EFFECT OF MEDICAL FOODS USED IN DIETARY MANAGEMENT OF SUBJECTS WITH PROPIONIC ACIDEMIA (PROP)

by

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis entitled:

Effect of Medical Foods Used in Dietary Management of Subjects with Propionic Acidemia (PROP)

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submitted by Haneen Saleemani in partial fulfillment of the requirements for the degree of Master of Science in Human Nutrition

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Abstract

Introduction: Propionic Acidemia (PROP) is an inborn errors of metabolism disorder, caused by a defect in the enzyme propionyl-CoA carboxylase (PCC). PCC catalyzes two of the branched-chain amino acids (BCAA), valine, isoleucine. The management of PROP depends on dietary protein restriction and medical food consumption. Recently, concerns have been raised about medical foods due to imbalanced content of BCAA (high leucine – another BCAA, and no valine/isoleucine). It has been suggested that this imbalanced mixture of BCAA negatively impacts plasma concentrations of valine and isoleucine, and therefore growth in children with PROP. Studies on long-term growth outcomes in subjects with PROP are limited. Thus, a comprehensive assessment of dietary intake and its impact on growth in children with PROP is needed. Furthermore, since subjects with PROP depend on medical foods as an easily tolerable source of energy and protein, there is a need to determine the optimal BCAA ratio in medical foods to optimize protein synthesis and growth.

Methods & Results: A retrospective chart review was conducted on four subjects with PROP; longitudinal data on dietary intake and growth outcomes for 1999-2018 were collected. Results suggest that subjects had persistently low height Z scores, despite consuming protein intakes higher than guidelines. However, the high consumption of medical foods protein relative to intact protein impacted growth. A prospective study to test different BCAA (LEU: ILE: VAL) ratios was conducted using the indicator amino acid oxidation method. Eight healthy children participated at 7 different test intakes with the use of L-1-^{13}C-Phenylalanine oxidation to $^{13}CO_2$ as a marker of protein synthesis. ANOVA showed significant differences with different test

intakes, with a ratio between 1: 0.26: 0.28 and 1:0.35:0.4 observed to be associated with optimal protein synthesis.

Conclusion: Our results indicate that intact protein restriction together with overusing medical foods could have affected growth in children with PROP**.** Currently used medical foods are formulated to provide an imbalanced BCAA (1:0:0), which was associated with the highest oxidation rates (indicating low protein synthesis). Future studies should examine the effect of BCAA ratios between (1:0.26:0.28) and (1:0.35:0.4) in subjects with PROP to measure adequacy for protein synthesis.

Lay Summary

Propionic Acidemia (PROP) is a genetic disorder in which the body is unable to process certain amino acids in protein rich foods. In order to provide a safe alternative to the regular diet for children with PROP, special medical foods have been developed. Recently, medical food consumption was reported to cause growth failure in children. In the first stage of this study, we reviewed dietary and growth charts of four children with PROP at BC Children's Hospital. We found that children were consuming high amounts of medical foods and had poor growth outcomes. In the second stage, we showed that the currently used medical foods for PROP limit total body protein synthesis, and therefore restrict growth. To ensure normal growth for children with PROP we propose: 1) Reformulating the amino acid mixture in the medical foods; 2) Shifting the practice from using medical foods as the primary source of nutrition.

Preface

I have written this thesis under the supervision of Dr. Rajavel Elango. My committee members, Drs. Sylvia Stockler, Gabriella Horvath and Crystal Karakochuk also provided substantial input regarding study design and methodology.

The current thesis consists of two studies which were approved by the University of British Columbia and BC Children's Hospital Research Ethics Board (H19-02912) and (H18-00439). In the first study, Dr. Gabriella Horvath and the dietitians in the Biochemical Disease Clinic at BC Children's Hospital helped with the data collection. In the second study, all procedures including pre-study assessments, test diet preparation, sample collection and statistical analyses were done by me with the help of Dr. Rajavel Elango. Breath sample analysis (isotope ratio mass spectrometer (IRMS, Isoprime Ltd, Cheadle, UK) and urine sample analysis using the Amino Acid Analyzer (AAA) (Hitachi L8900, Tokyo, Japan) and HPLC (Chromaster 5430 Diode Array Detector, Hitachi, Tokyo, Japan) were done by Katia Caballero, Madeleine Ennis, Betina Rasmussen (researchers within the Elango Lab) and myself.

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List of Abbreviations

AAA- Amino Acid Analyzer

AI- Adequate Intake

ANOVA- Analysis of Variance

APE- Atom Percent Excess

BCAA- Branched-Chain Amino Acids

BCKA- Branched-Chain Keto Acids

BCKD- Branched-Chain Keto Dehydrogenase

BIA- Bioelectrical Impedance Analysis

BMI- Body Mass Index

DRI- Dietary Reference Intakes

ECO_2-$^{13}CO_2$ Isotopic Enrichment (APE)

$F^{13}CO_2$ -Rate of L-[1-^{13}C] Phenylalanine or Leucine Tracer Oxidation

EER- Estimated Energy Requirements

FAO -Food and Agriculture Organization

FCO_2 -CO_2 Production Rate Using Indirect Calorimetry

FFM-Fat Free Mass

FM- Fat Mass

GMDI- Genetic Metabolic Dietitians International

HT- Height

IAAO- Indicator Amino Acid Oxidations

IB-CoA- Isobutyryl-CoA

IEM- Inborn Error of Metabolism

ILE- Isoleucine

IRMS- Isotope Ratio Mass Spectrometry

IV-CoA- Isovaleryl-CoA

KIC- ketoisocaproic Acid

KIV- keto- Isovalerate

KMV- keto-Methylvalerate

LEU- Leucine

MB-CoA- a- methylbutyryl-CoA

MET- Methionine

NBS- Newborn Screening

OMIM- Online Mendelian Inheritance in Man

PCC- Propionyl-CoA Carboxylase

PFD- Protein Free Powder

PHE- Phenylalanine

PKU- Phenylketonuria

PROP- Propionic Acidemia

RDA- Recommended Dietary Allowance

SD- Standard Deviation

TCA- Tricarboxylic Acid Cycle

TEE- Total Energy Expenditure

THR-Threonine

VAL- Valine

VCO_2 - Rate of Carbon Dioxide Production

WHO- World Health Organization

WT- Weight

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I would like to express my very great appreciation and gratitude to my advisor Dr. Rajavel Elango, who has been a great mentor through my studies. I thank him for the ongoing guidance, encouragement and the kind advice he has provided throughout my time in his lab. I would also like to thank my committee members, Dr. Sylvia Stockler, Dr. Gabriella Horvath and Dr. Crystal Karakochuk, for their time and support during my research. I would like to acknowledge the physicians and dietitians in the Biochemical Diseases Clinic at both BC Children's Hospital and Vancouver General Hospital for all their guidance and contributions to my research. Very special thanks to all my wonderful participants and their families, without whom this research could not have been completed. I would like to specially thank my colleagues in the Elango lab: Katia Caballero, Madeleine Ennis, Abrar Turki, Zoe Schmidt, Betina Rasmussen, Maggie Kinshella, and Kerri Scherbinsky for their help, constructive suggestions, and all the inspirational coffee breaks. I would also like to express my gratitude to King Abdulaziz University and the Saudi Arabian Cultural Bureau for their generous funding throughout my study.

Finally, special thanks are owed to my family, who have supported me throughout my years of education, both morally and financially. No words can express how grateful I am to my parents for all their love and emotional support.

Dedication

This thesis is dedicated to

My wonderful parents, Rabab & Hassan who have raised me to be the person I am today

My brothers, Rami & Hazem

My sisters Rasha, Futoon & Yara

My friends

All who have supported me through this journey

Chapter 1: Introduction

1.1 Introduction and Overview

Propionic Acidemia (PROP) is an inherited metabolic disorder, caused by a defect in the mitochondrial enzyme propionyl-CoA carboxylase (PCC). PCC converts propionyl-CoA to methylmalonyl-CoA, an intermediate in the catabolism of isoleucine (ILE) and valine (VAL), two of the three branched-chain amino acids (BCAA include leucine, isoleucine and valine), as well as threonine (THR), methionine (MET), and odd-chain fatty acids. A block in the catabolic pathway of these compounds leads to an accumulation of excess propionic acid metabolites that can disrupt the tricarboxylic acid cycle (TCA), as well as the urea cycle, causing hyperammonemia (Baumgartner et al., 2014). PROP is considered an ultra-rare disorder, with similar rates across all regions estimated to be 1 in 100,000, except for regions in the Middle East, where most inherited metabolic disorders are more frequent (Almási et al., 2019).

The goal of nutritional management of PROP is to reduce the accumulation of toxic metabolites by restricting dietary protein sources of the propiogenic amino acids (ILE, VAL, MET, and THR), while maintaining their normal plasma concentrations (Jurecki et al., 2019). In addition, subjects with PROP are usually supplemented with special medical foods that are formulated to contain no propiogenic precursors (ILE, VAL, MET, and THR) and normal to high amounts of other amino acids to ensure sufficient protein intake for optimal growth (Manoli et al., 2016). Specifically, medical foods for PROP are formulated to contain minimal or no valine and isoleucine, and high amounts of leucine. Due to the imbalanced content of the three BCAA in these formulations, there have recently been arguments against their use (Manoli et al., 2016;

Myles et al., 2018). High leucine intakes can negatively impact the other two BCAA (isoleucine and valine), by suppressing their plasma concentrations below normal ranges, limiting total protein synthesis and restricting growth (Harper et al., 1984). Studies on the long-term growth outcomes of individuals with PROP are limited. Thus, the objective of the first study in this thesis was to describe dietary therapeutic practices and growth outcomes in the management of subjects with PROP through a natural history.

Recent dietary guidelines for PROP were released in 2019 and the recommendation is to use medical foods as supplementation only in subjects who tolerate less than 100% RDA from intact protein (Jurecki et al., 2019). However, most subjects with PROP are at risk for malnutrition and depend on medical foods as an easily tolerable source of energy and protein (Daly et al., 2017). Therefore, there is a need to determine the optimal ratio of BCAA in medical foods to optimize protein synthesis and growth, and to prevent the accumulation of toxic metabolites. The objective of the second study in this thesis was to determine a BCAA ratio at which total body protein synthesis is optimized using a stable isotope-based method: the indicator amino acids oxidation method (IAAO).

Chapter 2: Background

2.1 Inborn Errors of Metabolism

Inborn errors of metabolism (IEMs) are a large, diverse group of disorders caused by a gene mutation that can result in total or partial impairment of a certain enzyme or cofactor. A greater number of IEMs are inherited in an autosomal recessive manner, due to a defect in single or multiple enzymes (Baker, 2015). As a result of this defect, a block in the metabolic pathway of some compounds may cause an accumulation of toxic metabolites. This accumulation can cause a wide range of symptoms in one or multiple organs, including neurological problems, disabilities, and even death in some cases. Most IEMs symptoms often start at or soon after birth, but also may appear at any time during adulthood (Nasser et al., 2012). The basic principle of management is to reduce the plasma and tissue accumulations of these toxic metabolites, either by limiting the intake of nutrients that produces them, or by increasing their excretion from the body (Boyer et al., 2015).

Considering that IEMs are extremely diverse, it is challenging to classify them. A single universal classification system for IEMs does not exist. However, there are approaches used to classify IEMs according to different criteria (Lanpher et al., 2006). Pathophysiologically, IEMs are divided into three groups. The first group includes IEMs that occur due to defects in the intermediary metabolic pathways, which result in accumulation of toxic compounds; these are mostly referred to as disorders of intoxication (**Figure 2-1**). The second group includes IEMs that result in an energy deficiency (disorders of energy metabolism). The third group comprises IEMs caused by defects in the synthesis or catabolism of complex molecules in certain cellular organelles (El-Hattab, 2015; Jameson and Morris, 2011). IEMs can also be classified as treatable

verses untreatable inborn metabolic diseases. Whereas most of the intoxication disorders are treatable, they require immediate removal of the accumulated toxins by extracorporeal procedures, cleansing drugs or vitamins (Saudubray et al., 2006), and lifelong medical and dietary management.

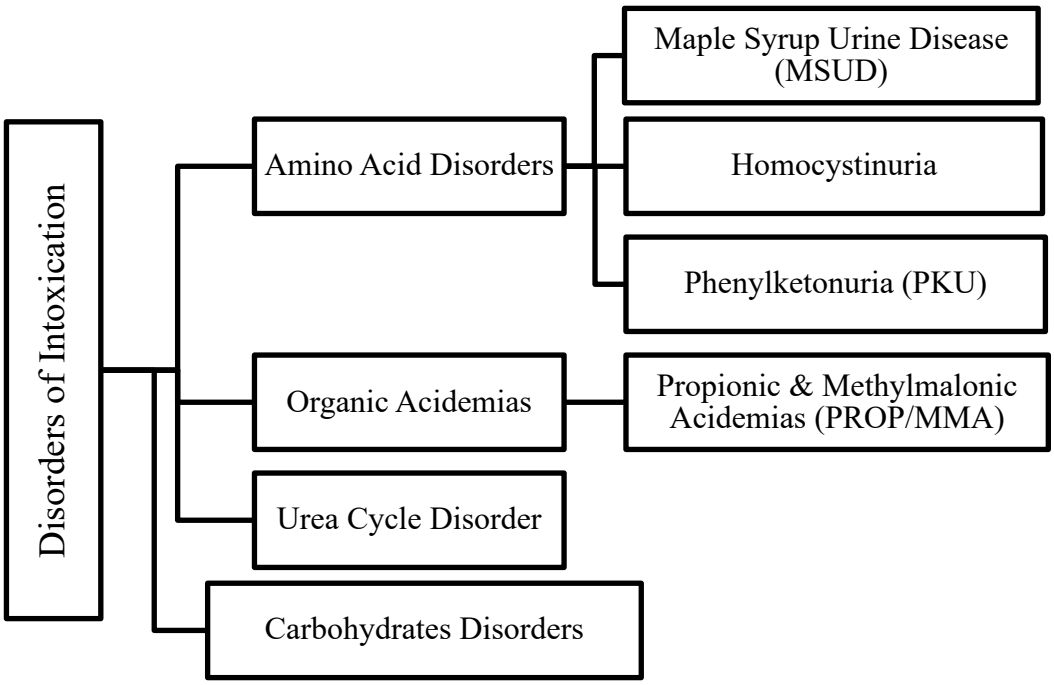


Figure 2-1 Disorders of Intoxication
Adapted from (Jameson and Morris, 2011)

2.2 Propionic Acidemia

Propionic Acidemia (PROP) (Online Mendelian Inheritance in Man (OMIM) number #606054) is an autosomal recessive, inherited metabolic disorder that is serious and life-threatening. It is one of the most common forms of organic acidemias. PROP is caused by a defect in the mitochondrial enzyme propionyl-coenzyme A (CoA) carboxylase (PCC) (EC 6.4.1.3), which results in the accumulation of toxic metabolites such as propionic acid and methylcitrate. PCC catalyzes the reversible biotin-dependent conversion of propionyl-CoA to D-methylmalonyl-CoA. Propionyl CoA is an intermediate in the catabolism of isoleucine (ILE) and valine (VAL), two of the three branched-chain amino acids (BCAA include leucine, isoleucine and valine), as well as threonine (THR), methionine (MET), and odd-chain fatty acids. When the catabolic pathway of the propiogenic amino acids is blocked by the loss of the catalytic activity of PCC, the excess propionic acids will accumulate and disrupt the tricarboxylic acid cycle (TCA), as well as the urea cycle, causing hyperammonemia **(Figure 2-2)**. The defect in the PCC enzyme can occur due to a deficiency in the enzyme, or its cofactor biotin, as a result of mutations in either the PCCA or PCCB gene (Almási et al., 2019; Baumgartner et al., 2014; Jurecki et al., 2019; Shchelochkov et al., 1993).

The onset of the disease varies depending on the disorder phenotypes. Early neonatal onset occurs frequently and can be severe with high mortality rates. The first days of life are characterized by hypotonia, lethargy, vomiting and poor feeding. If left untreated, it can progress to encephalopathy and death. While late onset PROP is usually milder, it can present with a variety of symptoms including developmental delay, intellectual disability, failure to thrive, movement disorders and protein intolerance (Shchelochkov et al., 1993). Prognosis of PROP is

poor, and severe cases may result in death in the newborn period or later, due to metabolic decompensations. Newborn screening (NBS) is used for early diagnosis, and can be an effective approach in reducing overall mortality rate. NBS can identify cases of PROP before it shows any symptoms, which allows for treatment to prevent metabolic decompensation (Heringer et al., 2016). In addition to NBS, another means of diagnosing PROP is to use acylcarnitine analysis by tandem mass spectrometry (MS/MS) on dried blood spots, which will report high levels of propionylcarnitine (C3) (Almási et al., 2019).

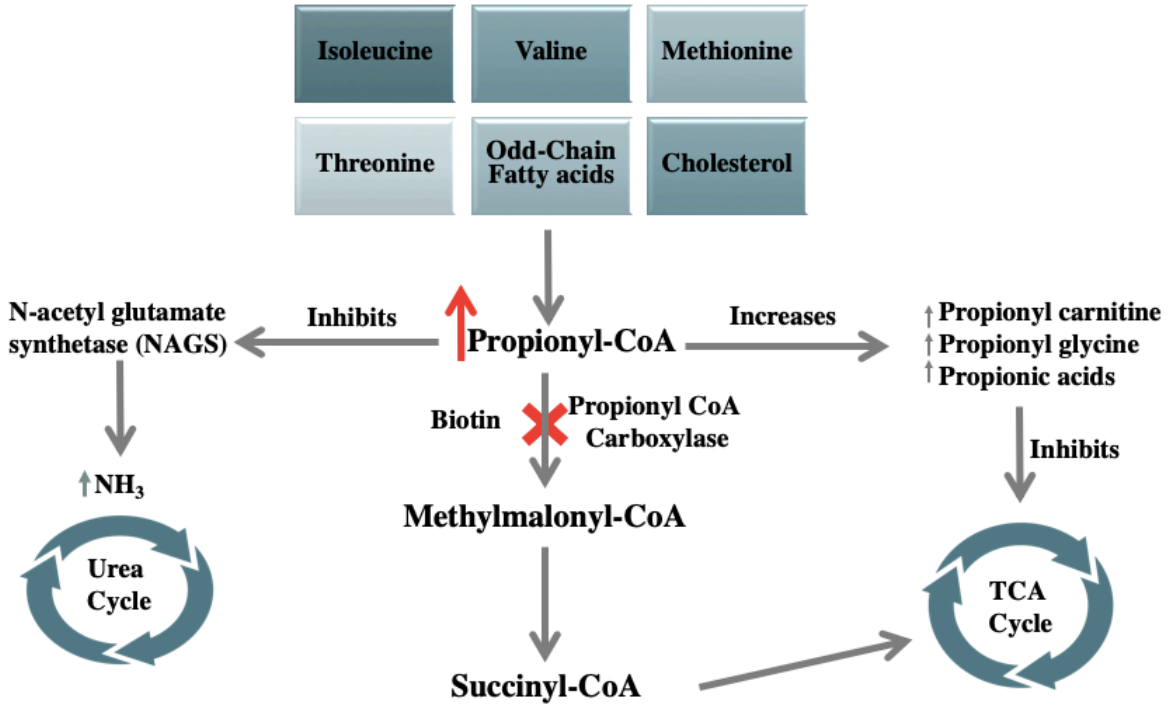


Figure 2-2 Metabolic Pathway of Propionic Acidemia
Adapted from (Jurecki et al., 2019)
TCA- Tricarboxylic Acid Cycle

2.3 Prevalence of PROP

Although individual IEM disorders are rare, collectively they represent a large and diverse group of genetic diseases, with new disorders and disease mechanisms being described regularly (Vernon, 2015). Their cumulative incidence is believed to be high, with very high mortality rates if not diagnosed early. The global birth prevalence is 50.9 per 100,1000 live births (Waters et al., 2018). However, in some populations, where consanguinity is very common, the incidence rate can be much higher. For instance, in Saudi Arabia the overall incidence is 1:1043 births (Alfadhel et al., 2016). Moreover, in a 13-year retrospective cohort study in Saudi Arabia 2001-2014, researchers reported an IEM incidence rate of (1:591). Among the cases, 64.7% were small molecule IEMs, where organic acidemia was the second most common category, with PROP as the most common in that group. This rate is the highest reported so far (Mak et al., 2013). Combining these results with another epidemiological study done in Saudi Arabia by Moammar et al., an IEM incidence rate of (1:667) was reported (Moammar et al., 2010). Another study from the Middle East done in the Emirates between January 1995 and May 2013, concluded a birth prevalence of IEM among the Emiratis to be 1 per 1329 live births (Al-Shamsi et al., 2014).

Due to the rarity of PROP, prevalence-based studies are not available. However, reports from newborn screening programs across different regions provided valuable data on the incidence rate of the disorder (**Table 2-1**). According to the most recent systematic literature review and meta-analysis, PROP is considered an ultra-rare disorder. PROP had similar rates across all regions, estimated to be 1 in 100,000, except for regions in the Middle East and North

Africa, where most inherited metabolic disorders are more frequent (Almási et al., 2019; Chapman and Summar, 2012).

Table 2-1 Incidence Rate of PROP by Population

Population	Incidence Rate	Reference
US	1:105,000 - 1:130,000	(Couce et al., 2011)
Italy	1:166,000	(Dionisi-Vici et al., 2006)
Germany	1:250,000	(Schulze et al., 2003)
United Arab Emirates	~1:20,000 -1:45,000	(Al-Shamsi et al., 2014)
Saudi Arabia	1: 12,500 1:2000 - 1:5000	(Alfadhel et al., 2016; Zayed, 2015)
Japan	1:17,400	(Yorifuji et al., 2002)

2.4 Overall Management of PROP

The goal of managing PROP is to prevent/minimize the accumulation and production of propionyl-CoA and its metabolites. Propionyl-CoA is derived from three main sources: 1- the catabolism of the amino acids ILE, VAL, MET, and THR, 2- catabolism of odd-chain fatty acids and cholesterol side chains, 3- bacterial anaerobic fermentation of carbohydrates in the gut. Precisely, using stable isotope techniques to measure propionate production in individuals with PROP showed that the catabolism of the offending amino acids represents approximately 50% of propionate, while 25% represents propionyl-CoA derived from catabolism of odd-chain fatty acids, and the last 25% derives from bacterial production of propionate. Therefore, to improve

overall metabolic control in individuals with PROP, it is necessary to reduce and control propionate production from all sources (Daly et al., 2017; Jurecki et al., 2019; Leonard, 1997)

Dietary therapy (which will be discussed in detail below) along with carnitine supplementation, antibiotics and ammonia scavengers, represent the main components of management. Carnitine plays a crucial role in the body. Not only does it transport long-chain fatty acids across the inner mitochondrial membrane to be oxidized via β-oxidation to produce energy, but it is also essential in the detoxification and removal of the accumulated acylcarnitine esters, wherein PROP will be found in the form of propionylcarnitine. Therefore, carnitine supplementation is used in PROP to enhance removal of accumulated propionic acid by converting it to propionylcarnitine, which is then excreted in urine (Sugiyama et al., 1990). This will increase carnitine requirements in the body, which, if not compensated for, can lead to secondary carnitine deficiency, which is not uncommon in PROP (Mardach et al., 2005). Carnitine supplementation is generally safe and the recommended dose ranges from 50-100 mg/kg/d. However, in critically ill individuals the dose can range from 100-300 mg/kg/d divided into 2-4 doses. The amount of carnitine provided should always be adjusted according to plasma total and free carnitine levels (Baumgartner et al., 2014; Jurecki et al., 2019).

Metronidazole is commonly used to prevent propionate production from the bacterial fermentation in the gut. Although no data are available on clinical outcomes compared between patients on metronidazole and those not managed with antibiotics, the recommended dose of 20 mg/kg/d in 2-3 doses can be used for 1-2 weeks alternating with few weeks off (De Baulny et al., 2005; Jurecki et al., 2019). To treat and/or prevent hyperammonemia associated with acute decompensation in PROP, ammonia scavengers such as sodium benzoate are cautiously used

(Baumgartner et al., 2014; Shchelochkov et al., 1993). Moreover, since the PCC enzyme is biotin-dependent, biotin supplementation is recommended to be used until definitive diagnosis is made that rules out biotin-responsive PROP (Jurecki et al., 2019). Finally, for severely affected individuals with PROP, liver transplantation may be a potential treatment option to provide increased enzyme capacity (Baumgartner et al., 2014; Shchelochkov et al., 1993).

2.4.1 Nutritional Management of PROP

Nutritional intervention is the cornerstone of management for most IEM disorders. The goal of nutritional management of PROP is to reduce the accumulation of toxic metabolites by restricting dietary protein sources of the propiogenic amino acids (ILE, VAL, MET, and THR), while maintaining their normal plasma concentrations (Jurecki et al., 2019). Moreover, it is essentially important to prevent endogenous catabolism, particularly protein catabolism, by providing sufficient energy to meet metabolic demands (Feillet et al., 2000). Sometimes, individuals with PROP need tube feeding to supplement their oral intake of nutrients and fluids, and to reduce fasting (Jurecki et al., 2019). Nutrient and energy intakes should be adjusted to ensure optimal growth and development in early years of infancy and childhood, and to maintain positive clinical outcomes during adulthood.

Given the lack of suitable nutrition-based studies on PROP, the dietary recommendations are provided based on an individualized clinical and laboratory assessment (Sutton et al., 2012). As with most IEMs, nutrient and energy requirements for individuals with PROP have not been studied fully, which is partly due to the variability of individuals' tolerance and clinical status (Evans et al., 2017). The absence of such recommendations therefore leads to the application of

the current Dietary Reference Intakes (DRI) for energy and protein in healthy populations, adapted to address the needs of individuals with PROP (**Table 2-2,Table 2-3,Table 2-4**).

Table 2-2 Dietary Reference Intakes for Energy (DRI 0-36 months)
(Institute of Medicine, 2005)

Age	**EER = TEE + Energy Deposition[1]**
0-3 months	(89 x weight [kg] – 100) + 175 kcal
4-6 months	(89 x weight [kg] – 100) + 56 kcal
7-12 months	(89 x weight [kg] – 100) + 22 kcal
13-36 months	(89 x weight [kg] – 100) + 20 kcal

[1] Estimated Energy Requirement EER (kcal/day) = Total Energy Expenditure TEE + Energy Deposition

Table 2-3 Dietary Reference Intakes for Energy (DRI 3-18 years)
(Institute of Medicine, 2005)

Estimated Energy Requirement (EER)	**Physical Activity Coefficient (PA)**			
Boys **3-8 years** EER = 88.5 - (61.9 x age [y]) + PA x {(26.7 x weight [kg]) + (903 x height [m])} + 20	Sedentary 1.00	Low Active 1.13	Active 1.26	Very Active 1.42
Boys **9-18 years** EER = 88.5 - (61.9 x age [y]) + PA x {(26.7 x weight [kg]) + (903 x height [m])} + 25	1.00	1.11	1.25	1.56
Girls **3-8 years** EER = 135.3 - (30.8 x age [y]) + PA x {(10.0 x weight [kg]) + (934 x height [m])} + 20	1.00	1.11	1.25	1.48
Girls **9-18 years** EER = 135.3 - (30.8 x age [y]) + PA x {(10.0 x weight [kg]) + (934 x height [m])} + 25	1.00	1.12	1.27	1.45

Table 2-4 Dietary Reference Intakes for Protein (DRI)
(Institute of Medicine, 2005)

Age	Protein Requirements g/kg/day EAR	Protein Requirements g/kg/day AI/RDA
0-6 months	N/A	1.52
7-12 months	1	1.2
1-3 y	0.87	1.05
4-8y	0.76	0.95
9-13 14-18 19-30	0.76 M: 0.73/ F: 0.71 M: 0.66/ F: 0.66	0.95 0.85 0.80

EAR- Estimated Average Requirement
AI- Adequate Intake
RDA- Recommended Dietary Allowance

2.4.1.1 Energy Requirements in PROP

Energy intake should always be provided in an adequate amount to prevent catabolism, which can lead to the release of propiogenic amino acids from endogenous protein, as well as the release of odd-chain fatty acids from lipid stores. However, it is equally important to avoid overfeeding, especially with inactive individuals, to prevent overweight and obesity (Phyllis B.Acosta and Steven Yannicelli, 2001). According to Feillet et al., using open circuit indirect calorimetry showed a 20% reduction in resting energy expenditure (REE) when compared to the energy requirement calculated using the Schofield equation among well PROP children. The Schofield equation has been used previously to determine the energy requirements for healthy

and diseased children (Kaplan et al., 1995). One reason for this reduction is that PROP children tend to have low muscle mass because they are consuming a restricted protein intake compared to healthy children, where up to 30% of REE is related to muscle mass. Another reason is that since most individuals with PROP have a neurological deficit, their neurological status may have an impact on their REE. On the other hand, energy requirements during illness can rise up to 30% of REE, especially during acute decompensation (Feillet et al., 2000). In 2004, van Hagen and colleagues refuted the findings of reduced REE in children with PROP, as they measured REE using indirect calorimetry in 6 PROP children and compared it to the predicted REE using the Schofield equation. The researchers did not find any major differences in the percentage of predicted REE versus measured REE. In fact, there was slight elevation in measured REE. Van Hagen et al. linked the REE increase with the high consumptions of synthetic amino acids mixtures, which could have been associated with higher muscle mass, leading to a rise in REE (van Hagen et al., 2004).

