Hormone Research in Paediatrics

Research Article

Horm Res Paediatr
DOI: 10.1159/000534560

Received: July 26, 2023
Accepted: September 30, 2023
Published online: October 13, 2023

Longitudinal Changes in Acylated versus Unacylated Ghrelin Levels May Be Involved in the Underlying Mechanisms of the Switch in Nutritional Phases in Prader-Willi Syndrome

Lionne N. Grootjen[a, b, c] Gwenaelle Diene[d, e] Catherine Molinas[e] Véronique Beauloye[f] T. Martin Huisman[g] Jenny A. Visser[g] Patric J.D. Delhanty[g] Gerthe F. Kerkhof[a, c] Maithe Tauber[d, e] Anita C.S. Hokken-Koelega[a, b, c]

[a]Dutch Reference Center for Prader-Willi Syndrome, Rotterdam, The Netherlands; [b]Dutch Growth Research Foundation, Rotterdam, The Netherlands; [c]Subdivision of Endocrinology, Department of Pediatrics, Erasmus University Medical Center-Sophia Children's Hospital, Rotterdam, The Netherlands; [d]Unité d'Endocrinologie, Obésités, Maladies osseuses et Gynécologie médicale, Hôpital des Enfants, CHU Toulouse, Université Toulouse III, Toulouse, France; [e]Centre de Référence Maladies Rares PRADORT (syndrome de PRADer-Willi et autres Obésités Rares avec Troubles du comportement alimentaire), Hôpital des Enfants, CHU Toulouse, Université Toulouse III, Toulouse, France; [f]Unité d'Endocrinologie pédiatrique, Cliniques Universitaires Saint-Luc, Université Catholique de Louvain, Brussels, Belgium; [g]Department of Internal Medicine, Erasmus University Medical Center, Rotterdam, The Netherlands

Keywords
Prader-Willi syndrome · Children · Infants · Growth hormone · Ghrelin

Abstract

Introduction: Prader-Willi syndrome (PWS) is characterized by a switch from failure to thrive to excessive weight gain and hyperphagia in early childhood. An elevated, more unfavorable ratio between acylated and unacylated ghrelin (AG/UAG ratio) might play a role in the underlying mechanisms of this switch. We aimed to assess the evolution of the appetite-regulating hormones acylated ghrelin (AG) and unacylated ghrelin (UAG) and the AG/UAG ratio and their association with the change in eating behavior in children with PWS, compared to healthy age-matched controls. ***Methods:*** A longitudinal study was conducted in 134 children with PWS and 157 healthy controls, from the Netherlands, France, and Belgium. Levels of AG and UAG and the AG/UAG ratio were measured and nutritional phases as reported for PWS were scored. ***Results:*** The AG/UAG ratio was lower in the first years of life in PWS than in controls and started to increase from the age of 3 years, resulting in a high-normal AG/UAG ratio compared to controls. The AG levels remained stable during the different nutritional phases ($p = 0.114$), while the UAG levels decreased from 290 pg/mL in phase 1a to 137 pg/mL in phase 2b ($p < 0.001$). The AG/UAG ratio increased significantly from 0.81 in phase 2a to 1.24 in phase 2b ($p = 0.012$). ***Conclusions:*** The change from failure to thrive to excessive weight gain and hyperphagia in infants and children with PWS coincides with an

karger@karger.com
www.karger.com/hrp

Correspondence to:
Lionne N. Grootjen, l.grootjen@kindengroei.nl

increase in AG/UAG ratio. The increase in AG/UAG ratio occurred during phase 2a, thus before the onset of hyperphagia.

Introduction

Prader-Willi syndrome (PWS) is a rare syndrome caused by the lack of expression of the paternally derived chromosome 15q11-q13, due to a paternal deletion or maternal uniparental disomy, and in rare cases by an imprinting center defect or paternal chromosomal translocation [1–3]. The clinical findings characterizing PWS are divided into the different nutritional phases that change during life [4]. The first phase after birth is characterized by failure to thrive with poor feeding and severe hypotonia. This phase is followed by a period of appropriate growth without feeding problems. Around the age of 18–36 months, children start to gain excessive body weight without an increase in calorie intake or interest in food. Thereafter, the appetite and interest in food start to increase abnormally. This phase turns into a state of hyperphagia with food-seeking behavior and obesity when leaving the children unattended [4, 5]. This switch from failure to thrive to hyperphagia remains to be unraveled, but hyperghrelinemia is thought to be involved [6–10].

There are two types of ghrelin in the circulation, acylated ghrelin (AG) and unacylated ghrelin (UAG) [11, 12]. While AG is known for stimulating appetite and its diabetogenic and obesogenic functions [13–15], UAG is known for its protective effects on the beta cells and muscle cells, and UAG improves glycemic control [16–20]. It has been suggested that UAG is a functional inhibitor of AG, having antagonistic effects [17, 20]. Since AG and UAG have these opposite effects, the ratio of AG and UAG levels (AG/UAG ratio) might be important. In previous studies, elevated AG/UAG ratios have been associated with obesity, diabetes, and hyperphagia [6, 7, 21–23].

Contradictory results have been reported for plasma ghrelin levels in children with PWS, with some presenting normal ghrelin levels [9, 24–27], while others describe hyperghrelinemia [8, 20]. In most of these studies, total ghrelin levels were measured, instead of AG and UAG levels. In our previous cross-sectional study, we found that the AG/UAG ratio was increased in children and young adults with PWS, while the UAG levels were similar to those of controls [6]. In particular, the AG/UAG ratio in children and young adults with PWS and hyperphagia and/or weight gain was higher compared to those without weight gain and/or hyperphagia, whose AG/UAG ratios were similar to controls. In infants, high levels of circulating UAG were found, while AG levels remained stable until the age of 48 months [7]. These studies indicated the presence of different AG/UAG ratios at various nutritional phases. Since both studies did not have a longitudinal follow-up, the association between AG, UAG, AG/UAG ratio and the change in weight gain and eating behavior from infancy into childhood is still unknown.

