Accepted Manuscript

Anti-Müllerian hormone (AMH) concentration in sheep and its dependence of age and independence of *BMP15* genotype: an endocrine predictor to select the best donors for embryo biotechnologies

B. Lahoz, .J.L. Alabart, .M.J. Cocero, .D. Monniaux, .E. Echegoyen, .P. Sánchez, .J. Folch

PII: S0093-691X(13)00402-0

DOI: 10.1016/j.theriogenology.2013.09.033

Reference: THE 12611

To appear in: *Theriogenology*

Received Date: 26 June 2013

Revised Date: 16 September 2013

Accepted Date: 30 September 2013

Please cite this article as: Lahoz B, Alabart JL, Cocero MJ, Monniaux D, Echegoyen E, Sánchez P, Folch J, Anti-Müllerian hormone (AMH) concentration in sheep and its dependence of age and independence of *BMP15* genotype: an endocrine predictor to select the best donors for embryo biotechnologies, *Theriogenology* (2013), doi: 10.1016/j.theriogenology.2013.09.033.

Anti-Müllerian hormone (AMH) concentration in sheep and its dependence of age and independence of *BMP15* genotype: an endocrine predictor to select the best donors for embryo biotechnologies

Lahoz B.[a*], Alabart J.L.[a], Cocero M.J.[b], Monniaux D.[c], Echegoyen E.[a], Sánchez P.[a], Folch J.[a]

[a]*Unidad de Tecnología en Producción Animal, Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón, 50059 Zaragoza, Spain*

[b]*Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), 28040 Madrid, Spain*

[c]*INRA, UMR85 Physiologie de la Reproduction et des Comportements, F-37380 Nouzilly, France; CNRS, UMR7247, F-37380 Nouzilly, France; Université François Rabelais de Tours, F-37041 Tours, France; IFCE, F-37380 Nouzilly, France*

[*] Corresponding author. Tel.:+34 976716448; fax: +34 976713503.

E-mail address: blahozc@aragon.es (B. Lahoz).

Abstract

Embryo biotechnologies contribute significantly to the genetic enhancement of livestock, although their efficiency is still limited in sheep, mainly due to variable ovarian responses to gonadotropins. At present, anti-Müllerian hormone (AMH), which is produced by the granulosa cells of the small antral follicles, is a reliable endocrine marker of the ovarian follicle reserve in many species. The expression of AMH in granulosa cells was shown to be stimulated by bone morphogenetic proteins (BMPs) in vitro, so a mutation affecting the *BMP15* gene might modulate AMH production in vivo. The present study aimed to assess plasma AMH concentrations before puberty in two groups of Rasa Aragonesa ewes that were

carrying (R+) or not carrying (++) the prolific *FecX*^{R} allele and to relate them with their AMH concentrations at adulthood. Additionally, we sought to establish in both genotypes whether AMH measurements during a LOPU programme could be predictive of the number of ovarian follicles (≥3 mm) and recovered cumulus-oocyte complexes (COCs). No differences in AMH were found between the R+ and ++ ewes before puberty or during the adult age. Before puberty, the AMH concentration tended to increase from 3 to 4.5 months and to decline at 6 months to levels similar to those observed later in adults (333.8 ± 73.3, 483.2 ± 135.5 and 184.1 ± 38.2 pg/mL, respectively; $P < 0.1$), showing a large variability between individuals and between ages. A relationship between the AMH concentrations before puberty and during adulthood was not found, likely reflecting different follicular growth dynamics. In adults, the AMH concentration at the beginning of the FSH treatment was strongly correlated with the number of punctured follicles at LOPU in R+ and ++ ewes ($r = 0.75$ and 0.78, respectively; $P < 0.001$), and it was possible to accurate determine AMH cut-off values for both genotypes to identify high-responding ewes. On average, 5.1 extra follicles and 2.7 extra COCs were expected per each 100 pg/mL increase in AMH ($P < 0.0001$ and $P < 0.01$, respectively). The repeatability of AMH concentration from session to session was 0.70 ($P < 0.0001$). Our results demonstrated that, regardless of age, the presence of the *FecX*^{R} allele did not affect plasma AMH levels. During adulthood, AMH proved to be a good predictor of the ovarian response to FSH stimulation. Such an indicator could therefore be used to improve the performance of embryo biotechnologies in sheep.

Keywords: COCs, *FecX*, follicle, FSH, ovarian reserve, ovum pick-up

1. Introduction

Reproductive efficiency in sheep is of great importance to optimise labour, improve the income of farmers and the viability of farms, and contribute to optimising the use of resources. The use of embryo biotechnologies contributes to an increase in the selection intensity and shortens the generation interval, allowing for the production of a large number of descendants from ewes with high genetic merit. In sheep, embryo biotechnologies have developed considerably in recent years due to a higher demand and an increased efficiency of the protocols and techniques. Apart from artificial insemination (AI) and multiple ovulation and embryo transfer (MOET), laparoscopic-ovum pick up (LOPU) and in vitro production of embryos (IVP) represent the next generation of techniques to improve the number of offspring in small ruminants [1], presenting many advantages over traditional MOET. Using OPU-IVP, more embryos can be produced in the same period of time per donor animal, as the procedure can be performed over a long period, including pregnancy, prepuberty, or anestrus, without detrimental effects on the fertility of the donor. LOPU-IVP is a versatile technique with a much greater potential for the future compared with MOET, and it could therefore significantly contribute to enhancing genetic improvement and preserving endangered breeds.

However, the overall efficiency of LOPU-IVP in sheep is still far from the yields reached in cattle [2]. Apart from the aspects related to IVP and the conservation of the produced embryos, a major limiting factor is the large between-individual variability in the ovarian response to stimulation treatments using exogenous FSH [3], resulting in high variability in the final number of transferable embryos in sheep. Variations in the ovarian response to FSH reflect the variability of the follicular population present at the beginning of the treatment in small ruminants [4–6].

Anti-Müllerian hormone is a glycoprotein belonging to the transforming growth factor (TGF)-β family that is only expressed in the gonads [7,8]. In women and female animals, AMH is a reliable marker of the size of the ovarian pool of growing follicles. At present, AMH is the

best predictive endocrine marker of the number of available follicles in Assisted Reproductive Technologies (ART) in response to ovarian stimulatory treatment in humans [9], cows [10], goats [11], and mares [12]. In the sheep ovary, AMH has recently been found to be highly produced by the antral follicles from 1 to 3 mm in diameter [13], but its ability to predict the number of follicles available for puncture in a LOPU programme has not been investigated yet. While the use of AMH in adulthood is of great interest, it would be even more interesting if it could be determined earlier in life. In a previous work, we investigated the ability of plasma AMH to predict the ovarian follicle reserve before puberty and to correlate it with early fertility at the first lambing for the early selection of replacement ewe lambs. Our results suggested that AMH could be a marker of the ovarian follicle reserve before puberty related to sexual precocity [14]. Thus, in the present work, we aimed to assess the usefulness of AMH determination at an early age for the precocious selection of the best future donors for embryo biotechnologies.

In recent years, several sheep breeds have been identified with different mutations in the bone morphogenetic protein 15 (*BMP15*) gene, which are responsible for an increase in the ovulation rate and prolificacy in heterozygous ewes [15–19]. One of the mentioned polymorphisms corresponds to the $FecX^R$ allele in the Rasa Aragonesa breed [18]. We recently found that the presence of the $FecX^R$ allele in heterozygosity led to a decrease in follicle diameter, with no effects on the developmental competence of their oocytes [20]. The *BMP15* gene, like AMH, belongs to the TGF-β superfamily [21]. Other BMPs belonging to this superfamily have recently been shown to stimulate *AMH* mRNA expression in granulosa cells in vitro, both in cattle (BMP4 and BMP6) [22] and sheep (BMP4) [13]. These recent results strongly suggest an important role for BMPs in enhancing AMH production in small antral follicles in vivo. Many questions remain to be addressed regarding regulation of AMH expression, including the potential effects of other members of the BMP family, such as BMP15 or GDF9. For this reason, the potential influence of a decrease in BMP15 protein expression in the oocyte due to a mutation affecting the *BMP15* gene, on AMH production in

follicles and therefore, on its endocrine level, should not be disregarded. Due to the reproductive advantages of the *FecX*^{R} allele, ewes heterozygous for *FecX*^{R} coexist with wild-type ewes in Rasa Aragonesa flocks. Therefore, potential differences in plasma AMH concentrations between genotypes should be assessed to determine whether the predictive ability of AMH could be extended or not to both genotypes and therefore if distinctions between the genotypes when using AMH as a selection tool should be made or not.

