

Dynamics of serum anti-Müllerian hormone levels during the luteal phase of controlled ovarian hyperstimulation

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BACKGROUND: To investigate the dynamics of serum anti-Müllerian hormone (AMH) levels during the luteal phase of controlled ovarian hyperstimulation (COH) and its possible association with follicle development. **METHODS:** We prospectively studied 34 women undergoing COH with GnRH agonist and FSH. On the day of hCG (dhCG), serum AMH, estradiol (E₂), progesterone and hCG levels were measured, and ovarian follicles were sorted into three size classes: <12, 12–15 and 16–22 mm. Hormonal measurements were repeated 4 days (hCG + 4) and 7 days (hCG + 7) after hCG. **RESULTS:** From dhCG to hCG + 4, we observed a decline in serum AMH levels ($-64 \pm 3\%$; $P < 0.0001$), which paralleled that of E₂ levels. From hCG + 4 to hCG + 7, an increase in AMH levels occurred ($82 \pm 28\%$; $P < 0.02$), whose magnitude was correlated with the number of < 12 mm follicles ($r = 0.68$; $P < 0.0001$) but not with other follicle size classes nor with the remaining hormone levels. **CONCLUSIONS:** After hCG, AMH levels initially decline, presumably as an effect of follicle luteinization, then increase during the mid-luteal phase. Although the mechanisms implicated in the mid-luteal AMH increase are unclear, its positive association with small follicle count, but not with luteal progesterone and E₂ levels, supports the hypothesis that AMH levels might reflect luteal follicle development.

Key words: anti-Müllerian hormone/controlled ovarian hyperstimulation/follicle size/hCG/luteal phase

Introduction

Anti-Müllerian hormone (AMH) is a glycoprotein that belongs to the transforming growth factor β superfamily (Cate *et al.*, 1986). In adult women, AMH is exclusively produced by the granulosa cells of ovarian follicles (Vigier *et al.*, 1984) and, according to recent data obtained in animals, it partakes in the inhibition of both the primordial to primary follicle growth (Durlinger *et al.*, 2002) and the responsiveness of preantral follicles to FSH (Durlinger *et al.*, 2001). However, the precise mechanisms involved in the regulation of granulosa cell AMH production remain undetermined. The observation that AMH expression increases significantly from the primary through the early antral stages of folliculogenesis (Durlinger *et al.*, 2002; Weenen *et al.*, 2004), and declines during the final follicular maturation process (Baarends *et al.*, 1995; Fanchin *et al.*, 2003a), indicates that the degree of follicular development influences AMH expression by ovarian follicles. Incidentally, its characteristic production by follicles at different developmental stages singled out AMH as a promising 'holistic' biomarker of the ovarian follicular status. In agreement with this, some clinical studies reported a quantitative association between peripheral AMH levels and the number of early antral follicles (De Vet

et al., 2002; van Rooij *et al.*, 2002; Pigny *et al.*, 2003), whose reliability outdoes that of serum inhibin B, estradiol (E₂) and FSH measurements on cycle day 3 (Fanchin *et al.*, 2003b).

Beyond the pre-ovulatory stage, the possible effects of granulosa cell luteinization and corpus luteum formation on AMH production are less well documented. Basic research studies conducted in rats indicated that isolated corpora lutea express much less AMH than small and large antral follicles (Baarends *et al.*, 1995). Yet, AMH levels measured in follicular fluids from women undergoing controlled ovarian hyperstimulation (COH) for IVF and embryo transfer remained detectable 32–34 h after hCG administration (Seifer *et al.*, 1993; Fallat *et al.*, 1997).

An interesting experimental model that could be helpful to clarify the effects of follicle luteinization and corpora lutea activity on peripheral AMH levels is that of COH. Indeed, contrary to the menstrual cycle in which only a single follicle ovulates and is converted into corpus luteum, COH is characterized by an extensive, hCG-driven transformation of maturing follicles into multiple corpora lutea. This supraphysiological process may be instrumental in amplifying the possible consequences of follicle luteinization

and corpora lutea activity on peripheral AMH levels. Hence, the present study was conducted to clarify this issue by analysing peripheral AMH levels during the early to mid-luteal phase in pituitary-desensitized COH cycles.

