

The follicular excess in polycystic ovaries, due to intra-ovarian hyperandrogenism, may be the main culprit for the follicular arrest

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This review exposes the follicular abnormalities responsible for anovulation in polycystic ovary syndrome (PCOS). The putative pathophysiological explanations involve the principal intra- and extra-ovarian regulators which intervene during normal folliculogenesis to control the initial recruitment and growth and then the cyclic recruitment. We propose the hypothesis that the follicular problem in PCOS is 2-fold, but with the two abnormalities being linked. First, the intra-ovarian hyperandrogenism may promote early follicular growth, leading to a 2–5 mm follicle excess. Second, the ensuing excessive number of selectable follicles would inhibit the selection process, presumably through follicle–follicle interaction involving granulosa cell (GC) products such as the anti-Müllerian hormone (AMH). These factors would induce a reversible refractoriness to the FSH-induced differentiation of GC. This explanation challenges but does not exclude other hypotheses about the follicular arrest, such as the premature LH action on the GC of selectable follicles. Hyperinsulinism or insulin resistance would act as a second hit, worsening the follicular arrest either through amplification of the intra-ovarian hyperandrogenism or through dysregulation of the GC. The loss of cyclic rhythm would prevent the inter-cycle elevation of FSH, thus perpetuating the impairment of the ovulation process.

Key words: anti-Müllerian hormone/androgens/follicular arrest/folliculogenesis/polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common causes of anovulation, infertility and hyperandrogenism in women, affecting between 5 and 10% of women of reproductive age worldwide (Franks, 1995). Despite substantial effort to define the cause of PCOS, its pathophysiology remains poorly understood. Consequently, determining the mechanisms that cause PCOS is a major goal of medical research in endocrine gynaecology and reproductive medicine.

The PCOS phenotype can be structured into three components: anovulation, hyperandrogenism and the metabolic syndrome (of which hyperinsulinism, secondary to insulin resistance, is the central abnormality). Only the first component, i.e. the anovulation, will be addressed in this review but it is clear that it is intimately intermingled with the others. There is evidence suggesting that the mechanism of PCOS involves a primary ovarian dysfunction (Jacobs, 1987; Webber *et al.*, 2003). One important line of evidence is the observation by Hughesdon (1982) that PCOS ovaries contain 2–3-fold the normal number of follicles, from the time when they start growing to a size of 2–5 mm (antral follicles). These data about early follicular develop-

ment have been recently confirmed by Webber *et al.* (2003). Another line of evidence is that PCOS follicles stop growing and developing when they reach 4–7 mm in diameter. Therefore, we hypothesized that the follicular problem of PCOS is 2-fold (Dewailly *et al.*, 2003): first, early follicular growth is excessive; second, the selection of one follicle from the increased pool and its further maturation to a dominant follicle does not occur (follicular arrest). Whether the primary defect(s) lie(s) within the theca, granulosa or oocyte is presently unknown.

In this review, we will expand on the follicular abnormalities in PCOS with regard to the recent literature data obtained in the mono-ovulatory species and dealing with the events leading to the selection of the dominant follicle. The putative pathophysiological explanations proposed so far will be reviewed, including our own hypothesis of a self-inhibitory effect within the pool of selectable follicles, due to their excessive number.

Normal folliculogenesis: a brief overview

Folliculogenesis is a very long process, during which a cohort of follicles grows from the primary stage to the size at which they

become visible at ultrasonography (from 2 mm in diameter). It proceeds further to the selection of the dominant follicle and culminates with ovulation. Thus, folliculogenesis is a dynamic process, comparable to a theatre play, where the appearance and the disappearance of the different characters are exquisitely determined and synchronized in order to ensure the ovulation of a mature follicle at each cycle.

Stem cell factor (or kit-ligand), basic fibroblast growth factor (bFGF), growth differentiation factor-9 (GDF-9) and anti-Müllerian hormone (AMH)—a member of the transforming growth factor- β (TGF- β) superfamily, also known as Müllerian inhibiting substance (MIS)—enter on stage during the first act, that of ‘initial recruitment and autonomous growth’. kit-ligand (Parrott and Skinner, 1999) and bFGF (Nilsson *et al.*, 2001) stimulate some primordial follicles to enter growth, while AMH seems to inhibit initiation of follicle growth (Durlinger *et al.*, 1999). GDF-9 is involved in the regulation of the development of early growing follicles from the entry into the growth phase to the early antral stage (1–2 mm in diameter) (Dong *et al.*, 1996).

During the second act, the selectable follicles start to grow more quickly during the luteal phase of the cycle preceding ovulation. This is the ‘regulated growth’ act. Indeed, these follicles become highly sensitive to FSH, and one of them will become a pre-ovulatory follicle. The selective rise in FSH levels that occurs during the luteal–follicular transition appears as a potent stimulus for follicle selection. AMH may be one of the gate-keepers for the cohort, preventing multiple selection.

The third act is the early ‘differentiation’ act. Differentiated functions progressively appear, joining the proliferate activities. Granulosa cells (GC) begin to secrete inhibins, which is a well-established FSH-induced response. Inhibins, also members of the TGF- β superfamily, are dimeric non-steroidal glycoprotein hormones that selectively inhibit FSH production and/or release from the pituitary. Ovarian activity then becomes visible, as the inhibin B serum level starts to increase in the early follicular phase (Groome *et al.*, 1996). When biologically active FSH first enters the follicle at ~6–7 mm, the inhibition of aromatase activity by AMH would decrease, allowing GC to express aromatase cytochrome P450 mRNA and protein and to secrete estradiol (Zelevnik and Fairchild-Benyo, 1994). Inhibins and estradiol cause a small but significant and progressive decline in the circulating FSH concentration, due to their inhibitory effects on pituitary secretion (Zelevnik and Fairchild-Benyo, 1994). The ‘FSH window’ closes at the end of third act (i.e. the mid-follicular phase). In association with putative local inhibitory factors, this results in a negative selection of the remaining cohort, leading to its ultimate demise and to mono-ovulation.

