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Anti-Mullerian hormone and ovarian morphology in women with isolated hypogonadotropic hypogonadism/Kallmann syndrome. Effects of recombinant human FSH.

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Context: Isolated hypogonadotropic hypogonadism (IHH), characterized by gonadotropin deficiency and absent puberty, is very rare in women. IHH prevents pubertal ovarian stimulation, but anti-Mullerian hormone (AMH) and antral follicle count (AFC), have not been studied.
Objectives: 1) Compare, in IHH versus controls, AMH, ovarian volume (OV) and AFC. 2) Compare in IHH, ovarian responses to recombinant human FSH (rhFSH) and rhFSH plus rhLH.
Subjects: 68 IHH women, 51 matched healthy women.
Methods: Were compared, serum LH, FSH, sex steroids, inhibin B (InhB), AMH and OV and AFC (sonography). Ovarian response during rhFSH administration was assessed in twelve IHH with low AMH levels and low AFC and compared to hormonal changes observed in six additional IHH women receiving rhFSH plus rhLH.
Results: InhB was lower in IHH than in controls. AMH levels were also significantly lower in the patients, but two-thirds had normal values. Mean OV, total, larger and smaller AFC were lower in IHH than in controls. Ovarian stimulation by rhFSH led to a significant increase in serum E2 and InhB levels and in the number of larger AF. AMH and smaller AFC increased early during rhFSH stimulation but then declined despite continued stimulation. rhFSH plus rhLH stimulation led to a significantly higher increase in E2 levels but to similar changes in circulating InhB and AMH, than rhFSH alone.
Conclusions: IHH women have both low AMH levels and low AFC. However, their decrease can be reversed by FSH. Serum AMH and AFC should not serve as prognostic markers of fertility in this population.

PRECIS: We measured AMH and AFC in IHH/KS women and studied effect of FSH. IHH/KS had low AMH and low AFC but their decrease can be reversed by FSH and are not prognostic markers of fertility in IHH/KS.

Isolated hypogonadotropic hypogonadism (IHH) and Kallmann syndrome (KS) are rare genetic diseases due to deficient GnRH secretion or action (1). Their prevalence appears to be lower in females (estimated at 1/10 000 to 1/50 000) than in males (1,2). Patients with IHH/KS have a profound chronic deficit, both prenatally and postnatally, in the two pituitary

gonadotropins (LH and FSH), which generally persists throughout life (3). In females, IHH/KS is usually diagnosed in the teenage years, because of primary amenorrhea with absent or partial breast development, depending on the severity of the deficit in gonadotropins and, thus, in ovarian steroids (1,2). Apart from two isolated cases (4,5), anti-Mullerian hormone (AMH) has not been evaluated in women with IHH/KS. Similarly, ovarian morphology, and especially the number of antral follicles, have not been precisely studied in this setting. Yet IHH/KS is an ideal model for analyzing the ovarian consequences of chronic gonadotropin deficiency. It also allows the physiological role of pituitary gonadotropins to be studied in terms of both ovarian peptide and steroid hormone secretion and follicle growth (6-8). The aims of this study were 1) to evaluate ovarian AMH and inhibin B levels (InhB) and the number of antral follicles (AF) in a significant number of well-phenotyped women with IHH/KS compared with healthy matched controls. 2) We evaluated the response of sex steroids and of these two ovarian peptides to recombinant human FSH (rhFSH) administration, and studied their relationship with the number and growth of antral follicles in a subset of these patients affected by severe gonadotropin deficiency. We also had the opportunity to compare the ovarian hormonal response in these latter patients to that of similar IHH/KS women having received combined rhFSH plus rhLH therapy.

Controls, Patients and Methods

Healthy controls

Healthy female volunteers aged over 18 years, recruited through the press, were included after a thorough history and clinical examination between March 2010 and October 2015. This control population consisted of 51 healthy women aged from 21–36 years with normal thyroid hormone, IgF1 and PRL levels, and a normal body mass index (Table 1). All healthy control women had a normal age of pubertal onset (12-14 years) and regular spontaneous menstrual cycles (27–31 days), and had ovulated in the cycle preceding the evaluation, as confirmed by progesterone levels above 6.0 ng/mL. None of these normal women had clinical evidence of androgen excess and none exercised excessively/intensively. These healthy controls were studied between days 3 and 6 after menses onset of a spontaneous cycle, at which time clinical examination, blood sampling, and transvaginal ovarian ultrasound were completed.

IHH/KS patients

All 68 women with IHH/KS included in this study were at least 18 years old at the time of evaluation (Table 1). At diagnosis, they had low to low-normal estradiol (E2) levels, low or inappropriately normal gonadotropin levels, no other pituitary hormone deficiencies (normal FT4, TSH, IGF1, and normal cortisol and GH responses to hypoglycemic challenge), normal to low serum prolactin levels, and no neuroanatomic (normal hypothalamo-pituitary MRI) or functional cause of hypogonadotropic hypogonadism. None of the IHH/KS women had an eating disorder, and all had BMI values $\geq$ 22 kg/m^2 (range: 22-26 kg/m^2). Their body fat composition, determined by dual-energy X-ray absorptiometry (DEXA), was also normal (21-25%/weight). None of the patients exercised excessively/intensively. None had clinical evidence of androgen excess. Thirty-eight of the patients were considered to have KS as olfactory testing (a rapid computer-assisted olfactory test to measure detection thresholds for five pure odorants) showed anosmia or hyposmia, and/or because MRI showed olfactory bulb aplasia or hypoplasia (9). Six additional IHH/KS (25-28 years) having received combined gonadotropin therapy were also included.

