gram Weight	Water	Energy	Protein	Total lipid (fat)
Breakfast	4093 Beneprotein Powder	20	gram	20	0	71.43	17.14	0
Breakfast	4093 GF Lemon Sugar Cookie	240	gram	240	20.17	1182.15	4.83	61.79
Breakfast	4093 Lophlex LQ Orange	280	gram	280		234.51	40.43	0
Breakfast	Orange-flavor drink, KRAFT, TANG Drin	80	gram	80	0.24	292.8	0	0
Breakfast	Water	1560	gram	1560	1558.44	0	0	0
Breakfast	4093 Camino Pro Complete Bar 12+ PB	80	gram	80		316	15.2	7.92
Breakfast Total				2260	1578.85	2096.89	77.6	69.71
Diet Total :				2260	1578.85	2096.89	77.6	69.71

Meal	Food Description	Amount	Measure	Gram Weight	Phenylalanine	Tyrosine	Valine	Arginine
Breakfast	4093 Beneprotein Powder	20	gram	20	0.48	0.48	0.96	0.36
Breakfast	4093 GF Lemon Sugar Cookie	240	gram	240	0.25	0.18	0.28	0.36
Breakfast	4093 Lophlex LQ Orange	280	gram	280	0	3.8	2.79	4.04
Breakfast	Orange-flavor drink, KRAFT, TANG Drin	80	gram	80				
Breakfast	Water	1560	gram	1560				
Breakfast	4093 Camino Pro Complete Bar 12+ PB	80	gram	80	0.04	1.28	0.89	1.58
Breakfast Total				2260	0.77	5.74	4.92	6.35
Diet Total :				2260	0.77	5.74	4.92	6.35

4093-02

Meal	Food Description	Amount	Measure	Gram Weight	Ash	Carbohydrate, by difference	Fiber, total dietary	Tryptophan
Breakfast	4093 Beneprotein Powder	20	gram	20		0	0	0.29
Breakfast	4093 GF Lemon Sugar Cookie	240	gram	240	2.2	152.5	3.46	0.06
Breakfast	4093 Lophlex LQ Orange	280	gram	280		18.8	1.01	0.83
Breakfast	Orange-flavor drink, KRAFT, TANG Drin	80	gram	80	1.04	78.72	0.24	
Breakfast	Water	1560	gram	1560	1.56	0	0	
Breakfast	4093 Camino Pro Complete Bar 12+ PB	80	gram	80		44.8	3.2	0.18
Breakfast Total				2260	4.8	294.82	7.91	1.36
Diet Total :				2260	4.8	294.82	7.91	1.36

Meal	Food Description	Amount	Measure	Gram Weight	Histidine	Alanine	Aspartic acid	Glutamic acid
Breakfast	4093 Beneprotein Powder	20	gram	20	0.27	0.87	1.8	2.93
Breakfast	4093 GF Lemon Sugar Cookie	240	gram	240	0.12	0.28	0.45	0.98
Breakfast	4093 Lophlex LQ Orange	280	gram	280	1.6	2.35	3.54	0
Breakfast	Orange-flavor drink, KRAFT, TANG Drin	80	gram	80				
Breakfast	Water	1560	gram	1560				
Breakfast	4093 Camino Pro Complete Bar 12+ PB	80	gram	80	0.38	0.57	0.87	1.88
Breakfast Total				2260	2.37	4.07	6.66	5.79
Diet Total :				2260	2.37	4.07	6.66	5.79

Meal	Food Description	Amount	Measure	Gram Weight	Threonine	Isoleucine	Leucine	Lysine
Breakfast	4093 Beneprotein Powder	20	gram	20	1.11	1.06	1.83	1.9
Breakfast	4093 GF Lemon Sugar Cookie	240	gram	240	0.18	0.2	0.4	0.18
Breakfast	4093 Lophlex LQ Orange	280	gram	280	2.1	2.51	4.31	3.3
Breakfast	Orange-flavor drink, KRAFT, TANG Drinl	80	gram	80				
Breakfast	Water	1560	gram	1560				
Breakfast	4093 Camino Pro Complete Bar 12+ PB	80	gram	80	1.6	1.03	3	0.57
Breakfast Total				2260	4.99	4.8	9.54	5.96
Diet Total :				2260	4.99	4.8	9.54	5.96

Meal	Food Description	Amount	Measure	Gram Weight	Glycine	Proline	Serine	Alcohol, ethyl
Breakfast	4093 Beneprotein Powder	20	gram	20	0.27	0.94	0.75	0
Breakfast	4093 GF Lemon Sugar Cookie	240	gram	240	0.24	0.23	0.25	1.84
Breakfast	4093 Lophlex LQ Orange	280	gram	280	3.8	4.04	2.2	
Breakfast	Orange-flavor drink, KRAFT, TANG Drinl	80	gram	80				
Breakfast	Water	1560	gram	1560				0
Breakfast	4093 Camino Pro Complete Bar 12+ PB	80	gram	80	0.12	1.28	0.65	0
Breakfast Total				2260	4.43	6.5	3.86	1.84
Diet Total :				2260	4.43	6.5	3.86	1.84

4093-02

Meal	Food Description	Amount	Measure	Gram Weight	Methionine	Cystine
Breakfast	4093 Beneprotein Powder	20	gram	20	0.38	0.41

Breakfast	4093 GF Lemon Sugar Cookie	240	gram	240	0.11	0.06
Breakfast	4093 Lophlex LQ Orange	280	gram	280	0.69	1.03
Breakfast	Orange-flavor drink, KRAFT, TANG Drinl	80	gram	80		
Breakfast	Water	1560	gram	1560		
Breakfast	4093 Camino Pro Complete Bar 12+ PB	80	gram	80	0.15	0.02
Breakfast Total				2260	1.32	1.52
Diet Total :				2260	1.32	1.52