Therefore, the aims of this study were the following: 1) to assess whether the presence of the *FecX*^{R} mutation in the *BMP15* gene could affect the AMH plasma concentrations; 2) to characterise the plasma AMH concentrations before puberty and to establish the relationship between AMH measured during this period of life and AMH and LOPU performance at the adult age; and 3) to establish whether plasma measurements of AMH during adulthood could be used to determine the number of ovarian follicles and cumulus-oocyte complexes (COCs) that a given sheep can produce during repeated LOPU sessions, using FSH or not.

2. Material and Methods

All experimental procedures were performed in accordance with the guidelines of the European Union (2003/65/CE) and Spanish regulations (RD 1201/2005, BOE 252/34367-91) for the use and care of animals in research. The experiments were carried out at the facilities of the research centre (CITA de Aragón, Spain). The *BMP15* genotype was determined in all animals by PCR genotyping, as previously described by Martínez-Royo et al. [23].

2.1. Experiment 1: Plasma AMH concentration before puberty

Twelve ewe lambs of the Rasa Aragonesa breed, including six heterozygous for the *FecX*^{R} allele (R+) and six wild-type ewe lambs (++), were used. The animals were born in April, with a maximum age difference between animals of 3 days. They were provided feed and water *ad libitum* and reared under the same conditions. Ewe lambs were submitted to blood sampling at 3, 4.5, and 6 months. The experimental protocol aimed to compare the plasma AMH concentrations depending on age and genotype.

2.1.1. Blood sampling

For AMH determination, three blood samples were obtained from each ewe lamb before puberty as follows: first sampling at 90 days of age (July), second sampling at 135 days of age (August), and third sampling at 180 days of age (October). All samples were taken at 9 a.m., with a maximum interval from the first to the last sampling of 10-15 minutes. In order to determine whether puberty had occurred by the time of AMH sampling, two blood samples at nine days interval were taken at 3, 4.5 and 6 months of age for P4 determination. The samples were collected by jugular puncture using 5-mL vacuum tubes with lithium heparin that were immediately centrifuged at 2100 X g for 25 min. The plasma was stored at -20 ºC until it was assayed for AMH and P4. After that, the ewe lambs were kept in irrigated pastures with *ad libitum* access to water and mineral supplements.

2.2. Experiment 2: Plasma AMH concentration at LOPU

When the ewe lambs from experiment 1 were adults (19 months), ten of them were submitted to four LOPU sessions, together with six additional ewes of similar age (8 R+ and 8 ++ in total), and blood samples were taken during each session for AMH measurement. The AMH samples were taken in the second LOPU trial of a previous work, consisting in two LOPU-IVP trials performed to evaluate the effects of the $FecX^R$ allele and FSH on follicle population and oocyte quality [20]. The present experiment aimed to assess the ability of the plasma AMH concentrations to predict the performance of an individual ewe submitted to a LOPU program, taking into account the potential effect of the R+ genotype and LOPU session on the AMH concentration. In addition, the relationships between the AMH concentrations of the ewe lambs before puberty with the AMH concentrations and performance (number of punctured follicles and COCs) of adult ewes submitted to LOPU were investigated.

2.2.1. Hormonal treatment and blood sampling

Four LOPU sessions were carried out 7 days apart, as follows (Fig. 1). The first and second sessions were carried out without FSH treatment, and the third and fourth sessions took place following FSH treatment. Prior to the first session, the ewes received two doses of 125 μg of cloprostenol (Estrumate, Schering-Plough Animal Health, Madrid, Spain) 8 days apart. Five days after the second injection, 30 mg fluorogestone acetate sponges (FGA; Sincropart 30 mg, CEVA Animal Health SA, Barcelona, Spain) were inserted and replaced by new ones at each LOPU session. The first LOPU session took place 12 days after the insertion of the first sponge, and the second took place one week after. Prior to the third and forth LOPU sessions, the ewes received a total of 32 mg of FSH (ULG, Liège, Belgium) administered in decreasing doses as follows: 8 mg (60 h), 8 mg (48 h), 6 mg (36 h), 6 mg (24 h), and 4 mg (12 h). Coinciding with the first injection of FSH, 125 μg of cloprostenol was injected. All FSH used in this study was highly purified porcine FSH, supplied by the Laboratory of Endocrinology of the Faculty of Veterinary Medicine, University of Liege, Belgium. The quantities are expressed in mg of the NIH-FSH-P1 standard.

(Approximated location Fig. 1)

A total of four blood samples for plasma AMH determination were taken from each ewe in different moments to establish the relationship between follicle and COC numbers with AMH. In sessions 1 and 2, samples were taken at the time of LOPU, and in sessions 3 and 4, the samples were taken coinciding with the first FSH injection in each session (60 h prior to LOPU). Blood sampling and storing was performed as in experiment 1.

2.2.2. Laparoscopic Ovum Pick Up (LOPU) and recovery of oocytes

Follicular aspiration was carried out as previously described [20]. Briefly, the donors were fasted for 12 hours before the LOPU session, anesthetised with 3 mg / kg body weight of propofol (Propofol ®Lipuro 1%, Braun, Spain) and submitted to inhalation anaesthesia with

isofluorane (IsoFlo®, Veterinary Esteve, Spain). An endoscope and an atraumatic grasping forceps were inserted into the abdominal cavity, and a 23G needle was introduced by a third incision to aspirate the contents of all the antral follicles ≥ 3 mm in diameter present on the ovaries, using a vacuum pump adjusted to -25 mm Hg (V-MAR 5100; Cook Ltd, Eight Mile Plains, Australia). The number and diameter of the punctured follicles was recorded for each donor and session. After oocyte collection, the ovaries were washed with sterile physiological saline solution (0.9% NaCl) supplemented with 5 IU heparin / mL. A staple and topical spraying with chlortetracycline hydrochloride (Pederol® spray, Syva, León, Spain) were applied to each of the three 1-cm incisions, and 1 mL/10 kg im of oxytetracycline (Oxycen-200 L.A. ®, S.P.veterinaria, s.a., Tarragona, Spain) was administered to each ewe. The Falcon tubes were brought to the laboratory and poured into a petri dish to recover the COCs, that were morphologically scored into five categories as previously described by Stangl et al. [24]. Only those surrounded by continuous multilayer unexpanded cumulus cells and showing a homogeneous cytoplasm were considered as suitable for in vitro maturation (IVM, categories I, II and III).

2.3. Assay for anti-Müllerian hormone

The plasma concentrations of AMH were measured with the AMH GenII ELISA Kit (Beckman Coulter France, Roissy CDG, France). This kit is an enzymatically amplified two-site immunoassay, providing materials for the quantitative measurement of AMH in human serum. For validation of the assay in sheep, serial dilutions of different ovine plasma and follicular fluid samples in steer plasma were analysed using the kit. The results showed that the follicular fluid dilution curves were linear and parallel to the standard curve (data not shown). Just before the assay, the frozen plasma samples were thawed in a warm water bath, vortexed and centrifuged (3200 g, 10 min, 4 °C) to remove any cell fragments that could interfere with the reagents of the assay. The anti-Müllerian hormone concentrations were determined in 50-µL undiluted plasma samples. The samples were incubated overnight at 4 °C in the presence of the primary antibody and then for 1.5 h at room temperature in the

presence of the secondary antibody. Dilutions of the points of the standard curve were made in steer plasma. With these conditions, the limit of detection of the assay was found to be 15 pg/mL. The intra-assay coefficients of variation were 9.9, 6.0, and 2.7% for the three quality control blood samples that were tested, containing 80, 160, and 400 pg/mL of AMH, respectively.

2.4. Assay for progesterone

Progesterone was analyzed by a direct competitive ELISA kit intended for ovine plasma (Ridgeway Science, St. Briavels, Gloucestershire, UK), following the manufacturer's instructions. The sensitivity was 0.28 ng/mL. All samples were run in the same assay. Intra-assay coefficients of variation for plasma pools of 0.5 and 1 ng/mL were 11.5 and 15.0%, respectively. The threshold considered for luteal activity was 0.5 ng/mL.

