

Postnatal pituitary and follicular activation: a revisited hypothesis in a sheep model

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Abstract

The importance of postnatal pituitary activation as regards female reproductive development is not yet understood. By taking advantage of the experimental model developed in a previous study, i.e. ewe lambs expressing markedly different ovarian phenotypes at 50 days of age, we designed this study to determine whether differences found in ovarian status during the early prepubertal period are due to different patterns of postnatal pituitary activation, and to assess whether these differences have long lasting effects on subsequent reproductive performance. Results showed that ewe lambs with high antral follicle count (AFC) at 50 days of age had significantly lower plasma FSH concentrations and higher anti-Mullerian hormone (AMH) concentrations during the first 9 weeks of age compared with low AFC ewe lambs ($P < 0.0001$). With a longitudinal experiment we showed that a high AFC in the early prepubertal period is associated with consistently higher AMH concentrations and numbers of antral follicles up to the postpubertal period, and with higher pregnancy rates in the first breeding season. In addition, the effect of age in decreasing AMH concentrations was more marked in the low AFC group. Results of the present study demonstrate that ewe lambs undergo different patterns of postnatal pituitary activation. A high AFC at 50 days of age indicates an advanced phase of ovarian maturation, which was accompanied by constantly higher AMH concentrations up to the postpubertal period, a greater ovarian response to FSH stimulation and by higher pregnancy rates at first mating, as compared with the low AFC group.

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Introduction

The reproductive axis and its hormonal control systems are largely established in fetal life, thus the *in utero* environmental conditions influence their programming and consequently the phenotype expressed later in life (Zambrano *et al.* 2014). However, according to the perinatal programming theory, early postnatal conditions also have long-lasting physiological effects (Gluckman *et al.* 2008), and recent studies evidenced that they can influence the newborn's future reproductive performance (Sloboda *et al.* 2009).

The first months of life provide a window of opportunity to examine the function of the

hypothalamic–pituitary–gonadal (HPG) axis before puberty (Kouri-Hänninen *et al.* 2014). Babies born small for gestational age have decreased ovarian volume, reduced ovulation rate and increased follicle stimulating hormone (FSH) concentrations when reaching puberty denoting that prenatal growth has endocrine effects that affects reproduction later in life (Chellakooty *et al.* 2003). In the context of the perinatal programming theory, this period might be important for further reproductive health and disease and therefore our group proposes a sheep model to the study of this relevant period and its later consequences.

During the first postnatal months an important phase in normal reproductive development takes place.

The HPG axis is transiently activated, and elevated concentrations of gonadotropins are produced (Forest *et al.* 1980, Schmidt & Schwarz 2000, Kuiri-Hänninen *et al.* 2011a,b) in a phenomenon referred to as 'minipuberty'. These hormonal changes are probably triggered by the decline after birth in sex steroid-mediated inhibition of gonadotropin secretion exerted by maternal placental and gonadal steroids (Lee 2003). In infant boys, the postnatal pituitary activation is associated with testicular testosterone secretion, penile and testicular growth, and an increase in the number of Sertoli and germ cells (Kuiri-Hänninen *et al.* 2011a), and this period is therefore considered to be an important phase in reproductive development in males. In infant girls even though reproductive hormones, especially FSH, exhibit large variations between diverse individuals (Chellakooty *et al.* 2003), circulating FSH concentration transiently increase during postnatal pituitary activation (Beck-Peccoz *et al.* 1991, Kuiri-Hänninen *et al.* 2011b) reaching concentrations similar to those observed in periovulatory women. Anti-Müllerian hormone (AMH) concentrations also increase, peaking a few months after birth (Hagen *et al.* 2010). A similar situation has been seen in cattle suggesting that the heightening in the follicular recruitment was produced by the remarkable increase in FSH concentration that occurs early after birth, then later falls due to an increase in the secretion of steroid and inhibin from the numerous antral follicles that have grown during the previous weeks (Rawlings *et al.* 2003, reviewed by Hernandez-Medrano *et al.* (2012)). Likewise, high plasma FSH concentrations have been observed also in ewe lambs in the first 2 months of life (Savoie *et al.* 1979, McNatty *et al.* 1998, Mahdi & Khallili 2008). However, the possible importance of this activation as regards female reproductive development is not yet understood.

It is assumed that AMH has some role in folliculogenesis (Gruijters *et al.* 2003). AMH is produced in granulosa cells of developmentally advanced growing follicles, preantral, and small antral follicles (Weenen *et al.* 2004), and declines at the time of follicle selection (Jeppesen *et al.* 2013). In adult ewes, AMH controls the rate at which follicles progress to the gonadotropin-dependent stage from the gonadotropin-responsive stage (Campbell *et al.* 2012). FSH may play a role in the down-regulation of AMH expression when small antral follicles differentiate into large antral follicles (Baarends *et al.* 1995) with numerous studies reporting a negative correlation between concentrations of FSH and AMH (Fanchin *et al.* 2003, Silberstein *et al.* 2006) with decreased AMH production when high FSH levels are present (Rico *et al.* 2011, reviewed by Monniaux *et al.* (2013)). The effect of AMH over follicles sensitivity to FSH is contradictory. For some authors AMH seems to inhibit follicles sensitivity to FSH (Pellatt *et al.* 2011), while other authors point that AMH enhances the effect of FSH (McGee *et al.* 2001).

Despite the advanced knowledge in adult physiology, many unknowns regarding the link between AMH, follicular development, and FSH in the early prepubertal period need to be elucidated.

