Anti-Müllerian Hormone Is an Endocrine Marker of Ovarian Gonadotropin-Responsive Follicles and Can Help to Predict Superovulatory Responses in the Cow

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ABSTRACT

The major limitation to the development of embryo production in cattle is the strong between-animal variability in ovulatory response to FSH-induced superovulation, mainly due to differences in ovarian activity at the time of treatment. This study aimed to establish whether anti-Müllerian hormone (AMH) was an endocrine marker of follicular populations in the cow, as in human, and a possible predictor of the ovarian response to superovulation. Anti-Müllerian hormone concentrations in plasma varied 10-fold between cows before treatment and were found to be highly correlated with the numbers of 3- to 7-mm antral follicles detected by ultrasonography before treatment \((r = 0.79, P < 0.001)\) and the numbers of ovulations after treatment \((r = 0.64, P < 0.01)\). Between-animal differences in AMH concentrations were found to be unchanged after a 3-mo delay \((r = 0.87, P < 0.01)\), indicating that AMH endocrine levels were characteristic of each animal on a long-term period. The population of healthy 3- to 7-mm follicles was the main target of superovulatory treatments, contained the highest AMH concentrations and AMH mRNA levels compared with larger follicles, and contributed importantly to AMH endocrine levels. In conclusion, AMH was found to be a reliable endocrine marker of the population of small antral gonadotropin-responsive follicles in the cow. Moreover, AMH concentrations in the plasma of individuals were indicative of their ability to respond to superovulatory treatments.

AMH, assisted reproductive technology, follicular development, granulosa cells, ovary

INTRODUCTION

In cattle, multiple ovulation and embryo transfer (MOET) programs have become a large international business. The technology is well established and participates in genetic selection strategies and crossbreeding schemes to improve zootechnical characteristics of dairy and beef breeds by enabling the amplification of the lineage of chosen females. More than 500 000 embryos are produced annually from superovulated (FSH-stimulated) cows worldwide [1]. However, the number of transferable embryos has not changed markedly in the last 20 yr, and the use of MOET technology in the animal industries is approaching a plateau [2]. The major limitation to its development is its reliance on FSH-induced superovulation and the large between-animal variations observed in response to treatments [3]. Despite improvements in superovulatory treatments, ovarian responsiveness to gonadotropins remains highly variable between individuals and difficult to predict. It is now well established that the major source of variability is the status of ovarian follicles at the time of initiation of FSH treatment. When a superovulatory treatment is applied, cows with a low number of growing small antral follicles have a low ovulatory response to this treatment [4–8].

A high between-individual variability in ovarian responses to gonadotropins, linked with differences in ovarian activity, also constitutes a limit to the improvement of assisted reproductive technologies (ARTs) in the human species [9]. Attention has been focused recently on the anti-Müllerian hormone (AMH) in the context of treatment of anovulatory infertility in woman, since it throws new light on the ovarian follicular pool and responsiveness to gonadotropins. Numerous clinical studies have shown that women with polycystic ovary syndrome have high AMH concentrations in plasma and, conversely, low AMH concentrations are indicative of ovarian aging [10–17]. Presently, AMH is the best endocrine marker of the ovarian follicular reserve in human [18–20], and similar observations have been reported recently in the mouse [21]. Anti-Müllerian hormone is also the best predictive marker of the ovarian response to a stimulatory treatment, as defined by the number of oocytes retrieved in ART [22–27].

Anti-Müllerian hormone is a glycoprotein of 140 kDa belonging to the transforming growth factor beta family (TGFβ) that is expressed only in the gonads [28]. It was originally identified in connection with its role in male fetal sex differentiation during embryonic development [29], but later studies have shown that AMH exerts inhibitory effects on the development and function of reproductive organs in both sexes (for review, see Teixeira et al. [30] and Josso and Clemente...
[31]). In the ovary, AMH expression is restricted to a single cell type, that is, granulosa cells (bovine [32–34], sheep [35], rat [36–38], human [39, 40], mouse [41, 42], and hen [43]). AMH expression is the highest in granulosa cells of preantral and small antral follicles, decreases during terminal follicular growth [44], and low AMH concentrations have been found in the follicular fluid of large antral and preovulatory follicles (human [45–47] and bovine [34]). This pattern of expression in the granulosa cells of growing follicles makes AMH an ideal marker for the size of the ovarian follicle pool. However, this question has not yet been raised in the bovine species.

