A Randomized Trial of the Effect of a GnRH Analogue Injection on Ghrelin Levels in Girls

Maria Rodanaki*a, Eva Raskb, Maria Lodefalka, b

*Department of Paediatrics, Faculty of Medicine and Health, Örebro University, Örebro, Sweden;
 bUniversity Health Care Research Centre, Faculty of Medicine and Health, Örebro University, Örebro, Sweden

Keywords
Acylated ghrelin · Central precocious puberty · Desacylated ghrelin · GnRH analogue · Protease inhibitor

Abstract
Introduction: Ghrelin concentrations decline during puberty by an unclear mechanism. Acylated ghrelin (AG) is unstable in sampling tubes, but no standardized sampling protocol exists. We hypothesized that ghrelin levels decrease as a consequence of increased gonadotropin-releasing hormone (GnRH) signalling and that the addition of a protease inhibitor to sampling tubes preserves the AG levels. Methods: In this randomized, placebo-controlled, cross-over study, 13 girls with suspected central precocious puberty were included. They performed an adjusted GnRH stimulation test twice and were given Relefact LHRH® (100 μg/m2) or saline in a randomized order. Blood was sampled repeatedly for 150 min for the analysis of hormone concentrations. Oestradiol levels were only measured at baseline. The protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) was added to the sampling tubes. Specific ELISA kits were used for the analysis of AG and desacylated ghrelin (DAG) levels. Results: Neither AG nor DAG levels changed after GnRH analogue injection in comparison to saline. The addition of AEBSF preserved AG levels (650.1 ± 257.1 vs. 247.6 ± 123.4 pg/mL, p < 0.001) and decreased DAG levels (51.9 [12.5–115.7] vs. 143.5 [71.4–285.7] pg/mL, p < 0.001). Both AG and DAG levels were inversely associated with insulin levels (r = −0.73, p = 0.005, and r = −0.78, p = 0.002, respectively). AG levels were inversely associated with oestradiol levels (rho = −0.57, p = 0.041). Conclusion: Ghrelin levels do not decrease following a pharmacological dose of a GnRH analogue in the short term in girls. Addition of a protease inhibitor to the sampling tubes decreases AG degradation, resulting in preserved AG and decreased DAG levels.

Introduction

Puberty starts when the hypothalamic gonadotropin-releasing hormone (GnRH) activates the maturation of gonadotropin-producing cells in the pituitary gland and subsequently the gonads. Precocious puberty is defined...
as the onset of puberty before the age of 8 years in girls and 9 years in boys and mostly affects girls [1]. The timing of the start of puberty is influenced by genetic, metabolic and lifestyle factors [2, 3], and even though the exact underlying mechanisms have not yet been clarified it is clear that multiple peptides are involved in the pubertal onset and one of them is ghrelin.

Ghrelin is produced mainly in the stomach transmitting a hunger signal to the hypothalamus [4–7]. It also plays a role in reproductive physiology, possibly transferring metabolic information to the reproductive system [8]. Previous data have shown that ghrelin inhibits gonadotropin secretion, lowering the circulating luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels, and exerts a direct effect on the gonads [7, 9–11]. However, during puberty, the circulating levels of ghrelin decline successively regardless of sex [12–15]. The mechanism behind this decline is not fully known, but the gonads may play a role since a negative correlation between serum testosterone and ghrelin levels has been found in boys [16]. However, studies in girls have shown a positive association between oestrogen and ghrelin levels [17–19]. Other potential mechanisms for decreasing ghrelin levels include increased GnRH signalling, which is in agreement with a finding of lower ghrelin levels in girls with central precocious puberty (CPP) treated with a long-acting GnRH analogue [19]. Increased body mass index (BMI) and potential insulin resistance are other potential drivers of the pubertal decline in ghrelin levels, as reciprocal levels of insulin and ghrelin have been frequently reported [14, 20], and puberty is associated with increased insulin levels [21]. Taken together, GnRH may be directly involved in the pubertal decline of ghrelin levels, but this suggestion needs further investigation.

The unique acylation of the ghrelin peptide renders ghrelin able to cross the blood-brain barrier and is crucial for binding to the receptor GHS-R type 1a (GHSR1a) [20]. Both acylated (AG) and desacylated ghrelin (DAG) are found in the circulation. As DAG is unable to bind and activate GHSR1a, it was initially considered to be biologically inactive. However, DAG has been reported as a functional antagonist of AG or may in some cases work independently [22]. AG is easily degraded in blood sampling tubes, hampering accurate measurements of its concentrations. Different methods for the preservation of AG have been suggested, such as cold handling of the tubes and the addition of the protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) [23–25] or aprotinin with or without plasma acidification [26]. There is no current consensus on a standardized method of handling sampling tubes for the analysis of AG and DAG levels, especially not in children, limiting accurate measurement and interpretation of ghrelin levels in both clinical practice and research.