In a previous study in ewe lambs (Torres-Rovira *et al.* 2014) we described significant inter-individual differences in antral follicle count (AFC) in the early prepubertal period. These differences were linked to differences in circulating AMH concentrations, follicular response to exogenous FSH administration, and oocyte quality. On the other hand, and in contrast to major findings in adult ovaries, AFC was not predictive of differences in either the number of healthy follicles or the size of the primordial follicle pool in prepubertal ovaries. We speculated that during the early prepubertal period follicular recruitment and development reflect the changes in the endocrine milieu and are preparatory to the subsequent development of the reproductive function.

As the early postnatal development of antral follicles could be linked to FSH stimulation, we hypothesize that the observed inter-individual differences in ovarian phenotype and function in the early prepubertal period might be related to differences in the pattern of postnatal pituitary activation. In our opinion, sheep is a suitable animal model for the study of the 'minipuberty' phenomenon with a lower cost, shorter gestation length and faster development than larger animals and superior developmental similarities to humans in terms of prenatal programming and developmental stages than the usual rodent model. Thus, the present study was carried out to determine if differences found in ovarian status during the early prepubertal period are due to different patterns of postnatal pituitary activation, and to assess whether these differences have long lasting effects on the subsequent reproductive performance. To achieve these objectives, we took advantage of the experimental model developed in a previous study, i.e. ewe lambs expressing markedly different ovarian phenotypes at 50 days of age (Torres-Rovira *et al.* 2014). In the present study we studied pituitary (FSH concentrations) and ovarian (AMH concentrations) function during the first weeks after birth in ewe lambs with a high (≥ 30 follicles) and a low (≤ 15 follicles) AFC at 50 days of age. Then, to determine eventual long lasting effects, in the same experimental model we assessed the response to the exogenous FSH ovarian reserve test (EFORT) at 50, 195 (6.5 months – peripubertal) and 496 days of age (16.5 months – postpubertal). Finally, we assessed pregnancy rates at first breeding season.

Materials and methods

The experimental procedures with animals (sheep, *Ovis aries*) were approved by the Animal Care and Use Committee of the University of Sassari, Italy. All experimental procedures were carried out at the experimental facilities of the Department of

Animal Production, AGRIS Sardegna, Bonassai, Sassari, Italy (latitude: 40° 40' 26" – longitude: 8° 22' 1"). These facilities meet the requirements of the European Union for Scientific Procedure Establishments. The experimental procedures followed ethical guidelines for care and use of agricultural animals for research (EC Directive 86/609/EEC for animal experiments). All the animals used were Sarda ewes and lambs housed outdoors with indoor access, and fed with a live-weight maintenance ration.

All reagents and media were from Sigma Chemical Co. unless otherwise specified.

Study 1. Pituitary and ovarian function during the postnatal period in ewe lambs with high and low AFC

This study was conducted to assess whether the expression of different ovarian phenotypes in the early prepubertal period is linked to differences in the pattern of postnatal pituitary activation in ewe lambs. Blood samples from December 2012/January 2013 born Sarda ewe lambs ($n=146$) were taken weekly from the week of birth until 9 weeks of age with vacuum blood evacuation tubes containing Lithium Heparin (Vacutainer Systems Europe, Becton Dickinson, Meylan Cedex, France). Immediately after recovery, blood samples were centrifuged at 1500 *g* for 10 min at 4 °C and plasma was removed and stored at –20 °C. At 6 weeks old ewe lambs were classified by transrectal ultrasonography according to the number of ≥ 2 mm follicles in the ovaries and assigned into two experimental groups: high AFC (≥ 30 follicles) and low AFC (≤ 15 follicle), as described in a previous study (Torres-Rovira *et al.* 2014). Plasma samples from 50 ewe lambs in the categories high AFC ($n=25$) and low AFC ($n=25$) were analyzed to study circulating hormone concentrations (AMH and FSH) from birth to 9 weeks of age.

Hormone analyses

To measure FSH concentrations the FSH (sheep) ELISA kit (Abnova, Neihu District, Taipei City, Taiwan), specific for sheep FSH, was used following the manufacturer instructions in an automatic ELISA analyzer (Personal LAB, Adaltis, Italy). The intra- and inter-assay coefficients of variability were <15% for both.

AMH concentration was measured in sheep plasma by using the AMH Gen II ELISA kit (Beckman Coulter, Inc., Brea, CA, USA) as previously described (Lahoz *et al.* 2012, Estienne *et al.* 2015). AMH concentrations were determined in 50 μ l aliquots of undiluted plasma. The sensitivity (limit of detection) of the assay was 0.08 ng/ml, while the inter- and intra-assay variation coefficients were 5.6 and 5.4% respectively. For AMH assay in samples recovered during the EFORT tests, EDTA (1.8 mg/ml, final concentration) was added to plasma samples and to the standards before assay, thus allowing an improvement of the sensitivity of the assay to 0.02 ng/ml (Rico *et al.* 2012). The AMH concentration given to animals when AMH was not detectable was assay's limit of detection, 0.02 ng/ml.

Study 2. Responsiveness of follicular population to the exogenous EFORT in the pre and postpubertal period

Forty eight Sarda ewe lambs from the same flock, with a mean age of 49.77 ± 1.15 day-old (born in December–January) and a mean body weight of 11.82 ± 0.34 kg at the beginning of the study, were selected according to their ovarian phenotype (low AFC, $n=24$; high AFC, $n=24$) determined by characterizing the AFC by transrectal ultrasonography as described in a previous study (Torres-Rovira *et al.* 2014). Animals were maintained together under the same conditions and diet throughout the experimental procedure, being housed outdoors with indoor access. Age and birth weight, were analyzed to confirm that there were no significant differences that may justify *per se* different ovarian phenotypes. Initial weight, when ewe lambs were classified into the two experimental groups, and peripubertal weight were also recorded.