Protocols

The study was approved by the Institutional Review Board of Bicêtre teaching hospital (P081216/IDRCB 2009 A00892-55 and PHRC-2009-2015), and all participants provided informed consent before inclusion.

The 68 IHH/KS patients were enrolled between November 2009 and March 2016. In 21 cases, initial basic hormonal profiling was performed at diagnosis, before any replacement therapy. In the remaining 47 cases, hormonal profiling and sonography were performed at least 3 months after discontinuation of estrogen/progestagen replacement. Only women who did not develop spontaneous menses after discontinuing replacement therapy were included, to exclude those whose gonadal axis might have recovered (reversible forms) (10). In addition to the 68 KS/IHH women discussed above, were included six IHH/KS patients wishing to conceive who were referred between March 2012 and June 2015 for ovulation induction.

Ovarian stimulation with recombinant human FSH (rhFSH)

With their informed consent, rhFSH was administered to 12 patients wishing to assess their fertility. This subset had low AMH levels (4.1 to 11.4 pmol/L, see Results), and their previous gynecologists had informed them that they had little chance of conceiving based on AMH cut-offs in women with an intact, hypothalamic-pituitary-ovarian axis (11,12). RhFSH (GONAL-f$^{(R)}$, Laboratoires Merck-Serono, France; or Puregon$^{(R)}$, Laboratoires MSD, France) was administered daily at a dose of 150 IU s.c. (13,14) for 5 days, followed by 75 IU/day for the next 7 days. None of these patients received exogenous recombinant LH or human chorionic gonadotropin.

Induction of ovulation in 6 additional women with KS and small ovaries associated with low AFC and low serum AMH

These six IHH/KS women had initially consulted other centers and had been refused gonadotropin treatment because of their low AMH levels (range: 4.2-11.2 pmol/L) associated with low ovarian volume (range: 1.25-2.6 mL) and low AF (range: 2.2-4.4). Their spouses had normal sperm counts (>15x10^6/mL, normal morphology and mobility percentages). In these 6 patients, treatment initially comprised 75 to 150 IU of rhFSH (GONAL-f$^{(R)}$ or Puregon$^{(R)}$) and 75 IU of rhLH (Luveris$^{(R)}$, Laboratoires Merck-Serono, France) (13,14), the doses then being titrated to the patients' responses. Treatment was individualized and monitored by serum E2 assay and ovarian ultrasound every 4 days: briefly,the mean daily dose (IU) regimens were for rhFSH and rhLH respectively: 131±31 (range 75-150) and 75±0.0 from day 0 to day 4, 144±15 IU (112.5-150) and 137.5±19 (112.5-150) from day 5 to day 8, 81±14 (75-112.5) and 150±41 (112.5-225) from day 9 to day 12.

Recombinant human hCG (rhCG; Ovitrelle$^{(R)}$, Laboratoires Merck-Serono, France) (250-500 µg s.c.) was given when a single dominant follicle larger than 16 mm was detected, with a concomitant E2 level above 350 pg/mL (mean: 449±62; range: 350-512 pg/mL). Luteal-phase support consisted of subcutaneous rhCG or vaginal progesterone. The cycle was cancelled if there were more than two dominant follicles on ovarian ultrasonography.

Ovarian ultrasound examination

Transvaginal ultrasonography was not possible in 29 of the 68 IHH/KS patients, because of virginity or refusal. Ovarian ultrasonography (OvUS) was performed in the remaining 39 untreated patients at an unspecified time (amenorrheic women) and in 41 healthy controls in the early follicular phase, 3 to 6 days after menses onset. OvUS was always performed on the same day as blood sampling.

Transvaginal OvUS was performed in patients and controls by the same experienced ovarian ultrasonographer (JML), using a Voluson E8 Expert device (Voluson E8 EXP BT13, 3D-4D, General Electric Systems, VELIZY, France) equipped with a 6–12 MHz transvaginal transducer. Ultrasound measurements were made in real time, using a standardized protocol. The highest possible magnification was used to examine the ovaries. Each ovarian volume (OV) was estimated as π/6 (transverse diameter) x (anteroposterior diameter) x (longitudinal diameter)(ellipsoid). Each antral follicle (AF) (≥ 2mm) was identified and then measured as

the mean of two diameters. The OV and the AF count data used for statistical analysis were the mean of the values for the left and right ovaries.

Hormone assays

Fasting blood samples were drawn between 08h00 and 10h00 from an antecubital vein and immediately centrifuged at 4°C. Serum was stored at -80°C until assayed. To avoid inter-assay variability, all hormone assays were performed during the same run with frozen (-80°C) samples. Serum InhB levels were measured with the Beckman Coulter GenII assay (Marseilles, France), with a detection limit of 3 pg/ml and intra- and inter-assay CVs of respectively 6% and 11% at 15 pg/ml. Serum AMH was measured using an enzymatically amplified two-site immunoassay (AMH Gen II ELISA; Beckman Coulter, Marseilles, France) according to the manufacturer's recommendations. The detection limit was 0.7 pmol/L. The mean inter-assay coefficient of variation (CV) was 8.6% and the mean intra-assay CV was 4.0% at 22 pmol/L.

