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# Reductions in the number of mid-sized antral follicles are associated with markers of premature ovarian senescence in dairy cows

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**Abstract.** High-producing dairy cows are subfertile; however, the mechanisms responsible for the decreased fertility are unknown. The aim of the present study was to test the hypothesis that culled dairy cows (4–8 years old) characterised by 'Lo' ovaries (i.e. those with <10 mid-antral follicles) are affected by premature ovarian senescence. Cows in which both ovaries were 'Lo' ovaries represented 5% of the total population analysed, and exhibited reduced ovarian size ($P < 0.001$) and increased perifollicular stroma ($P < 0.05$) compared with age-matched controls (i.e. cows in which both ovaries had >10 mid-antral follicles; 'Hi' ovaries). The total number of follicles, including healthy and atretic primordial, primary, secondary and small antral follicles, was lower in Lo ovaries ($P < 0.01$). Interestingly, the primordial follicle population in Lo ovaries was lower ($P < 0.05$) than in the control. Finally, the follicular fluid of mid-antral follicles from Lo ovaries had reduced oestradiol and anti-Müllerian hormone levels ($P < 0.05$), but increased progesterone concentrations ($P < 0.05$). Together, these data account for the reduced fertility of cows with Lo ovaries and are in agreement with previous observations that oocytes isolated from Lo ovaries have reduced embryonic developmental competence. Cows with a specific Lo ovary condition may represent a suitable model to address the causes of low fertility in high-yielding dairy cows, as well as the condition of premature ovarian aging in single-ovulating species.

**Additional keywords:** anti-Müllerian hormone, infertility, ovarian aging, ovary, premature ovarian failure.

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## Introduction

The antral follicle count (AFC) is considered one of the most reliable, non-invasive methods of determining ovarian reserve and is a good indicator of the size of the remaining primordial pool in women with proven natural fertility (Hansen *et al.* 2011). Diminished ovarian reserve, associated with a drop in ovarian volume and vascularity, generally predicts a poor response to gonadotropin, limits the possibility of successful pregnancy and is correlated with the occurrence of menopausal transition (Lass and Brinsden 1999; Kwee *et al.* 2007; La Marca *et al.* 2012). A low AFC has been described in women during the perimenopausal period (Hansen *et al.* 2008; Broekmans *et al.* 2009), as well as in young infertile and subfertile women affected by premature ovarian failure (POF; De Vos *et al.* 2010; Monget *et al.* 2012), suggesting that the factors affecting the size of the follicular reserve also affect the quality of the remaining oocytes and the likelihood of conception (Rosen *et al.* 2011).

Chronological age also affects fertility in cattle. Mature cows (13–16 years old) have reduced follicle numbers (Malhi *et al.* 2005, 2006) and decreased oocyte competence (Malhi *et al.* 2007) compared with young cows (1–6 years old). Interestingly, in young adult heifers (1–3 years old), the AFC is positively correlated with ovarian size, the total quantity of healthy follicles and healthy oocytes (Ireland *et al.* 2007, 2008) and pregnancy rate (Cushman *et al.* 2009; Mossa *et al.* 2012). Moreover variations in biomarkers of human follicular differentiation and function, as well as those for ovarian aging, such as serum concentrations of FSH, oestradiol (E2), anti-Müllerian hormone (AMH) and inhibin-B (Lambalk *et al.* 2009; La Marca *et al.* 2012), have been shown to be associated with the AFC and reproductive potential in cattle (for a review, see Ireland *et al.* 2011).

In both dairy and beef cows, reduced fertility is due to a high incidence of abnormal ovarian activity (Macmillan *et al.* 2003; Yimer *et al.* 2010). In previous studies, we demonstrated that the oocytes of 4- to 8-year-old dairy cows that had ovaries with fewer than 10 mid-antral follicles (2–6 mm in diameter) and no follicles ≥10 mm in diameter ('Lo' ovaries) exhibited reduced developmental capability compared with age-matched controls in which both ovaries had >10 mid-antral follicles ('Hi' ovaries; Modina *et al.* 2007). Moreover, defective endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) signalling was found

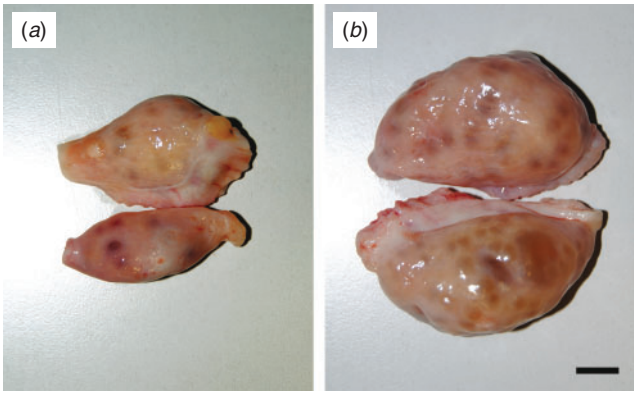


**Fig. 1.** Gross morphology of bovine ovaries with (*a*) fewer than 10 mid-antral follicles (2–6 mm in diameter) and no follicles ≥10 mm in diameter ('Lo' ovaries) and (*b*) >10 mid-antral follicles ('Hi' ovaries). Scale bar = 1 cm.

in Lo ovaries in addition to reduced follicle vasculature, which could adversely affect oocyte quality and early embryonic *in vitro* development (Tessaro *et al.* 2011). Therefore, the aim of the present study was to assess whether in a population of culled dairy cows of reproductive age those cows with Lo ovaries had morphological and endocrine markers commonly associated with premature ovarian aging that could, in turn, lead to a premature decline in fertility.