The EFORT test (Fanchin *et al.* 1994, Kwee *et al.* 2006) was made before puberty at an early prepubertal age (50 days of age), at a peripubertal age (195 days of age) since Sarda sheep usually reach puberty at 7–8 months of age (Carcangiu *et al.* 2005) and at a postpubertal age (496 days of age) during non-breeding season for this breed and latitude. Briefly, on Day 0 the AFC was determined by transrectal ultrasonography, with a real-time B-mode scanner (Aloka SSD 500, Aloka Co., Tokyo, Japan) fitted with a 7.5 MHz linear-array probe appropriate to the size of the animal (rigid laparoscopic transducer UST-5526L-7.5, Aloka Co. at 50 days of age and 82 mm prostate transducer UST-660-7.5, Aloka Co. at 195 and 496 days of age). Ultrasounds were carried out as previously described and validated (Gonzalez-Bulnes *et al.* 1994). The number of follicles for each category (total, 2, 3 and ≥ 4 mm in diameter) was written down. Afterwards, one-shot intramuscular dose of 105 IU of porcine FSH (Folltropin, Bioniche Animal Health, Minitublbérica, S.L., Reus, Spain) was administered. In order to accommodate to the larger size of the animals, for the EFORT performed after puberty (496 days of age), the intramuscular FSH dose was increased to 175 IU. Twenty-four hours later (Day 1), the growth of the antral follicles was assessed by a second ultrasonographic scanning of the ovaries. The response to ovarian stimulation was evaluated by the difference in the number of follicles between Day 1 and 0 for each follicular category, understood as the number of new follicles. The assessment of AFC has inter-observer and intra-observer variations (Jayaprakasan *et al.* 2008), for that reason in order to minimize the operator-dependent variability the same expert operator performed the ultrasonographic scanning in the three EFORTs.

Blood samples were drawn with vacuum blood evacuation tubes containing lithium heparin (Vacutainer Systems Europe) on Day 0 and Day 1. Immediately after recovery, blood samples were centrifuged at 1500 *g* for 15 min at 4 °C and plasma was removed and stored at –20 °C until assayed for AMH determination, as described in Study 1.

For the EFORT test performed after puberty (496 days of age), the number of animals was reduced to nine animals in the high AFC group and 15 animals in the low AFC group because only animals that did not delivered were used due of the impossibility of hormonal injection in lactating animals.

Study 3. Reproductive function in adulthood

This study was conducted to determine if the differences in plasma AFC and AMH concentrations seen at an early prepubertal age in Sarda ewe lambs with high or low AFC would entail differences in pregnancy rate at first breeding season. Animals from Study 2 were used in the current experiment. Two ewes from the low AFC group died and two more (one from each AFC group) were momentarily excluded from the experiment at first breeding season due to health problems, thus, pregnancy rate at first breeding season was assessed in 44 ewes ($n=23$ high AFC group, $n=21$ low AFC group).

Following the usual reproductive practices for ewe lambs that will replace the base sheep population of the centre (Department of Animal Production, AGRIS Sardegna), rams were introduced into the ewe flock from August to the end of September. Twenty days after the removal of the rams from the flock, pregnancy diagnosis was performed using transrectal ultrasonography (Aloka SSD 500, fitted to 82 mm prostate transducer UST-660-7.5, Aloka Co.). Pregnant sheep displayed enlargement of the uterine horns, embryo heartbeat was evidenced and in more advanced stages of pregnancy placentomes were seen.

Statistical analyses

Statistical analysis was performed using the statistical software program Statgraphic Centurion XV (Version 15.2.06 for Windows; StatPoint, Inc., Herndon, VA, USA) and a probability of $P<0.05$ was considered to be the minimum level of significance. Results are expressed as mean \pm S.E.M.

Study 1

Differences between high and low AFC groups in birth weight were determined by ANOVA. Differences in plasma AMH and FSH concentrations between the two experimental groups were assessed by general linear model (GLM) where: $Y = \mu + \text{week of age} + \text{group} + \text{week of age} \times \text{group} + \text{ewe lambs}$. Age and group were considered fixed factors and ewe lambs a random factor. The method used to discriminate between the means was Fisher's least-significant-difference (l.s.d.) procedure. The probabilities obtained by the l.s.d. test were corrected by Bonferroni's correction for multiple comparisons. Possible correlations between plasma AMH and FSH concentrations were determined.

Study 2

Differences between high and low AFC groups in age and weight at the beginning of the study, birth and peripubertal weight were determined by ANOVA. Differences in follicle numbers and AMH concentrations at different ages, between the two experimental groups, and between Days 0 and 1 of treatment, were assessed by GLM. The method used to discriminate between the means was Fisher's l.s.d. procedure. The probabilities obtained by the l.s.d. test were corrected by Bonferroni's correction for multiple comparisons.

In addition, we used Generalized Linear Mixed Models (GLMMs) to analyze which factors influence the AMH concentration variability. Specifically, the expected values of AMH in each measure were related to independent variables, namely age of the individual, the group, and the day of treatment. The remaining potential source of variation on the AMH values could be due to the individual-specific differences in treatment. The specific individual behaviour caused by random aspects could have caused some of the variation in the data. Ignoring such non-independence of the data may lead to an invalid statistical inference. To remove any bias caused by individual-specific differences in the sample, a random effect was included in the model.