Serum FSH and LH were measured using a sensitive immunoradiometric assay (CIS bio international, GIF sur Yvette, France), with a detection limit of 0.05 IU/L for both hormones (IU/L, 2nd IRP WHO 78/549 for FSH; IU/L, 1st IRP 68/40 for LH). The intra- and inter-assay CVs were 1.5% and 5.2% for LH, and 2.7% and 5.5% for FSH, using quality control (QC) sera measuring 3.5 and 3.9 IU/L, respectively. Estradiol (E2), androstendedione (ADIONE) and testosterone (T) were assayed simultaneously in the two groups of subjects using GCMS, after extraction and derivatization, as described in detail by Giton et al (15). The lower limits of detection (LLD) and the intra-assay and inter-assay CVs were 2.4 pg/mL, and 7.9/9.5%, respectively for E2 at using QC sera measuring 2.7 pg/mL; 0.05 ng/mL and 2.2/3.1% for T using QC sera measuring 0.48 ng/mL; 0.049 ng/ml, and 4.6/5.2% for ADIONE using QC sera measuring 0.15 ng/mL.

Statistical analyses

All results are reported as individual values in the figures and as mean ± SD in Table 1 and in the text. Hormonal parameters were compared by using a parametric t test or the Mann-Whitney, Wilcoxon, or Kolmogorov-Smirnov nonparametric test as appropriate. We used one-way repeated-measures ANOVA to assess the ovarian response to rhFSH or to rhFSH plus rhLH. Using ovarian hormone or AFC as dependent variables. Appropriate post-hoc pairwise comparisons were performed. Spearman's rank correlation procedure was used to define the serum AMH values that correlated with OV and AFC. *P* values <0.05 were considered to denote statistical significance.

Results

Basal gonadotropin and sex steroid levels

Individual serum FSH, LH, E2, ADIONE, T, InhB and AMH values are shown in Fig.1. Both FSH and LH were significantly lower in the IHH/KS patients than in the controls. E2 levels were also significantly lower in the patients than in the controls, but were nonetheless well above the detection limit of this sensitive steroid assay. Both ADIONE and T levels were slightly but significantly lower in the patients than in the controls.

Serum InhB levels were significantly lower in the patients than in the controls (Fig.1, panel E). Only 11 of 68 patients had InhB values above the lowest observed control value. The average InhB level (pg/mL) was lower among patients with serum FSH levels below 1 IU/L than among patients with higher FSH levels (7.4±3.8 versus 16.1±9.5; p<0.001). Likewise, the mean serum InhB level was significantly lower among patients with estradiol levels of 12 pg/mL or less than among patients with higher E2 levels (8.1±4.2 versus 19.2±10.9, p<0.001).

AMH levels (pmol/L) were also significantly lower in the patients than in the controls (Fig.1, panel F). However, it must be stressed that two-thirds of the patients (44/68) had AMH levels above the lowest observed control value. The mean AMH level was lower among patients with FSH levels below 1 IU/L than among patients with higher FSH levels (14.1±7.2 vs 32.5±10.7, p<0.001).

Ovarian ultrasonography

Mean (± SD) ovarian volume (OV) was very significantly lower in the patients than in the controls (1.54±0.55 versus 6.02±2.05 ml, p<0.0001)(Fig.2A). Mean OV was lower among patients with serum FSH levels below 1.0 IU/L than among patients with higher FSH levels (1.1±0.2 vs 1.9±0.5 ml, p<0.001). The mean total AF number/ovary was significantly lower in the patients than in the controls (3.67±1.9 vs 12.0±4.8; p<0.001)(Fig.2B). Further, the total AF number/ovary was lower in patients with FSH levels below 1 IU/L than in patients with higher levels (2.1±1.2 vs 4.4±1.3; p<0.001). Likewise, the numbers of small (≤6 mm) follicles (3.2±1.9 versus 6.9±3.9; p<0.001) and large follicles (>6 mm) (0.45±0.7 versus 5.0±2.7; p<0.001) were lower in the patients than in the controls (Fig.2 panels C-D). The number of small AF number/ovary was lower in patients with circulating FSH <1.0 IU/L than in patients with higher FSH levels (1.4±1.4 versus 3.9±1.6, p<0.001).

Ovarian aspect and circulating AMH

In IHH/KS serum AMH levels correlated positively with ovarian volume (r = 0.543, P < 0.001) and with the total number of follicles (r=0.553, P <0.001). Serum AMH also correlated positively with the number of small (≤6 mm) follicles (r =0.41, P <0.001).

Effect of recombinant human FSH on ovarian hormone levels and follicle counts

Individual changes in serum FSH, E2, T, InhB and AMH in the 12 IHH/KS patients with severe gonadotropin deficiency after treatment with rhFSH are shown in Fig.3, panels A-E. RhFSH administration induced a sustained elevation of plasma FSH levels (from 0.15±0.08 to a peak of 9.4±2.57 lU/L; F 103.4, P <0.0001) in the treated women, confirming their adherence to FSH therapy (Fig.3A). From the 8th day of FSH therapy, serum FSH levels stabilized as a result of lowering of doses administered.

FSH administration did not affect LH levels (0.34±0.28 before, 0.31±0.23 IU/L during).