The aim of the present study was to investigate the evolution of plasma levels of AG and UAG and AG/UAG ratio in children with PWS and to assess their association with the change in eating behavior. We, therefore, conducted a longitudinal study in children with PWS from infancy to the age of 7 years. We hypothesized that the switch from failure to thrive to increased weight gain in PWS coincides with an increase in AG/UAG ratio, even prior to the start of hyperphagia.

Methods

Patients

A total of 134 children with at least two blood samples were included in the study. They were followed at PWS reference centers in Rotterdam (the Netherlands), Toulouse (France), or Brussels (Belgium) and had PWS genetically confirmed. Growth hormone (GH) treatment was prescribed at a dose of 1 mg/m^2/day (~0.035 mg/kg/day).

The healthy controls were children with a normal body mass index (BMI), who underwent a minor surgical procedure. Their medical records were checked by the study team to exclude endocrine, metabolic, and neurological diseases. Control infants were recruited in Belgium and France and control children in the Netherlands.

Anthropometrics

Standing height was measured with a calibrated Harpenden stadiometer or, when appropriate, supine length with a Harpenden infantometer (Holtain Ltd.). Weight was determined on a calibrated scale (Servo Balance KA-20-150S; Servo Berkel Prior). Height, weight, and BMI of both controls and children with PWS were expressed as standard deviation scores (SDSs), adjusted for age and sex based on national reference data [28–31]. All SDSs were calculated with Growth Analyser RCT 4.1 (www.growthanalyser.org).

DXA Scan

Fat mass percentage (FM%) and lean body mass were measured by DXA (Lunar Prodigy; GE Healthcare). All DEXA scans were made on the same machines, either in Rotterdam, the Netherlands, or in Toulouse, France. Daily quality assurance was performed. FM% SDS was calculated according to age- and sex-matched reference values [32, 33]. As no reference value for FM% exists for children

below the age of 2 years, we could not describe SDS values for these children. Lean body mass index was calculated as lean body mass (kg)/m^2.

Design

A longitudinal, prospective study was conducted to investigate the long-term evolution of plasma AG and UAG levels and AG/UAG ratio and the change in eating behavior in infants and young children with PWS, according to the nutritional phases [4]. All participants visited either the Dutch PWS Reference Center in Rotterdam, the French PWS Reference Center in Toulouse, or the hospital in Brussels. They received multidisciplinary care from a specialized PWS team. The study protocol was approved by the Medical Ethics Committees of the participating centers. Written informed consent was obtained from parents of both children with PWS and the controls. Assent was obtained from children younger than 12 years of age.

Eating Behavior

The nutritional phases according to Miller were used to score the eating behavior of the subjects with PWS [4]: 1a = hypotonia with difficult feeding, 1b = no difficult feeding and growing appropriately on growth curve, 2a = excessive weight gain without an increase in appetite or excessive calories, 2b = weight increasing with an increase in appetite or excessive calories, 3 = hyperphagia, feels rarely full, 4 = appetite no longer insatiable. For each subject with PWS at each visit, the nutritional phase was assessed during an interview by the experienced physicians and nurses of the multidisciplinary teams. Questions about hunger, food-seeking behavior, and food intake were all included in the interview.

Collection of Blood and Plasma Preparation

In children >2 years of age, blood samples were collected in the morning after a 12-h overnight fast. Infants <2 years were fasted for at least 4 h. To stabilize the plasma ghrelin levels, blood samples were collected in EDTA tubes and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma-Aldrich Chemicals) was added at a concentration of 2 mg/mL at the time of collection. Blood was centrifuged at 4°C to prepare plasma, which was quickly frozen on dry ice. Samples were stored at −80°C and assayed within 6 months following collection. Plasma AG and UAG levels were assessed in duplicate in the laboratory of the Erasmus University Medical Center in Rotterdam, using two-step double antibody sandwich EIAs, obtained from SPIBio (Bertin Pharma, France; A05306 and A05319, respectively). The assay and its intra- and interassay coefficient of variation used in this study have been described [6]. Samples with an extremely low AG/UAG ratio (<0.01) were excluded due to stabilization concerns.

Statistics

Statistical analysis was performed by the Statistical Package for Social Sciences (version 24.0; SPSS, Chicago, IL, USA). Data are expressed as median (interquartile range [IQR]), as not all data were normally distributed. Plasma AG and UAG levels and AG/UAG ratios were log-transformed (natural logarithm), to obtain a normal distribution. One blood sample of thirty children was also included in our previous cross-sectional study [6]. All nutritional phases were compared to an age-matched control group. Many children remained in the same phase for a longer period, explaining the discrepancy between the number of children with PWS and the observations in Table 2. Comparative analyses were conducted using Mann-Whitney, Kruskal-Wallis, or χ^2 tests. The course of the ghrelin levels over time was calculated using linear mixed model analysis with AG, UAG, or AG/UAG ratio as dependent variable and age as independent variable. Differences were considered significant if the p value was <0.05.