Materials and methods

Subjects

We studied prospectively 34 infertile women, aged 24–39 years. All of them met the following inclusion criteria: (i) both ovaries present and with no morphological abnormalities; (ii) regular menstrual cycle lengths ranging between 25 and 35 days; (iii) no current or past diseases affecting ovaries or gonadotrophin or sex steroid secretion, clearance, or excretion; (iv) no clinical signs of hyperandrogenism; (v) body mass indexes ranging from 18 to 25 kg/m²; (vi) no current hormone therapy; (vii) adequate visualization of both ovaries in transvaginal ultrasound scans; (viii) no endometriosis. Infertility was due to sperm abnormalities (39%), tubal abnormalities (35%), or was unexplained (26%). An informed consent was obtained from all women and this investigation received the approval of our internal Institutional Review Board.

COH protocol

All women received a time-release GnRH agonist, leuprolide acetate (1 mg/day s.c., Lucrin; Abbott-France Pharmaceuticals, France) from cycle day 21 onwards. On day 3 of the subsequent cycle, complete pituitary desensitization was confirmed by the detection of low serum levels of E₂ and gonadotrophins. Patients also underwent a conventional ultrasound examination to exclude ovarian cysts and to verify that endometrial thickness was <5 mm. Recombinant (r)FSH therapy (Gonal-F; Serono Pharmaceuticals, France) was then initiated at a dosage of 225 IU/day whereas daily GnRH agonist administration was continued until the day of hCG administration (Gonadotrophine Chorionique 'Endo', 10000 IU i.m.; Organon

Pharmaceuticals, France). Daily FSH doses and timing of hCG administration were adjusted according to the usual criteria of follicular maturation. Administration of hCG was performed when at least five follicles were >16 mm in diameter and E₂ levels per mature follicle (>16 mm in diameter) were >200 pg/ml. Oocytes were retrieved 36 h after hCG administration by transvaginal ultrasound-guided aspiration. All embryo transfers were performed 2 days after oocyte retrieval using Frydman catheters (CCD Laboratories, France). Luteal phase was supported with micronized progesterone (Estima, 600 mg/day; Effik Pharmaceuticals, France) administered daily by vaginal route starting on the evening of embryo transfer.

Hormonal and follicular measurements

On the day of hCG administration (dhCG), 4 days later (hCG + 4), and 7 days later (hCG + 7), women underwent serum AMH, E₂, progesterone and hCG measurements at ~09:00. Blood samples drawn on hCG + 4 and hCG + 7 are usually performed to monitor the effectiveness of hCG administration and luteal progesterone support of IVF cycles in our centre. Serum AMH levels were determined using a 'second generation' enzyme-linked immunosorbent assay (reference A16507; Immunotech Beckman Coulter Laboratories, France). Intra- and inter-assay coefficients of variation (CV) were <6 and <10% respectively, lower detection limit at 0.13 ng/ml, and linearity up to 21 ng/ml for AMH. Serum E₂, progesterone and hCG levels were determined by an automated multi-analysis system using a chemiluminescence technique (Advia-Centaur; Bayer Diagnostics, France). For E₂, lower detection limit was 15 pg/ml, linearity up to 1000 pg/ml, and intra- and inter-assay CV were 8 and 9% respectively. For progesterone, lower detection limit was 0.1 ng/ml, linearity ≤60 ng/ml, and intra- and inter-assay CV were 8 and 9% respectively. For hCG, lower detection limit was 2 mIU/ml, linearity ≤1000 mIU/ml, and intra- and inter-assay CV were 4 and 5% respectively.

