The ovarian markers of the FSH insufficiency in functional hypothalamic amenorrhoea

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BACKGROUND: The purpose of this work was to revisit the gonadotrophin insufficiency of functional hypothalamic amenorrhoea (FHA) with the use of relevant ovarian markers. METHODS: Serum anti-Müllerian hormone (AMH), estradiol (E₂), inhibin B, LH and FSH were immunoassayed in 31 women with FHA and in 30 healthy women in early follicular phase. The ovarian antral follicle number (FN) was determined within two distinct diameter ranges (2–5 and 6–9 mm) by ultrasound in real time, the same day as the blood sampling. RESULTS: The 2–5 mm FN was similar between the two groups, while the 6–9 mm FN was significantly less in FHA than in controls, in relation with lower serum FSH levels (r = 0.428; P < 0.024). Nine (29%) FHA patients had a low serum basal FSH level (i.e. <4.5 IU/l, 5th percentile of control values). In the 22 (71%) patients with apparently normal FSH, the mean 6–9 mm FN was similar to controls. However, in this sub-group, the mean AMH serum level and the AMH:2–5 mm FN ratio were significantly higher and the mean inhibin B serum level was significantly lower than in controls. No significant relationship was found between the serum LH levels and the FN, AMH or inhibin B values. CONCLUSION: Only a minority of patients with FHA have a low serum basal FSH level, and we show that this is associated with fewer 6–9 mm follicles at the ovarian level. Despite a normal serum FSH level and 6–9 mm FN in the majority of patients with FHA, the functional follicle markers are abnormal. This suggests that the FSH action on the ovary is incomplete and is not properly reflected by its serum level nor by FN at ultrasound.

Key words: anti-Müllerian hormone/FSH/functional hypothalamic amenorrhoea/inhibin B/ovarian follicle

Introduction

Functional hypothalamic amenorrhoea (FHA) is a common and theoretically reversible form of anovulation that accounts for up to 48% of secondary amenorrhoea (Speroff et al., 1994). The clinical syndrome of FHA is characterized by hypoestrogenic amenorrhoea in women with low body weight and low body fat owing to excessive exercise or disordered eating, such as severe restriction of dietary fat intake (Couzinet et al., 1999; Warren and Fried, 2001).

The primum movens of FHA is a disordered frequency pattern of hypothalamic GnRH secretion (Santoro et al., 1986; Perkins et al., 2001). Consequently, the LH pulse frequency and amplitude are significantly reduced compared to controls leading to a low serum level of this hormone, while its response to the GnRH stimulation test is variable (Leyendecker and Wildt, 1983). Although the LH pattern has been extensively evaluated in FHA, less is known about the FSH secretion. It is commonly thought that FSH deficiency acts in concert with the disruption of LH pulsatility to impair ovarian function in FHA. However, most of the authors reported normal serum FSH levels in FHA (Alvero et al., 1998; Couzinet et al., 1999; Tschugguel and Berga, 2003).

In one study about recreational women runners, a situation close to FHA but with fewer menstrual disturbances, FSH insufficiency was restricted to a blunted elevation of the serum level during the luteal-follicular transition (De Souza et al., 1998). So, the question arises as to whether there is truly a quantitative FSH deficiency in FHA.

Knowing the physiological effects of FSH on antral follicle growth, if there were such a deficiency, one would expect the ovaries of women with FHA to be depleted from visible non-dominant follicles (i.e. 2–9 mm in diameter) at ovarian ultrasound (ultrasound). Although the data available in the literature are scarce, the ovaries actually appeared to be multifollicular in some studies (Adams et al., 1985; Futterweit et al., 1988), making them difficult to distinguish from polycystic ovaries (Futterweit et al., 1988). It is only in severe states of FHA, such as anorexia nervosa, that the ovaries appear small and amorphous at ultrasound (Treasure et al., 1988). In such patients, weight gain leads to the appearance of multifollicular ovaries (Treasure et al., 1988). Therefore, we hypothesize that the FSH deficiency in FHA is most often subtle and does not impair the antral follicle growth, until follicles reach the stage of being selectable at a diameter...
of ~5 mm (Gougeon, 1996). Ultimately, however, it would impair the shift from non-selectable to selectable follicles, which occurs in normal women during the luteal–follicular transition (so-called FSH window) (Macklon and Fauser, 2001). This phenomenon is highly FSH dependent and it precedes the selection process which is under the influence of LH (Gougeon, 1996). The multifollicular appearance of the ovaries in women with FHA could thus be explained by the accumulation of non-selectable follicles, due to the lack of inter-cycle FSH rise.