The present study aimed to identify AMH as an endocrine marker of the status of the population of antral follicles and as a possible predictive tool of the ovarian response to a superovulatory treatment in the cow. Experiments were designed to study the relationships existing between AMH concentrations in plasma, populations of gonadotropin-responsive follicles in ovaries, their AMH intrafollicular concentrations and expression levels in granulosa cells, and the superovulatory responses of cows.

MATERIALS AND METHODS

Animals and Experimental Design

Twenty-seven Prim’Holstein dairy cows, 4–9 yr old, were allocated to two different experiments (18 and 9 animals in experiments 1 and 2, respectively) conducted during the fall of 2006 and 2007. Animals were housed in free stalls and provided feed and water ad libitum. All procedures were approved by the agricultural and scientific research agencies (approval number C37-175-2) and conducted in accordance with the guidelines for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Experiment 1

This experiment aimed to establish the relationships existing between AMH concentrations in plasma on the one hand, and antral follicular populations and ovulatory responses to a superovulatory treatment on the other. Eighteen cows received subcutaneous progesterone implants (Crestar; Intervet, Angers, France) during 10 days. Eight days after implant insertion, cows were superovulated with a total of 32 mg FSH (Stimufol; Merial, Lyon, France), which was given as twice-daily injections over 4 days on a decreasing dose schedule [3]. An intramuscular injection of 22.5 mg prostaglandin F$_2$ (P$_2$: Prostrovit, Intervet) was administered at the time of the fifth FSH injection to induce luteolysis, and progesterone implants were removed at the time of the sixth FSH injection. Ovaries from each cow were analyzed by ultrasonography scanning using transrectal probe LV 513 (6.0/8.0-MHz transducer; MyLab30; ESAOTE), allowing detection and measurement of corpora lutea (CL) and antral follicles with an accuracy of 0.1 mm. Each ovary was scanned from end to end, and video images of the scan were captured and digitalized. For each follicle, antrum size was estimated by measurement of two perpendicular diameters when the antral cavity appeared at its maximal size on the video images. Thus, all of the follicles with an antrum larger than 3 mm in diameter were counted, measured, and allocated to three size classes—that is, small (3–5 mm), medium (5–7 mm), and large (>7 mm) follicles—according to antrum size. Ovaries were scanned at the time of the first injection of FSH (T$_0$), after treatment at time of estrus (T$_E$), and 7 days after estrus during the luteal phase following superovulatory treatment (T$_L$). The number of ovulations induced by the superovulatory treatment was assessed by counting CL on ovaries at T$_E$. Blood samples were recovered at T$_0$, T$_E$, and T$_L$, and plasmas were stored at −20°C until AMH assays.

