Serum anti-Müllerian hormone expression in women with premature ovarian failure

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BACKGROUND: Premature ovarian failure (POF) is generally irreversible. However, developing follicles up to the antral stage are reported in POF and anti-Müllerian hormone (AMH) might be a good indicator of follicular presence. This study analysed serum AMH, ovarian histology and AMH immunoexpression in POF patients. METHODS: A cross-sectional study of 48 POF patients in an Endocrinology Department setting. Patients had an ovarian biopsy simultaneously with serum AMH sampling and/or ovarian AMH immunostaining. RESULTS: Mean serum AMH was $1.04 \pm 1.66$ ng/ml. Serum AMH was significantly higher in women with 15 or more follicles at ovarian histology ($P = 0.001$). Comparison of ovarian AMH immunostaining from POF patients and 10 normal controls revealed a normal AMH expression in POF pre-antral follicles, but a decreased expression at the early antral stages. Serum AMH was undetectable in 77% of the patients with 0–5 AMH immunopositive follicles and detectable in 100% of the patients with more than 15 AMH immunopositive follicles. CONCLUSIONS: AMH levels in POF patients could identify women with persistent follicles. The decrease of AMH immunoexpression in POF antral follicles could suggest a defect of antral development.

Key words: anti-Müllerian hormone/Müllerian inhibiting substance/ovary/premature ovarian failure

Introduction

Premature ovarian failure (POF) is a syndrome defined as the cessation of ovarian function before the age of 40 and characterized by amenorrhoea associated with elevated gonadotrophin levels (Coulam et al., 1986; Anasti, 1998; Nelson et al., 2005). The aetiology of POF includes genetic disorders, autoimmune disease, and iatrogenic causes such as chemotherapy or radiotherapy. However, in most women with a normal 46, XX karyotype, the aetiology is generally unknown, and the condition is defined as idiopathic POF. In most cases, POF is an irreversible condition leading to infertility in women at reproductive age. Follicular development may persist in some patients as demonstrated by ultrasonography (Taylor et al., 1996) and at ovarian biopsy (Nelson et al., 1994). A spontaneous pregnancy may occur in this subgroup of patients with intermittent ovarian function (Massin et al., 2004).

Plasma levels of FSH, estradiol ($E_2$) or inhibin B are of limited value in predicting the presence of an ovarian reserve in patients with POF (Massin et al., 2004). The serum level of Müllerian-inhibiting substance, also known as anti-Müllerian hormone (AMH), has recently been suggested as a more accurate indicator of the presence of ovarian follicles (Durlinger et al., 2002b). In women, AMH appears in serum after birth, increases till puberty and progressively decreases in parallel with ovarian ageing (de Vet et al., 2002; van Rooij et al., 2005). In addition, serum AMH shows a negative correlation with age and a positive correlation with antral follicle count at ultrasound (de Vet et al., 2002; van Rooij et al., 2005) and, to a lesser extent, with plasma levels of inhibin B and FSH. Moreover, for a few years now, serum AMH levels have been presented as a sensitive marker for the presence of ovarian follicles and ovarian ageing (van Rooij et al., 2002; Fanchin et al., 2003; van Rooij et al., 2005), for the response to gonadotrophins in women undergoing assisted fertilization procedures (Seifer et al., 2002; Fanchin et al., 2003; Hazout et al., 2004) and for the number of antral follicles in normal and polycystic ovaries (PCOs) (Cook et al., 2002; Laven et al., 2004; van Rooij et al., 2005). In light of this, we hypothesized that the
determination of serum AMH levels in POF patients could help in evaluating the persistence of follicles and, possibly, of the fertility potential and, in some patients, could also help in clarifying the mechanisms of ovarian dysfunction.

The objectives of this study were to analyse, in a cohort of POF patients, (i) the relationship of serum AMH levels with ovarian histology and (ii) the pattern of immunoexpression of AMH in POF ovaries.

Patients and methods

**Patients**

We analysed the phenotypes of 16- to 39-year-old patients presenting with idiopathic POF in the Department of Endocrinology at Necker Hospital, Paris, France, between 1997 and 2005. The inclusion criteria were amenorrhea with elevated gonadotrophin levels (FSH >40 IU/l) before the age of 40, a normal karyotype and absence of iatrogenic causes (Coulam et al., 1986; Anasti, 1998; Nelson and Bakalov, 2005). The data were collected retrospectively before 2000 and prospectively afterwards. Two hundred and fifty POF patients were referred to our department. An ovarian biopsy was systematically proposed and, after informed consent, 93 patients (37%) accepted the biopsy. The local ethics committee of Necker Hospital approved this study.