The validity of ultrasound to analyse the above-mentioned shift had been previously reported by Pache et al. (1990) in normal women. They showed that the 2–5 mm (non-selectable) follicles are observed at each stage of the cycle while the 6–9 mm (selectable) follicles appear only in the early follicular phase (EFP). Accordingly, in our study, we considered that a follicle size of 5 mm at ultrasound represented the threshold between selectable and non-selectable follicles, as also suggested by our previous studies documenting the follicular arrest in polycystic ovary syndrome (PCOS) (Pigny et al., 2003; Jonard et al., 2003). Therefore, we used again this stratification of ultrasound data to document the putative FSH insufficiency in FHA.

Besides ultrasound, some biological ovarian markers may also be helpful in evaluating the gonadotrophin defect of FHA. In normal women in EFP, inhibin B is secreted by granulosa cells in response to the inter-cycle FSH rise, just before the induction of estradiol (E2) secretion (Hayes et al., 1998). Only two studies have previously addressed the inhibin B status in FHA (Petraglia et al., 1998; Casper et al., 2000). The basal level was considered as normal in the study by Petraglia et al. (1998), but their norms were significantly lower than those published later in a larger series (Pigny et al., 2000). In the study by Casper et al. (2000), the increase of inhibin B was not significant after GnRH stimulation while FSH levels doubled and LH levels increased 12-fold. However, the studied population did not exclusively contain patients with FHA. Lastly, there are no data concerning the anti-Müllerian hormone (AMH) in FHA. Recent studies indicated that it is a good marker of the small (i.e. 2–5 mm in diameter) antral follicle number (FN), both in normal women in EFP (Van Rooij et al., 2002; Fanchin et al., 2003) and in patients with PCOS (Pigny et al., 2003). In these studies, the serum AMH level also appeared to be negatively linked to the FSH level, which is in agreement with experimental data showing a negative effect of FSH on AMH secretion (Baanrends et al., 1995).

In this study, we combined biological follicle markers to ultrasound FN within two different size ranges to test the hypothesis that the putative FSH deficiency in FHA impairs the terminal growth and/or functions of antral follicles.

Materials and methods

Patient population

This study was approved by the Institutional Review Board of the Lille University Hospital and informed consent was obtained from all patients and controls before entry into the study. All patients and controls included in this series were consecutively recruited in our Infertility Clinic and submitted to our routine investigative protocol which was the same for all subjects, as previously described (Jonard et al., 2003). Clinical, hormonal and ultrasound data used in this study were taken from our general database and analysed retrospectively.

Controls

The control population consisted of 30 healthy women, whose mean age and body mass index (BMI) are indicated in Table I. They were referred for IVF because of tubal and/or male infertility. Exclusion criteria included a history of menstrual disturbances (i.e. cycle length either <25 days or >35 days), hirsutism, abnormal serum level of prolactin or androgens (i.e. serum testosterone and/or androstenedione >0.7 or 2.2 ng/ml respectively), polycystic ovaries (PCO) at ultrasound according to our criteria (Jonard et al., 2003), BMI >25 kg/m2 and hormonal treatment during the 3 months prior to the study.

