The Impact of Dietary Protein on Appetite-Regulating Hormones and Energy Metabolism in Children with Prader-Willi Syndrome

by

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Abstract

Prader-Willi syndrome (PWS) is a unique model of childhood obesity characterized by disordered satiety. The excessive weight gain caused by an imbalance between energy intake and expenditure associated with PWS is of concern to healthcare professionals and caregivers who acknowledge that weight management is an essential but challenging aspect of care for these children. To improve the effectiveness of treatments to curb the development of obesity in PWS, a more comprehensive understanding of the underlying mechanisms associated with altered energy balance in these children is needed. Therefore, the overall objective of this research was (1) to determine the impact of food intake (FI), higher protein (HP) meals and standard meals (SM) on postprandial regulation of ghrelin and asprosin; (2) other satiety factor concentrations; and (3) energy balance in children with PWS.

In study 1, two test meals were compared to a SM meal in 10 children with PWS and 7 body mass index (BMI) z-score matched children in a randomized, crossover study design. The first test meal had a higher protein–lower carbohydrate (HP-LC) content and the second test meal had a higher protein–lower fat (HP-LF) content. Under fasting conditions, the PWS group had higher concentrations of both acyl ghrelin (AG) ($p = 0.02$) and desacyl ghrelin (DAG) than controls, but a comparable ratio of AG:DAG. AG and DAG were reduced in both groups following all meals, but concentrations of AG and DAG remained higher in PWS across all postprandial time points ($p = 0.002$ and $p < 0.001$, respectively). Glucagon-like peptide 1 (GLP-1) concentrations were higher after the HP-LC meal than the SM at 2 and 4 hours ($p = 0.027$ and $p = 0.044$, respectively) and at hour 4 ($p = 0.02$) following the HP-LF in the PWS group only; peptide tyrosine tyrosine (PYY) responses were comparable.

In study 2, fasting and 1 hour post-meal serum concentrations of asprosin were measured in 52 children, 23 with PWS, 8 with obesity, and 21 healthy weights. The decrease in serum asprosin relative to baseline was not different between children with PWS and BMI-

z score matched children. In children with PWS, fasting asprosin was positively correlated with AG and 1-hour postprandial asprosin was negatively correlated with insulin. Additionally, fasting asprosin was negatively correlated with age and insulin in children with obesity and with age in healthy weight children. After adjusting for age, sex and BMI z-score, asprosin showed a positive correlation with glucose in children with obesity but not in children with PWS or healthy weight children. In study 3, in a randomized, crossover study design, 5 youth with PWS were randomly allocated to two isocaloric arms: a) standard diet (SD); b) high-protein (HP) diet. Participants received the prescribed diets (three meals plus two snacks per day accompanied by either a powder supplement (HP) or an extra snack (SD) for one day prior to each study visit and a breakfast meal inside a whole-body calorimetry unit (WBCU). Resting energy expenditure (REE), postprandial energy expenditure (PEE) and respiratory exchange ratio (RER) were assessed. PEE calculated as "fixed REE" was higher after the HP meal compared to SM. A lower RER was observed after the HP diet in comparison to the SD (0.80 ± 0.2 vs 0.86 ± 0.2; $p < 0.009$). However, no significant difference in subjective appetite assessment between the HP meal and the SM was found.

The major findings of this research were that higher concentrations of total ghrelin in children with PWS were due to higher concentrations in both AG and DAG, with no change in the AG:DAG ratio. Meal consumption also suppressed both forms of ghrelin to a greater extent in children with PWS. Higher protein meals stimulated greater increases in GLP-1 and PYY in PWS children compared to controls. RER after the HP diet was significantly lower compared to SD. In addition, this research highlights the heterogeneity in PEE in youth with PWS in response to HP diet and will contribute to the conceptualization of further research exploring PEE; considering, sex, puberty statues and body composition factors that influence response to energy metabolism in children with and without PWS.

Preface

This preface is an overview of the work completed in partial fulfillment of the requirements of a Ph.D.; it is complemented by more detailed and extensive prefaces at the beginning of each chapter. Some of the research conducted for this thesis uses data that was previously collected by other researchers.

Data from the dietary macronutrient regulation of acyl and desacyl ghrelin concentrations in children with Prader-Willi Syndrome at the Pediatric Endocrinology clinic, Stollery Children's Hospital, Pediatric Centre for Weight and Health, and Edmonton General Continuing Care Centre (Edmonton, Alberta, Canada) was used in several chapters ('Study 1' in **Chapter 3**; 'Study 2' in **Chapter 4**). Research from that study was approved by the University of Alberta's Health Research Ethics Board: 'Dietary macronutrient regulation of active ghrelin levels in children with Prader-Willi Syndrome (PWS)' (ID: RES0003532).

Drs. Andrea Haqq and Michelle MacKenzie [Research Associate, Haqq's lab] wrote the grant that was funded by the Women Children Health Research Institute (WCHRI). Recruitment and data collection for **Chapter 3** was completed by Dr. Michelle MacKenzie and other members of the research team before I joined. I was responsible for data entry of assayed blood samples from this research and also wrote the first draft of the manuscript. Plasma samples for acyl ghrelin and desacyl ghrelin were assayed using a specific two-site assay in the lab of Dr. Gaylinn at the Department of Medicine, University of Virginia Health System (Charlottesville, Virginia, United States).

Data from 'Study 2' in **Chapter 4** was collected as part of several investigations led by Dr. Andrea Haqq and approved by the University of Alberta's Health Research Ethics Board: 'Autonomic nervous system activity and metabolic profiling in children with Prader-Willi Syndrome compared to controls' (ID: Pro00009903): 'Relationship between brain-derived neurotrophic factor (BDNF) concentration, BDNF haplotypes and neurocognitive performance in children with Prader-Willi Syndrome (PWS)' (ID: Pro00011653). Participants were recruited from the Pediatric Endocrinology clinic, Stollery Children's Hospital (Edmonton, Alberta, Canada), National Institutes of Health, Bethesda, (Bethesda, Maryland, United State) and the Pediatric Centre for Weight and Health, Edmonton General Continuing Care Centre, Child Health Clinic, Misericordia Hospital (Edmonton, Alberta, Canada). Data was collected by other individuals as part of an investigation I designed in consultation with my supervisor Dr. Andrea Haqq.

I conducted the research that generated data on resting energy expenditure, postprandial energy expenditure, subjective appetite assessment, body composition and anthropometrics used in **Chapter 5**. Research from this study was approved by the University of Alberta's Health Research Ethics Board: 'Assessing the impact of dietary protein on energy metabolism and appetite in children with Prader-Willi Syndrome: a pilot project' (ID: Pro00066276).

I wrote the first draft of the grant that was funded by WCHRI with ongoing discussion with Drs Michelle Mackenzie, Sarah Elliott, Carla Prado and Andrea Haqq (PI). Research from this study was used in **Chapters 3 and 4** ('Study 2 and Study 3') combined with research collected by other individuals.

All work presented in this thesis was critically assessed for intellectual content by my supervisors, Drs. Andrea Haqq and Carla Prado, supervisory committee member, Dr. Catherine Field, and external committee members, Dr. Sarah Cawsey and Dr. Nick Bellissimo. Versions of some chapters have led to submitted or published journal articles:

Alsaif, M., Elliot, S. A., MacKenzie, M. L., Prado, C. M., Field, C. J., & Haqq, A. M. (2017). Energy Metabolism Profile in Individuals with Prader-Willi Syndrome and Implications for Clinical Management: A Systematic Review. *Adv Nutr, 8*(6), 905-915. doi:10.3945/an.117.016253. Published Nov 7, 2017 Located in Chapter 2.

Alsaif, M., Pakseresht, M., Mackenzie, M. L., Gaylinn, B.,Thorner, M.O., Michael Freemark, M., Field, C. J., Prado, C. M & Haqq A. H. (2019). Dietary macronutrient regulation of acyl and desacyl ghrelin concentrations in children with Prader-Willi syndrome. Submitted to *Clinical Endocrinology.* Located in chapter 3.

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Besides my supervisors, I am also grateful to Dr. Catherine Field for her insightful comments and encouragement, but also for asking the hard questions that encouraged me to widen my research from various perspectives.

Thank you to Dr. Nick Bellissimo and Dr. Sarah Cawsey for taking the time to serve on my defense committee. Also to Drs. Justine Turner and Donna Vine for serving on my candidacy exam committee and for helping me throughout the process. Thank you Dr. Rene Jacobs for serving as my candidacy exam chair and Dr. Noreen Willows for serving as my defense chair.

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List of Abbreviations

AEBSF: esterase inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride

AEE: activity energy expenditure

AG: acylated ghrelin

AgRP: agouti-related peptide

ARC: hypothalamic arcuate

BC: body composition

BEE: basal energy expenditure

BIA: bioelectrical impedance analysis

BMI: body mass index

BodPod: air displacement plethysmography

CO_2: carbon dioxide

DAG: desacyl ghrelin

DEL: deletion

DIT: diet-induced thermogenesis

DLW: doubly labeled water

DXA: dual-energy x-ray absorptiometry

EE: energy expenditure

EI: energy intake

FFM: fat-free mass

FM: fat mass

GH: growth hormone

GLP-1: glucagon-like peptide 1

HD: hypothalamic damage

HMW: high molecular weight

HO: hypothalamic obesity

HOMA-IR: homeostatic model assessment insulin resistance

HP: high protein

HP-LC: higher protein-lower carbohydrate

HP-LF: higher protein-lower fat

IC: indirect calorimetry

kcal: kilocalorie

kg: kilogram

M: methylation

Magel2: MAGE-like 2

MRI: magnetic resonance imaging

NPY: neuropeptide tyrosine

O_2: oxygen

PEE: postprandial energy expenditure

POMC: proopiomelanocortin

PWS: Prader-Willi syndrome

PYY: peptide tyrosine tyrosine

REE: resting energy expenditure

RER: respiratory exchange ratio

SD: standard diet

SEE: sleeping energy expenditure

SM: standard meal

T2D: type 2 diabetes

TBK: total body potassium

TEE: total energy expenditure

TSH: thyroid-stimulating hormone

UPD: uniparental disomy

WBCU: whole-body calorimetry unit

WHO: World Health Organization

y: year

Chapter 1 Introduction

1.1 Thesis Organization

This thesis has been prepared as a paper format according to specifications provided by the Faculty of Graduate Studies and Research at the University of Alberta. Following Chapter 1, Chapter 2 provides a literature review, and Chapters 3, 4 and 5 are individual manuscripts. Parts of Chapter 2 have been published in *Advances in Nutrition* (Alsaif et al., 2017; 15;8(6):905-915.doi:10.3945/an.117.016253.). Chapter 3 is being submitted to the journal *Clinical Endocrinology*. Chapter 4 is being prepared for submission to *Pediatric Obesity*. Chapter 5 is being prepared for submission to *Metabolism: Clinical and Experimental*, with a brief description of each study. Chapter 6 is a summary and final discussion of the thesis.

1.2 Rationale

As a growing global epidemic, childhood obesity is a major health concern (WHO, 2019). According to the World Health Organization (WHO, 2019), in 2016, the worldwide number of children with overweight under the age of 5 was estimated to be over 41 million, and over 340 million children and adolescents aged 5 to 19 years presented with overweight or obesity. Overall, the prevalence of overweight and obesity among children and adolescents aged 5 to 19 years has risen dramatically from just 4% in 1975 to over 18% in 2016. Both boys and girls are similarly affected: in 2016, 18% of girls and 19% of boys presented with overweight (WHO, 2019). In Canada, one in seven 6- to 17-year-olds presented with overweight or obesity (Rao, Kropac, Do, Roberts, & Jayaraman, 2016). Children with overweight and obesity are likely to remain as adults with obesity and are more likely to develop non-communicable diseases such as diabetes and cardiovascular diseases at a younger age (Nadeau, Maahs, Daniels, & Eckel, 2011).

Treatments for childhood obesity have had limited success. To improve the effectiveness of treatment, a greater understanding of the interaction between environmental factors (e.g., sleep duration, sedentary behaviors, exercise, availability and cost of food and hedonic eating) and the genetic and neuroendocrine underpinnings of the disease is required. As obesity defined as unbalance between energy intake (EI) and energy expenditure (EE); currently, the critical underlying neuroendocrine factors that regulate energy balance are not fully understood (Hill, Wyatt, & Peters, 2012).

Genetic mutations or chromosomal abnormalities can lead to syndromic obesity (Haqq, 2010). One of the most well described forms of syndromic obesity is Prader-Willi syndrome (PWS). First described in 1956 and formerly known as Prader-Labhart-Willi syndrome, PWS is a rare genetic disorder in which multiple genes on the paternal chromosome 15 (q 11-13) are deleted or unexpressed (Bekx, Carrel, Shriver, Li, & Allen, 2003a). Prader-Willi syndrome occurs in 1 in 10,000 to 16,000 live-born infants, and is characterized by dysmorphic features, muscular hypotonia, short stature, low fat-free mass (FFM), cognitive delay, and behavioral abnormalities (Cassidy & Driscoll, 2009), which develops insidiously and is often the catalyst for the development of obesity in this population (Burman, Ritzen, & Lindgren, 2001). Excessive weight gain can be observed by the age of 2 years; and at ~3–5 years, obesity becomes conspicuous (Bekx, Carrel, Shriver, Li, & Allen, 2003b). Furthermore, as a result of profound obesity (many individuals weigh > 200% of their ideal body weight), obesity-related morbidity and mortality are high in this population (Coplin, Hine, & Gormican, 1976; Haqq, 2010; Schoeller, Levitsky, Bandini, Dietz, & Walczak, 1988).

Children with PWS have a different metabolic profile compared to children with other forms of obesity. Lower fasting insulin, lower homeostasis model assessment of insulin resistance (HOMA-IR) scores, and higher concentrations of fasting total and high molecular weight (HMW) adiponectin have been reported in children with PWS when compared to body mass index (BMI) z-scores and leptin concentrations of matched children (Haqq et al., 2007). In addition, children with PWS showed significantly higher fasting ghrelin concentrations and total peptide tyrosine tyrosine (PYY), an anorexigenic peptide secreted primarily from the intestinal mucosa of the ileum and large intestine, compared to children with non-syndromic obesity (Haqq et al., 2007). Therefore, children with PWS exhibit a surprisingly different endocrine profile than BMI z-score matched children.

Children with PWS have high fasting and postprandial concentrations of total ghrelin (Bizzarri et al., 2010). Ghrelin is an orexigenic peptide produced primarily in the enteroendocrine cells of the stomach (Levin et al., 2006). In contrast, total ghrelin l concentrations are suppressed in children with “exogenous” obesity or with obesity caused by mutations in leptin or the melanocortin-4 receptor (Cummings et al., 2002; DelParigi et al., 2002; Haqq et al., 2003). Serum ghrelin concentrations were negatively correlated with BMI z-scores in children with healthy weight and children with other forms of obesity, but not in children with PWS (Haqq, Farooqi, et al., 2003). Ghrelin was also negatively correlated with insulin concentrations in all control groups (Haqq, Farooqi, et al., 2003). Infants with PWS

who have not yet developed hyperphagia or obesity have median fasting total ghrelin concentrations similar to age- and sex-matched individuals; however, one-third of PWS subjects had ghrelin concentrations greater than the 95th percentile for ghrelin values in the control individuals (Haqq et al., 2008).

Ghrelin administration to humans or rodents stimulates food intake, leading to weight gain in a dose-dependent manner (Toshinai et al., 2007). In humans, circulating ghrelin concentrations demonstrate a diurnal rhythm, increasing during fasting and decreasing after meals; these findings suggest that ghrelin plays a role in meal initiation in humans and in long-term regulation of energy storage (Atalayer, Gibson, Konopacka, & Geliebter, 2013). Circulating ghrelin concentrations in humans also correlate inversely with BMI and are, therefore, low in individuals with obesity and high in individuals with healthy weight (Haqq, Farooqi, et al., 2003). During weight loss or in conditions associated with anorexia or cachexia, ghrelin concentrations rise as a counter-regulatory mechanism, presumably to increase food intake and restore body weight (Blauwhoff-Buskermolen et al., 2017). In addition, asprosin, a newly discovered hormone produced by the white adipose tissue, stimulates glucose production and is correlated with insulin resistance (Greenhill, 2016). Like ghrelin, asprosin acts as an orexigenic hormone, increasing after fasting and decreasing with food intake and using the same signalling pathways—namely, neuropeptide tyrosine (NPY)/agouti-related peptide (AgRP) (Beutler & Knight, 2018). Concentrations of asprosin have been reported to be higher in individuals with obesity, individuals with type 2 diabetes mellitus and women with polycystic ovary syndrome (Acara, Bolatkale, Kiziloglu, Ibisoglu, & Can, 2018; Li et al., 2018; Zhang, Chen, Zhou, Fu, & Cheng, 2019). To date, the concentrations of asprosin have not been elucidated in individuals with PWS.

The excessive weight gain associated with PWS is of concern to health-care professionals and caregivers who acknowledge that weight management is an essential but challenging aspect of care for individuals with PWS. To improve the effectiveness of treatments to curb the development of obesity in PWS, a more complete understanding of the underlying mechanisms associated with altered energy balance in these individuals is needed. Excess energy intake associated with insatiable hyperphagia would contribute to energy imbalance. However, the reported lower energy requirements of individuals with PWS to prevent excessive weight gain (Butler, 2006) suggests that their energy expenditure (EE) is lower than predicted. Therefore, weight maintenance in individuals with PWS typically requires ~60% of the recommended intake in the general population (Cassidy & Driscoll, 2009; Mihalache et al., 2016).

The weight gain and subsequent obesity associated with this condition is caused by a chronic imbalance between energy intake and EE (Coplin et al., 1976; Holm & Pipes, 1976). A number of factors are responsible for this imbalance in children with PWS, including hyperphagia, decreased physical activity, lower FFM, reduced metabolic rate and hormonal dysregulation (Butler, 2011). Consequently, energy metabolism in these individuals is likely to be altered, when compared to other obesity states. Currently, knowledge surrounding the impact of altered energy metabolism on total energy expenditure (TEE) and its components is conflicting. There is evidence to suggest that individuals with PWS have a lower resting energy expenditure (REE), the primary determinant of TEE (Davies & Joughin, 1993; Hill, Kaler, Spetalnick, Reed, & Butler, 1990). However, research groups report inconsistent findings regarding a lower REE in PWS when compared to healthy weight and obese individuals, which highlights the need for further research using state-of-the art techniques to address these discrepancies (Alsaif et al., 2017). The varied results may be due to the control groups being not matched for age, sex and BMI z-score to individuals with PWS, and discrepancies in methods used to measure body composition. Also, an inconsistency in statistical methods may be responsible for conflicting results between studies that adjusted REE for FFM (Baum, Gray, & Binns, 2015; Maffeis et al., 2001; Maffeis, Schutz, Zoccante, Micciolo, & Pinelli, 1993).

An additional component of TEE that may be altered in individuals with PWS is diet-induced thermogenesis (DIT). This component of EE accounts for approximately 10% to 15% of a person's TEE and is reflective of the amount of energy expended during the processing and storage of food (Blasco Redondo, 2015). It has been hypothesized that increased body temperature, caused by heat released during the digestion of food (e.g., DIT), could result in a reduced food intake (Strominger & Brobeck, 1953). It has previously been documented that individuals with obesity have lower DIT compared to healthy weight individuals; this reduction in EE could explain the weight gain (De Palo et al., 1989; Maffeis et al., 2001; Marrades, Martinez, & Moreno-Aliaga, 2007; Schutz, Bessard, & Jequier, 1984; Segal, Edano, & Tomas, 1990; Steiniger, Karst, Noack, & Steglich, 1987). However, there is a paucity of information on DIT in children with PWS. To date, no study has assessed DIT in children with PWS. Therefore, it is important to determine if there is a mismatch in DIT between children with PWS and children with healthy weight that could explain the progressive weight gain in children with PWS. Only a few studies have measured DIT in children with healthy-weight and obesity, but they have also yielded conflicting results (Baum et al., 2015; Maffeis et al., 2001; Maffeis et al., 1993). These findings suggest that

adiposity and macronutrient composition may have an influence on DIT response. However, the varied results may be due to inconsistencies in methods used to obtain DIT values (including duration of measurement) and meal size. Other reasons for these inconsistencies remain unclear.

To improve the effectiveness of obesity treatment in children with PWS, a more complete understanding of the interaction between energy intake and EE as well as hormonal dysregulation is required. Therefore, suppression of ghrelin concentrations and increase DIT may be an effective treatment in the regulation of appetite and body weight in individuals with PWS. To develop future strategies that employ specific ghrelin antagonists, leading to sustained ghrelin suppression and weight loss in this population, it is essential to better understand the physiologic and nutritional regulation of active ghrelin in individuals with PWS. As well, it has been challenging to accurately measure the amount of acyltated (active) ghrelin in plasma. However, the development of an assay validation method that measures active ghrelin (Liu et al., 2008), which is used in this study, is exciting. The postprandial suppression of ghrelin and DIT in response depends on the total caloric content, as well as the macronutrient composition (fat, carbohydrates and protein) of the meal. It is possible that meals of similar caloric content but different macronutrient composition may impact DIT, potentially influencing TEE. Previous research has suggested that carbohydrate-based meals generally have a larger impact on the DIT than fat; however, protein has recently been found to have the greatest impact on DIT in both healthy weight and obese cohorts (Crowder, Neumann, & Baum, 2016; Maffeis et al., 2001).

1.3 Purpose

The overall objective of this research was to determine and compare the impact of higher protein versus standard meals on postprandial regulation of ghrelin and asprosin; (2) other satiety factor concentrations[1]; and (3) DIT in children with PWS.

1.4 Objectives and Hypotheses

1.4.1 Dietary Macronutrient Regulation of Acyl and Desacyl Ghrelin Concentrations in Children with Prader-Willi Syndrome (Chapter 3)

The objective of this chapter is as follows:

[1] Glucose, insulin, leptin, glucagon-like peptide 1[GLP-1] and peptide tyrosine tyrosine [PYY].

i. To determine the acute postprandial impact of lower and higher protein meals on plasma active ghrelin and insulin sensitivity in children with PWS compared to BMI z-score matched children.

The primary hypotheses of this chapter are as follows:

i. Fasting and postprandial active ghrelin will be higher in children with PWS.
ii. The meal-related decline in plasma active ghrelin will be greater in children with PWS, but the rebound in plasma active ghrelin back to baseline after a meal will be more rapid in children with PWS.
iii. A higher protein/lower fat meal will suppress plasma active ghrelin to a greater degree and increase insulin concentrations in both study groups compared to the higher protein/lower carbohydrate meal.

1.4.2 Serum Concentrations of Asprosin in Children Prader-Willi Syndrome: Association with Glucose and Insulin Resistance (Chapter 4)

The objectives of this chapter are as follows:

i. To determine if there is a difference in serum asprosin concentrations between children with PWS and BMI z-score matched children.
ii. To determine the impact of a meal on postprandial asprosin.
iii. To examine the association between serum asprosin and parameters of glucose, insulin resistance, ghrelin, leptin, and percentage of body fat.

The primary hypotheses of this chapter are as follows:

i. Fasting and postprandial asprosin will be higher in children with PWS.
ii. A breakfast meal will suppress asprosin concentrations in both groups.
iii. Serum asprosin will be positively and significantly associated with glucose and insulin resistance.

1.4.3 Effect of High Protein Diet on Diet-Induced Thermogenesis in Children with Prader-Willi Syndrome (Chapter 5)

The objectives of this chapter are as follows:

i. To determine the impact of a higher protein intake compared to a lower protein intake on DIT in children with PWS.
ii. To examine the association between a higher protein intake compared to a lower protein intake on subjective appetite sensations in children with PWS.

The primary hypotheses of this chapter are as follows:

i. A higher protein intake will result in higher DIT and satiety compared to a lower protein intake.

ii. A higher protein intake will result in reduced and prolonged appetite compared to a lower protein intake.

Chapter 2 Literature Review

2.1 Preface

Sections of this chapter that describe energy metabolism in children with Prader-Willi syndrome have been adapted from a published article in Advances in Nutrition (Alsaif et al., 2017; 15;8(6):905-915. doi: 10.3945/an.117.016253.).

2.2 Regulation of Food Intake and Energy Expenditure

In healthy individuals, energy homeostasis is regulated by food intake (FI) and energy expenditure (EE) to maintain energy balance (Woods, Schwartz, Baskin, & Seeley, 2000). Homeostasis of energy balance requires the brain to maintain energy levels by regulating metabolites, fuel stores or hormone secretion (Woods et al., 2000). These requirements demand first the ability to detect metabolic changes in the body energy by sensing the concentrations of plasma metabolic hormones and nutrients to integrate information from afferent signals projecting to the brain (Roh, Song, & Kim, 2016). The brain, particularly the hypothalamus, is responsible for this regulation (Morton, Meek, & Schwartz, 2014). The hypothalamic arcuate (ARC) is a hypothalamic area that primarily regulates food intake and metabolism by acting on different types of neurons, including orexigenic neuropeptide tyrosine (NPY)/agouti-related peptide (AgRP) and anorexigenic proopiomelanocortin (POMC) neurons (Roh et al., 2016). Adaptive changes in metabolic response and energy expenditure are regulated by neuronal network signals the brain to maintain body weight (Roh et al., 2016). Those signals include adiposity signals (e.g., leptin, insulin), metabolites (e.g., glucose, fatty acids, amino acids) or hormones satiety signals (e.g., glucagon-like peptide 1[GLP-1], peptide tyrosine tyrosine [PYY]) and hunger signals (e.g., ghrelin) (Kampe, Tschop, Horvath, & Widmer, 2000). Hormonal regulation of energy homeostasis is shown in (**Figure 2.1**).

2.2.1 Peripheral Signals Modulating Appetite

The modulation of food intake and glucose metabolism is controlled by pre- and postprandial physiological responses, regulated by orexigenic (ghrelin) and anorexigenic (leptin, insulin, GLP-1 and PYY) hormones (Steinert et al., 2017). Ghrelin is a 28-amino acid peptide hormone with an octanoyl group on the serine in position 3, which accounts for its biological function. It is produced mainly by the stomach and circulates as acylated (AG) and desacyl (DAG) forms (Date et al., 2000; Kojima et al., 1999). Acylated ghrelin is an active form that stimulates appetite and induces a positive energy balance, which can lead to weight gain (Yang, Brown, Liang, Grishin, & Goldstein, 2008). Desacyl ghrelin is an inactive form of ghrelin and can improve glycemic control (Allas & Abribat, 2013). This suggests that the ratio of AG and DAG concentrations (AG:DAG) plays an important role in maintaining weight balance and metabolic control. Circulating ghrelin consists of less than 10% AG and more than 90% DAG. Acylated ghrelin is unstable and is rapidly deacylated to DAG by esterases, which makes it more difficult to measure the active form of ghrelin (Date et al.,

2000; De Vriese et al., 2004; Delhanty et al., 2015). Therefore, the immediate addition of an esterase inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) is needed at the time of blood collection to determine reliable active ghrelin concentrations (Kuppens et al., 2015).

Leptin is a protein hormone secreted by adipocytes, and plasma leptin concentration increases in proportion to body fat mass (Campfield, Smith, Guisez, Devos, & Burn, 1995; Halaas et al., 1995; Pelleymounter et al., 1995). Insulin is produced by pancreatic islets and plays an important role in glucose regulation (Fu, Gilbert, & Liu, 2013). Both leptin and insulin regulate long-term food intake and energy expenditure to maintain body fat storage (Paz-Filho, Mastronardi, Wong, & Licinio, 2012). Glucagon-like peptide 1 and PYY hormones are secreted by the gut and act on other tissues/organs to reduce food intake (Zhou et al., 2015).

The postprandial suppression of these hormones is dependent on the macronutrient (fat, carbohydrates, protein) composition and distribution of the particular meal ingested. Meals high in carbohydrates (≥ 65% of total energy intake) are generally more potent than meals high in fat in suppressing ghrelin concentrations (Erdmann, Lippl, & Schusdziarra, 2003; Erdmann, Topsch, Lippl, Gussmann, & Schusdziarra, 2004; Monteleone, Bencivenga, Longobardi, Serritella, & Maj, 2003; Tannous dit El Khoury, Obeid, Azar, & Hwalla, 2006), while meals high in protein (≥ 30% of total energy) produce variable effects (Foster-Schubert et al., 2008; Leidy, Mattes, & Campbell, 2007). Meals with a mixed macronutrient composition stimulate an increase in plasma GLP-1 concentrations, which peak after 1 to 2 hours (Carr et al., 2010; Elliott et al., 1993). By contrast, oral glucose raises plasma GLP-1 concentrations after 5 to 15 minutes (Carr et al., 2010; Elliott et al., 1993; Hojberg et al., 2009; Kong et al., 1999), while protein- and fat-based meals produce slower and more sustained increases in plasma GLP-1 (Bowen, Noakes, & Clifton, 2006; Calbet & Holst, 2004; Carr et al., 2008). Plasma PYY increases 15 to 30 minutes after mixed macronutrient composition meals, peaking at approximately 1 to 1.5 hours (Ballantyne, 2006; Batterham et al., 2003; Degen et al., 2005). Meals high in fat (≥ 30% of total energy) increase PYY secretion more effectively than carbohydrates (Adrian et al., 1985; Brennan et al., 2012; Gumus Balikcioglu et al., 2015). However, the effect of dietary protein on postprandial PYY secretion is not fully understood (Adrian et al., 1985; Ballantyne, 2006; Heden et al., 2013). Understanding the role of the macronutrients on the regulation of ghrelin and other satiety factors will help guide the design of optimal diets for individuals with PWS and obesity in general.

2.3 Fasting and Postprandial Hormonal Response in Prader-Willi Syndrome

Fasting comparable leptin and higher fasting ghrelin, total adiponectin and high molecular weight adiponectin concentrations and insulin sensitivity have been reported in children with Prader-Willi syndrome (PWS) when compared to children with non-syndromic obesity (Eiholzer et al., 1998; Haqq, Muehlbauer, Newgard, Grambow, & Freemark, 2011; Krochik, Ozuna, Torrado, Chertkoff, & Mazza, 2006; Sohn et al., 2010; Talebizadeh & Butler, 2005). Goldstone et al., (2004) reported comparable fasting GLP-1 concentrations in adults with PWS and adults with and without obesity. Purtell et al., (2011) found comparable concentrations of GLP-1 and PYY in adults with PWS when compared to adults with obesity and healthy weight. In contrast, Butler et al., (2004) reported lower PYY concentrations in infants and children with PWS compared to previously published literature on infants and children without PWS. However, their study has several limitations that could affect their results, including the lack of a control group to compare their result to and differences in blood collection and processing methods, as well as the timing of the blood draws.

