

## NARRATIVE REVIEW

### Folliculogenesis and corresponding abnormalities

Giuseppe **Loverro**<sup>1</sup>, Maria **Bardi**<sup>1</sup>, Antonio Maurizio **Pellegrini**<sup>2</sup>, Matteo **Loverro**<sup>3</sup>, Edoardo **Di Naro**<sup>1</sup>, Gianluca Raffaello **Damiani**<sup>1,\*</sup>

<sup>1</sup> Department of Biomedical and Human Oncological Science (DIMO), University of Bari, Policlinico of Bari, Italy.

<sup>2</sup> Istituto San Raffaele, University of Milan, Milan, Italy.

<sup>3</sup> Department of Woman, Child and Public Health, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy.

\* **Corresponding author:** Gianluca Raffaello **Damiani**, M.D. Department of Biomedical and Human Oncological Science (DIMO), University of Bari, Policlinico of Bari, Piazza Giulio Cesare 11, 70124, Italy.

Email: [damiani14@alice.it](mailto:damiani14@alice.it).

ORCID: 0000-0003-0614-8068.

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## ABSTRACT

Despite Research progress in the last decades, many mechanisms involved in the formation, growth, and survival of oocytes remain poorly understood.

This article focuses on recent findings related to the creation of the founding cells of the follicular structure, the evolution of follicular structures during folliculogenesis, and to the pathological conditions that can affect follicular maturation. Indeed, not only the number and functionality of primordial follicles (PFs) is important for fertility, but also the process of activation and maturation of the follicles.

Folliculogenesis is the process by which ovarian follicles mature from a primordial stage to a mature one. The primordial follicles are the only source of oocytes during a woman's reproductive life. The maturation of oocytes is influenced by several autocrine and paracrine signals.

Folliculogenesis occurs through several stages, from primordial to primary, secondary, and tertiary follicles. Follicular maturation is regulated by a significant number of genes, transcription factors, and hormones secreted by the hypothalamic-pituitary-ovarian axis. Primordial follicles contain oocytes arrested in the prophase I of meiosis and are surrounded by a layer of flattened granulosa

cells. As follicles progress to primary, secondary, and tertiary stages, they accumulate follicular fluid and acquire the ability to produce estrogen.

Abnormalities in folliculogenesis can lead to reproductive dysfunctions, as seen in polycystic ovary syndrome (PCOS), which can cause anovulatory infertility. Women with PCOS show an increased number of follicles but abnormal growth and maturation, not allowing proper ovulation. Women with PCOS often show a reduction in serum levels of circulating FSH and increased serum LH levels.

Recent discoveries on the regulation of folliculogenesis and the pathological mechanisms affecting follicular maturation offer new treatment perspectives, particularly for women with PCOS. The aim is to regulate underlying folliculogenesis mechanisms, promoting proper follicular maturation. However, several challenges persist in identifying and correcting factors that limit ovulation and oocyte quality, so further studies are needed.

### **Key words**

Folliculogenesis; folliculogenesis abnormalities; PCOS; anti-Mullerian hormone; fertility impairment.

## **INTRODUCTION**

Dealing scientifically with follicles and their related oocytes is no longer as abstract as it was two decades ago.

In order to integrate what we currently know about this topic with the insights provided by recent research, we considered it useful to focus on the new knowledge about the creation of the founding cells of the follicular structure, on the origins and evolution of follicular structures during folliculogenesis, on the limits and diseases underlying pathological conditions of follicular maturation, and on what limits the use of the abundant number of new follicles, which still appears to be the only therapeutic strategy able to act on the ovarian reserve's decrease, despite the large availability of founding follicles such as primordial follicles (PFs).

## **THE FOLLICULOGENESIS**

### **The primordial follicle**

The intrauterine development of the ovary in mammals begins on the 5th day of pregnancy (E5) and ends two days after birth (P2). This process is guided by a series of signaling pathways that, in turn, regulate the activation of complex and thorny processes.

