Assessment of Basal and Gonadotropin-Releasing Hormone-Stimulated Gonadotropins by Immunochemiluminometric and Immunofluorometric Assays in Normal Children

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Context: Recently, new methodologies have been applied to commercial immunofluorometric (IFMA) and immunochemiluminometric (ICMA) LH and FSH assays.

Objective: The objective of the study was to use ICMA to establish basal and GnRH-stimulated LH and FSH reference values in normal subjects of different ages and sexual development, compared with IFMA.

Design and Methods: We established basal and GnRH-stimulated LH and FSH levels of 315 prepubertal and pubertal children (170 males and 145 females) divided into five groups according to Tanner stage. Of these, 106 subjects (59 males and 47 females) were submitted to GnRH test. The prepubertal upper limit of normal for basal LH, determined by the 95th percentiles of the prepubertal population, were 0.2 IU/liter (ICMA) and 0.6 IU/liter (IFMA) in both genders.

Results: No overlap of basal LH levels determined by ICMA was observed between prepubertal and pubertal males, but basal LH determined by IFMA overlapped in 11.8% of subjects. In girls, both methods yielded overlapping values (10.4%, ICMA; and 84.6%, IFMA). The LH peak after GnRH stimulation that defined puberty was 4.1 IU/liter (ICMA) and 3.3 IU/liter (IFMA) in boys and 3.3 IU/liter (ICMA) and 4.2 IU/liter (IFMA) in girls. After GnRH stimulation, values determined by the two methods overlapped in both genders.

Conclusions: We conclude that ICMA is more sensitive and precise than IFMA, permitting differentiation of pubertal and prepubertal stage in boys under basal conditions. However, in girls the overlap of basal values was marked, indicating the need for the GnRH test to establish maturity of the hypothalamus-pituitary-gonadal axis.

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The determination of basal LH, FSH, and gonadal steroids is sufficient for the diagnosis of the main gonadal disorders, but in some situations such as hypogonadotropic hypogonadism, pituitary tumors, and precocious puberty, evaluation of the hypothalamus-pituitary-gonadal (HPG) axis by a GnRH test becomes necessary. Some investigators have compared hormone assays with more sensitive methods in an attempt to avoid the use of the GnRH test and to diagnose maturation of the HPG axis based only on the measurement of basal gonadotropins. However, in prepubertal children, basal LH and FSH concentrations still overlapped with those of pubertal children, and the former therefore need to be submitted to a GnRH stimulation test (1–9).

Recently new methodologies have been applied to commercial immunofluorometric (IFMA) and immunochemiluminometric (ICMA) LH and FSH assays. The advantages of ICMA, compared with other standard techniques, include higher sensitivity, precision, and reproducibility, which requires a lower amount of reagent, thus reducing the cost per test (10–15). The objective of this study was to use ICMA to establish basal and GnRH-stimulated LH and FSH reference values in normal subjects of different ages and sexual development to compare this method with IFMA.

Subjects and Methods

Subjects

The research protocol was approved by the Ethical Committees of the Faculdade de Medicina da Universidade Federal do Triângulo Mineiro. All participants and/or their legal representatives received detailed information about the project and signed an informed consent form. The subjects of this study were selected from the Pediatric Endocrinology and Gonad and Pediatric outpatient clinics of Universidade Federal do Triângulo Mineiro. All participants were clinically evaluated by anamnesis and physical examination to exclude possible endocrinopathies, chronic diseases, and possible medication use. Weight and height were measured in the orthostatic position using a Prader stadiometer. Pubertal development was evaluated by two endocrinologists according to Tanner’s criteria (breast staging in females and testicular size in males) (16, 17).

Basal LH and FSH were measured in 315 subjects, 145 females and 170 males. Of these, 104 subjects (59 males and 47 females) were submitted to the GnRH stimulation test. These subjects were divided into five groups according to gender, age, and stage of sexual development: Tanner stage I subdivided into T1a (children aged 0–2.6 yr) and T1b (children aged 2.7–10 yr), TII, TIII, TIV, and TV. LH and FSH were measured by IFMA and ICMA.

