# Premature Ovarian Failure

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**Abstract:** Using autoradiography, transmission and raster electron microscopy, this review shows how oocytes disappear in human ovaries.

Clinical, hormonal and ultrasound (using 3D, vocal and inverse mode) parameters used in the diagnosis of early ovarian aging are described on the light of the most recent knowledge.

## INTRODUCTION

In the past decade, Spain, with only 1.36 children per family, has become the country with the lowest birthrate in the world.[1]

The integration of women into the labor force and the desire for procreation at more advanced ages are two of the factors that contribute to this state of affairs.

Health care professionals who work in assisted reproduction technology (ART) have known that long before menopause there is a decline in fecundity with advancing age.

Because many Spanish couples postpone childbearing to the third or even fourth decade of life, the number of patients 37 years of age or older requesting *in vitro* fertilization-embryo transfer (IVF-ET) services are larger every year.

Because the success rate is very low in this population, many of these patients avail themselves of oocyte donation, an illegal practice in several European countries.

The reasons for a low success rate with IVF-ET in women older than 37 years of age is due to a low ovarian reserve and to the poor quality of the remaining oocytes.

We stimate that 70 to 80% of remaining oocytes in these women are carriers of chromosomal or genetic defects.[2,3]

The success rate of 60 to 70% when women who are 45 to 55 years old (and even older) use donated oocytes indicates that uterine aging is not the cause of low fecundity in older women.

Menopause occurs when only about 1000 follicles remain, but sub-fertility or infertility precedes this event by about 13.5 years.

Menopause due to dysfunctional ovaries is also determined by the reserve of germ cells and by the rate of depletion during life.[4]

## Ovarian Aging

Follicular depletion starts at the onset of ovarian development and is complete about five years after menopause. The concentration of primordial follicles in the ovarian cortex undergoes steady depletion from fetal age onward. Follicular depletion starts during embryonic development when germ cells arrive at the *crista genitalis*.

This phenomenon is definitely evident before the 12th week of gestation.[5]

The primordial germinal cells come from a specific portion of the embryonic mass and they descend from the yolk sac (day 24) on the roof of the developing intestine through the celomic angle (day 28) across the germinal or Kaimbahn way to arrive at the urogenital ridge on day 31 (Fig. 1).

The urogenital ridge is a thickening of the germinal epithelium that is in a specific area of the celomic epithelium next to what will become the Wolffian and Müllerian ducts and close to the area where the kidneys and adrenal glands will develop.
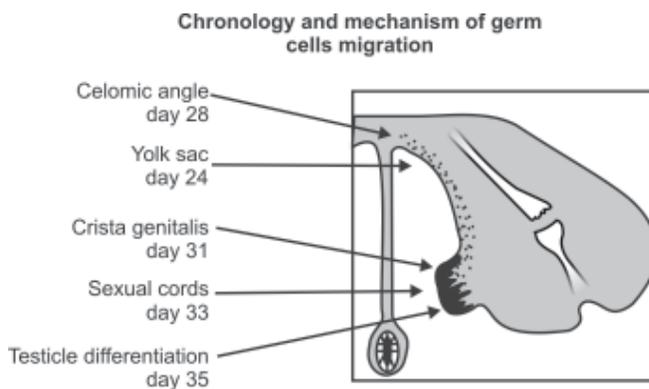


**Fig. 1:** Schematic representation of the origin and progress of human germinal cells until their arrival at the urogenital ridge

There is a triple mechanism of migration:

1. Germ cells possess ameboid movements.
2. There is chemotactic attraction between germ cells and the urogenital ridge.
3. Blood pressure speeds migration of germ cells to the urogenital ridge. There is a direct relationship between blood pressure and the speed with which germ cells arrive at the urogenital ridge.

Many germ cells do not arrive at the urogenital ridge during the process of migration and descent. Most germ cells that do not arrive at the urogenital ridge degenerate and disappear.

Those that cease to progress and implant at inappropriate sites give rise to teratomas or pilonidal cysts.

Once germ cells arrive at the urogenital ridge they go through a stage of florid cellular multiplication that result in cells arrested at the dictiotene phase of the second meiotic division.

This division process is so aggressive that an eight-week embryo has a supply of 600,000 primordial germ cells but by 7 months the supply exceeds 7 million.[6-8]

The cells remain in the dictiotene phase of the second meiotic division during reproductive life until moments before ovulation, when the cell in the dominant follicle will resume the meiotic process, a necessary step if fertilization is to take place (Fig. 2).

Once the division process is complete (between 5 and 12 weeks of embryonic life), the primitive germinal cells enter structures from the germinal epithelium known as Pflügger cords (see Fig. 1) and surround themselves with a thin layer of their own cells, which then become the granulosa layer (Figs 2 and 3). This is a critical immunologic defense mechanism since in its absence germinal cells will be identified as foreign and destroyed (see Figs 2 and 3).

From 7 months fetal age on, the rate of primordial germ cell depletion increases to the extent that at term one million primordial follicles are present, by the 6th postnatal month 600,000 remain, and at puberty only 300,000 of the primordial germ cells are left[5] (see Fig. 3).

Although the phenomenon of germ cell depletion continues throughout the reproductive life of women, at about age 37 follicle depletion accelerates with rapid loss of reproductive potential resulting thereafter.[9,10]

The problem of decreased reproductive potential with advancing age is compounded since in addition to the accelerated rate of follicle loss, the follicles that remain are of poorer quality. The best follicles are the ones likely to be recruited earlier during the reproductive years.

The decline in fertility is, therefore, exponential and doubles when supply falls below the critical level of 25,000 primordial and primary follicles, usually around the age of 37 years. There is the suggestion that follicles in perimenopausal women are of very poor quality and less sensitive to high levels of
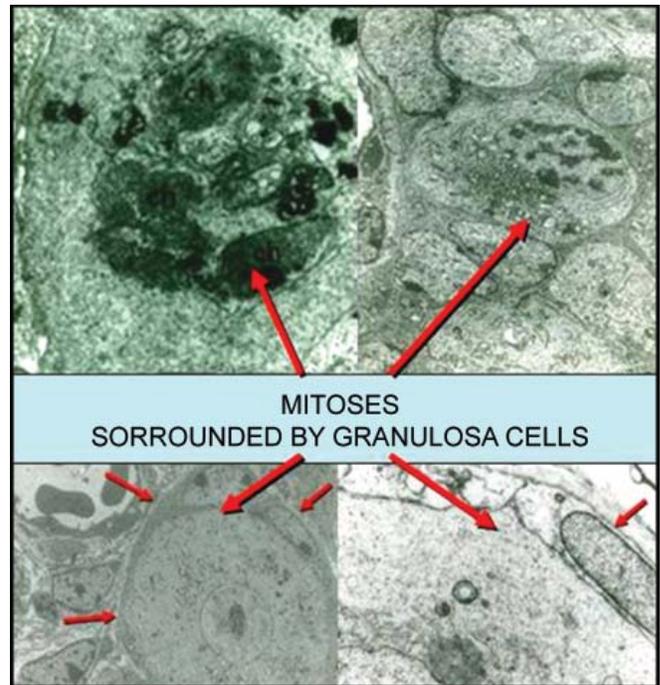


**Fig. 2:** Upper image is a transmission electron microscope picture of two embryonic germinal cells in division phase. The chromosomes can be seen in black. The lower picture shows the germinal cells which after dividing are surrounded by a fine layer of protective granulosa cells (red arrows)
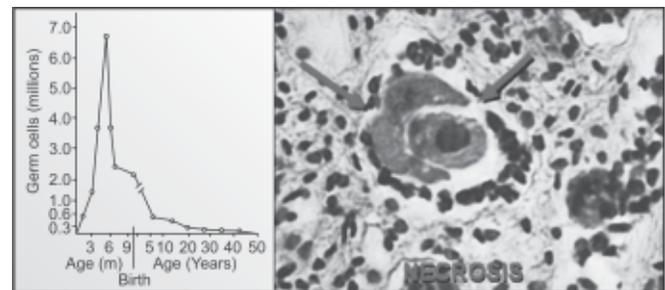


**Fig. 3:** Schematic representation of germinal cell reserve throughout life. On the right side there is a degenerating primordial cell in a fetal ovary due to damage of the protective granulosa cell layer

gonadotropins.[11,12] Up to 10% of women are already menopausal by age 45.