Following the interruption of these signaling pathways, negative reproductive outcomes can be observed, such as smaller primordial follicles, incomplete follicle development, and failure of sexual differentiation [1, 2, 3] even if they are unlikely to be found before adult life.

It is therefore necessary to implement the most suitable methods to early identify the recognition of an abnormal molecular signaling, which may result in anomalies in folliculogenesis, a process that is crucial for both hormonal homeostasis and the failure of reproductive capacity [4].

Folliculogenesis consists of a series of events that occur sequentially during follicular development and is regulated by a significant number of genes, transcription factors, and hormones secreted by the hypothalamic-pituitary-ovarian axis. During the early phase of fetal development, oocytes (called primordial germ cells, PGCs) gather into structures known as "nests", to ensure uniform

conditioning of the germ cells within them. When these nests break down, most of the oocytes enter meiosis and begin to form primordial follicles, each containing a single PGC [5].

The maturation of PGCs underpins oogenesis, which is closely associated with follicular development, due to factors produced by the oocytes themselves, which significantly impact the development of the granulosa and theca cell layers. Folliculogenesis always begins in the innermost part of the ovarian cortex, and primordial follicles consist of primary oocytes surrounded by a layer of flattened squamous cells known as pre-granulosa cells. Primordial follicles are the only source of oocytes available during a woman's reproductive years.

These primordial follicles have a diameter of approximately 25  $\mu\text{m}$  and are surrounded by a layer of flattened granulosa cells and a basal membrane. The oocyte within these follicles is arrested at the dictyate stage of the first meiotic division. The number of primordial follicles in the ovary during the intrauterine life represents the ovarian reserve available for both spontaneous reproduction and assisted reproduction, keeping in mind that from the sixth to the ninth month of pregnancy there is a significant loss of primordial follicles due to persistent apoptotic events.

Despite this, primordial follicles have intrinsic autocrine and paracrine factors capable of coping with the depletion of the ovarian reserve during intrauterine life, which in this phase is only due to the aforementioned apoptosis.

These factors, indeed, induce a negative regulation of primordial follicle activation and include PTEN, Foxo3a, and SDF-1 (stromal cell-derived factor-1), which act to limit this activation. This regulation is supported by the action of AMH (anti-Müllerian hormone) and SDF-1 from surrounding follicles that attempt to limit the activation of the follicles.

At the same time, primordial oocytes secrete platelet-derived growth factor (PDGF) and bFGF, which stimulate granulosa cells to secrete Kit-ligand, keratinocyte growth factor, BMP-4, and BMP-7. These factors positively influence the activation of primordial follicles, while granulosa cells produce GDF-9 and BMP-15, which promote granulosa cell proliferation [6].

### *Primary follicles*

Ovarian follicles that begin their maturation with the development of primordial follicles go through several stages before reaching reproductive maturity. However, not all follicles go through all the existing stages, which are instead essential to achieve full maturity.

Primordial follicles consist of a primary oocyte arrested in the meiotic prophase, surrounded by a single layer of pregranulosa cells without a basement membrane. These follicles can remain in this state for long periods, even beyond 40 years, and are found in the stromal cortex. Although defined as "quiescent," primordial follicles are metabolically active. Some of them undergo a very slow transformation into an intermediate stage before becoming primary follicles. These intermediate follicles contain a mixture of squamous and cuboidal granulosa cells, but with no change in the size of the oocyte or follicle [7].

Primary follicles are, therefore, the next stage of functional maturation. In fact, only a few original primordial follicles progress to primary follicles each day. This process begins at puberty and goes on every day, regardless of other modulating factors, independent of pregnancy, ovulation, until menopause.

As primordial follicles develop into primary follicles, changes occur in the oocytes. The oocyte increases in diameter and further develops the zona pellucida. Reactivation of the primordial oocyte genome causes the oocyte to secrete growth factors that play a crucial role in follicle

growth. Primary follicles consist of two layers of mitotically active granulosa cells, which change from flat into cuboidal in shape and reach a size of approximately 0.1 mm.

When the primordial follicle is stimulated, it expands and increases the number of granulosa cell layers, thus becoming a primary follicle. Finally, this type of follicle also develops a glycoprotein layer between the oocyte and the granulosa cells, called the zona pellucida [8].

