Human Reproduction Update, Vol.22, No.6 pp. 709–724, 2016
Advanced Access publication on August 27, 2016 doi:10.1093/humupd/dmw027

human reproduction update

Interactions between androgens, FSH, anti-Müllerian hormone and estradiol during folliculogenesis in the human normal and polycystic ovary

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Submitted on December 7, 2015; resubmitted on June 2, 2016; accepted on July 12, 2016

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BACKGROUND: Androgens, FSH, anti-Müllerian hormone (AMH) and estradiol (E2) are essential in human ovarian folliculogenesis. However, the interactions between these four players is not fully understood.

OBJECTIVES AND RATIONALE: The purpose of this review is to highlight the chronological sequence of the appearance and function of androgens, FSH, AMH and E2 and to discuss controversies in the relationship between FSH and AMH. A better understanding of this interaction could supplement our current knowledge about the pathophysiology of the polycystic ovary syndrome (PCOS).

SEARCH METHODS: A literature review was performed using the following search terms: androgens, FSH, FSH receptor, anti-Mullerian hormone, AMHRII, estradiol, follicle, ovary, PCOS, aromatase, granulosa cell, oocyte. The time period searched was 1980–2015 and the databases interrogated were PubMed and Web of Science.

OUTCOMES: During the pre-antral ('gonadotropin-independent') follicle growth, FSH is already active and promotes follicle growth in synergy with theca cell-derived androgens. Conversely, AMH is inhibitory by counteracting FSH. We challenge the hypothesis that AMH is regulated by androgens and propose rather an indirect effect through an androgen-dependent amplification of FSH action on granulosa cells (GCs) from small growing follicles. This hypothesis implies that FSH stimulates AMH expression. During the antral ('gonadotropin-dependent') follicle growth, E2 production results from FSH-dependent activation of aromatase. Conversely, AMH is inhibitory but the decline of its expression, amplified by E2, allows full expression of aromatase, characteristic of the large antral follicles. We propose a theoretical scheme made up of two triangles that follow each other chronologically. In PCOS, pre-antral follicle growth is excessive (triangle 1) because of intrinsic androgen excess that renders GCs hypersensitive to FSH, with consequently excessive AMH expression. Antral follicle growth and differentiation are disturbed (triangle 2) because of the abnormally persisting inhibition of FSH effects by AMH that blocks aromatase. Beside anovulation, this scenario may also serve to explain the higher receptiveness to gonadotropin therapy and the increased risk of ovarian hyperstimulation syndrome (OHSS) in patients with PCOS.

WIDER IMPLICATIONS: Within GCs, the balance between FSH and AMH effects is pivotal in the shift from androgen- to oestrogen-driven follicles. Our two triangles hypothesis, based on updated data from the literature, offers a pedagogic template for the understanding of folliculogenesis in the normal and polycystic ovary. It opens new avenues for the treatment of anovulation due to PCOS.

Key words: androgens / FSH / anti-Müllerian hormone / estradiol / folliculogenesis / granulosa cell / PCOS

Introduction

Folliculogenesis in the ovary still arouses a passionate interest from researchers and clinicians. It is indeed a phenomenon of extraordinary complexity and knowledge about it is constantly changing. Even if incomplete, pathophysiological schema focusing on the main actors involved are required to direct research and to assist in the care of patients. Androgens, FSH, anti-Müllerian hormone (AMH) and estradiol (E2) are all essential in human ovarian folliculogenesis. However, the interactions between these four players are not fully understood. The purpose of this review is to try to highlight the chronological sequence of their appearance and function and to discuss controversies in the relationship between FSH and AMH. A better understanding of this interaction could improve our current knowledge about the pathophysiology of the polycystic ovary syndrome (PCOS).

Methods

A literature review was performed using the following search terms: androgens, FSH, FSH receptor, anti-Müllerian hormone, AMHRII, estradiol, follicle, ovary, PCOS, aromatase, granulosa cell, oocyte. The time period searched was 1980–2015 and the databases interrogated were PubMed and Web of Science.

Androgens-FSH-AMH interactions in pre-antral follicle development ('gonadotropin-independent' follicle growth)

This part of the folliculogenesis depends mainly on numerous intra-ovarian factors (Hsueh *et al.*, 2015) and will not be reviewed in full. The aim of this review is to focus on the interplay involving three actors that are central in the pathophysiology of PCOS, namely, androgens, FSH and AMH.

The role of androgens

Twenty years ago, androgens were suspected of increasing follicle atresia (Hsueh *et al.*, 1994). Using a mouse model, some authors showed that androgens enhanced apoptosis of granulosa cells (GCs) (Billig *et al.*, 1993). However, more recent studies have shown rather a stimulating role of androgens on follicle development. For example, Vendola *et al.* (1998) reported an increased number of growing follicles as well as a greater proliferation of GCs and theca cells (TCs) in adult female Rhesus monkeys treated with testosterone and dihydrotestosterone (DHT). Conversely, mice deficient for the androgen receptor (AR) have more apoptotic GCs in their pre-ovulatory follicles and display a lower ovulation rate (Hu *et al.*, 2004), ultimately developing premature ovarian failure (Shiina *et al.*, 2006).

Since the AR is maximally expressed in murine preantral follicles (Tetsuka *et al.*, 1995; Lenie and Smitz, 2009), the main androgen action might take place during the early stages of folliculogenesis. Hillier *et al.* (1997) reported that in primates (marmosets), AR is most abundant in preantral and early antral follicles and virtually absent in pre-ovulatory follicles. We have also reported a lower expression of the AR gene in human GC in the large pre-ovulatory follicles compared to small ones (Catteau-Jonard *et al.*, 2008). This result is consistent with the decline in the expression of AR along with human follicular maturation (Rice *et al.*, 2007). Furthermore, a differential expression of the AR was reported in mature follicles, with low expression in mural GCs and preferential expression in cumulus cells (Szoltys and Slomczynska, 2000).

The role of FSH

Although gonadotropins are not supposed to act on the so-called gonadotropin-independent follicle growth, the question actually arises as to the possible effect of FSH on GCs at an earlier stage than the small antral follicle (Hsueh *et al.*, 2015). Some data indicate that GCs express the FSH receptor (FSHR) as soon as the pre-antral follicle stage (Gougeon, 1996). By autoradiography, on histological sections of human ovary, Yamoto *et al.* (1992) revealed the presence of the FSHR on the surface of the GCs of preantral follicles (at the secondary follicle stage) and antral follicles at different stages of follicular growth.

Despite the early expression of the FSHR during folliculogenesis, the effect of FSH on initial follicular growth remains controversial. In knockout mice deficient for the β-FSH or the FSHR gene (Kumar *et al.*, 1997; Durlinger *et al.*, 2001), follicles develop up to the preantral stage. In women who have mutations in one of these genes, follicles grow up to the stage of selectable follicles (2–5 mm) (Matthews *et al.*, 1993; Aittomaki *et al.*, 1995; Touraine *et al.*, 1999). These data imply initial follicular growth would be FSH-independent (Gulyas *et al.*, 1977; Hillier, 1994; Fauser and Van Heusden, 1997). Conversely, other groups have shown, in animal experiments, that although FSH is not essential in initiating follicular growth, it would support quantitatively and qualitatively this process. For instance, transplantation of neonatal ovaries to hypophysectomized (low FSH) or castrated (high FSH) rats showed that FSH supports the development of preantral follicles (Arendsen de Wolff-Exalto, 1982). In hypophysectomized rats and mice, different follicle classes are observed up to the stage of preantral follicles (McGee *et al.*, 1997). Nevertheless, the number of morphologically normal preantral follicle appears fewer when compared to controls. Similar observations were made in mouse models of hypogonadotropic hypogonadism (Halpin *et al.*, 1986) and in juvenile rats treated with a GnRH antagonist (Halpin *et al.*, 1986; Van Cappellen *et al.*, 1989). Xenografts of human ovaries transplanted under the kidney capsule of immunodeficient and hypogonadotropic mice suggest that FSH would be required for early follicle growth, from the stage of follicles with two GC layers (Oktay *et al.*, 1998). Experiments conducted in sheep, using the model of ovarian cortical tissue autograft, showed that endogenous FSH resulted in a clear stimulation of preantral follicle growth (Campbell *et al.*, 2004). Thus, it is possible that FSH stimulates follicle growth moderately during the basal follicle growth phase, in synergy with other stimulating factors such as androgens, kit-ligand (or stem cell factor), leukaemia inhibiting factor (LIF) and growth differentiation factor-9 (GDF9) (Hsueh *et al.*, 2015). In the absence of FSH, these stimulating factors would maintain 'FSH-independent' basal follicle growth which would, however, be less effective.

