HORMONAL ANALYSES IN INDIVIDUALS WITH PRADER-WILLI SYNDROME AND OTHERS WITH EARLY-ONSET MORBID OBESITY

By

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To my grandparents whose love and support I have never been without.

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Prader-Willi syndrome (PWS) is a rare genetic disorder characterized by infantile hypotonia and failure-to-thrive, developmental and intellectual disabilities, hypogonadism, short stature, behavioral problems, and early-onset morbid obesity. PWS is caused by failure of expression of paternally inherited genes in chromosomal region 15q11.2-q13. In PWS obesity is the major cause of morbidity and mortality; it typically begins at age 1-4 years and is further compounded by the development of hyperphagia, resulting from a lack of sense of satiety. The mechanism(s) behind development of obesity and hyperphagia in PWS remain unclear.

Here I report that ghrelin, a potent appetite-stimulating hormone, is significantly elevated in infants and young children with PWS before the onset of obesity and hyperphagia. Ghrelin levels were the highest in PWS infants still in the poor appetite phase. Given this fact, it is unlikely that elevated ghrelin levels are causing the switch to the hyperphagic phases of PWS. However, it has been shown in mice that ghrelin can also act to increase fat mass independent of its effect on appetite (Perez-Tilve et al., 2011). Therefore, it is likely that the elevated ghrelin levels are causing the increased

fat mass seen in PWS infants compared to normal infants with similar body mass indices (BMI).

I also report here that peripheral leptin levels are elevated in obese PWS individuals while alpha-melanocyte stimulating hormone (alpha-MSH) and brain-derived neurotrophic factor (BDNF) are not elevated. Given the fact that BDNF is primarily produced in the brain, and hypothalamic leptin signals through alpha-MSH to promote BDNF expression, it is therefore possible that obese individuals with PWS have low brain leptin levels resulting from inadequate brain leptin uptake, and thus suffer from leptin resistance.

CHAPTER 1
INTRODUCTION

Prader-Willi Syndrome

Prader-Willi Syndrome (PWS) is a genomic imprinting obesity disorder caused by lack of expression of paternally inherited genes in the PWS region of chromosome 15q11.2 - q13 [Figure 1-1]. PWS was first described by Andrea Prader, Heinrich Willi, and Alexis Labhart in 1956; it was the first disease to be linked with the phenomenon of genomic imprinting and also the first identified disorder resulting from maternal uniparental disomy (Prader-Willi Syndrome Association, USA; Bittel and Butler, 2005; Cassidy and Driscoll, 2009). PWS occurs in 1 in every 20,000 births and equally affects males and females of all races and ethnic groups. The clinical features of PWS include infantile lethargy and hypotonia causing poor feed and failure-to-thrive, developmental and intellectual disability, hypogonadism, morbid obesity if uncontrolled, hyperphagia, behavioral and psychiatric disturbances, short statue, temperature and pain insensitivity, characteristic facial appearance and body habitus (Bittel and Butler, 2005; Cassidy and Driscoll, 2009).

The loss of paternally expressed genes in PWS occurs via three major genetic mechanisms: a 5-7 Mb deletion of the paternally inherited chromosome 15q11.2-q13 region, maternal uniparental disomy (UPD) 15, or a defect in the imprinting process in the 15q11.2-q13 region on the paternally inherited region. However most cases of PWS result from the sporadic 5-7 Mb deletion with one of two proximal break points (BP1 and BP2) and a distal break point (BP3) [Figure 1-1]. In families where the father carries imprinting deletions in the PWS region, the risk is much higher (Bittel and Butler, 2005; Cassidy and Driscoll, 2009).

The Nutritional Phases of Prader-Willi Syndrome

Individuals with PWS are typically grouped in to two classic nutritional phases: before and after onset of obesity and hyperphagia. Most recently, children and adults with PWS have been grouped in to four major nutritional phases with sub-phases occurring in the first two (Miller et al., 2011) [Table 1-1]. Nutritional phase 1 occurs from birth to infancy and usually the affected infant is hypotonic and not obese. Infants in sub-phase 1a are characterized by poor appetite and failure-to-thrive while infants in sub-Phase 1b appear to grow steadily along at a normal rate and with a normal appetite.

Nutritional phase 2 generally occurs between 18-36 months of age and is characterized by significant weight increase across growth percentile lines. Affected children in sub-phase 2a have significant weight increase, crossing 1-2 or more growth percentile lines without significant increase in calories or appetite. In sub-phase 2b the affected child is typically overweight and daily caloric intake has increased along with an abnormally increased interest in food. However, the child can still feel full at this stage after a meal.

Nutritional phase 3 involves the development of hyperphagia, accompanied by aggressive food seeking and lack of satiety. The onset of phase 3 is quite variable and may appear as early as 3 years of age or as late as 15 years or, in a small minority, never. Most individuals in this phase are typically obese.

Nutritional phase 4 occurs when an individual previously in phase 3 no longer has insatiable appetite and can feel full. This phase does not start until adulthood and even though patients may still have a greater than normal appetite, it is not as aggressive and unrelenting as in phase 3 (Miller et al., 2011).

Development of Obesity in Prader-Willi Syndrome

In PWS, obesity is the major cause of morbidity and mortality. It typically begins between 1-4 years of age, if food intake is not strictly controlled, and is further compounded by the development of hyperphagia later on in childhood (Cassidy and Driscoll, 2009; Miller et al., 2011).

The etiology of obesity in PWS remains unclear: the mechanism(s) behind failure-to-thrive and poor appetite early on, and the subsequent onset of obesity well before any significant increase in appetite and food consumption, remain unknown. So far, no obesity-associated gene has been identified in the PWS region (15q11.2-q13).

Ghrelin and leptin have been reported by many groups to be significantly elevated in obese adults with PWS, however their roles in PWS obesity remain unclear (Cummings et al., 2002; Delparigi et al., 2002; Goldstone, 2005). A recent study of a small group of PWS patients reported low levels of brain-derived neurotropic factor (BDNF), an anorexigenic neuropeptide that signals downstream of the hypothalamic leptin-melanocortin pathway to regulate food intake and energy expenditure (Han et al., 2010).

Here we investigate the onset and consequences of hyperghrelinemia in individuals with PWS. We also investigate hypothalamic leptin signaling in obese PWS individuals by assaying for serum concentrations of the melanocortin peptide alpha-melanocyte stimulating hormone (α-MSH) and the neuropeptide BDNF.

Ghrelin: The Hunger Gene

Ghrelin is a pleiotropic hormone that is secreted at different developmental stages by a wide variety of organs including the pancreas, duodenum, stomach, hypothalamus, pituitary, bone, ovary, testis, and cartilage (Steculorum and Bouret, 2011). It is the first

identified hunger hormone and it was discovered in the rat and human stomach shortly after the discovery of its receptor (Kojima et al., 1999).

Originally, ghrelin was described as a 28 amino acid peptide that was secreted primarily from the stomach and that bound the growth hormone secretagogue receptor 1a (GHS-R1a) to stimulate the release of growth hormone (Kojima et al., 1999). Subsequent studies however revealed ghrelin to be a pleiotropic hormone secreted by a variety of organs with strong appetite-inducing effects (Steculorum and Bouret, 2011). Ghrelin's effects on appetite and energy homeostasis are potentially long-lasting; it modulates development of appetite-related brain centers early on in life by counteracting the effects of leptin, an anorexigenic hormone secreted by adipocytes which play an important regulatory role in the development of hypothalamic neurons that regulate feeding and energy homeostasis (Bouret et al., 2004a; 2004b; Steculorum and Bouret, 2011).

Ghrelin is significantly elevated in adults with PWS (Cummings et al., 2002; Delparigi et al., 2002) however the consequence of this hyperghrelinaemia is unclear; sustained reduction of ghrelin levels with pharmacological agents in PWS subjects did not reverse hyperphagia or significantly alter body composition (Tan et al., 2004; De Waele et al., 2008). Thus the high ghrelin levels observed in adult PWS subjects do not appear to be the cause of their hyperphagia.

The impact of hyperghrelinaemia in PWS however, may occur at an earlier stage such as the perinatal period, impacting development of appetite-regulatory neurons in the hypothalamus, and resulting in long lasting effects. Currently, ghrelin levels in infants and young children with PWS, as reported in the literature, are not well

described and appear controversial. Cummings et al. reported that ghrelin was not elevated in young non-obese children with PWS, while Haqq et al. reported that only a subset of young non-obese PWS children had hyperghrelinaemia (Erdie-Lalena et al., 2006; Haqq et al., 2008). However Tauber et al. reported that hyperghrelinaemia was present in PWS children at any age, and preceded the onset of obesity (Feigerlová et al., 2008).

I have analyzed serum ghrelin levels in infants, young children and adults with PWS and at the different nutritional phases of PWS. I report here that ghrelin is elevated early on in infants with PWS before the onset of obesity and hyperphagia (Chapter 3).

The Hypothalamic Leptin-Melanocortin Signaling Pathway

Dysfunction of the hypothalamus in PWS is thought to play an important role in the development of obesity and hyperphagia in individuals with PWS. Since the PWS region (15q11.2-q13) does not contain any known obesity-associated genes, dysfunction of the hypothalamic leptin-melanocortin signaling pathway (Figure 1-3) and its downstream effector, brain-derived neurotrophic factor (BDNF), bears exploring as a cause of the obesity.

Leptin is an anorexigenic hormone that is critical in the regulation appetite, energy homeostasis and body weight. A large part of leptin's effects on energy homeostasis and body weight is mediated in the hypothalamus, the site of highest mRNA expression of the long isoform of the leptin receptor (Ob-Rb), through the hypothalamic melanocortin signaling pathway (Oswal and Yeo, 2007; Farooqi and O'rahilly, 2008). Leptin diffuses into the arcuate nucleus (ARC) of the hypothalamus and acts directly as a transcription factor in two distinct classes of primary leptin-responsive neurons: one

class co-expresses the anorexigenic peptides pro-opiomelanocortin (POMC) and Cocaine and Amphetamine Related Transcript (CART) which inhibit appetite, while the other co-expresses the melanocortin antagonists neuropeptide Y (NPY) and agouti-related protein (AgRP) which induce appetite (Oswal and Yeo, 2007). Leptin, upon binding the leptin receptor, triggers a signaling cascade leading to transcriptional activation of the *POMC* and *CART* genes, while inhibiting the release of NPY and AgRP (Oswal and Yeo, 2007; Farooqi and O'rahilly, 2008). The full length *POMC* protein is cleaved by prohormone convertase 1 (PC1) and prohoromone convertase 2 (PC2) in a tissue-specific manner to produce an array of smaller bioactive peptides including β-endorphin, β-lipotrophin, and the melanocortin peptides adrenocorticotrophic hormone (ACTH), alpha-, beta- and gamma- melanocyte stimulating hormones (α-, β-, γ- MSH).

The melanocortins mediate their effect through a family of five G-protein coupled receptors known as the melanocortin receptors (Oswal and Yeo, 2007; Farooqi and O'rahilly, 2008). The melanocortin receptors (MCRs) signal primarily through the cyclic AMP transduction pathway via a Gs protein and adenyl cyclase. The agouti-related protein (AgRP), which is inhibited by leptin, competes with alpha-MSH for the receptors and acts as an antagonist and an inverse agonist to the receptors (Ollmann et al., 1997; Yang et al., 1999; Nijenhuis et al., 2001). Activation of the melanocortin-3 (MC3R) and the melanocortin-4 (MC4R) receptors also stimulates extracellular signal-regulated kinases (ERK) activation, suggesting multiple signaling pathways may be involved in addition to the cAMP pathway (Farooqi and O'rahilly, 2008).

Of the five melanocortin receptors, only *MC3R* and *MC4R* have been linked with regulation of energy homeostasis. Mutations in the *MC4R* gene result in childhood

obesity, and are the most common monogenic cause of human obesity (Adan et al., 2006; Butler, 2006; Shimizu et al., 2007; Lee, 2009; Roth et al., 2009).

Primary leptin-responsive neurons in the hypothalamus make numerous connections with second order hypothalamic nuclei in the lateral hypothalamus (LH), the paraventricular nucleus (PVN), the ventromedial nucleus (VN), and the dorsomedial nucleus (DMN), all of which highly express MC3R and MC4R (Oswal and Yeo, 2007). Transcriptional activation of *POMC* by leptin leads to stimulation of MC4R receptors by alpha-MSH in the hypothalamus, resulting in decreased appetite and increased energy expenditure [Figure 2-1].

The functional role of the leptin-melanocortin signaling pathway in the development of obesity and hyperphagia in individuals with PWS remains unclear. A decrease in circulating leptin levels, such as during fasting, is accompanied by simultaneous reduction in transcription of *POMC* and *CART* genes, and marked increase in *NPY* and *AgRP* mRNA levels. Peripheral leptin is appropriately elevated with the degree of adiposity in individuals with PWS (Butler et al., 1998; Proto et al., 2007) and expression of both *NPY* and *AgRP* genes appear to be normal in obese individuals with PWS (Goldstone et al., 2002). Leptin induction of *POMC* gene transcription also appears unhindered in mouse models of PWS (Ge et al., 2002; Bittel et al., 2007). Thus the immediate mediators of leptin signaling appear to be preserved in PWS. However, hypothalamic leptin levels and anorexigenic leptin signaling through alpha-MSH and MC4R in the brain remains unclear and unexplored in PWS.

The development of morbid obesity early on in PWS is reminiscent of young children with *MC4R* dysfunction. Mutations in the *MC4R* gene causing intracellular

retention of the receptor associate with early age of onset and greater severity of obesity (Lubrano-Berthelier et al., 2003). Expression of *MC4R* is not known to be aberrant in individuals with PWS however dysfunction through internalization and desensitization of the receptor due to continuous stimulation by elevated levels of alpha-MSH remains a possibility. Recent studies in mouse hypothalamic GT-7 cells (Shinyama et al., 2003), HEK 293 cells (Gao et al., 2003) and N2A cells (Mohammad et al., 2007) demonstrate that continuous stimulation of MC4R with high concentrations of alpha-MSH causes intracellular retention and desensitization of the receptor.

