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Direttore Prof. Sergio Ledda

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Comparative study on the potential reproductive differences of ewe lambs with diverse antral follicle count at an early prepubertal age

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Laura Torres Rovira

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A mi familia

La cortesía es la principal muestra de cultura

Baltasar Gracián (1601-1658)

Laura Torres Rovira. Comparative study on the potential reproductive differences of ewe lambs with diverse antral follicle count at an early prepubertal age. PhD thesis in Riproduzione, Produzione, Benessere Animale e Sicurezza degli Alimenti di Origine Animale, Università degli Studi di Sassari.

Departamento de
Producción Animal
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DOCTORAL DISSERTATION EX ANTE EVALUATION REPORT

PH. D. STUDENT: Laura Torres Rovira

TITLE: Comparative study on the potential reproductive differences of ewe lambs with diverse antral follicle count at an early prepubertal age

In Accordance whit Article 16 of the Regulations for Doctoral Studies at the University of Sassari (approved by the Governing Council dated October 12, 2012), Dr./a FERNANDO FORCADA MIRANDA, with ID / Passport No 17704160W at the request of the Doctoral Program Coordinator of the University of Sassari ISSUES THE FOLLOWING REPORT ON THE DOCTORAL DISSERTATION CONTENT

Additional pages can be used, if necessary

1. Research lines in which the thesis is rooted:

The Thesis deals with Physiology of Reproduction in sheep, being focused in the potential of ewe lambs at an early prepubertal age on oocyte production, and therefore on Biotechnology of Reproduction in that species. The criteria of early evaluation (40 days of age) of a higher antral follicle count (AFC) and plasma anti-Müllerian hormone (AMH) concentration have been used to predict the response at different ages of the female (prepubertal, peripubertal and adult) to exogenous hormonal treatments for ovarian stimulation.

The research group has a wide experience in this field of knowledge, which is absolutely necessary to set out the different experiments and to carry out the technology involved in the different assays of the study.

2. Scientific, Technical and Humanistic contributions of the doctoral dissertation:

The Thesis is a high value scientific document, very well organized, with a very accurate objectives set out in a logical sequence of research. The review of the previous knowledge in the literature is precise, very documented and focused on the main aspects investigated in the Thesis.

The main scientific contribution of the Thesis is that the AFC at an early prepubertal age (40 days) is a very variable parameter in ewe lambs, being related to the oocyte quality for in vitro embryo production procedures, to the response to hormonal stimulation and even to the fertility in the peripubertal period. However, these effects disappear in the adult age. The document contains a rich methodology, high a number of technics and hormonal determinations, showing the competence of the research group in the Physiology of Reproduction in sheep and other farm and companion animals.

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In my opinion, another great value of the document is the possibility to extend some of the results to other species like human, where the reproductive potential of young women is being studied as influenced by several factors as undernutrition, for example.

Finally and also in my opinion, a Doctoral Thesis has to find new open doors to further research as is the case of the present document. Author states that further research is needed to elucidate the implications in the adult life of the differences demonstrated in the very early prepubertal period. I think that the fact of the lack of differences found after puberty could be related to the main cause of the puberty, the lower hypothalamic sensitivity to the negative effect of ovarian estradiol. During the prepubertal period such sensitivity is high, being the endogenous opioids clearly involved in the inhibition of the GnRH/LH secretion in presence of estradiol. As such involvement is clearly reduced also after puberty, it is possible that some neuropeptids can be involved in the result of the present Thesis. This can be also a future field of study.

3. The doctoral dissertation compiles with the required quality standards:

YES X

NO ☐

Date: 29 October, 2013

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DOCTORAL DISSERTATION EX ANTE EVALUATION REPORT

PH. D. STUDENT: Laura Torres Rovira

TITLE: Comparative study on the potential reproductive differences of ewe lambs with diverse antral follicle count at an early prepubertal age

In Accordance whit Article 16 of the Regulations for Doctoral Studies at the University of Sassari (approved by the Governing Council dated October 12, 2012), Dra. Mª Teresa Encinas Cerezo, with ID / Passport No 50707071Y at the request of the Doctoral Program Coordinator Prof. Sergio Ledda, of the University of Sassari ISSUES THE FOLLOWING REPORT ON THE DOCTORAL DISSERTATION CONTENT

Additional pages can be used, if necessary

1. Research lines in which the thesis is rooted:
   - Biomakers of ovarian function
   - Reproductive potential
   - Animal and human fertility

2. Scientific, Technical and Humanistic contributions of the doctoral dissertation:
   The main scientific contributions of the doctoral dissertation are the implementation of the knowledge about the antral follicle count and anti-Mullerian hormone levels have on the reproductive potential in sheep. The influence of these parameters on the fertility at a young age and on the reproductive response to superovulatory treatments, oocyte competence. So, a single AMH measurement or a antral follicle count performed on ewe lambs early in age could be useful to select for replacement ewes with a higher predicted fertility at first mating.
   Technically, the dissertation standarizes the antral follicle count and the anti-mullerian hormone determination as indicators of a female's total follicular reserve.
   The application of the results obtained to human reproduction are the most relevant humanistic contribution, as it could be applied to solve the human infertility described after antitumoral treatment in breast cancer.

3. The doctoral dissertation compiles with the required quality standards:

   YES ☒

   NO ☐

Date: 29th October 2013

Signed: Mª Teresa Encinas Cerezo

FACULTAD DE VETERINARIA
DPTO. TOXICOLOGÍA Y FARMACOLOGÍA

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INDEX

SUMMARY

Numerous studies link the antral follicle count (AFC) and anti-Müllerian hormone (AMH) levels with the ovarian reserve and a wide variety of fertility indices in adult animals and women. In adulthood both parameters seem to remain relatively constant for each individual during long periods of time but show a high variability between individuals. However, little is known about differences in AFC and AMH during the prepubertal period and their eventual implication on ovarian function or future reproductive life. Thus, this dissertation aimed to determine differences in the early prepubertal period, in the evolution from the prepubertal period to adulthood and in adulthood between ewe lambs classified by their AFC at an early prepubertal age. In the early prepubertal period animals with a low AFC showed, although not accompanied by a reduction in the size of the primordial follicle pool, a lower response to gonadotrophin stimulation, poor oocyte in vitro developmental competence and delayed increase in AMH levels. Differences in the AFC and in the response to ovarian stimulation tent to disappear with age. In adulthood we could not find differences in the response to a multiple ovulation and embryo transfer treatment or in the oestrus cycle. However, ewe lambs with higher AFC and AMH in the early prepubertal age still had higher pregnancy rates at first breeding season. Hence, AFC and AMH might represent an interesting tool for ewe selection at an early age.

1. INTRODUCTION

1. INTRODUCTION

Despite of the decrease of sheep flock in the last decades, the ovine sector continues to be of remarkable importance. Sheep meat and milk production has significantly increased at world-wide scale. In Europe – the region with major sheep milk production in 2007 (FAO) – sheep production acquires special importance in the Mediterranean countries, such as Italy, mainly because is linked to multiple high quality traditional dairy products (e.g. cheese). Italy milked more than 5.5 million quintals of sheep milk in 2008 (Istat), representing nearly 5% of milk production in the country. In 2009, Italy was the sixth country in the European Union with greater sheep meat production (Eurostat). Within Italy, Sardinia is the leader region in sheep production with over 47% of the Italian sheep population concentrated in the island, thus, sheep in Sardinia have a notable economic impact. In general, both goats and sheep, by its rusticity, are very useful in countries with fewer economic and natural resources since small ruminants are a cheap source for production of protein-rich foods even from limited feed resources.

Reproduction is a key factor in sheep production. Sheep need to deliver to be productive, in order to get milk from lactation and meat from the offspring. However, there are still many unknowns concerning reproductive physiology, especially at pre- and peripubertal ages, critical periods for the establishment of the reproductive function. Therefore, the study of sheep reproduction is an issue of essential importance in the design of new selection strategies – particularly for females for which the selection process is slower than for rams – and to get a more depth knowledge about basic physiology.

Sheep are good animal models to experimental research and have been used primarily in biomedical research areas of foetal development, pregnancy and reproduction, and endocrinology. Like sheep most women have single ovulations, albeit multiple ovulations can occur, making the sheep an animal model closer to human reproduction than other polyovular species. Similar to women, they also have well characterized waves of

growth and atresia of antral follicles. Unlike larger animals, their maintenance is relatively easy and inexpensive, which allows to increase the number of individuals at an acceptable cost. Also, their shorter gestation length and shorter time to the onset of puberty can reduce the study time respect to large ruminants. Their size allows taking frequent multiple blood samples from the same individuals without compromising the animal's survival, and the use of techniques and equipments employed in humans (e.g.: ultrasound scanners, laparoscope, etc.). Defined synchronization protocols, enable to concentrate births around the same date, together with the gregarious character that characterizes the flock help to decrease the individual factor in favour of group factor.

Antral follicle count and anti-Müllerian hormone levels have been linked to high reproductive outcomes in adulthood and seem to be highly variable between individuals but relatively constant for each individual during long periods of time. However, little is known about differences in antral follicle number and anti-Müllerian hormone during the prepubertal period and their eventual implication on ovarian function or future reproductive life. Therefore, considering that sheep are important animals for the research in reproduction and animal production both in order to study new selection criteria as to deepen the knowledge of physiology in sheep as a model that may be applied to women, we decided to further study ewe lambs classified by their antral follicle count during the early prepubertal period which could have an impact during the prepubertal, peripubertal and postpubertal ages. This might be useful to the design of new strategies for early animal selection, to better understand possible differences in ovarian reserve, follicular recruitment and oocyte quality. To fulfil this purposes this research was divided in 4 main experiments in order to find the answers to the objectives specified in next section:

Preliminary experiment: aimed to determine whether there are differences in the antral follicle count at an early prepubertal age in Sarda ewe lambs as it has been seen in adults. This experiment pursues to respond to objective 1.

Experiment 1: is focused on the study of the early prepubertal period of ewe lambs with different antral follicle count. This experiment pursues to respond to objectives 2, 3 and 4.

Experiment 2: encompasses the study of the variations with age between three different moments in the reproductive life: the early prepubertal period, the peripubertal period and the postpubertal period of ewe lambs with different antral follicle count at an early prepubertal age. This experiment pursues to respond to objective 5.

Experiment 3: is focused on the study of some postpubertal reproductive characteristics of ewe lambs with different antral follicle count at an early prepubertal age. This experiment pursues to respond to objectives 6, 7 and 8.

2. OBJECTIVES

2. OBJECTIVES

1) To determine whether in Sarda ewe lambs there are differences in the antral follicle count at an early prepubertal age and evaluate if these entail differences in the response to exogenous gonadotrophin stimulation and anti-Müllerian hormone plasma concentration.

2) To verify whether differences in the antral follicle count at an early prepubertal age are accompanied by changes in oocyte and embryo developmental capacity.

3) To assess the ovarian reserve at an early prepubertal period in animals with diverse antral follicle count.

4) To follow the evolution of anti-Müllerian hormone and FSH plasma concentrations throughout the first weeks of life in groups with different antral follicle count.

5) To study if there are variations in the antral follicle count, anti-Müllerian hormone plasma levels and the response to exogenous gonadotrophin stimulation at an early prepubertal age, a peripubertal age and a postpubertal age between and within groups with diverse antral follicle count at an early prepubertal age.

6) To ascertain if fertility at first breeding season change between ewe lambs with high and low antral follicle count at an early prepubertal age.

7) To find out if animals with high or low antral follicle count at an early prepubertal age show differences in the oestrus cycle in adult life.

8) To investigate the effect of a high or low antral follicle count at an early prepubertal age on the response to a multiple ovulation and embryo transfer protocol in adulthood.

3. LITERATURE REVIEW

3. LITERATURE REVIEW

3.1. Reproductive physiology in ewes

Sheep (*Ovis aries*) are usually classified as seasonally polyoestrous animals which means that most sheep have multiple oestrus cycles during the breeding season alternated with an anoestrus period during which no sexual behaviour is expressed. Thus, ewes are not generally cycling throughout the whole year; seasonality is more marked in the higher altitudes and latitudes (reviewed by Rosa and Bryant, 2003) in the Frigid and Temperate zones, and less marked and even without presenting an anoestrus period as we approach the equator in the subtropics and the tropics, where the duration of the hours of light and darkness come to equal (Yenikoye, 1984; Carles and Kipngeno, 1986; Chemineau et al., 2007).

Sheep were already seasonal animals in the Late Stone Age (Balasse et al., 2003); this breeding strategy, adopted also by other farm animals species including fish, poultry and mammals (Chemineau et al., 2007), expects to fit the time of births to the season with better conditions (food and water availability, temperature, variations in the predatory conduct of other species, etc.) for the survival of the mother and her progeny (Ortavant et al., 1985; Wayne et al., 1989; Lindsay, 1996). Thereby lambing normally occurs at the end of the winter and early spring. However, domestication and artificial selection have reduced the effect of seasonality on the reproductive activity (Rosa and Bryant, 2003) resulting in variations in the timing of the reproductive season among breeds (O'Callaghan et al., 1992; Goff et al., 2013). Rustic breeds exhibit a more marked seasonality when compared with the most selected ones, similarly, wild and feral animals have a greater seasonality than domestic animals (Lincoln et al., 1990).

Cyclicity is deeply influenced by the amount and timing of light; in fact, sheep are so-called short-day breeders because they mate when daylight hours shorten while darkness hours increase; this corresponds mainly to late summer, autumn and early winter in the northern hemisphere. Photoperiod is considered as the main factor in the seasonality of the breeding season (Rosa and Bryant, 2003); other factors such as nutrition and nutritional status (Forcada and Abecia, 2006; Menassol et al., 2012), environmental temperature, social interactions (e.g.: male effect) and lactation period may modulate seasonality. The pineal gland is involved in the control of the seasonal reproductive activity linking the annual rhythm of photoperiod with the reproductive axis through a rhythmic pattern of melatonin section (Reiter, 1993; Wagner et al., 2008). The retina sends information about daylight hours through neural pathways to the pineal gland. Pineal gland modulated by day length information, releases melatonin only at night which causes the long and short days have a different duration of melatonin secretion. Melatonin acts in the hypothalamus modulating the pulsatile secretion of GnRH according to variations in melatonin release (Malpaux et al., 1993).

3.1.1. Oestrous cycle in sheep

During reproductive season ewes have repeated 16- to 17-day long oestrus cycles. Although the duration of the oestrous cycle in sheep has been reported between 14 and 19 days the mean duration of 16-17 days is remarkably constant. Abnormally long cycles may be related to prolonged lifespan of the corpus luteum (Bartlewski et al., 1999) while short cycles are related to a short lifespan of the corpus luteum and insufficient luteinisation coincident with the reactivation of the ovarian ovulatory function after the anoestrus or after delivery (Hunter, 1991). Oestrus cycles are classically divided into two phases:

- **Follicular phase:** with an average duration of 2 or 3 days includes prooestrus and oestrus phases and it is characterized by the final follicular growth and ovulation.

The prooestrus, the phase that immediately precedes the oestrus, is marked by reduced serum levels of progesterone and an important increase in the secretory activity of the whole reproductive system. The oestrus is the period of acceptance of the male under natural conditions, and it can be considered as day 0 of the cycle since it constitutes a good marker for the beginning of a new cycle. This behaviour is principally due to high oestradiol levels. Sheep have a period of sexual receptivity, also named as heat, whit a mean duration of 30 to 36 hours. Ovulation occurs before the end of this phase, around 36-40 hours after the onset of oestrus.

- **Luteal phase**: with an average duration of 14 or 15 days includes metoestrus and dioestrus and it is characterized by the formation, presence and activity of the corpus luteum. The metoestrus is the period immediately succeeding oestrus when the male is no longer accepted and the corpus luteum is being formed. The dioestrus correspond to the period in which there is a functional mature corpus luteum releasing progesterone. Corpus luteum only becomes active 2 or 3 days after ovulation.

3.1.1.1. Endocrine control and hormonal patterns of the oestrous cycle

Endocrine control

The endocrine system plays a fundamental role in the onset of puberty, restore and control of the reproductive cycle, formation of mature oocytes and establishment and maintenance of gestation. The neuroendocrine control of the sexual cycle in ewes performed by the hypothalamic-pituitary-ovarian-uterine axis through the release of different types of hormones (Scaramuzzi et al., 1993) is outlined in Figure 1.

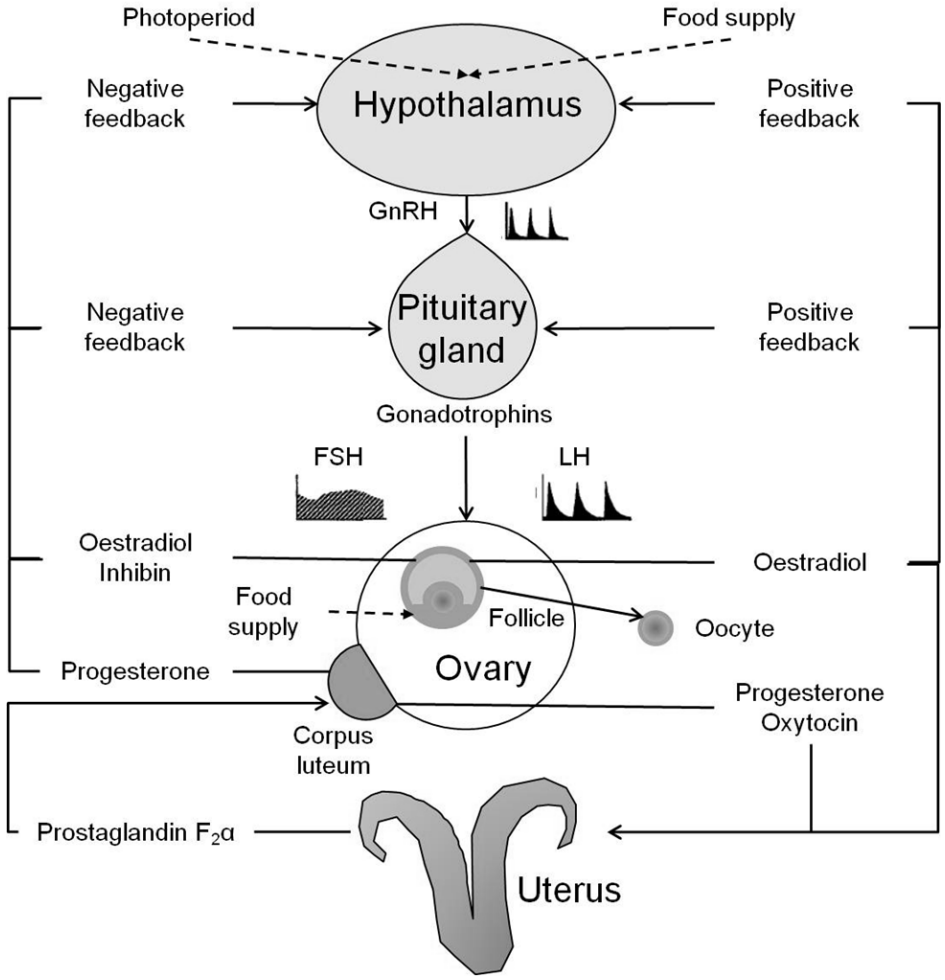

Figure 1: Schematic illustration on the neuroendocrine control performed by the hypothalamic-pituitary-ovarian-uterine axis in ewes during the oestrus cycle. Published in Scaramuzzi et al.,1993.