The GnRH stimulation test was always performed between 0800 and 1000 h, using 100 μg GnRH (Relisorm L, gonadorelin; Serono, Rockland,
MA) administered iv at time zero, and blood samples were drawn 15 min before and at 0, 15, 30, 45, 60, 90, and 120 min after GnRH administration for serum LH and FSH measurements. Blood was centrifuged and the samples obtained at each time interval were divided into two 2-ml aliquots and stored at −20°C until the time of the assay.

**Gonadotropin assay**

LH and FSH were measured by ICMA using an Immulite 1000 apparatus and commercial kits (Diagnostic Products Corp.-Medlab, Los Angeles, CA) and by IFMA using an AutoDELFIA apparatus and the AutoDELFIA LH Spec and FSH commercial kits (Wallac Oy, Turku, Finland). The ICMA for LH was standardized with the First International Reference Preparation 68/40 and the Second International Standard 80/552, and the standard curve ranged from 0.1 to 200 IU/liter. The IFMA for LH was standardized with the World Health Organization Second International Reference Preparation 78/549, and the standard curve ranged from 0.1 to 170 IU/liter. The IFMA was standardized with the World Health Organization International Standard LH 80/552 and the World Health Organization Second International Reference Preparation FSH 78/549, and the standard curves ranged from 0.6 to 250 IU/liter for LH and from 1.0 to 256 IU/liter for FSH. Lymphochek controls (Bio-Rad Laboratories, Hercules, CA) were used to obtain high, medium, and low controls for the AutoDELFIA LH Spec and AutoDELFIA FSH assays. All samples were measured in duplicate.

The working range of assays was established by the precision profile, which is a plot of intraassay variation vs. concentration in the samples and was defined by the interval in which the intraassay variation was less than 8%. By ICMA, this interval was from 0.1 to 200 IU/liter for LH and 0.1 to 170 IU/liter for FSH, and by IFMA, it was from 0.6 to 250 IU/liter for LH and 1.0 to 256 IU/liter for FSH. Because of this, the minimal detectable concentration (MDC) was set at 0.1 IU/liter for LH and 0.1 IU/liter for FSH by ICMA and 0.6 IU/liter for LH and 1.0 IU/liter for FSH by IFMA. By ICMA, concordance was obtained for the MDC calculated according to manufacturer’s recommendation, which was 0.1 IU/liter for LH and 0.1 IU/liter for FSH. This was confirmed by measuring 50 samples with serum levels below these MDC values. Regarding to IFMA, although the MDC calculated by the manufacturer of the commercial kits using the traditional method of various replicates of the zero standard (mean − 2 sd) is 0.05 IU/liter for both LH and FSH, yet the coefficient of variation at these levels was high (> 10%), and therefore, the MDC was set at 0.6 IU/liter for LH and 1.0 IU/liter for FSH. Characteristics of the hormone assays are shown in Table 1.

**Statistical analysis**

Results obtained by ICMA and IFMA were analyzed by the Kolmogorov-Smirnov normality test. The Bartlett test was used to test the homogeneity of variances. Peak LH and FSH levels between poststimulation times (15, 30, and 45 up to 120 min) were determined in both genders for each method and stage by repeated-measures ANOVA followed by the Tukey test. The Student t test was used to determine differences in basal and peak of LH and FSH between the ICMA and IFMA methods. For statistical analysis, hormone values that were below the lowest value established by the precision profile were regarded as the lowest values of the standard curve, i.e., 0.1 IU/liter (ICMA) and 0.6 IU/liter (IFMA) for LH and 0.1 IU/liter (ICMA) and 1.0 IU/liter (IFMA) for FSH. Overlapping of values between groups were determined by comparing the 95th and fifth percentiles, which were used as diagnostic cutoffs. In the prepubertal upper limit of normal for the TI group, we used only children from the T1–2 group (age ≥ 6 yr) to avoid the inclusion of young infants (age < 6 yr) who could possibly be experiencing the minipuberty of neonatal puberty. P < 0.05 was considered to be significant in all tests.