## Materials and methods

### *Collection of Hi and Lo ovaries and evaluation of gross ovarian anatomy*

Unless stated otherwise, all chemicals and reagents were purchased from Sigma Chemical (St Louis, MO, USA). Bovine ovaries were obtained from a local abattoir (INALCA, Ospedaletto Lodigiano, Italy) from 4- to 8-year-old dairy cows, subjected to routine veterinary inspection and consistent with specific health requirements as stated in EEC Directive 89/556 and the following modifications. The ovaries were transported, on ice, to the laboratory within 2 h from collection.

The ovaries from each cow were isolated and classified into one of two categories, as described previously (Modina *et al.* 2007; Tessaro *et al.* 2011). Briefly, ovaries were classified as Lo (= low efficiency) ovaries when they were found to contain <10 follicles between 2 and 6 mm in diameter and no follicles ≥10 mm or as Hi (= high efficiency) ovaries when they contained >10 follicles between 2 and 6 mm in diameter (Fig. 1).

Each ovary was subjected to gross examination, with mid-antral follicles visible on the ovarian surface counted and measured using a ruler. In addition, ovaries were weighed and ovarian length and height were determined using a ruler.

In the present study, we only used ovaries isolated from cows containing either both Lo or both Hi ovaries. Ovaries from cows with one Lo and one Hi ovary were not used. The Hi ovaries were used as the age-matched control group. In both categories, the presence or absence of a corpus luteum was not taken into account, as stated previously (Gandolfi *et al.* 1997; Modina *et al.* 2007; Tessaro *et al.* 2011). It has been demonstrated that the AFC is not affected by the stage of the oestrous cycle (Cushman *et al.* 2009). In addition, the corpus luteum does not exert a local effect on the developmental competence of oocytes derived from subordinate follicles (Vassena *et al.* 2003).

### *Microscopic evaluation of ovaries*

Ovarian sections were evaluated microscopically to: (1) confirm that the number of mid-antral follicles ('mid-AFC' hereafter) reflects the actual number of antral follicles in the entire organ; (2) count the number of follicles at earlier stages of folliculogenesis (i.e. primordial, primary, secondary and small antral follicles) in the entire organ; (3) evaluate follicular health status; and (4) evaluate the organisation of collagen fibres in the cortical and perifollicular ovarian stroma.

Ovaries were cut into eight equal longitudinal strips using a scalpel, as described previously (Ireland *et al.* 2008) with slight modifications. All strips were processed for histological evaluation as follows: strips were fixed in B5 fixative (Bio-Optica, Milan, Italy), dehydrated in a graded series of ethanol, cleared with xylene, embedded in paraffin and sectioned at 8 μm. The sections were then placed on glass microscope slides that had been treated with Vectabond (Vector Laboratories, Burlingame, CA, USA) to enhance the adherence of tissues, stained with haematoxylin and eosin and then analysed as described below.

All slides were examined under a microscope and the mid-AFC was determined by counting the number of mid-antral follicles present throughout the entire organ. The number of follicles at earlier stages of folliculogenesis was determined in every 40th section of a randomly chosen strip, as described previously (Tilly 2003; Ireland *et al.* 2007). Only follicles that contained a cross-section of the oocyte nucleus were counted. To obtain an estimate of the entire follicular population in the ovary, the number of follicles counted was multiplied for 320, a correction factor that takes into consideration the counting follicles in every 40th section in one of the eight strips ($40 \times 8 = 320$). It has been demonstrated previously that this is a reliable estimate of the total number of follicles at early stages of development (Ireland *et al.* 2008).

Oocytes surrounded by a single layer of flattened granulosa cells were classified as primordial follicles. Primary follicles consisted of an oocyte surrounded by one layer of cuboidal granulosa cells. Secondary follicles contained an oocyte surrounded usually by two to six layers of cuboidal granulosa cells and no antrum. Small antral follicles consisted of an oocyte surrounded by several layers of cuboidal granulosa cells with a fully formed theca interna and an antral cavity (<2 mm; Ireland *et al.* 2008; Rodgers and Irving-Rodgers 2010). A follicle was considered morphologically healthy if it exhibited an intact basal membrane, organised granulosa cell layers with only occasional pyknotic nuclei or atretic bodies in the granulosa cells or follicular antrum, and an intact oocyte and nucleus (Lussier *et al.* 1987; Yang and Rajamahendran 2000; Ireland *et al.* 2008; Tessaro *et al.* 2011).

Additional fragments of Lo and Hi ovaries were fixed and processed as described above and sections were stained with Heidenhain's Azan Trichrome (Bio-Optica), which specifically stains collagen fibres, to assess the density of collagen fibres in the ovarian cortical stroma and perifollicular stroma of healthy

mid-antral follicles. The ovarian and perifollicular stroma of Hi and Lo ovaries were analysed and the percentage of follicles enclosed in thick and thin layers of collagen fibres was determined.