Meal	Food Description	Amount	Measure	Gram Weight	Protein (%)	Fat (%)	Carbohydrate (%)
Breakfast	4093 Beneprotein Powder	20	gram	20	100	0	0
Breakfast	4093 GF Lemon Sugar Cookie	240	gram	240	1.61	46.41	50.91
Breakfast	4093 Lophlex LQ Orange	280	gram	280	68.26	0	31.74
Breakfast	Orange-flavor drink, KRAFT, TANG Drinl	80	gram	80	0	0	100
Breakfast	Water	1560	gram	1560	0	0	0
Breakfast	4093 Camino Pro Complete Bar 12+ PB	80	gram	80	19.53	22.9	57.57
Breakfast Total				2260	14.57	29.46	55.37
Diet Total :				2260	14.57	29.46	55.37

Study ID: 4093

Study Date:	12/18/2017	Subject ID:	4093-01		
Height (cm)	155.5	Weight (kg)	62.2	Age(yrs)	14

Energy Requirements

Indirect Calorimetry	1811
Activity Factor (1.5)	2716.5

Total Diet Constituents: (calculated by ProNutra using IC data)

	grams	kcal	Phe	Tyrosine	Lysine
Lophlex LQ Orange Formula	352	294.82	0	4780	4140
Beneprotein Powder	24	85.71	580	580	2280
Tang Drink Mix	104	380.64	0	0	0
Lemon Sugar Cookie	316	1556.5	330	240	240
Camino Pro Peanut Butter Bar	100	395	50	1600	720
Total	896	2712.67	960	7200	7380

Total Diet Constituent Energy Distribution:

Energy (kcal)	Protein			Fat			Carbohydrates	
2712.67	96.76	grams		91.26	grams		382.76	grams
	387.04	kcals	2325.6	821.34	kcals		1531.04	kcals
	14.27%	% of total kcals		35.32%	%kcals after protein		65.83%	%kcals after protein

Hourly Diet Constituents: (Total Diet/8 hourly meals)

	grams	kcal	Phe	Tyrosine	Lysine
Lophlex LQ Orange	44	36.8525	0	597.5	517.5
Beneprotein Powder	3	10.71375	72.5	72.5	285
Tang Drink Mix	13	47.58	0	0	0
Lemon Sugar Cookie	39.5	194.5625	41.25	30	30
Camino Pro Peanut Butter Bar	12.5	49.375	6.25	200	90

Total Diet Constituent HOURLY Energy Distribution:

Energy (kcal)	Protein			Fat			Carbohydrates	
339.08375	12.095	grams		11.4075	grams		47.845	grams
	48.38	kcals	290.7	102.6675	kcals		191.38	kcals
	14.27%	% of total kcals		35.32%	%kcals after protein		65.83%	%kcals after protein

[1-13C] Lysine:

Lysine Bolus at Meal #4 (2.5mg/kg)	155.5		
Oral Infusion 1-13C Lysine dose each 1/2 hour:	43.54	Total 1-13C Lysine after 9 doses	391.86
Total Dietary Lysine provided with meals	7380		
Total Oral Infusion 1-13C Lysine	547.36		
Total Lysine provided (diet and infusion)	7927.36	127.4495177	mg/kg
Total Lysine intake per meal (for 1st 3 meals)	922.5		
Total Lysine intake at meal #4 (bolus & infusion	1121.54		
Total Lysine intake meals #5-8 (meal & infusion	1009.58		

Phenylalanine Intake

Total Phe intake	960	15.43408	mg/kg
Hourly Phe intake	120		

Amino Acid Composition of Meal and Infusion

	Meal 1 9:00	Meal 2 10:00	Meal 3 11:00	Meal 4 12:00	Meal 5 13:00	Meal 6 14:00	Meal 7 15:00	Meal 8 16:00	Daily Total
Phe	120	120	120	120	120	120	120	120	960
Tyr	900	700	900	900	900	900	900	900	7000
Lys	922.5	922.5	922.5	922.5	922.5	922.5	922.5	922.5	7380
1-13C Lys Bolus				155.5					155.5
1-13 C Lys Infusion				43.54	87.08	87.08	87.08	87.08	391.86

Formula Requisition (formula = Lophlex, Tang, Beneprotein mixed with water)

Formula required per study day:		
(from ProNutra)	352	g Lophlex (1 Sachet = ~145g / 125 mL)
	24	g Beneprotein
	104	g Tang
Add water to yield desired final volum	1560	mL
Formula required per meal	195	mL
Free water rinse per meal	50	mL
Total Free water consumed	400	mL

Food Requisition
(from ProNutra)

Total Sugar Cookie	316	g/day		
Hourly Sugar Cookie	39.5	g/meal		
Total Camino Pro Peanut Butter Bar	100	g/day	1.23	Whole Bars
Hourly Camino Pro Peanut Butter Bar	12.5	g/meal	0.15	Bar/ meal
(1 bar = 81g)				

Study ID: 4093

Study Date:	2/23/2018	Subject ID:	4093-02		
Height (cm)	167.64	Weight (kg)	57.27	Age(yrs)	17

Energy Requirements

Indirect Calorimetry	1400
Activity Factor (1.5)	2100

Total Diet Constituents: (calculated by ProNutra using IC data)

	grams	kcal	Phe	Tyrosine	Lysine
Lophlex LQ Orange Formula	280	234.5159	0	3802.273	3293.182
Beneprotein Powder	20	71.425	435	435	1710
Tang Drink Mix	80	292.8	0	0	0
Lemon Sugar Cookie	240	1182.152	245.41136	178.481	178.481
Camino Pro Peanut Butter Bar	80	316	40	1280	570
Total	700	2096.893	720.41136	5695.754	5751.663