In a multicenter study on 13 children with PROP, an increase in length centile was reported in children who received 98% of the EER for age, while children who received about 87% of the recommended energy needs either remained the same or had a decrease in length centile. All children had received ≥ 100% RDA of total protein, which suggests that adequate energy intake is needed to spare protein in order to support overall growth and development (Yannicelli et al., 2003). Using the protein to energy ratio (P:E ratio) -the proportion of energy derived from protein- can provide clinicians with additional guidance when making dietary prescriptions. However, this can be challenging in PROP, since protein intake may be low. Furthermore, the value of medical foods as a protein alternative should also be considered, given

the differences in their absorption and bioavailability compared with natural sources. Failure to consider this can lead to height reduction and higher incidence of overweight. Evans et al concluded that using a protein: energy ratio ranging between >1.5 - <2.9 g protein/100 kcal/day correlated with optimal growth among most patients with inborn errors of intermediate protein metabolism including PROP (Evans et al., 2017)

The evidence for energy needs in PROP is based on limited research. However, the recent 2019 PROP guidelines state that energy requirement should meet 80-120% of the DRI age (**Table 2-5**) in order to spare protein catabolism. The guidelines take into consideration each individual's energy goals for physical activity and medical condition, as well as for normal growth and weight management (Jurecki et al., 2019).

Table 2-5 Recommended Energy Intakes for Well Individuals with PROP[1]
(Jurecki et al., 2019)

Age	**Energy (Kcal/kg/day)[2]**
0-6 months	M: 72-109 / F: 72-108
7-12 months	M: 65-97 / F: 64-96
1-3 years	M: 66-99 / F: 66-99
4-8 years	M: 59-88 / F: 56-84
9-13 years	M: 43-65 / F:39-58
14-18 years	M: 36-53 / F:30-45
≥ 19 years	80-100% of EER

1 Adapted from the Institute of Medicine
2 Represents 80-100% of estimated energy requirements (EER)for energy

2.4.1.2 Protein and Amino Acid Requirements in PROP

Although a protein-restricted diet is the primary foundation for nutritional management in PROP, there is little evidence for how much protein to prescribe, and what percentage of total protein should be provided through natural sources or medical foods. By restricting the consumption of intact protein intake, the propiogenic amino acid intake will be limited. However, these propiogenic amino acids are essential; they cannot be synthesized in the body, but must be obtained from food, in sufficient amounts to maintain growth and anabolism. The dietary prescription should be adjusted based on clinical and laboratory parameters. Severely affected individuals may require greater restriction of intact protein; therefore, they will depend more on medical foods to provide all other essential amino acids and nutrients. On the other hand, individuals with milder mutations may tolerate more intact protein, and such cases will not need to consume medical foods (Sutton et al., 2012). In a report of 49 PROP individuals from 18 metabolic centers across Europe, patients with mild phenotypes were treated with only natural protein, while severely affected patients were given only one third of their requirements from intact protein and the rest was supplemented using medical foods. The authors did not report any differences in outcomes between the two groups. In fact, they concluded that higher protein intakes, whether from natural sources or medical foods, may have been beneficial (Sass et al., 2004). Moreover, Sass et al. provided protein intake recommendations based on their experience during long-term management of PROP patients rather than on confirmation from a large study population (**Table 2-6**). In 2014, a cross-sectional survey was used to collect dietary data from 47 centers across Europe, where 186 individuals with PROP were followed. Most centers provided below the RDA for natural protein, especially those treating adult patients

supplemented with medical foods. Medical foods provided almost half of the requirement for age, and with this supplementation, about 83% of centers provided more than 120% of the RDA for total protein. No clinical or biochemical outcomes were reported (Daly et al., 2017).

Total protein intake for disorders of amino acid metabolism should be higher than the DRI for a number of reasons. DRI was not intended for children with lifelong illnesses including infections and stress that can impact both energy and protein requirements. Protein sources used by PROP individuals are often of low biological value, which negatively affect protein status (Yannicelli, 2006). Moreover, it's been suggested that the current Dietary Reference Intakes (DRI) recommendation for protein requirements in both adults and children is underestimated. Compared to the DRI (2005), and WHO (2007), protein requirements for school-aged children (6-10 y) has been determined by Elango et al. to be 1.3g/kg/day (EAR), and 1.55g/kg/day (RDA) using the indicator amino acid oxidation technique, which is significantly higher than DRI previously determined using nitrogen balance technique (Elango et al., 2011; Humayun et al., 2007). According to the most recent 2019 PROP guidelines, protein and amino acid requirements should be met by providing 60-100% of the age appropriate DRI from sources of intact protein. For individuals who only tolerate < 100% of the DRI from intact protein, there should be supplementation with medical foods to meet 100-120% of the total protein requirements (Jurecki et al., 2019) (**Table 2-6)**. Requirements may be greater than AI/RDA when L-amino acids supply the majority of protein, because there will be a rapid amino acid absorption that can result in an early and high peak of plasma amino acid concentrations, leading to rapid catabolism of amino acids (**Table 2-6)** (Phyllis B.Acosta and Steven Yannicelli, 2001).

Table 2-6 Recommended Protein Intakes for Subjects with PROP

GMDI Guidelines (Jurecki et al., 2019) [1-3]			
Age	**Total Protein g/kg/d**	**Intact Protein g/kg/d**	**Protein from Medical Food g/kg/d**
0-6 months	1.52-1.82	0.91-1.52	N/A
7-12 months	1.20-1.44	0.72-1.2	
1-3 y	1.05-1.26	0.63-1.05	
4-8y	0.95-1.14	0.57-0.95	
9-13 y	0.95-1.14	0.57-0.95	
14-18 y	0.85-1.02	0.51-0.85	
≥ 19 years	0.80-0.96	0.66-1.10	
Sass Recommendation (Sass et al., 2004)			
Age	**Total Protein g/kg/d**	**Intact Protein g/kg/d**	**Protein from Medical Food g/kg/d**
0-12 months	1.8-2.2	0.7-1.5	0.7-1.5
1-4 years	1.5-2.0	1-1.5	0.5-1.0
4-7 years	1.2-1.5	1-1.5	0.2-0.5
> 7 years	1.2-1.5	0.8-1.2	0.0-0.4
The Ross Nutrition Support Protocol Recommendation (Phyllis B.Acosta and Steven Yannicelli, 2001)			
Age	**Total Protein g/kg/d**	**Intact Protein g/kg/d**	**Protein from Medical Food g/kg/d**
0 - < 3 months	2.5-3.5	N/A	N/A
3 - < 6 months	2.5-3.5		
6 - <9 months	2.5-3.0		
9 - <12 months	2.5-3.0		
1 - <4 years	≥ 30.0 *		
4 - <7 years	≥ 35.0 *		
7 - <11 years	≥ 40.0 *		
11- <15 years	M: ≥ 50/ F: ≥ 55 *		
15- <19 years	M: ≥ 65/ F:≥ 55 *		
≥ 19 years	M: ≥ 65/ F:≥ 50 *		

[1] Adapted from the Institute of Medicine

[2] Represents 60-100% of the adequate intake/ recommended dietary allowance AI/RDA

[3] if < 100% AI/DRI from intact protein, supplement with medical food to provide 100-120% of AI/RDA for total protein.

*g/d

2.4.1.3 Medical Foods for PROP

The term medical food is defined by the Food and Drug Administration in section 5 (b) [3] of the Orphan Drug Act (21 U.S.C 360ee (b) [3]) as "food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation" (Office of Regulatory Affairs) (Office of Regulatory Affairs). Amino acid supplementation/mixtures, medical formula/food, and precursor-free amino acids supplements, all refer to the same type of supplementation, that is intended to be used as safe alternative to a regular diet in individuals with inborn errors of metabolism. These supplementations are formulated to contain no offending amino acids, in order to prevent the accumulation of toxic precursors metabolized through a blocked disease pathway. Medical foods specially formulated for individuals with PROP are produced to contain no propiogenic precursors, including valine, isoleucine, methionine, threonine, and to include normal amounts of the other amino acids to ensure sufficient protein intake for optimal growth and protein synthesis by meeting overall protein needs (Manoli et al., 2016). The most commonly used medical foods in PROP are: Propimex1/2 (Abbott), Maximaid/Maxamum (Nutricia) and OA1/2 (Mead Johnson (**Table 2-7**)

Although there are limited efficacy studies that support the use of medical foods in PROP, reports from the European survey stated that about 81% of 47 centers across Europe are prescribing medical foods regularly, with more than half of centers prescribing medical foods to provide more than 50% of total protein (Daly et al., 2017; Touati et al., 2006). The role of medical food use in PROP is still questionable, due to their formulation with imbalanced BCAA

content: that is, with minimal or no valine and isoleucine, and with high amounts of leucine (Myles et al., 2018). An average of 161, 164, and 184 mg of leucine per g of protein is found in three of the most commonly used medical foods [OA2 (Mead Johnson), Propimex 2 (Abbott), and Maxamum (Nutricia), respectively], compared to only 85 mg of leucine in 1 g of egg protein. Leucine appears to be the critical amino acid in most supplements, and until recently there have been no largely reported side effects to leucine-enriched supplements, other than a short-term rise in blood ammonia levels, a complication commonly reported in PROP. However, due to the lack of long-term side effect studies, especially studies related to the safe upper-intake levels of leucine in different health conditions, high leucine intake is still controversial (Elango et al., 2016; Komar et al., 2015). Moreover, high leucine intakes can negatively impact the other two BCAA (isoleucine and valine), by suppressing their plasma concentrations below normal ranges. This is referred to as BCAA antagonism, a well-known phenomenon that was first observed in animals when the addition of L-leucine to a low protein diet was found to reduce plasma concentration of isoleucine and valine, and cause growth restriction. These side effects were partially controlled with isoleucine and valine supplementation. These observations indicated that the consumption of high leucine in a low protein diet can increase the requirements for the other two BCAA (Harper et al., 1984). The practice of supplementing ILE and VAL is questionable, as these amino acids are the main contributors to the toxic metabolite pool and is likely not ideal for PROP patients (Myles et al., 2018).

Therefore, the most recent guidelines for the management of PROP are to discourage the use of medical food as a sole source of energy, and to only recommend their use as an additional source of energy and protein in patients with feeding difficulties, when protein

tolerance is less than 100% of DRI from intact protein (Jurecki et al., 2019). In a study using Propimex1(Abbot) as an additional source of protein for sixteen infants and toddlers diagnosed with PROP, they found that using medical foods for 6 months improved growth, which was measured using anthropometric measurements (weight, height centile), and improved nutritional status by measuring mean plasma indices for protein status (Yannicelli et al., 2003). However, others have reported that adding medical foods to a patient's diet has not shown any additional effect or did not play an important role in nutritional or developmental status (Nyhan et al., 1973; Touati et al., 2006). Clearly, further research is needed to determine what the optimal intake of medical food is, relative to intact protein.

Table 2-7 BCAA Content in Regular milk, Eggs and Common Medical Foods for PROP

Product Name	Leucine g/100g	Isoleucine g/100g	Valine g/100g	LEU: ILE: VAL
Milk[1]	0.333	0.173	0.207	1: 0.5: 0.6
Eggs[2]	1.086	0.671	0.858	1: 0.6: 0.7
XMTVI Maxamum[3]	6.4	0.14	nil	1: 0.002: 0
Propimex2[4]	2.76	0.24	0	1: 0.08: 0
OA2[5]	3.4	0	0	1: 0: 0

[1]USDA (01106) milk, whole (3.25 milkfat), with added vitamin D
[2] USDA (01123) Egg, whole, raw, fresh
[3] Nutricia, an unflavored powder free from methionine, threonine and valine, low in isoleucine but containing a balanced mixture of the other essential and non-essential amino acids, carbohydrate, vitamins, minerals and trace elements, for MMA/PROP children over 8 years and adults.
[4] Abbott, Nutrition support for children and adults with propionic or methylmalonic acidemia. Methionine- and valine-free; low in isoleucine and threonine. Use under medical supervision.
5 Mead Johnson, Medical food powder for children and adults with propionic or methylmalonic academia.

2.5 Growth Outcomes in PROP

Poor growth outcomes in children with inborn errors of protein metabolism is well described (Evans et al., 2017). Severely restricted dietary protein consumption is probably the main contributor to growth failure in children with PROP. While the body needs adequate protein intake for optimal protein synthesis and growth, optimal growth can also improve protein tolerance and therefore reduces metabolic decompensation (Molema et al., 2019a). A review of evidence regarding long-term dietary and growth outcomes in subjects with PROP is found in (**Table 2-8).** Data from subjects with methylmalonic acidemia (MMA) -another form of organic acidemia- is also used in the review, because of similarities to disease pathway and dietary treatments with PROP.

Table 2-8: Review of Evidence for Long-term Dietary Management and Growth Outcomes in PROP

Subjects	Intact Protein	Medical Foods	Growth outcomes	Reference
12 subjects with PROP (early onset)	• Severely restricted • Reported in g/d • Mean ranges between (5-10.7 g/d) for 0-6 years	• Reported in g/d • Mean ranges between (4.2-11.9 g/d) for 0-6 years	• Almost all subjects showed delay in at least one growth parameter (weight, height) • No difference seen in growth outcomes between time of onset • Mean height -2SD • Mean weight -1SD	(van der Meer et al., 1996)
5 subjects with PROP (Late onset)	• Reported in g/d • Mean ranges between (7.3-27.6 g/d) for 0-6 years	• Reported in g/d • Mean ranges between 2-1.3 g/d) for 0-4 years • Supplemented with medical foods only until age 4 years		
41 subjects with MMA 29 subjects with PROP	• Severely restricted • Reported in g/d • Mean ranges between (5-11 g/d) for 0-6 years	• Not reported	• Mean height below -2SD in girls by age 10 years • Results in boys are not clear	(De Baulny et al., 2005)
3 subjects with MMA 13 subjects with PROP	• Reported in g/d • Mean ranges between (15.3-25.1 g/d) for 0-4 years	• Medical foods supplemented almost 50% of protein intake	• Mean weight centile increased from 26 to 49% • Mean length centile increased from 25 to 33%	(Yannicelli et al., 2003)

7 subjects with MMA 9 subjects with PROP	• Reported in g/kg/d • Mean ranges between (0.92-0.77 g/kg/d) for 3-11 years	• No medical foods	• Most subjects had close to normal growth outcomes, with the exception of a few. • At 3 years: height: -0.47SD/ weight: 0.14SD • At 6 years: height: 0.45SD /weight:0.13SD • At 11 years: height: -0.75SD /weight: -0.04SD	(Touati et al., 2006)
15 subjects with MMA 8 subjects with PROP	• Reported in g/kg/d • Mean ranges between (0.75-0.54 g/kg/d) for 3-11 years	• Reported in g/kg/d • Mean ranges between (0.58-0.34 g/kg/d) for 3-11 years		
29 subjects with MMA	• Reported in g/kg/d • Subjects had highly variable intakes • Mean ranges between (0.29-2.12 g/kg/d) for 2-35 years	• Reported in g/kg/d • Subjects had highly variable intakes • Mean ranges between: (0.09-0.82 g/kg/d) for 2-35 years	• Mean height: -1.04SD • Mean weight: 0.01SD • Mean BMI: 0.77SD	(Hauser et al., 2011)
55 subjects with PROP	• Reported in g/kg/d • Median ranges between (0.8 to1.1 g/kg/d) for 0-5 years	• Reported in g/kg/d • Median ranges between (0.7 to 0.9g/kg/d) for 0-5 years	• 91% of subjects had median height ranges: -2SD to 2 SD • 6% had median height below -2SD • Median weight ranges: -3.8SD	(Grünert et al., 2013)

			to 3.7SD • Median BMI ranges:1SD to 1.5SD	
61 subjects with MMA	• Reported in g/kg/d • Mean ranges between (0.99g/kg/d) for 2-18 years	• Reported in g/kg/d • Mean ranges between (0.78 g/kg/d) for 2-18 years	• Mean height: -2SD • Mean weight: -0.81SD • Mean BMI: 0.72SD	(Manoli et al., 2016)
14 subjects with MMA/PROP	• Reported in g/kg/d • Mean ranges between (1.5-0.95 g/kg/d) for 3-11 years	• No medical foods	• Mean height: -1SD	(Evans et al., 2017)
263 subjects with MMA/PROP	• Reported as g protein/100kcal • Median: 1.23g/100kcal	• Reported as g protein/100kcal • Median: 0.6g/100kcal	• Mean height below -2SD in 33% of subjects	(Molema et al., 2019a)

SD- Standard Deviation
MMA- Methylmalonic Acidemia

2.6 Branched-Chain Amino Acids

Catabolism of the offending amino acids in PROP represents approximately 50% of propionate production, primarily from BCAA (Leonard, 1997). Leucine, isoleucine, and valine are collectively known as the branched-chain amino acids (BCAA) and are quantitatively by far the most important single group among the dietary indispensable amino acids, as they comprise about 35% of the indispensable amino acids in muscle proteins. These amino acids are "essential" amino acids, they cannot be synthesized in the body, necessitating their adequate intake from dietary sources like meat and dairy products. They make up almost 50% of the indispensable amino acids in the food supply, therefore deficiencies of them do not occur naturally (Cole, 2015). The principal roles of BCAA include tissue protein synthesis; carbon precursors for synthesis of tricarboxylic acid (TCA) cycle intermediates, ketone bodies and fat; carbon and nitrogen precursors for synthesis of alanine, glutamate and glutamine; and energy source via oxidation to CO_2. The catabolic pathways of the three BCAA have some features in common, where the initial step for each is transamination by the enzyme branched-chain amino transferase. This is a reversible step that converts each amino acid into its corresponding branched-chain keto acid (BCKA). Each BCKA then undergoes an irreversible oxidative decarboxylation, controlled by the enzyme branched-chain a-ketoacid dehydrogenase, which yields acyl-CoA metabolites. After that, the pathways will yield end products that can enter the TCA cycle. The end products of isoleucine catabolism are propionyl-CoA and acetyl-CoA; leucine catabolism yields acetoacetate and acetyl-CoA; and valine catabolism yields succinyl-CoA (**Figure 2-3**).

Although the three BCAA have similar metabolism, leucine is the key regulator among the BCAA (Harper et al., 1984). Excessive intakes of leucine, in a protein inadequate diet, causes not only a marked depression in growth, but also a drop in the plasma and tissue pools of isoleucine and valine, along with their corresponding ketoacids, keto-methylvalerate (KMV) and keto-isovalerate (KIV), respectively. The antagonistic effect of leucine is unique in that the effects are not observed with an adequate protein intake. Also, increased concentrations of isoleucine and valine do not decrease plasma leucine concentrations. Although the exact mechanism for this is yet to be elucidated, overall BCAA oxidation leading to depressed levels seems the most probable. Increased leucine supplies transaminase to form ketoisocaproic acid (KIC), and increased KIC levels stimulate transamination of valine and isoleucine. This increases the branched-chain keto dehydrogenase (BCKD) activity, thereby cell channeling of the keto acids of valine and isoleucine into their catabolic pathways takes place (Harper et al., 1984; Torres et al., 1998).

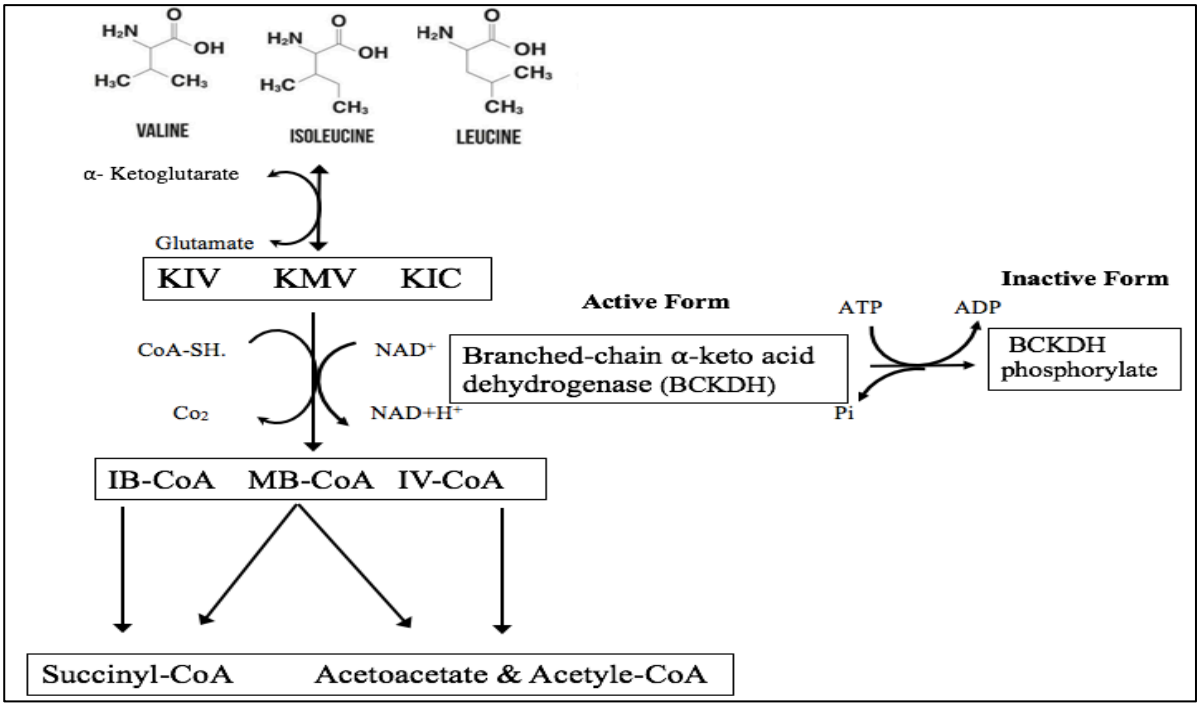


Figure 2-3 Catabolism of Branched-Chain Amino Acids
Adapted from (Shimomura et al., 2001)
KIV- keto- Isovalerate / **IB-CoA-** Isobutyryl-CoA
KMV- keto-Methylvalerate / **MB-CoA-** a- methylbutyryl-CoA
KIC- ketoisocaproic Acid **/ IV-CoA-** Isovaleryl-CoA

2.6.1 Branched-Chain Amino Acid Requirements in Humans

The FAO/WHO/UNU 1985 recommendations for amino acid intakes were based on the original technique using nitrogen balance studies in adults with total BCAA requirements of 34 mg/kg/d. The nitrogen balance technique involves the determination of the difference between the intake of amino acid nitrogen and the amount excreted in urine, faeces, and sweat, together with minor losses by other routes. This technique has many limitations, including overestimation of nitrogen intake and an underestimation of nitrogen excretion, leading to an overly positive balance, and therefore, underestimation of requirements, especially of the BCAA requirements. This led to the application of stable isotope-labeled amino acid studies, which have proven to be more rapid and sensitive to changes in amino acid intakes, and more importantly, have resulted

in higher requirements than those determined by the nitrogen balance studies (Elango et al., 2012a). The current Dietary Reference Intakes (DRI 2005) report and FAO report (2007) on protein and amino acid intake recommendations are based on requirement estimates for adults using stable isotope studies(Institute of Medicine, 2005, 2007) (**Table 2-9**). However, to reduce the possibility of interaction between the BCAA in the mixture used to determine the estimate of their requirements, Riazi et al. determined the total BCAA requirements in healthy adult men using indicator amino acid oxidation (IAAO), where the participants received a balanced mixture of the BCAA based on egg protein pattern (38.5, 32.5 and 29% for leucine, valine and isoleucine, respectively). They concluded that 144 mg/kg/d is the mean requirement of the total BCAA (55.4, 46.8 and 41.8 mg/kg/d for leucine, valine, and isoleucine, respectively), which is higher than both (FAO/WHO/UNU 1985) and (DRI 2005)(Riazi et al., 2003a)

Table 2-9 Dietary Reference Intakes for Branched-Chain Amino Acid Requirements (DRI) (Institute of Medicine, 2005)

Amino acids	**Adults 19 years and older (mg/kg/d)**	
	EAR	**RDA**
Leucine	34	42
Isoleucine	15	19
Valine	19	24

EAR- Estimated Average Requirement
RDA- Recommended Dietary Allowance

In children, the current WHO/FAO/UNU recommendation for BCAA requirements are also based on nitrogen balance studies with low estimates. On the other hand, the DRI recommendations for BCAA are determined using factorial approach based on the adult requirements plus the additional needs for growth in children with the assumption that maintenance rates are the same in adults and children. The current DRI recommendations for the mean intake of total BCAA for school-aged children is 81 mg/kg/d. The IAAO method was used to determine total BCAA requirements in healthy, school-aged children with the same model in adults, where the dietary BCAA were fed in the same proportion as those in egg protein, and concluded that mean requirements of total BCAA is 147 mg/kg/d (56.5, 42.6, and 47.7 mg/kg/d for leucine, isoleucine, and valine respectively) (Mager et al., 2003). Moreover, using the same IAAO method and BCAA dietary model, Mager et al. determined the total BCAA requirements in children with chronic liver disease to be 209 mg/kg/d, significantly higher when compared to healthy children, which suggests that children with liver disease may have an increased dietary needs for BCAA (Mager et al., 2006). Although, this model assumes that BCAA proportion in egg protein is optimal for total body protein synthesis in healthy children and adults, further research is needed to determine whether these proportions are truly optimizing protein synthesis (Mager et al., 2003).

2.6.2 Importance of Branched-Chain Amino Acid Ratio

Due to antagonism among BCAA, dietary supplementation with high levels of leucine might not only enhance metabolism of all BCAA and further increase the nutritional needs for isoleucine and valine, but may also aggravate consequences of their deficiencies. Therefore, an

appropriately balanced ratio of BCAA is very important to maintain overall health, especially when a low protein diet is used (Duan et al., 2018b). Wessels et al. examined the effect of excessive leucine intake in three different BCAA ratios on BCAA plasma and tissue concentrations in pigs. They fed the pigs either a control diet with BCAA ratio of (LEU: ILE: VAL = 1: 0.5: 0.7), or 2-fold and 4-fold higher in leucine associated with these ratios (1: 0.3: 0.4) and (1: 0.1: 0.2), respectively. They showed that pigs fed high leucine diets (both 2&4 fold) had lower food intake, which may be associated with their low growth rates. Moreover, leucine plasma concentrations in both groups fed high leucine were 2-4-fold higher compared to the control group. Conversably, plasma concentration of isoleucine and valine and their corresponding keto acids were low in response to high leucine diets, and they were deficient in almost all tissues (Wessels et al., 2016). On the other hand, Duan et al. showed that a BCAA ratio of (LEU: ILE: VAL= 1: 0.75: 0.75) in a protein reduced diet improved growth performance in growing piglets (Duan et al., 2016), and also improved their intestinal health and absorption area (Duan et al., 2018a), when compared to different ratios of (1:1:1) (1:0.51:0.63) and (1:0.25: 0.25). Another study using the IAAO technique to determine BCAA requirements in piglets fed enterally and parentally, where Elango et al. used a fixed BCAA ratio of (LEU: ILE: VAL= 1: 0.6: 0.7). The researchers showed different responses in plasma BCAA concentration during enteral and parental feedings. In parentally fed piglets, all BCAA plasma concentration were low until they reached the total BCAA requirement. At the requirement intake, all BCAA plasma concentrations increased and continued to increase with higher intakes. However, in enterally fed piglets, leucine concentration was low until requirements were met, then it started to increase, while isoleucine and valine were high even with low intakes. This might be explained by the

high demand for leucine in the gut, which made it unavailable for protein synthesis. In contrast, the other two BCAA were excreted into circulation, and were high in plasma (Elango et al., 2002). In another study, Elango et al. used IAAO to test the effect of providing deficient intake of all the BCAA in the fixed ratio of (LEU: ILE: VAL = 1: 0.6: 0.7), then supplementing each BCAA individually to test the effect of different BCAA ratios. They did not find any significant differences in the oxidation of the amino acid indicator before and after supplementing with all three BCAA, which suggests that during enteral feeding, the ratio of (LEU: ILE: VAL = 1: 0.6: 0.7) may be the most appropriate, compared to intravenous route (Elango et al., 2004).