While *POMC* has been shown to be elevated in the hypothalamus of newborns of PWS mouse models (Ge et al., 2002; Bittel et al., 2007), and peripheral leptin is known to be significantly elevated in individuals with PWS, not much is known about *POMC* expression or leptin levels in the hypothalamus of individuals with PWS. Thus it is possible that leptin activation of *POMC* mRNA expression in obese PWS patients is dysfunctional.

In this dissertation, I examined serum alpha-MSH levels in non-obese and obese individuals with PWS along with lean normal controls and non-PWS individuals with EMO. I report here that serum alpha-MSH levels decrease with onset of obesity and hyperphagia in PWS subjects (Chapter 4).

Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (*BDNF*) is a neuropeptide that is widely expressed in the central nervous system (CNS) and is best known for its role in regulating brain development and plasticity (Hofer and Barde, 1988; Carter et al., 2002; Chan et al., 2006). *BDNF* plays a critical role in neuronal survival and differentiation during development of the CNS and in regulating synaptic activity, neurotransmission

and plasticity in mature neurons (Fariñas, 1999; Cohen and Greenberg, 2008). Its levels in the brain are dramatically increased during postnatal development with the highest levels of mRNA and protein in the hippocampus, amygala, cerebral cortex and hypothalamus (Bartkowska et al., 2010; Ji et al., 2010).

The human *BDNF* gene is localized on chromosome 11p14.1 and spans over ~70 kb. It contains 11 exons (I-V, Vh, VI-VIII, VIIh and IX) and 9 functional promoters that are used in a tissue specific and brain-region specific manner (Jones and Reichardt, 1990; Pruunsild et al., 2007). Alternative splicing of the 5' exons generate at least 17 transcripts, producing three precursor pro-BDNF protein isoforms (a,b,c) that differ in length of their signal peptide. Pro-BDNF binds preferentially the p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor superfamily. Pro-BDNF is proteolytically cleaved extracellularly by plasmin and matrix metalloproteinase, or in the golgi network by furin and proconvertase to give rise to mature BDNF protein, a 120 amino acid peptide that binds preferentially to the tyrosine kinase receptor B (TrkB) receptor, promoting development and differentiation of neurons, cell survival, long-term potentiation and synaptic plasticity (Hofer and Barde, 1988; Carter et al., 2002; Chan et al., 2006).

Current research in animals has implicated BDNF and its receptor TrkB in modulation of energy balance downstream of the melanocortin pathway (Nakagawa et al., 2000; Xu et al., 2003; Unger et al., 2007). Mice lacking either one copy of the Bdnf gene or with a tissue-specific conditional deletion of Bdnf in the post-natal brain developed obesity and hyperphagia (Rios et al., 2001). Similarly, mice with a hypomorphic mutation in TrkB, resulting in 25% normal levels of expression, also

develop obesity (Xu et al., 2003). Bdnf mRNA expression in the ventromedial hypothalamus (VMH), an important satiety center in the brain, was shown to be increased in mice in the fed versus the fasted state. Central administration of BDNF reduced obesity in obese lepr (db/db) mice (Nakagawa et al., 2000), and reversed hyperphagia and obesity in heterozygous Bdnf knockout mice and in Mc4r deficient mice (Xu et al., 2003) suggesting BDNF plays a role in mediating energy balance downstream of the leptin-melanocortin pathway. Nicholson et al showed that activation of MC4R with an agonist in mice leads to the release of BDNF in the brain and regulation of appetite, body temperature and cardiovascular function (Nicholson et al., 2007). Heterozygous Bdnf knockout mice also displayed diminished pain sensitivity and behavioral disturbances (MacQueen et al., 2001).

In humans, the clinical phenotypes associated with BDNF deficiency are similar to those observed in individuals with PWS. BDNF deficiency, as observed in WAGR (Wilms tumor, aniridia, genitourinary anomalies, mental retardation) syndrome patients with heterozygous deletion of *BDNF* (Han et al., 2008) and in an 8-year-old girl with disruption of *BDNF* expression caused by interstitial 11p inversion (Gray et al., 2006), is associated with severe hyperphagia, childhood obesity, developmental delays, and decreased circulating BDNF levels. A de novo TrkB mutation was detected in an 8-year-old boy with severe hyperphagia, obesity, and impaired learning and memory (Yeo et al., 2004).

Extremely obese but otherwise healthy children were reported in a 2006 study to have decreased serum BDNF concentration (Areeg H El-Gharbawy, 2006), suggesting inappropriately low BDNF may associate with the pathophysiology of morbid obesity.

Most recently, a study of 13 PWS patients reported lower serum and plasma BDNF concentrations, suggesting insufficient central BDNF production in individuals with PWS (Han et al., 2010).

In this dissertation, I examined a larger cohort of PWS patients and their normal sibling counterparts along with non-PWS individuals with early-onset morbid obesity to determine their serum BDNF levels. I report here that BDNF levels decrease with onset of obesity and hyperphagia in PWS subjects (Chapter 5).

Significance of This Work

Morbid obesity and hyperphagia are the major medical concerns for individuals with Prader-Willi syndrome. The mechanism(s) behind their development in PWS remains unclear. Hyperghrelinaemia is present in adult PWS patients but its role or onset remains unclear and ghrelin levels in PWS children remain controversial. Peripheral leptin is significantly elevated in obese PWS individuals but its hypothalamic levels and signaling remain unknown in PWS.

Here, I show that ghrelin is significantly elevated in infants and young children with PWS before the onset of obesity and hyperphagia and that this may contribute to the increased adiposity observed in PWS infants, and the subsequent weight increase and onset of obesity early on in childhood.

I also show that obese PWS individuals may suffer from leptin resistance despite elevated peripheral leptin levels, as their serum alpha-MSH and BDNF decrease with onset of obesity, suggesting low brain leptin levels. I present a model of a potential mechanism by which obesity and hyperphagia develops in hyperghrelinaemic PWS subjects with elevated peripheral leptin.

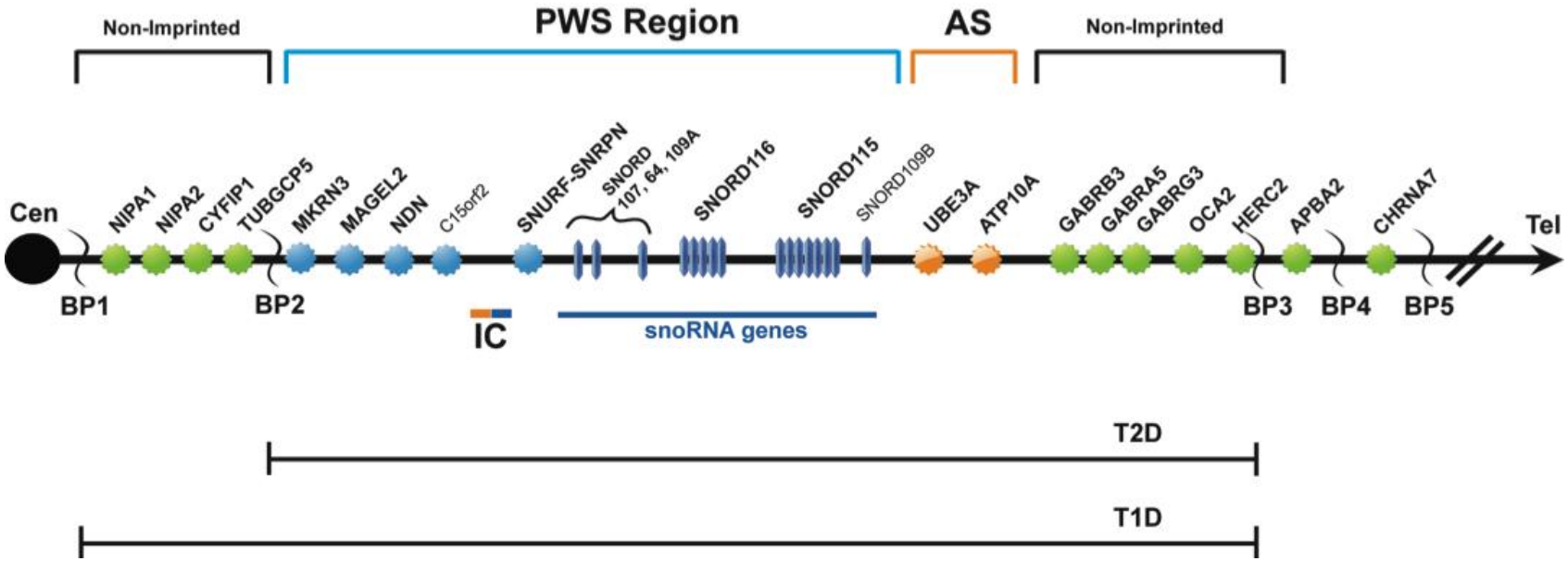


Figure 1-1. PWS chromosomal region 15q11.2-q13 with breakpoints (BP1, BP2, BP3), genes and their imprinting status indicated. Non-imprinted genes are in green and imprinted genes are in blue. Angelman syndrome (AS) genes are in orange.

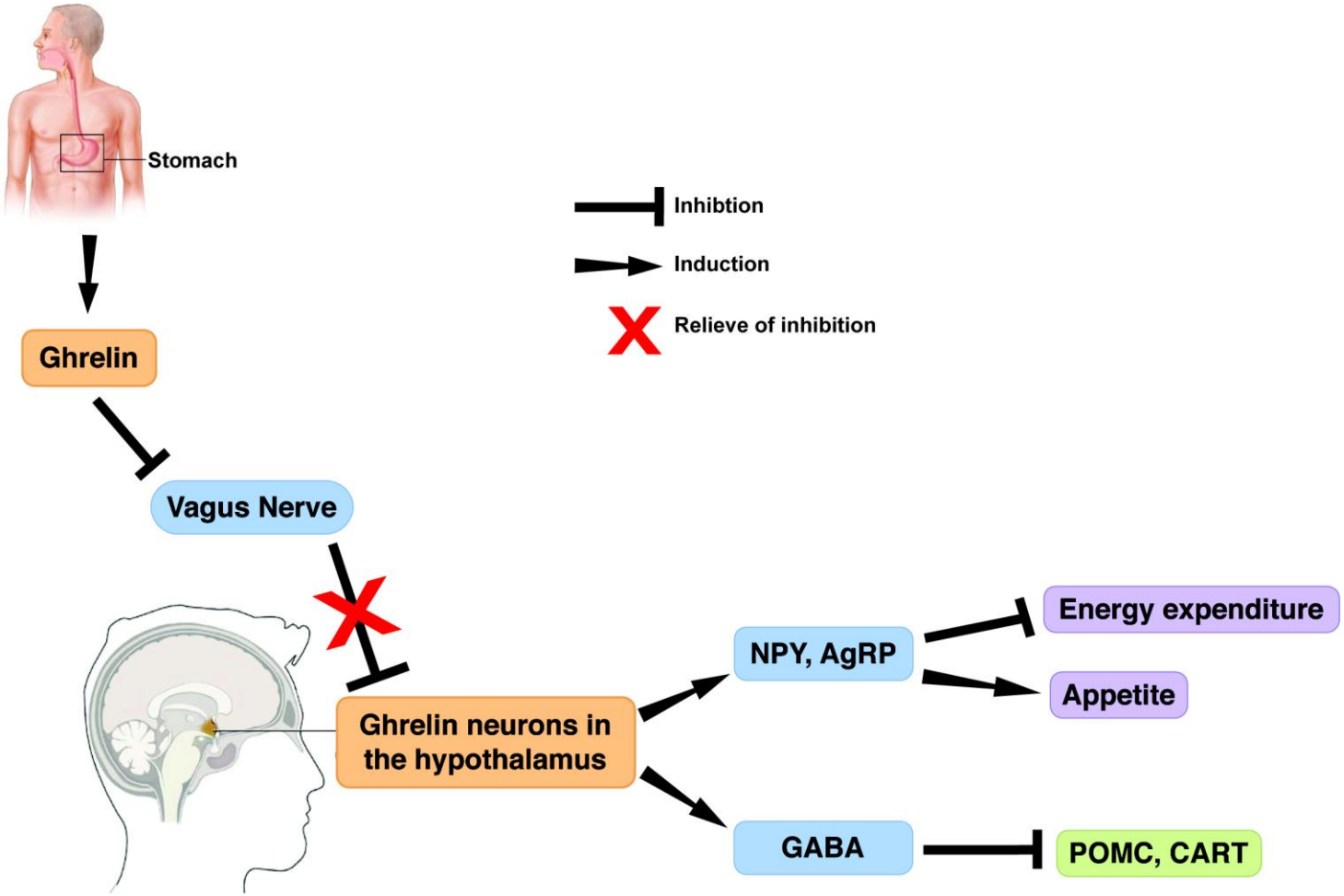


Figure 1-2. Ghrelin induction of appetite. Ghrelin secreted in the stomach inhibits the vagus nerve, thereby relieving repression of hypothalamic ghrelin neurons. This results in local release of ghrelin in the hypothalamus, stimulation of NPY/AgRP/GABA neurons, induction of appetite, inhibition of the POMC/CART neurons, and a decrease in energy expenditure.