Experiment 2

This experiment aimed 1) to further study AMH as a possible long-term endocrine marker of ovarian activity in the cow and 2) to determine which follicular populations contributed most importantly to AMH endocrine levels. Nine animals were submitted to a standard superovulatory treatment as described above. Blood samples were recovered at the time of the first injection of FSH (T$_1$), and plasmas were stored until AMH assays. At T$_1$, the ovarian response of each animal to treatment was assessed by counting large follicles and CL on ovaries using ultrasonography as described above. Then, after a 2-mo resting period without hormonal treatment, the nine animals were treated with two intramuscular injections of 15 mg P$_2$ spaced 11 days apart with the aim of inducing luteolysis and synchronizing occurrence of oestrus. Occurrence of ovulation was checked by ovarian ultrasound scanning 5 days after each P$_2$ injection. Cows were slaughtered 8 days after the second P$_2$ injection (T$_2$). Daily blood samples were recovered for 20 days on each cow, between the time of the first injection of P$_2$ and T$_L$, and plasmas were stored until AMH and steroid assays. At slaughter, all follicles larger than 3 mm in diameter present on the ovaries of the nine cows were carefully dissected with scissors and dissecting forceps and counted (total number of dissected follicles, n = 492). Follicular size was estimated by measurement of two perpendicular diameters of each intact follicle on a millimeter scale. Then follicles were allocated to the three size classes of small, medium, and large. In a validation study, total follicle counts obtained by dissection and by ultrasonography were found to be highly correlated (n = 9; r = 0.96, P < 0.001). As a 3-mm follicle by dissection represents a smaller follicle by ultrasonography due to the thickness of the follicular wall, the number of dissected follicles was found to be about 1.8-fold higher than the number of follicles detected by ultrasonography. Follicular fluids were recovered individually and stored at −20°C until AMH and estradiol-17β assays. Granulosa cells were recovered from all individual follicles as described previously [48], and they were stored at −80°C until reverse transcription and quantitative PCR (qPCR) analysis. For each follicle, a sample of granulosa cells was smeared on a histological slide, then Feulgen stained to determine follicular quality [49, 50]. Follicular quality was assessed by microscopic examination of the smears using classical histological criteria: healthy (presence of mitosis and absence of pycnosis in granulosa cells), early atretic (slightly pycnotic granulosa cells with persistence of mitosis), and late atretic (no mitosis in granulosa cells and presence of numerous pycnocytes).

Hormonal Assays

Plasma from experiments 1 and 2 was measured for AMH concentrations, and plasma from experiment 2 also was measured for estradiol-17β and progesterone. Follicular fluids from experiment 2 were measured for estradiol-17β and AMH concentrations. For small and medium follicles, due to small size, the recovered follicular fluids were pooled (between four and eight follicular fluids per pool) per cow, follicular size and follicular quality before analysis so that a total number of 160 follicular fluids were finally analyzed. For steroids and AMH, relevant comparisons were made within the same assay. For steroid assays, 30 μl follicular fluid or 600 μl plasma was extracted with 3 ml ethyl acetate-cyclohexane (v/v), and the dried extracts containing steroids were recovered in 1 ml PBS and then measured by radioimmunoassay (RIA). The percentage of steroid recovery varied between 98% and 100%. Estradiol-17β was assayed with the estradiol-2 RIA kit following the manufacturer’s specifications (DiaSorin SA, Antony, France). The antiserum of the kit cross-reacted slightly with estrone (0.6%), estradiol (0.6%), and other steroids (<0.1%). The limit of detection of the assay was 0.08 pg per tube, and the intraassay coefficients of variation (Diabotest) was lower than 7%. Progesterone was assayed as described previously [51]. The antiserum only cross-reacted with 20-hydroxyprogesterone (1.7%) and other steroids (<1%). The limit of detection of the assay was 12 pg per tube, and the intraassay coefficient of variation was lower than 10%.

Anti-Müllerian hormone was measured with the Active MIS/AMH ELISA kit (DSL, Cergy-Pontoise, France) as previously validated for bovine follicular fluid [34]. Anti-Müllerian hormone was measured on 50 μl undiluted plasma and 20 μl follicular fluid diluted at 1:500 for small follicles, 1:100 for medium follicles, and 1:50 for large follicles. Anti-Müllerian hormone was undetectable in bovine serum from castrated animals. Anti-Müllerian hormone concentrations were higher than the limit of detection of the assay (1 pg per well, corresponding to 0.020 ng/ml in plasma samples) in all of the plasma and follicular fluids tested. AMH concentrations were found to be 11.8% and 3.6% for plasma samples containing 0.033 ng/ml and 0.125 ng/ml AMH concentrations, respectively. For comparison of AMH in follicular fluid and in plasma of cows from experiment 2, the amount of AMH present in healthy follicles of a given follicular size class (small, medium, or large) was estimated from the formula: Q = C × N × N, where C is the AMH average intrafollicular concentration of the class (expressed in ng/ml), V the average follicular volume (expressed in milliliters), and N is the total number of healthy follicles of a given follicular size class.

