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Research article

Girls with premature thelarche younger than 3 years of age might have stimulated LH greater than 10 IU/L.

Running Head: GnRH stimulation test in young girls

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What is already known?
- A GnRH stimulation test is the gold standard for the diagnosis of central precocious puberty and a peak stimulated LH level of 5 mIU/L is considered pubertal by many endocrinologists.
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What is new?
- GnRH stimulation test references differ in young girls with premature thelarche compared to older girls.
- In all girls with premature thelarche under three years of age, peak LH/FSH ratio is ≤ 0,43 regardless of peak LH levels which are usually > 5 IU/L or even > 10 IU / L.

ABSTRACT
INTRODUCTION: Premature thelarche (PT) is the isolated breast development in girls prior to 8 years of age. Gonadotropin-releasing hormone (GnRH) stimulation test is sometimes used to distinguish between PT and central precocious puberty (CPP) although the interpretation of the test in early ages is challenging. The objective of this study is to determine the FSH and LH responses to GnRH stimulation in girls below 3 years of age with PT.
METHOD: A standardized GnRH stimulation test, bone age and pelvic ultrasound were evaluated and those without pubertal progression after a minimum of one-year follow up were included in the study.
RESULTS: In the GnRH stimulation test, the baseline median LH was 0.29 (0.10-0.74) IU/L, baseline median FSH was 4.96(3.18-7.05) mIU/mL, the peak median LH was 5.75 (3.31-8.58) IU/L, the peak mean FSH was 40.38 ± 20.37 mIU/mL. Among

the patients, 33.3% (n=10) had baseline LH > 0.3 IU/L, 67% (n=20) had peak LH > 5 IU/l and 16.6% (n=5) >10 IU/L. The peak LH/FSH ratio was 0.17 ± 0.09 and ≤ 0.43 in all participants.
CONCLUSION: Although consensus statements usually define baseline LH >0.3-0.5 IU/L, peak LH >5 IU/L, and LH/FSH ratios >0.66-1.0 as diagnostic cut-offs for CPP, in children below 3 years of age, the baseline and peak LH values may be detected pubertal, possibly due to mini-puberty. The dominant FSH response in the GnRH stimulation test is more valuable than the peak LH response in the diagnosis of PT.

Keywords: GnRH Stimulation Test, central precocious puberty, young girls

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Introduction Precocious puberty (PP) has been an actual issue in recent years due to both an increase in the number of related outpatient visits and the challenges in determining the patients who require treatment. Most girls with signs of puberty in the first 3 years of life are diagnosed with premature thelarche; a benign and non-progressive condition. While early skeletal maturation, increase in height growth rate and decrease in adult height are seen in CPP these findings are not seen in premature thelarche. How often such girls progress to central precocious puberty (CPP) and how much testing is required is a source of debate. Certain clinicians perform gonadotropin-releasing hormone (GnRH) stimulation tests based on the clinical findings and/or randomly requested baseline hormonal tests in these patients (1, 2). The consensus statements on PP recommend to use the same threshold values in the interpretation of the GnRH stimulation tests in all children below 8 years of age (e.g., stimulated LH>5 IU/L or a LH/FSH ratio above 0.66 or 1.0 supports CPP (3-5). However, the patients in early age group present challenges in the interpretation of diagnostic tests due to the impact of the of activation of the hypothalamic-pituitary-gonadal (HPG) axis in the first months of life termed ‘mini-puberty’ (6). Although the limited number of studies in the literature report that higher stimulated LH responses may be observed in the GnRH stimulation tests conducted in children below 3 years (7), this information is not pointed out in the consensus statements (1, 8).
In the present study, the GnRH stimulation test results among the children diagnosed with premature thelarche under the age of 3 years are evaluated and the validity of the criteria used in pediatric age groups to distinguish between pubertal and prepubertal responses has been investigated.

Methods

Thirty girls under the age of 3 years with premature thelarche, admitted to the Kocaeli University, Pediatric Endocrinology Department outpatient clinic between January

2010 and June 2016, were prospectively evaluated. All parents received oral and written information before signing a consent form. The study was approved by the Local Ethics Committee of the Kocaeli University Institutional Review Board (KÜ GÖKAEK 2016/70).