The role of AMH

AMH is a glycoprotein belonging to the TGF-β family that is secreted by the GCs as soon as the primary follicle stage (Durlinger *et al.*, 2002b; Weenen *et al.*, 2004). Its secretion is maximal at the stage of the selectable follicle (pre-antral stage in mice and antral stage in human). Then the secretion of AMH by the GCs decreases when the follicles begin their FSH-dependent cyclic recruitment process (Weenen *et al.*, 2004). AMH is considered to have two windows of action on folliculogenesis. First, it negatively regulates the initial follicular recruitment in mice and in humans by exerting an inhibitory effect on the transition from primordial follicle to primary follicle (Durlinger *et al.*, 2002a,b; Carlsson *et al.*, 2006). Second, it inhibits the FSH-dependent cyclical recruitment. AMH seems to play a role throughout gonadotropin-independent follicle growth.

Experimental in vitro data on the prepubescent mouse model knockout for the AMH gene (Durlinger *et al.*, 1999, 2001) support this continuous action of AMH. Firstly, in vivo, the knockout mice deficient for the AMH gene (*AMH* -/-) display a significant increase in the number of growing follicles at all stages, including those beyond the stage of preantral follicle, compared to control mice (Durlinger *et al.*, 1999). Secondly, the addition of AMH in cell culture media containing follicles from *AMH* -/- mice slowed the follicular growth, even in the presence of FSH, suggesting an inhibiting effect of AMH on FSH-dependent GCs proliferation (Durlinger *et al.*, 2001).

Interactions between AMH, FSH and androgens

Some of interrelationships between androgens, FSH and AMH are well demonstrated while others are much more controversial.

Androgens stimulate FSH effects on granulosa cells via enhanced FSH receptor expression

Knockout mice for the AR (*AR* -/-) have reduced mRNA levels coding for the FSHR, which suggests a positive effect of androgens on FSHR expression (Shiina *et al.*, 2006). This hypothesis was confirmed in an in vitro model of bovine GCs. Luo and Wiltbank (Luo and Wiltbank, 2006) showed an increased transcription of the FSHR gene under the action of testosterone and DHT, that involves the AR. By using bicalutamide, a specific antagonist of the AR, they were able to block this effect. An indirect effect through androgen to oestrogen conversion was ruled out since the FSHR mRNA levels were not increased in cells stimulated by oestrogens at different concentrations. In mouse cultured follicles, testosterone and non-aromatizable DHT amplified the follicular growth initiated with FSH (Wang *et al.*, 2001). Murray *et al.* (1998) also reported that DHT accelerated growth of murine follicles at very low FSH concentrations. The same findings were observed in other species. By in situ hybridization, Weil *et al.* (1999) have reported a strong positive correlation between AR and FSHR mRNA levels in GCs from primates treated or not with testosterone. In this model, androgen treatment increased the transcription of FSHR in GCs and these authors secondarily showed that the expression of AR was increased in these CGs exposed to testosterone (Weil *et al.*, 1998). This strongly suggests that androgens stimulate the expression of their own receptors. More recently, Nielsen *et al.* (2011) confirmed these results by studying small human follicles (3 to 9 mm in diameter) obtained during ovarian surgery to preserve fertility. They also found that AR mRNA and intrafollicular androgen levels were positively correlated with FSHR mRNA levels. Using RT-PCR quantification, we ourselves have shown a strong positive correlation between AR and FSHR mRNA levels in GCs collected in the follicular fluid of follicles from control and PCOS women stimulated for IVF (Catteau-Jonard *et al.*, 2008). Thus, also in primates and humans, the

expression of FSHR is most likely increased by androgens via enhanced expression of AR, as recently reviewed by Lebbe and Woodruff (Lebbe and Woodruff, 2013). The parallel increased expression of AR and FSHR, that we have observed in GCs from our PCOS women compared to controls, suggests this physiological effect is amplified in PCOS (Catteau-Jonard et al., 2008).

Altogether, these results are in favour of direct action of androgens on the transcription of FSHR gene and it is likely that this phenomenon is an early event during folliculogenesis (Lebbe and Woodruff, 2013). It remains, however, to elucidate whether the AR acts directly on the FSHR gene promoter and/or through other transcription factors and/or by stabilizing mRNA and/or through non-genomic effects. Recently, Sen et al. (2014) have shown androgens increase FSHR by an extra-nuclear mechanism independent of transcription, suggesting non-genomic mechanisms. This has already been suggested by Kousteni et al. (2001) to explain the anti-apoptotic effect of androgens that occurs very fast and is dissociated from genomic activities. In the neonatal mouse ovary, Yang et al. (2010) described activation of folliculogenesis almost immediately after administration of testosterone. This effect was reversed when adding flutamide (an AR antagonist). Binding of testosterone to its receptor was capable of inducing phosphorylation and activating very rapidly signalling pathways, which is not consistent with a genomic effect.

Thus, more and more arguments converge toward the promoting action of androgens on the transcription and translation of FSHR through genomic and non-genomic effects. This action improves the follicular growth and development mediated by FSH.

AMH counteracts FSH growth-promoting effects on granulosa cells

Knowing the necessity of a precise timing for folliculogenesis, and in particular prevention of premature GC differentiation, one might speculate that any accelerating factor such as FSH must have its counterparts. Anti-Müllerian hormone, secreted primarily by the GCs of pre-antral and small antral follicles, could be one of these. Indeed, FSH administration to *AMH -/-* mice induced a higher rate of early follicular growth than in wild mice (Durlinger et al., 2001). These data suggest that in the absence of AMH growing follicles are more responsive to FSH. Therefore, AMH could inhibit the sensitivity of preantral follicles to FSH and thus regulate negatively the follicle growth. These experimental data were confirmed first in the prepubertal mice where follicle growth was greater in *AMH -/-* than in wild-type mice (Durlinger et al., 2001). Moreover, in *bFSH -/-* mice having low FSH levels, a significantly higher number of growing follicles was observed when the AMH gene was also invalidated. In human GC cultures, Pellatt et al. (2011) observed a decrease in the expression of the FSHR gene when AMH was added to the culture media. In humans, some polymorphisms in AMH and its type II receptor genes partially reduce the ligand-receptor interaction, leading to a better sensitivity of the growing follicles to FSH, as suggested by the study of Kevenaar et al. (2007) showing significantly higher serum E2 levels in women bearing these polymorphisms. Altogether, these findings argue for a counterbalancing effect of AMH that might be seen as a physiological necessity against precocious exhaustion of the primordial follicle pool and/or against premature selection by FSH (Lebbe and Woodruff, 2013). Conversely, in pathological situations with excessive production of AMH, such as in PCOS, this protective effect might be exaggerated leading to slower follicle growth and thus follicle accumulation at every growing stage (a 'stockpiling effect') (Hughesdon, 1982; Maciel et al., 2004). The question then arises as to whether this putative protective phenomenon (i.e., AMH expression) is part of an auto-regulation loop that would involve androgens, FSH or both. This brings the issue of a possible effect of androgens and/or FSH on AMH expression.

