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ORIGINAL ARTICLE

Leuprolide acetate gonadotrophin response patterns during female puberty

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Summary

Objective To assess normative data and the usefulness of spontaneous and LHRH analogue-stimulated serum LH and FSH levels measured by immunoradiometric assays (IRMA) in the evaluation of normal puberty.

Design Prospective. Healthy girls in Tanner I and Tanner II from the local community were invited to participate ($n = 47$).

Methods A leuprolide acetate test (500 mcg/m^2; sc) was performed. LH and FSH levels were determined using IRMA. Tanner II girls were assessed every 6 months until Tanner V. Girls who progressed from Tanner II to Tanner III in the next 6 months were called Tanner II-2; otherwise, they were called Tanner II-1.

Results The prepubertal upper limit (CI 95%) was 0·49 IU/l for basal LH and 5·1 IU/l for stimulated LH. Taking into account these LH cut-off limits, 72·2% and 66·7% of Tanner II-1 and 41·6% and 41·7% of Tanner II-2 subjects presented overlapping values for basal and stimulated LH, respectively, as compared with the Tanner I group. The cut-offs for basal and stimulated LH to predict progression from Tanner II to Tanner III in the next 6 months were a basal LH level ≥0·49 IU/l (Sensitivity = 0·58; 1-Specificity = 0·33) and a poststimulated LH level ≥4·75 IU/l (Sensitivity = 0·67; 1-Specificity = 0·44).

Conclusion According to an IRMA, the basal and leuprolide acetate gonadotrophin response patterns during the beginning stages of puberty overlapped between Tanner I and Tanner II, and the cut-offs of basal and stimulated LH levels to predict progress from Tanner II to Tanner III had low sensitivities for the following 6 months.

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Introduction

The classical GnRH stimulation test has been considered the gold standard for the diagnosis of pubertal disorders. However, the lack of commercially available synthetic GnRH has made this test difficult to perform.[1] A single basal LH measurement could be adequate to document pubertal hypothalamic–pituitary–ovarian (HPO) axis activation in most, but not all, girls who have reached puberty when a chemiluminescent third-generation immunoassay is used.[2] In girls, a marked overlap was observed for basal LH measured by immunochemiluminometric assay (ICMA) between the Tanner I and Tanner II stages (53·8%), and LH levels were even higher for values obtained by immunofluorometric assay (IFMA) (84·6%).[3]

Therefore, in most cases, to define hypothalamic–pituitary ovarian activation, the patient needs to undergo a GnRH stimulation test. Leuprolide acetate is a synthetic analogue of naturally occurring GnRH that possesses greater theoretical potency than the natural hormone. The leuprolide acetate test has been considered to be an alternative and a better discriminator than a GnRH stimulation test for the diagnosis of precocious or delayed puberty.[4]

The aim of this study was to characterize pituitary (measured by immunoradiometric assays, IRMA) gonadal secretory responses to leuprolide acetate sc (subcutaneous) in prepubertal and pubertal normal girls recruited from the local community.

Subjects and methods

A prospective study was designed. All girls were evaluated at the Institute of Maternal and Child Research from 2003 to 2008.

Subjects

Girls from different schools in Santiago de Chile aged 3–12 years were invited to participate in this study. These girls, born at an appropriate gestational age and at term, were not taking any chronic medications and did not have histories of premature sexual development. Two groups of girls were studied. The prepubertal group (Tanner I) was transversally evaluated. The pubertal group was followed up for 3 years from the Tanner II stage of breast development. Girls who developed from Tanner II to Tanner III

over the next 6 months were classified as Tanner II-2. All other patients were classified as Tanner II-1 (see Fig. 1).

Study protocol

A complete physical exam was performed by one of two paediatric endocrinologists (I.H. and A.M.). Height and weight were measured by one nurse (A.A.). Height was measured using a wall-mounted Harpenden stadiometer (Holtain, UK). Weight was measured using a manual scale with a 10-g gradation (Seca; Quick-Medical, Snoqualmie, WA, USA). Pubertal development was assessed according to the method of Marshall and Tanner.[5] Bone age was determined by the Greulich and Pyle method.[6]

Basal blood samples were obtained in the morning at 8:00 in the supine position and after an overnight fast. Leuprolide acetate (500 μg) was then administered sc, and blood samples were drawn from an indwelling catheter positioned in an antecubital vein at 3 h for gonadotrophins and at 24 h after stimulation for oestradiol. The leuprolide acetate dose was previously validated by Ibáñez *et al.*[7] and used in our previous report.[8] The timing of blood sampling was based on previously published data showing that the gonadotrophin peak occurs 1–6 h after stimulation and that the steroid peak occurs 20–24 h later.[9] Blood samples were separated immediately after collection, and the serum was stored at −70 °C until analysis.