Premature thelarche was diagnosed based upon the criteria; isolated breast development without other signs of puberty, bone age within mean ± 2 SD of chronologic age. The diagnosis of premature thelarche was confirmed by lack of pubertal progression in at least one-year follow-up. Signs suggestive of pubertal progression were accepted as growth acceleration with height velocity above 1 SDS and/or progression to Tanner stage for breast development > stage 3 and/or bone age acceleration.

During the initial assessment of the girls presented with isolated breast development, the detailed patient history was taken and a physical examination was performed. The length of the children aged < 2 years of age was measured in the supine position on the head-foot board, while the height of the children > 2 years of age was measured in standing position with the help of the Harpenden Stadiometer. The SDS for height was calculated based on the Turkish Children growth standards (9). Height velocity SDS was calculated using Tanner's growth charts (10). Puberty stage was assessed by physical examination according to Tanner's criteria for breast development in females (11).

The left hand and wrist X-rays, pelvic ultrasonography (USG), and GnRH stimulation tests were performed at the time of the diagnosis.

Bone age was assessed using the Greulich & Pyle method by the same pediatric endocrinologist and repeated every 6 to 12 months (12). Bone age acceleration was defined as Δ bone age/Δ calendar age >1. Longitudinal diameter of the uterus >34 mm at pelvic ultrasonography was defined as the effect of estrogen exposure (13).

GnRH stimulation test procedure

An intravenous (IV) cannula was inserted into the antecubital region for blood sampling and GnRH analogue injection. After the baseline blood was drawn for the LH and FSH measurement, gonadorelin acetate (LH-RH Ferring ampul, 0.1mg/mL) 0.1 mg/m2 body surface area (max 0.1 mg) was injected as an IV bolus and a second blood sample was obtained at the 40th minute (14). LH and FSH were tested using the immune-chemiluminescense assay (ICMA) (15). For LH, the intra- and inter-assay coefficients of variation (CVs) were 4.8% and 10.7%, respectively. For FSH, the intra- and inter-assay CVs were 3.4% and 5.4%, respectively. The minimum detectable concentration was 0.1 IU/L for both FSH and LH.

Statistical Analysis

Statistical evaluation was performed using the IBM SPSS 20.0 (SPSS Inc., Chicago, IL, USA) software package. The normality of the distribution was assessed based on the Kolmogorov-Smirnov Test. Numeric variables were expressed in mean +/- standard deviation and median [25. percentile - 75. percentile] and frequency (percentage). Independent t-tests were used when comparing continuous variables that were normally distributed. Continuous variables that were not normally distributed were assessed using the Mann-Whitney U test. Categorical variables were presented as numbers and percentages. Differences between categorical variables such as Tanner Stage were assessed using a chi-square test. The relationship between the

variables within the normal distribution was evaluated using Pearson's Correlation analysis, while the relationship between the variables outside the normal distribution was analysed through Spearman's correlation analysis. Statistical significance was based on a value of $p<0.05$.

Results

Thirty girls with premature thelarche in whom the diagnosis was confirmed by lack of pubertal progression in at least one-year follow-up were enrolled in the study. Clinical characteristics of patients are shown in Table 1. The bone age was within mean ± 2SD of chronologic age range in all the patients. The pelvic USG results showed that the uterine sizes and ovarian volumes were consistent with the ages of the patients and no pathologies were observed. The results of the GnRH stimulation tests are presented in Table 2. The distribution of the baseline LH and FSH, peak LH and FSH, and the peak LH/FSH ratios according to the age are shown in Figure 1.The baseline LH value was >0.3 IU/L in 10/30 patients (33.3%). In 67% of the patients (n=20), the peak LH value was >5 IU/l; while it was >10 IU/L in 16.6% (n=5). In all the patients, the peak LH/FSH ratio was □(less than or equal to) 0.43.
No significant relationship was observed between the baseline LH and the peak LH ($r=0.054$, $p=0.776$).
There was a positive correlation between the stimulated FSH and the stimulated LH ($r=0.647$, $p< 0.001$).
There was no significant difference between subjects with basal LH values > 0.3 IU/l and < 0.3 IU/L in terms of Tanner stage, bone age, basal FSH, estradiol, peak LH and peak FSH values (Table 3).
There was no significant difference in Tanner stage, bone age, basal LH, basal FSH, estradiol and peak FSH values between the groups with peak LH value > 5 IU/L and peak LH value < 5IU/L (Table 4).