Briefly, Gonadotrophin-releasing hormone (GnRH) secretion depends on a complex interaction between internal factors, mainly through feedback mechanisms, and external factors (van den Hurk and Zhao, 2005) such as photoperiod, nutrition, male pheromones, and stress. The hypothalamus with the pulsatile secretion of GnRH stimulates the synthesis and release of gonadotrophins (FSH and LH) by the anterior pituitary gland (Pawson and McNeilly, 2005). These gonadotrophins will act on the ovaries. Follicle-stimulating hormone (FSH) stimulates the recruitment, growth and selection of follicles (Baird and McNeilly, 1981), while Luteinizing hormone (LH) stimulates the further development of the follicle, increases

the secretion of oestradiol (Baird and McNeilly, 1981) and the ovulation with the subsequent development of a corpus luteum in which formation and maintenance is involved. Each pulse of GnRH causes the secretion of a pulse of LH, of greater amplitude and frequency during the follicular phase; whereas the relationship between GnRH pulses with FSH secretion is not yet well established (Millar, 2005).

In the ovary the granulosa and theca cell from the follicles produce oestradiol (E_2) from androstenedione by aromatase activation (Hsueh et al., 1984). Granulosa cells stimulated by FSH increase in number and size, also the number of receptors for FSH increases making its effect bigger (Labadía Mazuecos, 1996). When E_2 concentration is high enough to reach a certain threshold level the preovulatory surges of GnRH and gonadotrophins are triggered (Moenter et al., 1991; Scaramuzzi et al., 1993). Although E_2 exert a positive feedback on the hypothalamic GnRH secretion when its concentration is high, it has also an inhibitory action at basal levels as it increases the negative feedback produced by progesterone, increases GnRH pulse frequency and alters GnRH pulse shape (Goodman et al., 1981, 2002).

Activin and inhibin are peptides with opposite action produced by granulosa cells in the follicles; activin enhances the production and secretion of FSH (Ying, 1988) while inhibin has a negative feedback role on FSH (Burger, 1993). Follistatin binds activin with high affinity modulating pituitary FSH secretion by endocrine feedback (reviewed by Sarraj and Drummond, 2012).

Once ovulation occurs and corpus luteum is formed and becomes active it begins to secrete progesterone (P_4) under the influence of LH. P_4 inhibits GnRH pulse frequency (Goodman et al., 2002) preventing further ovulation and preparing the uterus for a possible incoming pregnancy. When there is no embryo or in the absence of a positive recognition of pregnancy, the oxytocin released from the corpus luteum induces the secretion of $PGF_{2\alpha}$ by the uterine endometrial glands which is transported to the ovary through a countercurrent

mechanism exerting a luteolytic action (McCracken et al., 1972) by decreasing local blood flow.

Hormonal patterns

Main hormonal patterns during the oestrus cycle are schematized in Figure 2. FSH –that promotes the recruitment, growth and selection of follicles– presents a low basal blood concentration with elevations generally matching with the follicular waves (Evans et al., 2000) that will be later explained. The largest increases in FSH blood concentrations were observed before ovulation synchronously with the LH-surge and 20 to 30 hours after ovulation (Wheaton et al., 1984; Souza et al., 1997). LH is released in pulses in response to GnRH stimulating the final growth of follicles.

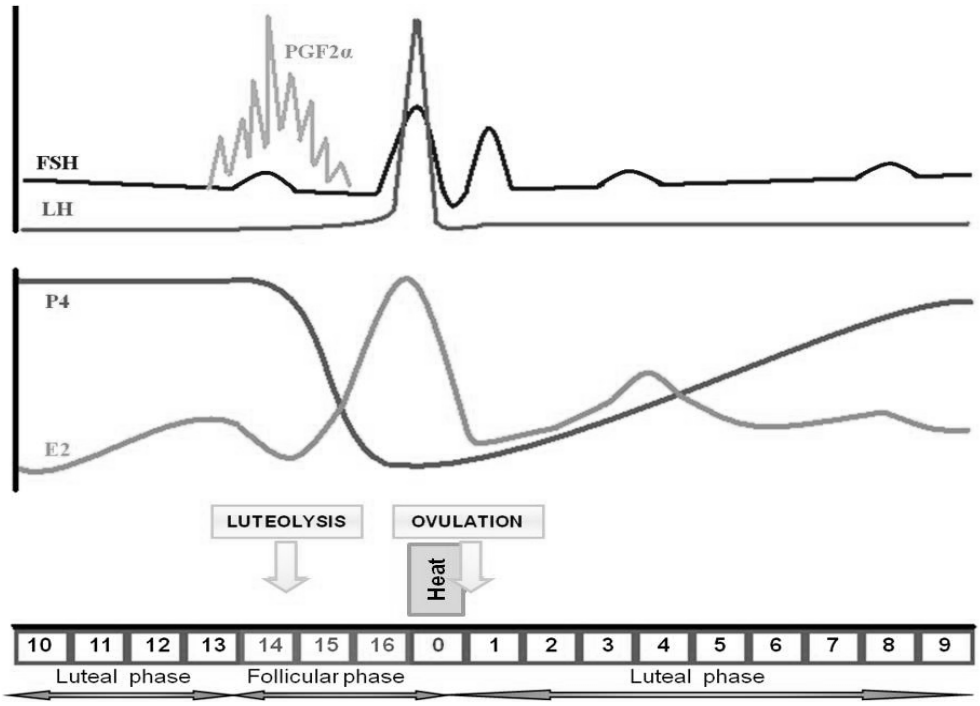

Figure 2: Schematic representation of the main hormonal patterns during the oestrus cycle in sheep in relation to major events. FSH (Follicle-stimulating hormone); LH (Luteinizing hormone); PGF2α (Prostaglandin F2 alpha); E2 (oestradiol); P4 (progesterone).

As follicles grow the levels of oestrogens secreted by them progressively increase, especially the oestradiol, which have its maximum concentration at the LH-surge (Souza et al., 1997). During the cycle 3 or 4 E_2 peaks can be identified coinciding with the end of the growth phase of the largest follicle from each follicular wave (Bartlewski et al., 1999; Bister et al., 1999). When E_2 levels rise and there are low levels of progesterone signs of heat (flushed and swollen vulva with acceptance of mate) appear.

Oestradiol in turn induces a large increase in the release of luteinizing hormone (LH) concentrations known as the preovulatory LH-surge that happens between 2 and 6 hours after the beginning of the oestrus and triggers ovulation -about 18 to 24 hours after the preovulatory surge of LH- and formation of a corpus luteum.

Progesterone concentrations increase as the corpus luteum develops, remaining elevated during the luteal phase and having its maximum concentrations from day 6 to days 10 or 12 of the cycle. High levels of progesterone prevent a new ovulation (Goodman et al., 1981) although during the luteal phase follicular waves still occur. If the sheep is not pregnant, progesterone levels decrease after luteolysis, which occurs around day 14-15 of the cycle (Scaramuzzi et al., 1993) in response to the uterine secretion of $PGF_{2,}$ then a new cycle can start. The preovulatory surge of LH occurs about 4 days after luteolysis begins since with the decrease in progesterone levels during luteolysis the secretion of LH begins to increase due to a higher frequency of pulses (Wallace et al., 1988).

3.1.2. Oogenesis and folliculogenesis

Oogenesis is defined as the process of forming the female gametes, oocytes, in sexual reproduction. Sheep are diploid animals (2n) so in somatic cells there is a maternal and a paternal copy of each chromosome (54 chromosomes, 27 pairs of homologous chromosomes) inherited from the oocyte of the mother and the spermatozoon of the father

(Hyttel, 2010). Thus, gametes need to be haploid - containing only half the number of chromosomes (n) - in order to get a diploid offspring after fertilization, they have to enter meiosis in order to reduce the number of chromosomes.

Meiosis requires two cell divisions, one reductive (meiosis I) and one equational (meiosis II). Meiosis is initiated during foetal life then becomes arrested at the dictyate stage of meiosis I which is only resumed shortly before ovulation when in response to the preovulatory LH-surge there are progressive structural and functional changes in the oocyte occurring nuclear and cytoplasmic maturation and cell division (Monget and Monniaux, 1995). Prior to the meiosis process the cell's chromosomes undergo a DNA replication, creating two exact copies (sister chromatids) from each chromosome of the homologous pair, each chromosome pair therefore consists of four chromatids. Pairs of homologous chromosomes exchange parts by recombination leading to crossovers of DNA between the maternal and paternal versions of the chromosome increasing genetic variability. Then, the members of each pair of homologous chromosomes, not their chromatids, are distributed into different nuclei of the now haploid daughter cells at first reductive division. In meiosis II, the sister chromatids forming each chromosome are separated and distributed in the nuclei of the successive daughter cells.

Once the female germ cell has initiated meiosis I is referred to as a primary oocyte, even during the meiosis arrest. At the end of meiosis I one of the daughter cells is smaller and almost deprived of organelles (first polar body) while the other daughter cell is larger with a greater number of organelles (secondary oocyte). First polar body degenerates without dividing whereas the secondary oocyte initiates meiosis II. When oocyte is fertilized the process will be reactivated (McNatty et al., 1995). At the end of meiosis II the secondary oocyte is divided unequally into the second polar body and a large cell whose genetic material will give rise to the female pronucleus.

Oocytes, as spermatozoa do, derived from primordial germ cells (PGCs) which are pluripotent cells. Primordial germ-cells develop during gastrulation becoming recognizable in the posterior rim of the embryonic disc. Then they move into the newly formed mesoderm and endoderm. Later, they can be seen in the visceral mesoderm and surround the yolk sac and the allantois, probably to be protected from the differentiation signals driving gastrulation within the embryo. Finally they migrate via the primitive mesentery into the still undifferentiated developing gonad (genital ridge) around day 23 of gestation in sheep (McNatty et al., 1995). PGCs proliferate first by mitosis - a process whereby a parental cell can give rise to two genetically identical daughter cells- during and after its migration, making larger the number of these cells, later they will enter meiosis. PGCs in the foetal ovarian develop in cyst-like clusters (reviewed by Sarraj and Drummond, 2012). Flat somatic cells - that will become follicular cells- starts to surround PGCs, turning the last ones into oogonia which proliferate into primary oocytes. This proliferation is followed by apoptosis (Sawyer et al., 2002) to which only a small population near the surface of the developing ovary will survive, and by which each survival oocyte gains additional pre-granulosa cells (Sawyer et al., 2002). At day 70 of gestation the foetal sheep ovary exhibits numerous oogonia and primary oocytes in meiotic prophase (Bezard et al., 1987). Primary oocytes become arrested at the dictyate stage and covered by flat follicular pre-granulosa cells, originated from the ovarian surface epithelium in sheep (Sawyer et al., 2002), giving rise to primordial follicles (Hyttel, 2010). First primordial follicles are seen around day 75 of gestation (McNatty et al., 1995; Sawyer et al., 2002).

Follicular growth is the succession of a series of stages in which the follicles acquire different states of maturation, or suffer degeneration and atresia. It consist of the enlargement of the oocyte itself and the development of the follicle envelope and later the antrum (Peters, 1969). Therefore, in the adult ovary, we find follicles at different stages: resting, growing and atretic follicles (Drummond and Findlay, 1999). The vast majority of follicles that enter a growth phase will degenerate through atresia, only a minority will complete their growth and ovulate. Folliculogenesis in sheep takes about 6 months (Hunter

et al., 2004) and includes the development from primordial follicle to ovulation or atresia , regulating ovulation rate as the number of follicles that passes from one state to the next development state decreases. At the end of the process the number of ovulatory follicles corresponds to the ovulation rate characteristic of the breed. Folliculogenesis starts as early as embryonic life, so that females, at birth, have follicles that have stopped growing. This growth is restarted after birth and peaks before puberty and during the reproductive life of the animal. This activation is regulated by gonadotrophins and intraovarian factors; the number of growing follicles is determined by factors such as race, season and nutrition (Armstrong and Webb, 1997). The follicular development can be divided into two phases: a first phase of slow development, explained in the next section, in which primordial follicles evolve to preantral follicles; and a second phase of much faster growth explained in the "Final growth phase: Follicular waves" section that occurs following a wave-like pattern when follicles become gonadotrophin-dependent till ovulation or atresia.

3.1.2.1. Early follicular development

Primordial follicles constitute the resting pool of follicles from which follicles are recruited for growth. First primordial follicles are seen at about day 75 of gestation (McNatty et al., 1995; Sawyer et al., 2002) formed by an immature and quiescent oocyte without zona pellucida surrounded by a single layer of flattened pre-granulosa cells enclosed by a basement membrane that separates them from the surrounding stromal cells. Thenceforward, primordial follicles are activated by multiple activator and repressor pathways controlled by a bidirectional communication between the oocyte and the surrounding somatic cells (Kim, 2012). Bone morphogenetic proteins, particularly BMP-4 and BMP-7, have been proposed as positive regulators of the primordial-to-primary follicle transition (Knight and Glister, 2006).

After activation they become primary follicles with a diameter of 23 μm to 53 μm in the case of sheep foetuses (Palma et al., 2012) in which the single layer of pre-granulosa cells is transformed into a monolayer of cuboid cells now called granulosa cells. Epithelium proliferates increasing the number of layers of cuboid granulosa cells and therefore follicular size. Both the oocyte and follicular cells secrete a refractive glycoproteinaceous translucent matrix that surrounds the oocyte called zona pellucida with glycoproteins responsible for the species-specificity that bind to spermatozoa and induce the acrosome reaction (Gupta et al., 2012). Despite the presence of the zona pellucida the oocyte maintains direct contact through gap junctions with the projections of the innermost granulosa cells that traverse the glycoproteinaceous matrix. These gap junctions are important for communication and transfer of low molecular weight substances, metabolites and signals, for normal cell development and function (Senbon et al., 2003; Thomas and Vanderhyden, 2006). Granulosa cell factors stimulate the differentiation of theca cells from the cortical stromal cells that surround them, at the same time thecal factors promote granulosa cell proliferation and suppress granulosa cell apoptosis stimulating the increased number of layers (Palma et al., 2012). Theca cells are divided into an inner layer of steroid-producing cells – theca interna – and outer concentric layers with supportive functions – theca externa. Theca interna contributes to oestradiol synthesis by producing androgens that are transported to the granulosa cells where they are aromatized into oestrogens (Hyttel, 2010). This new follicle with numerous layers of granulosa cell layers, zona pellucida and theca interna and externa is known as secondary follicle or preantral follicle. It takes about 130 days for the primordial follicle to reach an early preantral state of 0.2 mm in diameter (Cahill, 1981) and about 24-35 days for the secondary follicle to reach 0.5 mm in diameter. Even though FSH is a potent growth and differentiation factor for preantral follicle (Hsueh et al., 2000), follicles are not yet gonadotrophin-dependent being regulated by other autocrine and paracrine factors (Findlay et al., 2000; Senbon et al., 2003; Thomas and Vanderhyden, 2006).

As development continues in response to FSH the granulosa cells proliferate and secrete mucopolysaccharides into the follicle resulting in fluid-filled spaces between the

granulosa cells that will fuse to form a single cavity, the antrum. The antrum characterizes the tertiary follicle or early antral follicle. The antrum continues to expand, early antral follicles need an average of 5 days to reach the 2 mm in size and enter the terminal developmental stage. Early antral follicle evolves to preovulatory follicle or Graafian follicle with an expanded antrum fully formed and the cumulus oophorus which is a protrusion of the granulosa cells, now cumulus cells, that contains the oocyte. As the follicle develops so does the oocyte undergoing numerous morphological changes such as nuclear and cytoplasmic maturation with the development of cortical granules, becoming competent to resume the meiotic process and support the early embryonic development after fertilization (Hyttel, 2010), with the microenvironment of the antral follicle being responsible for the integrity of these processes to obtain high quality oocytes (Hennet and Combelles, 2012).

3.1.2.2. Final growth phase: Follicular waves

The last phase of follicular development occurs following a wave-like pattern (Bartlewski et al., 1999; Evans et al., 2000; Driancourt, 2001) when follicles become gonadotrophin-dependent. Follicular waves were first discovered thanks to the availability of transrectal ultrasonography (reviewed by Singh et al., 2003), that has become a useful tool for the study of reproduction in small ruminants since it provides an indispensable method to perform repeated non-invasive examinations of the ovarian structures and allows the number and size of antral follicles to be determined regardless of their depth inside the ovary (Gonzalez-Bulnes et al., 2010). Follicular wave is defined as the synchronously growth of a group of follicles through the following steps:

- **Recruitment:** a cohort of healthy follicles initiates a fast growth under the influence of FSH action. Changes in the duration and amplitude of the elevations of FSH previous to the interovulatory waves do not influence the characteristics of the following follicular wave although FSH peaks are essential in the emergence of the follicular

wave (Toosi et al., 2010). Only gonadotrophin-dependent follicles are recruited. In sheep, follicles become gonadotrophin-dependent when they reach about 2 mm in diameter (Webb et al., 2003). Recruitment lasts 1 day in the ewe (Driancourt, 2001).

- **Selection:** from the initial cohort of follicles recruited to grow only one to three follicles - according to ovulation rate for each breed (Hunter et al., 2004)- are selected for a further growth, the rest will become a cohort of subordinate follicles and enter atresia (Driancourt, 2001). Follicles that are further developed secrete high amounts of oestradiol and inhibin making FSH levels decline during the follicular phase, while the frequency of LH pulses increases, starting the selection process which culminates in the process of dominance. Increased release of inhibin by the selected dominant follicle may up-regulate the androgen LH-induced secretion that is required to sustain a high level of oestradiol secretion during the pre-ovulatory phase (Knight and Glister, 2006). Meanwhile, atretic antral follicles contain low concentrations of E2 and are less capable of releasing it (Rouillier et al., 1996). Antral follicles that will be selected can also cause a direct inhibitory effect on the growth of the rest of the cohort through the secretion of substances in the blood which reduce the sensitivity to FSH of the less developed follicles (Gonzalez-Bulnes et al., 2004c; Knight and Glister, 2006). Only those follicles with receptors for LH and capable of transfer its gonadotrophic requirements from FSH to LH can survive the reduced levels of FSH and reach the dominant stadium (Campbell et al., 1995; Webb et al., 2003). Without an adequate supply of FSH the activity of the aromatase -enzyme that synthesizes oestradiol from androstenedione- is reduced, decreasing the secretion of E_2 and increasing the accumulation of androgens within the follicle, fact that triggers atresia.