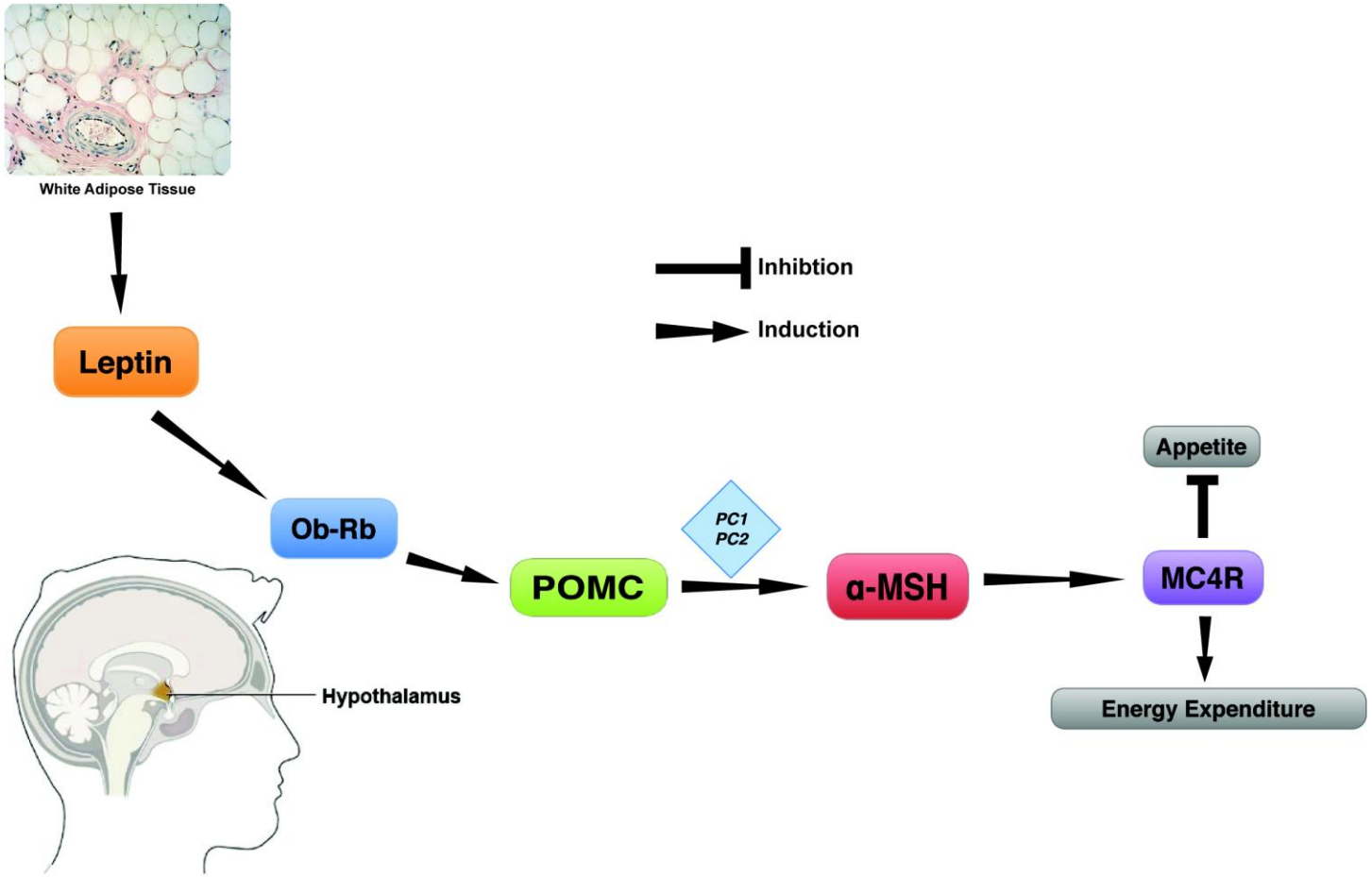


Figure 1-3. The hypothalamic leptin-melanocortin signaling pathway. Leptin secreted by the adipose tissue diffuses into the hypothalamus, binds the leptin receptor (Ob-Rb) and trigger POMC transcription. POMC prohormone is cleaved by prohormone convatase (PC1 & PC2) to release alpha-MSH that activates MC4R, leading to decreased appetite and increased energy expenditure.

Table 1-1. Nutritional Phases in Prader-Willi syndrome

Nutritional Phase	Description
0	Decreased fetal movements, birth weight 15% < normal sibs
1a	Hypotonia with difficulty feeding & decreased appetite (0 - 9 mo)
1b	Improved feeding, appetite & growth (9 - 25 mo)
2a	Weight increases without increase in appetite or calories (2.1 – 4.5 yr)
2b	Increased appetite, but can feel full (4.5 – 8 yr)
3	Insatiable appetite, rarely feels full (8 yrs – adulthood)
4	Appetite no longer insatiable & can feel full (some adults)

CHAPTER 2
METHODOLOGY

Study Subjects

Subjects with Prader-Willi syndrome before and after growth hormone therapy were recruited to the University of Florida Natural History Study (2001 – 2011) and admitted in our 2-day intensive research study at the General Clinical Research Center (GCRC) at Shands Hospital at the University of Florida. Non-PWS subjects with early-onset morbid obesity (EMO) as well as normal weight sibling controls (Sib.C) were also recruited to serve as comparison group for the study. A medical geneticist and an endocrinologist examined each research subject, and a nutritionist also performed a thorough nutritional assessment. Each subject had blood taken after an overnight fast for collection of plasma and serum, some of which was stored in multiple aliquots at -80^{o}C until further assay. A total of 60 PWS subjects, 39 EMO subjects and 95 normal sibling controls from 0-36 years of age were recruited in this study (Table 2-1). Most subjects had blood drawn more than once at different time points during the course of the study, thus accounting for the discrepancy between subject number and sample number.

Blood Sample Collection and Processing

Blood was taken from each research subject between 8 – 9 AM in the morning after an overnight fast. For isolation of serum samples, blood was collected in a vacutainer tube lacking anti-coagulants and allowed to sit at room temperature for 15-30 minutes until clot. The samples were then centrifuged at 3000 rpm (1,800 x g) for 10 minutes at +4^{o}C. Both serum and plasma samples were aliquot into 1mL cryo-tubes and stored at -80^{o}C until use.

Hormone Assays

For ghrelin analysis, serum samples were analyzed in triplicate using a fluorescent Enzyme-linked Immunosorbent Assay (ELISA) kit from Phoenix Pharmaceuticals, Inc., California, USA. The kit measures total ghrelin based on the principle of "competitive" enzyme immunoassay. Briefly, the immunoplate is pre-coated with a secondary antibody and the nonspecific binding sites blocked. Primary ghrelin antibody and unknown serum samples are incubated overnight in the immunoplate, followed by incubation with a biotinylated ghrelin peptide. The Fc fragment of the primary antibody binds the secondary antibody in the immunoplate, while its Fab fragment competitively binds ghrelin peptide in the unknown samples or the biotinylated ghrelin peptide. The biotinylated peptide interacts with streptavidin-horseradish peroxidase (SA-HRP) that catalyzes the substrate. The fluorescence intensity is inversely proportional to the amount of ghrelin peptide in the unknown sample. The ghrelin concentration in the unknown sample is determined by extrapolation from a standard curve of known concentrations.

For leptin analysis, plasma samples were diluted 2x and assayed with the Luminex assay system using the metabolic panel per instructions of the manufacturer (Millipore Inc, CA, USA).

Statistical Analysis

ELISA data was normalized for inter-assay variability to four internal controls that were present in every assay that was performed. Two-tailed Student's t-test was used to statistically compare groups after adjusting for age and sex differences. P-values

less than 0.05 were considered to be significant. Spearman's correlation analysis was used to further analyze interactions in groups.

CHAPTER 3
ANALYSIS OF GHRELIN LEVELS IN YOUNG AND OLD INDIVIDUALS WITH PRADER-WILLI SYNDROME

Ghrelin Level in Young PWS Children Is Unclear and Controversial

The consequences of hyperghrelinemia in adult individuals with PWS remain unclear. The current literature on ghrelin in young PWS children is also unclear and controversial. Studies from the laboratories of Merlin Butler and David Cummings describe ghrelin as normal in children with PWS (Butler et al., 2004; Erdie-Lalena et al., 2006). Cummings et al. looked at both acylated and total ghrelin in PWS children and did not find any significant difference from normal controls. Andrea Haqq later reported elevated ghrelin levels in a subset (33%) of young PWS children (Haqq et al., 2008). However, another study by Maithe Tauber reported that hyperghrelinaemia was present in PWS children at any age and preceded the onset of obesity (Feigerlová et al., 2008).

In this study, I investigate ghrelin levels in infants and young children with PWS and how it relates to obesity and hyperphagia in PWS.

Ghrelin Is Elevated in Infants and Young Children with PWS (0 – 1.99 Years)

A competitive ghrelin ELISA assay was used to analyze serum ghrelin levels of PWS infants and young children, and normal control infants and young children between the ages of 0 – 1.99 years [Table 3-1]. Ghrelin was significantly elevated in PWS infants and young children relative to normal control infants and young children of the same age (5521 ± 3696 pg/ml vs 2883 ± 1172 pg/ml; $p=0.016$) [Figure 3-1; Table 3-2].

There was no significant difference in plasma leptin levels between PWS and normal control infants and young children of this age (272 ± 231 pg/ml vs 216 ± 145 pg/ml; $p=0.48$).

PWS infants and young children at this age (0-1.99y) had significantly lower weight-for-length percentile relative to their normal counterparts of the same age. However, body fat percentile as determined by DEXA did not differ significantly between the two groups at this age; PWS infants had as much body fat as normal sibling control infants [Figure 3-7] despite their low weight-for-length percentile.

Ghrelin Is Not Elevated in PWS Children 2 – 4.99 Years Old

Ghrelin levels of PWS children 2 – 4.99 years old did not differ significantly from levels in normal lean control children (Sib.C) and EMO children of the same age (*p=0.12 & p=0.71* respectively). Also, ghrelin levels in EMO children 2 – 4.99 years old did not differ significantly from levels in normal lean children (*p=0.30*) of the same age [Figure 3-2]. However, PWS and EMO children of this age had significantly higher plasma leptin levels than normal lean control children of this age (*p<0.001*) [Table 3-2]. The BMI-Z score of PWS children was not significantly higher than that of Sib.C but was significantly less than that of EMO children [Table 3-1].

Ghrelin Is Not Elevated in PWS Children 5 – 11.99 Years Old

Ghrelin was not significantly elevated in PWS children 5-11.99 years old relative to normal lean control children of the same age (*p=0.21*); however their ghrelin was significantly elevated relative to that of EMO children of same age (*p=0.012*) [Figure 3-3].

Plasma leptin was significantly elevated in both PWS and EMO children within this age group relative to normal lean controls (*p<0.001***) [Table 3-2]. There was no significant difference between leptin levels in PWS and EMO subjects in this age group (*p=0.56*).

Ghrelin Is Elevated in Teenagers and Young Adults with PWS (12-20.99 Years)

Teenagers and adults with PWS (12-20.99y) had significantly higher ghrelin levels than normal lean controls (*p=0.011*) and EMO subjects (*p=0.0085*) of the same age [Figure 3-4]. They also had significantly elevated leptin levels (*p=0.0094*) and BMI-Z scores relative to normal lean controls [Table 3-2].

EMO teenagers and young adults had ghrelin levels similar to normal lean controls (*p=0.49*) but significantly elevated leptin levels relative to both PWS and normal lean control subjects (*p=0.0060*** & *p<0.001*** respectively).

Hyperghrelinemia Precedes Obesity and Hyperphagia in PWS

The nutritional phases in PWS were more predictive of ghrelin levels in PWS individuals than was age or any other factor. Individuals in nutritional phase 1a, which is characterized by poor appetite and failure-to-thrive, had the highest ghrelin levels relative to all the other nutritional phases combined [Figure 3-5]. Nutritional phase 3, which is characterized by hyperphagia and obesity, had some of the lowest observed ghrelin levels.

Correlation of Ghrelin with Leptin, BMI z-Score, DEXA, and Growth Hormone Therapy in PWS

PWS individuals on growth hormone therapy had serum ghrelin levels about 1202 ± 534 pg/ml less than individuals not on growth hormone therapy [Figure 3-6]. This difference was significant after adjusting for age and sex (*p=0.043**).

Ghrelin correlated significantly with leptin in normal control infants and young children 0-1.99 years old (*p=0.0063***) but not in PWS children (*p=0.98*) of the same age. However, leptin correlated significantly with weight-for-length in PWS infants and

young children 0-1.99 years old (*p=0.0025***) but not in normal control infants and young children (*p=0.90*) of the same age [Table 3-3].

Above the age of 2 years, ghrelin did not significantly correlate with leptin, BMI z-score or DEXA in PWS (*p=0.74*), normal lean controls (*p=0.089*), and EMO subjects (*p=0.71*) [Table 3-4]. However leptin correlated significantly with DEXA and BMI z-score in PWS (*p<0.001*** *& p=0.0013*** respectively) and normal lean control subjects (*p<0.001 & p=0.0013*** respectively). DEXA correlated with BMI z-score in all three groups (PWS, *p<0.001***; Sib.C, *p<0.001***; EMO, *p=0.0082***) [Table 3-4].

There was no significant difference in ghrelin levels of PWS individuals with Type 1 or Type 2 deletions (*p=0.67*), or between UPD and deletion PWS subjects (*p=0.46*).

The significance of this work in relationship to the current state of the field is discussed in chapter 6. The ghrelin assay used in this study had intra-assay variability less than 4% and an inter-assay variability less than 15%.