Results

Baseline Characteristics

Table 1 presents the clinical characteristics at first visit of 134 children with PWS and 157 controls. Median (IQR) age was 1.5 (0.6; 3.4) years at first visit in children with PWS and 2.3 (1.2; 3.6) years in controls ($p = 0.002$). Height SDS and weight SDS were significantly lower in children with PWS compared to controls ($p < 0.001$). Sixty-four children (48%) had a deletion, 59 had a maternal uniparental disomy (44%), 1 had a translocation, 2 had an abnormal methylation defect, 4 had an imprinting mutation, and of 1 child the specific subtype was unknown. Seventy-five children with PWS received GH treatment. The others were, at baseline, prior to GH treatment due to their young median (IQR) age of 0.6 (0.5; 0.9) years, but started GH treatment at the age of 0.72 (0.59; 1.27) years. At the baseline visit, 45.5% of children with PWS were in nutritional phase 1b.

Longitudinal Ghrelin Levels from Infancy into Childhood

Figure 1a–c shows the course of plasma AG and UAG levels and AG/UAG ratio over time, until the age of 7 years, in 134 children with PWS. The AG levels in children with PWS increased in the first 6 months of life, albeit not significantly. After this initial increase, AG levels decreased from 216 pg/mL at 6 months to 120 pg/mL at 3.5 years ($p < 0.001$). Following the decrease in the first years of life, AG levels remained stable until the age of 7 years. When compared to controls, plasma AG levels were within the normal range until the age of 4 years, but thereafter, AG levels were higher in children with PWS.

The UAG levels increased in the first 6 months of life from 176 pg/mL to 319 pg/mL ($p = 0.013$). After this increase, UAG levels decreased to 85 pg/mL at 4.5 years ($p < 0.001$). At the age of 5 years, UAG levels increased to 166 pg/mL ($p = 0.008$) and remained stable thereafter. When compared to the controls, UAG levels in children with PWS were higher during the first 3 years of life, but thereafter, UAG levels were within the normal range when compared to the controls.

Table 1. Baseline characteristics at the first visit

	PWS (*N* = 134)	Controls (*N* = 157)	*p* value
Age, years	1.5 (0.6; 3.4)	2.3 (1.2; 3.6)	0.002
Sex (male), *n* (%)	67 (50.0)	71 (45.2)	0.416
Genetic subtype, *n* (%)	64 (47.8)		
Deletion mUPD	59 (44.0)		
Translocation	1 (0.7)		
Abnormal methylation profile	2 (1.5)		
Other atypical form	1 (0.7)		
GH use, *n* (%)	75 (56.0)		
Age at start of GH, years	0.76 (0.62; 1.17)		
IGF-1 SDS	1.07 (−0.77; 2.74)		
Height SDS	−0.52 (−1.52; 0.15)	0.27 (−0.47; 1.24)	<0.001
Weight SDS	−0.73 (−1.72; 0.42)	0.00 (0.78; 0.79)	<0.001
BMI SDS	−0.40 (−129; 0.90)	−0.13 (−0.97; 0.68)	0.910
FM percentage	29.4 (23.0; 37.2)		
FM (%) SDS	−0.55 (−2.43; 2.25)		
FM SDS	0.02 (−2.18; 1.48)		
LBM SDS	−0.18 (−0.104; 0.91)		
Nutritional phase*, *n* (%)			
1a	26 (19.4)		
1b	61 (45.5)		
2a	14 (10.4)		
2b	13 (9.7)		
3	1 (0.7)		

Data are expressed as median (IQR) or *n* (%). GH, growth hormone; SDS, standard deviation score; FM, fat mass; LBM, lean body mass; phase, nutritional phase according to Miller et al. [4]; mUPD, maternal uniparental disomy. *Unknown for 19 patients.

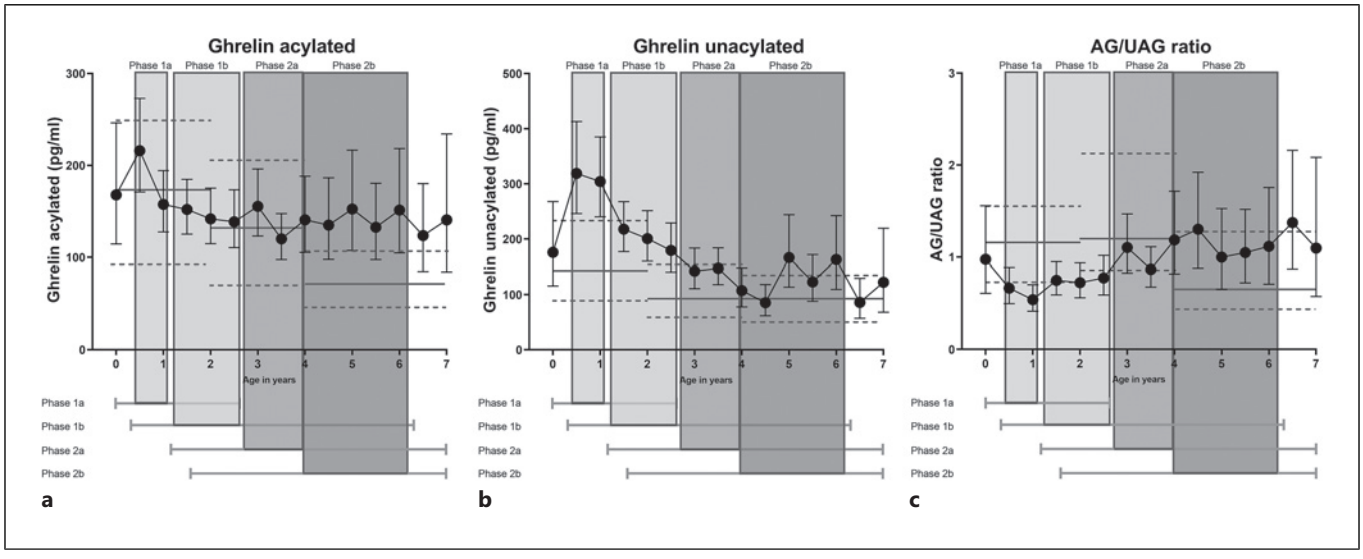

Fig. 1. Longitudinal changes in estimated marginal means with 95% CI in AG (**a**), UAG (**b**), and the AG/UAG ratio (**c**) in children with PWS. The IQR of age of nutritional phase is depicted with the gray areas and the total range with the gray bars below the graph. The AG, UAG, and AG/UAG ratio levels in controls are presented as median (gray lines) and IQR (dotted lines) in the graph for ages 0–2 years, 2–4 years, and 4–7 years.