- **Dominance:** Dominance is known as the process by which the growth stops in the subordinate follicles cohort driving them into atresia, while the preovulatory follicular growth and maturation of the selected follicles occurs. Selected antral follicle evolves

to preovulatory Graafian follicle, characterized by being estrogenically active, having a diameter greater than 4 mm (Driancourt, 2001) and a greater number of layers of granulosa cells, and a large number of receptors for LH. A selected follicle of around 2 mm in diameter needs approximately 4 days to reach the preovulatory size of 4.5 to 5 mm in diameter (McNeilly, 1984). The dominant follicle is characterized by its progressive increase in diameter due to increase in antral fluid volume as well as an increased number of granulosa cells (Baird, 1983) reaching 5 mm in diameter before regression or ovulation in anovulatory and ovulatory waves respectively. The presence of one or more dominant follicles growing should prevent the follicular recruitment from the next cohort till the end of the growing phase (reviewed by Evans et al., 2000, 2003). However, the fact that more than one follicle can reach the ovulatory size during the same wave and that follicles belonging to two consecutive waves are able to ovulate at the same time (Bartlewski et al., 1999), even if follicles from the penultimate wave are less viable that follicles from the final wave (Seekallu et al., 2010), suggests that dominance in sheep is weak (Bartlewski et al., 2011). During reproductive season when there is no corpus luteum coinciding with the presence of a dominant follicle this may ovulate.

At the beginning, there was a lack of consensus among authors about the pattern of follicular development in sheep; some described a continuous growth independent of cycle phase (Lopez-Sebastian et al., 1997), while others observed growth in waves, varying the number of waves per cycle with breed and individual (reviewed by Evans et al., 2000). These findings can be explained by distinguishing two types of waves that occur continuously in the cycle of small ruminants: primary or major follicular waves and secondary or minor follicular waves. Secondary waves are produced during the mid-luteal phase (dioestrus) without a dominant follicle clearly differentiated due to suppression of LH induced by progesterone. On the other hand in primary waves a dominant follicle (> 5 mm) is clearly developed. Primary waves occur during the early luteal phase (metoestrus) in which there is less suppression of LH because of a relative lack of progesterone as the corpus luteum is still

young, and during the follicular phase (proestrus and oestrus) since after luteolysis progesterone levels decline being unable to suppress LH secretion (Adams, 1999). Similarly, during anoestrus, LH levels are low and ovulation doesn't occur, yet ovaries remain active with follicular waves in which antral follicles growth to reach the preovulatory size - 5mm or more in diameter in the sheep- before regression (Webb et al., 1992; Bartlewski et al., 1999).

During this final growth phase before ovulation is triggered by the preovulatory LH-surge oocyte acquires its competence to be fertilized and support initial embryonic development through a process called oocyte capacitation. The follicle switches steroid synthesis from oestradiol to progesterone and prepares the wall for the beak that will release the cumulus-oocyte complex (COC). In the oocyte there is a nuclear maturation whereby meiosis is resumed when oocyte is ovulated and a cytoplasmic maturation whereby cortical granules migrate to a peripheral position in preparation for exocytosis at fertilization (Hyttel, 2010).

In sheep the intervals between waves usually have a mean duration of 3 to 5 days, which means that during the oestrus cycle 3 or 4 follicular waves will occur in each interovulatory follicular interval, as depicted in Figure 3 (reviewed by Evans et al., 2000; Bartlewski et al., 2011). Similarly, in women, 2 or 3 follicular waves per cycle with dominant follicles and subordinate cohorts and major or minor waves are being described (Baerwald et al., 2003, 2012). In Sarda sheep in most individuals it has been described a mean of 4 follicular waves per cycle and a smaller number of animals having 5 waves per oestrus cycle (Spezzigu, 2010). In pre- and peripubertal ewe lambs the pattern of regular follicular wave emergence seems to not be yet fully established (Bartlewski et al., 2006).

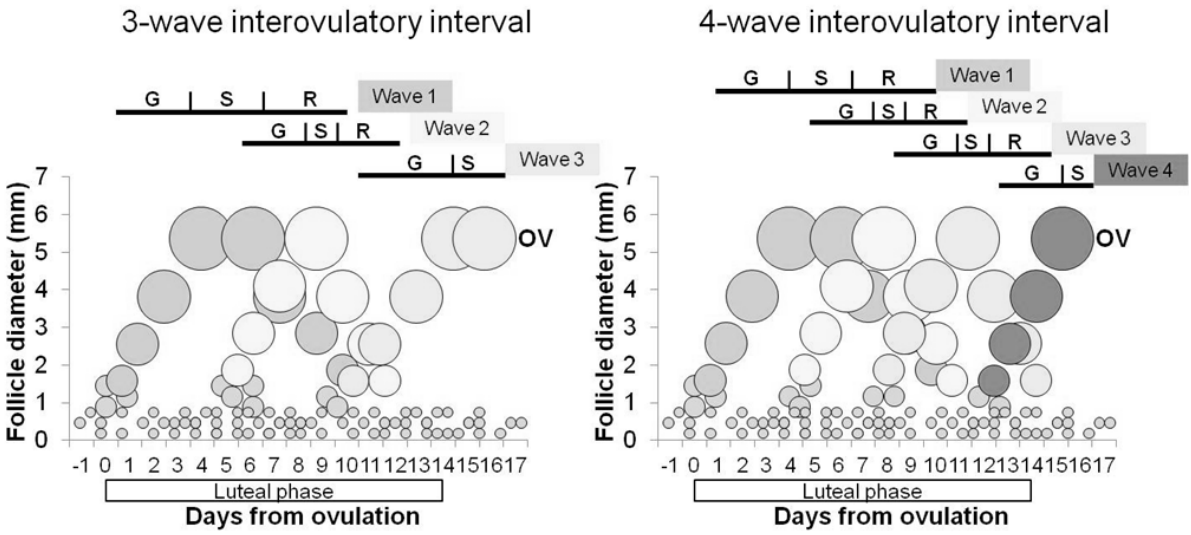

Figure 3: Schematic illustration of the interovulatory follicular growth and regression of dominant follicles during the luteal phase, and growth of the ovulatory follicle in ewes with 3 or 4 waves per oestrus cycle. OV= ovulation. Approximate duration of the growing (G), static (S) and regressing (R) phases of the lifespan of the largest follicles of waves are shown in the upper chart areas. Adapted from Bartlewski et al., 2011.

3.2. Ovarian reserve

The complete follicle pool in the ovaries, that is the amount and quality of primordial follicles containing a quiescent oocyte - the pool of non-growing follicles (NGFs)- and growing follicles, is known as ovarian reserve (OR; Gleicher et al., 2011). Sometimes the term ovarian reserve is misused in assisted conception literature referring only to the number of growing follicles that can be recruited to grow by exogenous FSH which should be better named functional ovarian reserve (FOR; Gleicher et al., 2011; Anderson et al., 2012; Anderson and Wallace, 2013). The most widespread theory holds that the number of oocytes formed during foetal development is the maximum pool of follicles available along reproductive life, being a non-renewable pool. However, the discovery in adults of ovarian germline stem cells (GSCs) capable of proliferation and meiotic maturation to form new

oocytes (Johnson et al., 2004) has brought into discussion the possibility that OR could be renew (Bukovsky et al., 2005; Tilly et al., 2009; Kerr et al., 2013), but even if there is some follicular renewal it is finite too.

The number of follicles around birth is widely variable. In lambs some authors have described about 100,000 to 200,000 follicles in the ovary (Land, 1970). However, as occurs in other species, this pool is lost while aging (Gougeon et al., 1994; McNatty et al., 1995; Hansen et al., 2008; Wallace and Kelsey, 2010; Anderson and Wallace, 2013) . Even before birth there is an important follicle depletion by atresia mediated by autophagy (Hulas-Stasiak and Gawron, 2011). Later ovarian atresia is characterized by apoptosis. The vast majority of follicles will be lost in the younger years even before puberty (Wallace and Kelsey, 2010) by this spontaneous degeneration, either at primordial follicle stage or at any subsequent stage of development, while only a few will reach ovulation (Gougeon et al., 1994).

It is not well understood yet how activation to enter early follicle growth is regulated to have enough primordial follicles to sustain the reproductive lifespan despite of follicular reduction by atresia, while providing an adequate supply of mature oocytes for ovulation. Primordial follicles itself are proposed to inhibit the activation of others into primary follicles (Da Silva-Buttkus et al., 2009) and complex signalling processes between oocyte and its surrounding somatic cells are needed (McLaughlin and McIver, 2009). The combination of different studies investigating models for determining functional ovarian reserve during childhood, adolescence, and adult life (Hagen et al., 2010; Nelson et al., 2011a, 2011b) has provided a new perspective on follicular behaviour and challenged some previous hypotheses about follicular development (Fleming et al., 2012). In human the rate of NGF recruitment from the primordial follicle pool shows a steady increase during childhood and adolescence for reaching a maximum at 14 years-old which (Wallace and Kelsey, 2010) is coincidental with postpubertal establishment of adult ovarian function, afterwards declines with age until menopause (Faddy et al., 1992; Faddy and Gosden, 1996). Hence, both the

number of primordial follicles recruited into the growing follicle pool and the rate of follicular growth are higher during the infant period than in adulthood (Hage et al., 1978).

The OR is very important since it is the only source of oocytes needed to form the embryo, unlike in male the number of female gametes is finite so a poorly stocked initial reserve or one in which primordial follicles are precociously depleted, will result in a shortened reproductive lifespan, infertility and in the human early menopause (Nelson et al. 2013). Hence the boom in studies trying to find markers able to determine accurately the OR and FOR to assess an estimation of the remaining reproductive lifetime or to predict how it will respond to fertility treatments in order to improve ovarian stimulation protocols and vaticinate the success of assisted reproductive techniques (ART). In human the measurement of FSH levels at day 3 of the menstrual cycle is one of the most common biomarkers as it gives a quick insight of how is functioning the hypothalamic-pituitary-gonadal axis, albeit it still requires standardization to ensure reproducibility. FSH levels should be low, same as oestradiol levels, with levels higher than normal indicating a diminished OR. Some studies point the increasing in FSH levels as a sign of reproductive aging in sheep (Gonzalez-Bulnes et al., 2004b) as in humans (de Koning et al., 2008). FSH is consider a late indirect indicator of decreased fertility being more advisable the search of an early direct indicator (reviewed by Roudebush et al., 2008). The administration of exogenous FSH has been used for ovarian reserve testing (EFORT; Fanchin et al., 1994; Kwee et al., 2006). It is thought by some that stimulation tests for ovarian reserve determination are more sensitive indicators than basal testing alone (reviewed by Roudebush et al., 2008). Oestradiol and the FSH:LH ratio have also been used as indirect biomarkers as they depend on the feedback action of others hormones. The Inhibin B, a peptide hormone produced by small antral follicles that inhibits FSH release, is a promising direct biomarker for OR (Roudebush et al., 2008). It shows a positive association with the oocyte yield after superovulation but requires measurement in the early follicular phase and is an indicator that decreases late (reviewed by Anderson et al., 2012).

Since the number of early-growing follicles has been proportionally related to the follicular stock they come from (Gougeon, 1996; Kevenaar et al., 2006), antral follicles have acquired a major role in estimating the ovarian reserve. For that reason the study of antral follicles through antral follicle count (AFC) and anti-Müllerian hormone (AMH) levels determination, both equally useful in determination of OR status and/or ovarian responsiveness (van Rooij et al., 2002; Gruijters et al., 2003; Roudebush et al., 2008; Aflatoonian et al., 2009; Broer et al., 2009, 2011), is becoming increasingly important as will be indicated below. In fact, serum AMH levels may reflect better the ovarian follicular status than inhibin B, oestradiol, FSH and LH, even with a single measurement, since are more strongly correlated with the number of early antral follicles (Fanchin et al., 2003; Fanchin et al., 2005) and levels remain relatively constant along the whole cycle because there is a continuous growth of small follicles (Cook et al., 2000; La Marca et al., 2004, 2007; Hehenkamp et al., 2006; Tsepelidis et al., 2007) as well as they exhibit very little changes with season in seasonal breeding animals (goat, Monniaux et al., 2011). At the same time, AFC has proved to be better predictor of ovarian response than inhibin B, oestradiol and FSH (Bancsi et al., 2002; Hendriks et al., 2005).

3.2.1. Antral follicle count (AFC)

The antral follicle count is the number of antral follicles, which can be classified by size, present in both ovaries of one female at a given time. It can be determined by histological quantification, which prevents repeated measures on the same animal and affects reproductive capacity in the case of ovarian amputation, or using a non-invasive ultrasound technique for identifying the antral follicles $\geq$2 mm in diameter, which allows repeated short- and long-term studies on the same subject without alteration in reproductive capacity or need for surgical intervention being easy and inexpensive.

The AFC is highly repeatable within the same animal while differs between individuals (Burns et al., 2005; Ireland et al., 2007; Ireland et al., 2008), which allows to phenotype adult cattle according to their individual differences in AFC during follicular waves (Evans et al., 2010) in animals with a high number of antral follicles and animals with a low number of antral follicles in the ovaries (Ireland et al., 2008; Ireland et al., 2009) in which circulating FSH levels are greater (Burns et al., 2005; Ireland et al., 2007; Mossa et al., 2010). A similar high variation has been observed in the AFC in adult Sarda ewes with sheep presenting high or low number of antral follicles (Mossa et al., 2008).

The AFC and the response to exogenous hormonal stimulation have been used as suitable markers to determine the ovarian reserve (Ireland et al., 2008; Hansen et al., 2011), to predict the number of healthy follicles and oocytes in the ovaries (Ireland et al., 2008) and the number of retrieved oocytes after exogenous stimulation (Kawamata, 1994; Kwee et al., 2008; Broer et al., 2009; Melo et al., 2009; Hsu et al., 2011), and to predict oocyte quality in adults (Ireland et al., 2009; Holte et al., 2011) or transferable embryos (Kawamata, 1994). Several studies have associated the presence of relatively high numbers of antral follicles with a wide variety of fertility indices in animals (cow: Ireland et al., 2007, 2009; Jimenez-Krassel et al., 2009; Evans et al., 2010; sheep: Mossa et al., 2008) and women (Chang et al., 1998; Nahum et al., 2001; Scheffer et al., 2003; Maseelall et al., 2009; Holte et al., 2011), such as higher oocyte quality, higher progesterone production, higher pregnancy rates, higher live-birth rates and shorter calving to conception intervals, although other studies could not establish the AFC as a predictor for embryo quality (Aflatoonian et al., 2009; Melo et al., 2009; Hsu et al., 2011); in a like manner, other authors describe a reduced fertility in cows with low AFC (Cushman et al., 2009). AFC is also related to the follicular response after COS for ARTs (Tomas et al., 1997; Chang et al., 1998; Bancsi et al., 2002) to the extent that it can be used as a reliable predictor of the potential fertility of a woman (Chang et al., 1998). Similarly, cattle with high number of antral follicles have greater number of medium and large follicles (≥5 mm in diameter) after a stimulation with gonadotrophins than cattle

with low number of antral follicles (Singh et al., 2004), having a better quantitative (Rico et al., 2009) and qualitative (Ireland et al., 2007) ovulatory response after the treatment .

Although the number of antral follicles has been linked to ovarian reserve (Hansen et al., 2011) and oocyte quality (Holte et al., 2011) in the adulthood, little is known about differences in antral follicle number during the prepubertal period – taking into consideration that the prepubertal endocrine environment is markedly different from that in the adult – and their eventual implication on ovarian function or future reproductive capacity.

Antral follicles are already visible in heifer calves at 2 weeks of age (Evans et al., 1994). Some authors point that a great increase in AFC and in the diameter of the largest follicle occurred within the first 2 months of life (Bergfeld et al., 1994), while others indicate a peak in the total number of follicle ≥3 mm in diameter that extends until later between 4 and 12 weeks of age, and an increase in the size of the largest follicle between 8 and 14 weeks after birth (Honaramooz et al., 2004). The heightening in the follicular recruitment is a result of a remarkable increase in FSH concentration that lately falls due to an increase in the secretion of steroid and inhibin from the numerous antral follicles that have grown during the previous weeks. Meanwhile, LH levels are maintained relatively low until puberty (reviewed by Hernandez-Medrano et al., 2012). All this is accompanied by an increase in ovarian dimensions from 2 to 14 weeks and again after 34 weeks of age. Later, AFC in cattle begins to decrease from 5 years of age (Cushman et al., 2009).

Regarding sheep, already in the ovaries of newborn lambs an enhanced number of antral follicles can be found (Land, 1970). A study based on post-morten observations of Merino ewe lambs showed an increase in the number of antral follicles at 4 weeks of age with an increase in the ovarian weight at 4-8 weeks of age (Kennedy et al., 1974), then decline to relatively stable numbers till the end of the study at 33 weeks when animals not yet had their first oestrus cycle (Kennedy et al., 1974). Similarly subsequent studies noted

that the maximum number of antral follicles occurs at 4 weeks after birth (Tassell et al., 1978) and that ovarian weight increased very quickly till 4 weeks and there is a further rise within the 6 following weeks till 10 weeks of age (Mahdi and Khallili, 2008). In ewe lambs of a non-seasonal breed, through ovarian histology, the number of follicles ≥3 mm in diameter rose gradually from birth to 14 weeks of age to peak again at 24 weeks of life a peripubertal age for this breed; moreover, the number of follicles <3 mm in diameter increased rapidly from birth to 10 weeks of age and then declined (Mahdi and Khallili, 2008). Some authors, using transrectal ovarian ultrasonography and therefore making repeated measurements on the same animals, have pointed the existence of an early prepubertal peak in the AFC of April-born cross-bred ewe lambs and February-born Suffolk ewe lambs from 14 to 16 weeks and 12 to 14 weeks respectively, probably due to changes in FSH release and clearance rates. Also, another increase was detected as puberty and first ovulation approach because of changes in LH pulse frequency and an increase in biological FSH activity associated with changes in the distribution pattern of FSH isoforms that may lead to the recruitment of a larger number of antral follicles in the peripubertal period (Bartlewski et al., 2002, 2006 ; Rawlings et al., 2003). The size of the largest ovarian follicles increased from 8 to 14 (Bartlewski et al., 2006; Mahdi and Khallili, 2008) or 16 (Bartlewski et al., 2002) weeks of age, then drop from 14 to 22 weeks of age to later rise between 32 and 36 weeks of age in Suffolk and cross-breed with Suffolk ewe lambs (Bartlewski et al., 2006), or peaked 24 weeks after birth in Ouled Djellel ewe lambs (Mahdi and Khallili, 2008). In a study in Sarda ewe lambs undergoing ovarian stimulation with gonadotrophins every month the highest number of follicles was obtained at 4 weeks, also around puberty an enlargement in growth rate of ovarian follicles was seen (Ptak et al., 2003).