Forty-eight patients who had an ovarian biopsy with histological analysis matched the following criteria and were included in this study: (i) absence of history of chemotherapy and/or pelvic radiotherapy and normal 46, XX karyotype and (ii) available frozen serum sample taken on the day of ovarian biopsy for serum AMH analysis (n = 35) or presence of sufficient biopsy material for AMH immunostaining (n = 40) or both (n = 27). In the first part of the study, we analysed the relationship between serum AMH levels and pathological phenotype. In the second part of this study, we analysed AMH expression in POF ovaries by immunohistochemistry and used 10 normal archival ovarian samples as control. The control ovarian samples were obtained from 18- to 40-year-old women (mean age, 35 years) ovariotomized for small, benign ovarian tumours. Finally, we compared serum AMH levels and ovarian AMH immunostaining.

**Laparoscopy and ovarian sampling**

After giving informed consent, the patients underwent ovarian biopsies by laparoscopy. There were no complications, and the hospitalization time did not exceed 24 h; for each patient, at least two biopsies of 3–5 mm, one on each ovary, were sampled and fixed in neutral formal. After embedding in paraffin, biopsies were serially sectioned at 5 μm, then stained with haematoxylin/eosin and Masson’s trichrome stains. Histological examination was performed with a conventional optical microscope (Provis, Olympus, Japan). One of every 20 sections was observed, and the number of resting and growing follicles were counted. The sections were carefully examined to detect fragments of large follicles that could be present on the edges of the biopsy and atretic follicles in the ultimate stages of atresia and degenerated corpora lutea. The control ovaries were surgically removed; the fixation, embedding and immunostaining procedures were the same as those used for POF ovarian biopsies.

**Hormone assays**

Plasma levels of FSH and LH (Immunotech Beckman, France) were measured by radioimmunoassay. Plasma E2 concentration (DiaSorin, Italy) was determined after previous plasma extraction. The FSH, LH and E2 intra-assay coefficients varied from 2.6 to 6.7%, whereas the inter-assay coefficients varied from 3.7 to 6.3%. Inhibin B was measured in serum by an enzyme immunometric assay using Oxford Bio-Innovation reagents (Serotec, Oxford, UK) with a detection limit of 10 pg/ml and intra- and inter-assay coefficients of variation of 6 and 12%, respectively. The normal ranges, determined in women at reproductive age with regular menstrual cycles, are as follows: FSH 3–9 IU/l, LH 1–5 IU/l, E2 70–1100 pmol/l and inhibin B 60–200 ng/l. Serum AMH (AMH/MIS Elisa® Immunotech, Beckman-Coulter) concentrations in serum samples were measured in duplicate using a sandwich enzyme-linked immunosorbent assay (ELISA) method. The range of the assay was 0.4–21 ng/ml. The intra- and inter-assay coefficients for AMH determination were 5.3 and 8.7%, respectively.

**Immunohistochemistry**

In brief, serial tissue sections were deparaffinized by successive washes in xylene and alcohol, and antigen retrieval was performed in a commercial microwave oven at full power in pH 6 citrate buffer for 15 min. The sections were then incubated overnight with the primary rabbit anti-AMH antibody (Rey et al., 2000) (dilution 1/2000) at 4°C in a humid chamber. After antibody incubation, endogenous peroxidases were quenched with 3% H2O2 in phosphate-buffered saline (PBS) (pH 7.4) for 5 min, and the bound immunoglobulins were revealed with a secondary biotinylated antibody and peroxidase-labelled streptavidin (LSAB2 immunostaining kit. DAKO Corp., Carpinteria, CA, USA) according to the manufacturer’s instructions. Aminoethylcarbazol (Sigma-Aldrich Chemical Co., France) was used as a chromogen. The sections were counterstained with Mayer’s haematoxylin.

Each experiment was performed three times on non-consecutive sections. The 10 normal ovaries were used as controls. No labelling was found, except for endothelial cells, when the sections were incubated with an irrelevant polyclonal antibody (anti von Willebrand factor, Sigma-Aldrich Chemical Co.) at the same concentration as the primary anti-AMH antibody or with PBS.

**Statistical analysis**

Analysis was performed with Statview version 5 (Abacus concepts, USA). Descriptive statistics was performed for each variable; quantitative results are presented as mean ± SD, and qualitative results are presented as a distribution of a number of patients. Hormonal parameters within the three groups were compared using analysis of variance or Kruskal–Wallis test, and proportions were compared using the χ2 test. P values <0.05 were accepted as significant. The value 0.2 ng/ml was given to concentrations of AMH below the detection limit.