2.5. Statistical analyses

The effects of *FecX*^{R} genotype and age on AMH concentrations at a prepubertal age were tested by repeated-measures analysis of variance (ANOVA) using the MIXED procedure of SAS [25]. The following terms were included in the model: genotype, age, and their interaction as fixed effects and the donor ewe within genotype as random.

Correlations between the variables were assessed by the Pearson's correlation coefficient. The repeatability of the AMH concentrations at different times was calculated as the ratio of the between-animal variance to the sum of the between-animal and the within-animals variance. The variance components were estimated by the method of restricted maximum likelihood using the VARCOMP procedure of SAS. The statistical significance from zero of the repeatability estimates were assessed using confidence intervals [26].

The effects of *FecX*^{R} genotype and FSH treatment on the total number of punctured follicles ($\geq$ 3 mm), recovered COCS and COCs suitable for IVM per ewe and session were analysed by repeated-measures ANOVA using the MIXED procedure of SAS. The following fixed

effects were included in the models: genotype, treatment, and session, as well as all the possible interactions among them. The treatment and the session within each treatment were treated as repeated factors. The donor ewe within the genotype group was included as a random term.

The effects of the *FecX*^{R} genotype and the number of sessions on the plasma AMH concentrations, which were measured per ewe and per session, were analysed by repeated-measures ANOVA using the MIXED procedure of SAS. The following fixed effects were included in the model: genotype, session, and their interaction. Session was treated as the repeated factor. The donor ewe within the genotype group was included as a random term.

Between-groups comparisons were performed by pairwise contrasts and the raw probabilities were adjusted for multiple comparisons by the step-down Shaffer-simulated method incorporating logical constraints [27,28] using the LSMESTIMATE statement [29].

The effect of the *FecX*^{R} genotype on the linear relationship between the variables recorded in the LOPU sessions (number of punctured follicles, recovery rate, numbers of recovered COCs and COCs suitable for IVM per ewe and per session) and AMH was analysed by repeated-measures analysis of the variance-covariance (ANCOVA). Separate models were fitted for untreated sessions (first and second) and treated sessions (third and fourth). The terms included in the models were the following: *FecX*^{R} genotype (allowing for different intercepts for each genotype), AMH (included as covariate), and their interaction (allowing for different slopes for each genotype), as fixed effects; and the donor ewe within the genotype as random. If the interaction between genotype and AMH was not significant, a common slope could be assumed (two parallel lines were fitted, one for each genotype). In addition, if the genotype effect was not significant, a common intercept could be assumed (a common line was fitted for both genotypes) [30].

The performance of the plasma AMH concentration prior to FSH treatment to predict donor ewes with higher numbers of punctured follicles (≥ 3 mm) at the day of LOPU was tested by

receiver-operating characteristic (ROC) analysis, using the LOGISTIC procedure of SAS. Donors with more than 18 punctured follicles per session were considered as "high-responding" and those with 18 follicles or less as "low-responding" ewes, as the median number in all ewes (both genotypes pooled) was 18.5 follicles per session in treated sessions. Sensitivity (Se) was defined as the ratio between the donors correctly predicted as "high-responding" and the total number of "high-responding" donors. Specificity (Sp) was defined as the ratio between the donors correctly predicted as "low-responding" and the total number of "low-responding" donors. The ROC curve is a plot of sensitivity (the true positive rate) versus one minus specificity (the false positive rate). The area under the ROC curve (AUC) was used as a measure of the predicting ability of AMH. As it is known that the values of AUC are too higher when obtained from the data set used to fit the model, AUC values obtained by the leave-one-out cross-validation method are also reported. Between-genotypes comparisons of the AUC values were carried out using the method described by Greiner et al. [31]. The maximum value of the Youden index ($J = Se + Sp - 1$) was used to determine the optimal value of plasma AMH (cut-off value) to discriminate between "high" and "low responding" donors to FSH.

The normality of the ANOVA and ANCOVA residuals were tested by the Jarque–Bera test, applying a correction for finite samples [32]. A Box–Cox transformation was applied to variables failing to meet the requirement of residual normality. The significance level was set at $P < 0.05$. Otherwise indicated, results are reported as means ± standard errors.

3. Results

3.1. Experiment 1: Plasma AMH concentration before puberty

Progesterone was lower than 0.5 ng/mL in the two samples taken from all ewe lambs at 3, 4.5 or 6 months, so all animals were assumed to have not reached puberty at these ages. Plasma AMH was detected in all the ewe lambs, although one of them showed a non-detectable value at 6 months, preceded by concentrations higher than 250 pg/mL at 3 and 4.5 months, and with

AMH values higher than 100 pg/mL at the adult age. Therefore, the value at 6 months was not taken into account in this ewe lamb.

Effect of the $FecX^R$ genotype on plasma AMH concentration before puberty

No significant differences in plasma AMH concentrations were found between R+ and ++ ewes at 3 months (355.3 ± 118.0 vs. 312.2 ± 86.4 pg/mL), at 4.5 months (490.5 ± 205.4 vs. 476.0 ± 176.6 pg/mL), or at 6 months (145.1 ± 56.5 vs. 223.1 ± 65.5 pg/mL), respectively (Fig. 2). The interaction between genotype and age was not significant, indicating that variations in AMH with age were similar in both genotypes.

Effect of age on plasma AMH concentration before puberty

Plasma AMH levels at 3, 4.5 and 6 months were 333.8 ± 73.3, 483.2 ± 135.5 and 184.1 ± 38.2 pg/mL, respectively. Plasma AMH concentrations at 6 months were lower than those found at 3 or 4.5 months ($P < 0.05$ for both), although significance was not attained when probabilities were adjusted for multiple comparisons ($P < 0.1$ for both). The AMH profile throughout this period was different between animals, so the maximum AMH values were found at different times in different ewe lambs at 3 months (n = 4), at 4.5 months (n = 6), or at 6 months (n = 2). The plasma AMH concentrations ranged from 20.4 to 829.1 pg/mL at 3 months, from 69.3 to 1370.8 pg/mL at 4.5 months, and from 69.3 to 471.4 pg/mL at 6 months, demonstrating high between-individual variability. Overall, the within-animal repeatability in the plasma AMH concentration at 3, 4.5, and 6 months was found to be low (0.17; NS). When only those ewe lambs that presented the AMH peak at the same time (4.5 months) were considered, the overall within-animal repeatability at 3, 4.5, and 6 months was higher, although significance was not attained (0.39; $P < 0.1$).

(Approximated location Fig. 2)

3.2. Experiment 2: Plasma AMH concentration at LOPU

Plasma AMH was detected in all the animals and sessions, ranging from 19.6 to 446.0 pg/mL.

Effect of the FecX^R genotype and LOPU session on plasma AMH concentration at LOPU

No significant differences in the plasma AMH concentration were found between R+ and ++ ewes (Table 1). The interaction between genotype and session was not significant, indicating that variations in AMH within a session were similar in both genotypes. The interaction between genotype and treatment was not significant.

In sessions 1 and 2, the plasma AMH concentrations were similar (213.8 ± 19.9 vs. 213.6 ± 25.8 pg/mL), and both were higher than those in the subsequent sessions 3 and 4 ($P < 0.02$). The plasma AMH concentrations were slightly higher in the third than in the fourth session, although significance was not attained (154.4 ± 21.0 vs. 109.9 ± 14.9; $P < 0.1$). A trend to significance was found for the interaction between session and FSH treatment ($P < 0.1$).

Table 1. Plasma AMH concentrations (pg/mL) in adult ewes that were carrying (R+) or not carrying (++) the *FecX^R* allele of the *BMP15* gene, submitted to four LOPU sessions, with or without FSH treatment, one week apart.

Effect			AMH (pg/mL)	P-value
Genotype (G)				
	R+		166.2 ± 14.9	0.71
	++		179.8 ± 17.8	
Session (S)				
		1	213.8 ± 19.9 [a]	0.0001
		2	213.6 ± 25.8 [a]	
		3	154.4 ± 21.0 [b]	
		4	109.9 ± 14.9 [b]	
G X S				
	R+	1	200.9 ± 30.5	0.88
		2	214.9 ± 28.3	
		3	149.8 ± 26.5	
		4	99.1 ± 17.0	
	++	1	227.3 ± 26.7	
		2	212.3 ± 45.4	
		3	158.9 ± 34.5	
		4	120.8 ± 25.1	

[a,b]: $P < 0.02$

Repeatability of AMH concentrations during LOPU sessions

The individual repeatability of plasma AMH concentration during repeated sessions of LOPU was 0.57 ($P < 0.0001$), increasing up to 0.70 when only considering sessions 3 and 4 (with FSH; $P < 0.0001$ for both).