Reverse Transcription and qPCR

Granulosa cell samples from experiment 2 were measured for AMH, CYP19A1, and RPL19 mRNA. Due to the small size of samples for small and medium follicles, cells recovered from different follicles were pooled (four to eight follicles per pool) per cow, follicular size, and follicular quality before analysis. Total RNA was extracted from granulosa cell samples, then reverse transcription, and reverse transcription with quantitative PCR (qPCR) was performed. The percentage of RNA recovery was estimated at 90% for small and medium follicles and 75% for large follicles. For all qPCRs, the expression of the endogenous control housekeeping gene GAPDH was determined, and the expression level of AMH was calculated using the ΔΔCt method.
FIG. 1. Numbers of follicles and CL on cow ovaries before and after administration of superovulatory treatment in experiment 1. Follicles of different size classes (small [S]: 3–5 mm, medium [M]: 5–7 mm, and large [L]: >7 mm) and CL were counted by ovarian ultrasonography on cow ovaries (n = 18 cows) at T₀ (before treatment, white bars), Tₑ (at time of estrus after treatment, gray bars) and T₀ (in the luteal phase after treatment, black bars). A) Changes in the numbers of follicles of different size classes with time. For each follicular size class, different letters indicate significant differences between T₀, Tₑ, and T₀ resulting from analysis by repeated-measures ANOVA. B) The relationship between the number of small and medium follicles at T₀ and the number of large follicles at T₀. Each circle represents data from one cow.

FIG. 2. Anti-Müllerian hormone concentrations measured in plasma before and after administration of superovulatory treatment in experiment 1 (n = 18 cows). A) Anti-Müllerian hormone average concentrations at T₀, Tₑ, and T₀. Different letters indicate significant differences between means, resulting from analysis by repeated-measures ANOVA. A vs. b: P < 0.001. B) Relationships between AMH concentrations at T₀ and at Tₑ (black circles) or T₀ (white circles). Each circle represents data from one cow.

**Results**

**Relationships Between AMH Concentrations in Plasma, Antral Follicular Populations, and Ovarian Responses to Superovulation**

As assessed by ovarian ultrasound scanning in the 18 studied cows from experiment 1, high between-animal variations were observed for the numbers of follicles and the ovarian response (CL and nonovulated large follicles) to the superovulatory treatment. The total number of follicles larger than 3-mm diameter varied in the range of 4–45 at T₀ (before transcribed, and PCR reactions were performed using SYBR Green Supermix (Bio-Rad, Marnes la Coquette, France) as described previously [34]. The following specific primer sequences were used: for AMH, forward 5'-GTGGTGCTGCTGCTAA/AGATG-3' and reverse 5'-TCGGACAGGCTGTATGGAGGAG-3'; for CYP19A1, forward 5'-TGCTTGCTGTCACCCCCTTG-3' and reverse 5'-GCGTCCTGGTGCTGCTGCT-3'; and for RPL19, forward 5'-AATGCCAATGCCAACTC-3' and reverse 5'-TCGGACAGGCTGAT-3'. For each primer pair, efficiency curves were generated using serial dilutions of the cDNA of granulosa cells in abscissa and the corresponding Ct (cycle threshold) in ordinate. The slope of the log-linear phase reflects the amplification efficiency (E) derived from the formula E = 10^[1/slope]. Amplification efficiency was found to be E_AMH = 1.88, E_CYP19A1 = 1.95, and E_RPL19 = 1.95. For quantification analysis, the Ct of the target gene (AMH or CYP19A1) was compared with the internal reference gene RPL19 encoding a ubiquitous ribosomal protein according to the ratio R = E^Ct_RPL19 / E^Ct_target, where E is the average coefficient calculation was allowed following the two nonsignificant z and Jarque-Bera hypothesis tests [53].

**RESULTS**

For correlation studies, the significance was considered according to the Bravais-Pearson r critical values. For all analyses, differences with P > 0.05 were considered not significant.