In the following fourth act, that of ‘terminal maturation’, the dominant follicle alone continues to grow. In the absence of pregnancy, luteolysis occurs, the negative feedback of inhibin A and estradiol decreases and the inter-cycle elevation of FSH is again set off. The second act can start again, while first act continues backstage in an acyclic fashion, to ensure the well-ordered entry of players.

In PCOS, several factors prevent the characters from playing their roles properly, thus disturbing the play (the folliculogenesis). Although a large body of evidence points out that theca interna cells (TIC) and GC dysregulations are the main culprits, oocyte defect(s) may also participate in abnormal folliculogenesis of

PCOS. We previously proposed to divide the follicular problem of PCOS into two main components (Dewailly *et al.*, 2003): first, the early follicular growth is excessive; second, the selection of one future dominant follicle from this increased pool does not proceed (‘follicular arrest’).

Early follicular growth in PCOS

Polycystic ovaries (PCO) are endowed with an abnormally rich pool of growing follicles, the number of which is 2–3-fold that of normal ovaries, from classes 1 to 5, except the pool of primordial follicles which is normal (Hughesdon, 1982). Very recently, Webber *et al.* (2003) revisited this data by examining ovarian cortical biopsies from normal and PCOS women. They found an even greater (6-fold) increase in the number of primary growing follicles in PCO from anovulatory women, in comparison to normal ovaries. The materials did not allow examination of secondary follicles.

This excess of follicles could be the consequence of an increase in initiation and/or in subsequent follicle growth.

Is the initiation of follicular growth excessive?

The first recruitment (or ‘initiation of follicular growth’) is believed to be a continuous process. Just after follicle formation, during fetal life, some follicles enter growth, but the vast majority of them remain at a quiescent stage. Factors triggering initial recruitment are not completely identified but kit-ligand (Parrott and Skinner, 1999) and bFGF (Nilsson *et al.*, 2001) appear to be attractive candidates. These factors stimulate some primordial follicles to enter growth, whereas the rest of the follicles remain quiescent for months or years. Follicles then enter the growing pool of large primary follicles, reach the preantral stage after some months, and ultimately need ~70 additional days to reach 2 mm in diameter (mid-antral stage).

Initiation is also under a negative control, exerted by AMH. Indeed, ovaries of AMH-knockout (AMHKO) mice are depleted of their primordial follicles earlier than they are in control mice (Durlinger *et al.*, 1999). This decrease is caused by increased recruitment of primordial follicles in AMHKO females, since more preantral and small antral follicles are found in pre-pubertal and adult AMHKO mice. These results suggest that AMH may inhibit initiation of follicle growth. Durlinger *et al.* (2002) confirmed this hypothesis by a study in which neonatal mouse ovaries were cultured *in vitro* in the absence/presence of AMH. AMH caused a 40–50% decrease in the number of growing follicles after 2 or 4 days of culture. Therefore, AMH may be considered as an inhibitory factor of initiation. However, since it is not produced by resting follicles but by early growing follicles (Weenen *et al.*, 2004), it appears likely that AMH acts on resting follicles through a paracrine effect from neighbour growing follicles.

Is initiation of follicle growth excessive in PCOS? In both studies from Hughesdon (1982) and Webber *et al.* (2003), it was reported that the primordial follicle pool was normal in size, compared to control ovaries. However, the latter authors suggested that the initiation of follicle growth could be stimulated to excess in PCO. Indeed, the mean proportion of primordial follicles was lower than in normal ovaries while the mean proportion of primary follicles was higher. Theoretically, this would induce premature

follicle depletion and would accelerate the onset of menopause, which is the case in the AMHKO mice but not in PCOS (Dahlgren *et al.*, 1992). In addition, the ovarian production of AMH is excessive in PCOS, as will be discussed below. It is therefore still unclear whether initiation of folliculogenesis is altered or not in PCOS.

Excessive early follicle growth

After initial recruitment, GC in primary follicles proliferate. The oocyte continues to grow, the zona pellucida is formed, TIC differentiate, and the vascular supply develops. Compared with the initial recruitment process, substantially more is known about this phenomenon. Oocyte–GC–TIC interactions may play an essential role in the development of early follicles, via their secretions.

In PCOS, the studies from Hughesdon (1982) and Webber *et al.* (2003) indicated a generalized ‘multifollicularity’ beyond the primordial stage, which appears as the salient features of PCO. With regard to their important effects on the small follicle growth, the intra-ovarian hyperandrogenism, which is the cardinal feature of PCOS (Strauss and Dunaif, 1999), is designated as the main culprit for this follicle excess.

Relationship between androgens and follicle excess

Although the role of sex steroids in preantral follicle development remains unclear, recent studies highlight the effect of androgens in early follicle growth. In cultured mouse preantral follicles, androgen treatment stimulates follicle growth (Murray *et al.*, 1998). In intact monkeys, androgen treatment increases the number of preantral and small antral follicles up to 1 mm in diameter, by acting through androgen receptors (Hillier *et al.*, 1997; Vendola *et al.*, 1998; Weil *et al.*, 1998). Androgens promote both TIC and GC proliferation, as measured by cell proliferation-specific antigen Ki67, and inhibit apoptosis, as determined by *in situ* detection of DNA fragmentation (Takayama *et al.*, 1996; Vendola *et al.*, 1998). This effect on folliculogenesis predominates in small follicles, due probably to their richness in androgen receptors. Indeed, Weil *et al.* (1998) showed that the androgen receptor gene expression is the highest in GC of preantral and antral follicles. It is positively correlated with GC proliferation and follicular growth, and is negatively correlated with GC apoptosis and follicular atresia. Similarly, in a non-androgenized primate model, Hillier *et al.* (1997) found that the average androgen receptor level in GC of immature follicles was 4.2-fold higher ($P < 0.01$) than in GC of pre-ovulatory follicles. Furthermore, evidence for a trophic effect of androgens on primate GC is provided by *in vitro* studies showing that androgens promote gonadotrophin responsiveness and steroidogenesis in these cells (Hillier, 1994a). In addition, the finding that testosterone augments follicular FSH receptor expression suggests that androgens promote indirectly follicular growth and estrogen biosynthesis, by amplifying the actions of FSH (Weil *et al.*, 1999). Also, in the primate ovary, Vendola *et al.* (1999) showed that androgens promote the IGF-I and IGF-I receptor gene expression. Such an effect is likely to intervene in the promoting action of androgens on follicle growth.

