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Evaluation of different markers of the ovarian reserve in patients presenting with Premature Ovarian Failure

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Summary

Premature Ovarian Failure (POF) is a heterogeneous syndrome, possibly due to mutations of genes involved in the normal development of the ovary and/or the follicles. Based essentially on animal models, these mutations are associated with various ovarian histological phenotypes, from a complete absence of to a partial follicular maturation. The aims of our work were in one hand to determine if ovarian histology, compared to pelvic ultrasonography, would be helpful either in identifying which patients display an impaired follicular growth or in the orientation of the POF etiology; on the other hand, since developing follicles up to the antral stage are reported in POF and that Anti-Müllerian Hormone (AMH) might be a good indicator of follicular presence, we decided to determine whether AMH should be a better marker to determine the presence of an ovarian reserve in POF patients. To try to answer to the first question, we studied first 166 patients suffering from POF with a normal karyotype. Vaginal ultrasonography (US) was performed in 134 patients and an ovarian biopsy was obtained in 67 women. The presence of follicles suggested at US was confirmed at histology in only 56% of the patients. Ovarian histology led to the distinction of two phenotypes, (a) small-sized ovaries, deprived of follicles, and (b) normal-sized ovaries with partial follicular maturation. To confirm the value of ovarian biopsies, samples from 20 normal women have been studied, confirming that ovarian biopsy at random allow reliable assessment of follicular activity. To try to answer to the second question of our work, a cross sectional study analyzing serum AMH, ovarian histology and AMH immunoexpression in 48 POF patients, was performed. Serum AMH was significantly higher in women with more than 5 follicles at ovarian histology. Ovarian AMH immunostaining revealed a normal AMH expression in POF preantral follicles but a decrease

expression at the early antral stages. In conclusion, ovarian histology appears to be a reliable tool to appreciate the follicular reserve, and helpful and complementary to clinical and hormonal phenotyping in order to orient the search for various genetic causes of POF syndrome. Finally, AMH levels in POF patients could identify women with persistent follicles.

Key words: Premature Ovarian Failure/ Ovary/ AMH

Introduction

Premature ovarian failure (POF) is defined as the complete cessation of menses before the age of 40 (Coulam *et al*, 1986). In the absence of surgical oophorectomy, chemotherapy and pelvic radiation, POF encompasses a heterogeneous spectrum of conditions, from ovarian dysgenesis to gonadotropin resistance (Anasti, 1998). In molecular terms, most of the data is obtained from animal models with various ovarian phenotypes - from the complete deprivation to the partial maturation of folliculogenesis- depending on the gene defect (Elvin and Matzuk, 1998; Burns and Matzuk, 2002). In humans, the Follicle Stimulating Hormone Receptor (FSHR) gene has been the particular focus of most teams involved in the management of patients presenting with POF (Aittomaki *et al* 1995, Doherty *et al* 2002, Allen *et al* 2003). We currently follow-up women presenting a POF syndrome associated with a normal karyotype. Among them, 3 patients also presenting different FSHR gene mutations have been already described, leading to a partial or an almost complete loss of function of FSHR (Beau *et al* 1998, Touraine *et al* 1999, Meduri *et al* 2003). However, this cause of POF is probably rare since it has not been found by other groups and, in most cases, the etiology of POF remains unknown. Therefore, a diagnosis strategy must be defined to orientate for the genetic causes of POF. The absence or the presence of follicles may lead to the distinction of two different situations: an absence of follicles likely due to defects of genes involved in oogenesis, or an impaired follicle growth, due to defects of genes involved in follicular maturation (Anasti 1998). To date, the presence of follicles is most often assessed by vaginal ultrasonography (US), which is unable to detect follicles smaller than 2 mm. Therefore, we

decided to ask our patients to undergo a laparoscopy for an ovarian biopsy, to compare both methods (Massin *et al.*, 2004).