Despite all the work described above on BCAA ratios in adult humans and in animals, there is no evidence for the optimal BCAA ratio in children's diets. This still needs to be experimentally determined.

2.7 Indicator Amino Acid Oxidation (IAAO) Technique to Measure Total Body Protein Synthesis

The Indicator amino acid oxidation (IAAO) technique has been used recently to determine protein and amino acid needs in healthy adults and children, as well as in disease, such as in PKU, and MSUD (Courtney-Martin et al., 2002; Elango et al., 2007, 2011; Humayun et al., 2007; Riazi et al., 2004). It can also be used with multiple test intakes to compare relative protein synthesis. The IAAO method is based on the concept that when one essential amino acid is limited in the body, all other amino acids, including the indicator amino acid, are oxidized. This is because amino acids cannot be stored in the body, and the amount of the limiting amino acid controls the partitioning of the other essential amino acids between protein synthesis and

oxidation. Therefore, when the limiting amino acid is provided, protein synthesis will increase, and the oxidation of the indicator amino acid will decrease (Elango et al., 2008).

The IAAO technique is minimally invasive, as it uses hourly oral doses of stable isotope (1-^{13}C-labeled essential amino acid) and breath sampling in order to measure isotopic enrichment. Therefore, it can be used to determine the effect of multiple test amino acid intakes on total body protein synthesis in vulnerable populations such as school-aged children and pregnant women (Elango et al., 2008; Stephens et al., 2015).

2.7.1 Choice of Indicator Amino Acid in PROP

The choice of the indicator amino acid is critical, and its selection relies on the following criteria:

1- The indicator amino acid must be an essential amino acid.

2- The indicator amino acid must have a carboxyl-labeled carbon unit that is irreversibly oxidized upon catabolism and is released to CO_2 that can be measured in breath.

3-The indicator amino acid should not have a large pool in the body, and is not involved in main pathways other than being used for protein synthesis or oxidized to CO_2 (Zello et al., 1995).

Lysine and leucine fulfill the first and second criteria, but lysine has a large pool in the body, and leucine has been shown to stimulate protein synthesis and insulin secretion (Casperson et al., 2012; Columbus et al., 2015). However, phenylalanine in the presence of excess tyrosine is the preferable indicator amino acid, as it fits the criteria required for choosing the indicator amino acid (Elango et al., 2012b; Zello et al., 1995).

Chapter 3: Rationale, Objective and Hypothesis

3.1 Rationale

Propionic Acidemia (PROP) is an inborn error of metabolism disorder, caused by a defect in the enzyme propionyl-CoA carboxylase (PCC), resulting in the accumulation of propionic acid metabolites. PCC catalyzes propionyl-CoA, oxidative product of two of the branched-chain amino acids (valine, isoleucine), as well as methionine, threonine, odd-chain fatty acids and cholesterol (Baumgartner et al., 2014). The management of PROP primarily depends on dietary protein restriction to prevent the accumulation of propiogenic amino acids. However, to ensure optimal protein synthesis and proper growth, special medical foods were developed for PROP that are formulated to contain all essential amino acids and nutrients, but no propiogenic compounds. Recently, concerns have been raised about the use of medical foods, due to imbalanced content of amino acids; in particular, medical foods containing high leucine contents, and minimal or no valine and isoleucine. It has been suggested that this imbalanced mixture of BCAA negatively impacts plasma concentrations of valine and isoleucine, and therefore restricts growth in children with PROP (Manoli et al., 2016; Myles et al., 2018). Although PROP is one of the most frequent forms of organic acidemias, studies on the long-term growth outcomes of affected individuals are limited. Thus, a comprehensive assessment of dietary intake (including intact protein vs medical foods) and impact on anthropometric data through a natural history is needed. A cohort of subjects with PROP at BC Children's Hospital treated from 1990-2018 will be included in this analysis.

Recently the PROP dietary guidelines (2019) were released and the recommendation is to reduce reliance on medical foods as a primary source of energy and protein. However most individuals with PROP are at risk of malnutrition and depend on medical foods as an easily tolerable source of energy and protein (Jurecki et al., 2019). Thus, there is a need to determine the optimal ratio of BCAA in medical foods to optimize protein synthesis and growth, and to prevent the accumulation of toxic metabolites. With the application of stable isotope-based minimally invasive methods in children, the effect of different BCAA ratios (LEU: ILE: VAL) on whole body protein synthesis can be tested. The indicator amino acid oxidation technique (IAAO), already established to determine amino acid requirements, uses oxidation of L-^{13}C-Phenylalanine as an indicator for protein synthesis, in response to different test amino acid intakes. Different BCAA ratios ranging from the current high leucine: no/minimal valine and isoleucine to a more balanced ratio found in intact protein foods will be tested as part of a proof-of-concept study using healthy children. This approach allows for a safe method to examine multiple test BCAA ratios, determine outcomes based on protein synthesis and help design future studies on subjects with PROP.

3.2 Objectives

The specific objectives of the thesis were:

Objective 1: To describe dietary therapeutic practices and their impact on anthropometric (growth) outcomes in the management of four subjects with PROP at BC Children's Hospital Biochemical Diseases Clinic from 1990 - 2018.

Objective 2: To examine the effect of different **LEU: ILE: VAL** ratios on whole body protein synthesis in healthy children using the IAAO method (oxidation of L-^{13}C-Phenylalanine to $^{13}CO_2$), by reducing leucine levels from the current high doses in medical foods, sequentially, while providing isoleucine and valine at PROP recommended intake levels.

Objective 3: To examine the effect of different **LEU: ILE: VAL** ratios on urinary concentrations of BCAA, by reducing leucine levels from the current high doses in medical foods, sequentially, while providing isoleucine and valine at PROP recommended intake levels.

3.3 Hypothesis

1- We hypothesize that subjects with PROP would have poor growth outcomes (low height for age and weight for age/height Z scores) associated with high intake of medical food compared to intact protein consumption, during childhood

2- We hypothesize that IAAO (oxidation of L-^{13}C-Phenylalanine to $^{13}CO_2$) will be high (suggesting low protein synthesis) when BCAA ratio is imbalanced and will be low (suggesting optimal protein synthesis) when leucine levels are reduced and at a better-balanced ratio

3- We hypothesize that participants will have low urinary concentrations of isoleucine and valine, with high urinary leucine when BCAA ratio is imbalanced and would normalize at a better-balanced ratio

Chapter 4: Natural History Study on Subjects with Propionic Acidemia

Subjects with PROP, treated with protein-restricted diets, are prone to growth failure (Grünert et al., 2013). Numerous factors, including physiological, genetic, and environmental aspects, can influence growth outcomes. However, consuming a diet that is severely restricted in protein is probably the main contributor to growth failure in children with PROP (Lui et al., 2015). Even though PROP is one of the most frequent forms of organic acidemias, information on the long-term growth outcomes of affected individuals is limited. Thus, a comprehensive assessment of dietary and anthropometric data of children with PROP at BC Children's Hospital through a natural history study was needed. In this chapter, I discuss the methods and results of conducting a retrospective chart review for the years 1999-2018, on four subjects with PROP.

4.1 Subjects and Methods

This study was approved by the University of British Columbia and BC Children's Hospital Research Ethics Board (H19-02912). A retrospective chart review was conducted on a sample of four pediatric patients with propionic acidemia followed in the Biochemical Diseases Clinic at BC Children's Hospital. These were two sibling pairs, with the older siblings diagnosed in the newborn period with an acute metabolic decompensation, and the younger siblings screened right after birth. We collected longitudinal data on dietary intake and growth outcomes for the years 1999-2018, following the cohort from age 0 to 18 years via a natural history study. Data were collected from medical and dietetic clinic records when subjects were metabolically stable.

4.1.1 Data Collection

4.1.1.1 Growth Data

Anthropometric data were collected from medical and dietetics records during clinic visits. Weight and length for children under 2 years of age were obtained by standard techniques using digital baby weighing scales and crown-heel length on a scale length board. Weight and height for children greater than 2 years of age were measured using digital scale and a stadiometer, respectively. Body mass index (BMI) was calculated using the equation kg/m^2. Measurements were performed by the dietitian or clinic nurse. Anthropometric measurements were expressed as age-and sex-specific Z-scores, using the WHO Anthro and Anthroplus software for 0-5 years of age and 5-19 years of age, respectively. Indicators used between 0-5 years of age includes: weight for age, weight for height, and height for age. Between 5 and 10 years of age, indicators used include: weight for age, height for age, and BMI for age (WHO AnthroPlus for personal computers Manual: Software for assessing growth of the world's children and adolescents. Geneva: WHO, 2009) (http://www.who.int/growthref/tools/en/)..

4.1.1.2 Dietary Data

Dietary data was collected on the basis of formula recipes delivered via tube feeding and food records for the oral intake, which was analyzed by the metabolic dietitian using the MetabolicPro software from Genetic Metabolic Dietitian International (GMDI). MetabolicPro is the only web-based nutrient analysis software program designed specifically for the metabolic nutritionist. Dietary data was only collected when subjects were metabolically stable. We excluded data during sick days as subjects were consuming a special sick day formula, which

was supplying 120% EER for energy and no or very low intact protein. Number of sick days for each subject was calculated and presented with subject characteristics (**Table 4-1**). Formula composition information was obtained from the respective manufacturers. The data represent reported, rather than prescribed intake. Dietary intake of protein was expressed in grams per kilogram body weight per day (g/kg/d) as total protein, which was calculated by adding the intake of intact protein, protein from medical food, as well as single amino acid supplements (L-isoleucine and L-valine). Protein intake was also reported as g/kg/d of intact protein vs. protein from medical food separately. Energy intake was collected as kcal/kg/d. The protein to energy (P: E) ratio was calculated on the basis of the amount of total protein in g per 100 kcal per day. The calculated P: E ratio values were compared with P: E values associated with optimal growth in subjects with inborn errors of protein metabolism described by Evans et al (> 1.5 - < 2.9g/100kcal/day) (Evans et al., 2017). Since subjects were nutritionally managed using different guidelines at different time periods, subjects' actual intakes were compared with the Ross recommendation 2001(Phyllis B.Acosta and Steven Yannicelli, 2001), the Sass recommendation 2004 (Sass et al., 2004) and with the most recent guidelines for PROP from GMDI 2019 (Jurecki et al., 2019). For comparison purposes, protein and energy intakes were grouped for all four subjects according to age (0-6 months, 7-12 months, 1-3 years, 4-8 years, 9-13 years, and 14-18 years).

4.1.2 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software Inc, CA). Descriptive statistics were used to compare actual energy and protein intakes with different

recommendation guidelines; all data were expressed as median and range (minimum - maximum). Different growth Z scores were reported according to age groups, for 0-5 years old (Weight for height Z scores, Weight for age Z scores, and Height for age Z scores). For 5 -10 years old (Height for age Z scores, Weight for age Z scores, and BMI Z scores). For 10-18 years old (Height for age Z scores, and BMI Z scores). Growth data were also expressed as median and range (minimum -maximum).

4.2 Results

4.2.1 Subject characteristics

Four subjects with PROP were followed in the Biochemical Disease Clinic at BC Children's Hospital. PROP-01 and PROP-02 are a girl and boy sibling, respectively, and PROP-03 and PROP-04 are two sisters. All subjects had gastrostomy tubes, receiving part or all of their daily nutritional needs though bolus or continuous feeds; one subject (PROP-03) had received growth hormone treatment at 9 years of age for documented growth hormone deficiency. Although all subjects were well controlled with no major metabolic crises, one subject (PROP-04) died of cardiomyopathy at 10 years of age. Characteristics of the four subjects including age, time of diagnosis and mutations are presented in (**Table 4-1)**

Table 4-1 Subjects with PROP Characteristics

Subjects no.	Age (Years)	Age at Diagnosis	Gender	Number of Sick Days [1]	Mutation
PROP-01	19	4 weeks	F	• Birth-1 year (45 days) • 1-3 years (22 days) • 4-8 years (165 days)	PCCA[2] Homozygous c.134_135delTA p. Leu45TyrX
PROP-02	18	2 weeks	M	• Birth-1 year (12 days) • 1-3 years (42 days) • 4-8 years (28 days)	PCCA Homozygous c.134_135delTA p. Leu45TyrX
PROP-03	19	6 months	F	• Birth-1 year (43 days) • 1-3 years (13 days) • 4-8 years (47 days)	PCCB[3] Homozygous c.337C>T p. Arg113X
PROP-04	18	prenatal	F	• Birth-1 year (39 days) • 1-3 years (63 days) • 4-7 years (26 days)	PCCB Homozygous c.337C>T p. Arg113X

[1] Sick day formula was used to supply 120%EER for age, no or low protein intake
[1] Calculated as number of days from data points collected
[2] Propionyl Co-A Carboxylase Alpha subunit
[3] Propionyl Co-A Carboxylase Beta subunit

4.2.2 Growth Data

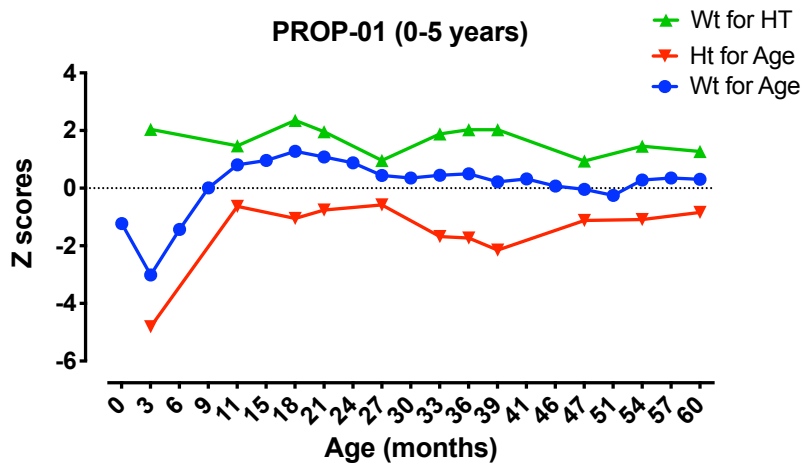
All four children had poor growth outcomes with persistently reduced height Z scores, and elevated weight and BMI Z scores. During the first 5 years of life, all subjects had a median Z score of 1.6, ranging from 1.04 to 2 for weight for height, and -0.717 (range: -1.36 to -0.2) for height. From 5 to 10 years of age, height Z scores declined to a median of -1.03 (range: -1.78 to -0.23) for all subjects. However, their median BMI Z scores were at 1.35 with a range of 1.01 to 2.03 that translates to a BMI percentile of (> 84 and < 95) and classify them as overweight. Between the ages of 10 and 18 years, height Z scores decreased even more to a median of -1.4 ranging from -1.84 to -4.6. All growth Z scores are presented in more detail for each subject individually in (**Table 4-2) and (Figure 4-1Figure 4-2Figure 4-3Figure 4-4).** After 10 year of age, the WHO recommends stopping using weight for age, as it does not distinguish between height and BMI during period of age where children are experiencing pubertal growth spurt. Although the Canadian Pediatric Endocrine Group extended the WHO growth charts to include weight for age beyond age 10y, WHO indicated that the new extended charts are not considered WHO charts (**Appendix H, I &J**)**.** Thus our data are only presented as BMI for age from 10-18y. PROP-03 Subject, who had received growth hormone replacement therapy at age 9, had an improvement in height and BMI Z sores **(Figure 4-3)**.

Table 4-2 Subjects with PROP Growth Z Scores *

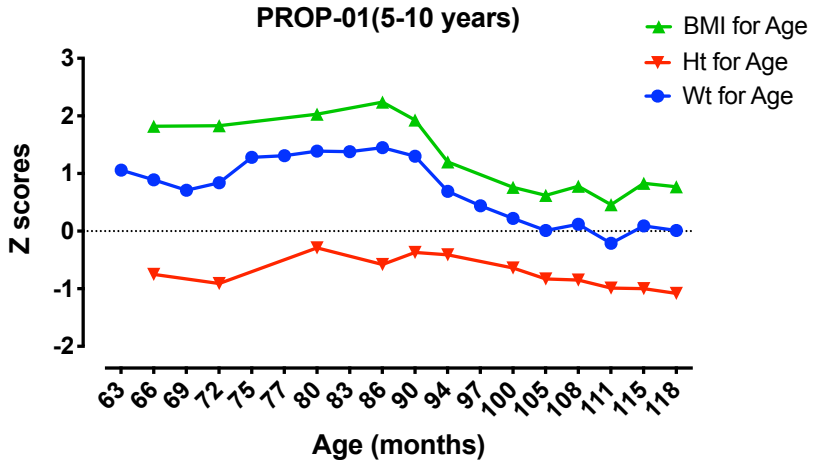
Age	Variable	Median (Range)
PROP-01	Weight for Age	0.32 (-3.01– 1.28)
0-5 years	Height for Age	-1.09 (-4.81– -0.8)
	Weight for Height	1.88 (0.94 – 2.35)
5-10 years	Weight for Age	0.775 (-0.21– 1.45)
	Height for Age	-0.79 (-1.08- -0.29)
	BMI for Age	1.015 (0.46 – 2.24)
10-18 years	Height for Age	-1.4 (-1.82 – -1.18)
	BMI for Age	0.85 (0.39 – 1.28)
PROP-02	Weight for Age	0.48 (-3.23 – 1.61)
0-5 years	Height for Age	-0.20 (-1.97 – 0.48)
	Weight for Height	1.04 (-2.61 – 2.4)
5-10 years	Weight for Age	0.94 (0.56 – 1.85)
	Height for Age	-0.23 (-0.58 – 0.08)
	BMI for Age	1.38 (1.26 – 2.46)
10-18 years	Height for Age	-1.405 (-1.69 – -0.86)
	BMI for Age	1.655 (0.96 – 2.04)
PROP-03	Weight for Age	0.42 (-0.25 – 1.54)
0-5 years	Height for Age	-1.365 (-1.72 – 1.21)
	Weight for Height	1.365 (0.44 – 1.95)
5-10 years	Weight for Age	-0.11 (-0.38 – 0.49)
	Height for Age	-1.78 (-2.16 – -1.42)
	BMI for Age	1.33 (0.82 – 1.89)
10-18 years	Height for Age	- 1.84 (-2.21 – -0.8)
	BMI for Age	0.435 (-0.72 – 0.62)
PROP-04	Weight for Age	1.535 (0.24 – 2.79)
0-5 years	Height for Age	-0.345 (-1.25 – 1.82)
	Weight for Height	2.015 (1.54 – 2.86)
5-10 years	Weight for Age	1.03 (0.61 – 1.28)
	Height for Age	-1.275 (-1.53 – -0.91)
	BMI for Age	2.035 (1.67 – 2.61)

***Assessed using sex-specific growth charts**

(A)



(B)



(C)

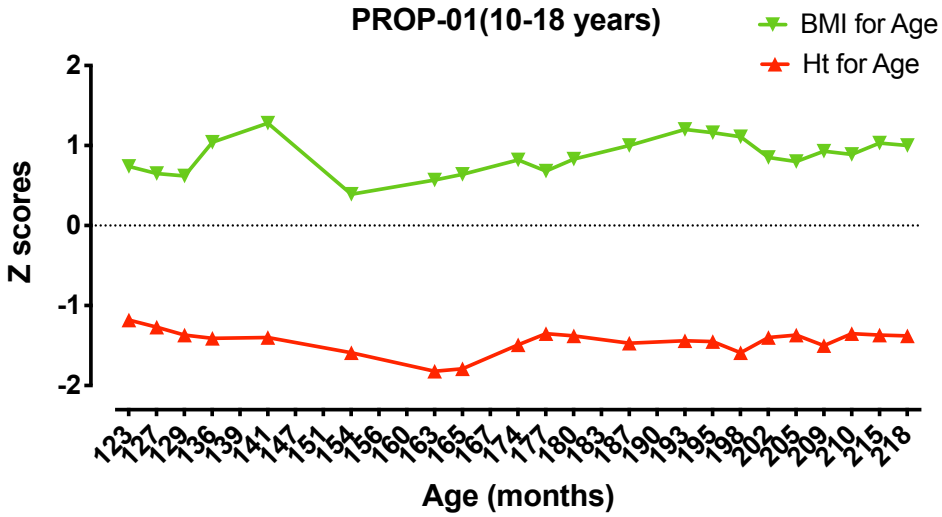
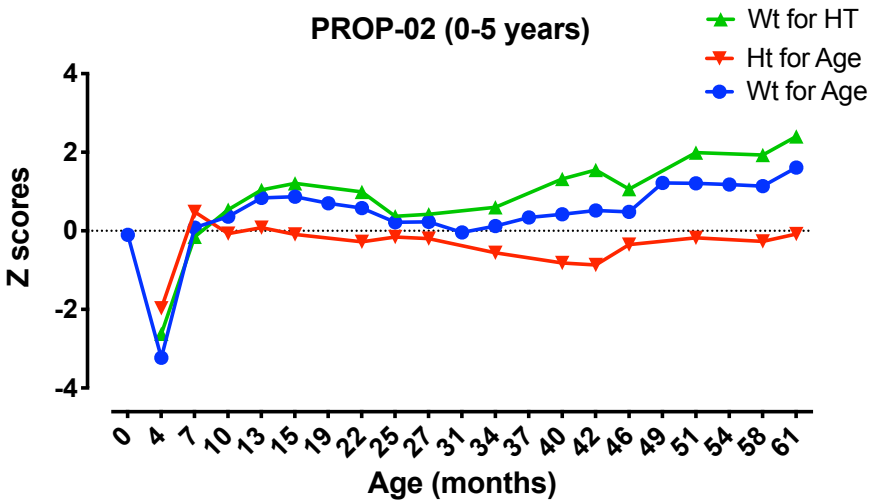
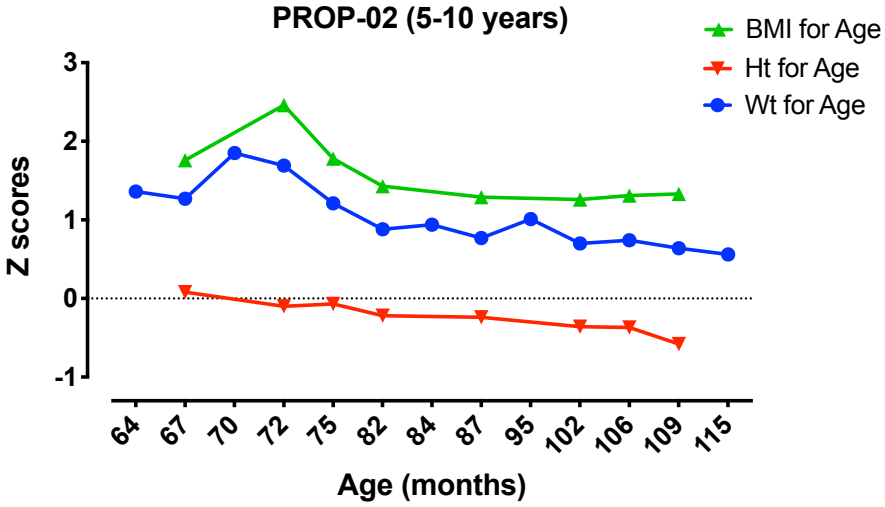


Figure 4-1 Growth Data Z scores for PROP-01 (sex-specific charts)
A) Weight for Height, Height for Age, and Weight for Age from 0-5 years old. B) BMI, Height and Weight for age from 5-10 years old. C) BMI and Height for age from 10-18 years old**.**

(A)



(B)



(C)

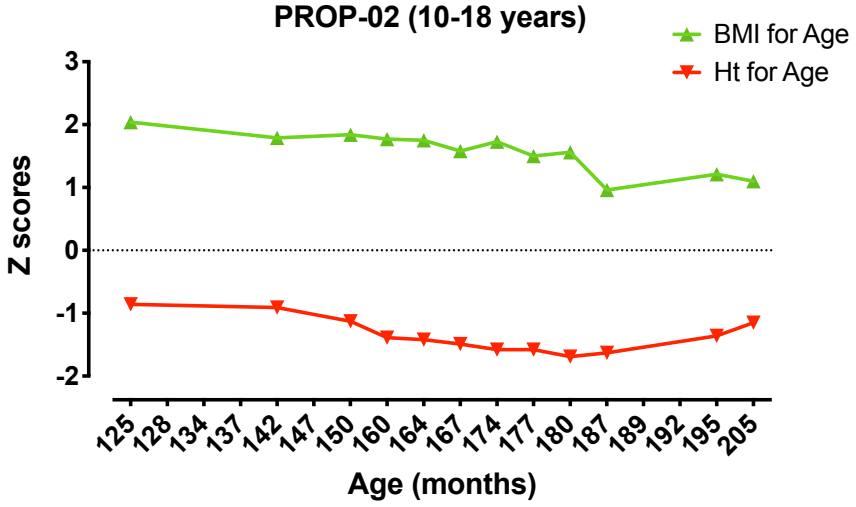
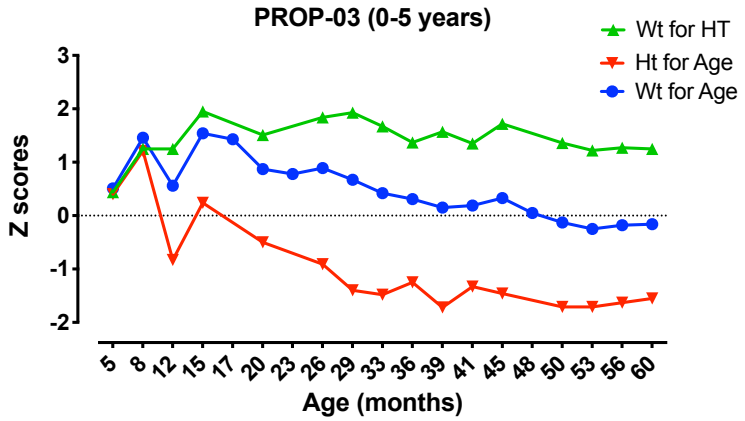
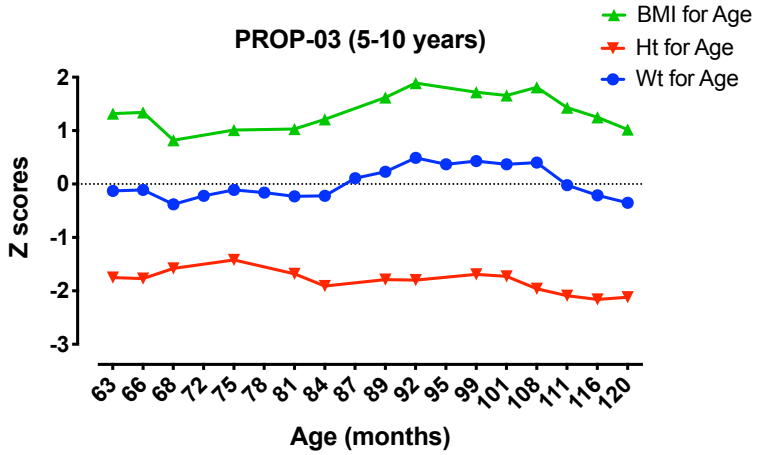


Figure 4-2 Growth Data Z Scores for PROP-02 (sex-specific charts)
A) Weight for Height, Height for Age, and Weight for Age from 0-5 years old. B) BMI, Height and Weight for age from 5-10 years old. C) BMI and Height for age from 10-18 years old.