Postprandial response to feeding has been reported in handful of studies and has yielded conflicting results (Bizzarri et al., 2010; Butler, Bittel, & Talebizadeh, 2004; Gumus Balikcioglu et al., 2015; Purtell et al., 2011). Gumus Balikcioglu et al., (2015) found that high-carbohydrates and high-fat meals suppressed ghrelin concentrations in children with PWS as well as in BMI z-score-, age- and sex-matched controls. These findings correspond to those of Bizzarri et al., (2010), who showed that a mixed liquid meal suppressed plasma ghrelin more in children with PWS than in children with obesity.

Several reports have described the role of PYY in PWS, but again with conflicting results. Butler et al., (Butler et al., 2004) reported lower PYY concentrations in infants and children with PWS and a higher plasma PYY response to meals in children with PWS compared to children with and without obesity (Bizzarri et al., 2010). Gumus Balikcioglu et al., (2015) reported higher fasting PYY and a blunted postprandial response to high-fat meals in children with PWS compared to BMI z-score-matched children. In adults, Purtell et al., (2011) found comparable fasting and postprandial PYY concentrations in PWS subjects when compared to adults with obesity. Similarly, Purtell et al., (2011) reported comparable fasting GLP-1 and postprandial GLP-1 concentrations in adults with PWS following high-carbohydrates and high-fat meals (50% carbohydrates, 35% fat, 15% protein) compared to adults with and without obesity.

The exact role of macronutrients in regulating appetite and metabolic function in children with PWS is poorly understood. Individuals with PWS have preference for sweet

food, which could explain the weight gain (Hinton, Holland, Gellatly, Soni, & Owen, 2006; Martinez Michel, Haqq, & Wismer, 2016), suggesting that a high-carbohydrates diet could influence hyperphagia and weight gain (Irizarry et al., 2019). A high-protein diet has been shown to increase satiety hormones, reduce hunger, decrease energy intake and improve weight loss and glycemic control in individuals with non-syndromic obesity (Paddon-Jones et al., 2008; Pasiakos, 2015; Pesta & Samuel, 2014). High-protein diets stimulate the secretion of satiety hormones GLP-1 and reduce orexigenic hormone secretion of ghrelin as well as improve glycemic control through increases in hepatic gluconeogenesis (Pesta & Samuel, 2014). Therefore, we postulate that high-protein diets might reduce orexigenic and increase anorexigenic drive, increase satiety and improve glucose homeostasis in children with PWS.

2.4 Energy Metabolism in Prader-Willi Syndrome

In order to further explore the topic on energy metabolism in individuals with PWS a literature searches were performed on Pubmed, Web of Science, SCOPUS and Medline using the following keywords: "energy metabolism," "energy expenditure," "resting energy expenditure," "resting metabolic rate," "basal metabolic rate," "basal energy expenditure," "activity energy expenditure," "total energy expenditure," "daily energy expenditure," "diet-induced thermogenesis," "thermic effect of food," "postprandial thermogenesis," "indirect calorimetry," "doubly labeled water," and "Prader-Willi syndrome." Studies published between time of inception and February 2017 were included in this review. Studies were included if they measured energy expenditure (EE) in individuals with PWS compared to matched controls using indirect calorimetry (IC) or doubly labeled water (DLW) Methods of EE measurement will be explained later in section **2.5.1 Measurement Protocol**. A total of 1,310 articles were found. After excluding duplicates (N=899), irrelevant articles (not related to the aim of this review) (N=393), and articles that did not meet the inclusion criteria (N=8), a total of 10 articles were included in this review.

A flowchart of the literature review process is given in (**Figure 2.2)** and relevant terms related to energy metabolism are defined in (**Table 2.1)**. As explained elsewhere, (Carneiro et al., 2016), multiple terms to describe specific components of energy expenditure are used interchangeably in the literature (e.g., resting and basal energy expenditure [BEE]). However, differences in the specific measurement conditions for obtaining REE and BEE exist, and should be used to clarify which component (REE or BEE) is actually being assessed (**Table 2.1**). These components were hereby classified as either BEE or REE according to their measurement conditions as described in the methodology sections of the

reviewed papers, even if the study authors chose different terms. Additionally, to ensure consistency in reporting and ease of comparison, all energy expenditure values were expressed in kcal/day. Study details, including the population investigated and methods employed to assess the specific components of TEE, are presented in (**Table 2.2)**.

2.4.1 Total Energy Expenditure (TEE)

Despite the concerns for weight management in the PWS population, very few studies investigate the energy expenditure of individuals with PWS. To date, only a handful of studies have examined TEE and it is components among this cohort (Bekx et al., 2003a; Butler, Theodoro, Bittel, & Donnelly, 2007b; Davies & Joughin, 1993; Goldstone et al., 2002; Hill et al., 1990; Lloret-Linares et al., 2013; Purtell et al., 2015; Schoeller et al., 1988; van Mil, Westerterp, Kester, et al., 2000) and few have investigated differences between PWS and healthy weight subjects or individuals with obesity to understand the propensity for development of obesity in persons with PWS.

In the 1980s, evidence to suggest that there was a reduction in energy expenditure in PWS first emerged with studies showing that the TEE expenditure in PWS was approximately 30% lower than control subjects. To date, five studies measured TEE in individuals with PWS. All studies found a lower TEE (kcal/day) compared to matched (for either age or BMI) individuals ranging from 20 to 46% lower in individuals with PWS. Differences in the magnitude of reduction may be related to the methods employed and/or the control group used for comparison.

The majority of studies assessed “free-living” energy expenditure using the gold standard method, DLW (Bekx et al., 2003a; Davies & Joughin, 1993; Schoeller et al., 1988; van Mil, Westerterp, Kester, et al., 2000). The use of DLW allows all components of energy expenditure (REE/BEE, AEE, SEE, DIT) to be captured over a 7- to 14-day period. The highest reduction in TEE was reported by Schoeller and colleagues (Schoeller et al., 1988) in the late 1980s using DLW (under free-living conditions) for 7 days in adolescents with PWS (1980 ± 580 kcal/day). Energy expenditure in PWS was 47% lower compared to age-and sex-matched individuals (3700 ± 820 kcal/day, $p < 0.05$). However, mean body mass index (BMI) was higher in the control group, with a measured BMI of 36 compared to a BMI of 29 in individuals with PWS. This finding would lead one to expect a higher TEE based on higher body weight in the controls, assuming a higher amount of fat-free mass (FFM); thus, reported differences between the cohorts might have been exaggerated. Another study conducted by van Mil et al., (2000) measured TEE using DLW for 14 days, and reported a 28% lower TEE

in PWS children/adolescents (1705 ± 411 kcal/day) compared to the control group matched for age, sex, bone age and BMI (2374 ± 631 kcal/day, $p < 0.01$). Furthermore, a 28% lower in TEE was reported by Davies and Joughin (1993) using DLW (duration not provided) in PWS children/adolescents (1758 ± 569 kcal/day) compared to age- and sex-matched individuals (2474 ± 724 kcal/day, $p < 0.01$). Interestingly, Bekx et al. (2003b) measured TEE in infants with PWS using DLW for 7 days and reported results that were 24% lower in PWS infants (587 ± 189 kcal/day) compared to normative data for age and sex (775 ± 150 kcal/day, $p < 0.001$). These latter findings suggest that changes in the energy metabolism profile in PWS originate early on in development. To date, only one study has measured TEE using whole-body calorimetry unit (WBCU). TEE was measured for only 8 hours, and the results extrapolated to a full day (Butler et al., 2007b). Under these "controlled environment" conditions the authors also reported that PWS adults had a 20% lower TEE (2346 ± 465 kcal/day) compared to age-matched adults; however, BMI was not matched in this study (2973 ± 708 kcal/day, $p < 0.001$).

The overall impact of this lower TEE in individuals with PWS, compared to both healthy age-matched and BMI-matched individuals, is that they would be expected to have reduced energy requirements. Available energy recommendations and published predictive equations are not specific for PWS and would therefore overestimate the energy requirement needs of individuals with PWS. A 30 to 40% reduction in overall energy requirements is the clinical recommendation for individuals with PWS (Miller, Lynn, Shuster, & Driscoll, 2013); however; the lack of precise energy recommendations for individuals with PWS makes it difficult to establish baseline values from which adjustments can be made. The current energy requirement recommendation for individuals with PWS is to lower energy intake to maintain a healthy body weight. However, this strategy does not take into consideration hyperphagia, dysfunction in satiety, body composition and food-seeking behaviors that are inherent to PWS (Martinez Michel et al., 2016). Considering these additional factors is crucial when deriving energy needs and assessing satiety to facilitate the development of optimal diets for weight maintenance in children with PWS.

2.4.2 Resting Energy Expenditure (REE) and Basal Energy Expenditure (BEE)

The majority of studies captured in this review examined differences in REE using IC via a ventilated hood system (Davies & Joughin, 1993; Hill et al., 1990; Lloret-Linares et al., 2013; Purtell et al., 2015) or via a WBCU system (Butler, Theodoro, Bittel, & Donnelly, 2007a; Goldstone et al., 2002) with measurement periods ranging from 10 to 30 minutes.

Ventilated hood systems are used to determine REE, a process that involves using a face mask, mouthpiece or hood to measure oxygen (O_2) and carbon dioxide (CO_2) (Psota & Chen, 2013). The quantity of CO_2 gas is measured and connects to a gas analyzer system (Psota & Chen, 2013). Open-circuit ventilated hood systems can provide rapid estimates when correctly calibrated and positioned on the participants (Psota & Chen, 2013). The smaller "dead spaces" in the ventilation system improves response times compared to open-circuit IC system (Levine, 2005). Even though the set up and use of open-circuit ventilated hood systems is readily comparable to an open-circuit IC system, participants still have limited mobility, the procedure is uncomfortable, and this method makes communication and eating impossible (Duffield, Dawson, Pinnington, & Wong, 2004; McLaughlin, King, Howley, Bassett, & Ainsworth, 2001). These systems are therefore only appropriate for studies that last from 20 minutes to six hours (Psota & Chen, 2013).

Comparable results to the open-circuit ventilated hood systems can be achieved using portable open-circuit spirometry systems (Duffield et al., 2004; McLaughlin et al., 2001). However, participants must also wear a face mask and mouthpiece (Psota & Chen, 2013). The gas analyzer and the power supply are in a backpack, which must be worn during the measurement process (Brychta, Wohlers, Moon, & Chen, 2010). The increased portability of this system allows for greater mobility and more flexibility to be used in free-living conditions. However, the equipment remains cumbersome due to the added weight of the analyzers (Brychta et al., 2010). The majority of the portable devices, such as the MedGem and Fitmate devices, are not equipped with a CO_2 analyzer; instead, they only measure O_2 and assume that respiratory exchange ratio (RER) is equal to 0.85 (Hipskind, Glass, Charlton, Nowak, & Dasarathy, 2011). In addition, the power supply limits the recording time to approximately four hours (Brychta et al., 2010).

Measurements in a WBCU system work in a similar manner. They are designed as airtight rooms that allow for all of the gases respired by a person to be captured (Levine, 2005). The room is ventilated with a constant or known gas composition to facilitate accurate measurements, using paramagnetic analyzers to analyze O_2 concentrations, infrared analyzers to analyze CO_2 concentrations and a mass spectrometer to measure the gas concentrations (Levine, 2005). Energy expenditure is calculated from the net flow rate of O_2, CO_2 and respiratory gas exchange within the room (Eknoyan, 1999). Open-circuit IC systems provide measurements of all components of the energy metabolism, such as REE, DIT and activity energy expenditure (AEE) (Brychta et al., 2010). The open-circuit IC system consists of a large comfortable room that interferes less often with typical physical activity, thus allowing

continuous measurements of TEE up to several days (Smith et al., 2000). Open-circuit IC systems are considered the gold standard for measuring TEE and its components, owing to their high accuracy and precision (<2% error) (Rising, Whyte, Albu, & Pi-Sunyer, 2015). However, there are still several challenges to achieve accurate measurements of human metabolism using open-circuit IC systems. First, only a few institutions in the world have access to an open-circuit IC system; therefore, the large majority of researchers do not have access to this state-of-the-art technique due to the high cost and skills required for operation (Brychta et al., 2010). Given the strict conditions associated with its measurements, such as fasting and remaining at rest during measurements, accommodating children under the age of eight years is difficult (Brychta et al., 2010). Studies have reported that children become restless during the measurement period; therefore, the use of shorter protocols has been suggested to decrease fidgeting and boredom (Compher, Frankenfield, Keim, & Roth-Yousey, 2006; Ventham & Reilly, 1999).

All studies found lower REE or BEE (kcal/day) in PWS compared to controls, ranging from 3 to 37%. Differences in the magnitude of reduction may be related to the varied methods of measuring REE compared to BEE (which is measured under standard conditions), length of the measurement and/or the characteristics of the control group to which it was compared.

Two studies used the IC hood system to determine REE. Hill et al. (1990) measured REE for 15 to 20 minutes in PWS children/adolescents (1104 kcal/day), and found REE was 37% lower compared to individuals with obesity (1752 kcal/day) and 20% lower compared to healthy weight individuals (1392 kcal/day, $p < 0.05$). Davies and Joughin (1993) also assessed REE for 20 minutes and reported an 18% difference between PWS children/adolescents (1324 ± 408 kcal/day) and age- and weight-matched individuals (1615 ± 376 kcal/day, $p < 0.05$). However, studies measuring REE using IC hood systems in adults reported different findings. Lloret-Linares et al. (2013) measured REE in adults with PWS compared to lesional genetic hypothalamic obesity (HO) and healthy individuals with obesity. Measured REE in PWS women (1758 ± 360) was comparable to that seen in HO and BMI-matched control individuals; however, REE in PWS men (1946 ± 428 kcal/day) was reported to be 19% lower compared to BMI-matched individuals (2405 ± 423 kcal/day, $p < 0.05$), yet similar to the HO group (Lloret-Linares et al., 2013). PWS had 25% lower lean mass compared to BMI-matched individuals, but comparable to the HO group. Purtel et al. (2015) measured REE for 30 minutes in adults with PWS compared to individuals with obesity and healthy-weight and also reported that REE was comparable to age-matched

individuals. In contrast to studies that utilized an IC hood system, studies measuring REE using a WBCU system reported a reduced REE in adults with PWS (Butler et al., 2007a; Goldstone et al., 2002). Goldstone et al., (2002) measured REE and reported an 8% reduction in REE in PWS individuals (1584 ± 108 kcal/day) compared to BMI-matched individuals (1716 ± 69 kcal/day $p < 0.01$). Butler et al. (2007a) reported a 16% lower REE in PWS individuals (2074 ± 360 kcal/day), compared to age-matched individuals (2448 ± 475 kcal/day, $p < 0.05$). It is important to highlight that Butler et al. (2007a) measured REE while participants were seated (due to feasibility issues for PWS patients). Therefore, this approach may have incorrectly elevated the reported readings since standardized REE protocols require the patient to be supine, with minimal body motion, and to remain awake during testing. It is also not clear if this adapted seated REE protocol was applied to individuals without PWS as well. Finally, differences in REE might exist between children with PWS as compared to adults with the syndrome. Two studies have assessed BEE using an IC hood system. One study by van Mill et al. (2000a) measured BEE using an IC hood system. They reported results for only 10 minutes of measurement, and concluded that BEE was reduced by up to 16% in PWS children/adolescents (1280 ± 282 kcal/day) compared to age-, sex- and BMI-matched controls (1524 ± 370 kcal/day, $p < 0.05$). Another study conducted by Schoeller et al. (1988) measured BEE in PWS and found that measured BEE values in PWS adolescence/adults (1160 ± 95 kcal/day) were 3 to 12% lower than values obtained from various predictive equations: Harris-Benedict (1310 ± 82 kcal/day); Passmore (1400 ± 120 kcal/day); and Cunningham (1200 ± 78 kcal/day, $p < 0.05$). However, Schoeller et al. (1988) did not compare measured BEE in PWS individuals to matched controls.

2.4.3 Sleeping Energy Expenditure (SEE)

Excessive sleepiness, daytime hypersomnolence and sleep apnea (both central and obstructive) have been reported in individuals with PWS (Camfferman, McEvoy, O'Donoghue, & Lushington, 2008; Gillett & Perez, 2016; Weselake et al., 2014), possibly leading to alterations in sleeping metabolic rate (SMR). Only one study has measured SEE in PWS using WBCU. Van Mil et al. (2000b) reported that SEE was significantly lower in individuals with PWS than in controls (1103 ± 257 kcal/day vs. 1337 ± 63 kcal/day, $p < 0.05$). This finding is in agreement with TEE and REE findings, which are reported to be lower in PWS compared to their counterparts. In summary, absolute values of REE, BEE and SEE were found to be lower in PWS individuals when PWS was compared to obese individuals. This finding might be explained by their abnormal body composition (reduction

in FFM), which could impact energy expenditure values. Appropriately adjusting for variability in FFM between PWS and controls, and the impact this adjustment has on the inferences drawn from these studies is discussed in detail in section **2.6 Contribution of Body Composition to Lower Energy Expenditure**

2.4.4 Activity Energy Expenditure (AEE)

Four studies measured AEE in individuals with PWS and all reported a lower AEE (13–66%) in PWS compared to controls. Three of these studies assess TEE in "free-living" conditions using DLW and derived AEE. Van Mil et al. (van Mil, Westerterp, Kester, et al., 2000) estimated AEE by subtracting BEE and DIT from TEE; Davies and Joughin (1993) estimated AEE by dividing TEE by REE; and Schoeller et al. (1988) estimated AEE based on a predictive equation that included FFM based on Ravussin et al. (1986) methods, with daily energy expenditure calculated as (667 + 20.5 FFM). The greatest difference in AEE was reported by Schoeller et al. (1988). In individuals with PWS, AEE (650 ± 310 kcal/day) was 66% less than AEE measured in age- and sex-matched controls (1940 ± 640 kcal/day, $p < 0.05$). A similar difference of 58% in AEE was reported by van Mil et al. (van Mil, Westerterp, Kester, et al., 2000). AEE was reported to be (256 ± 165 kcal/day) in individuals with PWS compared to age-, sex- and BMI-matched controls (611 ± 246 kcal/day, $p < 0.001$). Furthermore, Davies and Joughin (1993) reported daily activity to be 13% lower in individuals with PWS compared to age- and weight- matched individuals, $p < 0.05$. Physical activity levels were reported to be lower (1.33 ± 0.21) in individuals with PWS compared to age- and weight-matched individuals (1.53 ± 0.23, $p < 0.05$). The data for AEE was not given and estimated based on total mean given for TEE and REE. The estimated AEE was found to be lower (526 kcal/day) in individuals with PWS compared to age- and weight-matched individuals (859 kcal/day).

Only one study measured AEE directly using a whole-body calorimetry unit (Butler et al., 2007a); the energy expended during physical activity during the eight hours was 38% lower in individuals with PWS (1.9 ± 0.61 vs 3 ± 0.99 kcal/minute, $p < 0.001$). Also, standing energy expenditure was reported to be lower in PWS (1.5 ± 0.3 kcal/minute) compared to age-matched subjects (1.8 ± 0.4 kcal/minute), $p < 0.001$. In summary, the literature to date suggests that adults and children with PWS tend to have lower absolute AEE. Again, these differences may be explained by muscle hypotonia and lower skeletal muscle mass, leading to lower values of FFM (Butler et al., 2007a).

2.4.5 Diet-Induced Thermogenesis (DIT)

Only one study has investigated DIT in 11 adults with PWS were compared with 12 individuals with BMI matched and 10 with healthy-weight. Purtell et al. (2015) found no difference in DIT between PWS and control groups after consumption of a breakfast meal high in carbohydrates and fat (600 kcal, 50% carbohydrates, 35% fat, 15% protein). As examination of DIT was not the primary objective of the aforementioned study, details concerning the measurement protocol for DIT were not reported. Therefore, it is unclear how these differences were assessed, and whether or not baseline adjustments for body composition were made.

Exploring the impact of DIT on PWS is important because dietary macronutrient manipulation may have an impact on energy metabolism in PWS. It is well known that meals of similar energy content but different macronutrient composition will impact DIT, which could influence the overall estimates of TEE. Protein has the greatest impact on DIT in both normal-weight and obese cohorts (Baum et al., 2015; Maffeis et al., 2001).

Studies have now demonstrated that higher-protein diets are able to increase satiety hormones, decrease hunger and increase DIT, and thereby promote maintenance of FFM in the setting of low energy intake (Nguo et al., 2019; Westerterp-Plantenga, Nieuwenhuizen, Tome, Soenen, & Westerterp, 2009) (Austin, Ogden, & Hill, 2011; Buchholz & Schoeller, 2004; Gosby, Conigrave, Raubenheimer, & Simpson, 2014; Pesta & Samuel, 2014; Quatela, Callister, Patterson, & MacDonald-Wicks, 2016; Simpson & Raubenheimer, 2005). We speculate that DIT is lower in individuals with PWS, which also contributes to their lower REE. The hierarchy of macronutrients with respect to their impact on DIT is protein, then carbohydrates, and, lastly, fat. It has been documented that protein plays an important role in body weight regulation through satiety related to DIT (Westerterp, 2004a). In conclusion, macronutrient composition should be taken into consideration when estimating the energy needs of individuals with PWS. Although DIT contributes to only 5 to 15% of daily energy expenditure, alterations in DIT could translate into energy imbalance, leading to significant excessive weight gain over the longer term. Future research should clarify additional means to favourably manipulate DIT to improve overall energy balance.

2.5 Methodological Considerations in the Measurement of DIT

2.5.1 Measurement Protocol

The measurement of DIT is performed in three steps: 1) a baseline measure of energy expenditure (resting energy expenditure; REE), measured during fasting state, 2) the

ingestion of a test meal, and 3) measurement of postprandial energy expenditure rate for several hours after the meal (Dabbech, Aubert, Apfelbaum, & Boulier, 1994; Weststrate, 1993). DIT is then calculated as postprandial EE minus fasting EE, and can be further expressed as a percentage of meal energy content (Houde-Nadeau, de Jonge, & Garrel, 1993; Nguo, Huggins, Truby, Brown, & Bonham, 2018; Piers, Soares, Makan, & Shetty, 1992; Westerterp, 2004a). Measurements of REE and DIT are generally performed using IC with a mouthpiece or mask, a ventilated hood, or a WBCU system (Brychta et al., 2010; Granata & Brandon, 2002).

Energy expenditure can be estimated from predictive equations or it can be measured using calorimetry (Brychta et al., 2010). Calorimetry is an approach to directly and indirectly measure EE (Gupta et al., 2017; Scott, 2005). Direct calorimetry measures changes in body temperature (heat production) (Jequier, 1986), whereas IC estimates heat production by measuring the amount of O_2 uptake and CO_2 production by the body (Gupta et al., 2017; Scott, 2005). However, despite being accurate, the application of direct calorimeter methods remains scant because this technique is both expensive and difficult to obtain due to the strict experimental conditions required for accurate results (Brychta et al., 2010; Levine, 2005). Indirect calorimetry techniques have been proven a more accessible and cost-efficient alternative (Brychta et al., 2010; Levine, 2005).

Protein, carbohydrates and fat produce different amount of CO_2 in relation to their required O_2 consumption during their oxidative process, and the total CO_2 produced in relation to the O_2 consumed is termed the RER (Albouaini, Egred, Alahmar, & Wright, 2007; Gupta et al., 2017). During carbohydrate oxidation, CO_2 produced equals O_2 consumed, resulting in a RER value of 1.0. During the oxidization of pure fat, less CO_2 is produced for a given amount of O_2 consumed, leading to RER being reduced to 0.7. The RER for a mixed diet, proteins and alcohol are approximately 0.85, 0.82 and 0.67, respectively (McClave et al., 2003).

The majority of studies examined differences in DIT using indirect calorimetry via a hood canopy system (Nguo et al., 2018; Reed & Hill, 1996) or via a WBCU system (Acheson et al., 2011; Kassis et al., 2019). In some of the ventilated hood systems studies, DIT measurements are 30 minutes, with 30 minutes intervals allowing for sanitary activities (Nguo et al., 2018). Studies using a WBCU to measure DIT generally average the DIT response from a number of meals provided over periods often up to and exceeding 24 hours.

Studies that measured DIT using a ventilated hood system reported shorter measurements of DIT showed a higher early peak of DIT in healthy weight adults compared

to adults with obesity following a meal that contained 15% of total energy from protein, suggesting that a measurement lasting an additional 2 hours may wipe out that difference; therefore, they recommended that measurements last ≥ 5 hours (Reed & Hill, 1996). Another study of adolescents with obesity and healthy weight measured DIT for 4 hours after high-protein (whey) (55% protein, 30% carbohydrate and 15% fat) or high-carbohydrate (maltodextrin) (79% carbohydrate, 5% protein, and 16% fat) liquid meals (Nguo et al., 2018). They reported no differences in DIT between adolescents with obesity and healthy weight; however, DIT was significantly greater after the high protein compared with the high-carbohydrates meal ($p < 0.001$) (Nguo et al., 2018).

Among the studies that measured DIT using WBCU systems, Acheson et al. (2011) measured DIT for 5.5 hours to determine the differential effects of three protein sources in healthy weight adults. Three meals consisting of 50% protein (whey, casein or soy), 40% carbohydrates and 10% fat were compared to a meal consisting of 95.5% carbohydrates. They reported that the DIT effect was greater after the whey than after the casein or soy meals and was greater after the whey, casein and soy meals than after the high-carbohydrates meal (Acheson et al., 2011). More recently, Kassis et al. (2019) aimed to evaluate the efficacy of two different doses and types of protein 30 g of whey protein, 50 g of whey or 50 g of casein where protein and carbohydrates were exchanged on DIT for 7 hours in overweight adults. They reported that the 50 g of whey protein resulted in greater DIT than 30 g of whey protein and 50 g of casein (Kassis et al., 2019).

The variation in methodological approaches for measuring DIT may explain the conflicting findings between studies. Even within studies, thermogenic responses to meals are highly variable between individuals and subgroups of populations. For example, differences in the thermogenic response of healthy weight adults compared to adults with obesity are commonly reported (Reed & Hill, 1996). In contrast, Nguo et al. (2018) did not find a significant difference in DIT between adolescents with obesity and healthy weight, but found that a high-protein meal has a greater effect on DIT than a carbohydrate meal. The latter observation is consistent with Acheson et al. (2011) and Kassis et al. (2019) findings, both of which found that both the protein source (e.g., whey protein) and the dose affected DIT response. In summary, the DIT response is highly variable, depending on meal composition and duration of measurement as well as variation between people.

2.5.2 Duration of the DIT Measurement

The majority of studies measured DIT response between 3 and 6 hours (D'Alessio et al., 1988; Davies et al., 1989; Katzeff & Danforth, 1989; Schutz, Golay, Felber, & Jequier,

1984; Tentolouris et al., 2008; Welle & Campbell, 1983). However, the total DIT response may take between 8 to 10 hours for complete digestion (D'Alessio et al., 1988; Melanson, Saltzman, Vinken, Russell, & Roberts, 1998). The duration of the DIT response may be affected by individual differences in the rate of gastric emptying, nutrient digestion and storage, meal size and macronutrients distribution of test meal (Scott, Fernandes, & Lehman, 2007). However, given that the long duration of measurement can be burdensome to participants, Ruddick-Collins et al. (2013) sought to determine whether shorter measures of DIT correlate with the total DIT response, and reported that the three-hour measures provide sufficient information about the magnitude of the DIT response and may be applicable for testing individuals' response.

2.5.3 Minimizing Variability Arising from Movement in Children during the DIT Measurements

The DIT measurement requires participants to remain still in the same position throughout the duration of the pre-meal REE and DIT (Dietz, Bandini, Morelli, Peers, & Ching, 1994; Weststrate, Van der Kooy, Deurenberg, & Hautvast, 1990). The measurements of DIT assume that postprandial REE over the baseline REE remains constant throughout the entire duration of its measurement. However, movement and activities during the REE and DIT measurement could influence the overall measurements and result in incorrectly higher or lower REE or DIT. Therefore, the majority of studies that assess DIT allowed their participants to watch movies, listen to music, play games or read books (den Besten, Vansant, Weststrate, & Deurenberg, 1988; Scott & Devore, 2005; Vasilaras, Raben, & Astrup, 2001).

Weststrate et al. (1990) reported a significant increase in DIT measurements after watching a horror movie compared to a romance, which indicated that stress can increase energy expenditure and cause a falsely elevated DIT. Koepp et al. (2016) compared energy expenditure using an under-the-table leg-fidget bar or a fidget-promoting chair, compared to the standard office chair. They found that energy expenditure increased by ~20 to 30% using chairs and devices that promote fidgeting (Koepp, Moore, & Levine, 2016). Another study measured REE in children as they watched television, read or sat quietly for 15 minutes (Dietz et al., 1994). Movement was assessed using activity monitors, and a manual count of movements was observed on videotape. The researchers found that children were fidgeted more when sitting quietly than when they read or watched television, which suggests that television viewing does not alter REE (Dietz et al., 1994).

These findings highlight that the possibility that an individual's level of fidgeting and his or her postural position may affect energy expenditure measurements, which could produce discrepancies in the energy expenditure measurements between days and throughout the baseline REE and DIT measurements. An increase in baseline REE may unfavourably influence DIT response, making it appear lower; fidgeting during postprandial REE measurement, on the other hand, may falsely increase DIT measurement. Therefore, it is important to control participants' activities, reduce fidgeting, facilitate a calm emotional state, and have them maintain a consistent sitting position during the baseline REE and DIT measurements and between days to minimize variability.

2.6 Contribution of Body Composition to Lower Energy Expenditure

A unique altered body composition consisting of higher fat mass (FM) and a lower FFM for body weight is described in PWS infants, even before hyperphagia manifests (Bekx et al., 2003a). This suggests a possible genetic or developmental origin to the altered body composition phenotype documented in PWS. As mentioned previously, a major determinant of energy expenditure is body composition, specifically, the metabolically active tissues that make up the FFM compartment. The amount of FFM (kg) can vary considerably between PWS, obese and non-obese individuals (Bedogni, Grugni, Tringali, Agosti, & Sartorio, 2015). Overall, PWS individuals tend to have lower FFM. Therefore, the absolute value of REE tends to be lower in PWS individuals due to their reduced FFM; however, once FFM is taken into consideration, these differences between PWS and controls mainly disappear (Bekx et al., 2003a; Davies & Joughin, 1993; Hill et al., 1990; Lloret-Linares et al., 2013; Purtell et al., 2015; Schoeller et al., 1988; van Mil, Westerterp, Kester, et al., 2000; van Mil, Westerterp, Gerver, et al., 2000), which suggests that there might not be a major disruption to energy metabolism at a cellular level in PWS.

In most studies included in this review, the adjustment method was performed by simply dividing REE/BEE by FFM. However, this method is not optimal, since FFM includes bone, skeletal mass and highly metabolically active organs such as the brain, heart, liver, kidney and gastrointestinal tract (Carneiro et al., 2016; Heymsfield et al., 2002). When body weight instead of FFM is used to calculate BEE and SEE, these remained significantly lower in PWS compared to age, sex and BMI (Colditz, 2010). However, the use of body weight for adjustment might not be ideal due to unique differences in body composition in PWS (Orsso et al., 2017). Finally, Butler et al. (2007a) (Butler et al., 2007a) reported that REE remained significantly ($p < 0.001$) lower when adjusting for FM between the two

groups. The justification for expressing energy expenditure data adjusted for FM is questionable and the authors offer no rationale for choice of their adjustment methods. Additionally, a major limitation of the above study is that PWS individuals were not compared to a BMI-matched group.