Total Diet Constituent Energy Distribution:

Energy (kcal)	Protein			Fat			Carbohydrates	
2096.8927	77.6	grams		69.71	grams		294.82	grams
	310.4	kcals	1786.5	627.39	kcals		1179.28	kcals
	14.80%	% of total kcals		35.12%	%kcals after protein		66.01%	%kcals after protein

Hourly Diet Constituents: (Total Diet/8 hourly meals)

	grams	kcal	Phe	Tyrosine	Lysine
Lophlex LQ Orange	35	29.31449	0	475.2841	411.6477
Beneprotein Powder	2.5	8.928125	54.375	54.375	213.75
Tang Drink Mix	10	36.6	0	0	0
Lemon Sugar Cookie	30	147.769	30.67642	22.31012	22.31013
Camino Pro Peanut Butter Bar	10	39.5	5	160	71.25

Total Diet Constituent HOURLY Energy Distribution:

Energy (kcal)	Protein			Fat			Carbohydrates	
262.111588	9.7	grams		8.71375	grams		36.8525	grams
	38.8	kcals	223.31	78.42375	kcals		147.41	kcals
	14.80%	% of total kcals		35.12%	%kcals after protein		66.01%	%kcals after protein

[1-13C] Lysine:

Lysine Bolus at Meal #4 (2.5mg/kg)	143.175		
Oral Infusion 1-13C Lysine dose each 1/2 hou	40.089	Total 1-13C Lysine after 9 do	360.801
Total Dietary Lysine provided with meals	5751.663		
Total Oral Infusion 1-13C Lysine	503.976		
Total Lysine provided (diet and infusion)	6255.639	109.2306	mg/kg
Total Lysine intake per meal (for 1st 3 meals)	718.9578		
Total Lysine intake at meal #4 (bolus & infusi	902.2218		
Total Lysine intake meals #5-8 (meal & infusi	799.1358		

Phenylalanine Intake

Total Phe intake	720.41136	12.57921	mg/kg
Hourly Phe intake	90.05142		

Amino Acid Composition of Meal and Infusion

	Meal 1 9:00	Meal 2 10:00	Meal 3 11:00	Meal 4 12:00	Meal 5 13:00	Meal 6 14:00	Meal 7 15:00	Meal 8 16:00	Daily Total
Phe	90.05142	90.05142	90.05142	90.05142	90.05142	90.05142	90.05142	90.05142	720.4114
Tyr	711.9692	551.9691988	711.9692	711.9692	711.9692	711.9692	711.9691988	711.9692	5535.754
Lys	718.9578	718.957825	718.9578	718.9578	718.9578	718.9578	718.957825	718.9578	5751.663
1-13C Lys Bolus				143.175					143.175
1-13 C Lys Infusion				40.089	80.178	80.178	80.178	80.178	360.801

4093-02

Formula Requisition (formula = Lophlex, Tang, Beneprotein mixed with water)

Formula required per study day:		
(from ProNutra)	280	g Lophlex (1 Sachet = ~145g / 125 mL)
	20	g Beneprotein
	80	g Tang
Add water to yield desired final volum	1560	mL
Formula required per meal	195	mL
Free water rinse per meal	50	mL
Total Free water consumed	400	mL

Food Requisition
(from ProNutra)

Total Sugar Cookie	240	g/day		
Hourly Sugar Cookie	30	g/meal		
Total Camino Pro Peanut Butter Bar	80	g/day	0.99	Whole Bars
Hourly Camino Pro Peanut Butter Bar	10	g/meal	0.12	Bar/ meal
(1 bar = 81g)				

References

1. Vockley, J., et al., *Phenylalanine hydroxylase deficiency: diagnosis and management guideline.* Genet Med, 2014. **16**(2): p. 188-200.
2. Ozalp I, C.T., Tokatli A, Kalkanoğlu HS, Dursun A, Tokol S, Köksal G, Ozgüc M, Köse R, *Newborn PKU screening in Turkey: at present and organization for future.* Turk J Pediatr, 2001. **43**(2): p. 97-101.
3. Singh, R.H., et al., *Recommendations for the nutrition management of phenylalanine hydroxylase deficiency.* Genet Med, 2014. **16**(2): p. 121-31.
4. van Calcar, S.C. and D.M. Ney, *Food products made with glycomacropeptide, a low-phenylalanine whey protein, provide a new alternative to amino Acid-based medical foods for nutrition management of phenylketonuria.* J Acad Nutr Diet, 2012. **112**(8): p. 1201-10.
5. Olsson, G.M., S.M. Montgomery, and J. Alm, *Family conditions and dietary control in phenylketonuria.* J Inherit Metab Dis, 2007. **30**(5): p. 708-15.
6. Crone, M.R., et al., *Behavioural factors related to metabolic control in patients with phenylketonuria.* J Inherit Metab Dis, 2005. **28**(5): p. 627-37.
7. Bekhof J, v.R.M., Sauer PJJ, Ten Vergert EM, Reijngoud DJ, van Spronsen FJ, *Plasma phenylalanine in patients with phenylketonuria self-managing their diet.* Arch Dis Childh, 2005. **90**: p. 163-164.
8. Bernstein, L.E., et al., *Nutrition education tools used in phenylketonuria: clinician, parent and patient perspectives from three international surveys.* J Hum Nutr Diet, 2014. **27 Suppl 2**: p. 4-11.
9. Fujita, S., et al., *Nutrient signalling in the regulation of human muscle protein synthesis.* J Physiol, 2007. **582**(Pt 2): p. 813-23.
10. Burd, N.A., et al., *Low-load high volume resistance exercise stimulates muscle protein synthesis more than high-load low volume resistance exercise in young men.* PLoS One, 2010. **5**(8): p. e12033.
11. Dreyer, H.C., et al., *Resistance exercise increases AMPK activity and reduces 4E-BP1 phosphorylation and protein synthesis in human skeletal muscle.* J Physiol, 2006. **576**(Pt 2): p. 613-24.
12. Elango, R., R.O. Ball, and P.B. Pencharz, *Recent advances in determining protein and amino acid requirements in humans.* Br J Nutr, 2012. **108 Suppl 2**: p. S22-30.
13. (database)., N.C.f.B.I.O.-O.M.I.i.M., http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim. 2013.
14. Acosta, P.B., *Nutrition Management of Patients with Inherited Metabolic Disorders*, ed. P.B. Acosta. 2010: Jones and Bartlett Publishers, LLC. 476.
15. Centerwall, *<Pediatrics-2000-Centerwall-89-103.pdf>.*
16. Christ, S.E., *Asbjorn Folling and the discovery of phenylketonuria.* J Hist Neurosci, 2003. **12**(1): p. 44-54.