Is there a direct relationship between androgens and AMH?. Some data indirectly suggest the existence of this relationship but contrary to data in males (Rey et al., 2003), direct evidence is lacking in females. The paradigm of PCOS led some authors to postulate that androgens increase AMH expression in GCs as serum levels of both hormones are tightly and positively associated (Pigny et al., 2003). However, a comparison of PCOS phenotypes indicates that the increase of serum AMH levels is more closely related to the ovulation disorder than to the hyperandrogenism of the syndrome (Alebić et al., 2015). This will be discussed below.

Data from in vitro cultured bovine small follicles showed an effect of androgens on AMH expression, but it was negative (Crisosto et al., 2009). This finding would be difficult to reconcile with the above hypothesis if this negative effect were physiologically relevant in female species. In the pharmacological situation of androgen treatment with high doses in female to male transsexuals, a strong decline of serum AMH levels was recently observed (Caanen et al., 2015). This model apparently contradicts a previous report suggesting induction of PCOS in such a situation (Balen et al., 1993), although this finding was more recently contradicted (Mueller et al., 2008). In fact, in this study, the subjects also received a GnRH agonist that suppressed their serum FSH level. If AMH cannot rise and even decreases in the absence of FSH, despite the increase in follicle number induced by androgens, does that mean that FSH is involved in the expression of AMH? This would imply that in fact androgens have no direct action and that they rather regulate AMH expression indirectly, via amplification of GC sensitivity to FSH (see legend of Fig. 1). However, although TC-derived androgens do not seem involved, other products from TCs may control AMH expression by GCs. For instance, TCs synthesize bone morphogenetic proteins (BMPs) whose stimulating effect on AMH production has been shown in vitro in human (Shi et al., 2009) and sheep (Estienne et al., 2015) GCs. This deserves particular attention since so far, a putative paracrine effect from TC to GC through BMPs, has not been specifically demonstrated in humans. If it were, it remains to be demonstrated whether thecal BMPs would act directly on the AMH gene (Estienne et al., 2015), or indirectly by enhancing FSHR expression as shown in the hen (Kim et al., 2013). The putative FSH-enhancing effect on AMH expression is discussed next.

FSH stimulates AMH expression in small growing follicles. Although we propose that FSH induces AMH expression, this concept remains controversial with many conflicting results; some support a positive effect while others indicate a negative one.

The first in vivo studies on the regulation of AMH by FSH were originally made in the male. For example, Kuroda et al. (1990) demonstrated in male rats treated with FSH that AMH levels in the testes were greatly reduced. This first study was therefore in the sense of an inhibitory effect of FSH on AMH expression. Some in vivo studies in female agree with this. In the female rat, Baarends

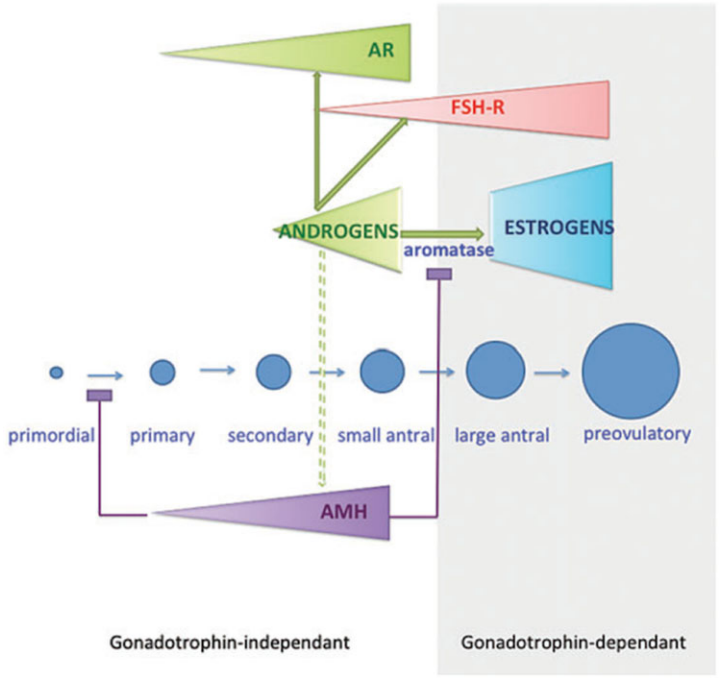

Figure 1 Working model for androgen action in granulosa cells from pre-antral follicles. This figure is reproduced, with permission, from Lebbe and Woodruff (2013). Growing follicles from the secondary stage onwards secreted androgens which amplify their effects by increasing the expression and activity of their own receptors. Androgens also induce the expression of FSHR, supporting FSH-driven follicular growth and maturation. However the follicle is protected from premature selection by FSH through a negative effect from AMH, which inhibits FSH-induced aromatase expression preventing the conversion of androgens to oestrogens. Lebbe and Woodruff (2013) postulated that androgens directly stimulate AMH secretion (dotted green arrow) to maintain a predominantly androgenic intrafollicular milieu. Our hypothesis agrees with this interpretation except that we suggest that androgens stimulate AMH secretion not directly but through enhancement of FSH-stimulated AMH expression. Reproduced from Lebbe and Woodruff (2013), with permission.

et al. (1995) demonstrated that combined administration of FSH at a daily dose of 10 IU for 4 days and GnRH antagonists led to a decrease in AMH expression within follicles. However, the absence of quantification precluded any measure of the degree of this decrease. In women, serum AMH levels decline over 50% during ovarian stimulation treatments for IVF using exogenous FSH and GnRH agonists or antagonists (Fanchin *et al.*, 2003; Weintraub *et al.*, 2014). Likewise, during low-dose FSH stimulation, serum AMH decreases in control (La Marca *et al.*, 2004) or PCOS (Catteau-Jonard *et al.*, 2007) women. Another clinical situation suggesting a negative effect of FSH on AMH is the pubertal transition during which a minor decrease in serum AMH is observed along with the rise of serum FSH and LH levels (Kelsey *et al.*, 2011; Hagen, Aksglaede *et al.*, 2012; Lashen *et al.*, 2013). However, these results need to be discussed. Indeed, in vivo studies cannot determine whether FSH directly or indirectly alters the AMH secretion and/or expression. For instance, in the male, testosterone secreted by the Leydig cells in the testis has a potent negative effect on AMH expression, which may mask the FSH effect on AMH (Crisosto *et al.*, 2009). Indeed, in the study of Kuroda *et al.* (1990) performed on whole testis, it is possible that the intratesticular testosterone has masked the action of FSH. This is why Al-Attar *et al.* (1997), as well as Lukas-Croisier *et al.* (2003), instead used pre-pubertal or bFSH-KO rats, respectively, thus having no intratesticular testosterone. They concluded that FSH stimulates testicular and serum AMH in the absence of testosterone. This is corroborated by the study of Young *et al.* (2005) in neonates with hypogonadotropic hypogonadism, showing a rise of serum AMH levels under recombinant FSH administration, in the absence of androgen secretion from the testis. However, such a bias cannot be evoked in women since testosterone is secreted by TCs that are not directly regulated by FSH. Conversely, E2 rather than testosterone may be an important confounder that will be discussed below. Also, it is well described that the expression and secretion of AMH by the follicles is maximal during the pre-antral and small antral stages and that it gradually decreases in the later stages to be almost undetectable in the pre-ovulatory and luteinized follicles (Bezard *et al.*, 1987; Baarends *et al.*, 1995; Weenen *et al.*, 2004). Since serum AMH does not reflect the AMH status of small growing follicles (Kristensen *et al.*, 2014), the changes in the follicular pool during FSH stimulation may be reducing the serum AMH levels without involving a direct negative effect of FSH. Therefore, in vivo data obtained during FSH stimulation could have been biased for several reasons.