Instead, it has been suggested that the adjustment of energy expenditure for FFM should be carried out by calculating residuals from regression models, including other covariates (sex, age, FM, height) as described by Carneiro et al. (2016). Such an adjustment method using multiple liner regression analysis was employed in the study by Goldstone et al. (2002), which reported REE to be lower in PWS individuals in comparison to normal weight and BMI-matched individuals, after adjustment for FFM and age; REE was higher after adjustment for age and FFM. These findings highlight the importance of correctly accounting for differences in FFM when comparing groups with different body composition, because, without proper adjustment, results may be misinterpreted. Another study conducted by Hill et al. (1990) using multiple linear regression analysis reported no difference in REE adjusted for FFM in PWS compared to controls. A limitation of this study was the use of bioelectrical impedance analysis (BIA) to measure FFM. BIA assesses total body water and then estimates FFM and fat mass using algorithms. These algorithms may not be suitable for use in subjects with PWS since they have not been validated in this clinical population. Additionally, BIA assumes a constant FFM hydration factor (Deurenberg, 1996), which varies considerably with age and clinical condition (Wang et al., 1999). Specifically, as body composition is altered in individuals with PWS, alterations in body water distribution (van Mil et al., 2001), the underlying assumptions the BIA technique relies upon, may be violated. This would result in an overestimation of the amount of FFM and an underestimation of fat mass in PWS (Bedogni et al., 2015; Deurenberg, 1996). Therefore, REE may be overestimated in PWS, which would lead to the erroneous conclusion that obese individuals have a lower REE than PWS individuals due to the inadequate normalization for FFM.

It is important to highlight the importance of the use of adequate body composition tools in PWS to better account for FFM in analysis. The use of total body potassium (TBK) to estimated whole-body cell mass/metabolically active tissue (Wang et al., 2007) has not yet been utilized to examine body composition in PWS, but this would provide a more accurate measure of metabolically active lean tissue, without the adverse impact of potential hydration changes.

Overall, these studies suggest that PWS individuals have lower absolute REE than obese individuals due to their lower FFM. Efforts to increase FFM in PWS may therefore be an effective strategy to increase overall EE in PWS individuals.

2.7 Contribution of Endocrine Dysfunction to Lower Energy Expenditure

The hormonal abnormalities observed in PWS, such as growth hormone (GH), and thyroid-stimulating hormone (TSH) deficiencies, and testosterone insufficiency, would contribute to lower EE due to their effects on FFM (Aycan & Bas, 2014). The altered body composition (e.g., lower FFM values) observed in individuals with PWS has been a point of interest for clinicians and has resulted in the increased use of GH therapy and thyroid and testosterone supplementation (Haqq, Stadler, Jackson, et al., 2003; Kido et al., 2013). Growth hormone treatment during childhood and in adults with PWS has been shown to increase FFM (Carrel et al., 2004; Haqq, Stadler, Jackson, et al., 2003; Hoybye, 2004). In addition, testosterone supplementation in adults with PWS has been shown to increase the amount of FFM (Kido et al., 2013). The resultant increased FFM likely increases energy expenditure and may help individuals with PWS obtain and maintain a healthy body weight. GH has potent protein anabolic and lipid and carbohydrates metabolic effects. In general, GH stimulates lipolysis, hyperinsulinemia, and stimulation of insulin-like growth factor 1 activity. This results in potent protein anabolic effects, increased amino acid uptake, increased protein synthesis, and decreased protein oxidation (Moller et al., 1995). Finally, controlled studies have reported significant increases in REE with the use of GH in PWS (Haqq, Stadler, Jackson, et al., 2003). Of the previous studies reviewed, only two reported the use of GH (Bekx et al., 2003a; Lloret-Linares et al., 2013) in the assessed subjects with PWS. Lloret-Linares et al. (2013) reported that 22% of individuals (6 out of 27 subjects) with PWS were receiving GH therapy. Bekx et al. (2003b) reported that 87.5% of individuals (14 out of 16 subjects) with PWS were receiving GH treatment. The use of GH in the remainder of the examined studies is unknown due to a lack of reporting. Additionally, details as to whether GH status was considered as a covariate in the final analysis are unknown, which may also explain the inconsistent findings between studies.

2.8 Summary and Gaps in Understanding of Energy Expenditure in PWS

From the literature to date, the findings suggest that PWS individuals have lower absolute energy expenditure than age-, sex-, and BMI-matched controls as a consequence of reduced REE, SEE and AEE; these differences may be secondary to altered body

composition and hormone dysfunction. However, due to the paucity of information on DIT in individuals with PWS, the relative contribution of specific components of TEE to the altered metabolism in PWS is not clear. Once body composition is taken into consideration, many differences disappear, which suggests that there might, in fact, be no overall disturbance in whole-body energy metabolism.

However, these results should be interpreted with caution because of the inherent limitations in the use of adjustment methods and the discrepancies in the methods used to measure body composition in PWS. Additionally, DIT needs to be assessed in individuals with PWS and compared with individuals with non-syndromic obesity, whereas differences in energy expenditure estimates according to genetic subtype and impact of hormonal therapies need further study. The mechanism of action for the significant overall reduction in energy expenditure in individuals with PWS is likely multifactorial. Approximately one-third of children with PWS have hypothyroidism due to hypothalamic-pituitary dysfunction, resulting in low or low-normal concentrations of thyroid-stimulating hormone and low concentrations of total or free thyroxine. Therefore, central hypothyroidism may play a role in the reduced energy expenditure demonstrated in children with PWS (Emerick & Vogt, 2013). A PWS mouse model [mice lacking MAGE-like 2 (Magel2)] demonstrated normal leptin sensitivity in proopiomelanocortin neurons into the early post-weaning period; however, thereafter, the mice demonstrated progressive leptin insensitivity, which is predicted to impair the release of the melanocortin receptor agonist a-melanocyte stimulating hormone, with downstream effects to reduce energy expenditure (Pravdivyi, Ballanyi, Colmers, & Wevrick, 2015). It is speculated that a similar reduction in leptin responsiveness occurs over time in children with PWS; this would account for a reduction in energy expenditure in individuals with PWS, which would worsen with aging. Previous research has examined whether individuals with PWS exhibit reduced fat oxidation. One study by Purtell et al. (2015) did not detect metabolic defect in respiratory quotient or fat oxidation in individuals with PWS after a mixed high-carbohydrates and high-fat meal. However, Rubin et al. (2017) examined the effect of PWS on the hormonal and metabolic response to resistance exercise; individuals with PWS demonstrated earlier increases in fatty acids during recovery and exhibited higher glycerol and ketone concentrations than controls, suggesting incomplete fat oxidation (Rubin et al., 2017). Previous longitudinal studies in Pima Indians report that low fat oxidizers (90th percentile for respiratory quotient) had a 2.5 times greater risk of gaining 5 kg of body weight than high fat oxidizers (Zurlo et al., 1990). Furthermore, those successful at maintaining weight loss tend to have higher fat oxidation rates than those

experiencing weight relapse (Froidevaux, Schutz, Christin, & Jequier, 1993). Thus, lower fat oxidation in individuals with PWS might also contribute to the imbalance between intake and energy expenditure. Finally, lower spontaneous physical activity and sympathetic activity have been reported in individuals with PWS (Butler et al., 2007a; Rubin et al., 2017). Children with PWS demonstrated no exercise-induced increase in catecholamines and a 40% lower heart rate elevation in response to exercise compared with controls. These findings suggest a sympathetic autonomic deficit in PWS (Castner, Rubin, Judelson, & Haqq, 2013; DiMario, Dunham, Burleson, Moskovitz, & Cassidy, 1994; Richer et al., 2011).

In summary, altered endocrine function (thyroid and leptin deficits) and lower fat oxidation, sympathetic activity and spontaneous physical activity are multiple factors that might play an etiologic role in the reduction in energy expenditure that is characteristic of individuals with PWS. Further longitudinal studies are required to determine if this defect in EE progressively worsens with age. Finally, it is important to highlight that although no metabolic differences have been documented to date, accurate assessments of FM and FFM could certainly affect the calculation of energy requirements for those with PWS compared with healthy controls. Currently, energy requirements are based on predictive equations used to assess REE. These equations incorporate the use of body weight and assume a "healthy or reference" body composition, which is problematic in this clinical cohort in which disturbances in body composition have been clearly documented (Orsso et al., 2017). The abnormal body composition in individuals with PWS (characterized by reduced FFM and increased FM) would lower EE and thus lower energy requirements compared with healthy individuals. Thus, assessing body composition accurately with the use of a valid techniques and determining energy requirements taking body composition into consideration are integral parts of dietary management in individuals with PWS. Increasing FFM and maximizing physical activity should be considered to increase energy expenditure in PWS.

2.9. Tables

Table 2.1. Relevant Terms and Definitions Related to Energy Metabolism

Term	Definition
Basal Energy Expenditure (BEE)	Minimum energy required to maintain vital body functions. It represents approximately 50 to 75% of TEE. It is measured under standard conditions, which include a full night's sleep in the metabolic unit, optimal fasting condition for 12 to 14 hours, laying down, remaining awake but motionless, abstaining from exercise for at least 12 hours, and no physical exercise on the day of testing (Pinheiro Volp, Esteves de Oliveira, Duarte Moreira Alves, Esteves, & Bressan, 2011)
Resting Energy Expenditure (REE)	Energy required to support body's basic metabolic activities; REE can be 3 to 10% higher than BEE
Activity Energy Expenditure (AEE)	Energy expended to support physical activity (exercise and non-exercise) (Westerterp, 2013)
Diet-Induced Thermogenesis (DIT)	Energy required after food intake for digestion, absorption, usage, and storage of nutrients (Blasco Redondo, 2015).
Sleeping Energy Expenditure (SEE)	Energy required to maintain vital body functions during sleep; it is 5 to 10% lower than BEE (Garby, Kurzer, Lammert, & Nielsen, 1987)

Table 2.2. Summary of Studies Investigating Differences in the Components of Energy Expenditure in Individuals with or without Prader-Willi Syndrome (PWS)

Author, Year	Study Design and Purpose	Study Population (mean±standard deviation)	Measurements	Outcomes
(Schoeller et al., 1988)	Cross-sectional study to compare total daily energy expenditure, basal metabolic rate, and body composition.	PWS n=10; 5F/5M Age 16±4 years BMI 28.9±8.1(*lower) FFM 29.8±9.6 kg (*lower) %FM 48±7 No information on GH treatment PWS subtype: 5DEL/ 5 not reported PWS for BMR n=6; 3F/3M Age 24±4 years BMI 22.7±1.8 PWS subtype: not reported Controls n=10; 5F/5M Age 15 ± 3.6 years BMI 36.3±7.5 FFM 56.4±13.8 kg %FM 45±6	TEE: DLW, 7 days BEE: IC ventilated hood, 30 minutes AEE: estimated based on predictive equation that includes FFM BC: total body water	TEE compared to controls:: PWS 47% lower kcal/day BEE compared to predictive equations: Measured 3 to 12% lower than predictive equations. Predictive equation that includes FFM not different from measured BMR. Estimated AEE compared to controls: PWS 66% lower kcal/day; no difference kcal/day/kg BW.
(Davies & Joughin, 1993)	Cross-sectional study to assessed total energy expenditure and resting energy expenditure.	PWS n=10; 5F/5M Age 11.65±3.5 BMI not reported FFM 23.9±10.3 kg %FM 43±10.5 No information on GH treatment PWS subtype: 7DEL/ 8 not reported Control n=60 no information on how many M/F Age 12.56±4.2 BMI not reported FFM 34.2±15.1 kg %FM 23.8±9 (*lower)	TEE: DLW, details not provided REE: IC hood, 20 minuets AEE: TEE/REE BC: total body water	TEE compared to controls: PWS 28% lower kcal/day unadjusted, no difference with adjustment for age, FFM and sex. REE compared to controls: PWS 18% lower kcal/day unadjusted, no difference with adjustment for age, FFM and sex. Estimated AEE compared to controls: PWS 13% lower kcal/day unadjusted, lower with adjustment for age.
(van Mil, Westerterp, Kester, et al., 2000)	Cross-sectional study to measure activity related energy expenditure corrected for body size.	PWS n=17; 10F/7M Age 11.9±3.4 years BMI 23.5±6 FFM 27.5±9.9 kg (*lower)	TEE: DLW, 14 days BEE: ventilated hood, 10 minutes	Absolute TEE compared to controls: PWS 28% lower kcal/day unadjusted TEE/FFM (kcal/day/kg FFM):

		FM 22.4±11.7 kg %FM 43.7±7.9 Bone age 12.7±2.9 years PWS subtype: DEL or UPD GH: ever received GH Controls n=17; 10F/7M Age 11.3±2.6 years BMI 26±6.5 FFM 35.9±13.4 kg FM 25,6±12.7 kg %FM 39.1±8.8 Bone age 12.7±3.2 years	AEE: 0.9 x (TEE-BEE), correcting for 10% DIT BC: total body water	PWS 42% and 38% lower with BW and BW co-variates, respectively. BEE compared to controls: PWS 16% lower kcal/day unadjusted; 21% lower with BW as co-variate; no difference with FFM as co-variate. AEE compared to controls: PWS 58% lower kcal/day; PWS 50% lower kcal/kg BW/day
(Bekx et al., 2003a)	Cross-sectional study to evaluate body composition in relationship to energy expenditure in infants and toddlers with PWS.	PWS n=16; 8F/8M Age 12.4±6 months DXA FFM 5.7±1.4 kg %FM 28.8±6 DLW %FM 34.9±7 PWS subtype: 10/DEL/5UPD/1M GH: 14 current GH	TEE: DLW, 7 days BC: DXA, DLW	Absolute TEE compared to published normative data: PWS 24% lower kcal/day TEE adjusted as liner regression for FFM (kcal/day kg FFM): PWS comparable to published normative data.
(Butler et al., 2007a)	Cross-sectional study to determine the relationship among body composition, activity levels and metabolic rates.	PWS n=48; 27F/21M Age 23±9 years BMI 34±9 (*lower) FFM 35±7 kg (*lower) FM 39±14 kg (*lower) %FM 51±8 PWS subtype: 27DEL/21UPD GH: non receiving GH Controls n=24; 15F/9M Age 27±13 years BMI 41±8 FFM 51±12 kg FM 50±14 kg %FM 50±7	TEE: WBIC, 8 hours REE: whole-body IC, 30 minutes AEE: energy expended during the periods when the participants were instructed and encouraged to do exercises BC: DXA	PWS 20% lower kcal/8hr unadjusted; lower with adjustment for FM; no difference with adjustment for FFM REE compared to controls: PWS 16% lower kcal/min; lower with adjustment for FM; no difference with adjustments for FFM or BW. AEE compared to controls: 38% lower kcal/min with a 35% reduction in mechanical work over 8 hrs.
(Hill et al., 1990)	Cross-sectional study to determine whether the relationship between REE	PWS n=22; 13F/9M Age 13±1 years BMI 24±1	REE: IC hood, 15–20 minuets BC: BIA	REE compared to controls: PWS 37% lower than obese and 20% lower than lean controls unadjusted, no difference with

	and body weight/body composition was different in individuals with PWS.	No information on PWS subtype and GH Obese controls n=11; 7F/4M Age 10±1 years BMI 28±1 Lean controls n=20; 9F/11M Age 10±1 years BMI 19±1 Missing information on FM and FFM FM and FFM data not reported.		adjustment for FFM
(van Mil, Westerterp, Gerver, et al., 2000)	Cross-sectional study to measure SEE adjusted for FFM.	Same participants as reference (van Mil, Westerterp, Kester, et al., 2000)	SEE: respiratory camper, overnight	Absolute SEE compared to controls: PWS 17% lower kcal/day unadjusted, lower with adjustment for body weight, no difference with adjustment FFM.
(Goldstone et al., 2002)	Cross-sectional study to assess REE.	PWS n= 8F Age 25±2 years BMI 42.1±3 (*higher) FFM 46.1±3.7 kg (*lower) FM 49.6±6.3 kg (*higher) %FM 50.8±2 PWS subtype: not reported GH: non receiving GH Obese controls n=13F Age 26±3 years BMI 37.1±1.6 FFM 58.5±1.8 kg FM 43.2±2.9 kg %FM 42.1±1.2 Lean controls n=28F Age 31±1 years BMI 23.7±0.5 FFM 46.1±0.9 kg FM 19.5±0.9 kg %FM 29.1±0.8	REE: WBIC, ≥ 20 minutes BC: MRI	Absolute REE compared to controls: PWS 8% lower than obese controls kcal/day unadjusted, lower with adjustment for age, height and weight and after adjustment for age and FM; higher after adjustment for age and FFM no difference with adjustment for age, FFM and FM.

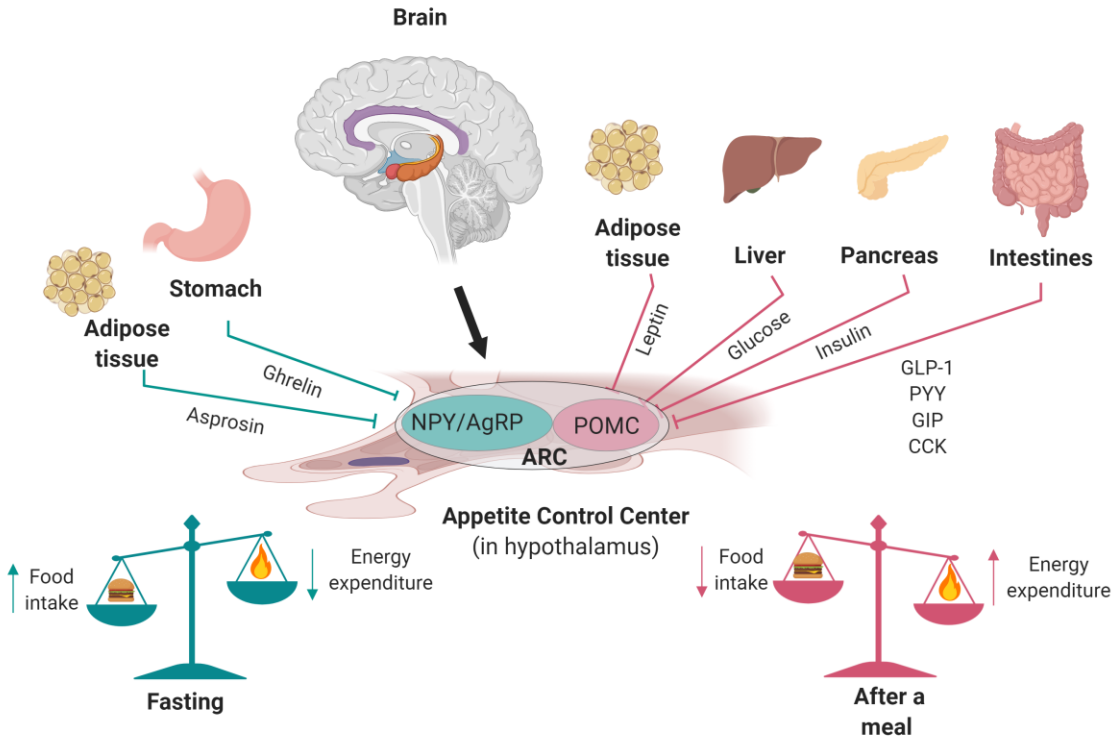
(Lloret-Linares et al., 2013)	Cross-sectional study to compare REE of adults with PWS or lesional genetic hypothalamic obesity compared to obese controls.	Female PWS n=16 Age 25.8±4.9 years BMI 46.6±8.5 FFM 46.7±10 kg (*lower than female HD) FM 53±13.2 kg % FM 52.5±4 Male PWS n=11 Age 25.5±10.4 years BMI 46.6±9.1 FFM 58.6±12.6 kg (* lower than male HD and controls) FM 58.6±19.2 kg %FM 48.9±7.3 All PWS subtype: 18DEL/8UPD/1IM 1 current GH, 5 GH in childhood Female HD n=5 Age 42.2±16.1 years BMI 49±7.6 FFM 61.7±11.7kg FM 57.9±13.4 kg %FM 47±1.6 Male HD n=10 Age 33.8±13.2 years BMI 47.2±7.7 FFM 75±12.3 kg FM 58±14 kg %FM 40±7.4 All HD: 1 current GH, 5 GH in childhood Female controls n=176 Age 36±8.4 years BMI 46.8±6.3 FFM 61.7±8.3 kg FM 60.5±12.3 %FM 47.5±4.5	REE: IC hood, details not provided BC: DXA	Absolute REE compared to controls: Female PWS similar to controls and HD unadjusted and adjusted for age, PWS 13% higher than controls kcal/day and similar to HD with adjustment for FFM. Male PWS 19% lower than controls kcal/day unadjusted and similar to HD; no differences when adjusted for age and FFM.

		Male controls n=30 Age 41.7±7.9 years BMI 46.3±5.7 FFM 78.8±8.3 kg FM 58.5±14%FM 40.4±4.6		
(Purtell et al., 2015)	Cross-sectional study to measure changes in energy expenditure in response to meal	PWS n=11; 4F/7M Age 27.5±2.7 years BMI 37.35±2.9 (*higher than controls) FFM 43.21 kg (*lower than obese controls) FM 3.06 kg %FM 47.68 PWS subtype: 6DEL/5UPD GH: non ever received GH Obese controls n=12; 5F/7M Age 32.25±2.5 years BMI 34.21±1.2 FFM 52.67 kg FM 40.33 kg %FM 46.25 Lean controls n=10; 5F/5M Age 28.8±1.1 years BMI 21.4±0.4 FFM 71.54 kg FM 14.63 kg %FM 24.32	REE: IC hood, 30 minutes Postprandial REE IC hood, 240 minutes. BC: DXA	Absolute REE compare to controls: PWS were comparable to obese and lean controls kcal/day unadjusted, no differences when adjusted for FFM and FM. Absolute postprandial REE compared to controls: PWS were comparable to obese and lean controls kcal/day unadjusted, no differences when adjusted for FFM and FM.

AEE: Activity Energy Expenditure; BEE: Basel Energy Expenditure; BIA: Bioelectrical Impedance Analysis; BMI: Body Mass Index; BC: Body Composition; DEL: Deletion; DXA: Dual-energy X-ray absorptiometry; FFM: Fat-Free Mass; %FM: Fat Mass; F: Female; GH: Growth Hormone; HD: Hypothalamic Damage; IC: Indirect Calorimetry; kg: kilogram; M: Male; MRI: Magnetic Resonance Imaging; M: Methylation; PWS: Prader-Willi Syndrome; SEE: Sleeping Energy Expenditure; TEE: Total energy Expenditure; UPD: Uniparental disomy; WBUC: Whole Body Calorimetry Unit

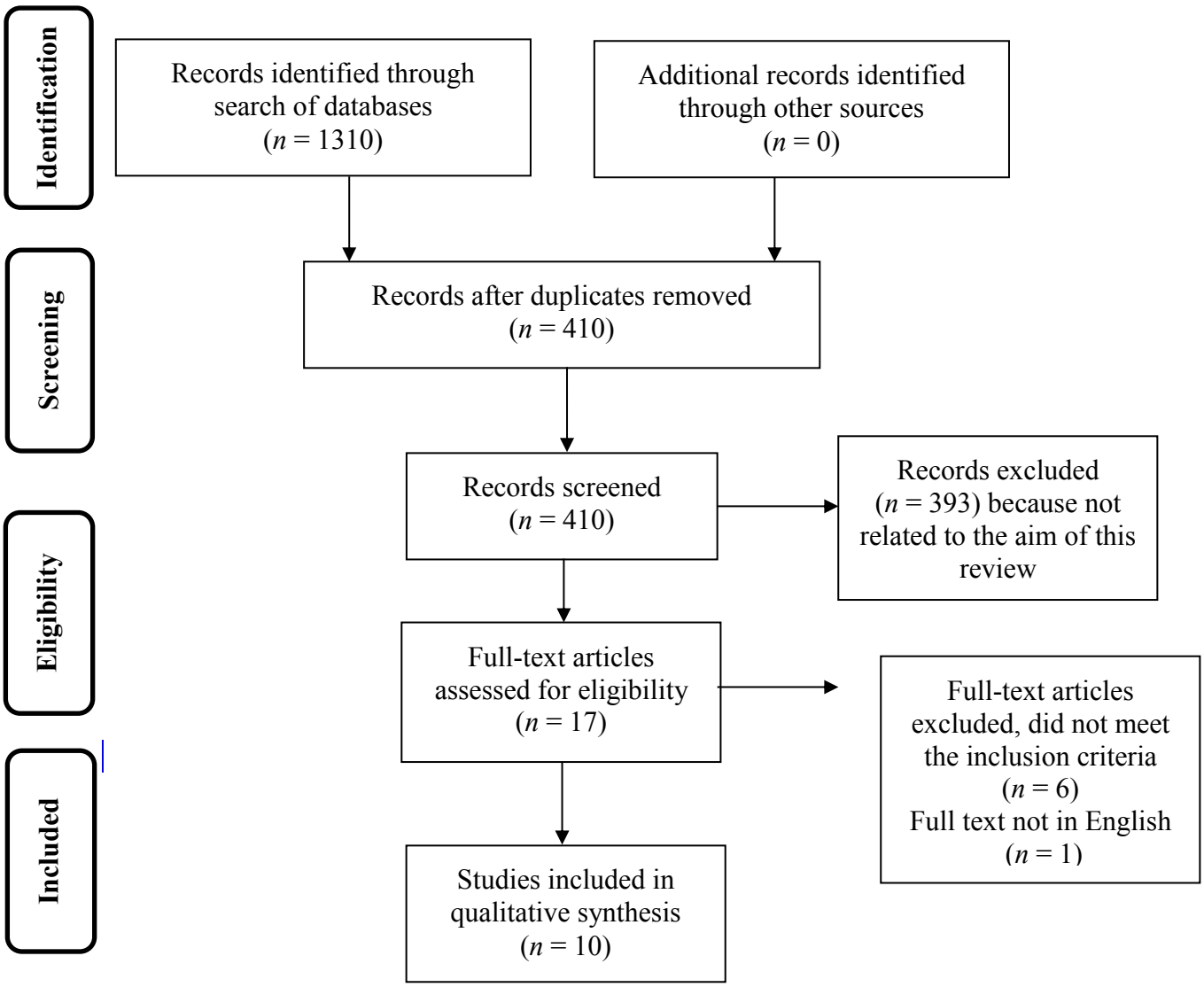
2.10. Figures

Figure 2.1. Hormonal Regulation of Energy Homeostasis



The brain integrates metabolic signals from peripheral tissues such as the stomach, adipose tissue, liver, pancreas and intestine. Adaptive changes in the brain coordinate food intake and energy expenditure in response to altered metabolic condition. ARC: Hypothalamic Arcuate; NPY: Neuropeptide Tyrosine; AgRP: Agouti-related Peptide; POMC: Proopiomelanocortin; GLP-1: Glucagon-like Peptide; PYY; Peptide Tyrosine Tyrosine; GIP: Glucose-dependent Insulinotropic Peptide; CCK: Cholecystokinin. Figure by Maha Alsaif©

Figure 2.2. A flowchart of Literature Review Process



Chapter 3 Dietary Macronutrient Regulation of Acyl and Desacyl Ghrelin Concentrations in Children with Prader-Willi Syndrome

3.1 Preface

The following chapter is based on data from 17 children, 10 with Prader-Willi syndrome (PWS) and 7 BMI z-score matched children at University of Alberta. The study aimed to compare the effects of variation in protein content in meals matched for calorie content on the concentrations of acyl ghrelin (AG), desacyl ghrelin (DAG), glucose, insulin, glucagon-like peptide 1 (GLP-1), and peptide tyrosine tyrosine (PYY). Ms. Maha Alsaif was responsible for assaying the blood samples, analyzing the data and writing the first draft of the manuscript, supported by ongoing discussions with Dr. Andrea Haqq. Dr. Mohammadreza Pakseresht performed the statistical analysis and contributed to the data interpretation. Dr. Michelle Mackenzie recruited the data. Drs. Haqq and Mackenzie conceived the study design. Dr. Michael Thorner contributed to interpretation and critical review of the manuscript. Drs. Andrea Haqq, Michelle Mackenzie, Carla Prado, Mohammadreza Pakseresht, Bruce Gaylinn, Michael Thorner, Michael Freemark and Catherine Field contributed to concept of formation, interpretation and editing of the final manuscript. This manuscript is being submitted for publication to *Clinical Endocrinology*.

3.2 Introduction

Prader-Willi syndrome (PWS) is a clinical model of severe childhood obesity with a prevalence approximating 1 in 10,000 to 16,000 live births (Nicholls, Knoll, Butler, Karam, & Lalande, 1989). Infants with PWS present with hypotonia and failure to thrive, and tube feeding is often required. This is followed by hyperphagia and progressive weight gain in childhood. Relative to children with obesity, children with PWS have elevated fasting and postprandial concentrations of ghrelin (Haqq et al., 2008). The high circulating concentrations of ghrelin may be critical for the pathogenesis of obesity in PWS because ghrelin stimulates food intake and weight gain (Atalayer et al., 2013). Thus, studies of the regulation of ghrelin secretion may provide important insights into the control of appetite and weight gain in children with PWS.

In a recent study, by Irizarry et al. (2019) compared the effects of low-fat, high carbohydrate (LF-HC) and low carbohydrate, high-fat (LC-HF) intake in children with PWS using meals matched for calorie and protein content. They found that the LC-HF diet resulted in lower postprandial insulin concentration, increased fasting and postprandial glucagon-like peptide 1 (GLP-1) concentrations, and increased fatty acid oxidation compared to LF-HC diet (Irizarry et al., 2019).

In the current investigation, we enrolled children with PWS and BMI z-score matched controls in a randomized crossover study and compared the effects of variation in protein content in meals matched for calorie content. Two test diets were compared to a standard meal (SM) containing 55% carbohydrate, 30% fat, and 15% animal protein. The first test meal (HP-LC) contained higher concentrations of animal protein (30%), lower concentrations of carbohydrate (40%), and equivalent concentration of fat (30%); this allowed us to test the effect of substituting protein for carbohydrate in a calorie and fat-balanced meal. The second (HP-LF) contained higher concentrations of animal protein (30%), lower concentrations of fat (15%), and equivalent concentrations of carbohydrate (55%); this allowed us to test the effect of substituting protein for fat in a calorie and carbohydrate-balanced meal. We compared the effects of the three meals on the concentrations of acyl ghrelin (AG), desacyl ghrelin (DAG), glucose, insulin, GLP-1, and peptide tyrosine tyrosine (PYY), all of which regulate food intake in humans and experimental animals. Understanding

the role of the macronutrients on the regulation of ghrelin and other satiety factors will help guide the design of more optimal diets for individuals with PWS and obesity in general.