During rhFSH therapy there was a marked increase in serum E2 from 7.6±2.9 to 104.4±22.0 pg/mL; p< 0.0001 (Fig 3B) with no increase in plasma T (Fig 3C). InhB increased with FSH treatment from 6.8±2.9 to 190.8±21.7 pg/mL; p<0.0001 (Fig. 3D). A marked increase in AMH was evident by day 4 of rhFSH administration, followed by a slight but significant decline on day 12 (Fig.3E).

Changes in the mean AFC during rhFSH therapy are shown in Fig.3F. Interestingly, the number of larger follicles (>6 mm) rose significantly from 0.4±0.7 before rhFSH to 4.2±1.8 on day 4, and continued to increase significantly on day 8 (6.2±1.9), reaching a maximum on day 12 (8.0±1.4) in spite of decrease in FSH doses administered. The number of smaller follicles (≤6 mm) also rose significantly between days 0 and 4 from 2.8±1.4 to 5.9±1.8 but then fell to 4.4±1.2 on day 8 and 4.0±1.0 on day 12.

Comparative effects of rhFSH alone and combined rhFSH plus rhLH on ovarian hormone levels in women with IHH/KS

Comparison of changes in mean (±SD) serum FSH, LH, E2, T, Inhibin B and AMH in the twelve IHH/KS patients having received rhFSH alone versus the six IHH/KS patients treated with combined rhFSH plus rhLH for ovulation induction are shown in Fig.4, panels A-F. A significant and similar increase in mean serum FSH levels was observed during both rhFSH and rhFSH plus rhLH administration. Whereas rhFSH administered alone did not affect, neither mean LH nor mean T levels, in IHH/KS patients who undergone the combined rhFSH plus rhLH treatment, mean serum LH and mean T increased significantly (Fig.4, panels B and

C). In parallel, when compared to IHH/KS patients having received rhFSH alone, the increase in mean serum E2 levels was significantly and sharply higher in patients who received rhFSH plus rhLH combination therapy (Fig.4, panel D). In contrast, the significant increase in both mean serum inhibin B and mean AMH levels was similar during both rhFSH alone and during combined rhFSH plus rhLH administration (Fig.4, panels E and F).

Ovulation and conception in the 6 KS patients with low serum AMH levels and low AF numbers
In the 6 KS patients with low AMH levels who underwent ovulation induction with rhFSH and rhLH, ovulation occurred after 2-4 cycles of stimulation. Five of these patients, who had programmed intercourse before and at the time of triggered ovulation, conceived and gave birth to a healthy baby (no twin pregnancies).

Discussion

The relation between circulating AMH levels and ovarian morphology, including the number of antral follicles, has mainly been studied in women with polycystic ovary disease (11) or ovarian failure (12, 16), during ovulation stimulation with or without GnRH analogs (17,18) and, more rarely, in healthy prepubescent girls (17,19). In contrast, only anecdotal reports (4,5) are available for women with IHH/KS. This lack of published data is probably due to the rarity of IHH/KS in females (1,2). Yet this disease represents a unique model, given the presence of isolated gonadotropin deficiency and normal levels of other anterior pituitary hormones as it begins in the antenatal period and continues through the neonatal and postnatal periods, persisting into adulthood with rare exceptions (3,6-8).

Thus, the first aim of this study was to study AMH and other ovarian hormones, as well as ovarian volume, and the number of antral follicles in a large group of women with IHH/KS compared to healthy cycling control matched women.

In line with a previous report that used a RIA assay (2) we observed here very low estradiol levels in untreated IHH/KS women. This deficiency in ovarian estradiol secretion was caused by the combined effects of FSH and LH insufficiencies. First, FSH is essential to stimulate aromatase in ovarian granulosa cells. Secondly, LH deficiency in women with IHH / KS can be responsible for a relative androgen deficiency. Indeed, low circulating androstenedione and testosterone levels demonstrated here, are probably consecutive to inadequate stimulation of internal theca cells by low circulating LH. This hypothesis was confirmed by the increase in testosterone levels during combined rhLH plus rhFSH stimulation of IHH/KS women whereas FSH had no effect. Data provided here also showed a larger increase in serum E2 in IHH/KS women receiving rhFSH plus rhLH when compared to those stimulated with rhFSH alone. This reinforce the importance of LH deficiency in IHH/KS and its replacement requirement (6-8,13,14). A sufficient elevation of estradiol, during ovulation induction is indeed essential to ensure both sufficient development of the endometrium and adequate secretion of cervical mucus; both being mandatory for sperm passing through the cervix and for a embryo implantation and necessary for obtaining an ongoing pregnancy (13,14).

In addition to low levels of FSH, LH and estradiol, circulating InhB levels were also very low in KS/IHH women. Moreover, InhB levels were particularly low in the subgroup of women with the lowest FSH levels. This suggests that InhB reflects the severity of FSH deficiency in IHH/KS women, as previously reported in men with this disease (1). The low InhB concentrations observed in these patients are similar to those described in normal girls before the pubertal increase in gonadotropins (19-21). Although the patients with IHH/KS have concomitant deficiencies in both FSH and LH, our findings indicate that abnormal ovarian inhB secretion in these patients is due mainly to their FSH deficiency. This assumption is consistent almost undetectable serum InhB observed in women with isolated complete FSH deficiency due to FSH-β gene mutation, despite elevated LH levels (4). The

mainly dependency of ovarian inhB secretion on FSH in the IHH/KS women studied here was also clearly suggested by the inhB response to rhFSH administration in the absence of an increase in LH and was reinforced by the lack of additional inhB stimulation in IHH/KS women having received both rhFSH plus rhLH. Further, these results are consistent with the early follicular phase dependency of inhB on FSH, but not LH, in normal women in whom endogenous gonadotropins were suppressed by a GnRH agonist (22,23). The low inhB levels in our patients were also associated with low estradiol levels, as the average inhB level was significantly lower in the subgroup of patients with the most pronounced estradiol deficiency. This *in vivo* finding is consistent with the positive correlation reported between E2 and inhB concentrations in antral follicular fluid (24).