Discussion

Signs of puberty, especially breast development in girls up to 3 years of age are a source of concern both for the parents and physicians. At such an early age, isolated PT is the most likely diagnosis, often all that is needed is patience and observation. On the other hand, CPP, however rarely, is also observed in this age group or cases diagnosed initially as isolated PT may progress to CPP (1, 16). Clinically, breast development in patients with isolated PT is unaccompanied by areola and nipple maturation, breast development shows fluctuations, and no growth spurt is observed (16). But there is an exception to every rule. In a study from Israel comprising the follow up of 139 patients with PT for a decade, CPP was reported in 13% regardless of the age of the diagnosis and the clinical progression (17). In this study, progressive cases were significantly less frequent in patients under the age of 2 in comparison to the patients over 2 years of age (3.8% vs. 52.6%). Other studies from Italy and Denmark also showed 14% of girls with the diagnosis of premature thelarche progressed to CPP in the follow-up (18, 19). Therefore, advanced studies including GnRH stimulation tests can be performed by physicians in certain cases. However,

the lack of established reference values on the GnRH stimulation test responses in girls below 3 years considering the effect of mini-puberty leads to confusion. In a study from Italy including 450 patients, progression to CPP was observed in only 2% of the patients diagnosed with PT below the age of 2 and the baseline hormone levels including the GnRH test were found to be insignificant to predict the progression (2). In this study, 97 patients were evaluated through endocrine tests and imaging methods in addition to the 3-month clinical follow up and 85 patients were diagnosed with PT, 9 patients were diagnosed with central precocious puberty and 3 patients received the diagnoses of peripheral precocious puberty. Among the patients with the final diagnosis of premature thelarche, 36.4% had peak LH levels >5 IU/L (100% among the patients with CPP). On the other hand, baseline LH values >0,2 IU/L among the patients with premature thelarche was observed in 1.17%. All girls with isolated thelarche had a FSH predominant response with peak LH/FSH ratio < 1, while girls with complete sexual development showed a ratio > 1. A study from Taiwan reported in the literature also gave similar results (7). In this study, 36 patients with the final diagnosis of isolated PT were classified into 2 groups as patients under the age of 4 (group A) and over the age of 4 (group B) and their GnRH stimulation test results by ELISA were compared. In Group A, the peak mean LH was 13.0±6.06 IU/L, while it was 8.5±4.10 in Group B and the peak LH response was significantly higher in Group A compared to Group B (p<005). In addition, the peak mean FSH in Group A was 120.5± 45.87, while it was 48.7±24.05 IU/L in Group B and the peak FSH response was significantly higher in Group A compared to Group B (p<0,001). The peak LH/FSH ratio was < 1 in all the patients.

In a study, Vestergaard et al. investigated the physiological LH, FSH and LH/FSH response to GnRH stimulation test in healthy girls below 6 years of age with no signs of precocious puberty (20). The study showed an age-dependent response to the GnRH test with larger LH and FSH responses in girls aged from 10 months to 3 years compared to girls aged from 3 to 6 years (the 30-min LH response 5.2 ± 4.0 and 2.9 ± 2.5 IU/L, the 30-min FSH response 23.3 ± 16.2 and 14.5 ± 10.3 IU/L). The peak LH/FSH ratio was 0.23 ± 0.19 (range 0.06-0.43).

In our study, the ratio of both the baseline LH values >0.3 IU/L (33%) and the peak LH values >5 IU/L (67%) were high. Also, 16.6% of the patients (n=5) had values of peak LH >10 IU/L. Especially these patients can easily be misdiagnosed with CPP with a cliché approach. Indeed, certain patients referred to our pediatric endocrinology clinic were suggested treatment with GnRH analogues based on these results by former physicians, whereas from the point of view regarding the peak LH/FSH ratio suggested by certain authors, all the patients had values ≥0.43.

The main focus of our study was to analyse GnRH stimulation test results among the patients with a confirmed diagnosis of isolated PT. The study demonstrates that high gonadotropin response even if peak LH values >10 IU/L is not associated with progression to true precocious puberty and treatment decision should not be based upon these criteria solely. The peak LH/FSH ratio seems to be a reliable parameter to define pubertal activation.