**Results**

**Patient characteristics**

The clinical and hormonal characteristics of the patients are summarized in Table I, for the total population of POF patients referred to our department (n = 250), for the patients who agreed to have an ovarian biopsy (n = 93) and, finally, for the patients meeting the inclusion criteria in this study (n = 48). These 48 patients presented with two different clinical patterns with regard to pubertal development: primary amenorrhoea with lack of puberty or partial pubertal development and secondary amenorrhoea with complete pubertal development. As expected, the mean serum FSH and LH levels were high (>40 IU/l), and the mean serum AMH (1.04 ± 1.66 ng/ml), E2 and inhibin B levels were low, compared with normal range.
Clinical and hormonal characteristics of the premature ovarian failure (POF) patients in the total group (n = 250), in the group with ovarian biopsy (n = 93) and in the patients who met our criteria and were included in the study (n = 48)

<table>
<thead>
<tr>
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<th>Mean ± SD [range (n)]</th>
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<tr>
<td></td>
<td>Total (n = 250)</td>
</tr>
<tr>
<td>Age at diagnosis (year)</td>
<td>26.9 ± 7.8 [12–39 (242)]</td>
</tr>
<tr>
<td>Age at the day of evaluation (year)</td>
<td>29.1 ± 7.9 [12–42 (241)]</td>
</tr>
<tr>
<td>Primary amenorrhea (%)</td>
<td>19.6% (49)</td>
</tr>
<tr>
<td>Secondary amenorrhea (%)</td>
<td>80.4% (201)</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>72.5 ± 40.2 [3.6–284 (250)]</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>20.5 ± 17.3 [3.2–159 (237)]</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>24.7 ± 37.2 [5–308 (238)]</td>
</tr>
<tr>
<td>Inhibin B (pg/ml)</td>
<td>13.7 ± 21.0 [5–160 (168)]</td>
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<tr>
<td>Anti-Müllerian hormone (ng/ml)</td>
<td>0.73 ± 1.23 [0.2–7.5 (73)]</td>
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Serum AMH and ovarian phenotype in POF patients

Serum AMH concentration on the day of ovarian biopsy was available for 35 patients. Serum AMH was below the detection threshold (0.4 ng/ml) in 20 women (57%). For the remaining 15 women, the mean serum AMH level was below the normal range [normal range in cycling women for days 3–5 of the cycle is 2.2–6.8 ng/ml (Pigny et al., 2003)] in nine women (26%) and within the normal range in six women (17%). Serum AMH values were not significantly correlated to the age of the patients (r = -0.21, P = 0.22, data not shown).

Regarding ovarian histology, there were two different ovarian phenotypes, as we described in a previous study (Massin et al., 2004): biopsies totally devoid of follicles, n = 17 (48.6%) and biopsies with presence of at least one follicle (n = 18). According to the number of small follicles (primordial, inter- mediar y and primary stage), the latter group could be divided into two groups: women with no more than 5 follicles at biopsy, n = 11 (31.4%) and women with >15 follicles at biopsy, n = 7 (20%). There were no patients with an intermediate number of follicles (between 6 and 14). Serum AMH values according to the ovarian phenotype are shown in Figure 1. Among patients with absence of follicles at biopsy, serum AMH levels were below the detectable threshold in 12 patients (70.6%) and below the normal range in three patients (17.6%). AMH level was within the normal range in two patients (11.8%) at 2.8 and 4.7 ng/ml. Among patients with five follicles or less at biopsy, AMH levels were below the detectable threshold in seven patients (63.6%), below normal range in three patients (27.3%) and normal in one patient at 2.7 ng/ml (9.1%). Among patients with 15 follicles or more, AMH level was undetectable in only one subject (14.2%) and detectable in the other six women (85.8%). In this group of seven patients, three (42.9%) had low levels of AMH between 0.7 and 1.27 ng/ml and three (42.9%) had normal values. Detailed histological analysis of the biopsies of these three patients with normal AMH levels revealed between 40 and 80 small follicles.