Horm Res Paediatr
DOI: 10.1159/000534560

Table 2. Comparison of variables between the nutritional phases and controls

	Phase 1a (*N* = 35)	Controls (*N* = 50)	*p* value	Phase 1b (*N* = 185)	Controls (*N* = 79)	*p* value	Phase 2a (*N* = 80)	Controls (*N* = 59)	*p* value	Phase 2b (*N* = 100)	Controls (*N* = 11)	*p* value	*p* value*
Age, years	0.5 (0.4; 1.1)	0.9 (0.5; 1.2)	0.281	1.9 (1.2; 2.7)	1.8 (1.3; 2.4)	0.276	3.5 (2.7; 4.1)	3.1 (2.6; 3.6)	0.137	5.0 (3.9; 6.2)	4.8 (4.3; 6.4)	0.640	<0.001
Age at start of GH, years	0.67 (0.56; 0.75)			0.64 (0.52; 0.75)			0.69 (0.60; 1.08)			0.83 (0.68; 1.20)			<0.001
AG, pg/mL	187 (100; 345)	178 (94; 253)	0.261	151 (85.3; 235.0)	164 (86; 238)	0.994	127 (77; 205)	115 (72; 182)	0.686	161 (89; 227)	72 (46; 107)	0.002	0.114
UAG, pg/mL	290 (160; 561)	150 (86; 235)	<0.001	218[a] (128; 366)	111 (71.8; 198)	<0.001	174 (97; 289)	93 (58; 149)	<0.001	136.8[a] (91.6; 207.0)	93 (50; 135)	0.054	<0.001
AG/UAG ratio	0.60 (0.33; 1.93)	1.10 (0.72; 1.63)	0.050	0.72 (0.40; 1.27)	1.20 (0.81; 1.74)	<0.001	0.81 (0.38; 1.53)	1.23 (0.86; 2.13)	<0.001	1.24[a] (0.73; 1.88)	0.65 (0.44; 1.28)	0.045	<0.001
Height SDS	−1.38 (−1.76; −0.54)	0.20 (−0.74; 1.06)	<0.001	−0.49 (−1.05; 0.24)	0.42 (−0.28; 1.35)	<0.001	−0.42 (−1.16; 0.53)	0.61 (−0.03; 1.47)	<0.001	0.00 (−0.73; 0.64)	−0.14 (−1.3; 0.5)	0.745	<0.001
Weight SDS	−1.85 (−2.65; −0.97)	−0.13 (−0.84; 1.00)	<0.001	−0.98[c] (−1.73; −0.18)	−0.08 (−0.81; 0.7)	<0.001	0.13[c] (−0.49; 0.84)	0.06 (−0.85; 0.82)	0.331	0.80[c] (−0.07; 2.09)	−0.47 (−0.91; 0.46)	0.004	<0.001
BMI SDS	−1.67 (−2.42; −0.68)	−0.21 (−1.19; 0.92)	<0.001	−0.92[c] (−1.55; 0.01)	−0.21 (−0.15; 0.49)	0.006	0.88[c] (0.11; 1.34)	−0.20 (−1.11; 0.36)	<0.001	1.16[a] (0.17; 1.73)	−0.27 (−1.19; 0.86)	0.001	<0.001
FM%	32.1 (28.4; 37.7)			27.5[a] (22.1; 35.3)			35.3[c] (26.7; 41.0)			34.0 (27.7; 40.8)			<0.001
FM% SDS				−0.82 (−2.14; 1.70)			1.83[b] (0.10; 2.48)			2.16[b] (1.40; 2.77)			<0.001
LBMI	10.1 (9.6; 10.9)			11.1 (10.1; 12.0)			10.7 (10.1; 11.9)			10.9 (10.0; 12.2)			0.029

AG, acylated ghrelin; UAG, unacylated ghrelin; SDS, standard deviation score; BMI, body mass index; FM%, fat mass percentage; LBMI, lean body mass index. **p* value for the difference in variables between the nutritional phases (according to Miller et al. [4]). [a]*p* value for the difference between phases 1a and 1b, 1b and 2a, or 2a and 2b <0.05. [b]*p* value for the difference between phases 1a and 1b, 1b and 2a, or 2a and 2b <0.01. [c]*p* value for the difference between phases 1a and 1b, 1b and 2a, or 2a and 2b <0.001.

The AG/UAG ratio decreased significantly from 0.97 to 0.54 during the first year of life ($p = 0.030$). After this decrease, the ratio started to slowly increase to an AG/UAG ratio of 1.09 at 7 years. From the age of 3 years until the age of 7 years, the AG/UAG ratio was significantly higher than the ratio at 1 year (all p values <0.046). In comparison to controls, the AG/UAG ratio was lower in the first 2.5 years of life but was within the normal range until the age of 4 years. After the age of 4 years, the AG/UAG ratio was in the high-normal range of controls.

Figure 1a–c shows the IQR of the age per nutritional phase as the gray areas and the total range as the gray bars below the graph. As the phases 1b, 2a, and 2b were overlapping, we also investigated the data per phase in Table 2.

