Like other members of the transforming growth factor β superfamily (1) such as activins and inhibins, anti-müllerian hormone (AMH) is mainly expressed by the granulosa cells of ovarian follicles (2). In adult women, the exact physiologic role of AMH has not been well established. Studies conducted in rodents have suggested that AMH might be involved in both the control of primordial to primary follicle growth (3) and the follicular responsiveness to FSH (4). The AMH secretion by ovarian follicles probably is modulated by the degree of gonadic development, as it barely is detectable at birth (5) and reaches higher levels after puberty (6). Also, it is likely to be influenced by the stage of follicular growth, with increasing production from the primary through the early antral stages (3, 7).

Beyond the early antral stage, follicles seem to progressively lose their capacity to produce AMH. Previous experiments conducted in isolated follicles from adult rats have indicated that AMH and its type II receptor mRNA expression are markedly decreased in large as compared with small antral follicles (8). Also, pooled follicular fluid AMH measurements performed in bovines have shown that AMH concentrations are higher in small as compared with large follicles (2). In support of this, the thorough transformation of small antral follicles into maturing follicles as a result of controlled ovarian hyperstimulation (COH) was associated with a dramatic decrease in peripheral AMH levels (9). However, these indirect data should be challenged through direct assessment of the production of AMH by individual follicles at different stages of development.

In adult women, the possible effects of granulosa cell luteinization on AMH production are less well documented. Previous data indicate that isolated corpora lutea from rats express minimal amounts of AMH and its type II receptor mRNA as compared with small and large antral follicles (8).
Moreover, in a cohort of women undergoing COH for in vitro fertilization and embryo transfer (IVF-ET), AMH levels in pooled follicular fluid remained detectable 34 hours after human chorionic gonadotropin (hCG) administration (10). Conclusive clinical data on the possible influence of the degree of follicle luteinization on AMH production is currently not available.

Besides these uncertainties concerning the influence of follicular development and luteinization on AMH production, some clinical studies have reported a quantitative relationship between peripheral AMH levels and the number of early antral follicles (11–13), the reliability of which surpasses conventional hormonal biomarkers of ovarian follicular status (14). However, it still remains unclear whether increased peripheral AMH levels reflect exclusively the number of early antral follicles or are also due to increased per-follicle AMH secretion. Insights into this sensitive issue could help to clarify the role of AMH not only as a quantitative but also as a qualitative indicator of ovarian follicular status.

Hence, the present investigation analyzed serum and follicular fluid samples obtained from IVF-ET candidates’ follicles at two different development stages to assess [1] the possible modulating role of follicular maturation and luteinization on AMH secretion, and [2] the relationship of per-follicle AMH levels, ovarian follicular status, and responsiveness to COH.

### MATERIALS AND METHODS

#### Patients

We prospectively studied 37 female volunteers, ages 23 to 41 years, who met the following criteria: [1] both ovaries present; [2] regular menstrual cycle lengths ranging between 25 and 35 days; [3] no current or past diseases affecting the ovaries or the secretion, clearance, or excretion of gonadotropin or sex steroids; [4] no clinical signs of hyperandrogenism; [5] body mass index (BMI) ranging between 18 and 35 days; [3] no current or past diseases affecting the present; [2] regular menstrual cycle lengths ranging between 41 years, who met the following criteria: [1] both ovaries

The ultrasound measurements were performed by a single operator, using a 3.6–8.0 MHz multi-frequency transvaginal probe (EC9-4, Sonoline Antares; Siemens S.A.S., Saint-Denis, France) in accordance with methodology previously described elsewhere (14, 18). In brief, during the baseline follicular status examination, on cycle day 3 preceding the initiation of the COH protocol, all early antral follicles that measured 3–10 mm in mean diameter were carefully counted in both ovaries. At the end of the COH protocol on the day of hCG administration, the number of growing antral follicles, defined as those whose mean diameter equaled or surpassed 12 mm, was determined.

#### Controlled Ovarian Hyperstimulation Protocol

Women received a time-release gonadotropin releasing-hormone (GnRH) agonist, (3 mg IM of Decapeptyl; Beau-four Ipsen Pharma, Paris, France) on cycle day 2. Three weeks later, complete pituitary desensitization was confirmed by the detection of low serum E\textsubscript{2} and gonadotropin levels. Patients also had a conventional ultrasound examination to exclude ovarian cysts and to verify that endometrial thickness was <5 mm. Therapy was then initiated with combined subcutaneous recombinant follicle-stimulating hormone (FSH), 225 IU/day, and subcutaneous luteinizing hormone (LH), 75 IU/day (Gonal-F and Luveris, respectively; Serono Pharmaceuticals, Boulogne, France). Daily FSH and LH doses and the timing of hCG administration were further adjusted according to the usual criteria of follicular maturation. Administration of hCG (10,000 IU, IM, Gonadotrophine Chorionique “Endo”; Organon Pharmaceuticals, Saint-Denis, France) was performed when at least three follicles exceeded 17 mm in diameter and the E\textsubscript{2} levels per mature follicle (>17 mm in diameter) were higher than 300 pg/mL. Oocytes were retrieved approximately 36 hours after hCG administration by transvaginal ultrasound-guided aspiration. All ETs were performed 2 days after oocyte retrieval using Frydman catheters (CCD Laboratories, Paris, France). The luteal phase was supported with micronized progesterone (600 mg/day of Estima; Effik Pharmaceuticals, Bièvres, France) administered daily by vaginal route starting on the evening of ET.