### Hormone concentrations in follicular fluid

Follicular fluid was aspirated from mid-antral follicles of Lo and Hi ovaries using a 2-mL syringe with a 26.5-gauge needle. For each ovary, five follicles were aspirated and the follicular fluid recovered was pooled. Each pool was centrifuged for 30 s at 5000*g* to separate the follicular cells. The supernatant was stored at −20°C until use.

Quantitative analyses of E2 (sensitivity 10 pg mL$^{-1}$) and progesterone (P4; sensitivity 0.1 ng mL$^{-1}$) were performed on 400-μL samples of follicular fluid using an Architect i1000SR Immunoassay Analyzer (Abbott Diagnostics, Abbott Park, IL, USA) according to the manufacturer's instructions (Stricker *et al.* 2006; Plati *et al.* 2010).

Concentrations of AMH were determined using the Active MIS/AMH ELISA kit (Diagnostic Systems Laboratories, Webster, TX, USA; sensitivity 0.006 ng mL$^{-1}$), according to the manufacturer's instructions. This kit is an enzymatically amplified two-site immunoassay containing materials for the quantitative measurement of AMH in human serum; it recently has been validated in bovine follicular fluid (Monniaux *et al.* 2008; Ireland *et al.* 2009; Rico *et al.* 2009). Concentrations of AMH were determined in 10-μL samples of follicular fluid diluted 1 : 200 using a protein-based buffer included in the kit. Analyses of the colorimetric reaction were performed using the Multiskan Reader MS plate reader (MTX Laboratory Systems, Vienna, VA, USA; sensitivity 0.006 ng mL$^{-1}$) at a wavelength of 450 nm.

### Statistical analysis

Data are presented as the mean ± s.e.m. Mean values of two groups were compared using *t*-tests, preceded by the Levene test to assess the equality of variances in the different samples. For correlation studies, the significance of differences was determined using Pearson's correlation coefficient (*r*). All analyses were performed using SPSS version 17.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered significant.

## Results

### Evaluation of gross ovarian anatomy: mid-AFC and ovarian size and weight

In the present study, a total of 475 cows aged 4–8 years were examined. Ovaries were obtained from cows culled at the local abattoir in four different slaughtering sessions. Of the 475 cows examined, 4.66 ± 1.79% were found to have both Lo ovaries (<10 mid-antral follicles 2–6 mm in diameter and no follicles ≥10 mm in diameter).

Gross ovarian anatomy (i.e. confirmation of mid-AFC and evaluation of ovarian size and weight) was assessed in 28 Lo and 48 Hi ovaries. Figure 1 shows representative images of the gross ovarian anatomy of Lo (Fig. 1*a*) and Hi (Fig. 1*b*) ovaries. As indicated in Table 1, there was a significant difference in the mean number of mid-antral follicles between Lo and Hi ovaries (4.51 ± 0.32 vs 31.93 ± 1.67, respectively; $P < 0.001$). Similarly, there was a significant reduction in ovarian wet weight and size (length and height) in Lo compared with Hi ovaries ($P < 0.001$).

**Table 1. Total number of mid-antral follicles (as determined by assessing gross anatomy) and organ size of 28 bovine 'Lo' (i.e. <10 mid-antral follicles) and 48 bovine 'Hi' (i.e. >10 mid-antral follicles) ovaries**

Data are the mean ± s.e.m. *$P < 0.001$ compared with Hi ovaries (*t*-test preceded by the Levene test). Mid-AFC, number of mid-antral follicles

| | Lo ovaries | Hi ovaries |
|---|---|---|
| Mid-AFC (*n*) | 4.51 ± 0.32* | 31.93 ± 1.67 |
| Ovary wet weight (g) | 5.07 ± 0.28* | 13.29 ± 0.68 |
| Ovary height (cm) | 1.79 ± 0.06* | 2.69 ± 0.06 |
| Ovary length (cm) | 3.82 ± 0.10* | 4.67 ± 0.09 |

**Table 2. Number of mid-antral follicles as determined by microscopic evaluation in 20 bovine 'Lo' (i.e. <10 mid-antral follicles) and 20 bovine 'Hi' (i.e. >10 mid-antral follicles) ovaries**

Data are the mean ± s.e.m. *$P < 0.01$, **$P < 0.001$ compared with Hi ovaries (*t*-test preceded by the Levene test)

| | No. follicles | |
|---|---|---|
| | Lo ovaries | Hi ovaries |
| Total healthy + atretic follicles | 5.61 ± 0.60** | 27.89 ± 3.05 |
| Total healthy follicles | 1.78 ± 0.47* | 13.05 ± 2.69 |
| Healthy follicles per g ovary | 0.34 ± 0.10* | 1.07 ± 0.22 |
| % Healthy/total follicles | 34.86 ± 8.90 | 44.10 ± 6.49 |

### Microscopic evaluation of the ovaries: follicular population count and health status

Twenty ovaries from each of the Lo and Hi groups were evaluated histologically. The results of the microscopic evaluation of mid-antral follicles are given in Table 2 and are in agreement with the results obtained by counting mid-antral follicles on the ovarian surface, confirming the accuracy of the experimental approach used in the present study. The number of mid-antral follicles differed significantly between Lo and Hi ovaries (5.61 ± 0.60 vs 27.89 ± 3.05 respectively; $P < 0.001$). Interestingly, the number of healthy mid-antral follicles and the number of healthy mid-antral follicles per g ovary were lower in Lo compared with Hi ovaries ($P < 0.01$). However, this difference was no longer significant if the number of healthy follicles as a proportion of the total number of follicles (healthy + atretic) was considered, rather than the absolute number or the number of follicles per g ovary.