Hormone assays

Serum LH and FSH levels were measured by IRMA assays from Diagnostic System Laboratories. Intra-assay coefficients of variation (CVs) were 6·5% for LH and 3·6% for FSH. Inter-assay CVs were 7·6% for LH and 6·2% for FSH. Serum oestradiol was determined by competitive specific binding RIA (Diagnostic System Laboratories, Webster, TX, USA). The intra- and inter-assay CVs were 4·1% and 6·7%, respectively. The lower limits of detection were 0·05 and 0·06IU/l for LH and FSH, respectively; and 18 pmol/l for oestradiol.

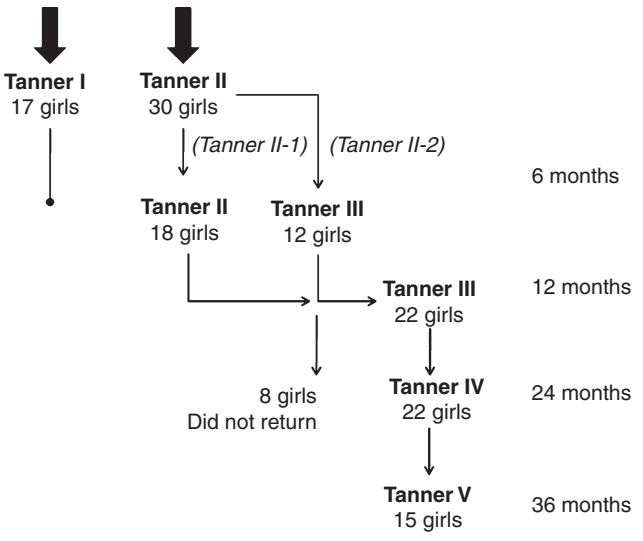

Fig. 1 Prepubertal and Pubertal groups, follow-up over 3 years.

Ethics

The protocol was approved by the Ethical Committee of the Hospital San Borja Arriarán and the Faculty of Medicine, University of Chile (August 8 2002), in accordance with the Helsinki Declaration. All parents signed informed consent forms, and girls (volunteers) gave their consent before entering the study.

Statistical analysis

Results are expressed as medians (CI 95%). Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc, Chicago; Illinois, EEUU). Differences between groups were assessed by nonparametric tests (Mann–Whitney *U*-tests) for variables that were not normally distributed. *P*-values <0·05 were considered statistically significant. The area under the curve was calculated to determine which basal and stimulated LH cut-off point could predict the progress from Tanner II to Tanner III over the following 6 months.

Results

Anthropometrics and general characteristics

Seventeen girls without secondary sexual characteristics and bone ages within the normal limits for chronological age were categorized in the Tanner I group. The remaining 30 girls with Tanner II breast development were followed using the same study protocol. During the next 6 months, 18 girls showed no progression of their secondary sexual characteristics and were defined as the Tanner II-1 group. Twelve girls progressed to Tanner III breast development and were defined as Tanner II-2. From the initial 30 girls with Tanner II breast development, 22 girls returned periodically for medical examination at least once a year, and were evaluated during the Tanner III and Tanner IV stages. After a 3-year follow up, 15 girls have reached Tanner V (Fig. 1).

Girls in Tanner I were younger than girls in the Tanner II-1 and Tanner II-2 groups. Tanner II-1 and II-2 did not differ with regard to chronological, height standard deviation score (height-SDS) or BMI percentile. However, girls in Tanner II-2 had an older bone age than girls in Tanner II-1. Baseline clinical characteristics of these patients are reported in Table 1.