In the present analysis, ovarian responsiveness to COH was evaluated according to the following recognized parameters (19): total exogenous FSH dose used for COH, number of growing follicles (>12 mm in diameter on the day of hCG administration), and number of oocytes obtained.

#### Follicular Measurements

Within the 3 months preceding COH, all women underwent an ultrasonographic count of early antral follicles on day 3 of their menstrual cycles. The objective of this examination was to document the ovarian follicular status (16, 17) and to further test its relationship with AMH levels. Because the number of early antral follicles reportedly is steady from one cycle to another (15), measurements performed within the 3 months preceding COH were considered acceptable for the present study.

#### Serum and Follicular Fluid Collection

On the day of oocyte retrieval, the women underwent blood sampling by venipuncture at approximately 9:00 AM. Serum was separated and frozen in aliquots at −80 °C for subsequent centralized analysis. Just before regular oocyte retrieval, follicular fluids from two distinct follicles whose mean diameters ranged from 8 to 12 mm (small follicles) and from two other distinct follicles whose mean diameter
ranged from 16 to 20 mm (large follicles) were individually aspirated using a 10-mL syringe under transvaginal ultrasound guidance.

We elected to retrieve follicular fluids from more than one follicle in each size class to reduce the risk that individual follicle characteristics might bias the comparison of hormonal production between large and small follicles. The choice of each follicle was arbitrary, but preference was given to those whose transvaginal accessibility was straightforward. This methodology was set to ensure complete follicular emptying and to minimize the risk of blood contamination of follicular fluids. Follicular diameters (mean of the two perpendicular diameters) were recorded for all follicles.

Follicular fluids were maintained at steady temperature conditions (37 °C) until oocytes were identified and isolated. They were, thereafter, centrifuged at 3000 × g for 15 minutes at 4 °C to eliminate cellular elements, and then frozen at −80 °C for centralized hormonal analysis. The elapsed time between follicular aspiration and follicular fluid cryopreservation did not exceed 30 minutes. Unfortunately, practical reasons linked to our routine biologic protocol for IVF-ET prevented us from examining the fate of oocytes and embryos from the two sets of small and large follicles obtained for the present study.

Hormonal Measurements in Serum and Follicular Fluids

Serum AMH levels were determined using a “second generation” enzyme-linked immunosorbenent assay (reference A16507; Immunotech Beckman Coulter Laboratories, Villepinte, France). Intra-assay and interassay coefficients of variation were <6% and <10%, respectively, with the lower detection limit at 0.13 ng/mL and linearity up to 21 ng/mL for AMH. Serum E2 and P4 levels were determined with an automated multi-analysis system using a chemiluminescence technique (Advia-Centaur; Bayer Diagnostics, Puteaux, France). For E2, the lower detection limit was 15 pg/mL, linearity up to 1000 pg/mL, and intra-assay and interassay coefficients of variation were 8% and 9%, respectively. For P4, the lower detection limit was 0.1 ng/mL, linearity up to 60 ng/mL, and intra-assay and interassay coefficients of variation were 8% and 9%, respectively.

For the AMH, E2, and P4 measurements in follicular fluids, we used a similar methodology. To avoid possible bias due to follicular fluid volume variability, hormone concentrations in the follicular fluid were adjusted to its protein content, as previously reported elsewhere (20). Proteins were measured according to the conventional Biuret reaction (21) using an automated multi-analysis system (AU640; Olympus, Rungis, France). Follicular fluid hormone levels were expressed as ng/g of protein for AMH and as μg/g of protein for E2 and P4.

Statistics

The measures of central tendency and variability were, respectively, the mean and standard error of the mean when data distribution was normal, and the median and the ranges when normality could not be ascertained. Paired data were compared with the paired Student’s t-test or the Wilcoxon signed rank test, where appropriate. Relationships between two continuous variables were assessed by correlation when they were independent from each other and by simple regression when one of them depended on the other. The Fisher r to z test was used to determine if coefficients of correlation (r) were significantly different from zero. Comparison of strength of correlations was performed using Hotelling’s t-test (22) to assess the difference between coefficients of correlation for correlated samples that share a common variable. P <.05 was considered statistically significant.