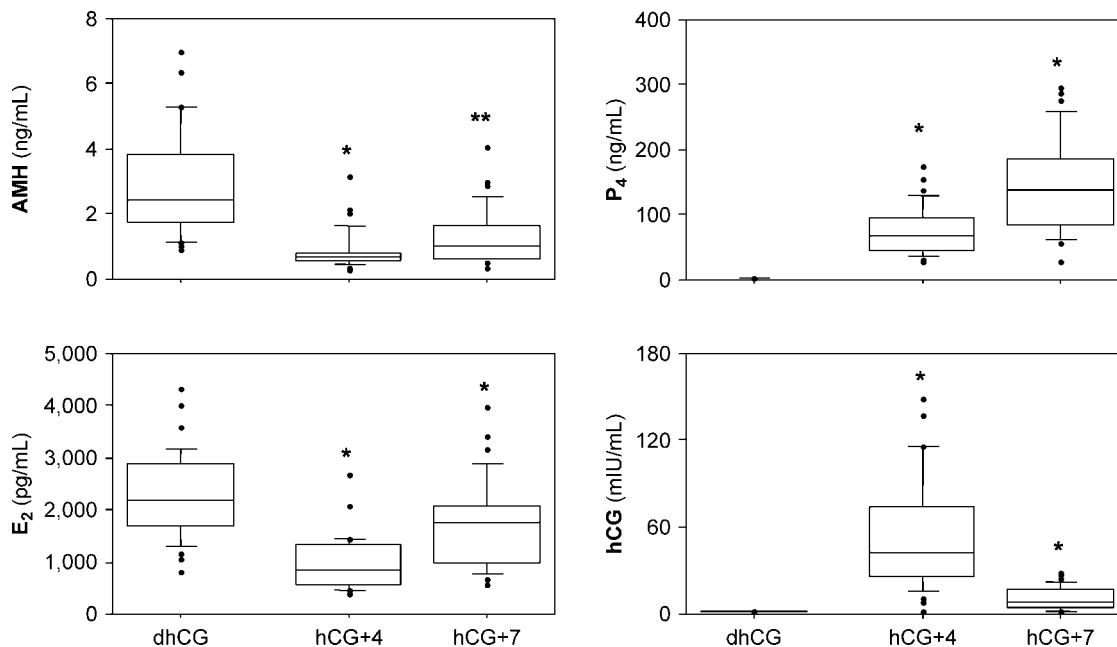


Figure 1. Box-and-whisker plots showing serum anti-Müllerian hormone (AMH), estradiol (E₂), progesterone (P₄) and hCG levels on the day of hCG administration (dhCG) and 4 and 7 days later (hCG +4 and hCG +7 respectively). Horizontal lines inside the boxes represent median levels. Upper and lower limits of the boxes and whiskers represent the 75th and 25th centiles and 90th and 10th centiles. Values outside the 90th and 10th centile limits are represented as dots. All longitudinal hormone changes reached statistical significance (**P* < 0.0001 and ***P* < 0.02).

On dhCG, ovarian ultrasound scans were performed using a 3.6–8.0 MHz multi-frequency transvaginal probe (EC9-4, Sonoline Antares; Siemens S.A.S., France) to evaluate the number and sizes of ovarian antral follicles. For the purposes of the present study, antral follicles were sorted into three size classes: small (3–11 mm in diameter), intermediate (12–15 mm in diameter), and large (16–22 mm in diameter) follicles. The choice of these thresholds to define follicle size classes was arbitrary and based on the fact that, in the menstrual cycle, the sizes of non-dominant follicles remain <12 mm (Pache *et al.*, 1990) whereas follicle maturation is putatively achieved from 16 mm onwards (Dubey *et al.*, 1995).

Statistics

Measures of central tendency and variability used were, respectively, the mean and SEM when data distribution was normal, and the median and the ranges when normality could not be ascertained. Longitudinal changes in hormone levels from dhCG to hCG + 7 were assessed by the Wilcoxon signed rank test. The association between two continuous variables was assessed by correlation when they were independent from each other and by simple regression when there was a dependency. The Fisher *r* to *z* test was used to determine if coefficients of correlation (*r*) were significantly different from zero. *P* < 0.05 was considered statistically significant.

Results

Hormone dynamics

Hormone dynamics during the luteal phase of COH are shown in Figure 1. Serum AMH levels decreased by $-64 \pm 3\%$ from dhCG (median, 2.43 ng/ml; range, 0.89–7.00 ng/ml) to hCG + 4 (median, 0.65 ng/ml; range, 0.26–3.17 ng/ml, *P* < 0.0001). Thereafter, AMH levels increased by $82 \pm 28\%$ from hCG + 4 to hCG + 7 (median, 0.99 ng/ml; range, 0.31–4.03 ng/ml, *P* < 0.02). Serum E₂ dynamics followed a pattern similar to that of AMH, with an initial decrease of $-58 \pm 2\%$ and secondary increase of $97 \pm 12\%$. As expected, serum progesterone levels increased rapidly from dhCG to hCG + 4, presumably due to a massive follicle luteinization. A further increase in progesterone levels occurred between hCG + 4 and hCG + 7 (median, 138.65 ng/ml; range, 26.80–295.80, *P* < 0.0001), probably because of the combined effect of corpora lutea activity and exogenous progesterone administration used for luteal support of COH. Primary and secondary increases in progesterone levels were $9993 \pm 1095\%$ and $106 \pm 14\%$ respectively. Finally, following hCG administration (10 000 IU), serum hCG levels became detectable on hCG + 4 but declined significantly on hCG + 7. Primary increase and secondary decrease of hCG levels were $2732 \pm 318\%$ and $-78 \pm 2\%$ respectively.