In the androgenized monkey model, the gross anatomic appearance of ovaries was very close to the one observed in ovaries from women with PCOS, after only 10 days of exposure to androgens (Vendola *et al.*, 1998). In line with these experimental data, congenital adrenal hyperplasia, virilizing tumours and

exogenous androgen treatment (e.g. in female-to-male transsexuals) are associated with increased numbers of non-ovulatory antral follicles similar to those seen in women with ‘idiopathic’ PCOS (Kase *et al.*, 1963; Pache and Fauser, 1993). Many of these ‘cystic’ follicles have healthy steroidogenic and growth characteristics (Pache *et al.*, 1992; Takayama *et al.*, 1996).

Furthermore, we reported that the 2–5 mm follicle number at ultrasound was positively correlated to the serum testosterone and androstenedione levels in our patients with PCOS (Jonard *et al.*, 2003a). This strengthens the hypothesis that the increased number of small follicles is due to the trophic effects of androgens, whether increased locally in the ovary as in PCOS, or systemically as in the other conditions.

All these studies support the concept that androgens are not in fact atretogenic in the primate and human ovary. These findings are in contrast with previous data in rats, which have contributed largely to the view that androgens are atretogenic (Daniel and Armstrong, 1986; Billig *et al.*, 1993), a cornerstone of the PCOS pathophysiology for a long time. However, a recent study in mice showed that androgen treatment stimulated the growth of cultured mouse preantral follicles (Murray *et al.*, 1998). This discrepancy may be explained by significant interspecies differences in ovarian follicular development to different experimental conditions, such as the use of hypophysectomized rats leading to absence of FSH.

How to explain the intra-ovarian hyperandrogenism?

Androgen biosynthesis in the human ovary takes place primarily in TIC, whose function is excessive in PCOS. The excess of androgen production can be explained by extra- as well as intra-ovarian factors.

Extra ovarian factors. A common feature in PCOS is an overall increase in plasma LH concentrations, consisting of both increased LH pulse frequency and LH pulse amplitude (Taylor *et al.*, 1997). The resulting elevated serum LH concentration promotes ovarian TIC steroidogenesis (Gilling-Smith *et al.*, 1994). The mechanism for the LH hypersecretion remains unclear, but fewer workers now consider it to be a primitive phenomenon. Recent data suggest that it results from an impaired negative feedback on LH secretion, due to excessive androgen action on the hypothalamic–pituitary axis (Eagleson *et al.*, 2000).

Hyperinsulinaemia provides another extra-ovarian determinant of hyperandrogenism by enhancing the effects of LH on TIC steroid production, as extensively reported in the literature. Experimental evidence (reviewed by Dunaif, 1997) was recently confirmed by the clinical observation that serum androgen concentrations decrease in women in whom insulin concentrations are lowered with insulin-sensitizing agents or by weight loss (Nestler and Jakubowicz, 1996).

Intra-ovarian factors. (i) *Intrinsic theca dysregulation.* Primary cultures of freshly isolated TIC from PCOS ovaries produce more dehydroepiandrosterone, progesterone, 17 β -hydroxyprogesterone and androstenedione than TIC isolated from normal ovaries (Gilling-Smith *et al.*, 1994). Using a system of propagating human TIC in long-term culture, Nelson *et al.* (1999) have shown that enhanced production of these steroids is a persistent biochemical phenotype of TIC from PCO. Since these TIC cultures can be maintained through multiple population doublings, the increased steroidogenic activity of TIC from PCO compared with normal TIC is unlikely to reflect the influence of *in vivo* hormonal

stimulation (i.e. increased LH and/or insulin levels associated with PCOS). Consequently, these observations suggest that dysregulation of androgen biosynthesis is an intrinsic property of TIC from PCO. Studies have demonstrated that these TIC have increased 3 β -hydroxysteroid dehydrogenase, 17 α -hydroxylase/17,20 lyase (CYP17) activities under basal and forskolin-stimulated conditions (Nelson *et al.*, 2001). Northern blot analyses have shown that CYP17 and CYP11A (P450 side chain cleavage enzyme) mRNA were more abundant in TIC from PCO than normal TIC, while there were similar levels of steroidogenic acute regulatory protein (StAR) in both PCOS and normal TIC (Nelson *et al.*, 1999). Consistent with Northern blot analyses, transient transfection experiments have indicated that the CYP17 promoter is more active in TIC from PCO than in normal TIC, while the StAR promoter was not differentially regulated (Wickenheisser *et al.*, 2000). These experiments suggest that transcription of genes encoding specific steroidogenic enzymes are naturally up-regulated in PCOS TIC, but not all components of the steroidogenic machinery are concerned. It leads to increased production of progestins and androgens. This suggests that hyperandrogenaemia is genetically determined, in line with the result of familial studies indicating that hyperandrogenism clusters as a dominant genetic trait (Legro *et al.*, 1998). However, it is unlikely that the hyperandrogenaemia of PCOS is principally determined by polymorphisms or mutations in the genes encoding a single steroidogenic enzyme activity, such as CYP17 (Gharani *et al.*, 1996) or CYP11a (Gharani *et al.*, 1997; Urbanek *et al.*, 1999; San Millan *et al.*, 2001). Novel studies using new molecular techniques (i.e. micro-array analysis, serial analysis of gene expression and suppression subtractive hybridization) and genetic analysis of PCOS families (i.e. affected sib pair analysis and transmission/disequilibrium tests) are actually in progress for the identification of PCOS genes leading to hyperandrogenism (Strauss *et al.*, 2002). Recently, Wood *et al.* (2003) found increased mRNA abundance in PCOS TIC corresponding to the genes of aldehyde dehydrogenase-6 and retinol dehydrogenase-2, which both increase the expression of 17 α -hydroxylase.