Table 3-1. List of subjects, observations and characteristics

	PWS	Sib.C	EMO
Age group 1	0-1.99 years	0-1.99 years	0-1.99 years
Subjects	18 (9M, 9F)	12 (7M, 5F)	N/A
Observations	25 (15M, 10F)	14 (8M, 6F)	N/A
Average age (years)	1.1 ± 0.5	0.91 ± 0.5	N/A
Mol. Class (Del/UPD/ID)	14/9/2	N/A	N/A
GH treatment (Yes/No)	14/11	N/A	N/A
Age group 2	2-4.99 years	2-4.99 years	2-4.99 years
Subjects	41 (22M, 19F)	26 (11M, 15F)	9 (6M, 3F)
Observations	53 (27M, 26F)	28 (11M, 17F)	9 (6M, 3F)
Average age (years)	3.7 ± 0.7	3.7 ± 0.9	4.1 ± 0.9
Mol. Class (Del/UPD/ID)	33/18/2	N/A	N/A
GH treatment (Yes/No)	49/4	N/A	N/A
Age group 3	5-11.99 years	5-11.99 years	5-11.99 years
Subjects	29 (12M, 17F)	54 (25M, 29F)	20 (10M, 10F)
Observations	41 (17M, 24F)	74 (31M, 43F)	28 (16M, 12F)
Average age (years)	7.5 ± 1.8	8.0 ± 1.7	8.2 ± 1.8
Mol. Class (Del/UPD/ID)	26/13/2	N/A	N/A
GH treatment (Yes/No)	36/5	N/A	N/A
Age group 4	12-20.99 years	12-20.99 years	12-20.99 years
Subjects	12 (7M, 5F)	23 (14M, 9F)	12 (5M, 7F)
Observations	17 (9M, 8F)	31 (18M, 13F)	15 (7M, 8F)
Average age (years)	16.2 ± 2.8	15.5 ± 2.0	15.7 ± 2.7
Mol. Class (Del/UPD/ID)	15/2/0	N/A	N/A
GH treatment (Yes/No)	14/3	N/A	N/A

Age is expressed as Mean ± SD; N/A = Not Applicable; M = Male; F = Female
Sib.C = Normal-weight Sibling Controls
PWS = Prader-Willi Syndrome
EMO = Early-onset Morbid Obesity
GH treatment = Growth Hormone treatment
Molecular class and GH treatment are descriptive characteristics for observations

Table 3-2. Results of biological assays and measurements

	PWS	Sib.C	EMO	P1	P2	P3
Age group (years)	0-1.99	0-1.99	0-1.99			
Ghrelin (pg/ml)	5521 ± 3696	2883 ± 1172	N/A	0.016*	N/A	N/A
Leptin (pg/ml)	272 ± 231	216 ± 145	N/A	0.48	N/A	N/A
Weight-for-length	25.07 ± 28.17	57.41 ± 37.50	N/A	0.025*	N/A	N/A
DEXA	21.36 +/-7.89	19.64 ± 6.33	N/A	0.57	N/A	N/A
Age group (years)	2-4.99	2-4.99	2-4.99			
Ghrelin	3113 ± 1898	2556 ± 927	3430 ± 2320	0.12	0.71	0.30
Leptin	1389 ± 1785	150 ± 99	2248 ± 1107	<0.001**	0.098	<0.001**
BMI z-score	0.93 ± 1.55	0.32 ± 1.19	4.29 ± 0.79	0.074	<0.001**	<0.001**
DEXA	24.98 ± 10.47	18.61 ± 6.39	44.04 ± 5.78	0.005**	<0.001**	<0.001**
Age group (years)	5-11.99	5-11.99	5-11.99			
Ghrelin (pg/ml)	2476 ± 1332	2111 ± 1013	1645 ± 983	0.21	0.021*	0.10
Leptin (pg/ml)	2107 ± 1572	397 ± 720	2408 ± 1569	<0.001**	0.56	<0.001**
BMI z-score	1.62 ± 1.17	0.35 ± 0.92	2.72 ± 0.22	<0.001**	<0.001**	<0.001**
DEXA	35.08 ± 12.75	20.39 ± 8.05	45.83 ± 4.97	<0.001**	<0.001**	<0.001**
Age group (years)	12-20.99	12-20.99	12-20.99			
Ghrelin (pg/ml)	2086 ± 885	1233 ± 509	1053 ± 847	0.011*	0.0085 **	0.49
Leptin (pg/ml)	2837 ± 1839	1138 ± 1485	5459 ± 2289	0.0094**	0.0060**	<0.001**
BMI z-score	2.10 ± 0.66	0.50 ± 1.08	2.73 ± 0.34	<0.001**	0.0051**	<0.001**
DEXA	47.95 ± 8.72	26.00 ± 10.79	54.28 ± 6.09	<0.001**	0.049*	<0.001**

All data expressed as Mean ± SD; * = $p<0.05$, ** = $p<0.01$
P1 = *p*-value for comparison of PWS vs Sib.C
P2 = *p*-value for comparison of PWS vs EMO
P3 = *p*-value for comparison of Sib.C vs EMO
Sib.C = Normal-weight Sibling Controls
PWS = Prader-Willi Syndrome
EMO = Early-onset Morbid Obesity

Table 3-3. Mean change in Dependent Variable (DV) per Unit Change in Independent Variable (IV): Age 0-1.99: Slope (SE)[N]{P-value}

DV	IV	PWS	Sib.-C
Ghrelin	Leptin	0.09(4.7)[14]{0.98}	7.2(1.6)[7]{0.0063}
Ghrelin	Weight for Length	17.6(34.5)[16]{0.62}	-6.6(12.9)[9]{0.62}
Ghrelin	Dexa	110.5(138.4)[15]{0.44}	66.6(59.2)[6]{0.32}
Leptin	Dexa	13.4(7.21)[14]{0.088}	5.39(8.28)[7]{0.54}
Leptin	Weight for Length	4.55(1.80)[15]{0.025}	-0.19(1.37)[9]{0.90}
Dexa	Weight for Length	0.17(0.058)[16]{0.0096}	0.086(0.053)[8]{0.16}

Table 3-4. Mean change in Dependent Variable (DV) per Unit Change in Independent Variable (IV): Age 2+: Slope (SE)[N] {P-value}

DV	IV	PWS	Sib.C	EMO
Ghrelin	Leptin	-0.032(0.094)[83]{0.74}	-0.23(0.13)[87]{0.089}	0.034(0.087)[40]{0.71}
Ghrelin	BMI-Z	-142.1(123.8)[118]{0.26}	-36.9(92.4)[135]{0.69}	227.1(251.7)[52]{0.38}
Ghrelin	DEXA	-13.2(15.4)[106]{0.40}	-9.2(11.6)[122]{0.42}	-51.5(30.2)[44]{0.12}
Leptin	DEXA	74.2(16.0)[86]{<0.001}	61.0(8.8)[85]{<0.001}	263(38.1)[39]{<0.001}
Leptin	BMI-Z	664(123)[100]{<0.001}	352(85.8)[94]{0.0013}	692(418)[46]{0.13}
DEXA	BMI-Z	5.69(0.54)[120]{<0.001}	5.17(0.61)[127]{<0.001}	3.70(1.17)[50]{0.0082}

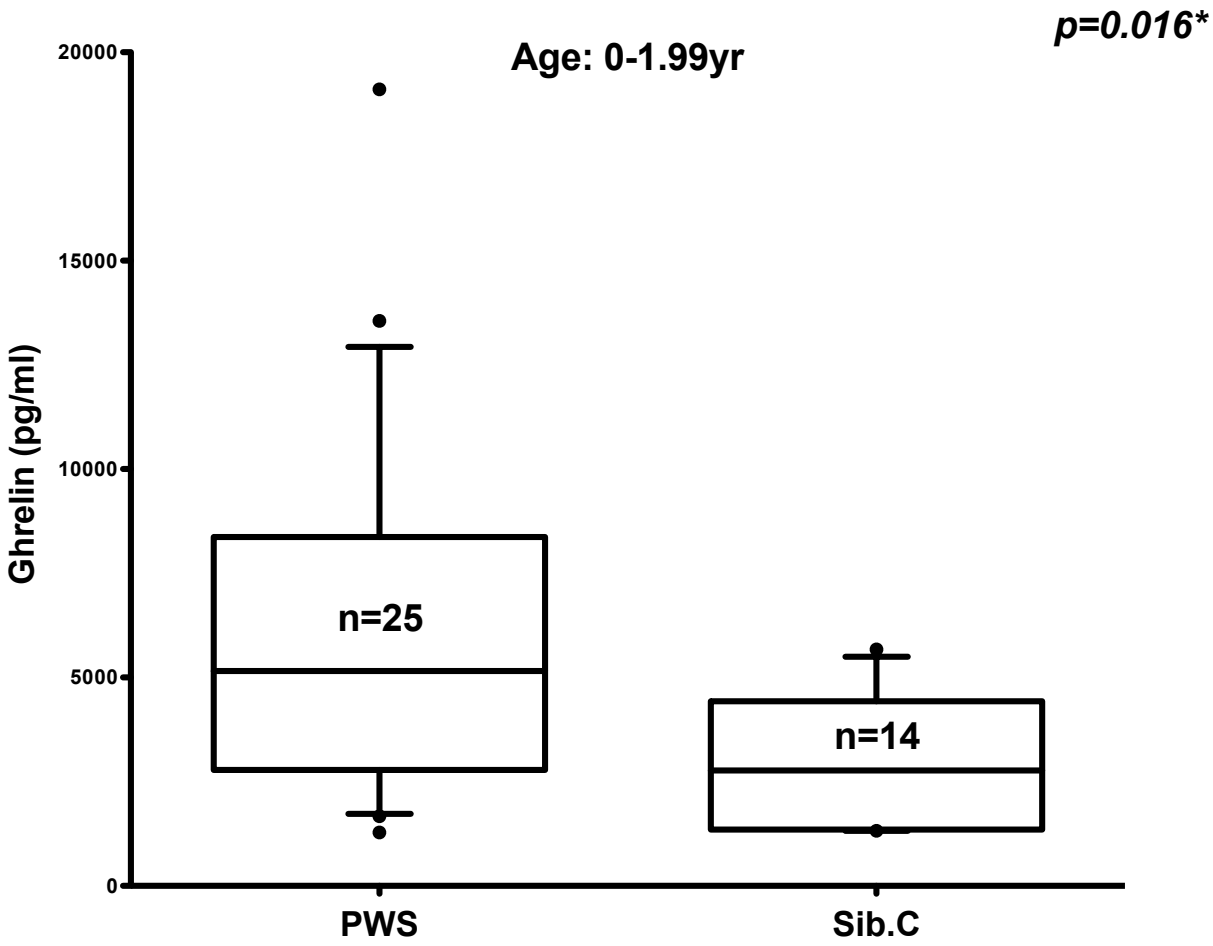


Figure 3-1. Box plot of ghrelin levels in infants and young PWS and normal control (Sib.C) children 0-1.99 years old. The tails of the box plot represent the 10th and 90th percentiles; the bars represent the 25th, 50th and 75th percentiles. The dots represent outliers.

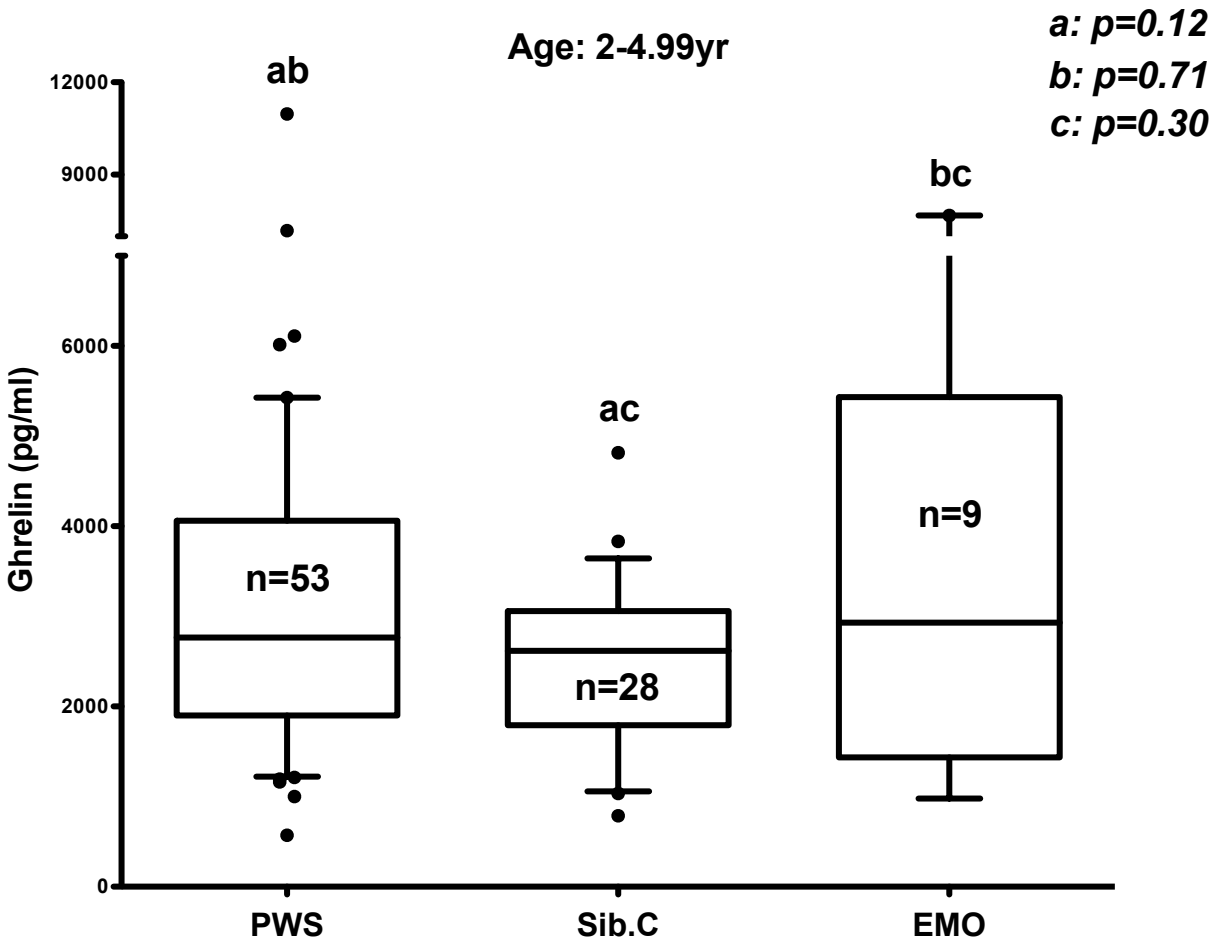


Figure 3-2. Box plot of ghrelin levels in PWS, Sib.C and EMO children 2-4.99 years old. The tails of the box plot represent the 10th and 90th percentiles; the bars represent the 25th, 50th and 75th percentiles. The dots represent outliers.