Hormonal and follicular associations

The associations between serum hCG and remaining hormone levels on hCG + 4 and hCG + 7 are detailed in Table I. As shown, hCG levels on hCG + 4 were positively correlated with AMH levels on hCG + 4 but not hCG + 7. In contrast, a significant association between hCG levels on hCG + 4 and E₂ and progesterone levels on hCG + 7 was observed. On hCG + 7, serum hCG levels were correlated with progesterone levels but not with AMH and E₂ levels.

Table I. Regression analysis of serum hCG, anti-Müllerian hormone (AMH), progesterone and estradiol (E₂) levels on day 4 and day 7 after hCG administration (hCG + 4 and hCG + 7 respectively)

	Serum hCG levels	
	hCG + 4	hCG + 7
Serum AMH levels	hCG + 4 <i>r</i> = 0.45, <i>P</i> < 0.008 hCG + 7 <i>r</i> = 0.24, NS	– <i>r</i> = 0.22, NS
Serum E ₂ levels	hCG + 4 <i>r</i> = 0.17, NS hCG + 7 <i>r</i> = 0.36, <i>P</i> < 0.04	– <i>r</i> = 0.19, NS
Serum progesterone levels	hCG + 4 <i>r</i> = 0.03, NS hCG + 7 <i>r</i> = 0.51, <i>P</i> < 0.002	– <i>r</i> = 0.53, <i>P</i> < 0.002

NS = not significant.

Serum AMH levels on dhCG showed a positive correlation with progesterone levels on hCG + 4 (*r* = 0.51, *P* < 0.003) and hCG + 7 (*r* = 0.35, *P* < 0.05). Conversely, AMH and progesterone levels failed to show any relationship on hCG + 4 and hCG + 7. Furthermore, serum AMH and E₂ levels showed a correlation only on hCG + 4 (*r* = 0.41, *P* < 0.02). The magnitudes of serum E₂ and progesterone changes from hCG + 4 and hCG + 7 were positively correlated with each other (*r* = 0.55, *P* < 0.001) but not with that of AMH levels.

Associations between the number of follicles in each size class and AMH levels on dhCG, hCG + 4 and hCG + 7 are shown in Table II. On dhCG, the mean number of follicles was 17.7 ± 1.0 , of which 4.9 ± 0.5 were small (3–11 mm in diameter), 5.5 ± 0.4 were intermediate (12–15 mm in diameter) and 7.4 ± 0.5 were large (16–22 mm in diameter). The strength of the association between AMH levels and follicle counts on dhCG decreased progressively as follicle sizes increased. It is noteworthy that absolute AMH levels on hCG + 7 and their percentage of increase from hCG + 4 to hCG + 7 were positively correlated with the number of small follicles on dhCG (*r* = 0.71, *P* < 0.0001, Table II; and *r* = 0.67, *P* < 0.0001 respectively), but not with the number of intermediate and large follicles. Incidentally, as expected, serum E₂ levels were positively related to the total number of follicles (*r* = 0.44, *P* < 0.009) on dhCG, but the strength of such a relationship tended to decrease as the follicle size increased (small follicles, *r* = 0.35, *P* < 0.04; intermediate follicles, *r* = 0.32, *P* < 0.05; large follicles, *r* = 0.21, not significant).

Table II. Associations between the number of small (3–11 mm in diameter), intermediate (12–15 mm in diameter), and large (16–22 mm in diameter) follicles on the day of hCG administration (dhCG) and serum anti-Müllerian hormone (AMH) levels on the three observation days (dhCG, hCG + 4 and hCG + 7) during the luteal phase of controlled ovarian stimulation

No. of follicles	Serum AMH levels		
	dhCG	hCG + 4	hCG + 7
Small	<i>r</i> = 0.63, <i>P</i> < 0.0001	<i>r</i> = 0.02, NS	<i>r</i> = 0.71, <i>P</i> < 0.0001
Intermediate	<i>r</i> = 0.37, <i>P</i> < 0.03	<i>r</i> = 0.15, NS	<i>r</i> = 0.26, NS
Large	<i>r</i> = 0.25, NS	<i>r</i> = 0.10, NS	<i>r</i> = 0.16, NS

NS = not significant.