Newborn baby girls have thin, elongated ovaries that measure $1.5 \times 0.5 \times 0.4$ mm. On histologic examination they are covered with a monolayer of poorly active cells derived from celomic epithelial rests. These cells disappear progressively, although they may persist in a few areas of the ovary (ovarium giratum) and over the years may give rise to 85% of the most common ovarian tumors, cystadenomas and cystadeno-carcinomas (Fig. 4).

However, when compared with the medulla, the cortical layer of newborn baby girls is thickened since it still contains many hundred thousands primordial and primary follicles (Fig. 4).
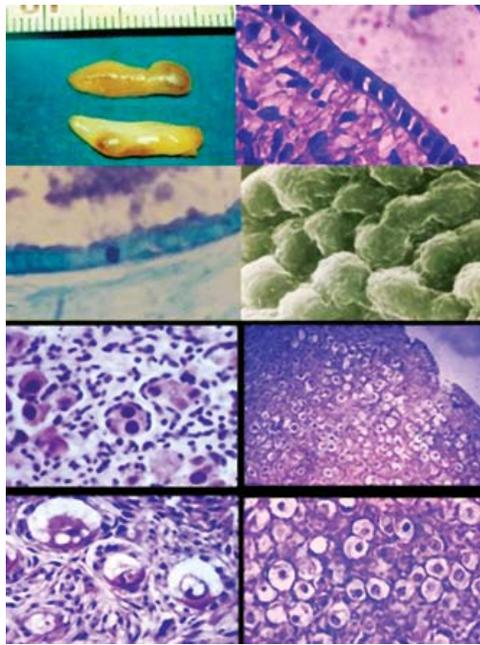
**Fig. 4:** Image of an ovary of a newborn baby girl (upper left). On the right side we can see a histologic cut of the ovarian surface covered with germinal epithelium. The germinal epithelium shows little activity and for this reason it barely has cells in G1 phase of mitosis (the left central image shows only one cell labeled with tritiated thymidine which was identified by radioautography). These cells were observed with raster electron microscopy. They barely show surface microvilli (right center). Histologic cuts at the cortical level show hundreds of primordial follicles with their complete fine layer of granulosa cells. Several degenerating or already degenerated primordial follicles can be seen



**Fig. 5:** Optic electron microscopy and radioautography using tritiated thymidine, cytidine, and uracil that show the onset of primordial and primary follicle growth. Only those with an intact granulosa layer will start the growing process (schematic drawing). Radioautography shows how the initial growth is in the oocyte mass for subsequent growth in the granulosa to take place

Once menstrual cycles are established, about 80 days prior to ovulation recruitment starts of about 450 to 500 primordial and primary follicles. This event occurs independently of FSH and LH levels and is dependent on local growth and vascular factors, i.e. insulin growth factor, interleukin 1, interleukin 6, vascular endothelial growth factor, etc.[13,14] (Fig. 5).
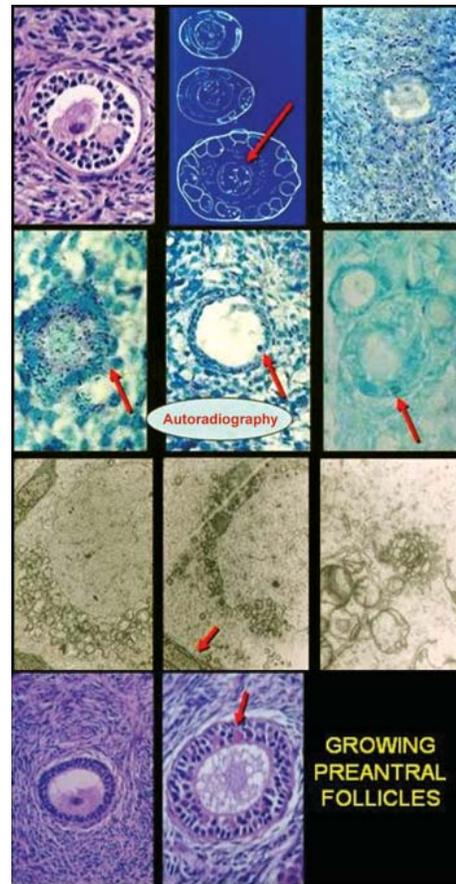
Of the recruited follicles only 20 to 25 arrive at the peak of intercyclic FSH. The remainder degenerate and disappear. Of the 20 to 25 arrivals only 1 to 3 mature and acquire the status of preovulatory follicle. Nevertheless, this is enough since the average woman gets to ovulate 400 to 450 times during her reproductive years.

We will now examine the main mechanisms that result in millions of germinal cells degenerating throughout life, especially at the beginning of postnatal life, and why in some women germ cell degeneration accelerates with time.

There are three mechanisms.[8]

## Necrosis

Necrosis has a vascular origin. It comes about because of loss or reduction of the perifollicular vascular net[49,77] (Fig. 6). This phenomenon is important during prenatal life but much more so during the reproductive period. Once follicles become necrotic they disappear. The process speeds up when there is primary vascular failure, infections, inflammation, etc. For this reason the evaluation of vascular flow in the medulla of the ovary of adult women is important.

## Autolysis

Autolysis and phagocytosis occurs when a protective layer of granulosa cells surrounding completely the follicle is lacking. When this layer is altered oocytes degenerate and eliminated by phagocytosis. This phenomenon takes place when pathologic conditions are the result of immunologic or genetic causes, as is the case with gonadal dysgenesis. In these cases, the primordial germinal cells do not get a protective layer of granulosa cells when they arrive at the urogenital ridge and they are then removed by phagocytosis (Fig. 7).
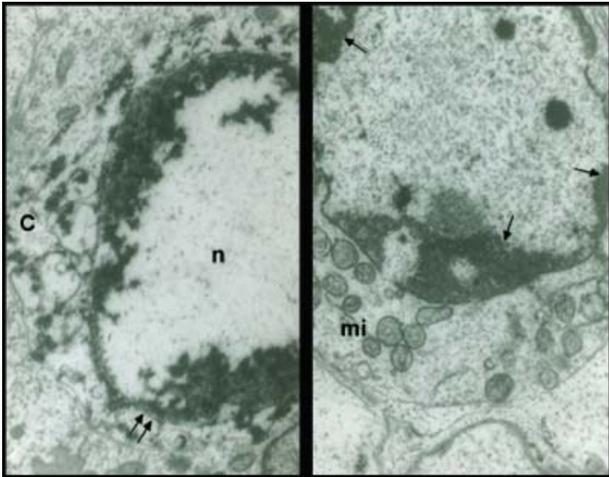
**Fig. 6:** Electron microscopy of two necrotic, human, fetal primordial follicles. The oocyte shows the concentrated and irregular chromatin on the periphery of the nucleus. Intranuclear vacuolization is already present
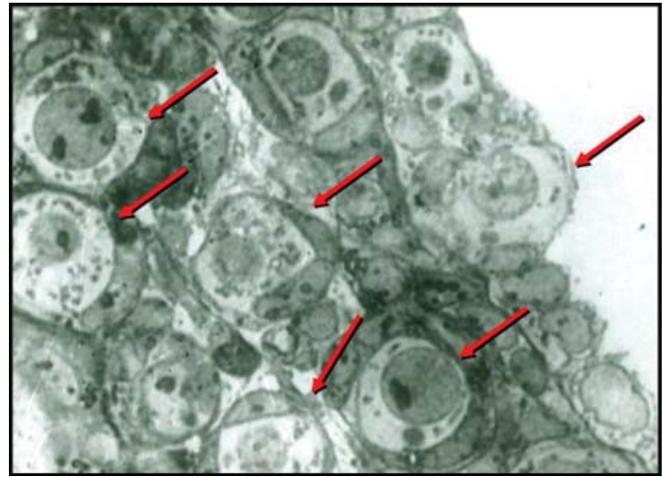