3.3 Materials and Methods

3.3.1 Population

We studied ten children with PWS (9 females, 1 male; mean age, 8.5±4.2 years; BMI z-score, 0.93±0.5) and seven BMI z-score matched children (1 female, 6 male, mean age 12.1±3.6 years; BMI z-score, 1.14±0.89). We excluded individuals with a medical condition known to affect body composition, such as diabetes mellitus, chronic inflammatory bowel disease, chronic severe liver or kidney disease or neurologic disorders, and those who were taking an investigational drug in the past year.

The diagnosis of PWS was confirmed by DNA methylation and fluorescence in situ hybridization genetic analysis. The control group was comprised of otherwise healthy individuals with BMI z-score comparable to the PWS children. Some controls were siblings or friends of the participants with PWS, while others were recruited from the local Pediatric Centre for Weight and Health. The study was approved by the University of Alberta's Health Research Ethics Board. Written informed consent and assent was obtained from all participants and parents.

3.3.2 Experimental Design

In a randomized crossover study design, patients were asked to arrive after an 8-hour fast. At each of three study visits, fasting blood samples and anthropometric measurements (height and weight) were obtained. Participants were randomly selected to consume one of the three breakfast meals at each visit. The macronutrient content of the SM, HP-LC, and HP-LF meals is shown in (**Table 3.1)**.

Blood samples were obtained every hour for 4 hours after the meal. Samples were assayed for AG and DAG, glucose, insulin, GLP-1, and PYY. Additional analysis of leptin, total and high-molecular weight (HMW) adiponectin were done in fasting samples only. No physical exercise was allowed until testing was complete. The period between test meals was two weeks. Subjective appetite was assessed

immediately before every blood draw using visual analog scales (VAS) (100mm). The assessment contained six questions that required the participant to rate their level of hunger, fullness, desire to eat, amount of food they could eat, urge to eat, and preoccupation with food (Stubbs et al., 2000) (**Appendix D. Example for VAS**).

3.3.3 Preparation of Samples

Blood samples for the determination of AG and DAG concentrations were collected into polypropylene tubes containing K_2 EDTA (3.6 mg) and AEBSF (4mM final), and centrifuged. The separated plasma was immediately treated with 1N HCl (200uL 1N HCl per 1mL plasma) and stored at -80°C until analysis. The remaining blood samples were collected into polypropylene tubes containing K_2 EDTA (3.6 mg) or serum separator tubes supplemented with aprotinin (10 uL per 1mL plasma or serum). Following centrifugation, plasma and serum were stored at -80°C until assayed. All analyses were measured in duplicate.

3.3.4 Hormone and Adipocytokine Assays

Plasma samples were assayed using a novel sensitive and specific two-site assay for AG and DAG, in the lab of Dr. Gaylinn (Liu et al., 2008). Glucose was measured by AHS (Alberta Health Serves lab) total and HMW adiponectin, GLP-1, leptin and insulin were measured using RIAs (Millipore), total PYY was measured using MSD (MesoScale Discovery), and proinsulin was measured using EMD (Millipore), according to manufacturer's instructions. Total and HMW adiponectin, GLP-1 leptin and insulin had coefficients of variation (CVs) <10%. The total PYY had intra- and inter-assay CVs < 9%. Proinsulin had CV < 15%. Glucose had CVs <10%. Homeostatic model assessment insulin resistance (HOMA-IR) was calculated as fasting glucose (mg/dL) × fasting insulin (μIU/mL) ÷ 405 (Conwell, Trost, Brown, & Batch, 2004).

3.3.4 Statistical Analysis

Baseline demographic and biomarker data were described as median and interquartile range (IQR). All null hypotheses for between-group comparisons at baseline were examined using Two-sample Wilcoxon Rank-Sum test Sum (Mann-

Whitney). Repeated measures were performed for each participant in the PWS and control groups for three types of diets at four time points after meal intake. An independent linear mixed model was used to assess changes in each postprandial biomarker outcome (AG and DAG, insulin, glucose, PYY, and GLP-1) over time and examine for group differences and dietary effects. The relative change of biomarker values after meal intake was calculated as a logarithm (base 2) of the fold change of values with respect to the baseline; changes were reported as percentages. Meal types and times were included as fixed effects in linear mixed models. For each model, an appropriate covariance structure was selected to fit the data while keeping models parsimonious. All models were adjusted for age, sex, and BMI z-score. Null hypotheses were rejected at p value ≤ 0.05. All statistical analyses were performed using Stata 13.1 software (College Station, TX: StataCorp LP.) and GraphPad Prism 7 software (GraphPad Software Inc.).

3.4 Results

3.4.1 Baseline Anthropometric and Metabolic Characteristics

The baseline characteristics of study individuals are shown in (**Table 3.2)**. The PWS and control groups were not different in BMI z-scores; however, the PWS group included more females and was younger ($p = 0.05$). One PWS participant had uniparental disomy; all others had a chromosome 15 deletion. All PWS individuals had free thyroxine (T4) and thyroid stimulating hormone (TSH) concentrations in the normal range. Eight patients were taking growth hormone (GH) treatment prior to study enrollment; no changes in GH treatment were made during the course of the study. Of the other two patients, one had been previously treated with GH, but was not taking GH at the time of the study; one patient was never treated with GH.

Under fasting conditions, the PWS group had higher concentrations of total and HMW adiponectin ($p = 0.005$ and $p = 0.02$, respectively), AG ($p = 0.02$), and DAG ($p = 0.01$) than the BMI z-score matched controls. The ratio of fasting AG:DAG was similar in both groups. The PWS group had lower fasting concentrations of glucose ($p = 0.04$) and HOMA-IR ($p = 0.05$) than the control group but fasting concentrations of insulin, proinsulin, GLP-1, PYY and leptin were comparable. The fasting proinsulin to insulin ratio was higher in the PWS group ($p = 0.05$).

3.4.2 Acyl Ghrelin, Desacyl Ghrelin and Acyl Ghrelin:Desacyl Ghrelin Ratio Responses to SM, HP-LC and HP-LF meals

3.4.2.1 Acyl Ghrelin and Desacyl Ghrelin—PWS vs. control Group

Absolute concentrations of AG and DAG were higher in PWS than in BMI z-score matched children across all postprandial time points ($p = 0.002$ and $p < 0.001$ respectively) (**Figure 3.1, A and B**). AG and DAG concentrations decreased in both groups following the SM, HP-LC and HP-LF meals. The time course and magnitude of AG and DAG suppression, as assessed by change relative to baseline, were comparable following SM, HP-LC and HP-LF meals in the PWS group at one ($p = 0.31$, ns and $p = 0.24$, ns), two ($p = 0.4$, ns and $p = 0.26$. ns), three ($p = 0.35$, ns and $p = 0.30$, ns) and four hours ($p = 0.99$, ns and $p = 0.13$, ns) and in the control group at one ($p = 0.46$, ns and $p = 0.67$, ns), two ($p = 0.28$, ns and $p = 0.74$. ns), three ($p = 0.50$, ns and $p = 0.35$, ns) and four hours ($p = 0.67$, ns and $p = 0.61$, ns) (**Figure 3.1, C and D**). However, the magnitude of AG and DAG suppression following all three meals was more pronounced in children with PWS at one ($p = 0.012$ and $p = 0.15$, ns), two ($p = 0.001$ and $p = 0.002$), three ($p = 0.001$ and $p < 001$) and four hours ($p = 0.001$ and $p = 0.001$) and the time return to baseline to recovery was delayed (**Figure 3.1, C and D**).

3.4.2.2 Acyl Ghrelin and Desacyl Ghrelin—PWS Group

The rate and magnitude of return to baseline of ghrelin in children with PWS was similar following all three meals.

3.4.2.3 Acyl Ghrelin and Desacyl Ghrelin—Control Group

The rate and magnitude of return to baseline of ghrelin in control group was similar following all three meals.

3.4.2.4 Acyl Ghrelin:Desacyl Ghrelin Ratio—PWS vs. control Group

The ratio of AG:DAG was higher in the PWS group than in the control group at hour 2 after the SM ($p = 0.03$) (**Figure 3.1, E**).

3.4.2.5 Acyl Ghrelin:Desacyl Ghrelin Ratio—PWS Group

No differences were observed in the ratio of AG:DAG in children with PWS in response to HP-LC and HP-LF meals.

3.4.2.6 Acyl Ghrelin:Desacyl Ghrelin Ratio—Control Group

No differences were observed in the ratio of AG:DAG in control group in response to HP-LC and HP-LF meals.

3.4.3 Glucose, Insulin, and Gut-peptide Responses to SM, HP-LC and HP-LF meals

3.4.3.1 Glucose—PWS vs. Control Groups

Blood glucose concentrations were higher in PWS children than in BMI z-score matched children 1-hour after the SM (106.2 % vs. 94.3 %) and HP-LC (109.1 % vs 89.5 %) meals ($p = 0.03$ and $p = 0.002$ respectively); glucose responses were comparable following the HP-LF meal (**Figure 3.2, A**).

3.4.3.2 Glucose—PWS Group

Glucose responses were comparable in the PWS after all three meals.

3.4.3.3 Glucose—Control Group

Blood glucose concentrations increased more at 1-hour after the HP-LF meal (98.94 %) than after the SM and HP-LC meals (94.3 % and 89.5 % of baseline respectively) ($p = 0.02$ and $p = 0.04$ respectively) in controls (**Figure 3.2, A**).

3.4.3.4 Insulin—PWS vs. Control Group

The fasting proinsulin to insulin ratio (0.08 vs. 0.05) was higher in the PWS group ($p = 0.05$). Insulin concentrations were higher in PWS children compared to BMI z-score matched children following all meals. Following the SM, insulin concentrations were higher in children with PWS than in controls at hours 1, 2 and 3 ($p = 0.002$, $p = 0.008$ and $p < 0.001$, respectively) (**Figure 3.2, B**). HP-LC and HP-LF meals stimulated a greater release of insulin in children with PWS at hours 1, 2 and 3 hours ($p = 0.006$, $p = 0.007$, $p = 0.221$, ns $p < 0.001$, $p = 0.04$ and $p = 0.126$, ns, respectively) than in BMI z-score matched children (**Figure 3.2, B**). The HP-LF meal stimulated a greater release of insulin at hour 1 than the SM in the both children with

PWS and BMI z-score matched controls (p = 0.002 and p = 0.03 respectively) (**Figure 3.2, B**).

3.4.3.5 Insulin—PWS Group

Insulin concentrations were higher following the HP-LC meal at hours 1 and 2 (p = 0.02 and p = 0.03 respectively) in children with PWS (**Figure 3.2, B**). Also, the HP-LF meal stimulated a much greater release of insulin at hour 1 compared to the SM (p = 0.002) (**Figure 3.2, B**).

3.4.3.6 Insulin—Control Group

The HP-LF meal stimulated a much greater release of insulin at hour 1 than the SM (p = 0.03) (**Figure 3.2, B**). There were no differences in insulin concentrations after the HP-LC meal when compared to the SM.

3.4.4 GLP-1

3.4.4.1 GLP-1—PWS vs. Control Group

GLP-1 responses were comparable in PWS and BMI z-score matched children after the SM and HP-LC meals. Overall, for both groups, GLP-1 concentrations were higher than the baseline at hours 1 and 2 following the SM ($p < 0.001$ and $p = 0.007$ respectively) and at hours 1, 2 and 3 following the HP-LC meal ($p < 0.001$, $p < 0.001$ and $p = 0.01$ respectively). GLP-1 concentrations declined in PWS subjects by 3 hours after the SM (**Figure 3.3, A**).

3.4.4.2 GLP-1—PWS Group

GLP-1 concentrations were higher at hours 2 and 4 after HP-LC meal (p = 0.03 and p = 0.04 respectively) and at hour 4 (p = 0.02) following the HP-LF meal in PWS but not in control subjects (**Figure 3.3, A**).

3.4.4.3 GLP-1—Control Group

No differences were observed in GLP-1 response following SM, HP-LC, and HP-LF meals in the control group.

3.4.5 PYY

3.4.5.1 PYY—PWS vs. control Group

PYY responses were comparable in PWS and BMI z-score matched children after the SM, HP-LC, and HP-LF meals. Overall, for both groups, PYY concentrations were higher than baseline at hour 2 after the HP-LC meal ($p = 0.01$) and at hours 1 and 2 after the HP-LF meal ($p = 0.003$ and $p = 0.03$ respectively) (**Figure 3.3, B**).

3.4.5.2 PYY—PWS Group

There were no differences in PYY responses to SM, HP-LC and HP-LF meals among children in PWS group.

3.4.5.3 PYY—Control Group

There were no differences in PYY responses to SM, HP-LC and HP-LF meals among children in control groups.

3.4.6 Appetite Assessment using Visual Analog Scale

3.4.6.1 Control Group

As assessed by VAS, appetite scores were lower at hour 1 compared to baseline after all three meals ($p = 0.02$) (**Figure 3.4, A**). Ratings for appetite were lower after the HP-LC meal than the SM or HP-LF meals at hours 3 and 4 ($p = 0.007$ and $p = 0.03$ respectively) (**Figure 3. 4, A**).

3.5 Discussion

The roles of macronutrient content of the diet in regulating ghrelin and metabolic function in children are poorly understood. Our randomized, crossover study of children with PWS and BMI-matched controls compared the effects of substituting protein for carbohydrate or fat in a calorie balanced meal. Outcome measures included circulating AG, DAG, GLP-1, PYY and indices of insulin secretion and sensitivity.

A summary of the effects of the various diets and the key differences between the PWS children and controls is shown in (**Table 3.3)**. First, fasting and postprandial

AG:DAG ratios were found to be comparable in children with PWS and controls. This study indicates that the higher concentration of ghrelin in children with PWS is due to an increase in both AG and DAG without a change in the ratio of the two forms.

Second, the fasting ratio of proinsulin:insulin ratio was higher in children with PWS than controls, suggesting differential proinsulin processing.

Finally, the two high protein meals HP-LC and HP-LF stimulated a much greater release of GLP-1 compared to the SM in the PWS group only. This suggests that higher protein diets stimulated GLP-1 secretion; suggesting higher protein meals could provide a favorable influence on biomarkers of satiety and possibly, degree of hyperphagia.

Our fasting results are consistent with our previous work and that of other researchers (Eiholzer et al., 1998; Haqq et al., 2011; Krochik et al., 2006; Sohn et al., 2010; Talebizadeh & Butler, 2005). Our AG:DAG ratios findings are in agreement with work done by Kuppens et al. (2015) who found no differences in fasting AG:DAG ratios in stable weight individuals with PWS (with or without hyperphagia) and age-matched individuals (Kuppens et al., 2015). Our participants were overweight and had stable body weight. Paik et al. (2006) reported that fasting AG concentrations were higher but fasting DAG concentrations comparable in children with PWS (BMI between 90^{th} and 95^{th} percentiles) relative to children with obesity (BMI > 95^{th} percentiles) (Paik et al., 2006). Park et al. (2007) also reported higher fasting AG concentrations in children with PWS when compared to children with obesity (Park et al., 2007). None of these studies reported the ratio between AG and DAG. The conflicting results between Paik et al. (2006) and Park et al. (2007) could be attributed to blood sample collection methodology, and the lack of esterase inhibitor to prevent AG deacylation. In the present study, the addition of AEBSF to the blood samples inhibited the deacylation of AG before centrifuging the samples.

The higher fasting proinsulin:insulin ratio in children with PWS in the current study agrees with findings from Burnett et al. (2017). The Burnett et al. (2017) study reported elevated fasting plasma proinsulin:insulin ratios in patients with PWS compared with age- and BMI-matched individuals (Burnett et al., 2017). Elevated fasting plasma proinsulin:insulin ratios in humans with PWS suggests impaired proinsulin processing (Burnett et al., 2017). Proinsulin was found to be increased in a

Snord116 mouse model that mimics a number of neuroendocrine phenotypes of PWS, such as hyperphagia, impaired motor learning, hypoinsulinemia, and hyperghrelinemia (Ding et al., 2008; Skryabin et al., 2007).

Fasting and postprandial AG and DAG concentrations were higher in children with PWS than in BMI z-score matched children. However, meal intake suppressed both forms of ghrelin in both groups. AG and DAG suppression was more pronounced in children with PWS, delaying the return to baseline of ghrelin concentrations. There were no effects of HP-LC or HP-LF meals on AG, DAG, the AG:DAG ratio within or between groups. Our finding is consistent with those of Gumus Balikcioglu et al. (2015) who found that both high-carbohydrate and high-fat meals suppressed ghrelin concentrations in children with PWS as well as BMI-, age- and sex-matched children (Gumus Balikcioglu et al., 2015). These findings also concord with those of Bizzarri et al. (2010) who showed that a mixed liquid meal suppressed plasma ghrelin more in children with PWS than in children with obesity (Bizzarri et al., 2010). In our study, the delayed recovery to baseline in AG and DAG ghrelin in children with PWS compared to BMI z-score matched children may be attributed to the interrelationship between ghrelin and insulin. Several studies showed that insulin reduces circulating concentrations of ghrelin independent of glucose (Chabot, Caron, Laplante, & St-Pierre, 2014; Flanagan et al., 2003; Mohlig et al., 2002; Paik et al., 2007; Saad et al., 2002). Suppression of AG and DAG ghrelin is associated with insulin sensitivity in children with PWS (Broglio et al., 2004; Saad et al., 2002). In the current study, postprandial glucose and insulin concentrations were higher in children with PWS across all the postprandial time points. However, the exact mechanism of suppression ghrelin by insulin is not fully understood. Gumus Balikcioglu et al. (2015) speculated that insulin might modulate neuroendocrine inputs that regulate ghrelin secretion or have an impact on the ghrelin-secretion cells of the stomach (Gumus Balikcioglu et al., 2015).

In the present study, postprandial glucose and insulin concentrations were higher in children with PWS across all postprandial time points. The prolonged insulin and glucose response in PWS implies lower tissue uptake, possible due to lower muscle mass (Alsaif et al., 2017). Another explanation could be due to the GH treatment; previous studies have shown that GH suppresses glucose uptake in the

adipose tissue (Kim & Park, 2017); In the present study, 9 out of 10 children with PWS were on GH treatment; which may explain prolonged insulin and glucose response. As insulin sensitivity reported to be higher in individuals with PWS (Haqq et al., 2011). Blood glucose concentrations were higher in PWS children when compared to controls following SM and HP-LC meal but showed little difference after the HP-LF meal. Studies have shown that GH treatment stimulates glucose release through gluconeogenesis and glycogenolysis from the liver and kidney (Kim & Park, 2017), which may explain the higher blood glucose concentrations in children with PWS. This observation suggests reduced insulin sensitivity and glucose tolerance in the children with PWS group. Yet HMW adiponectin, a marker of insulin sensitivity (Haqq et al., 2011; Irizarry et al., 2019) was higher in children with PWS compared to controls. There were no effects of variations in meal protein content on glucose in children with PWS and no effect of HP-LC meal on glucose and insulin in control group. However, blood glucose concentration increased after HP-LF in the control group meal due to higher carbohydrate content.

Goldstone et al. (2004) reported comparable fasting GLP-1 concentrations in adults with PWS and adults with and without obesity (Goldstone et al., 2004); however, they did not measure postprandial GLP-1. Similarly, Purtell et al. (2011) reported comparable fasting GLP-1 and postprandial GLP-1 concentrations in adults with PWS following high carbohydrate and high fat diet meals (50% carbohydrate, 35% fat, 15% protein) compared to adults with and without obesity (Purtell et al., 2011). The latter observation is consistent with our SM findings. However, the two high protein meals in the current study stimulated much greater increases in the concentrations of GLP-1 in children with PWS. Since GLP-1 inhibits food intake through effects on vagal and CNS function (Dailey & Moran, 2013), this finding suggests that substitution of protein for carbohydrate or protein for fat might limit food intake in children with PWS.

Several reports have described the role of PYY in PWS, but with conflicting results. Our findings regarding PYY differ from previous work. Butler et al. (2004) reported lower PYY concentrations in infants and children with PWS and a higher plasma PYY response to meals in children with PWS compared to children with and without obesity (Butler et al., 2004). Gumus Balikcioglu et al. (2015) reported higher

fasting PYY and a blunted postprandial response to high fat meals in children with PWS compared to BMI z-score matched children (Gumus Balikcioglu et al., 2015). In adults, Purtell et al. (2011) found comparable fasting and postprandial PYY concentrations in PWS individuals when compared to adults with obesity (Purtell et al., 2011). This is in agreement with our findings, where fasting and postprandial PYY were comparable among children with PWS and BMI z-score matched children. However, postprandial PYY concentrations were higher in both groups at hour 2 following the HP-LC meal and at hours 1 and 2 following the HP-LF meals. This suggests that higher protein diets stimulated PYY secretion. Thus higher protein meals might promote satiety in PWS through effects on PYY as well as GLP-1.

Major strengths of our study include its randomized crossover design in which all participants served as their own controls, and its use of specific assays to measure AG and DAG concentrations; however, there might be cross reactivity in the assay that potentially may recognize proghrelin, the precursor of AG and DAG. Limitations of our study include the size and relative heterogeneity of the cohort. There were more females in the PWS group and more males in the control group; we also did not collect pubertal data, which could have influenced some of our hormone findings in the PWS group. Haqq et al. (Haqq et al., 2008) reported that ghrelin concentrations are lower in older children with PWS and control compared to younger children. The control group in the current study was older than the children with PWS. Other studies have shown that ghrelin concentrations are higher in healthy females than in males, and that the concentration decreases with puberty (Whatmore, Hall, Jones, Westwood, & Clayton, 2003). Subjective appetite assessment was not assessed in children with PWS in this study. The use of VAS depends on age and cognitive development (Shields, Palermo, Powers, Grewe, & Smith, 2003), which limited its use in the current study. The children with PWS were younger than those in the control group and lacked the understanding and communication ability to complete VAS questionnaires. We, therefore, used VAS ratings only for the control group.

In the control group, the VAS ratings paralleled our ghrelin suppression findings; appetite was lower at one-hour post-meal compared to baseline for all three meals. Also, appetite was lower after the HP-LC meal compared to the SM at hours 3

and 4. In other studies, adults with PWS rated high hunger scores and high satiety scores immediately after a meal (Purtell et al., 2011). Such results, when compared to our results for VAS ratings, bring into question the reliability of using subjective appetite assessment in PWS individuals. Furthermore, our study meal was 350 kcal and was not personalized to meet each individual's energy needs. It is possible that the young children were overfed and the older children underfed in this study.

Despite these limitations, present study's findings suggest that the higher concentration of total ghrelin in children with PWS was due to an increase in both AG and DAG, with no change in the ratio. Meal consumption also suppresses both forms of ghrelin to a greater extent in children with PWS. An elevated fasting plasma proinsulin:insulin ratio in PWS children suggests impaired processing of proinsulin. Higher protein meals stimulated greater increases in GLP-1 and PYY in children with PWS than in controls. This could provide a favorable influence on biomarkers of satiety and, possibly, degree of hyperphagia. Testing this hypothesis will require long-term studies of the effects of high protein intake on food intake and weight gain.

3.6 Tables

Table 3.1. Study meals

Meal	A standard meal	A higher protein/lower carbohydrate	A higher protein/lower Fat
Protein	13 g, 15% of total kcal	26 g, 30% of total kcal	26 g, 30% of total kcal
Fat	12 g, 30% of total kcal	12 g, 30% of total kcal	6 g, 15% of total kcal
Carbohydrate	48 g, 55% of total kcal	35 g, 40% of total kcal	48 g, 55% of total kcal
Total Energy	350 kilocalories (kcal)	350 kilocalories (kcal)	350 kilocalories (kcal)
Foods	• whole milk with chocolate syrup • toast • cheddar cheese • sliced regular ham • reduced-fat mayonnaise	• skim milk with chocolate syrup • tortilla • cheddar cheese • sliced ham regular and extra lean • reduced fat mayonnaise	• skim milk with chocolate syrup • toast • cheddar cheese reduced fat • extra lean sliced ham • reduced fat mayonnaise

Table 3.2. Baseline characteristics of Prader-Willi syndrome (PWS) and (body mass index) BMI z-score matched children

Characteristic	PWS	BMI z-score matched children	*P* Value
Age, y	6.63 (5.1-17.9)	12.54 (6.8-17.1)	**0.05**
Females/males	9/1	1/6	**0.01**
BMI z-score	1.05 (0.34-1.28)	0.95 (0.72-1.95)	0.81
Total adiponectin, ng/mL	1.5 (0.91-1.60)	0.64 (0.57-0.88)	**0.005**
HMW adiponectin, ng/mL	0.97 (0.72-1.11)	0.59 (0.42-0.62)	**0.02**
Leptin, ng/mL	7684 (3435-27730)	5058 (1908-9850)	0.33
Acyl ghrelin, pg/mL	189.85 (105.00-336.10)	88.20 (36.80-140.20)	**0.02**
Desacyl ghrelin, pg/mL	152.65 (119.90-208.80)	89.80 (50.60-112.00)	**0.01**
Acyl:desacyl ghrelin	122 (1.02-1.49)	1.03 (0.86-1.21)	0.2
Glucose, mmol/L	4.35 (4.20-4.60)	4.70 (4.60-4.75)	**0.04**
Insulin, pg/mL	91.90 (74.80-99.50)	201.90 (90.40-315.20)	0.13
Proinsulin	7.63 (7.38-9.02)	8.46 (5.15-12.93)	0.63
Proinsulin:insulin	0.08 (0.02-0.12)	0.05 (0.04-0.08)	**0.05**
HOMA-IR	0.43 (0.35-0.62)	0.87 (0.74-1.16)	**0.05**
GLP-1, pg/mL	11.5 (7.90-15.30)	9.10 (7.60-14.80)	0.85
PYY, pg/mL	152.17 (109.73-210.95)	121.44 (84.66-158.80)	0.53

y: year; BMI: body mass index; HMW: High molecular weight; HOMA-IR: Homeostatic model assessment of insulin resistance; GLP-1: Glucagon-like peptide-1; PYY: peptide tyrosine tyrosine

All data reported as values are presented as mean ± standard deviation except sex that is number of male/female. p values for statistically significant differences are shown in bold. Two-sample Wilcoxon Rank-Sum test Sum (Mann-Whitney) was used

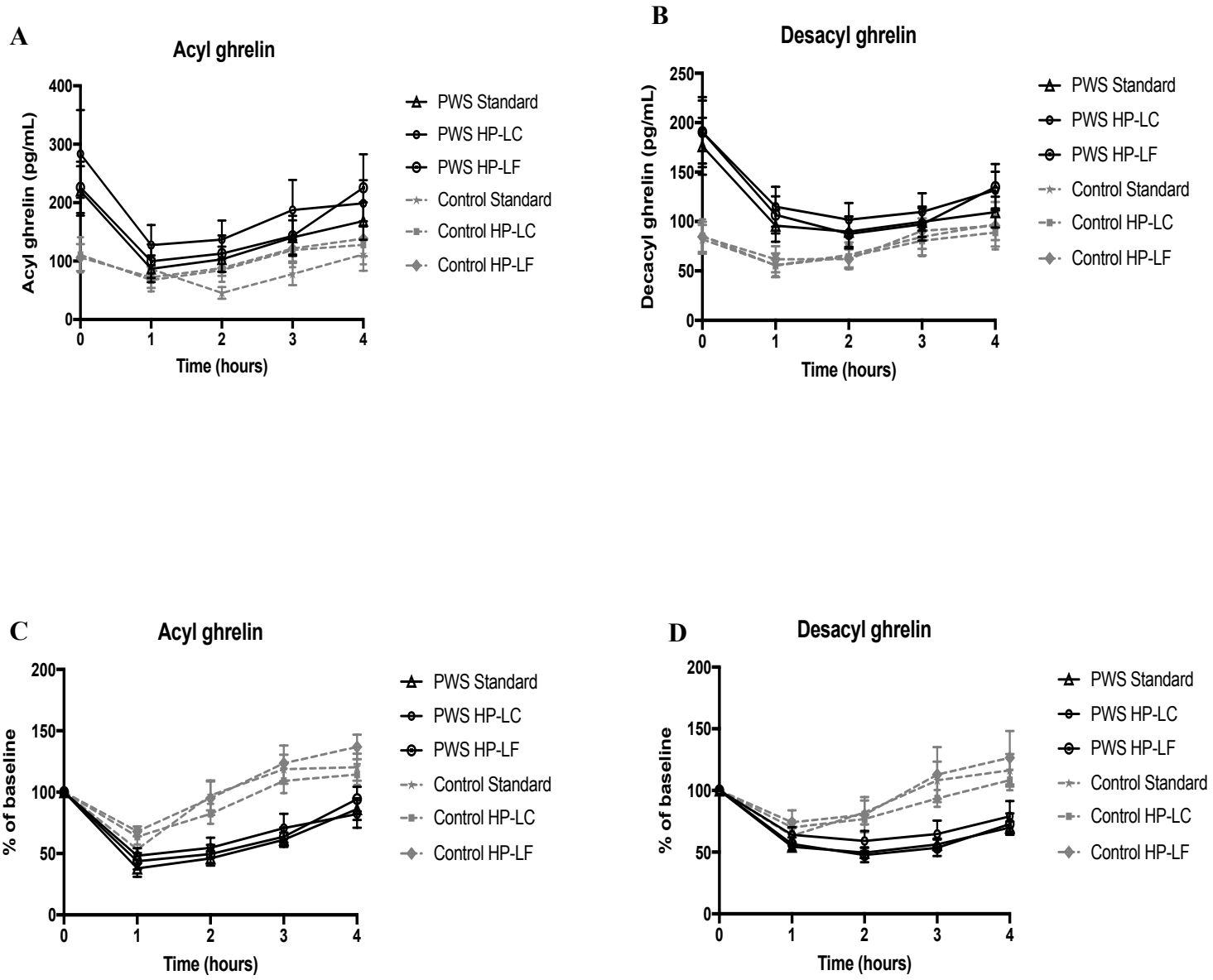
Table 3.3. Summary of the key differences between children with Prader-Willi syndrome (PWS) and the controls

Biomarkers	PWS	Controls	PWS vs. Controls
AG and DAG	The time course and magnitude of suppression of AG and DAG were comparable following all three meals.	The time course and magnitude of suppression of AG and DAG were comparable following all three meals.	Suppression following all three meals was more pronounced in children with PWS following all three meals at all the time points.
AG:DAG	No differences were observed in the ratio of AG:DAG in response to HP-LC and HP-LF meals.	No differences were observed in the ratio of AG:DAG in response to HP-LC and HP-LF meals.	The Ratio of AG:DAG was higher in the PWS group than in the control group at hour 2 after the SM.
Glucose	Glucose responses were comparable after all three meals.	Glucose concentrations increased more at 1-hour after the HP-LF meal than after the SM.	Glucose was higher in PWS children than controls at 1-hour after the SM and HP-LC meal.
Insulin	The HP-LF meal stimulated a greater release of insulin at hour 1 and the HP-LC meal at hours 1 and 2 than the SM.	The HP-LF meal stimulated a greater release of insulin at hour 1 than the SM	Insulin concentrations were higher in PWS children compared to controls following all three meals.
GLP-1	GLP-1 concentrations were higher at hours 2 and 4 after HP-LC meal and hour 4 following the HP-LF meal.	No differences in GLP-1 responses following all three meals.	GLP-1 responses were comparable following all three meals.
PYY	No differences in PYY response following all three meals.	No differences in PYY response following all three meals.	PYY concentrations were higher than baseline at hour 2 after the HP-LC meal and at hours 1 and 2 after the HP-LF meal for both groups.