Women with FHA

Data collected in 31 women with FHA were used for this study. Mean age and BMI are indicated in Table I. The diagnosis of FHA was based on the association of: (i) a history of primary amenorrhea (with evidence of some pubertal development) or secondary amenorrhea ≥6 months (without pregnancy), along with a history of decreased caloric intake and/or extreme physical exercise and/or emotional or stressful events, with a low or a normal weight; (ii) low or normal LH and FSH levels; (iii) normal hypothalamic and pituitary magnetic resonance imaging; (iv) absence of elevated FSH (i.e. >10 IU/l) or LH (i.e. >6.5 IU/l) serum level. Exclusion criteria were hirsutism and/or elevated serum levels of androgens (i.e. serum testosterone and/or androstenedione >0.7 or 2.2 ng/ml respectively) and/or PCOS and/or PCO features at ultrasound (Jonard et al., 2003), hyperprolactinaemia, pituitary insufficiency, idiopathic hypogonadotrophic hypogonadism, Kallmann’s syndrome, premature ovarian failure and a history of drug abuse or hormonal treatment during the 3 months prior to the study. Any patient had ultrasonic exclusion criteria (see below).

Blood sampling was performed in the EFP (i.e. between days 2 and 7 after beginning of bleeding) in control women, as previously described (Pigny et al., 2000). All FHA patients received diidrogesterone (10 mg/day for 7 days). In the case of uterine bleeding (39% of patients), blood sampling was performed between days 2 and 7.

Table I. Clinical, hormonal and ultrasound data in controls and in patients with functional hypothalamic amenorrhea (FHA)

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 30)</th>
<th>FHA (n = 31)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>29.1 (24.5–32.7)</td>
<td>26.9 (19.8–33.0)</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>21.9 (19.3–24.2)</td>
<td>18.3 (15.1–22.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>Ovarian area (cm2)</td>
<td>3.9 (3.0–4.75)</td>
<td>3.4 (2.4–4.8)</td>
<td>0.03</td>
</tr>
<tr>
<td>2–5 mm follicles</td>
<td>5.0 (2.5–8.0)</td>
<td>5.4 (2.5–10.0)</td>
<td>NS</td>
</tr>
<tr>
<td>6–9 mm follicles</td>
<td>2.3 (0–5.0)</td>
<td>1.1 (0–3.0)</td>
<td>0.007</td>
</tr>
<tr>
<td>Ovarian area (cm2)</td>
<td>3.8 (2.2–5.4)</td>
<td>1.9 (0.5–3.9)</td>
<td>0.0001</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>6.2 (4.8–7.7)</td>
<td>4.8 (1.6–7.4)</td>
<td>0.004</td>
</tr>
<tr>
<td>Inhibin B (pg/ml)</td>
<td>87 (47–130)</td>
<td>48 (15–79)</td>
<td>0.0001</td>
</tr>
<tr>
<td>AMH (pmol/l)</td>
<td>23.7 (6.8–37.5)</td>
<td>35.4 (19.0–61.4)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Values are expressed as mean with 10–90th range in parentheses.

aStudent’s t-test.

bMean of both ovaries.

NS = not significant; BMI = body mass index; AMH = anti-Müllerian hormone.
after the beginning of the bleeding. In the absence of bleeding, blood sampling was performed 7 days after didrogesterone withdrawal.

**Hormonal immunoassays**

E₂, inhibin B, testosterone, and rostenedione, LH and FSH were measured prospectively by immunoassays as described previously (Pigny et al., 2000).

Serum AMH levels were measured retrospectively after thawing stored serum samples. They were performed in duplicate using an ultra-sensitive enzyme-linked immunosorbent assay (AMH-EIA; Beckman Coulter, France) according to the supplier’s instructions. Results are expressed in pmol/l using human recombinant AMH as a standard. The detection limit of this assay using the ultra-sensitive protocol is 0.7 pmol/l (Pigny et al., 2003). This assay could not be performed in 10 patients with FHA, due to the absence or insufficient quantity of stored serum sample.