Representative ewes of both genotypes are shown in Fig. 3.

(Approximated location Fig. 3)

Relationships between plasma AMH concentrations and LOPU yields

No significant differences were found between R+ and ++ ewes in the number of punctured follicles (16.9 ± 1.1 vs. 14.8 ± 0.9), total COCs (10.2 ± 0.8 vs. 8.3 ± 0.6) and COCs suitable for IVM (8.8 ± 0.8 vs. 7.2 ± 0.7; respectively).

In sessions 1 and 2 (without FSH), the number of punctured follicles was lower than in sessions 3 and 4, with FSH (12.9 ± 0.9 vs. 18.8 ± 1.1; $P < 0.001$). We found a weak correlation between the AMH concentrations and the number of punctured follicles (≥ 3 mm) at LOPUs 1 and 2 ($r = 0.30$; $P < 0.1$). As shown in Fig. 4, a common regression line for both genotypes could be assumed. The correlations between AMH and recovery rate, total COCs recovered, or COCs suitable for IVM were not significant ($r = -0.08$; $r = 0.09$ and $r = -0.08$; respectively). For each of these variables, a common regression line for both genotypes could be assumed (not shown).

Conversely, in sessions 3 and 4 (with FSH), the number of punctured follicles (≥ 3 mm) at LOPU was strongly correlated with the AMH concentrations measured at the beginning of the FSH treatment in both R+ and ++ ewes ($r = 0.75$ and $r = 0.78$, respectively; $P < 0.001$ for both). In this way, 5.1 ± 1.0 extra follicles were expected to be punctured per 100 pg/mL increase in AMH in both genotypes ($P < 0.0001$). In this case, two parallel regression lines, one for each genotype, were fitted (Fig. 4). For the same AMH concentrations, 4.5 ± 1.6 extra follicles were expected to be punctured in the R+ genotype than in ++ genotype ($P < 0.05$). As

in sessions 1 and 2, the recovery rate of COCs was not correlated with plasma AMH measured at the beginning of FSH treatment ($r = -0.13$; $P < 0.49$) and a common regression line could be assumed for both genotypes (not shown). Conversely, correlations with plasma AMH were also found for total COCs recovered or COCs suitable for IVM ($r = 0.41$ and 0.39, respectively; both each $P < 0.05$). For these variables two parallel regression lines, one for each genotype, were fitted (not shown). In this way, 2.7 ± 0.7 extra COCs were expected to be recovered per 100 pg/mL increase in AMH in both genotypes ($P < 0.01$). In addition, for the same AMH concentrations, 4.0 ± 1.2 extra COCs were expected to be recovered in R+ than in ++ genotype ($P < 0.01$). Likewise, 2.3 ± 0.8 extra COCs suitable for IMV were expected to be recovered per 100 pg/mL increase in AMH in both genotypes ($P < 0.01$). For the same AMH concentrations, 3.7 ± 1.2 extra COCs suitable for IVM were expected to be recovered in R+ than in ++ genotype ($P < 0.01$).

(Approximated location Fig. 4)

Determination and predicting performance of AMH cut-off values to detect "high-responding" ewes

The AUC values were 0.867 ± 0.107 and 0.883 ± 0.086 for the R+ and ++ genotypes, respectively. These values were different from 0.5, the AUC value of a test without discriminatory capacity (both $P < 0.001$). After cross-validation, these were lowered to 0.783 ± 0.129 and 0.767 ± 0.132, respectively, also different from 0.5 (both $P < 0.05$). The AUC values were similar for both genotypes, either prior to ($P < 0.90$) or after cross-validation ($P < 0.93$). The optimal cut-offs for selecting "high-responding" ewes were 112 pg/mL for the R+ genotype (corresponding to 80.0% sensitivity and 83.3% specificity) and 169 pg/mL for the ++ genotype (corresponding to 66.7% sensitivity and 100% specificity; Fig. 5).

(Approximated location Fig. 5)

Relationship between plasma AMH concentration at puberty and LOPU performance at the adult age

There was no significant relationship between the plasma AMH concentrations before puberty (at 3, 4.5 and 6 months) and the plasma AMH at the adult age (at the first, second, third, and fourth LOPU sessions) for any genotype. In the same way, the relationships between plasma AMH concentrations at 3, 4.5, and 6 months and the follicle yield at the LOPU sessions at the adult age were not significant (Fig. 6). The within-animal repeatability of AMH at 3, 4.5, or 6 months with AMH at the adult age was close to zero.

(Approximated location Fig. 6)

4. Discussion

The present study shows that: 1) the presence of the $FecX^R$ mutation in the *BMP15* gene did not affect the plasma AMH concentrations; 2) the plasma AMH concentration in the prepubertal age was demonstrated to be highly variable between individuals and months, and it did not demonstrate a relationship with the plasma AMH or follicle number at the adult age; 3) In adults, the plasma AMH proved to be a good predictor of the response to FSH stimulation, and therefore it could be used to improve the performance of the LOPU-IVP procedures in both R+ and ++ ewes.

In the present study, we aimed to investigate whether the presence of the $FecX^R$ allele, with a deletion in the sequence encoding the *BMP15* gene, influences the plasma AMH concentrations, as other BMP factors (BMP4 and BMP6) have been shown to promote in vitro *AMH* expression in granulosa cells in cattle [22] and sheep [13]. We found no differences in the average plasma AMH concentrations between R+ and ++ ewes, neither before puberty, nor at the adult age. Nevertheless, when ewes with similar AMH concentrations at the time of FSH administration were compared, we found that R+ ewes developed a higher number of follicles at LOPU in response to FSH than ++ ewes. In fact, we

proposed a different AMH cut-off value for each genotype to select the best-responding ewes. We found similar indications of an increased response of R+ ewes to exogenous gonadotropins in previous works. First, the advantage on the ovulation rate of the R+ ewes with respect to the ++ ewes was doubled when applying a standard dose of eCG [33]. Second, in LOPU experiments, the punctured follicles from R+ ewes were smaller than those from ++ ewes, and the application of FSH leads to approximately three extra follicles in R+ ewes, while similar numbers of follicles were punctured without FSH in both genotypes [20]. Third, we found a positive correlation in the untreated R+ ewes, but not in ++ ewes, between the LH peak area and ovulation rate, suggesting that the same LH concentration can trigger the ovulation of an increased number of follicles in R+ ewes [34]. This increased ovulatory response of R+ ewes could be explained by the "window" theory proposed by Scaramuzzi et al. [35]. According to that theory, the multiple ovulations could be due to an increase in the pool of gonadotropin-dependent follicles and/or to a lowering of the FSH threshold, or an increase in circulating FSH levels. The most accepted model for ewes carrying the Booroola mutation or other mutations in *BMP15* or *GDF9* relies on an increased size of the pool of gonadotropin-dependent follicles of smaller size, without apparent changes in endocrine patterns [36,37]. We can therefore hypothesise that R+ ewes could have an increased number of small growing follicles able to respond to gonadotropins, contributing to the secretion of AMH, that we have not taken into account in the present study. In addition, in other prolific breeds (Finn ewes) the increased ovulation rate was found to be achieved by rescuing some follicles from atresia, which came from the preceding follicular wave [38]. The rescue of some follicles undergoing atresia would be another hypothesis to explain the increased ovulatory response of R+ ewes. Our results are a first and gross analysis at the plasma level of differences in AMH between animals carrying or not carrying a deletion in the *BMP15* gene. Therefore, further studies are needed to address whether R+ ewes could have an increased number of small antral follicles able to respond to gonadotropins, as well as the AMH secretory capacity of R+ follicles in relationship with their diameter.