**Fig. 8:** The arrows point to germinal cells migrating to the ovarian surface. It is likely that these are the cells currently recovered in cultures as "mother or truncal follicular cells" and not the stromal cells as reported in the literature
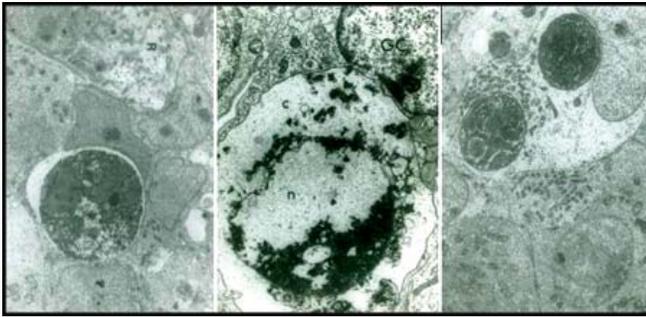


**Fig. 7:** Electron microscopy of three degenerated human fetal oocytes. The two lateral ones are already phagocytized and the central necrotic one is in the process of undergoing phagocytosis



**Fig. 9:** Raster electron microscopy of the surface of a 28-week fetal ovary

### Oocyte Migration to the Ovarian Surface

An important mechanism during fetal life is oocyte migration to the ovarian surface. It is a phenomenon that continues only for a few months after birth. By this mechanism oocytes that migrate to the ovarian surface separate from the ovary and disappear. It has been observed in histologic samples prepared for transmission electron microscopy (Fig. 8). As fetal age increases the number of migrating oocytes that are close to the ovarian surface increases to. Oocyte migration has been confirmed by scanning electron microscopy observation of fetal ovaries of different ages (see Figs 9 to 13).

The surface of the fetal ovary at 28 weeks gestation is smooth and has invaginations (ovarium giratum) with coelomic epithelium rests that persist into postnatal life (Fig. 9).

Nevertheless, when scanning electron microscope images are enlarged there are numerous surface oocytes which may be released. Surface oocytes are released in two manners:

a. They may occur in strawberry-shaped or blackberry-shaped clusters (Fig. 10) typically seen between weeks 24 and 32 of gestation.

b. They may occur as isolated cells over large areas of the ovarian surface, many of them disengaging from the ovary (Fig. 11).

This phenomenon continues in postnatal life, predominantly in the isolated oocyte release form, although in a less intense and more selective manner. The areas from where follicles separated in clusters from the surface of the ovary remain as crater-like lesions (Fig. 12).
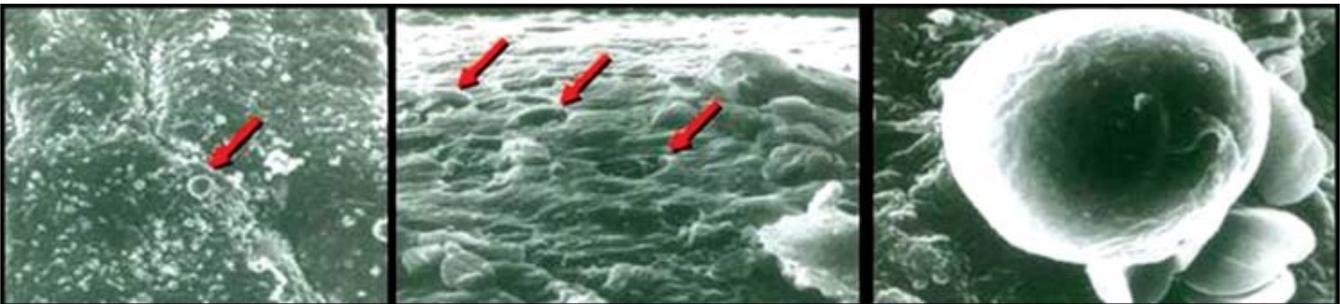
In summary, the decline in number of oocytes is permanent, more pronounced during fetal life, but persistent up to five years after menopause.
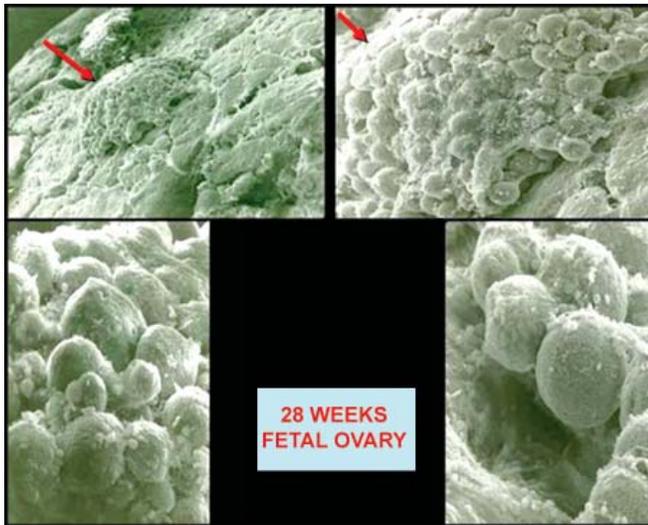
**Fig. 10:** Raster electron microscopy of the surface of the ovary of a 28-week fetus. Numerous groups of primordial follicles emerging in clusters can be observed. Some of these clusters (upper left) resemble strawberry or blackberry clusters
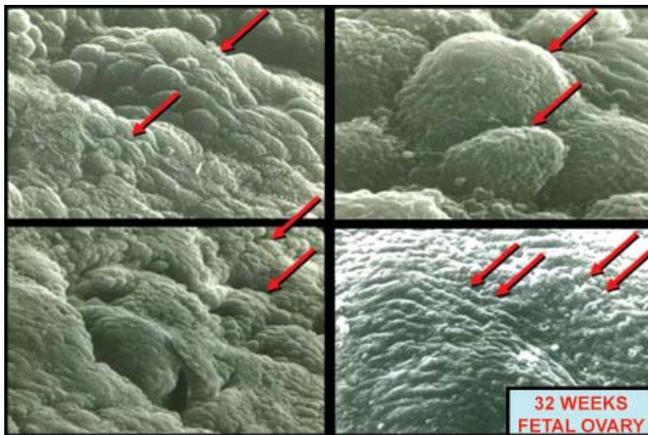


**Fig. 11:** Raster electron microscopy of the surface of the ovary of a 32 week fetus. Numerous isolated primordial follicles can be seen emerging from the ovarian surface (arrows)

However, the last remaining oocytes have qualitative differences and different rates of decline, especially in the period of about thirteen years prior to onset of menopause.

Prenatal and postnatal follicular atresia, which takes place by a process of programmed cell death, or apoptosis, is therefore an important determinant of the quality and density of the remaining follicular pool.

It is now known that gonadotropins, estrogens, growth factors, cytokynes, the reorganization of cytoskeleton actin, and nitric acid are protective against apoptosis. On the other hand, tumor necrosis factor alpha, androgens, and BCL2 and ICE genes promote apoptosis.[12]

A follicular pool that is diminished at the outset or an increased rate of follicular atresia will result in premature follicular depletion.[15]

## ETIOLOGY OF PREMATURE OVARIAN FAILURE

In 1967, De Moraes-Ruehsen defined premature ovarian failure as non-physiologic, postmenarchal cessation of menstruation prior to 40 years of age.[16]

Castelo-Branco *et al* classified the following etiologic agents of premature ovarian failure.