### *Secondary follicles*

As the follicle grows, the granulosa cells (which are somatic in origin, not germinal) split mitotically, so that secondary follicles have two to six layers of granulosa cells, cuboidal - shaped. Secondary follicles also acquire additional somatic layers, which are called the theca cells. The formation of the theca layer depends on the presence of differentiation factors, such as growth differentiation factor-9 (GDF-9), produced by the oocyte. The theca layer forms around the basement membrane in secondary follicles and eventually differentiates into the internal and external theca layers. The growth of follicles from primordial to secondary is gonadotropin-independent.

Secondary follicles represent the most advanced stage in the middle phase of folliculogenesis, where the layer of cells outside the follicle becomes evident and contributes to estrogen production. Secondary follicles are very similar to primary follicles, except that they are larger, contain many follicular cells, and have several stockpile of fluid in the intercellular spaces, which serve to nourish the oocyte. These fluids gradually merge to generate the antrum, while the theca cells contribute to estrogen production.

The granulosa cells surrounding the oocyte are called the cumulus oophorus, from the Greek meaning "pile of eggs", and in this structure two layers differentiate: the inner theca (made up of round cells that secrete androgens and follicular fluid) and a more fibrous outer layer, called the external theca, whose cells are spindle-shaped.

The androgens are converted into estrogens by the granulosa cells [9, 10].

### *Tertiary follicles*

Tertiary follicles are also called antral follicles. These contain a cavity known as the antrum, which is filled with fluid. When follicles reach this stage of development, they are much larger and can be observed on ultrasound.

During the formation of tertiary or pre-antral follicles, the follicles continue to grow. As they progress from secondary follicles to antral follicles, the granulosa cells secrete fluid that accumulates between the cells. A large amount of additional fluid diffuses from the thecal blood vessels and joins a fluid, known as follicular fluid. This fluid contains steroids, protein hormones, anticoagulants, enzymes, and electrolytes, and from a content perspective, it is similar to blood serum. Follicles filled with follicular fluid are tertiary or preovulatory follicles. These follicles have a layer of mural granulosa cells consisting of four layers, while the thecal layer differentiates into an intermediate position between the internal and external layers of the theca.

About follicular functionality, the mechanistic role of growth factors and signaling pathways in the early stages of folliculogenesis involves several important molecules, such as the mechanistic target of rapamycin (mTOR), phosphoinositide 3-kinase (PI3K), and components of the mammalian Hippo signaling pathways [11]. The early stages of folliculogenesis do not rely on gonadotropins. After the early activation, the synthesis of growth factors, activins, and anti-

Müllerian hormone (AMH) begins in the follicles and can act on them both locally and via the hypothalamic-pituitary system. These components are secreted by granulosa cells. As the antral cavity forms, follicular growth increasingly depends on gonadotropins [12].

The preantral follicles increase in size, and at 0.1 to 0.5 mm they will form an antral follicle filled with fluid, also known as tertiary or Graafian follicles. These early stages of folliculogenesis occur in the absence of gonadotropin support and are likely regulated by intraovarian growth factors such as insulin-like growth factors (IGF), members of the transforming growth factor beta (TGF- $\beta$ ) superfamily, and vascular endothelial growth factor (VEGF) [13].

### *Tertiary follicle's oocytes*

The oocyte plays an active role in regulating folliculogenesis and that there is a significant exchange between the oocyte, granulosa cells and the theca cells. It has been widely recognized that the oocyte secretes two important growth factors: growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15).

The oocytes in tertiary follicles are suspended in the follicular fluid by a bridge of granulosa cells, called the *cumulus oophorus*, which keeps it attached to the future placenta. Surrounding the oocyte there is a thin ring of granulosa cells, known as the *corona radiata*. At this stage, the follicle is called "Graafian follicle" and appears as a transparent vesicle that bulges out from the surface of the ovary.