In the present study, we hypothesized that the circulating levels of AG decrease during puberty as a direct consequence of the increased GnRH activity and that the addition of AEBSF to sampling tubes significantly alters both AG and DAG concentrations in children by blocking AG degradation. The specific aims were to investigate AG and DAG levels during a GnRH stimulation test in girls with suspected CPP and to compare these concentrations in samples with and without AEBSF and in samples with and without plasma acidification. Furthermore, we aimed to analyse potential associations between ghrelin and insulin, glucose, gonadotropins, and oestriadiol levels.

Materials and Methods

Study Design
This was a randomized, placebo-controlled, cross-over, single-blinded, multicentre experimental study on 13 girls.

Study Population
We aimed at studying the potential effects of a GnRH analogue injection on ghrelin levels in children while at the same time minimizing the number of medical procedures performed without a clear clinical benefit to the child. Therefore, we identified the patient group that is subjected to most GnRH stimulation tests [27] at our institution, and that was girls with suspected CPP. Thus, eligible patients for this study were girls with suspected CPP at the Departments of Paediatrics at Örebro University Hospital, Örebro, and at Uppsala University Hospital, Uppsala, Sweden. The clinical suspicion of CPP was raised by physical examination, medical history or both. The clinical situation indicated to the responsible paediatric endocrinologist that a GnRH stimulation test was clinically justified. Precocious puberty was defined as breast development to at least Tanner stage B2 before the age of 8 years [1]. A diagnosis of CPP did not have to be confirmed prior to inclusion in the study nor was inclusion restricted to a specific Tanner stage. Exclusion criteria were age <1 year, body weight <12 kg, any known syndrome, a previously diagnosed hypothalamic or pituitary tumour or malformation, untreated thyroid disease, diabetes mellitus, obesity (defined as BMI >3 standard deviation scores according to Swedish reference data [28]), GH treatment, and previous surgery or disease affecting the stomach. The girls, as well as their guardians, gave written informed consent to participate in the study. The study was conducted in accordance with the Declaration of Helsinki, approved by the Regional Board of Ethics in Uppsala, Sweden (2015/028), and registered at ClinicalTrials.gov (NCT02431416).

Procedures
Pubertal staging was performed by a paediatric endocrinologist in accordance with Tanner staging [29]. All girls performed an ad-
justed GnRH stimulation test twice. In a randomized order, they received the active substance, the GnRH analogue (Relefact LHRH®), on one of the test occasions, and on the other occasion, they received a placebo injection (NaCl 9 mg/mL). The Relefact LHRH® dose was 100 μg/m² body surface (maximum dose: 100 μg). Saline was given at the same volume as the volume used for each girl when Relefact LHRH® was injected. The randomization was performed in Örebro by the use of prearranged closed envelopes containing a paper in each envelope with either the word “GnRH” or the word “placebo,” which stated what substance should be given on the first occasion. The patient and her guardians were blinded to what substance was injected. The tests were performed in the morning (starting at 8:00 a.m.) in the fasting state. Each test was prolonged by 30 min compared to an ordinary GnRH stimulation test [27]. Blood samples were collected immediately before (time 0) and at 30, 60, 90, 120, and 150 min after the injection of either Relefact LHRH® or NaCl. The following cut-off levels were used for diagnosing CPP: baseline LH >0.3 IU/L, maximum stimulated LH >5 IU/L, or a ratio of maximum stimulated LH/FSH >0.66 [30–32].

Weight, height, and abdominal circumference were measured, and an intravenous cannula was placed in each forearm on each test occasion. If the test was performed >1 month after the last pubertal assessment, the pubertal stage was reassessed. The body weight had to be stable between the two test occasions (defined as <10% difference). The time period between the two test occasions

Fig. 1. The procedures followed for the tubes used for the analysis of acylated and desacylated ghrelin levels.
had to be ≥1 week but <1 month. Girls were asked to avoid excessive physical exercise and had to be free from gastrointestinal infection on the days before a test was performed.