Indeed, other in vivo studies favour of a stimulating effect of FSH on AMH. FSH treatment in male prepubescent mice (Al-Attar *et al.*, 1997)

and in the knockout mice deficient for FSH gene (Lukas-Croisier et al., 2003) stimulates the expression of AMH in the testis and its secretion in serum. In the female primate, in vivo administration of GnRH antagonists, which result in a fall in serum FSH and LH levels, causes a decrease of AMH secretion in pre-antral and small antral follicles (Thomas et al., 2007). In women, other studies also support a positive effect of FSH on AMH expression. In particular, several groups have shown a decrease in serum AMH levels secondary to inhibition of the gonadotropin axis with GnRH agonists (Anderson et al., 2006; Hagen, Sorensen et al., 2012) or oestrogen-progestin pills (Arbo et al., 2007; Kallio et al., 2013). Similarly, during pregnancy, a situation with virtually no circulating FSH, there is also a decrease in serum AMH (Koninger et al., 2013). Finally, in patients with hypogonadotropic hypogonadism, the serum AMH level is low, presumably because of the absence of FSH-dependent growing follicles. When a stimulation treatment with gonadotropins is started, the AMH level rises (Chan and Liu, 2014).

Measuring the concentration of AMH in the culture medium of in vitro studies is theoretically the most convincing approach. In GCs from cows, Rico et al. (2011) demonstrated with the Immunotech assay that FSH at a concentration of 5 or 50 ng/ml inhibits the AMH secretion by about 50% in cultured GCs from 3 to 5 mm follicles. In cultured human GCs, two studies concluded the same inhibitory effect of FSH on AMH secretion. The first one, performed on 2 to 10 mm follicles, found this negative effect only in GCs from women with PCOS, while no effect was observed in controls (Pellatt et al., 2007). The second study, conducted in GCs from 8 to 10 mm follicles collected only in women with PCOS, claimed an inhibitory effect of FSH on the AMH secretion by about 50% (Li et al., 2011). Conversely, a third study reported upregulation of both AMH expression and secretion by human lutein GCs exposed to FSH (Pierre et al., 2013). Altogether, data from culture medium are controversial and this is due in part to technical issues. For instance, in one study (Pellatt et al., 2007), testosterone was added to the culture medium, which may have had by itself an inhibitory effect on AMH (see above). Second, studies were all conducted in luteinized mural GCs, constituting a model distant in physiology, in particular, with respect to expression of AMH. Therefore, the assay kits used for the measurement of AMH in the culture media might have been not sensitive enough and some results could have been distorted. The recent marketing of ultra-sensitive AMH assays should help clarifying this issue. Last but not least, as will be discussed below, some FSH-dependent confounding factors intervening in these cultured GCs may have abrogated the true FSH effect on AMH.

An alternative approach is the search for changes in the expression of AMH gene, by measuring intracellular mRNA by qRT-PCR. Experiments using human luteinized GCs and immortalized GCs lines concluded that FSH stimulates the expression of AMH by 50% to 100% depending on the study (Taieb et al., 2011; Grynberg et al., 2012; Pierre et al., 2013). Conversely, in the study of Rico et al. (Rico et al., 2011) in cows, FSH inhibited the AMH gene expression in GCs from 5 to 10 mm follicles by about 30% but had no effect in 3 to 5 mm follicles. Again, the models used are not the most appropriate to elucidate what really happens in small growing follicles.

The most appropriate approach to address the issue of AMH regulation by FSH, halfway between in vivo and in vitro studies, remains culture of whole ovaries or follicles. In cultures of salmon ovaries, a moderate negative effect of FSH on AMH expression was demonstrated (Luckenbach et al., 2011) to lasted up to 24 hours of treatment but disappeared thereafter. Conversely, in the media of cultured follicles from monkeys and women, an increase in AMH secretion was observed in growing follicles in response to FSH administration (15 ng/ml recombinant human FSH), which was more marked in fast- than in slow- or non-growing follicles (Xu et al., 2011) (Fig. 2). The stimulatory effect of FSH on the AMH secretion was at a maximum at 20 days of culture and then decreased after 28 days of culture. According to these authors, this increase would be indirect and would involve oocyte factors such as BMPs (Rico et al., 2011). Indeed oocytes have FSH receptors (Meduri et al., 2002), allowing FSH to stimulate the secretion of some BMPs that are known to enhance the expression and secretion of AMH in women (Shi et al., 2009; Estienne et al., 2015). This influence of the oocyte cannot be addressed in most in vitro models which are based on mural GC culture (Baumgarten et al., 2014). Although evidence is still lacking, it must be kept in mind that the oocyte may also be involved in the regulation of AMH by FSH. In this regard, the authors did not discuss their observation of time-dependent opposite curves of AMH and E2 levels in the culture media, i.e., the AMH level started to plateau and then to decline when the E2 level began to rise (Fig. 2).

Thus, in vivo and in vitro data are still conflicting primarily because the ideal experimental model does not exist. However, beside limitations and criticisms about each model, one other major reason for discrepancies could be the ignorance, in many experiments, of the crucial role of a fourth player coming into the game at the time of antral follicle development ('gonadotropin-dependent' follicle growth), namely E2.

FSH-AMH-estradiol interactions in antral follicle development ('gonadotropin-dependent' follicle growth)

At this follicular stage, the effects of androgens on GCs wane, as do the effects of AMH, while FSH promotes the differentiation functions of GCs such as the capacity to synthesize E2. Therefore, the role of androgens and their interaction with FSH and AMH are no longer relevant while those of E2 take more and more importance up to terminal follicle maturation.

Aromatase expression

E2 is synthetized by GCs through the action of aromatase, the enzyme that converts androgens (coming from TCs) to oestrogens. Aromatase is already expressed by foetal GCs in small growing follicles (di Clemente et al., 1992; Stocco, 2008) but, up to birth, its expression remains relatively low. It gradually increases in the preantral and small antral follicles in the immediate neonatal period. Then, gradually, the expression of aromatase decreases in small growing follicles and preantral follicles and is then only present in the large antral follicles during childhood (Guigon et al., 2003; Stocco, 2008). This increased expression during the postnatal period is dependent on gonadotropins and in particular on FSH (Gray et al., 1995). However,