(A)



(B)



(C)

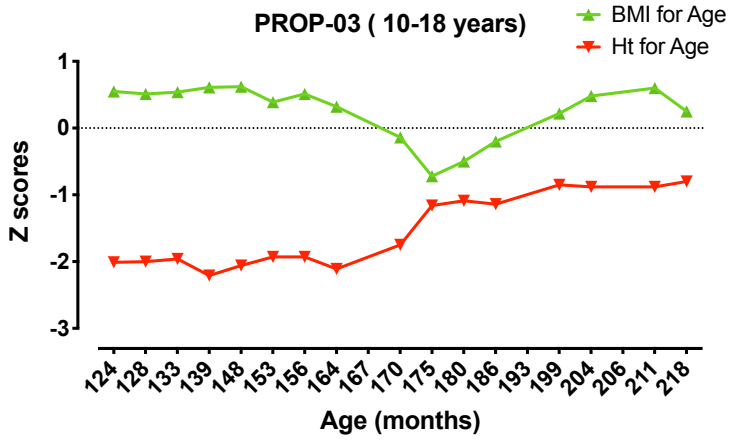
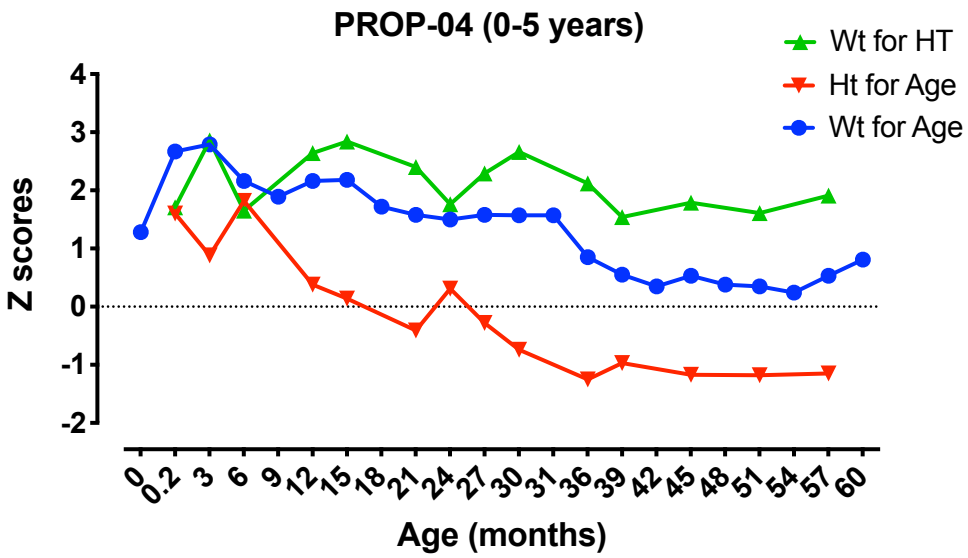


Figure 4-3 Growth Data Z Scores for PROP-03 (sex-specific charts)
A) Weight for Height, Height for Age, and Weight for Age from 0-5 years old. B) BMI, Height and Weight for age from 5-10 years old. C) BMI and Height for age from 10-18 years old**.**

(A)



(B)

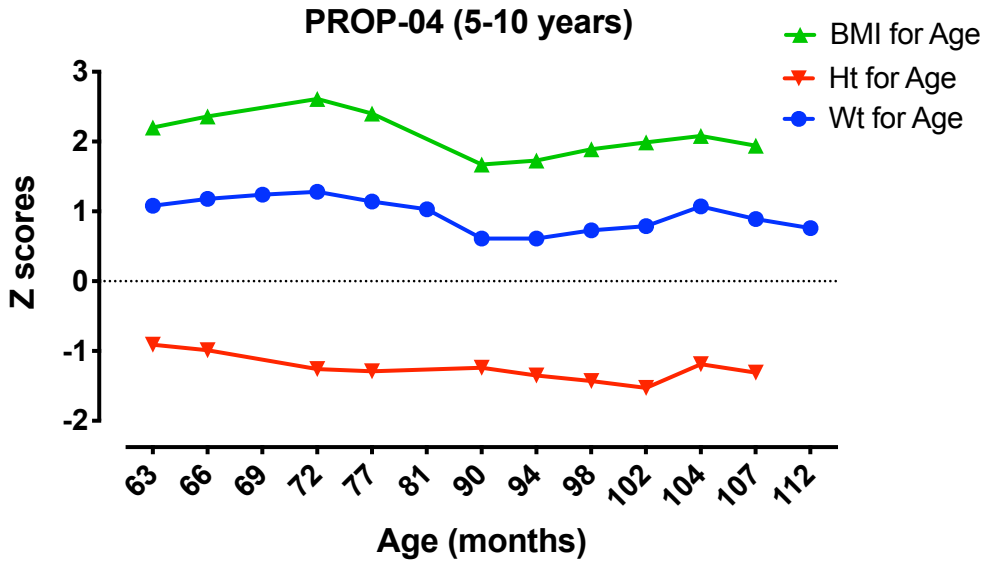


Figure 4-4 Growth Data Z Scores for PROP-04 (sex-specific charts)
A) Weight for Height, Height for Age, and Weight for Age from 0-5 years old. B) BMI, Height and Weight for age from 5-10 years old.

4.2.3 Dietary Data

Energy and protein intakes, including total, intact, and protein from medical foods in g/kg/d are all presented for each subject in (**Table 4-3**). Energy intake for all subjects was within 80-120% of the EER for age, as defined in the National Research Council Washington DC, 2005 (Institute of Medicine, 2005) (**Figure 4-5**). Protein intake was compared with three different guidelines; The Ross recommendation guidelines for total protein intake for 0-12 months of age (Phyllis B.Acosta and Steven Yannicelli, 2001), the Sass recommendation for both total and intact protein intake for all ages (Sass et al., 2004), and lastly with the Genetic Metabolic Dietitian International (GMDI) guidelines in PROP (Jurecki et al., 2019). A comparison among these guidelines for total protein intake is presented in (**Figure 4-6)**. There are no major differences in total protein recommendations between the (GMDI.2019, and Sass et al.2004) guidelines. However, the Ross recommendations for total protein is 92-108% higher than GMDI recommended intakes, and 59-50% higher than Sass recommendations for 0-6 months and 7-12 months, respectively. This indicates that compared to the most recent guidelines, the Ross recommendations were significantly higher in total protein intakes.

All subjects had low intakes of intact protein compared with both guidelines (GMDI.2019, and Sass et al.2004), and were supplemented with medical foods and single L-amino acids (Valine and/or Isoleucine), which led to the excess consumption of total protein in comparison to both guidelines. It must be noted here that only the Ross recommendation guidelines were used to manage infants with PROP at BC Children's Hospital between 1999 and 2003. Median percentages of intact protein vs. protein from medical foods varied for each patient and for each age group (**Figure 4-7**

Figure 4-8**Figure 4-9Figure 4-10**). However, as children grew older, their intakes from intact protein increased and, accordingly, medical food consumption decreased. Median intact protein consumption in g/kg/d for 1-3 years was 0.74 (0.62-0.85) 70% of RDA for intact protein. Whereas median total protein intake in g/kg/d for the same age was 2.26 (2.02-2.37) 179% of RDA for total protein (GMDI.2019). This indicates that for 1-3 years of age, 67% of total protein consumption was supplied from medical foods rather than intact protein sources. **(Figure 4-7**

Figure 4-8**Figure 4-9Figure 4-10 Breakdown of Protein Intake**).

The protein to energy ratio (P: E) in g/100kcal/d was calculated for both intact vs. total protein, and presented for all subjects in (**Table 4-4**). A protein to energy ratio of (> 1.5 to <2.9 g/100kcal) was found by Evans at el. to be associated with optimal growth outcomes. In our results, median total protein: energy ratio for 1-3 years was 2.75/100kcal, within the optimal ratio. However, the median intact protein: energy ratio for the same age was 0.9g/100kcal; well below the reference for optimal growth.

Table 4-3 Subjects with PROP Dietary Intake

Total Protein g/kg/d							
Age	**Subjects' Actual Intakes [1]**				**Recommended Intakes**		
	PROP-01	**PROP-02**	**PROP-03**	**PROP-04**	**ROSS [2]**	**SASS [3]**	**GMDI [4]**
0-6 months	2.67 (0.44-3.23)	1.96(1.47-2.83)	1.8(0.51-2.25)	1.92(1.31-2.54)	2.50 - 3.50	1.8-2.2	1.52-1.82
7-12 months	2.25(1.93-2.61)	2.08(1.53-2.13)	1.52(1.46-1.71)	1.96(1.3-2.3)	2.50 - 3		1.20-1.44
1-3 years	2.02(1.18-2.18)	2.29(1.62-2.51)	2.37(1.52-2.61)	2.23(2.06-2.5)	≥30*	1.5-2	1.05-1.26
4-8 years	2.07(1.26-2.24)	1.76(1.21-2.22)	2.33(1.22-2.51)	1.84(0.88-2.34)	≥35*	1.2-1.5	0.95-1.14
9-13 years	1.25(0.96-1.46)	1.02(0.92-1.21)	1.23(1.1-1.44)	0.98(0.95-1.05)	≥40*		0.95-1.14
14-18 years	1.04(0.77-1.13)	0.93(0.82-1.06)	1.13(1.05-1.3)	N/A	M: ≥65 / F: ≥55*		0.85-1.02
Intact Protein g/kg/d							
Age	**Subjects' Actual Intakes [1]**				**Recommended Intakes**		
	PROP-01	**PROP-02**	**PROP-03**	**PROP-04**	**ROSS [2]**	**SASS [3]**	**GMDI [4]**
0-6 months	0.95(0.44-1.41)	1.44(0.5-1.72)	1.2(0. .32-1.36)	1.17(0.53-1.21)	N/A	0.7-1.5	0.91-1.52
7-12 months	0.67(0.58-0.83)	0.87(0.21-0.97)	1.06(0.76-1.29)	0.87(0.43-1.03)			0.72-1.2
1-3 years	0.62(0.31-0.71)	0.85(0.42-0.93)	0.72(0.42-0.81)	0.77(0.67-0.88)		1-1.5	0.63-1.05
4-8 years	0.76(0.43-1.50)	0.86(0.75-1.01)	0.91(0.75-1.18)	0.76(0.58-0.86)			0.57-0.95
9-13 years	0.66(0.56-0.80)	0.70(0.55-0.96)	0.78(0.7-0.87)	0.63(0.61-0.69)		0.8- 1.2	0.57-0.95
14-18 years	0.75(0.54-0.89)	0.77(0.65-1.01)	0.75(0.73-0.84)	N/A			0.51-0.85
Protein from Medical Foods g/kg/d							
Age	**Subjects' Actual Intakes [1]**				**Recommended Intakes**		
	PROP-01	**PROP-02**	**PROP-03**	**PROP-04**	**ROSS [2]**	**SASS [3]**	**GMDI [4]**
0-6 months	1.18(1.23-3.06)	0.63(0-1.8)	0.83(0.22-1.15)	0.74(0.44-1.32)	**N/A**	0.7-1.5	N/A

7-12 months	1.58(1.36-1.77)	1.12(0-1.2)	0.48(0.22-0.81)	1.10(0.65-1.42)			
1-3 years	1.37(1.22-1.63)	1.43(1.15-1.7)	1.63(0.76-1.89)	1.46(1.3-1.72)		0.5-1	
4-8 years	1.30(0.62-1.54)	0.86(0.46-1.37)	1.24(0.47-1.64)	1.03(0.30-1.54)		0.2-0.5	
9-13 years	0.61(0.40-0.68)	0.38(0-0.46)	0.44(0.38-0.57)	0.34(0.32-0.37)		0-0.4	
14-18 years	0.22(0.19-0.36)	0.15(0-0.19)	0.34(0.33-0.44)	N/A			

Energy Kcal/kg/day

Age	**Subjects' Actual Intakes [1]**				**Recommended Intakes**		
	PROP-01	**PROP-02**	**PROP-03**	**PROP-04**	**ROSS [2]**	**SASS [3]**	**GMDI [4]**
0-6 months	97(97-97)	95(95-95)	95(95-95)	92.76(78.36-120.4)	95-145	N/A	M: 72-109/F: 72-108
7-12 months	91.2(93.9-111.6)	94.1(90-95.2)	90.34(84.30-100)	70.59(65.96-88.24)	80-135		M: 65-97/F: 64-96
1-3 years	75.9(72.9-87.6)	89.5(72.7-97.9)	83.7(77.30-95.48)	74.31(65.36-85.54)	900-1800†		M: 66-99/F: 66-99
4-8 years	66.8(40.4-80.7)	56.9(51-66.1)	57.08(49.03-82.16)	56.96(50.65-70.8)	1300-2300†		M: 59-88/F: 56-84
9-13 years	46(34.4-56.9)	40(34.96-65.8)	47.07(42-55.05)	48.82(45.71-52.52)	1650-3300†		M: 43-65/F:39-58
14-18 years	34.6(32.1-40.7)	34.9(30.2-37.2)	37.97(33.28-44.70)	N/A	M:2100-3900 / F:1200-3000†		M: 36-53/F:30-45

[1] Subjects' actual intakes reported in median and range (Minimum- Maximum)
[2] Recommended intakes adapted from the Ross Nutrition Support Protocol (Phyllis B.Acosta and Steven Yannicelli, 2001)
[3] Recommended intakes based on the Sass Recommendation (Sass et al., 2004)
[4] Recommendedn intakes based on the GMDI guidelines (Jurecki et al., 2019)
* g/d
† kcal/d

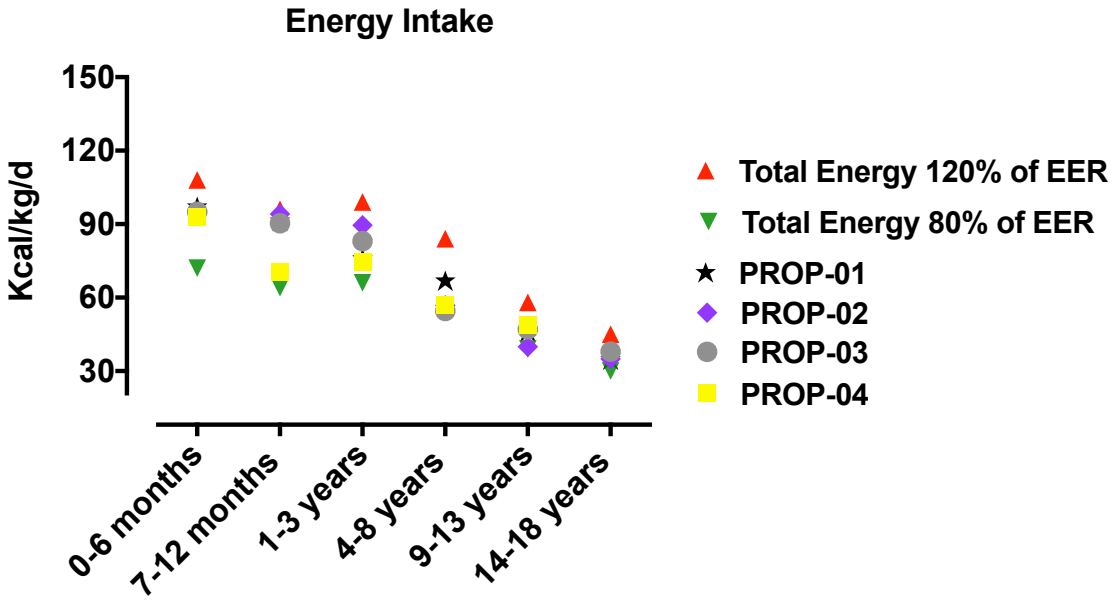


Figure 4-5 Energy Intake for Subjects with PROP Compared with 80-120% of RDA

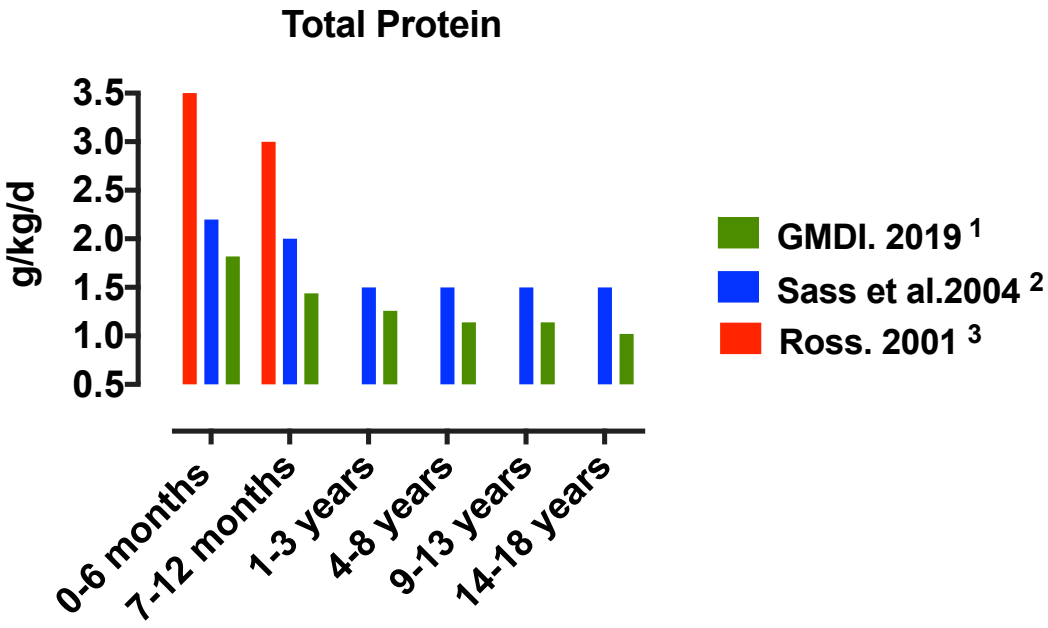


Figure 4-6 A Comparison Among Different Guidelines for Total Protein Intakes for Subjects with PROP

[1] (Jurecki et al., 2019)

[2] (Sass et al., 2004)

[3] (Phyllis B.Acosta and Steven Yannicelli, 2001). Recommendations were reported in g.kg/d only for 0-12 months of age.

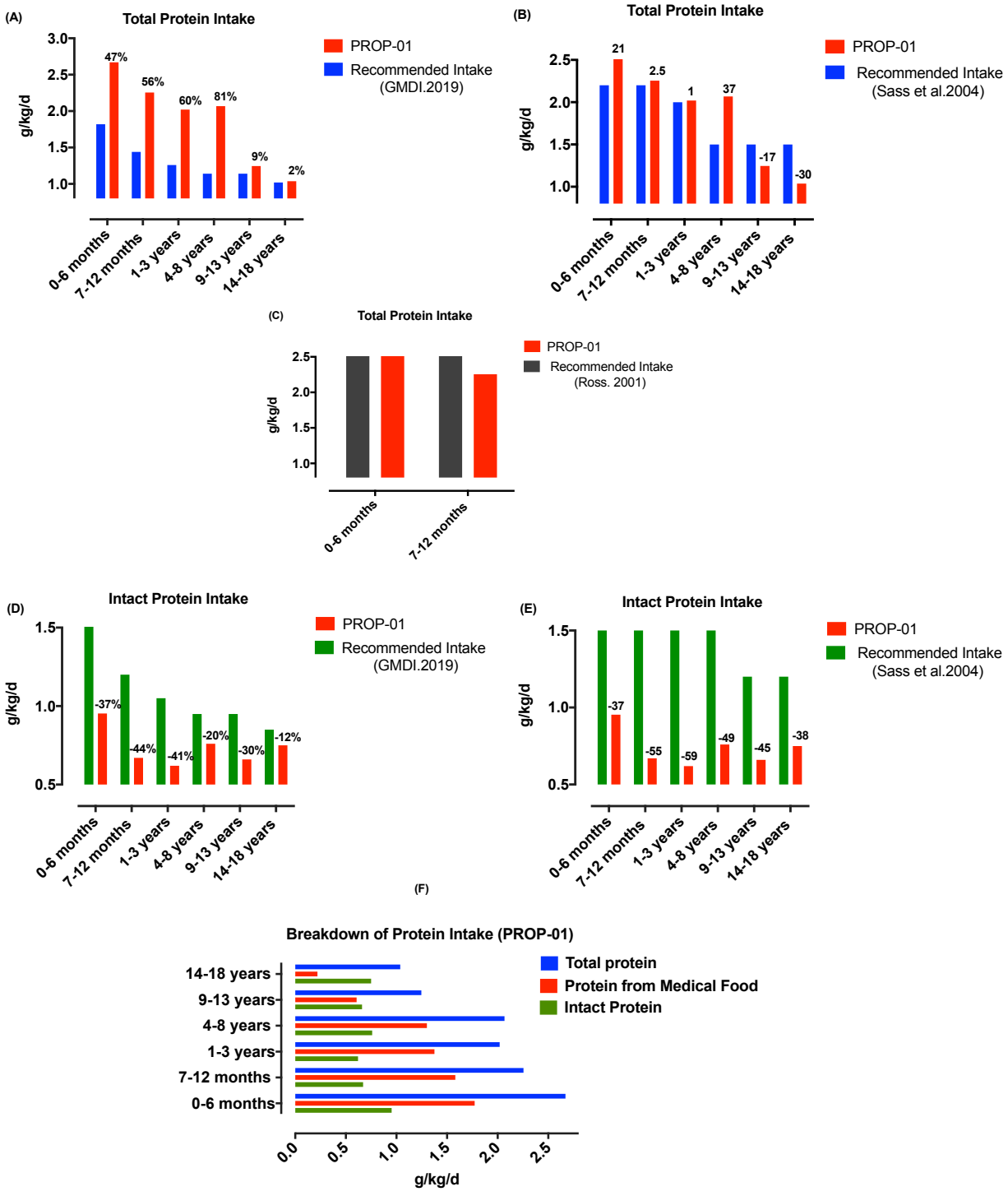


Figure 4-7 PROP-01 Subject Protein Intakes

A) Total Protein Intake in Comparison with GMDI 2019. B) Total Protein Intake in Comparison with Sass et.2004. C) Total Protein in Comparison with Ross.2001. D)Intact Protein in Comparison with GMDI.2019. E). Intact Protein in Comparison with Sass et al.2004. F) Breakdown of Protein Intake

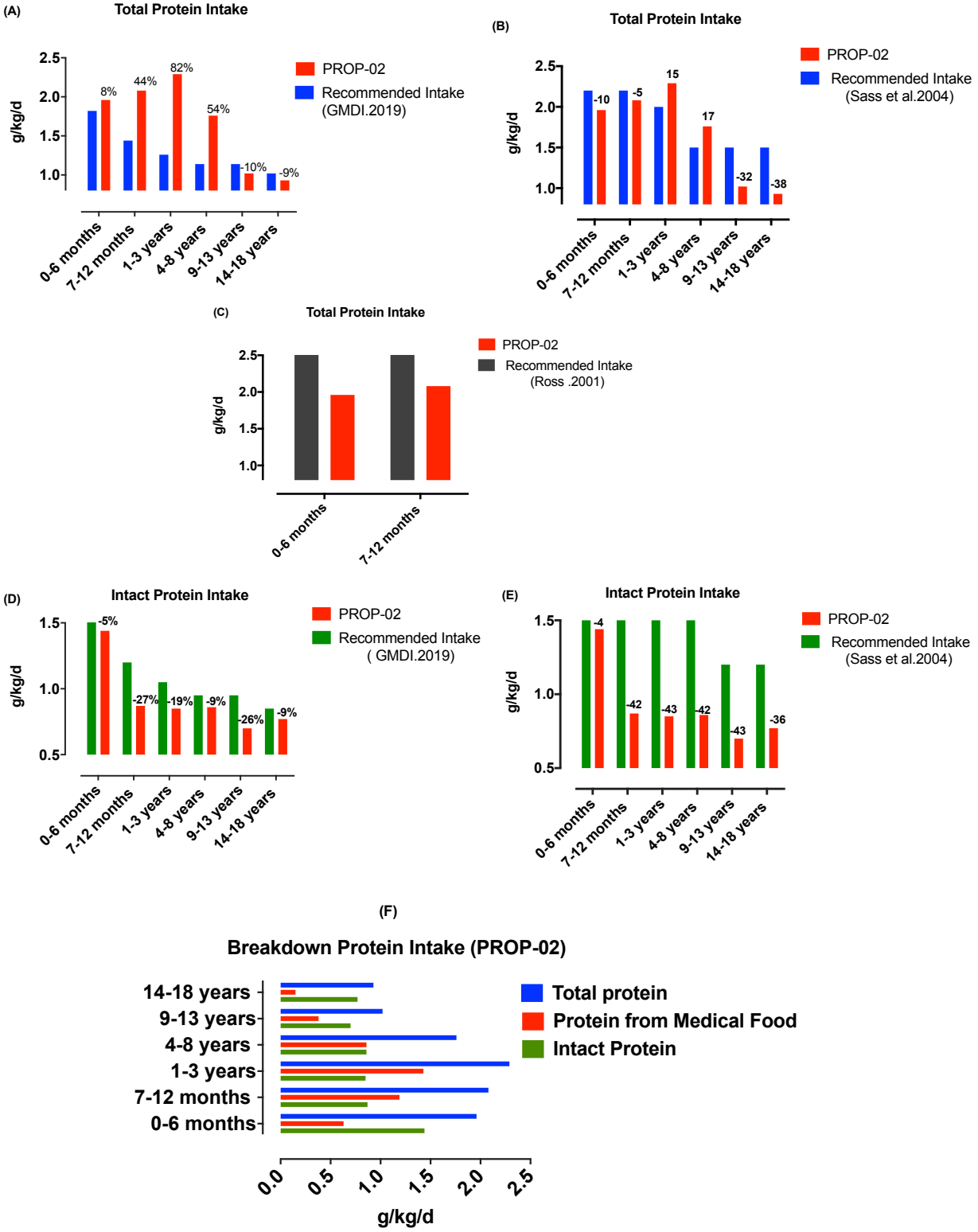


Figure 4-8 PROP-02 Subject Protein Intakes

A) Total Protein Intake in Comparison with GMDI 2019. B) Total Protein Intake in Comparison with Sass et.2004. C) Total Protein In comparison with Ross.2001. D)Intact Protein in Comparison with GMDI.2019. E). Intact Protein in Comparison with Sass et al.2004. F) Breakdown of Protein Intake

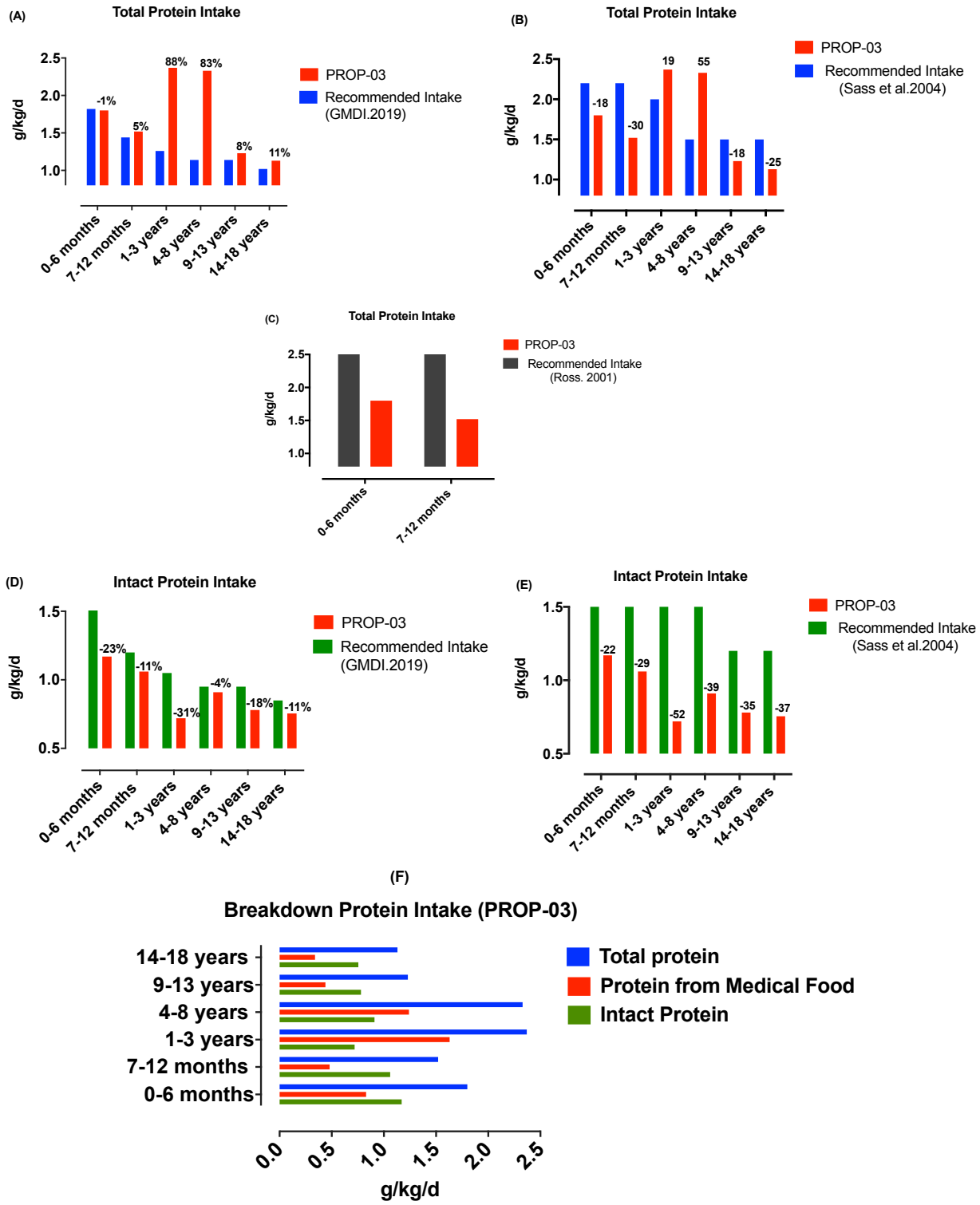


Figure 4-9 PROP-03 Subject Protein Intakes

A) Total Protein Intake in Comparison with GMDI 2019. B) Total Protein Intake in Comparison with Sass et.2004. C) Total Protein in Comparison with Ross.2001. D)Intact Protein in Comparison with GMDI.2019. E). Intact Protein in Comparison with Sass et al.2004. F) Breakdown of Protein Intake