(ii) *Granulosa dysregulation.* Although theca dysregulation seems to be the main culprit of intra-ovarian hyperandrogenism, granulosa dysregulation may also play a role, via GC secretions.

Ovarian GC produce inhibins which are thought to modulate directly follicular steroidogenesis. Hillier *et al.* (1991) showed that recombinant inhibin A enhances both basal and LH-induced androgen production by cultured human TIC. Thus, inhibins might be involved in the excess of intra-ovarian androgens in PCOS through a paracrine effect from GC (Udoff and Adashi, 1997). The use of immunoassays that detect serum dimeric inhibins has allowed us to unravel a peculiar dysfunction of the inhibin system in PCOS. These abnormalities include a net increase in the serum level of α -inhibin precursor proteins (pro- α C), which was positively and significantly related to the androgen serum levels (Pigny *et al.*, 1997). These data could strengthen the hypothesis that inhibins participate in the hyperandrogenism of PCOS but we also reported minimal differences in the levels of inhibin B between controls and patients with PCOS and a decrease in mature dimeric inhibin A (Pigny *et al.*, 2000). Therefore, our results left us with the question whether the increase of pro- α C is only a marker of theca hyperactivity in PCOS or whether it reflects the exaggeration of a putative relationship between inhibin α -SU

and androgen production by TIC. Further basic studies are required to shed light on this enigma.

Finally, recent data about AMH, which is produced by GC, may suggest that it could be implicated in the hyperandrogenism of PCOS. Indeed, the ovarian production of AMH is excessive in PCOS (Cook *et al.*, 2002; Pigny *et al.*, 2003). We found a positive and significant relationship between AMH and serum testosterone and androstenedione levels in our PCOS patients but not in our controls (Pigny *et al.*, 2003). This could suggest a paracrine positive effect of AMH on TIC. The detection by *in situ* hybridization of AMH type II receptor (AMHRII) in TIC of maturing follicles (Ingraham *et al.*, 2000) could lend support to a paracrine effect of AMH on TIC, as reported for Leydig cells. However, further experimental studies are required before speculating on implications of AMH in the theca dysregulation of PCOS. It especially remains to be demonstrated that this ovarian action of AMH would be the opposite of the one on the testis. Indeed, Laurich *et al.* (2002) have shown in a mouse Leydig cell line that the AMH effect was to inhibit both LH- and cAMP-induced expression of CYP17.

(iii) *Oocyte factors.* It has also been proposed that the oocyte may play a role in regulating TIC activity but the available data are still controversial. GDF-9 stimulates both basal and LH-stimulated androgen biosynthesis by rat TIC (Solovyeva *et al.*, 2000). In contrast, the treatment of human TIC *in vitro* with GDF-9 increased their proliferation but blocked forskolin-stimulated progesterone and androgen synthesis (Yamamoto *et al.*, 2002). The level of GDF-9 expression has been reported to be reduced in polycystic ovaries (Teixeira Filho *et al.*, 2002) and one could speculate that a low level of GDF-9 is one the causes enhancing androgen synthesis in PCOS follicles. However, these data are too scarce presently to cast the oocyte for a role in the pathophysiology of hyperandrogenism in PCOS.

To conclude this section: the first follicular abnormality in PCOS is an increased number of early-growing and selectable follicles, which is presumably the consequence of intra-ovarian hyperandrogenism. The accumulation of 2–5 mm follicles gives the typical aspect of multifollicular ovaries at ultrasonography, which is in close relationship with the androgen serum level (Takayama *et al.*, 1996; Jonard *et al.*, 2003a).

Follicular arrest

'Follicular arrest' means that the selection of one dominant follicle is impaired, despite the excess in the number of selectable follicles. This second abnormality in the folliculogenesis explains the anovulation of PCOS. Before addressing this issue, a brief review of the normal selection process is necessary.

The normal selection process

In mono-ovulatory species, the aim of the selection process is to allow the maturation of a single follicle until ovulation and to prevent the growth of the others. It occurs after puberty onset and it depends mostly on the inter-cycle FSH rise. This cyclic recruitment (selection) is a very quick phenomenon because it takes only 2 weeks for a selectable follicle to become a pre-ovulatory Graafian follicle. The rise followed by the decrease in FSH have to occur in a timely fashion, during a short period which is designated

as the 'FSH window', as recently reviewed by Macklon and Fauser (2001).

The FSH window

The increase in circulating FSH during the peri-menstrual period allows a cohort of selectable follicles (2–5mm in diameter) to escape apoptotic demise. Indeed, FSH is an important trophic factor involved in proliferation, survival of GC and cyclic recruitment of a dominant follicle. Then, among this selectable follicle pool, a leading follicle emerges as dominant at the time of the inter-cycle FSH rise. Once selected, its responsiveness to gonadotrophins increases by acquiring both FSH and LH receptors in GC (Harlow *et al.*, 1988). This provides an auto-amplification mechanism to ensure the superiority of the selected follicle and its evolution until ovulation. However, this mechanism has not yet been proved to operate in women.

During the subsequent mid-follicular phase, the release of high levels of inhibin B by the selectable follicles and then estradiol and inhibin A by the dominant follicle suppresses pituitary FSH release (Zelevnik and Fairchild-Benyo, 1994). This closes the FSH window and the ensuing relative FSH defect results, at least partly, in a negative selection of the subordinate follicles from the remaining cohort, leading to their ultimate demise.

Thus, the wave of FSH reaching the ovary during the inter-cycle rise has a major role in the process of selection, but it has to be precisely dammed up at the ovarian level by positive and negative selectors, in order to ensure that only one follicle is selected.