We also found that circulating AMH concentrations were significantly lower in women with IHH/KS than in healthy controls. This clearly shows the dependency of circulating AMH levels on gonadotropins, as suggested by the decrease in AMH levels in women whose gonadal axis is inhibited by GnRH antagonists or agonists (17). However, almost two-thirds of our IHH/KS patients had serum AMH levels within the control range. Interestingly, the subgroup of IHH/KS women with the lowest FSH levels had a significantly lower AMH levels than those with higher FSH levels, indicating that the low levels of AMH are also linked to the severity of FSH deficiency. The correlation between ovarian volume and the AMH concentration also favors a relationship between the severity of gonadotropin deficiency and the AMH level.

In the current study we have shown that AMH levels in KS/IHH patients in the absence of FSH stimulation are lower than in cycling control women in contrast to AMH levels in women with acquired gonadotropin deficiency secondary to functional hypothalamic amenorrhea or to hyperprolactinemia (25,26), in whom AMH concentrations are similar to those of normal women. Two factors could account for this difference. The first is the greater severity of chronic gonadotropin (especially FSH) deficiency in IHH/KS. Second, the very prolonged (pre- and postnatal) gonadotropin deficiency in women with IHH/KS might also have a more pronounced impact on ovarian follicle development and, thus, on AMH secretion, than would an acquired deficit. This is consistent with results from Deubzer et al., who reported very low AMH levels in three teenage girls with severe congenital panhypopituitarism, whereas girls with global hypopituitarism acquired after birth had AMH levels closer to those of healthy women (27).

Average ovarian volume was significantly less in women with IHH/KS than in the healthy controls and similar to ovarian healthy pre-pubertal girls (19). The low OV observed here is in agreement with that reported by Shaw et al. in 39 women with IHH/KS (2). We also found that the subgroup of women with the lowest FSH concentrations had the smallest ovaries.

For the first time we determined the number of antral follicles in a significant number of women with IHH/KS. The total AF number/ovary was lower than in healthy controls studied with the same ultrasound method. Low follicle counts appear to be related to the severity of gonadotropin (especially FSH) deficiency, as the subgroup of women with the lowest FSH levels had also the lowest counts. However, the number of larger follicles was more severely affected than the number of smaller follicles. This suggests that the FSH deficit in women with IHH/KS preferentially affects terminal follicle development responsible for larger follicles and is consistent with published data showing that the development of larger follicles, and the selection of a dominant follicle, requires sufficient, near-normal FSH secretion (28). The relative sparing of small follicles suggests that a degree of follicle growth can occur despite very inadequate FSH secretion, as was also true in a woman with isolated FSH deficiency due to mutation of the FSH-β subunit gene (4). The lower number of small AF among IHH/KS women than among healthy controls suggests that follicle growth to the small antral stage also requires a certain threshold level of FSH secretion. The total AF

number in patients with IHH/KS may be even lower than those reported in pre-pubertal girls measured using ovarian MRI (19). While this difference may suggest a specific ovarian impact of both prenatal and early postnatal gonadotropin deficiency as is seen in men with IHH/KS (3), confirmation requires comparisons using the same methodology in both groups.

As expected, rhFSH administration caused a significant increase in estradiol (4-8). It is noteworthy that the estradiol elevation was smaller than that described in similar patients treated concomitantly with rhFSH and recombinant LH (12,13). RhFSH stimulation also allowed us to specify, in vivo, the relationship between the growth of ovarian follicles and the secretion of inhB and AMH. The sharp increase in inhB levels coincided with the increase in the number of large (>6 mm) antral follicles. This is in agreement with reports that inhB is secreted by granulosa cells present in larger dominant follicles (29-31). However, it is noteworthy that E2 and larger antral follicles increased despite a decrease in FSH doses while Inh B increased and then plateaued. These data suggest that terminal follicular growth and estradiol secretion by the larger antral follicles could require less FSH than inhibin B secretion.

Interestingly, in IHH/KS circulating AMH levels increased rapidly after rhFSH stimulation. This strongly suggests that FSH plays a role in ovarian AMH secretion. Even more interestingly, we found that serum AMH levels normalized in the majority of IHH/KS women treated with rhFSH, showing that the low basal AMH levels in these patients are reversible. The relatively early increase in AMH was associated with an early increase in the overall number of antral follicles and in particular those ≤ 6 mm. The subsequent decrease in AMH levels and small follicle number could be related to the decreased FSH doses administered. Alternatively and most likely, the delayed decrease in AMH levels could mainly be related to the relative increase in the number of large antral follicles gradually induced by rhFSH therapy. This latter hypothesis is in agreement with both the demonstrated negative correlation between human follicular fluid AMH concentrations and the increase in size of antral follicles and also the with the decrease in AMH levels observed during puberty (30, 31,33).