**Pelvic ultrasound examination**

ultrasound examination was performed on the same day as blood sampling with a 7 MHz transvaginal transducer (Logic 400; General Electric, USA). Ultrasound measurements were taken in real time, according to a standardized protocol, as previously reported (Jonard et al., 2003). All follicles of <9 mm but >2 mm were counted according to our previously reported method (Jonard et al., 2003).

Patients in whom transvaginal ultrasonography was inappropriate (virgin or refusing patients) were excluded, as well as those in whom no follicle was seen in either the right or the left ovary and/or in whom the ovarian area was below the lower normal limit, i.e. 2.5 cm². Patients with at least one follicle >9 mm in diameter at ultrasound, or a serum E₂ level >80 pg/ml, were also excluded from the study so as not to confound the data with the presence of a dominant follicle.

**Statistical methods**

Within each follicle size range (i.e. 2–5 and 6–9 mm), the data used for statistical analysis was the mean of observed FN for the left and right ovaries. Statistical significance between mean values was attributed to two-tailed P < 0.05. Comparisons of two independent groups were made using Student’s t-test or the χ²-test, as appropriate. Significant relationships between the various parameters were evaluated by the non-parametric Spearman correlation coefficient. Influence of the day of sampling was assessed by analysis of variance (ANOVA). All statistical procedures were run on Statview 4.5 (Abacus Concepts Inc., USA).

**Results**

Table I summarizes the main clinical, ultrasound and biological data obtained in the control and FHA groups. As expected, mean BMI, ovarian area, serum LH and FSH levels were significantly lower in the FHA group than in controls. Serum E₂ level was below the detection limit (20 pg/ml) in 79% of patients versus 34% of controls (P < 0.002).

The separate counts of the 2–5 and the 6–9 mm follicles indicated that the mean value of the former was similar between the two groups, while the mean value of the latter

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**Figure 1.** Box-and-whisker plots showing the values of: (A) the 2–5 and 6–9 mm follicle number (FN) and serum FSH and LH levels (in IU/l); and (B) the serum anti-Müllerian hormone (AMH) and inhibin B levels, in patients with functional hypothalamic amenorrhea (FHA) (n = 21 and 31 respectively) and in controls (n = 30). Horizontal small bars represent the 10–90th percentile range, and the boxes indicate the 25–75th percentile range. The horizontal line in each box corresponds to the median. *Significantly different from controls (see Table I).
was significantly less in FHA than in the control group (Figure 1A and Table I). Univariate analysis using the Spearman correlation test indicated significant negative and positive relationships between FSH and the 2–5 and 6–9 mm FN respectively in the FHA group exclusively (Table II and Figure 2A and B respectively).

The mean serum level of inhibin B was lower in the FHA group than in controls (Table I and Figure 1B). It was not related to FSH, in any group, whereas it was significantly and positively related to the 6–9 mm FN in controls exclusively (Table II and Figure 3B).

The serum level of AMH was assayed in only 21 patients with FHA (see Materials and methods) and in all controls. The mean value was significantly higher in the FHA group than in controls (Table I and Figure 1B). It was negatively related to FSH, in controls exclusively (Table II), whereas it was positively related to the 2–5 mm FN in both groups, albeit non-significantly in controls, according to Bonferroni correction (Table II and Figure 3A).

Nine (29%) and 22 (71%) patients with FHA had a basal serum FSH level below and above 4.5 IU/l (5th percentile of control values) respectively. The mean value of the 6–9 mm FN was significantly lower in the low FSH group than in the normal FSH group (0.3 ± 0.7 versus 1.5 ± 1.5 IU/l respectively, \( P = 0.02 \)), the latter being similar to controls (Figure 4A). On the other hand, no significant difference was found for the mean AMH and inhibin B serum levels between the two subgroups, every mean value remaining significantly higher and lower than in controls, in both subgroups, respectively (Figure 4B). In patients with normal FSH (>4.5 IU/l), the ratio AMH:2–5 mm FN was significantly higher than in controls (8.4 ± 3.8 versus 5.4 ± 2.9 respectively, \( P = 0.007 \)), whereas it was similar to controls in the low FSH group (5.3 ± 1.6, not significant).