### Follicular Depletion

a. *Deficient initial follicular pool*
   - Pure gonadal dysgenesis
   - Thymic aplasia or hypoplasia
   - Idiopathic
b. *Accelerated follicular atresia*
   - Chromosomal alterations
     — Numeric
       – Turner's syndrome
       – Mosaicisms
     — Structural
       – Chromosome X deletions



**Fig. 12:** Raster electron microscopy of the ovarian surface of a three-month old baby girl. Isolated primordial follicles can still be seen emerging from the surface (right and left), but in lesser numbers. Holes resembling craters are seen in the areas where follicle emigration has taken place (arrows in central picture)

c. *Galactosemia*
d. *External agents*
   • Iatrogenic (tubal and ovarian surgery, chemotherapy, radiotherapy)
   • Others (smoking, endometriosis, infections)
e. *Immunologic alterations*
f. *Idiopathic*

## Follicular Dysfunction

a. *Immunological alterations*
   • Autoimmunity
   • Lymphocytic oophoritis
b. *Enzyme deficits*
   • 17α-hydrolase deficiency
   • 17-20 desmolase deficiency (20)
   • Cholesterol desmolase deficiency
   • Galactose-1-phosphate-uridiltransferase deficiency
c. *Gonadotropic abnormalities*
d. *Resistant ovary syndrome*

## Idiopathic Failure

It is estimated that idiopathic ovarian failure occurs in less than 1% of women younger than 40 years of age.[17,18]

Based on anatomy and functionality two types of premature ovarian failure have been described.

### Afollicular Ovarian Failure

Seen in gonadal dysgenesis, gonadoblastomas, and hermaphroditism.

There is a total depletion of follicles in these cases.

### Follicular Ovarian Failure

Some follicular structures can be detected. Theoretically some degree of ovarian function may reappear.

Among this type of premature ovarian failure is follicular oophoritis, ovaries with only a few follicles, and ovaries with many follicles (also resistant ovary syndrome).

It is evident that there are multiple etiologies for premature ovarian failure, a condition in which at times there is fast reduction and disappearance of follicles, or a lack of response to normal hormonal stimulation.

In some cases there is family history of premature ovarian failure,[17] which underscores the importance of knowing antecedents when investigating the medical history.

Among the above mentioned etiologic factors of follicular depletion, the reduction of the original pool during embryonic and fetal phase is not well understood.

Chromosomal factors such as gonadal dysgenesis as well as enzymatic, immunological, and external agents have been considered. But in any case, both prenatal and postnatal accelerated follicular atresia result in gonads with very few or no follicles.

This outcome almost certainly results from the adverse effect of genetic, enzymatic, immunological, or external agents. The presence of two X chromosomes is necessary for normal development of ovaries. Absence of an X chromosome does not interfere with migration of primordial germinal cells, but it does accelerate follicular atresia enough to result in dysgenetic gonads without follicles at birth.

Numeric or structural abnormalities of chromosomes occur in 20 to 50% of cases of premature ovarian failure. Examples are Turner's syndrome, alterations of short and long arms of chromosomes, isochromosomes, and the fragile X chromosome syndrome.

But the most frequent alterations associated with premature ovarian failure are mosaics such as 45XO/46XX, 46XX/47XXX. In these cases gonadal damage tends to occur at the time of differentiation and development of the ovary and for this reason some degree of follicular function frequently remains.

Galactosaemia, a hereditary autosomal recessive disorder of carbohydrate metabolism due to galactose-1-phospate-uridinetransferase deficiency, causes alterations in ovarian function of 30 to 60% of patients with the condition, but the mechanisms that result in these alterations are unknown.

The presence of follicles, when there is follicular dysfunction, is not synonymous with normal ovarian function. Although many causes of follicular dysfunction remain unknown, we know some of the causes such as enzymatic alterations of steroidogenesis.[13]

Several studies have identified autoimmunity as a potential cause.[18] Endocrine autoimmune diseases produce aberrant regulation of immune response both in cellular immunity (T cells and NK cells) as in humoral immunity (antibodies), so that endocrine tissue becomes the target of autoimmune attack, which results eventually in glandular destruction. It seems that genetic and environmental factors combine in the pathogenesis of these endocrine failures.[19]

There are numerous autoimmune illnesses capable of having an adverse effect on ovarian tissue. Among these are hypothyroidism, Addison's disease, Crohn's disease, vitiligo, pernicious anemia, mucocutaneous candidiasis, myasthenia gravis, diabetes mellitus, rheumatoid arthritis, and systemic lupus erythematosus, to name a few. There are, for example, known cases of circulating antibodies against FSH and LH receptors, and against the zona pellucida among other reproductive tissue targets, in individuals with autoimmune disorders. Unfortunately, the mechanisms involved in autoimmunity against ovarian tissue remain unknown.

Finally, when a cause for ovarian failure cannot be identified, and there are many cases in this group, they are delegated to

the idiopathic category. Some cases may be transitory and patients recover ovarian function spontaneously and even become pregnant, even if several of these relapse into idiopathic ovarian failure.

## DIAGNOSIS

Several methods can be used to confirm the diagnosis of premature ovarian failure. The diagnosis is made by a correlation of the following:
1. Clinical evaluation
2. Basal hormone determinations
3. Hormonal tests
4. Ultrasonography.

We will consider the role each of these methods in the establishment of a diagnosis of premature ovarian failure.

## Clinical Evaluation

Although it is listed prominently in virtually all protocols, clinical evaluation is of limited value for a definitive diagnosis of premature ovarian failure.

Women with premature ovarian failure frequently have the following:
- They are of advanced reproductive age, usually older than 33 to 35 years of age
- They are typically asymptomatic. However, some authors[16] report that up to 50% of affected women may have physical and psychological symptoms such as hot flashes, anxiety, and vaginal dryness that are compatible with accelerated ovarian aging
- They have normal menstrual cycles, but flow is shortened anywhere from three to five days.[9,10] The length of the follicular phase is shortened from the 16.9 days seen in women 18 to 20 years of age to the 10.4 days seen in women who are 40 to 45 years of age[15]
- They are inexplicably infertile
- Those who conceive have a higher rate of spontaneous abortion, and paradoxically, have bizygotic twins more frequently.

Although age is an important factor, it cannot accurately predict reproductive potential. While some affected women become pregnant in their forties, others are unable to conceive in their thirties.

It is widely regarded that the ideal age to conceive is between 22 and 24 years of age.[9] Fertility start to decline at the age of 30 and accelerates after the age of 35. The reproductive potential at age 35 is less than 5% the reproductive potential of normal women in their twenties.

For this reason the Spanish Law of Assisted Reproduction, the least restrictive in the world, is available to workers, and covers all expenses of assisted reproduction, including medicines, because of cost/benefit considerations forbids initiation of ART in women who are 38 years of age or older as well as continuation of treatment after a woman arrives at her 40th birthday.

Many studies have been done to evaluate the relationship between age and pregnancy rates, both in the general population as well as with patients entering ART programs. Age was found to be an important prognostic factor for pregnancy rate in the general population. The results showed a marked decrease in fertility after the age of 35 years.

Female age may therefore be considered a marker for ovarian reserve. In all prognostic models female age was shown to be an important factor that was inversely related to fertility potential.

However, age is only a rough marker of ovarian reserve which should be replaced by one or more objective tests of ovarian reserve.[1]

## Basal Hormonal Determinations

### *Serum FSH Concentration on day 3 of the Menstrual Cycle*

Cycle day 3 serum FSH concentration is an indirect estimate of ovarian reserve. It is the measure of the amount of inhibin B produced by granulosa cells which specifically inhibits FSH as well as the $17\alpha$-estradiol that a cohort of follicles is producing. This inhibition has a negative feedback effect in the pituitary.[1,21-25] Patients with low FSH concentrations respond better to ovulation induction.

This marker is a better predictor of ovarian follicular reserve than age. Patients undergoing IVF-ET with basal cycle day 3 FSH serum concentrations > 20 mIU/ml never become pregnant[26] and those in natural cycles showed poorer follicular growth.[27,28]

Although upper limits fluctuate between 10 and 20 mIU/ml in the literature, although different concentrations have been observed in different cycles of a given woman, and although laboratory variations must be taken into account,[1] a concentration > 15 mIU/ml should still be considered evidence of low ovarian reserve.