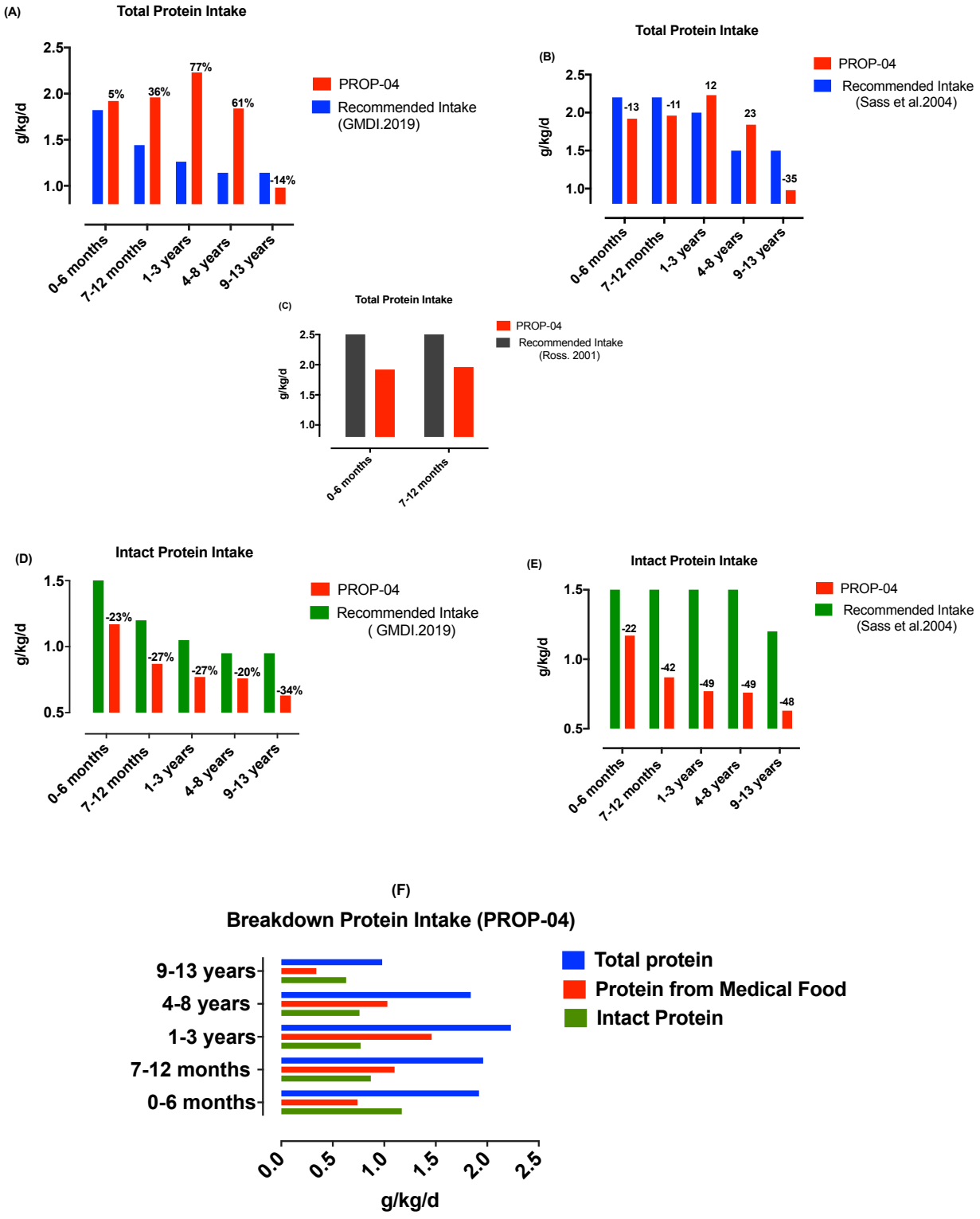


Figure 4-10 PROP-04 Subject Protein Intakes

A) Total Protein Intake in Comparison with GMDI 2019. B) Total Protein Intake in Comparison with Sass et.2004. C) Total Protein in Comparison with Ross.2001. D)Intact Protein in Comparison with GMDI.2019. E). Intact Protein in Comparison with Sass et al.2004. F) Breakdown of Protein Intake

Table 4-4 Protein to Energy Ratio for Subjects with PROP[1]

Age	Subjects' Actual Intakes[2]		Recommended Protein to Energy Ratio[3] (Evans et al., 2017)
	Total Protein: Energy	Intact Protein: Energy	
PROP-01 0-6 months	2.75	0.98	>1.5 to < 2.9
7-12 months	2.47	0.73	
1-3 years	2.66	0.81	
4-8 years	3.09	1.13	
9-13 years	2.7	1.4	
14-18 years	2.99	2.1	
PROP-02 0-6 months	2.06	1.5	>1.5 to < 2.9
7-12 months	2.21	0.92	
1-3 years	2.55	0.94	
4-8 years	3.09	1.5	
9-13 years	2.55	1.7	
14-18 years	2.66	2.2	
PROP-03 0-6 months	1.89	1.23	>1.5 to < 2.9
7-12 months	1.68	1.17	
1-3 years	2.85	0.86	
4-8 years	4.2	1.66	
9-13 years	1.61	1.65	
14-18 years	2.97	1.98	
PROP-04 0-6 months	2.06	1.26	>1.5 to < 2.9
7-12 months	2.77	1.23	
1-3 years	3	1.03	
4-8 years	2.23	1.33	
9-13 years	2.06	1.29	

[1] g of protein/100 kcal/day

[2] Subjects' Actual Intakes are reported as medians

[3] Protein to Energy ratio associated with optimal growth in subjects with inborn error of metabolism

4.3 Discussion

Poor growth outcomes with respect to height rather than weight in subjects with PROP has been well described (De Baulny et al., 2005; Evans et al., 2017; Grünert et al., 2013; van der Meer et al., 1996; Molema et al., 2019a; Touati et al., 2006). Although physiological, genetic, and environmental factors can influence growth patterns, severely restricted intact protein consumption, especially during the first years of life, is probably the main contributor to growth failure in PROP (Lui et al., 2015). The purpose of this retrospective chart review was to describe dietary practices in the management of subjects with PROP and to establish their effects on growth outcomes. We collected longitudinal dietary and growth data for four pediatric patients for 1999-2018, following the cohort for 18 years through a natural history study. Results from the current study confirmed that all children had poor growth outcomes, with persistently reduced height-for-age Z scores, and elevated weight and BMI Z scores. Energy intakes for all children were within 80-120% of the EER for age. All children had low intakes of intact protein compared with most recent guidelines and were supplemented with medical foods and single L-amino acids (Valine and/or Isoleucine), which lead to the excess consumption of total protein in comparison to guidelines.

Our results showed that with the current dietary intakes, all children had persistently low height Z scores. However, height data could be skewed by subject PROP-03 who received growth hormone treatments at age 9 in response to documented growth hormone deficiency. Low height for age Z score reflects a process of failure to reach linear growth potential due to suboptimal health and/or nutritional conditions, which is also an indication of stunting (Lui et al.,

2015). In agreement with our results, many reported a tendency toward a decrease in height Z scores and an overall growth failure in subjects with PROP (**Table 2-8**) (De Baulny et al., 2005; Evans et al., 2017; Grünert et al., 2013; van der Meer et al., 1996; Molema et al., 2019a; Sass et al., 2004). However, the one exception is the study, where Yannicelli et al. reported an improved weight and moderate improvement in height centile in infants and toddlers with PROP when medical food provided 50% of total protein intake (Yannicelli et al., 2003). It is unknown why these results differ, and may be related to the clinical severity of the disease. The strong restriction of intact protein seen in our subjects, which persisted beyond the first two years of life could have limited their linear growth. The association between severely restricted intact protein consumption and low height Z scores was also previously reported (De Baulny et al., 2005; Evans et al., 2017; Grünert et al., 2013; van der Meer et al., 1996; Molema et al., 2019a; Sass et al., 2004). In addition to low height Z scores, we also observed an elevated weight for height/age Z scores during the first five years of life, as well as elevated BMI Z scores after 5 years of age: a surprizing observation, since all children had an energy intake within 80-120% of EER at different ages. This discrepancy can be explained by two reasons; subjects could have been physically inactive due to their neurological deficits and accordingly would have needed less energy, but with the lack of data on physical activity from the charts, we cannot conclude whether or not their energy consumption was appropriate. In one study on 6 children with PROP, measured resting energy expenditure by indirect calorimeter was noted to be 20% less than calculated requirements (Feillet et al., 2000). Thus, subjects with PROP could only require 80% of their EER, except during periods of illness. Another explanation is the exclusion of dietary data from sick days, where subjects were supplied with energy at 120% of EER for age, which

could have affected their overall weight and BMI. The number of sick days varied among children: on average all children had 32 sick days in the first 6 months of life, and 35 days from 1-3 years (**Table 4-1**). During those days, children were consuming high energy (120%EER), none to low protein, which was increased gradually after the first two days. The impact of sick days and subsequent impact on growth cannot be quantified, however it is likely that the eventual outcomes were influenced by the episodes of sick days.

Furthermore, protein requirements assume an adequate energy intake to ensure efficient protein utilization, thus protein and energy are interdependent (Uauy, 2013). This can be difficult to maintain in patient population with highly modified diets, which lead to the application of protein to energy ratio (P: E). This concept describes the proportion of energy derived from protein, if an individual is consuming a diet that meets energy needs, will the amount of protein be high enough to meet protein needs? Evans et al. showed that a ratio of > 1.5-<2.9 g of protein/100 kcal is associated with optimal growth in subjects with inborn errors of metabolism (Evans et al., 2017). In a natural history study on organic acidemia patients, the median intact protein to energy ratio was 1.23g/100kcal/d and it was positively associated with height Z score (Molema et al., 2019a). In the current study, calculated P: E ratios from total protein showed a median ratio that was within the optimal ratio. However, when intact protein intake was used to calculate the ratio, the results for all subjects were lower than the optimal ratio (**Table 4-4**). This further indicates that one of the reasons for the low height for age observed in all children were due to inadequate protein in relation to the energy supply. As stated above, protein and energy needs both need to be met for normal linear growth (Evans et al., 2017)

This suggests that the highly modified diets with the excess consumption of the nutritionally imbalanced medical food could have resulted in low P: E ratio, which can cause an increase in body weight and BMI, predisposing subjects to overweight. Although high weight for height is an indicator of obesity, in this case, with the lack of an adiposity measure, obesity should not be used to describe high weight for height seen in these children (de Onis and Blössner, 1997). Future studies could include body composition assessment in PROP subjects and observe changes longitudinally.

In comparison to total protein consumption, intact protein intake was significantly lower than current guidelines, which indicates that the argument of increasing intact protein intake was translated into increasing protein from medical foods. In fact, the percentage of protein derived from medical foods was higher than intact protein (**Figures 4-7 to 4-10 (E) Breakdown of total protein intake**).However, it is also possible that these subjects did not tolerate higher than prescribed intact protein specially during infancy, and therefore medical foods were used as an alternative to increase total protein intake. It was taken into consideration that the Ross recommendation guidelines were used to manage children with PROP at BC Children's Hospital between 1999 and 2003. The guidelines suggested 3.5 and 3 g/kg/d as the recommended intake values for total protein during infancy and childhood, respectively, especially if L-amino acids were used as the major source of protein intake. The Ross guidelines also advised clinicians to either supply all or half of the protein intake from medical foods (Phyllis B.Acosta and Steven Yannicelli, 2001). Therefore, total protein intake among all subjects were high in comparison to both the Sass and the GMDI guidelines. This high protein consumption can cause high nitrogen load especially for a patient cohort with a risk of chronic kidney disease and hyperammonemia.

It is also worth mentioning that with new guidelines, all subjects' intakes of intact protein increased with age, and accordingly, medical food consumption decreased.

The use of medical food specially formulated for subjects with PROP can result in chronic imbalanced supplementation of leucine in comparison to the other BCAA, which can induce unintended effects on amino acid metabolism and transport (Myles et al., 2018). The effect of this imbalanced content of the BCAA was reported by many. In a cross-sectional study, Molema et al. reported that subjects receiving medical food had significantly lower plasma values of valine and isoleucine compared to subjects not receiving medical food. Moreover, plasma valine was positively associated with the amount of intact protein consumption and negatively associated with the amount of leucine in medical food used (Molema et al., 2019a, 2019b). Another study presenting the long-term outcomes and dietary data on PROP, reported low to very low plasma valine and isoleucine in all subjects (Touati et al., 2006). In this study, we did not report plasma amino acid concentrations, but the fact that subjects needed to be supplemented with single amino acids (valine and isoleucine) indicates that their plasma values were deficient. This can be explained by the antagonistic interaction among the BCAA (Harper, 1984). BCAA are essential for maintaining anabolism and supporting normal growth and development. A decrease in their plasma values can lead to an acute metabolic crisis, decompensation and growth impairment.

In conclusion, despite adequate amount of total protein and energy intake, growth outcomes in all subjects were below population standards. Although various dietary protocols with different ranges of intact protein and medical foods were used, most studies reported growth outcomes similar to our results. This suggests that the restricted intact protein intake together

with the overuse of medical food could have affected subjects' overall growth. Therefore, optimizing dietary management is the primary means to improving outcome in subjects with PROP. The imbalanced BCAA ratio in medical foods needs to be corrected by determining the ideal balance among LEU: ILE: Val to ensure children who do need to use medical foods are not compromised.

4.4 Limitations

There are some limitations associated with this study, including the small sample size of four patients. Due to the rarity of the disease, there were only six subjects with PROP being treated at BC Children's Hospital. Two of them transitioned to adulthood in the early 2000's; thus, collecting their data after 1999 would not have provided enough data for making any comparisons. Although all four subjects were treated at the same clinic, there was high variability in their dietary treatments. Therefore, we could not perform any statistical correlations between diet and growth outcomes. While the dietary data represented actual intakes rather than prescribed, it did not describe intakes of the branched-chain amino acids. In this study, we assumed that subjects had low plasma isoleucine and/or valine concentrations that prompted the clinic to supplement them with single amino acids. Although the longitudinal growth data over 18 years in four subjects with PROP enabled us to confidently document growth pattern, there was no information on body composition. Moreover, one of the subjects (PROP-03) was found to be growth hormone deficient, which prompted the clinic to treat with growth hormones. This could have been the reason for their skewed height data.

Chapter 5: Determining Ideal Balance among BCAA as a Proof of Concept Study in Healthy Children

PROP is an inherited metabolic disorder, caused by a defect in the mitochondrial enzyme propionyl-CoA carboxylase (PCC), resulting in the accumulation of propionic acid metabolites. PCC catalyzes two of the branched-chain amino acids (valine, isoleucine), as well as methionine, threonine, odd-chain fatty acids and cholesterol (Baumgartner et al., 2014). The goal of nutritional management for PROP is to prevent the accumulation of toxic metabolites derived from propiogenic amino acids by restricting natural protein intake, while also maintaining normal plasma concentrations. Moreover, subjects with PROP usually need to supplement their diet with special medical foods, which are formulated to contain all essential amino acids and nutrients, but no propiogenic compounds. Due to the imbalanced content of BCAA (high leucine; minimal or no valine and isoleucine) in medical foods specially formulated for PROP, concerns have been raised about their use. An imbalanced mixture of BCAA reduces plasma concentrations of valine and isoleucine to below normal ranges, restricting total body protein synthesis and limiting growth in children with PROP (Manoli et al., 2016; Myles et al., 2018). Although recent dietary guidelines are recommending that medical foods only be used for individuals who cannot tolerate their RDA from intact protein, most of individuals with PROP are at risk for malnutrition and are depending on these medical foods as an easily tolerable source of energy and protein (Jurecki et al., 2019). Thus, there was a need to determine the ideal ratio of BCAA in medical foods to optimize protein synthesis and growth, and to prevent the accumulation of toxic metabolites. In this chapter, I discuss the methods and results of using the

stable isotope based minimally invasive technique in children, to determine the effect of different BCAA ratios (LEU: ILE: VAL) on body protein synthesis.

5.1 Methods and Materials

5.1.1 Study Principle

The experimental design was based on the oxidation of the stable isotope L-[1-^{13}C] phenylalanine to $^{13}CO_2$ to compare protein synthesis in healthy children, under different test intakes (leucine) while other amino acids, including isoleucine and valine, are kept constant. This study was done as a proof of concept in healthy children, to allow for characterization of the metabolic response to different leucine test intakes, which will help us design different BCAA ratios to test in individuals with PROP. All procedures were reviewed and approved by the Committee for Ethical Review of Research involving Human Subjects at the University of British Columbia (H18-00439).

5.1.2 Subjects

This study examined 8 healthy, school-aged children between 6 and 10 years. The upper limit for age range (10 y) was selected to avoid influence of hormones (puberty) on our results. Use of the IAAO to determine lysine requirement in women provided evidence that F$^{13}CO_2$ oxidation rate and therefore lysine requirement was affected by different menstrual cycle phases (luteal vs. follicular phase). Lysine requirement was found to be higher in the luteal phase, which may be attributed to the higher oxidation of amino acids during luteal phase compared to follicular phase (Kriengsinyos et al., 2004). Previous IAAO studies in children and adults have demonstrated that reliable results can be obtained by studying 5-6 subjects in a repeated measure

design (Elango et al., 2007; Mager et al., 2003; Rasmussen et al., 2016). Moreover, The ratio of BCAA was studied previously in adult men using the IAAO, where five healthy men were studied 7 times for a total of 35 studies (Riazi et al., 2003b).

5.1.2.1 Recruitment

Eight healthy children were recruited to participate in the study. Recruitment posters (**Appendix C**) were posted in local coffee shops, community centers, and doctors' offices to recruit children. The poster included contact information such as cellphone, email address, and office number where interested parents could contact us for additional information. Participants were invited to participate in 7 studies per child for a total of 42 studies. A master list of participants and their assigned alphanumeric code was kept in a locked cabinet at BC Children's Hospital Research Institute (**Appendix F**). Participants were compensated for transportation costs, including public transit passes or parking passes, and offered an honorarium ($100/ study day) for their participation.

5.1.2.2 Inclusion and Exclusion Criteria

Inclusion Criteria

- Healthy children 6-10 years old.
- Normal weight (3rd -85th percentiles for weight according to the World Health Organization (WHO)
- Normal eating habits (no food allergies)

Exclusion Criteria

- Children under 6 years old, or over 10 years old.
- Children who are currently ill with a fever, cold, vomiting or diarrhea.
- Children outside of normal weight parameters (3rd -85th percentiles for weight according to the World Health Organization (WHO)).
- Children with claustrophobia.
- Children currently or recently taking prescription medication or antibiotics.
- Children with food allergies.

5.1.3 Experimental Design

Each subject was studied at seven different BCAA ratios (LEU: ILE: VAL). A negative control BCAA ratio of (1: 0: 0) was chosen to resemble the BCAA ratio in medical foods for subjects with PROP. Following this ratio, subjects received five different BCAA ratios: (1: 0.14: 0.15), (1:0.19: 0.20), (1: 0.21: 0.24), (1: 0.26: 0.28), (1:0.35:0.4), during which isoleucine and valine intake were kept constant based on recommended intakes for subjects with PROP (Blau, 2006; Phyllis B.Acosta and Steven Yannicelli, 2001). Leucine was provided in high amounts based on observed intakes of subjects with PROP (Manoli et al., 2016; Myles et al., 2018) and then gradually decreased by 25%. Lastly, the BCAA ratio found in egg protein was tested as a positive control, where we reduced leucine more than 25% to reach a ratio of (1:0.6:0.7) while also keeping isoleucine and valine constant (**Figure 5-1**). All study days were separated by a minimum of 1 week to ensure sufficient washout period between intakes.

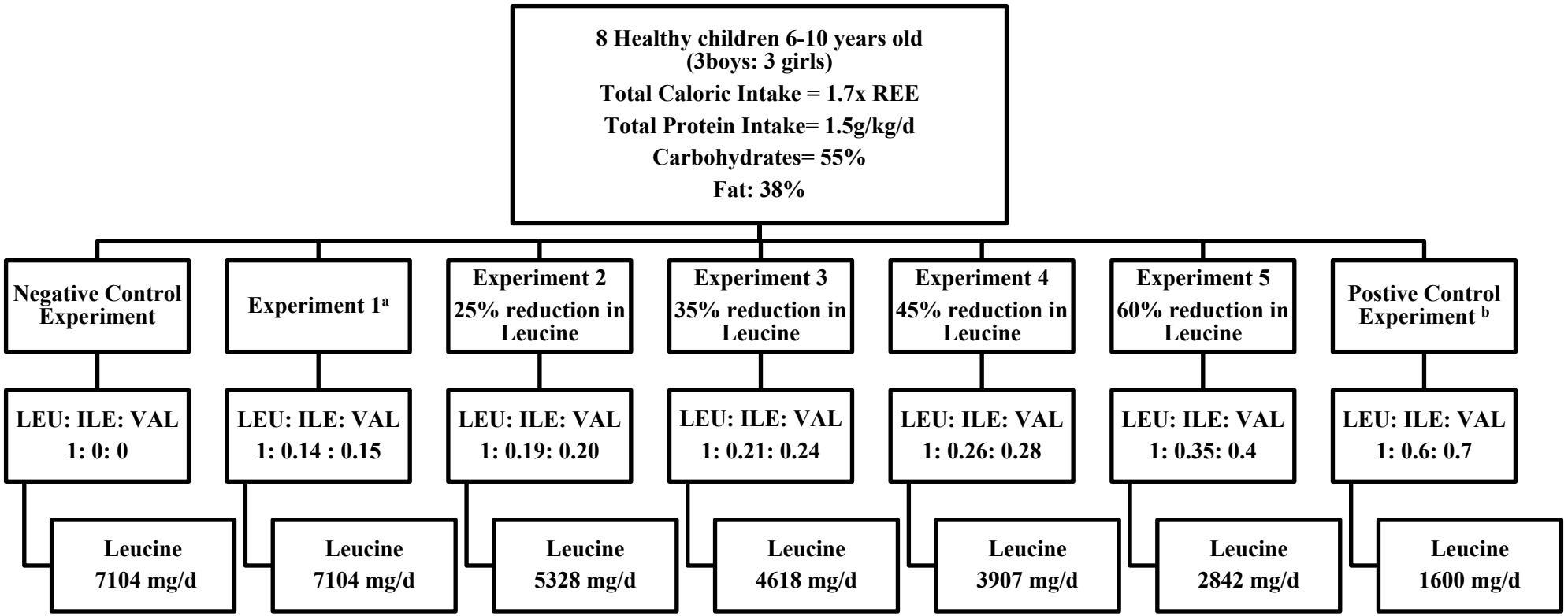


Figure 5-1 Experimental Design

[a] Isoleucine and Valine intakes were based on the recommended intakes for subjects with PROP (Isoleucine:1000 mg/day, Valine:1100 mg/day). Leucine intake was based on the observed intake of subjects with PROP (Leucine:7104 mg/day)

[b] based on egg protein pattern.

5.1.3.1 Pre-Study Day Protocol

Before the studies were started, all participants had an initial assessment to determine eligibility. All pre-study day assessment was conducted after an overnight fast (~12 hours), and took place at the Clinical Research Evaluation Unit (CREU), BC Children's Hospital Research Institute. During this visit, the following measurements were taken: anthropometric measurements (weight and height), body composition analysis, and resting energy expenditure (REE). Weight and height were measured using a digital scale and a stadiometer, respectively. Body composition was measured by bioelectrical impedance analysis (BIA model Quantum IV; RJL Systems), and, using the manufacturer's software system (RJL Systems, Body Composition Analysis V.2.1), we calculated fat mass (FM) and fat free mass (FFM). REE, which describes the caloric requirement for body functions in the absences of any physical activity, was measured by continuous, open-circuit indirect calorimetry (Vmax Encore, Viasys, CA). REE was used to calculate total energy content of the study diet in order to ensure that caloric needs for each child were met. A general questionnaire was used during the pre-study day assessment to collect information about medical history, nutritional status, and physical activity (**Appendix D**). Participants were screened to have no history of recent weight loss or illness. The purpose of the study and the potential risks involved were explained in detail to each participant's parent or guardian and written informed consent (**Appendix A**) and assent (**Appendix B**) forms were obtained for all children from their parents or guardians. A multivitamin was provided for each child to ensure adequate intake of vitamins and minerals (Centrum Junior Complete). A three-day food record was collected from each subject to allow the prescription of a standardized diet

to be consumed prior to each study day. (**Appendix G**). We used food models to advise parents or guardians on how to record food intakes.

5.1.3.2 Study Day Protocol

Two days before each study day, subjects consumed a maintenance diet supplying 1.7 x REE energy and 1.5 g protein/kg/day. Maintenance diet was used to standardize participants' protein intake, which was determined by analysing the food record collected during the pre-study day using a nutrient analysis database (Food Processor SQL 11, ESHA Research). Participants were also asked to keep a two-day food record during their maintenance diet to ensure consistency of dietary protein intake among subjects. Parents were advised to increase, decrease or maintain their children's protein intake in order to achieve a total of 1.5g/kg/d. Food choices were based on each child's food preferences.

Each study was carried out at the Clinical Research Evaluation Unit (CREU), BC Children's Hospital Research Institute, after an overnight fast (~12 hours). Weight and height measurements were taken, then each study proceeded in accordance with the minimally invasive IAAO model (**Figure 5-2**) (**Appendix E**) (Bross et al., 1998). The study day diet was consumed as hourly isocaloric meals Each meal was formulated to represent one-twelfth of each subject's daily energy requirements, depicting a 12-hour feed condition. The experimental diet consisted of protein-free liquid formula made with protein-free powder (PFD1, Mead Johnson, Evansvile, IN), flavored with added drink (Tang and Kool-Aid, Kraft Foods, Toronto, Canada), corn oil and protein-free cookies. Energy was be provided at 1.7 x REE, based on each subject's measured REE after 12-hour fast, as described earlier. The diet provided 55% of the energy as

carbohydrates, 38% as fat and 7-10% as protein. Protein was added as a crystalline L-amino acid mixture based on egg protein pattern, except for BCAA, which followed a special pattern determined according to the recommended BCAA intakes in individuals with PROP. The test leucine doses were provided as crystalline L-leucine graded into 7 different doses in 7 different BCAA ratios.

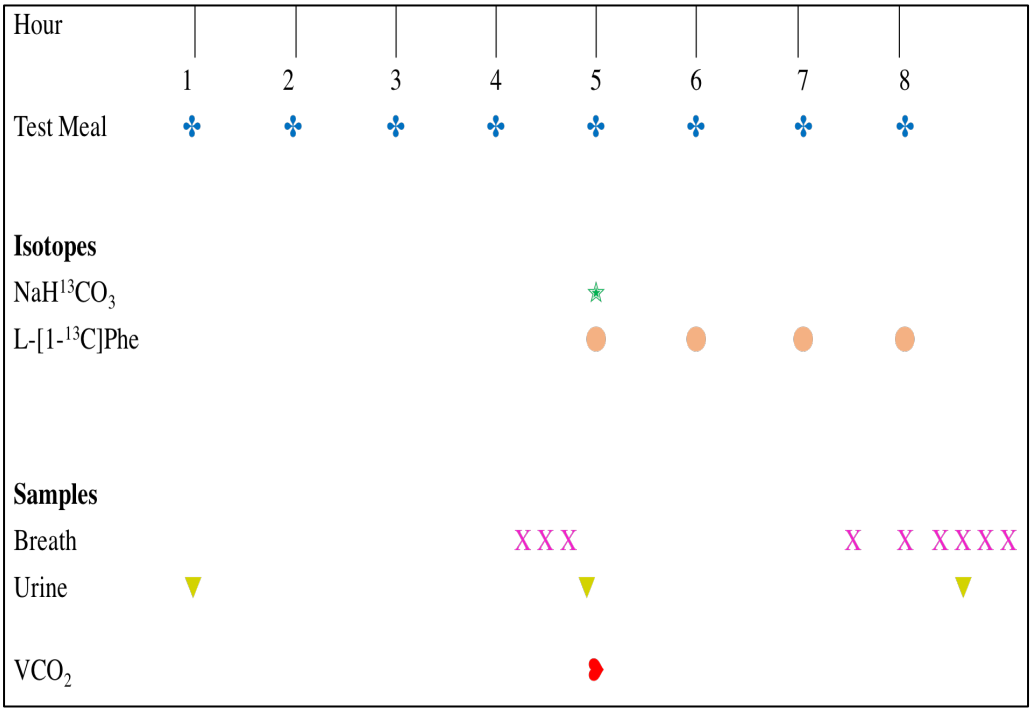


Figure 5-2 Study Day Protocol

5.1.3.3 Stable Isotope Protocol

On each study day, participants consumed 4 hourly meals, before the consumption of the tracer isotope. The oral isotope consumption protocol started at the fifth meal on each study day. Subjects consumed a priming oral dose of 2.5 mg/kg of L-[1-^{13}C] phenylalanine, a priming dose of $NaH^{13}CO_3$ of (0.176mg/kg/day) and a continuous dose of (1.4 mg/kg/h) of L-[1-^{13}C] phenylalanine (99 atom percent excess, Cambridge Isotope Laboratories Inc., Andover, MA) until the end of the study. The quantity of phenylalanine provided in the isotope was subtracted from the diet to provide total amount of 30.5 mg/kg/day phenylalanine. Tyrosine was given at a total of 61 mg/kg/day to ensure that an excess amount of tyrosine was available to separate the carboxyl carbon of phenylalanine between incorporating into protein synthesis or oxidations (Elango et al., 2011). The priming dose of $NaH^{13}CO_3$ of (0.176mg/kg/day) was given with the fifth meal to prime the body pool to more rapidly achieve steady state ^{13}C enrichment (Hoerr et al., 1989).