In another hand, plasma levels of follicle-stimulating hormone (FSH), estradiol, 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 (MIS), 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 aging (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 (AFC) 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 aging (Fanchin *et al.*, 2003; van Rooij *et al.*, 2005; van Rooij *et al.*, 2002) regarding the response to gonadotropins in women undergoing assisted fertilization procedures (Fanchin *et al.*, 2003; Hazout *et al.*, 2004; Seifer *et al.*, 2002) and the number of antral follicles in normal and polycystic ovaries (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 (Meduri *et al.*, 2006).

PATIENTS AND METHODS

We analysed the phenotypes of 16 to 39 year old patients presenting with idiopathic premature ovarian failure in the Department of Endocrinology at Necker Hospital, Paris, France, between 1997 and 2003. The inclusion criteria were an amenorrhea with elevated gonadotropin levels (FSH > 40 UI/L) before the age of 40, a normal karyotype and absence of iatrogenic causes (Anasti, 1998; Coulam *et al.*, 1986; LM Nelson and Bakalov, 2005). The study began in 2000; therefore, all data concerning patients referred before this date, were collected retrospectively whereas the data were collected prospectively after 2000. Therefore, 166 women with a normal karyotype were included in our retrospective study, to analyze the value of ovarian histology. Clinically, the median age at diagnosis was 28 yrs. Patients presented with two different clinical patterns with regard to pubertal development: primary amenorrhea with lack of puberty or partial pubertal development and secondary amenorrhea with complete pubertal development. As expected, the mean serum FSH and LH levels were high, and the mean serum estradiol and inhibin B levels were low.

All patients gave a written informed consent before participating to this study. Vaginal Ultrasonography was performed in 134 patients by the same radiologist. After informed consent, 67 patients underwent ovarian biopsies per laparoscopy. For each patient two biopsies of 3 to 5 mm, one on each ovary, were sampled and fixed in neutral formalin. After embedding in paraffin, biopsies were serially sectionned at 5 μ m, then stained with hematoxylin/eosin/safran. One section out of every 20 was observed and the number of resting and growing follicles were counted (Massin *et al.*, 2004).

Presence of available frozen serum sample on the day of ovarian biopsy for serum AMH dosage (N=35) or presence of sufficient biopsy material for AMH immunostaining (N=40) enabled to perform those specific studies, in a second time (Meduri *et al.*, 2006).

RESULTS

Ovarian histology in POF patients

The results of ovarian histological examination led to classification of the patients into two categories: 1) patients showing nongrowing follicles and/or evidence of follicular growth consisting in the presence of follicles at various stages of maturation/involution and/or corpora lutea at various stages of their involution (Fig. 1A); and 2) patients showing no follicles (Fig.1B). Twenty-nine patients exhibited follicles - from primordial to antral stages- whereas 38 patients did not.

FSH, LH, E2 and inhibin B levels were compared in patients with histological evidence of follicular maturation and those without presence of follicles (Fig.2) No difference was seen for gonadotropin levels between the two groups. Inhibin B and E2 levels were significantly higher in patients for whom biopsy showed the presence of follicular structures.

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 in 9 women (26%) and within the normal range in 6 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 devoided of follicles, N=17 (48.6%),

and biopsies with presence of at least one follicle; N=18 (543%). According to the number of small follicles (primordial, intermediary, and primary stage), the latter group could be divided in two groups: women with no more than 5 follicles at biopsy, N=11 (31.4%) and women with more than 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 3. Among patients with absence of follicles at biopsy, AMH levels were below the detectable threshold in 12 patients (70.6%) and below the normal range in 3 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 5 follicles or less at biopsy, AMH levels were below the detectable threshold in 7 patients (63.6%), very low in 3 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 6 women (85.8%). In this group, 3 patients (42.9%) had low levels of AMH between 0.7 to 1.27 ng/mL and 3 (42.9%) had normal values. Detailed histological analysis of the biopsies of these 3 patients with normal AMH levels revealed between 40 and 80 small follicles.

The predictive positive value of follicular presence was 66.7% for AMH and 60% for the predictive negative value.