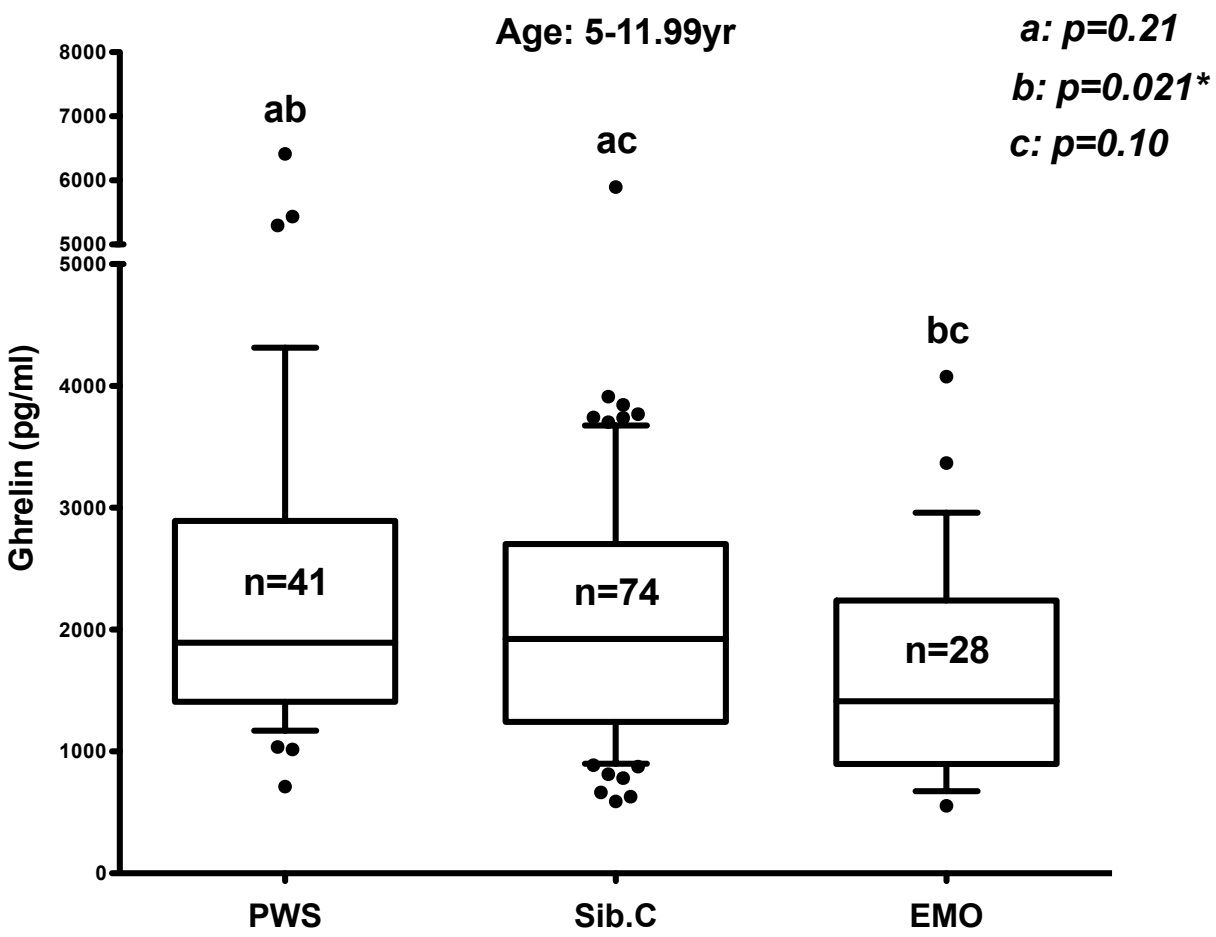


Figure 3-3. Box plot of ghrelin levels in PWS, Sib.C and EMO children 5-11.99 years old. The tails of the box plot represent the 10th and 90th percentiles; the horizontal bars are the 25th, 50th and 75th percentiles. The dots represent outliers.

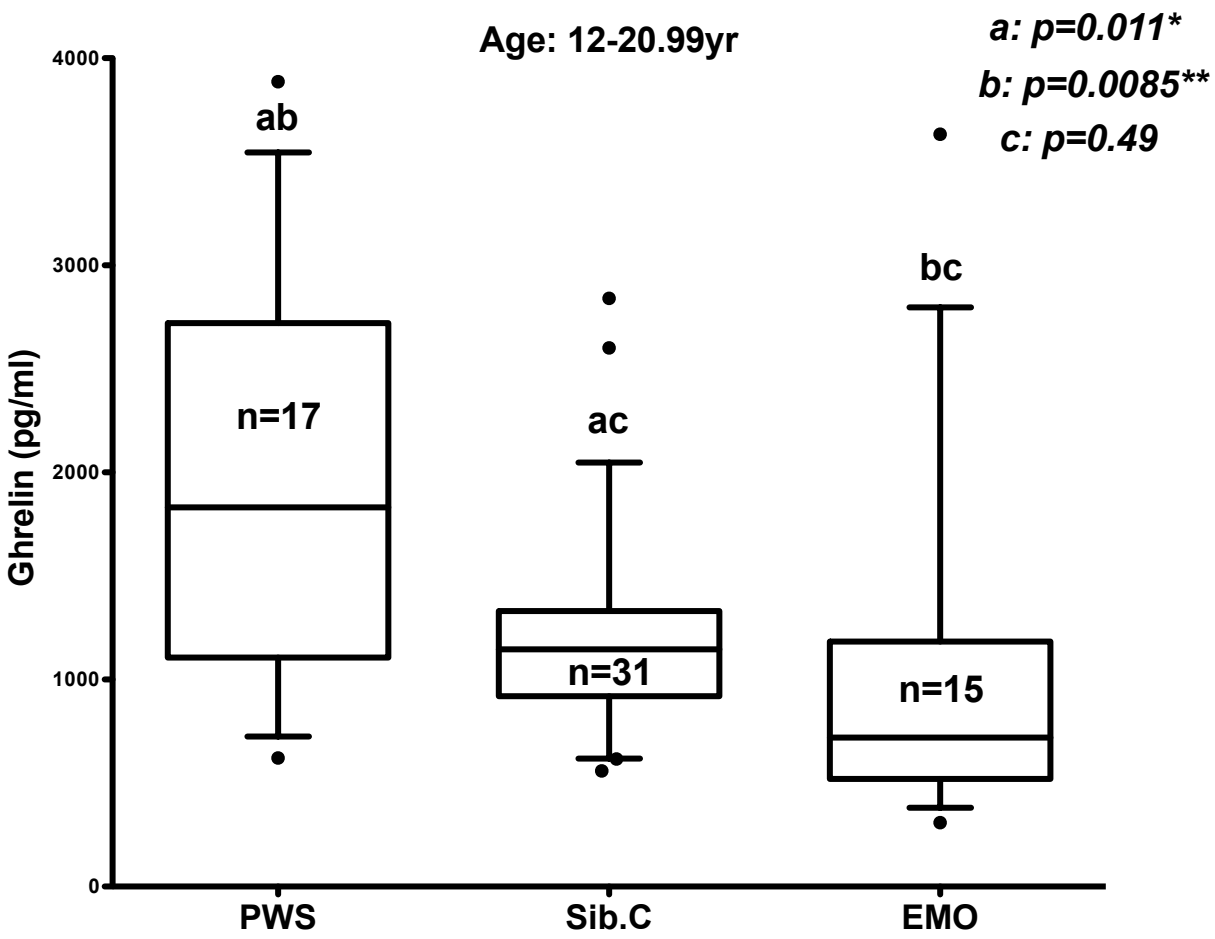


Figure 3-4. Ghrelin is elevated in teenagers and adults with PWS. Box plot of ghrelin levels in PWS, Sib.C and EMO teenagers and young adults 12-20.99 years old. The tails of the box plot represent the 10th and 90th percentiles; the bars are the 25th, 50th and 75th percentiles. The dots represent outliers.

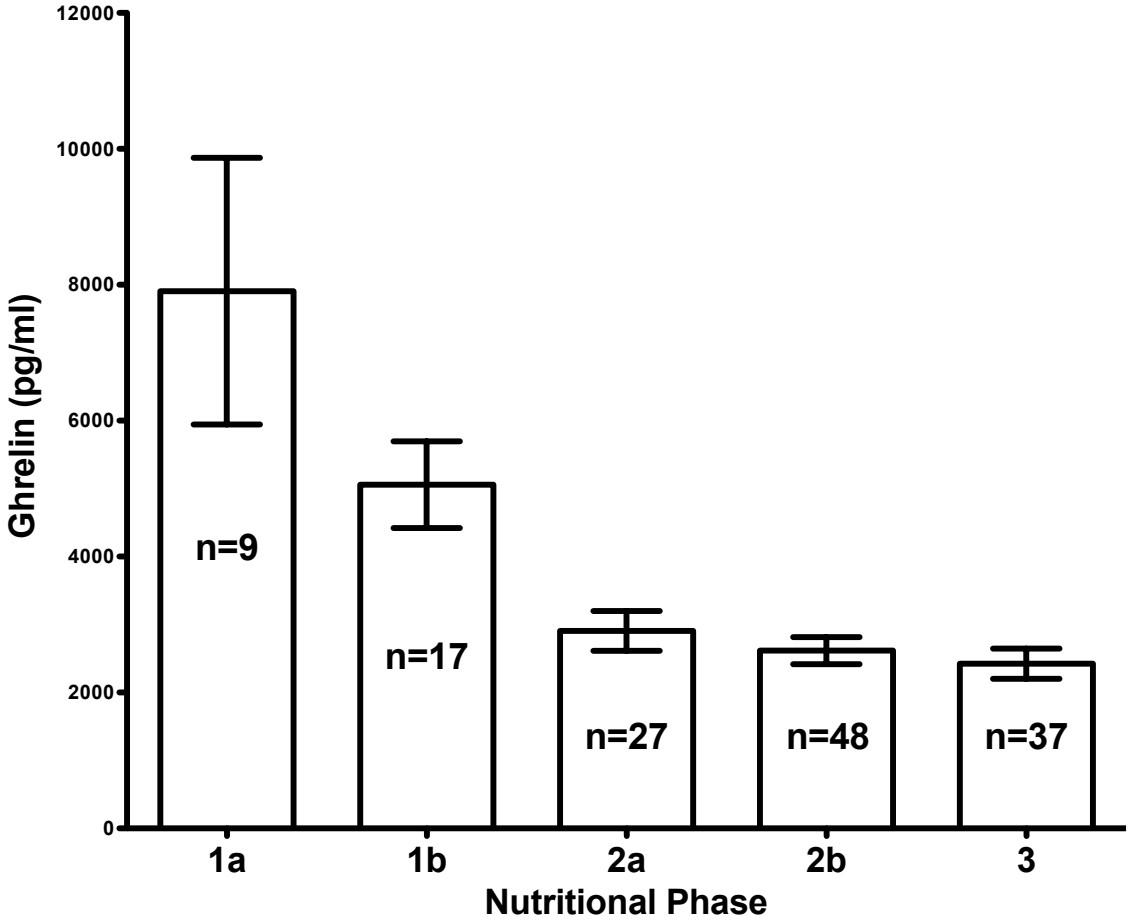


Figure 3-5. Bar chart of average ghrelin levels in PWS nutritional phases. Nutritional phase is more prognostic of ghrelin levels in PWS.

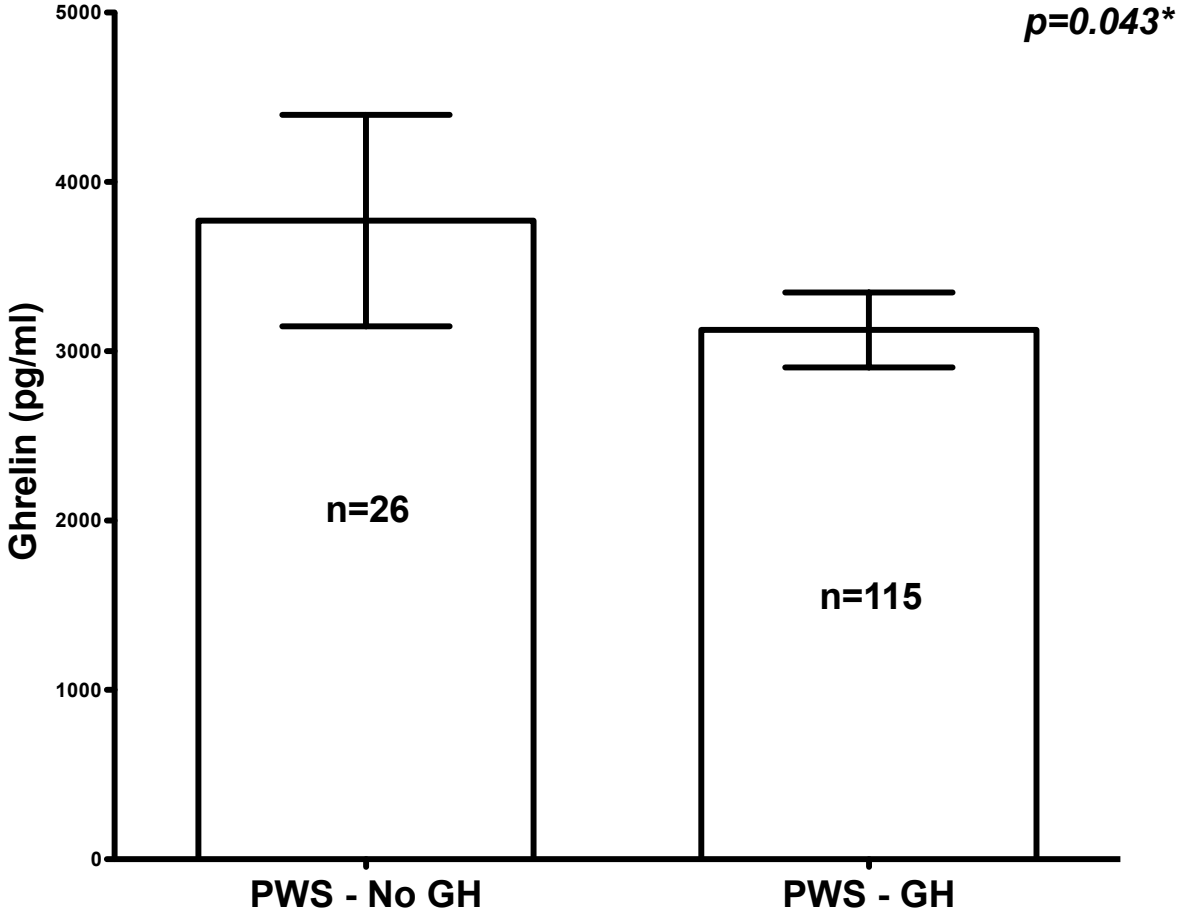


Figure 3-6. Growth hormone therapy decreases ghrelin levels in PWS. Bar chart of mean ghrelin levels of PWS individuals not on growth hormone therapy (PWS – No GH) versus PWS individuals on growth hormone therapy (PWS-GH)