Positive local selectors

A growing body of evidence shows that the selection of the dominant follicle is mainly driven by concomitant increases in local growth factors that stimulate follicle vasculature and FSH responsiveness. Multiple studies have demonstrated the importance of insulin-like growth factors (IGF) in the amplification of FSH action (Gougeon, 1996; Mazerbourg *et al.*, 2003). Among their wide range of actions, there are promotion of growth, inhibition of apoptosis (Chun *et al.*, 1994) and differentiating effects on antral follicle GC, making them the most potent co-gonadotrophic factors. However, Zelevnik *et al.* (2002) have recently shown that infusion of IGF-I in female rhesus monkeys did not have gonadotropin-amplifying actions on the primate ovary *in vivo*, in contrast to its marked effects *in vitro* on both GC and TIC steroidogenesis.

Other local factors also act as survival factors, particularly growth hormone (GH) (Eisenhauer *et al.*, 1995), fibroblast growth factor-2 (Tilly *et al.*, 1992) or interleukin-1 β (Chun *et al.*, 1995). Actually, all these factors are important once the dominant follicle has been selected, to ensure follicle survival for ovulation.

Negative local selectors

Besides the negative regulation of selection through the endocrine feedback effect of inhibin B, which occurs once the dominant follicle is selected, local inhibitors may play a role during the selection process to prevent multifollicular development. Even more, it is tempting to speculate that the escape of one selectable follicle from these inhibitors is the key event of the selection process.

Indeed, some growth factors may exert a direct inhibiting effect on FSH actions. Several studies have raised the possibility of the

presence of specific FSH receptor-binding inhibitors in human serum and follicular fluid (Reichert *et al.*, 1979; Lee *et al.*, 1993; Schipper *et al.*, 1997). However, these factors remain unknown. Also, epidermal growth factor (EGF) and TGF α (Steinkampf *et al.*, 1988; Masson *et al.*, 1990) have been reported to inhibit FSH-induced differentiation of antral follicles. However, studies in the last decade have more particularly highlighted the role of insulin-like growth factor-binding proteins (IGFBP). By trapping IGF, they act as anti-FSH molecules *in vitro* (Masson *et al.*, 1992) and would then be involved in FSH inhibition *in vivo*. In turn, these inhibitors are negatively regulated by proteases which neutralize their activity. Indeed, some authors (Rivera and Fortune, 2003; Mazerbourg *et al.*, 2003) hypothesize that a FSH-induced increase in pregnancy-associated plasma protein-A (PAPP-A, an IGFBP protease) in the future dominant follicle decreases the levels of inhibitory IGFBP-4 and -5. This makes IGF able to bind to their receptors and to synergize with FSH to promote an increase in estradiol secretion and follicular dominance (see above).

Finally, besides its inhibiting effect on the initial recruitment of primordial follicles via a paracrine effect (see above), AMH may be one of the local inhibitors of FSH action (Josso *et al.*, 2001). Indeed, AMH has been shown to decrease aromatase activity in the fetal ovary (di Clemente *et al.*, 1992) and to inhibit granulosa-luteal cell proliferation and progesterone production (Kim *et al.*, 1992). AMH presumably exerts these effects by decreasing the GC sensitivity to FSH, since follicles from AMH knockout mice are more sensitive to FSH than those from the wild type (Durlinger *et al.*, 2001). Conversely, Baarends *et al.* (1995) previously reported that FSH down-regulates the AMH and AMH type II receptor expression in adult rat ovaries. In line with these experimental data, several authors including ourselves reported a negative correlation between AMH and FSH serum levels in normal women in early follicular phase (Seifer *et al.*, 2002; Van Rooij *et al.*, 2002; Fanchin *et al.*, 2003; Pigny *et al.*, 2003). This balance between AMH and FSH might be crucial for aromatase activity at the time of the selection process. In the future selected follicle, the acquisition of an exquisite sensitivity to FSH would lower the AMH expression. This would allow aromatase to escape from a putative AMH inhibition, thus conferring to the selected follicle the ability to secrete estradiol. Thus, we can speculate that AMH repression would be one of the ultimate goals of the FSH inter-cycle peak.

Does the selection process involve follicle-follicle interactions?

In addition to our uncertainty about the factor(s) that are locally involved in the positive and negative regulation of selection, the way they act is an unsolved issue. Since they are presumably secreted by the GC, one may believe that they act through an autocrine intra-follicular mechanism. Alternatively, they may produce their stimulatory or inhibitory effects through inter-follicular (or follicle-follicle) influences. This old concept (Goodman and Hodgen, 1983; Gougeon and Testart, 1990) has not yet been confirmed, but it fits with the spatial rearrangement of follicles during the selection process, as shown by computer modelling of ultrasound images in women (Gore *et al.*, 1997). These authors reported that the dominant follicle moves away from its neighbouring subordinate ones, which could suggest that the former exerts a suppressive effect on the latter's growth through FSH-inhibiting factors. Conversely, some factor(s) in the

microenvironment of the latter might exert an inhibiting effect resulting in an auto-blocking phenomenon, from which the dominant follicle would have to escape in order to ensure its continuing growth.

Therefore, the number of growing follicles could be an important negative regulator of the selection process. Its strength could be decreased in situations of partial follicle depletion, such as the menopausal transition, thus explaining why several follicles can be selected in the early follicular phase of ageing women (Prior, 1998). On the other hand, it could be increased when follicle growth and number are excessive, such as in PCOS.

Mechanisms of the follicular arrest in PCOS

The follicular arrest has not received yet a clear and unanimous explanation. Furthermore, in contrast to the excess in small follicles, it does not always occur in patients with PCOS and some of them do even ovulate monthly (Franks *et al.*, 1998; Carmina and Lobo, 1999). Lastly, the follicular arrest can be easily reversed in most cases by pharmacological manipulations aiming at increasing the amount of FSH reaching the ovaries. Many selectable follicles can therefore be rescued, which suggests that in PCOS, unknown factor(s) protect(s) them partially from atresia, which normally should happen because of their stagnation (Homburg and Amsterdam, 1998). The main clinical consequence of this is the well-known increased risk for ovarian hyperstimulation syndrome (OHSS) in PCOS.

Several mechanisms can be hypothesized to explain the follicular arrest in PCOS. Any one excludes the others, but one or the other can be prevalent in different situations.