Baseline and leuprolide acetate-stimulated gonadotrophin levels

Basal LH and FSH levels rose progressively with advancing pubertal stage as shown in Table 2 and Fig. 2. There were no statistical differences in basal LH between girls in Tanner II-1, Tanner II-2 or Tanner III as compared with girls in Tanner I. The median basal LH level was statistically different for Tanner IV as compared with Tanner I (3·4 *vs.* 0·45 IU/l; $P < 0·01$).

In Tanner I, the upper limit of the CI 95% for the basal level of LH was 0·49 IU/l, and that for FSH was 2·37 IU/l. Taking into

Table 1. Clinical features in 47 girls at enrolment, grouped according to Tanner I, Tanner II-1 and Tanner II-2 stages of breast development

	n	CA (years)	BA (years)	Ht (SDS)	BMI (percentile)
Tanner I	17	6·0 (5·2–6·9)*,†	6·8 (5·4–7·8)*,†	−0·3 (−0·2 to 0·9)*	73 (58–88)
Tanner II-1	18	9·3 (8·6–9·8)	8·8 (8·6–9·8)‡	−0·7 (−1·2 to −0·7)	63 (49–76)
Tanner II-2	12	9·8 (9·4–10·4)	10·0 (9·5–10·6)	0·4 (−0·5 to 0·6)	63 (48–77)

Results are expressed as the median (CI 95%).
Tukey test.
*Tanner I *vs.* Tanner II-1; $P < 0·05$
†Tanner I *vs.* Tanner II-2; $P < 0·05$
‡Tanner II-1 *vs.* Tanner II-2; $P < 0·05$
CA, Chronological age; BA, Bone age; Ht-SDS, Height standard deviation score; BMI, Body Mass Index.

Table 2. Basal and 3-h postleuprolide acetate-stimulated gonadotrophin levels and oestradiol in girls grouped according to Tanner stages of breast development

		LH (IU/l)		FSH (IU/l)		Oestradiol (pmol/l)		
	n	Basal	Post	Basal	Post	Basal	Post	Stimulated LH/FSH ratio
Tanner I	17	0·45s (0·43–0·49)	3·8 (3·5–5·1)	1·9 (1·76–2·37)	23·1 (20·8–27·8)	23·1 (19·5–38·9)*	107·9 (92·1–185·8)*	0·19 (0·15–0·23)*
Tanner II-1	18	0·42 (0·36–0·55)	3·9 (2·1–12·9)	2·2 (1·80–2·72)	22·5 (18·35–24·93)	67·9 (54·3–80·4)†	228·0 (170·3–351·8)†	0·24 (0·11–0·65)
Tanner II-2	12	0·58 (0·43–0·93)	6·8 (5·6–17·0)	3·8 (2·87–4·39)	19·0 (15·7–22·7)	75·6 (54·3–127·4)‡	343·2 (196–521·6)	0·42 (0·27–1·61)
Tanner III	22	0·98 (0·73–1·44)	15·5 (13·1–29·7)	5·4 (4·39–6·16)	22·9 (19·25–25·48)	235·0 (195·7–271)	613·1 (594.7–978·0)	0·78 (0·56–1·78)§
Tanner IV	22	3·40 (2·56–3·91)	66·1 (50·8–118·1)	6·6 (5·23–7·12)	27·6 (22·0–30·75)	211·1 (194·9–346·5)	831·5 (739·0–997·8)	2·76 (2·4–5·6)
Tanner V	15	2·60 (2·48–8·06)	109·6 (97·5–294·5)	5·9 (4·52–6·91)	26·6 (22·7–44·73)	292·6 (244·1–450·8)	664·5 (664·5–1197·1)	3·73 (3·2–9·7)
P-value		<0·001	<0·001	<0·001	0·168	<0·001	<0·001	<0·001

Results are expressed in median (CI 95%).
To convert oestradiol from the SI unit (pmol/l) to the conventional unit (pg/ml), divide by 3·671 (conversion factor).
Dunn's multiple comparison test.
*Tanner I *vs.* Tanner III; $P < 0·05$.
†Tanner II-1 *vs.* Tanner III; $P < 0·05$.
‡Tanner II-2 *vs.* Tanner III; $P < 0·05$.
§Tanner III *vs.* Tanner IV; $P < 0·05$.

account this basal LH cut-off limit, 72·2% (13 of 18) of Tanner II-1, 41·6% (five of 12) of Tanner II-2 and 14·2%· (three of 21) of Tanner III subjects presented values that overlapped with those of girls in Tanner I. For basal FSH, 61·1% (11 of 18) of Tanner II-1, 8·3% (three of 12) of Tanner II-2 and 4·7% (one of 21) of Tanner III subjects presented values that overlapped with those of girls in Tanner I.