For clinical and therapeutic purposes, it was important to show here that the low AMH levels and low antral follicle counts in women with IHH/KS can be reversed by FSH administration, and that fertility can be achieved despite data suggesting an “impaired ovarian reserve". The low AMH levels and low antral follicle counts in women with complete IHH/KS should therefore not be considered predictive of a poor response to ovarian stimulation with gonadotropins, or as a sign of a poor fertility prognosis. Indeed, it is clearly established that, in the vast majority of cases, ovulation and fertility can be achieved by pulsatile GnRH administration or by gonadotropin combination therapy in these patients (12,13,32).

Conclusion

We show that women with IHH/KS have variably low circulating AMH levels, and that the severity of AMH deficiency is related to the severity of FSH deficiency. In addition, the low AMH levels in these patients are associated with low antral follicle counts. However, both these latter parameters can be reversed by FSH administration. These patients' low ovarian volume, low AMH and or antral follicle count should not be considered to indicate a poor childbearing prognosis, as fertility can be effectively restored by both pulsatile GnRH and gonadotropin administration.

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Fig. 1: Serum FSH and LH (panel A), estradiol (E2) (panel B), androgens androstenedione (ADIONE, panel C) and testosterone (panel D), and serum ovarian peptides inhibin B (panel E), and AMH (panel F) levels in healthy women (Controls) (n=52; 21–36 y) and in untreated women with IHH/KS (n= 68; 18-34 y). ** p< 0.01 and *** p < 0.0001. To convert T values from nanograms per milliliter to nanomoles per liter, multiply by 3.467, and to nanograms per deciliter multiply by 100. To convert ng/mL to nanomoles per liter, multiply by 3.491 for 4-ADIONE. To convert E2 values to picomoles per liter, multiply by 3.671.

Fig.2: Mean ovarian volume (panel A), total mean antral follicle (AF) number /ovary (panel B), mean small (≤6 mm) AF number/ovary (panel C) and larger (>6 mm) AF number/ovary (panel D) in healthy women (Controls) (n=41) and in untreated women with IHH/KS (n=39). ** p< 0.01 and *** p < 0.0001.

Fig.3: Panels A-E: Effect of recombinant human FSH (rhFSH) administration on individual levels of circulating FSH, estradiol (E2), testosterone (T), inhibin B and AMH in 12 women with complete IHH/KS. *p<0.05, ** p< 0.01 and *** p < 0.0001. ULN: upper limit of

normal; LLN: lower limit of normal. **Panel F**: Effect of FSH on the number of small (≤6 mm) and large (>6 mm) follicles per ovary in the same patients (value is mean of right and left ovary). [a]D4 vs D0, [b]D8 vs D0, [c]D8 vs D4, [d]D12 vs D0, [e]D12 vs D8. RhFSH was administered daily and subcutaneously at a dose of 150 IU s.c. for 5 days (D0 to D5), followed by 75 IU/day for the next 7 days (from D5 to D12), see methods section.

Fig.4: Comparison of mean (±SD) circulating FSH and LH (panels A and B), testosterone (T) and estradiol (E2) (panels C and D) and the ovarian peptides inhibin B and AMH (panels E and F) levels in women with IHH/KS treated with either rhFSH alone (n=12; black circles), or with combined rhFSH plus rhLH (n=6; open triangles). In the subgroup receiving rhFSH alone, IHH/KS patients and rhFSH dose regimen are the same than those described in Fig.3 (are shown here mean (±SD) serum hormones levels instead of individual values). In the 6 IHH/KS women having received combined gonadotropin therapy, the detail of rhFSH and rhLH dose regimens are indicated in patients and methods section. ULN: upper limit of normal; LLN: lower limit of normal.

Table 1. Characteristics of normal and untreated IHH/KS women

	Controls	IHH/KS
Number	51	68
Age (years)	28.5 ± 6 (21-36)	29.1 ± 7 (18-33)
BMI (Kg/m2)	22.8 ± 1.5 (21-27)	23.6 ± 1.4 (22-26)
Primary amenorrhea	0	64
Oligomenorrhea	0	4
Secondary amenorrhea	0	0
Regular spontaneous menses	51	0
Ovarian volume [a] (mL)	6.0±2.0 (2.6-10)	1.5±0.6 [b] (0.7-2.8)
FSH (IU/L)	5.1 ± 1.3 (2.4-7.9)	1.2±1.4 [c] (0.05-5.1)
LH (IU/L)	4.5 ± 1.6 (2.2-8.2)	0.9±1.3 [c] (0.05-5.3)
Estradiol (pg/mL)	36.4±18.1 (13-97)	9.6±6.0 [a] (2.0-28)
SHBG (nmol/L)	48±17 (32-85)	42±15 (25-77)
ADIONE (ng/ml)	1.72±0.5 (0.8-2.6)	1.15±0.40 [c] (0.12-1.9)
T (ng/ml)	0.38±0.14 (0.17-0.59)	0.27±0.13 [c] (0.1-0.55)
DHEAS (ng/mL)	1924±539 (1256-3985)	1813±604 (1133-4023)
Cortisol (µg/100 mL, at 8h00 AM)	14±2.6 (8.9-22.5)	13.6±2.9 (8.7-21.1)
Free T4 (pmol/L)	16.0±2.8 (12.5-22.4)	14.8±3.2 (12.7-21.6)
TSH (mIU/L)	2.6±1.2 (1.1-3.9)	2.2±1.5 (1.2-4.1)
Prolactin (ng/mL)	16±2.3 (9-21)	11±2.9 [d] (7-16)
IGF1 (µg/L)	242±3 (170-367)	258±38 (212-392)

Data are expressed as means ± SD (range).
[a] Evaluated in 39 IHH/KS and in 41 healthy women. [b]p<0.001; [c] p<0.01; [d]p<0.05
To convert E2 in pg/mL to pmoles/L multiply by 3.671.
To convert the testosterone values in ng/mL to nanomoles/L, multiply by 3.467 and to ng/dL multiply by 100.
To convert ng/mL to nanomoles per liter, multiply by 3.491 for ADIONE.
To convert the prolactin values in ng/mL to nanomoles/L, multiply by 0.043.
For DHEAS, to convert ng/mL to nanomoles per liter, multiply by 2.714.