Table 3 gives results of the microscopic evaluation of follicles at earlier stages of folliculogenesis (as the sum of primordial, primary, secondary and small antral follicles). The absolute number of follicles at these stages of folliculogenesis and the number of healthy follicles per g ovary were lower in Lo compared with Hi ovaries, but the number of healthy follicles as a percentage of total follicles did not differ between the two groups.

**Table 3. Total number of follicles at earlier stages of folliculogenesis, as the sum of primordial, primary, secondary and small antral follicles, in 20 bovine 'Lo' (i.e. <10 mid-antral follicles) and 20 bovine 'Hi' (i.e. >10 mid-antral follicles) ovaries**

Data are the mean ± s.e.m. *$P < 0.05$, **$P < 0.01$ compared with Hi ovaries (*t*-test preceded by the Levene test)

| | No. follicles | |
|---|---|---|
| | Lo ovaries | Hi ovaries |
| Total healthy + atretic follicles | 7436.19 ± 2484.69** | 33 680.00 ± 6133.13 |
| Total healthy follicles | 2270.48 ± 496.19** | 15 888.00 ± 4081.88 |
| Healthy follicles per g ovary | 486.18 ± 87.93* | 1248.63 ± 272.48 |
| % Healthy/total follicles | 42.26 ± 6.79 | 46.88 ± 5.72 |

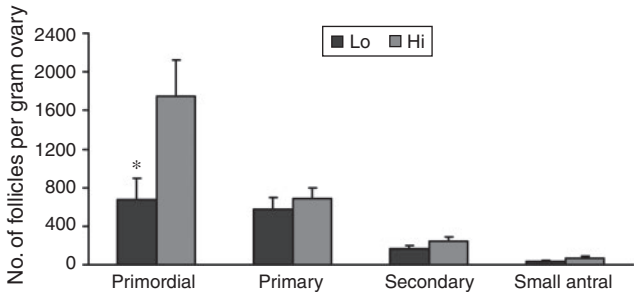


**Fig. 2.** Total number of primordial, primary, secondary and small antral follicles per g ovary in bovine 'Lo' (i.e. <10 mid-antral follicles) and 'Hi' (i.e. >10 mid-antral follicles) ovaries. Data are the mean ± s.e.m. ($n = 20$ in each group). *$P < 0.05$ compared with Hi ovaries (*t*-test, preceded by the Levene test).

Interestingly, comparing the number of follicles at each stage of folliculogenesis between the two groups revealed that the total number of primordial follicles per g ovary was lower in Lo compared with Hi ovaries ($P < 0.05$; Fig. 2), whereas there were no differences in follicle numbers in the other follicle classes. In addition, the percentage of primordial healthy follicles per g ovary was lower in Lo compared with Hi ovaries, whereas there were no differences in the health status between the two groups of primary and secondary follicles. At the small antral stage, there was a lower percentage of healthy follicles in the Hi compared with Lo ovaries ($P < 0.01$; Fig. 3). Variations in mid-AFC exhibited significant positive correlations with ovarian weight ($r = 0.86$; $P < 0.01$), the number of healthy mid-antral follicles ($r = 0.90$; $P < 0.01$) and the number of atretic mid-antral follicles ($r = 0.86$; $P < 0.01$; Table 4). Finally, variations in mid-AFC were positively correlated with the number of healthy ($r = 0.57$; $P < 0.01$) and healthy plus atretic ($r = 0.52$; $P < 0.01$) follicles at earlier stages of development (as the sum of primordial, primary, secondary and small antral follicles; Table 5).

### *Evaluation of ovarian cortical and perifollicular stroma*

Histochemical evaluation of ovarian cortical and perifollicular stroma was conducted on 22 ovaries in each of the Lo and Hi groups. In Lo ovaries, the cortical stroma was generally characterised by dense and compacted collagen fibres arranged in thick bundles (Fig. 4*a*). In contrast, in Hi ovaries, the ovarian stroma had mostly a fine organisation (Fig. 4*b*). Further analysis revealed that the perifollicular stroma of mid-antral follicles was organised in a network of collagen fibres of various densities. Thus, mid-antral follicles were classified as enclosed in a thick

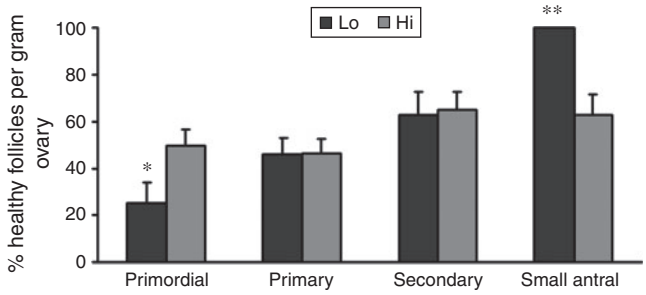


**Fig. 3.** Percentage of morphologically healthy primordial, primary, secondary and small antral follicles in bovine 'Lo' (i.e. <10 mid-antral follicles) and 'Hi' (i.e. >10 mid-antral follicles) ovaries. Data are the mean ± s.e.m. ($n = 20$ in each group). *$P < 0.05$, **$P < 0.01$ compared with Hi ovaries (*t*-test preceded by the Levene test).