A similar test is determination of serum FSH/LH ratio elevation on day 3 of the cycle.[29]

### *Cycle Day 3 Serum 17β-estradiol Concentration*

A more advanced rate of follicular recruitment and selection may be responsible for the relatively high 17β-estradiol concentration on day 3 of the menstrual cycle that is observed in certain infertile women.[30]

No pregnancy was observed when serum values were > 75 pg/ml, which indicates that this is a good test for evaluation of ovarian reserve, but not as sensitive as the cycle day 3 FSH concentration and elevation of FSH/LH ratio tests.

### Cycle Day 3 Serum Inhibin B Concentrations

The inhibins are dimeric peptides composed of an α-subunit with a βA-subunit (inhibin A), or with a βB-subunit (inhibin B). Inhibin A seems to be secreted by the dominant follicle, since it increases during the late follicular phase.[31,32] It may then be a marker for follicular maturity. Inhibin B is secreted by the developing cohort of follicles, since its concentration rises across the luteal-follicular transition and peaks in the mid-follicular phase.[33] It may then be a marker for the number and quality of developing follicles.

Follicles of older women contain less granulosa cells and for this reason have lower concentrations of serum inhibin B (< 45 pg/ml). This lower concentration leads to a specific increase in FSH concentration due to a decrease in selective pituitary inhibition.

Decreased inhibin B concentrations were found in women with diminished ovarian reserve, despite not having elevated FSH concentrations. This suggests that a fall in inhibin B concentration may be an earlier marker of poor ovarian reserve than an elevation in FSH concentration.[34]

### Other Markers

Investigators[35-38] have recently proposed other markers of diminishing ovarian reserve. These markers are expensive hormonal assays which are not yet part of routine practice.

1. Lower circulation of gonadotropin surge-attenuating factor bioactivity (GnSAF)[35]
2. Low insulin-like growth factor 1 (IGF-1) concentrations in follicular fluid[36]
3. Serum concentration reduction of anti-Müllerian hormone, which correlates well with age and with the number of antral follicles. This reduction occurs before the other markers emerge[37,38]
4. DNA footprints.

## Hormonal tests

### The Clomiphene Citrate Challenge Test

This test for predicting ovarian reserve has been validated by several authors.[39-44] The test is carried out by the administration of 100 mg clomiphene citrate on cycle days 5 to 9 and determination of FSH concentration (and if desired, 17β-estradiol concentration) on days 3 and 10 of the cycle. With normal responding ovaries the clomiphene citrate-dependent rise in FSH will be suppressed by 17β-estradiol and inhibin B produced by the follicles.

An abnormal test is defined as an abnormally high FSH (and of one wishes, of 17β-estradiol) on day 3 and/or day 10 of the cycle. Threshold values have been established in 25 IU/L of FSH when the values of days 3 and 10 are added up or if the values of FSH on day 10 of the cycle are greater than 10 IU/L.

One investigator[45] has found the relationship between the number of follicles counted by ovarian biopsy and the results of this test.

### The GnRH Agonist Stimulation Test

This test (also known as the Lupron screening test) evaluates the 17β-estradiol serum concentration change from cycle day 2 to day 3 after administration of GnRH agonist, which causes a temporary increase in pituitary secretion of FSH and LH. In response, the ovaries produce 17β-estradiol.[24,46]

Pathologic values were considered when the threshold value of day 2/day 5 estradiol ratio was 2,[46] or when there was an increase of estradiol above 100 pg/ml.[24]

The ability of this test to demonstrate diminished ovarian reserve seems to be limited and not very useful.

### The Exogenous FSH Ovarian Reserve Test

This test, originally introduced as a screening method for poor responders in IVF cycles,[47] use cycle day 3 FSH and 17β-estradiol serum concentration determinations as well as 17β-estradiol response on day 4 following 300 IU FSH injection, or following daily injections, response on cycle day 8.[48]

The addition of the dynamic component threshold value of increased 17β-estradiol > 30 pg/ml and of basal FSH < 11 IU/L may be an improvement of the predictive value of good response to ovarian stimulation.

However, this test has not been confirmed by other investigators.

### Ultrasound

It is inconceivable that anyone would attempt to control or treat infertility at this day and age without the use of ultrasonography.

The use of 2D, 3D, 4D, Doppler, and transvaginal ultrasonography have become fundamental techniques in state-of-the-art infertility services. Ultrasonography is readily available and has been used in many ways. But literature is so extensive that it is almost impossible for an interested reader to cover all of it. We will summarize the most relevant findings related to early ovarian aging based on our own experience.[49-52]

### Ovarian Volume

Throughout life the volume of the ovary changes. It increases up to the end of fertile age and diminishes as the complement of follicles diminish.

Ovarian volume increases from 0.7 cm$^3$ at 10 years of age to 6 cm$^3$ by the age of 18 years to arrive at a maximum volume of 9 cm$^3$ during the reproductive years, followed by a subsequent decline in volume.

Studies of ovarian volume were done originally at Kings College in London and were subsequently confirmed by Brazilian investigators who correlated ultrasound measurements with the volume of water displaced by the same ovaries following surgical removal due to uterine pathological conditions. These findings have been used for early diagnosis of ovarian cancer and for ART.[52]

A postmenopausal ovarian volume greater than 3 cm$^3$ or 6 ml must be considered suspicious for tumor. On the other hand, a small volume during the reproductive years would indicate a reduction in ovarian reserve and a reduced probability of success in ART.[53-55] An ovarian volume of less than 3 cm$^3$ has been associated with poor response to ovarian stimulation and high risk of cycle cancellation.[56,57]

Ovarian volume has been shown to be a good predictor of the number of follicles undergoing growth.[58] For this reason determination of ovarian volume is considered a good method for evaluation of ovarian reserve.

However, other ultrasound paramenters are more sensitive.

### Antral Follicle Counts

Measurement of the number and growth of follicles with 2D ultrasonography and even better with 3D[49] at the beginning of the estrogenic phase has proven to be one of the best measures of ovarian reserve and of the probability of success with ART, both for subfertile women (Fig. 13) as well as for women undergoing ovulation induction (Fig. 14).

The use of high-frequency transducers (7.5 to 10 MHz) affords observation and counting of follicles from the time they attain a size of 2 to 3 mm.[51,58,59] There is a relationship between the number of follicles observed and the number of oocytes recovered for ART as well as with the number of pregnancies attained.

Ultrasound evaluation of the number and growth of follicles provides better prognostic information about the likelihood of poor response during hormonal stimulation for IVF than does the chronological age of patients or the results of available endocrine markers.[60]

A number below 3 to 6 antral follicles on cycle day 3 would be a poor prognostic index. There is evidence indicating that the number of follicles observed on ultrasound are smaller in women with scant reserve. The number of follicles also decrease with advancing age.[51,61,62]

Ultrasound evaluation of follicle number and growth is therefore an excellent test of ovarian reserve.

### Color Doppler and Doppler Energy

These modes have been used for evaluation of follicular development, for diagnosing ovulatory follicles, for vascular evaluation of the corpus luteum, and for the vascular study of patients with dysovulation (Fig. 15).
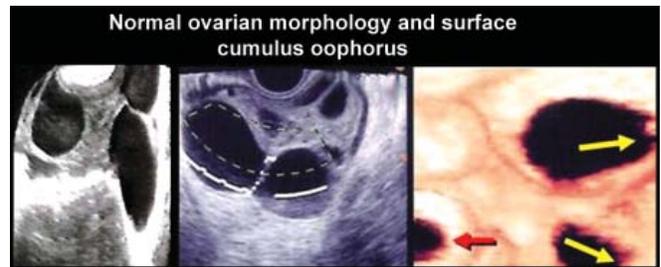


**Fig. 13:** Three-dimensional (3D) ultrasound image of the ovarian surface (left) of a subfertile woman showing three antral follicles of different sizes. To the right is a clomiphene citrate-stimulated ovary showing numerous follicles of different sizes, three of them showing a cumulus oophorus. The cumulus contains metaphase II oocytes of the best quality. Ultrasonography allows evaluation of i) number, ii) growth, and iii) in some ways, quality of the antral follicles
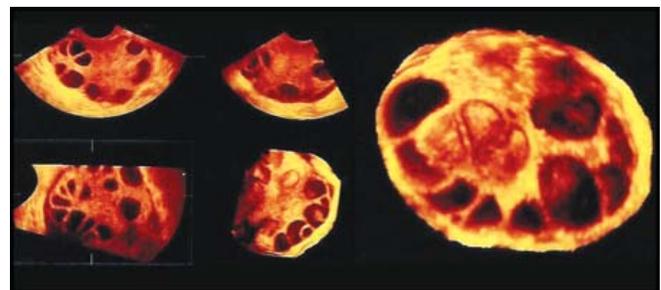