The absence of inter-cycle FSH rise in PCOS

Although normogonadotrophic anovulatory patients (a situation we can assimilate to PCOS) display bioactive and immunoreactive FSH levels in the range of those during the normal menstrual cycle (Fauser *et al.*, 1991; Van Dessel *et al.*, 1996), these patients lack the inter-cycle FSH rise. The enlargement of the pool of selectable follicles could explain this, via an excessive production of inhibin B, leading to a suppression of FSH release by negative feed-back effect. However, there is no clear-cut increase in the inhibin B serum levels in PCOS, and we and others have previously reported that the serum FSH and inhibin B levels do not correlate negatively (Pigny *et al.*, 2000; Laven *et al.*, 2001). Therefore, the absence of inter-cyclic FSH rise is more likely due to the absence of ovulation and the subsequent absence of luteum corpus and luteolysis during a preceding cycle. Hence, it is rather a secondary phenomenon than a primary defect.

Is there a defect in positive local selectors?

Although it is well recognized that the IGF system is highly involved in the dominant follicle growth (see above), there is as yet no convincing evidence to confirm the hypothesis that a derangement in the IGF system is the central abnormality in PCOS follicular arrest. This is understandable since the mechanisms of the latter apply to selectable and not to selected follicles (Magoffin *et al.*, 1995; Cataldo, 1997; Giudice, 1999).

Is there an excess of negative local selectors in PCOS?

Several experimental and clinical arguments give support to the hypothesis that the follicular arrest is due to an excess of local

inhibitor(s) of FSH activity (i) To induce ovulation with hMG or FSH in women with PCOS, dedicated protocols are recommended, the most-used being the so-called 'chronic step-up low dose' (Hamilton-Fairley and Franks, 1990). This protocol has been designated to take into account the well-known temporarily ovarian refractoriness to FSH. (ii) A recent dose study fits well with this clinical observation. Coffler *et al.* (2003a) reported that the estradiol response to increasing doses of human recombinant FSH occurred at a higher threshold in PCOS subjects compared to normal controls. (iii) *In vitro*, the GC from PCO antral follicles produce normal or increased estradiol amounts (Almahbobi *et al.*, 1996), suggesting clearly that the *in vivo* functional abnormality is not due to an intrinsic factor lying in GC, and that, conversely, the *in vivo* environment of GC exert an inhibitory effect.

Among the potential inhibitors mentioned above, the presence in serum and follicular fluid of an excessive level of factors inhibiting FSH receptor activation has not been confirmed (Schipper *et al.*, 1997). On the other hand, IGFBP-4 may be an attractive candidate. Indeed, Zhou *et al.* (2003) have recently shown that its expression depends on the expression of the LH receptor, and the latter is thought to be prematurely expressed in the GC of PCOS follicles (see below). However, Magoffin *et al.* (1995) have shown that the IGFBP concentrations in the 5–7 mm diameter follicles from the polycystic ovaries were comparable with androgenic cohort follicles from normal women. In addition, PCOS pre-ovulatory follicles contained a normal pattern of IGFBP expression. Likewise, for Cataldo (1997), alterations in IGFBP may sustain the anovulatory steady state in PCOS but are unlikely to initiate development of the syndrome.

One of the still unknown factors secreted locally by the selectable follicles and inhibiting the FSH effects could be the AMH. In two previous studies from the same group, it has been shown that women with PCOS have significantly higher AMH levels in both serum and follicular fluid than normal women (Fallat *et al.*, 1997; Cook *et al.*, 2002). We recently confirmed this result in our PCOS population compared to a large control group (Pigny *et al.*, 2003). Moreover, we showed that the marked increase in the serum AMH level was positively and tightly related to the excess of 2–5 mm follicle number at ultrasound, in agreement with the findings by Laven *et al.* (2004). Conversely, we observed a negative correlation between AMH and FSH serum levels, both in our patients and controls. In addition, others reported an inverse relationship between AMH and estradiol (E₂) serum levels in women with PCOS (Cook *et al.*, 2002). Thus, we can speculate that the AMH excess is involved in the lack of FSH-induced aromatase activity, which characterizes the follicular arrest of PCOS (Jakimiuk *et al.*, 1997). That the ratio AMH:follicle number was not increased in our patients suggests that each follicle produces a normal amount of AMH (Pigny *et al.*, 2003). Therefore, the inhibitory effect of AMH would not result from an intra-follicular excess of this hormone but rather from an excessive AMH tone within the microenvironment of the selectable follicles, acting through follicle–follicle interactions. Alternatively, an endocrine action of AMH could be suggested since the increase in the circulating AMH levels in PCOS patients is 2–3-fold the one of normal women (Pigny *et al.*, 2003). Nevertheless, the strong and independent positive correlation that has been found between follicle number and serum AMH level argues in favour of the hypothesis that the excess in 2–5 mm

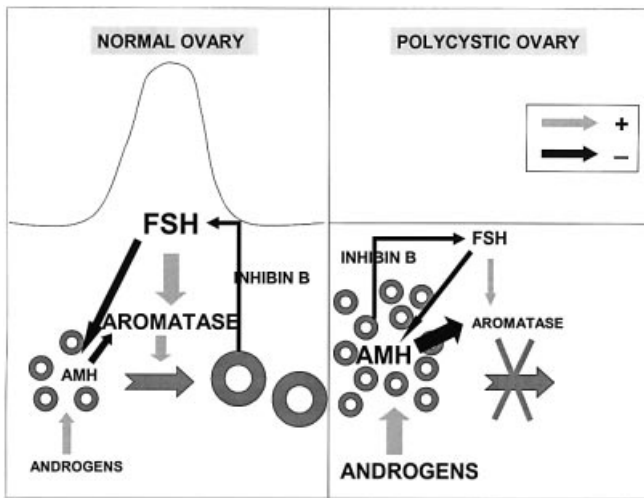


Figure 1. Interactions between intra-ovarian androgens, number of selectable follicles, anti-Müllerian hormone (AMH) production and FSH effect on aromatase and on the selection of the dominant follicle, within the normal ovary (left panel, displaying the FSH inter-cycle peak) and PCO (right panel). In PCOS, the balance between FSH and AMH would turn towards AMH, which leads to *in vivo* defect of aromatase activity and follicular arrest. The excess of AMH would be the consequence of the excess of antral follicles. In turn, this follicle excess would be the result of intra-ovarian hyperandrogenism. The negative feed-back exerted by inhibin B would not be the culprit. A proper balance between FSH and AMH can be restored by cautious increase of FSH in PCOS. Indeed, the inhibiting physiological effect of FSH on AMH is maintained in patients with PCOS (from Pigny *et al.*, 2003 and Jonard *et al.*, 2003b).