17. Penrose L, Q.J., *Metabolic Studies in Phenylketonuria.* Biochemistry, 1936. **31**(2): p. 266-74.
18. Bickel H., G.A.J., Hickman E.M. , *Influence of phenylalanine intake on Phenylketonuria.* Lancet, 1953. **2**: p. 812-19.
19. Guthrie R, S.A., *A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants.* Pediatrics, 1963. **32**: p. 332-43.
20. Chace DH, M.D., Terada N, Kahler SG, Roe CR, Lindsay FH., *Rapid Diagnosis of Phenylketonuria by Quantitative Analysis for Phenylalanine and Tyrosine in Neonatal Blood Spots by Tandem Mass Spectrometry.* Clin Chem, 1993. **39**(1): p. 66-71.
21. PItt DB, D.D., *The natural history of untreated phenylketonuria over 20 years.* J Paediatr Child Health, 1991. **27**: p. 189-190.
22. Gonzalez, M.J., et al., *Neurological complications and behavioral problems in patients with phenylketonuria in a follow-up unit.* Mol Genet Metab, 2011. **104 Suppl**: p. S73-9.
23. Horner FA, S.C., Alejandrino LL, Reed LH, Ibbott F., *Termination of Dietary Treatment of Phenylketonuria.* New England Journal of Medicine, 1962. **266**(11): p. 79-81.
24. FP, H., *Termination of Dietary Treatment of Phenylketonuria.* Arch Dis Childh, 1967. **42**: p. 198-200.
25. DYY, H., *Phenylketonuria: A Study of Human Biochemical Genetics.* Pediatrics, 1965. **38**(2): p. 173-184.
26. Vandeman, P., *Termination of Dietary Treatment for Phenylketonuria.* Arch J Dis Child, 1963. **106**: p. 492-495.
27. Cerone R, S.M., Di Stefano S, Veneselli E, *Phenylketonuria: diet for life or not?* Acta Paediatr, 1999. **88**: p. 664-6.
28. Smith I, L.M., Stevenson JE, Wolff OH, Schmidt H, Grubel-Kaiser S, Bickel H, *Effect of stopping low-phenylalanine diet on intellectual progress of children with phenylketonuria.* British Medical Journal, 1978. **9**: p. 723-36.
29. Seashore MR, F.E., Novelly RA, Bapat V, *Loss of Intellectual Function in Children with Phenylketonuria after Relaxation of Dietary Phenylalanine Restriction.* Pediatrics, 1985. **75**(2): p. 226-32.
30. Viau, K.S., et al., *Correlation of age-specific phenylalanine levels with intellectual outcome in patients with phenylketonuria.* J Inherit Metab Dis, 2011. **34**(4): p. 963-71.
31. Bik-Multanowski, M., et al., *Quality of life in noncompliant adults with phenylketonuria after resumption of the diet.* J Inherit Metab Dis, 2008. **31 Suppl 2**: p. S415-8.
32. *National Institutes of Health Consensus Development Conference Statement: Phenylketonuria: Screening and Management, October 16-18, 2000.* Pediatrics, 2001. **108**(4): p. 972-982.

33. Loeber, J.G., *Neonatal screening in Europe; the situation in 2004.* J Inherit Metab Dis, 2007. **30**(4): p. 430-8.
34. Zschocke J, M.J., Eiken HG, *Phenylketonuria and the peoples of Northern Ireland.* Hum Genet, 1997. **100**: p. 189-194.
35. Zschocke, J., *Phenylketonuria mutations in Europe.* Hum Mutat, 2003. **21**(4): p. 345-56.
36. Guldberg P, H.K., Sipila K, Guttler F, Chapelle A, *Phenylketonuria in a low incidence population: molecular characterisation of mutations in Finland.* J Med Genet, 1995. **32**: p. 976-978.
37. *Neonatal screening in Europe. Abstracts of the 7th ISNS European Neonatal Screening Regional Meeting. Geneva, Switzerland. August 28-30, 2011.* J Inherit Metab Dis, 2011. **34 Suppl 2**: p. 17-48.
38. Borrajo, G.J., *Newborn screening in Latin America at the beginning of the 21st century.* J Inherit Metab Dis, 2007. **30**(4): p. 466-81.
39. Cornejo, V., et al., *Past, present and future of newborn screening in Chile.* J Inherit Metab Dis, 2010.
40. Jian-Ying Zhan, Y.-F.Q., Zheng-Yan Zhao, *Neonatal screening for congenital hypothyroidism and phenylketonuria in China.* World Journal of Pediatrics, 2009. **5**(2): p. 136-139.
41. Maitusong R, J.R., Zheng-yan Z, Ru-lai Y, Xiao-lei H, Hua-qing M., *Newborn screening in Zhejiang, China.* Chin Med J, 2012. **125**(4): p. 702-204.
42. Pangkanon S, C.W., Janejai N, Boomwanich W, Chaisomchit S, *Detection of Phenylketonuria by the Newborn Screening Program in Thailand.* Southeast Asian J Trop Med Public Health, 2009. **40**(3): p. 525-529.
43. Desviat LR, P.B., Gamez A, Sanchez A, Garcia MJ, Martinez-Pardo M, Marchante C, Boveda D, Baldellou A, Arena J, Sanjurjo P, Fernandez A, Cabello ML, Ugarte M., *Genetic and phenotypic aspects of phenylalanine hydroxylase deficiency in Spain: molecular survey by regions.* European Journal of Human Genetics, 1999. **7**: p. 386-392.
44. Hardelid, P., et al., *The birth prevalence of PKU in populations of European, South Asian and sub-Saharan African ancestry living in South East England.* Ann Hum Genet, 2008. **72**(Pt 1): p. 65-71.
45. Scriver CR, K.S., *Hyperphenylalanemia: phenylalanine hydroxylase deficiency.*, ed. B.A. Scriver CR, Sly WS, Valle D, Childs B, Kinzler K, Vogelstein B. Vol. 8. 2001, New York: McGraw-Hill.
46. *PAH Mutation Database*. 2013 [cited 2013 February 8]; Available from: http://www.pahdb.mcgill.ca.
47. Hanley WB, L.L., Davidson W, Moes CAF., *Malnutrition with Early Treatment of Phenylketonuria.* Pediat Res, 1970. **4**: p. 381-327.
48. Thony, B. and N. Blau, *Mutations in the BH4-metabolizing genes GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase, sepiapterin reductase,*