**Fig. 14:** Good ovarian reserve in a recombinant gonadotropin-stimulated ovary studied with 3D. The use of orthogonal planes and 3D rendering allows a much better visualization of the antral follicles. This image shows the typical asynchrony
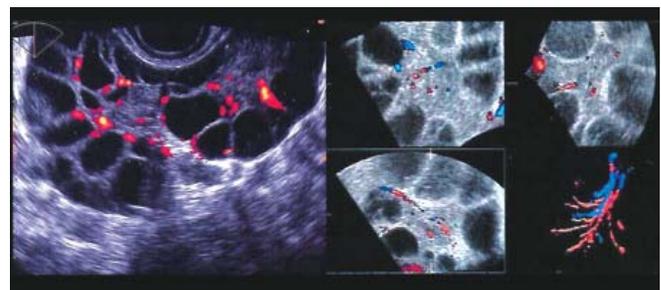


**Fig. 15:** Vascularization of preovulatory follicles in a case of ovarian hyperstimulation studied with 3D Doppler color. The presence of perifollicular flow indicates immediate ovulation

However, Doppler technique is not capable of predicting ovarian hyperstimulation.

Analysis of ovarian stromal blood flow has been used to predict ovarian reserve. Menopausal patients and patients with low ovarian reserve have monotonous, high impedance stromal flow with scant variability.[63]

So far, however, the results have been unimpressive and inconclusive (Fig. 16).
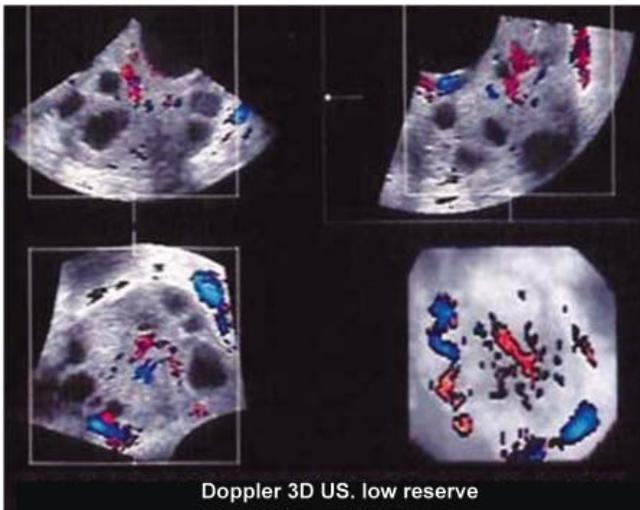
Fig. 16: Color Doppler imaging in a case of low ovarian reserve studied with 3D and orthogonal planes. Only medullary flow without impedance changes and with elevated resistive and pulsatility indices are detected

### 3D/4D Evaluation

Our group has pioneered the use of 3D and 4D transvaginal Doppler instead of 2D ultrasonography for the evaluation of ovarian reserve.[49,51,52] The high reproducibility of measurements obtained with transvaginal 3D ultrasonography for ovarian volumes and antral follicle counts has been demonstrated recently.[64]

While 3D ultrasonography has been shown to be more reliable than 2D for ovarian volume measurements,[65] some authors have yet to confirm its advantages for antral follicle counting.[66]

The results of our group have shown the following:
1. By using three orthogonal planes, the number of antral follicles observed at the onset of the cycle is different and more exact than the number obtained with 2D. These numbers have been confirmed by histologic analysis of ovaries obtained from patients operated for different pathologies (i.e. myomas) within 24 hours of 3D ultrasound counts.
2. The number of selectable follicles and the total number of follicles within an antrum was found to be significantly reduced in women with low response and/or low reserve to ovarian stimulation in the prior cycle, compared to the numbers obtained in normal responders.
3. In patients with low ovarian reserve, there are two differences in antral follicles when compared with those of normal responders:
   a. In both ovaries a very small number of antral follicles is found in all three orthogonal planes.
   b. The antral follicles of patients with low ovarian reserve were a bigger (5 to 7 mm) than the antral follicles of normal responders (2 to 3 mm).
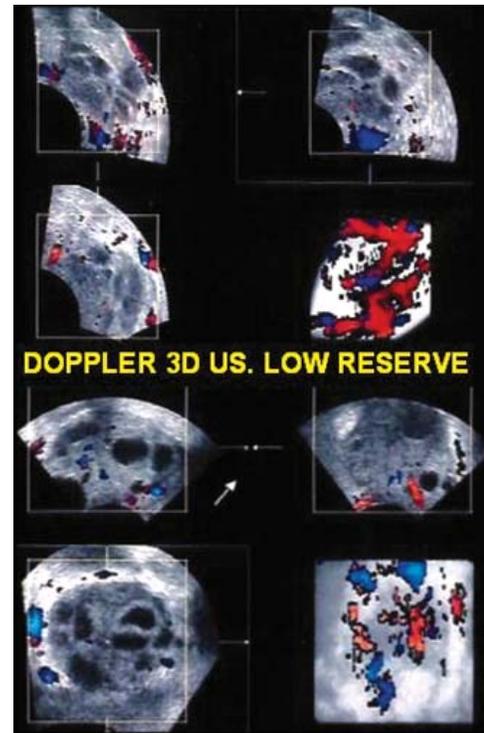


Fig. 17: Three-dimensional transvaginal ultrasonography, orthogonal planes, 3D rendering, and 3D Doppler of an ovary with scant reserve. Orthogonal planes afford an exact determination of the number of recruited follicles in each ovary. It can be observed that follicles are very scarce and that they are larger than those seen in normal fertile women on cycle day 3. Doppler reveals stromal vascularization

This phenomenon probably represents the early cohort recruitment alluded to above, as well as the elevated threshold levels of FSH observed in patients with low ovarian reserve.

Three-dimensional Doppler was used in this study, but except for detection of high impedance vascular stromal flow no significant differences were observed. Ongoing studies with 3D/4D ultrasonography are of great interest (Fig. 17).

### Volume Mode, Virtual Organ Computer-aided Analysis (VOCAL)

This mode differs from 3D ultrasonography by making use of a histometric evaluation program that is available in the machine's software which is capable of analyzing tissues of different ultrasound densities at the same time that it calculates volume based on numerous tomographic cuts. The technique can also be used in inverse mode, allowing in this way determination of the volume of any structure with greater accuracy[52] (Fig. 18).

As of yet, there are no published studies of ovarian reserve, but intuitively we believe that this is a promising technique.
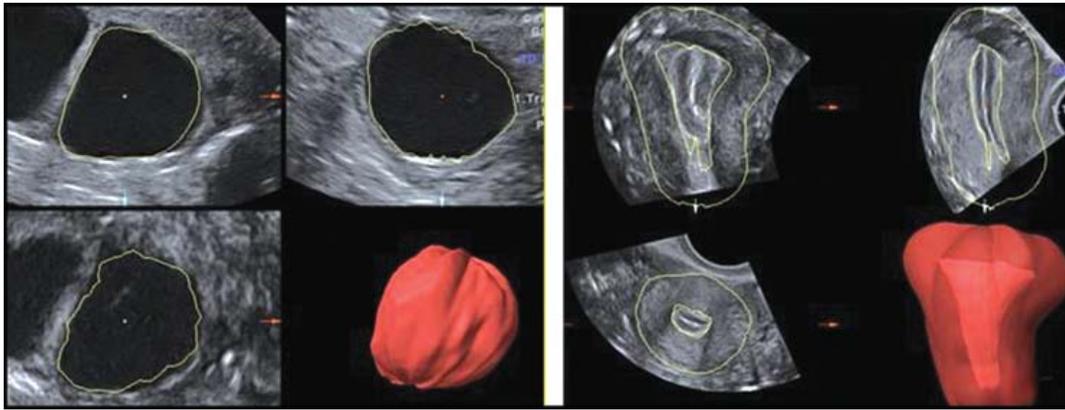
**Fig. 18:** VOL mode of a follicle, left image, and of a uterus, right image, clearly showing the difference between endometrium and myometrium. This technique has been used to calculate endometrial volume, which seems to be an excellent parameter for success in assisted reproduction. Volumes less than 3 cm$^3$ are associated with a low gestation rate (Raga 2003)

### Inverse Mode and Multi-view Slice

This mode enables automatic and immediate demonstration of the follicles within the volume acquired. If VOL is used to manually define the ovarian cortex in a series of planar rotations, the surrounding tissue is removed and the follicles alone are displayed, facilitating an even quicker objective assessment of their number.