Differences between the Nutritional Phases

Table 2 presents the different nutritional phases in the PWS group, compared with age-matched controls. The AG levels remained stable during the different phases ($p = 0.114$), while UAG levels decreased from 290 pg/mL in phase 1a to 137 pg/mL in phase 2b ($p < 0.001$). The AG/UAG ratio increased from phase 1a to phase 2b, but the strongest increase was observed from phase 2a to phase 2b (0.81 vs. 1.24, $p = 0.012$, respectively). Median BMI SDS and FM% SDS increased during the nutritional phases, with the highest median BMI and FM% SDS found in phase 2b (both p values <0.001). Lean body mass index was different between the nutritional phases, being lowest in phase 1a ($p = 0.029$).

Nutritional Phase 1a and Age-Matched Controls

Due to matching for age, the median age was similar between the infants with PWS and their controls. While plasma AG levels were not different between the groups, UAG levels were significantly higher in infants with PWS ($p < 0.001$). The median (IQR) AG/UAG ratio in infants with PWS was 0.60 (0.33; 1.93) and 1.10 (0.72; 1.63) in the controls ($p = 0.050$). Median BMI SDS was significantly lower in the children with PWS ($p < 0.001$).

Nutritional Phase 1b and Age-Matched Controls

Median age was similar between the infants with PWS and their controls. Plasma AG levels were not different between the groups, but UAG levels were significantly higher in children with PWS ($p < 0.001$). The median (IQR) AG/UAG ratio was 0.72 (0.40; 1.27) in children with PWS and 1.20 (0.81; 1.74) in the control group ($p < 0.001$). Median BMI SDS was significantly lower in children with PWS.

Nutritional Phase 2a and Age-Matched Controls

There was no difference in median age between the children with PWS and their controls. Plasma AG levels were not different between the groups, while UAG levels were significantly higher in the children with PWS ($p < 0.001$). The median (IQR) AG/UAG ratio was 0.81 (0.38; 1.53) in children with PWS and 1.23 (0.86; 2.13) in the controls ($p < 0.001$). Median BMI SDS was for the first time higher in children with PWS, being 0.88 (0.11; 1.34) in children with PWS and −0.20 (−1.11; 0.36) in controls ($p < 0.001$).

Nutritional Phase 2b and Age-Matched Controls

There was no difference in median age between the groups. Plasma AG levels were significantly higher in children with PWS compared to their controls ($p = 0.002$), and there was a trend toward a higher UAG level in children with PWS ($p = 0.054$). The median AG/UAG ratio was 1.24 in children with PWS and 0.65 in the controls ($p = 0.045$). Median BMI SDS was significantly higher in children with PWS, 1.16 (0.17; 1.73) compared to −0.27 (−1.19; 0.86) in controls ($p = 0.001$).

Ghrelin Levels, Sex, and Genotypes

There was neither a difference in the course of the AG/UAG ratio over time between boys and girls ($p = 0.154$) nor between the various genetic subtypes ($p = 0.948$).

Discussion

This study presents the changes in the appetite-regulating hormones AG, UAG, and the AG/UAG ratio from infancy into childhood, linked to changes in the nutritional phases, in a large cohort of children with PWS in comparison with healthy controls. During the first years of life, the AG/UAG ratio was lower in PWS patients than in controls and started to increase from the age of 3 years, resulting in a high-normal AG/UAG ratio compared to controls from the age of 4 years onward. Our findings demonstrate that there was a significant and striking increase in the AG/UAG ratio when children with PWS progressed from nutritional phase 2a to phase 2b.

From infancy into childhood, plasma AG levels increased in the first 6 months, and after a decrease, they remained stable in the children with PWS. The decrease in AG levels was greater in controls, leading to higher plasma AG levels in the children with PWS after the age of 4 years. In contrast, plasma UAG levels were higher in the children with PWS during the first 3 years of life;

thereafter, plasma UAG levels were similar to controls. Together, this resulted in a low-normal AG/UAG ratio in the first 3 years of life, followed by an increasing AG/UAG ratio until the age of 4 years. From the age of 4 years, the AG/UAG ratio remained stable in the high-normal range of the controls.

The older children with PWS were heterogeneous with regard to their nutritional phases. As this might have influenced the average plasma AG and UAG levels and the AG/UAG ratio over time, we also investigated the longitudinal plasma AG and UAG levels and the AG/UAG ratio per nutritional phase from early infancy to the age of 7 years.

During early infancy, in phase 1a, we found higher plasma levels of UAG in children with PWS compared to controls, while the AG levels were normal, which resulted in a lower AG/UAG ratio in infants with PWS. This finding confirms the findings by Beauloye et al. [7], which also found elevated levels of UAG in infants with PWS, with normal AG levels (some infants in the present study were also included in that study). UAG is associated with a decreased food intake in mice and humans [16, 17]. Also, a high UAG and a low AG/UAG ratio have been found in adults with restrictive anorexia nervosa [34]. Nutritional phase 1a is characterized by failure to thrive and is thought to be related to hypotonia [35]. However, our findings suggest that the high UAG levels and low AG/UAG ratio might play a role in the cause of the failure to thrive, as these may lead to decreased appetite and thereby decreased food intake.

During phase 1b, we found a stable, low-normal AG/UAG ratio. This is in line with our expectations, as phase 1b is characterized by a period of appropriate growth without feeding problems. We found in phase 1b, a higher UAG compared to healthy controls, which is in line with previous findings [6], indicating that there are abnormalities in the ghrelin system, even during phase 1b.