Except for the variable 'age of the individual', which is continuous, the other explanatory variables are all categorical: number of days of treatment ('0' and '1') and group ('high AFC' and 'low AFC').

Effects of categorical variables are considered for $k-1$ of the k factor levels, with the remaining one being considered as the base level. Hence the estimated coefficient of each factor level will indicate the deviation with respect to the value of the base level.

The fixed effects quantify the overall effects of factors on AHM concentrations; the random effects quantify the variation across individuals of the fixed-effect parameters.

To fit GLMMs we used the 'lme4' library of the R-software (RC Team 2014).

All the resulting models obtained from combining the mentioned variables and the respective interactions were fitted and compared. The Akaike information criterion (AIC) (Akaike 1974) was used as a measure for the goodness-of-fit. The smaller the AIC, the better the compromise between fit and parsimony.

Study 3

The χ^2 -test was used to determine differences in pregnancy rates at the first breeding season between high and low AFC groups.

Results

Study 1. Pituitary and ovarian function during the first weeks after birth in ewe lambs with high and low AFC

In the first week of age, AMH was detected in the 48% of the ewe lambs (12/25) from the high AFC group, while it was under the limit of detection for all ewe lambs from the low AFC group (25/25). At 4 weeks of age, AMH plasma concentrations from all ewe lambs from the high AFC group could be detected, while, for the low AFC group, the detection of AMH concentrations in all animals could not be reached until the 9th week.

Circulating FSH and AMH concentrations proved to be negatively correlated during the first 9 weeks of age in ewe lambs ($P<0.001$, correlation coefficient: -0.184). During this period, mean plasma FSH concentrations were significantly higher in the low compared with the high AFC group, while the opposite was found for

plasma AMH concentrations, being higher in the high compared with the low AFC group ($P < 0.0001$, Fig. 1). In addition, being an interaction between groups and age ($P < 0.0001$), FSH and AMH surge patterns differed significantly between the two experimental groups.

In ewe lambs with high AFC, the postnatal FSH surge was barely detected, as its concentrations reached values higher than those recorded at birth in the 2nd week of age ($P < 0.01$), and then remained relatively stable up until the 9th week of age (Fig. 1). On the other hand, in ewe lambs with low AFC, circulating FSH concentrations increased sharply soon after birth, peaked between the 2nd and 5th weeks of age, and then declined gradually until the 9th week of age ($P < 0.01$).

Regarding the trend of AMH concentrations during the study, in the low AFC group, although values progressively increased, this elevation was not significant in the first 9 weeks of age. However, in the high AFC group, there was a marked increase in AMH plasma concentrations that peaked in the 5th week of age (1.065 ± 0.095 ng/ml) to decline gradually from the 6th week ($P < 0.001$, Fig. 1).

Study 2. Responsiveness of follicular population to the exogenous EFORT in the pre and postpubertal period

There were no significant difference between animals from the high and low AFC groups either in the mean age (49.56 ± 1.72 day-old and 49.96 ± 1.55 day-old, for high AFC vs low AFC) and weight (12.38 ± 0.43 kg and 11.27 ± 0.51 kg, for high AFC vs low AFC) at the beginning of the study, or in the mean birth weight (3.58 ± 0.15 kg and 3.45 ± 0.10 kg, for high AFC vs low AFC) or peripubertal weight (27.73 ± 0.80 kg and 26.21 ± 1.00 kg, for high AFC vs low AFC) that could *per se* justify differences in the response to the EFORT.

The EFORTs performed in the peripubertal and postpubertal periods showed that the differences in the ovarian phenotypes observed in the early prepubertal period (50 days of age) between the high and low AFC groups, although less marked, were maintained at least during the first 16 months of age (Fig. 2). In the peripubertal period (195 days of age), although the number of total follicles (≥ 2 mm) did not differ between the two experimental groups at Day 0, follicular response to FSH administration was higher in the high AFC group compared with the low group. This difference was mainly due to a higher number of 2 mm follicles ($P < 0.0001$). In the postpubertal period (496 days of age), the high AFC groups still showed a higher number of total follicles, both before and after FSH administration ($P < 0.0001$). Differences in the follicular population were accompanied by differences in the circulating concentration of AMH. At every time point analyzed, its values were always higher in the high AFC group compared with the low group, both before and after FSH administration ($P < 0.0001$).

It should be pointed out that in the early prepubertal period the follicular population before FSH administration differed significantly in most follicular categories to that found in the peripubertal and postpubertal periods for both groups (Fig. 2). However, while in the high AFC group the total number of follicles observed at 50 days of age was drastically reduced at 195 and 496 days of age, mainly due to the decrease in the most abundant follicular category (2 mm follicles), in the low AFC group the opposite trend was seen, with an increase in the number of total follicles at 195 and 496 days of age compared with 50 days of age (Fig. 2). In both groups, follicles ≥ 4 mm in diameter increased with age ($P < 0.0001$). In both groups, circulating AMH concentrations dropped from the early prepubertal to the