The temporal relationships between AMH and estradiol-17β or progesterone concentrations were studied using cross-correlation analysis. The cross-correlation analysis estimates the correlation between one time series at time t and the other time series at the time t ± x lags [52]. Cross-correlation coefficients were computed using the Matlab `corr` function after subtraction of the means from the time series. Cross-correlation coefficients were computed for all lags, with each lag corresponding to a day blood sampling interval. The correlation coefficients of the unlagged data stand at the midpoint (lag 0). For positive lags, variations in AMH concentrations preceded variations in estradiol or progesterone concentrations; conversely, for negative lags, variations in estradiol or progesterone concentrations preceded variations in AMH concentrations. To obtain an average estimate of the correlation, the individual correlation coefficients of the nine cows were averaged for each lag. This average coefficient calculation was allowed following the two nonsignificant z and Jarque-Bera hypothesis tests [53].

**Data Analysis**

Experimental data are presented as means ± SEM, except for correlation studies. Data were analyzed using t-test or one-way ANOVA followed by Newman-Keuls multiple comparison tests for comparisons between two or several means, respectively. Repeated-measures ANOVA or paired t-tests were used to analyze changes in the average numbers of follicles and AMH average concentrations with time. When variances were heterogeneous, data were log-transformed or were analyzed by the nonparametric Mann-Whitney test (for comparison of two means) or the Kruskal-Wallis test (for comparison of several means).
administration of the superovulatory treatment), 15–55 at \( T_E \) (time of estrus), and 4–37 at \( T_L \) (in the luteal phase after treatment), and the number of CL at \( T_L \) varied in the range of 4–27. Following administration of the superovulatory treatment, the average number of small (3–5 mm in diameter) follicles dropped between \( T_0 \) and \( T_E \) \( (P < 0.01) \), whereas the average numbers of medium (5–7 mm) and large (7–11 mm) follicles increased strikingly at \( T_E \) \( (P < 0.001; \text{Fig. 1A}) \). A highly significant correlation was found between numbers of small and medium follicles at \( T_0 \) and numbers of large follicles at \( T_L \) \( (r = 0.75, P < 0.001; \text{Fig. 1B}) \). Moreover, numbers of large follicles at \( T_E \) were significantly correlated with numbers of CL \( (r = 0.70, P < 0.01) \) and numbers of CL + large follicles at \( T_L \) \( (r = 0.56, P < 0.05) \). These results indicate that small and medium follicles were targets of the stimulatory treatment, and cows with high numbers of small and medium (3–7 mm) follicles at \( T_0 \) had high ovarian responses to the superovulatory treatment.

High between-animal variations were observed for AMH concentrations in plasma in the range of 0.025–0.228 ng/ml at \( T_0 \), 0.049–0.359 ng/ml at \( T_E \), and 0.026–0.212 ng/ml at \( T_L \). Following administration of the superovulatory treatment, AMH average concentrations in plasma increased significantly between \( T_0 \) and \( T_E \) \( (P < 0.001) \), then decreased between \( T_E \) and \( T_L \) \( (P < 0.001) \), reaching their initial values (Fig. 2A). AMH concentrations measured in plasma at \( T_0 \) were highly correlated with AMH concentrations measured in plasma both at \( T_0 \) and \( T_L \) \( (r = 0.87 \text{ and } r = 0.93, \text{ respectively}, P < 0.001; \text{Fig. 2B}) \).

The relationships between AMH concentrations in plasma, follicular populations, and superovulatory responses to treatment were investigated. Anti-Müllerian hormone concentrations measured in plasma at \( T_0 \) were highly correlated with numbers of small and medium follicles detected by ultrasonography at \( T_0 \) \( (r = 0.79; P < 0.001; \text{Fig. 3A}) \) and numbers of large follicles at \( T_E \) \( (r = 0.83, P < 0.001; \text{Fig. 3B}) \), and significant correlations also were found with numbers of CL at \( T_L \) \( (r = 0.64, P < 0.01; \text{Fig. 3C}) \) and numbers of CL + large follicles at \( T_L \) \( (r = 0.58, P < 0.05; \text{Fig. 3D}) \). Similar results were obtained with AMH concentrations measured in plasma at \( T_E \) and at \( T_L \) (data not shown). These results indicate that cows with high numbers of 3- to 7-mm follicles before a superovulatory treatment and high ovarian responses to treatment had high AMH concentrations measured just before and after treatment.