Although the AG/UAG ratio in phase 2a was still lower compared to age-matched controls, the BMI was, for the first time, higher than in the age-matched controls in this phase. Phase 2a is characterized by a period with an excessive increase in body weight without an increase in calorie intake or interest in food. In line with our expectations, FM% SDS also increased significantly in phase 2a compared to phase 1b, suggesting impaired fat mass metabolism. The AG/UAG ratio in children with PWS started to increase during this phase, mainly due to a decrease in plasma UAG levels. Even stronger was the increase in AG/UAG ratio from phase 2a into phase 2b. Phase 2b is characterized by an increased interest in food and an increased appetite, leading to overweight when the child is unattended. BMI SDS in phase 2b was the highest BMI of all nutritional phases in our study and higher than in the age-matched controls. Additionally, FM% SDS was the highest in phase 2b and above the normal range. Interestingly, we also found a higher AG/UAG ratio in phase 2b compared to the controls. After entering phase 2b at the age of 4 years, the AG/UAG ratio remained stable until the age of 7 years. The higher ratio was mainly related to the higher AG levels in the children with PWS. As AG stimulates appetite and can induce a positive energy balance, the higher AG levels might play a role in the underlying mechanisms of the hyperphagia known in PWS.

The strength of this study is both the large cohort of 134 children with PWS and the longitudinal follow-up. To date, there is one other longitudinal study [27], but in that study, total ghrelin levels were measured instead of making a distinction in plasma AG and UAG levels. In that study, elevated total ghrelin levels were found in young children with PWS, long before the onset of hyperphagia. We found high UAG levels in early infancy and a low AG/UAG ratio. Later in childhood, we showed that the high total ghrelin can be explained by the higher AG levels, which resulted in a higher AG/UAG ratio. Given the opposite effects of AG and UAG [17, 20], the balance between the two forms of ghrelin is crucial.

At the start of our study, 56% of the children with PWS were already on GH treatment. The remaining children had not yet started GH treatment due to their young age, but treatment began soon after the start of the study (median age: 0.75). In previous studies, it was postulated that GH could influence ghrelin concentrations [36, 37]. In our previous cross-sectional study, there was no difference in the AG/UAG ratio between GH-treated and untreated patients with PWS [6]. Since children in our study population were very young at the start of GH treatment (0.76 years) and they all continued treatment during childhood, we believe that, if GH influences the course of the AG/UAG ratio, this effect is negligible in this study.

The nutritional phases according to Miller et al. [4] were used to score the feeding problems, in relation to the natural history of PWS, but in practice, the clinical presentation of children with PWS has significantly changed in today's population. Some children have hyperphagia without increased weight or food-seeking behavior, due to early diagnosis and GH treatment, improved health care, physiotherapy, diet regimen, and the awareness of parents to limit the food intake. Although the hyperphagia in a more controlled environment makes it difficult to categorize the child according to

nutritional phase, we could still, based on our expertise with PWS, determine the nutritional phases based on growth charts, food behavior, and consultation records. However, development of a more appropriate scoring system for today's population is warranted.

In our present study, none of the 134 children were in or had entered nutritional phase 3. As phase 3 is characterized by hyperphagia, it would have been useful to have information about the AG and UAG levels in this nutritional phase. In addition, having other appetite regulation hormone measured in present study would have provided a broader view of the pathogenesis of the development of hyperphagia in children with PWS. Although our findings are interesting and give more insight into the mechanism behind the switch in nutritional phases, more research on this topic is needed.

Although a higher nutritional phase was on average accompanied by an older age, an older age did not necessarily coincide with a higher nutritional phase, as several older children were still scored in nutritional phase 1b. The lower phase could have been due to underscoring of the hyperphagia in a well-controlled environment, but we tried to unravel why these children stayed in phase 1b. We could not link it to the genetic subtype, age at start of GH treatment, or other factors. It might be that a combination of factors (e.g., having more severe autistic characteristics, being on a strict diet, etc.) can influence the natural course of changing into different nutritional phases in PWS. It might also be that these children change to another nutritional phase at an older age.

The specific hyperphagia in PWS is best explained by the effects of AG, UAG, and especially the AG/UAG ratio [10]. The development of medication that interferes with the ghrelin system is, therefore, of great interest. Unfortunately, there is still no specific drug found that can treat the hyperphagia in PWS. There is one study investigating an UAG-analog [38], which might lower the AG/UAG ratio. Despite providing promising results in the beginning, the phase 2b study was terminated earlier than planned, due to a lack of significant improvement in hyperphagia and food-related behaviors after 3 months, particularly in adults. Since this UAG-analog did not influence the hyperphagia, it may be speculated that the high UAG levels in early life, play a role in the programming of the appetite system. Recently, an inhibitor of ghrelin O-acyltransferase (GOAT), the enzyme that converts UAG into AG, was investigated. Although treatment with the GOAT inhibitor resulted in a decrease of AG levels and increase of UAG levels, no effect on hyperphagia was found [39]. Further research for a solution for treating the hyperphagia in PWS is still needed and it might be that therapies targeting the ghrelin system should be tested in very young children with PWS.

In conclusion, this longitudinal study shows the course of the appetite-regulating hormones AG and UAG and the AG/UAG ratio from early infancy into childhood in a large cohort of children with PWS compared to a control group. We found that the change from failure to thrive to excessive weight gain and hyperphagia in infants and children with PWS coincides with an increase in AG/UAG ratio. In phase 1a, the plasma UAG levels were higher in children with PWS compared to the controls, while the AG levels were similar. This changed during childhood, leading to an increase in the AG/UAG ratio, which occurred even before the onset of hyperphagia. At the start of phase 2b, a significantly higher AG/UAG ratio was found, caused by higher plasma AG levels. Our results suggest that the longitudinal changes in the AG and UAG levels may be involved in the underlying mechanisms causing the switch in nutritional phases in PWS from the anorectic stage during phase 1a to the hyperphagia in phase 2b.