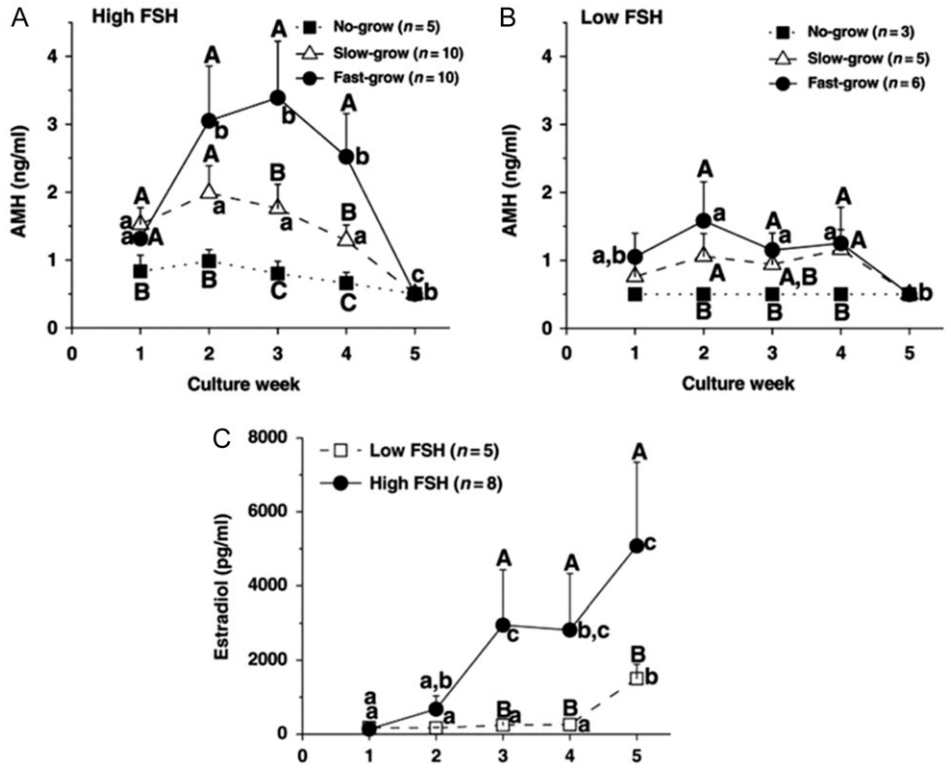

Figure 2 AMH and estradiol responses to FSH in growing follicles. AMH concentrations from non-, slow- and fast-growing follicles during culture in media containing high (**A**) or low (**B**) levels of FSH (15 or 0.3 ng/ml recombinant human FSH, respectively) at 5% O_2 without LH supplementation at Day 30. **C**: Dose–response of FSH on E2 production by fast growing follicles in vitro in media containing either low- or high-dose FSH at 5% O_2 without LH supplementation at Day 30. Significant differences over time (lower case) or between the FSH dosage groups (upper case) are indicated by different letters ($p < 0.05$). Data are presented as the mean + SEM. *n*, number of follicles. In fast-growing follicles, note timing of the curves for AMH and E2 in presence of high or low FSH. This suggests that, chronologically in growing follicles, FSH first stimulates AMH production and then, in more mature follicles, it stimulates E2 secretion that turns off AMH synthesis. Reproduced from Xu *et al.* (2011), with permission.

increased aromatase expression does not cause, at this point, a significant rise in plasma E2 levels due to the absence of implementation of optimal steroidogenic process (i.e., according to the model of the two-cell theory, the rest of the enzymatic equipment is still insufficient in the TCs and GCs). Steroidogenesis can only occur optimally in the post-pubertal period. From that time, the expression of aromatase increases in large antral and preovulatory follicles (Turner *et al.*, 2002; Guigon *et al.*, 2003; Stocco, 2008).

FSH stimulates aromatase expression

The expression of the aromatase gene (CYP19) is significantly stimulated by FSH. Binding of FSH to its receptor induces phosphorylation of the transcription factor CREB, secondary to increased cyclic AMP and therefore activation of protein kinase A. The phosphorylated factor CREB (*trans*-activator factor) will bind to the *cis*-activating sequences CRE (cyclic AMP responsive element) upstream of the CYP19 gene and will thereby stimulate the transcription of this gene (Mendelson *et al.*, 2005; Mendelson and Kamat, 2007; Stocco, 2008).

AMH inhibits FSH-induced aromatase activity

In humans, a negative correlation between AMH and estradiol levels has been observed in follicular fluid of small antral follicles, suggesting a close interdependence between AMH expression and FSH activity inside each follicle (Andersen and Byskov, 2006; Dumesic *et al.*, 2009). Grossman *et al.* (2008) showed an inhibitory effect of AMH on the catalytic activity of aromatase in partially luteinized cultured human GCs from follicular fluid of normo-ovulatory patients undergoing IVF; this explained by a repressive effect on CYP 19 gene expression (Grossman *et al.*, 2008). In the follicular fluid of small antral follicles from human ovaries collected for fertility preservation, Nielsen *et al.* (2011) confirmed a significant negative correlation between AMH levels and the CYP19 gene expression. It seems that AMH represses the FSH effect through inhibition of the catalytic activity of adenylate cyclase (Chang *et al.*, 2013). AMH administration in culture media of GCs induces a significant decrease in cyclic AMP production which might secondarily reduce transcription of the CYP19 gene (Chang *et al.*, 2013). Pellatt *et al.* (2011) also confirmed

in human GC cultures that AMH inhibits the catalytic activity of FSH-dependent aromatase through repression of the CYP19 gene. More recent studies confirm these data and suggest that AMH does not affect the basal expression of aromatase but specifically attenuates the FSH stimulating effect (Sacchi et al., 2014; Prapa et al., 2015).

Estradiol represses AMH expression

In vivo data argue in favour of E2 repressing the expression of AMH. First, during the pubertal transition, gonadotropin recovery is associated with rising levels of E2 (Kelsey et al., 2011; Hagen, Aksglaede et al., 2012; Lashen et al., 2013) that could explain the slight decrease in serum AMH at that time, instead of it being caused by a direct inhibiting effect of FSH (see above). Second, all studies in women during ovarian stimulation for IVF report a significant decrease in plasma levels of AMH, with a nadir on the day of triggering by hCG (Fanchin et al., 2003; La Marca et al., 2004; Catteau-Jonard et al., 2007; Lee et al., 2010; Weintraub et al., 2014). Each of these studies found a negative correlation between serum AMH and estradiol levels on the day of hCG. One of them even showed a negative correlation between the relative increase and decrease of serum E2 and AMH, respectively, from the start of stimulation up to the day of hCG (Lee et al., 2010). Other studies investigating the relationship between E2 and AMH concentrations in follicular fluid from mature follicles have shown similar results (Andersen and Byskov, 2006; Andersen and Lossl, 2008; Nielsen et al., 2011). Conversely, such a negative relationship between serum E2 and AMH levels was not observed by La Marca et al. (2004) in the follicular phase of spontaneous cycles. Similarly, Liberty et al. (2010) questioned whether the repressive effect of E2 on AMH is physiologically relevant and not exclusively iatrogenic. They compared the longitudinal variations of serum AMH in the following situations: spontaneous cycle, cycle induced by clomiphene citrate or gonadotropins, ovarian stimulation for IVF, and high dose oral or transvaginal oestrogen therapy in preparation for frozen embryo transfer. The AMH levels were unchanged in each group, except in the group with ovarian stimulation. However, more recently, a statistically significant decrease in AMH was observed from 5 days before to 2 days after ovulation in spontaneous cycles (Gnoth et al., 2015). This discrepancy could be explained by better sensitivity of the AMH assay used in the more recent study (Gnoth et al., 2015) and by the fact that the systemic effect of exogenous E2 would be less than that induced by locally produced E2 within the GCs under the influence of FSH.

In vitro data agree with E2 repression of AMH. E2 has a response element in the promoter of AMH gene (Guerrier et al., 1990). More specifically, according to the work of Grynberg et al. (2012), E2 would have a stimulating effect on AMH via the oestrogen-receptor (ER) α and an inhibitory effect on AMH via ER β. ER β is the principal receptor in GCs from growing follicles while ER α then becomes predominant in the corpus luteum (Couse et al., 2005). The effect of estradiol on AMH during the late follicular growth phase would therefore be mainly inhibitory and would thus overcome the stimulatory effects of FSH on AMH that prevails in smaller follicles expressing no or little E2. Therefore, it seems the FSH-dependent E2 secretion is the major confounding factor in the effect of FSH on AMH, making it either directly positive or indirectly negative depending on the absence or presence, respectively, of E2 in the model used.