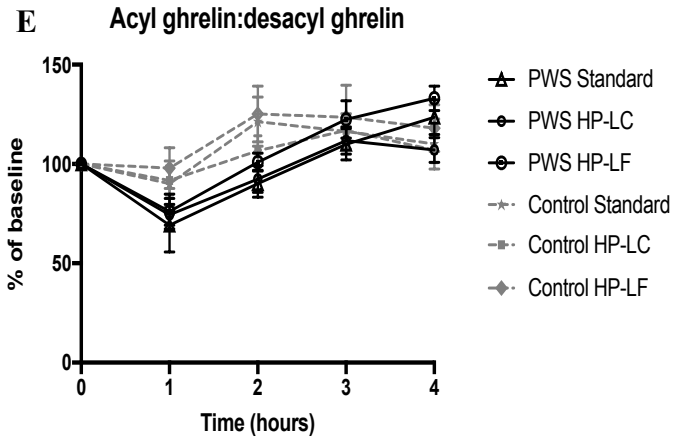
PWS: Prader-Willi syndrome: AG: acyl ghrelin; DAG: desacyl ghrelin; HP-LC: high protein-low carbohydrate meal; HP-LF: high protein-low fat meal; SM: standard meal; GLP-1: glucagon-like peptide 1; PYY: peptide tyrosine tyrosine

3.7 Figures

Figure 3.1. Acyl ghrelin, desacyl ghrelin and acyl ghrelin:desacyl ghrelin ratio response to isocaloric standard, high protein-low carbohydrate and high protein-low fat breakfast meals



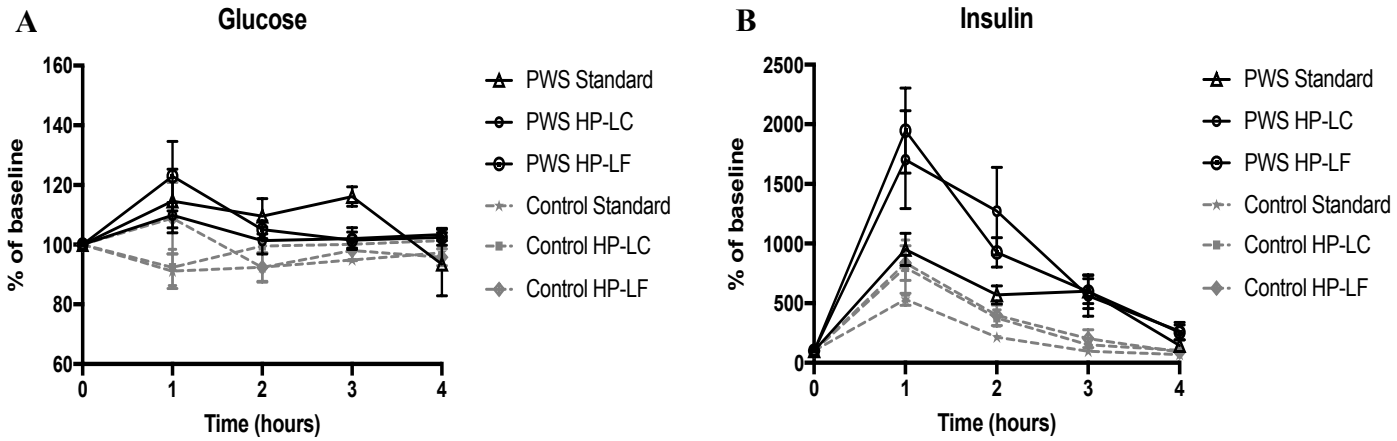
AG and DAG	Between groups		Within group	
	PWS	Control	PWS	Control
Absolute concentrations	Higher at 1, 2, 3 and 4 hours	$p < 0.05$		
% change of baseline				
SM	Higher at 1, 2, 3 and 4 hours	$p < 0.05$	ns	ns
HP-LC	Higher at 1, 2, 3 and 4 hours	$p < 0.05$	ns	ns
HP-LF	Higher at 1, 2, 3 and 4 hours	$p < 0.05$	ns	ns



Predicted values and SEs of percentage changes relative to baseline (time = 0) of each group at each time point were based on linear mixed models. SM, standard meal; HP-LC, high protein-low carbohydrate meal; HP-LF, high protein-low fat meal; AG, acyl ghrelin; DAG, desacyl ghrelin.

AG:DAG	Between groups		Within group	
% change of baseline	PWS	Control	PWS	Control
SM	Higher at hour 2	$p < 0.05$	ns	ns
HP-LC	ns	ns	ns	ns
HP-LF	ns	ns	ns	ns

Figure 3.2. Glucose and Insulin response to isocaloric standard, high protein-low carbohydrate and high protein-low fat breakfast meals

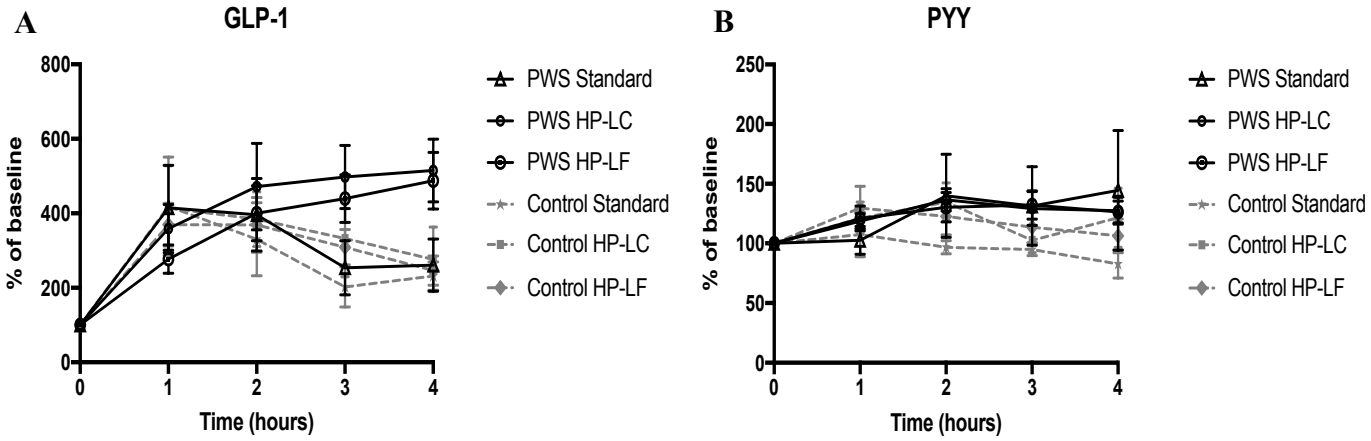


Predicted values and SEs of percentage changes relative to baseline (time = 0) of each group at each time point were based on linear mixed models. SM, standard meal; HP-LC, high protein-low carbohydrate meal; HP-LF, high protein-low fat meal.

Glucose	1-hour between groups		1-hour within group	
% change of baseline	PWS	Control	PWS	Control
SM	Higher compared to control	$p < 0.05$	ns	$p < 0.05$
HP-LC	ns	ns	ns	$p < 0.05$
HP-LF	Higher compared to control	$p < 0.05$	ns	Higher compared to SM and HP-LC

Insulin	Between groups		Within group	
% change of baseline	PWS	Control	PWS	Control
1-hour	Higher after HP-LF meal compared to SM in both group $p < 0.05$ for both groups			
SM	Higher compared to control at 1, 2 and 3 hours	$p < 0.05$	$p < 0.05$	$p < 0.05$
HP-LC	Higher compared to control at 1, 2 and 3 hours	$p < 0.05$	Higher at 1 and 2 hours compared to SM	ns
HP-LF	Higher compared to control	$p < 0.05$	Higher at 1-hour compared to SM	Higher at 1-hour compared to SM

Figure 3.3. Glucagon-like peptide 1 (GLP-1) and peptide tyrosine tyrosine (PYY) response to isocaloric standard, high protein-low carbohydrate and high protein-low fat breakfast meals

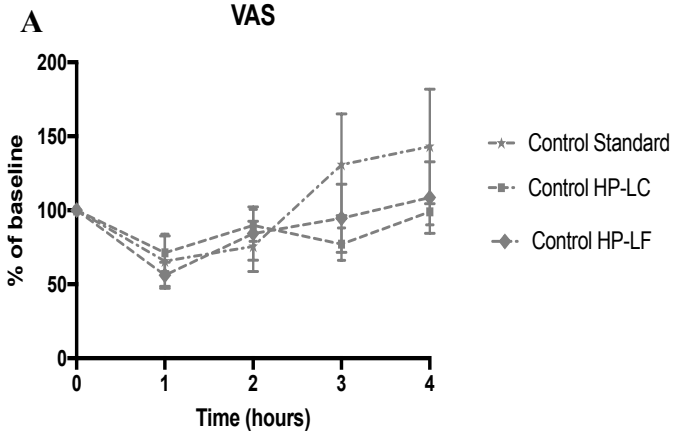


Predicted values and SEs of percentage changes relative to baseline (time = 0) of each group at each time point were based on linear mixed models. SM, standard meal; HP-LC, high protein-low carbohydrate meal; HP-LF, high protein-low fat meal; GLP-1, glucagon like peptide1; PYY, peptide

GLP-1	Between groups		Within group	
% change of baseline	PWS	Control	PWS	Control
Both groups	Higher after the SM at 1 and 2 hours $p < 0.05$ Higher after the HP-LC meal at 1, 2 and 3 hours $p < 0.05$			
SM	ns	ns	$p < 0.05$	ns
HP-LC	ns	ns	Higher at 2 and 4 hours compared to SM	ns
HP-LF	ns	ns	Higher at hour 4 compared to SM	ns

PYY	Between groups		Within group	
% change of baseline	PWS	Control	PWS	Control
Both groups	Higher after the HP-LC at 1 and 2 hours $p < 0.05$ Higher after the HP-LC meal at 1, 2 and 3 hours $p < 0.05$			
SM	ns	ns	ns	ns
HP-LC	ns	ns	ns	ns
HP-LF	ns	ns	ns	ns

Figure 3.4 Visual analog scale responses (VAS) to isocaloric standard, high protein-low carbohydrate and high protein-low fat breakfast meals



Predicted values and SEs of percentage changes relative to baseline (time = 0) of each group at each time point were based on linear mixed models. SM, standard meal; HP-LC, high protein-low carbohydrate meal; HP-LF, high protein-low fat meal.

Appetite scores	Within group
% change of baseline	
All three meals	Lower at 1-hour $p < 0.05$
SM	$p < 0.05$
HP-LC	$p < 0.05$
HP-LF	Lower at 3 and 4 hours compared to SM and HP-LF

Chapter 4 Serum Asprosin Concentrations in Children with Prader-Willi Syndrome: Correlations with Adiposity-Related Parameters

4.1 Preface

The following chapter is based on data collected from 52 children [23 children with Prader-Willi syndrome (PWS) and 29 children with obesity and healthy weight]. Children with PWS were recruited from the Pediatric Endocrinology clinic, Stollery Children's Hospital (Edmonton, Alberta, Canada) and National Institutes of Health, Bethesda, (Bethesda, Maryland, United State). Controls were recruited from the Pediatric Centre for Weight and Health, Edmonton General Continuing Care Centre, Child Health Clinic, Misericordia Hospital (Edmonton, Alberta, Canada). The orexigenic hormone ghrelin was reported to be higher in individuals with PWS at fasting and postprandially, which Chapter 3 also confirms. Asprosin is a newly discovered hormone, considered a second orexigenic hormone. Asprosin use the same neuropeptide tyrosine (NPY)/agouti-related peptide (AgRP) pathway as ghrelin to stimulate appetite. Therefore, this chapter aimed to measure serum asprosin concentrations in children with PWS compared to children with obesity and healthy weight. In a subgroup analysis, we matched children with PWS with body mass index (BMI) z-score-matched children and measured the correlation to glucose, insulin, insulin resistance, percentage of body fat, acyl ghrelin (AG) and leptin. Ms. Maha Alsaif contributed to the study design formation, data analysis, performed the statistical analysis, and was responsible for data interpretation and writing the initial manuscript. She was supported by ongoing discussions with Drs. Andrea Haqq and Carla Prado. This manuscript is being prepared for submission to *Pediatric Obesity*.

4.2 Introduction

Prader-Willi syndrome (PWS) is a unique clinical model of disordered satiety and paradoxical fasting and postprandial hyperghrelinemia (Haqq et al., 2008). PWS is characterized by a failure to thrive and low muscle tone during infancy, followed by severe obesity in childhood (Cassidy, Schwartz, Miller, & Driscoll, 2012; Curfs & Fryns, 1992). The pathogenesis of obesity in children with PWS has been ascribed to several factors, including high circulating concentrations of ghrelin because ghrelin stimulates food intake and weight gain (Atalayer et al., 2013).

Asprosin is a newly-discovered protein hormone produced by the white adipose tissue discovered by identifying two patients with neonatal progeroid syndrome (NPS) who were insulin sensitive despite having lipodystrophy (Romere et al., 2016). In addition, they consumed less food than age matched controls. These findings raised the question of why these NPS patients do not display a typical lipodystophic phenotype. To address this question, Romere et al. (2016) performed genetic analysis of the two NPS patients. These patients had a truncating mutation in FBN1 (which encodes profibrillin). This mutation resulted in the loss of the C-terminal cleavage product of profibrillin, which later named asprosin (Romere et al., 2016). Romere et al., (2016) observed that FBN1 mRNA expression levels were highest in adipose tissue across all tissues, which suggests that adipose tissue is the source of asprosin. Immunoblotting techniques have previously shown Asprosin to be a single protein (found to be around 30 kDa when run on SDS-PAGE). This hormone is predicted to have three N-linked glycosylation sites in humans (Romere et al., 2016). It stimulates hepatic glucose production via the G protein-coupled receptor (GPCR)-activated cAMP signaling pathway and is correlated with insulin resistance (Romere et al., 2016). This explains why NPS patients lacking this hormone appear to be protected from insulin resistance. Additionally, circulating asprosin concentrations increased with fasting in mice, rats, and humans, and decreases with food intake (Beutler & Knight, 2018). This suggested that asprosin may act as a circulating orexigenic signal that stimulates appetite and food intake similar to ghrelin (Beutler & Knight, 2018). Asprosin interacts with the neuropeptide tyrosine (NPY)/agouti-related peptide (AgRP) pathway to exert its effects on appetite (Beutler & Knight, 2018).

Fasting asprosin has been measured only in one study in adults with obesity and in one study in children with obesity (Long et al., 2019; Wang et al., 2018). Wang et al., (2018) reported significantly higher concentrations of fasting asprosin in adults with obesity than in healthy weight participants (Wang et al., 2018). By contrast, in children with obesity, fasting

asprosin is lower than in children with healthy weight (Long et al., 2019). However, it is not clear whether asprosin concentrations are altered in children with PWS and children with non-syndromic obesity and healthy weight. Therefore, the current study measured the concentrations of serum asprosin in children with PWS, children with obesity and children with healthy weight, and assessed the relationship of asprosin concentrations to glucose, insulin, insulin resistance, acyl ghrelin (AG), leptin, and percentage of body fat.

4.3 Materials and Methods

4.3.1 Population

The study population consisted of 52 children (23 children with PWS and 29 children with obesity and healthy weight). Seventeen participants (10 children with PWS and 7 body mass index (BMI) z-score matched children) had participated in the previously described study of dietary macronutrient regulation of AG and desacyl ghrelin concentrations in children with PWS (**Chapter 3**). Three participants (1 child with PWS and 2 controls) had participated in the study of the effect of high protein diet on diet-induced thermogenesis in children with PWS (**Chapter 5**). Thirty-two children (12 children with PWS and 20 controls) were recruited from the Pediatric Endocrinology Clinics, Stollery Children's Hospital, Pediatric Centre for Weight and Health, Edmonton General Continuing Care Centre, Child Health Clinic, Misericordia Hospital, and National Institutes of Health, Bethesda, Maryland. We excluded individuals with medical conditions known to affect body composition, such as diabetes mellitus, chronic inflammatory bowel disease, chronic severe liver, kidney disease or neurologic disorders, and those individuals who have been taking an investigational drug in the past year. The diagnosis of PWS was confirmed by DNA methylation and fluorescence in situ hybridization genetic analysis. The University of Alberta's Health Research Ethics Board and National Institutes of Health research ethics board approved the study. Written informed consent and assent were obtained from all participants and parents.

4.3.2 Anthropometry and Body Composition

Weight was measured to the nearest 0.1kg using the same calibrated scale. Height was measured to the nearest 0.1cm using a wall-mounted stadiometer for children. BMI standard deviation (SD) score was calculated using *EpiInfo* (CDC, Atlanta, GA). Body composition (percentage of body fat) was measured by air displacement plethysmography (BOD POD, Life Measurement Inc.) Waist circumference (WC) was measured in centimetre (cm) at the top of the iliac crest using standardized techniques.

4.3.3 Experimental Design

In a cross-sectional study design, participants arrived to the research unit following an 8-hour fast; weight and height were recorded. Body composition was measured in 17 participants (10 children with PWS and 7 controls). Blood samples were collected at baseline. In a subgroup of participants (10 children with PWS and 7 controls), blood samples were collected 60 minutes after consuming a 350 kcal standard meal (SM) breakfast containing 55% carbohydrate, 30% fat and 15% protein, which represents a typical Canadian diet (Garriguet, 2007).

4.3.4 Preparation of Samples

Blood samples for the determination of asprosin concentrations were collected into serum separator tubes supplemented with aprotinin (10 uL per 1mL serum). Following centrifugation, serum was stored at -80°C until assayed. Blood samples for the determination of glucose, insulin, AG and leptin were described elsewhere (see **Chapter 3, section 3.3.3: Preparation of Samples**). All analyses were measured in duplicate.

4.3.5 Hormone and Adipocytokine Assays

Fasting and 1-hour post-meal serum concentrations of asprosin were measured in duplicate using an enzyme-linked immunosorbent assay kit, with intra-assay coefficients of variation (CVs) < 10% and inter-assay CVs < 12% (Catalogue No. abx257694; Abbexa, Cambridge, UK). Asprosin was assayed concurrently on previously unthawed frozen serum. The serum samples were diluted to 1:2 with the diluent (0.01 mol/L PBS). Data from additional hormones (glucose, insulin, AG and leptin) were described in (**Chapter 3 section 3.3.4: Hormone and Adipocytokine Assays)** and have been included here. Homeostatic model assessment insulin resistance (HOMA-IR) was calculated as fasting glucose (mg/dL) × fasting insulin (μIU/mL) ÷ 405.

4.3.6 Statistical Analysis

Data are presented as median and interquartile range, because of the non-normal distribution for many variables. The Shapiro-Wilk Test was used to test for normality. All null hypotheses for between-group comparisons for age, sex, BMI z-score, fasting asprosin, glucose, insulin, HOMA-IR and WC were examined using Kruskal-Wallis H test (for three group

comparisons). In the subgroups analysis, between-group comparisons for percentage of body fat, asprosin, 1-hour post-meal asprosin, and AG were examined using the nonparametric. Two-sample Wilcoxon Rank-Sum test Sum (Mann-Whitney) (for two group comparisons). The Wilcoxon Signed-Rank Test was used for within-group comparison of fasting asprosin and 1-hour post-meal asprosin. The correlation between age, BMI z-score, percentage of body fat, glucose, insulin, HOMA-IR, WC, leptin and AG, and fasting were determined using a Spearman's Rank-Order correlation. Partial correlation analyses were performed to evaluate the relationships between fasting asprosin and 1-hour postprandial asprosin and percentage of body fat, glucose, insulin, HOMA-IR, WC, leptin and AG adjusted for potential confounders (age, sex and BMI z-score). All statistical analyses were performed using SPSS version 24 for Windows (SPSS Inc., Chicago, IL) and GraphPad Prism 7 software (GraphPad Software Inc.). A value of $p \leq 0.05$ was considered statistically significant.

4.4 Results

4.4.1 Baseline Anthropometric and Metabolic Characteristics

Baseline characteristics of study participants are shown in (**Table 4.1)**. We studied a total of 52 children—23 with PWS, 8 with obesity, and 21 with healthy weight. Groups were comparable for sex distribution, asprosin, glucose, insulin and HOMA-IR. Median age values were comparable among children with obesity and children with healthy weight, but lower in children with PWS ($p = 0.05$). Median BMI z-score values were lower in children with PWS compared to children with obesity ($p = 0.008$) and lower in children with healthy weight compared to children with PWS and obesity ($p = 0.007$ and $p < 0.001$, respectively). Waist circumference values were comparable between children with PWS, children with obesity, and children with healthy weight. However, WC was lower in children with healthy weight when compared to children with obesity ($p = 0.008$). Five participants with PWS had uniparental disomy; all others had a chromosome 15 deletion. All PWS individuals had free thyroxine (T4) and thyroid stimulating hormone (TSH) concentrations in the normal range. Eighteen participants with PWS were taking growth hormone (GH) treatment prior to study enrollment; no changes in GH treatment were made during the course of the study. Of the other five participants, three had been previously treated with GH, but were not taking GH at the time of the study; two participants had never been treated with GH. In the subgroups of study participants, there were 10 children with PWS and 7 BMI z-matched children; characteristics of study participants are shown in (**Table 4.2**). The children with PWS group included more females and were younger ($p = 0.002$ and $p = 0.05$, respectively) and had

lower fasting concentrations of glucose (p = 0.04) and showed a lower HOMA-IR (p = 0.05) compared to BMI z-matched children. Fasting asprosin, insulin, percentage of body fat, WC and leptin were comparable between groups. However, children with PWS had higher fasting concentrations of AG (p = 0.02) compared to BMI z-matched children.

4.4.2 Postprandial Asprosin

Fasting serum asprosin and 1-hour post-meal serum asprosin did not differ between children with PWS and BMI-z matched children (p = 0.60 and p = 0.87, respectively) (**Figure 4.1 A and B**). The decrease in serum asprosin relative to baseline was not different between children with PWS and BMI-z matched children (p = 0.80).

4.4.3 Correlation Between Asprosin and Metabolic Parameters

Correlation analyses were performed between asprosin concentration and clinical and biochemical parameters in children with PWS, children with obesity and healthy weight children (**Table 4.3**). The analyses showed a negative correlation of asprosin concentration with age and insulin and HOMA-IR (rs = -0.71, p = 0.05, rs = -0.95, p = 0.001 and rs = -0.95, p = 0.001) in children with obesity and with age in healthy weight children (rs = -0.45, p = 0.04) (**Table 4.3**). No significant correlations were found between asprosin and age, BMI z-score, WC, glucose, insulin and HOMA-IR in children with PWS. After adjusting for age, sex and BMI z-score, asprosin showed a positive correlation with glucose (rs = 0.92, p = 0.02) in children with obesity but not in children with PWS or healthy weight children (**Table 4.4**).

In the subgroup analyses, children with PWS and BMI z-matched children correlation analyses were performed between fasting asprosin and body composition (BMI z-score, WC and percentage of body fat), and clinical and biochemical parameters (age, glucose, insulin, HOMA-IR, leptin and AG) (**Table 4.5**). Fasting asprosin was positively correlated with AG (r_s = 0.62, p = 0.05) in children with PWS, but not in BMI z-matched children (**Table 4.5**). Furthermore, 1-hour postprandial asprosin was negatively correlated with insulin (r_s = -0.62, p = 0.05) in children with PWS but not in BMI z-matched children (**Table 4.5**). However, when adjusting for age, sex and BMI z-score, the correlation between fasting asprosin and AG and 1-hour postprandial asprosin and insulin were no longer existed (**Table 4.6**).

4.5 Discussion

This is the first study to compare fasting asprosin in children with PWS, children with obesity, and healthy weight children, and to analyze its relationship to glucose, insulin, HOMA-IR, and WC. In the subgroup of study participants, we assessed fasting and 1-hour postprandial asprosin in children with PWS and BMI z -score matched children and its relationship to glucose, insulin, HOMA-IR, ghrelin, leptin, WC and percentage of body fat.

In children with PWS, fasting asprosin was positively correlated with AG and 1-hour postprandial asprosin was negatively correlated with insulin. Both asprosin and ghrelin are orexigenic hormones that stimulate appetite and food intake (Beutler & Knight, 2018). Additionally, fasting asprosin was negatively correlated with age and insulin in children with obesity and with age in healthy weight children. After adjusting for age, sex and BMI z-score, asprosin showed a positive correlation with glucose in children with obesity but not in children with PWS or healthy weight children. This is in disagreement with Long et al. (2019) who reported that fasting asprosin was negatively correlated with BMI z-score, insulin, and HOMA-IR in children with obesity and healthy weight.

Excessive adiposity leads to dysfunction of adipokines and metabolic disorders (Clark & Hoenig, 2016; Czech, 2017). This present study indicated that serum asprosin was positively correlated with insulin and glucose in children with obesity. Following the increased in asprosin concentrations, glucose was gradually increased. Children with obesity are at higher risk of developing type 2 diabetes (T2D) compared to healthy weight children (Abbasi, Juszczyk, van Jaarsveld, & Gulliford, 2017). This finding is in agreement with Zhang et al. (2019) who found a positive correlation between serum asprosin and glucose in adults with T2D (Zhang et al., 2019). These findings suggest that asprosin might be a risk factor associated with the development of T2D.

There is a relationship between increased adiposity and insulin resistance (Mokdad et al., 2003). In adults with insulin resistance, asprosin concentrations were higher and positively correlated with insulin resistance (Li et al., 2018). The insulin and HOMA-IR concentrations in children with PWS and control children in the current study were low, suggesting they are metabolically healthy (Iacobini, Pugliese, Blasetti Fantauzzi, Federici, & Menini, 2019). It has been well documented that children with PWS are insulin sensitive (Haqq et al., 2011; Irizarry et al., 2019). This may explain why a relationship between asprosin and HOMA-IR was not observed.

We did not detect significant difference in fasting asprosin concentrations among children with PWS, children with obesity, and healthy weight children, or in the subgroup

analysis between children with PWS and BMI z-score matched children. However, a previous study of 117 adults with obesity (BMI 41.6 ± 6.3 kg/m2) and 57 healthy weight adults (BMI = 25.3 ± 3.6 kg/m^2) reported that fasting asprosin concentrations were significantly higher in adults with obesity when compared to healthy weight participants (2360 ± 5094 ng/ml vs. 307 ± 833 ng/ml, $p < 0.000$) (Wang et al., 2018). The finding of higher asprosin concentrations in adults with obesity is in disagreement with Long et al. (2019) who reported lower fasting asprosin concentrations in 47 children with obesity (BMI z-score = 2.09 ±0.47 for boys and 2.22 ± 1.08 for girls) than in the 40 children with healthy weight (BMI z-score = –0.52 ± 0.84 for boys and –0.27 ± 0.95 for girls) (9.24 ± 4.11 ng/mL vs. 12.33 ± 4.18 ng/mL, $p < 0.001$) (Long et al., 2019).

This discrepancy can be attributed to the degree of obesity, and may also be an important factor affecting asprosin concentrations. In Wang et al. (2018) the average BMI for adults with obesity was > 35 kg/m^2, which is 3 SD higher than in the Long et al. (2019) study. Another possibility may be that the discrepancies are due to the use of BMI and BMI z-score to define obesity; however, BMI and BMI z-scores do not accurately measure fat mass or muscle mass (Prado & Heymsfield, 2014). It has been reported that asprosin is secreted from adipocytes (Romere et al., 2016); it is not clear whether the high BMI and BMI z-score in previous studies is due to increase in fat mass or fat-free mass. In the current study, in the subgroup analysis, children with PWS and children with BMI z-score matched children had a comparable percentage of fat mass, which could explain why we did not observe any differences in fasting asprosin between the two groups.

Also, the difference between our findings and those of Wang et al. (2018) and Long et al. (2019) may due to the inconsistency of the methods used to obtain asprosin measurement. Wang et al. (2018) diluted serum samples in 1:4 with the diluent (2% non-fat milk, 0.05% Tween 20, PBS) and used an in-house assay. In Long et al. (2019) it was not clear whether the samples were diluted and the asprosin concentrations were determined by a commercial ELISA kit according to the manufacturer's instructions (Human ELISA Kit; Wuhan EIAab Science Co. Ltd., China).

In the current study, serum samples were diluted to 1:2 with the diluent (0.01 mol/L PBS) and measured in duplicate using an enzyme-linked immunosorbent assay kit (Catalogue No. abx257694; abbexa, Cambridge, UK). Therefore, differences in blood collection and processing methods, as well as the timing of the blood draw may explain the conflicting findings between studies

A previous study has shown that asprosin increases with fasting and reduces after feeding, and that higher asprosin concentrations stimulates appetite in mice models with obesity (Duerrschmid et al., 2017). In the current study, we did not observe any significant difference between fasting and postprandial asprosin in both groups. Postprandial asprosin was measured an hour after a SM was consumed, and the peak in asprosin may have occurred outside the window of our measurement. Currently, no final conclusion can be drawn on the impact of food intake on asprosin suppression. In addition, the small sample size does not provide sufficient power to test for differences due to the high variation among the groups. Much remains to be learned about this hormone, specifically its duration of action. It remains to be seen if asprosin is involved in long-term regulation of food intake, or if it is a fast-acting hormone with a role in meal initiation (Beutler & Knight, 2018). Our findings suggest that asprosin might be a mediator of long-term regulation of energy balance and of food intake suppression (Romere et al., 2016), rather than a rapid agonist of AgRP neurons (Beutler & Knight, 2018).

Major strengths of our study include relatively large sample size for a rare genetic disease with a prevalence 1 in 10,000 to 16,000 live births (Nicholls et al., 1989). This is the first study to measure asprosin in children with PWS, children with obesity and healthy weight children, as well as the measurement of postprandial concentration of asprosin. Limitations of our study include not measuring body composition in the entire cohort as the data collected from two different sites. This is a cross-sectional designed study and therefore causality between serum asprosin concentrations and other parameters cannot be established.

After adjusting for age, sex and BMI z-score, the concentrations of serum asprosin correlated positively with glucose in children with obesity. Thus, the circulating asprosin might be a predictor of early weight gain in childhood and might be a potential therapeutic target for obesity and T2D.