For the analysis of AG and DAG levels, all handling and processing of sampling tubes were performed on ice. Three millilitres of blood were collected in precooled K2-EDTA sampling tubes when AEBSF was added (Fig. 1; Table 1). AEBSF was added within 2 min after blood sampling to a final concentration of 2 mg/mL as suggested previously [24]. To minimize the volume of blood collected from each girl, only 2 mL of blood was collected in K2-EDTA sampling tubes when no inhibitor was added (Fig. 1; Table 1). Cooled centrifugation (4°C, 2,000 g for 15 min) was then performed within 30 min of blood sampling. Hydrochloric acid (HCl, 1 mol/L) was added to approximately half of the plasma sample derived from the AEBSF blood to a final concentration of 50 μmol/L for plasma acidification, as suggested previously [26]. Finally, plasma with and without acidification was allocated to precooled microtubes and stored frozen (−80°C) until further processing (Fig. 1).

Laboratory Analyses
AG, DAG, LH, FSH, insulin, and glucose concentrations were analysed at every time point, while oestradiol levels were analysed at time point 0 min only (Table 1). Plasma glucose concentrations were analysed immediately after each sampling using a bedside method (MediSense Precision PCx, Abbott®, in Örebro and FreeStyle Precision Pro, Abbott®, in Uppsala). The other samples were stored frozen at −80°C until analysis. Serum FSH, LH, and insulin concentrations were analysed at the Department of Laboratory Medicine, Örebro University Hospital, using the Architect i2000 immunoassay analyser (Abbott Laboratories®, Chicago, IL, USA) in accordance with the manufacturer’s instructions and clinical routines. Serum oestradiol concentrations were analysed using an ultrasensitive radioimmunoochemical assay, the Modified Cisbio® Oestradiol RIA with pre-extraction (Cisbio Bioassays®, Codolet, France), at the Gothenburg University Laboratory of Growth, Gothenburg, Sweden, with a detection limit of 4 pmol/L. Plasma AG concentrations were analysed using the Millipore® (Missouri, USA) Human Ghrelin (Active) ELISA kit.

Table 1. Time points for blood collection for the analysis of oestradiol, glucose, insulin, FSH, LH, and ghrelin concentrations

| Time, min | Oestradiol | Glucose | Insulin, FSH, LH | Ghrelin (with AEBSF) | Ghrelin (without AEBSF) |
|-----------|------------|---------|-----------------|----------------------|------------------------|
| 0         | X          | X       | X               | X                    | X                      |
| 30        | X          | X       | X               | X                    |                        |
| 60        | X          | X       | X               | X                    |                        |
| 90        | X          | X       | X               | X                    |                        |
| 120       | X          | X       | X               | X                    |                        |
| 150       | X          | X       | X               | X                    |                        |

FSH, follcle-stimulating hormone; LH, luteinizing hormone; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride. ¹Both acylated and desacylated ghrelin levels were analysed.

Table 2. Clinical characteristics, baseline, and GnRH stimulated hormone concentrations in every girl participating in the study

| Patient number | Age, years | BMI z-score | Pubertal stage | Basal LH, IU/L | Peak stimulated LH, IU/L | Basal FSH, IU/L | Peak stimulated FSH, IU/L | Maximum stimulated LH/FSH ratio | Basal oestradiol, pmol/L | Basal acylated ghrelin (with AEBSF), pg/mL |
|----------------|------------|-------------|----------------|----------------|--------------------------|----------------|-----------------------------|-------------------------------|---------------------|------------------------------------------|
| 1              | 7.5        | −0.8        | B2             | 0.09           | 3.71                     | 2.32           | 15.65                       | 0.29                          | 11.0                | 996.07                                   |
| 2              | 6.6        | +1.0        | B2             | 0.47           | 15.36                    | 2.14           | 7.84                        | 2.41                          | 16.0                | 1,252.10                                 |
| 3              | 8.4        | +1.0        | B3             | 1.92           | 9.89                     | 5.90           | 11.22                       | 1.07                          | 94.0                | 513.82                                   |
| 4              | 7.4        | +1.8        | B3             | 0.06           | 6.05                     | 2.98           | 22.52                       | 0.35                          | 8.5                 | 463.04                                   |
| 5              | 6.5        | −0.5        | B3             | 0.03           | 2.81                     | 1.83           | 14.07                       | 0.25                          | 17.0                | 541.84                                   |
| 6              | 8.0        | −1.0        | B3             | 0.08           | 1.42                     | 1.69           | 9.79                        | 0.17                          | 11.0                | 1,047.25                                 |
| 7              | 7.5        | +1.3        | B2             | 0.06           | 3.34                     | 1.66           | 10.03                       | 0.42                          | 9.0                 | 650.70                                   |
| 8              | 7.5        | +0.2        | B3             | 0.16           | 12.14                    | 2.38           | 16.65                       | 0.90                          | 9.5                 | 577.53                                   |
| 9              | 9.0        | +0.5        | B4             | 2.63           | 11.71                    | 4.70           | 6.88                        | 1.88                          | 159.0               | 277.79                                   |
| 10             | 10.1       | −0.3        | B4             | 2.64           | 12.95                    | 5.73           | 10.1                        | 1.49                          | 104.0               | 299.99                                   |
| 11             | 9.0        | +2.3        | B3             | 1.02           | 28.85                    | 3.16           | 13.73                       | 2.37                          | 15.0                | 349.46                                   |
| 12             | 9.1        | −0.7        | B2             | 0.42           | 3.95                     | 5.60           | 14.35                       | 0.33                          | 65.0                | 626.73                                   |
| 13             | 8.2        | +2.6        | B2             | 0.12           | 2.42                     | 4.13           | 15.66                       | 0.20                          | 8.0                 | 759.81                                   |