Studies on plasma AMH at the prepubertal age in domestic animals are scarce. In beef heifers, plasma AMH concentrations were found to increase markedly between 1 and 3 months of age, remaining high at 6 months, and then declining slowly until 12 months, which corresponds with their age of puberty [13]. In the present study, using Rasa Aragonesa ewes in which puberty occurs at approximately 7 to 8 months [39], we found a similar profile for AMH, showing an increase at 4.5 months, and low AMH concentrations at 6 months, similar to those observed later in adulthood. This increase in AMH concentrations at 4.5 months would be coincident with a period where a peak in the total number of follicles ≥ 3 mm has been observed in other sheep breeds at approximately 16 weeks [40]. However, in the present study the maximum AMH concentrations were not found at the same time in all the ewe lambs, so individuals were not consistently ranked, which could explain the low repeatability found at these ages. In Sarda sheep, the plasma AMH concentrations determined at 40 and 110 days have been related to the total antral follicle count (AFC) [41]. It therefore appears that AMH at these early ages reflects changes in the ovarian follicle status; however, future studies are needed to assess the causes of these AMH variations before puberty and to evaluate whether these observed changes in AMH reflect changes in the number of small antral follicles or are due to changes in the amount of AMH secreted by each follicle.

In the present study, the AMH concentration in the plasma before puberty was not related with AMH concentration or the number of follicles that could be punctured after FSH treatment at the adult age. In a previous study, we found that plasma AMH measured at three months in prepubertal ewe lambs was a marker of 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 [14]. According to both studies, it appears that AMH is a quantitative marker of the number of ovarian follicles at any age, although its implications before puberty may be different than later in life. The large between-animal variations observed in the pattern of plasma AMH concentrations before puberty appear to reflect that ovarian maturity has occurred at an earlier age in some ewe lambs than in others. During the prepubertal phase, the

AMH levels have been found to be negatively correlated with the FSH levels in cattle [13]. In our opinion, this relationship should be further investigated with a larger number of animals, a higher sampling frequency, and determination of other hormones related to puberty.

In the present study, the plasma AMH concentration at LOPUs without FSH were not fully predictive of the number of follicles ≥ 3 mm present in the ovary at these time points, although a tendency was found. In follicles from sheep, AMH concentrations are the highest in small antral follicles and decrease markedly when follicles increase to their preovulatory size. In Romanov ewes, the highest intrafollicular AMH concentrations were found in follicles from 1 to 3 mm, where it was approximately three-fold higher than the intrafollicular AMH found in 3 to 5-mm follicles, while it was almost negligible in follicles greater than 5 mm [13]. This pattern of production, together with the fact that the population of follicles from 1 to 3 mm is usually larger than the other populations, could explain the trend observed in the present study: when puncturing follicles from 3 mm onwards, we are only accounting for one small part of the AMH-producing follicles.

On the other hand, AMH prior to FSH treatment was highly predictive of the ovarian response to FSH treatment at LOPU, being correlated with the number of punctured follicles, recovered COCs and COCs suitable for IVM. As expected, correlations between AMH and punctured follicles were stronger than AMH and COCs, as the number of retrieved oocytes is affected by the recovery rate, which usually varies among animals and sessions, and which can be affected by several technical aspects external to the animal itself. Regarding the performance of AMH determination to detect high or low-responding ewes, the AUC values were greater than 0.7 in both genotypes, even after cross-validation. According to an arbitrary rule that classifies tests as non-informative (AUC = 0.5), less accurate (0.5 < AUC < 0.7), moderately accurate (0.7 < AUC < 0.9), highly accurate (0.9 < AUC < 1) and perfect tests (AUC = 1) [31], we can rate this AMH test as “moderately accurate”. Moreover, AMH was demonstrated to be highly repeatable between the sessions. Our results are in agreement with those previously found by Rico et al. [42] in cows undergoing repeated OPUs. Variations in the

ovarian responses to FSH treatments are known to reflect the ovarian follicular population present at the beginning of treatment in small ruminants [5,43], so according to our results in sheep, it appears that the follicle population that produces the highest amounts of AMH is the same population able to be stimulated to grow by FSH. In conclusion, the use of AMH as a predictive method to select the best-responding donor ewes for embryo biotechnologies could improve the poor performance of the technique, and the cost of the analyses are expected to be largely offset by the reduced spending on stimulatory treatments, labour, or the optimised use of recipients.

In goats, the administration of exogenous FSH induces a decrease in the AMH concentrations, which occurs 3 to 4 days after treatment, apparently due to the temporary depletion of gonadotropin-responsive follicles, which return to the initial values before 3 weeks [11]. In agreement with this work, in the present study we found a diminution of plasma AMH after the administration of FSH. In the conditions of our work, this occurred seven days after the last FSH injection, in the last session. The diminution of AMH observed in the third session, which was not preceded by FSH treatment, is likely due to the short interval between follicle puncture in session 2 and sampling in session 3, separated only by 4.5 days, suggesting that the population of AMH-producing follicles was not fully restored after puncture. During the first two sessions, when FSH was not used, the plasma AMH concentrations remained constant, despite the AMH sampling in session 2 was preceded by ovarian puncture in session 1.

5. Conclusions

In conclusion, regardless of age, the presence of the *FecX*^{R} allele did not affect plasma AMH concentrations. Therefore, for its application on farms as a selection tool for early fertility, distinction between genotypes would not be needed. However, when applied to select the best-responding ewes to FSH for LOPU-IVP technologies, the *FecX*^{R} genotype should be taken into account. At the adult age, we propose that the determination of the plasma AMH

concentration in a single blood sample prior to FSH treatment is a suitable method for selecting the best oocyte donors for embryo biotechnologies. However, the precocious selection of such animals by AMH sampling at their prepubertal age would not currently be feasible, as the plasma AMH concentrations before puberty varied significantly between months and between individuals.

Acknowledgements

This work was supported by INIA (RTA2011-128 project and fellowship for B. Lahoz).

References

[1] Tervit HR. Laparoscopy/laparotomy oocyte recovery and juvenile breeding. Anim Reprod Sci 1996;42:227–38.

[2] Merton JS, de Roos AP, Mullaart E, de Ruigh L, Kaal L, Vos PL, et al. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology 2003;59:651–74.

[3] Gibbons A, Bonnet FP, Cueto MI, Catala M, Salamone DF, Gonzalez-Bulnes A. Procedure for maximizing oocyte harvest for in vitro embryo production in small ruminants. Reprod Domest Anim 2007;42:423–6.

[4] Driancourt MA. Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. Theriogenology 2001;55:1211–39.

[5] Veiga-Lopez A, Gonzalez-Bulnes A, Garcia-Garcia RM, Dominguez V, Cocero MJ. The effects of previous ovarian status on ovulation rate and early embryo development in response to superovulatory FSH treatments in sheep. Theriogenology 2005;63:1973–83.

[6] Mossa F, Duffy P, Naitana S, Lonergan P, Evans ACO. Association between numbers of ovarian follicles in the first follicle wave and superovulatory response in ewes. Anim Reprod Sci 2007;100:391–6.

[7] Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NM, Cheung A, et al. Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the human gene in animal cells. Cell 1986;45:685–98.

[8] Josso N, Di Clemente N. Transduction pathway of anti-Müllerian hormone, a sex-specific member of the TGF-β family. Trends Endocrinol Metab 2003;14:91–7.

[9] La Marca A, Sighinolfi G, Radi D, Argento C, Baraldi E, Artenisio AC, et al. Anti-Mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART). Hum Reprod Update 2010;16:113–30.

[10] Rico Ch, Fabre S, Medigue C, di Clemente N, Clement F, Bontoux M, et al. Anti-müllerian hormone is an endocrine marker of ovarian gonadotropin-responsive follicles and can help to predict superovulatory responses in the cow. Biol Reprod 2009;80:50–9.

[11] Monniaux D, Baril G, Laine AL, Jarrier P, Poulin N, Cognie J, et al. Anti-Mullerian hormone as a predictive endocrine marker for embryo production in the goat. Reproduction 2011;142:845–54.

[12] Vernunft A, Schneider F, Tuchscherer A, Becker F, Kanitz W. Anti-Muellerian Hormone (AMH) can help to predict follicular growth in mares. PFERDEHEILKUNDE 2011;27:293–6.

[13] Monniaux D, Drouilhet L, Rico C, Estienne A, Jarrier P, Touze JL, et al. Regulation of anti-Mullerian hormone production in domestic animals. Reprod Fertil Dev 2013;25:1–16.