**Table 4. Pearson's correlation coefficients for the number of mid-antral follicles, ovary weight and mid-antral follicles status in cattle ovaries**

*$P < 0.01$. Mid-AFC, number of mid-antral follicles

| | Mid-AFC | Ovarian weight | No. healthy mid-antral follicles | No. atretic mid-antral follicles |
|---|---|---|---|---|
| Mid-AFC | 1 | 0.86* | 0.90* | 0.86* |
| Ovarian weight | | 1 | 0.70* | 0.82* |
| No. healthy mid-antral follicles | | | 1 | 0.55* |
| No. atretic mid-antral follicles | | | | 1 |

**Table 5. Pearson's correlation coefficients for the number of mid-antral follicles and the population of primordial, primary, secondary and small antral follicles in cattle ovaries**

*$P < 0.05$, **$P < 0.01$. Mid-AFC, number of mid-antral follicles

| | Mid-AFC | No. healthy early stage follicles | No. atretic early stage follicles | Total no. healthy and atretic early stage follicles |
|---|---|---|---|---|
| Mid-AFC | 1 | 0.57** | 0.28 | 0.52** |
| No. healthy early stage follicles | | 1 | 0.34* | 0.80** |
| No. atretic early stage follicles | | | 1 | 0.83** |
| Total no. healthy and atretic early stage follicles | | | | 1 |

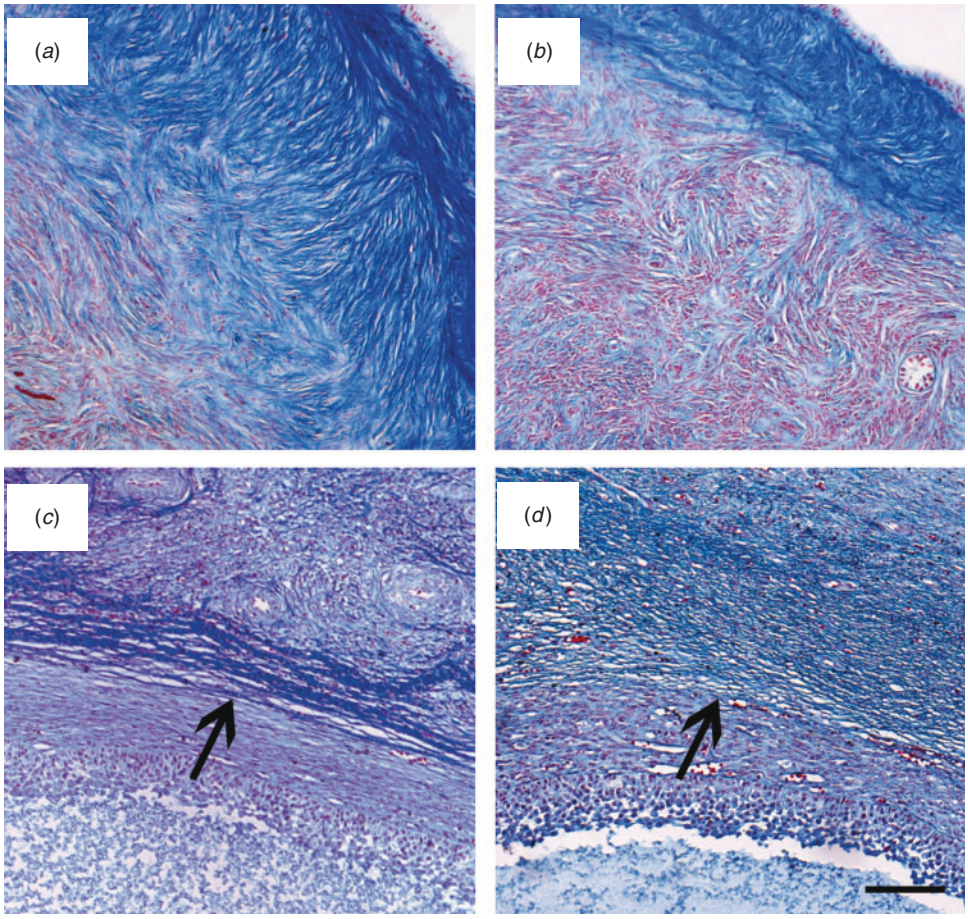


**Fig. 4.** Evaluation of ovarian (*a*, *b*) cortical stroma and (*c*, *d*) perifollicular stroma, stained with Heidenhain's Azan Trichrome in (*a*, *c*) 'Lo' (i.e. <10 mid-antral follicles) and (*b*, *d*) 'Hi' (i.e. >10 mid-antral follicles) ovaries. Nuclei are stained dark red and connective tissue collagen fibres are stained blue. (*a*) Collagen fibres are arranged in thick bundles and the cortical stroma is thick and compact. (*b*) The collagen fibres are thinner and they are arranged in a fine network. (*c*, *d*) Representative images of healthy mid-antral follicles enclosed in a thick (*c*) or thin (*d*) layer of collagen fibres (arrows). Scale bar = 100 μm.