AMH expression in the ovaries of 40 patients with POF

(Figure 4)

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 the ovarian samples of 8 of these patients while the others

had no evidence whatsoever of folliculogenesis. AMH immunostaining was negative in all 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. Similarly 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 4-B). All of the primary follicles were labeled except in two patients. Granulosa cells of both small (Figure 4-D) and large (Figure 4-F) 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 aspect and AMH immunolabeling 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 in the hypertrophic luteinized granulosa cells of these follicles was focal (Figure 4-H) and dissimilar from the diffuse labeling 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. This aspect was associated, in half of the cases, with a hypertrophic theca interna (data not shown). The follicles with early atresia exhibited focal AMH immunolabeling of the granulosa cells (Figure 4-J) while 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.

DISCUSSION

The POF etiology in women with normal karyotype remains poorly understood (Anasti 1998). However, more and more genes involved in the normal follicular and ovarian development are becoming known, which are also likely to be involved in this disease, based on the data obtained from animal models (Burns and Matzuk 2002). In such context, the first study presented herein was conducted to evaluate whether the ovarian biopsy, when compared with data provided by pelvic US, could be a reliable tool to individualize patients with an ovarian phenotype suggestive of a particular gene mutation, therefore suggesting the usefulness of laparoscopy and ovarian histology in orientating the search for the possible genetic etiologies of the POF syndrome (Massin et al., 2004).

In our experience, when women had no follicle at US, histological examination did not detect follicles in most of our patients. At the contrary, only 56% patients with normal size ovaries with the presence of follicles suggested at US, displayed follicles when histological examination of an ovarian biopsy was performed. Consequently, it can be assumed that, US is not predictive of the presence of follicular structures within the ovary.. However, to ensure that follicular content in ovarian biopsies from POF patients was representative of the whole ovarian follicular content, counts were randomly performed in ovaries from 20 normal patients, without POF, and considered as controls in this study (Massin *et al*, 2004). Ovarian biopsies appear effective to discriminate between patients having or not having resting follicles and it can be assumed, in our hands, that the error in estimating the presence or the absence of resting follicles in a given ovary is only 0.8 %. Less accurate but still informative is the reliability of small ovarian biopsies to detect follicular growth. Indeed, 12.1 % of the sections showed the presence of resting follicles without any presence of either growing or apoptotic follicles. Therefore, the small biopsy appears very instructive to distinguish

ovarian phenotypes marked by the presence of a follicular reserve from ovaries deprived of such primordial follicles.

We also wanted to know if hormonal levels were sufficient to point towards the presence or not of an ovarian activity and to possibly avoid surgical laparoscopy. Even if E2 and inhibin B medians were significantly higher in patients with the presence of follicles at histology, the overlap between the two groups appears considerable

We also presented data concerning AMH plasma levels and AMH expression in POF patients. As expected, the mean AMH level in 16 to 39 year old women with POF was low, 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. 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 and in women with polycystic ovary syndrome. However, the fact that the positive or negative predictive values are around 60 to 70% do not permit to consider AMH as a sole marker of the presence or the absence of an ovarian reserve. AMH levels are used in reproductive medicine to predict ovarian responsiveness to controlled ovarian hyperstimulation during assisted reproduction and as a marker of ovarian tumors. 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 past 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 the patients more likely of possessing follicles available for development and, eventually, ovulation.

Moreover, 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.*, 2002a; Durlinger *et al.*, 1999). Recently, defects in early follicular development through an altered expression of AMH in small growing follicles have been implicated in polycystic ovary syndrome (Stubbs *et al.*, 2005). This prompted us to compare AMH expression in normal ovarian follicles and follicles from POF ovaries.

We studied AMH immunoexpression in 40 ovaries of patients with premature ovarian failure and compared it with that found in 10 normal cycling ovaries of comparable age. AMH immunolabeling 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 preantral 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. Antral follicles in the biopsies of POF patients under study were never healthy; our histological results showed POF antral follicles undergoing either atretic changes, often accompanied by thecal hypertrophy, or, less often, premature luteinization of both granulosa and theca cells. Antral AMH immunoexpression in POF biopsies was always inferior to that in the control ovaries. This reduction of AMH production in POF ovaries can have different causes according to the antral pathology. In atretic follicles of both normal and POF ovaries, the much reduced or negative AMH

expression is probably secondary to the apoptotic changes in the granulosa cells. The focal expression of AMH in luteinized POF antral follicles is probably secondary to the inappropriate luteinization of granulosa cells. In conclusion, the dysfunction of AMH production in the antral follicles of POF women observed in our series of ovarian biopsies suggests the possibility of the presence of a defect in antral follicular development, particularly concerning the granulosa cells. A decreased antral granulosa cell AMH production could alter the mechanism of follicular recruitment in these patients.