**Long-Term Variations in AMH Concentrations in Plasma and Relationships with Antral Follicular Populations**

The results of experiment 1 suggested that AMH might be an endocrine marker of gonadotropin-responsive follicles and of ovarian responsiveness to a superovulatory treatment when measured just before administration of treatment. In experiment 2, we investigated whether AMH could also be predictive of the ovarian status of cows over a long-term period. To answer this question, nine cows were first studied for their AMH concentrations at \( T_0 \) before treatment or (E–D) the ovarian response to superovulatory treatment in experiment 1 \( (n = 18 \text{ cows}) \). Data represent the relationships between AMH concentrations at \( T_0 \) and the numbers of small (S) and medium (M) follicles at \( T_E \) (A), the numbers of large (L) follicles at \( T_L \) (B), the numbers of CL at \( T_L \) (C), and the numbers of CL + L follicles at \( T_L \) (D). Each circle represents data from one cow.
After superovulatory treatment, the number of CL and the number of CL + large follicles varied in the ranges of 5–31 and 8–31, respectively. The numbers of CL + large follicles after treatment were significantly correlated with AMH concentrations measured in plasma at T₀ just before treatment (r = 0.70, P < 0.05), confirming results obtained in experiment 1. Moreover, the numbers of CL + large follicles were also correlated with AMH concentrations measured in plasma at Tₛ, 3 mo later (r = 0.72, P < 0.05; Fig. 5A). At slaughtering, high between-animal variations were observed in the numbers of healthy, early atretic, and atretic follicles of different sizes recovered by dissection on ovaries (Table 1). The numbers of healthy small and medium follicles present on ovaries at Tₛ were highly correlated with AMH concentrations measured in plasma at T₀, 3 mo before slaughtering (r = 0.93, P < 0.001), but also at Tₛ, 3 mo before slaughtering (r = 0.95, P < 0.001; Fig. 5B). Together, these results indicate that AMH was a good marker of between-animal differences in follicular populations and ovarian responsiveness to a superovulatory treatment, even after a 3-mo interval.

**Daily Variations in AMH and Steroid Concentrations in Plasma**

Anti-Müllerian hormone concentrations measured in plasma of the same animals at 3-mo intervals were highly correlated, indicating a strong animal effect, but whether the stage of the estrus cycle influenced AMH concentrations was unknown. To address this question, daily changes in AMH, progesterone, and estradiol-17β concentrations were studied concomitantly in plasma during the 20 days that preceded slaughtering. During this period, animals received two Pg injections at 11-day intervals for cycle synchronization.

As expected, progesterone concentrations dropped after each Pg injection, and this decrease was followed by an increase in estradiol-17β concentrations within 1 or 2 days. These changes did not seem to be accompanied by clear variations in AMH concentrations, as illustrated for three different animals in Figure 6, A–C. Further cross-correlation analyses aimed to study whether AMH daily fluctuations were associated with progesterone and/or estradiol-17β variations. In analysis of individual animals, cross-correlation coefficients were not found to be consistently significant at any lag (Fig. 6, D and E). For averaged values, no cross-correlation coefficient was significant at any lag. Thus, no systematic relationship between AMH and estradiol-17β or progesterone could be established.

**AMH Intrafollicular Concentrations and Relationships with Antral Follicular Populations**

With the aim of determining which follicular populations contributed to AMH endocrine levels, AMH intrafollicular concentrations were measured in follicles with various sizes and degrees of atresia that were from the ovaries of the nine cows in experiment 2. Estradiol-17β, a well-established marker of follicular maturation, also was measured in the same follicular fluid samples. As expected, estradiol-17β concentrations were about 100-fold higher in healthy large follicles compared with healthy small and medium follicles (P < 0.001) and decreased with atresia in all follicular size classes (healthy vs. early atretic...
follicles, \( P < 0.01 \); healthy vs. late atretic follicles, \( P < 0.001 \); Fig. 7A). In contrast, AMH intrafollicular concentrations were the highest in healthy small follicles and decreased with follicular growth (small vs. medium healthy follicles, \( P < 0.001 \); medium vs. large healthy follicles, \( P < 0.001 \); Fig. 7B). Anti-Müllerian hormone concentrations decreased significantly with atresia in small and medium follicles only (healthy vs. late atretic follicles, \( P < 0.001 \); Fig. 7B).