The shift from androgen- to oestrogen-driven follicles

The interrelationships between androgens, AMH, FSH and E2 vary depending on the cycle and follicular development stage.

Until the pre-antral stage, in the absence of E2, the expression of AMH is maximal, under the influence of androgens acting through enhancement of FSH action within GCs and through growth factors such as BMPs, secreted by TCs, GCs and the oocyte. As follicular growth progresses, androgen effects wane and E2 production will gradually increase in follicles until it reaches the threshold at which the suppression of AMH production will occur (Fig. 2). Therefore, E2 takes control of follicle transformation, including for instance the increase in FSHR expression and acquisition of LH receptors (LHR), making follicles able to undergo their cyclic recruitment process and to be part of the cohort of selectable follicles.

Obviously, not all follicles follow this scenario and AMH could be one of the factors that modulate the shift from androgenic to oestrogenic tone, allowing only a limited number of follicles to undergo the cyclic recruitment process. Indeed, in rat ovaries, there seems to be a differential expression of AMH gene within non-atretic large pre-antral and small antral follicles (Baarends et al., 1995). Although these follicles are hardly distinguishable morphologically, some strongly express the AMH gene while expression is lower in others. Those expressing AMH the least are the most sensitive to FSH and are therefore more prone to be selected during the cyclic recruitment process. Conversely, those expressing the most AMH are more resistant to FSH, which would facilitate their entry into atresia (Durlinger et al., 2002b; Visser and Themmen, 2005). Consequently, the sharp decrease in AMH expression in larger antral follicles (6–8 mm average diameter) might be seen as a physiological prerequisite allowing only a limited number of follicles to be recruited by FSH during the inter-cycle rise of this gonadotropin (Fauser and Van Heusden, 1997; Broekmans et al., 2008; Jeppesen et al., 2013). That AMH would act as a gate keeper is supported by our observation that the appearance of a dominant follicle is preceded by the decline in serum AMH level in anovulatory women with PCOS under a low-dose step-up protocol using recombinant FSH (Catteau-Jonard et al., 2007).

Sensitivity to LH may be another factor conditioning the cyclic recruitment process and the establishment of dominance (Franks et al., 2008). However, LHR acquisition by GCs occurs late. In one study (Jeppesen et al., 2012), LHR expression in antral follicles (3–10 mm in diameter) was approximately 10% of the maximum observed in pre-ovulatory follicles. Interestingly, however, LHR expression in small follicles seems to be negatively associated with follicular fluid AMH levels (Eilso Nielsen et al., 2010; Jeppesen et al., 2012). This is in line with the hypothesis that AMH plays a primary role in negatively controlling the cyclic recruitment process and the establishment of dominance.

The two triangles hypothesis

New schemes with a wide panoramic view over the whole of folliculogenesis are needed. The 'two cells, two gonadotropins' theory is still relevant but it concerns only large antral follicles (Erickson and

Shimasaki, 2001). Therefore, to illustrate and to summarize what happens before and during folliculogenesis, we propose a scheme made up of two triangles that follow each other chronologically (Fig. 3). This paradigm of two triangles leads to consideration of four vertices (androgens, E2, FSH and AMH, the latter two being common to both triangles) and six sides. Beside other clinical applications, such as poor response to ovarian stimulation for in vitro fertilization (IVF), this working hypothesis, while certainly not exhaustive, can help in understanding some issues in the pathophysiology of the polycystic ovary syndrome.

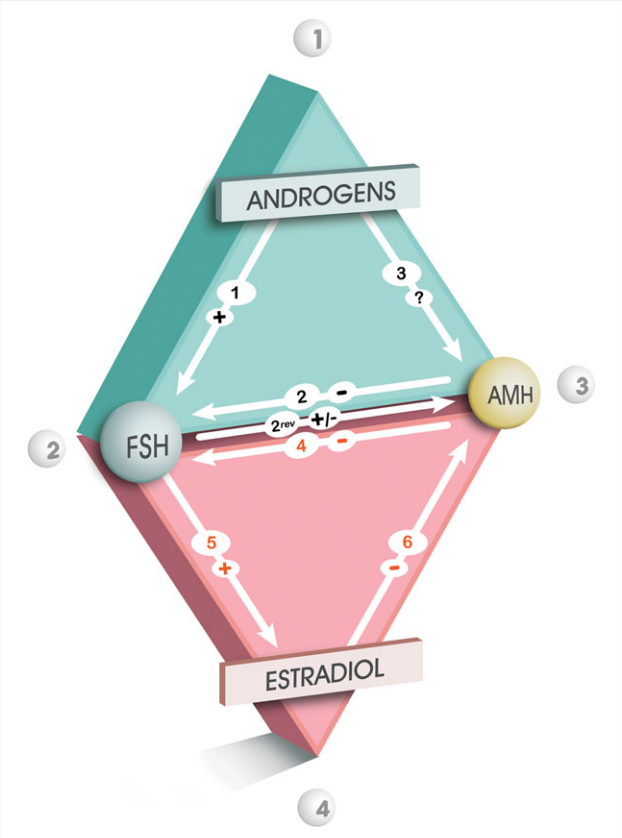

Figure 3 Relationships between androgens, FSH and AMH during the gonadotropin-independent follicular growth phase (green triangle) and between FSH, AMH and estradiol during the gonadotropin-dependent follicular growth phase (red triangle 2). '+', '−' or '?' indicate a positive, negative or uncertain effect, respectively, from one of the factors on the other. During the gonadotropin-independent follicular growth phase, the inhibitory effect of AMH mainly influences the promoting effect of FSH on follicular growth (arrow 2). According to our theory, FSH, whose receptors are enhanced by androgens (arrow 1), would stimulate the AMH production during this phase (arrow 2 rev), in the absence of estradiol. A direct effect from androgens on AMH production (arrow 3) is unlikely (see text for details). During the gonadotropin-dependent follicular growth phase, AMH is also involved in a triangular relationship with FSH and estradiol. During this phase, the inhibitory effect of AMH influences mainly the cell differentiation functions induced by FSH (arrow 4), in particular the induction of aromatase (arrow 5). This inhibitory effect will gradually subside, which will allow induction of aromatase by FSH, with consequent synthesis of estradiol which will in turn accelerates the extinction of AMH secretion in large antral follicles (arrow 6) (see text for details).

The paradigm of the polycystic ovary syndrome

PCOS is the most common cause of anovulation, infertility and hyperandrogenism in women. Indeed, 5–10% of women of reproductive age are affected (Norman *et al.*, 2007). The cardinal features of PCOS are hyperandrogenism and oligo-anovulation. Despite considerable efforts to determine the cause, the pathophysiology of PCOS remains poorly understood, but evidence is accumulating to suggest that the central abnormalities of PCOS are primarily ovarian. More specifically, the hyperandrogenism is becoming more like the 'heart' of PCOS and the first impact would be disturbed folliculogenesis (Catteau-Jonard and Dewailly, 2013). For presumably genetic and/or epigenetic reasons (Franks and Berga, 2012), the TCs are intrinsically overactive, thus creating an intra-ovarian androgen excess. Knowing the importance of androgens on small follicle growth, as described above (vertex1 of triangle 1), intra-ovarian hyperandrogenism is designated as primarily responsible for the follicle excess that characterizes the disease (Jonard and Dewailly, 2004; Homburg, 2009). This follicle excess plays by itself an important role on oligo-anovulation, through mechanisms that are not fully understood but seem to be independent from any systemic hormonal influence (Dewailly *et al.*, 2007; Alebić *et al.*, 2015). Indeed, the negative correlation between the number of small (2–5 mm) and large (6–9 mm) antral follicles, in PCO compared to that in normal ovaries (Dewailly *et al.*, 2007), suggests an inhibiting action of the former on the latter, through follicle-to-follicle interactions that have not been elucidated so far but would be exacerbated in PCOS. The fact is that the more small follicles there are, the more they are growth arrested and the chance of ovulation is lower. Interestingly, serum AMH in PCOS is in close connection with the number of 2–5 mm follicles but not with the one of 6–9 mm follicles detected by ultrasound (Pigny *et al.*, 2003). This is expected since this class of follicles is precisely those in which the AMH expression by the GCs is maximal (Weenen *et al.*, 2004). We therefore hypothesized that the follicular arrest might result from an excessive AMH tone within the microenvironment of the selectable follicles (Jonard and Dewailly, 2004), but no data has supported this assertion so far.