5.1.3.4 Sample Collection

On each study day, breath and urine samples were collected as baseline and plateau samples. On the study day, after the fifth meal was consumed, the indirect calorimeter (Vmax Encore, Viasys, CA) was used for 20 minutes to measure the rate of carbon dioxide production (VCO_2).

Breath samples were collected three times as a baseline 45, 30, and 15 minutes before the consumption of the isotope, and 2.5 hours after the start of the tracer protocol, six breath samples were collected before study end (**Figure 5-2**) (Elango et al., 2007). Breath samples were collected in disposable vacuum Exetainer tubes (Labco Ltd) by using breath bags (Single use collection bags, EasySampler System, QuinTron, Terumo Medical). Participants were taught to keep their mouth closed over the mouthpiece and take a normal breath then blow the air into air bag, at the same time the Exetainer tube was pressed into the needle, which is located in the lower part of the mouthpiece till the rubber of the Exetainer was punctured. The Exetainer tubes were prepared by using a vacuum needle pump to bring the interior pressure down to a very low internal pressure. This ensured that the exetainers draw in a ~10 ml breath sample during the sampling process. These samples were then labeled with individual subject ID, breath sample number, and set ID, and stored at room temperature following collection, until analyzed with isotope ratio mass spectrometry (IRMS, IsoPrime 100)

In accordance with the minimally invasive method, we collected urine samples in place of plasma samples to measure amino acid concentration (Rasmussen et al., 2016). Urine samples were collected at the beginning of the study day before the first meal, 15 minutes prior to the oral isotope consumption, and at the end of the study day (**Figure 5-2**). Samples were collected in

urine hats (Specimen Container w/pour spout, 6.5 oz, Medegen), and 10 mL aliquot of urine was transferred into 15 mL conical tubes (BD Falcon, Mississauga ON) which contained 200 µL 10% HCL to suppress bacterial growth. One mL of HCL urine mix was transferred to microcentrifuge tubes. In the event that participants were unable to produce the small amount of urine per sample time, participants were offered a cup of water, and asked to try again after a few minutes. Water was made freely available throughout the course of the study day to ensure proper hydration, and starting 20 minutes before an upcoming urine sample collection, participants were reminded to drink plenty of water. All urine samples were stored at -80°C for later analysis. Urinary concentrations of BCAA were determined by ion exchange chromatography with post-column ninhydrin derivatization using an Amino Acid Analyzer (AAA) (Hitachi L8900, Tokyo, Japan), and using a modified procedure as previously described (Le Boucher et al., 1997). 100 µL of 1% TCA was added to 1000 µL of urine, followed by centrifugation for 15 min at 10000 rpm at 4 °C. The resulting supernatant was filtered and then 440 µL dried down using a speed vac (Savant, Thermo Electron Corporation, SPD 131DDA). After the sample had dried, it was diluted with 55 µL water, vortexed, and 100 µL was aliquoted into glass vile insert, and 25 µL was injected into the AAA. The amino acids were separated using an ion exchange column (Hitachi Packed Column #2622 6.0 Å~ 40 mm Li Type, Tokyo, Japan,) and analyzed against an amino acid standard mix (AA-S- 18, Sigma, St Louis, MO). The areas under the peaks were integrated using the EZChrom Elite software (version 3.3.2 SP2; Agilent, ON, Canada). From the same aliquot of urine, urinary creatinine concentrations were measured using HPLC (Chromaster 5430 Diode Array Detector, Hitachi, Tokyo, Japan) using a modified procedure as previously described (Tsikas et al., 2004), on a C18 Agilent 2.1 x mm column (EC 125/2

Nucleosil 100-3 C18, Phenomenex, CA, USA), to allow standardization of urinary amino acid concentrations on a g creatinine basis. 375 µL of buffer (10 mmol/L of sodium salt of 1-octanesulfonic acid, pH 3.2) was added to 125 µL of filtered urine and vortexed, and 50 µL was injected into the HPLC.

5.1.4 Sample Analysis

Expired $^{13}CO_2$ enrichment was measured using a continuous flow isotope ratio mass spectrometer (CF-IRMS IsoPrime100, Cheadle, UK). $^{13}CO_2$ enrichment was represented as atom percent excess (APE) compared with a reference CO_2 gas standard. Co-efficient of variant < 5% in breath $^{13}CO_2$ enrichment was ensured at isotopic steady state on all study days. (Elango et al., 2007; Humayun et al., 2007).

5.1.5 Data Calculations

$F^{13}CO_2$ is the rate of $^{13}CO_2$, that was released in the breath after L-[l-^{13}C] phenylalanine oxidation (µmol/kg/h), and it was calculated as:

$$\boldsymbol{F^{13}CO_2=(FCO_2)\ (ECO_2)\ (44.6)\ (60)/(W)\ (0.82)\ (100)}$$

Where FCO_2 is the CO_2 production rate(mL/min), ECO_2 is the $^{13}CO_2$ enrichment in the expired breath at isotope steady state atom percent excess (APE), W is the subject body weight (kg). The constants 44.6 (µmol/mL) and 60(min/h) will be used to convert FCO_2 to micromoles per hour. The factor 0.82 is the correction for CO_2, and the factor 100 converts the APE to a fraction (Hoerr et al., 1989).

5.1.6 Statistical Analysis

Subject characteristics are expressed as means ± SDs. The Shapiro Wilk test was used to check for normality. Repeated Measures ANOVA was used to measure the difference among means of $F^{13}CO_2$ oxidations in response to 7 test intakes (BCAA ratios), as well as means of BCAA urinary excretions using GraphPad Prism 4.0 (GraphPad Software Inc, CA). In all cases, the difference was considered significant at $P < 0.05$. When warranted, post hoc analysis was performed using Tukey's multiple comparisons test to confirm where the differences occur between different conditions (BCAA ratios).

5.2 Results

5.2.1 Subject Characteristics

A total of 8 healthy children were studied, completing 7 study days ($N_{Subjects} = 8$, $N_{Studies} = 42$). Four subjects participated in all seven test intakes, two subjects participated in 4 test intakes and two subjects participated in three test intakes, completing a total of 42 oxidation study days. During the study period, two children moved out of the city and therefore we had to recruit additional children to complete all study test intakes. Children's ages ranged from 7-9 years old with a mean of (8.3 ± 0.81). They were all in good health and had normal body mass index (BMI) for their age, mean BMI 16.02 ± 1.59. One participant was taking 30 mg of VYVANS (Lisdexamfetamine), a treatment for attention deficit hyperactivity disorder in children aged 6-10 years. This compound is bonded to L-lysine, an essential amino acid, that, after oral ingestion, realizes lysine into the circulation (K.A et al., 2007). The amount of lysine in the drug was

considered very low and did not significantly increase total protein intake during study day. The medication was not consumed on study days. Subjects had a mean of 1.78± 0.3 for protein intake during the two-day adaptation prior to study day. This variation in protein intake was not expected to affect our results. Thorpe et al., showed that consuming 0.8, 1.4 and 2 g/kg/d protein for two days prior to study, did not affect $F^{13}CO_2$ oxidation rates (Thorpe et al., 1999) (**Table 5-1**).

Table 5-1 Subject Characteristics

Characteristic	**Value[1]**
Age (years)	8.4 ± 0.74
Male: Female	4: 4
Weight (kg)	29.4 ± 5.3
Height (cm)	132.8 ± 5.6
BMI (kg/m^2)	16.7 ± 1.6
Fat Free Mass (%)[2]	76.5 ± 6.8
Fat Mass (%)[2]	23.4 ± 6.8
REE (Kcal/d)[3]	1073 ± 138
Protein Intake g/kg[4]	1.78± 0.3

[1] Values are mean ± SD (N=8)
[2] Determined by electrical impedance analysis (BIA)
[3] Resting Energy Expenditure determined by open-circuit indirect calorimeter
[4] Values derived from two-day food records prior to each study day

5.2.2 $F^{13}CO_2$ Oxidation

$F^{13}CO_2$ data were normally distributed among all test intakes (Shapiro Wilk test, p value >0.05). Repeated measures ANOVA showed significant differences in $F^{13}Co_2$ with different test intakes (BCAA ratios); P value < 0.001, $R^2 = 0.9281$. $F^{13}Co_2$, which represents the rate of the release of $^{13}CO_2$ from phenylalanine oxidation that reflects total body protein synthesis, was significantly higher when a ratio of 1:0:0 (LEU: ILE: VAL) was tested compared to all other ratios (p value <0.001), using Tukey's multiple comparison. This indicates that total body protein synthesis at this ratio was low. Oxidation rates associated with these ratios (1:0.19: 0.20), (1: 0.21: 0.24), (1: 0.26: 0.28) were low with no significant differences between them. However, when the ratio of (1:0.35:0.4) was tested, oxidation rate as indicated by $F^{13}CO_2$ increased significantly compared with (1:0.19: 0.20), (1: 0.26: 0.28) p value <0.05. The data taken together suggests that a BCAA ratio between 1: 0.26: 0.28 and 1:0.35:0.4 may enhance total body protein synthesis (**Figure 5-3**).

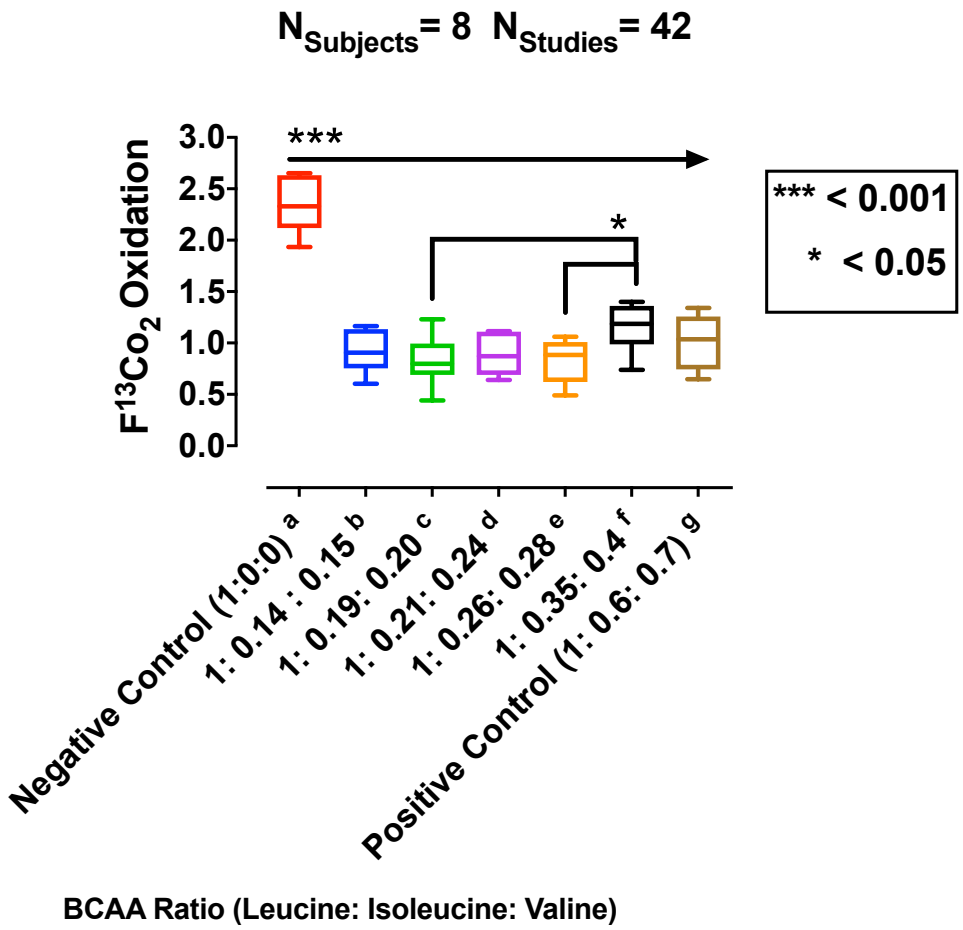


Figure 5-3 $F^{13}Co_2$ Oxidation Rate at Different Test

[a] leucine (**7100** mg/d), Isoleucine and Valine (**0** mg/d)

[b] Leucine (**7100**mg/d), Isoleucine (*1000*mg/d), Valine (*1100* mg/d)

[c] Leucine (**5380**mg/d), Isoleucine (*1000*mg/d), Valine (*1100* mg/d)

[d] Leucine (**4618**mg/d), Isoleucine (*1000*mg/d), Valine (*1100* mg/d)

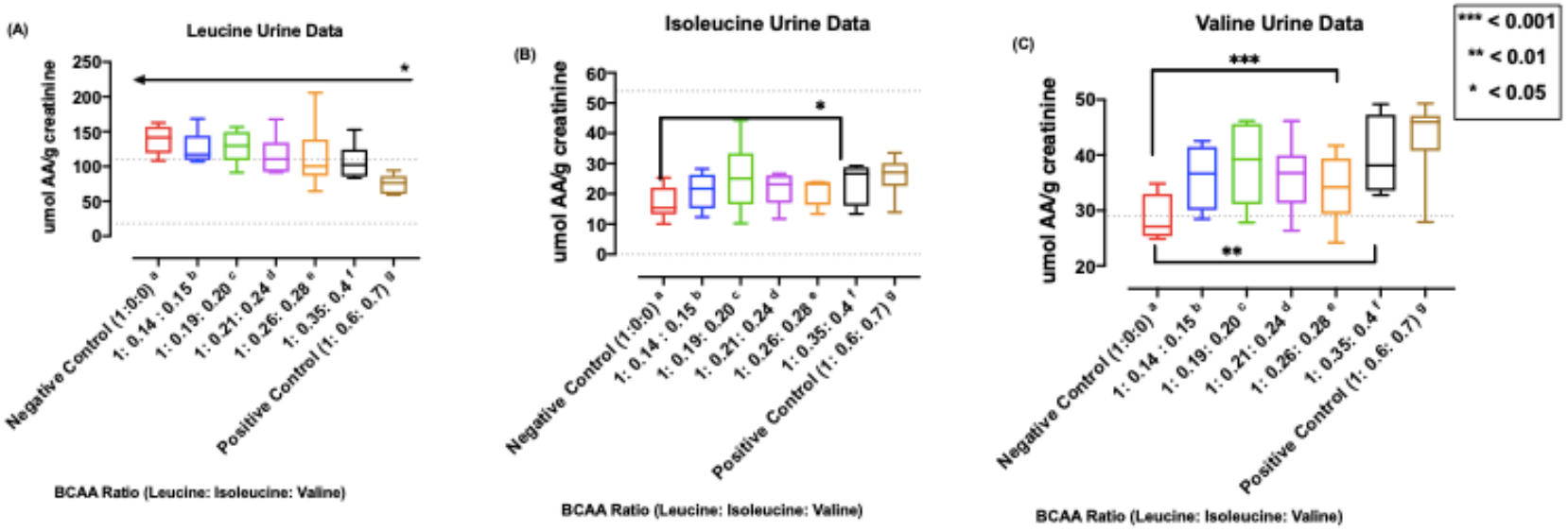
[e] Leucine (**3907**mg/d), Isoleucine (*1000*mg/d), Valine (*1100* mg/d)

[f] Leucine (**2842**mg/d), Isoleucine (*1000*mg/d), Valine (*1100* mg/d)

[g] Leucine (**1600**mg/d), Isoleucine (*1000*mg/d), Valine (*1100* mg/d)

5.2.3 Urine BCAA Concentrations

Urinary BCAA concentrations were measured from the last time point sample at the end of each study day. Urinary BCAA data were all normally distributed according to the Shapiro Wilk test, p value > 0.05. ANOVA showed significant differences between urinary leucine concentrations (p value < 0.001). With decreasing intakes of leucine, urinary leucine concentrations (standardized to per g creatinine) continued to decrease (P value <0.05) (**Figure 5-4A**). However, urinary leucine concentrations were higher than reference values for 4-13-year-old children (BC Children's Hospital Laboratories), except when a ratio of (1:0.6:0.7) was tested, where leucine intake was 1600 mg/d, equivalent to 57 mg/kg/d for a child weighing 28kg: in this case, urinary leucine concentration reduced significantly compared to all test intakes. Isoleucine urinary concentrations were always within normal reference values, with significant lower concentrations seen when a ratio of (1:0:0) was tested compared to (1:0.19: 0.20), and (1: 0.35:0.4) (**Figure 5-4B**). Valine urinary concentration was below normal reference values when a ratio of (1:0:0) was tested, and that was significantly lower than concentrations associated with a ratio of (1: 0.35:0.4), where valine urinary concentration increased to be within normal reference values (**Figure 5-4C**).



Reference values: for 4-13 years-old children; values were btained from BC Children's Hospital Laboratories
A (Leucine: 18-124 nmolAA/g creatinine)
B (Isoleucine: 0-54 nmolAA/g creatinine)
C (Valine 29-124 nmolAA/g creatinine)

Figure 5-4 Urinary Amino Acids Concentrations

A) Leucine Urinary Concentration. B) Isoleucine Urinary Concentration. C) Valine Urinary Concentration

[a] leucine (**7100** mg/d), Isoleucine and Valine (**0** mg/d)

[b] Leucine (**7100**mg/d), Isoleucine (*1000*mg/d), Valine (*1100* mg/d)

[c] Leucine (**5380**mg/d), Isoleucine (*1000*mg/d), Valine (*1100* mg/d)

[d] Leucine (**4618**mg/d), Isoleucine (*1000*mg/d), Valine (*1100* mg/d)

[e] Leucine (**3907**mg/d), Isoleucine (*1000*mg/d), Valine (*1100* mg/d)

[f] Leucine (**2842**mg/d), Isoleucine (*1000*mg/d), Valine (*1100* mg/d)

[g] Leucine (**1600**mg/d), Isoleucine (*1000*mg/d), Valine (*1100* mg/d)

5.3 Discussion

The purpose of this proof of concept study was to determine a BCAA ratio at which total body protein synthesis is optimized. We used the minimally invasive indicator amino acid oxidation technique (IAAO), and L-1-^{13}C-Phenylalanine as the indicator amino acid for protein synthesis to test the effect of different BCAA ratios in healthy children. Results from the current study confirmed that a BCAA ratio of (LEU: ILE: VAL =1:0:0), similar to the BCAA ratio in medical foods specially formulated for PROP, limits total body protein synthesis as indicated by $F^{13}CO_2$. Moreover, a balanced BCAA ratio at which total protein synthesis is optimized may be found between (1: 0.26: 0.28) and (1: 0.35:0.4). By using the IAAO, this study provides the first direct test of the hypothesis that the current BCAA mixture found in medical foods for PROP is inadequate.

The IAAO is a functional method based on the concept that the amount of the limiting amino acid controls the partitioning of the other essential amino acids between protein synthesis and oxidation. Therefore, when the limiting amino acid is provided, protein synthesis will increase, and the oxidation of the indicator amino acid will decrease (Elango et al., 2008). In this study, we did not limit the intake of any amino acids directly (except in the negative control experiment) **(Figure 5-1**). Instead, we provided leucine in excess amounts that may increase the oxidation of isoleucine and valine and therefore limit their availability for protein synthesis (Harper et al., 1984). Thus, we hypothesized that the oxidation of the indicator amino acid indicated by $F^{13}CO_2$ would be high when leucine is given in high amounts and it would decrease when leucine intake decreased, reaching a balanced ratio with the other BCAA. The study design

included seven different test intakes (BCAA ratios LEU: ILE: VAL). We gradually decreased leucine intakes while keeping isoleucine and valine constant at the recommended levels for children with PROP.

When a ratio of (LEU: ILE: VAL=1:0:0) was tested, the oxidation rate of the indicator amino acid increased as indicated by $F^{13}CO_2$ compared to the rates associated with all other ratios tested (P value <0.001); this suggests that protein synthesis was extremely limited. To our knowledge, this is the first study to test the effect of BCAA ratio of (LEU: ILE: VAL =1:0:0) on total body protein synthesis using the IAAO. Moreover, isoleucine and valine urinary concentrations at this imbalanced ratio were significantly lower than concentrations associated with other ratios (P value <0.05). Although, isoleucine urinary concentration was still within normal reference values, valine was reduced to less than normal reference values at (LEU: ILE: VAL =1:0:0). In this study, urinary amino acids concentrations were compared with values obtained from the BC Children's Hospital Laboratories for children 4-13 years of age. Although children consumed adequate protein diets (1.5 g/kg/d) including high leucine (~250mg/kg/d for a child weighing 28 kg) and all other essential and non-essential amino acids, their total body protein synthesis was still limited due to the absence of isoleucine and valine from the diet. Despite the fact that isoleucine and valine are considered among the offending compounds for PROP, that are metabolized to produce propionate, they are essential amino acids and the body needs them in an adequate amount to maintain homeostasis, protein synthesis, and growth. Therefore, if subjects with PROP were to depend on medical foods as a sole source of nutrition, they may have low plasma and urinary isoleucine and valine, limited body protein synthesis and restricted growth. In fact, many studies have reported that excess consumption of medical foods

was associated with low plasma isoleucine and valine concentrations in subjects with PROP, which prompted their clinics to supplement with single amino acids (L-isoleucine and/or valine) (Manoli et al., 2016; Molema et al., 2019a). Supplementing with isoleucine and valine to ameliorate side effects caused by excess leucine consumption was reported in animals fed low protein diet (Harper, 1984). However, in the case of PROP, supplementing with single L-isoleucine and valine can be harmful (Jurecki et al., 2019).

The BCAA have similar structures and share common enzymes for metabolism and transportations. Interactions between disproportionate BCAA intakes have been reported in human and animals. The addition of excess leucine amounts to a low protein diet depleted isoleucine and valine pools, and depressed food intake and growth in animals (Harper, 1984). High intakes of leucine enhanced the activity of the branched-chain keto acid dehydrogenase enzyme in various tissues, that increased the oxidation of isoleucine and valine, which limited their availability for protein synthesis (Harper, 1984). Similarly, excess leucine supplementations resulted in reducing plasma and urinary isoleucine and valine in healthy elderly men (Elango et al., 2016b). In subjects with PROP, the effect of high leucine intake associated with excess consumption of medical food on plasma valine and isoleucine and overall growth outcomes was reported by many (Molema et al., 2019a, 2019b; Touati et al., 2006). Just because leucine is not metabolized into propionic acids, we cannot assume that any amount of leucine is safe for subjects with PROP; as clearly shown by our 1:0:0 of LEU: ILE: VAL diet and negative impact on whole body protein synthesis. In this study, by gradually decreasing leucine intakes by 25%, starting with high amounts, like those found in medical foods, while keeping isoleucine and valine constants, we tested the effect of the following BCAA ratios on total body protein

synthesis; LEU: ILE: VAL = (1:0.14: 1.15) , (1:0.19: 0.20), (1: 0.21: 0.24), (1: 0.26: 0.28), (1: 0.35:0.4) and (1:0.6: 0.7 reflecting egg protein pattern). Oxidation rates associated with these ratios (1:0.19: 0.20), (1: 0.21: 0.24), (1: 0.26: 0.28) were low with no significant differences between them. However, when the ratio of (1: 0.35:0.4) was tested, oxidation rate as indicated by $F^{13}CO_2$ increased significantly compared with (1:0.19: 0.20), and (1: 0.26: 0.28) p value <0.05. This may indicate that a BCAA ratio between (1: 0.26: 0.28) and (1: 0.35:0.4) enhanced total body protein synthesis. Moreover, by gradually decreasing leucine intakes, leucine urinary concentration decreased from higher than refence values to within normal values for healthy children 4-13 years old. As well, valine urinary concentrations improved when leucine intakes were reduced.

We can speculate that when we tested a ratio of (1:0.14: 1.15), there was a relative excess of leucine that stimulated the oxidation of isoleucine and valine through the branched-chain keto acid dehydrogenase enzyme, limiting their availability for protein synthesis. This ratio was also associated with leucine urinary concentration higher than normal reference values. The ratio of (1: 0.6 :0.7) derived from egg protein pattern was associated, though not significantly, with a numeric increase in oxidation rate. This may be explained by the fact that total BCAA intake at this ratio was low in some subjects (106-150 mg/kg/d), compared to the total BCAA requirement in healthy school-aged children, determined by the IAAO 147 mg/kg/d (Mager et al., 2003). Furthermore, at this ratio while leucine urinary concentration significantly decreased, isoleucine and valine urinary concentrations increased compared to other test intakes. Similarly, Elango et al. showed that plasma leucine concentration was low, while isoleucine and valine concentrations were high in piglets fed low BCAA test intakes. This indicated that while leucine

was extracted and used by the gut, thus limiting protein synthesis, isoleucine and valine were passing to the circulation. While leucine limited protein synthesis, isoleucine and valine were excreted in the urine (Elango et al., 2002). Another explanation would be that the BCAA ratio in egg protein (LEU: ILE: VAL = 1:0.6:0.7) is not optimal for protein synthesis. Using the IAAO, Riazi et al. tested different BCAA ratios to determine whether the BCAA ratio in egg protein was optimal for protein synthesis. They concluded that valine may be limited in a ratio of (LEU: ILE: VAL = 1:0.6:0.7), and that this could limit total body protein synthesis (Riazi et al., 2003b).

In the current study, the effect of the imbalanced BCAA ratios on $F^{13}CO_2$ oxidation rate was less than expected, and that may be explained by the small reduction in leucine between test intakes (25%). In addition, our subjects consumed adequate protein diets during study days (1.5g/kg/d), so the effect of the imbalanced BCAA ratio was smaller in comparison to that in other studies, where they supplied a deficient protein diet that could have exacerbated the BCAA antagonism interactions (Harper, 1984). In conclusion, the effect of different BCAA ratios on total body protein synthesis was tested for the first time on healthy children in a non-invasive repeated measure and it was concluded that the current BCAA ratio found in medical foods for PROP is inadequate. A ratio between (1:0.26: 0.28) and (1: 0.35:0.4), where leucine intake can be provided between (3907-2842 mg/d equivalent to 102-140 mg/kg/d for a child weighing 28 kg) could improve plasma and urinary BCAA concentration, total body protein synthesis, and therefore growth. Thus, with the current isoleucine and valine recommendations for PROP, metabolic dietitians are encouraged to gradually reduce leucine intake to 102-140 mg/kg/d for a child weighing 28 kg, by limiting the use of medical foods for subjects who cannot tolerate their RDA from intact protein. Moreover, reformulating the medical foods by reducing leucine content

by 50% would be a step forward for the manufacturers. Further research is needed to determine the optimal BCAA ratio in subjects with PROP to optimize their total protein synthesis.

5.3.1 Limitations

This was a proof of concept study in healthy children to establish an adequate BCAA ratio that optimizes protein synthesis. Therefore, results from this study cannot be generalized to patient populations. This was a short-term with an acute dose response study, where each subject was studied 7 times with different BCAA ratios. Thus, future studies are needed to assess the long-term effect of these BCAA ratios on growth pattern. Another limitation is the small sample size (4 boys and 4 girls). We studied healthy children to characterize the metabolic response of different BCAA ratios in order to design different BCAA ratios to test in subjects with PROP.