Leuprolide acetate-stimulated gonadotrophin levels are shown in Table 2. The stimulated LH level increased dramatically during puberty ($P < 0·001$). However, the median leuprolide acetate-stimulated LH levels in girls in Tanner II-1, Tanner II-2 and Tanner III were not statistically different as compared with Tanner I. The median leuprolide acetate-stimulated LH level was only statistically different in Tanner IV girls as compared with Tanner I girls (66 IU/l *vs.* 3·8 IU/l; $P < 0·05$).

In Tanner I, the upper limit (CI 95%) after leuprolide acetate stimulation was 5·1 IU/l for LH and 27·4 IU/l for FSH. Considering this LH cut-off limit, 66·7% (12 of 18) of Tanner II-1, 41·7% (five of 12) of Tanner II-2 and 9·5% (two of 21) of Tanner III subjects presented values that overlapped with those of the Tanner I group. No overlap was observed between either Tanner IV or Tanner V and Tanner I poststimulated LH values. For stimulated FSH levels, there were no differences among groups (ANOVA; $P = 0·168$). In addition, there was an important overlap of Tanner I stimulated FSH values as compared with values from all of the Tanner stages, 83·3% (15 of 18) of Tanner II-1, 91·7% (11 of 12) of Tanner II-2, 80·9% (17 of 21) of Tanner III, 54·5% (12 of 22) of Tanner IV and 60% (nine of 15) of Tanner V subjects.

Leuprolide acetate-stimulated LH/FSH ratio

The leuprolide acetate-stimulated LH/FSH ratio rose progressively with advancing pubertal stage as shown in Table 2. However, the median leuprolide acetate-stimulated LH/FSH ratio in girls in Tanner II-1 and Tanner II-2 was not significantly different from the values observed for Tanner I subjects. However, the measure did differ in Tanner III girls as compared with Tanner I girls (0·78 *vs.* 0·17; $P < 0·05$).

In Tanner I, the upper limit of the CI 95% for LH/FSH ratio after leuprolide acetate stimulation was 0·23. Taking into account this LH/FSH ratio cut-off limit, there was an important overlap of Tanner I values with those from Tanner II to III stages: 50% (nine