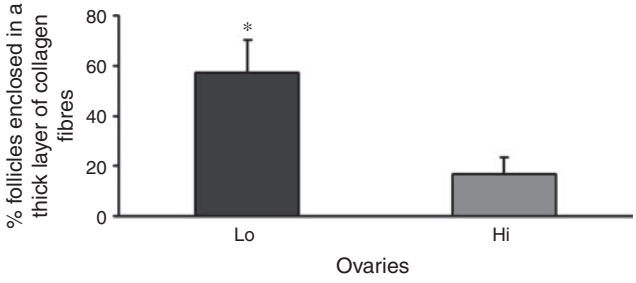


**Fig. 5.** Percentage of healthy mid-antral follicles enclosed in a thick layer of collagen fibres in bovine 'Lo' (i.e. <10 mid-antral follicles) and 'Hi' (i.e. >10 mid-antral follicles) ovaries. Data are the mean ± s.e.m. ($n = 66$ follicles from 22 ovaries in each group). *$P < 0.05$ compared with Hi ovaries (*t*-test preceded by the Levene test).

(Fig. 4*c*) or thin (Fig. 4*d*) layer of collagen fibres. The percentage of follicles enclosed in a thick layer of collagen fibres was significantly higher in Lo compared with Hi ovaries ($P < 0.05$; Fig. 5).

### *Hormone concentrations in follicular fluid*

Hormone concentrations were determined in the follicular fluid aspirated from mid-antral follicles of 44 Lo and 60 Hi ovaries. Lower concentrations of E2 and AMH were detected in Lo compared with Hi ovaries, in addition to a decreased E2/P4 ratio ($P < 0.05$). In contrast, P4 concentrations were higher in Lo compared with Hi ovaries ($P < 0.05$; Fig. 6).

## Discussion

The present study demonstrates a direct relationship between reductions in mid-AFC and markers of ovarian premature senescence in dairy cows of reproductive age. In the present study, 4- to 8-year-old dairy cows with Lo ovaries exhibited decreased ovarian size and an increase in the ovarian stroma, a decline in the ovarian reserve and a reduction in AMH concentrations in the follicular fluid compared with the age-matched control group (Hi ovaries). Remarkably, these features, in addition to decreased E2 and increased P4 concentrations in the follicular fluid, correlate with embryonic developmental