follicle number *per se* is responsible for this excess of AMH production. Altogether, these data concerning AMH make it a good candidate to explain the auto-inhibiting effect of selectable follicles, more particularly on aromatase, thus checking the selection process. Despite the subnormal serum FSH level in PCOS, the negative effect exerted by FSH on AMH would not be sufficient to permit the follicles to escape from the AMH tone and to start to express aromatase (Figure 1).

This hypothesis is supported by the negative correlation that we found recently between the small (2–5 mm) and the larger (6–9 mm) antral follicle number (FN) at ultrasound, both in normal and PCOS women (Jonard *et al.*, 2003b). In our opinion, this negative relationship reflects an inhibitory mechanism exerted by the selectable follicles on their further maturation. This physiological phenomenon involving local inhibitory factors would be exaggerated in PCOS.

If the presence of inhibitors of FSH (such as AMH) within the cohort are believed to participate in follicular arrest by lessening the FSH effects on GC differentiation, other mechanisms may involve LH.

The premature action of LH in PCOS

Physiologically, GC develop their own LH receptors in the mid-late follicular phase (Erickson *et al.*, 1979). Thus, LH takes the control of terminal follicular growth and enhances estradiol and progesterone production by GC, while inhibiting their proliferation. Although an excessive serum LH level is not always observed in patients with PCOS and does not seem to be a pre-

requisite to anovulation (see above), premature LH action on GC is supported by some experimental data. This hypothesis provides an alternative explanation for the follicular arrest.

Willis *et al.* (1998) have cultured GC from anovulatory PCOS patients, ovulatory PCO patients and control patients in the presence of LH. The GC from anovulatory PCOS patients responded much earlier with estradiol secretion to LH (at a follicle size of 4 mm instead of 9.5/10 mm) than those from the other two patient categories. Furthermore, mRNA expression of LH receptor is excessive in GC of antral follicles from PCO (5 mm) (Jakimiuk *et al.*, 2001). These results suggest an earlier LH receptor gain in anovulatory patients. These authors hypothesized that GC from anovulatory women with PCOS are at a prematurely advanced stage of development, leading to arrest of cell proliferation, stagnation of follicle growth, and anovulation. Indeed, *in vitro* premature exposure of normal human GC to LH inhibited their proliferation, such that development of the dominant follicle was arrested (Yong *et al.*, 1992; Hillier, 1994b). In line with these experimental data, we have reported a positive and significant relationship between serum LH and inhibin B levels in women with PCOS but not in controls (Cortet-Rudelli *et al.*, 2002), suggesting that in the former, LH is operating earlier on GC function than in the latter.

What is the role of hyperinsulinism and/or insulin resistance?

Hyperinsulinism resulting from insulin resistance has been suspected to influence the process of premature differentiation of GC (Franks *et al.*, 1998). Willis *et al.* (1996) have previously shown *in vitro* that insulin increases the ability of GC to respond to LH and they suggested that raised insulin levels in anovulatory women with PCOS may be a major factor causing the follicular arrest. In this situation, hyperinsulinism may disturb the FSH-to-LH shift that triggers the correct follicular development until ovulation.

On the other hand, Coffler *et al.* (2003b) incriminate insulin resistance at the GC level rather than hyperinsulinism. Indeed, they examined GC responsiveness to recombinant human FSH in women with PCOS before and during insulin infusion using the hyperinsulinaemic–euglycaemic clamp method. They found that this responsiveness was enhanced by insulin after having improved the sensitivity to this hormone by treatment with pioglitazone.

However, rather than being the primary cause of anovulation in PCOS, hyperinsulinism and/or insulin resistance may be viewed as a ‘second hit’ that non-specifically worsens the follicular arrest (see below).

Oocyte abnormalities

To date, this part of the puzzle is certainly the least documented. Dumesic *et al.* (2002) have shown that in prenatally androgenized monkeys undergoing ovarian stimulation for IVF, oocyte developmental competence is impaired, as indicated by a decreased percentage of zygotes developing into blastocysts. A plausible explanation is an impaired acquisition of maternally derived proteins and/or transcripts that are important for embryonic gene activation.

A primary oocyte abnormality may also be involved in PCOS aberrant folliculogenesis since the level of GDF-9 mRNA appeared to be reduced in primary oocytes from PCO during their growth and differentiation phase (Teixeira Filho *et al.*, 2002).

However, it remains to be determined whether the reduced GDF-9 mRNA levels participate in the PCOS follicular arrest or are simply the consequence of another primary abnormality. Nevertheless, the possibility of intrinsic oocyte abnormalities opens a new track, and GDF-9 as well as other oocyte factors, such as the bone morphogenic protein-15 (BMP-15), may be added to the list of suspects.

Why is the follicular arrest inconstant?

Actually, PCOS is a cause of oligo-ovulation, rather than anovulation. From time to time, for unknown reasons, a dominant follicle is able to escape from the inhibitory intra-ovarian influence and proceeds towards ovulation and formation of a corpus luteum. Because of these random ovulations, the fertility rate in untreated patients is not null, although it is less than in normal women. Moreover, some patients with PCOS ovulate regularly and have a normal fertility, despite the presence of a clinical and/or biological hyperandrogenism (Carmina and Lobo, 1999).

Attempts to unravel the factor(s) which differentiate(s) ovulatory from anovulatory women with PCOS can provide important clues, but they are seldom in the literature.