Although the function of the ovary is to produce oocytes, most of the oocytes are never ovulated. Backwards it should be noted that the number of oocytes peaks after the ovaries have formed. After this early phase, the number of oocytes decreases irreversibly.

At birth, a woman has already formed all the oocytes she will ever have during her reproductive life, with no evidence of neo-oogenesis. Most of the oocytes, enclosed in follicles, are eliminated before ovulation through a process called atresia, which is triggered by the activation of apoptosis, a phenomenon that occurs both in the oocyte and in the granulosa cells. Atresia is present, although with varying intensities, at all stages of follicular atresia [14].

### *Graaf's follicle*

The secondary follicles, and especially the tertiary ones, develop into a Graafian follicle. In this way, meiosis I is now completed, and the oocyte becomes a secondary oocyte, which then can begin the second meiotic division.

After the first meiotic division, most of the cytoplasm is transferred to one of the two daughter cells. The other cell becomes the polar body, which remains invisible until it is eventually reabsorbed.

The follicular fluid fills a single space, called the antrum, which is surrounded by the follicular cells, forming the granulosa membrane. The granulosa cells surrounding the oocyte and extending into the antrum are called the cumulus oophorus. There is also a basal membrane between the granulosa cells and the internal theca, while the outer fibrous theca merges with the surrounding stroma.

The oocyte, the zona pellucida, and the follicular cells surrounding the oocyte (also known as the corona radiata) are expelled during ovulation and are drawn into the fallopian tube.

Once released, the oocyte resumes the second meiotic division, progressing only to metaphase II. The division of the oocyte and the formation of the embryonic cells occurs only when the oocyte is fertilized [8, 12].

### *Corpus Luteum*

After ovulation, the ruptured follicle collapses and fills with a blood clot (*corpus haemorrhagicum*), which then forms the *corpus luteum*. The granulosa cells enlarge and become vesicular and are now called luteinized granulosa cells, almost folded.

The spaces between the folds are filled with cells from the internal theca, which expand and become glandular, now known as luteinized theca cells.

The cells of the granulosa zone begin to secrete progesterone (luteinized granulosa cells). The *corpus luteum* also secretes estrogen (which inhibits FSH) and relaxin (which relaxes the fibrocartilage of the pubic symphysis).

If pregnancy does not occur, the *corpus luteum* degenerates into the *corpus albicans*, and the levels of estrogen and progesterone decrease, allowing the release of FSH and LH.

If pregnancy occurs, the syncytiotrophoblasts of the placenta release human chorionic gonadotropin (hCG) and the corpus luteum persists.

Approximately 20 primordial follicles begin to develop each cycle, but only ONE succeeds! [15].

### Abnormalities in follicular developments: PCOS and beyond

Folliculogenesis is the process by which female germ cells develop within the somatic cells of the ovary and mature into fertilizable oocytes. Typically, follicular development is complex and involves the integration of signals from various organs and systems.

Anomalies of folliculogenesis can occur when a follicle, in normal development, is no longer able to complete its follicular maturation, resulting in premature ovulation, as well as in cases in which the follicle stops its steroidogenic production or when follicle growth is intrinsically halted due to chromosomal or metabolic alterations. These aspects are only sparsely reported in the literature but need to be codified, considering the new genetic and molecular investigative techniques [16].

The ovarian pathology in which anomalies of folliculogenesis were most commonly observed is polycystic ovary syndrome (PCOS) [17].

PCOS affects 10 – 15% of fertile women in Western Countries and, according to Rotterdam criteria (the most widely used and worldwide accepted) it is diagnosed when at least two of the following patterns can be observed: chronic oligo/anovulation (six or fewer menstruations in one year), hyperandrogenism (clinically established cause of the presence of hirsutism, acne or alopecia or biochemically established as the presence of elevated blood levels of total or free testosterone) and ultrasound evidence of polycystic ovaries ( $\geq 12$  follicles with a 2-9 mm diameter and/or increased ovarian volume  $\geq 10$  mL); these diagnostic criteria do not take into account the insuline resistance and hyperinsulinemia that very often affect women with PCOS, and that are strongly related to the hyperandrogenism. Four phenotypes of PCOS were recognized, defined with letters from A to D, depending on the different presenting complaints and clinical presentations in these patients. The impairment of insulin activity in PCOS could be due to defects in the insulin second messenger pathway. In PCOS, there is a tissue – specific insuline resistance, being liver, fat and muscular tissue insulin-resistant and ovary insulin – sensitive. For this reason, the oral administration of molecules like myo – inositol could lead to beneficial effects in terms of increased