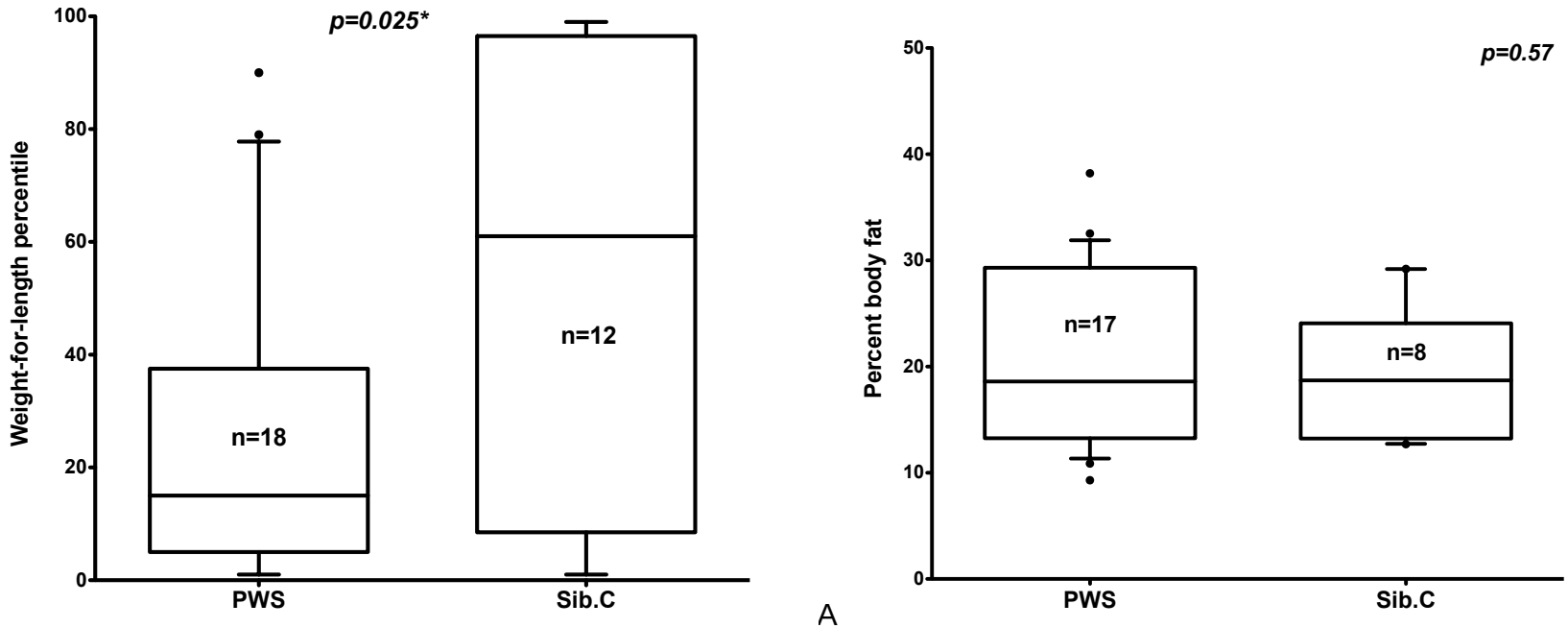


Figure 3-7. PWS children have low weight-for-length and normal body fat; A) Box plot of weight-for-length percentile in PWS children relative to normal control children (0-1.99y); B) Box plot of percent body fat in PWS children relative to normal controls (0-1.99y). The tails of the box plots represent the 10th and 90th percentiles; the bars are the 25th, 50th and 75th percentiles. The dots represent outliers.

CHAPTER 4
ANALYSIS OF LEPTIN SIGNALING IN INDIVIDUALS WITH PRADER-WILLI SYNDROME THROUGH ANALYSIS OF CIRCULATING ALPHA-MELANOCYTE STIMULATING HORMONE LEVELS

Hypothalamic Leptin Signaling Is Unknown in PWS

The hypothalamic leptin-melanocortin signaling pathway consists of leptin and the melanocortin peptides POMC, alpha-MSH and MC4R. Leptin signals through its receptor in the brain to up-regulate POMC mRNA expression and translation in the hypothalamus. The POMC peptide is processed to release alpha-MSH, which activates the melanocortin-4 receptor (MC4R) leading to decreased appetite and increased energy expenditure.

Little is known about leptin levels in the hypothalamus of individuals with PWS. Obese adults with PWS have elevated peripheral leptin levels that are appropriate for their degree of obesity and fall well within the range of the normal obese population (Butler et al., 1998). However, while direct delivery of leptin into the brain has been shown to reduce feeding, peripheral injection of leptin fails to reduce feeding and does not significantly alter leptin levels in the cerebrospinal fluid (Schwartz et al., 1996; Van Heek et al., 1997; Ramsey et al., 1998). Thus it remains possible that PWS subjects may suffer from inadequate hypothalamic leptin signaling despite high peripheral leptin levels.

The aim of this study was to analyze hypothalamic leptin signaling in individuals with Prader-Willi syndrome by measuring circulating alpha-MSH levels. Because the brain is the major source of circulating alpha-MSH in the body, we reasoned that peripheral changes in alpha-MSH level would reflect changes in anorexigenic leptin signaling in the brain. We hypothesized that if hypothalamic leptin signaling was

appropriate in PWS, then obese and hyperphagic children with PWS will have circulating alpha-MSH levels significantly higher than normal lean control children.

To test this hypothesis we measured plasma leptin levels and serum alpha-MSH levels in normal lean control children (Sib.C), in non-PWS children with early-onset morbid obesity (EMO), in PWS children before the onset of obesity and hyperphagia (PWS-BOH), and in PWS children after the onset of obesity and hyperphagia (PWS-AOH) [Table 4-1]. Multiple serum and plasma samples were obtained more than once from subjects with repeated visits to our clinic, thus accounting for the discrepancy between subjects and samples (observations) in Table 4-1.

Leptin Is Appropriately Elevated in Obese Hyperphagic PWS Children

We used the LUMINEX assay system (Millipore Inc., CA, USA) to measure plasma leptin levels in 21 non-obese children with PWS (PWS-BOH), 17 obese PWS children (PWS-AOH), 17 normal lean control children (Sib.C), and 11 children with early-onset morbid (EMO) [Table 4-1].

Peripheral leptin levels in non-obese non-hyperphagic PWS children (PWS-BOH) did not differ significantly from normal lean control children (194.9 ± 194.9 pg/ml vs 222.5 ± 163.0 pg/ml; $p=0.64$) but was significantly lower relative to leptin levels in obese hyperphagic PWS children (194.9 ± 194.9 pg/ml vs 2464 ± 1475 pg/ml; $p<0.001$**) and EMO children (194.9 ± 194.9 pg/ml vs 2817 ± 1737 pg/ml; $p<0.001$**) [Figure 4-1].

Obese hyperphagic PWS children had significantly elevated plasma leptin relative to normal lean control children (2464 ± 1475 pg/ml vs 194.9 ± 194.9 pg/ml; $p<0.001$**) however their leptin did not differ significantly from leptin levels in EMO children (2464 ± 1475 pg/ml vs 2817 ± 1737 pg/ml; $p=0.55$).

Leptin levels were significantly elevated in EMO children relative to normal lean control children (2817 ± 1737 pg/ml vs 194.9 ± 194.9 pg/ml; *p<0.001***).

Alpha-MSH Is Not Elevated in Obese Hyperphagic PWS Children

I used a competitive ELISA assay (Phoenix Pharmaceuticals Inc., USA) to measure serum alpha-MSH levels in the same group of 21 non-obese PWS children, 17 obese PWS children, 17 normal-weight control children, and 11 children with early-onset morbid described above.

Obese PWS children had peripheral alpha-MSH levels similar to normal lean control children (209.6 ± 85.09 pg/ml vs 216.1 ± 75.34 pg/ml; *p=0.82*) but significantly lower than non-obese PWS children (209.6 ± 85.09 pg/ml vs 282.7 ± 111.9 pg/ml; *p=0.033**) and EMO children (209.6 ± 85.09 pg/ml vs 5883 ± 1137 pg/ml; *p=0.048**) [Figure 4-2].

Non-obese non-hyperphagic PWS children had significantly higher peripheral alpha-MSH levels than normal lean control children (282.7 ± 111.9 pg/ml vs 216.1 ± 75.34 pg/ml; *p=0.043**). However their serum alpha-MSH was significantly lower relative to EMO subjects (282.7 ± 111.9 pg/ml vs 5883 ± 1137 pg/ml; *p=0.029**) [Figure 4-2].

EMO children had significantly elevated alpha-MSH levels than normal lean control children (5883 ± 1137 pg/ml vs 216.1 ± 75.34 pg/ml; *p=0.048**) [Figure4-2].

Table 4-1. Leptin and alpha-MSH levels in PWS, Sib.C & EMO subjects

Group	Subjects	Observations	Age (years)	BMI-Z	Leptin (pg/ml)	α-MSH (pg/ml)
PWS-BOH	5M, 4F	13M, 8F	2.0 ± 1.1	-0.58 ± 0.99	194.9 ± 194.9	282.7 ± 111.9
PWS-AOH	5M, 5F	8M, 9F	7.1 ± 2.9	2.4 ± 0.47	2464 ± 1475	209.6 ± 85.09
Sib.C	3M, 4F	6M, 11F	5.5 ± 3.9	0.44 ± 0.57	222.5 ± 163.0	216.1 ± 75.34
EMO	8M, 3F	10M, 4F	6.6 ± 3.0	3.4 ± 0.99	2817 ± 1737	5883 ± 1137

Data are expressed as Mean ± SD.

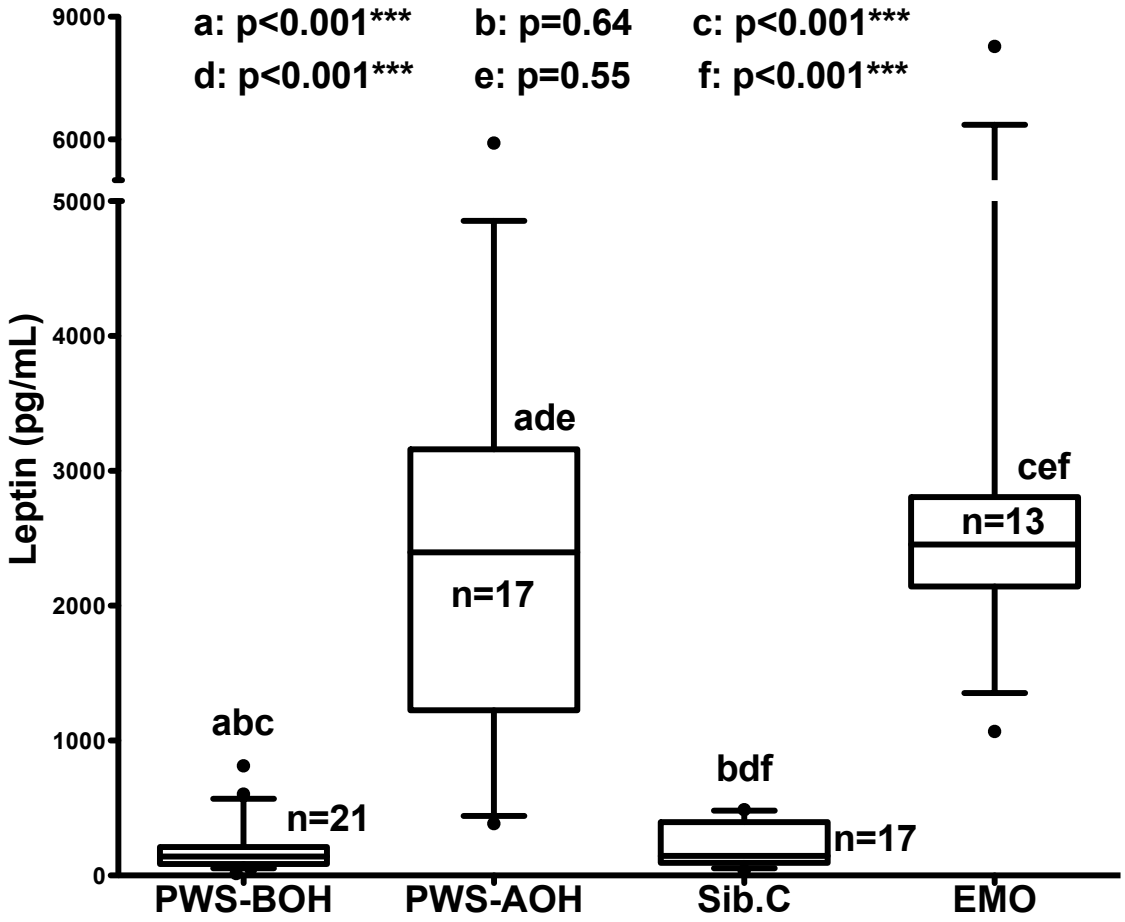


Figure 4-1. Box plot of peripheral leptin levels in obese-hyperphagic PWS children (PWS-AOH), non-obese non-hyperphagic PWS children (PWS-BOH), normal control children (Sib.C), and children with early-onset morbid obesity (EMO). The tails of the box plot represent the 10th and 90th percentiles; the bars are the 25th, 50th and 75th percentiles. The dots represent outliers.