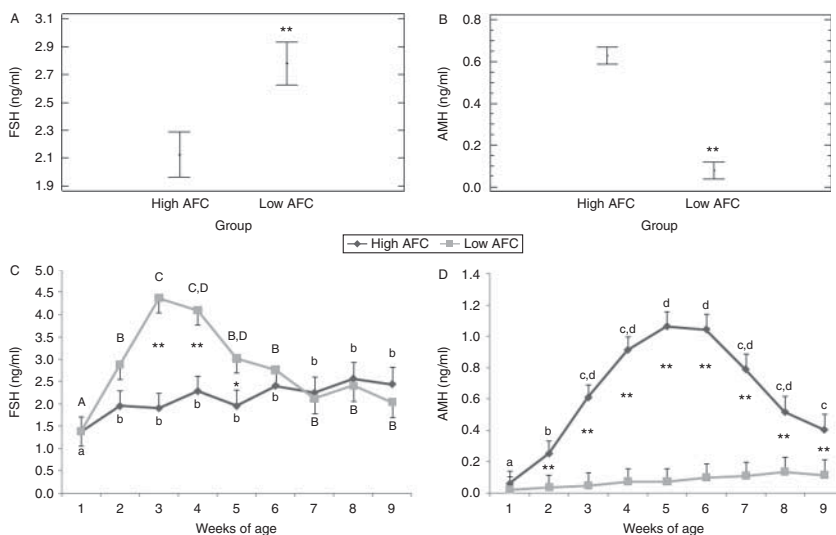


Figure 1 Differences in FSH (A and C) and AMH plasma levels (B and D) during the first 9 weeks of age in ewe lambs assigned to high AFC (≥ 30 follicles, $n = 25$) or low AFC group (≤ 15 follicles, $n = 25$) according to the ovarian phenotype expressed at 50 days of age. A and B represent Bonferroni's CI and the overall mean FSH (A) and AMH (B) concentrations regardless of age during the first 9 weeks for both AFC groups. Asterisks indicate significant differences between groups: ANOVA $*P < 0.05$; $**P < 0.0001$. Letters indicate significant differences among the weeks within the same group: ANOVA $P < 0.0001$. Upper case letters: low AFC group; lower case letters: high AFC group.