**Results**

**Basal gonadotropin levels**

Basal LH and FSH levels rose progressively with advancing pubertal stage in both males and females. (Tables 2 and 3 and Figs. 1 and 2). LH and FSH levels measured by the two methods (ICMA and IFMA) did not differ significantly (P > 0.05) between males of group TI-1 and those of group TI-2. In contrast, FSH measured in females using the two methods was lower in the group TI-2 than in the group TI-1 (P < 0.05).

By ICMA, basal LH was less than 0.1 IU/liter (MDC) in 85.3% (87 of 102) of TI males (Fig. 1A). For FSH, the values was less than 1.1 IU/liter, with FSH being less than 0.1 IU/liter (MDC) in 51.0% (52 of 102). In girls, a significant difference in LH measured by ICMA was observed between the TI groups and the other groups (P < 0.05) (Table 2). All TI girls presented basal LH less than 0.7 IU/liter and LH less than 0.1 IU/liter (MDC) in 93.3% (73 of 80) of them. Regarding FSH, all girls presented values less than 8.0 IU/liter, with FSH being less than 0.1 IU/liter (MDC) in 8.7% (seven of 80) (Table 2).

By ICMA, in boys the prepubertal upper limit of normal was 0.3 IU/liter for LH and 0.9 IU/liter for FSH. No overlapping LH values were observed between stages TII, TIII, TIV, and TV and stage TI (P < 0.05) (Fig. 1A). Basal FSH levels differed significantly between group TI and groups TII, TIII, TIV, and TV (P < 0.05) (Table 2), with no significant difference among the last four groups (P > 0.05). In girls, this upper limit of normal was 0.1 IU/liter for LH and 8.0 IU/liter for FSH in TI-1 group and 0.2 IU/liter for LH and 2.1 IU/liter for FSH in T1–2 group and 0.2 IU/liter for LH and 2.1 IU/liter for FSH in T1–2 group and 0.2 IU/liter for LH and 2.1 IU/liter for FSH in T1–2 group and 0.2 IU/liter for LH and 2.1 IU/liter for FSH in T1–2 group and 0.2 IU/liter for LH and 2.1 IU/liter for FSH in T1–2 group.
Tukey): TI

LH levels, in male groups, measured by ICMA (ANOVA-F; Tukey): TI

TIII; TI

(18.3–27.0) (1.5–6.3) (2.4–8.2) (0.6–5.0) (1.2–5.7) (16.9–27.2) (0.8–12.1) (0.6–15.4) (0.1–6.7) (1.0–7.3)

Statistical difference between the Tanner stage for basal LH levels in male groups, measured by ICMA (ANOVA-F; Tukey): TI × TII; TI × TIII; TI × TV; TI × TV (<0.001); TII × TV (0.04); TIII × TV (0.03). By IFMA (ANOVA-F; Tukey): TI × TII; TI × TIII; TI × TV; TI × TV (<0.001); TII × TV (0.01). Statistical difference for basal LH levels, in female groups, measured by ICMA (Kruskal-Wallis; Dunn): TII × TII (0.04); TI × TII; TI × TV: TI × TV (<0.001); TII × TII (<0.01); TII × TV (0.02); TII × TV (<0.01); by IFMA (Kruskal-Wallis; Dunn): TI × TII; TI × TV; TI × TV (<0.001); TII × TII; TII × TV; TI × TV; TI × TV (<0.001).

* P < 0.05 for ICMA × IFMA comparison using Student’s t test.

Feminines

<table>
<thead>
<tr>
<th>Tanner stage</th>
<th>n</th>
<th>LH (IU/liter)</th>
<th>FSH (IU/liter)</th>
</tr>
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<tbody>
<tr>
<td>TI-1</td>
<td>20</td>
<td>1.6 ± 0.7</td>
<td>0.1 ± 0.02</td>
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<tr>
<td>TI-2</td>
<td>82</td>
<td>7.1 ± 2.4</td>
<td>0.1 ± 0.1</td>
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<tr>
<td>TII</td>
<td>17</td>
<td>12.5 ± 1.5</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>TIII</td>
<td>14</td>
<td>13.6 ± 1.1</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>TV</td>
<td>16</td>
<td>14.7 ± 8.3</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>TV</td>
<td>21</td>
<td>22.1 ± 2.8</td>
<td>3.5 ± 1.7</td>
</tr>
</tbody>
</table>

Baseline LH levels were different significantly between stages TI, TII, and TV (see Table 2).