GnRH, gonadotropin-releasing hormone; BMI, body mass index; LH, luteinizing hormone; FSH, follicle-stimulating hormone; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride.
Unacylated Ghrelin (human) Easy Sampling EIA Kit (#501190) from Cayman Chemicals® (Michigan, USA) with a limit of detection of 2 pg/mL, inter- and intra-assay CV (%) of 3.2–13.2, and <0.06% cross-reactivity with human AG. ELISA analyses were performed by trained personnel at the Clinical Research Laboratory, Örebro University Hospital, according to the manufacturers’ instructions.

Statistical Analysis

Power analyses were performed prior to the study based on previously published ghrelin concentrations in children and adults, but the available data on children was limited. When using \( \alpha = 0.05 \), power = 85%, and assuming a mean difference in the change from baseline to nadir for AG concentrations of 150 pg/mL between the two test occasions and SD = 110 pg/mL [13, 33, 34], at least seven girls would be needed in the study. Due to the uncertainty in the estimation, 13 girls were included in the study.

Data are presented as absolute numbers (percent), mean (SD) or median (min-max), as appropriate. The areas under the curve (AUC) for AG and DAG concentrations were calculated according to the trapezoid rule. Delta values were calculated as the maximal change from each baseline value (time 0 min). Comparisons in AUC and delta values between the Relefact LHRH® test and the NaCl test were performed using the paired-samples t test and Wilcoxon signed-ranks test, as appropriate. Pearson’s correlation and Spearman’s rank tests were used, as appropriate, to investigate associations between variables. To compare the effect of different types of additives on the levels of AG and DAG, one-way ANOVA for repeated measurements with Bonferroni correction for post hoc pairwise comparisons was used. As the variables were not normal-

Fig. 2. FSH (IU/L) (a), LH (IU/L) (b), glucose (mmol/L) (c), and insulin (mIU/L) (d) levels (mean ±1 SD) in 13 girls with suspected central precocious puberty before and after intravenous injection of Relefact LHRH® (100 μg/m²) or placebo (NaCl, 9 mg/mL).
ly distributed, the natural logarithms were used in this comparison. Significance was set at \( p < 0.05 \) for two-sided tests. Calculations were performed in the statistical software package IBM® SPSS Statistics for Windows, version 26 (IBM Corp.®, Armonk, NY, USA).

**Results**

The 13 included girls performed the adjusted GnRH stimulation tests between August 2015 and November 2017. Their individual ages, BMI \( z \)-scores, pubertal stages, baseline, and stimulated hormone concentrations are presented in Table 2. A diagnosis of CPP was established in eight girls after the performance of the stimulation test using the definition mentioned above (under procedures). The efficiency of Relefact LHRH\(^®\) in inducing a gonadotropin response in the study population is clearly shown in Figure 2. The glucose and insulin concentrations gradually declined during both occasions as fasting proceeded (Fig. 2).

**Effect of GnRH Analogue Injection on AG and DAG Levels**

Neither AUC nor delta values for AG concentrations differed between the two test occasions. The type of additive (AEBSF only or AEBSF + HCl) did not influence these findings (Fig. 3a, b). Dividing the study period into shorter periods did not change this finding. Neither AUC for AG (0–90 min) nor AUC for AG (0–120 min) differed significantly between the two test occasions. There were no significant differences in AG concentrations between the two test occasions when two outliers were excluded.
Stratifying the study population for CPP diagnosis did neither effect the results. When comparing the AUCs for DAG levels and the ratio of DAG/AG levels between the two test occasions, no significant differences between the test occasions were found (Fig. 3c, d).