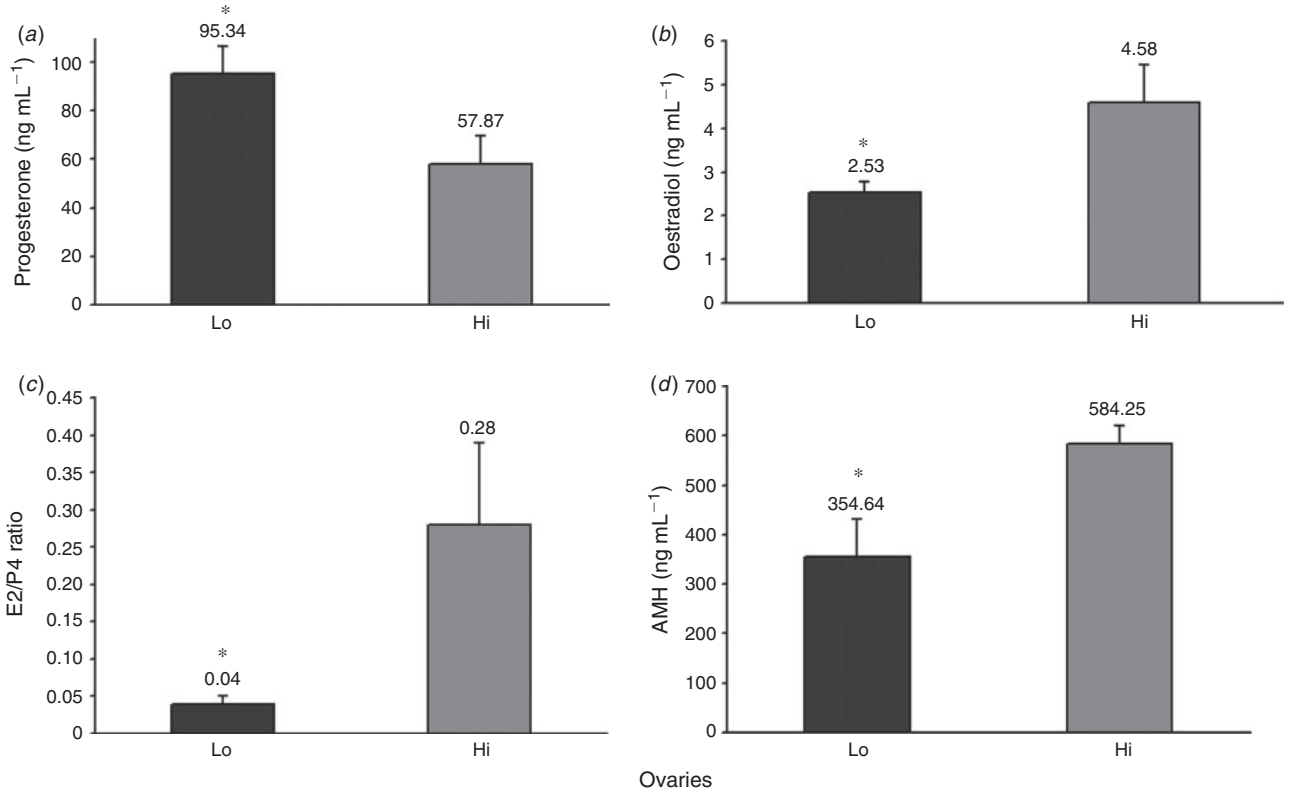


**Fig. 6.** (*a*) Progesterone (P4), (*b*) oestradiol (E2), (*c*) E2 : P4 ratio and (*d*) anti-Müllerian hormone (AMH) concentrations in the follicular fluid of mid-antral follicles from bovine 'Lo' (i.e. <10 mid-antral follicles) and 'Hi' (i.e. >10 mid-antral follicles) ovaries. Concentrations of E2 and P4 were determined in 10 Lo and 10 Hi ovaries; AMH concentrations were determined in 34 Lo and 50 Hi ovaries. Data are the mean ± s.e.m. $*P < 0.05$ compared with Hi ovaries (*t*-test preceded by the Levene test).

competence because oocytes collected from animals with Lo ovaries have a significantly lower *in vitro* developmental competence than oocytes from animals with Hi ovaries (Gandolfi *et al.* 1997; Modina *et al.* 2007; Tessaro *et al.* 2011).

Over the past two decades, significant fertility reduction in dairy cows has been described. Currently, up to 50% of dairy cows exhibit abnormal post partum oestrous cycles and ovarian dysfunction resulting in an increase in the calving to first insemination interval (Sakaguchi 2011; Walsh *et al.* 2011) and a decrease in conception rates (Garnsworthy *et al.* 2009). This is an important issue because reproductive inefficiency has serious economic impacts by decreasing milk yield and the number of calves born, and by increasing the cost of veterinary services and the culling rate (Gröhn and Rajala-Schultz 2000; Sakaguchi 2011).

Several lines of evidence indicate that ovarian dysfunction and/or reduced oocyte competence may play an important part in reducing fertility (Lucy 2001; Lucy 2007). For example, Opsomer *et al.* (1998) analysed ovarian dysfunction in 335 high-yielding dairy cows of reproductive age (after calving) and found that a high percentage of cows (almost 23%) suffered delayed cyclicity or anovulation and a prolonged luteal phase. Interestingly, most of these animals had inactive ovaries that were, as determined by rectal palpation, small and hard, with limited or absent follicular development. In the present study, approximately 5% of the dairy cows examined were found to have comparable macroscopic characteristics: they had ovaries with a reduced number of mid-antral follicles and were fibrous. In the present study, specific histochemical analyses were used to better characterise the Lo phenotype ovary. The analyses revealed the presence of a compact stroma encapsulating the few healthy mid-antral follicles localised in Lo ovaries; this increased perifollicular ovarian stroma may contribute to the scarce ingrowth of capillaries into the theca, as reported recently for these follicles (Tessaro *et al.* 2011), and contributes to the isolation of the mid-antral follicles from ovarian environment.

Interestingly, healthy mid-antral follicles from Lo ovaries are also characterised by reduced eNOS expression, which may be related to increased oxidative stress due to vascular defects, and reduced NO availability in the follicular fluid (Tessaro *et al.* 2011). Thus, we can hypothesise that changes in the uptake of nutritional and regulatory molecules may contribute to the low quality of oocytes from Lo ovaries of dairy cows of reproductive age, as suggested for menopausal woman (Tatone *et al.* 2008). Conversely, the depletion of ovarian follicles would likely result in an increase in cortical ovarian stroma, accounting for the changes in ovarian morphology of Lo ovaries.

In the present study, Lo ovaries exhibited a phenotype resembling menopausal gonads that have been described in

young women affected by POF (Haidar *et al.* 1994). POF is a heterogeneous disorder characterised by hypergonadotropic hypogonadism before the age of 40, which is preceded by a phase of accelerated ovarian senescence (Kokcu 2010) that results in follicle reduction (Shelling 2010). Two major reasons for the development of POF have been proposed: (1) failure to acquire an adequate number of initial primordial follicles, which normally takes place during fetal life; and (2) excessive clearance of primordial follicles, together with the suppressed activation and further development of primordial follicles (Jagarlamudi *et al.* 2010). The aetiology of most of cases of POF is idiopathic; however, this condition has a heterogenous background (Nelson 2009; De Vos *et al.* 2010) and, in the past decade, an increasing number of genes has been implicated in premature ovarian failure (Goswami and Conway 2005; Skillern and Rajkovic 2008; van Dooren *et al.* 2009; Persani *et al.* 2010; Cordts *et al.* 2011).

Light and transmission electron microscopy studies have led to the identification of two POF phenotypes: follicular and afollicular POF ovaries (Haidar *et al.* 1994; Massin *et al.* 2004, 2008). Massin *et al.* (2004) described POF follicular ovaries as normal-sized ovaries characterised by the presence of both non-growing and growing follicles at various stages of development, whereas POF afollicular ovaries are smaller in size and do not contain any follicles. In contrast, Haidar *et al.* (1994) described both follicular and afollicular ovaries as diminished in volume, fibrous in consistency and rich in irregular spirals of cells and fibres in the cortical region. Regardless of these classifications, our observations suggest that bovine ovaries with low mid-AFC are comparable to POF ovaries, indicating early ovarian senescence. This idea is supported by the fact that, in our model (population of cows with Lo ovaries), the quantity of mid-antral follicles on the ovarian surface exhibited a significant positive correlation with the total number of primordial follicles, as described previously in dairy cows (Cushman *et al.* 1999) and in women during the perimenopausal period (Hansen *et al.* 2008; Broekmans *et al.* 2009) or affected by POF (De Vos *et al.* 2010; Monget *et al.* 2012). This observation is important because exhaustion of the pool of primordial follicles within the ovarian cortex is the cause of primary ovarian insufficiency in most young women (De Vos *et al.* 2010).