[14] Lahoz B, Alabart JL, Monniaux D, Mermillod P, Folch J. Anti-Mullerian hormone plasma concentration in prepubertal ewe lambs as a predictor of their fertility at a young age. BMC Vet Res 2012;8:118.

[15] Galloway SM, McNatty KP, Cambridge LM, Laitinen MPE, Juengel JL, Jokiranta TS, et al. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat Genet 2000;25:279–83.

[16] Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R, et al. Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (*Ovis aries*). Biol Reprod 2004;70:900–9.

[17] Bodin L, Di Pasquale E, Fabre S, Bontoux M, Monget P, Persani L, et al. A novel mutation in the bone morphogenetic protein 15 gene causing defective protein secretion is associated with both increased ovulation rate and sterility in Lacaune sheep. Endocrinology 2007;148:393–400.

[18] Martinez-Royo A, Jurado JJ, Smulders JP, Marti JI, Alabart JL, Roche A, et al. A deletion in the bone morphogenetic protein 15 gene causes sterility and increased prolificacy in Rasa aragonesa sheep. Anim Genet 2008;39:294–7.

555 [19] Demars J, Fabre S, Sarry J, Rossetti R, Gilbert H, Persani L, et al. Genome-Wide
556 Association Studies Identify Two Novel BMP15 Mutations Responsible for an Atypical
557 Hyperprolificacy Phenotype in Sheep. PLoS Genet 2013;9:e1003482.
558 [20] Lahoz B, Alabart JL, Folch J, Sanchez P, Echegoyen E, Cocero MJ. Influence of the
559 *FecX*^{R} allele in heterozygous ewes on follicular population and outcomes of IVP and ET
560 using LOPU-derived oocytes. Reprod Domest Anim 2013;48:717–23.
561 [21] Knight PG, Glister C. TGF-beta superfamily members and ovarian follicle development.
562 Reproduction 2006;132:191–206.
563 [22] Rico C, Medigue C, Fabre S, Jarrier P, Bontoux M, Clement F, et al. Regulation of anti-
564 Mullerian hormone production in the cow: a multiscale study at endocrine, ovarian,
565 follicular, and granulosa cell levels. Biol Reprod 2011;84:560–71.
566 [23] Martínez-Royo A, Dervishi E, Alabart JL, Jurado JJ, Folch J, Calvo JH. Freemartinism
567 and *FecX*^{R} allele determination in replacement ewes of the Rasa Aragonesa sheep breed
568 by duplex PCR. Theriogenology 2009;72:1148–52.
569 [24] Stangl M, Kühholzer B, Besenfelder U, Brem G. Repeated endoscopic ovum pick-up in
570 sheep. Theriogenology 1999;52:709–16.
571 [25] SAS Institute Inc. 2011. SAS OnlineDoc® 9.3. Cary, NC: SAS Institute Inc.
572 [26] Turner HN, Young SSY. Quantitative Genetics in Sheep Breeding, Melbourne:
573 MacMillan of Australia, 1969;332.
574 [27] Shaffer JP. Modified Sequentially Rejective Multiple Test Procedures. J Am Stat Assoc
575 1986;81:329–35.
576 [28] Westfall PH. Multiple Testing of General Contrasts Using Logical Constraints and
577 Correlations. J Am Stat Assoc 1997;92:299–306.
578 [29] Westfall PH, Tobias RD, Wolfinger RD. Multiple Comparisons and Multiple Tests
579 Using SAS®, 2nd edition, 2011. Cary, NC: SAS Institute Inc., pp. 282-284.
580 [30] Dunn OJ, Clark VA. Applied Statistics. Analysis of Variance and Regression. Shewhart
581 WA, Wilks SS (eds). John Wiley & Sons, Inc., NY, USA: 2nd edition, 1987;445.

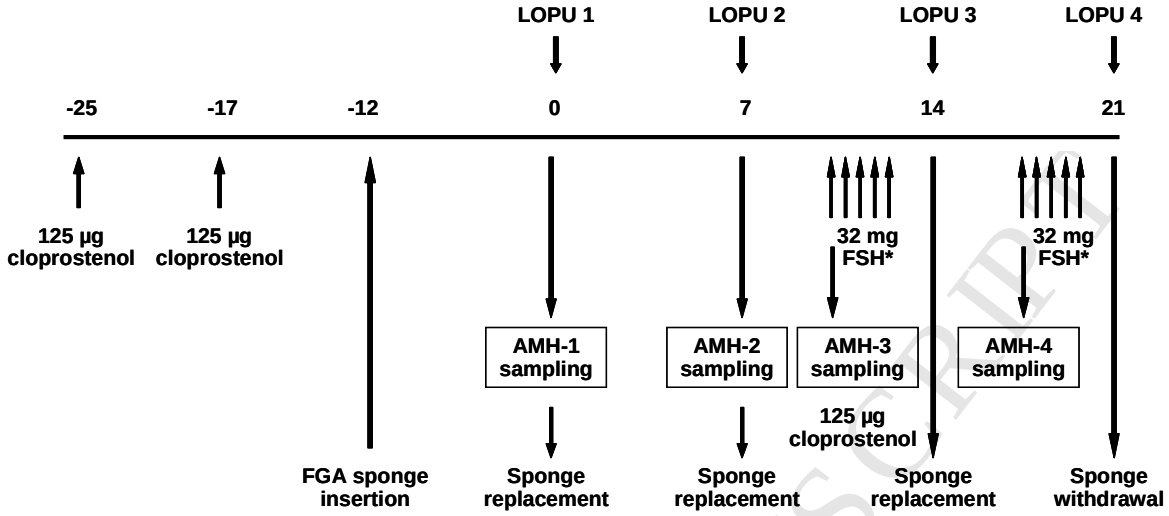
582 [31] Greiner M, Pfeifferb D, Smith RD. Principles and practical application of the receiver-
583 operating characteristic analysis for diagnostic tests. Prev Vet Med 2000;45:23–41.
584 [32] Lawford S. Finite-sample quantiles of the Jarque-Bera test. Appl Econ Lett 2005;12:351–
585 4.
586 [33] Lahoz B, Alabart JL, Jurado JJ, Calvo JH, Martinez-Royo A, Fantova E, et al. Effect of
587 the *FecX*^{R} polymorphism in the bone morphogenetic protein 15 gene on natural or equine
588 chorionic gonadotropin-induced ovulation rate and litter size in Rasa Aragonesa ewes and
589 implications for on-farm application. J Anim Sci 2011;89:3522–3530.
590 [34] Lahoz B, Folch J, Sanchez P, Alabart JL. Preovulatory LH surge of Rasa Aragonesa
591 ewes carrying or not the *FecX*^{R} allele (ROA®) [Caracterización del pico preovulatorio de
592 LH en ovejas Rasa Aragonesa portadoras o no del alelo *FecX*^{R} (ROA®)]. ITEA-
593 Información Técnica Económica Agraria 2013; Vol. 109-4,
594 http://dx.doi.org/10.12706/itea.2013.025.
595 [35] Scaramuzzi RJ, Baird DT, Campbell BK, Driancourt MA, Dupont J, Fortune JE, et al.
596 Regulation of folliculogenesis and the determination of ovulation rate in ruminants.
597 Reprod Fertil Dev 2011;23:444–67.
598 [36] Fabre S, Pierre A, Mulsant P, Bodin L, Di Pasquale E, Persani L, et al. Regulation of
599 ovulation rate in mammals: contribution of sheep genetic models. Reprod Biol Endocrinol
600 2006;4:20.
601 [37] Vinet A, Drouilhet L, Bodin L, Mulsant P, Fabre S, Phocas F. Genetic control of multiple
602 births in low ovulating mammalian species. Mamm genome 2012;23:727–40.
603 [38] Bartlewski PM, Baby TE, Giffin JL. Reproductive cycles in sheep. Anim Reprod Sci
604 2011;124:259–68.
605 [39] Folch J, Alabart JL. Características reproductivas de la ovejas Rasa Aragonesa. Revista
606 OVIS. 2000;68:27–36.
607 [40] Bartlewski PM, Beard AP, Cook SJ, Rawlings NC. Ovarian activity during sexual
608 maturation and following introduction of the ram to ewe lambs. Small Rumin Res
609 2002;43:37–44.