**Associations between Ghrelin and Other Hormone Concentrations**

Both mean AG and DAG levels were inversely correlated with mean insulin levels at baseline when using AEBSF only ($r = -0.73, p = 0.005$, and $r = -0.78, p = 0.002$, respectively). Similar associations were found when analysing separate and not mean values (from the two test occasions), when using values from tubes with both AEBSF and HCl, when using values from the 150 min time point and when analysing the corresponding AUC values. Mean AG levels were inversely correlated with mean FSH levels at baseline ($r = -0.57, p = 0.043$), but AUC values for AG and FSH (after the saline injection) did not correlate, nor did AG and FSH levels at the 150 min time point after the saline injection. Mean AG levels were inversely correlated with mean oestradiol levels at baseline (rho = −0.57, $p = 0.041$).

**The Effects of Additives on AG and DAG Levels**

The addition of AEBSF to sampling tubes significantly altered baseline AG concentrations (650.1 ± 257.1 pg/mL vs. 247.6 ± 123.4 pg/mL, $p < 0.001$, Fig. 4a) and decreased baseline DAG concentrations (51.9 pg/mL [12.5–115.7] vs. 143.5 pg/mL [71.4–285.7], $p < 0.001$, Fig. 4b). Similar results were obtained using the concentrations at 150 min. The addition of HCl to the plasma did not significantly alter the baseline AG levels compared to AEBSF only (681.2 ± 299.1 pg/mL vs. 650.1 ± 257.1 pg/mL, ns.) but increased the baseline DAG levels compared to the addition of AEBSF only (63.8 pg/mL [25.6–124.8] vs. 51.9 pg/mL [12.5–115.7], $p < 0.001$, Fig. 4b).

**Discussion**

In this study, we showed for the first time that the GnRH analogue administered during a GnRH stimulation test did not affect the plasma levels of AG or DAG up to 150 min after the injection. In contrast, adding a protease inhibitor to the sampling tubes, with or without plasma acidification, profoundly affected the levels of...
both AG and DAG. The addition of AEBSF led to significantly higher levels of AG and significantly lower levels of DAG, consistent with reduced AG degradation. However, despite the careful handling of the sampling tubes in this study, DAG levels were still detectable, indicating that DAG is probably not just an artefact created in sampling tubes, as suggested previously [35]. Finally, we confirmed the negative association between ghrelin and insulin levels repeatedly found previously [14, 20, 36, 37], but to the best of our knowledge, we showed for the first time a negative association between AG and oestradiol levels.

Although our findings do not support an inhibitory effect of GnRH on ghrelin secretion, we cannot completely exclude the possibility that GnRH signalling may be directly involved in the pubertal decline in ghrelin levels. In the present study, one pharmacological dose of a GnRH analogue was studied for only 150 min and only in girls. Future studies should investigate the effects of physiological GnRH signalling for longer periods in larger study populations including both sexes. Other potential mechanisms behind the pubertal decline in ghrelin levels include increased sex steroid levels, but previous findings have been discordant. During normal puberty, the levels of sex steroids rise, whereas the levels of ghrelin decline [38], and a negative correlation between serum testosterone and ghrelin concentrations has been found in boys [16]. Although oestrogen replacement therapy increased the levels of ghrelin in adult postmenopausal women [17], sex hormone administration did not affect the levels of ghrelin in peripubertal girls [18], whereas during treatment with a long-acting GnRH analogue, the levels of both oestradiol and ghrelin decreased in girls [19], indicating a possible positive correlation between ghrelin and oestradiol concentrations. On the other hand, in the present study, a negative correlation between oestradiol and AG levels was observed. A negative correlation between DAG and oestradiol levels has been reported previously in both normal weight and obese children, but the association for AG was only weak and not statistically significant in a study including 76 girls [14]. However, no protease inhibitor or other measures to prevent AG degradation were used in that study, which probably affected the ghrelin results. It is possible that increased sex steroid levels during early puberty, potentially in conjunction with other pubertal changes such as increased insulin levels, decrease AG secretion through a negative feedback mechanism. The resulting reduced plasma AG concentrations may signal to the hypothalamus that the energy stores and the metabolic status of the body are sufficient for the acquisition of full reproductive function, in line with earlier suggestions [11], permitting continued pubertal development. Future studies including larger sample sizes with the possibility to perform adjustments for potential confounding factors are needed to confirm our finding of a negative association between oestradiol and AG levels. Future studies are also needed to further investigate mechanisms involved in the pubertal ghrelin decline.

