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Hyperghrelinemia in Prader-Willi Syndrome Begins in Early Infancy Long Before the Onset of Hyperphagia

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Abstract

Circulating total ghrelin levels are elevated in older children and adults with Prader-Willi syndrome (PWS). However, the presence or absence of hyperghrelinemia in young children with PWS remains controversial. We hypothesized that a more robust way to analyze appetite-regulating hormones in PWS would be by nutritional phases rather than age alone. Our objectives were to compare total serum ghrelin levels in children with PWS by nutritional phase as well as to compare total ghrelin levels in PWS (5 weeks to 21 years of age) to normal weight controls and individuals with early-onset morbid obesity (EMO) without PWS.

Fasting serum total ghrelin levels were measured in 60 subjects with PWS, 39 subjects with EMO of unknown etiology, and in 95 normal non-obese sibling controls of PWS or EMO subjects (SibC) in this 12 year longitudinal study. Within PWS, total ghrelin levels were significantly ($P < 0.001$) higher in earlier nutritional phases: phase 1a ($7,906 \pm 5,887$); 1b ($5,057 \pm 2,624$); 2a ($2,905 \pm 1,521$); 2b ($2,615 \pm 1,370$) and 3 ($2,423 \pm 1,350$). Young infants with PWS also had significantly ($P = 0.009$) higher total ghrelin levels than did the sibling controls.

Nutritional phase is an important independent prognostic factor of total ghrelin levels in individuals with PWS. Circulating ghrelin levels are elevated in young children with PWS long before the onset of hyperphagia, especially during the early phase of poor appetite and feeding. Therefore, it seems unlikely that high ghrelin levels are directly responsible for the switch to the hyperphagic nutritional phases in PWS.

Keywords

Hyperghrelinemia; hyperphagia; nutritional phase; Prader-Willi; ghrelin; obesity

INTRODUCTION

Prader-Willi syndrome (PWS) is a rare neurogenetic disorder caused by a lack of expression of paternally inherited imprinted genes in chromosomal region 15q11.2 – q13 by three main mechanisms: paternal deletion of the region (65–75% of individuals), maternal uniparental disomy 15 (20–30%), and imprinting defect (1–3%) [Cassidy et al., 2012]. PWS is the most frequently diagnosed known genetic cause of early childhood obesity, occurring in approximately 1 in 10,000 to 25,000 live births [Butler et al., 2002; Cassidy et al., 2012]. Obesity in PWS is further compounded by the development of hyperphagia and lack of satiety later in childhood [Butler et al., 2010; Miller et al., 2011].

Ghrelin, an orexigenic hormone secreted primarily by the stomach and to a lesser extent the pancreas and hypothalamus [Dezaki et al., 2004; Kojima et al., 1999; Kojima and Kangawa 2010; Scerif et al., 2011], is significantly elevated in older children and adults with PWS [Cummings et al., 2002; Goldstone et al., 2005; Scerif et al., 2011; Tauber et al., 2004]. Ghrelin is pleiotropic in action and is known to have other important biological, physiological and metabolic functions besides food intake and growth hormone release [Korbonits et al., 2004]. It plays important roles in neurogenesis, memory, learning, behavior, sleep, pituitary hormone (especially growth hormone) secretion, and glucose and lipid metabolism [Claudia Theander-Carrillo 2006; Kojima and Kangawa 2010; van der Lely et al., 2004]. It is also important in early embryonic development and perinatal growth [Steculorum and Bouret 2011] and is thought to be involved in the process of ‘catch-up’ growth in small for gestational age infants [Fidanci et al., 2010; Iñiguez et al., 2002].

The biological consequences of hyperghrelinemia in PWS are unclear and its onset is controversial, with conflicting reports in the literature regarding ghrelin levels in young children with PWS before the onset of obesity and hyperphagia. Several investigators have found that ghrelin levels were not elevated as a group in young children with PWS [Butler et al., 2004; Erdie-Lalena et al., 2006; Haqq et al., 2008a]. However one study reported elevated ghrelin levels in young children with PWS as early as in infancy, long before the onset of hyperphagia [Feigerlová et al., 2008], while another reported hyperghrelinemia only in a subset of young children with PWS [Haqq et al., 2008a]. We hypothesized that the reliance on age alone in determining the onset of hyperghrelinemia in individuals with PWS may be a flawed approach and that a more robust approach would be to utilize the nutritional phase information and correlate this with ghrelin levels and other appetite regulating hormones in PWS.

We have previously reported on the complexity of the various nutritional phases and their varied age ranges in PWS [Miller et al., 2011]. In this study we examined total ghrelin levels in a large cohort of infants, young children, and adolescents with PWS categorized by nutritional phase as part of our 12-year longitudinal natural history study in PWS. We show that total serum fasting ghrelin is significantly elevated early in infancy in PWS, long before

the onset of obesity and hyperphagia. As part of our study, we also sought to determine whether leptin and insulin levels, GH therapy and PWS molecular class have an effect on ghrelin levels in individuals with PWS. Normal weight siblings, as well as others (i.e., non-PWS) with early childhood obesity, were utilized as control groups.