No significant relationship was found between the serum LH levels and the FN, AMH or inhibin B values. Likewise, these parameters did not differ between patients with either low (<2 IU/l) (66% of patients) or normal LH serum level (34% of patients) (data not shown). By ANOVA in controls and in patients who experienced bleeding after didrogesterone, the day of sampling (i.e. days 2–7 after beginning of bleeding) did not significantly influence the hormonal and ultrasound parameters (data not shown).

### Table II. Relationships between the serum FSH, inhibin B and anti-Müllerian hormone (AMH) levels and the 2–5 and 6–9 mm follicle number (FN) in patients with FHA and in controls

<table>
<thead>
<tr>
<th></th>
<th>FHA (n = 31)</th>
<th>Controls (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \rho )</td>
<td>( p )</td>
</tr>
<tr>
<td>AMH, 2–5 mm FN</td>
<td>0.618(^*)</td>
<td>0.006</td>
</tr>
<tr>
<td>AMH, FSH</td>
<td>-0.122(^*)</td>
<td>NS</td>
</tr>
<tr>
<td>Inhibin B, 6–9 mm FN</td>
<td>0.113</td>
<td>NS</td>
</tr>
<tr>
<td>FSH, 2–5 mm FN</td>
<td>-0.424</td>
<td>0.025</td>
</tr>
<tr>
<td>FSH, 6–9 mm FN</td>
<td>0.428</td>
<td>0.024</td>
</tr>
</tbody>
</table>

\(^*\)Spearman correlation coefficient.
\(^*\)In 21 patients.
\(^*\)Non-significant after Bonferroni correction.

NS = not significant.

### Discussion

The markers that we used in our patients with FHA (FN, serum AMH and inhibin B levels) enabled us to document the FSH insufficiency better than the LH one. That the serum LH level was not related to these follicular markers, neither in controls nor in patients with FHA, was expected. Indeed, granulosa cells (GC) have not yet acquired their LH receptors at the stage of follicular growth when our patients and controls were investigated (i.e. no follicle >9 mm) (Willis et al., 1998). As our study did not address the LH-dependent events which intervene later in the cycle, once a dominant follicle is selected, our data do not contradict the major role of LH.
insufficiency in the anovulation of FHA. This role includes impairing the dominant follicle selection, as well as its further growth and function. It is better addressed in studies comparing treatments with or without LH (hMG and recombinant FSH respectively) for ovulation induction in patients with FHA or hypogonadotrophic hypogonadism (Shoham et al., 1991).

Our ultrasound data document for the first time that the ovarian marker best related to low FSH serum levels (i.e., <4.5 IU/l) in FHA patients is the paucity in 6–9 mm follicles. Presumably, this finding reflects the impaired shift from the 2–5 mm to the 6–9 mm follicles, which is known to be highly FSH dependent and occurs during the FSH intercycle peak in normal women (Macklon and Fauser, 2001). That the 2–5 mm FN was normal in our patients with FHA having a low basal FSH level could seem paradoxical since the growth of these small antral follicles is also FSH dependent (McGee and Hsueh, 2000). To explain this, we hypothesize that the aforementioned impaired shift was responsible for the stagnation of these follicles. The negative relationship that we found between the FSH serum level and the 2–5 mm FN in our patients with FHA argues further for the responsibility of the relative FSH insufficiency in this impaired shift. This phenomenon may explain why ovaries in FHA frequently appear as multifollicular (Adams et al., 1985; Futterweit et al., 1988). Also, that no relationship was found between serum FSH level and the 2–5 mm or the 6–9 mm FN in controls could be explained by the fact that most of them were investigated during the second part of their FSH window; a time when the role of FSH on follicle maturation declines (Macklon and Fauser, 2001). This emphasizes the need for an early work-up in the cycle (no later than day 3) if one wishes to analyse the relationships between FSH and follicle maturation or number during the FSH window in normal women.