carbinolamine-4a-dehydratase, and dihydropteridine reductase. Hum Mutat, 2006. **27**(9): p. 870-8.
49. Gropper SS, S.J., Groff JL, *Advanced Nutrition and Human Metabolism*. 5th ed, ed. P. Adams. 2009, Belmont, California: Wadsworth Cengage.
50. Lichter-Konecki U, H.C., Konecki DS., *Human Phenylalanine Hydroxylase Gene Expression in Kidney and Other Nonhepatic Tissues.* Mol Genet Metab, 1999. **67**: p. 308-316.
51. Bross R, B.R., Clarke JTR, Pencharz PB, *Tyrosine requirements in children with classical PKU determined by indicator amino acid oxidation.* Am J Physiol Endocrinol Metab, 2000. **278**: p. E195-E201.
52. Kayaalp E, T.E., Waters PJ, Byck S, Nowacki P, Scriver CR., *Human Phenylalanine Hydroxylase Mutations and Hyperphenylalaninemia Phenotypes: A Metanalysis of Genotype-Phenotype Correlations.* Am J Hum Genet, 1997. **61**: p. 1309-1317.
53. van Spronsen, F.J., et al., *Large neutral amino acids in the treatment of PKU: from theory to practice.* J Inherit Metab Dis, 2010. **33**(6): p. 671-6.
54. WM, P., *Blood-Brain Barrier Carrier-Mediated Transport and Brain Metabolism of Amino Acids.* Neurochemical Research, 1998. **23**(5): p. 635-644.
55. de Groot, M.J., et al., *Pathogenesis of cognitive dysfunction in phenylketonuria: review of hypotheses.* Mol Genet Metab, 2010. **99 Suppl 1**: p. S86-9.
56. Surtees R, B.N., *The neurochemistry of phenylketonuria.* Eur J Pediatr, 2000. **159**(S2): p. S109-S113.
57. QR, S., *Glutamate and Glutamine in the Brain.* J Nutr, 2000. **130**: p. 1016S-1022S.
58. Brumm VL, A.C., Moats RA, Stern AM, Broomand C, Nelson MD, Koch R, *Neuropsychological outcomes of subjects participating in the PKU Adult Collaborative Study: A preliminary review.* J Inherit Metab Dis, 2004. **27**: p. 549-566.
59. Christ, S.E., et al., *Decreased functional brain connectivity in individuals with early-treated phenylketonuria: evidence from resting state fMRI.* J Inherit Metab Dis, 2012. **35**(5): p. 807-16.
60. PR, H., *The neuropathology of phenylketonuria: human and animal studies.* Eur J Pediatr, 2000. **159**(Suppl 2): p. S102-S106.
61. Anderson, P.J. and V. Leuzzi, *White matter pathology in phenylketonuria.* Mol Genet Metab, 2010. **99 Suppl 1**: p. S3-9.
62. Schoemans, R., et al., *Oligodendrocyte development and myelinogenesis are not impaired by high concentrations of phenylalanine or its metabolites.* J Inherit Metab Dis, 2010. **33**(2): p. 113-20.
63. van Spronsen FJ, R.D., Smit GPA, Stellaard NF, Berger R, Heymans HSA, *Phenylketonuria: The in Vivo Hydroxylation Rate of Phenylalanine into Tyrosine is Decreased.* J Clin Invest, 1998. **101**(12): p. 2875-2880.