PATIENTS AND METHODS

PWS Nutritional Phases

There are six nutritional phases (Supplemental Table I – see supporting information online) observed in PWS after birth as previously described in detail and validated by the Prader-Willi syndrome consortium in the NIH funded Rare Disease Clinical Research Network [Miller et al.,2011]. Briefly, phase 1a is characterized by poor appetite, suck, and growth while infants in phase 1b are growing steadily along the growth curve with an improved and relatively normal appetite. Children in phase 2a begin to climb on the weight curve without a significant increase in calories or appetite, whereas in phase 2b hyperphagia begins, but there is still a sense of satiety after meals. Individuals in phase 3 have a nearly insatiable appetite and a lack of satiety. Phase 4 occurs when an individual previously in phase 3 has a decrease in the appetite drive and can feel full.

Participants

Sixty subjects with PWS between the ages of 5 weeks and 36 years were recruited to the University of Florida Natural History Study and admitted for a 2-day intensive phenotyping research study at the Clinical Research Center, Shands Hospital, University of Florida. In addition, 39 non-PWS subjects with early-onset morbid obesity (EMO) of unknown etiology and 95 normal weight control siblings (SibC) of PWS or EMO subjects were recruited to serve as comparison groups for the study. Characteristics of these three groups are shown in Table I for ages 0–21 years. Appropriate genetic testing was used to classify individuals with PWS into the appropriate molecular class – deletion (del), uniparental disomy (UPD) or an imprinting defect (ID) [Cassidy et al.,2012]. This study was approved by the University of Florida Institutional Review Board, and all adult participants or guardians provided written informed consent and, where appropriate, participants provided assent.

An individual was determined to be in the EMO group if they became obese (>97th centile) on the Centers for Disease Control (CDC) body mass index (BMI) curve (www.cdc.gov) before 4 years of age and remained obese. All subjects with EMO included in this study had a normal chromosomal microarray (CMA), normal DNA methylation analysis for PWS, detectable serum leptin, and absence of a melanocortin-4 receptor (MC4R) deficiency by mutational analysis. BMI z-scores were calculated from body weight and height measurements using the United States Centers for Disease Control (CDC) criteria. Since the CDC does not have BMI z-scores for children less than 2 years in age, we determined weight-for-length centiles based on CDC data. Body composition was determined by dual-energy x-ray absorptiometry (DEXA). The data were analyzed with PC PAL Growth XP v1.0 software and the total body fat was expressed as a percentage of body weight.

Most subjects had multiple visits with blood sampling during the course of the study, accounting for the discrepancy between subject number and observations in Table I. The ages of the PWS subjects in the study ranged from 5 weeks to 36 years old; however because there were few controls and EMO subjects beyond the age of 21, comparisons between PWS, EMO and sibling controls were limited to less than 21 years of age. In total there were 136 serum samples from PWS subjects below the age of 21 years; 113 of these subjects were receiving growth hormone therapy. In addition there were 52 EMO serum samples and 147 SibC serum samples from individuals less than 21 years old (Table I).

Hormone Assays

Blood samples were collected from each study participant between 08:00 and 09:00 after an overnight fast for serum and plasma isolation to be used for ghrelin, leptin, and insulin analyses. Serum blood samples were collected in serum separator tubes containing 100 μ l aprotinin and allowed to sit at room temperature for 15–30 minutes until clotting, while plasma blood samples were collected in EDTA blood collection tubes with 100 μ l aprotinin, and then centrifuged at 3000 rpm ($1,800 \times g$) for 10 minutes at $+4^{\circ}\text{C}$. All samples were stored at -80°C until use.

Total serum ghrelin was measured in triplicates using a fluorescent ELISA kit from Phoenix Pharmaceuticals, Inc., California, USA, per instructions of the manufacturer. The serum samples were diluted $20\times$ with assay buffer and assayed in triplicates. The intra- and inter-assay variabilities were 5–10% and $<15\%$, respectively. The minimum detectable concentration of the assay was 11.9 pg/ml.

Plasma leptin was measured in duplicates using the metabolic panel of the Luminex assay system per instructions of the manufacturer (Millipore Inc, CA, USA). The intra- and inter-assay variabilities of the assay were 5–10% and $<15\%$ respectively. The minimum detectable concentration of the assay was 122 pg/ml.

Both the ELISA and Luminex assay data were normalized for inter-assay variability to four internal controls that were included with every assay performed.