RESULTS

Overall Data

The mean women’s ages were 33.2 ± 0.6 years, the mean menstrual cycle lengths were 28.4 ± 0.3 days, and the mean BMI values were 21.0 ± 0.3 kg/m2. At baseline (on cycle day 3 before COH), transvaginal ultrasonography identified 17.2 ± 0.8 early antral follicles. Controlled ovarian hyperstimulation lasted 12.3 ± 0.2 days and required 2673 ± 124 IU of recombinant FSH and 922 ± 18 IU of recombinant LH. On the day of hCG administration, 16.8 ± 0.9 follicles exceeded 11 mm in diameter.

Serum and Follicular Fluid Hormone Levels

On the day of oocyte retrieval, median serum level of AMH was 1.44 ng/mL (range 0.38–4.96 ng/mL); E2 was 1206 pg/mL (range 168–3835 pg/mL); and P4 was 14.0 ng/mL (range 5.8–41.9 ng/mL). As expected by design, the median concentrations were almost three times as high in small follicles (10.0 mm, range: 8–12 mm; and 10.5 mm, range: 8–12 mm) and large follicles (18.2 mm, range: 16–20 mm; and 18.2 mm, range: 16–20 mm) within the same woman were similar. Given that hormone levels between two small follicles and between two large follicles for the same woman were comparable as well, we decided to average them to simplify further analysis.

The hormonal concentrations in small and large follicles are depicted in Figure 1. As shown, median AMH concentrations were almost three times as high in small follicles (111.0 ng/g of protein, range: 21.7–656.2 ng/g of protein) as in large follicles (40.6 ng/g of protein, range: 14.4–108.0 ng/g of protein; P <.0001). In contrast, small follicles secreted statistically significantly less P4 (0.11 μg/g of protein, range: 0.03–0.36 μg/g of protein; versus 0.20 μg/g of protein, range: 0.11–0.33 μg/g of protein, respectively; P <.0001) but secreted comparable E2 levels (7742 μg/g of protein, range: 1186–18753 μg/g of protein; versus 8011 μg/g of protein, range: 4010–20,098 μg/g of protein, respectively) as compared with large follicles.

Serum and Follicular Fluid Hormone Relationships.

Relationships between follicular fluid AMH, P4, and E2 levels are
depicted in Figure 2. We observed a negative correlation between follicular fluid P₄ and AMH levels in both small (r = −0.61, P < .0001) and large (r = −0.36, P < .03) follicles. No correlation was observed between follicular fluid E₂ and AMH levels in small or large follicles. Also, we found a positive correlation between follicular fluid P₄ and E₂ levels in small (r = 0.34, P < .04) but not large follicles (data not plotted). In addition, we observed a remarkable correlation between serum and follicular fluid AMH levels both in large (r = 0.89, P < .0001) and small (r = 0.48, P < .003) follicles. In contrast, serum and follicular fluid levels of E₂ and P₄ failed to correlate in either follicular groups.

Relationships with Baseline Follicular Status and Ovarian Response to COH

Relationships between follicular fluid AMH levels and baseline follicular status and ovarian response to COH are shown in Figure 3. In both small and large follicles, follicular fluid AMH levels correlated positively with the number of early antral follicles on cycle day 3 before COH (r = 0.37, P < .03; and r = 0.63, P < .0001, respectively), growing follicles on the day of hCG administration (≥12 mm, r = 0.32, P < .05; and r = 0.45, P < .005, respectively) and oocytes retrieved (r = 0.31, P < .05; and r = 0.56, P < .0003, respectively), but correlated negatively with total recombinant FSH dose required for COH (r = −0.30, P < .05; and r = −0.48, P < .003, respectively). The strength of relationships between follicular fluid AMH and follicular status and COH parameters was not statistically significantly different between the two follicular classes. In contrast, follicular fluid P₄ and E₂ levels were not statistically significantly related to the same parameters. We did not observe any relationship between AMH levels and the recombinant LH dose used for COH.

Further, as had been observed for follicular fluid AMH levels, we observed a positive relationship between serum AMH levels on the day of oocyte retrieval and the number of early antral follicles on cycle day 3 before COH (r = 0.67, P < .0001), the number of growing follicles on the day of hCG administration (≥12 mm; r = 0.62, P < .0001) and the number of oocytes retrieved (r = 0.68, P < .0001) (data not plotted). This relationship was negative with total FSH requirement (r = −0.54, P < .0006) (data not plotted). Incidentally, as expected, the number of early antral follicles on cycle day 3 before COH, the number of growing follicles on the day of hCG administration (≥12 mm), and the number of oocytes retrieved were positively and statistically significantly correlated to each other. These parameters correlated negatively and statistically significantly with the total recombinant FSH dose required for COH. Furthermore, women’s ages, menstrual cycle lengths, and BMI values were not statistically significantly related to serum or follicular fluid hormone levels on the day of oocyte retrieval. Finally, clinical indications for IVF-ET did not statistically significantly influence serum and follicular fluid hormone levels.