3.2.2. Anti-Müllerian hormone (AMH)

Anti-Müllerian hormone (AMH) is a glycoprotein dimer of the transforming growth factor β (TGF- β) superfamily that includes other structurally related peptides such as

inhibins, activins, growth and differentiation factors (GDFs) and bone morphogenetic proteins (BMPs), many of which are also involved in reproductive functions (Itman et al., 2006; Knight and Glister, 2006). AMH is a 140 kDa glycoprotein dimer composed of two 72 kDa monomers linked by disulfide bridges (Picard and Josso, 1984; Lane and Donahoe, 1998; Teixeira et al., 2001; Visser and Themmen, 2005). It is synthesised as a pro-hormone afterward, at the site of action, undergoes cleavage to give rise a biologically active C-terminal homodimer of 25 kDa and a 110 kDa N-terminal homodimer (reviewed by Pellatt et al., 2010; reviewed by Monniaux et al., 2013). AMH is also called Müllerian inhibiting factor (MIF), Müllerian-inhibiting hormone (MIH) or Müllerian-inhibiting substance (MIS) and is best known for its effects on male sexual differentiation.

Members of the TGF- β superfamily exert their actions through transmembrane-spanning serine/threonine kinase receptors classified as type I, which is shared with the BMP family, and type II which is specific. Type I receptor for AMH remains still unidentified (Josso et al., 2001), but type I receptors ALK2, -3 and -6 from the BMP family are the most likely candidates (Gouedard et al., 2000; Teixeira et al., 2001; Visser and Themmen, 2005). AMH acts on its own ligand-specific type II receptor, AMHRII (Baarends et al., 1994; Imbeaud et al., 1996; Mishina et al., 1999), then forms a tetraheteromer of two type I and two type II receptors. The phosphorylation of type I receptor by the AMHRII triggers the downstream signalling through a BMP-like signalling pathway mediated by intracellular cytoplasmic effectors known as R-Smad proteins (R-Smad1, -5 and -8) which become phosphorylated and form a complex with the collaborating Smad4 protein, that is common for BMP and TGF-β/activin signalling pathways. This complex traslocates into the nucleus to regulate gene transcription (reviewed by Kretzschmar and Massague, 1998; Visser, 2003; Visser and Themmen, 2005). The process is depicted in Figure 4.

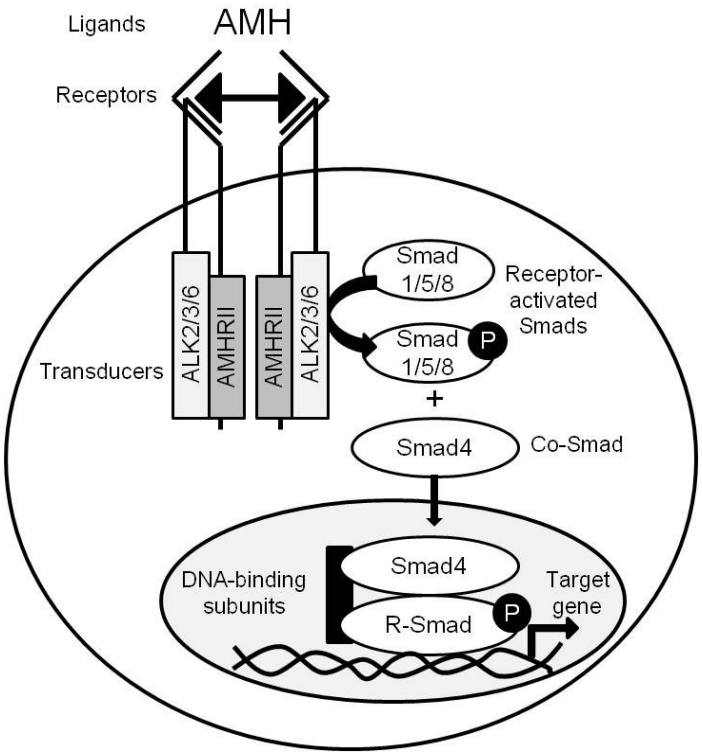

Figure 4: Signalling of AMH through the BMP-like signalling pathway. AMH phosphorylates Smad1, Smad5 or Smad8 through a receptor complex containing AMHRII and ALK2, ALK3 or ALK6. Then the receptor-activated Smads form a complex with the common Smad4. This complex translocates to the nucleus and regulates transcription of the AMH gene. Adapted from Kretzschmar and Massague, 1998 and Visser et al., 2005.

In 1916 Frank R. Lillie to explain the freemartin effect suggested the presence of soluble factors produced by the male gonads causing in the twin female calf foetus to have masculinised external organs and regression of Müllerian ducts. Müllerian ducts were first described by Johannes Müller and are the anlagen of the Fallopian tubes, uterus and upper vagina (reviewed by Teixeira et al., 2001). One of these substances will be later identified as AMH. The existence of these factors produced by male gonads was later shown in an experiment performed by Alfred Jost in rabbits in which an embryonic testicular fragment was implanted in rabbit female embryos before sexual differentiation resulting in animals showing stimulation of the Wolffian ducts – that in normal females degenerate while in males

give rise to the vasa deferens, epididymes and seminal vesicles- and degeneration of the Müllerian ducts. When testicular fragment was replaced by pellets of testosterone - hormone required for Wolffian duct differentiation - female embryos did not present regression of the Müllerian ducts despite became masculinised (reviewed by Teixeira et al., 2001). These findings led Jost to propose the existence of another substance different from the testosterone produced by the testicles causing inhibition of Müllerian ducts, nowadays known as AMH (Josso et al., 1993).

AMH, produced by Sertoly cells (Josso, 1973) once the gonad differentiates into a testis under the influence of SRY gene (Koopman, 1992; Wilhelm et al., 2007), causes regression of the Müllerian ducts in the male meanwhile in its absence in normal females Müllerian ducts develop to form the oviducts, uterus and upper one-third of the vagina. Failure in the male for mutation of the AMH gene or the AMHRII (Imbeaud et al., 1996) leads to persistent Müllerian duct syndrome (PMDS) in otherwise normally virilised males having underdeveloped cervix, uterus and fallopian tubes (reviewed by Lane and Donahoe, 1998; Rey and Picard, 1998; Nef and Parada, 2000; Josso et al., 2005). AMH may also have a role in male gonad development because can redirect ovaries into a more testis-like gonad. Its postnatal production (reviewed by Nef and Parada, 2000) suggest other roles such us regulation of testicular descent, lung development or gonadal function (reviewed by Lane and Donahoe, 1998). AMH seems to exert some influence over spermatogenesis as it controls Leydig cell proliferation and steroidogenesis (Racine et al., 1998) as well as its seminal concentration have been positively correlated with sperm concentration being a good marker for Sertoli cell development (Fujisawa et al., 2002).

Once sexual differentiation is completed AMH can be also found in the female. Actually AMH expression in the ovaries has already been described during gestation in humans (Rajpert-De Meyts et al., 1999; Teixeira et al., 2001; reviewed by La Marca et al., 2009) and sheep (Bezard et al., 1987; Veiga-Lopez et al., 2012). Ovaries in females seem to be the only source of AMH production since levels wane drastically to become undetectable

in women and monkey undergoing bilateral oophorectomy (La Marca et al., 2005; Appt et al., 2009). It is assumed that AMH has some role in folliculogenesis (Gruijters et al., 2003; da Silva et al., 2010). AMH holds back initiation of previous stages of follicle growth, avoiding ovarian primordial follicle recruitment in mice and rat (Durlinger et al., 2002a; Durlinger et al., 2002b; Nilsson et al., 2007; Park et al., 2011), making AMH-null mice lacking AMH having an earlier depletion of primordial follicles (Durlinger et al., 1999; Visser et al., 2007). Similarly, it has been described as an inhibitor of bovine follicle activation (Gigli et al., 2005). In women contradictory results have been published, some author reported an inhibitory role on primordial follicle growth (Carlsson et al., 2006) while others reported and enhancer role (Schmidt et al., 2005). In ewes AMH controls the rate at which follicles progress to the gonadotrophin-dependent stage from the gonadotrophin-responsive stage (Campbell et al., 2012). AMH is produced in granulosa cells of developmentally advanced growing follicles, preantral and small antral follicles of multiple species (sheep, Bezard et al., 1987; rat, Hirobe et al., 1994; mouse, Salmon et al., 2004; human, Weenen et al., 2004; cow, Monniaux et al., 2008, 2013; reviewed by Sarraj and Drummond, 2012), and declines at the time of follicle selection (Jeppesen et al., 2013).

Even though variations in AMH levels seem to display follicles dynamics rather than regulation exerted by gonadotrophins (Durlinger et al., 2002b; van Rooij et al., 2002; Visser et al., 2006), FSH may play a role in the down-regulation of AMH and AMHRII mRNA expression in cells in vivo when small antral follicles differentiate into large antral follicles (Baarends et al., 1995) with numerous studies reporting a negative correlation between levels of FSH and AMH (Seifer et al., 2002; Fanchin et al., 2003; Piltonen et al., 2005; Silberstein et al., 2006) and decreased AMH production when high FSH levels are present (Rico et al., 2011; reviewed by Monniaux et al., 2013). Other studies show opposite results with FSH increasing AMH expression (Xu et al., 2009; Taieb et al., 2011). These contradictory results of FSH action on AMH expression may be explained by a dose-dependent action of FSH, increasing AMH expression at low concentrations (Scheetz et al., 2012) and decreasing it at higher FSH concentrations (Scheetz et al., 2012; Monniaux et al., 2013), as it has been

seen in cows. On the contrary, no inhibitory effect of high FSH concentrations on AMH was seen in sheep (Monniaux et al., 2013), nor in goat, which present similar AMH concentrations in autumn and spring despite of FSH concentrations being higher during autumn (Monniaux et al., 2011). The modulation of responsiveness to FSH by AMH could be relevant during the cyclic recruitment of follicles (Durlinger et al., 2002b). At the same time, the effect of AMH over follicles sensitivity to FSH is contradictory. For some author AMH seems to inhibit follicles sensitivity to FSH (Durlinger et al., 2002b; Pellatt et al., 2010) with a diminished follicular diameter in mouse in vitro FSH-stimulated preantral follicles (Durlinger et al., 2001) and null-mice containing more growing follicles than their wild type littermates despite low FSH levels (Durlinger et al., 1999); while other authors point that AMH enhances the effect of FSH on inducing increase in both follicle diameter and cell number in rat preantral follicles (McGee et al., 2001). In addition, AMH shows a negative effect on oestradiol (Fanchin et al., 2007), inasmuch as it reduces the expression of the aromatase enzyme involved in converting androgens into oestrogens (Vigier et al., 1989; Eilso Nielsen et al., 2010; Monniaux et al., 2011; Chang et al., 2013), such, that the expression of AMH is markedly reduced with increasing follicle size while expression of aromatase is enhanced (Campbell et al., 2012).

AMH and its type II receptor are mainly expressed in granulosa cells from non-atretic developing preantral and small antral follicles, little or no expressed in large antral follicles, atretic follicles and corpora lutea and absent in primordial follicles (Baarends et al., 1995; Weenen et al., 2004; Visser et al., 2006). Yet, the causes for activation or inhibition of the expression of AMH are still unknown. In view that it has been displayed a relationship between AMH levels or expression and the number of 5-8 mm follicles in humans (Jeppesen et al., 2013), 3-7 mm follicles in cows (Rico et al., 2009) and 1-5 mm follicles in goats (Monniaux et al., 2011), is thought that these follicle sizes contribute significantly to the AMH circulating levels. Contrary to its proapoptotic action on Müllerian ducts AMH seems to have no effect on preantral follicle cell apoptosis (McGee et al., 2001); however, it might play some role in preovulatory follicular atresia (Kedem-Dickman et al., 2012). The co-

localization of both the ligand and the receptor in the same cell suggests a paracrine action of AMH in the ovary (Baarends et al., 1995). Even though AMH is only produced in granulosa cells, AMHRII has been isolated in extraovarian tissues (endometrium, Wang et al., 2009) and some cancer cell lines (Segev et al., 2000; Renaud et al., 2005; Chang et al., 2011), so further research is needed to elucidate new AMH roles.

AMH is heterogeneously distributed in granulosa cell population as antral follicle grows being less expressed in the granulosa cells in the periphery and more intensively expressed in the innermost granulosa cell layers, close to the oocyte (cumulus cells) and lining the antral cavity (sheep: Bezard et al., 1987; mouse: Munsterberg and Lovell-Badge, 1991; rat: Hirobe et al., 1994; Baarends et al., 1995; human: Weenen et al., 2004; cow: Monniaux et al., 2013) reflecting possible functional differences between granulosa cells surrounding the oocyte and granulosa cells in the periphery. AMH continues to be expressed in granulosa cells from the cumulus of late antral and preovulatory follicles (Grondahl et al., 2011) being less expressed as the oocyte matures (Kedem-Dickman et al., 2012). In fact, the oocyte itself seem to participate in the up-regulation of AMH expression in the granulosa cells of early and late preantral follicles and the cumulus cells of preovulatory follicles since AMH expression in these cells increased when co-cultured with oocytes (Salmon et al., 2004).

Within the follicular fluid in antral follicles AMH concentration is greater than its circulating levels (Yding Andersen et al., 2008), but preovulatory follicles contain low levels of AMH, even though women with higher concentrations of AMH in the follicular fluid of the preovulatory follicles have better pregnancy and embryo implantation rates (Fanchin et al., 2007). In ruminants, as in humans, AMH concentration in follicular fluid droops when follicles reach the preovulatory size (Monniaux et al., 2013). Also, cysts in women with PCOS contain higher amounts of AMH (reviewed by Pellatt et al., 2010) but have worse reproductive outcomes. Meanwhile, in cysts of cows, neither expression nor intrafollicular

concentration of AMH seem to be affected when compared with antral follicles from ovaries without cysts (Monniaux et al., 2008).

As AMH is produced by granulosa cells of healthy developing preantral and small antral follicles, its circulating levels have been directly related to the number of these follicles with an excellent correlation with AFC both humans (de Vet et al., 2002; van Rooij et al., 2002; Gruijters et al., 2003) and animals (Kevenaar et al., 2006; Rico et al., 2009). For this reason, AMH has emerged as a suitable marker for ovarian function (reviewed by La Marca et al., 2009; Loh and Maheshwari, 2011) with its serum concentrations reflecting, during the adult life, both primordial follicle pool and the number of small growing follicles in human (Hansen et al., 2011) and animal species (Kevenaar et al., 2006) strengthening the study of the relationships between ovarian reserve, number of antral follicles and fertility.

The number of oocytes obtained after ovarian stimulation treatments is highly correlated with AMH levels (Seifer et al., 2002; Gruijters et al., 2003; Fleming et al., 2006; Rico et al., 2009; Monniaux et al., 2011), particularly, women with high AMH levels are likely to respond better, even excessively, to stimulation with exogenous gonadotrophins than women with low AMH levels (van Rooij et al., 2002; Ficicioglu et al., 2006; La Marca et al., 2007, 2010; Anderson et al., 2012) with the exception of women with PCOS who have high AMH levels with worse responses to ART with a higher number of immature oocytes (Fallat et al., 1997; Mulders et al., 2004; Eldar-Geva et al., 2005; Pigny et al., 2006; Pellatt et al., 2010). Thence, AMH may become a useful tool in clinical practice to define poor ovarian response. AMH levels seem to be not only strongly associated with the number of oocytes but also with their quality after ovarian stimulation (Ebner et al., 2006; Silberstein et al., 2006; Brodin et al., 2013) and consequently with the number of high-quality embryos obtained (Monniaux et al., 2011) and even with higher pregnancy rates (Hazout et al., 2004). Therefore AMH levels could be used to identify animals that respond poorly to MOET or OPU protocols and discard them as donor females (Rico et al., 2012; Monniaux et al., 2013).

As AFC does, AMH levels in adults are highly variable between different individuals but also tent to remain quite constant for the same animals over long periods (Rico et al., 2012; reviewed by Monniaux et al., 2013). In addition, AMH levels change across lifespan although only one third of variation in AMH concentrations can be attributed to the variations which occur with age (Kelsey et al., 2011). Some studies show undetectable levels of AMH during childhood using ELISA, that do not rise until puberty to become undetectable again after menopause (reviewed by Teixeira et al., 2001). In contrast, more recent studies detect this hormone during childhood. In particular, AMH is barely detected at birth (Hudson et al., 1990), then plasma AMH levels show an increase during the postnatal period and childhood (Hagen et al., 2010; Kelsey et al., 2011), later a distinct fluctuation around the time of puberty, followed by a secondary increase during the next decade to a maximum at around 25 years-old (Kelsey et al., 2011) to decline progressively (5.6 % per year, Bentzen et al., 2013) to values below the levels of assay sensitivity when menopause is reached (de Vet et al., 2002; Piltonen et al., 2005; Kelsey et al., 2011). Hence, women under 25 years old have higher AMH levels that more aged ones (Piltonen et al., 2005). From 25 years-old it appears that the relationship between the decline in the size of the pool, the number of follicles undergoing recruitment and the serum AMH concentrations closely resemble each other (Hagen et al., 2010). Thus, AMH has emerged as a suitable marker for ovarian function (Loh and Maheshwari, 2011). In the cow, it has been described a markedly increase between 1 and 3 months of age; then after 6 months of age, AMH levels drop slowly until puberty (Monniaux et al., 2013).

There is a strong positive correlation between circulating AMH and follicular recruitment prior to the onset of puberty (Fleming et al., 2012), which contrasts with evidences indicating its inhibitory role on follicle recruitment at postpubertal stages (mice: Durlinger et al., 2002a; sheep: Campbell et al., 2012). Theoretically, low pre- and peripubertal AMH levels may have a different clinical implication than later in life, and the interpretation of low serum AMH level as a marker of a lower ovarian reserve may not apply to the prepubertal period since mechanisms regulating follicle recruitment and ovarian

function may differ from those acting in the adulthood. Indeed, the relationships between AMH levels with follicle recruitment and the number of NGF are changing during puberty and early adult life (Fleming et al., 2012).

3.3. Puberty

Puberty in females is a coordinated and complex not fully understood process that integrates both internal and external factors to achieve complete gonadal maturation, full sexual maturity and reproductive capacity with the establishment of cyclic ovulatory reproductive activity.