AMH immunostaining in the ovaries of 40 patients with POF and in 10 normal control women

Normal ovaries

AMH was expressed by granulosa cells only. In normal follicular structures, AMH expression appeared at the intermediary stage. No AMH immunostaining was found in primordial follicles, which consisted of an oocyte surrounded by flat granulosa cells (data not shown). About two-thirds of intermediary follicles exhibited immunostaining of one or more epithelioid granulosa cells (Figure 2A). Primary follicles, consisting of an oocyte surrounded by a layer of cubical granulosa cells, were labelled. Granulosa cells of both small (Figure 2C) and large follicles (Figure 2G) were all immunopositive for AMH. No pre-ovulatory follicles were found in the control samples. The corpora lutea of the control ovaries were always AMH immunonegative (data not shown). The follicles with histological evidence of early atresia, shown by the presence of several granulosa cells with pycnotic nuclei, disorganization of the granulosa layer and desquamation of granulosa cells in the antrum, exhibited a focal immunolabelling of the granulosa cells still adherent to the basal lamina (Figure 2I). The atretic follicles with a totally sloughed membrana granulosa were negative (data not shown).

POF ovaries

The biopsies of the 40 patients could be split into two subgroups according to the presence of follicular structures. Twenty-six patients presented biopsies without follicular structures. Some signs of past follicular development such as remnants of hyalinized basal laminae or corpora albicantia were found in eight of these patients, whereas the others had no
evidence whatsoever of folliculogenesis. AMH immunostaining was negative in all 26 patients. The second subgroup of 14 patients (with presence of ovarian follicles) exhibited a variable number of follicular structures at different stages of development, ranging from the primordial to the antral stage. Similar to normal control ovaries, none of the primordial follicles were immunostained (data not shown). About 60% of the intermediary follicles exhibited immunostaining of one or more granulosa cells (Figure 2B). All the primary follicles were labelled except in two patients. Granulosa cells of both small (Figure 2D) and large (Figure 2F) secondary follicles of POF ovaries were immunopositive for AMH, as observed in the normal ovaries.

All antral follicles were histologically abnormal in POF biopsies of this study. Their histological appearance and AMH immunolabelling were dissimilar from that of healthy antral follicles in the normal ovaries. Some antral follicles contained large, luteinized theca and granulosa cells with a clear, vacuolated cytoplasm, without histological evidence of atresia. The expression of AMH protein in the hypertrophic luteinized
granulosa cells of these follicles was focal (Figure 2H) and unlike the diffuse labelling found in granulosa cells of normal antral follicles. However, most POF antral follicles showed signs of atresia ranging from a partial sloughing of pyknotic granulosa cells into the antrum to a total absence of granulosa. In half of the cases, this feature was associated with a hypertrophic theca interna (data not shown). The follicles with early atresia exhibited focal AMH immunolabelling of the granulosa cells (Figure 2J), whereas the late atretic follicles with a totally sloughed membrana granulosa were negative (data not shown).

No luteal structures were found in the ovarian sections of these POF patients.

**Serum AMH level and AMH immunostaining according to ovarian phenotype in POF patients**

Data for 27 POF patients with both serum AMH levels on the day of ovarian biopsy and ovarian AMH immunostaining are summarized in Table II. AMH was detectable in the serum in only five patients (23%) of the 22 with five or less follicles or absence of follicle and in all the patients with 15 or more follicles ($P = 0.004$). In the two groups with the lower number of follicles, the mean serum AMH levels were significantly lower than in the group with 15 or more follicles ($P = 0.001$). As expected, there was no AMH immunostaining in patients with no follicles. AMH immunostaining was present in one third of the patients with five or less small follicles, and in 100% of the patients with 15 or more follicles ($P < 0.001$). According to these data, AMH expression was significantly higher in the group of POF patients with $>15$ follicles at ovarian biopsy.

**Discussion**

This is the first study that reports AMH protein expression and secretion in patients with POF. As expected, the mean AMH level in 16- to 39-year-old women with POF was low compared to non-POF women, as in normal post-menopausal women (La Marca et al., 2005) and below the detection threshold in about 60% of the patients, as in women with bilateral oophorectomy (La Marca et al., 2005). When detectable in the serum of POF patients, AMH level was mostly below the normal range, and within the normal range in only a few patients.