The levels of AG found in the present study were in good agreement with the levels found previously in healthy adolescent boys [39] but considerably higher than the levels found in children of similar ages [12, 40] and lower than the levels described previously in girls with CPP [19]. These differences may depend on differences in food intake, as higher levels are expected in the fasting state, which was the case in our study but not in other studies [12, 40]. Other explanations include differences in sampling procedures, inhibitors and assays used, but also the fact that our study population included girls in different pubertal stages, hampering comparisons.

The role of DAG has been debated. It was initially proposed to be a degradation product of AG only since it is unable to bind and activate GHSR1a [4]. However, more recent studies indicate that DAG acts like a separate hormone, either interacting with AG or having AG-independent effects [41]. Consequently, accurate measurements of both AG and DAG levels are important, and the findings of the present study clearly show the effects of different handling of sampling tubes on both AG and DAG levels. The low levels of AG, together with the high levels of DAG found in samples without the protease inhibitor in the present study, confirm that AG is unstable and that degradation occurs rapidly in blood sampling tubes [26, 42]. This finding underlines the need for special procedures to minimize AG degradation. Following the protocol proposed by Blatnik and Soderstrom [24] and used in previous studies [23], also including paediatric populations [25], we confirmed that the addition of AEBSF was critical to decreasing the degradation of AG. However, the acidification of plasma did not result in significantly higher AG levels compared to AEBSF only, even though it has been recommended for the preservation of plasma ghrelin [26]. We suggest that cool handling, the addition of AEBSF to the sampling tubes and centrifugation within 30 min should be considered in future standardized protocols for proper measurements of AG and DAG levels in children.

The strengths of the present study included the randomized, placebo-controlled, single-blinded design and

DOI: 10.1159/000526147
the use of the study subjects as their controls, minimizing the impact of large inter-individual variations in ghrelin concentrations. Other strengths were the careful handling of the sampling tubes and the standardized condition applied for each subject on the test occasions. Limitations were the small study population and the inclusion of girls in different pubertal stages and ages, some of them having CPP and some of them not, possibly leading to a larger variation in ghrelin levels. However, the use of a cross-over design decreased the impact of this limitation. Another limitation of the study was the use of two different methods for the analysis of plasma glucose levels, but it was unfortunately not possible to change the methods used at the hospitals involved in the study. On the other hand, plasma glucose levels obtained in the present study did not contribute to our main results.

In conclusion, the circulating levels of ghrelin were not affected in the short term by an intravenous injection of a GnRH analogue in girls. A negative correlation between AG and oestriadiol levels was found, which ought to be validated in other populations. Special procedures, including the addition of a protease inhibitor such as AEBSF, are needed to minimize the degradation of AG in sampling tubes. We recommend such handling for proper quantification of AG and DAG levels in future studies and clinical practice.

Acknowledgments

We thank Maria Halldin, MD, PhD, a paediatric consultant at Uppsala University Hospital at the time of the study, for the inclusion of some of the patients. We thank the specialist nurses Petra Renholm (Uppsala) and Elisabeth Särnblad, Anna Onelöv, and Ewa Bergenwall (Örebro) for performing the stimulation tests, including the demanding blood sampling, as well as Elisabet Tina and Seta Kurt for their valuable work with the ELISA analyses at the Clinical Research Laboratory, Örebro University Hospital.

References

1 Klein KO. Precocious puberty: who has it? Who should be treated? J Clin Endocrinol Metab. 1999 Feb;84(2):411–4.
2 Palmert MR, Boepple PA. Variation in the timing of puberty: clinical spectrum and genetic investigation. J Clin Endocrinol Metab. 2001 Jun;86(6):2364–8.
3 Parent AS, Teilmann G, Juul A, Skakkebaek NE, Toppari J, Bourguignon JP. The timing of normal puberty and the age limits of sexual precocity: variations around the world, secular trends, and changes after migration. Endocr Rev. 2003 Oct;24(5):668–93.
4 Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 1999 Dec 9;402(6762):656–60.
5 Kojima M, Kangawa K. Ghrelin: structure and function. Physiol Rev. 2005 Apr;85(2):495–522.
6 Perchard R, Clayton PE. Ghrelin and growth. Endocr Dev. 2017;32:74–86.
7 Akalu Y, Molla MD, Dessie G, Ayelign B. Physiological effect of ghrelin on body systems. Int J Endocrinol. 2020;2020:1385138.
8 Tena-Sempere M. Exploring the role of ghrelin in as novel regulator of gonadal function. Growth Horm IGF Res. 2005 Apr;15(2):83–8.
9 Fernandez-Fernandez R, Tena-Sempere M, Navarro VM, Barreiro ML, Castellano JM, Aguilar E, et al. Effects of ghrelin upon gonadotropin-releasing hormone and gonadotropin secretion in adult female rats: in vivo and in vitro studies. Neuroendocrinology. 2005;82(5–6):245–55.