64. Stemerdink B.A., K.A.F., van der Meere J.J., van der Molen M.W., Huisman J., de Jong W.A., Slijper F.M.E., Verkerk P.H., van Spronsen F.J. , *Behaviour and school achievement in patients with early and continuously treated phenylketonuria.* J. Inherit Metab Dis, 2000. **23**(6): p. 548-562.
65. Burgard P, B.H., Buhrdel P, Clemens PC, Monch E, Przyrembel H, Trefz FK, Ullrich K, *Rationale for the German recommendations for phenylalanine level control in phenylketonuria 1997.* Eur J Pediatr, 1999. **158**: p. 46-54.
66. Rohde, C., et al., *Unrestricted consumption of fruits and vegetables in phenylketonuria: no major impact on metabolic control.* Eur J Clin Nutr, 2012. **66**(5): p. 633-8.
67. Weetch E, M.A., *Determination of phenylalanine content of foods suitable for phenylketonuria.* J Hum Nutr Dietet, 2006. **19**: p. 229-36.
68. MacDonald A, R.G., Davies P, Asplin D, Hall SK, Booth IW, *Free use of fruits and vegetables in phenylketonuria.* J Inherit Metab Dis, 2003. **26**: p. 327-338.
69. van Spronsen, F.J., et al., *Phenylalanine tolerance can already reliably be assessed at the age of 2 years in patients with PKU.* J Inherit Metab Dis, 2009. **32**(1): p. 27-31.
70. MacLeod, E.L., et al., *Breakfast with glycomacropeptide compared with amino acids suppresses plasma ghrelin levels in individuals with phenylketonuria.* Mol Genet Metab, 2010. **100**(4): p. 303-8.
71. Ney, D.M., R.D. Blank, and K.E. Hansen, *Advances in the nutritional and pharmacological management of phenylketonuria.* Curr Opin Clin Nutr Metab Care, 2014. **17**(1): p. 61-8.
72. Belanger-Quintana, A., et al., *Up to date knowledge on different treatment strategies for phenylketonuria.* Mol Genet Metab, 2011. **104 Suppl**: p. S19-25.
73. Singh, R.H. and M.E. Quirk, *Using change in plasma phenylalanine concentrations and ability to liberalize diet to classify responsiveness to tetrahydrobiopterin therapy in patients with phenylketonuria.* Mol Genet Metab, 2011. **104**(4): p. 485-91.
74. Sarkissian, C.N., et al., *Preclinical evaluation of multiple species of PEGylated recombinant phenylalanine ammonia lyase for the treatment of phenylketonuria.* Proc Natl Acad Sci U S A, 2008. **105**(52): p. 20894-9.
75. Gamez, A., *Toward PKU Enzyme Replacement Therapy: PEGylation with Activity Retention for Three Forms of Recombinant Phenylalanine Hydroxylase.* Molecular Therapy, 2004. **9**(1): p. 124-129.
76. Robert J.J, B.D., Zhao XH, Matthews DE, Young VR, *Glucose and Insulin Effects on de novo amino acid synthesis in young men: Studies with stable isotope labeled alanine, glycine, leucine and lysine.* Metabolism, 1983. **31**(2): p. 1210-1218.
77. Zello GA, P.P., Ball RO, *The Design and Validation of a Diet for Studies of Amino Acid Metabolism in Adult Humans.* Nutr Res, 1990. **10**: p. 1353-1365.

78. Kim, K., McMillan I., Bayley H.S., *Determination of amino acid requirements of young pigs using an indicator amino acid.* Br J Nutr, 1983. **50**: p. 369-382.
79. Ball RO, B.H., *Tryptophan requirement of the 2.5 kg piglet determined by the oxidation of an indicator amino acid.* Am Inst Nutr, 1984: p. 1741-1746.
80. Zello GA, P.P., Ball RO, *Dietary lysine requirement of young adult males determined by oxidation of L-[1[13C] phenylalanine.* Am J Physiol Endocrinol Metab, 1993. **264**: p. E677-E685.
81. Elango R, B.R., Pencharz PB, *Indicator Amino Acid Oxidation: Concept and Application.* J Nutr, 2008. **138**: p. 243-246.
82. Courtney-Martin G., B.R., Raffi M., Clarke J.T.R., Ball R.O., Pencharz P.B., *Phenylalanine requirement in children with classical PKU determined by indicator amino acid oxidation.* Am J Physiol Endocrinol Metab, 2002. **283**: p. E1249-E1256.
83. Bross R, B.R., Pencharz PB, *Development of a minimally invasive protocol for the determination of phenylalanine and lysine kinetics in humans during the fed state.* J Nutr, 1998. **128**: p. 1913-1919.
84. Zello GA, P.P., Ball RO, *Phenylalanine flux, oxidation and conversion to tyrosine in humans studied with L-[1-13C]phenylalanine.* Am J Physiol Endocrinol Metab, 1990. **259**: p. E835-E843.
85. Roberts SA, T.J., Ball RO, Pencharz PB, *Tyrosine requirement of healthy men receiving a fixed phenylalanine intake determined by using indicator amino acid oxidation.* Am J Clin Nutr, 2001. **73**: p. 276-82.
86. Di Buono M, W.L., Cole DEC, Ball RO, Pencharz PB., *Regulation of Sulfur Amino Acid Metabolism in Men in Response to Changes in Sulfur Amino Acid Intakes.* Human Nutrition and Metabolism, 2003. **133**: p. 733-739.
87. di Buono M, W.L., Ball RO, Pencharz PB, *Total sulfer amino acid requirement in young men as determined by indicator amino acid oxidation with L-[1-13C]phenylalanine.* Am J Clin Nutr, 2001. **74**(74): p. 756-60.
88. Turner JM, H.M., Elango R, Rafii M, Langos V, Ball RO, Pencharz PB, *Total sulfer amino acid requirement of healthy school-age children as determined by indicator amino acid oxidation technique.* Am J Clin Nutr, 2006. **83**: p. 619-23.
89. Humayun MA, T.J., Elango R, Rafii M, Langos V, Ball RO, Pencharz PB, *Minimum methionine requirement and cystein sparing of methionine in healthy school-age children.* Am J Clin Nutr, 2006. **84**: p. 1080-5.
90. Wilson DC, R.M., Ball RO, Pencharz PB, *Theronine requirement of young men determined by indicator amino acid oxidation with use of L-[1-13C]phenylalanine.* Am J Clin Nutr, 2000. **71**: p. 757-64.
91. Lazaris-Brunner G, R.M., Ball RO, Pencharz PB, *Tryptophan requirement in young adult women as determined by indicator amino acid oxidation with L-[13C]phenylalanine.* Am J Clin Nutr, 1998. **68**: p. 303-10.

