Short Communication

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Effect of a GnRH injection on kisspeptin levels in girls with suspected precocious puberty: a randomized-controlled pilot study

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Abstract

Objectives: Kisspeptin plays a major role in the onset of puberty by stimulating the gonadotropin-releasing hormone (GnRH) neurons. The aim of this study was to investigate whether GnRH inhibits kisspeptin secretion via a negative feedback mechanism and potential associations between kisspeptin levels and other hormones of importance for pubertal onset.

Methods: Thirteen girls with suspected central precocious puberty underwent a GnRH stimulation test twice in a randomized, placebo-controlled manner. Blood was sampled up to 150 min after an IV injection of either Relefact LHRH® or saline. The levels of kisspeptin, acylated ghrelin, ultrasensitive oestradiol, luteinizing hormone (LH), follicle-stimulating hormone (FSH), insulin and glucose were analysed.

Results: Baseline kisspeptin levels ranged from 9.9 to 201.6 pg/mL. Neither area under the curve for kisspeptin levels nor peaks were significantly lower after the GnRH injection compared to placebo. Baseline kisspeptin and glucose levels tended to be associated (rho=0.55, p=0.051) but no other associations were found between kisspeptin and other hormones.

Conclusions: Basal levels of kisspeptin vary widely in young girls. We found no evidence of a negative feedback mechanism of GnRH on kisspeptin in this small pilot study. The suggested association between kisspeptin and glucose levels needs further investigations.

Keywords: gonadotropin-releasing hormone; hypothalamic-pituitary-gonadal axis; kisspeptin; precocious puberty

Puberty, controlled by the hypothalamic–pituitary–gonadal (HPG) axis, starts when the pulsatile secretion of gonadotropin-releasing hormone (GnRH) is initiated, stimulating the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary leading to activation of gonads [1]. The initiation of GnRH secretion has been linked to kisspeptin, a peptide hormone mainly secreted by specific neurons in the hypothalamus. It is also involved in the negative feedback mechanism of sex steroids on gonadotropin release and is important for general fertility [2].

In addition, the well-established association between energy balance and puberty appears to be mediate, at least in part, by kisspeptin signalling. Various metabolic modulators, such as ghrelin, leptin, insulin, are involved in the regulation of kisspeptin activity. Kisspeptin neurons seem to be inhibited by ghrelin but stimulated by leptin and insulin [3]. However, the exact pathways connecting metabolic state and puberty require further investigations.

In accordance with the function of kisspeptin, higher serum levels of kisspeptin have been found in pubertal compared to prepubertal children [4, 5]. Further, kisspeptin levels may be useful when diagnosing central precocious puberty (CPP) and when monitoring the efficacy of CPP treatment [6, 7]. However, there is considerable variation in reported plasma kisspeptin levels among peripubertal children, possibly due to lack of standardized protocols for blood sampling and analysis, and our understanding of the regulation of kisspeptin secretion remains incomplete.

Our group previously investigated ghrelin levels in response to a GnRH analogue injection in girls with suspected CPP [8]. In this randomized controlled pilot study, we

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aimed to investigate the short-term effect of GnRH on circulating kisspeptin levels in the same study population. We hypothesized that GnRH reduces kisspeptin secretion through a negative feedback mechanism, leading to lower plasma kisspeptin levels. Moreover, we aimed to study potential associations between kisspeptin and other relevant hormones, such as ghrelin, as well as glucose.

This was a randomized, placebo-controlled, crossover, single-blinded, multicentre study conducted at the Department of Paediatrics at Örebro and Uppsala University Hospitals, Sweden, between August 2015 and November 2017. 13 girls with suspected CPP who underwent a diagnostic GnRH stimulation test were included in the study. CPP was defined as pubertal signs before the age of 8 years and suspected either by medical history or findings during the clinical assessment. Further inclusion criteria were age >1 year and weight >12 kg. Exclusion criteria were presence of a syndrome, hypothalamus or pituitary tumour or malformation, untreated thyroid disease, diabetes mellitus, body mass index (BMI) >3 standard deviation scores (SDS)), previous surgery or disease affecting the stomach, and treatment with growth hormone. All girls and their guardians provided written informed consent to participate and the study was approved by the Regional Board of Ethics in Uppsala, Sweden (2015/028), and registered at ClinicalTrial.gov (NCT02431416).