Role of hyperinsulinism and/or insulin resistance. In their experiments with cultured GC, Willis *et al.* (1998) have shown that premature LH receptor expression in GC harvested from small follicles was restricted to anovulatory patients. They suggested that this was related to hyperinsulinism (see above), which effectively does not affect all patients with PCOS. From clinical data, it is clear that obesity and/or hyperinsulinism have a negative effect on the ovulation rate (Kiddy *et al.*, 1990). However, even though the use of insulin-sensitizing drugs effectively improved the ovulation rate in several studies, certainly not all patients become ovulatory with such treatments (Costello and Eden, 2003). Moreover, no marker of hyperinsulinism could clearly predict the treatment outcome (Nestler *et al.*, 2002). In addition, some ovulatory patients also display features of hyperinsulinism and insulin resistance (Carmina and Lobo, 2001). Conversely, lean oligomenorrhoeic women with PCOS may display no feature of hyperinsulinism and/or insulin resistance, despite the use of sophisticated measures (Ovesen *et al.*, 1993). Therefore, rather than being the primary cause of anovulation in PCOS, hyperinsulinism and/or insulin resistance may be viewed as a 'second hit' that non-specifically worsens the follicular arrest. The precise target of this hit remains to be ascertained. Is it at the time of early follicular growth, through enhancement of the follicle number *via* insulin-induced TIC hyperfunction and ovarian hyperandrogenism, and/or is it at the time of selection, through deleterious effects of hyperinsulinism and/or insulin resistance on GC?

Role of the small follicle excess. According to our hypothesis of an auto-blocking effect within the pool of selectable follicles, one could speculate that the excess in the follicle number and/or in AMH is less in ovulatory patients. The recent histomorphometric data from Webber *et al.* (2003) agree with this, although this study was restricted to the primordial and primary follicle density. These authors showed that the primary follicle excess was clearly higher in ovarian biopsies from anovulatory than from ovulatory women with PCOS. In our database including 350 patients with PCOS, we have observed by ultrasound that the 2–5 mm follicle number was less in regularly menstruating women than in the cases of irregular cycles or amenorrhoea (11.5 ± 6.2 versus 16.1 ± 8.0 respectively,

$P < 0.0005$, unpublished data). In a sub-set of the former, we also observed a trend to a lower mean serum AMH level than in the latter, but our limited series precluded appropriate statistical analysis. In their larger series of women with WHO II oligo-ovulation, Laven *et al.* (2004) found that serum concentrations of AMH were strongly and positively correlated with the interval between bleeding periods.

Patients' age could also differentiate ovulatory from anovulatory women with PCOS. Indeed, Elting *et al.* (2003) reported recently that the decrease in the size of the follicle cohort due to ovarian ageing seemed to be largely responsible for the higher incidence of regular menstrual cycles in women with PCOS aged >35 years. Likewise, in our database, patients with regular cycles were significantly older than those with either oligo- or amenorrhoea (29.1 ± 3.8 versus 26.4 ± 5.1 years, $P < 0.0005$, unpublished data). We also recently reported that the age was a negative determinant of the 2–5 mm follicle number in women with PCOS (Jonard *et al.*, 2003b). Similarly, Laven *et al.* (2004) have found a significant negative relationship between age and AMH level in their patients with PCOS.

The preliminary results of our ongoing prospective study comparing PCOS women ovulating or not under clomiphene citrate (CC) indicate that the probability of being resistant to CC is much higher when the baseline 2–5 mm follicle number is >20 (71 versus 11%, unpublished data). Likewise, Van Der Meer *et al.* (1998) reported that the higher sensitivity for gonadotrophin stimulation in patients with PCOS compared with women with regular menstrual cycles was not dependent on differences in FSH threshold, but rather on the larger size of the FSH-sensitive cohort of small antral follicles.

From all these data, it appears therefore that ovulation in PCOS, either spontaneous or induced, is highly conditioned by the degree of small follicle excess. This strengthens our hypothesis that the latter is principally responsible for the follicular arrest, and that its negative effect might be exerted through excessive AMH production.

Conclusion

This review began with the postulate that the follicular problem in PCOS is 2-fold. Firstly, the intra-ovarian hyperandrogenism promotes early follicular growth and leads to a 2–5 mm follicle excess. Secondly, an impaired action of FSH and/or a premature LH action prevent the selection of a dominant follicle from this increased pool.

Actually, these two abnormalities may be only one, at least in some patients with PCOS. With regard to recent pathophysiological data, the intra-ovarian hyperandrogenism may be the main culprit for the follicular arrest. Indeed, it seems to lead to follicle excess, which in turn increases the AMH intra-ovarian level and then could exert an inhibiting effect on the FSH-induced aromatase activity. Thus, the follicular arrest may be the consequence of the follicle excess, via a phenomenon of auto-inhibition within the cohort due to follicle–follicle interactions (Jonard *et al.*, 2003b).

This hypothesis does not exclude that hyperinsulinism may play a role in prematurely advancing GC differentiation, leading to a worsening of follicular arrest. Intrinsic oocyte abnormalities, such as GDF-9 expression, might be involved in PCOS follicular arrest

as well as in defective embryo survival. Finally, the improvement of ovulation rate following laparoscopic ovarian surgery, even when unilateral and without any other treatment (Amer *et al.*, 2003), raises much uncertainty as to whether the reversibility of the follicular arrest depends on local or extra-ovarian factors.

The current therapeutics for PCOS dysovulation consist of decreasing the hyperinsulinism and of correcting the FSH relative insufficiency. With regard to the recent pathophysiological data, the treatments which have been used empirically for many years are still the most adequately suited. If we wished to act more specifically by neutralizing the main culprit, i.e. intra-ovarian hyperandrogenism, we would have to wait for anti-androgens deprived of any anti-gonadotrophic and teratogenic effects, or for a negative modulator of TIC activity not altering GC functions. So far, no promising candidate has appeared.

Finally, research in genetics, which has not contributed very much so far to this specific topic, perhaps will allow the play entitled 'The Disturbed Folliculogenesis of PCO' to proceed again in the near future, with new actors and a new plot!

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