Chapter 6: Conclusions and Future Directions

Growth failure is one of the most common complications in subjects with propionic acidemia (Baumgartner et al., 2014). Although numerous factors can affect growth outcomes, including physiological, genetic and environmental factors, dietary protein restriction is probably the main contributor to growth failure in subjects with PROP (Lui et al., 2015). In the first study, we confirmed that despite adequate intake of total protein and energy, all four subjects with PROP had poor growth outcomes. All subjects had persistently low height for age Z scores combined with high weight and BMI Z scores, which are indications of stunting, overweight and an abnormal body composition. Analyzing dietary treatments in the management of four subjects with PROP demonstrated that subjects were consuming a diet that is restricted in intact protein. However, they were consuming total protein that exceeded the recommendations, due to their high intake of medical foods.

High consumption of medical foods could have resulted in a chronic and imbalanced supplementation of leucine relative to the other two BCAA (isoleucine and valine), therefore restricting overall growth. In the second study, we confirmed that the BCAA ratio of (LEU: ILE: VAL = 1:0:0), which is similar to the ratio found in the medical foods, limited total body protein synthesis. Using the IAAO method in healthy children, we tested for the first time the effect of BCAA ratio of (LEU: ILE: VAL = 1:0:0) on total body protein synthesis. Furthermore, a balanced BCAA ratio that optimized protein synthesis using the IAAO method was found to be between (1: 0.26: 0.28) and (1: 0.35:0.4). By decreasing leucine intake from the current high doses found in medical foods, results demonstrated a significant reduction in leucine urinary

concentrations to normal ranges, with a subsequent increase in both valine and isoleucine urinary concentrations within normal ranges.

To optimize dietary management in subjects with PROP, further research is needed to determine the optimal intake of medical foods relative to intact protein. Furthermore, in a cohort of subjects with PROP, the effect of different distributions between intact protein and medical foods should be studied with a focus on long-term clinical outcomes including growth and metabolic control. In addition, since the current isoleucine and valine requirements in subjects with PROP are based on an individualized clinical and laboratory assessment, there is a need to determine isoleucine and valine requirements using a direct measure like the IAAO. Future research is also needed to determine the optimal BCAA ratio to optimize total body protein synthesis in subjects with PROP.

In summary, with the findings from this thesis, when current isoleucine and valine recommendations for PROP management are being followed, metabolic dietitians are encouraged to decrease leucine intake by limiting the use of medical foods. In subjects who tolerate less than 100%of the RDA from intact protein, medical foods should be added only to complement protein needs as a secondary source to achieve 100-120% of RDA. Finally, we propose reformulating the BCAA mixture in medical foods, by reducing leucine content by 50%.

Bibliography

Alfadhel, M., Benmeakel, M., Hossain, M.A., Al Mutairi, F., Al Othaim, A., Alfares, A.A., Al Balwi, M., Alzaben, A., and Eyaid, W. (2016). Thirteen year retrospective review of the spectrum of inborn errors of metabolism presenting in a tertiary center in Saudi Arabia. Orphanet Journal of Rare Diseases *11*.

Almási, T., Guey, L.T., Lukacs, C., Csetneki, K., Vokó, Z., and Zelei, T. (2019). Systematic literature review and meta-analysis on the epidemiology of propionic acidemia. Orphanet J Rare Dis *14*, 40.

Al-Shamsi, A., Hertecant, J.L., Al-Hamad, S., Souid, A.-K., and Al-Jasmi, F. (2014). Mutation Spectrum and Birth Prevalence of Inborn Errors of Metabolism among Emiratis. Sultan Qaboos Univ Med J *14*, e42–e49.

Baker, P.R. (2015). Pathophysiology of Inherited Metabolic Disease. In Nutrition Management of Inherited Metabolic Diseases, (Springer, Cham), pp. 35–45.

Baumgartner, M., Horster, F., Dionisi-Vici, C., Haliloglu, G., Karall, D., and Chapman, kimberly A. (2014). Proposed guidelines for the diagnosis and management of methylmalonic and propionic acidemia. Orphanet Journal of Rare Diseases *9*.

Blau, N. (2006). Physician's guide to the treatment and follow-up of metabolic diseases (Berlin: Springer).

Boyer, S.W., Barclay, L.J., and Burrage, L.C. (2015). Inherited Metabolic Disorders: Aspects of Chronic Nutrition Management. Nutr Clin Pract *30*, 502–510.

Bross, R., Ball, R.O., and Pencharz, P.B. (1998). Development of a Minimally Invasive Protocol for the Determination of Phenylalanine and Lysine Kinetics in Humans during the Fed State. The Journal of Nutrition *128*, 1913–1919.

Casperson, S.L., Sheffield-Moore, M., Hewlings, S.J., and Paddon-Jones, D. (2012). Leucine supplementation chronically improves muscle protein synthesis in older adults consuming the RDA for protein. Clinical Nutrition *31*, 512–519.

Chapman, K.A., and Summar, M.L. (2012). Propionic acidemia consensus conference summary. Molecular Genetics and Metabolism *105*, 3–4.

Cole, J.T. (2015). Metabolism of BCAAs. In Branched Chain Amino Acids in Clinical Nutrition, R. Rajendram, V.R. Preedy, and V.B. Patel, eds. (New York, NY: Springer New York), pp. 13–24.

Columbus, D.A., Steinhoff-Wagner, J., Suryawan, A., Nguyen, H.V., Hernandez-Garcia, A., Fiorotto, M.L., and Davis, T.A. (2015). Impact of prolonged leucine supplementation on protein synthesis and lean growth in neonatal pigs. American Journal of Physiology-Endocrinology and Metabolism *309*, E601–E610.

Couce, M.L., Castiñeiras, D.E., Bóveda, M.D., Baña, A., Cocho, J.A., Iglesias, A.J., Colón, C., Alonso-Fernández, J.R., and Fraga, J.M. (2011). Evaluation and long-term follow-up of infants with inborn errors of metabolism identified in an expanded screening programme. Molecular Genetics and Metabolism *104*, 470–475.

Courtney-Martin, G., Bross, R., Raffi, M., Clarke, J.T.R., Ball, R.O., and Pencharz, P.B. (2002). Phenylalanine requirement in children with classical PKU determined by indicator amino acid oxidation. American Journal of Physiology - Endocrinology and Metabolism *283*, E1249–E1256.

Daly, A., Pinto, A., Evans, S., Almeida, M.F., Assoun, M., Belanger-Quintana, A., Bernabei, S.M., Bollhalder, S., Cassiman, D., Champion, H., et al. (2017). Dietary practices in propionic acidemia: A European survey. Molecular Genetics and Metabolism Reports *13*, 83–89.

De Baulny, H.O., Benoist, J.F., Rigal, O., Touati, G., Rabier, D., and Saudubray, J.M. (2005). Methylmalonic and propionic acidaemias: Management and outcome. J Inherit Metab Dis *28*, 415–423.

Dionisi-Vici, C., Deodato, F., Röschinger, W., Rhead, W., and Wilcken, B. (2006). 'Classical' organic acidurias, propionic aciduria, methylmalonic aciduria and isovaleric aciduria: Long-term outcome and effects of expanded newborn screening using tandem mass spectrometry. J Inherit Metab Dis *29*, 383–389.

Duan, Y., Tan, B., Li, J., Liao, P., Huang, B., Li, F., Xiao, H., Liu, Y., and Yin, Y. (2018a). Optimal branched-chain amino acid ratio improves cell proliferation and protein metabolism of porcine enterocytesin vivo and in vitro. Nutrition *54*, 173–181.

Duan, Y.H., Zeng, L.M., Li, F.N., Li, Y.H., Tan, B.E., Ji, Y.J., Kong, X.F., Tang, Y.L., Zhang, Y.Z., and Yin, Y.L. (2016). Effects of dietary branched-chain amino acid ratio on growth performance and serum amino acid pool of growing pigs1. Journal of Animal Science *94*, 129–134.

Duan, Y.H., Li, F.N., Wen, C.Y., Wang, W.L., Guo, Q.P., Li, Y.H., and Yin, Y.L. (2018b). Branched-chain amino acid ratios in low-protein diets regulate the free amino acid profile and the expression of hepatic fatty acid metabolism-related genes in growing pigs. J Anim Physiol Anim Nutr *102*, e43–e51.

Elango, R., Pencharz, P.B., and Ball, R.O. (2002). The branched-chain amino acid requirement of parenterally fed neonatal piglets is less than the enteral requirement. The Journal of Nutrition *132*, 3123–3129.

Elango, R., Goonewardene, L.A., Pencharz, P.B., and Ball, R.O. (2004). Parenteral and enteral routes of feeding in neonatal piglets require different ratios of branched-chain amino acids. The Journal of Nutrition *134*, 72–78.

Elango, R., Humayun, M.A., Ball, R.O., and Pencharz, P.B. (2007). Lysine requirement of healthy school-age children determined by the indicator amino acid oxidation method. Am J Clin Nutr *86*, 360–365.

Elango, R., Ball, R.O., and Pencharz, P.B. (2008). Indicator Amino Acid Oxidation: Concept and Application. J. Nutr. *138*, 243–246.

Elango, R., Humayun, M.A., Ball, R.O., and Pencharz, P.B. (2011). Protein requirement of healthy school-age children determined by the indicator amino acid oxidation method. Am J Clin Nutr *94*, 1545–1552.

Elango, R., Ball, R.O., and Pencharz, P.B. (2012a). Recent advances in determining protein and amino acid requirements in humans. The British Journal of Nutrition; Cambridge *108*, S22-30.

Elango, R., Ball, R.O., and Pencharz, P.B. (2012b). Recent advances in determining protein and amino acid requirements in humans. The British Journal of Nutrition; Cambridge *108*, S22-30.

Elango, R., Rasmussen, B., and Madden, K. (2016a). Safety and Tolerability of Leucine Supplementation in Elderly Men. Journal of Nutrition *146*, 2630S-2634S.

Elango, R., Rasmussen, B., and Madden, K. (2016b). Safety and Tolerability of Leucine Supplementation in Elderly Men. The Journal of Nutrition *146*, 2630S-2634S.

El-Hattab, A.W. (2015). Inborn errors of metabolism in infancy and early childhood: an update. Clinics in Perinatology *42*, 413–439.

Evans, M., Truby, H., and Boneh, A. (2017). The Relationship between Dietary Intake, Growth, and Body Composition in Inborn Errors of Intermediary Protein Metabolism. The Journal of Pediatrics *188*, 163–172.

Feillet, F., Bodamer, O.A., Dixon, M.A., Sequeira, S., and Leonard, J.V. (2000). Resting energy expenditure in disorders of propionate metabolism. The Journal of Pediatrics *136*, 659–663.

Grünert, S.C., Müllerleile, S., De Silva, L., Barth, M., Walter, M., Walter, K., Meissner, T., Lindner, M., Ensenauer, R., Santer, R., et al. (2013). Propionic acidemia: clinical course and outcome in 55 pediatric and adolescent patients. Orphanet Journal of Rare Diseases *8*, 6.

van Hagen, C.C., Weber, E.C., van den Hurk, Th.A.. M., Oudshoorn, J.H., Dorland, L., Berger, R., and de Koning, T. (2004). Energy expenditure in patients with propionic and methylmalonic acidaemias. J Inherit Metab Dis *27*, 111–112.

Harper, A. (1984). Branched-Chain Amino Acid Metabolism. Annual Review of Nutrition *4*, 409–454.

Harper, A.E., Miller, R.H., and Block, K.P. (1984). Branched-Chain Amino Acid Metabolism. Annual Review of Nutrition *4*, 409–454.

Hauser, N.S., Manoli, I., Graf, J.C., Sloan, J., and Venditti, C.P. (2011). Variable dietary management of methylmalonic acidemia: metabolic and energetic correlations. The American Journal of Clinical Nutrition *93*, 47–56.

Hoerr, R.A., Yu, Y.-M., Wagner, D.A., Burke, J.F., and Young, V.R. (1989). Recovery of 13C in breath from NaH13CO3 infused by gut and vein: effect of feeding. American Journal of Physiology-Endocrinology And Metabolism *257*, E426–E438.

Humayun, M.A., Elango, R., Ball, R.O., and Pencharz, P.B. (2007). Reevaluation of the protein requirement in young men with the indicator amino acid oxidation technique. Am J Clin Nutr *86*, 995–1002.

Institute of Medicine (2005). Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Washington DC: The National Academy Press).

Jameson, E., and Morris, A.A.M. (2011). Nutrition in metabolic disease. Paediatrics and Child Health *21*, 401–405.

Jana, Heringer, Lund, A.M., Wijburg, F.A., Freisinger, P., Barić, I., Baumgartner, M.R., Burgard, P., Burlina, A.B., Chapman, K.A., i Saladelafont, E.C., et al. (2016). Impact of age at onset and newborn screening on outcome in organic acidurias. J Inherit Metab Dis *39*, 341–353.

Jurecki, E., Ueda, K., Frazier, D., Rohr, F., Thompson, A., Hussa, C., Obernolte, L., Reineking, B., Roberts, A.M., Yannicelli, S., et al. (2019). Nutrition management guideline for propionic acidemia: An evidence- and consensus-based approach. Molecular Genetics and Metabolism *126*, 341–354.

K.A, S., Keating, B., and Keating, G.M. (2007). Blick-Keating2007_Article_Lisdexamfetamine.pdf.

Kaplan, A.S., Zemel, B.S., Neiswender, K.M., and Stallings, V.A. (1995). Resting energy expenditure in clinical pediatrics: Measured versus prediction equations. The Journal of Pediatrics *127*, 200–205.

Komar, B., Schwingshackl, L., and Hoffmann, G. (2015). Effects of leucine-rich protein supplements on anthropometric parameter and muscle strength in the elderly: A systematic review and meta-analysis. J Nutr Health Aging *19*, 437–446.

Kriengsinyos, W., Wykes, L.J., Goonewardene, L.A., Ball, R.O., and Pencharz, P.B. (2004). Phase of menstrual cycle affects lysine requirement in healthy women. American Journal of Physiology-Endocrinology and Metabolism *287*, E489–E496.

Lanpher, B., Brunetti-Pierri, N., and Lee, B. (2006). Inborn errors of metabolism: the flux from Mendelian to complex diseases. Nature Reviews Genetics *7*, 449–459.

Le Boucher, J., Charret, C., Coudray-Lucas, C., Giboudeau, J., and Cynober, L. (1997). Amino Acid Determination in Ciological Fluids by Automated Ion-Exchange Chromatography: Performance of Hitachi L-8500A.

Leonard, J.V. (1997). Stable isotope studies in propionic and methylmalonic acidaemia. Eur J Pediatr *156*, S67–S69.

Lui, J.C., Garrison, P., and Baron, J. (2015). Regulation of body growth: Current Opinion in Pediatrics *27*, 502–510.

Mager, D.R., Wykes, L.J., Ball, R.O., and Pencharz, P.B. (2003). Branched-chain amino acid requirements in school-aged children determined by indicator amino acid oxidation (IAAO). The Journal of Nutrition *133*, 3540–3545.

Mager, D.R., Wykes, L.J., Roberts, E.A., Ball, R.O., and Pencharz, P.B. (2006). Branched-chain amino acid needs in children with mild-to-moderate chronic cholestatic liver disease. The Journal of Nutrition *136*, 133–139.

Mak, C.M., Lee, H.-C.H., Chan, A.Y.-W., and Lam, C.-W. (2013). Inborn errors of metabolism and expanded newborn screening: review and update. Critical Reviews in Clinical Laboratory Sciences *50*, 142–162.

Manoli, I., Myles, J.G., Sloan, J.L., Shchelochkov, O.A., and Venditti, C.P. (2016). A critical reappraisal of dietary practices in methylmalonic acidemia raises concerns about the safety of medical foods. Part 1: isolated methylmalonic acidemias. Genetics in Medicine *18*, 386–395.

Mardach, R., Verity, M.A., and Cederbaum, S.D. (2005). Clinical, pathological, and biochemical studies in a patient with propionic acidemia and fatal cardiomyopathy. Molecular Genetics and Metabolism *85*, 286–290.

van der Meer, S.B., Poggi, F., Spada, M., Bonnefont, J.P., Ogier, H., Hubert, P., Depondt, E., Rapoport, D., Saudubray, J.M., Rabier, D., et al. (1996). Clinical outcome and long-term management of 17 patients with propionic acidaemia. Eur J Pediatr *155*, 205–210.

Moammar, H., Cheriyan, G., Mathew, R., and Al-Sannaa, N. (2010). Incidence and patterns of inborn errors of metabolism in the Eastern Province of Saudi Arabia, 1983-2008. Annals of Saudi Medicine *30*, 271–277.

Molema, F., Gleich, F., Burgard, P., van der Ploeg, A.T., Summar, M.L., Chapman, K.A., Lund, A.M., Rizopoulos, D., Kölker, S., Williams, M., et al. (2019a). Decreased plasma l-arginine levels in organic acidurias (MMA and PA) and decreased plasma branched-chain amino acid levels in urea cycle disorders as a potential cause of growth retardation: Options for treatment. Molecular Genetics and Metabolism *126*, 397–405.

Molema, F., Gleich, F., Burgard, P., van der Ploeg, A.T., Summar, M.L., Chapman, K.A., Barić, I., Lund, A.M., Kölker, S., Williams, M., et al. (2019b). Evaluation of dietary treatment and amino acid supplementation in organic acidurias and urea-cycle disorders: On the basis of information from a European multicenter registry. J Inherit Metab Dis.

Myles, J.G., Manoli, I., and Venditti, C.P. (2018). Effects of medical food leucine content in the management of methylmalonic and propionic acidemias: Current Opinion in Clinical Nutrition and Metabolic Care *21*, 42–48.

Nasser, M., Javaheri, H., Fedorowicz, Z., and Noorani, Z. (2012). Carnitine supplementation for inborn errors of metabolism. In Cochrane Database of Systematic Reviews, The Cochrane Collaboration, ed. (Chichester, UK: John Wiley & Sons, Ltd), p.

Nyhan, W.L., Fawcett, N., Ando, T., Rennert, O.M., and Julius, R.L. (1973). Response to dietary therapy in B12 unresponsive methylmalonic acidemia. Pediatrics *51*, 539–548.

Office of Regulatory Affairs Medical Foods Guidance Documents & Regulatory Information.

de Onis, M., and Blössner, M. (1997). WHO Global Database on Child Growth and Malnutrition. 74.

Phyllis B.Acosta, and Steven Yannicelli (2001). The Ross Metabolic Formula System.Nutrition Support Protocol.

Rasmussen, B., Gilbert, E., Turki, A., Madden, K., and Elango, R. (2016). Determination of the safety of leucine supplementation in healthy elderly men. Amino Acids *48*, 1707–1716.

Riazi, R., Wykes, L.J., Ball, R.O., and Pencharz, P.B. (2003a). The Total Branched-Chain Amino Acid Requirement in Young Healthy Adult Men Determined by Indicator Amino Acid Oxidation by Use of l-[1-13C] Phenylalanine1. The Journal of Nutrition *133*, 1383–1389.

Riazi, R., Rafii, M., Wykes, L.J., Ball, R.O., and Pencharz, P.B. (2003b). Valine May Be the First Limiting Branched-Chain Amino Acid in Egg Protein in Men. The Journal of Nutrition *133*, 3533–3539.

Riazi, R., Rafii, M., Clarke, J.T.R., Wykes, L.J., Ball, R.O., and Pencharz, P.B. (2004). 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. American Journal of Physiology - Endocrinology and Metabolism *287*, E142–E149.

Sass, J.O., Hofmann, M., Skladal, D., Mayatepek, E., Schwahn, B., and Sperl, W. (2004). Propionic Acidemia Revisited: A Workshop Report. Clinical Pediatrics *43*, 837–843.

Saudubray, J.-M., Sedel, F., and Walter, J.H. (2006). Clinical approach to treatable inborn metabolic diseases: An introduction. Journal of Inherited Metabolic Disease *29*, 261–274.

Schulze, A., Lindner, M., Kohlmuller, D., Olgemoller, K., Mayatepek, E., and Hoffmann, G.F. (2003). Expanded Newborn Screening for Inborn Errors of Metabolism by Electrospray Ionization-Tandem Mass Spectrometry: Results, Outcome, and Implications. PEDIATRICS *111*, 1399–1406.

Shchelochkov, O.A., Carrillo, N., and Venditti, C. (1993). Propionic Acidemia. In GeneReviews(®), M.P. Adam, H.H. Ardinger, R.A. Pagon, S.E. Wallace, L.J. Bean, H.C. Mefford, K. Stephens, A. Amemiya, and N. Ledbetter, eds. (Seattle (WA): University of Washington, Seattle), p.

Shimomura, Y., Obayashi, M., Murakami, T., and Harris, R.A. (2001). Regulation of branched-chain amino acid catabolism: nutritional and hormonal regulation of activity and expression of the branched-chain α-keto acid dehydrogenase kinase: Current Opinion in Clinical Nutrition and Metabolic Care *4*, 419–423.

Stephens, T.V., Payne, M., Ball, R.O., Pencharz, P.B., and Elango, R. (2015). Protein Requirements of Healthy Pregnant Women during Early and Late Gestation Are Higher than Current Recommendations. J. Nutr. *145*, 73–78.

Sugiyama, N., Kidouchi, K., Kobayashi, M., and Wada, Y. (1990). Carnitine Deficiency in Inherited Organic Acid Disorders and Reye Syndrome. Pediatr Int *32*, 410–416.

Sutton, V.R., Chapman, K.A., Gropman, A.L., MacLeod, E., Stagni, K., Summar, M.L., Ueda, K., Mew, N.A., Franks, J., Island, E., et al. (2012). Chronic management and health supervision of individuals with propionic acidemia. Molecular Genetics and Metabolism *105*, 26–33.

Thorpe, J.M., Roberts, S.A., Ball, R.O., and Pencharz, P.B. (1999). Prior Protein Intake May Affect Phenylalanine Kinetics Measured in Healthy Adult Volunteers Consuming 1 g Protein · kg−1 · d−1. The Journal of Nutrition *129*, 343–348.

Torres, N., López, G., De Santiago, S., Hutson, S.M., and Tovar, A.R. (1998). Dietary Protein Level Regulates Expression of the Mitochondrial Branched-Chain Aminotransferase in Rats. The Journal of Nutrition *128*, 1368–1375.

Touati, G., Valayannopoulos, V., Mention, K., de Lonlay, P., Jouvet, P., Depondt, E., Assoun, M., Souberbielle, J.C., Rabier, D., Ogier de Baulny, H., et al. (2006). Methylmalonic and propionic acidurias: Management without or with a few supplements of specific amino acid mixture. Journal of Inherited Metabolic Disease *29*, 288–298.

Tsikas, D., Wolf, A., and Jürgen C, F. (2004). Simplified HPLC Method for Urinary and Circulating Creatinine.

Uauy, R. (2013). Improving Linear Growth without Excess Body Fat Gain in Women and Children. Food Nutr Bull *34*, 257–260.

Vernon, H.J. (2015). Inborn Errors of Metabolism: Advances in Diagnosis and Therapy. JAMA Pediatrics *169*, 778.

Waters, D., Adeloye, D., Woolham, D., Wastnedge, E., Patel, S., and Rudan, I. (2018). Global birth prevalence and mortality from inborn errors of metabolism: a systematic analysis of the evidence. Journal of Global Health *8*, 021102.

Wessels, A.G., Kluge, H., Hirche, F., Kiowski, A., Schutkowski, A., Corrent, E., Bartelt, J., König, B., and Stangl, G.I. (2016). High Leucine Diets Stimulate Cerebral Branched-Chain Amino Acid Degradation and Modify Serotonin and Ketone Body Concentrations in a Pig Model. PLoS ONE *11*, e0150376.

Yannicelli, S. (2006). Nutrition therapy of organic acidaemias with amino acid-based formulas: Emphasis on methylmalonic and propionic acidaemia. Journal of Inherited Metabolic Disease *29*, 281–287.

Yannicelli, S., Acosta, P.B., Velazquez, A., Bock, H.-G., Marriage, B., Kurczynski, T.W., Miller, M., Korson, M., Steiner, R.D., Rutledge, L., et al. (2003). Improved growth and nutrition status in children with methylmalonic or propionic acidemia fed an elemental medical food. Molecular Genetics and Metabolism *80*, 181–188.

Yorifuji, T., Kawai, M., Muroi, J., Mamada, M., Kurokawa, K., Shigematsu, Y., Hirano, S., Sakura, N., Yoshida, I., Kuhara, T., et al. (2002). Unexpectedly high prevalence of the mild form of propionic acidemia in Japan: presence of a common mutation and possible clinical implications. Hum Genet *111*, 161–165.

Zayed, H. (2015). Propionic acidemia in the Arab World. Gene *564*, 119–124.

Zello, G.A., Wykes, L.J., Ball, R.O., and Pencharz, P.B. (1995). Recent advances in methods of assessing dietary amino acid requirements for adult humans. J. Nutr. *125*, 2907–2915.

(2007). Protein and amino acid requirements in human nutrition: report of a joint WHO/FAO/UNU Expert Consultation ; [Geneva, 9 - 16 April 2002] (Geneva: WHO).

Appendices

Appendix A : Subject Consent Form

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PARTICIPANT INFORMATION AND CONSENT FORM

Determining the Optimal Ratio of Branched-Chain Amino Acids (BCAA) in Medical Foods for Methylmalonic and Propionic Acidemias (MMA/PROP)

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Site: Oak Street Campus, UBC
Child & Family Research Institute

1. INVITATION

If you are a parent or legal guardian of a child who may take part in this study, permission from you and the assent (agreement) of your child may be required. When we say "you" or "your" in this consent form, we mean you and/or your child; "we" means the researchers and other staff.

You are being invited to take part in this research study because you are a healthy individual between the ages of 6-10 years, free of any medical condition and are currently free from fever or having a cold. You will act as a control participant for this research study. This study is being conducted as part of Haneen Saleemani's MSc dissertation research.

2. YOUR PARTICIPATION IS VOLUNTARY

Your participation is entirely voluntary, so it is up to you to decide whether or not to take part in the study. Before you decide, it is important for you to understand what the research involves. This consent form will describe the study, why the research is being done, what will happen to you during the study and the possible benefits, risks, and discomforts. If you wish to participate, you will have a minimum of 7 days to sign the forms, this will give you enough time to consult with your family, friends, and physician if you want to. If you decide to take part in this study, you are still free to withdraw at any time and without giving any reasons for your decision(s). If you do not wish to participate, you do not have to provide any reason for your decision(s) nor will you lose the benefit of any medical care to which you are entitled or are presently receiving. Please take time to read the following information carefully and to discuss it with your child, family and friends before deciding.

3. WHO IS CONDUCTING THE STUDY?

The Principal Investigator [*Rajavel Elango*] will receive financial compensation from the sponsor [*Saudi Arabian Cultural Bureau*] for the work required in doing this clinical research and/or for providing advice on the design of the study/travel expenses/etc. Financial compensation to researchers for conducting the research is associated with obligations defined in a signed contractual agreement between the researchers and the sponsor. Researchers must serve the interests of the participant and also abide by their contractual obligations. For some, the payment of financial compensation to the researchers can raise the possibility of a conflict of interest. You are entitled to request any details concerning this compensation from the Principal Investigator.

4. BACKGROUND

Methylmalonic and propionic acidemias (MMA/PROP) are diseases caused by some enzymes (chemical substances in our cells which help breakdown food) not working properly. They are caused by a defect in the enzymes responsible for the degradation of some essential amino acids. These essential amino acids include, isoleucine, valine, methionine, and threonine. Our body cannot make these amino acids and we need to get them from food that has proteins (meat, dairy, cereals, and legumes).

Children with MMA/PROP cannot tolerate large amount of protein foods. To help these children meet their energy and protein requirements, special medical foods are used as a supplementation. But these medical foods contain imbalanced ratio of three essential amino acids called branched-chain amino acids BCAA (leucine, isoleucine, and valine). This imbalanced content of the BCAA is affecting the children's protein synthesis and growth. With the results in this study, we hope to determine a balanced ratio of these three amino acids to be added to the medical food.

5. WHAT IS THE PURPOSE OF THE STUDY?

The purpose of the study is to determine the optimal ratio of BCAA in six healthy school-aged children (6-10 years; 3 girls: 3 boys). This will help us to determine the optimal BCAA ratio in medical foods specially formulated for MMA/PROP children to ensure protein synthesis.

6.WHO CAN PARTICIPATE IN THE STUDY?

- ☐ Healthy children between 6 and 10 years of age
- ☐ Normal weight and normal eating habits.
- ☐ English speaking children

7.WHO SHOULD NOT PARTICIPATE IN THE STUDY?

- ☐ Children under 6 years old, or over 10 years old.
- ☐ Children who are currently ill, with a fever, cold, vomiting or diarrhea.
- ☐ Children outside of normal weight parameters (3^{rd}-85^{th} percentiles for weight according to Dieticians of Canada).
- ☐ Children with claustrophobia.
- ☐ Children currently or recently taking medication or antibiotics.
- ☐ Children with food allergies.
- ☐ Children who cannot speak, write, and read in English

8. WHAT DOES THE STUDY INVOLVE?

This study will be conducted at Clinical Research Evaluation Unit (CREU), Child and Family Research Institute (CFRI). If you agree to participate in this study, then you will be asked to complete the study procedures described below. You may participate in 6 separate study days and 1 pre-study visit (1 hour). Study days can take place on non-school days, weekends, holidays, during the summer break, or as per your convenience.