Indeed, both our study results and the other studies have demonstrated that after mini-puberty although the baseline LH and FSH values are undetectable, the GnRH test may induce “pubertal gonadotropin response” in girls under the age of three years (2, 6). Although the association of this response with the etiology of PT is yet to be cleared, it should be considered as a crucial diagnostic factor.

In conclusion, a peak LH value even if > 10 IU/L is inadequate to make an accurate therapy decision solely in patients with PT below 3 years of age. Elevated LH

responses to GnRH stimulation test are common, but not related to precocious puberty. The diagnosis of CPP solely based on the response to GnRH test is often misleading in the first three years of life, leading to overestimation of CPP. The peak LH/FSH ratio is more valuable to distinguish between the pubertal and prepubertal response. As strongly emphasized in recent years, there is still uncertainty regarding the diagnosis and treatment of early puberty, and the evaluation of the biochemical results with the clinical findings and the age of the patient is one of the most important points to avoid unnecessary treatment (21, 22).

Study Limitations

As the study was conducted as an observation of young girls with premature thelarche, the number of participants were unfortunately restricted. Expansion of the sample size may yield more beneficial results. Also we could not compare the patients with central precocious puberty or healthy girls without pubertal progression in the same age group because of insufficient numbers and ethical issues.

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Tables

Table 1. Clinical characteristics of Premature Thelarche Girls.

No of girls	30
Age (months)	20 ± 7[a]
Bone age (months)	17.0 [12.0-24.0][b]
Bone age advancement (months)	4.0 [1.0-16.0][b]
BMI-SDS	0.78 (-0.94±0.98)[a]
Breast stage II	8 (26.6)[c"]
Breast stage III	22 (73.3)[c]
Height SDS	1.5 [0.5-3.0][b]
Height velocity SDS	-0.19 [-2.1-3.7][b]

[a]Mean SD, [b] median [25.-75.percentile],[c] number (%).

Table 2. The hormonal characteristics of GnRH stimulation test in premature thelarche girls.

	Statistics
Basal LH (IU/L)	0.29 [0.10-0.74]
Basal FSH (mIU/mL)	4.96 [3.18-7.05]
Peak LH (IU/L)	5.75 [3.31-8.58]
Peak FSH (mIU/mL)	40.38 [20.37
Peak LH/Peak FSH ratio	0.17 [0.09

mean SD/median [25.-75.percentile]

Table 3. The comparison of girls with basal LH value < 0.3 IU/L and > 0.3 IU/L.

	Basal LH <0,3 IU/L	Basal LH >0,3 IU/L	p-value
Number of girls (n)	15	15	

Tanner stage (n, II/III)	5-11	3-11	0.367
Bone age (years)	12 [9-24][b]	24 [12-24][b]	0.539
Basal FSH (mIU/mL)	4.57 [3.1-5.1][b]	6.91 [4.3-8][b]	0.102
Estradiol (pg/ml)	13.3 [5-20][b]	17.4 [12.5-20][b]	0.886
Peak LH (IU/L)	5.77 [3.2-7.04][b]	5.6 [3.9-14.5][b]	0.325
Peak FSH (mIU/mL)	35.9 (±16.1)[a]	46.2(±24.3)[a]	0.089

[a]Mean±SD, [b] median [25.-75.percentile]

Table 4. The comparison of girls with peak LH value <5 IU/L and >5 IU/L.

	Peak LH <5 IU/L	Basal LH >5 IU/L	p-value
Number of girls	12	18	
Tanner stage (n, II-III)	4-8	4-14	0.14
Bone age (years)	19[12-27][b]	12[9.75-24][b]	0.689
Basal LH (IU/L)	0.32 [0.16-0.54][b]	0.24[0.1-0.82][b]	1.000
Basal FSH (mIU/mL)	4.7 [3.31-5.03][b]	5.6 [3.3-7.96][b]	0.156
Estradiol (pg/ml)	11.5 [2-19.5][b]	17.4 [7.72-20][b]	0.472
Peak FSH (mIU/mL)	27.2(±9.32)[a]	43.1(±21.2)[a]	0.342

[a]Mean±SD, [b] median [25.-75.percentile]