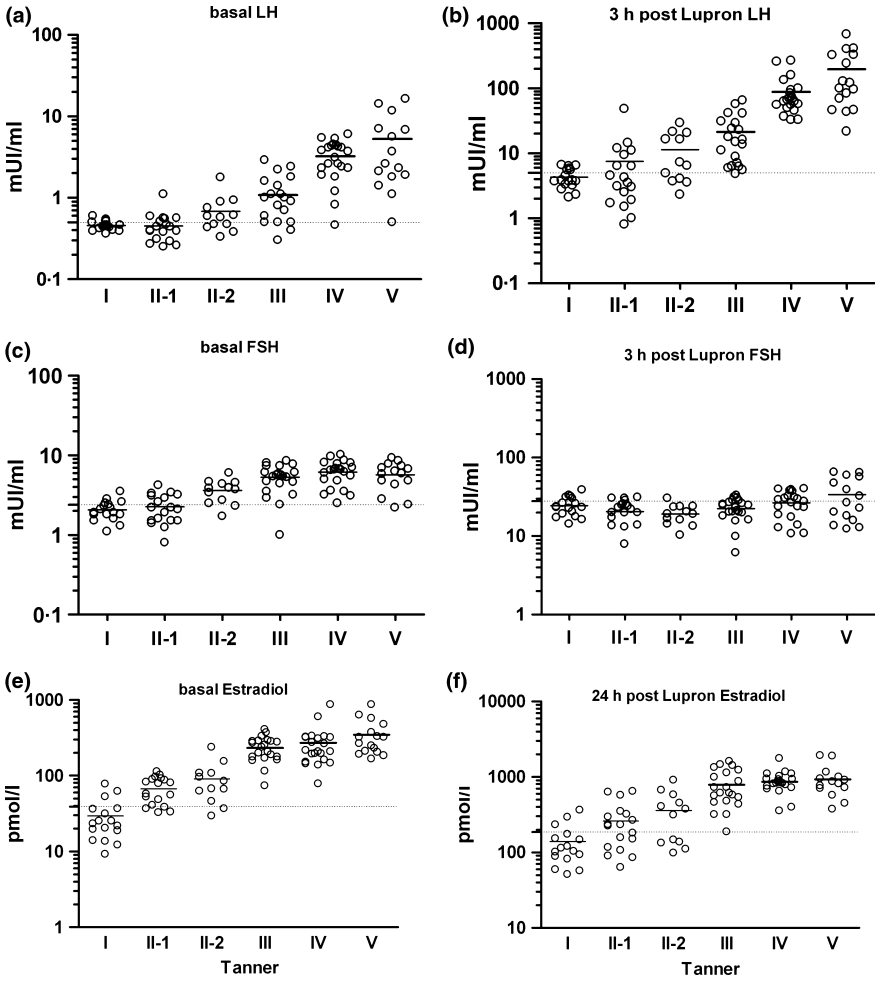

Fig. 2 Basal and 3-h postleuprolide acetate-stimulated gonadotrophin and oestradiol levels in girls grouped according to Tanner stages of breast development. Basal and poststimulated serum LH, luteinizing hormone (a,b); FSH, follicle-stimulating hormone (c,d) and oestradiol levels (e,f). The upper limit (CI 95%) levels of these hormones in the Tanner I group are represented by a dotted line. To convert oestradiol from the SI unit (pmol/l) to the conventional unit (pg/ml), divide by 3·671 (conversion factor).

of 18) of Tanner II-1, 41·7% (five of 12) of Tanner II-2, 4·8% (one of 21) of Tanner III.

Baseline and leuprolide acetate-stimulated oestradiol levels

The median basal plasma oestradiol levels were lower in girls in Tanner I as compared with those in Tanner III (22·7 *vs.* 253 pmol/l; $P < 0·001$). Basal median oestradiol did not differ significantly between girls in Tanner I and II-1. Taking into account the upper limit (CI 95%) for the basal plasma oestradiol in girls in Tanner I (38·9 pmol/l), the overlapping values with Tanner II-1 represented 22·2% (four of 18) and 16·2% (two of 12) of Tanner II-2 subjects. There was no overlap between Tanner III, Tanner IV, Tanner V and Tanner I plasma oestradiol.

The median 24-h-stimulated oestradiol level varied between Tanner I and Tanner III girls (108 *vs.* 613 pmol/l; $P < 0·001$), but did not differ between girls from the Tanner II-1 and Tanner II-2 groups ($P > 0·05$). Taking into account the upper limit (CI 95%) of 24-h-stimulated oestradiol obtained from girls in Tanner I (185·8 pmol/l), 44% (eight of 18) of girls in Tanner II-1 and 41·6% (five of 12) of Tanner II-2 subjects presented overlapping values with girls in Tanner I. No overlap for 24-h-stimulated oestradiol was observed when comparing Tanner I and Tanner III, Tanner IV or Tanner V subjects.

Area under the curve (ROC) to predict progress from Tanner II to Tanner III over the next 6 months

For basal LH in Tanner II, the area under the curve was 0·745, SE of the area was 0·094, and the confidence limits for the area were between 0·562 and 0·929 (P-value of a hypothesis test = 0·025). For stimulated LH in Tanner II, the area under the curve was 0·713, SE of the area was 0·095, and the confidence limits for the area were between 0·526 and 0·900 (P-value of a hypothesis test = 0·051). For stimulated LH/FSH ratio in Tanner II, the area under the curve was 0·741, the standard error of the area was 0·094, and the confidence limits for the area were between 0·557 and 0·924 (P-value of a hypothesis test = 0·028) (Fig. 3).

In Table 3, the x-axis and y-axis co-ordinates of the ROC Curve for basal LH and stimulated LH and LH/FSH ratio are displayed. These values were used to predict Tanner II progress to Tanner III over the next 6 months.