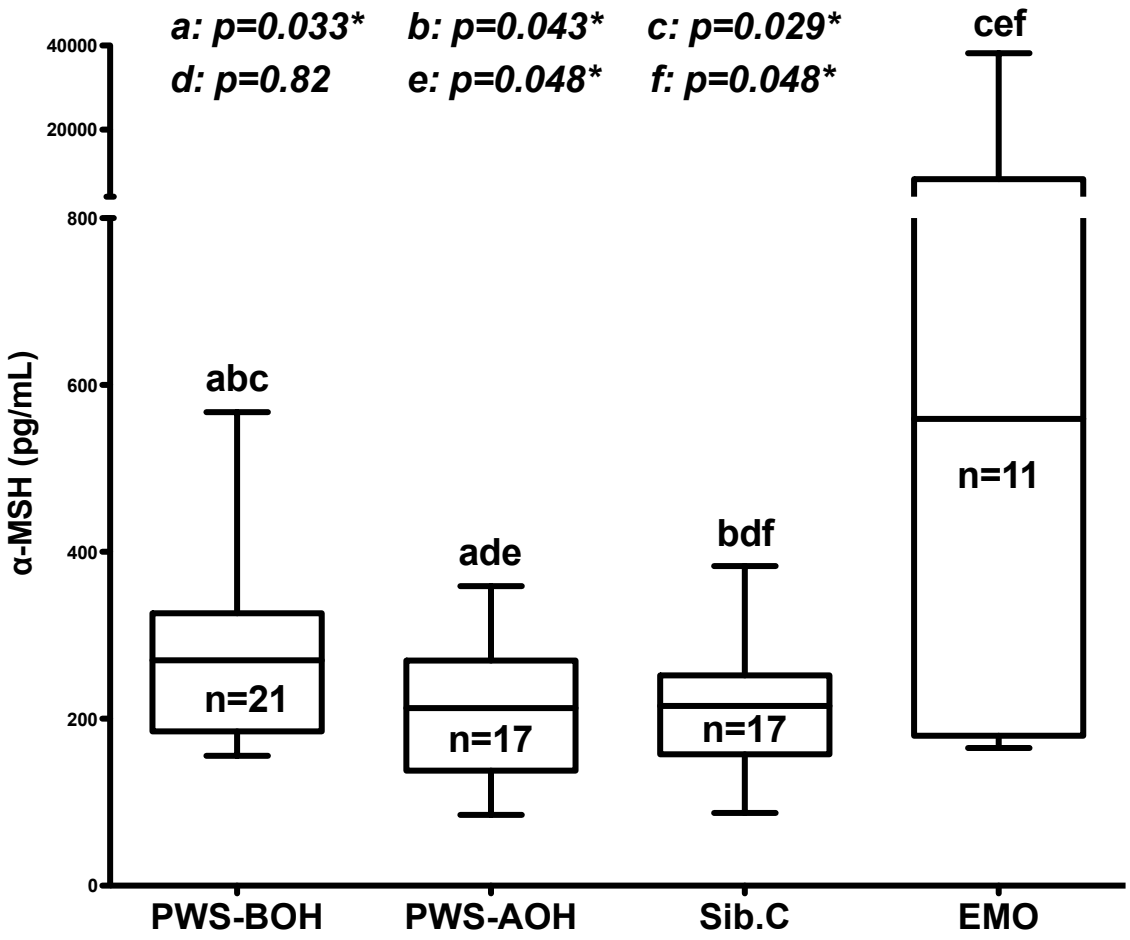


Figure 4-2. Box plot of peripheral alpha-MSH levels in obese PWS (PWS-AOH) and non-obese PWS (PWS-BOH) children, normal-weight control children (Sib.C) and children with early-onset morbid obesity (EMO). The tails of the box plot represent the 10th and 90th percentiles; the bars are the 25th, 50th and 75th percentiles.

CHAPTER 5
ANALYSIS OF CIRCULATING BRAIN-DERIVED NEUROTROPHIC FACTOR LEVELS IN INDIVIDUALS WITH PRADER-WILLI SYNDROME

Brain-Derived Neurotrophic Factor and Prader-Willi Syndrome

Brain-derived neurotrophic factor (BDNF) is a neuropeptide that is highly expressed in the brain and plays an important role in energy homeostasis. BDNF functions downstream of the hypothalamic leptin-melanocortin signaling pathway and modulates food intake and energy expenditure.

BDNF haploinsufficiency is associated with low peripheral BNDF levels, severe hyperphagia, obesity, and developmental delay (Han JC et al., 2008; Gray J et al., 2006). Mutations in the BDNF receptor *TrkB* result in morbid obesity and hyperphagia.

Since BDNF is produced primarily in the brain, peripheral BDNF concentrations are thought to reflect cerebral BDNF output. A recent pilot study reported low peripheral BDNF levels in 13 obese PWS patients relative to obese and lean control subjects, suggesting insufficient central BDNF production in individuals with PWS (Han et al., 2010). In this study, we investigate serum BDNF levels in a larger cohort of obese PWS subjects {28 subjects, 47 observations; age, 10.5 ± 7.5yr; BMI-Z, 2.6 ± 0.5}, in lean control subjects (Sib.C) {66 subjects, 81 obeservations; age, 9.1 ± 6.1yr; BMI-Z, -0.06 ± 0.7} and in non-PWS individuals with early-onset morbid obesity (EMO) {36 subjects, 48 observations; age, 10.8 ± 5.8yr; BMI-Z, 3.0 ± 0.7} [Table 5-1; Figure 5-1].

Serum BDNF Is Elevated in PWS Subjects

PWS subjects as a group had significantly higher mean serum BDNF levels relative to lean control subjects (9846 ± 7894 pg/ml vs 5623 ± 7808 pg/ml; *p=0.0011***). However, their serum BDNF concentration was not significantly different from levels in non-PWS individuals with early-onset morbid obesity (9846 ± 7894 pg/ml vs 7808 ±

8135 pg/ml; *p=0.22*). EMO subjects did not have significantly higher serum BDNF levels relative to lean controls (7808 ± 8135 pg/ml vs 5623 ± 7808 pg/ml, *p=0.089*) [Figure 5-2].

There was no significant correlation between BDNF and age in PWS subjects (*r = -0.05248; p=0.7261*), in lean controls (*r = 0.1842; p=0.082*) or in EMO subjects (*r = 0.04125; p=0.78*). There was also no significant correlation with BMI z-scores in PWS, Sib.C or EMO subjects (*p=0.75, 0.19 & 0.68* respectively). However, serum BDNF levels correlated significantly with nutritional phase in obese PWS subjects (*r = -0.3243; p=0.026**).

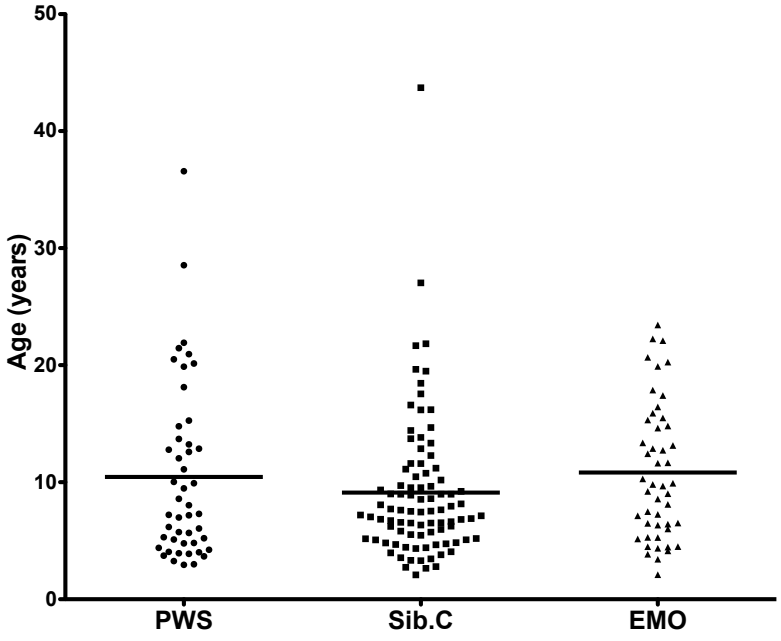
BDNF Levels Decrease with Onset of Hyperphagic Nutritional Phases in PWS

To assess the effect of PWS nutritional phases on circulating BDNF levels in PWS subjects, I analyzed serum BDNF levels of obese PWS subjects in nutritional phase 2a, 2b and 3. Serum BDNF levels decreased from 12643 ± 3280 pg/ml in nutritional phase 2a, to 11018 ± 2012 pg/ml in nutritional phase 2b and then to 7823 ± 1550 pg/ml in nutritional phase 3. Serum BDNF of PWS individuals in nutritional phase 2a was significantly elevated relative to lean controls (12643 ± 3280 pg/ml vs 5623 ± 691 pg/ml; *p=0.0091***). PWS individuals in nutritional phase 2b also had significantly elevated serum BDNF levels relative to lean controls (1108 ± 5012 pg/ml vs 5623 ± 691 pg/ml; *p=0.0050***). However serum BDNF level of PWS subjects in nutritional phase 3 was not significantly higher than BDNF levels in lean controls (7823 ± 1550 pg/ml vs 5623 ± 691 pg/ml; *p=0.17*) [Figure 5-3].

Table 5-1. Subjects, observations and biological values for BDNF analysis

Group	Subjects	Observations	Age (yr)	BMI-Z	BDNF	P1	P2	P3
PWS	16M, 12F	25M, 22F	10.5 ± 7.5	2.6 ± 0.5	9846 ± 7894	0.0011**	0.22	0.089
Sib.C	30M, 36F	40M, 36F	9.1 ± 6.1	-0.06 ± 0.7	5623 ± 6561			
EMO	17M, 19F	25M, 23F	10.8 ± 5.8	3.0 ± 0.7	7808 ± 8135			

Data are expressed as Mean ± SD. Groups were compared using student's t-test.
(*P1= PWS vs Sib.C; P2 = PWS vs EMO; P3 = Sib.C vs EMO*)



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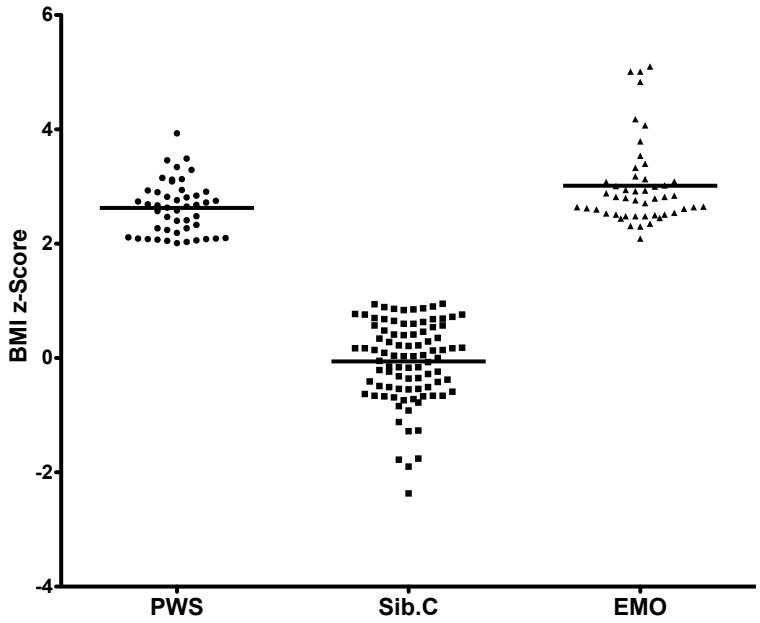

B

Figure 5-1. Graphical representation of subject ages and BMI z-scores; A) Scatter plot of ages with Mean; B) Scatter plot of BMI z-scores with Mean

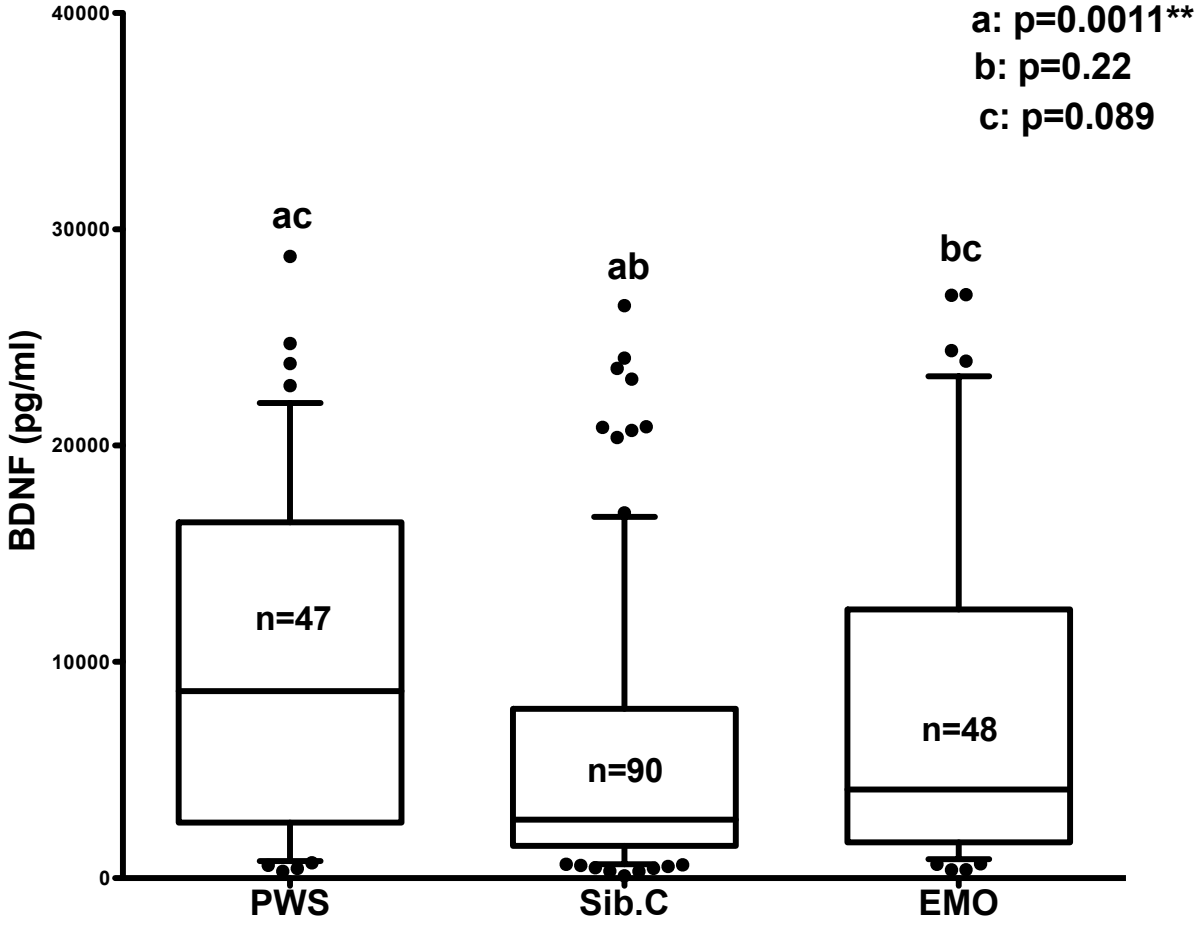

Figure 5-2. Box plot of serum BDNF levels in PWS, Sib.C and EMO subjects. The tails of the box plot represent the 10th and 90th percentiles; the bars are the 25th, 50th and 75th percentiles. The dots represent outliers.