Statistical Methods

There were two separate sets of analyses conducted. The first was to look within PWS, and determine if ghrelin levels differ by nutritional phase, and if so, how the phases segregate. Once this was determined, we then asked if other factors were significantly prognostic for ghrelin level, after adjusting for nutritional phase. The second set of analyses contrasted ghrelin and other markers by group (PWS vs. EMO vs. Control).

Analysis of Nutritional Phase—A linear mixed model, with subjects as a random factor and an autoregressive correlation matrix, was employed with dependent variable ghrelin level and the following independent variables: (1) Phase 1a vs. not; (2) [Phases 1a and 1b] vs. Phase [2a, 2b, 3, and 4]; (3) [Phase 1a, 1b, and 2a] vs. Phase [2b, 3, and 4]; (4) [Phases 1a, 1b, 2a, 2b] vs. Phase [3 and 4]; (5) Phase 4 vs not. The first step was to control the study-wide false discovery rate by testing the single null hypothesis that there was no difference in any of the five partitions. Had that been non-significant ($P>0.05$), the analysis would

terminate. We then applied a recursive partition to the data, partitioning on the most significant cut as long as it had a P -value below 0.05 (two-sided). Further subdivision within each cut was planned in a like manner, but after the initial subdivision, no subdivision resulted in $P < 0.05$. Once this partition was obtained we further analyzed the independent prognostic importance of age (continuous variable), insulin levels, and HOMA-IR.

Contrasting PWS with SibC and EMO—We utilized two methods, which target slightly different endpoints. The first method (Mean of Mean) takes the personal averages within the group being analyzed (e.g., ages 2–4.99 years), and contrasts these personal means in an analysis of variance. This analysis is aimed at making the inference at the subject level, equalizing the weight of each contributing subject regardless of how many observation the subject contributes to the cell (e.g., age group). The second method uses a Mixed Model analysis of variance with subject as a random effect, and used an autocorrelational covariance structure to account for repeated measures. This analysis is targeted to approximately equalizing the weights given to the observations, with those contributing more observations being weighted higher than those contributing less. Where the two analyses qualitatively agree, this gives strength to the robustness of the conclusions.

RESULTS

Analysis of PWS Ghrelin Levels by Nutritional Phases

Taken together, the six nutritional phases (five partitions) were significantly related to ghrelin ($P < 0.001$). This justifies the recursive partition to determine how the phases segregate on ghrelin. The analysis resulted in nutritional phase being subdivided into early phases (1a+1b) and later phases (2a+). No subdivision of the early nutritional phases or the later nutritional phases in PWS by nutritional phase reached statistical significance at $P < 0.05$, making it the first partition. Table IIa provides point estimates and standard error for the association of ghrelin level with the key independent variables. We estimate that PWS subjects in the later nutritional phases (2a+) average about 3,300 units lower than those in the early phases (1a+1b; $SE = 543$), $P < 0.001$. The slopes for ghrelin against age, insulin, and HOMA-IR were not close to significant when adjusted for our nutritional phase partition. Nutritional phase was the most predictive of PWS ghrelin levels and ghrelin levels decreased in childhood with advancing phases. Since we only had two patients with PWS (both adults) in phase 4 they were not included in any further analyses (Table IIb; Fig 1).

Analysis of PWS vs EMO vs SibC Ghrelin Levels

After adjusting for age, the mean ghrelin level for the PWS group was 975 pg/ml higher relative to the SibC group ($SE = 237$, $P < 0.001$), and 1,147 pg/ml higher relative to the EMO group ($SE = 316$, $P < 0.001$). However, there was considerable overlap of ghrelin levels in all three groups at all ages (Fig 2). Ghrelin levels decreased on average 76 pg/ml per year of age ($SE = 15$, $P < 0.001$) with no statistical evidence of an age by group (PWS vs EMO vs SibC) interaction ($P = 0.16$). Thus, the rate at which ghrelin decreased with age showed no significant difference among the three groups.

Clinical and Hormonal Assay by Age Groups

In order to compare individuals with PWS to those in the EMO and sibling control groups, we divided our subjects into four major age groups: infants (0–1.99 years), young children (2–4.99 years), older children (5–11.99 years), adolescents and young adults (12–21 years). Since there were few SibC and EMO subjects beyond the age of 21 years further comparisons among PWS, EMO and SibC ghrelin levels were limited to subjects less than 21 years.

Infants with PWS (0–1.99 years old) as a group had significantly higher ghrelin levels than their counterparts in the SibC group by both the mean of means and mixed model analyses after correcting for weight-for-length, insulin and HOMA-IR (Table III).

Serum ghrelin levels in children 2–4.99 years old with PWS were significantly elevated relative to their counterparts in the SibC group by the mixed model, but not by the mean of means (Table III). Ghrelin levels in EMO children within this age group were higher than in the PWS and SibC groups, but did not reach statistical significance using either statistical model.