Although the precise mechanisms triggering the onset of puberty are still being investigated, the endocrine and nervous systems play interwoven roles in this phenomenon. It is accepted that an increase in GnRH pulse generation affecting frequency and amplitude and, as a consequence, an increase in the pulsatile release of FSH and LH is produced (Foster et al., 1985). Although high frequency GnRH pulses can be already observed from an early age in ewe lambs, the extreme sensitivity to the inhibitory feedback action of gonadal steroids avoid ovulation (Foster et al., 2006). The negative feedback of oestradiol decreases when the hypothalamic-pituitary-gonadal axis matures, increasing the frequency of release of LH pulses (Foster et al., 1985; Kinder et al., 1995) which raises the antral follicle development and oestrogen production (Rawlings et al., 2003) and give rise to a preovulatory LH surge (Mahdi and Khallili, 2008). Different neuronal pathways are involved in the control of frequency of GnRH release during the peripubertal period. The major events involved in the onset of puberty are an increase in glutamatergic and kisspeptin stimulation of GnRH neurones contemporaneously with a decrease in GABA-ergic and opiatergic neuronal inhibition (Ojeda et al., 2006).

Puberty onset is the result of a complex interaction of endogenous and environmental factors including age, breed, body weight, nutrition, season of the year and social cues. The age at onset of puberty is different among breeds (Young and Dickerson, 1991); Sarda ewe lambs usually reach puberty at 7-8 months of age (Carcangiu et al., 2005), but puberty can be delayed if other factors are not suitable. Time of puberty onset can be accelerated by social cues such as the "ram effect" by which gonadotrophin secretion can be induced in peripubertal ewe lambs (reviewed by Ebling and Foster, 1989) stimulating further follicular development (Bartlewski et al., 2002) when a sexual mature ram is introduced into the group. Young peripubertal ewes only start their reproductive activity during the optimal season of the year, determined through the information provided by the photoperiod (Ebling and Foster, 1989), to ensure that possible offspring is born at the most favourable time of the year.

Animals must also achieve a certain appropriate stage of body growth, nutrition and body weight that would guarantee the metabolic conditions and the amount of energy reserves required for successful pregnancy and lactation. Young Sarda ewes need to reach a body weight exceeding 26 kg (Bini et al., 2000) or 30 kg (Carcangiu et al., 2005) to trigger puberty. Postnatal nutrition affects future reproductive function, puberty is advanced in overfed animals while undernourished animals present a lower frequency of GnRH and LH pulses and reach puberty later. Even maternal nutrition through the developmental programming impacts in the puberty onset of the offspring (reviewed by Valasi et al., 2012).

The mechanisms linking timing of puberty with optimal metabolic and energy conditions involve complex regulatory pathways in which leptin and kisspeptin seem to participate. Kisspeptin plays a key role in the pubertal maturation of the gonadotropic axis, being an essential gatekeeper in the control of puberty onset (Tena-Sempere, 2006; Navarro et al., 2007; Sanchez-Garrido and Tena-Sempere, 2013), while leptin levels reflect the size of fat stores signalling energy status to the brain and low leptin levels are linked to delayed puberty onset (Roa et al., 2008; Sanchez-Garrido and Tena-Sempere, 2013). Leptin affect

somehow the expression of kisspeptin (Roa et al., 2008; Sanchez-Garrido and Tena-Sempere, 2013). Also ghrelin, which is involved in the control of energy balance and reproduction, is being supposed to be implicated in the pubertal process of ewes (Valasi et al., 2012). Thus, in the relationship between body energy status and the onset of puberty, kisspeptin might act as a sensor and signal for transmitting neuroendocrine metabolic information to the GnRH releasing neurons in the reproductive centres of the brain.

4. EXPERIMENTS

4. EXPERIMENTS

All the experimental procedures with animals (sheep, Ovis aries) were approved by the Animal Care and Use Committee of the University of Sassari. All experimental procedures were carried out at the experimental facilities of the Department of Veterinary Medicine at the University of Sassari, Italy (latitude 40°43' N) or at the experimental facilities of the Department of Animal Production, AGRIS Sardegna, Bonassai, Sassari, Italy (latitude 40°40' N). 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 reagents and media were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified.

4.1. PRELIMINARY EXPERIMENT

4.1. PRELIMINARY EXPERIMENT

4.1.1. Abstract

In adult, AFC remains relatively constant within the same animal but there is a high inter-individual variability between animals. It is well established that adult cattle can be reliably phenotyped based on robust individual differences in AFC during follicular waves. A similar high variation in the AFC in sheep has previously been observed. Furthermore, AFC has been highly correlated with AMH levels and differences in AFC have been linked to variations in the response to ovarian stimulation with exogenous gonadotrophins. However, little is known about differences in AFC before puberty. Thus, the current experiment aimed to study if high inter-individual variations in the AFC are present in 6 week-old Sarda ewe lambs at an early prepubertal period with theoretically high follicular development, if AFC is linked to AMH levels and whether differences in the AFC influence the response to an exogenous FSH ovarian reserve test. Using transrectal ultrasonography a highly variable number of ≥2 mm antral follicles was detected in ewe lambs making possible to classify them by their AFC into Low (≤15 follicles), Intermediate (16-29 follicles) or High (≥30 follicles) AFC. Differences between groups were mainly due to the number of 2 mm follicles. There were differences in total, 3 mm and ≥4 mm follicles and new 2, 3 and ≥4 mm follicles 24 hours after a 105 IU FSH administration between the High AFC and the remainder groups, while the differences between the intermediate and low disappeared. Circulating AMH plasma levels detected by ELISA were positively correlated to AFC, and to the development of large follicles (≥3 mm in diameter) in response to FSH stimulation during the early prepubertal period. We concluded that differences in AFC are present in ewe lambs at an early prepubertal age and either AFC an AMH were predictive of an enhanced response to ovarian stimulation treatment in animals with high AFC.

4.1.2. Specific objectives

To determine in Sarda ewes lambs if there are differences at an early prepubertal age in the AFC, if differences in the AFC are accompanied by differences in AMH, and ascertain if there is a distinct response to the exogenous FSH ovarian reserve test (EFORT) according to the AFC.

4.1.3. Material and Methods

4.1.3.1. Assessment of the AFC by transrectal ultrasonography

A group of 39 Sarda ewe lambs, around 35-45 days-old and 8-10 kg of body weight was used. Animals were maintained under the same conditions throughout the experimental procedure, being housed outdoors with indoor access. In all the ewe lambs, the ovarian phenotype was determined by characterizing the AFC by transrectal ultrasonography, with a real-time B-mode scanner (Aloka SSD 500, Aloka Co., Tokyo, Japan) fitted to a 7.5 MHz linear-array probe (laparoscopic rigid transducer UST-5526L-7.5, Aloka Co., Tokyo, Japan; Figure 5, right side) done by the same single person. Scanning was performed as previously described and validated Gonzalez-Bulnes et al. 1994. In brief, observations were conducted with the ewe lamb placed in dorsal recumbence (Figure 5, left side). After introducing a hydrosoluble contact gel into the rectum, the probe was introduced so that the transducer was perpendicular to the abdomen wall. When the urinary bladder was surpassed and the uterine horns were located, the probe was rotated laterally 90° clockwise and 180° counter-clockwise to observe both ovaries and their structures.

Figure 5: Top image shows how transrectal ultrasound scanning was performed with the ewe lambs in dorsal recumbence (photography taken by Gallus, M.). Bottom image shows a detail of the 7.5 MHz linear-array probe with a suitable diameter to the size of the animals used for transrectal ovarian ultrasound scanning in ewe lambs.

Each ovary was scanned several times from different angles in order to determine number and size of all follicles ≥2 mm. Animals were divided, at first ultrasound observation, in three experimental groups accordingly to the number of ≥2 mm follicles (Figure 6): Low AFC group (≤15 follicles; n=13), Intermediate AFC group (16-29 follicles; n=15) and High AFC group (≥30 follicles; n=11).

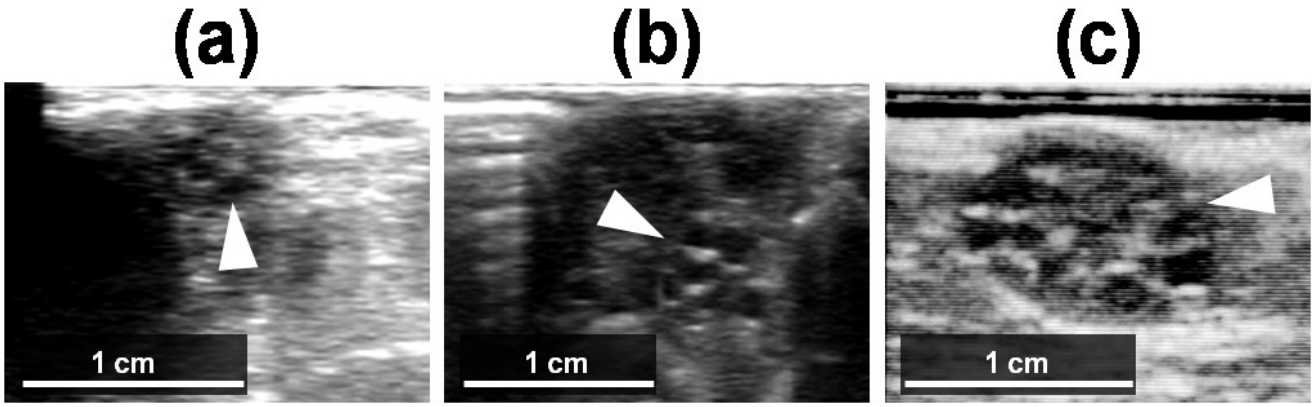

Figure 6: Ovary images of 35-45 days-old Sarda ewe lambs by transrectal ultrasound. The lambs were classified according to the number of follicles ≥2 mm in diameter present in the ovaries into: (a) Low AFC group (≤15 follicles), (b) Intermediate AFC group (16-29 follicles) and (c) High AFC group (≥30 follicles). White arrows are pointing the ovaries. The follicles are seen as pseudo-circular anechoic structures.

4.1.3.2. Exogenous FSH ovarian reserve test

Immediately after determining AFC, Day 0, the responsiveness of the follicular population to the exogenous FSH ovarian reserve test (EFORT: Fanchin et al., 1994; Kwee et al., 2006) was determined. First, blood samples were drawn with 10 ml vacuum blood evacuation tubes containing EDTA (Vacutainer® Systems Europe, Becton Dickinson, Meylan Cedex, France). Immediately after recovery, blood samples were centrifuged at 1500 x *g* for 10 min at 4 ºC and plasma was removed and stored at -20 ºC until assayed for AMH and 17β-oestradiol. Afterwards, all the animals were administered one-shot intramuscular dose of 105 IU porcine FSH (Folltropin, Bioniche Animal Health, Minitub Ibérica, S.L., Reus, Spain). Twenty-four hours later, Day 1, the growth of the antral follicles was assessed by a second ultrasonographic scanning and a second blood sample was drawn. Analysis of the EFORT included changes in follicular population and in plasma concentrations of AMH and 17β-oestradiol.

4.1.3.3. Assessment of hormonal plasmatic levels

Plasma AMH concentration was measured by using the AMH Gen II ELISA kit (Beckman Coulter Inc., Brea, CA), following the manufacture instructions. Briefly, the test is based on the binding of AMH from samples, calibrators and controls with an anti-AMH antibody with which the microtitration wells are coated. After incubation and washing an anti-AMH detection antibody labelled with biotin, which will bind to the AMH trapped by the anti-AMH antibody from the well, is added to each well. After another incubation and washing, streptavidin-horseradish peroxidase which will bind the former antibody is added to the wells, followed again by other incubation and washing. Then the substrate TMB is added to the wells and incubated. Colour will develop in the wells as a result of the transformation of the substrate by the horseradish peroxidase action. Lastly, an acidic stopping solution is added. The absorbance measured at 450 nm with 600-630 nm correction is directly proportional to the concentration of AMH. The AMH concentrations in the samples can then be calculated from the calibration curve obtained from representation of the logarithm of the absorbance versus the logarithm of the concentration of the calibrators. The kit was validated for use in ovine plasma by determining the parallelism of different dilutions of plasma from lambs and ovariectomized sheep. Sensitivity of the assay was 0.08 ng/ml, whilst the inter- and intra-assay variation coefficients were 5.6% and 5.4%, respectively.

Oestradiol was measured using the Spectria radioimmunoassay kit (Orion Diagnostic Corp, Espoo, Finland), as described by Romeu et al. (1995) and adapted for use in ovine plasma (Gonzalez-Bulnes et al., 2003). Sensitivity of the assay was 0.5 pg/ml, whilst the inter- and intra-assay variation coefficients were 6.1% and 3.5%, respectively.

4.1.3.4. Statistical analysis

Differences in follicle numbers and hormone concentrations among the three experimental groups and between Days 0 and 1 of treatment were assessed by General

Lineal Model where: $Y = \mu + day + group + day \times group + ewe\ lambs$. Day and group were considered fixed factor and ewe lambs a random factor. The method used to discriminate among the means was Fisher's least significant difference (LSD) procedure. The probabilities obtained by the LSD test were corrected by the Bonferroni's correction for multiple comparisons. New follicles stimulated to growth after FSH administration were calculated by subtracting for each animal in each size category the follicles found on Day 0 of the follicles found on Day 1. Possible correlations among follicle population and AMH levels were determined. Statistical analysis was performed using the statistical software program Statgraphic Centurion XV (version15.2.06 for Windows; StatPoint, Inc., Herndon, VA, USA) and a probability of $p \leq 0.05$ was considered to be the minimum level of significance. All results are expressed as mean ± S.E.M.

4.1.4. Results

Ovarian ultrasound scanning performed before exogenous FSH administration indicated that the initial follicular population in ewe lambs aged 40 days was mostly represented by follicles with 2 mm in diameter (Figure 7, panel a). The highest number of total and 2 mm follicles was observed in the High AFC group (40.8 ± 1.5 and 36.6 ± 2.2, respectively), followed by the Intermediate (20.5 ± 0.9 and 16.6 ± 1.2) and then by Low AFC group (14 ± 1.8 and 11.5 ± 2.5; $p<0.0001$). The mean number of follicles of 3 mm and ≥4 mm in diameter did not differ among the three groups.

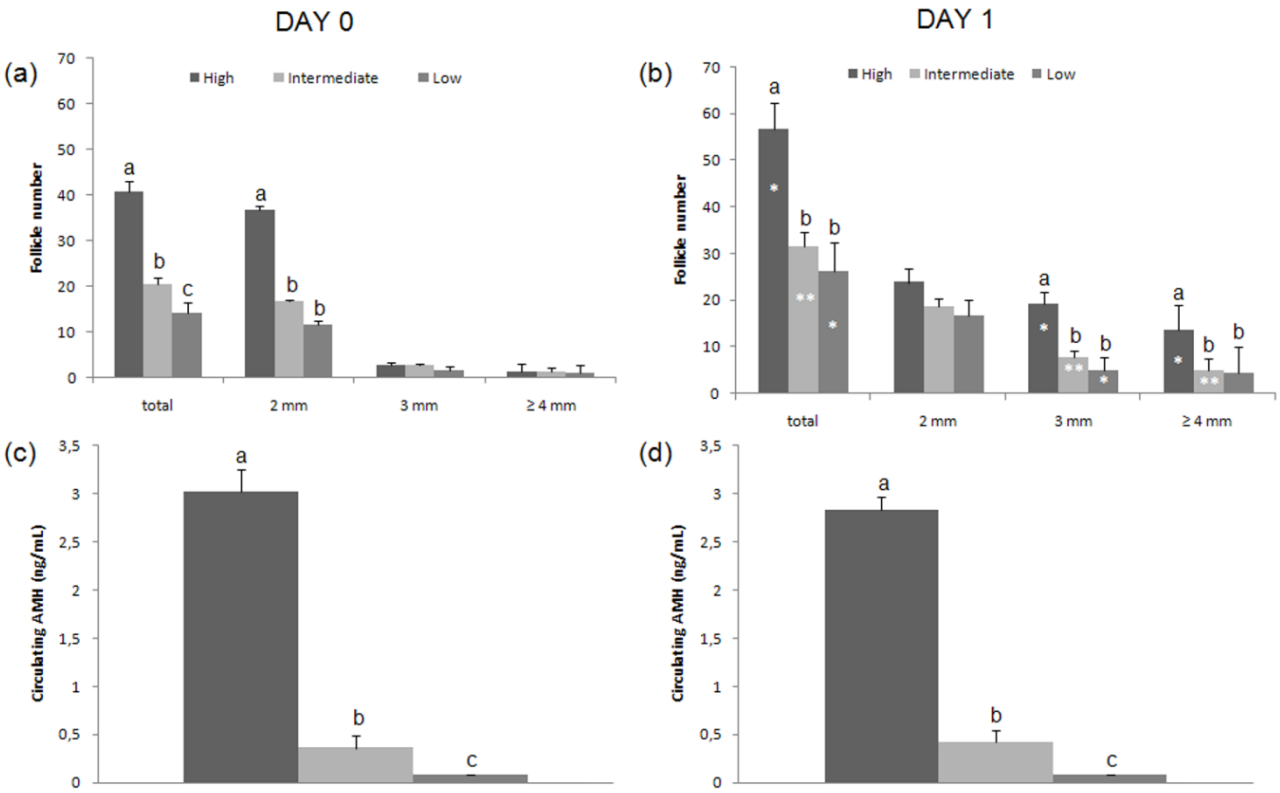

Figure 7: Follicle population and anti-Müllerian hormone plasma levels in 40 day-old ewe lambs with different ovarian phenotypes before and after FSH administration. Mean number of total follicles and follicles of 2 mm, 3 mm, ≥4 mm in diameter (A and B) and mean circulating AMH concentrations (C and D) before (left hand; Day 0) and 24 h after FSH treatment (right hand; Day 1) in 40 day-old ewe lambs assigned to High (≥30 follicles), Intermediate (16 – 29 follicles), and Low group (≤15 follicles) according with the ovarian phenotype expressed.

[a,b,c] Indicate a statistical difference among the three experimental groups: a ≠ b ≠ c $p<0.0001$; a ≠ b $p<0.05$.

* Asterisks in panel (b) indicate a statistical difference from values observed at day 0 in the same experimental group: * $p<0.05$; ** $p<0.01$.

After exogenous FSH administration (Day 1), the mean number of total follicles rose within each group ($p<0.05$), mainly due to an increase in the number of 3 mm and ≥4 mm follicles ($p<0.05$). The number of 2 mm follicles did not vary significantly from the initial values within none of the groups (Figure 7, panel b). Analyzing the difference in the follicular

population among the three experimental groups after exogenous FSH administration, a higher number of total, 3 and ≥4 mm follicles was found in the High group compared to the Intermediate and Low ones.

In addition, the growth dynamics of the follicle differed among the three experimental groups (Figure 8). By comparing the new follicles grown after exogenous FSH administration (whose number was obtained by subtracting follicles recorded on Day 1 from those recorded on Day 0), we found that the increase in the number of large follicles (≥3 mm) was accompanied by a decrease in the number of 2 mm ones only in the High AFC group.