frequency of ovulation, weight decrease, increased serum high density lipoproteins (HDL), reduction in blood testosterone levels and better IVF outcomes in women with PCOS [18].

The lack of regular ovulatory function in these women designates PCOS as the leading cause of anovulatory infertility, with intrinsic anomalies of folliculogenesis [19]. It is of utmost importance to underline that PCOS does not only affect ovulation, but it is very often linked to cardiovascular and metabolic anomalies with possible long – term diseases, even in the menopausal age [18].

The mechanisms responsible for anovulation are unknown, although anomalies in the follicle and its respective cellular compartments have been well documented.

Women with PCOS have:

- Granulosa cells (GC) with signs of degeneration, which may lead to issues in the response to gonadotropins, although this response is not entirely excluded. Among the degeneration and inflammatory molecules involved in PCOS discovered in recent studies, long Pentraxin 3 (PTX 3) seems to be a promising target. Indeed, this glycoprotein is expressed in many tissues in the body, and also in the ovaries, in the cumulus cells, after the stimulation of pre – ovulatory follicles by LH or human chorionic gonadotropin (hCG). Serum PTX3 levels are lower in non – fertile PCOS women compared to fertile PCOS women, suggesting a possible role of this molecule in PCOS – related fertility and leading to a possible use of PTX3 as a diagnostic marker for PCOS - related infertility. It was also demonstrated an inverse relationship between PTX3 serum levels and AMH levels [20].
- A primary defect in steroidogenesis in the theca cells (TC), which could explain the excess of androgens (defects in steroidogenesis in PCOS could explain the significant association between the expression of the acute regulatory protein gene, StAR, which controls the steroidogenesis in the adipous tissue, and better fertility outcomes after intracytoplasmic sperm injection in PCOS women [21].
- The abnormal growth and development of follicles do not, however, preclude a good ovarian response to appropriate gonadotropin stimulation during ovulation induction.
- In PCOS, there is a pronounced increase in the number of follicles, typically 2-3 times higher than in normal ovaries [22].

The number of healthy primordial follicles, as a percentage of the total number of follicles present, was lower in anovulatory polycystic ovaries compared to normal ovaries [23].

- The growth of preantral follicles occurs more slowly in PCOS, resulting in the accumulation of growing follicles [22].
- There is no evidence that women with PCOS experience early menopause [23], as we might expect with accelerated recruitment of primordial follicles.
- These data suggest that in PCOS, there is a higher number of growing follicles compared to normal ovaries, without any consistent effect on the number of primordial follicles.

In PCOS a prolonged follicle survival was noticed, suggesting an underlying increase in follicle entry or a decrease in exit from the growing follicle pool.

Observations from the culture of ovarian tissue fragments taken from women with PCOS have shown that:

- Normal follicles showed increases in both diameter and growth during culture.
- Examination of atresia revealed a higher percentage of degenerated follicles in normal ovaries compared to those observed in PCOS follicles.
- PCOS follicles in culture underwent less atresia compared to follicles at similar stages from normal ovaries.

Another disease in which, similarly to PCOS, folliculogenesis impairment is present is endometriosis. PCOS is characterized by poor oocyte quality and scarce endometrial receptivity. Endometriosis, defined as the presence of ectopic endometrial tissue, is an estrogen – dependent disease, very debilitating, which can cause chronic pelvic pain, infertility, dyspareunia and/or dysmenorrhea. Recent research has shown that endometriosis and PCOS are united by oestrogen, progesterone and androgen receptors involvement, being both these conditions linked to subfertility/infertility. Both in endometriosis and in PCOS there is an abnormal (increased) expression of G – protein oestrogen receptor (GPER), another oestrogen – binding receptor; GPER exerts an inhibition effect of oocyte maturation in PCOS, while in endometriosis it is involved in the proliferation of endometrial ectopic cells and growth of endometrial lesions. Further studies are needed to establish the exact link between these two conditions, folliculogenesis abnormalities, and to reach new therapeutic targets [24].