DISCUSSION

The present study was designed to examine, in individual follicles, the hypothesis that the degree of follicular matura-
tion and luteinization influences AMH production. Also, it aimed at clarifying whether the reported quantitative relationship between peripheral AMH levels and the number of early antral follicles might be affected by their individual ability to produce AMH. For achieving these objectives, COH for IVF-ET represented a unique model to quantify not only the antral follicle responsiveness to exogenous FSH but also the AMH production in large and small follicles in the same patient.

Some inherent methodological limitations should be taken into account in the interpretation of our results. First, cutoffs used to sort follicles into small (8–12 mm) and large (16–20 mm) classes were merely indicative and based, respectively, on the sizes of nondominant and preovulatory follicles during the late follicular phase of the menstrual cycle (23). Retrieval of fluids from follicles measuring less than 8 mm was judged technically cumbersome and was not performed. Second, it is possible that small follicles on the day of oocyte retrieval presented some degree of developmental incompetence in response to FSH, a phenomenon that could affect their hormonal production (24). Conversely, it is also conceivable that small and large follicles were comparably healthy, and size differences were attributable to discrepant pretreatment sizes and/or to a late recruitment in response to exogenous FSH. Unfortunately, because the present study did not track the development of individual follicles, we could not clarify these issues.

Our results showed that, in adult women, AMH levels are roughly three times as high in small as in large follicles. These data are consistent with the hypothesis that AMH production by granulosa cells probably declines during final follicular maturation (2, 8), providing direct confirmation to our previous findings that showed a progressive decline in serum AMH levels during the evolution of ovarian follicles from the early antral stage to the preovulatory stage during COH (9). Both the physiologic mechanisms implicated in the reduction of AMH production by maturing follicles and the possible consequences of this phenomenon on the regulation of folliculogenesis remain unclear. It is possible that the increase in granulosa cell sensitivity to FSH that occurs during the ultimate stage of folliculogenesis and the down-regulation of AMH and its type II receptor mRNA are interrelated phenomena (8). Further studies are needed to clarify these issues and to provide insights into the role of AMH during the final follicular maturation.

Our observation that $P_4$ levels were lower in small follicles compared with large follicles may be explained by the
fact that the expression of LH/hCG receptors in granulosa cells probably is less intense in small follicles compared with large follicles (25). Also, the similar follicular fluid E\textsubscript{2} levels between small and large follicles may be due to the potentially different luteinization status of the two follicular classes. Assuming that follicular luteinization probably is milder in smaller than larger follicles (25), the transient decrease in E\textsubscript{2} production that accompanies the luteinization process (26, 27) possibly was attenuated in small follicles, too.

The negative relationship between follicular fluid AMH and P\textsubscript{4} levels observed in both follicular classes supports the hypothesis that follicle luteinization exerts a negative effect on the production of AMH by granulosa cells. In line with this, Baarends et al. (8) have previously demonstrated that AMH and its type II receptor mRNA expression are markedly reduced in the isolated corpora lutea of rats, compared with small and large antral follicles. Expanding this observation, our present results suggest that the more advanced the process of luteinization of granulosa cells, as reflected by the magnitude of intrafollicular P\textsubscript{4} secretion, the lesser their ability to secrete AMH. If confirmed, these results may also constitute an alternative explanation to the overall increased AMH levels observed in small follicles.

The most striking of our findings is the relationship between AMH content in individual follicles and the surrounding follicular status, as represented by the number of early antral follicles on day 3 and their responsiveness to COH. Accordingly, the positive correlation between serum and follicular fluid AMH levels clearly indicates that peripheral AMH levels are not exclusively dependent on the number of follicles; they also are modulated by their individual ability to produce AMH. Hence, elevated peripheral AMH levels indicate not only that the number of antral follicles is increased, but also that each follicle probably produces more AMH individually. This offers us a new understanding of the reported association between peripheral AMH levels and the ovarian fertility potential, and leads us to speculate that serum AMH measurements could reflect not only quantitative but also qualitative ovarian responsiveness to COH.

The present study provides direct confirmation of the hypothesis that follicular maturation and luteinization interferes with the AMH production by granulosa cells in individual follicles. Yet the remarkable correlation between intrafollicular AMH content, ovarian follicular status, and ovarian response to COH indicates that peripheral AMH measurements may reflect not only follicle count but also per-follicle AMH production. Further studies, possibly looking at the fate of oocytes and embryos derived from follicles containing high or low AMH concentrations, are required to verify the hypothesis that serum AMH measurements might provide not only quantitative but also qualitative information about ovarian follicle status.

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