Overall population characteristics and COH results

Mean women's ages and menstrual cycle lengths were 33.0 ± 0.7 years and 29.0 ± 0.4 days. COH lasted 11.5 ± 0.2 days and required 2582 ± 112 IU of recombinant FSH. We observed a negative association between recombinant FSH requirement for COH and serum AMH levels on dhCG ($r = -0.40$; $P < 0.02$) but not hCG + 4 or hCG + 7. We did not observe any significant relationship between the remaining clinical parameters listed above and serum AMH levels on dhCG, hCG + 4 and hCG + 7. In addition, serum AMH levels on dhCG, hCG + 4, and hCG + 7 were comparable in patients whose infertility was due to sperm or tubal abnormalities, or it was unexplained. The mean numbers of total oocytes, available embryos and transferred embryos were 9.4 ± 0.7 , 5.8 ± 0.5 and 2.6 ± 0.1 respectively. Rates of clinical pregnancy (presence of intrauterine gestational sac with cardiac activity) and ongoing pregnancy (≥ 12 weeks of amenorrhea) per embryo transfer were 29 and 24% respectively. On dhCG, serum AMH levels were positively correlated with the number of oocytes ($r = 0.37$, $P < 0.03$) and embryos obtained ($r = 0.35$, $P < 0.05$) but not on hCG + 4 and hCG + 7. As the present study was not powered to evaluate the possible relationship between serum AMH levels and IVF outcome, descriptive data showed that patients who became pregnant ($n = 11$) tended to have higher serum AMH levels than those who did not ($n = 23$) on dhCG and hCG + 4 but not on hCG + 7. These differences did not reach statistical significance at any observation day.

Discussion

The foremost objective of the present investigation was to clarify the effects of follicle luteinization and corpora lutea activity on peripheral AMH levels. The extensive switch of multiple maturing follicles into corpora lutea that occurs in COH cycles constituted an attractive model to examine this issue, insofar as a similar phenomenon is not reproduced in the menstrual cycle. Overall, our results indicated that serum AMH levels first declined from dhCG to hCG + 4, then increased from hCG + 4 to hCG + 7. The exact physiological mechanisms implicated in both of these remarkable hormonal events are unclear.

The initial decrease in serum AMH levels is probably related to the putative adaptations that granulosa cells undergo during the follicle luteinization process. Indeed, the similarity between serum AMH and E_2 level dynamics from dhCG to hCG + 4 and the fact that AMH levels were positively correlated with E_2 levels on hCG + 4 support this hypothesis and suggests that both hormonal decreases shared the same origin. As in the menstrual cycle (Hoff *et al.*, 1983) and in COH (Fanchin *et al.*, 2000), granulosa cell luteinization induces a remarkable decline in E_2 levels due to the shift of follicular steroidogenesis from E_2 to progesterone. In addition, the scarce expression of AMH and its type II receptor mRNA in isolated corpora lutea as compared to non-luteinized antral follicles from rats (Baarends *et al.*, 1995) further indicates that granulosa cell luteinization alters the follicular ability to produce AMH. The physiological

mechanisms implicated in this striking phenomenon remain to be established. Also, if confirmed, these results suggest that AMH could be a potential marker of follicle luteinization after hCG in COH. Last, but not least, we observed a positive correlation between serum hCG and AMH levels on hCG + 4, which may suggest that hCG administration stimulates early luteal AMH production. In contrast, hCG levels on hCG + 4 failed to influence AMH levels on hCG + 7, thereby suggesting that hCG does not exert a significant influence on AMH production by corpora lutea. Further studies are needed to elucidate the role of hCG on the follicular and luteal production of AMH.