Statement of Ethics

The girls included in the study, as well as their guardians, gave written informed consent to participate in the study, which was approved by the Regional Board of Ethics in Uppsala, Sweden (2015/028), and registered at ClinicalTrials.gov (NCT02431416).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This study received financial support from the Research Committee and ALF funding, Region Örebro County, Sweden, and from the Regional Research Council Mid Sweden. The funders did not influence any part of the research project, the interpretation of the results, the preparation of the manuscript or the decision to submit the manuscript to a journal.

Author Contributions

Dr. Maria Lodefalk initiated the study, received funding and ethical permission, was responsible for the study design, and recruited patients. Dr. Maria Rodanaki participated in recruiting patients, controlled the accuracy of the data, performed the statistical analysis and all figures, and wrote the first draft of the manuscript. Dr. Eva Rask, Dr. Maria Rodanaki, and Dr. Maria Lodefalk interpreted the findings, revised the manuscript, agreed on the final version of the manuscript, and accept responsibility for the entire content of the manuscript and approve its submission.

Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.
GnRH Analogue and Ghrelin Levels in Girls

10 Martini AC, Fernandez-Fernandez R, Tovar S, Navarro VM, Vigo E, Vazquez MJ, et al. Comparative analysis of the effects of ghrelin and unacylated ghrelin on luteinizing hormone secretion in male rats. Endocrinology. 2006 May; 147(5):2374–82.

11 Garcia MC, Lopez M, Alvarez CV, Casanueva F, Tena-Sempere M, Dieguez C. Role of ghrelin in reproduction. Reproduction. 2007 Mar; 133(3):531–40.

12 Whatmore AJ, Hall CM, Jones J, Westwood M, Clayton PE. Ghrelin concentrations in healthy children and adolescents. Clin Endocrinol (Oxf). 2003 Nov;59(5):649–54.

13 Soriano-Guillen L, Barrios V, Chowen JA, Maffeis C, Franceschi R, Moghetti P, Camilot.

14 Bellone S, Pradom F, Savastio S, De Rienzo F, Demarchi I, Trovato L, et al. Acylated and unacylated ghrelin levels in normal weight and obesity. J Clin Endocrinol Metab. 2018 May;103(8):2851–60.

15 Cheng HL, Sainsbury A, Garden F, Sritharan M, Lauriola S, Tato L. Circulating ghrelin levels during treatment with LHRH analog. J Endocrinol Invest. 2006 Dec; 29(11):962–7.

16 Kellokoski E, Poykko SM, Karjalainen AH, Ukkola O, Heikkinen J, Kesaniemi YA, et al. Estrogen replacement therapy increases plasma ghrelin levels. J Clin Endocrinol Metab. 2005 May;90(5):2954–63.

17 Blatnik M, Soderstrom CI. A practical guide for the stabilization of acylghrelin in human blood collections. Clin Endocrinol. 2011 Mar; 74(3):325–31.

18 Ukkola O, Heikkinen J, Kesaniemi YA, et al. Ghrelin degradation by serum and tissue homogenates: identification of the cleavage site. Horm Res Paediatr 2022;95:442–451
DOI: 10.1159/000526147

19 van der Lely AJ, Tschop M, Heiman ML, Ghioto E. Biological, physiological, patho-physiological, and pharmacological aspects of ghrelin. Endocr Rev. 2004 Jun;25(3):426–57.

20 Kelsey MM, Zeitler PS. Insulin resistance of young men. J Clin Endocrinol Metab. 2008 May;93(5):1980–7.

21 Blatnik M, Soderstrom CI. A practical guide for the stabilization of acylghrelin in human blood collections. Clin Endocrinol. 2011 Mar; 74(3):325–31.