[41] Berlinguer F, Torres-Rovira L, Spezzigu A, Leoni G, Succu S, Gallus M, et al. Anti-Mullerian hormone as an age-related predictor of the antral follicle count and the response to exogenous FSH ovarian reserve test in sheep. 17th International Congress on Animal Reproduction (ICAR), Vancouver, Canadá jul 29-aug 02 2012. Reprod Domest Anim 47(SI:4): 493-493.

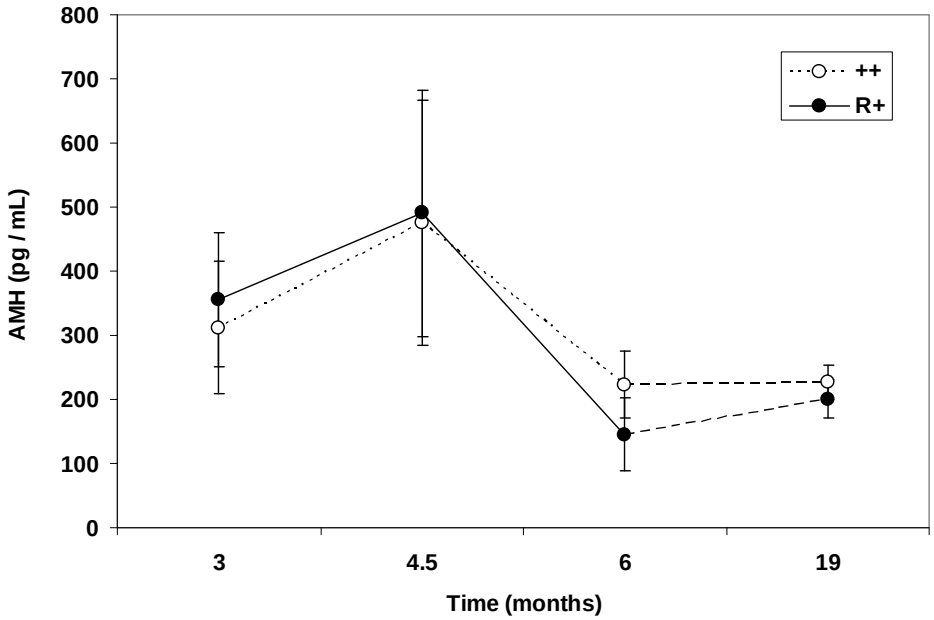
[42] Rico C, Drouilhet L, Salvetti P, Dalbies-Tran R, Jarrier P, Touze JL, Pillet E, Ponsart C, Fabre S, Monniaux D. Determination of anti-Mullerian hormone concentrations in blood as a tool to select Holstein donor cows for embryo production: from the laboratory to the farm. Reprod Fertil Dev 2012;24:932–44.

[43] Menchaca A, Vilarino M, Crispo M, de Castro T, Rubianes E. New approaches to superovulation and embryo transfer in small ruminants. Reprod Fertil Dev 2010;22:113–8.