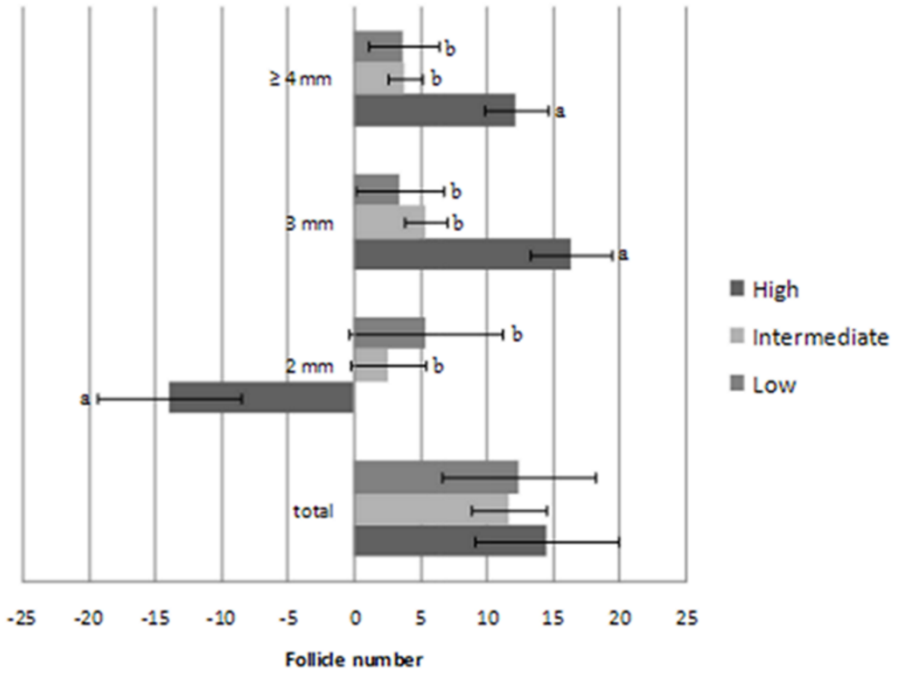

Figure 8: New follicles stimulated to growth after FSH administration in 40 day-old ewe lambs with different ovarian phenotype. Ewe lambs were assigned to High (≥30 follicles), Intermediate (16 – 29 follicles), and Low group (≤15 follicles) according with the ovarian phenotype expressed and the number of new follicles was recorded by ovarian ultrasound scanning 24 hours after FSH administration.

[a,b] Indicate statistical difference: $a \neq b$ $p<0.05$.

Before exogenous FSH administration, the mean plasma concentration of AMH in all the ewe lambs was 0.93 ± 0.42 ng/ml. Plasma AMH levels were significantly related to the number of total follicles ($r=0.967$; $p<0.001$), due to a high correlation with the number of follicles with 2 mm in size ($r=0.943$; $p<0.0001$). Thus, AMH concentrations were higher in the High AFC group (3.03 ± 0.22 ng/ml) than in the Intermediate (0.36 ± 0.13 ng/ml; $p<0.01$) and the Low AFC groups (0.08 ± 0.01 ng/ml; $p<0.05$; Figure 7, panel c). After exogenous FSH administration, the High AFC group maintained the higher AMH plasma levels (2.84 ± 0.13 ng/ml) compared with the Intermediate (0.42 ± 0.13 ng/ml; $p<0.05$) and the Low AFC groups (0.08 ± 0.01 ng/ml; $p<0.05$; Figure 7, panel d). Within each experimental group, no significant variation was found in the AMH plasma levels after FSH treatment. At day 1, AMH plasma levels were again significantly related to the number of total follicles ($r=0.794$; $p<0.005$) but, in this case, due to a high correlation with the number of follicles with 3 mm in size ($r=0.854$; $p<0.001$). A positive correlation was also found between AMH plasma levels at day 0 and the number of large follicles (3 and ≥4 mm in diameter) grown after exogenous FSH administration at day 1 ($r=0.572$, $p< 0.005$).

Plasma oestradiol levels were undetectable in all ewe lambs before and after FSH administration.

4.1.5. Discussion

Sarda ewe lambs at 40 days of age present a highly variable number of antral follicles (≥2 mm), as it was determined using transrectal ultrasonography making possible to classify them by their AFC in ewe lambs with Low, Intermediate or High AFC. Differences between groups in AFC continued after exogenous FSH administration except between the Intermediate and Low AFC groups. Circulating AMH was positively correlated to AFC, and to the development of large follicles (≥3 mm in diameter) in response to FSH stimulation.

It is noteworthy to stress that during the prepubertal period the hormonal milieu is markedly different form the adult life, and steroid production is minimal (Foster et al., 2006). In this study plasma oestradiol levels were undetectable in all the ewe lambs used for the in vivo trial. The full hierarchy of follicles within the ovary (i.e. large antral and dominant follicles) becomes established only after puberty, and follicle acquire their full (pituitary controlled) steroidogenic competence only at the end of development. The ovarian ultrasound scanning performed in this study revealed that, as expected, in the early prepubertal ovary the follicular population is mostly represented by follicles of 2 mm in diameter. The group with the highest number of follicles 2 mm, the High AFC, was the one that after treatment with FSH showed the greatest number of total follicles, medium-sized follicles (3 mm) and large follicles (≥4 mm). This is in agreement with that observed in cattle, in which the superstimulatory response can be predicted by the number of follicles ≥2 mm at wave emergence (Singh et al., 2004). Follicles growth to medium and large size in the High AFC group was accompanied by a drop in the number of small follicles, as it was described in cattle by Rico et al. (2009). This finding suggests that small and medium follicles were targets of the stimulatory treatment with the animals with the highest number of small and medium follicles having the best response to the ovarian stimulation treatment.

In our study, mean plasma AMH levels in 40 days-old ewe lambs are higher from values reported from other Authors in older ewe lambs (range from 0.043±0.015 to 0.163±0.04 at 3.6 months of age; Lahoz et al. 2012). In heifers, plasma AMH concentrations were found to increase markedly between 1 and 3 months of age, remaining high at 6 months, then declining again until puberty (Monniaux et al. 2013). Since no previous study has examined AMH across the lifespan in healthy ewes, this variation may be linked to several factors other than age (see also Kelsey et al. 2011).

Results of the in vivo trial evidenced a strong positive correlation between AFC and circulating AMH levels in the early prepubertal period. In this way, ovarian phenotypes expressing the highest number of 2 mm follicles had also the highest levels of AMH. Previous studies in adults have shown a close relationship between antral follicles and serum

AMH (de Vet et al., 2002; van Rooij et al., 2002; Gruijters et al., 2003; Laven et al., 2004; Kevenaar et al., 2006; Pigny et al., 2006; Jayaprakasan et al., 2010), confirming that AMH reflects the size of the growing follicle pool. In sheep, as in other species (mice: Durlinger et al. 2002a; human: Weenen et al. 2004), AMH is strongly expressed by the granulosa cells of preantral and small antral follicles (sheep: Campbell et al. 2012) even during the foetal and prepubertal period (Bezard et al., 1987). These stages of follicle development are gonadotrophin-responsive in sheep (Campbell et al., 2004) and other species (primates: Gougeon 1996; cattle: Webb et al. 1999).

In the current study, as it has been seen in studies in adults from other species (cattle: Rico et al., 2009; human: Jayaprakasan et al., 2010), the number of antral follicles counted by ultrasonography correlated positively with AMH plasma levels, both before and after FSH administration. In addition, the evaluation of AMH plasma levels prior to the EFORT test was positively correlated with the number of large follicles grown after exogenous FSH administration. The analysis of the response to the EFORT test in 40 days old ewe lambs expressing different ovarian phenotypes, revealed that in individuals with a high AFC a higher number of large follicles developed in response to FSH stimulation compared with individuals with intermediate and low AFC. In addition, in ewe lambs with high AFC the increase in the number of large follicles was accompanied by a decrease in the number of 2 mm ones. These results could suggest that in individuals with a high AFC small follicles were more sensitive to grow to larger categories in response to exogenous FSH stimulation than those from ewe lambs with lower AFC. Nevertheless, as AMH is mainly produced by preantral and early antral follicles, it is assumed that ewe lambs from the High AFC group should have a higher number of growing follicles, that did not growth till the gonadotrophin-dependent stage (2 mm) in response to the FSH treatment, since follicle recruitment did not differ between groups being the number of total new follicles similar. A previous study in ewes determined that AMH controls the rate at which follicles progress to the gonadotrophin-dependent stage from the gonadotrophin-responsive stage (Campbell et al., 2012), which could explain the similar follicle recruitment despite higher AMH levels. Thus, the highest

number of large follicles after FSH treatment could be explained not to a major sensibility to FSH but just to a higher initial number of gonadotrophin-dependent follicles. It is hypothesized that AMH attenuates antral follicle responsiveness to FSH in adults. Experiments conducted in mice have suggested that AMH not only inhibits growth initiation of primordial follicles (Durlinger et al. 1999, 2002a, 2002b), but also partakes in the regulation of the growth of preantral and small antral follicles by inhibiting their sensitivity to FSH (Durlinger et al. 2001). Both in vitro and in vivo studies seem to suggest that follicles are more sensitive to FSH in the absence of AMH (mice: Visser and Themmen 2005). In addition, a recent study evidenced that antral follicle responsiveness to FSH, as far as it takes into account the available pool of FSH-sensitive follicles, is negatively correlated to the circulating AMH levels in normo-cycling women (Genro et al., 2011). These data apparently stand in contradiction to the evaluation of AMH as a reliable marker of ovarian response to hormonal stimulation in women (La Marca et al. 2007), cows (Rico et al. 2012; Ireland et al. 2011), and goats (Monniaux et al. 2011). These findings may result from the putative positive correlation between peripheral AMH levels and the pre-treatment number of small antral follicles (Genro et al. 2011). In small ruminants variations in ovarian responses to FSH are known to reflect the follicular population present during the initiation of treatment (Gonzalez-Bulnes et al. 2004, Veiga-Lopez et al. 2005, Menchaca et al. 2010). At prepubertal stages, when the pituitary-ovarian axis is generally regarded as being quiescent and circulating sex steroid are minimal, the relationship between follicle recruitment and circulating AMH is less clear. In a previous study an increased rate of recruitment from the primordial pool was observed in AMH null prepubertal mice (Durlinger et al. 1999). On the other hand, a recent study in human indicates a strong positive correlation between circulating AMH and follicular recruitment before the onset of puberty (from birth to 9 years-old; Fleming et al. 2012). The possibility that AMH may play a permissive or even a stimulatory role in follicle recruitment and survival of early growing follicles has been also suggested by in vitro studies (human: Schmidt et al. 2005). These findings are in agreement with results of the present study, where the highest levels of AMH were found in individuals

showing the highest number of 2 mm follicles and the best response to the EFORT test in terms of follicle competence to grow in response to gonadotrophin stimulation.

4.2. EXPERIMENT 1: early prepubertal period

4.2. EXPERIMENT 1: early prepubertal period

4.2.1. Abstract

Several articles indicate that both the AFC and AMH levels are good markers for ovarian reserve, being specially related to healthy follicles. They have also been suggested to have some influence in the quantity and quality of oocytes and embryos produced, yet the vast majority of studies are made in adults. Besides, the sheep seems to show a fast increase in the number of antral follicles during the early prepubertal period that might be accompanied by changes in AMH and FSH levels. Still little is known about the prepubertal period and different criteria could apply during the early prepubertal period in which the hypothalamic-pituitary-ovarian-uterine axis does not have the same functionality as in the adult being the endocrine environment markedly different. Thus current study aimed to determine whether differences in the AFC at an early prepubertal age are accompanied by changes in oocyte and embryo developmental capacity, changes in the ovarian reserve and changes in the evolution of AMH levels during the first 9 weeks after birth. To achieve this objective, COCs recovered from animals with Low (≤15 follicles), Intermediate (16-29 follicles) or High (≥30 follicles) AFC were in vitro cultured up to the blastocyst stage, and the quality of the embryos in the three categories was assessed by evaluating their cryotolerance and total cell number. As well, total number of primordial, primary, secondary, antral, polyovular and atretic follicles was determined in histological sections of the ovaries classified as belonging to the High, Intermediate and Low AFC groups; Also, FSH and AMH plasma levels throughout the first weeks of life were determined by ELISA in ewe lambs with Low and High AFC. From our results we concluded that a low AFC, although not accompanied by a reduction in the size of the primordial follicle pool, was related to a lower number of early and late antral follicles and predictive of poor oocyte in vitro developmental competence. On the contrary animals with a high AFC showed higher AMH plasma levels peaking at 5 and 6 weeks of age, coinciding with a greater number of early and late antral

follicles in the histology and an increased oocyte quality in the in vitro culture from ovaries of ewe lambs of this age. FSH could not be analyzed because plasmatic levels were undetectable using a sheep-specific ELISA kit.

4.2.2. Specific objectives

To determine whether differences in the AFC at an early prepubertal age are accompanied by changes in oocyte and embryo developmental capacity, in the ovarian reserve and in the evolution of AMH and FSH levels throughout the first weeks of life.

4.2.3. Material and Methods

4.2.3.1. Collection and classification of the ovaries

Complete reproductive tracts (uterus plus ovaries) from Sarda ewe lambs of similar age and weights (40 days of age, body weight 8-10 kg) were obtained from a local slaughterhouse and transported to the laboratory, within 1 hour, in Dulbecco's phosphate balanced saline pH 7.2, osmolarity around 280 mOsm (2.7 mM KCl, 1.5 mM KH_2PO_4, 136.9 mM NaCl, 8.9 mM Na_2HPO_4-$7H_2O$; PBS) supplemented with penicillin and streptomycin (50 mg/ml) at 30-35 ºC. The pairs of ovaries were classified into the three AFC group categories according to the total number of follicles ≥2 mm in diameter on their surface (Figure 9) stabilized in the preliminary experiment: Low AFC group (≤15 follicles), Intermediate AFC group (16-29 follicles) and High AFC group (≥30 follicles). Ovaries assigned to the three experimental groups were used for evaluation of in vitro oocyte developmental competence (n=658) and for histological quantification of the ovarian reserve (n=22). Before being incorporated in the two experimental trails, ovaries were separated from the rest of the reproductive tract, then weighed and measured for length and height.

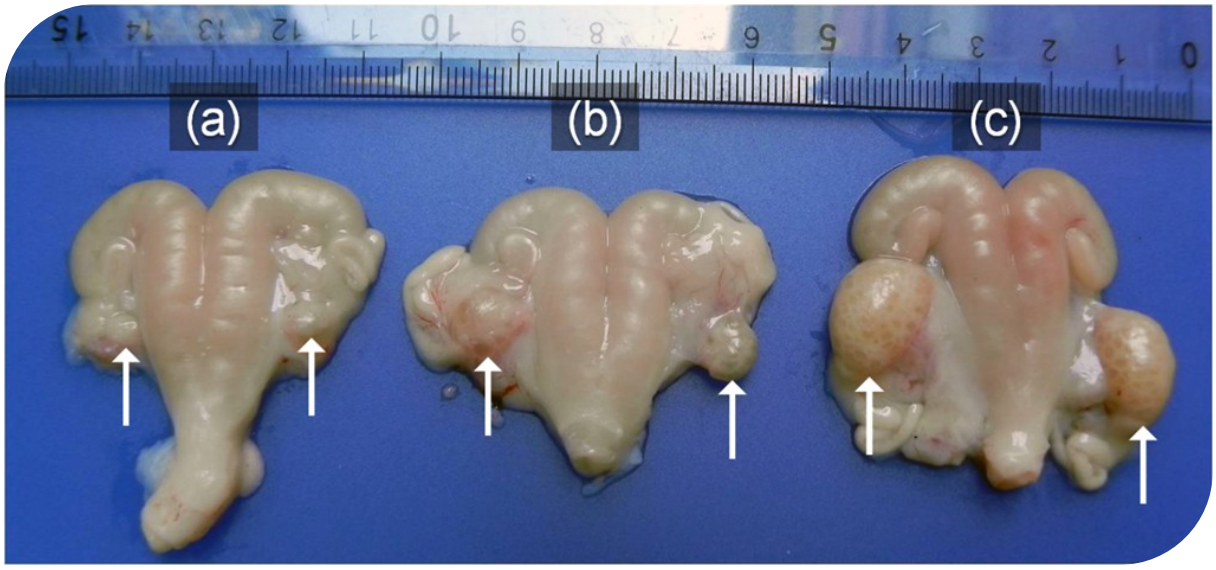

Figure 9: Reproductive tracts from ewe lambs aged 40 days. Ewe lambs were classified according to the total number of follicles ≥2 mm in diameter on the surface of the ovaries in (a) Low group ≤15 follicles, (b) Intermediate group between 16 and 29 follicles, and (c) High group ≥30 follicles. Ovaries are pointed with white arrows.

4.2.3.2. In vitro embryo production

The procedure for in vitro embryo production was repeated 11 times. Ovaries assigned to the three experimental groups were processed separately. Within each group, oocytes selected for in vitro maturation were pooled, cultured together, and considered as one batch.

After being washed in PBS fresh medium, the ovaries were sliced using a micro-blade and the follicle content was released in medium TCM199 (with Earle's salts and bicarbonate) supplemented with 25 mmol HEPES to maintain the physiological pH, 0.1 g/l penicillin, 0.1 g/l streptomycin and 0.1% (w/v) PVA. Cumulus-oocyte complexes (COCs) with 4-10 layers of granulosa cells, oocytes with a uniform cytoplasm, homogenous distribution of lipid droplets in the cytoplasm and outer diameter of about 90µm (mean) were selected for the experiment. The selected COCs, after three washes in the same fresh medium, were in vitro

matured in TCM199 supplemented with 10 % heat-treated foetal calf serum (FCS), 1 IU/ml ovine FSH, 1 IU/ml ovine LH, and 100μM of cysteamine. Thirty to 35 COCs were put in 600μL of the maturation medium in a four-well Petri dish (Nunclon, NalgeNunc International, Denmark), layered with 300μL mineral oil and cultured for 24 h in 5% CO_2 in air at 39 ºC.

After maturation, the COCs were stripped of the granulosa cells and fertilized in vitro at 39 ºC and 5% CO_2, 5% O_2 and 90% N_2 atmosphere in four-well Petri dishes (Nunclon). Frozen-thawed spermatozoa from the same ejaculate of one ram were used across all experimental procedures. The fertilization system was composed of 300μl of synthetic oviductal fluid medium (SOF) supplemented with 2% of oestrus ovine serum and swim-up derived motile spermatozoa at 1×10^6 spermatozoa/ml concentration layered with mineral oil. Finally, after 22 h, presumptive zygotes were mechanically denuded of their cumulus cells and cultured to blastocyst stage, in four-well Petri dishes containing SOF + essential and non-essential aminoacids at oviduct concentration (Walker et al., 1996) + 0.4% BSA under mineral oil in maximum humidified atmosphere with 5% CO_2, 5% O_2, 90% N_2 to blastocyst stage. The number of cleaved oocytes, showing two distinct blastomeres was recorded at 26 hours post-insemination. Culture dishes were observed daily starting from the sixth day of culture and newly formed blastocysts were recorded (Figure 10).