Acknowledgments

We express our gratitude to all children and their parents for their enthusiastic participation in this study. We want to thank ESPE for the financial support, and we thank M. van Eekelen, S. Faye, Dr. R. Kuppens (MD, PhD), Dr. N. Bakker (MD, PhD), Dr. L. Damen (MD, PhD), and Dr. S. Donze (MD, PhD) for all their help.

Statement of Ethics

The study was conducted in accordance with the Declaration of Helsinki and the ethical standards of Erasmus University Medical Center. The Dutch PWS Cohort study (MEC-2001-230, September 18, 2001) was approved by the Medical Ethics Committee of the Erasmus University Medical Center. Written informed consent was obtained from the participants' parent or legal guardian to participate in the study.

Conflict of Interest Statement

The authors declare no conflict of interest.

Funding Sources

This collaborative project by M.T. (coordinator), A.H.K., and V.B. was supported by a grant from the research unit of the European Society for Paediatric Endocrinology (ESPE) obtained in 2012.

Author Contributions

Conceptualization: Lionne Grootjen and Anita Hokken-Koelega; data curation: Lionne Grootjen, Gwenaelle Diene, Catherine Molinas, Véronique Beauloye, and Maithe Tauber; formal analysis and writing – original draft: Lionne Grootjen; funding acquisition and resources: Maithe Tauber and Anita Hokken-Koelega; investigation: Lionne Grootjen, Martin Huisman, Jenny Visser, Patric Delhanty, Maithe Tauber, and Anita Hokken-Koelega; methodology: Lionne Grootjen, Gerthe Kekhof, Maithe Tauber, and Anita Hokken-Koelega; project administration: Lionne Grootjen, Catherine Molinas, Gerthe Kerkhof, Maithe Tauber, and Anita Hokken-Koelega; supervision: Gerthe Kerkhof, Maithe Tauber and Anita Hokken-Koelega; and writing – review and editing: Lionne Grootjen, Gwenaelle Diene, Catherine Molinas, Véronique Beauloye, Martin Huisman, Jenny Visser, Patric Delhanty, Gerthe Kerkhof, Maithe Tauber, and Anita Hokken-Koelega.

Data Availability Statement

Data are not publicly available due to ethical reasons. Further inquiries can be directed to the corresponding author.

References

1 Cassidy SB, Driscoll DJ. Prader-Willi syndrome. Eur J Hum Genet. 2009;17(1):3–13.
2 Goldstone AP, Holland AJ, Hauffa BP, Hokken-Koelega AC, Tauber M; speakers contributors at the Second Expert Meeting of the Comprehensive Care of Patients with PWS. Recommendations for the diagnosis and management of Prader-Willi syndrome. J Clin Endocrinol Metab. 2008;93(11):4183–97.
3 Deal CL, Tony M, Hoÿbye C, Allen DB, Tauber M, Christiansen JS, et al. Growth hormone research society workshop summary: consensus guidelines for recombinant human growth hormone therapy in Prader-Willi syndrome. J Clin Endocrinol Metab. 2013;98(6):E1072–87.
4 Miller JL, Lynn CH, Driscoll DC, Goldstone AP, Gold JA, Kimonis V, et al. Nutritional phases in Prader-Willi syndrome. Am J Med Genet A. 2011;155A(5):1040–9.
5 Holm VA, Cassidy SB, Butler MG, Hanchett JM, Greenswag LR, Whitman BY, et al. Prader-Willi syndrome: consensus diagnostic criteria. Pediatrics. 1993;91(2):398–402.
6 Kuppens RJ, Diène G, Bakker NE, Molinas C, Faye S, Nicolino M, et al. Elevated ratio of acylated to unacylated ghrelin in children and young adults with Prader–Willi syndrome. Endocrine. 2015;50(3):633–42.
7 Beauloye V, Diene G, Kuppens R, Zech F, Winandy C, Molinas C, et al. High unacylated ghrelin levels support the concept of anorexia in infants with prader-willi syndrome. Orphanet J Rare Dis. 2016;11(1):56.
8 Feigerlová E, Diene G, Conte-Auriol F, Molinas C, Gennero I, Salles JP, et al. Hyperghrelinemia precedes obesity in Prader-Willi syndrome. J Clin Endocrinol Metab. 2008;93(7):2800–5.
9 Haqq AM, Grambow SC, Muehlbauer M, Newgard CB, Svetkey LP, Carrel AL, et al. Ghrelin concentrations in Prader-Willi syndrome (PWS) infants and children: changes during development. Clin Endocrinol. 2008;69(6):911–20.
10 Tauber M, Coupaye M, Diene G, Molinas C, Valette M, Beauloye V. Prader-Willi syndrome: a model for understanding the ghrelin system. J Neuroendocrinol. 2019;31(7):1–13.
11 Müller TD, Nogueiras R, Andermann ML, Andrews ZB, Anker SD, Argente J, et al. Ghrelin. 2015;4(6):437–60.
12 Van Der Lely AJ, Tschöp M, Heiman ML, Ghigo E. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocr Rev. 2004;25(3):426–57.
13 Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, et al. Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab. 2001;86(12):5992.
14 Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature. 2000;407(6806):908–13.
15 Theander-Carrillo C, Wiedmer P, Cettour-Rose P, Nogueiras R, Perez-Tilve D, Pfluger P, et al. Ghrelin action in the brain controls adipocyte metabolism. J Clin Invest. 2006;116(7):1983–93.
16 Asakawa A, Inui A, Fujimiya M, Sakamaki R, Shinfuku N, Ueta Y, et al. Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut. 2005;54(1):18–24.
17 Delhanty PJD, Neggers SJ, Van Der Lely AJ. Mechanisms in endocrinology: ghrelin: the differences between acyl- and des-acyl ghrelin. Eur J Endocrinol. 2012;167(5):601–8.
18 Inhoff T, Mönnikes H, Noetzel S, Stengel A, Goebel M, Dinh QT, et al. Desacyl ghrelin inhibits the orexigenic effect of peripherally injected ghrelin in rats. Peptides. 2008;29(12):2159–68.
19 Tam BT, Pei XM, Yung BY, Yip SP, Chan LW, Wong CS, et al. Unacylated ghrelin restores insulin and autophagic signaling in skeletal muscle of diabetic mice. Pflugers Arch. 2015;467(12):2555–69.
20 Özcan B, Neggers SJCMM, Miller AR, Yang HC, Lucaites V, Abribat T, et al. Does desacyl ghrelin improve glycemic control in obese diabetic subjects by decreasing acylated ghrelin levels? Eur J Endocrinol. 2014;170(6):799–807.
21 Cederberg H, Rajala U, Koivisto V-M, Jokelainen J, Surcel H-M, Keinänen-Kiukaanniemi S, et al. Unacylated ghrelin is associated with changes in body composition and body fat distribution during long-term exercise intervention. Eur J Endocrinol. 2011;165(2):243–8.
22 Pacifico L, Poggiogalle E, Costantino F, Anania C, Ferraro F, Chiarelli F, et al. Acylated and nonacylated ghrelin levels and their associations with insulin resistance in obese and normal weight children with metabolic syndrome. Eur J Endocrinol. 2009;161(6):861–70.
23 St-Pierre DH, Karelis AD, Coderre L, Malita F, Fontaine J, Mignault D, et al. Association of acylated and nonacylated ghrelin with insulin sensitivity in overweight and obese postmenopausal women. J Clin Endocrinol Metab. 2007;92(1):264–9.
24 Butler MG, Bittel DC. Plasma obestatin and ghrelin levels in subjects with Prader-Willi syndrome. Am J Med Genet A. 2007;143A(5):415–21.
25 Goldstone AP, Holland AJ, Butler JV, Whittington JE. Appetite hormones and the transition to hyperphagia in children with Prader-Willi syndrome. Int J Obes. 2012;36(12):1564–70.
26 Erdie-Lalena CR, Holm VA, Kelly PC, Frayo RS, Cummings DE. Ghrelin levels in young children with Prader-Willi syndrome. J Pediatr. 2006;149(2):199–204.
27 Kweh FA, Miller JL, Sulsona CR, Wasserfall C, Atkinson M, Shuster JJ, et al. Hyperghrelinemia in Prader-Willi syndrome begins in early infancy long before the onset of hyperphagia. Am J Med Genet A. 2015;167A(1):69–79.
28 Fredriks AM, Van Buuren S, Burgmeijer RJF, Meulmeester JF, Beuker RJ, Brugman E, et al. Continuing positive secular growth change in The Netherlands 1955-1997. Pediatr Res. 2000;47(3):316–23.