The percentage of atresia was higher in animals with Lo than Hi ovaries; it is thus reasonable to assume that, in our model, there is an accelerated clearance of the pool of primordial follicles and, in turn, the reduced follicle count could explain the decreased fertility in cows with Lo ovaries. However, the mechanisms responsible for ovarian insufficiency and primordial follicle depletion remain to be determined and several factors may be involved.

In a previous study, Ireland *et al.* (2008) observed that young adult cattle with a high AFC had a greater number of morphologically healthy follicles in all follicular stages analysed than their age-matched counterparts with a low AFC. In contrast, our data show that the percentage of healthy follicles in all follicular stages analysed was similar between the two groups, whereas the population of healthy primordial follicles was significantly compromised in Lo ovaries. However, in their study, Ireland *et al.* (2008) used cross-bred beef heifers that were 10–14 months old, previously synchronised. Thus, breed, lactating status, age and hormonal treatments may account for the discrepancies between these two studies, including the unexpected higher percentage of healthy early antral follicles in Lo compared with Hi ovaries.

Analysing the follicles as individual classes, as described previously (Rodgers and Irving-Rodgers 2010), we observed a certain level of atresia at all stages of folliculogenesis, including the mid-antral follicle stage, in both Lo and Hi ovaries. Moreover, at the mid-antral follicle stage, the percentage of healthy (and atretic) follicles was similar between the Lo and Hi ovaries. Thus, we would have expected the follicular fluid of mid-antral follicles from both categories to have similar concentrations of E2, P4 and AMH, because these hormones are known to be related to follicular health status (Irving-Rodgers *et al.* 2003; Rico *et al.* 2011). However, we found higher P4 and lower E2 concentrations (and a lower E2 : P4 ratio) in Lo compared with Hi ovaries. These observations are consistent with the main features of 'basal atresia' described by Irving-Rodgers *et al.* (2001), which has been defined as a form of atresia in which cell death commences within the basal regions of the membrana granulosa and differs from 'antral atresia', in which cell death is initiated within the antral compartment. In particular, basal atretic follicles have been described as those $<5$ mm in diameter that have substantially elevated P4 and decreased androstenedione, testosterone and E2 concentrations compared with healthy follicles and follicles affected by 'antral atresia' (Irving-Rodgers *et al.* 2003). It is important to mention that the early morphological features of basal atresia can be recognised unequivocally only at the ultrastructural level and that is difficult to distinguish these follicles using standard histological evaluation; in addition, oocytes collected from these follicles are of a significantly poorer quality than either healthy follicles or follicles affected by 'antral atresia' (Irving-Rodgers *et al.* 2010).

Analysis of the follicular fluid revealed lower AMH concentrations in mid-antral follicles from Lo ovaries, further supporting the hypothesis that these follicles were affected by basal atresia. We assume that the reduced AMH concentrations may be the consequence of decreased protein production by the mural cells lying immediately on the basal membrane of the follicles. In fact, it has been demonstrated that, in antral follicles, the outer layer of granulosa cells close to the theca is one of the regions of high AMH expression where expression declines sharply in follicles undergoing atresia (Rico *et al.* 2009, 2011).

There were no significant differences in AMH content in the follicular fluid of 3- to 5-mm diameter antral follicles from dairy cows with either a high or low number of this follicles size class in the ovaries (Rico *et al.* 2011). However, in that study, the cows were classified as 'high' or 'low' on the basis of their responses to superovulation and, using this classification, resulted in differences in the number of 3- to 5-mm follicle class. This approach and the absence of further information regarding the characteristics of the ovaries and the healthy and/or atretic status of 3- to 5-mm diameter antral follicles do not enable the results of that study to be compared with those of the present study.

Decreased AMH expression has been observed in granulosa cells of early antral follicles from women affected by POF (Meduri *et al.* 2007), in addition to low plasma AMH concentrations (Dumesic and Abbott 2008; La Marca *et al.* 2009), as a result of a reduction in the number of growing follicles (Knauff *et al.* 2009). Thus, we can speculate that, in our model, AMH concentrations in the follicular fluid are predictive of oocyte quality and, in turn, may provide further evidence of precocious ovarian aging. This condition may be involved in the premature decline in fertility in dairy cows of reproductive age.

The present study has shown, for the first time, that within the population of culled dairy cows, those cows with ovaries in which there is a reduced number of mid-antral follicles may be affected by POF. Moreover, the results of the present study contribute to the identification of the mechanisms and factors involved in reduced fertility. This is particularly relevant because, in the dairy industry, reproductive disorders are a major cause of economic losses. Finally, considering that the number of antral follicles in cattle is very highly repeatable and stable within individuals (Burns *et al.* 2005), dairy cows with a Lo phenotype ovary may represent a suitable model and be a readily accessible source of ovaries and oocytes for future studies into the mechanisms controlling premature ovarian aging and oocyte developmental competence in single-ovulating species.

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