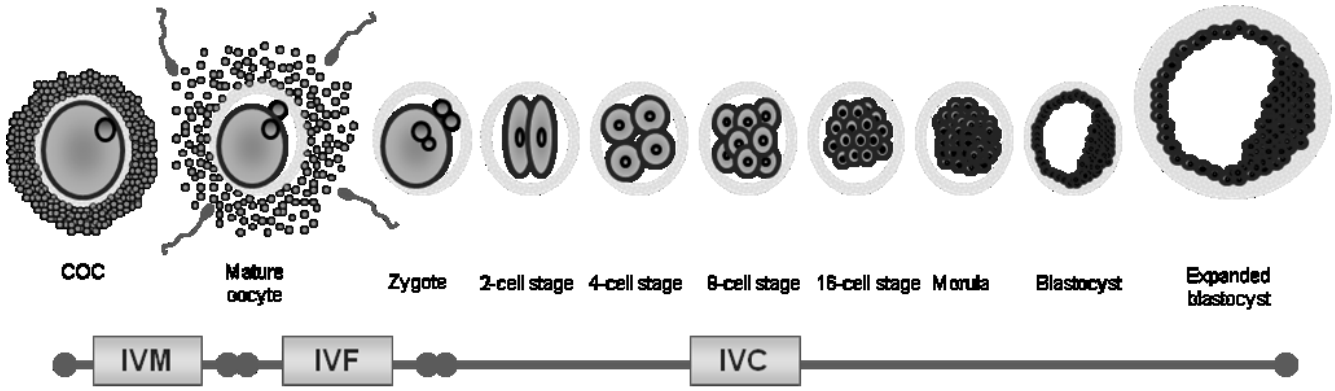

Figure 10: Diagram of the development stages in the in vitro culture. IVM: in vitro maturation; IVF: in vitro fertilization; IVC: in vitro culture

4.2.3.3. Vitrification and warming procedures

Vitrification and warming media were prepared using PBS supplemented with 20% (v/v) FCS as base media. Embryos were vitrified according to a simple method (Leoni et al., 2002). Briefly, blastocysts were put into 200 µl drops of 1.4 M glycerol for 3 min, then into 200 µl drops of 1.4 M glycerol and 3.6 M ethylene glycol for 3 min before being transferred into a 15 µl column of 3.4 M glycerol and 4.6 M ethylene glycol, and loaded into the centre of 0.25 ml plastic insemination straws using a fine glass capillary pipette. In the straws, the embryos and vitrification media were separated from two columns of 0.5 M sucrose solution. After sealing, the straws were transferred directly into LN_2 and kept in it.

For warming to a biological temperature, the straws were transferred from LN_2 into a water bath at 35 ºC for 10 s. The content of each straw was expelled into a Petri dish and stirred gently to facilitate the mixture of the two solutions. The embryos were retrieved and transferred into 200 µl drops of 0.25M sucrose solution supplemented with 20% FCS for 3 min for allowing the removal of intracellular cryoprotectants. Embryos were held 10 min in corresponding media of PBS containing 20% FCS for re-hydration and equilibration.

4.2.3.4. Assessment of embryo quality

We determined blastocyst cryotolerance after vitrification and warming to assess differences in embryo quality among the experimental groups, in terms of in vitro re-expansion and hatching rates during 72 hours post-warming culture. The embryos that re-expanded the blastocoelic cavity were considered to be viable (Leoni et al., 2006), as this parameter represents a reliable indicator of in vitro produced blastocysts quality (Rizos et al., 2003; Leoni et al., 2008).

After warming, blastocysts were cultured in TCM199 supplemented with 10% foetal calf serum in humidified atmosphere with 5% CO_2 in air at 39°C and examined every 12

hours during 72 hours to assess re-expansion and hatching rates. Blastocysts were considered completely re-expanded when their diameter was recovered to the original value before vitrification. Hatched blastocysts were fixed in a solution of methanol in PBS (40% v/v) and stained with propidium iodide (10 µg/ml). After washing in PBS/PVA, embryos were placed on glass slides, covered with cover slips and observed under a fluorescent microscope to count the total cell number (Figure 11).

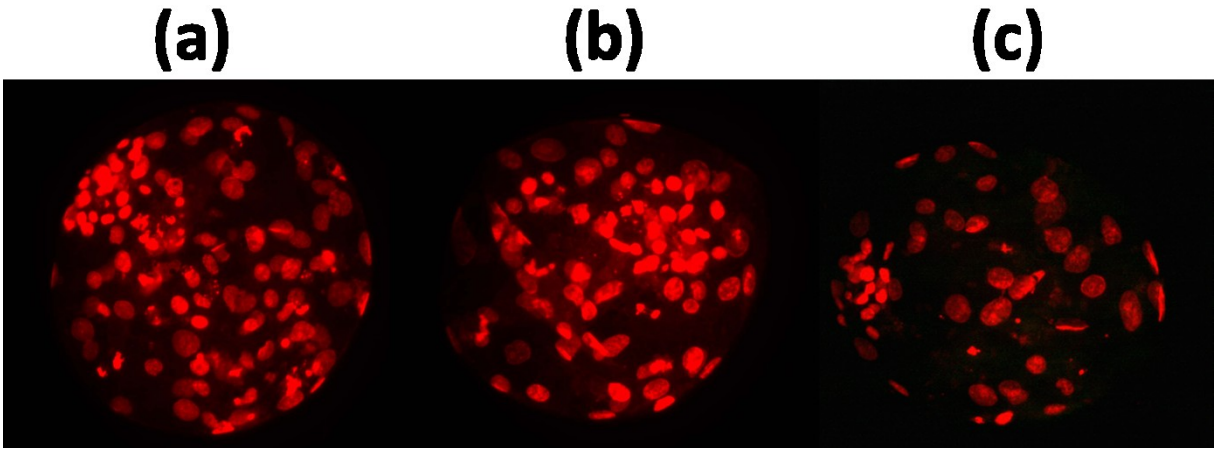

Figure 11: Blastocyst produced from 40 day-old ewe lambs oocytes from the (a) High AFC group (≥30 follicles), (b) Intermediate AFC group (16-29 follicles) and (c) Low AFC group (≤15 follicles), stained with propidium iodide when re-expanded after vitrification. Cellular nuclei appear stained in red.

4.2.3.5. Histological processing of the ovaries

Pairs of ovaries from 40 days-old ewe lambs were assigned to the High, Intermediate and Low AFC groups according to the total number of follicles, as above described. One of each pair of ovaries was then selected for histological analysis making a total of 6 ovaries in the Low AFC group, 8 ovaries in the Intermediate AFC group and 8 ovaries in the High AFC group. Before fixation, in order to allow an adequate penetration of the fixative, the ovaries from the Intermediate and High AFC groups were half-sectioned along the major diameter of

the ovary, while, for the Low group whole ovaries were used. Thereafter, ovaries were fixed overnight in Dietrich fixative [30% v/v Ethanol 95%, 10% v/v Formalin (Formaldehyde 37% solution containing 10-15% Methanol), 2% v/v Glacial Acetic Acid, 58% v/v distilled water] and transferred into PBS at 4°C until being dehydrated in an increasing Ethanol gradient (30% Ethanol 30 min, 70% Ethanol 30 min, 90% Ethanol 1 h, 100% Ethanol 1 h twice), followed by a 2-hours bath in Ethanol/Butanol (1:1 v/v), and a bath in butanol for 6 hours; then placed in paraffin for 3 hours. These steps were performed using an automatic tissue processor (UC-KD-TS3-Tissue Processor, nSquareTech). Afterwards, samples were embedded in a paraffin block.

Paraffin blocks were serially sectioned at 5µm intervals using a rotary microtome (Leica RM2245, Italy) and every 20th section was placed on a glass microscope slide. Slides were stained with hematoxylin and eosin (Leica ST5020 multistainer, Italy) and mounted. Then, observed under a microscope to determine the number of morphologically healthy primordial, primary, secondary, early antral, late antral follicles, as well as polyovular and atretic follicles. For Intermediate and High groups only sections from one of the ovary halves were analyzed.

4.2.3.6. Classification of follicles and follicle count

Only follicles of each 20th section with a cross-section of the oocyte nucleus were counted. Follicles were classified as morphological healthy follicles when presented an intact basal membrane, well-organized granulosa cell layers with only occasional pyknotic nuclei, none or few atretic granulosa cells in the follicular antrum, and intact oocyte and nucleus. The type of follicles is described in Figure 12. Due to the advanced stage of atresia of some follicles it was difficult to classify the atretic follicles into each follicle type; therefore, atretic follicles were classified only as atretic and were counted even when there was no nucleus. Follicles having more than one oocyte were classified as polyovular independently of the atretic or non atretic stage of the inside oocytes.

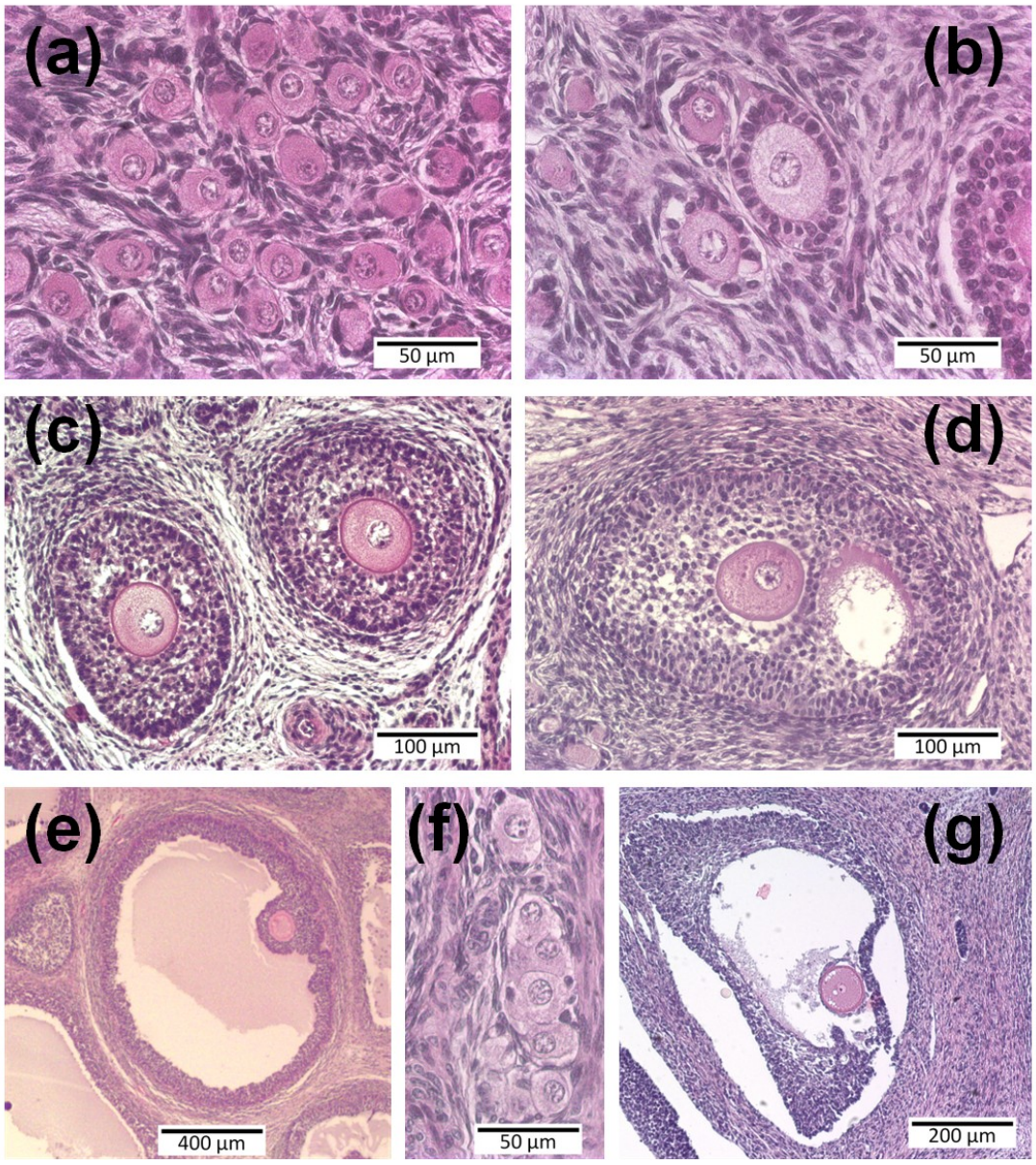

Figure 12: Histological sections of ovaries from 40 day-old ewe lambs. Primordial follicles (a) showed an oocyte surrounded by a flat single layer of follicular cells. Primary follicles (b) had an oocyte surrounded by a cuboidal epithelium of one to less than two layers of granulosa cells. Secondary follicles (c) presented the oocyte and zona pellucida surrounded by a variable number of concentric layers, two or more, of cuboidal granulosa cells without a discernible antrum. Once a small developing antrum was identified follicles were recorded as early antral follicles (d). Late antral follicles (e) exhibited a clearly formed antral cavity with an almost isolated oocyte surrounded by the cumulus oophorus. Follicles with two or more oocytes inside (f) were classified as polyovular follicles. (g) Example of atretic follicle with detachment of cells in the antrum and absence of cumulus oophorus.

In order to estimate the total number of follicles in the ovaries, as previously described by Ireland et al. (2008) in cattle ovaries, a correction factor was applied. For ovaries in the Low group the number of counted follicles for each type was multiplied by the correction factor 40 (20 X 2; 20 corrects for counting follicles in every 20th section and 2 corrects for counting follicles in a single ovary), while for ovaries in the Intermediate and High groups the correction factor used was 80 (20 X 2 X 2; 20 corrects for counting follicles in every 20th section, 2 corrects for counting follicles in only one half of the ovary and 2 corrects for counting follicles in a single ovary).

4.2.3.7. Evolution of FSH and AMH levels during the first weeks after birth

Blood samples from 146 December/January born Sarda ewe lambs from AGRIS Sardegna 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 x *g* for 10 min at 4 ºC and plasma was removed and stored at -20 ºC. The sixth week of life ewe lambs were classified by transrectal ultrasonography, as described in the preliminary experiment, according to the number of ≥2 mm follicles in the ovaries and assigned into one of the three experimental categories: High AFC, Intermediate AFC or Low AFC. Plasma samples from 50 ewe lambs in the categories High AFC (n=25) and Low AFC (n=25) were analyzed for AMH and FSH. AMH levels were determined with the AMH Gen II ELISA kit (Beckman Coulter Inc., Brea, CA) in an automatic ELISA analyzer (Personal LAB™, Adaltis, Italy) as described in the preliminary experiment.

To measure FSH levels the Sheep Follicle Stimulating Hormone (FSH) ELISA kit (Cusabio, China) was used, following the manufacture instructions and using an automatic ELISA analyzer (Personal LAB™, Adaltis, Italy). Briefly, standards, samples and controls, and after them a Horseradish Peroxidase conjugated antibody for FSH, were pipetted into a

microplate pre-coated with antibody specific for beta subunit chain of sheep FSH. Unbound reagent was eliminated with washes. Later, substrate solution was added and after incubation the reaction in stopped with a stop solution and colour intensity was measured. The intra- and inter- assay coefficients of variability were <15% for both.

4.2.3.8. Statistical analysis

Differences in oocyte developmental competence among the three experimental groups were assessed by ANOVA of the following ratios: oocytes selected for IVM/ovaries collected; fertilized oocytes/oocytes selected for IVM; cleaved oocytes/fertilized oocytes; blastocysts developed at Day 7 of culture/cleaved oocytes; blastocysts developed at Day 8 of culture/cleaved oocytes; blastocysts developed at Day 9 of culture/cleaved oocytes; total blastocysts/cleaved oocytes. Differences among the three groups in ovaries wet-weight, in the number of collected ovaries belonging to the three experimental groups, and total cell number in the blastocysts were assessed by ANOVA. When data were not normally distributed (Shapiro Wilk W test: $p<0.05$), the analysis of variance for non-parametric values (Kruskal-Wallis test) was used. In this case, between groups differences were analyzed by the post hoc paired comparison analysis (Wilcoxon's paired test). Fisher's LSD (Least Significant Difference) test was used for the mean separation. The chi-squared test, or Fisher exact test when appropriate, was used to determine differences on blastocyst re-expansion and hatching rates. Possible correlations among the following variables were determined: i) size of the ovaries and in vitro developmental competence of the oocyte, as evaluated in terms of fertilized and cleaved oocytes and subsequent embryo output: we considered, for each replicate, the in vitro developmental competence of each batch of oocytes and the sum of the wet-weights of all the ovaries assigned to each experimental group.; ii) size of the ovaries and numbers of growing and primordial follicles. Differences in AMH concentrations between the two experimental groups and during the first 9 weeks of age were assessed by general lineal model where: $Y = \mu + day + group - + day \times group + ewe\ lambs$. Day 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 (LSD) procedure. The probabilities obtained by the LSD test were corrected by Bonferroni's correction for multiple comparisons. Statistical analysis was performed using the statistical software program Statgraphic Centurion XV (version15.2.06 for Windows; StatPoint, Inc., Herndon, VA, USA) and a probability of $p \leq 0.05$ was considered to be the minimum level of significance. All results are expressed as mean ± S.E.M.

4.2.4. Results

4.2.4.1. Effects of the ovarian phenotype on oocyte in vitro developmental competence

Marked inter-individual differences in ovarian follicle number were evident in the collected ovaries. In addition, the proportion of ovaries among the three groups varied significantly ($p<0.0001$), and the lowest number of ovaries (8.5%) was assigned to the High group followed by the Intermediate (33.7%) and Low group (57.8%; Table 1). The number of COCs selected for IVM per ovary varied significantly among the groups. In particular, more COCs were obtained from the High group than from the Intermediate and Low ones ($p<0.0001$). Differences in oocyte developmental competence among groups are summarized in Table 1. A higher embryo output was obtained from oocytes collected from High and Intermediate groups when compared to the Low group ($p<0.05$). In addition, in the High and Intermediate groups a higher number of blastocyst developed on day 8 of culture compared to the Low one ($p<0.05$).

Table 1: Comparison of the proportion and sizes of ovaries assigned to High (≥30 follicles), Intermediate (16 – 29), and Low AFC group (≤15) according with the ovarian phenotype expressed, and number and in vitro developmental competence of oocytes collected from ewe lamb (40 days of age) ovaries.