The displays may be rotated, either manually by the user or automatically by using a cine loop setting of varying angles and speed to provide a virtual realtime examination of the follicles.[65,67] With this technique it is possible to eliminate images of echodense tissue such as muscle and stroma and transform less dense structures like cysts and hydrosalpinx into visible echo-positive images.

Although this mode has been used for the study of fetal malformations (i.e., Eagle-Barrett syndrome, pleural effusions, hydronephrosis, duodenal atresia, etc.),[67,68] there are still very few reports about its use in ART.

There is only one recent publication of sufficient interest to merit a journal cover.[69] It reports on the use of this technique in 100 consecutive ART cases obtaining increasingly reliable results. This technique allows better visualization of follicles, assessment of the number and size of existing follicles, their location, and determination of their volume (Figs 19 to 23).

Because of its potential advantages for the evaluation of follicles, this technique seems to have a promising future.

The capacity for counting antral follicles afforded by 3D, 3D VOL, and inverse mode[69] offers the potential to use them as ovarian reserve tests, although there are still only a few studies reported.

### IVF-ET

By the ovarian response to stimulation during ovulation induction in ART programs, follicular reserve can be measured.
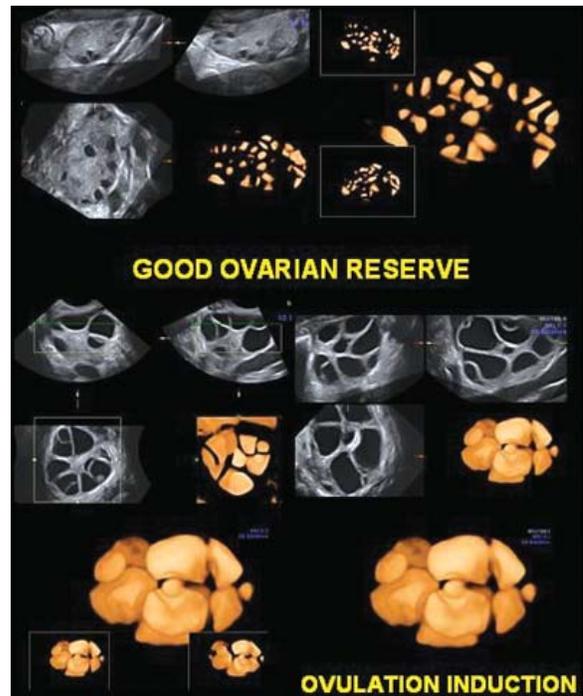


**Fig. 19:** Inverse mode of an ovary with good reserve on day 3, and of the same case following induction with recombinant FSH. Observe the beauty of the follicular images

A significant percentage of women (9 to 24% of patients undergoing IVF-ET) respond poorly to the usual gonadotropin protocols.[70] This type of response may reflect the pool of existing oocytes, as well as giving the best information of their quality and fertilization rate (Fig. 24).

Several studies have shown convincingly that poor ovarian response is the first sign of premature ovarian aging.[71-73]

Garcia[74] was the first to describe the patient with peak estradiol levels below 300 pg/ml and decreased follicular
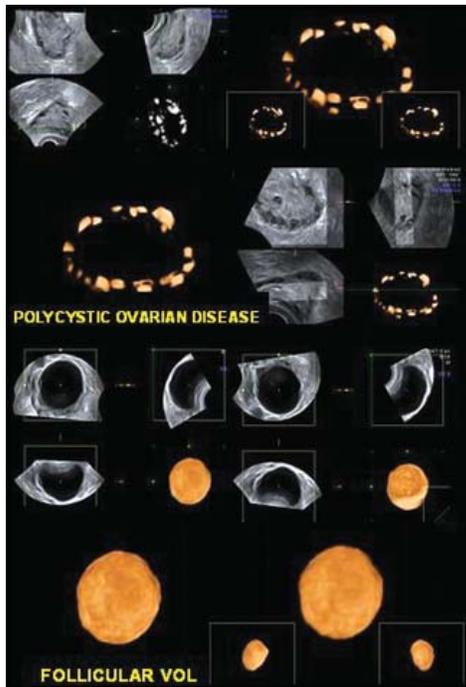
Fig. 20: Upper image, inverse system in case of polycystic ovaries. The follicles are arranged in the typical cortical "cart wheel" distribution. Lower image is VOL calculation of the volume of a preovulatory follicle using the inverse system
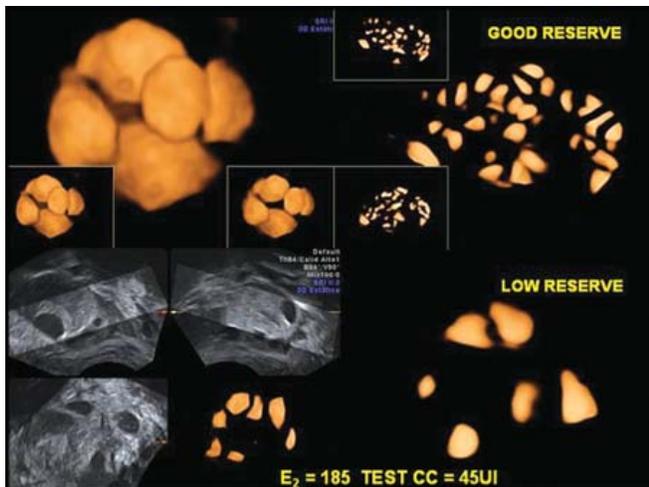


Fig. 21: Comparison of inverse system in a woman with excellent ovarian reserve (upper) with one with very low reserve (lower)

response as a poor responder. Since then, numerous criteria have been used to characterize poor response:
- The number (<3 to 6) of developed follicles[76,77]
- The number of oocytes retrieved (< 3 to 5)
- Peak 17$\alpha$-estradiol levels of less than 300 to 500 pg/ml
- Patient's age and abnormal tests as mentioned above.



Fig. 22: Two cases of very low ovarian reserve studied with inverse mode. There are few antral follicles that are larger than normal. On the left side the findings in the same ovary scanned with inverse mode can be compared with findings using the three orthogonal planes
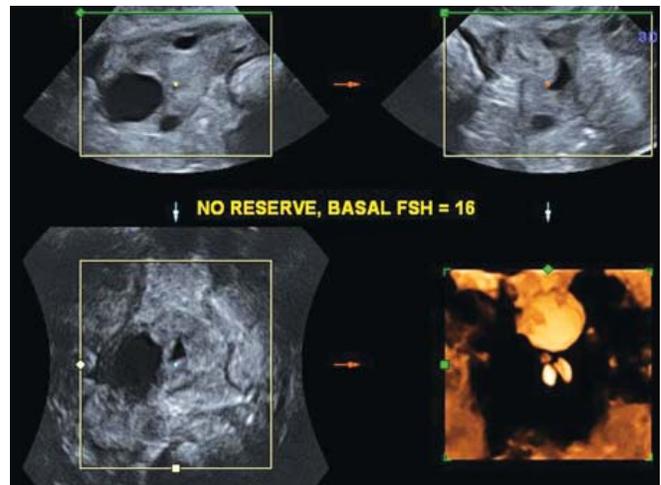


Fig. 23: Patient who practically has no ovarian reserve. There are only 3 antral follicles, one very large and two small central ones. The inverse mode allows them to be seen more clearly than with orthogonal planes

In fact, low ovarian response is confirmed only after the patient has failed ovarian stimulation following an accepted ovarian stimulation regimen.[28] In our opinion, this is one of the main disadvantages of IVF-ET, since for one to know that there is low reserve the patient must submit to expensive and uncomfortable ART procedures. We need a reliable, minimally invasive test that identifies non responders earlier.