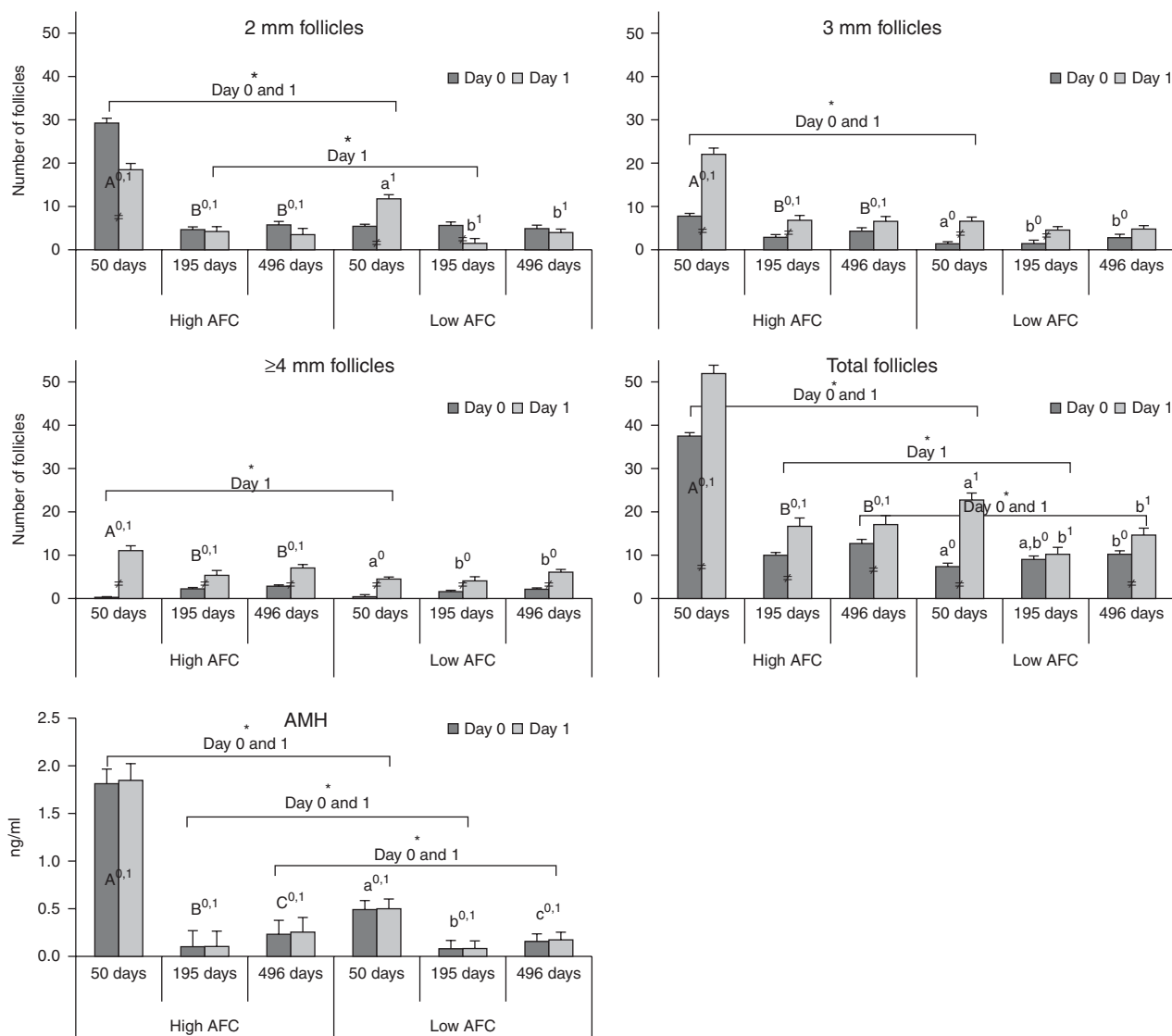


Figure 2 Follicle population and plasma AMH levels in ewe lambs assigned to high AFC (≥ 30 follicles) or low AFC group (≤ 15 follicles) according to the ovarian phenotype expressed at 50 days of age. Follicle population and AMH levels were determined both before (Day 0) and after (Day 1) FSH administration in the prepubertal (50 days of age; high AFC $n=24$, low AFC $n=24$), peripubertal (195 days of age; high AFC $n=24$, low AFC $n=24$), and postpubertal (496 days of age; high AFC $n=9$, low AFC $n=15$) period. Different letters indicate a statistical difference among different ages in the same group: GLM $P < 0.0001$. Upper case letters: high AFC group; lower case letters: low AFC group. The symbol \neq indicates differences in the number of follicles between Day 0 and Day 1 within the same group and at the same age. The lines indicate differences in the number of follicles between the two experimental groups at the same age.

peripubertal period, to rise again, even if not reaching values comparable with those recorded in the youngest age, in the postpubertal period.

The response to ovarian stimulation changed over time (Fig. 3). The major response to ovarian stimulation with exogenous FSH was obtained at 50 days of age with a greater number of total new follicles in both groups (21.92 ± 4.08 and 15.32 ± 4.04 for the high AFC group and the low AFC group respectively) than those obtained in subsequent EFORTs at 195 and 496 days of age (7.17 ± 1.06 and 4.22 ± 1.22 in the high AFC group at 195 and 496 days of age, 0.79 ± 0.74 and 4.07 ± 1.00 in

the low AFC group at 195 and 496 days of age). The number of new 3 mm and ≥ 4 mm follicles grown after FSH administration decreased at peripubertal and postpubertal age compared with the ones observed at an early prepubertal age. The decrease in the number of new 2 mm follicles was more marked in the high AFC group during the early prepubertal EFORT (-8.08 ± 1.91) than in the peripubertal (-1.58 ± 0.80) and postpubertal ones (-2.22 ± 1.27 , $P < 0.01$), but also the increase in the number of new follicles in larger categories was more marked during the early prepubertal EFORT. In the low AFC group there was an increase in

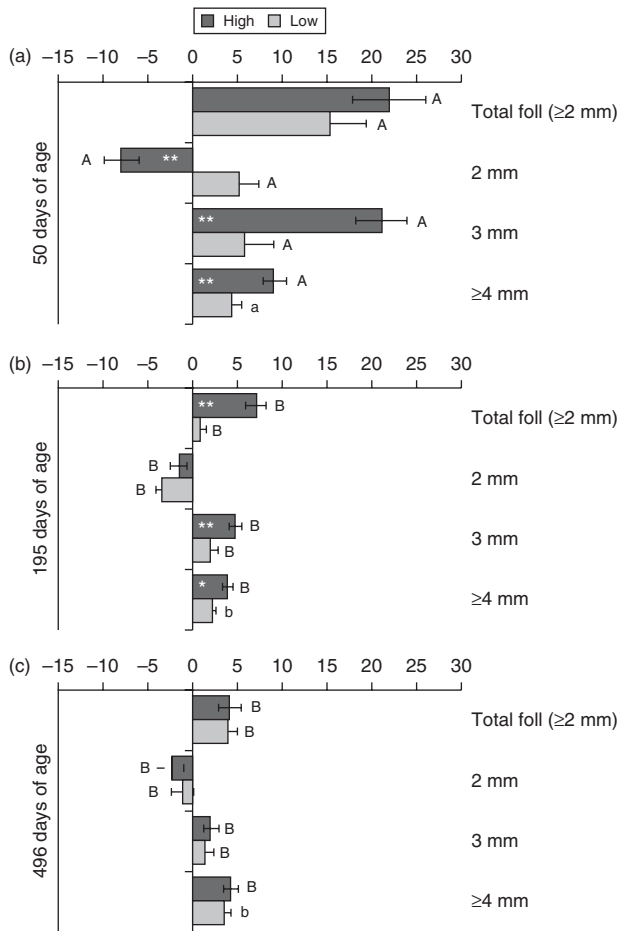


Figure 3 New follicles per follicular category 24 h after an ovarian stimulation test (EFORT) with FSH at 50 (a), 195 (b), and 496 (c) days of age in ewes classified according to their AFC (high ≥ 30 follicles, low ≤ 15 follicles) at an early prepubertal age (high AFC $n=24$, low AFC $n=24$ at 50 and 195 days of age; high AFC $n=9$, low AFC $n=15$ at 496 days of age). ^{a,b,A,B}Indicate a statistical difference in the same follicular category in the same group among different EFORTs (GLM): $a \neq b$ $P < 0.05$; $A \neq B$ $P < 0.01$. *Indicate a statistical difference between groups in the same follicular category from the same EFORT: * $P < 0.05$, ** $P < 0.01$.

the number of new 2 mm follicles recruited after exogenous gonadotrophin stimulation at 50 days of age (5.2 ± 2.20) while, at 195 and 496 days of age there was a reduction in the number of new 2 mm follicles (-3.46 ± 0.62 and -1.07 ± 1.19 respectively, $P < 0.01$).

The GLMM of AMH selected for its best fit (based on the lowest AIC) includes the group, the age, the random effect of the individual, and interaction between age and group as covariates. The proportion of deviance explained by this model was about 42%. Results showed that the low AFC group is the one with the lowest estimated circulating AMH concentrations (estimated mean = -483 , s.d. = (± 100)) with respect to the reference level (high AFC group). The age of the individual shows a negative relationship with estimated AMH (estimated mean = -1.5 , s.d. = (± 0.26)), i.e. plasma

AMH concentrations decrease with age. The interaction between the age and the group show a positive relationship (estimated mean = 2.33 , s.d. = (± 0.25)) for the low AFC group, with respect to the reference level (high AFC group), i.e. the decrease in plasma AMH concentrations with age is more marked in the low AFC group compared with the high one.