Children 5–11.99 years old with PWS had significantly elevated ghrelin levels relative to the EMO group by both statistical models and relative to the SibC group by the mixed model ($P=0.044$), but not the mean of means ($P=0.21$) statistical model (Table III). There was no significant difference between ghrelin levels in the SibC and EMO groups.

Among teenagers and young adults 12–20.99 years old, the average ghrelin level of the PWS group was significantly elevated relative to the SibC and EMO groups by both the mean of means and mixed model analyses (Table III). There was no significant difference between ghrelin levels in the SibC and the EMO groups.

Correlations Analysis

Since the CDC does not have BMI z-scores for children less than 2 years in age, correlation analysis for infants (0–1.99 years) were done separately using their weight-for-length values. There were no significant correlations between ghrelin and weight-for-length, percent body fat or insulin in the PWS infant group or the sibling control infant group (Table IVa). There was however a significant positive correlation between ghrelin and leptin in the sibling control infant group (slope = 7.2; $P=0.0063$), but not in the PWS infant group (slope = 0.09; $P=0.98$). Interestingly the PWS infant group had a significantly lower weight-for-length than the sibling control group ($P=0.015$), but there was no significant difference in body fat or leptin level (Fig 3) between the two groups.

For ages 2–20.99 years, there was a significant negative correlation (Table IVb) between ghrelin and insulin in all three groups (PWS, EMO and SibC). There were no significant correlations between ghrelin and leptin, BMI z-scores or percent body fat in the PWS, EMO or SibC groups.

Effect of Growth Hormone and PWS Molecular Classes

Growth hormone therapy (GHT) was associated with an average decrease of 1,202 pg/ml in ghrelin levels of individuals with PWS and this was significant after adjusting for age (SE=535; $P=0.043$). There were no significant differences in ghrelin levels between type 1 and type 2 deletion subjects. There was a trend towards lower ghrelin levels in PWS subjects with UPD and ID relative to subjects with deletions, but it was not significant.

DISCUSSION

Several groups have found elevated total ghrelin levels in older children and adults with PWS [Cummings et al., 2002; Goldstone et al., 2005; Haqq et al., 2003; Tauber et al., 2004]. Since ghrelin is a potent orexigenic hormone [Cummings 2006; Kamegai et al., 2000; Wren and Bloom 2007], it was a reasonable candidate to explain the switch to hyperphagia and lack of satiety that is reliably found in older children and adults with PWS. An abnormal increase in appetite is not seen in PWS until approximately a median age of 3.5–4.5 years [Butler et al., 2010; Miller et al., 2011]. Thus if hyperghrelinemia was responsible for the switch to hyperphagia in PWS then it would be expected that young infants with PWS who are not yet hyperphagic would have normal or low ghrelin levels compared to age matched controls. In fact several groups have reported that total ghrelin levels were not elevated as a group in young non-obese children with PWS compared to controls [Butler and Bittel, 2007; Erdie-Lalena et al., 2006; Goldstone et al., 2012; Haqq et al., 2008a] although one of these groups did report that a subset (33%) of young PWS children had hyperghrelinemia [Haqq et al., 2008a]. By contrast, the largest published cohort to date of young children found that hyperghrelinemia was present in PWS children at any age, and preceded the onset of obesity [Feigerlová et al., 2008].

In agreement with Feigerlová et al. [2008], our 12-year longitudinal study of individuals with PWS found that total ghrelin levels are highest in the youngest children with PWS (less than one year old) who are in nutritional phase 1a, which is characterized by poor appetite and feeding. Given that the total ghrelin was elevated as early as 5 weeks of age in infants with PWS in our study (several years before the median age of onset of hyperphagia in PWS [Butler et al., 2010; Miller et al., 2011]) and that there is some overlap in ghrelin levels between individuals in the PWS and sibling control groups at all ages, it seems unlikely that elevated ghrelin levels alone are responsible for the switch to the hyperphagic phases of PWS. Furthermore, in looking at individual data points from the literature, an overlap in total ghrelin levels in some PWS and control subjects can also be seen at all ages in studies done by others [Butler and Bittel 2007; Cummings et al., 2002; Feigerlová et al., 2008; Goldstone et al., 2012; Haqq et al., 2008b; Tauber et al., 2004]. In addition, reduction of total and acylated ghrelin levels with pharmacological agents in obese adults with PWS did not significantly reduce appetite, compulsive food-seeking or body weight [De Waele et al., 2008; Tan et al., 2004].