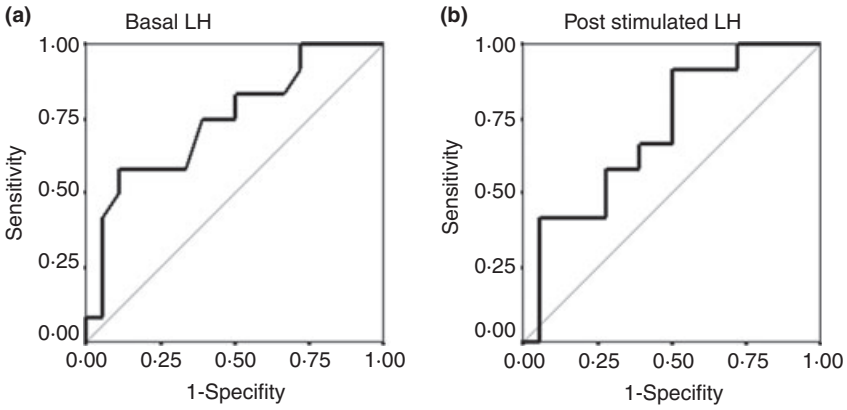

Fig. 3. Cut-off of basal and stimulated LH to predict progression from Tanner II to Tanner III over the next 6 months. (a) Basal serum LH and (b) poststimulated LH. The sensitivity (or probability of correctly identifying a positive) is plotted on the *y* axis. 1-specificity (where specificity is the probability of correctly identifying a negative) is plotted on the *x*-axis).

Table 3. Co-ordinates for each cut-off of basal and stimulated LH to predict progression from Tanner II to Tanner III over the next 6 months

	Positive if ≥	Sensitivity	1-Specificity
Basal LH (IU/l)	0·455	0·750	0·389
	0·490	0·583	0·333
	0·530	0·583	0·278
Poststimulated LH (IU/l)	3·52	0·917	0·500
	4·73	0·667	0·444
	5·61	0·583	0·389
Poststimulated LH/FSH (IU/l)	0·161	0·917	0·389
	0·220	0·750	0·278
	0·272	0·667	0·278

Sensitivity (probability of correctly identifying a positive) and 1-specificity (where specificity is the probability of correctly identifying a negative).

Discussion

To our knowledge, this is the first time that a longitudinal study during female puberty was performed utilising GnRHa leuprolide acetate. The contribution of this study is normative data in the context of the difficulty of evaluating disorders of pubertal development. The main result is that there is considerable overlap in the biological results observed at various clinical stages of puberty. If measured by IRMA, basal and stimulated LH and FSH levels as determined by leuprolide test are not sensitive enough to differentiate between prepubertal and pubertal levels.

A single basal LH measurement could be adequate to document pubertal hypothalamic–pituitary–ovarian (HPO) activation when chemiluminescent third-generation immunoassays are used.[2] However, when this assay is not available, there is a need for stimulated gonadotrophin values to assess whether hypothalamic–pituitary gonadal activation has occurred. Because of the lack of commercial availability of native GnRH, LHRH agonists, which have a longer half-life in the circulation, have been used as a means of gonadotrophin stimulation.[10] Using an IRMA, peak LH responses after acute leuprolide acetate stimulation clearly discriminated between Tanner III and Tanner II, but were not able to distinguish between the responses of girls in Tanner I or Tanner II stages of puberty. In addition, the cut-off for basal and stimulated LH to predict progress from Tanner II to Tanner III over the next 6 months had low sensitivity. Moreover, there was a significant overlap of basal LH levels in the various stages of puberty. Thus, despite improved sensitivity, IRMA as used for LH determination does not distinguish clearly between basal prepubertal and pubertal levels of this hormone. Mitamura *et al.*[11] used a more sensitive assay (DELFIA). The authors determined that LH and FSH exhibit night-day rhythms; these rhythms are already found to exist in 5- to 6-years-old girls.