92. Riazi, R., et al., *Total branched-chain amino acids requirement in patients with maple syrup urine disease by use of indicator amino acid oxidation with L-[1-13C]phenylalanine.* Am J Physiol Endocrinol Metab, 2004. **287**(1): p. E142-9.
93. Mager DR, W.L., Ball RO, Pencharz PB, *Branched-Chain Amino Acid Requirements in School-Aged Children determined by Indicator Amino Acid Oxidation (IAAO).* Am J Clin Nutr, 2003. **133**: p. 3540-3540.
94. Elango R, H.M., Ball RO, Pencharz PB, *Lysine requirement of healthy school-age children determined by the indicator amino acid oxidation method.* Am J Clin Nutr, 2007. **86**: p. 360-5.
95. Kriengsinyos W., W.L.J., Ball R.O., Pencharz P.B., *Oral and Intravenous Tracer Protocols for the Indicator Amino Acid Oxidation Method Provide the Same Estimate of the Lysine Requirement in Healthy Men.* J Nutr, 2002. **132**: p. 2251-2257.
96. Pillai, R.R. and A.V. Kurpad, *Amino acid requirements in children and the elderly population.* Br J Nutr, 2012. **108 Suppl 2**: p. S44-9.
97. Hsu JW, B.R., Pencharz PB, *Evidence that Phenylalanine may not provide the full needs for aromatic amino acids in children.* Ped Research, 2007. **61**(3): p. 361-365.
98. Elango, R., et al., *Protein requirement of healthy school-age children determined by the indicator amino acid oxidation method.* Am J Clin Nutr, 2011. **94**(6): p. 1545-52.
99. Zello GA, W.L., Ball RO, Pencharz PB, *Recent advances in methods of assessing dietary amino acid requirements for adult humans.* Am Inst Nutr, 1995: p. 2907-2915.
100. Elango, R., et al., *Available versus digestible amino acids - new stable isotope methods.* Br J Nutr, 2012. **108 Suppl 2**: p. S306-14.
101. Bell J.A., F.S., Volpi E., Cadenas J.G., Rasmussen B.B., *Short-term insulin and nutritional energy provision do not stimulate muscle protein synthesis if blood amino acid availability decreases.* Am J Physiol Endocrinol Metab, 2005. **289**: p. E999-E1006.
102. Truong, A.D., et al., *Bench-to-bedside review: mobilizing patients in the intensive care unit--from pathophysiology to clinical trials.* Crit Care, 2009. **13**(4): p. 216.
103. Chambers, M.A., J.S. Moylan, and M.B. Reid, *Physical inactivity and muscle weakness in the critically ill.* Crit Care Med, 2009. **37**(10 Suppl): p. S337-46.
104. Kress, J.P., *Clinical trials of early mobilization of critically ill patients.* Crit Care Med, 2009. **37**(10 Suppl): p. S442-7.
105. Puthucheary, Z., S. Harridge, and N. Hart, *Skeletal muscle dysfunction in critical care: wasting, weakness, and rehabilitation strategies.* Crit Care Med, 2010. **38**(10 Suppl): p. S676-82.
106. Williams BD, W.R., Bracy DP, Wasserman DH, *Gut proteolysis contributes essential amino acids during exercise.* Am J Physiol, 1996. **270**: p. E85-90.

107. Lavoie C, D.F., Bourgue J, Langelier H, Chiasson JL, *Glucose metabolism during exercise in man: the role of insulin and glucagon in the regulation of hepatic glucose production and gluconeogenesis.* Can J Physiol Pharmacol, 1997. **75**(26-35).
108. van Hall G, M.D., Saltin B, Wagenmakers AJM. , *Mechanisms of activation of muscle branched-chain keto acid dehydrogenase during exercise in man.* J Physiol, 1996. **494**: p. 899-905.
109. Felig P, W.J., *Amino acid metabolism in exercising man.* J Clin Invest, 1971. **50**: p. 2703-2709.
110. Halliday D, M.R., *Measurement of muscle protein synthetic rate from serial muscle biopsies and total body protein turnover in man by continuous intravenous infusion of L-alpha-15N Lysine.* Clin Sci Mol Med, 1975. **49**: p. 581-590.
111. Biolo G, T.K., Klein S, Wolfe RR, *An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein.* Am J Physiol, 1997. **273**: p. E122-E129.
112. Blomstrand E, S.B., *Effect of muscle glycogen on glucose, lactate, and amino acid metabolism during exercise and recovery in human subjects.* J Physiol London, 1999. **514**: p. 293-302.
113. Rennie MJ, T.K., *Protein and Amino Acid Metabolism during and after exercise and the effects of nutrition.* Ann Rev Nutr, 2000. **20**: p. 457-483.
114. Eriksson LS, B.S., Bjorkman O, Wahren J, *Ammonia metabolism during exercise in man.* Clin Physiol, 1985. **5**: p. 325-336.
115. Katz A, B.S., Sahlin K, Wahren J. , *Muscle ammonia and amino acid metabolism during dynamic exercise in man.* Clin Physiol, 1986. **6**: p. 365-379.
116. Sahlin K, K.A., Broberg S., *Tricarboxylic acid cycle intermediates in human muscle during prolonged exercise.* Am J Physiol Cell Physiol, 1990. **259**: p. C834-C841.
117. van Hall G, S.B., van der Vusse GJ, Soderland K, Wagenmakers AJM., *Deamination of amino acids as a source for ammonia production in human skeletal muscle during prolonged exercise.* J Physiol, 1995. **489**: p. 251-261.
118. MacLean DA, G.T., Saltin B. , *Branched-chain amino acids augment ammonia metabolism while attenuating protein breakdown during exercise.* Am J Physiol, 1994. **267**: p. E1010-E1022.
119. Bergstrom JP, F.P., Hultman E. , *Free amino acids in muscle tissue and plasma during exercise in man.* Clin Physiol, 1985. **5**: p. 325-336.
120. J, H., *Effect of Exercise on Amino Acid Concentrations in Skeletal Muscle and Plasma.* J Exp Biol, 1991. **160**: p. 149-165.
121. Wagenmakers AJ, B.J., Coakley JH, Reilly T, Edwards RH., *Exercise-induced activation of the branched-chain 2-oxo acid dehydrogenase in human muscle.* Eur J Appl Physiol Occup Physiol, 1989. **59**: p. 159-167.