In conclusion, the clinical presentation associated with the hormonal investigations, the ultrasonographic description constitute the global phenotyping of our POF patients and are mandatory to lead the search for the identification of such new genetic mutations. Besides such strategy, AMH could be useful in discriminating POF patients with a sizable follicular population from patients with no or few ovarian follicles, with a lesser potential for ovulation and pregnancy. Finally the use of the ovarian biopsy still remains interesting in our hands to help us to better understand the process of ovarian dysfunction and is mandatory in patients for whom anomalies of genes involved either in follicular growth or maturation are found.

Legends

Figure 1: Histological aspect of the ovary of 67 POF patients

1A. Ovaries with partial follicular maturation (n = 28): from primordial follicles (white arrow) up to the early antral follicle stage (black arrow).

1B. Ovarian cortex deprived of follicles (n = 39).

Figure 2: Hormonal evaluation of patients presenting with POF, depending on the presence or the absence of follicles at histology

Figure 3: Serum AMH distribution according to ovarian reserve

Figure 4: AMH expression in POF ovaries

No differences in granulosa cell AMH expression are evident between intermediary and small or large secondary follicles of control (Fig. **A**, **C**, **E**) or POF ovaries (Fig. **B**, **D**, **F**). The strong diffuse expression of AMH in the antral follicles of the normal ovaries (Fig. **G**) is dissimilar from that of antral follicles of POF ovaries exhibiting abnormally luteinized granulosa cells with a focal AMH immunolabeling (Fig. **H**). Early atretic antral POF follicles present a reduced expression of AMH in the early apoptotic granulosa cells (Fig. **I**): notice the sloughing of some cells in the antral cavity. The aspect is similar to that found in early atretic antral follicles of the control ovaries (Fig. **J**). A: antral cavity, GC: granulosa cells layer, TI: theca interna. Fig. **A**, **B**, **C**, **D**, **I**, **J**: original magnification X 200. Fig. **E**, **F**, **G**, **H**, original magnification X100.