4.6 Tables

Table 4.1. Baseline characteristics of children with Prader-Willi syndrome (PWS), children with obesity and children with healthy weight

Characteristic	PWS (n=23)	CWO (n=8)	HWC (n=21)
Age, y	8.4 (6.2, 12.5)[a]	12.9 (11.1, 15.0)[b]	12.8 (9.0, 14.0)[b]
Males/females	8/15	6/2	13/8
BMI z-score	1.02 (0.3, 1.3)[a]	1.7 (1.34, 2.1)[b]	-0.1 (-0.5, 0.4)[c]
WC, cm	67.7 (55.7, 82.0)[a,b]	81.43 (76.3, 94.1)[a]	63.8 (59.6, 70.6)[b]
Asprosin, pg/mL	3.5 (2.9, 4.5)	4.18 (1.5, 4.5)	3.71 (2.9, 4.4)
Glucose, mmol/L	4.0 (3.7, 4.3)	4.7 (3.2, 4.8)	3.94 (3.6, 4.2)
Insulin, pg/mL	471.5 (95.0, 1680.0)	840.0 (375.0, 5610.0)	915.5 (315.2, 1471.5)
HOMA-IR	2.0 (0.5, 7.1)	3.5 (2.1, 19.2)	3.7 (1.3, 5.9)

Data reported as medians (interquartile range); The Shapiro-Wilk Test was used. Groups with different letter superscripts are significantly different ($p \leq 0.05$) from each other. PWS: Prader-Willi syndrome; CWO: children with obesity; HWC: healthy weight children; y: year; BMI: body mass index; WC: waist circumference; HOMA-IR: homeostatic model assessment insulin resistance.

Table 4.2. Baseline characteristics of children with Prader-Willi syndrome (PWS) and body mass index (BMI) z-score matched children

Characteristic	**PWS (n=10)**		**BMI z-s MC (n=7)**		*p* Value
Age, y	6.6 (5.4, 10.1)		12.5 (10.2, 13.7)		**0.05**
Males/females	1/9		6/1		**0.002**
BMI z-score	1.0 (0.41, 1.2)		0.95 (0.8, 1.1)		0.81
WC, cm	60.0 (54.04, 69.5)		77.45 (68.4, 83)		0.07
Asprosin, pg/mL	3.2 (2.9, 4.3)		4.3 (3.7, 4.9)		0.13
1- hour asprosin, pg/mL	3.1 (2.4, 4.5)		4.9 (3.5, 4.9)		0.16
Glucose, mmol/L	4.7 (4.2, 5.1)		4.1 (3.9, 4.6)		**0.04**
Insulin, pg/mL	91.85 (77.0, 98.5)		201.9 (110.0, 210.8)		0.13
HOMA-IR	0.43 (0.37, 0.58)		0.9 (0.8, 0.9)		**0.05**
Acyl ghrelin, pg/mL	189.8 (110.2, 308.9)		88.2 (60.0, 92.6)		**0.02**
Leptin, ng/mL	7683.7 (43734.0, 24142.2)		5058 (2912.4, 5497.8)		0.36
Body fat, %	**Male**	**Females**	**Males**	**Female**	
	34.8	24.1 (20.7, 34.6)	25.8 (20.8, 28.7)	20.9	0.74

Data reported as medians (interquartile range); Two-sample Wilcoxon Rank-Sum test Sum (Mann-Whitney) used. p values for statistically significant differences are shown in bold. PWS: Prader-Willi syndrome; BMI z-s MC: body mass index z-score matched children; WC: waist circumference; HOMA-IR: homeostatic model assessment insulin resistance.

Table 4.3. Spearman correlations of fasting asprosin to other metabolic parameters in children with Prader-Willi syndrome (PWS), children with obesity and children with healthy weight

	PWS		**CWO**		**HWC**	
	r	***p***	**r**	***p***	**r**	***p***
Age	0.17	0.45	-0.71	**0.05**	-0.45	**0.04**
BMI z-score	0.32	0.14	-0.71	0.87	-019	0.34
WC, cm	0.24	0.26	-0.33	0.42	-0.32	0.16
Glucose, mmol/L	0.04	0.85	0.18	0.67	-0.70	0.76
Insulin, pg/mL	0.29	0.18	-0.95	**0.001**	-0.36	0.10
HOMA-IR	0.32	0.13	-0.95	**0.001**	-0.38	0.08

PWS: Prader-Willi syndrome; CWO: children with obesity; HWC: healthy weight children; BMI: body mass index; WC: waist circumference; HOMA-IR: homeostatic model assessment insulin resistance. p values for statistically significant differences are shown in bold.

Table 4.4. Partial correlations of fasting asprosin to other metabolic parameters adjusting for age, sex and BMI z-score in children with Prader-Willi syndrome (PWS), children with obesity and healthy weight children

	PWS		**CWO**		**HWC**	
	r	***p***	**r**	***p***	**r**	***p***
WC, cm	0.24	0.29	0.32	0.60	0.26	0.16
Glucose, mmol/L	-0.05	0.84	0.92	**0.02**	0.04	0.87
Insulin, pg/mL	-0.06	0.80	-0.77	0.12	-0.36	0.10
HOMA-IR	-0.08	0.73	-0.46	0.43	-0.14	0.59

PWS: Prader-Willi syndrome; CWO: children with obesity; HWC: healthy weight children; BMI: body mass index; WC: waist circumference; HOMA-IR: homeostatic model assessment insulin resistance. p values for statistically significant differences are shown in bold.

Table 4.5. Spearman correlations of fasting and 1-hour postprandial asprosin to other metabolic parameters in children with Prader-Willi syndrome (PWS), and children with body mass index (BMI) z-score matched children

	Fasting asprosin				**1-hour postprandial asprosin**			
	PWS		BMI z-s MC		PWS		BMI z-c MC	
	r	*p*	r	*p*	r	*p*	r	*p*
Age	0.50	0.14	-0.60	0.15	0.37	0.29	-0.61	0.15
% body fat	-0.21	0.56	-0.36	0.94	-0.08	0.83	-0.04	0.94
BMI z-score	-0.30	0.40	0.36	0.43	-0.26	0.47	0.36	0.43
WC, cm	0.13	0.73	-0.29	0.53	0.11	0.75	-0.29	0.43
Glucose, mmol/L	-0.29	0.41	-0.07	0.88	-0.24	0.49	-0.07	0.88
Insulin, pg/mL	-0.61	0.06	-0.32	0.48	-0.62	**0.05**	-0.32	0.48
HOMA-IR	0.23	0.54	-0.29	0.53	0.09	0.80	-0.29	0.53
Acyl ghrelin, pg/mL	0.62	**0.05**	0.39	0.38	0.56	0.09	0.39	0.38
Leptin, ng/mL	0.42	0.23	0.07	0.88	0.49	0.15	0.07	0.88

PWS: Prader-Willi syndrome; BMI z-s MC: body mass index z-score matched children; WC: waist circumference; HOMA-IR: homeostatic model assessment insulin resistance. p values for statistically significant differences are shown in bold.

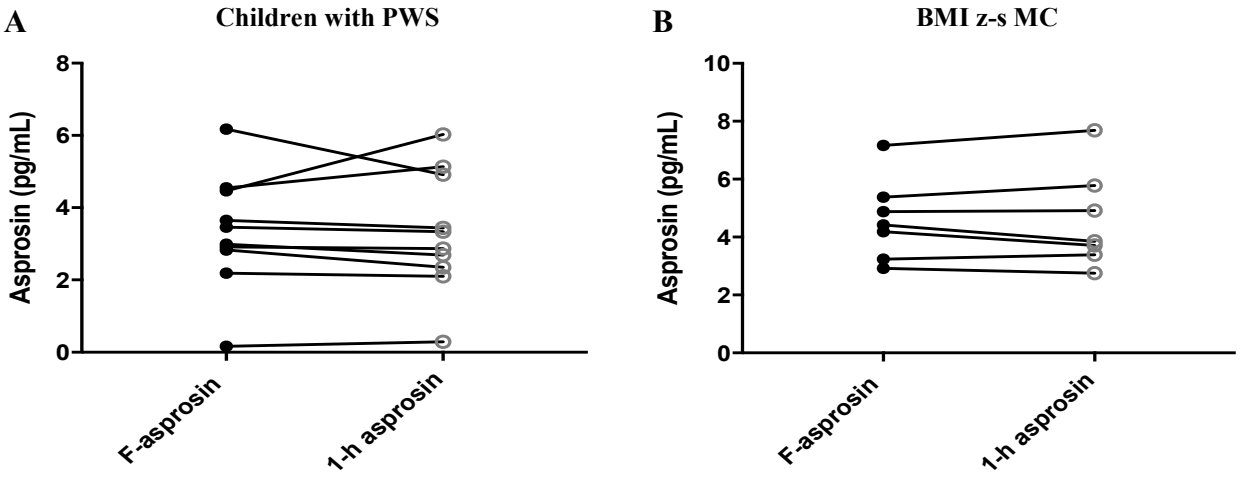
Table 4.6.
Partial correlation of fasting asprosin to other metabolic parameters adjusting for age, sex and BMI z-score in children with Prader-Willi syndrome (PWS), and children with body mass index (BMI) z-score matched children

	Fasting asprosin				**1-hour postprandial asprosin**			
	PWS		BMI z-s MC		PWS		BMI z-s MC	
	r	*p*	r	*p*	r	*p*	r	*p*
% body fat	0.02	0.97	0.17	0.83	-0.13	0.78	0.14	0.86
WC, cm	0.06	0.89	-0.46	0.55	0.12	0.79	-0.44	0.56
Glucose, mmol/L	-0.29	0.41	-0.41	0.59	0.47	0.29	-0.41	0.59
Insulin, pg/mL	-0.30	0.50	0.51	0.48	-0.25	0.58	0.48	0.52
HOMA-IR	0.10	0.83	-0.64	0.54	0.22	0.63	-0.51	0.49
Acyl ghrelin, pg/mL	0.59	0.16	-0.30	0.70	0.49	0.27	-0.29	0.71
Leptin, ng/mL	0.32	0.46	-0.40	0.60	0.36	0.43	-0.44	0.56

PWS: Children with Prader-Willi syndrome; BMI z-s MC: body mass index z-score matched children; WC: waist circumference; HOMA-IR: homeostatic model assessment insulin resistance. p values for statistically significant differences are shown in bold.

4.7 Figure

Figure 4.1. Fasting and 1-hour post-meal asprosin in children with Prader-Willi syndrome (PWS) and body mass index (BMI) z- score matched children



PWS: Prader-Willi syndrome; BMI z-s MC: body mass index z-score matched children; F-asprosin: fasting asprosin; 1-h asprosin: 1-hour asprosin. Wilcoxon Signed-Rank Test was used.

Chapter 5 Effect of High Protein Diet on Postprandial Energy Expenditure in Children with Prader-Willi Syndrome

5.1 Preface

The following chapter is based on data collected from five youth with Prader-Willi syndrome (PWS), at the University of Alberta. The study aimed to determine the impact of a high protein (HP) intake compared to low protein intake on postprandial energy expenditure (PEE) in children with PWS. This study also examined the correlation between PEE and subjective appetite sensations in children and youth with PWS. Ms. Maha Alsaif was responsible for designing the study, recruiting the participants and acquiring the data, performing the statistical analysis, analyzing the data and writing the first draft of the chapter. She had discussions and support from Drs. Andrea Haqq and Carla Prado. Ms. Maha Alsaif, Drs. Michelle Mackenzie, Sarah Elliott, Carla Prado and Andrea Haqq developed the study design. Drs. Andrea Haqq, Michelle Mackenzie, Sarah Elliott and Carla Prado contributed to concept of formation, interpretation and editing of the final chapter. This manuscript is being prepared for submission to *Metabolism: Clinical and Experimental.*

5.2 Introduction

Diet-induced thermogenesis (DIT) is the energy expended through digestion, absorption and storage of nutrients (Westerterp, 2004b). DIT contributes to approximately 10 – 15% of total energy expenditure (TEE) and this value varies within and between individuals (Donahoo, Levine, & Melanson, 2004; Scott & Devore, 2005). Even though DIT represents a small component of TEE, the cumulative impact of altered DIT over time could have a measurable influence on energy metabolism and metabolic health (Westerterp, 2004b). Individuals with obesity have lower DIT compared to healthy weight individuals; this reduction in TEE could explain the weight gain (De Palo et al., 1989; Maffeis et al., 2001; Marrades et al., 2007; Schutz, Bessard, et al., 1984; Segal et al., 1990; Steiniger et al., 1987).

The weight gain and subsequent obesity in children with Prader-Willi syndrome (PWS) (as described in Chapter 1) is thought to be caused by a chronic imbalance between energy intake (EI) and energy expenditure (EE) (Coplin et al., 1976; Holm & Pipes, 1976). However, there is a paucity of information on DIT in youth with PWS. Attempts to increase EE by increasing physical activity or DIT has been studied in healthy individuals (children and adults) (Hill et al., 2012; Nguo et al., 2019; Ruddick-Collins, King, Byrne, & Wood, 2013; Westerterp, 2004b) but not in youth with PWS.

Meals of similar caloric content but different macronutrient composition may impact DIT, potentially through TEE (Quatela et al., 2016). Substitution of one macronutrient, particularly protein, for another can positively impact both EI and EE (Austin et al., 2011; Buchholz & Schoeller, 2004). For example, protein is the most satiating macronutrient and promotes appetite control, which could help regulate food intake (FI), body-weight loss and body-weight maintenance (Austin et al., 2011; Gosby et al., 2014; Simpson & Raubenheimer, 2005). Also, higher protein intake is associated with a 50 % increase in fat oxidation (Labayen, Diez, Parra, Gonzalez, & Martinez, 2004), which could lead to a reduction in body fat. Studies have reported that increased dietary protein while maintaining energy balance produced a prolonged DIT, which contributed to a greater TEE (Westerterp-Plantenga et al., 2009).

However, the DIT measurements are inconsistent among studies (Bessard, Schutz, & Jequier, 1983; Granata & Brandon, 2002; Laville et al., 1993; Leibel, Rosenbaum, & Hirsch, 1995; Melanson et al., 1998; Nelson et al., 1992; Schutz, Golay, et al., 1984; Segal et al., 1992). Discrepancy in study protocols contributes to these inconsistencies, such as meal size, amount of protein, the duration of the post-meal measurement period and the methods used to

obtain data. Previous studies have demonstrated an increase in DIT when a meal consisting of ≥ 50% energy from protein was consumed (Johnston, Day, & Swan, 2002; Nguo et al., 2018).

Discrepancies in DIT study measurement protocols is a challenge for interpretation of findings in healthy-individuals (Westerterp, 2004b). The reported day-to-day in intra-individual coefficients of variation (CV) of DIT measurements ranges from 15% to 29% (Armellini et al., 2000; Dabbech et al., 1994; Houde-Nadeau et al., 1993; Piers et al., 1992; Weststrate, 1993). In addition, the method used to calculate DIT may contribute to the high variability. Various methods were used to obtained DIT values: calculating the difference between postprandial and fasting EE (Baum et al., 2015; Dabbech, Boulier, Apfelbaum, & Aubert, 1996; De Palo et al., 1989; Lloret-Linares et al., 2013; Maffeis et al., 2001; Maffeis et al., 1993; Purtell et al., 2015; Schutz, Bessard, et al., 1984; Segal et al., 1990; Tentolouris et al., 2011), subtracting activity energy expenditure (AEE) from TEE (Schutz, Bessard, et al., 1984), or by assessing the incremental increase in caloric expenditure over resting energy expenditure (REE) after a meal is ingested (D'Alessio et al., 1988). The majority of studies calculated DIT as the differences between postprandial energy expenditure (PEE) subtracted from REE. REE is measured in a fasted state immediately before meal intake (Katzeff & Danforth, 1989; Laville et al., 1993; Nelson et al., 1992; Schutz, Golay, et al., 1984; Tentolouris et al., 2008; Welle & Campbell, 1983) as these measurements are affected by FI. Therefore, differences in dietary intake in the day prior to the REE measurement can affect the calculated value for DIT (Ruddick-Collins et al., 2013). To minimize the effects of day-to-day variability in REE measurements in the current study, a baseline value for REE measured at baseline visit is being used to calculate PEE and articulated as (‘fixed REE’). There are two aims of the present study: (1) to determine the impact of a high protein (HP) intake compared to a lower protein (standard) intake on PEE in children with PWS, and (2) to examine the correlation between a protein meal and subjective appetite sensations in children with PWS.

5.3 Materials and methods

5.3.1 Population

We recruited five youth with PWS (four females and one male). We excluded individuals with medical conditions known to affect body composition, such as diabetes mellitus, chronic inflammatory bowel disease, chronic severe liver, kidney disease or neurologic disorders, and those individuals who have been taking an investigational drug in the past year. The diagnosis of PWS was confirmed by DNA methylation and fluorescence in situ hybridization

genetic analysis. The study was approved by the University of Alberta's Health Research Ethics Board. Written informed consent and assent were obtained from all participants and parents.

5.3.2 Experimental design and testing procedure

A randomized, not blinded crossover study, in youth with PWS, was performed over 3 study visits at the University of Alberta. The impact of a HP meal contains 50% of total energy form protein was compared to a standard protein meal contains 15% of total energy on PEE. Study visits were separated by a two to four-week washout period to control for menstrual cycle (Bisdee, James, & Shaw, 1989). Each participant served as his/her own comparison. Trial design for study visits 1, 2 and 3 is shown in (**Figure 5.1 Trial design)**. On the first visit (baseline) the participants were asked to come to the Human Nutrition Research Unit (HNRU) at University of Alberta between 0700 and 0800 after an 8-hour fast. Pubertal status was self-assessed using the Tanner Stage scale. (**Appendix A. example for the puberty assessment**). REE was measured continuously for 60 minutes in a whole-body calorimetry unit (WBCU). Values recorded during this measurement period were used to calculate each participant's REE, and 24-hour energy requirements were estimated using the factorial method (REE x activity factor). A physical activity factor of 1 was used to reflect a "sedentary lifestyle" commonly observed and previously documented in children with PWS (Butler et al., 2007a; Eiholzer et al., 2003; Morales et al., 2019).

On test days visit 2 and visit 3 (**Figure 5.1**. **Trial design**), anthropometric measurements were recorded; REE and 6 hours of PEE were measured. All testing was conducted in the morning after an 8-hour fast.

5.3.3 Test meals

Food consumed by participants was prepared at the HNRU metabolic kitchen by trained staff and designed with the use of the Food Processor Nutrition Analysis Software (ESHA Research, Inc., version 10.6.0). The same menu was used for all participants. All food and beverage servings were weighed so that calorie levels met the individual energy requirements to maintain current body weight. One day prior to visits 2 and 3, participants were asked to come the HNRU facility to consume breakfast [standard diet (SD) or HP diet a combined with whey protein] and received 2 meals (lunch and dinner) and 2 snacks (morning and evening snacks) to take with them (**Appendix B. Diet menu**). Two experimental diets were assessed in randomized order. The SD represents a typical Canadian diet of approximately

55% carbohydrate, 30% fat and 15% protein (Garriguet, 2007) and the HP diet consisted of 20% carbohydrate, 30% fat and 50% protein; diets were matching in total energy and fibre content. Bottled water was provided ad libitum. The macronutrient content of the SD and HP diet shown in (**Table 5.1)**. To assess adherence with the study diet, participants were asked to complete a FI track form (**Appendix C. Food intake track**).

On each of the two test mornings**,** participants consumed one of the isoenergetic standard meal (SM) (containing 55% carbohydrate, 30% fat and 15% protein or HP breakfast meal containing 20% carbohydrate, 30% fat and 50% protein). The breakfast meal with morning snack was calculated to provide 35% of each participant's 24-h energy requirements (Gibney et al., 2018; O'Neil et al., 2014). The morning snack was provided with their breakfast to reduce hyperphagia associated with PWS (Miller, Strong, & Heinemann, 2015); see (**Table 5.2)** for meal composition.

5.3.4 Anthropometry and body composition

Weight was measured at each visit to the nearest 0.1kg using the same calibrated scale. Height was measured to the nearest 0.1cm using a wall-mounted stadiometer for children. Body mass index (BMI) percentile was calculated using EpiInfo (CDC, Atlanta, GA). Waist circumference was measured at the top of the iliac crest using standardized techniques. Body composition (total fat-free mass (FFM), total body fat mass (FM) and percent body fat was assessed via the air displacement plethysmography (BodPod) measured at baseline visit.

5.3.5 Satiety and appetite

To assess subjective appetite, a motivation to eat visual analog scales (VAS) (100mm) was used (Stubbs et al., 2000). The VAS collects information through the following six questions: (1) thinking about food ("no thoughts of food" to "very preoccupied with thought of food and is difficult to concentrate on anything else"), (2) hunger ("not hungry at all" to "as hungry as I've ever been"), (3) fullness ("not full at all" to "full as I've ever been"), (4) desire to eat ("very little" to "very strong"), (5) prospective consumption ("nothing at all" to "a large amount"), and urge to eat ("no urge to eat" to "very strong urge to eat"). The following formula was used to calculate subjective average appetite [(desire to eat) + (hunger) + (100 − fullness) + (desire to eat) + (prospective food consumption) + (urge to eat)/6]. This appetite score indicates the overall perceived appetite sensation, as used in other studies (Anderson, Catherine, Woodend, & Wolever, 2002; Bellissimo, Pencharz, Thomas, & Anderson, 2007; Belza et al., 2013; Panahi, El Khoury, Luhovyy, Goff, & Anderson, 2013; Samra &

Anderson, 2007). VAS questionnaires were assessed in a fasted state, at 15, 30 minutes and every hour for 4 hours on test visits 2 and 3 (**Figure 5.2**. **Protocol visits 2 and 3**) (**Appendix D. Example for VAS**).

5.3.6 Blood samples

Blood samples (10 ml per sample) were drawn through an indwelling catheter immediately prior to meal consumption (after 8 hours of fasting) and at 15, 30 minutes and 1-hour intervals for 4 hours following consumption of the breakfast meal (**Figure 5.2**. **Protocol visits 2 and 3**) in order to determine the nutrient regulation of plasma active ghrelin and other hormones. Sample preparation has been described elsewhere (see **Chapter 3, section 3.3.3: Preparation of Samples**).

5.3.7 Measurement of postprandial energy expenditure

5.3.7.1 Baseline (visit 1). REE was measured for 60 minutes in a WBCU, with the first 30 min excluded from analyses to account for acclimatization after fasting for ≥8 hour and refraining from exercise for 24 h prior to the test. Participants were in a standardized semi-reclined position to avoid them falling asleep and were allowed to watch movies to avoid fidgeting (Dietz et al., 1994). Participants were requested to have only minimal physical activity on the morning of the test (e.g., get dressed; drive from home to the HNRU; and take the short walk from the parking lot to the HNRU).

5.3.7.2 Postprandial energy expenditure (test visits 2 and 3). The measurement of DIT is strict, and requires that participants remain supine but awake during 6 hours of measurements (Reed & Hill, 1996). Therefore, PEE was measured instead of DIT. Participants were in a standardized semi-reclined position and were allowed to watch movies (Dietz et al., 1994; Weststrate et al., 1990). An overview of the protocol is included (**Figure 5.2**. **Protocol visits 2 and 3**). Upon arrival at the HNRU, anthropometric measurements and fasting blood sample were taken and a catheter was inserted into an antecubital vein. The participants transferred to the WBCU and positioned in a semirecumbent state. The venous catheter was connected, via a small airtight connecting compartment in the chamber, to a physiologic saline line. REE (described above) and respiratory exchange ratio (RER) (ratio of carbon dioxide production (VCO_2) produced and oxygen consumption (VO_2) consumed) were measured in a fasted state for 1-hour. This REE measurement served as the 'baseline' energy expenditure for the

calculation of PEE. Immediately after this baseline REE measurement, participants consumed a test meal within 30 minutes. The metabolic measurement was resumed, and PEE was measured for a total of 6 hours, with two 10 minutes 'comfort breaks' at 2 and 4 hours. For all metabolic measurements, participants were in a semi-reclined position which was kept consistent between the test days. PEE was also expressed as a percentage of the total energy of the test meal.

To facilitate adherence to the test conditions, participants were allowed to watch movies, play video games or read during the metabolic measurement period (den Besten et al., 1988; Scott & Devore, 2005; Vasilaras et al., 2001). Subjective appetite was assessed in a fasted state and 15, 30 minutes and every hour for 4 hours using visual analog scales VAS.

5.4 Calculation of postprandial energy expenditure

PEE was calculated as the difference between 6-hour total energy measured after the breakfast consumed subtracted from REE, using REE measured in a fasted state immediately before meal intake. In the current study, participants consumed a SD one day prior to the SM test and HP diet one day prior to the HP meal test. Therefore, differences in the prior day diet can affect REE and alter the calculated value for PEE. To minimize the effect of prior day diet variability in REE on the calculation of PEE, PEE also calculated as the difference between 6-hour total energy measured after the breakfast consumed subtracted from REE measured at baseline visit and called as ('fixed REE').

5.5 Statistical analysis

All statistical analyses were performed using SPSS version 24 for Windows (SPSS Inc., Chicago, IL) and GraphPad Prism 7 software (GraphPad Software Inc.). A value of $P \leq 0.05$ was considered statistically significant. All values are reported as mean and standard deviation (SD). Data were verified as normally distributed using Shapiro–Wilk tests of normality before the analysis. A paired sample t-test was used to compare the mean of the RER, pre-meal REE and PEE (SM or HP) between two occasions for each participant in the study. Change from baseline subjective average appetite scores was analyzed using a two-factor repeated measure ANOVA to assess the effects of meal and time. Area under the curve for VAS was calculated taking into account areas above and below fasting baseline. Correlation between PEE and VAS_{AUC} was determined using Spearman's Rank-Order correlation.

5.6 Results

5.6.1 Baseline anthropometric and metabolic characteristics

The baseline characteristics of the study participants are shown in (**Table 5.3).** Five participants (four females and one male) completed both arms of the study; mean age: 15 ± 3.7 (11–20 years) BMI percentile: 85.7 ± 10.5 (70.2–98.3). One PWS participant had uniparental disomy; all others had a chromosome 15 deletion. All PWS individuals had free thyroxine (T4) and thyroid stimulating hormone (TSH) concentrations in the normal range. All participants were taking growth hormone (GH) treatment prior to study enrollment; no changes in GH treatment were made during the course of the study. Self-assessed pubertal status ranged from Tanner stage II–V.

5.6.2 Resting energy expenditure

REE was measured at baseline, visit study 1 and visit study 2. Mean REE value measured at baseline was 1686.1 ± 233.5 kcal. Mean REE measured at the following day after the intake of SM was 1590.3 ± 168 kcal and mean REE value measured the following day after the intake of HP diet was 1650.6 ± 188.4 kcal (**Figure 5.3**).

5.6.3 Postprandial energy expenditure using same day resting energy expenditure measurements

No differences were detected in mean PEE calculated using REE measured in a fasted state immediately before meal intake between the SM 184 ± 148 kcal and HP meal 200 ± 188 kcal (p = 0.74) (**Figure 5.4 A**). Mean PEE (% energy intake) were similar between the SM 9.8 ± 7.2% and HP meal 10.5 ± 9.2% (p = 0.8) (**Figure 5.4 B**).

5.6.3 Postprandial energy expenditure using (‘fixed REE’) measurements

No difference in mean PEE calculated as ‘fixed REE’ between the SM 89 ± 149 kcal and HP meal 165 ± 146 kcal (p = 0.2) were seen (**Figure 5.5 A**). Mean PEE (% energy intake) was 5.5 ± 9.7% after the SM and 10.4 ± 9.6% after HP meal (p = 0.18) (**Figure 5.5 B**).

5.6.4 Respiratory exchange ratio

Mean RER value was lower following the HP meal 0.80 ± 0.01 vs the SM 0.87 ± 0.02 (p = 0.009) (**Figure 5.6**).

5.6.5 Subjective appetite response

Change from baseline average appetite score was affected by time ($p = 0.02$), but the change from baseline was unaffected by type of meal ($p = 0.44$) and there was no meal/time interaction ($p = 0.99$) (**Figure 5.7**). Average appetite decreased after SM and HP meals consumed at 15 minutes ($p = 0.02$) and returned to baseline levels at 180 minutes ($p = 0.02$). Average appetite was higher than baseline after SM and HP meals at 240 minutes ($p = 0.04$).

5.6.6 Correlation between PEE and subjective appetite sensations

No correlation between PEE and VAS as expressed as the incremental area under the curve ($VAS_{AUC\ it}$) ($rs = 0.286$, $p = 0.64$ for SM and $rs = 0.005$, $p = 0.99$ for HP meal) was observed.

5.7 Discussion

The impact of macronutrient content of the diet on PEE in youth with PWS is poorly understood. This is the first study aimed at assessing PEE in youth with PWS. Our randomized, crossover study aimed to determine the impact of a high versus a low protein intake on PEE in youth with PWS. Outcome measurements included REE, PEE, RER. We examined the correlation between protein intake and subjective appetite sensations in youth with PWS.

DIT measurements depend on the meal size and macronutrient composition as well as the duration of the measurement (Quatela et al., 2016). We did not detect differences in PEE between the SM and HP meals calculated using REE measured in a fasted state immediately before meal intake. REE measured in a fasted state is affected by the prior day diet (Agus, Swain, Larson, Eckert, & Ludwig, 2000; Katzeff & Danforth, 1989; Laville et al., 1993; Nelson et al., 1992; Schutz, Bessard, et al., 1984; Schutz, Golay, et al., 1984; Tentolouris et al., 2008; Welle & Campbell, 1983). Johnston et al. (2000) measured postprandial thermogenesis following a high protein diet versus high carbohydrate diet. Participants consumed a SD providing (50% carbohydrate, 15% protein and 25% as fat) for two days prior to each test day to assure similar baseline measures (Johnston et al., 2002). Ruddick-Collins et al. (2013) measured DIT following a standard fixed breakfast meal contains (54 % carbohydrate, 14% protein and 32% fat) on two occasions and no standardized diet before each test day was provided. They used the lowest REE measured on both test days as defined as ('fixed REE') to calculate DIT (Ruddick-Collins et al., 2013). To minimize the effects of the prior day diet in REE measurements in the current study, a value for REE measured at baseline visit ('fixed REE') was used to calculate PEE for HP meal and SM. PEE calculated,

as ‘fixed REE’ was double after HP meal compared to SM; however, was not statistically significant.