NLRH-stimulated gonadotropin levels

In both genders, the peak response to the GnRH test occurred between 30 and 45 min in all groups. At these time intervals, gonadotropin levels were similar and differed significantly from the other times.

In boys, the upper limit of normal to distinguish prepubertal and pubertal LH responsiveness to GnRH stimulation (95th percentile) was 4.1 IU/liter by ICMA. However, 17.6% (three of 17) TI boys presented overlapping LH values with group TII (Table 3 and Fig. 1B). Peak LH levels differed significantly between group TII and the other groups (P < 0.05) but not between groups TII, TIII, TIV, and TV (P > 0.05) (Table 3).

No overlapping with group TII was observed with the stage TIII. The prepubertal upper limit of normal for FSH

* P < 0.05 for ICMA × IFMA comparison using Student’s t test.

Feminines

<table>
<thead>
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<th>Tanner stage</th>
<th>n</th>
<th>LH (IU/liter)</th>
<th>FSH (IU/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TI-1</td>
<td>25</td>
<td>1.2 ± 0.7</td>
<td>0.1 ± 0.0</td>
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<tr>
<td>TI-2</td>
<td>56</td>
<td>6.6 ± 2.2</td>
<td>0.1 ± 0.1</td>
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<tr>
<td>TII</td>
<td>13</td>
<td>10.8 ± 1.2</td>
<td>0.5 ± 1.1</td>
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<tr>
<td>TIII</td>
<td>12</td>
<td>11.7 ± 1.1</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>TV</td>
<td>11</td>
<td>13.0 ± 1.3</td>
<td>2.2 ± 0.9</td>
</tr>
<tr>
<td>TV</td>
<td>28</td>
<td>22.2 ± 2.7</td>
<td>4.5 ± 3.4</td>
</tr>
</tbody>
</table>

Baseline LH levels did not differ between TI and the other groups.

* P < 0.05 for ICMA × IFMA comparison using Student’s t test.

Feminines

<table>
<thead>
<tr>
<th>Tanner stage</th>
<th>n</th>
<th>LH (IU/liter)</th>
<th>FSH (IU/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TI-1</td>
<td>10</td>
<td>1.9 ± 1.1</td>
<td>2.1 ± 1.1</td>
</tr>
<tr>
<td>TI-2</td>
<td>10</td>
<td>4.4 ± 3.7</td>
<td>5.3 ± 3.9</td>
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<tr>
<td>TII</td>
<td>10</td>
<td>18.6 ± 6.5</td>
<td>21.0 ± 5.7</td>
</tr>
<tr>
<td>TII</td>
<td>9</td>
<td>18.6 ± 6.5</td>
<td>21.0 ± 5.7</td>
</tr>
<tr>
<td>TIII</td>
<td>9</td>
<td>37.3 ± 36.6</td>
<td>37.9 ± 32.5</td>
</tr>
</tbody>
</table>

TABLE 3. Peak values after GnRH for LH and FSH concentrations expressed as mean ± standard deviation and 5 and 95% percentiles, determined by ICMA and IFMA assays, in 316 normal subjects in different pubertal stages (TI–TV)

<table>
<thead>
<tr>
<th>Pubertal stage</th>
<th>n</th>
<th>LH (IU/liter)</th>
<th>FSH (IU/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TII</td>
<td>10</td>
<td>2.2 ± 0.9</td>
<td>2.2 ± 0.9</td>
</tr>
<tr>
<td>TII</td>
<td>14</td>
<td>15.3 ± 8.0</td>
<td>15.6 ± 8.7</td>
</tr>
<tr>
<td>TII</td>
<td>14</td>
<td>15.6 ± 6.2</td>
<td>15.6 ± 8.7</td>
</tr>
<tr>
<td>TII</td>
<td>11</td>
<td>17.3 ± 4.3</td>
<td>17.3 ± 4.7</td>
</tr>
<tr>
<td>TV</td>
<td>10</td>
<td>25.9 ± 14.8</td>
<td>28.9 ± 15.9</td>
</tr>
</tbody>
</table>