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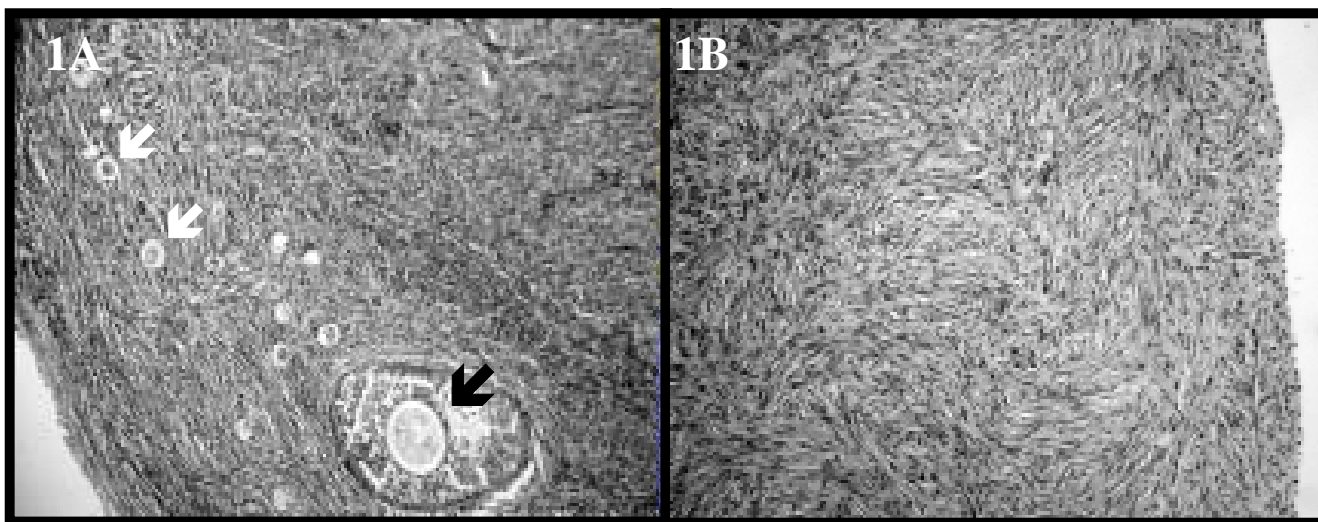
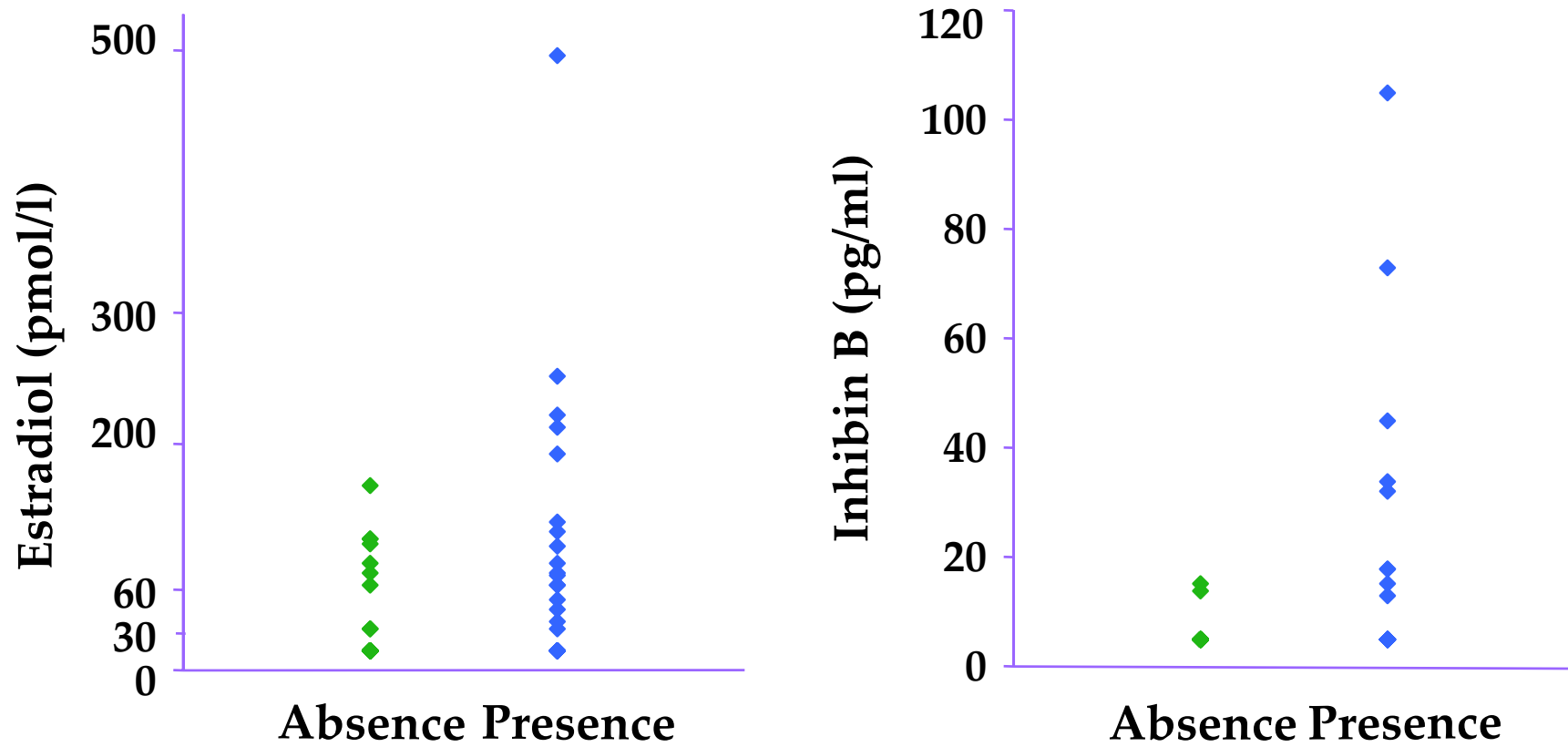