During the interval from hCG + 4 to hCG + 7, serum AMH, progesterone and E_2 levels increased simultaneously. The magnitudes of progesterone and E_2 increases were positively correlated, despite the possible interference exerted by progesterone administration used for luteal support on endogenous progesterone levels. This suggests that the elevation in both hormone levels from hCG + 4 to hCG + 7 was triggered by a common phenomenon, i.e. the corpus luteum steroidogenesis (Hoff *et al.*, 1983). The mechanisms involved in the subtle, yet significant, AMH increase observed from hCG + 4 to hCG + 7 are less evident. They are possibly linked to the hormonogenesis by corpora lutea and/or antral follicles during the luteal phase of COH. Yet, the first possibility is challenged by at least two reasons. First, corpora lutea have been shown to express negligible amounts of AMH (Baarends *et al.*, 1995). Second, the magnitude of AMH elevation was clearly dissociated from that of the putative markers of luteal activity, such as progesterone and E_2 .

Another possible explanation for the secondary increase in AMH levels is that it could result from the development of antral follicles during the luteal phase of COH. The positive relationship between the magnitude of AMH increase from hCG + 4 to hCG + 7 and the number of small follicles on dhCG is in agreement with this hypothesis. Recent well-designed studies have demonstrated that subtle waves of follicular growth sporadically occur during the luteal phase in the menstrual cycle despite the putative FSH suppression by luteal inhibin A and E_2 secretions (Baerwald *et al.*, 2003a,b). It is conceivable that, during COH, the steadily high FSH doses administered have generated additional waves of follicle growth that might persist after hCG administration. The lack of relationship between the total dose of gonadotrophins used for COH and AMH levels on hCG + 4 and hCG + 7 observed in the present study does not disprove this hypothesis but suggests that such a phenomenon may be triggered even in the presence of low recombinant FSH doses. Unfortunately, the mass effect by multiple corpora lutea and their ability to produce E_2 are factors that hamper the monitoring of possible follicle growth by ultrasound or E_2 levels during the luteal phase of COH and prevented us from testing this hypothesis. Since AMH is probably scarcely expressed in the corpus luteum (Baarends *et al.*, 1995), luteal AMH measurements could theoretically serve as a valuable endocrine probe into the dynamics of follicle development during the luteal phase. The possible effects of exogenous FSH doses used for COH on the characteristics of non-dominant follicle growth during

the luteal phase remain to be determined. Moreover, further studies aiming at monitoring serum AMH and follicular growth dynamics during the luteal phase in spontaneous menstrual cycles are needed to clarify this issue.

Furthermore, our observation that, on the day of hCG administration, serum AMH levels were strongly associated with the number of small but not large follicles confirms our own previous results (Fanchin *et al.*, 2003a) and further supports the hypothesis that, with final follicle maturation, granulosa cells lose their ability to produce AMH (Baarends *et al.*, 1995; Fanchin *et al.*, 2003a). In addition, women whose serum AMH levels were high on dhCG showed an improved responsiveness to COH as indicated by a reduced gonadotrophin requirement, a large number of antral follicles and oocytes, and a more intense increase in progesterone levels from dhCG to hCG + 7. These data further support the relationship between AMH and the ovarian follicular status (De Vet *et al.*, 2002; van Rooij *et al.*, 2002; Fanchin *et al.*, 2003b; Pigny *et al.*, 2003) and responsiveness to COH (Seifer *et al.*, 2002). Incidentally, on the day of hCG administration, we observed that serum AMH levels were higher in the present study than in an earlier report by our team (Fanchin *et al.*, 2003a). This difference presumably is related to the fact that, in the present investigation, serum AMH levels were determined using a 'second generation' enzyme-linked immunosorbent assay (reference A16507; Immunotech Beckman Coulter Laboratories, France), which included several analytical modifications as compared to the original assay. These aimed at minimizing the background and matrix effects and improving AMH detection at low concentrations.

In conclusion, the present results represent the first documentation on the dynamics of serum AMH levels during the luteal phase of COH. They indicate that, after hCG, AMH levels initially decline, then increase during the mid-luteal phase. The mechanisms implicated in these remarkable hormonal changes are unclear. The initial decline may be attributed to adaptations of granulosa cells to the follicle luteinization process. The secondary increase may reflect luteal follicle development, as the magnitude of AMH increase was positively related to the number of small follicles at the end of COH. Further studies aiming at elucidating the mechanisms implicated in both phenomena will help to clarify the regulation of AMH secretion during the luteal phase and to determine the clinical usefulness of AMH measurements as a marker of follicle luteinization and possibly luteal follicle development.

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