All girls went through an adjusted GnRH stimulation test twice, as previously reported [8]. In single blind, randomized order, during one of the test occasions, the GnRH analogue Relefact LHRH® (dose 100 µg/m^2, maximum 100 µg) was given IV, and during the other test occasion, the same volume of saline (NaCl 9 mg/mL) was injected. The tests were conducted in the fasting state with a pretest sample (time 0) taken, followed by blood sampling 30, 60, 90, 120 and 150 min after the IV injection, for the analysis of kisspeptin, ghrelin, FSH, LH, glucose, and insulin levels. Blood was collected in precooled EDTA tubes and processed on ice. A protease inhibitor (AEBSF) was added to sampling tubes within 2 min to a final concentration of 2 mg/mL as reported previously [8].

Plasma kisspeptin levels (KISS1 (metastin)) were analysed at the Clinical Research Laboratory, Örebro University Hospital, using a Sandwich-ELISA kit, LS-F8231, from LSBio® according to the manufacturer's instruction. The detection range was 31.25–2,000 pg/mL, and limit of sensitivity (LOS) was <13.8 pg/mL. The laboratory methods used for the other analyses have been reported previously [8]. Kisspeptin values < LOS were imputed by using the formula LOS/$\sqrt{2}$, as previously suggested for values below the limit of detection [9]. The area under the curve (AUC) for kisspeptin was calculated and comparisons between the test occasions were performed using the paired samples t-test. The Wilcoxon signed rank test, or the paired samples t-test was used to compare peaks with baseline or mean levels, as appropriate. Pearson´s correlation and Spearman´s rank tests were used to investigate associations. Significance was set at $p<0.05$. Calculations were performed using IBM® SPSS Statistics for Windows.

Baseline clinical characteristics and plasma kisspeptin levels are shown in Table 1. The baseline kisspeptin values ranged from 9.9 to 201.6 pg/mL, but the intraindividual variation between baseline kisspeptin levels was low, as shown by the high correlation between each girl's two baseline values ($r=0.899$, $p<0.001$) and a rather low coefficient of variation (CV=30 %). Eight girls were diagnosed with CPP using the criteria: baseline LH > 0.3 IU/L, maximum

Table 1: Clinical characteristics and baseline kisspeptin levels of the 13 included girls with suspected central precocious puberty.

Patient number	Age, years	Weight, kg	Height, cm	BMI, kr/m^2	BMI, SDS	Pubertal stage according to tanner	Kisspeptin levels at baseline[a], pg/mL
1	7.5	21.9	122.5	14.6	−0.8	B2 PH1	201.6
2[b]	6.6	17.4	101.5	16.9	+1.0	B2 PH1	22.8
3[b]	8.4	38.0	146.0	17.8	+1.0	B3 PH1	85.7
4[b]	7.4	35.1	134.0	19.6	+1.8	B3 PH2	67.8
5	6.5	20.6	118.8	14.6	−0.5	B2 PH1	95.7
6	8.0	21.8	124.5	14.1	−1.0	B3 PH1	14.5
7	7.5	29.4	127.2	18.2	+1.3	B2 PH2	82.1
8[b]	7.5	23.9	122.4	16.0	+0.2	B3 PH1	63.7
9[b]	9.0	37.1	146.9	17.2	+0.5	B4 PH4	26.5
10[b]	10.1	31.3	139.8	16.0	−0.3	B4 PH4	18.9
11[b]	9.0	41.9	136.0	22.7	+2.3	B3 PH1	9.9
12[b]	9.1	27.5	135.6	15.0	−0.7	B2 PH1	80.1
13	8.2	44.4	137.2	23.6	+2.6	B2 PH1	14.7

BMI, bone mass index; SDS, standard deviation score. [a]Mean baseline kisspeptin level from both test occasions (Relefact LHRH® or saline IV injection) for each girl. [b]Girls diagnosed with CPP.