621 **Figure 1.**

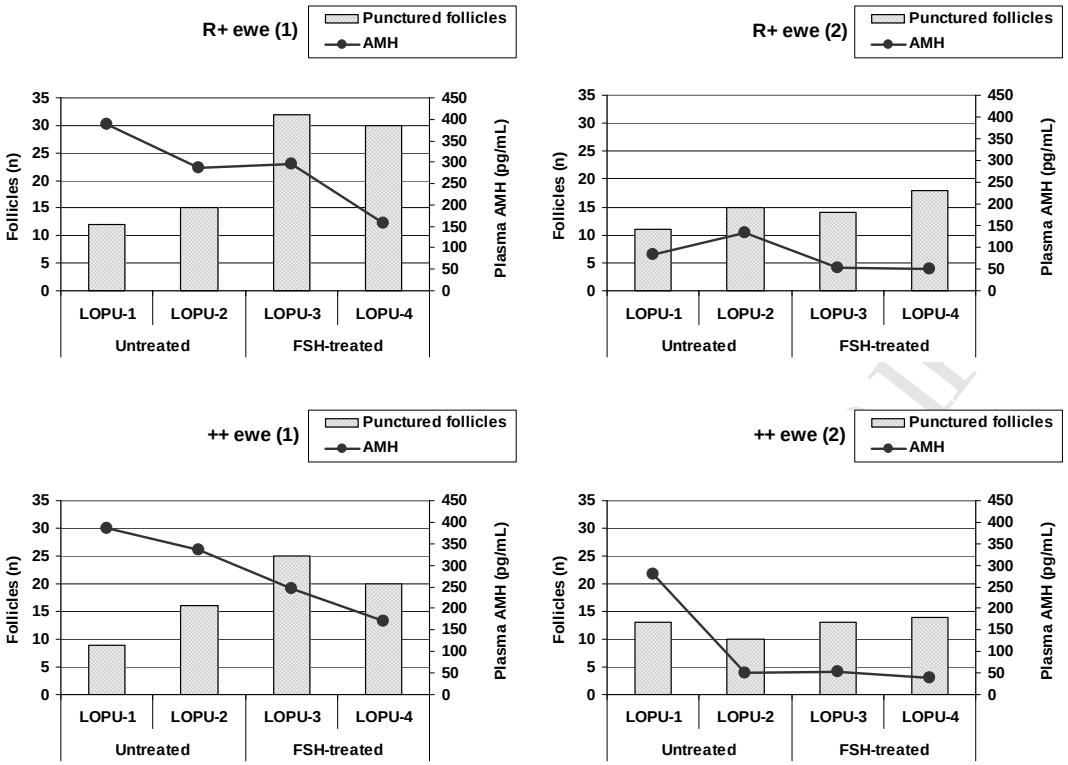


622 **Figure 2.**



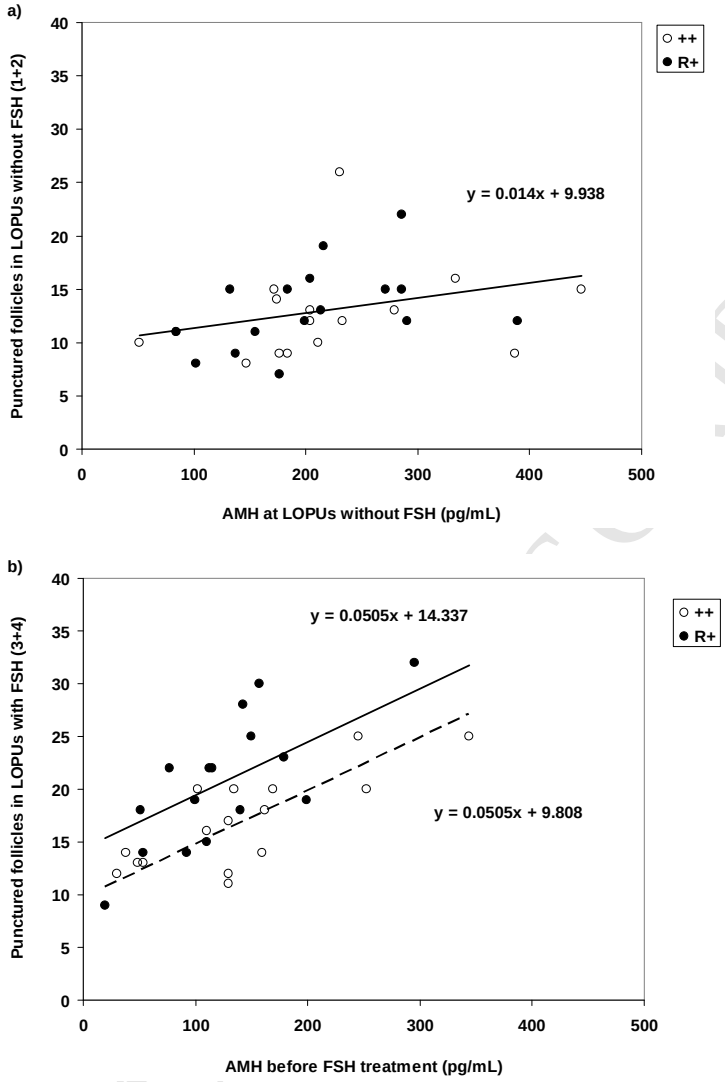
623

624 **Figure 3.**

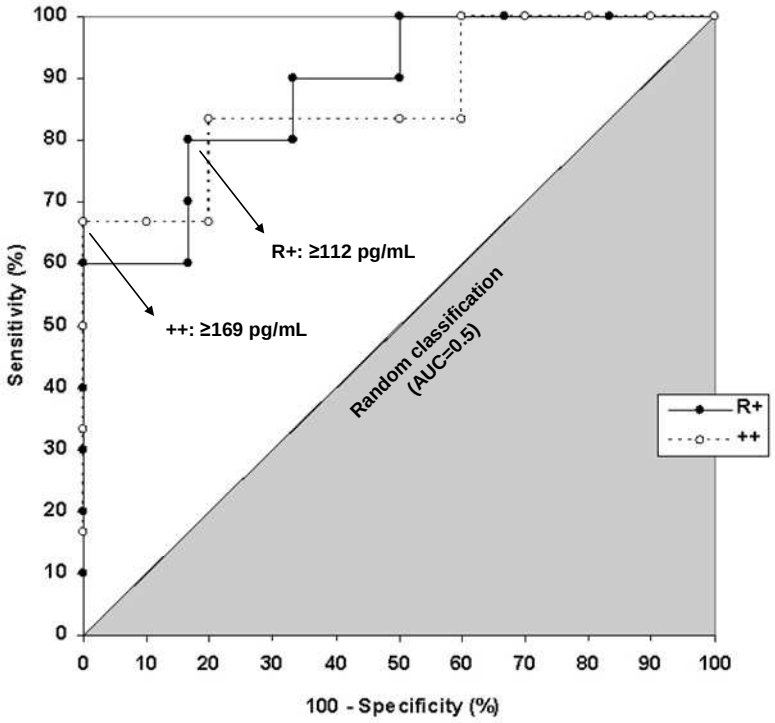


625

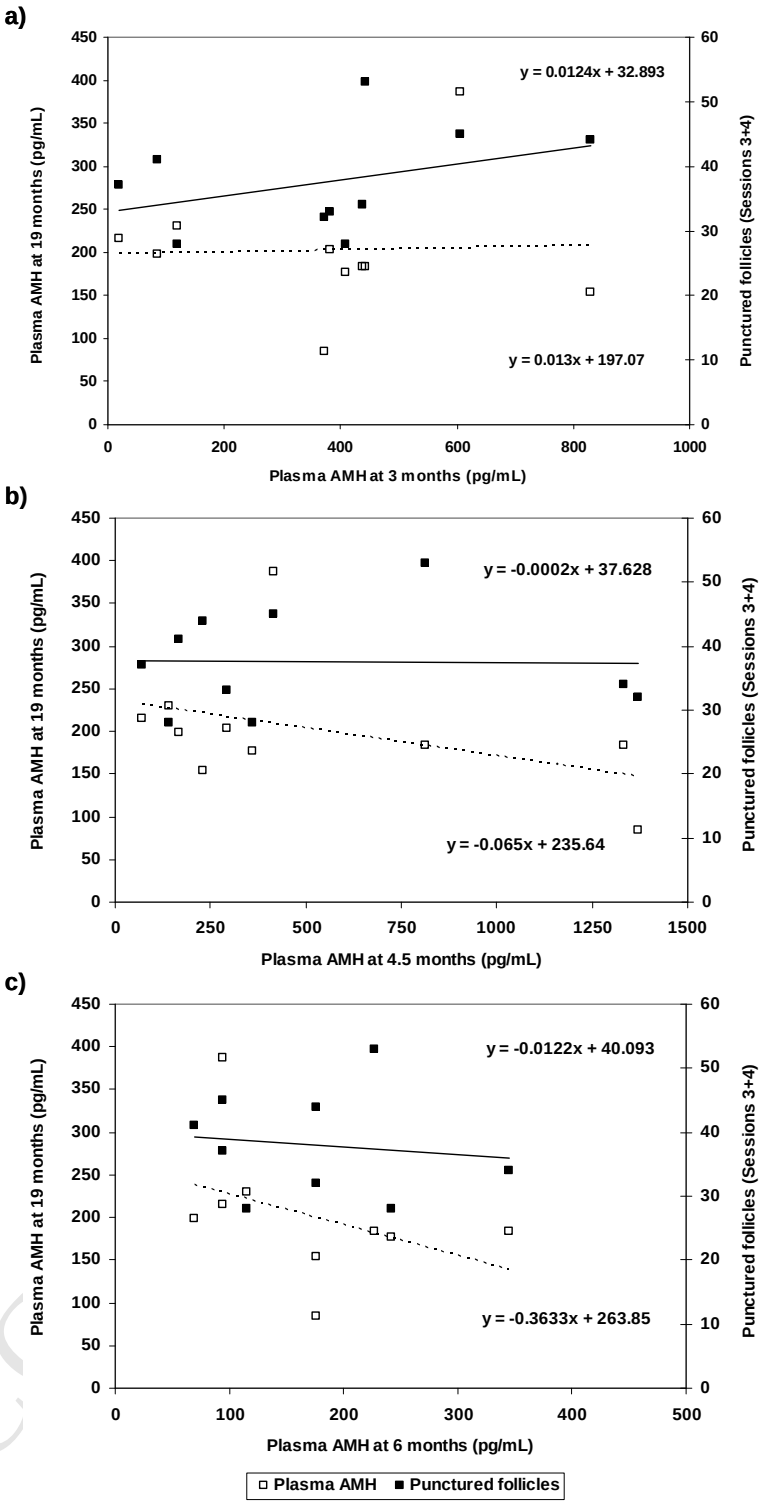
626 **Figure 4.**


a)
Punctured follicles in LOPUs without FSH (1+2)
AMH at LOPUs without FSH (pg/mL)
y = 0.014x + 9.938
++
R+
b)
Punctured follicles in LOPUs with FSH (3+4)
AMH before FSH treatment (pg/mL)
y = 0.0505x + 14.337
y = 0.0505x + 9.808
++
R+

627 **Figure 5.**



628 **Figure 6.**



629

630 In (c), nine points are represented both for AMH and punctured follicles, as there was one ewe lamb with non-detectable plasma AMH
631 concentration at 6 months not taken into account.

632 **Figure captions**

633 ***Figure 1.*** **Experimental design of laparoscopic ovum pick-up (LOPU).** Hormonal
634 treatment and blood sampling for AMH determination performed in 16 adult ewes, carrying
635 or not carrying the *FecX*^{R} allele (R+; n = 8, ++; n = 8).

636 ***Figure 2.*** **Changes in the plasma anti-Müllerian hormone (AMH) concentration before**
637 **puberty and the AMH concentrations at the adult age in ewes carrying or not carrying**
638 **the *FecX*^{R} allele**. Blood samples were taken at 3, 4.5, 6, and 19 months of age (Mean ±
639 S.E.M.) in 12 ewe lambs (R+; n = 6, ++; n = 6) and in 16 adult ewes (R+; n = 8, ++; n = 8).

640 ***Figure 3.*** **Plasma AMH concentrations and the number of punctured follicles (≥ 3 mm)**
641 **during each LOPU session**. Representative profiles with high and low AMH (above and
642 below the determined cut-off point to detect "high-responding" ewes) and follicle numbers
643 are shown for two ewes of each genotype; with (R+) and without (++) the *FecX*^{R} allele.

644 ***Figure 4.*** **Relationship between plasma AMH concentration and the number of**
645 **punctured follicles (≥ 3 mm) at LOPU**. Plasma AMH was measured in 16 adult ewes
646 carrying or not carrying the *FecX*^{R} allele (R+; n = 8, ++; n = 8) at the time of LOPU in non-
647 stimulated ewes (a; sessions 1+2), or at the beginning of the FSH treatment in LOPUs using
648 FSH (b; sessions 3+4).

649 ***Figure 5.*** **Receiver-operating characteristic (ROC) curves for predicting high (≥18**
650 **follicles) and low (<18 follicles) responding R+ and ++ ewes at LOPU.** The arrows show
651 the cut-off points for both genotypes that maximize the Youden index and their corresponding
652 AMH values. A test without capacity of discrimination (random test) is represented
653 (diagonal).

654 ***Figure 6.*** **Relationships between the plasma AMH concentrations before puberty and**
655 **during adulthood**. Plasma AMH at 3 (a), 4.5 (b), and 6 (c) months in relationship to the
656 plasma AMH concentration at 19 months (open squares, dotted line) and the total number of
657 follicles ≥ 3 mm to be punctured after FSH treatment at 19 months (closed squares, solid
658 line).