29 Fredriks AM, Van Buuren S, Wit JM, Verloove-Vanhorick SP. Body index measurements in 1996-7 compared with 1980. Arch Dis Child. 2000;82(2):107–12.
30 Sempé M, Pédron G, Roy-Pernot M. Auxologie: méthode et séquences. Paris: Théraplix; 1979.
31 Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ. 2000;320(7244):1240–3.
32 Boot AM, Bouquet J, De Ridder MAJ, Krenning EP, De Muinck Keizer-Schrama SMPF. Determinants of body composition measured by dual-energy X-ray absorptiometry in Dutch children and adolescents. Am J Clin Nutr. 1997;66(2):232–8.
33 van Beijsterveldt IALP, van der Steen M, de Fluiter KS, Spaans SAMJ, Hokken-Koelega ACS. Body composition and bone mineral density by Dual Energy X-ray Absorptiometry: reference values for young children. Clin Nutr. 2022;41(1):71–9.
34 Germain N, Galusca B, Grouselle D, Frere D, Tolle V, Zizzari P, et al. Ghrelin/obestatin ratio in two populations with low bodyweight: constitutional thinness and anorexia nervosa. Psychoneuroendocrinology. 2009;34(3):413–9.
35 Bacheré N, Diene G, Delagnes V, Molinas C, Moulin P, Tauber M. Early diagnosis and multidisciplinary care reduce the hospitalization time and duration of tube feeding and prevent early obesity in PWS infants. Horm Res. 2008;69(1):45–52.
36 Hauffa BP, Petersenn S. GH treatment reduces total ghrelin in Prader-Willi syndrome (PWS) and may confound ghrelin studies in young PWS children. Clin Endocrinol. 2009;71(1):155–6.
37 Hauffa BP, Haase K, Range IM, Unger N, Mann K, Petersenn S. The effect of growth hormone on the response of total and acylated ghrelin to a standardized oral glucose load and insulin resistance in children with Prader-Willi syndrome. J Clin Endocrinol Metab. 2007;92(3):834–40.
38 Allas S, Caixàs A, Poitou C, Coupaye M, Thuilleaux D, Lorenzini F, et al. AZP-531, an unacylated ghrelin analog, improves food-related behavior in patients with Prader-Willi syndrome: a randomized placebo-controlled trial. PLoS One. 2018;13(1):e0190849.
39 Miller JL, Lacroix A, Bird LM, Shoemaker AH, Haqq A, Deal CL, et al. The efficacy, safety, and pharmacology of a ghrelin O-acyltransferase inhibitor for the treatment of prader-willi syndrome. J Clin Endocrinol Metab. 2022;107(6):e2373–80.