Fig.1

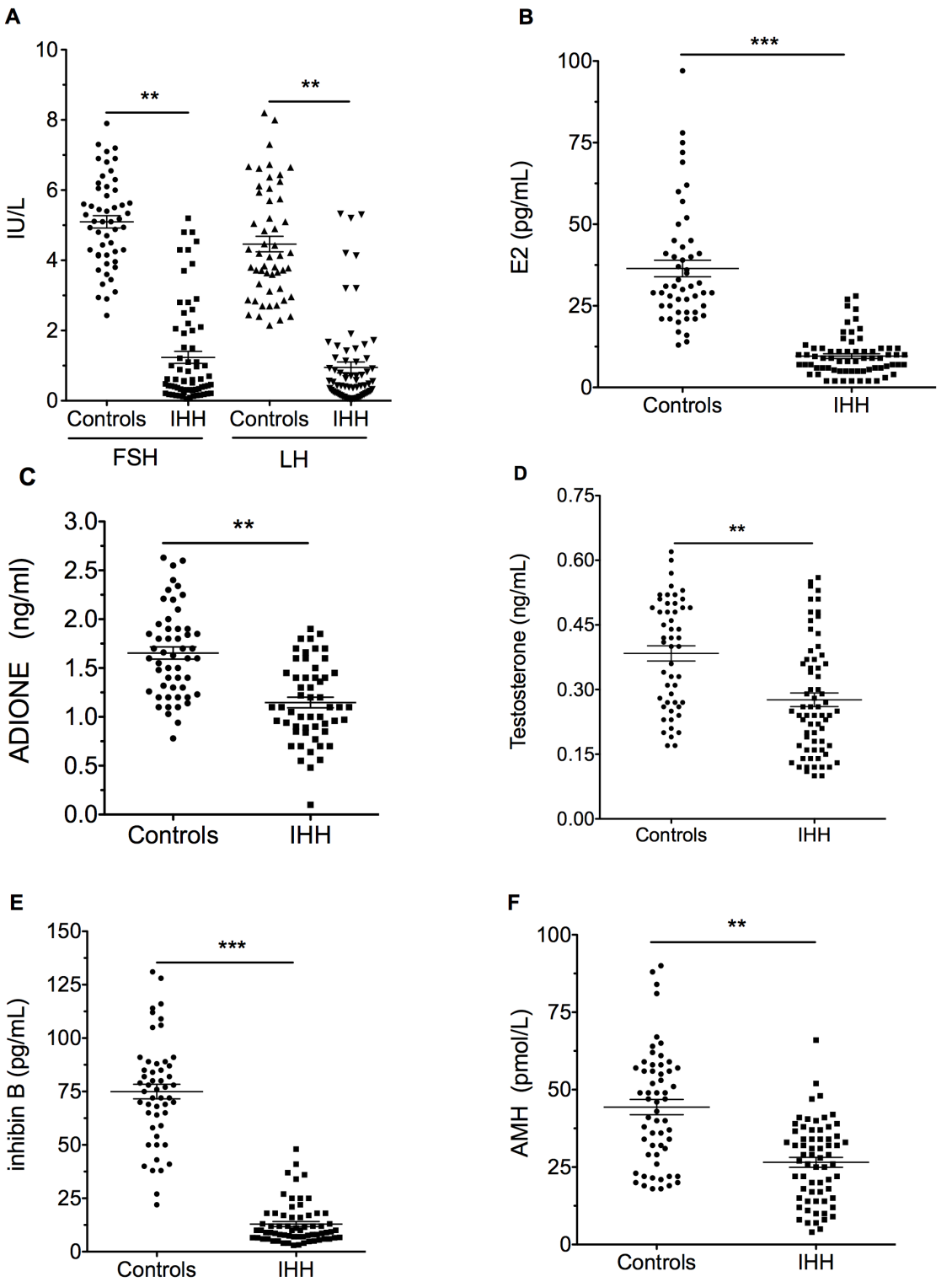
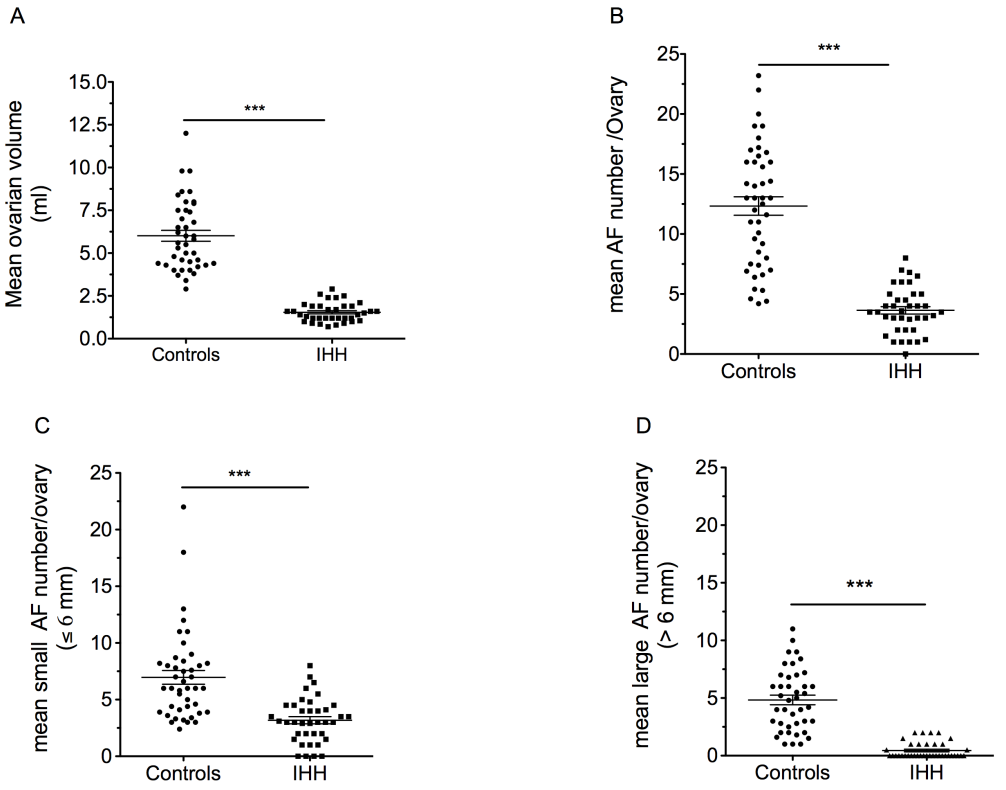