Although several etiologies have been suggested, our group suggests that a diminished ovarian reserve is the principal factor,
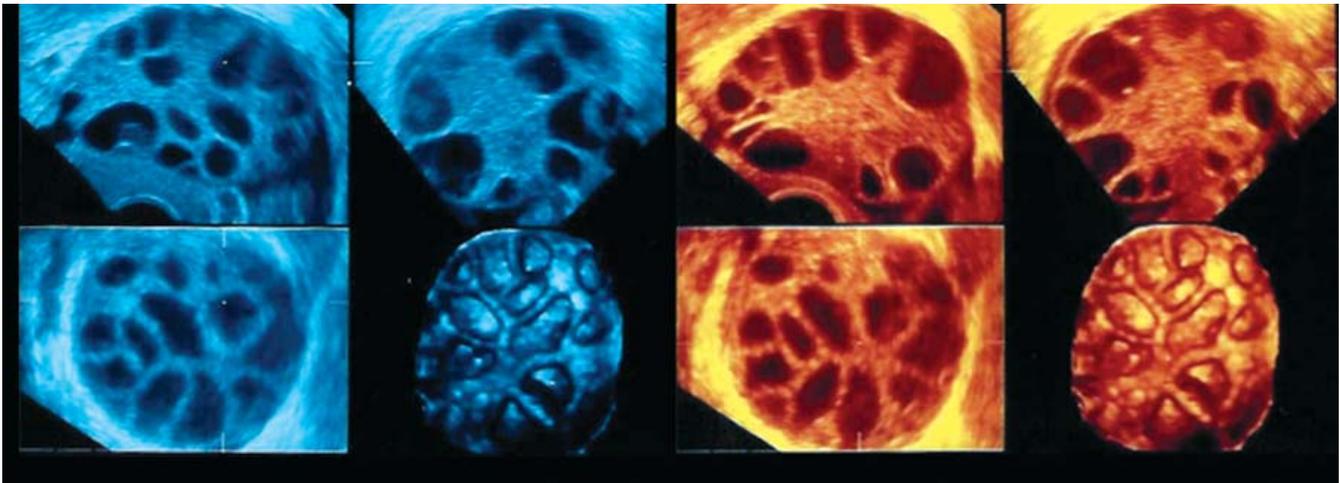
**Fig. 24:** Ovary with very good response to induction with rFSH

although an inappropriate local vascular network for the distribution of gonadotropins and the presence of autoantibodies against granulosa cells have also been observed.[49,77]

At present there is no definitive evidence for the predictive value of the above-mentioned hormone determinations and tests. Ultrasound evaluations and ovarian response to stimulation are clearly the best tests available.

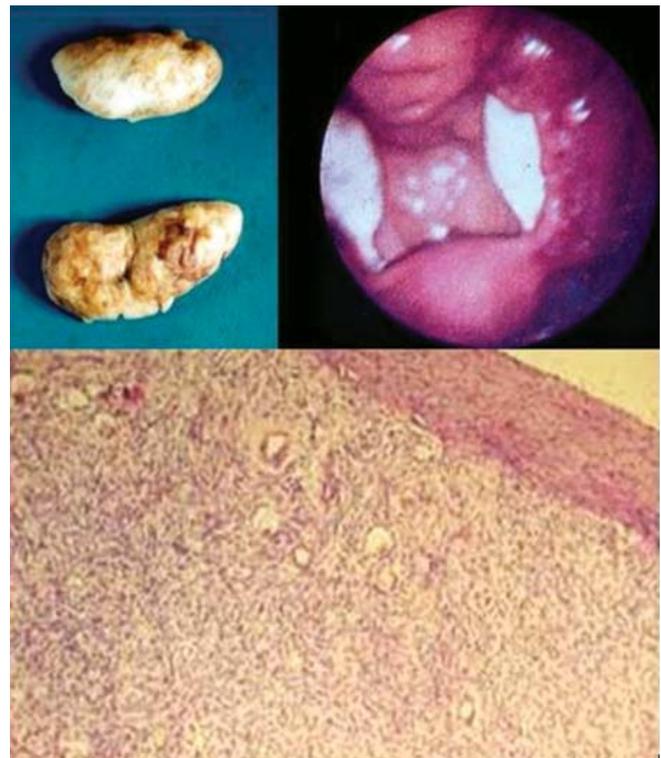## LAPAROSCOPY AND OVARIAN BIOPSY

Women with premature ovarian failure as well as infertile women of advanced reproductive age and women with scant ovarian reserve have diminished ovarian follicles have undergone laparoscopic ovarian biopsy[78] (Fig. 25).

Although biopsy is an important and necessary diagnostic step for some types of ovarian pathology such as premature ovarian failure and gonadal dysgenesis, it is used very infrequently due to the serious surgical and clinical implications entailed.

## COMMENTARY

There are numerous prognostic models that always use clinical evaluation and combine some of the tests discussed in this article and also adding vaginal ultrasonography, Doppler, and more recently, 3D and 4D ultrasonography to their diagnostic armamentarium. None of these models, including ultrasonography, has yet been validated singly or in combination.

A diagnostic model is surely needed which can guide clinicians to identify patients from a clinical population who will not benefit from ART, prior to submitting them to expensive and invasive screening methods. Health care professionals who work in ART programs would then be able to refer patients with



**Fig. 25:** Laparoscopy and macroscopic view of ovaries with scant reserve. Histology of biopsy specimen reveals the scant follicular endowment in the stroma well below a thickenned tunica albuginea

little or no possibility of success early on to an experienced and reputable oocyte donor program. At present none of the existing models is capable of reliably identifying such cases and for this reason continue performing IVF-ET procedures on women with, at best, doubtful probability of success.

Our recommendation for health care professionals involved in ART services is to assess the following parameters prior to subjecting women to IVF-ET:

1. The age of the woman
2. Family history of age at menopause (> 36 years old)
3. Basal FSH (> 15 mIU/ml)
4. Basal 17β-estradiol (> 75 pg/ml)
5. Clomiphene citrate test (Basal FSH + Day 10 FSH > 25 mIU/ml)
6. Basal inhibin B (> 45 pg/ml)
7. Transvaginal sonography with Doppler and inverse 3D mode to asses:
   a. Ovarian volume (< 3 ml or 6 cc)
   b. Ovarian stromal flow (scant and high impedance)
   c. Recruited follicles (< 6 between both ovaries and larger than normal).

We suggest that the results of this ovarian reserve screening panel will be of medical and clinical interest since it is available to all properly trained gynecologists. We no longer agree that assessment is complete when there is a failure of ovulation induction and less so when IVF-ET is offered to women with little or no chance of success.

We believe that the ideal situation would be to arrive at a diagnosis regarding existing ovarian reserve before submitting patients to IVF-ET, an expensive and complex procedure that requires specialized services equipped with high technology. Health care professionals must keep in mind that failure has serious economic, emotional, and psychological consequences for patients. We have yet to find a case of a patient who in spite of very detailed information about success rates is not convinced that she will be among those blessed with a take-home baby.

It is the duty of physicians to clearly inform couples if the existing ovarian reserve is scant (independently of the woman's age), and with very little (or no) probability of success. Physicians must also explain to such couples that there are other types of ART (such as oocyte donation) with a much better probability of success.

Physicians also have a duty to inform younger women with diminishing ovarian reserve of their status and apprise them that they should not postpone childbearing since (if they are interested in having children) postponing pregnancy could be an error with disastrous consequences.

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