The disordered folliculogenesis of PCOS is not only a matter of follicle number. Besides this excess, we hypothesize that there would also be an intrinsic GC dysregulation, involving androgens, FSH, AMH and E2. Thus PCOS is certainly the best pathological paradigm for the two triangles theory.

In PCOS, triangle 1 is oversized

According to this theory, because of the local excess of androgens, all vertices and sides of triangle 1 (Fig. 3) are amplified compared to normal ovaries. Indeed, since androgens from TCs up-regulate the expression of their own receptors in GC, there is, in polycystic ovaries, an excessive androgen activity in these cells (vertex 1), as suggested by the increase in their AR content (Catteau-Jonard *et al.*,

2008). Consequently, according to side 1 of triangle 1, the FSHR expression, number and activity is increased in these cells (vertex 2), as documented many years ago in cultured granulosa-lutein cells from polycystic ovaries (Erickson et al., 1992; Almahbobi et al., 1996) and more recently confirmed in two studies using PCR in granulosa-lutein cells from pooled follicles in PCOS women undergoing IVF (Catteau-Jonard et al., 2008; Gonzalez-Fernandez et al., 2011).

It can be questioned whether androgen excess is the only culprit for this FSH amplification in PCOS. The genetics of the FSHR in PCOS is the subject of an extensive literature. However, no mutation was found and only polymorphisms were reported. Whether the allelic variant [FSHR (Ser680)] is associated with an increased risk of PCOS is of great controversy, but more recent studies are negative (Fu et al., 2013; Singhasena et al., 2014). Recently, another variant (rs2268361-T) located in an intron of FSHR, was found to be associated with PCOS risk (Saxena et al., 2015). This variant was also associated with lower serum FSH levels and thus may influence FSH receptor responsiveness. However whether this variant could induce PCOS by itself is unlikely. Conversely, it could modulate the effect of hyperandrogenism on GC sensitivity to FSH and thus contribute to the phenotype variability of PCOS. So far, no genetic mutation or polymorphism of the FSHR gene seems involved in the link between FSH and AMH in PCOS women.

Several in vivo and in vitro studies support an increased AMH production within GCs from PCO (vertex 3). Indeed, concentrations of AMH in follicular fluid were much higher in the follicular fluid of small follicles derived from a polycystic ovary cultured compared to those from a normal ovary and were three times higher in oligo-anovulatory women with PCOS compared to those who ovulated (Das et al., 2008). Pellatt et al. (2007) reported a four times greater AMH production in culture media of GCs from anovulatory women with PCOS compared to that in GCs from ovulating women. Finally, within equivalently sized follicles, GCs demonstrated a two times higher expression of the AMH gene at the time of oocyte collection for IVF in patients with PCOS compared to controls (Desforges-Bullet et al., 2010). Therefore, contrary to what we claimed initially (Pigny et al., 2003), the increase in serum AMH in PCOS (Fallat et al., 1997; Cook et al., 2002; Pigny et al., 2003; Laven et al., 2004; Nardo et al., 2007) not only reflects the excess in follicle number but also indicates excessive AMH production by each follicle.

The reason(s) for this excessive AMH production in PCOS remain(s) elusive. No abnormality in the AMH gene resulting in overexpression of the protein has been reported (Sproul et al., 2010). The positive correlation between the serum AMH and testosterone or androstenedione levels, reported by several authors in patients with PCOS (Pigny et al., 2003; Pigny et al., 2006; Dewailly et al., 2010) was initially thought to reflect a direct stimulating effect of androgens on AMH expression (side 3). However, as discussed above, no experimental data supports this hypothesis. If it were true, the decrease in serum AMH level under cyproterone acetate (CPA), a potent anti-androgen with strong progestin anti-gonadotropic activity, would be greater than under other anti-gonadotropic drugs such as oral contraceptive pills. In our experience (Plouvier et al., 2016), this was not the case, suggesting that in patients under CPA, the low FSH level due to the anti-gonadotropic activity of the drug precludes any anti-androgen effect on AMH secretion. Indeed, according to the reverse arrow of side 2 of triangle 1 and in the absence of E2, it is the FSH hyperactivity on GCs that could be directly responsible for the excessive AMH expression in small follicles of PCOS patients.

Last, the overproduction of AMH per follicle appears to vary according to the PCOS phenotype as defined by the Rotterdam classification. Higher AMH levels have been found in hyperandrogenic than in normo-androgenic PCOS women (Eldar-Geva et al., 2005; Pigny et al., 2006; Piouka et al., 2009). Conversely, in another study, serum AMH levels were higher in anovulatory PCOS women, with or without hyperandrogenism, than in ovulatory PCOS women, having hyperandrogenism by definition (Alebić, et al., 2015). By multivariate analysis, the dysregulated AMH production by GCs in PCOS, as reflected by the ratio AMH to antral follicle count (AFC), was significantly associated with anovulation but not with hyperandrogenism. This issue is complex since principal component analysis showed that the markers of hyperandrogenism and oligo-anovulation are intimately linked (Dewailly et al., 2010). However, when both hyperandrogenism and anovulation are challenged to serum AMH excess, the association is significant with the latter, while the former would be merely a confounder (Alebić, et al., 2015).

Therefore, AMH excess seems to be particularly relevant in patients with oligo-anovulatory PCOS and could be one of the main factors disturbing triangle 2 (Fig. 3).

In anovulatory PCOS, triangle 2 is dysfunctional

In PCOS, aromatase expression and thus E2 synthesis (Fig. 3: vertex 4) in large antral follicles (i.e., >2 mm) is impaired (Jakimiuk et al., 1998). This is not the consequence of a genetic defect (Söderlund et al., 2005) but indicates rather insufficient aromatase stimulating bioactivity to increase CYP 19 mRNA expression (Jakimiuk et al., 1998). This could result from abnormally persisting inhibition of FSH effects by AMH (side 5), because of its accumulation in GCs during the previous follicular stages (triangle 1). In addition, the down regulation of the AMHRII that is physiologically induced by LH in GCs from large antral follicles seems to not occur normally in PCOS (Pierre et al., 2013).