If you decide to join this study:

a- Preliminary Study Day Procedures:

- ☐ The preliminary assessment is done to collect basic information about you, make sure you are informed about the study details, and to collect information about you to design the study diet specifically to meet your body needs.

- ☐ The preliminary assessment will be conducted at the Clinical Research Evaluation Unit (CREU) at the Child & Family Research Institute located in BC Children's Hospital. You will be asked to come at 8AM after having fasted overnight (10-12h). The whole procedure will take 1 hour to complete.

- ☐ During the preliminary assessment, a Research Assistant will measure your weight, height, body fat and muscle mass, and resting metabolic rate, which tells us how much energy your body needs. Body muscle will be measured using bioelectrical impedance which measures the passage of a small, safe amount of current (that cannot be felt) between four electrodes on the arms and legs while you lay still for a few minutes. The muscle measurements are completely safe and do not cause any discomfort or harm. Metabolic rate, which is the amount of energy used by the body is measured using an indirect calorimetry machine. This machine tells us the energy needed by the body, which we measure by the amount of oxygen you breathe in and the carbon dioxide

you breathe out. It consists of a clear hood that is placed over your head while you lay on a bed, breathing normally. You can see everything through the hood and breathe normally without any discomfort. This measurement takes about 20-30 minutes to complete. If you want to stop the test anytime, you can just pull the hood away. This test measures how much carbon dioxide you produce.

- ☐ You will also be asked health related questions to assess your medical history.

- ☐ You will also be provided a 3-day food record sheet to fill out and return. This consists of recording the food you eat for any 2 days during the week and 1 day on the weekend.

b- Study Day Procedures:

If you agree to participate in this study, then the following procedures will be followed:

- ☐ For the two days before each study day, we will recommend how much protein you should eat from foods. This will consist of foods typically consumed by you and will be developed based on your reported food intake from the above described 3-day food record. This diet is made to ensure that you have enough protein in your diet before our study day

- ☐ The study will be conducted in the Clinical Research Evaluation Unit (CREU) at the Child & Family Research Institute located in BC Children's Hospital. You will be asked to come at 8AM after an overnight fast of about 12 hours.

- ☐ Only water may be consumed prior to arriving on the study day, and during the study day. The study day test diet as described below will provide your child's daily energy and nutritional needs. At the end of the study day, your child's is free to consume his/her normal food intake.

- ☐ A Research Assistant will measure your weight and height.

- ☐ You will eat the test liquid diet as eight small hourly meals on the study day. Each meal is made up of 1) a mixture of amino acids, 2) an amino acid-free flavored liquid and amino acid-free cookies that provide energy and other nutrients, and 3) the labeled amino acid is added to the last four meals. This labeled amino acid is called stable isotope, which is colorless, odorless, tasteless, and is completely safe; they are present in the air we breathe, water we drink, and food we eat. Amino acids are made of mostly ^{12}C, a kind of carbon, however the isotope tracer contains ^{13}C, a different kind of carbon. The tracer can be detected in breath and urine samples with special equipment because it looks different than the rest of the amino acids in the body. You will take them with your meals to help us measure your total protein synthesis.
- ☐ The test meals will meet all your daily energy, vitamin and mineral needs.

- ☐ To measure how your body responds to the test diet we will collect your breath sample

9 times, and urine 3 times during the study day. To collect breath, you will have to breathe into a container – just like blowing through a straw into a bag. To collect urine, you will have to pass urine into a urine sample hat in the privacy of the washroom.

- ☐ The Research Assistant will also measure the rate at which you are breathing out carbon dioxide (VCO_2) using an indirect calorimetry machine. This will require you to relax on the examination table while a clear hood/canopy is placed on your face and head. This has room air freely flowing in and out. You can watch television/DVD player during the testing. The entire test will take about 20-30 minutes.

- ☐ You are invited to participate in 6 study days separated by at least 1 week (7 days) so the total study time will be at least 6 weeks.

- ☐ In total, you can expect to dedicate approximately 8 hours per study day you participate in. Parents can stay with the child in the Clinical Research Evaluation Unit (CREU) during the study period if they wish to, but they won't have to stay as there will be research assistants keeping the child company all the time. You are invited to participate in all 6 studies. If you choose to participate in all 6 studies, you will be asked to dedicate approximately 48 hours to this project.

8. WHAT ARE MY RESPONSIBILITIES?

In order to participate in this study:

- ☐ Participants must meet all inclusion criteria.
- ☐ On study days, only consume the test diet during study hours.
- ☐ Make sure you are fasted before each study day.
- ☐ Let us know of any negative food or health related events.

10. WHAT ARE THE POSSIBLE HARMS AND SIDE EFFECTS OF PARTICIPATING IN THIS STUDY?

The test requires you to consume a small dose of isotope. This is a safe form of isotope and is widely used in medical research even in children and pregnant women. During the indirect calorimetry test you are requested to lie down for 20-30minutes with an open hood/canopy over the face and head. Some children may feel uncomfortable during this period. The hood/canopy has outside air freely moving in and out. If you are uncomfortable, then the test will be stopped immediately. Our liquid test diet may cause a stomach upset, which usually goes away after one meal.There are no other known risks involved with participating in this research. We recognize that the length of each study day may pose an inconvenience to you.

11. WHAT ARE THE BENEFITS OF PARTICIPATING IN THIS STUDY?

There are no direct benefits to participating in the study. The test may help us determine the optimal ratio of BCAA in the medical foods specially formulated for children with MMA/PROP.

We hope that the results from this study can also be used in the future to improve management of patients with MMA/PROP.

12. WHAT HAPPENS IF I DECIDE TO WITHDRAW MY CONSENT TO PARTICIPATE IN THIS STUDY?

You may withdraw from this study at any time without giving reasons. If you choose to enter the study and then decide to withdraw at a later time, you have the right to request the withdrawal of your information collected during the study. This request will be respected to the extent possible. Please note however that there may be exceptions where the data will not be able to be withdrawn for example where the data is no longer identifiable (meaning it cannot be linked in any way back to your identity) or where the data has been merged with other data. If you would like to request the withdrawal of your data, please let your study researcher know. If your participation in this study includes enrolling in any optional studies, or long-term follow-up, you will be asked whether you wish to withdraw from these as well.

13. CAN I BE ASKED TO LEAVE THE STUDY?

If you are not able to meet the requirements of the study, the study researcher may withdraw you from the study. If you are asked to leave the study, the reasons for this will be explained to you and you will have the opportunity to ask questions about this decision.

14. WHAT WILL THE STUDY COST ME?

Participation in the study will not cost you anything. In appreciation of the time that it takes to complete this study you will receive $100 upon each study day completion to a maximum of $600 for 6 study days. Each participant will receive $100 upon completion of each study day, and they will not be asked to refund the money if they decided to withdraw afterward. Vehicle parking coupons or transit fare will also be provided depending on method of transportation. Receiving compensation of $500 or more is taxable and you will therefore be issued a T4A form. In order to issue the T4A form we will collect your SIN number. The SIN number will be used for tax purposes only and will be stored in a locked secure area separate from your study data. In addition, your SIN number will be given to the UBC financial department in order to compensate you and generate the T4A form.

15. WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

Your confidentiality will be respected. However, research records and medical records identifying you may be inspected in the presence of the Investigator or the University of British Columbia – Children's & Women's Health Centre of BC Research Ethics Board for the purpose of monitoring the research. No information or records that disclose your identity will be published without your consent, nor will any information or records that disclose your identity be removed or released without your consent unless required by law.

You will be assigned a unique study number as a participant in this study. Only this number will be used on any research-related information collected about you during the course of this study, so that your identity [i.e. your name or any other information that could identify you] as a participant in this study will be kept confidential. Information that contains your identity will remain only with the Principal Investigator and/or designate. The list that matches your name to the unique identifier that is used on your research-related information will not be removed or released without your consent unless required by law.

Your rights to privacy are legally protected by federal and provincial laws that require safeguards to ensure that your privacy is respected and also give you the right of access to the information about you that has been provided to the sponsor and, if need be, an opportunity to correct any errors in this information. Further details about these laws are available on request to your study doctor. Signing this consent form in no way, limits your legal rights against the sponsor, investigators, or anyone else.

16. WHO DO I CONTACT IF I HAVE QUESTIONS ABOUT THE STUDY DURING MY PARTICIPATION?

If you have any questions or desire further information about this study before or during participation, or if you experience any adverse effects, you can contact Dr. Rajavel Elango at 604-875-2000 ext. 4911, or his research assistant Haneen Saleemani at 604-875-2000 x4607.

17. WHO DO I CONTACT IF I HAVE QUESTIONS OR CONCERNS ABOUT MY/MY CHILD'S RIGHTS AS A PARTICIPANT DURING THE STUDY?

If you have any concerns or complaints about your rights as a research participant and/or your experiences while participating in this study, contact the Research Participant Complaint Line in the University of British Columbia Office of Research Ethics by e-mail at RSIL@ors.ubc.ca or by phone at 604-822-8598 (Toll Free: 1-877-822-8598). Please reference the study number (H18-00439) when contacting the Complaint Line so the staff can better assist you.

18. PARTICIPANT CONSENT TO PARTICIPATE
Determining the Optimal Ratio of Branched-Chain Amino Acids (BCAA) in Medical Foods for Methylmalonic and Propionic Acidemias (MMA/PROP)

My signature on this consent form means that I:

- ☐ have had this study explained to me and my child, read this form and understand the information concerning this study.
- ☐ have had sufficient time to consider the information provided, get advice and ask questions if necessary and I/my child have received satisfactory responses to our questions.
- ☐ understand that all of the information collected will be kept confidential and that the results will only be used for scientific objectives.
- ☐ understand that my/my child's participation in this study is voluntary and that I/my child am/is completely free to refuse to participate or to withdraw from this study at any time without giving any reason(s) and my/my child's decision to withdraw will not change in any way the quality of care that I/my child receive.
- ☐ understand that signing this consent form in no way limits my/my child's legal rights against the sponsor, investigators or anyone else.
- ☐ understand that there is no guarantee that this study will provide any benefits to myself/my child.
- ☐ understand that if I have any further questions or desire further information I should contact Dr. Rajavel Elango at [redacted] or email [redacted]
- ☐ have been told that I will receive a dated and signed copy of this form for my own record.

The parent(s)/guardian(s)/substitute decision-maker (legally authorized representative) and the investigator are satisfied that the information contained in this consent form was explained to the child/participant to the extent that he/she is able to understand it, that all questions have been answered, and that the child/participant assents to participating in the research.
I will receive a signed copy of this consent form for my own records.

I consent to participate in this study.

_____	_____	_____
Parent/Guardian Signature	Parent/Guardian Printed Name	Date

_____	_____	_____	_____
Signature of Person Obtaining Consent	Printed Name	Study Role	Date

Investigator Signature

_____	_____	_____
Investigator Signature	Printed name	Date

My signature above signifies that the study has been reviewed with the study participant by me and/or by my delegated staff. My signature may have been added at a later date, as I may not have been present at the time the participant's signature was obtained.

I consent to be contacted in the future, regarding participation in additional studies.
Yes [] No []

Appendix B : Subject Assent Form

Department of Pediatrics
950 West 28th Avenue, Room 170A
Vancouver, BC, V5Z 4H4
Tel: (604) 875-2000 x4911 Fax: (604) 875-3597

PARTICIPANT ASSENT FORM (7-10 years of age)

Determining the Optimal Ratio of Branched-Chain Amino Acids (BCAA) in Medical Foods for Methylmalonic and Propionic Acidemias (MMA/PROP)

1. INVITATION

I am being invited to be part of a research study. No one will make me be part of the study. Even if I agree now to be part of the study, I can change my mind later. No one will be mad at me if I choose not to be part of this study.

2. WHY ARE WE DOING THIS STUDY

Protein in our body is made of different amino acids (building blocks of protein). There are 3 amino acids called, "Branched chain amino acids" or "BCAA". Our body cannot make these amino acids and we need to get them from food that has protein (like - meat, milk, wheat, and chickpeas). Healthy children can eat these foods to help them grow. However, children with a disease called Methylmalonic and Propionic Acidemias MMA/PROP cannot eat lots of protein foods. To help these children special medical foods are available. But these medical foods do not have a good balance of the BCAA. So children do not grow well. With the help of this study, we hope to find out a good balance of BCAA to be added to the medical food.

3. WHAT WILL HAPPEN IN THIS STUDY?

Pre-Study day visit

- ✓ I will have to come to the Clinical Research Evaluation Unit (CREU) without eating anything for 12 hours.
- ✓ I will have to come one time for 1 hour before I start the study.
- ✓ At that time a researcher will measure my weight and height.
- ✓ How much muscle I have will be measured. This test will only take 30 seconds to 1 minute. While I lie down on a bed two wires will be put on my right hand and foot, like Band-Aids. I will not feel anything during this test.
- ✓ While I lie down on a bed, a clear hood (like a space-man's suit) will be placed on my head. This is a clear hood with air coming freely in and out. I can watch TV during the testing. This test will take about 20-30 minutes. If I want to stop the test anytime, I can just pull the hood away. This test measures how much carbon dioxide I produce. Carbon dioxide is a gas we produce when we breathe air.
- ✓ My parents can stay with me in the Clinical Research Evaluation Unit (CREU) during the study.

Study day visits

- ✓ I will have to come 6 times for the study days.
- ✓ Each of the 6 study visit will take all day (8 hours).
- ✓ Study days can take place on non-school days, weekends, holidays, or during the summer break.

- ✓ My parents can stay with me in the Clinical Research Evaluation Unit (CREU) during the study.
- ✓ I will drink a test liquid (1 small cup eight times during the day). The liquid drink will taste a bit sweet and a bit sour because it has pure protein powders and orange tang added in. Small cookies will be given with each liquid drink, which are regular cookies. The test liquid is very safe to drink.
- ✓ I will breathe into a container 9 times, like blowing through a straw into a bag.
- ✓ Urine samples (pee) will be collected in a container like a hat 3 times during the day.
- ✓ During the day I can watch television, listen to music, read, use a computer, play board games (Monopoly, Blockus, Connect4, Clue, Scrabble etc), card games (Uno, SkipBo, regular playing cards etc), color pictures (crayons, markers, coloring sheets will be provided), or do homework. I can also bring my electronic devices such as an iPad, as there will be wireless internet available.

4. WHO IS DOING THIS STUDY?

Dr. Rajavel Elango and his research assistant **Haneen Saleemani** will be doing this study. **Dr. Stockler** (Head, Division of Biochemical Diseases) is also involved. They will answer any questions I have about the study. I can also call them at [redacted] **(Dr. Elango)** or at [redacted] **(Haneen Saleemani)** or at [redacted] **(Dr. Stockler)**, if I am having any problems or if there is an emergency and I cannot talk to my parents.

5. CAN ANYTHING BAD HAPPEN TO ME?

There is nothing in this study, which will make anything bad happen to me. The drink on the study days is a bit sour and bit sweet and may make you feel like throwing up. We give lots of water and sugar cookies at the end of the drink to make you feel better.

6. WHAT SHOULD I DO IF I AM NOT FEELING WELL?

If I do not feel well, I should let the researchers Dr. Elango or Haneen Saleemani know immediately. I can also call the study supervisor **Dr. Elango** [redacted] **or Haneen Saleemani at** [redacted], anytime.

7. WHO WILL KNOW I AM IN THE STUDY?

Only the researchers who are involved in the study will know I am in it. When the study is finished, the researchers will write a report about what was learned. This report will not say my name or that I was in the study. My parents and I do not have to tell anyone I am in the study if we don't want to.

8. WHEN DO I HAVE TO DECIDE?

I have 7 days from today to decide if I want to be part of the study. When I come in for my next visit to the clinic at BC Children's Hospital I can be part of the study if I want to. I have also been asked to discuss my decision with my parents.

9. SIGNATURES

If I put my name at the end of this form, it means that I agree to be in the study.

_____	_____	_____
Printed Name	*Signature*	*Date*

Appendix C : Recruitment Poster

BC Children's Hospital Research Institute

Nutrition Research Study

Branched-Chain Amino Acids from Food in Children

UBC

Protein in our food is made of amino acids **(building blocks)**.Branched chain amino acids or BCAA are 3 amino acids (Valine, Leucine, Isoleucine) that are found in protein rich foods. Children with Methylmalonic and Propionic Acidemias do not have the special enzymes that breakdown two of these amino acids (valine and isoleucine). We are interested in measuring the correct balance of BCAA in the medical foods specially made for those children. We will study **healthy children** to test different ratios of BCAA which will help make protein in the body.

WE ARE LOOKING FOR:

- **Healthy children (6 -10years)**
- **Boys and girls**

Compensation parking, and transit passes will be offered

STUDY INCLUDES:

- Screening meeting to determine eligibility for 1 hour at BC children's hospital.
- 6 visits (8 hours each), where children will be given a special diet
  - Collection of 7 breath samples, and 3 urine samples.
  - Measuring body weight and muscle mass.

Principal Investigator

Dr. Rajavel Elango

Contact: [redacted]

Primary Contact

Haneen Saleemani

Contact: [redacted]

Appendix D : Pre-Study Day Assessment Form

Pre-Study Day Assessment
Determining the Optimal Ratio of Branched-Chain Amino Acids (BCAA) in Medical Foods

Principal Investigator: Dr. Rajavel Elango [redacted](office)
Student Investigator: Haneen Saleemani [redacted](office)
[redacted](cell)

PRELIMINARY ASSESSMENT

Subject ID: _____ Date: _____

Birthday: _____/_____ Age: _____ Gender: ___
(month) (year)
Height (cm): _____ Weight (kg): _____ BMI: _____

Bioelectrical Impedance Analysis

BIA:R_____ XC_____
(resistance) (reactance)

BIA: Z_____
(Impedance)

Body Composition Profile

% Body fat (BIA): _____ Lean body mass (BIA): _____

Indirect Calorimetry

Measured REE (kcal/day): _____ Estimated REE (kcal/day):_____

Daily energy requirement (kcal/day): _____

VCO2 (ml/min): _____ VO2 (ml/min): _____

RQ: _____

Medical History

Details of health condition(s) _____

Are you currently taking any prescription medications? Yes_____ No_____

List of medications: _____

Are you currently having vomiting episodes? Yes_____ How many/day _____ No_____

Are you currently having fever/cold? Yes_____ No_____

Nutritional Supplement Intake

Are you currently taking vitamins? Yes_____ No_____

Are you taking any other nutritional supplements? Yes_____ No_____

If yes, please list all nutritional supplements:

1._____ 3. _____

2._____ 4. _____

Activity Level

Daily exercise (minutes)_____

Sedentary_____ Moderate_____ High_____

Availability for 6 studies

Yes_____ No_____

Comments:

Appendix E : Study Day Form

Study Day Protocol
Determining the Optimal Ratio of Branched-Chain Amino Acids (BCAA) in Medical Foods

Subject ID: _____ Date:_____

Height (cm): _____ Weight (kg):_____

Protein intake (g/kg/d): _____ Energy intake (kcal/day): _____

Time	Sample Collection/ Anthropometry	Meals and isotope tracer	Comments
8:00		Meal #1	
9:00		Meal #2	
10:00		Meal #3	
11:00		Meal #4	
11:15	1st breath (3x) 1st urine		
11:30	2nd breath (3x)		
11:45	3rd breath (3x) 2nd urine		
12:00	VCO_2 measurement	Meal #5 – primer dose and 1st oral dose	
13:00		Meal #6 – 2nd oral dose	
14:00		Meal #7 – 3rd oral dose	
14:30	4th breath (3x)		
15:00	5th breath (3x)	Meal #8 – 4th oral dose **Collect breath 1st**	
15:15	6th breath (3x)		
15:30	7th breath (3x)		
15:45	8th breath (3x)		
16:00	9th breath (3x) 3rd urine		

Appendix F : Subject Code Form

Subject Code Master List

Determining the Optimal Ratio of Branched-Chain Amino Acids (BCAA) in Medical Foods

Subject Name	Code (Alpha-Numeric)	Comments

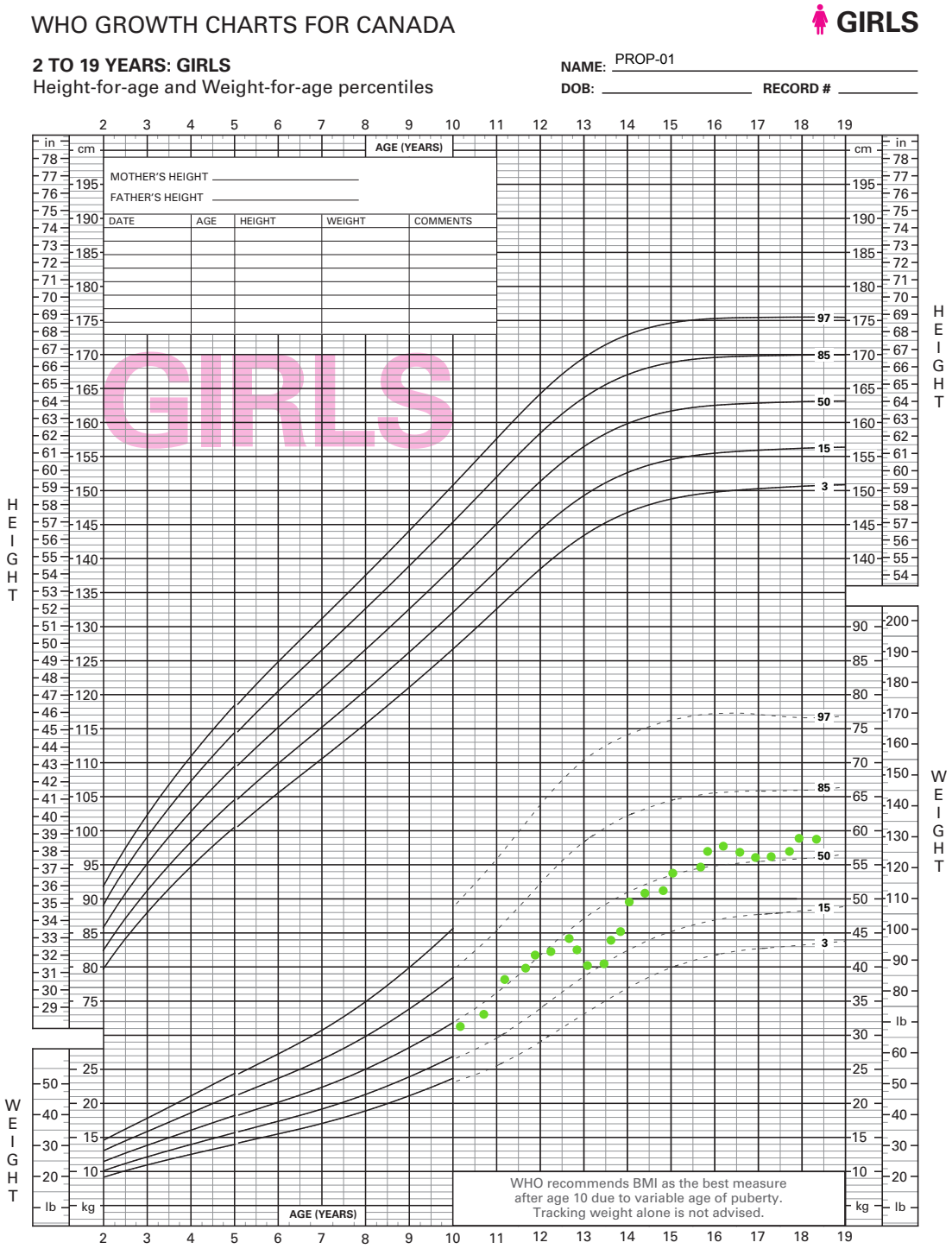
Appendix G : Dietary Record Sheets

Dietary Record Subject ID:_____

Date: ____/_____/_____ Mon Tues Wed Thu Fri Sat Sun

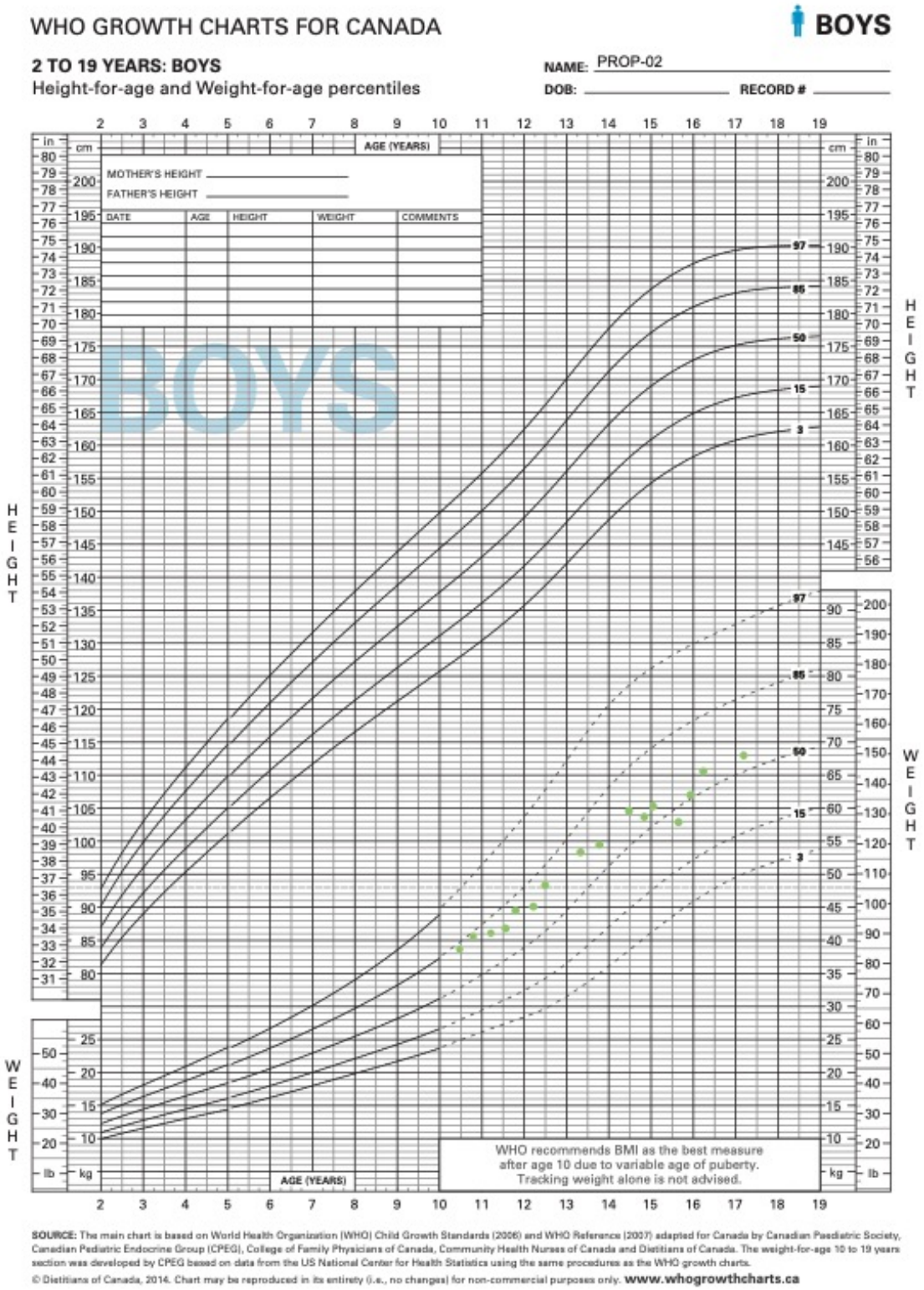
Item	Amount	Item	Amount
Breakfast		Snack	
Snack		Dinner	
Lunch			

Appendix H : WHO Growth Charts for Canada (PROP-01 Weight for Age >10 years)



SOURCE: The main chart is based on World Health Organization (WHO) Child Growth Standards (2006) and WHO Reference (2007) adapted for Canada by Canadian Paediatric Society, Canadian Pediatric Endocrine Group (CPEG), College of Family Physicians of Canada, Community Health Nurses of Canada and Dietitians of Canada. The weight-for-age 10 to 19 years section was developed by CPEG based on data from the US National Center for Health Statistics using the same procedures as the WHO growth charts.

Appendix I : WHO Growth Charts for Canada (PROP-02 Weight for Age >10 years)



Appendix J : WHO Growth Charts for Canada (PROP-03 Weight for Age >10 years)