We did find a significant negative correlation between nutritional phase and ghrelin levels in PWS after adjusting for differences in age, insulin and HOMA-IR. However, there was no significant correlation between ghrelin and age after adjusting for nutritional phase, insulin and HOMA-IR. Since the age of onset of the various nutritional phases varies among

individuals with PWS, analysis of ghrelin levels by age alone is misleading. For example, we observed young children with PWS in 4 different nutritional phases in the 2–4.99 years age group. We also observed that infants and young children with PWS in the early nutritional phases (1a, 1b) as a group had significantly more ghrelin than their normal sibling control counterparts of similar age. Thus it is possible that the inconsistencies in the literature on ghrelin levels in young children with PWS may be due to reliance on age alone and small sample sizes. Our study and that previously reported [Feigerlová et al., 2008] have the largest number of young infants in both the PWS and control groups. Furthermore, in the 2–4.99 and 5–11.99 year age groups we observed a significant difference between the PWS and sibling control group by the Mixed Model analysis, but we did not observe a significant difference by the Mean of Means analysis. Where the two analyses qualitatively agree, this gives strength to the robustness of the conclusions and when they disagree (as is the case here with the 2–11.99 year age range) it is difficult to make any strong conclusions about the data. Interestingly, the rate at which the total ghrelin decreased with age showed no significant difference among the PWS, SibC and EMO groups.

We also observed that GHT decreased total ghrelin levels on average by 1,202 pg/ml in PWS subjects. This difference was significant after adjusting for age differences. It is thus possible GHT affects ghrelin levels in PWS and may also be a source for discrepancies in ghrelin studies in young PWS children, as previously suggested in two other studies [Hauffa et al., 2007; Hauffa and Petersenn 2009].

Total ghrelin levels of non-PWS obese older children and adults have been shown to be significantly lower than lean controls [Cummings et al., 2002; Goldstone et al., 2005]. We found in our older children and adults (5–20.99 years) that the EMO group had lower ghrelin compared to the SibC group, but that this difference did not reach significance (Table III). However in younger children (2–4.99 years), the EMO group had a higher average ghrelin than both the PWS and SibC groups, but the difference was also non-significant. In comparison to other studies, our EMO sample is unique due to the very early onset of their obesity and the young age we were able to collect samples (i.e., 2–4.99 years). Given that leptin in the central nervous system can modulate ghrelin signaling [Friedman and Halaas 1998; van der Klaauw et al., 2013], central leptin resistance in the EMO group resulting from elevated peripheral leptin levels at a young age may negatively impact leptin-ghrelin dynamics in older children and adults with EMO.

The reason(s) for the presence of hyperghrelinemia in PWS as a group, especially in infancy, remain unclear. Ghrelin has been suggested to play a critical role in fetal adaptation to intrauterine malnutrition as well as in catch-up growth in small for gestational age (SGA) infants [Bellone et al., 2006; Fidancı et al., 2010; Sahin et al., 2012]. Significantly elevated total and active ghrelin levels have been observed in SGA and preterm infants [Fidancı et al., 2010; Sahin et al., 2012] as well as in the umbilical cord of SGA infants [Abdel Hakeem et al., 2012], leading us to hypothesize that intrauterine growth restriction and low birth weight are possible physiological stimulants for ghrelin secretion. It is therefore possible that the elevated ghrelin levels observed in infants with PWS are a physiological response to their being born on average about 15% underweight relative to their normal siblings [Miller et al., 2011]. Ghrelin acts as an anabolic drive during the early stages of life [Bellone et al.,

2006; Bellone et al., 2004] and can up-regulate gene expression of lipogenic enzymes and promote lipogenesis as well as inhibit fatty acid oxidation independent of its role as an orexigenic hormone [Perez-Tilve et al., 2011; Wren and Bloom, 2007]. Hyperghrelinemia may therefore serve to drive rapid weight gain in infants with PWS. It is probably the reason for the elevated body fat in PWS relative to their weight-for-length percentile as observed by us in this study and by others previously [Eiholzer et al., 1999; Goldstone et al., 2012; Haqq et al., 2008a]. Interestingly, out of the 11 infants in the SibC group, 4 had ghrelin levels greater than 3,000 pg/ml. Further analysis of these four infants revealed an average weight-for-length of 24.5%, similar to what is observed in the PWS infant group (25.07%). In contrast, the 7 other SibC infants with ghrelin levels less than 3,000 pg/ml had an average weight-for-length of 64.9%.

The lack of appetite in infants with PWS despite the presence of hyperghrelinemia may be the consequence of immature hypothalamic neurons [Swaab et al., 1995] and a poorly developed leptin-ghrelin-neuropeptide Y feedback loop [Bellone et al., 2006; Kalra et al., 2005]. Unbalanced serum leptin and ghrelin dynamics have been implicated in prolonged post-prandial satiety and reduced hunger in the elderly, contributing to anorexia in this age group [Di Francesco et al., 2006]. Alternatively, it is possible ghrelin's role in infants and young children is different from its role in adults. Ghrelin secretion in neonates and prepubertal children is refractory to the inhibitory effects of food [Bellone et al., 2006; Bellone et al., 2004], unlike in adults, suggesting its primary role early on may not be appetite regulation. It is also possible that elevated ghrelin levels in infancy disrupt the early development of the CNS centers controlling feeding behavior and energy homeostasis, leading to development of obesity and hyperphagia later in childhood.