Garibaldi *et al.*[12] reported a sensitivity of 67% in diagnosing Central Precocious Puberty (CPP) when using a random LH level in excess of 0·5 IU/l IRMA. Compared with our results, this value has a sensitivity of 58% with a specificity of 67% for puberty progression. However, when more sensitive methods are used, such as ICMA, the range of random LH values in normal girls 11- to 13-years old is very broad, but 100% of spontaneous LH values are above 0·3 IU/l by late puberty.[13]

In our group, the median levels of basal LH were significantly higher in girls in Tanner III or during more advanced stages of puberty (0·98 IU/l *vs.* 0·58 IU/l in Tanner II-2). However, although the stimulated LH levels overlapped significantly between Tanner I and Tanner II-1 (66·7%) and between Tanner I and Tanner II-2 (41·7%), displaying peak LH below 5·1 IU/l. Girls in Tanner III had a consistently stimulated LH exceeding the cut-off limit of 5·1 IU/l (upper CI 95% for Tanner I) or 12·9 IU/l (upper CI 95% for Tanner II-1).

Using AUTODELFIA, Brito *et al.*[14] reported a substantial overlap in basal and GnRH-stimulated gonadotrophin levels in normal individuals of both sexes with pubertal Tanner stages 1 and 2. The sensitivities of basal and peak LH for the diagnosis of central puberty were 62·7% and 92·2% in girls. The negative predicted values for basal and peak LH in girls were 40·6% and 76·5%, respectively. Thus, these authors concluded that when using AUTODELFIA, basal LH levels were sufficient to establish the diagnosis of gonadotrophin-dependent precocious puberty in 62·7% of girls. In the remaining patients, a GnRH stimulation test was still necessary to confirm this diagnosis.

As has been demonstrated previously, serum FSH values measured by RIA, IRMA, ICMA and IFMA under basal and stimulated conditions overlapped substantially in prepubertal and pubertal subjects. Therefore, these indexes do not allow effective discrimination between these two populations.[10,15]

Simulated FSH levels in all stages of puberty do not differ from those obtained in girls in Tanner I. Also, stimulated FSH levels

overlapped significantly in normal prepubertal and pubertal subjects, and for this reason, it was not possible to determine a cut-off limit that would allow effective discrimination between Tanner I and Tanner II-2.

The importance of FSH measurement is more closely related to differences in the hormone profile between prepubertal and pubertal children, in which a reduction in FSH and an increase in LH occur simultaneously.[11] In addition, high FSH levels can occur in girls with isolated thelarche who do not necessarily progress to puberty.[16]

In our study, basal and poststimulated oestradiol were also evaluated in an attempt to establish cut-off limits to distinguish between the prepubertal and pubertal states. Using a sensitive method, basal and poststimulated oestradiol cut-off values were 38·9 and 185·8 pmol/l, respectively. These values showed an overlap of 22·2% and 44%, respectively, between patients in Tanner I and Tanner II-1 and an overlap of 16·2% and 41·6%, respectively, with patients in Tanner II-1 and Tanner II-2. No overlap for basal or stimulated oestradiol was observed when comparing Tanner I with Tanner III, Tanner IV or Tanner V. These results preclude using serum oestradiol as a sensitive and specific measure to define pubertal staging.

Basal and stimulated oestradiol levels appear to increase before basal and stimulated LH level in Tanner II-I girls. It is well known that during late prepuberty girls begin to experience increasing diurnal production of gonadotrophins, with FSH predominance and a diurnal rise in oestradiol that peaks at midmorning.[16] Those girls in Tanner II-I were in this late prepubertal stage characterized by the transition from FSH-predominant to LH-predominant gonadotrophin secretion, which is characteristic of progressive puberty.[4,11,17]

As this result is not new, these confirmatory results are important and will provide a reference for clinicians using IRMA who do not have such normative data locally available. However, it should be noted that these results derived from a small sample and must be used with caution in the evaluation of female puberty. A new study to evaluate the validity of such suggested cut-offs must be designed.

In conclusion, using an IRMA, the basal as well as stimulated leuprolide acetate gonadotrophin response patterns during the beginning stages of puberty overlapped significantly with values obtained from girls in Tanner I and Tanner II. Moreover, the use of cut-off basal and stimulated LH values to predict progress from Tanner II to Tanner III over the subsequent 6 months had low sensitivity. It is important to have these method limitations in mind when evaluating the hypothalamic–pituitary gonadal axis.

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Competing interests/financial disclosure

The authors have nothing to disclose.

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