In addition, an association between serum AMH levels and the presence and number of small follicles in the ovarian biopsy was found in these patients. Serum AMH level was within the normal range in patients without follicles at biopsy in only one case. The ovarian sample of this patient consisted mainly of ovarian medulla, which is devoid of follicular structures and thus was not contributive. Our results find an association between number of follicular structures (at biopsy) and AMH levels, as previously found in several studies in normal women undergoing assisted reproductive technologies (ART), and in women with PCO syndrome (PCOS). In women, AMH is expressed in granulosa cells of pre-antral and antral ovarian follicles. Its expression begins in the perinatal period (Ueno et al., 1989), continues throughout sexual maturity, declines at the end of reproductive life (van Rooij et al., 2005) and finally disappears completely after menopause (La Marca et al., 2005). AMH levels are used in reproductive medicine to predict ovarian responsiveness to controlled ovarian stimulation during assisted reproduction and as a marker of ovarian tumours. In a substantial number of patients with premature idiopathic ovarian failure, there is evidence of follicular development at ultrasonography (Conway et al., 1996; Massin et al., 2004), and we found evidence of present or previous follicular development in about half of the ovarian biopsies of these patients. Sporadic cases of pregnancy (van Kasteren and Schoemaker, 1999) have been described in POF patients, and the identification of the presence of small ovarian follicles, invisible at ultrasonography, through the measurement of AMH levels could perhaps help discriminate those patients more likely of possessing follicles available for development and, eventually, ovulation.

Moreover, AMH-knock out (AMH-KO) mice show an increase in the number of early growing follicles and an equivalent decrease in the primordial pool, suggesting that AMH plays an important role in early follicle growth (Durlinger et al., 1999, 2002a). Recently, defects in early follicular development through an altered expression of AMH in small growing follicles have been implicated in PCOS (Stubbs et al., 2005); this prompted us to compare AMH expression in normal ovarian follicles and follicles from POF ovaries.

We studied AMH immunoeexpression in 40 ovaries of patients with POF and compared it with that found in 10 normal cycling ovaries from women of comparable age. AMH immunolabelling was always absent in primordial follicles. Normal and POF intermediary, primary and secondary follicles had comparable AMH expression in all cases but two. The similarity of expression of AMH in the pre-antral follicles of most POF patients and normal women suggests that the developing granulosa cells of most POF patients are functionally efficient and capable of AMH production. We are aware of some discrepancies between our findings and those reported by others (Weenen et al., 2004; Stubbs et al., 2005) regarding the AMH immunoeexpression of small and atretic follicles in normal ovaries. Stubbs et al. (2005) reports immunolabelling of primordial follicles, but we observed immunolabelling only from the intermediary stage of follicular growth, similar to the observations reported by Weenen et al. (2004). We also detected AMH immunoexpression in all the normal primary

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**Table II. Serum Anti-Müllerian hormone (AMH) level and AMH immunostaining according to ovarian phenotype in 27 POF patients**

<table>
<thead>
<tr>
<th>Ovarian biopsy</th>
<th>Serum AMH detectable (%)</th>
<th>AMH level [mean ± SD (ng/ml)]</th>
<th>Presence of AMH immunostaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence of follicles</td>
<td>18.7</td>
<td>0.42 ± 0.65</td>
<td>0/16 (0%)</td>
</tr>
<tr>
<td>Five or less follicles</td>
<td>33.3</td>
<td>0.33 ± 0.21</td>
<td>2/6 (33%)</td>
</tr>
<tr>
<td>Fifteen or more follicles</td>
<td>100*</td>
<td>2.16 ± 1.66**</td>
<td>5/5 (100%)***</td>
</tr>
</tbody>
</table>

AMH expression was significantly higher in the group with 15 or more follicles compared to the two other groups with absence of follicles, or five or less follicles.

* $P < 0.001$

** $P = 0.004$

*** $P = 0.001$

*** $P < 0.001$.  

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follicles and, focally, in the early atretic antral follicles. These differences could be due to a varying sensitivity of the antibodies used and their ability to recognize different antigenic determinants after tissue fixation.

Interestingly, some immunonegative primary follicles were found in two POF patients with undetectable serum AMH levels. The biopsies of these patients were characterized by a reduced ovarian reserve (between one and five follicles) and a fibrous cortical stroma (data not shown). This is similar to the ovaries of mature AMH-KO mice, which have an initially abundant ovarian reserve that is rapidly exhausted in mature females (Durlinger et al., 1999). The abnormal depletion of the ovarian cortex in these animals is attributed to an accelerated follicular recruitment secondary to the lack of AMH and the absence of its inhibitory effect on the development and recruitment of reserve follicles (Durlinger et al., 1999). Antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed P

We are aware of the fact that our results are, at best, preliminary and that a larger number of cases are required to draw firmer conclusions about the physiopathology of AMH secretion in POF. Moreover, the findings in ovarian biopsy samples might not correspond exactly to the histology of whole ovary (Lass, 2004) However, our observations raise a few questions about the correlation between AMH secretion and the presence of small follicles in the ovaries of POF patients and the relevance of serum AMH monitoring in POF.

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