There were no differences between ICMA × IFMA for both hormones and genders. Statistical difference between the Tanner stage for peak LH levels, in male groups, measured by ICMA (ANOVA-F; Tukey): TI × TII; TI × TIII; TI × TV; TI × TV (<0.001); by IFMA (ANOVA-F; Tukey): TI × TII; TI × TIII; TI × TV; TI × TV (<0.001). Statistical difference for peak LH levels, in female groups, measured by ICMA (ANOVA-F; Tukey): TI × TII; TI × TIII; TI × TV; TI × TV (<0.001); by IFMA (ANOVA-F; Tukey): TI × TII; TI × TIII; TI × TV; TI × TV (<0.001).
peak was 10.4 IU/liter for stage TI. There was important overlapping of TI values of the FSH peak with the other groups.

In girls, the prepubertal upper limit of normal after GnRH stimulation was 3.3 IU/liter for LH by ICMA. Taking into account this cutoff limit, there was no significant difference between groups TI and TII because 46.1% (six of 13) of TII subjects presented overlapping values with group TI. However, significant differences were found between group TI and the other groups (P < 0.05) (Fig. 2B and Table 3). No overlapping with group TI was observed with the stage TIII, but overlapping occurred between the other groups. FSH values were higher in the TI group, compared with the other groups, but this difference was not significant (P > 0.05). The prepubertal upper limit of normal for FSH peak was 23.7 IU/liter in the TI group. There was important overlapping of TI values with the other groups.

By IFMA, in boys, the upper limit of normal of LH peak level after GnRH stimulation was 3.3 IU/liter for the prepubertal stage. However, 5.9% (one of 17) of TII subjects presented overlapping with the TI group (Fig. 1B and Table 3). This upper limit of normal for FSH peak was 10.6 IU/liter for stage TI. TI values showed important overlapping among all groups.

In girls, the prepubertal upper limit of normal of LH peak value after GnRH stimulation was 4.2 IU/liter for the prepubertal stage. However, 30.8% (four of 13) of TII subjects presented overlapping with group TI (Fig. 2B). Peak LH levels did not differ significantly between groups TI and TII.

No overlapping was observed between TI and TIII groups, but LH levels overlapped within the other groups (Table 3).

**Comparison of ICMA and IFMA**

Comparison of basal LH levels in boys, measured by the ICMA and IFMA methods, showed a significant difference (P < 0.05, Student t test) for all stages, except stage TII (P > 0.05), with higher LH values being obtained by IFMA (Table 2). For basal FSH, significant differences between ICMA and IFMA were observed for all groups studied (P < 0.05).

Comparison of peak LH from ICMA and IFMA methods showed no significant difference (P > 0.05, ANOVA followed by the Tukey test) in either stage, although higher peak LH values were obtained by IFMA (Tables 2 and 3). No significant difference between the ICMA and IFMA methods was observed for FSH in all groups studied.

In girls, comparison of basal LH measured by the ICMA and IFMA methods showed a significant difference only for groups TI and TII (P < 0.05, Student t test) and no significant difference (P > 0.05) for groups TIII, TIV, and TV. A significant difference in basal FSH between the ICMA and IFMA methods was observed for groups TI, TII, and TIII (Table 2).

Similar to boys, no significant difference in peak LH or FSH measured by ICMA and IFMA was observed in any stages (P > 0.05, ANOVA followed by the Tukey test), although higher LH and FSH levels were obtained by IFMA (Table 3).
Discussion

The determination of gonadotropin reference values under basal conditions and after stimulation by exogenous GnRH by ICMA and IFMA is of great importance in clinical practice. Although manufacturers provide normative data for their methods, it is preferable for norms to be established in each population under study, especially for the stimulation test, with new values having been established for the different methods employed (7–9, 14, 15, 18–21).