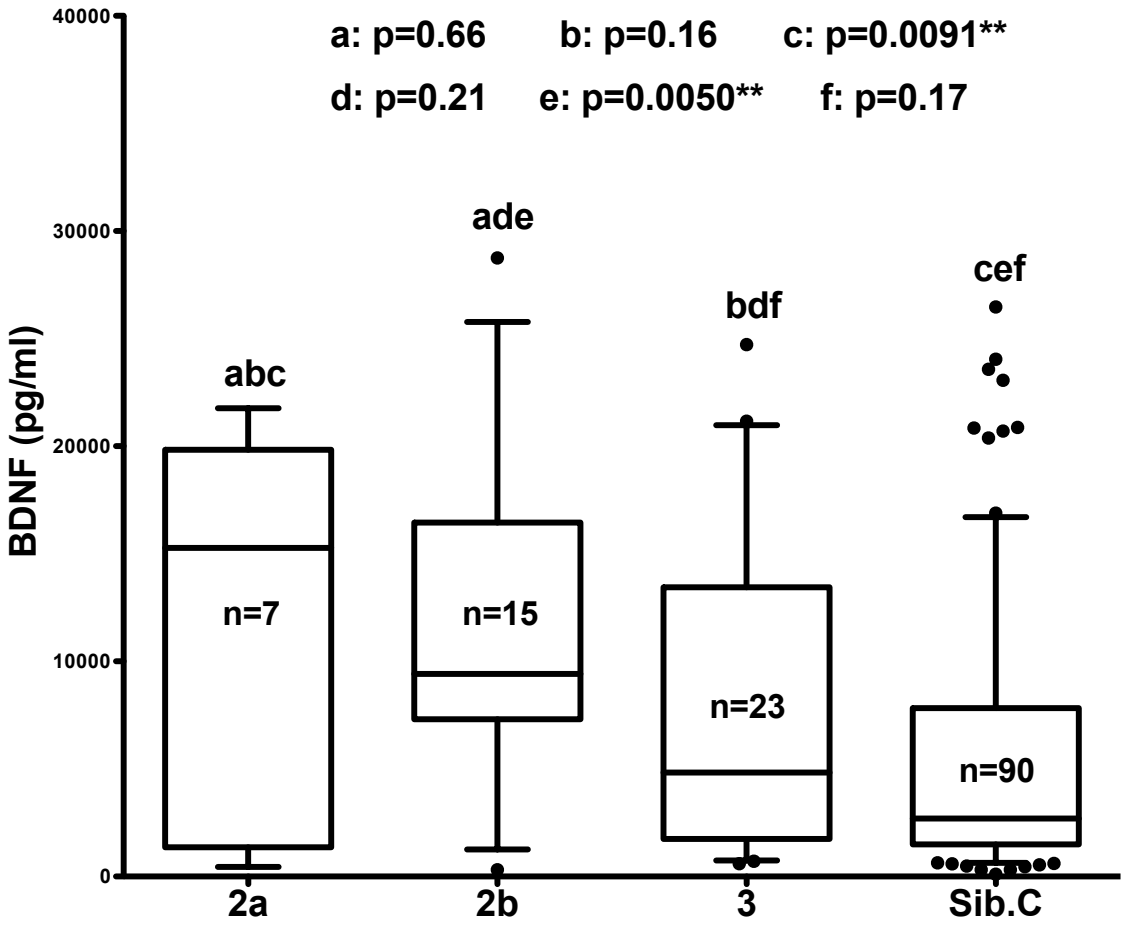

Figure 5-3. BDNF levels decrease with onset of hyperphagia in PWS. Box plot of serum BDNF levels in PWS nutritional phases 2a, 2b, and 3 and also in normal sibling control subjects of similar ages. The tails of the box plot represent the 10th and 90th percentiles; the bars are the 25th, 50th and 75th percentiles. The dots represent outliers.

CHAPTER 6
DISCUSION

Hyperghrelinemia Begins Early in Prader-Willi Syndrome

I measured serum ghrelin levels in a younger and larger group of PWS subjects and normal control subjects (2 months to 20.99 years) than has been previously analyzed by others [Table 3-1]. PWS infants and young children between 2 months – 2 years had significantly elevated ghrelin levels than their normal counterparts of similar age while the ghrelin of PWS children between 2 – 12 years was not significantly elevated than their normal counterparts within the same age group. However, we found that PWS nutritional phase was more prognostic of ghrelin levels in PWS subjects than age or BMI-Z. The onset of the hyperphagic nutritional phases in PWS patients correlated significantly with lower ghrelin levels: PWS children in nutritional phase 1a and 1b had significantly elevated ghrelin levels relative to normal control children of same age while PWS individuals in nutritional phase 3 did not have significantly elevated ghrelin levels relative to normal children of same age. Given that the age of onset of the nutritional phases varies among individuals with PWS, analysis of ghrelin levels in PWS by age alone may be misleading. Thus the inconsistencies in the literature on ghrelin levels in young PWS children may be due to a greater number of PWS subjects in the more advanced nutritional phases in the study group.

Given that ghrelin levels were the highest in PWS children with poor appetite (Phase 1a), it seems unlikely that elevated ghrelin levels are responsible for the switch to the hyperphagic phases of PWS. However, it has been demonstrated in mice that ghrelin can act to increase fat mass independent of its effect on appetite (Perez-Tilve et al., 2011). It is therefore likely that the elevated ghrelin levels are causing the increased

fat mass seen in infants with PWS compared to normal infants with similar body mass indices (BMI). This would explain why PWS infants have normal body fat even though they surfer from failure to thrive and have significantly lower weight-for-length percentile relative to normal infants.

We also observed that PWS individuals on growth hormone therapy had significantly lower ghrelin levels than PWS individuals not on growth hormone therapy. Thus part of the effect of growth hormone in promoting lean body mass in young PWS subjects may result from its ability to significantly decrease ghrelin levels early on.

PWS Individuals May Suffer From Leptin Resistance

Hypothalamic leptin signals through its receptor to induce *POMC* mRNA expression in the brain, consequently leading to increased alpha-MSH levels both centrally and peripherally. My data demonstrates that non-obese PWS subjects have significantly elevated serum alpha-MSH levels relative to normal lean controls even though their plasma leptin levels are not elevated. However, obese PWS subjects have significantly lower alpha-MSH levels than non-obese PWS subjects even though their plasma lepin is significantly elevated. Thus it appears serum alpha-MSH levels decrease with onset of obesity and hyperphagia in PWS.

Leptin levels in the cerebrospinal fluid (CSF) correlate strongly with peripheral leptin levels in a nonlinear manner and also with body mass index (Schwartz et al., 1996). It is thought that plasma leptin enters human CSF in proportion to body adiposity, however the efficiency of this process is significantly lower in obese individuals with high peripheral leptin levels. Schwartz et al. hypothesized that a saturable mechanism mediates CSF leptin transport and that leptin resistance may

occur in obese individuals with high peripheral leptin levels due to reduced efficiency of brain leptin delivery.

It is possible that the high peripheral leptin levels of PWS subjects have triggered a saturable mechanism, resulting in leptin resistance through inefficient leptin transport into the brain. Decreased BDNF levels in obese PWS subjects appear to support this hypothesis. BDNF functions downstream of the leptin-melanocortin signaling pathway (Xu et al., 2003); leptin's activation of MC4R through alpha-MSH induces BDNF expression through the cyclic AMP protein kinase A pathway (Caruso et al., 2012). Just like with alpha-MSH, peripheral BDNF levels decrease with onset of obesity and hyperphagia in PWS.

We hypothesize that elevated ghrelin levels early on in PWS infants leads to increased adiposity, which leads to elevated leptin levels in older children with PWS that trigger leptin resistance, leading to inadequate hypothalamic leptin-melanocortin signaling and low BDNF levels, resulting in development of obesity and hyperphagia in PWS children and adults [Figure 6-1].

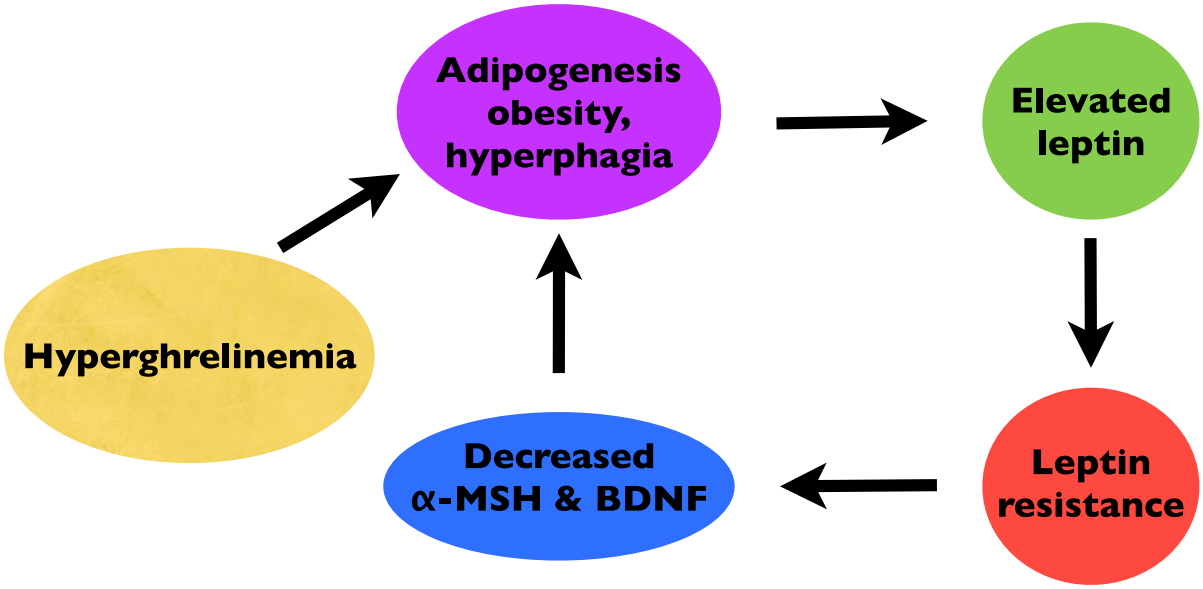

Figure 6-1. Working model for development of obesity and hyperphagia in PWS. Elevated ghrelin levels in PWS infants activate lipogenic genes early on, leading to increased adiposity and early-onset obesity. Consequently, peripheral leptin levels are elevated significantly, triggering a saturable mechanism that decreases the efficiency of leptin uptake by the brain (leptin resistance). Low brain leptin levels results in decreasd alpha-MSH and BDNF levels, which further promote obesity and trigger hyperphagia in PWS subjects.

CHAPTER 7
FUTURE DIRECTIONS

Analysis of POMC and PRCP Expression in PWS

We will further analyze alpha-MSH levels in the different nutritional phases in Prader-Willi syndrome with larger sample sizes. To determine whether changes in alpha-MSH levels in PWS stem from transcriptional regulation or regulation of the mature protein, we will evaluate *POMC* mRNA expression in each nutritional phase.

The enzyme prolylcarboxypeptidase (PRCP) degrades and regulates alpha-MSH protein levels and function and has been recently implicated in obesity. We will analyze expression of *PRCP* mRNA and protein levels in PWS individuals at different nutritional phases.

Analysis of Ghrelin-Responsive Pathways in Young Children with PWS

The inability of infants and young children with PWS to respond to high ghrelin levels may be the result of poorly developed ghrelin-responsive pathways early on. To this end, we intend to measure ghrelin-responsive proteins such as oxytocin, neuropeptide-y and agouti-related protein in infants and young children with PWS. We will also analyze frozen adult PWS brain samples for expression of ghrelin-responsive neurons in the hypothalamus by immunohistochemistry.

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BIOGRAPHICAL SKETCH

Frederick A. Kweh was born in Cameroon, West Africa. He moved to the United States of America in the fall of 1999 to live with his uncle Andrew Kweh in Florida. From the fall of 2000 to the spring of 2002 he attended Santa Fe Community college in Gainesville, during which time he was a student ambassador for the college. He graduated with an Associate of Arts degree in the spring of 2002 and was admitted at the University of Florida as a junior for the 2002 fall semester. He graduated with a Bachelor of Science degree in microbiology in May 2004 after which he worked as a biological technician in the laboratory of Dr. Margaret Wallace.

In the fall of 2006 he began his PhD work in the Interdisciplinary Program in Biomedical Science at the University Of Florida College Of Medicine. His graduate research in the Medical Sciences was done under the mentorship of Dr. Daniel J. Driscoll.