Study 3. Reproductive function in adulthood

Regardless of their AFC in the early prepubertal period, 22 ewes from the total of 44 used in Study 3 became pregnant in the first breeding season, which represents a 50% pregnancy rate in the flock. Pregnancy rate in the first breeding season significantly differs between experimental groups (65.2% for the high AFC group vs 33.3% for the low AFC group), with only seven of the 21 animals from the low AFC group becoming pregnant compared with 15 of the 23 ewes in the high AFC group ($P = 0.03$).

Discussion

The neonatal period is a critical stage in the process of sexual development and maturation. However, the significance of neonatal brain–pituitary–gonadal function has not been fully defined. The present study extends our knowledge on the linkage between FSH, AMH, and follicular dynamics during the postnatal period and on the effects of the stage of ovarian maturation during the early prepubertal period on subsequent reproductive function.

In the present study, by taking advantage of the experimental model developed in a previous work (Torres-Rovira *et al.* 2014), we found out that full term born ewe lambs with similar body weights show different patterns of postnatal pituitary activation. In particular, ewe lambs with high AFC at 50 days of age had significantly lower plasma FSH concentrations and significantly higher plasma AMH concentrations during the first 9 weeks of age compared with low AFC ewe lambs. These findings indicate a more advanced developmental stage of follicles in the group showing high AFC at 50 days of age. Moreover, they do not support the hypothesis that an increase in the postnatal FSH concentrations is needed to drive a greater follicular recruitment and stimulate the development of antral follicles during the first weeks after birth in sheep.

The interplay of FSH, AMH, and antral follicle development in the early prepubertal period has not been fully investigated to date. In a previous study, by using the same experimental model described here, we found that in 50 day-old ewe lambs, plasma AMH concentrations were positively related to AFC and to the number of large follicles grown after exogenous FSH administration (Torres-Rovira *et al.* 2014). The present

study further confirms these previous findings. During the first 9 weeks of age ewe lambs with an ovarian phenotype characterized by high AFC at 50 days of age had low circulating FSH concentrations and high circulating AMH concentrations, which peaked at 5–6 weeks of age. On the contrary, a low AFC phenotype at 50 days of age is accompanied by significantly higher FSH concentrations, which peaked at 3–4 weeks of age, and with constantly low circulating AMH concentrations.

During the prepubertal phase, AMH concentrations have been found to be negatively correlated with FSH concentrations also in cattle (Monniaux *et al.* 2013). Considering that the maturation of the negative feedback loop mediated by ovarian hormones on the hypothalamo–pituitary complex is attained *in utero* (Rhind *et al.* 2001, Kotsampasi *et al.* 2009), the low FSH concentration found in the high AFC group are likely to be caused by the production of inhibin and estrogen by the high number of growing follicles (Mann *et al.* 1992). Similar plasma FSH concentration would be found in the low AFC only 7 weeks later. Thus, the hormonal milieu during the first 6 weeks of age differs significantly in ewe lambs from the two experimental groups, and it is likely that FSH does not drive the first wave of follicular development that occurs after birth in lambs.

Contrary to what happens in adults (Rico *et al.* 2009), and as reported in the present study, AMH concentrations in the early prepubertal period are not constant. In girls, AMH is barely detected at birth (Hudson *et al.* 1990) and later shows an increase during the postnatal period (Hagen *et al.* 2010, Kelsey *et al.* 2011). Likewise, it has been shown that is a marked increase in AMH between 1 and 3 months of age in cattle also (Monniaux *et al.* 2013). In prepubertal Rasa aragonesa ewe lambs the maximum plasma AMH concentration has been seen at different ages (3, 4.5, or 6 months of age) in different ewe lambs (Lahoz *et al.* 2014). This finding may suggest the possibility of an increase in AMH concentrations later in life in the animals from the low AFC group. A recent study (Kuiri-Hänninen *et al.* 2011a,b) reported that in the postnatal period preterm girls had higher urinary FSH concentrations and lower serum AMH concentrations as compared with full term girls. In particular, authors evidenced that follicular development was delayed by ~9 weeks in preterm girls as compared with full-term girls, and that this delay was accompanied by an earlier and greater increase of serum AMH concentrations in full-term girls, indicating a more advanced developmental stage of follicles compared with preterm girls. These differences mirror what we observed in our two experimental groups, and suggest that full term ewe lambs are born with different stages of ovarian maturation, and that AMH concentrations in the postnatal period may represent a marker of ovarian maturation. Furthermore, in agreement to a more advanced stage of ovarian maturation in ewe lambs with a high AFC at 50 days, in a previous study of our research group

we showed that oocytes collected from ovaries with the highest AFC proved to be the most competent to develop into a blastocyst after incorporation into an *in vitro* production system (Torres-Rovira *et al.* 2014). It is important to stress that, considering folliculogenesis time-line in sheep (McNatty *et al.* 1995), the difference in ovarian maturation stage and follicle differentiation observed between the groups studied are likely to be initiated during gestation. In this regard, the importance of ovarian maturation during fetal life has already been underlined (Kuiri-Hänninen *et al.* 2014).