Strengths and Limitations

The strengths of this study are several. This is the first study to look at total ghrelin levels in PWS by nutritional phase. In addition, we were able to collect large number of children in the various age groups from each of the three cohorts – PWS, EMO and normal weight control siblings. This provided the PWS group with both an obese and non-obese comparison groups. In particular we were able to collect samples from 18 children with PWS (9 in nutritional phase 1a) and 12 controls less than 2 years of age. Longitudinal data on individuals were able to confirm the decreasing ghrelin levels as the individual progressed through the various nutritional phases. The novel aspect of this study was the ability to correlate ghrelin, leptin and insulin with age as well as by nutritional phase in PWS.

The limitations of this study are that we only had a few individuals in our natural history study that were in nutritional phase 4 and all were older than 21 years so they were not included in this analysis. Also our youngest infant with PWS was 5 weeks old. Ideally, we would have liked to have blood samples within the first 1–2 weeks after birth from all subjects with PWS. Several of our infants did not have hyperghrelinemia, but might have in the early days after birth. We did not evaluate acylated ghrelin. However previous studies have found that the ratio of total and acylated ghrelin remain constant in different samples tested [Ariyasu et al., 2002; Marzullo et al., 2004; Nakai et al., 2003] including in children

with PWS when compared to controls [Erdie-Lalena et al., 2006]. Thus, Erdie-Lalena and colleagues (2006) have concluded that total ghrelin levels are a “reasonable surrogate” for acylated ghrelin when comparing various subjects (e.g., PWS, controls and nutritional phases).

Summary and Future Work

Our data clearly show that total ghrelin levels are significantly elevated in PWS subjects beginning in the earliest nutritional phase (i.e., 1a), which is characterized by a period of poor appetite and poor feeding. We also show that total ghrelin levels in the PWS group correlate better with nutritional phase than age. In particular, young children in the 2–4.99 year age range could be in four different nutritional phases. The hyperghrelinemia in early infancy may result from intra-uterine abnormalities in development. The switch to hyperphagia in PWS is therefore not explained by an increase in circulating ghrelin. Future work should address other appetite regulating hormones and metabolic factors in fasting and post-prandial samples to identify biomarkers for the different nutritional phases in PWS. Understanding the hormonal and metabolic factors involved in the transition through the nutritional phases will provide valuable insight into the pathophysiology of appetite and obesity in PWS and therefore lead to rationale treatment strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BMI	Body Mass Index
CDC	Centers for Disease Control
Del	Deletion in the paternally inherited chromosome 15q11–q13 region
EMO	Early-onset Morbid Obesity
FTT	Failure to thrive
GH	Growth Hormone
GHT	Growth Hormone Therapy
HOMA-IR	Homeostasis Model Assessment-insulin resistance
ID	Imprinting Defect
NIH	National Institutes of Health

PWS	Prader-Willi syndrome
SibC	Normal non-obese Sibling Controls of PWS and EMO subjects
UPD	Maternal uniparental disomy of chromosome 15

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Figure 1.

Total fasting ghrelin levels at the various nutritional phases in PWS. A) Box plot of serum ghrelin at each nutritional phase (ages 0–36 years); The lower and upper whiskers in the boxplots represent the 10th and 90th centiles, the bars from bottom to top represent the 25th, 50th and 75th centiles respectively, while the plus (+) sign represents the mean. The sample sizes (n) represent repeated measures. The only significant transition in nutritional phases is from phase 1b to 2a ($P=0.0032$). B) Scatter plot of nutritional phase ghrelin by age in PWS (cut off at 21 years for illustrative purposes). Note that in the 2–4.99 year age range (denoted by the hashed red lines) individuals in four different nutritional phases (i.e., 1b, 2a, 2b and 3) can be found.

Figure 2.
Ghrelin levels of children and young adults belonging to the PWS, sibling control and EMO groups using repeated measures (cut off at 21 years for illustrative purposes).

Figure 3.

Comparisons of PWS with sibling controls (SibC) within age group 0 – 1.99 years for A) weight-for-length centiles, B) body fat centiles, and C) fasting plasma leptin levels. P -values derived from mixed model analysis and the sample sizes (n) represent observations.