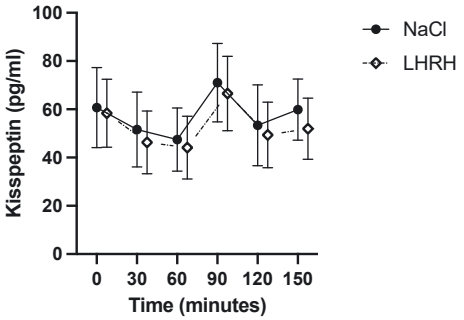

Figure 1: Plasma kisspeptin levels (pg/mL, mean and SEM) after an IV injection of Relefact LHRH® or NaCl. 13 girls with suspected central precocious puberty were examined twice in a fasting state and a randomized-controlled way using an adjusted gonatropin-releasing hormone stimulation test.

stimulated LH >5 IU/L or a ratio of maximum stimulated LH/FSH >0.66.

The kisspeptin levels after the Relefact LHRH® injection did not differ from those after the saline, either when comparing AUC or peak values (Figure 1). The concentration peaked at 90 min irrespective of substance given. Kisspeptin levels at 90 min were significantly higher than at baseline (p=0.019) and in comparison with mean of all other concentrations (p=0.011) when Relefact LHRH® was given. Similar findings were true when saline was given but they did not reach statistical significance.

Baseline plasma glucose tended to be correlated with kisspeptin levels (rho=0.55, p=0.051), without significant change after the exclusion of an outlier (Patient 1). No other correlations were found between kisspeptin and LH, FSH, insulin, ghrelin and oestradiol levels.

Kisspeptin is crucial for the activation of GnRH neurons and we hypothesized that GnRH could repress kisspeptin secretion by a negative feedback mechanism. However, we were not able to demonstrate such an effect, as an injection of a GnRH analogue did not affect circulating kisspeptin levels differently than placebo in girls with suspected CPP. We cannot exclude that a negative feedback mechanism exists at the hypothalamic level and not in periphery, or could have been detected in the circulation if we had sampled blood for a longer period than 150 min.

We found a large interindividual variation but a small intraindividual variation and a tendency towards an association between kisspeptin and glucose levels.

A large variation in kisspeptin levels has been shown before in pubertal girls and boys. The range of kisspeptin levels found in the present study (9.9–201.6 pg/mL) was similar to levels reported previously, though considerably higher levels have also been reported in peripubertal girls (range 10.2 pg/mL to 2.96 ng/mL) [5–7, 10–12]. Differences in handling of sample tubes, use of protease inhibitor, laboratory methods (ELISA or RIA) and type of kisspeptin isoform analysed probably influence the kisspeptin concentration. Our finding of only small intraindividual variation suggests that the measured levels are accurate and that some peripubertal girls have consistently higher kisspeptin levels than others.

A positive correlation between kisspeptin and LH and FSH has been observed before [13], as well as between kisspeptin and different metabolic factors, such as insulin, leptin, BMI, and body weight, HbA1c [14, 15]. Similarly, in our study, a positive correlation between kisspeptin and glucose levels was observed in line with the known close connection between puberty and metabolic state [3].

The strengths of our study are the randomized placebo-controlled study design, the practice of using the study subjects as their own controls and the careful sampling procedure. The main limitation is the small study population. Another limitation is missing or low kisspeptin levels leading to a need for imputed values. However, the number of replaced values did not differ between the two test occasions, suggesting that our main finding may not have been biased.

In conclusion, an IV injection of Relefact LHRH® to girls with suspected CPP did not affect the circulation levels of kisspeptin during a time period of 150 min. Our hypothesis that GnRH may reduce the secretion of kisspeptin through a negative feedback mechanism could not be confirmed. Future research, including larger study populations, should continue to study the regulation of kisspeptin.

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Research ethics: The study was conducted in accordance with the Declaration of Helsinki and was approved by the Regional Board of Ethics in Uppsala, Sweden (2015/028) and registered at ClinicalTrial.gov (NCT02431416).

Informed consent: All girls included in the study, as well as their legal guardians, gave written, informed consent to participate in the study.

Author contributions: ML had the main idea of the study and formatted the study design, received the first funding and ethical permission, recruited participants, and supervised the work. MR participated in recruiting participants, monitored the data, performed the statistical analyses, created the figures, and wrote the first draft of the

manuscript. All the authors were active during the study process and data analysis, as well as during the revision of the manuscript. All the authors agree on the final version of the manuscript, accept responsibility for the content of the manuscript and approve its submission.

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Conflict of interest: The authors have no conflicts of interest to declare.

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Data availability: Extra data are available by reasonable request by emailing maria.rodanaki@oru.se.

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