Fig. 2



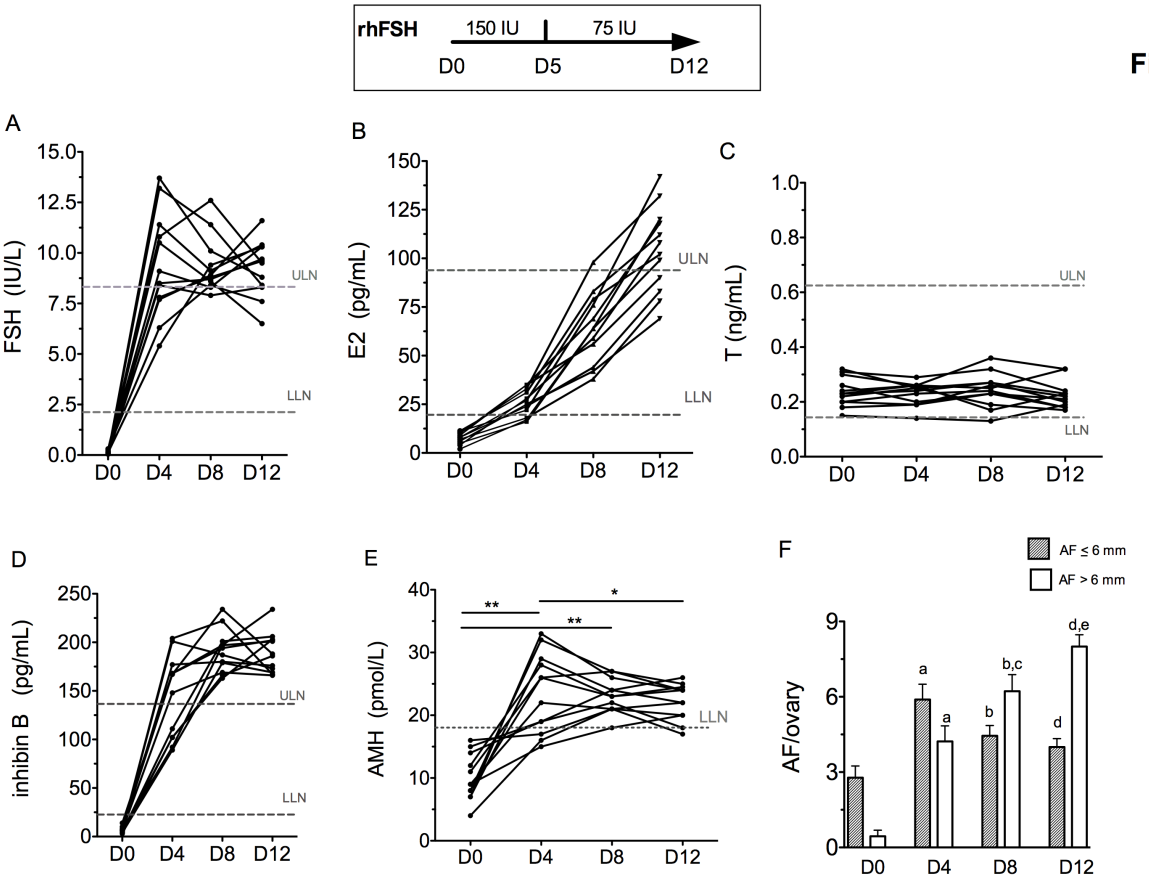


Fig. 3

Fig. 4