Figures

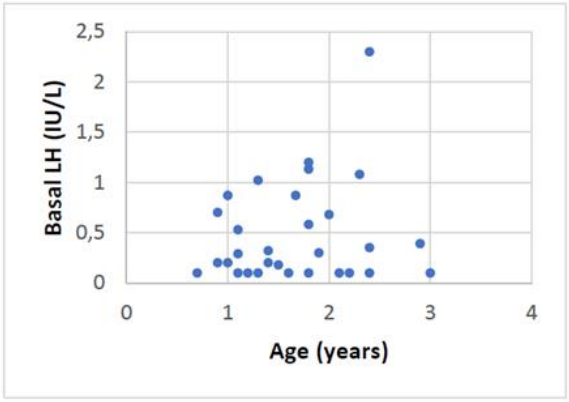

Figure 1a

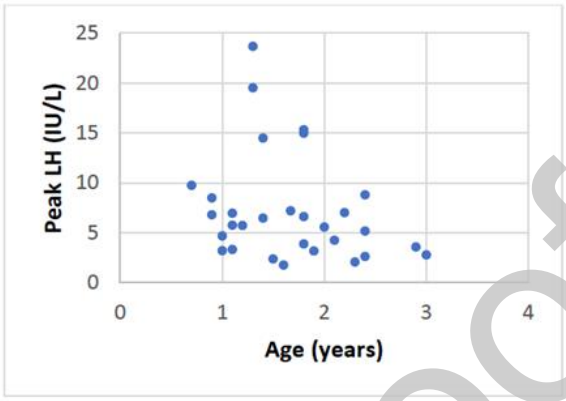

Figure 1b

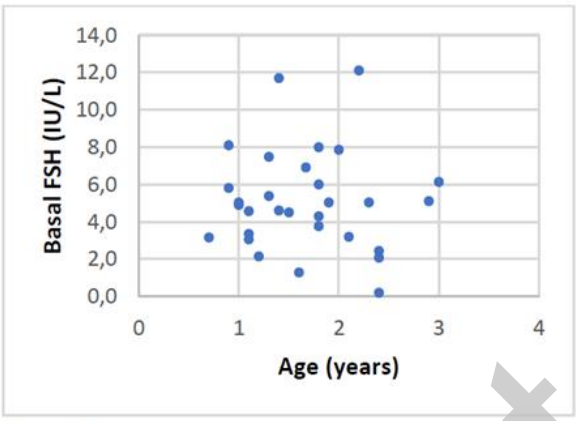

Figure 1c

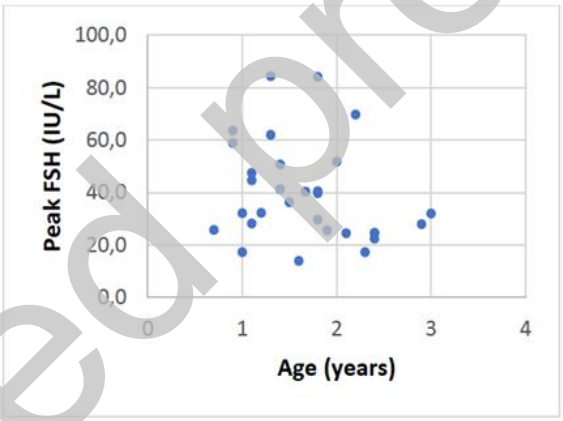

Figure 1d

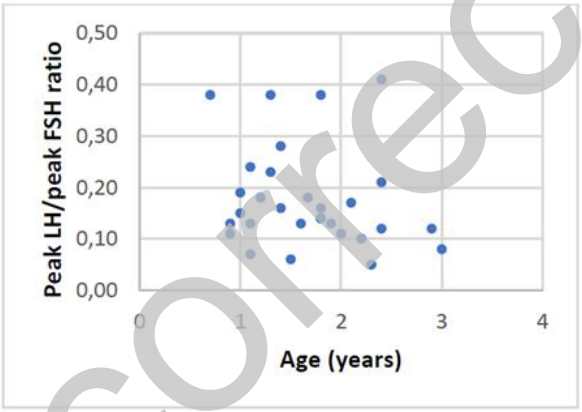

Figure 1e

Figure 1

a) The distribution of the baseline LH levels according to age.
b) The distribution of the peak LH responses according to age.
c) The distribution of the baseline FSH levels according to age.
d) The distribution of the peak FSH responses according to age.
e) The distribution of the peak LH/ peak FSH ratio according to age.