The longitudinal experiment carried out in the present study demonstrated that, in ewe lambs, having a high or low AFC phenotype during the early prepubertal period has long-lasting effects on ovarian status and responsiveness to gonadotropin stimulation. A low AFC was indeed associated with constantly lower number of total follicles and plasma AMH concentrations up to the postpubertal period (496 days of age), even if differences in the response to FSH stimulation in terms of new follicles stimulated to grow tend to decrease with age. In addition, the statistical model developed evidenced that the decrease in plasma AMH concentrations with age is more marked in the low AFC group compared with the high one. In this regard, AFC and AMH are widely used to predict ovarian reserve and ovarian response to controlled stimulation with exogenous gonadotropins for assisted reproductive technologies (ARTs) treatments (La Marca *et al.* 2007, Aflatoonian *et al.* 2009, Fleming *et al.* 2015); moreover, a better prediction of ovarian responsiveness is achieved when using AMH, AFC, and age together (Brodin *et al.* 2015). Taken together, these results immediately suggest that high AFC and AMH concentrations in the early prepubertal period may represent a precocious marker of the ovarian reserve of a given individual, considering that the quantity and quality of non-growing follicles in the ovary is positively associated with the number of growing follicles, plasma AMH concentrations, and reproductive life-span (Monniaux *et al.* 2014). However, the results obtained in a previous study (Torres-Rovira *et al.* 2014) allow us to rule out differences in the size of the primordial follicle pool, often known as ovarian reserve, as the cause of the observed differences in AFC and AMH in the two experimental groups (Torres-Rovira *et al.* 2014). AFC and AMH concentrations in the early prepubertal period were indeed only predictive of the functional ovarian reserve (Gleicher *et al.* 2011), i.e. the number of growing follicles that can be recruited to grow by exogenous FSH. We speculated that the significant differences in AFC observed in prepubertal ewe lambs would simply reflect the slow increase in follicular activity (recruitment and development) that is required throughout this period, and that follicular recruitment and development are preparatory to the subsequent development of reproductive function.

Some remarks on the post-pubertal EFORT results of our longitudinal study should be made. First, although the EFORT was performed during the anoestrus period for this breed and latitude, secondary follicular waves were not synchronized. Therefore, the response to FSH stimulation may slightly vary according to the phase of the follicular wave during which the gonadotropin stimulation occurs. However, AMH concentrations remain relatively constant along the whole cycle because there is a continuous growth of small follicles (La Marca *et al.* 2007) following a specific profile during the estrous cycle which occurs independently of the follicular waves (Rico *et al.* 2011); exhibit very little changes with season in seasonal breeding animals (goat, Monniaux *et al.* 2011), and are positively correlated to ovarian response to exogenous stimulation (Torres-Rovira *et al.* 2014). Thus, we believe that the differences seen in the basal concentrations of AMH on Day 0 between the high and low AFC groups support the existence of the differences seen between both groups at 16.5 months in the EFORT response despite the non-synchronization of the follicular wave. Secondly, we cannot completely rule out the possibility that our EFORT results at 16.5 months could somehow be influenced by the fact that only nonpregnant ewes at first breeding season, representing a smaller sample size and perhaps underlying not-detected reproductive problems, were used for this study.

With the longitudinal experiment we were able to show that a high AFC in the early prepubertal period is associated with consistently higher AMH concentrations and number of antral follicles, but also with higher pregnancy rates in the first breeding season. Some studies have associated higher AMH concentrations (Kamel *et al.* 2014, Sahmay *et al.* 2014) and AFC (Holte *et al.* 2011) with higher pregnancy rates in women undergoing reproductive treatments. However, longitudinal studies on plasma AMH at the prepubertal age and its implications in later reproductive life in girls and domestic animals are scarce.

A recent study reported that plasma AMH measured at 3 months in prepubertal ewe lambs was a marker for the number of ovarian follicles able to respond to gonadotropins at these early ages and was also positively correlated with fertility at their first mating (Lahoz *et al.* 2014). This result is in agreement with present findings, where ewe lambs with high AFC and AMH concentrations at 50 days of life showed higher pregnancy rates at the first breeding season, as compared with ewe lambs with low AFC and plasma AMH concentrations. The same research group reported in a subsequent study that the highest AMH value was found at different times (3, 4.5, and 6 months of age) in different ewe lambs (Lahoz *et al.* 2014). The authors speculated that the large variations between animals observed in the pattern of plasma AMH concentrations before puberty may suggest that ovarian maturity occurs at an earlier age in some

ewe lambs than in others. On the other hand, in contrast to present findings, plasma AMH concentration before puberty was not related to AMH concentration or the number of follicles grown after FSH treatment at the adult age (Lahoz *et al.* 2014).

In any case, the difference between our groups in the AFC and AMH points to a high individual variability in the occurrence of postnatal pituitary activation in sheep, as it occurs in human.

Conclusion

Our animal model helps to strengthen the knowledge of the perinatal period and its importance later in life, being a good model for the study of the 'minipuberty' occurrence and the delay in follicular development in girls and their possible implications in their later reproductive life. Results of the present study demonstrate that ewe lambs undergo different patterns of postnatal pituitary activation, and that a high AFC at 50 days of age indicates an advanced phase of ovarian maturation accompanied by high plasma AMH and low plasma FSH concentrations, although the mechanism driving the postnatal development of antral follicles remains unknown. In addition, in the high AFC group the more advanced stage of ovarian maturation in the early prepubertal period was accompanied by constantly higher AMH concentrations up to the postpubertal period, by a greater ovarian response to FSH stimulation, and by higher pregnancy rates at first mating, as compared with the low AFC group.

Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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