Because healthy small and medium follicles contained the highest AMH intrafollicular concentrations, it was hypothesized that they might contribute most importantly to AMH concentrations in plasma. The amount of AMH contained in the follicular fluid of the population of small and medium healthy follicles was calculated for each animal. According to the proposed hypothesis, AMH intrafollicular amounts were highly correlated with AMH concentrations measured in
AMH mRNA Expression in Granulosa Cells of Antral Follicles

AMH mRNA levels were studied in granulosa cells of follicles from ovaries of the nine cows of experiment 2. CYP19A1, a well-established marker gene of granulosa cell differentiation, was studied concomitantly in the same samples. As expected, CYP19A1 mRNA levels were strongly higher in healthy large follicles ($P < 0.001$ vs. healthy small and medium follicles) and decreased in early atretic (healthy vs. early atretic small and medium follicles, $P < 0.05$; healthy vs. early atretic large follicles, $P < 0.001$) and late atretic (healthy vs. late atretic small and medium follicles, $P < 0.01$; healthy vs. late atretic large follicles, $P < 0.001$) follicles of the three follicular size classes (Fig. 7C). AMH mRNA levels were higher in healthy small follicles compared with healthy medium and large follicles (both $P < 0.05$; Fig. 7D). AMH mRNA levels decreased significantly in late atretic follicles in all follicular size classes ($P < 0.001$, $P < 0.05$, $P < 0.01$ for small, medium, and large follicles, respectively).

**DISCUSSION**

Our results show for the first time that AMH concentrations in plasma could be used as a predictive marker of ovarian responsiveness to a superovulatory treatment in the cow. In the two groups of studied cows, AMH measured in plasma just before treatment was highly indicative of the number of gonadotropin-responsive follicles able to develop to the preovulatory stage after treatment. Cows with high AMH concentrations in plasma had the highest follicular and ovulatory responses to treatment and, conversely, cows with low AMH levels were poorly responsive to treatment. These results agree with data obtained in women submitted to ART protocols, indicating that AMH measured in plasma before ovarian stimulation was a predictive marker of the number of oocytes retrieved by follicular punction after treatment [22–27]. Interestingly, from our results, between-animal differences in AMH concentrations were found to be unchanged after a 3-mo time interval, indicating that AMH endocrine levels were characteristic of each animal on a long-term period. This observation reinforces the interest of AMH as a strong possible predictive tool to select highly responsive animals in MOET programs.
The results of this study 1) have allowed the identification of AMH as a very good endocrine marker of the population of 3- to 7-mm healthy follicles present on unstimulated cow ovaries and 2) have shown that this population of small antral follicles was the main target of the superovulatory treatment. Granulosa cells of these follicles expressed higher AMH mRNA levels and their follicular fluids contained higher AMH concentrations compared with larger antral follicles and atretic ones. Moreover, their AMH intrafollicular contents were strongly related to AMH concentrations in plasma, suggesting that these follicles contribute importantly to AMH endocrine levels. In agreement with previous results in the cow [4–8], this follicular population was very variable quantitatively and qualitatively between animals, and cows with high numbers of follicles in this size class before treatment had high ovarian responses to the superovulatory treatment. Interestingly, despite important variations in numbers of follicles among animals, the number of growing follicles larger than 3 mm in diameter is highly repeatable during each follicular wave of individuals [8, 54] and, accordingly, a strong within-animal repeatability in ovarian responses to superovulatory treatments also has been reported [55–57]. Together, these data are consistent with our observations that AMH concentrations in plasma were highly variable between animals but rather steady with time for each animal, and they reinforce the interest in AMH as a reliable endocrine marker of ovarian activity in the cow.