If one relied on their normal basal FSH level, the majority (71%) of our patients with FHA could be considered as having no FSH insufficiency. However, at the follicle level, they displayed features of FSH insufficiency, as reflected by the lower mean inhibin B serum level and the lack of significant relationship between this parameter and the 6–9 mm FN, whereas in controls this relationship tended to be significant. Indeed, the positive association between inhibin B and the 6–9 mm FN in our controls indicates that these follicles are the main source of this hormone, which is in agreement with physiological studies (Hayes et al., 1998). The fact that patients with a normal serum FSH level have a low serum inhibin B level despite the presence of a normal number of 6–9 mm follicles suggests that the FSH-dependent secretion and growth functions are dissociated in these follicles. It has been theorized that the FSH threshold is lower for inducing follicle growth than for inducing GC differentiation (Gougeon, 1996). According to this theory, such a finding could indicate a partial loss of FSH activity at the follicle level which is not properly reflected by the routine assay of its serum level.

AMH was positively related to the 2–5 mm FN in our whole group of patients with FHA, as previously reported in normal women (Seifer et al., 2002; Van Rooij et al., 2002; Fanchin et al., 2003) and in patients with PCOS (Cook et al., 2002; Pigny et al., 2003). However, the absolute mean AMH level was significantly higher than in controls despite similar 2–5 mm FN. One could relate this finding to FSH insufficiency since experimental data have shown a suppressive effect of FSH on the AMH secretion by GC from 2–5 mm follicles (Baarends et al., 1995). Nevertheless, the increased AMH serum levels were independent from the serum FSH levels, which is in contrast to controls. In addition, the mean AMH level was still excessive in the subgroup of patients with normal serum

![Figure 3. Relationship between (A) serum AMH level and the 2–5 mm FN and (B) serum inhibin B level and the 6–9 mm FN, in controls (n = 30) and in patients with FHA (n = 21). See Table II for the values of the Spearman coefficient of correlation. For each panel, the thicker and the thinner regression lines apply to FHA and control groups respectively.](https://academic.oup.com/humrep/article-abstract/20/1/101/671554)
FSH and the mean ratio AMH:2–5 mm FN was higher than in controls. Although we cannot exclude the possibility that higher AMH levels in FHA are due to an excess of follicles that are below the range of detection by ultrasound, our data rather suggest a higher AMH secretion by each follicle and therefore an impaired FSH activity at the follicle level. Whether both inhibin B and AMH abnormalities are secondary to alteration in the pulsatile secretion of FSH and/or in its bioactivity and/or in follicle sensitivity was not addressed in our study and would deserve further investigation. Nevertheless, the fact that the ratio AMH:2–5 mm FN was normal in patients with a low FSH serum level argues against an intrinsic FSH resistance in the follicles of women with FHA. These findings in FHA differ from those that we previously reported in PCOS (Pigny et al., 2003). Indeed, in the latter situation, the increase in AMH serum level could be explained by the excess in the 2–5 mm FN per se and it occurred despite the maintenance of the negative effect of FSH on AMH (Pigny et al., 2003). From both data in FHA and PCOS, we hypothesize therefore that the serum AMH level in women not only reflects the number of small antral follicles (2–5 mm), but also the maturation stage of these follicles, and more especially their degree of exposure and/or sensitivity to bioactive FSH.

In conclusion, our ultrasound data document for the first time that the paucity in the 6–9 mm FN directly reflects the low FSH level in FHA, which is infrequent, however. Despite a normal serum FSH level and 6–9 mm FN in the majority of patients with FHA, the functional follicle markers of the FSH sensitivity during the EFP (inhibin B, AMH) are abnormal, suggesting that FSH is not fully active.

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