TABLE I

Characteristics of the study participants by age groups

	PWS	SibC	EMO
0 – 1.99 years			
Subjects	18 (9M, 9F)	12 (7M, 5F)	N/A
Observations *	25 (15M, 10F)	14 (8M, 6F)	N/A
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
2 – 4.99 years			
Subjects	41 (22M, 19F)	26 (11M, 15F)	9 (6M, 3F)
Observations *	53 (27M, 26F)	28 (11M, 17F)	9 (6M, 3F)
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
5 – 11.99 years			
Subjects	29 (12M, 17F)	54 (25M, 29F)	20 (10M, 10F)
Observations *	41 (17M, 24F)	74 (31M, 43F)	28 (16M, 12F)
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
12 – 20.99 years			
Subjects	12 (7M, 5F)	23 (14M, 9F)	12 (5M, 7F)
Observations *	17 (9M, 8F)	31 (18M, 13F)	15 (7M, 8F)
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

* Observations describe total sample size analyzed including repeated measures from subjects with multiple visits

mol. = molecular; Del = deletion of paternal 15q11.2; UPD = maternal uniparental disomy 15; ID = imprinting defect; GH = growth hormone

a. Association of Ghrelin with Phase 1 (1a & 1b) vs Not (Phase 2+), Insulin, HOMA-IR, and Age in PWS

Effect	Estimate	SE	P-Value
Intercept	6678	434	
Phase 2+	-3341.5	543	<0.001
Insulin	-25.7	139.6	0.85
HOMA-IR	-138.8	596.3	0.82
Age	-19.6	25.1	0.44

b. Nutritional phase clinical and hormonal assay data in PWS

Variable	Phase 1a	1b	2a	2b	3
Ghrelin (pg/ml)	7,906(5,887)[9]	5,057(2,624)[17]	2,905(1,521)[27]	2,615(1,370)[48]	2,423(1,350)[37]
Leptin (pg/ml)	359(295)[9]	185(145)[16]	758(1,087)[20]	2,235(2,053)[33]	2,434(1,647)[41]
Insulin (mg/dl)	1.72(1.87) [10]	3.21(2.35)[16]	6.43(3.85)[28]	10.91(7.83)[50]	10.24(9.04)[45]
HOMA-IR	0.33(0.40)[10]	0.64(0.52)[16]	1.31(0.84)[28]	2.40(1.89)[49]	2.30(2.08)[44]
Weight-for-length (%)	21.27(26.52)[9]	22.77(23.43)[13]	N/A	N/A	N/A
BMI z-score	N/A	-0.96(0.77)[6]	0.84(1.42)[28]	1.44(1.23)[51]	2.07(0.92)[47]

Fixed Effects Estimates (*Method is via a mixed model that takes repeated measures into account*)

Interpretation: The fitted mean value is for ghrelin is 6678-25.7*Insulin-138.8*HOMA-IR-19.6*Age (In Years) for Phase 1a and 1b vs. is (6678-3341)-25.7*Insulin-138.8*HOMA-IR-19.6*Age (In Years) for Phase 2+. Only Phase remains significantly prognostic. After adjusting for Phase 1 vs. Phase 2+, none of insulin, HOMA-IR, or age is significantly prognostic for ghrelin.

Data expressed as mean with standard deviation (SD) and sample size [N].