Recent studies in mice showed a possible connection between folliculogenesis abnormalities and premature ovarian failure (POF). Indeed, the role of oestrogen receptors is well known; on the other hand, the role of androgen receptors is less clear but can be linked to impaired folliculogenesis and to the genesis of POF. Androgens are clearly known and studied about their role in male fertility, but their receptors are also expressed in female reproductive organs; in ovaries, follicular androgens (produced by theca cells under LH stimulation) are converted by the enzyme aromatase in oestrogens. Furthermore, androstenedione is converted to testosterone by 17 beta – hydroxysteroid dehydrogenase (17beta – HSD) in theca cells, and then testosterone is converted to dihydrotestosterone (DHT) by 5 alfa – reductase in granulosa cells; moreover, androgens receptors are expressed in theca cells, granulosa cells and stromal cells. Androgen receptors (AR) deficient mice are subfertile and develop premature ovarian failure, cause of a reduction in corpora lutea, in oocytes after superovulation and in granulosa cells in the ovaries, with an increasing number of atretic follicles as in a POF – like scenario, showing a possible role of AR during the luteal phase and during folliculogenesis. POF is characterized by primary or secondary amenorrhoea, hypoeestrogenism, increased serum gonadotropins and infertility under the age of 40 years, cause of a depletion of follicles. In many cases, this scenario underpins chromosomal or genetic abnormalities, which are often deletions or translocations in the X chromosome; the gene encoding for androgen receptors is located in the X chromosome, so an involvement of AR gene expression abnormalities can be possible in POF [25].

## *Conclusions*

The slowed follicular development during the primary stage, along with a prolonged survival model, can lead to an accumulation of preantral follicles in women with PCOS.

Women with PCOS often show a reduction in serum levels of circulating FSH due to increased chronic estrogen secretion and an increase in LH secretion, resulting from enhanced activity of the GnRH pulse generator.

Concerning the role of anti-Müllerian hormone (AMH), that inhibits aromatase, it has been proposed that excessive local production of this protein, combined with reduced FSH secretion, may promote follicular arrest and decrease ovarian estradiol (E2) production [26].

During controlled ovarian hyperstimulation, serum AMH levels decreased with the length of the treatment [16]. However, these observations could also be explained by the reduction of AMH in the final stages of follicular development. The potential role of AMH in follicular arrest, a hallmark of PCOS, remains uncertain, as the endogenous factors that regulate this protein are currently unknown.

AMH seems to not to be a reliable marker of fertility, as it reflects only the quantity (and not the quality) of ovarian follicles, but it could help to recognize women with mild forms of PCOS, menopause and POF [27].

In conclusion, in PCOS there is no confirmed evidence of abnormalities in the follicular activation and maturation. The only certainties we have concern the right number of primordial follicles contained in the polycystic ovary, knowing that this can limit their maturation towards the antral follicle. Indeed, we should not forget the high risk of apoptosis for each primordial follicle during its development. This need justifies the incessant search for therapeutic strategies to limit their loss during the intraovarian maturation.

#### **Authors contribution**

G.L.<sup>1</sup>: Conceptualization, project administration, writing – original draft

M.B.<sup>1</sup>: writing – review & editing

G.R.D.<sup>1</sup>: supervision

G.L.<sup>1</sup>, G.R.D.<sup>1</sup>: investigation, methodology

A.M.P.<sup>2</sup>, M.L.<sup>3</sup>, E.D.N.<sup>1</sup>: validation, visualization

G.L.<sup>1</sup>, M.B.<sup>1</sup>: resources, software

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#### **Disclosure of interest**

The authors declare they have no conflict of interests.

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## Data sharing

N/A.

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