In a perspective of prediction of superovulatory responses in the cow, it is important to know whether AMH levels should be measured in plasma at a given day of the estrus cycle or whether they can be measured anytime. From our results, no changes in AMH concentrations were found to be associated with the striking daily changes in progesterone and estradiol-17β concentrations observed in response to prostaglandin injections during cycle synchronization of cows before slaughtering. In women, the existence of variations in AMH concentrations during the menstrual cycle remains controversial. In some studies, no consistent fluctuation patterns in AMH levels were observed throughout a menstrual cycle [58–62], whereas other authors have reported higher AMH levels in the mid luteal [63] or in the late follicular [64] phase. During the cow estrus cycle, quantitative and qualitative changes in the population of small antral follicles are known to accompany growth and regression or ovulation of the dominant follicle [54, 65], and these changes are likely associated with fluctuations in AMH ovarian secretion rate. Considering that the half-life of AMH is about 2 days in bovine serum [66], whether these changes in AMH ovarian secretion rate are paralleled by detectable AMH endocrine changes now has to be investigated in the cow.

The mechanisms regulating AMH production by granulosa cells are poorly understood. In situ hybridization studies in the rat have shown that AMH expression is the highest in preantral and small antral follicles and decreases during terminal follicular growth [44]. Accordingly, a decrease in the protein content of granulosa cells of large antral follicles has been shown in humans by immunohistochemistry [40], and low AMH concentrations have been detected when measured in the follicular fluid of large antral and preovulatory follicles in humans [45–47] and recently in bovine species [34]. The results of this study showed that AMH intrafollicular concentrations decreased sharply in follicles larger than 7 mm in diameter compared with smaller antral follicles, and they were clearly affected by atresia. Variations in AMH intrafollicular concentrations did not always parallel those in AMH mRNA levels in granulosa cells. The strong drop in AMH intrafollicular concentrations observed between the small and the large healthy follicles was associated with only a slight decrease in AMH mRNA levels in granulosa cells. Moreover, in the large early atretic follicles, AMH mRNA levels in granulosa cells were clearly decreased compared with healthy follicles, whereas AMH intrafollicular concentrations were slightly increased. These results suggest that both transcriptional and posttranscriptional mechanisms, likely including secretion rate, binding to extracellular matrix proteins [67], and dilution in follicular fluid, may play a determinant role in regulating AMH accumulation in antrum. In plasma, AMH concentrations clearly increased at the following estrus when a superovulatory treatment was administered to animals, and this increase may reflect enhanced growth of small follicles that were not detected by ultrasonography. Alternatively, whether FSH treatment may have enhanced the AMH secretion rate by granulosa cells or the efficiency of AMH capture by follicular blood vessels of stimulated small antral follicles remains to be established.

From our results, AMH and CYP19A1 can be proposed as functional markers of immature and fully differentiated granulosa cells, respectively. Indeed, when comparing small and large healthy follicles, AMH and CYP19A1 mRNA levels varied in opposite ways. Accordingly, a clear inverse relationship was observed between AMH and estradiol-17β intrafollicular concentrations. Interestingly, AMH has been shown to inhibit the induction of CYP19A1 and LH receptors by FSH in rat and porcine granulosa cells in vitro [68]. A possible direct role for AMH in regulating antral follicular development in the cow has never been investigated. Further studies are needed to establish whether AMH might participate in regulation of terminal follicular development by inhibiting FSH-dependent CYP19A1 expression in granulosa cells of small antral follicles in the cow.

In conclusion, this study is the first report of the existence of a strong relationship between AMH endocrine levels and gonadotropin-responsive follicles in cow ovaries. The results of this study allow us to propose AMH as a reliable marker of ovarian activity and a possible predictor of the ovarian response to superovulation in the cow. As AMH concentration in the plasma of unstimulated cows seems to be strongly indicative of their ability to respond to superovulatory treatments, MOET programs might benefit from AMH measurement in plasma before treatment in order to discard poorly responding animals.

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