With respect to sensitivity and precision, the ICMA was superior to the IFMA because it was able to detect lower values with intra- and interassay coefficients of variation of less than 3.5%. This difference was significant for most pubertal stages, especially in boys who presented lower LH and FSH values by ICMA. On the other hand, in girls lower LH and FSH levels determined by the ICMA, compared with the IFMA, were found only in groups TI and TII. These data agree with those reported by Neely et al. (14), who measured LH and FSH levels by three different methods: RIA, IFMA, and ICMA. However, although we used a more sensitive and specific method, overlapping basal gonadotropin values were observed among the different pubertal stages as reported by several investigators (7–9, 14, 15, 18–21).

In girls, marked overlapping was observed for basal LH measured by ICMA between stages TI and TII (53.8%), and it was even higher for values obtained by IFMA (84.6%). However, a significant difference between pubertal stages was noted from stage TII on. These data agree with the finding of significantly lower basal LH levels in TI, compared with stages TIII, TIV, and TV, in both genders (P < 0.05) (7–9, 14, 15, 18–22). Therefore, our data suggest that girls in stage TIII and beyond are unlikely to require a GnRH test for the differential diagnosis between central precocious puberty and precocious thelarche or pubarche. The clinical context, advancing bone age and basal LH higher than 0.2 IU/liter suggest evidences of the maturity of the HPG axis.

Surprisingly, no overlapping for basal LH between TI and TII was observed for males when using the ICMA method, demonstrating that in this group, basal measurement might be sufficient to distinguish pubertal development in these stages. Therefore, our data suggest that boys are unlikely to require a GnRH test to differentiate the prepubertal from the pubertal stage because basal LH measured by ICMA alone is capable of distinguishing these stages.

With respect to basal FSH, substantial overlapping of absolute values was observed between all pubertal stages despite a significant difference between certain stages. 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 (14). From a physiological point of view, this observation confirms the fact that FSH is the main hormone secreted during the prepubertal period, whereas the expression of LH is higher during the pubertal period (3, 23). The LH and FSH concentrations observed in the present study agree with the literature in terms of the FSH response, which was higher than the LH response in stage TI. However, an inversion of hormone value became evident from stage TII on, i.e. LH showed an increased that was 5 times greater than FSH (9, 24). FSH values were found to be higher in prepubertal girls, a finding also
observed in other studies (7, 9, 14, 18, 25). Physiologically this observation is explained by both the absence of inhibin, which is found at a higher concentration in boys, and a differentiated sensitivity of the gonadotropin to GnRH in this condition (26). Another explanation would be that with the progression of puberty the increase in estradiol secretion selectively inhibits the secretion of FSH (27).

LH levels after GnRH stimulation test are considered the main marker of maturity of the HPG axis. In the present study, the upper limit of normal for LH after GnRH stimulation test by ICMA was 4.1 IU/liter for boys and 3.3 IU/liter for girls, and that measured by IFMA was 3.3 IU/liter for boys and 4.2 IU/liter for girls. These values were lower than those reported in the literature but more sensitive to the diagnosis of precocious puberty than those reported by Brito et al. (15). In that study, the cutoff limits for LH measured by IFMA were 6.9 IU/liter for girls and 9.6 IU/liter for boys. Neely et al. (14) found values of 5.0 IU/liter measured by ICMA and of 7.9 IU/liter measured by IFMA. In the latter study, the authors demonstrated a significant correlation (r = 0.96) between gonadotropin values obtained by ICMA and IFMA and the superiority of the ICMA method in terms of sensitivity and reproducibility. Furthermore, the authors suggested that only basal measurements of gonadotropins would be sufficient to diagnose puberty. In our study, we used the 95th percentile as a pubertal upper limit of normal. We could have chosen a more sensitive cutoff limit, thus reducing specificity. However, it was not convenient in this situation to reduce specificity because of the increase in the probability of false-positive tests.

We conclude that the ICMA was superior to the IFMA in terms of sensitivity and precision, permitting the differentiation between the pubertal and prepubertal stage under basal conditions in boys. However, marked overlapping of basal values was observed in girls, indicating the need for the GnRH test to establish activation of the HPG axis in the differential diagnosis of delayed and premature puberty.

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