Figure 2



Follicles at biopsy

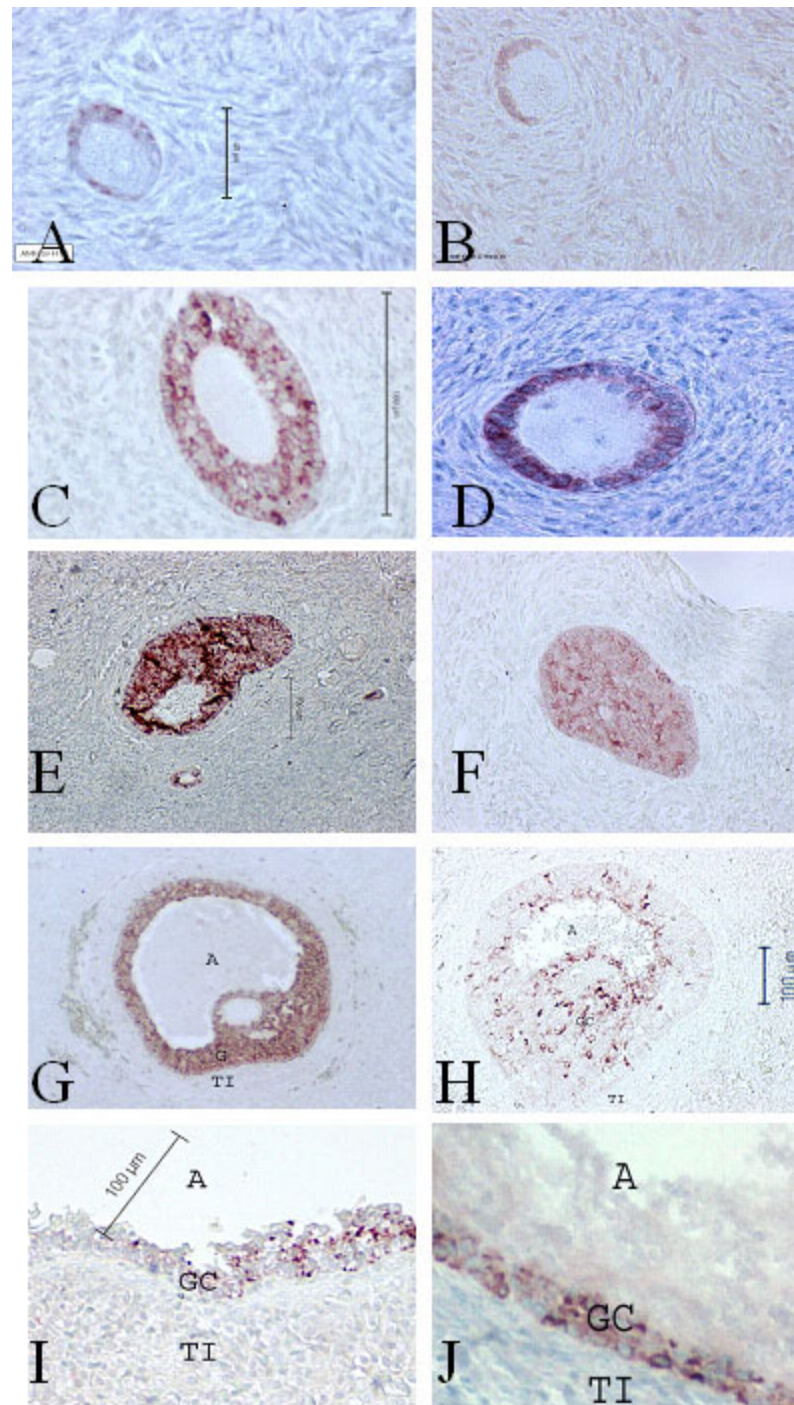


Figure 4