N/A = Not Applicable

TABLE III

Clinical and hormonal assay data by age groups

	PWS	SibC	EMO	P1	P2	P3
0 – 1.99 years						
Ghrelin (pg/ml)	5,521 ± 3696	2,883 ± 1172	N/A	0.016* [0.0087**]	N/A	N/A
Leptin (pg/ml)	272 ± 231	216 ± 145	N/A	0.48 [0.39]	N/A	N/A
Insulin (mg/dl)	2.29 ± 2.01	2.53 ± 2.54	N/A	0.73 [0.77]	N/A	N/A
HOMA-IR	0.45 ± 0.45	0.53 ± 0.52	N/A	0.65 [0.66]	N/A	N/A
Weight-for-length (%)	25.07 ± 28.17	57.41 ± 37.50	N/A	0.025* [0.015*]	N/A	N/A
DEXA	21.36 ± 7.89	19.64 ± 6.33	N/A	0.57 [0.48]	N/A	N/A
2 – 4.99 years						
Ghrelin (pg/ml)	3,113 ± 1,898	2,556 ± 927	3,430 ± 2,320	0.12 [0.041*]	0.71 [1.0]	0.30 [0.18]
Leptin (pg/ml)	1,389 ± 1,785	150 ± 99	2248 ± 1,107	<0.001** [0.0040*]	0.098 [0.15]	<0.001** [$<0.001^{**}$]
Insulin (mg/dl)	7.07 ± 5.79	4.04 ± 3.29	11.25 ± 7.36	0.0057** [0.013*]	0.15 [0.032*]	0.014* [$<0.001^{**}$]
HOMA-IR	1.52 ± 1.46	0.86 ± 0.83	2.75 ± 1.71	0.016* [0.028*]	0.092 [0.017*]	0.011* [$<0.001^{**}$]
BMI z-score	0.93 ± 1.55	0.32 ± 1.19	4.29 ± 0.79	0.074 [0.083]	<0.001** [$<0.001^{**}$]	<0.001** [$<0.001^{**}$]
DEXA	24.98 ± 10.47	18.61 ± 6.39	44.04 ± 5.78	0.005** [0.0066]	<0.001** [$<0.001^{**}$]	<0.001** [$<0.001^{**}$]
5 – 11.99 years						
Ghrelin (pg/ml)	2,476 ± 1,332	2,111 ± 1013	1,645 ± 983	0.21 [0.044*]	0.021* [0.016*]	0.10 [0.55]
Leptin (pg/ml)	2,107 ± 1,572	397 ± 720	2,408 ± 1,569	<0.001** [$<0.001^{**}$]	0.56 [0.25]	<0.001** [$<0.001^{**}$]
Insulin (mg/dl)	12.63 ± 8.61	6.86 ± 3.92	19.71 ± 14.24	<0.001** [$<0.001^{**}$]	0.11 [0.024*]	0.0011** [$<0.001^{**}$]
HOMA-IR	2.67 ± 1.81	1.51 ± 0.95	4.17 ± 3.06	<0.001** [$<0.001^{**}$]	0.11 [0.019*]	0.0011** [$<0.001^{**}$]
BMI z-score	1.62 ± 1.17	0.35 ± 0.92	2.72 ± 0.22	<0.001** [$<0.001^{**}$]	<0.001** [$<0.001^{**}$]	<0.001** [$<0.001^{**}$]
DEXA	35.08 ± 12.75	20.39 ± 8.05	45.83 ± 4.97	<0.001** [$<0.001^{**}$]	<0.001** [$<0.001^{**}$]	<0.001** [$<0.001^{**}$]
12 – 20.99 Years						
Ghrelin (pg/ml)	2,086 ± 885	1,233 ± 509	1,053 ± 847	0.011* [$<0.001^{**}$]	0.0085** [0.0056**]	0.49 [0.21]
Leptin (pg/ml)	2,837 ± 1,839	1,138 ± 1485	5,459 ± 2,289	0.0094** [0.0090**]	0.0060** [$<0.001^{**}$]	<0.001** [$<0.001^{**}$]

	PWS	SibC	EMO	P1	P2	P3
Insulin (mg/dl)	13.36 ± 9.26	11.08 ± 5.33	27.07 ± 18.79	0.37 [0.28]	0.015* [0.0082**]	0.0044** [$<0.001^{**}$]
HOMA-IR	3.10 ± 2.41	2.48 ± 1.23	5.63 ± 3.97	0.35 [0.25]	0.036* [0.026*]	0.0070** [$<0.001^{**}$]
BMI z-score	2.10 ± 0.66	0.50 ± 1.08	2.73 ± 0.34	$<0.001^{**}$ [$<0.001^{**}$]	0.0051** [0.051]	$<0.001^{**}$ [$<0.001^{**}$]
DEXA	47.95 ± 8.72	26.00 ± 10.79	54.28 ± 6.09	$<0.001^{**}$ [$<0.001^{**}$]	0.049* [0.090]	$<0.001^{**}$ [$<0.001^{**}$]

Note: All data expressed as Mean ± SD. *P*-values expressed for both Mean of Means and [Mixed Model] analyses. The [Mixed Model] analysis takes repeated measures (observations) into account while the Mean of Means does not.

{ * = *P*-value less than 0.05; ** = *P*-value less than 0.01 }

P1 = *P*-value for comparison of PWS vs SibC

P2 = *P*-value for comparison of PWS vs EMO

TABLE IV**a. Correlations, ages 0 – 1.99 years**

DV	IV	PWS	SibC
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}
Ghrelin	Insulin	-75.6(415.1)[15]{0.86}	-93.3(158.7)[9]{0.58}
Ghrelin	HOMA-IR	-383(1839)[15]{0.84}	-412(770)[9]{0.61}
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}

b. Correlations, ages 2 – 20.99 years

DV	IV	PWS	SibC	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-score	-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}
Ghrelin	Insulin	-59.4(17.9)[113]{0.0019**}	-95.1(18.1)[133]{<0.001**}	-28.4(10.7)[51]{0.020*}
Ghrelin	HOMA-IR	-249(76.8)[112]{0.0024**}	-397(76.6)[132]{<0.001**}	-111(53.7)[50]{0.062}
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-score	664(123)[100]{<0.001**}	352(85.8)[94]{0.0013**}	692(418)[46]{0.13}
DEXA	BMI z-score	5.69(0.54)[120]{<0.001**}	5.17(0.61)[127]{<0.001**}	3.70(1.17)[50]{0.0082**}

Mean change in Dependent variable (DV) per Unit Change in Independent Variable (IV): Slope (SE)[N]{*P*-value}.