In PCOS, FSH, although in low to normal serum concentrations, would not be able to induce a sufficient decrease in AMH secretion/action to allow aromatase expression (side 4) (Jonard and Dewailly, 2004; Catteau-Jonard and Dewailly, 2013). This hypothesis is reinforced by our study already mentioned (Catteau-Jonard et al., 2007) showing that the modest increase in serum FSH levels by recombinant FSH administration is inversely correlated to the decrease in serum AMH levels that precedes the emergence of a dominant follicle. Spontaneously, this does not occur presumably because of the absence of an inter-cycle FSH peak and also because GCs need to be exposed to FSH for a long enough time to clear the AMH excess. Furthermore, a negative correlation between serum FSH and AMH levels has been reported in women with PCOS, that remains unexplained to date (Pigny et al., 2003; Catteau-Jonard et al., 2007). Once GCs have received enough FSH for a sufficient time, the imbalance between FSH and AMH effects on the control of aromatase expression is corrected, leading to increased content of E2 within these cells. Then, according to side 6 of triangle 2, E2 represses AMH expression and thus allows full expression of aromatase. This theory

is supported by the in vitro findings of Xu *et al.* (2011), as discussed above (Fig. 2), in line with our in vivo data with recombinant FSH showing that serum E2 levels starts to rise once serum AMH level pass under some threshold (Catteau-Jonard *et al.*, 2007). This threshold is certainly variable from one patient to the next, but as groups, amenorrheic and oligomenorrheic women with PCOS have higher ranges of serum AMH levels than eumenorrheic PCOS patients (Catteau-Jonard *et al.*, 2012). One practical fallout of this threshold theory is the use of a serum AMH assay to calculate the dose of FSH for monofollicular ovulation induction. In a study sample of clomiphene-resistant PCOS patients, the serum AMH level was the only independent variable for which the effect on the required dosage of FSH to achieve monofollicular development was statistically significant (Koninger *et al.*, 2014).

This scenario may also serve to explain the increased risk of ovarian hyperstimulation syndrome (OHSS) under gonadotropin therapy in patients with PCOS (Delvigne and Rozenberg, 2002). When GCs are exposed to high amounts of FSH, their richness in FSHR causes them to over respond in terms of E2 production and the protective effect of AMH is lost. This illustrates the difficulty in manipulating triangle 2 at the level of vertex 2 (FSH) in patients with PCOS. Possibly in the future it will be possible to act rather on vertex 3 (AMH), for instance by using molecules having an antagonist activity at the AMHRII level (Seifer and Merhi, 2014). Presumably, this would lower the risk of OHSS by maintaining physiological and endogenously regulated serum FSH levels.

What about LH?

One may be surprised that LH is not part of our theory while it has been considered for a long time to be a central actor in the pathophysiology of PCOS. Indeed, the presence of LH is absolutely necessary for the expression of the intrinsic TCs dysregulation with its subsequent ovarian hyperandrogenism. Therefore, LH is implicitly involved in vertex 1 of triangle 1, both in normal and PCOS women, through its stimulating effect on androgen synthesis by TCs, which is amplified in PCOS. As our two triangle theory is restricted to GCs and to growing follicles up to the stage of small antral follicles, with physiologically no or little expression of LHR (Jeppesen *et al.*, 2012), no other role of LH is addressed. However, previous studies have suggested that expression of LHR is premature in GCs from PCO, an abnormality that could be responsible for the follicular arrest in anovulatory women with PCOS (Willis *et al.*, 1998; Jakimiuk *et al.*, 2001). This hypothesis has received little support since it was first proposed and is difficult to reconcile with the observed inverse relationship between follicular fluid AMH and LHR expression in GCs (Eilso Nielsen *et al.*, 2010; Jeppesen *et al.*, 2012). Last, a recent genetic study, in women suspected to have PCOS, has shown an association between oligo-anovulation and a polymorphism in the LHR gene (rs13405728) whose functional significance in TCs and/or GCs is presently unknown (Cui *et al.*, 2015). Therefore, LH should certainly still deserve full attention in our appraisal about anovulation linked to PCOS.

In addition, there is a positive association between LH and AMH. Indeed, in women with PCOS, the positive relationship between serum levels of both hormones was shown to be independent from serum androgen and FSH levels by multivariate analysis (Catteau-Jonard *et al.*, 2007). By principal component analysis, both hormones correlated positively and independently to the same component in women with PCOS but not in controls (Dewailly *et al.*, 2010). This could reflect a stimulating effect of LH on AMH secretion, as shown in cultured luteinized GCs (Pellatt *et al.*, 2007; Taieb *et al.*, 2011) thus not involving TC-derived androgens. Alternatively, some experimental data have recently raised the exciting issue of a possible stimulating effect of AMH on GnRH neuron activity/secretion at the median eminence level (Cimino *et al.*, 2016). This opens the revolutionary perspective that ovarian AMH and hypothalamic control of LH secretion might be both involved in a vicious circle in PCOS, with AMH having an endocrine action (i.e., positive feed-back) in addition to its autocrine ovarian effects described in this review.

Future avenues and perspectives

This model of the double triangle is necessarily restrictive and cannot claim to answer all the questions concerning the regulation of follicular growth in women. Many aspects remain insufficiently, or not, explored and will, hopefully, be the subject of future studies that will complement, or even possibly question, our model.

First the cross-talk between the oocyte and the GCs of the cumulus, in particular the relationships between FSH, AMH and oocyte growth factors such as BMP-15 or GDF-9 (Salmon *et al.*, 2004; Rico *et al.*, 2011) needs to be addressed.

Second, the role of AMH among the other factors that regulate follicular growth at all stages has yet to be specified. Whether mutation or polymorphism in the AMH or AMHRII gene confers a risk for ovarian failure in humans is controversial (Yoon *et al.*, 2013; Alvaro Mercadal *et al.*, 2015). Intriguingly, the phenotype of affected sisters of men with the persistent Mullerian duct syndrome due to mutations in the AMH or AMHRII gene has been very little described and seems normal (Carre-Eusebe *et al.*, 1992; Abduljabbar *et al.*, 2012).

Third, the role of LH, as mentioned above, has to be redefined, based on recent advances in genetics.

Fourth, the chronology of the dysregulation of AMH in PCOS is not known. Does this dysregulation already exist in the foetal ovary? Can it integrate into pathophysiologic models supporting ontogenesis of PCOS during foetal life (Dumesic *et al.*, 2014)? In support of this, increased serum AMH levels have been reported in infant and prepubertal girls at risk for PCOS (Sir-Petermann *et al.*, 2006).

Fifth, if one day they are available, will molecules endowed with an antagonistic action on AMHRII be of interest in treating PCOS? Probably they will, for a short-term use to facilitate ovulation. However, long-term use as background therapy seems more problematic because, according to our model, they would remove the regulatory role of AMH on the FSH effects and might induce accelerated folliculogenesis with ultimately early follicular depletion.

Finally, this model can be applied to situations other than PCOS, especially the reverse situation, namely the follicular depletion, responsible for poor response during ovarian stimulation for IVF. In particular, our model fits the rationale for the administration of androgens, but so far, the results of this therapeutic measure on IVF outcomes have still to be confirmed by well-designed studies (Nagels *et al.*, 2015).

Conclusion

Our model of the double triangle highlights the essential roles of FSH and AMH in the regulation of follicular growth, although many other factors are involved. This model promotes the hypothesis that the secretion of AMH would be FSH-dependent as long as GCs do not synthetize E2. However, this hypothesis remains to be validated by further studies.

Finally, this model is applicable to both normal follicular growth and the excessive follicular growth observed in PCOS. The abnormalities of folliculogenesis in PCOS would therefore not be the result of an 'aberration' but simply the exaggeration of a physiological process.

Acknowledgements

We thank Pr Marcelle CEDARS for her invaluable help in editing this manuscript.

Authors' roles

D.D. designed this review and participated in the literature analysis, manuscript drafting and critical discussion; G.R., M.P., C.D., P.P. and S.C.J. also participated in the literature analysis, manuscript drafting and critical discussion.

Funding

No funding was provided for this work.

Conflict of interest

There is no conflict of interest to disclose for any author.

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