22 Kuppens RJ, Diene G, Bakker NE, Molina 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 Dec;50(3):633–42.

23 Hosoda H, Doi K, Nagaya N, Okumura H, Nakagawa E, Enomoto M, et al. Optimum collection and storage conditions for ghrelin measurements: octanoyl modification of ghrelin is rapidly hydrolyzed to desacyl ghrelin in blood samples. Clin Chem. 2004 Jun; 50(6):1077–80.

24 Lee PA. Laboratory monitoring of children with precocious puberty. Arch Pediatr Adolesc Med. 1994 Apr;148(4):369–76.

25 Wildland KA, Luo ZC, Niklasson A, Karlberg J. Swedish population-based longitudinal reference values from birth to 18 years of age for height, weight and head circumference. Acta Paediatr. 2002;91(7):739–54.

26 Marshall WA, Tanner JM. Variations in pattern of pubertal changes in girls. Arch Dis Child. 1969 Jun;44(235):291–303.

27 Harrington J, Palmert MR, Hamilton J. Use of local data to enhance uptake of published recommendations: an example from the diagnostic evaluation of precocious puberty. Arch Dis Child. 2014 Jan;99(1):15–20.

28 Kleinwort P, Bloch C; Section on Endocrinology AAOA. Evaluation and referral of children with signs of early puberty. Pediatrics. 2016 Jan;137(1).

29 Blatnik M, Soderstrom CI, Dysinger M, Fra- ser SA. Preadial ghrelin attenuation provides evidence that des-acyl ghrelin may be an artifact of sample handling in human plasma. Bioanalysis. 2012 Oct;4(20):2447–55.

30 Chabot F, Caron A, Laplante M, St-Pierre DH. Interrelationships between ghrelin, insulin and glucose homeostasis: physiological relevance. World J Diabetes. 2014 Jun 15;5(3):328–41.

31 Kaplowitz P, Bloch C; Section on Endocrinology AAOA. Evaluation and referral of children with signs of early puberty. Pediatrics. 2016 Jan;137(1).

Ab Rahim SN, Omar J, Tuan Ismail TS. Go- nadotropin-releasing hormone stimulation test and diagnostic cutoff in precocious pu- berty: a mini review. Ann Pediatr Endocrinol Metab. 2020 Sep;25(3):152–5.

33 Murdolo G, Lucidi P, Di Loreto C, Parlati N, De Cicco A, Fatone C, et al. Insulin is required for prandial ghrelin suppression in humans. Diabetes. 2003 Dec;52(12):2923–7.

34 Lodefalk M, Carlsson-Skwirut C, Holst JJ, Aman J, Bang P. Effects of fat supplementation on postprandial GIP, GLP-1, ghrelin and IGFBP-1 levels: a pilot study on adolescents with type 1 diabetes. Horm Res Paediatr. 2010;73(5):355–62.

35 Blatnik M, Soderstrom CI, Dysinger M, Fra- ser SA. Preadial ghrelin attenuation provides evidence that des-acyl ghrelin may be an artifact of sample handling in human plasma. Bioanalysis. 2012 Oct;4(20):2447–55.

36 Chabot F, Caron A, Laplante M, St-Pierre DH. Interrelationships between ghrelin, insulin and glucose homeostasis: physiological relevance. World J Diabetes. 2014 Jun 15;5(3):328–41.

37 Razzaghy-Azar M, Nourbaksh M, Pour- motaeb A, Nourbaksh M, Ilbeigi D, Khos- ravi M. An evaluation of acylated ghrelin and obestatin levels in childhood obesity and their association with insulin resistance, metabolic syndrome, and oxidative stress. J Clin Med. 2016 Jun 23;5(7):E61.

38 Abreu AP, Kaiser UB. Preadial development and regulation. Lancet Diabetes Endocrinol. 2016 Mar;4(3):254–64.

39 Sen TA, Simsek DG, Darcan S, Coker M. Ghrelin levels in children with constitutional delay of growth and puberty. J Clin Res Pediatr Endocrinol. 2010;2(3):117–21.

40 Kurnaz E, Sen Y, Aydin S. Plasma kisspeptin and ghrelin levels in pubertry variant cases. J Pediatr Endocrinol Metab. 2017 May 1;30(5):569–73.

41 Delhanty PJ, Negers SJ, van der Lely AJ. Des-acyl ghrelin: a metabolically active peptide. Endocr Dev. 2013;25:112–21.

42 De Vriese C, Gregoire F, Lema-Kisoka R, Waelderbroeck M, Robberecht P, Delporte C. Ghrelin degradation by serum and tissue homogenates: identification of the cleavage sites. Endocrinology. 2004 Nov;145(11):4997–5005.