This, however, does not necessarily indicate that no difference exists in PEE between a SM and HP meal. Previous studies have shown significant differences in PEE comparing healthy children and adults using a standardized diet previous to the test day (Johnston et al., 2002) or with no change on the habitual diet occurring before the tests days (Nguo et al., 2018). Nguo et al, (2018) reported that the mean DIT (% of energy intake) was higher after a high protein diet (8.19 ± 0.709%) compared with the high carbohydrate meal (4.36 ± 0.48%) ($p < 0.001$) in an acute crossover study in adolescents with obesity and healthy-weight aged 11–19 years. Participants consumed either a high protein (whey) (55% protein, 30% carbohydrate, and 15% fat) or high carbohydrate (79% carbohydrate, 5% protein, and 16% fat) liquid meal in a random order (Nguo et al., 2018). Differences in findings between Nguo et al. (2018) and our study could be attributed to differences in meal composition. Nguo et al. (2018) provided a high carbohydrate meal containing almost 80% of total energy from carbohydrate and only 5% of total energy from protein, therefore a much higher carbohydrate and lower protein content compared to our diet. Importantly, 5% of total energy content from protein is very low and does not represent usual protein content of a meal (Garriguet, 2007).

In healthy adults, a high protein diet produces a greater DIT effect than a higher carbohydrate or fat content diet (Halton & Hu, 2004; Westerterp-Plantenga, Lemmens, & Westerterp, 2012). Only one study has investigated DIT in individuals with PWS compared with BMI-matched and healthy-weight individuals (Purtell et al., 2015). They found no difference in DIT between the two groups after consumption of a breakfast meal of moderate protein content (600 kcal, 50% carbohydrate, 35% fat, 15% protein) (Purtell et al., 2015). As examination of DIT was not the primary objective of their study, details concerning DIT’s measurement protocol were not described.

Following consumption of large meals (>1000 kcal), total DIT response may take between 8 to 10 hours (D'Alessio et al., 1988; Melanson et al., 1998). However, the majority of studies used meals with energy content range between 400 and 1000 kcal, and measured DIT until 3 and 6 hours; DIT response is often incomplete at the end of < 5 hours measurement period (D'Alessio et al., 1988; Jebb, 1995; Nelson et al., 1992; Schutz, Golay, et al., 1984; Tentolouris et al., 2008). Differences in gastric emptying, nutrient absorption and storage between individuals may affect the duration of the DIT response (Scott et al., 2007). Therefore, a general recommendation is for the measurement to last for > 5 hours to capture the complete DIT response (Reed & Hill, 1996; Ruddick-Collins et al., 2013). Therefore, in

the current study we measured PEE for 6-hour period following a 500 kcal meal; as a longer measurement period would have substantially increased participant burden. However, it is possible a 6-hour measurement was not sufficient to capture total DIT response due to a delay in gastric emptying previously reported in individuals with PWS (Arenz, Schwarzer, Pfluger, Koletzko, & Schmidt, 2010). Peak in DIT may have occurred outside the window of indirect calorimetry measurement.

We found no relationship between PEE and appetite measures using $VAS_{AUC\ it}$. Previous studies in healthy adults examined the relationship between DIT and subjective appetite assessment using VAS (Crovetti, Porrini, Santangelo, & Testolin, 1998; Westerterp-Plantenga, Rolland, Wilson, & Westerterp, 1999). Westerterp-Plantenga et al. (1999) reported that differences between satiety or hunger AUC (between high-protein high-carbohydrate diet and a high-fat diet) in women with healthy weight was correlated to differences in DIT between the two macronutrient compositions. Another study by Crovetti et al. (1998) reported a positive correlation in women with healthy weight between fullness sensation and DIT.

Protein intake and PEE were not found to relate to subjective measures of satiety. Previous studies in healthy adults and children have shown a relationship between satiety and DIT when a meal consisting of ≥ 50% energy from protein was consumed (Acheson et al., 2011; Nguo et al., 2019). In the current study, no difference in satiety was found between the SM and HP meal as assessed by VAS. Satiety scores were similar after the 2 meals, with a rapid return to fasting levels after meal termination. We found no evidence of impaired postprandial satiety hormone responses in children with PWS compared to BMI z-score matched children in study 1 (Chapter 3). The secretion of anorexigenic hormones PYY and GLP-1 was not reduced compared to BMI z-score match children. This is an agreement with Purtell et al. (2011) who found no differences in the secretion of PYY and GLP-1 in adult with PWS compared to control (Purtell et al., 2011). In study 1 (Chapter 3) we found that fasting and postprandial ghrelin concentrations were significantly higher in children with PWS compared to BMI z- score matched children. Ghrelin findings have been confirmed by previous studies (DelParigi et al., 2002; Goldstone, 2004; Goldstone et al., 2005; Haqq, Farooqi, et al., 2003; Purtell et al., 2011; Tauber et al., 2004). However, meal intake suppressed ghrelin concentrations in individuals with PWS (Haqq, Farooqi, et al., 2003; Paik et al., 2006; Purtell et al., 2011).

Blood withdrawn in individuals with PWS is difficult and quite invasive procedure due to low muscle tone and increased subcutaneous fat (Kuppens et al., 2015; Orsso et al.,

2017). In the current study, we were not able to collect blood samples from all the participants as the venous catheter was connected, via a small airtight connecting compartment in the chamber, to a physiologic saline line. We lost access to the venous catheter in some participants and due to the limited access we were not able to reinsert the venous catheter. Therefore, blood samples results are not available.

Satiety scores were similar after the two meals, with a rapid return to fasting levels after meal termination. In other studies, adults with PWS rated high hunger scores and high satiety scores immediately after a meal (Purtell et al., 2011). In healthy adults and typically developing children, hunger and satiety response to VAS is in balance; when satiety is high, hunger is low (Bennett et al., 2018; Lee, Brett, Chang, de Zepetnek, & Bellissimo, 2019; Purtell et al., 2011). In the current study, some participants rated high hunger scores and high satiety scores at the same time. Purtell el., (2011) reported a normal satiety response in adults with PWS as determine by an increase in postprandial VAS fullness scores with a rise in PYY and GLP-1 concentrations and a drop in plasma ghrelin concentrations. However, high VAS hunger scores were also rated immediately after a large meal was consumed. They suggested that the observed higher postprandial ghrelin concentration (compared to the control group) might be sufficient to stimulate appetite and lead to hyperphagia (Purtell et al., 2011). The similar VAS ratings following the HP meal or SM in the current study bring into question the impact of HP meal in suppressing appetite in individuals with PWS.

The major finding of this study was that RER was lower after the HP diet compared with the SD; suggesting a shift towards fat rather than carbohydrate as a fuel source. RER is affected by the availability of dietary and stored macronutrients. RER rises with a high carbohydrate meal and falls during fasting or after a high fat meal. During carbohydrate oxidation, the amount of CO_2 produced equals O_2 consumed, resulting in a RER value of 1.0. During fat oxidation, less CO_2 is produced for a given amount of O_2 consumed, leading to a RER of 0.7. The RER for a mixed diet, protein and alcohol, is approximately 0.85, 0.82 and 0.67, respectively (McClave et al., 2003). The transition from carbohydrate to fat oxidation (e.g. drop in RER) during an overnight fast or in response to nutritional state has been called metabolic flexibility (Galgani, Moro, & Ravussin, 2008). In contrast, Purtell et al. (2015) found no differences in RER between 11 adults with PWS and 12 BMI-matched individuals with healthy-weight in response to a standardized breakfast of mixed high carbohydrate and high fat content (600 kcal, 50% carbohydrate, 35% fat, 15% protein) (Purtell et al., 2015). However, the adaption to adjust fuel oxidation to fuel availability occurs between 1 to 7 days, may help to explain the differences in findings (Coyle, Jeukendrup, Wagenmakers, & Saris,

1997; el-Khoury et al., 1997; Forslund et al., 1999; Galgani & Ravussin, 2008; Galgani et al., 2008; Schrauwen, van Marken Lichtenbelt, Saris, & Westerterp, 1997).

Major strengths of our study include its randomized crossover design in which all participants served as their own controls. This is one of the first studies to comprehensively examine REE and PEE using a state-of-the-art technique of WBCU in children with PWS. Limitations of our study include the limited sample size due to the rarity of this disease and the lack of a control group. The sample size of the current study was calculated based on changes in PEE 22 ± 16 in high carbohydrate diet 69 % of total energy and 62 ± 14 kcal in a HP group up 68% of energy from protein over 7 hours PEE measurement from a previously published study (Crovetti et al., 1998) and accounted for a 20% attrition rate.

In conclusion, this study found that RER was lower after the HP meal than the SM meal in children with PWS. No difference in PEE was found between the SM and HP meals. This study highlights the heterogeneity in PEE in youth with PWS. The high variation in PEE metabolism response could be attributed to factors such as variability in pubertal maturation, difference in age ranges from 11 to 20 years within our study, body composition, and sex. Future studies are required to compare low protein-high carbohydrate and standard fat, standard/typical Canadian intake and high protein-low carbohydrate and standard fat in children with and without PWS; considering factors that influence response to energy metabolism (e.g., early puberty status to adult, body composition and sex).

5.8 Tables

Table 5.1. Study diets

Diet	**A standard diet**	**A higher protein diet**
Protein	15% of total kcal	50% of total kcal
Fat	30% of total kcal	30% of total kcal
Carbohydrate	55% of total kcal	20% of total kcal
Foods		
	BREAKFAST	***BREAKFAST***
	Milk	Milk with whey protein
	Egg wrap	Turkey egg scramble
	Orange fruit slice	Tortilla whole wheat
	MORNING SNACK	***MORNING SNACK***
	Milk	Milk with whey protein
	White toast with strawberry jam	
	LUNCH	***LUNCH***
	Steak wrap	Rice with turkey
	Wedges potato	Steak
	Garden salad	Garden salad with whey protein
	DINNER	***DINNER***
	Chicken stir-fry	Baked chicken
	Banana	Garden salad with whey protein
	Rice	
	Garden salad	
	EVENING SNACK	***EVENING SNACK***
	Milk	Milk with whey protein
	Apple	

Table 5.2. Study meals

Meal	A standard meal	A higher protein meal
Protein	15% of total kcal	30% of total kcal
Fat	30% of total kcal	30% of total kcal
Carbohydrate	55% of total kcal	40% of total kcal
Foods	• Milk • Egg wrap • Orange fruit slice • White toast with strawberry jam	• Milk with whey protein • Turkey egg scramble • Tortilla whole wheat

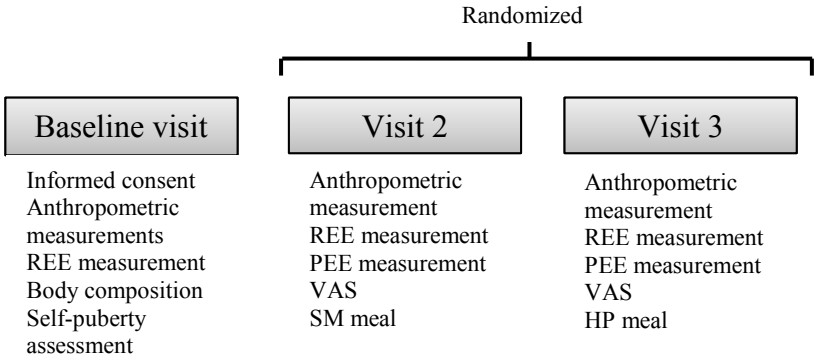
Table 5.3. Baseline characteristics of Prader-Willi syndrome (PWS)

Characteristic	Participants (n=5)	
Age, y	15±3.7 (11-20)	
Males/females	1/4	
BMI percentile	85.7±10.5 (70.2-98.3)	
Waist circumference (cm)	85.1±13.3 (74-105.7)	
Body composition	**Male**	**Females**
FFM (kg)	28.0	39.1±3.9 (34.2- 43.1)
FM (kg)	12.8	28±14.3 (18.9-48.1)
FM (%)	31.4	38.9±10.2 (30.8-53.9)
Baseline protein intake (%)	16.4±7 (13.7- 21.1)	
Baseline REE (kcal)	1686±234 (1538-2098)	
SM REE (kcal)	1590±168 (1532-1841)	
HP meal REE (kcal)	1651±188 (1392-1914)	

y: year; BMI: body mass index; FFM: fat-free mass; kg: kilogram; FM: fat mass; REE: resting energy expenditure; SM: standard meal; HP: high protein; kcal: kilocalorie. All data reported as values are presented as mean ± standard deviation (range) except sex that is number of male:female.

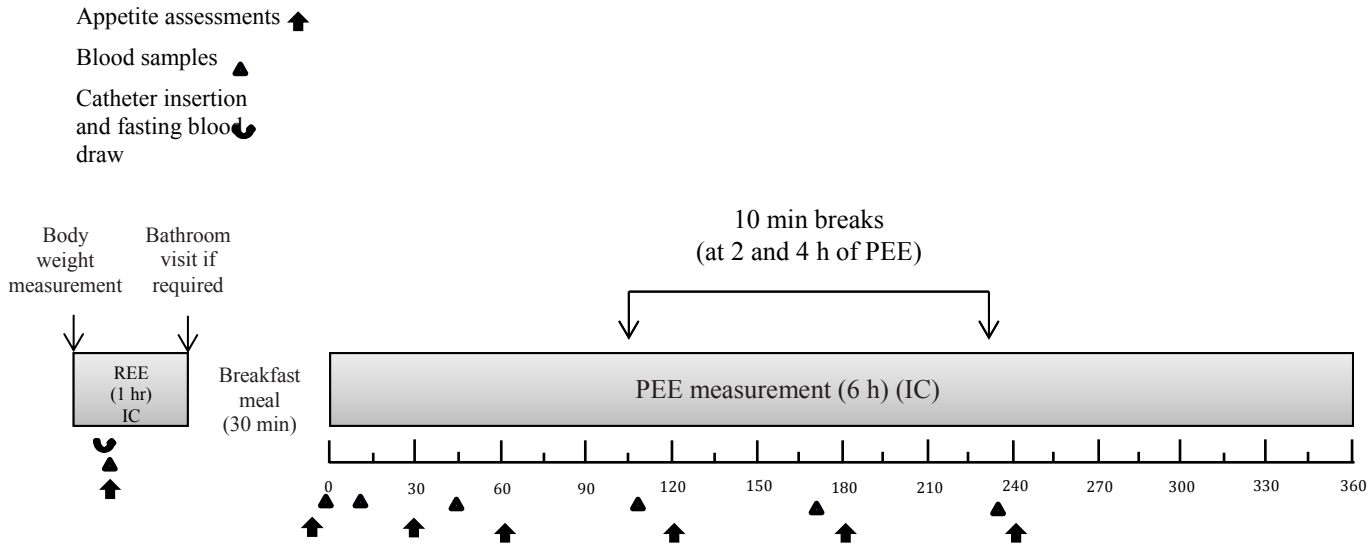
5.9 Figures

Figure 5.1. Trial design



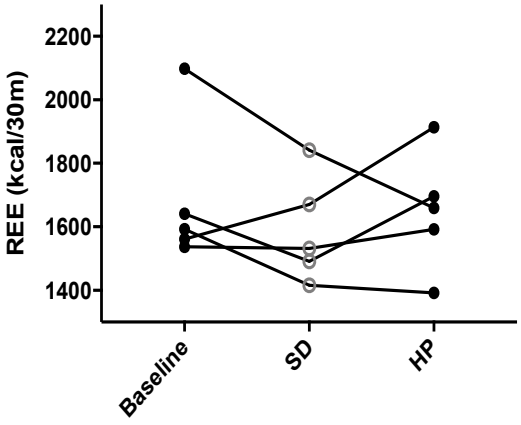
REE: resting energy expenditure; PEE: postprandial energy expenditure; VAS: visual analog scale; SD: standard meal; HP: high protein

Figure 5.2. Protocol visits 2 and 3



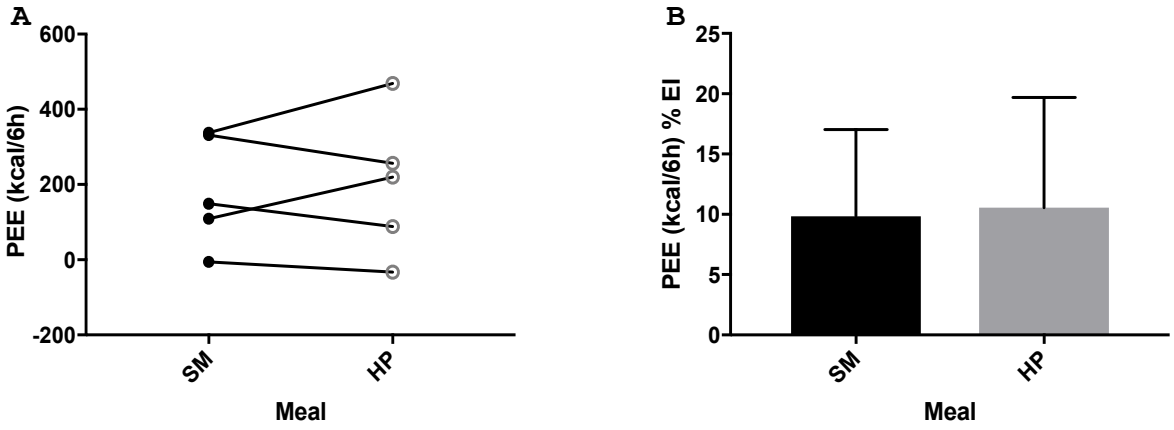
REE: resting energy expenditure; PEE: postprandial energy expenditure; hr: hour; IC: indirect calorimetry; min: minutes

Figure 5.3. Resting energy expenditure (REE) measurements



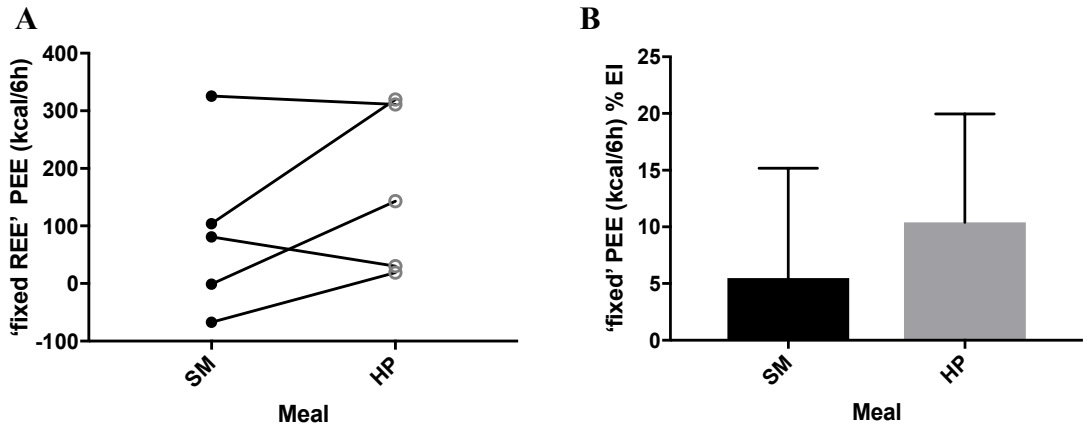
REE: resting energy expenditure; kcal: kilocalorie; m: minutes; SD: standard diet; HP: high protein

Figure 5.4. Postprandial energy expenditure (PEE) response



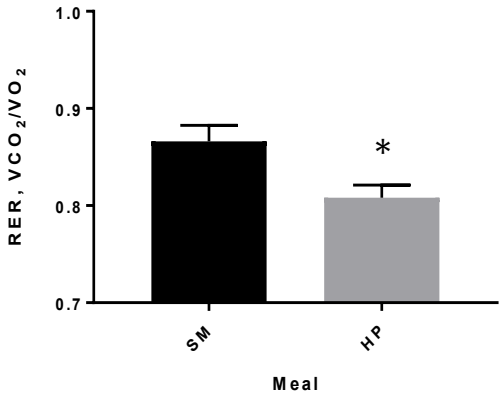
PEE: postprandial energy expenditure; kcal: kilocalorie; h: hour; SM: standard meal; HP: high protein meal; EI: energy intake. A paired sample t-test was used

Figure 5.5. Postprandial energy expenditure (PEE) response using 'fixed REE'



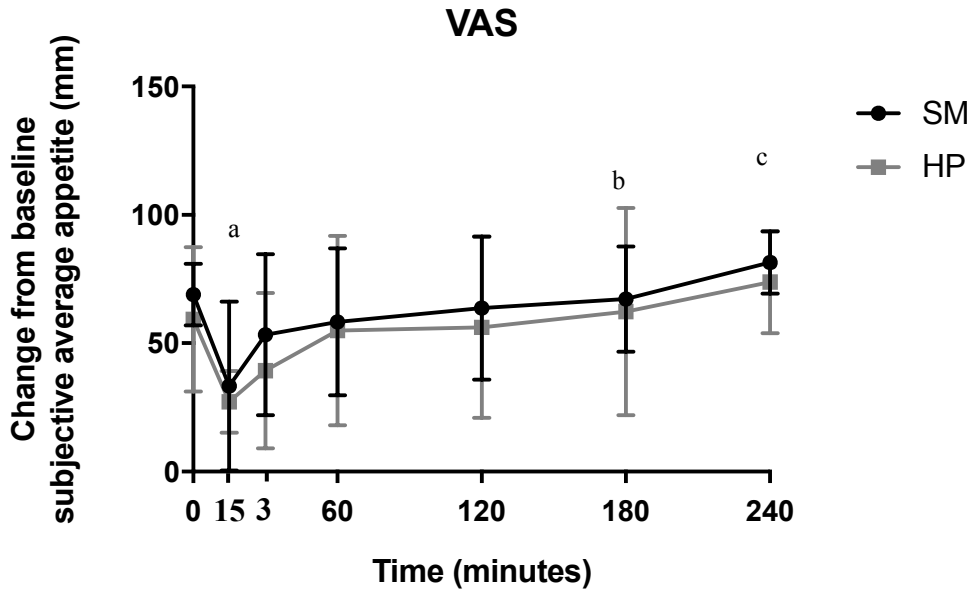
REE: resting energy expenditure; PEE: postprandial energy expenditure; kcal: kilocalorie; h: hour; SM: standard meal; HP: high protein meal; EI: energy intake. A paired sample t-test was used.

Figure 5.6. Respiratory exchange ratio (RER)



RER: respiratory exchange ratio; VCO_2: carbon peroxide production; VO2: oxygen consumption; SM: standard meal; HP: high protein meal
Significant difference *($p = 0.009$). A paired sample t-test was used.

Figure 5.7. Subjective appetite response



VAS: visual analog scale; SM; standard meal; HP: high protein
Significant difference between meals [a]($p = 0.017$), [b]($p = 0.023$) and [c]($p = 0.045$). A two-factor repeated measure ANOVA was used.

Chapter 6
Discussion and Conclusions

6.1 Introduction

To ensure an optimal body weight, energy intake (EI) has to match energy need; energy imbalance can lead to overweight or obesity (Hill et al., 2012). The regulation of energy balance and food intake (FI) are affected by multiple physiological, behavioral and environmental factors (e.g., diet) that can increase or decrease appetite (Anderson, Hunschede, Akilen, & Kubant, 2016; Carnell, Benson, Pryor, & Driggin, 2013; Hall et al., 2012; Stewenius et al., 1995). Efforts to develop effective strategies to maintain body weight and prevent weight gain attempt to understand how macronutrients, carbohydrate, protein and fat can impact EI and energy expenditure (EE) (Anderson & Moore, 2004; Botchlett & Wu, 2018; Carreiro et al., 2016). Researchers have paid attention to the potential beneficial outcomes associated with dietary protein in weight management, due to the following factors: 1) protein intake promotes satiety more than the isoenergetic ingestion of carbohydrate or fat (Astrup, 2005; Westerterp-Plantenga et al., 1999; Westman, Yancy, Edman, Tomlin, & Perkins, 2002; Yancy, Olsen, Guyton, Bakst, & Westman, 2004), as measured by an increase in satiety hormones (e.g., glucagon-like peptide 1 (GLP-1) and peptide tyrosine tyrosine (PYY) hormones) (Belza et al., 2013; van der Klaauw et al., 2013), and reduces the EI in the next meal as measured by subjective appetite assessment (Anderson et al., 2016; Baum et al., 2015; Bellissimo, Desantadina, et al., 2008); and 2) a high protein (HP) diet increases EE by increasing diet-induced thermogenesis (DIT) (Halton & Hu, 2004). (**Figure 6.1)** summarizes the role of protein intake in the regulation of FI and energy metabolism. Although this potential mechanism of HP has been studied in adults, research that focuses on the impact of a HP diet in regulating appetite and energy metabolism in children and adolescents, especially in children with syndromic obesity, is a neglected area. This thesis focused on the potential beneficial outcomes associated with dietary protein in children with Prader-Willi syndrome (PWS).

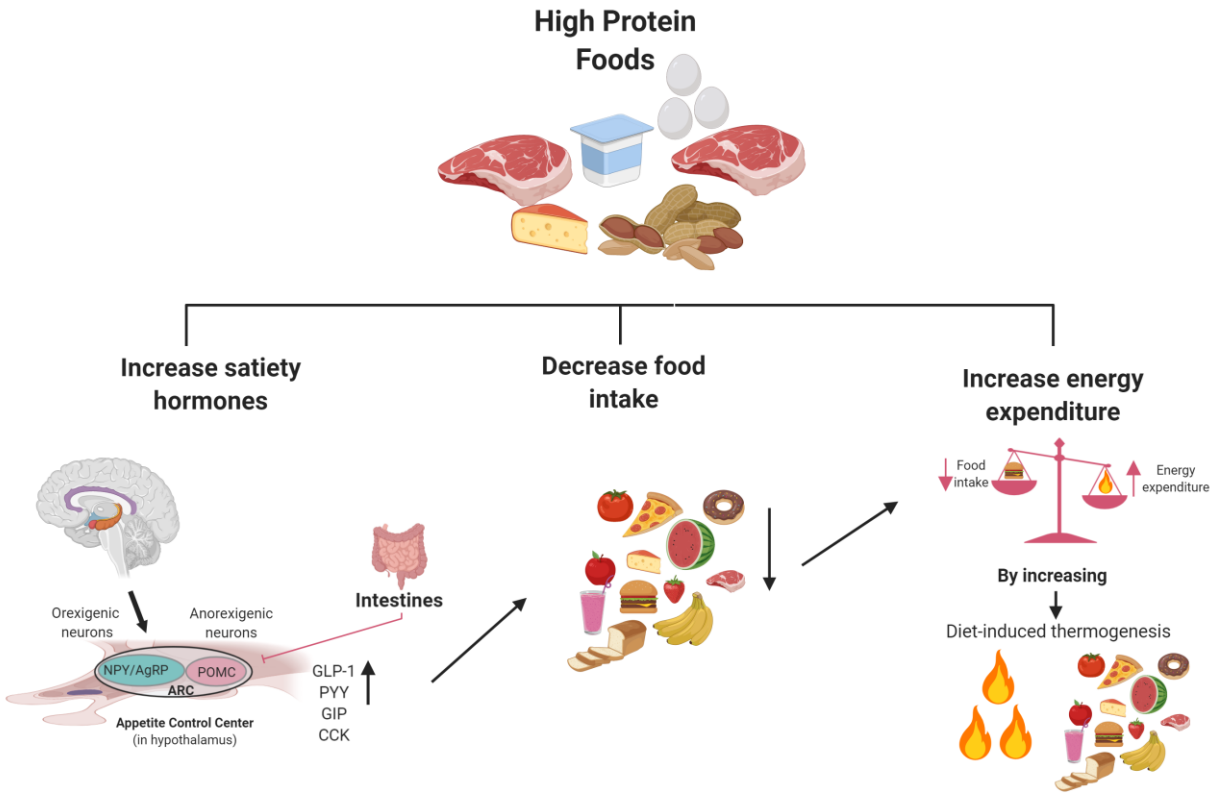


Figure 6.1. The role of protein intake in the regulation of food intake and energy metabolism. Figure by Maha Alsaif©

6.2 Summary of the Studies' Aims and Results

The excessive weight gain associated with PWS is caused by an imbalance between EI and EE (Coplin et al., 1976; Holm & Pipes, 1976). A number of factors are responsible for this imbalance in children with PWS, including hormonal dysregulation, hyperphagia, reduced resting energy expenditure (REE), decreased physical activity and fat-free mass (FFM) (Butler, 2011). I aimed to investigate the impact of a HP or/and a standard meal (SM), which represents a typical Canadian FI containing 55% carbohydrate, 30% fat and 15% protein (Garriguet, 2007) on postprandial regulation of orexigenic hormones (ghrelin and asprosin) and other satiety factor concentrations as well as energy balance in children with PWS. In Chapter 3, I hypothesized that: 1) fasting and postprandial active ghrelin will be higher in children with PWS; 2) postprandial active ghrelin suppression will be greater in children with PWS compared to body mass index (BMI) z-score matched children, but rapid return back to baseline in plasma active ghrelin will be greater in children with PWS; and 3) plasma active ghrelin will be suppressed to a greater degree and there will be higher insulin concentrations in both study groups following a higher protein–lower fat (HP-LF) meal containing (55%

carbohydrate, 15% fat and 30% protein) compared to the higher protein–lower carbohydrate (HP-LC) meal containing (40% carbohydrate, 30% fat and 30% protein). Children with PWS had higher fasting concentrations of both acyl ghrelin (AG) ($p = 0.02$) and desacyl ghrelin (DAG) ($p = 0.01$) than BMI z-score matched children. Absolute concentrations of AG and DAG were higher in PWS than in BMI z- score matched children across all post-prandial time points ($p = 0.002$ and $p < 0.001$ respectively). Thus, SM, HP-LC and HP-LF meals reduced AG and DAG in both groups, but the magnitude of AG and DAG suppression following all three meals was more pronounced in children with PWS at one ($p = 0.012$ and $p = 0.15$, ns), two ($p = 0.001$ and $p = 0.002$), three ($p = 0.001$ and $p < 001$) and four hours ($p = 0.001$ and $p = 0.001$) and the time return to baseline to recovery was delayed. No effects of HP-LC or HP-LF meals on AG, DAG and the AG:DAG ratio within or between groups were observed. Postprandial glucose concentrations were higher in PWS children than in BMI z-score matched children 1 hour after the SM (106.2 % vs. 94.3 %) and HP-LC (109.1 % vs 89.5 %) meals ($p = 0.03$ and $p = 0.002$ respectively); glucose responses were comparable following the HP-LF meal. Following the SM, insulin concentrations were higher in children with PWS than in controls at hours 1, 2 and 3 ($p = 0.002$, $p = 0.008$ and $p < 0.001$, respectively). HP-LC and HP-LF meals stimulated a greater release of insulin in children with PWS at hours 1, 2 and 3 hours ($p = 0.006$, $p = 0.007$, $p = 0.221$, ns $p < 0.001$, $p = 0.04$ and $p = 0.126$, ns, respectively) than in BMI z-score matched children. The HP-LF meal stimulated a greater release of insulin at hour 1 than the SM in the both children with PWS and BMI z-score matched children ($p = 0.002$ and $p = 0.03$ respectively). In Chapter 4, I hypothesized that: 1) fasting and postprandial asprosin will be higher in children with PWS than in children without the syndrome; 2) following a breakfast meal, asprosin concentrations will be decreased in both groups; and 3) a positive correlation between serum asprosin and glucose and asprosin and insulin resistance will be observed. No difference in fasting asprosin in children with PWS compared to BMI z-score matched children was observed.