122. Shimomura Y, F.H., Suzuki M, Murakami T, Fujitsuka N, Nakai N., *Branched chain alpha-keto acid dehydrogenase complex in rat skeletal muscle: regulation of the activity and gene expression by nutrition and physical exercise.* J Nutr, 1995. **125**(Suppl): p. S1762-1765.
123. van Hall G, M.D., Saltin B, Wagenmakers AJM., *Mechanisms of activation of muscle branched-amino acids as a source for ammonia production in human skeletal muscle during prolonged exercise.* J Physiol, 1996. **489**(251-261).
124. Wolfe RR, G.R., Wolfe MH, Royle GT, Nadel ER, *Isotopic analysis of leucine and urea metabolism in exercising humans.* J Appl Physiol, 1982. **52**: p. 458-466.
125. Bylund-Fellenius AC, O.K., Flaim KE, Li JB, Wassner SJ, Jefferson LS., *Protein synthesis versus energy state in contracting muscles of perfused rat hindlimb.* Am J Physiol Endocrinol Metab, 1984. **246**(E297-E305): p. E297.
126. Fujita S, D.H., Drummond MJ, Glynn EL, Volpi E, Rasmussen BB., *Essential amino acid and carbohydrate ingestions before resistance exercise does not enhance postexercise muscle protein synthesis.* J Appl Physiol, 2009. **106**: p. 1730-1739.
127. Chelsey A, M.J., Tarnopolsky MA, Atkinson SA, Smith K, *Changes in human muscle protein synthesis after resistance exercise.* J Appl Physiol, 1992. **73**: p. 1383-1388.
128. Ikemoto M, N.T., Kano M, Hirasaka K, Kitano T, Watanabe C, Tanaka R, Yamamoto T, Kamada M, Kishi K, *Cysteine supplementation prevents unweighting-induced ubiquitination in associated skeletal muscle.* Biol Chem, 2002. **383**(3-4): p. 715-21.
129. van Rijn M, H.M., Sauer P, Modderman P, Reijngoud DJ, van Spronsen FJ., *Adult patients with well controlled phenylketonuria tolerate incidental intake of phenylalanine.* Ann Nutr Metab, 2011. **58**(2): p. 94-100.
130. Mamerow, M.M., et al., *Dietary Protein Distribution Positively Influences 24-h Muscle Protein Synthesis in Healthy Adults.* J Nutr, 2014. **144**(6): p. 876-80.
131. Paddon-Jones, D. and B.B. Rasmussen, *Dietary protein recommendations and the prevention of sarcopenia.* Curr Opin Clin Nutr Metab Care, 2009. **12**(1): p. 86-90.
132. MacDonald A, R.G., Hall SK, Asplin D, Booth IW, *Factors affecting the variation in plasma phenylalanine in patients with phenylketonuria on diet.* Arch Dis Child, 1996. **74**: p. 412-417.
133. Hoeksma, M., et al., *Phenylketonuria: High plasma phenylalanine decreases cerebral protein synthesis.* Mol Genet Metab, 2009. **96**(4): p. 177-82.
134. Cleary, M., et al., *Fluctuations in phenylalanine concentrations in phenylketonuria: a review of possible relationships with outcomes.* Mol Genet Metab, 2013. **110**(4): p. 418-23.
135. MacDonald A, R.G., Davies P, Asplin D, Hall SK, Booth IW, *Administration of protein substitute and quality of control in phenylketonuria: A randomized study.* J Inherit Metab Dis, 2003. **26**(4): p. 319-326.

136. MacDonald A, R.G., Davies P, Asplin D, Hall SK, Booth IW, *Does a single plasma phenylalanine predict quality of control in phenylketonuria?* Arch Dis Child, 1998. **78**: p. 122-126.
137. van Spronsen FJ, v.R.M., van Dijk T, Smit GPA, Reijngoud DJ, Berger R, Heymans HSA., *Plasma Phenylalanine and Tyrosine Responses to Different Nutritional Conditions (Fasting/Postprandial) in Patients With Phenylketonuria: Effect of Sample Timing.* Pediatrics, 1993. **92**: p. 570.
138. Grunert SC, B.C., Krebs A, Clement HW, Rauh R, Fleischaker C, Henninghausen K, Sass JO, Schwab KO, *Diurnal variation of phenylalanine and tyrosine concentrations in adult patients with phenylketonuria: subcutaneous microdialysis is no adequate tool for the determination of amino acid concentrations.* Nutr J, 2013. **12**(60): p. 1-12.
139. Hussein MA, Y.V., Murray E, Scrimshaw NS., *Daily Fluctuation of Plasma Amino Acid Levels in Adult Men: Effect of Dietary Tryptophan Intake and Distribution of Meals.* J Nutr, 1971. **101**: p. 61-70.
140. Nasset ES, H.F., Calloway DH, Margen S, Schneeman P., *Amino Acids in Human Blood Plasma after Single Meals of Meat, Oil, Sucrose, and Whiskey.* J Nutr, 1979. **109**: p. 621-630.