In children with PWS, fasting asprosin was positively correlated with AG ($rs = 0.62$, $p = 0.05$), and 1-hour postprandial asprosin was negatively correlated with insulin ($rs = -0.62$, $p = 0.05$). In Chapter 5, I hypothesized that 1) a HP intake containing (20% carbohydrate, 30% fat and 50% protein) will result in higher postprandial energy metabolism (PEE) and satiety compared to a lower protein SM intake in children and youth with PWS; and 2) a HP intake will result in reduced and prolonged appetite compared to a lower protein intake as measured by subjective appetite assessment. The respiratory exchange ratio (RER) after the HP diet

was significantly lower compared to standard diet ($p = 0.009$). No significant difference in PEE and subjective appetite assessment were found between the HP meal and SM.

Collectively, these results can be used to inform future evidence-based nutrition guidelines for patients with PWS and have the potential to change the dietetic care that these individuals receive. The following sections discuss the implications and limitations of the present findings and provide suggestions for future research.

6.3 Hormonal Regulation

Findings from Chapter 3 confirmed previous reports that individuals with PWS have higher fasting and postprandial hyperghrelinemia compared to individuals with obesity and healthy weight (DelParigi et al., 2002; Goldstone et al., 2004; Gumus Balikcioglu et al., 2015; Haqq, Farooqi, et al., 2003; Rigamonti et al., 2017; Tauber et al., 2004). However, despite having overall higher concentrations of ghrelin, children with PWS exhibited normal postprandial suppression of AG and DAG (Chapter 3). This finding is consistent with the results of two studies in adults (Gimenez-Palop et al., 2007; Rigamonti et al., 2017) and four in children who have PWS (Gumus Balikcioglu et al., 2015; Haqq, Stadler, Rosenfeld, et al., 2003; Paik et al., 2006; Paik et al., 2007). Degrees of AG and DAG suppression in Chapter 3 were not affected by the type of meal ingested—SM, HP-LC or HP-LF. However, following the HP-LC and HP-LF meals, there was an increase in PYY in both children with PWS and BMI z-score matched children. The increase in GLP-1 secretion occurred after the higher protein meals in the PWS group only. Despite ghrelin suppression after meal intake, the concentrations of AG and DAG remained higher in children with PWS compared to children with BMI z-score children across all postprandial time points, and that may be sufficient to drive appetite leading to hyperphagia. Future trials are needed to elucidate the role of ghrelin in children with PWS using specific ghrelin antagonists. Current clinical trials are investigating the safety and efficacy of AZP-53 (Livoletide), a peptide analog of DAG, for the treatment of hyperphagia in adults with PWS (Allas et al., 2018).

6.4 Diet-Induced Thermogenesis Measurements

While DIT might be altered in children with PWS as described in this thesis, our understanding of DIT to date has been informed primarily by studies in adults and children without the syndrome. The measurement of DIT is strict, and requires that participants remain supine but awake during 6 hours of measurements (Reed & Hill, 1996). In Chapter 5, instead of measuring DIT, we measured PEE while participants were in a semi-reclined

position. (Therefore, we articulated our findings as PEE instead of DIT.) To facilitate adherence to the test conditions, participants were allowed to watch movies, play video games or read during the metabolic measurement period (den Besten et al., 1988; Scott & Devore, 2005; Vasilaras et al., 2001). Nguo et al. (2018) measured PEE for 30 minutes of every hour; between the measurements, non-strenuous sedentary activities were permitted. In their study, Purtell et al. (2015) did not report the measurement protocol for DIT, which makes it difficult to compare findings. Furthermore, multiple terms to describe specific components of EE have been used interchangeably in the literature (e.g., DIT/thermic effect of food (TEF)/meal-induced thermogenesis (MIT) thermic effect of meal (TEM) and PEE). DIT is the total energy expended throughout the day in digestive processes to a number of meals measured in a whole-body calorimetry unit (Verboeket-van de Venne et al., 1996; Westerterp, 2004b). MIT, TEF and TEM are a representation of an individual's thermogenic response to acute meal intake (Komai et al., 2016; Reed & Hill, 1996; Ruddick-Collins et al., 2013). The majority of studies measured thermogenesis in response to a single meal due to feasibility and cost of the measurements. However, differences in the specific measurement conditions for obtaining DIT/TEF/MIT/TEM and PEE exist and should be used to clarify which component (DIT/TEF/MIT/TEM or PEE) is actually being assessed.

6.5 Measures of Subjective Appetite Assessment

Developed by (Hill & Blundell, 1982), the subjective appetite assessment visual analog scale (VAS) is a 100 mm line affixed with contrasting statements at both ends. The assessment contains six questions that require the participant to rate their level of hunger, fullness, desire to eat, amount of food they could eat, urge to eat and preoccupation with food (Stubbs et al., 2000). Previous research studies have used the VAS to assess subjective appetite in children (Bellissimo, Thomas, Pencharz, Goode, & Anderson, 2008; Bennett et al., 2018; Gheller et al., 2019; Lee, Brett, Chang, et al., 2019; Lee, Brett, Wong, et al., 2019; Poirier et al., 2019; Vien et al., 2017) and adults (Bodinham, Hitchen, Youngman, Frost, & Robertson, 2011; Horner, Byrne, & King, 2014; Law, Lee, Vien, Luhovyy, & Anderson, 2017). Reproducibility of the VAS in measuring subjective appetite rating in adults and children has been reported (Bellissimo, Thomas, et al., 2008; Horner et al., 2014). In boys aged 9 to 14 years, subjective appetite rating before the test meal was strongly correlated with FI (Bellissimo, Thomas, et al., 2008). This finding indicates that children are able to understand the scales and rate their hunger (Bellissimo, Thomas, et al., 2008). However, the use of VAS depends on age and cognitive development. Generally, VAS can be used with children aged 7

and older (Shields et al., 2003). Findings from Chapters 3 and 5 are discussed below within this context.

In Chapter 3, the children with PWS were younger (5.1 to 17.9 years) than those in the control group and lacked the understanding and communication skills to complete the VAS questionnaire, which limited its use. Except for one child, who was not able to complete the assessment due to their young age (6.7 years), children in the control group were able to evaluate their level of hunger and satiety. We found that in the control group, the VAS ratings paralleled our ghrelin suppression findings; appetite was lower at one-hour post-meal compared to baseline for all three meals.

In Chapter 5, surprisingly, no differences were found between the HP meal combined with whey protein supplement and SM in subjective appetite response in children and youth with PWS. Protein is the most satiating macronutrient (Anderson & Moore, 2004). Studies in both healthy adults and children with obesity and healthy weight supported this hypothesis (Anderson & Moore, 2004; Baum et al., 2015; Marmonier, Chapelot, & Louis-Sylvestre, 2000; Paddon-Jones et al., 2008; Pasiakos, 2015). Baum et al. (2015) reported that a protein-based breakfast [344 kcal, 21% protein (18 g), 52% carbohydrate, and 27% fat] increased satiety and reduced hunger when compared with a carbohydrate-based breakfast [327 kcal, 4% protein (3 g), 67% carbohydrate, and 29% fat] among children (male and female) aged 8 to 12 years with obesity and healthy weight (Baum et al., 2015). Bellissimo et al. (2008) reported that FI was suppressed more after consumption of whey protein compared to a glucose drink in children with healthy weight. Moreover, a 200 kcal whey protein drink suppressed FI in boys with healthy weight (age 12.2 ± 0.3 years), but not in boys with obesity (age 11.4 ± 0.3 years) (Bellissimo, Desantadina, et al., 2008). These findings suggest that the impact of HP in children on satiety and subsequent FI is weight dependent (Anderson et al., 2016).

In their study, Marmonier et al. (2000) studied young adults with healthy body weight (age 20±26 years) consumed a high fat (58% of energy from fat), a HP (77%) or a high carbohydrate (84%) snack. They found that a HP snack delayed the request for dinner by 60 minutes, while the high-fat snack delayed dinner request by 25 minutes and the high-carbohydrate snack delayed dinner request by 34 minutes (Marmonier et al., 2000). The HP meal with whey protein supplement was expected to increase satiety for the following reasons. First, whey protein digests quickly compared to casein (Boirie et al., 1997), resulting in a fast release and sustained increase of plasma amino acid, which leads to increased satiety (Anderson, 1979). However, it possible that due to a delay in gastric emptying, which has

been reported in individuals with PWS (Arenz et al., 2010), the increase in plasma amino acids was delayed (Boirie et al., 1997). Also, whey protein stimulates the release of satiety hormones, including PYY and GLP-1 (Belza et al., 2013; van der Klaauw et al., 2013). This latter observation is in agreement with our findings in Chapter 3. Higher protein meals stimulated greater increases in GLP-1 and PYY in children with PWS than in controls. However, postprandial AG and DAG were higher in children with PWS than in BMI z-score matched children, which might be sufficient to stimulate appetite and lead to hyperphagia. No differences in the VAS rating following the HP meal and SM were observed.

6.6 Energy Metabolism and Requirements

While REE might be altered in children with PWS as described in this thesis, dietary energy requirements ultimately relate to total energy expenditure (TEE). To date, only five studies have examined TEE and its components among this cohort (Bekx et al., 2003a; Butler et al., 2007a; Davies & Joughin, 1993; Schoeller et al., 1988; van Mil, Westerterp, Kester, et al., 2000); three have investigated differences between PWS and healthy weight subjects or individuals with obesity and found lower absolute values (ranging from 20 to 46% lower) of TEE (kcal/day) compared to matched (for either age or BMI) individuals (Davies & Joughin, 1993; Schoeller et al., 1988; van Mil, Westerterp, Kester, et al., 2000). The overall impact of this lower TEE in individuals with PWS, compared to both healthy age-matched and BMI-matched individuals, is that they would be expected to have reduced energy requirements. In fact, current clinical energy recommendations for individuals with PWS suggest an estimated 30 to 40% reduction in overall energy requirements (Miller et al., 2013). This recommendation is based on energy metabolism predictive equations that are not specific to PWS and therefore may overestimate the energy needs in these individuals.

Energy needs are estimated by assessing REE. In clinical practice, REE is estimated based on individual characteristics (age, sex, weight, and height) using predictive equations when measurement of REE cannot be directly performed (Cancello et al., 2018). Henes et al. (2013) compare measured REE with estimated REE using published predictive equations with the Harris Benedict, Lazzer and Molnar equations in 80 youth with obesity aged 7 to 18 years. They found that the mean difference between measured REE and equation-estimated REE varied from 198 kcal/day to 308 kcal/day (Henes et al., 2013). Large discrepancies between measured and predicted REE could have significant negative implications if such equations are used, especially in children with PWS given their non-specificity.

Underestimation might lead to weight loss, while overestimation might contribute to weight gain.

Additionally, this strategy does not take into consideration hyperphagia, dysfunction in satiety, body composition (lower FFM) and food-seeking behaviors that are inherent to PWS (Martinez Michel et al., 2016). Considering these additional factors is crucial when deriving energy needs and assessing satiety to facilitate the development of optimal diets for weight maintenance in children with PWS. The results presented in Chapter 5 add to the small body of literature that investigates REE and demonstrate that non-specific predictive equations cannot be used to make energy intake recommendations for individuals with PWS. Furthermore, REE and PEE were highly variable within individual with PWS. These results collectively suggest the need for individualized energy requirements for individuals with PWS.

6.7 Limitations and Challenges

This research is highly novel and has shed light on the effect of macronutrient composition on orexigenic (ghrelin and asprosin) and an anorexigenic GLP-1 and PYY, subjective appetite assessment, and PEE in children with PWS compared to children without PWS. However, some limitations must be considered, in addition to those mentioned in Chapters 3, 4 and 5. First, findings presented in Chapter 4 are from a cross-sectional study; causation cannot be determined. Chapter 5 presented data on children and youth with PWS only; thus, findings should only be applied to individuals with PWS. Furthermore, the addition of a control group of children without the syndromic matching for age, sex and BMI z-score would have provided information about whether children with PWS metabolize food differently. In this thesis, the control group was not matched for sex with the PWS group. Recruiting adolescents with overweight and obesity is challenging (Nguyen et al., 2012). There are a number of complexities in adolescents with overweight and obesity research that create additional challenges for clinical research. These include retention, compliance with protocols, issues around consent and confidentiality, and participants' motivation to support the research (Nguyen et al., 2012). Our studies required participants to attend 3 to 5 visits of between 3 and 7 hours each; this significant time commitment is a barrier to the participation of control participants. Our controls were mainly boys, and siblings or friends of the participants with PWS. Another limitation of the study is that dietary intake was not standardized prior to each DIT measurement. DIT is calculated by subtracting PEE from REE. Differences in dietary intake the day prior to the REE measurement can affect the

calculated value for DIT (Ruddick-Collins et al., 2013). Therefore, to minimize the effects of day-to-day variability in REE measurements a run-in diet period may be appropriate. This could be achieved through the provision of a standardize diet prior to each test day to assure similar baseline measures. However, one of the advantages of providing the test diet prior to the test visit, as we did in our study (one day prior to visits 2 and 3, participants received [standard diet (SD) or HP diet a combined with whey protein] and received 3 meals [breakfast, lunch and dinner] and 2 snacks [morning and evening snacks]), was to allow for adaption to adjust fuel oxidation to fuel availability, which usually occurs between 1 to 7 days (Coyle et al., 1997; el-Khoury et al., 1997; Forslund et al., 1999; Galgani & Ravussin, 2008; Galgani et al., 2008; Schrauwen et al., 1997). Additionally, long-term feeding would have a measurable influence on metabolic hormones compared to acute meal challenge (Havel, 2001). The choice of methods should be specific to the particular research question, with practicality and quality of data as priorities for consideration. For example, measurement of REE, especially comparing two different diets, may be preferable under similar conditions by standardizing the diet prior to each study visits (Johnston et al., 2002).

In Chapters 4 and 5, air displacement plethysmography (BodPod) was used to measure body composition, which may also present some limitations. The BodPod uses age- and sex-specific prediction formulas to estimate FFM based on body density (Gómez-Ambrosi et al., 2012). However, most of the predictive formulas do not take into consideration puberty status; given that puberty is largely reported to be delayed or absent in individuals with PWS (Crino et al., 2003), body composition measured using the BodPod in children should be interpreted with caution as the FFM could be under- or overestimated. Another limitation of using the BodPod is its sensitivity to hydration levels and fluid distribution (Hames, Anthony, Thornton, Gallagher, & Goodpaster, 2014; Le Carvennec et al., 2007). Body composition is altered in individuals with PWS and that there are alterations in body water distribution (van Mil et al., 2001). Thus, the assumptions made for this method may not be true for individuals with PWS.

I faced challenges in working with rare disease research as PWS occurs in 1 in 10,000 to 16,000 live-born infants (Cassidy & Driscoll, 2009). The most frequent problem was the recruitment of the required number of study participants for a research study. Recruitment was limited to Edmonton and surrounding areas due to limited funding to cover participants' travel expenses. PWS is also associated with cognitive delay (borderline to mild/moderate) and behavioral abnormalities, including temper outbursts and obsessive and compulsive behaviors such as hoarding, picking at the skin, ordering and arranging objects, insistence on

routines, and repetitive speech (Cassidy & Driscoll, 2009). Participants' cognitive impairment and limited communication skills affected their ability to complete the study tasks and limited our final sample size. In addition, males with the deletion subtype (the most common phenotype) are at greater risk for aggressive behavior, depression, dependent personality disorder, and overall severity of psychopathology than females with PWS deletion (Hartley, Maclean, Butler, Zarcone, & Thompson, 2005). Thus, the majority of our participants were female. Multicenter and international collaborations would help provide greater support for clinical investigations of rare diseases such as PWS. These recruitment challenges and reduced study sample sizes also lead to the need for the adoption of specialized study designs, such as a randomized crossover design in which all participants serve as their own controls, as well as the use of biostatistical techniques to maximize data from a small numbers of participants (Griggs et al., 2009). Additionally, reporting participants' data on rare diseases requires greater vigilance in protecting the privacy of participants as publishing of family pedigree information or detailed clinical descriptions can lead to identification of specific individuals (Griggs et al., 2009).

Despite the challenges of working with a rare disease, knowledge and experience obtained from this opportunity increased our understanding of the impact of protein meals on regulating appetite hormones and energy metabolism on this population. Additionally, these results may shed light on personalized dietary interventions designed to curb hyperphagia and maintain body weight in individuals with PWS.

6.8 Translation and Future Research Directions

Understanding the role of dietary protein on appetite-regulating hormones, subjective satiety and energy metabolism in children with PWS is important for clinical translation and can direct future research aimed at developing an optimal diet for weight maintenance among children with PWS. There is evidence to suggest that REE, the main determinant of TEE, is lower in individuals with PWS (Alsaif et al., 2017). Alterations in FFM, lower muscle mass (Orsso et al., 2017) and lower physical activity appears to be responsible for the lower EE in children with PWS, rather than metabolic differences (Alsaif et al., 2017). Regardless of the underlying mechanism for lower TEE, the estimation of energy requirements with the use of equations derived for the general population would result in weight gain in individuals with PWS (Alsaif et al., 2017). The research presented in Chapter 5 suggests that there is a short-term impact of a HP diet on REE. In addition, PEE calculated as "fixed REE" (explained in Chapter 5) promoted approximately double the EE after the HP meal compared to the SM

(164.5 ± 146 vs 88.5 ± 149 kcal). An eight-week randomized controlled feeding trial among weight-stable males and females, aged 18 to 35 years, found that 25% of total energy from protein (high-protein intervention) resulted in an increase in REE, as measured by ventilated hood, and lean soft tissue, as measured by dual-energy x-ray absorptiometry, compared to 5% of total energy from protein (low-protein intervention) (Bray et al., 2012). Future research on long-term, HP intake interventions combined with exercise in children with PWS will shed light on the impact of high-protein intake on REE and FFM, and will help to facilitate the development of optimal diets for weight maintenance in children with PWS.

Chapter 3 highlights a current gap in the ability to assess appetite (the desire to eat) in young children. The development of this ability generally occurs at around age seven and is dependent on cognitive ability (Shields et al., 2003). Therefore, current validated tools to assess appetite do not provide accurate estimations in young children. Future research on validating tools to assess appetite in young children will facilitate researchers' ability to the investigation of the role of appetite in the regulation of FI and the impact of interventions on appetite. Ongoing study in our lab aims to validate a tool to assess appetite in children ages 4 to 10 years using a dual-component, picture-based appetite assessment tool. The first component assesses motivation to eat, while the second component assesses level of hunger or fullness.

Chapter 4 measured the suppression of asprosin one hour after a SM that represented a typical Canadian diet (Garriguet, 2007). Future studies on the impact of macronutrients distribution on asprosin regulation postprandial shortly after a meal and a few hours later will help us understand the role of asprosin in regulating FI.

Results from Chapter 3 demonstrated that higher protein meals stimulated greater increases in GLP-1 and PYY in PWS children. Results from Chapter 5 proved that average appetite was lower at 15 minutes after breakfast consumption. Previous studies revealed associations between protein intake and body composition. Dietary protein is needed for muscle anabolism and it occurs when protein synthesis exceeds its breakdown rate (Mitchell et al., 2016). Jen et al. (2018) showed that a higher protein intake in children is associated with a higher FFM (Jen, Karagounis, Jaddoe, Franco, & Voortman, 2018). Body weight might also change frequently and rapidly in children as they grow—hence the need for improved early nutritional interventions in these individuals. Based on results from this thesis and previous published studies (Acheson et al., 2011; Miller et al., 2013; Nguo et al., 2018), we recommend frequent, high quality protein meals and snacks as the optimal approach for children with PWS to curb hunger and improve body composition. Ideally, a total energy intake based on the individual's REE is recommended, with high quality protein

approximately every 2 to 3 hours. Miller et al. (2013) measured REE in children with PWS using a metabolic cart and recommended a total energy intake based on each individual's REE. Participants, who followed the recommendation for 5 years saw improvements in weight control, body composition (lower body fat) and lower RER (Miller et al., 2013). Additionally, studies in healthy adults and children have shown a relationship between satiety and PEE when a meal consisting of ≥ 50% energy from protein was consumed (Acheson et al., 2011; Nguo et al., 2018).

It is important to consider not only protein quantity but also the source of protein. Consumption of animal protein (especially red meat) was related to higher FFM in the pediatric population and young adults (Assmann et al., 2013; Harris et al., 2016). In men with overweight and obesity, animal protein (pork protein) increased EE compared to (plant protein) soy protein (Mikkelsen, Toubro, & Astrup, 2000). In a study of individuals with overweight and obesity who consumed supplemental whey protein, soy protein, and an isoenergetic amount of carbohydrate for 23 weeks, those consuming whey protein showed decreased body weight, fat mass, waist circumference and fasting ghrelin (Baer et al., 2011). Regarding appetite and energy intake, no differences between animal (beef) vs. plant (soy) source of protein on hunger, fullness, PYY, or GLP-1 responses in healthy adults were observed (Douglas, Lasley, & Leidy, 2015). These findings suggest that high quality protein, regardless of the source, has similar effects on appetite. Findings from this thesis revealed that average appetite decreased at 15 minutes ($p = 0.02$) after the meal was consumed and returned to baseline level at 3 hours ($p = 0.02$). Furthermore, average appetite was higher than baseline at 4 hours ($p = 0.04$). Findings from this thesis showed that an acute intake of HP diet was beneficial to these individuals. However, to help design targeted nutritional strategies to curb hunger drives, future studies must determine the impact of longer-term intake of HP diet of different macronutrient ratios, and its respective impact of frequent meals and snack consumption on subjective appetite, and satiety hormones, among others. Nonetheless, this thesis is an initial step in facilitating the development of optimal diets for weight maintenance in children with PWS.

6.9 Conclusion

The major findings of this research were that a higher concentration of total ghrelin in children with PWS was due to higher concentrations of both AG and DAG, with no change in the ratio. Meal consumption also suppresses both forms of ghrelin to a greater extent in

children with PWS. An elevated fasting plasma proinsulin:insulin ratio in PWS children suggests an impaired processing of proinsulin. Higher protein meals stimulated greater increases in GLP-1 and PYY in children with PWS than in controls. In addition, the RER after the HP diet was significantly lower compared to standard diet; and PEE, calculated as "fixed REE," was double after the HP meal compared to SM. Therefore, a HP diet could provide a favorable influence on biomarkers of satiety and, possibly, the degree of hyperphagia in children with PWS. Testing this hypothesis will require long-term studies of the effects of HP intake on FI and weight gain.

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Appendices

Appendix A. Example for the puberty assessment

Girls Tanner Scale

At your age, girls usually begin to experience many physical changes. Please mark any changes you have experienced.

	Breast	Pubic Hair
Stage 1	Small nipples. No breast. 	No pubic hair. 
Stage 2	Breast and nipples have just started to grow. The areola has become larger. Breast tissue bud feels firm behind the nipple. 	Initial growth of long pubic hairs. These are straight, without curls, and of light color. 
Stage 3	Breast and nipples have grown additionally. The areola has become darker. The breast tissue bud is larger. 	The pubic hair is more widespread. The hair is darker, and curls may have appeared. 
Stage 4	Nipples and areolas are elevated and form an edge towards the breast. The breast has also grown a little larger. 	More dense hair growth with curls and dark hair. Still not entirely as an adult woman. 
Stage 5	Fully developed breast. Nipples are protruding, and the edge between areola and breast has disappeared. 	Adult hair growth. Dense, curly hair extending towards the inner thighs. 

Boys Tanner Scale

At your age, boys usually begin to experience many physical changes. Please mark any changes you have experienced. Please choose only ONE answer for each stage.

	Genitals	Pubic Hair
Stage 1	No signs of puberty. Scrotum, testes, and penis as in childhood. 	No pubic hair. 
Stage 2	Initial growth of scrotum and testes. The skin on the scrotum has become redder, thinner, and more wrinkled. The penis may have grown a little in length. 	Few hairs around the root of the penis. The hairs are straight, without curls, an of light color. 
Stage 3	The penis has now grown in length. Scrotum and testes have grown. The skin of the scrotum has become darker and more wrinkled. 	Hairs are darker and curlier and still sparse, mostly located at the penis root. 
Stage 4	The penis has grown in both length and width. The head of the penis has become larger. The scrotum and testes have grown. 	More dense, curly, and dark hair. The hair growth is reaching the inner thighs. 
Stage 5	Penis and scrotum as an adult. 	Pubic hair extends upwards to the umbilicus. It is dense and curly. 

Girls and boys tanner scales adapted from "Validity of self-assessment of pubertal maturation," by Rasmussen, A. R., Wohlfahrt-Veje, C., Tefre de Renzy-Martin, K., Hagen, C. P., Tinggaard, J., Mouritsen, A., Main, K. M. (2015), Pediatrics, 135(1), 86-93. doi:10.1542/peds.2014-0793. Adapted with permission

Appendix B. Meals menu: example for the test meal

Diet 1

BREAKFAST
Milk
Milk, whey protein
Turkey egg scramble
Eggs, butter, onions, tomato, garlic powder, ground turkey
Tortilla whole wheat

MORNING SNACK
Milk
Milk, whey protein

LUNCH
Rice with turkey
White rice, ground turkey, butter
Steak
Garden salad
Lettuce, cucumber, tomato, lemon, olive oil, feta cheese, whey protein

DINNER
Baked chicken
Chicken breast, lemon, olive oil, parsley, garlic powder
Garden salad
Lettuce, cucumber, tomato, lemon, olive oil, feta cheese, whey protein

EVENING SNACK
Milk
Milk, whey protein

Diet 2

BREAKFAST
Milk
Egg wrap
Boiled Egg, butter, Tortilla white
Orange fruit slice

MORNING SNACK
Milk
Toast with jam
White toast, strawberry jam

LUNCH
Steak wrap
Tortilla white, beef, onions, parsley, butter
Wedges potato
Baked potato, rosemary, garlic, olive oil
Garden salad
Lettuce, cucumber, tomato, lemon, olive oil

DINNER
Chicken stir-fry
Chicken breast, olive oil, soy sauce, ground ginger, corn starch, broccoli, cauliflower
Banana
Rice
Garden salad
Lettuce, cucumber, tomato, lemon, olive oil

EVENING SNACK
Milk
Apple

DIT STUDY

For more information please contact
Maha at **(587) 938-8108** or alsaif@ualberta.ca

HP meal

Appendix C. Food intake track

Did you consume your breakfast?

Milk with whey protein	**Turkey egg scramble**	**Tortilla whole wheat**
0 None of it	0 None of it	0 None of it
0 25%	0 25%	0 25%
0 Half	0 Half	0 Half
0 75%	0 75%	0 75%
0 All of it	0 All of it	0 All of it

Comments:

_____

_____

_____

Did you consume your morning Snack?

Milk with whey protein

0 None of it
0 25%
0 Half
0 75%
0 All of it

Comments:

_____

_____

_____

Did you consume your lunch?

Rice with turkey	**Steak**	**Garden salad**
0 None of it	0 None of it	0 None of it
0 25%	0 25%	0 25%
0 Half	0 Half	0 Half
0 75%	0 75%	0 75%
0 All of it	0 All of it	0 All of it

Comments:

_____

_____

_____

Did you consume your dinner?

Baked chicken	**Garden salad**
0 None of it	0 None of it
0 25%	0 25%
0 Half	0 Half
0 75%	0 75%
0 All of it	0 All of it

Comments:

_____

_____

_____

Did you consume your evening snack?

Milk with whey protein

- 0 None of it
- 0 25%
- 0 Half
- 0 75%
- 0 All of it

Comments:

_____

_____

_____

SM

Did you consume your breakfast?

Milk	**Egg wrap**	**Orange fruit slice**
0 None of it	0 None of it	0 None of it
0 25%	0 25%	0 25%
0 Half	0 Half	0 Half
0 75%	0 75%	0 75%
0 All of it	0 All of it	0 All of it

Comments:

_____

_____

_____

Did you consume your morning Snack?

Milk	**Toast with jam**
0 None of it	0 None of it
0 25%	0 25%
0 Half	0 Half
0 75%	0 75%
0 All of it	0 All of it

Comments:

_____

_____

_____

Did you consume your lunch?

Steak wrap	**Wedges potato**	**Garden salad**
0 None of it	0 None of it	0 None of it
0 25%	0 25%	0 25%
0 Half	0 Half	0 Half
0 75%	0 75%	0 75%
0 All of it	0 All of it	0 All of it

Comments:

_____

_____

_____

Did you consume your dinner?

Chicken stir-fry	**Rice**	**Banana**	**Garden salad**
0 None of it	0 None of it	0 None of it	0 None of it
0 25%	0 25%	0 25%	0 25%
0 Half	0 Half	0 Half	0 Half
0 75%	0 75%	0 75%	0 75%
0 All of it	0 All of it	0 All of it	0 All of it

Comments:

_____

_____

_____

Did you consume your evening snack?

Milk

- 0 None of it
- 0 25%
- 0 Half
- 0 75%
- 0 All of it

Apple

- 0 None of it
- 0 25%
- 0 Half
- 0 75%
- 0 All of it

Comments:

_____

_____

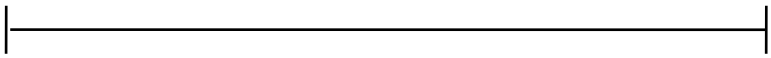
_____

ID#: _____ Date: _____
Time: _____

Appendix D. Visual analog scale

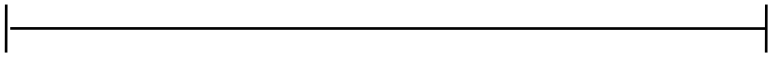
For each question, mark on the line how you feel.

1. **How much are you thinking about food?**



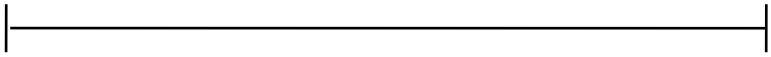
I have no thoughts of food. I am very preoccupied with thoughts of food and it is difficult to concentrate on anything else.

2. **How hungry do you feel?**

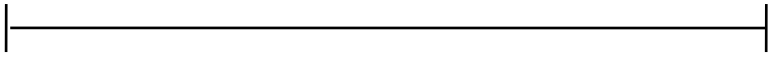


I am not at all hungry. I am as hungry as I've ever been.

3. **How full do you feel?**

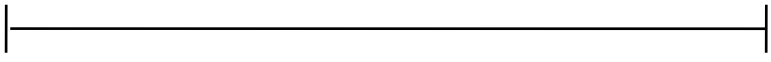


4. **How strong is your desire to eat?**



Very little desire to eat. Very strong desire to eat.

5. **How much do you think you could eat?**

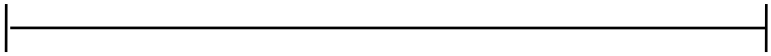


Nothing at all. A large amount.

6. **How strong is your urge to eat?**

ID#: _____ Date: _____

Time: _____



No urge to eat. Very strong urge to eat.