		Groups		
		High	Intermediate	Low
Ovaries	Ovaries (%)	56^{α} (8.5)	222^{β} (37.7)	380^{γ} (57.8)
	Ovary wet weight (mg)	609.9 ± 21^{a}	264.9 ± 21^{b}	54.8 ± 24.3^{c}
	Ovary height (mm)	1.3 ± 0.04^{a}	0.95 ± 0.04^{b}	0.65 ± 0.05^{c}
	Ovary lenght (mm)	1.01 ± 0.05^{a}	0.78 ± 0.05^{b}	0.5 ± 0.05^{c}
Oocytes	IVM	1832^{a}	1928^{b}	343^{c}
	(per ovary; mean ± SE)	(36.4 ± 2.9)	(9.9 ± 2.7)	(1.1 ± 2.8)
	IVF	1411	1355	274
	(%)	(79.6 ± 4.3)	(70.2 ± 3.9)	(81.6 ± 4.1)
Embryos (%)	Cleaved*	1087	1082	187
		(74.1 ± 5.7)	(81.3 ± 5.3)	(71.3 ± 5.5)
	Day 7**	99	68	14
		(6.3 ± 2.3)	(5.6 ± 2.2)	(3.9 ± 2.2)
	Day 8**	99^{a}	104^{a}	10^{b}
		(10 ± 2)	(9.7 ± 1.9)	(4.1 ± 1.9)
	Day 9**	9	17	3
		(1.1 ± 0.5)	(1.3 ± 0.5)	(0.4 ± 0.5)
	Total**	207^{a}	189^{a}	27^{b}
		(17.4 ± 2.8)	(16.6 ± 2.6)	(8.3 ± 2.7)

*Percentages are calculated on fertilized oocytes.

**Percentages are calculated on cleaved oocytes.

Different superscripts indicate statistical difference among the experimental groups: $\alpha \neq \beta \neq \gamma$ $p<0.0001$ (Chi-squared test); $a \neq b \neq c$ $p<0.0001$; $a \neq b$ $p<0.05$ (ANOVA).

Ovaries wet-weight proved to be strongly correlated with the number of collected oocytes per gram of ovarian tissue (correlation coefficient: 0.90; $p<0.0001$); a positive correlation was also found with the number of oocytes fertilized (0.53; $p<0.001$), cleaved

(0.49; $p<0.01$), with the number of blastocysts obtained at Day 8 of culture (0.45; $p<0.01$) and the total number of blastocysts obtained without regards of the day (0.48; $p<0.01$).

Blastocysts cryotolerance, as evaluated by in vitro re-expansion and hatching rates, did not differ among the experimental groups (Table 2). Total cell number was higher in blastocyst obtained from oocytes belonging to ovaries with high follicle number compared with the other groups ($p<0.05$).

Table 2: In vitro re-expansion, hatching rates, and total cell number of vitrified/warmed blastocysts obtained from ewe lamb ovaries (40 days of age) with different follicle numbers (High AFC: ≥30; Intermediate AFC: 16 – 29; Low AFC: ≤15).

Groups (n)	Re-expansion (%)	Hatching (%)	Total cell number* (mean ±SE)
High (130)	66 (50.7)	44 (33.8)	92 ± 4.3^{a}
Intermediate (134)	58 (43.2)	36 (26.9)	75.1 ± 4.6^{b}
Low (27)	17 (62.9)	7 (26)	72 ± 7.6^{b}

*Total cell number was determined on hatched blastocysts. Different superscripts indicate statistical difference: $a \neq b$ $p<0.05$ (ANOVA).

4.2.4.2. Determination of ovarian reserve in ewe lambs expressing different ovarian phenotypes

The largest ovaries, as determined by their wet weight, height and length, belonged to the High group, followed by the Intermediate and then by the Low group ($p<0.0001$; Table 1). However, the total number of morphologically healthy follicles and the number of morphologically healthy primordial, primary and secondary follicles did not vary in relation

with the ovarian phenotype expressed, as ovaries with the smallest size bore the highest number of healthy follicles per mg of tissue ($p<0.0001$; Table 3). Ovarian phenotype reflected the number of morphologically healthy early and late antral follicles, which reached the highest absolute value and proportion over healthy follicles in the High group, followed by the Intermediate and then by the Low group.

Table 3: Total number of follicles in ovaries of ewe lambs (40 days of age) assigned to High (≥30 follicles), Intermediate (16 – 29 follicles), and Low AFC (≤15 follicles) groups according with the ovarian phenotype expressed.

	Total n° of follicles in ovaries		
Follicle parameters	High (*n=8*)	Intermediate (*n=8*)	Low (*n=6*)
Healthy + atretic	237850 ± 43593	250270 ± 43593	206753 ± 50337
Healthy	219430 ± 40570	231685 ± 40570	194587 ± 46846
(%)	(93.9 ± 0.8)	(93.2 ± 0.7)	(92.1 ± 0.7)
Healthy per mg ovaries	190 ± 119[a]	437 ± 119[a]	1922 ± 138[b]
Primordial	207020 ± 39975	221915 ± 39975	184060 ± 46159
(%)*	(92.8 ± 1.2)	(95.3 ± 1.2)	(93.8 ± 1.4)
Primary	8920 ± 1052	6280 ± 1052	7547 ± 1215
(%)*	(4.9 ± 0.7)	(2.9 ± 0.7)	(4.5 ± 0.8)
Secondary	2490 ± 512	3170 512	2940 ± 591
(%)*	(1.6 ± 0.4)	(1.6 ± 0.4)	(1.7 ± 0.5)
Early antral	1310 ± 107[a]	680 ± 107[b]	333 ± 124[c]
(%)*	(0.8 ± 0.1)	(0.4 ± 0.1)	(0.2 ± 0.1)
Late antral	1000 ± 81[a]	320 ± 81[b]	40 ± 93[c]
(%)*	(0.7 ± 0.2)	(0.2 ± 0.2)	(0 ± 0.2)
Polyovular	1260 ± 316	1390 ± 316	767 ± 365
(%)**	(0.5 ± 0.1)	(0.5 ± 0.1)	(0.33 ± 0.1)

* Percentages are calculated on healthy follicles.

** Percentages are calculated on total follicles.

[a,b,c] Different letters indicate statistical difference among the experimental groups: $a \neq b \neq c$ $p<0.0001$ (ANOVA). Differences among the experimental groups in early and late antral follicles account for both their total number and their proportion over healthy follicles.

No correlation was found between ovarian sizes (weight, height and length) and primordial follicle number, nor between number of growing follicles (primary, secondary and antral follicles) and number of primordial follicles, nor between number of antral follicles (early antral plus late antral) and the total number of morphologically healthy follicles.

4.2.4.3. AMH and FSH levels during the first weeks after birth

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 levels from all ewe lambs from the High AFC group could be detected, whilst, for the Low AFC group, the detection of AMH levels in all animals could not be reached until the ninth week.

Both groups had similar values of AMH only the first week after birth, showing already since the second week and, at least, until the ninth week significantly different levels between the two groups (Figure 13). Regarding the evolution of AMH levels along the weeks, in the Low AFC although levels progressively increased this elevation was not significant in the first 9 weeks of age. Meanwhile, in the High AFC group, there was a clear increase in AMH plasma levels that peaked in the fifth week (1.065 $\pm$ 0.095 ng/ml) to decline gradually from the sixth week (Figure 13).

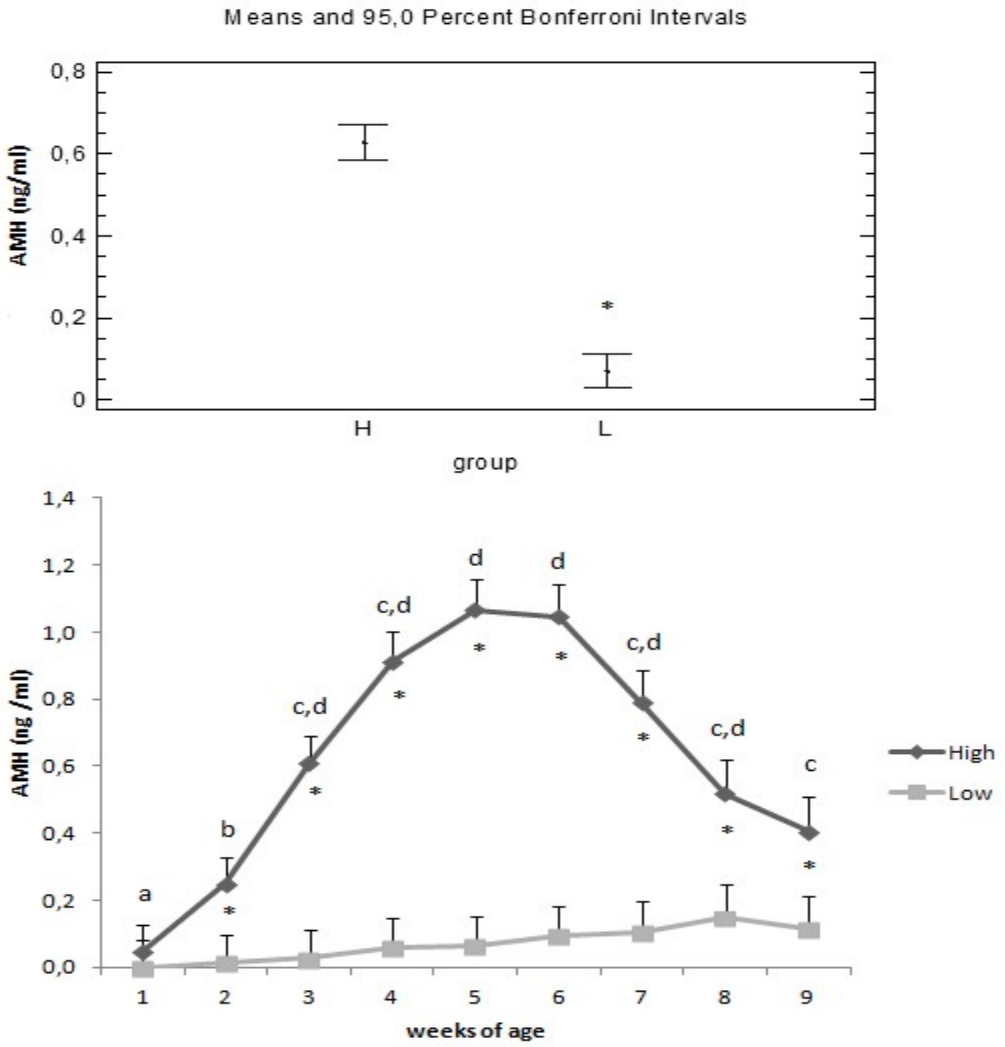

Figure 13: Mean AMH values throughout 9 weeks (upper part) and evolution of mean plasma levels from birth week till 9 week of age (lower part) in Sarda ewe lambs from the High AFC (n=25) and Low AFC (n=25) groups.

[a,b,c,d] Indicate a statistical difference in values from the same experimental group: a ≠ b ≠ c ≠ d $p<0.0001$.

* Asterisks indicate a statistical difference between the two experimental groups: $p<0.0001$.

Plasma FSH levels could not be detected in any of the ewe lambs using the Sheep Follicle Stimulating Hormone (FSH) ELISA kit (Cusabio, China).

4.2.5. Discussion

The results of the current experiments extend our knowledge of the relationships among AMH, oocyte quality and the ovarian reserve during the early prepubertal period. A low AFC, although not accompanied by a reduction in the size of the primordial follicle pool, was predictive of poor oocyte in vitro developmental competence. In the High AFC group plasma AMH levels peaked in the 5 and 6 weeks of age, coinciding with a greater number of early and late antral follicles in the histology and an increased oocyte quality in the in vitro culture from ovaries of ewe lambs of this age.

Our study evidenced that ovarian phenotype expressed during the early prepubertal period can predict oocyte quality, as evaluated by its in vitro developmental competence. Oocytes collected from ovaries with the highest AFC proved to be the most competent to develop into a blastocyst after incorporation into the in vitro production system. In addition, ovary weight was positively correlated with subsequent in vitro embryo output. These results confirm previous findings in adult ewes (Mossa et al. 2008). Follicle numbers have been positively associated with a variety of measures of fertility in single-ovulating species, like human (Kupesic et al., 2003; Scheffer et al., 2003), cattle (Cushman et al. 2009) and sheep (Mossa et al. 2008). In young adult cattle, antral follicle number has been positively related to AMH levels, number of healthy oocytes and follicles (Burns et al. 2005), corpus luteum functionality (Jimenez-Krassel et al. 2009), responsiveness to superovulation, oocyte quality and in vitro blastocyst development (Ireland et al. 2008). Cows with a high AFC had higher pregnancy rates, shorter calving to conception intervals and received fewer services during the breeding season, when compared with cows with low AFC (Ireland et al. 2011). It has been suggested that a low number of antral follicles would create a hormonal milieu that negatively affects ovarian function and oocyte quality (Jimenez-Krassel et al. 2009). Individual follicles from cattle with low AFC have higher follicular fluid concentrations and produce more oestradiol than follicles from animals with high AFC (Ireland et al. 2009) and it has been speculated that this difference may be a consequence of higher circulating FSH

concentrations in the low versus the high AFC groups. In addition, circulating androgen and progesterone concentrations are indeed higher in cattle with high compared with low AFC (Jimenez-Krassel et al. 2009).

The mechanisms of inter-follicular regulation are poorly understood (mice: Durlinger et al. 2002a; Da Silva-Buttkus et al. 2009), but it is likely that local autocrine and paracrine products, reflecting the different stages of follicular development, may influence the development of other follicles and thus oocyte quality. Further research is needed to elucidate the role of these putative paracrine factors during the very first stages of follicle development and how they can influence the ovarian milieu and the acquisition of oocyte developmental competence in a virtually steroid-free environment from the hormonal milieu of the prepubertal period.

The antral follicle count is currently regarded as a reliable marker of the ovarian reserve both in adult women (Hansen et al. 2011) and in animal models (cattle: Ireland et al. 2008). In our study in prepubertal ovaries, AFC was not predictive of differences in either the number of healthy follicles or the size of the primordial follicle pool and no correlation was found between numbers of growing (from primary to late antral) and primordial follicles. Previous studies have evidenced that the number of growing follicles is correlated to the size of the primordial follicle stock (Gougeon, 1996; Kevenaar et al., 2006). In addition, a striking correlation has been found between ovarian volume and non-growing follicle population (Wallace and Kelsey 2004) not evidenced in our animals. In our current experiment, no difference was appreciated in the non-growing follicle population but there was a difference in ovary weight and size, probably justified because the number of late antral follicles was greater in the High AFC group. More space is occupied by these larger follicles and follicular content contributed in getting a higher weight. Maybe these diverse ovarian densities are not maintained for a long time. Considering that an increase in ovarian weigh was seen in Ouled Djellel ewe lambs till 4 weeks of age and then a further increase from 4 weeks of age till 10

weeks of age (Mahdi and Khallili, 2008) the same increase might be retarded in the Low AFC group.

In mice, although the number of primordial follicles declined with aging the number of growing follicle did not decline till later (Kevenaar et al., 2006). A similar situation could be occurring in our lambs; perhaps, although the number of primordial follicles got reduced, antral follicles are not yet reflecting the actual primordial follicle population but a previous one. In particular, AFC could be reflecting the population of growing follicles and only indirectly the population of primordial follicles. It was manifest for AMH in mice that AMH levels do not change until when the effect of primordial follicles loss influences the recruitment of new growing follicles when dealing with 4 - vinylcyclohexene diepoxide, a toxic that affects primordial follicles (Sahambi et al., 2008). AFC at 40 days old could be not directly reflecting the actual primordial follicle population but a previous one, as previously proposed, since the ovary suffers loss of follicles by atresia before and after birth (Wallace and Kelsey, 2010; Hulas-Stasiak and Gawron, 2011).

In the data from our study there is great interindividual variability in ovarian reserve. AFC does not reflect significant differences in the number of primordial follicles but in the number of antral follicles between groups. Maybe without a larger follicular reserve but with a greater speed of follicle recruitment it may occur a further depletion of ovarian reserve in individuals from the High AFC group that could affect their future reproductive live. It is known that reproductive life is reduced, even leading to infertility, in women with a smaller ovarian reserve or excessive loss of follicles (Kerr et al., 2013; Nelson et al., 2013). Similarly, in infertile woman, the sooner they begin to cycle, having menarche at an earlier age, the greater is the risk of functional diminished ovarian reserve (Weghofer et al., 2013).

Contrary to what happens in adults (Rico et al., 2009), AMH levels in the early prepubertal period are not constant. AMH in Sarda sheep was almost undetectable the first week of live to progressively increase, especially in the High AFC group that showed a remarkable increase during the first five weeks after birth. This is in line with that found in

girls in which AMH is barely detected at birth (Hudson et al., 1990) to later show an increase during the postnatal period and childhood (Hagen et al., 2010; Kelsey et al., 2011). Likewise, it has been described a markedly increase in AMH between 1 and 3 months of age also in cattle (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., 2013). This finding may suggest the possibility of an increase in AMH levels later in life in the animals from the Low AFC group. A recent study evidenced that ewe lambs with the highest AMH plasma levels at 3.6 months were also the most precocious in terms of early puberty, resulting in the improved fertility observed when testing fertility of different ewes at the same age (Lahoz et al. 2012). In girls, a study comparing postnatal development described an earlier and greater increase of serum AMH levels in full term girls compared with preterm girls (Kuiri-Hanninen et al., 2011a). Thus, AMH could represent a sign of follicular maturation in the prepubertal ovaries, and its close relationship with the non-growing follicle pool could develop only after the full establishment of the reproductive activity.

The most likely explanation to our findings is 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 through this period. In infant boys and girls it has been described a postnatal pituitary activation sometimes called "minipuberty", probably produced by the decline after birth in sex steroid-mediated inhibition of gonadotrophin secretion exerted by maternal placental and gonadal steroids (Lee, 2003). In infant boys, it is associated with testicular testosterone secretion, penile and testicular growth, and an increase in the number of Sertoli and germ cells (Kuiri-Hanninen et al., 2011b), 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-Hanninen et al., 2011a) reaching levels similar to those observed in

periovulatory women, while AMH levels increase soon after birth, peaking a few months after birth (Hagen et al. 2010). However, the possible importance of this activation as regards female reproductive development is not understood. Similarly in our study data shows a peak in AMH levels in the group High AFC that could match the occurrence of this "minipuberty" in sheep.

We can thus speculate that during the prepubertal period follicular recruitment and development reflect the changes in the endocrine milieu and are preparatory to the subsequent development of the reproductive function. High plasma FSH concentrations have also been detected in the first few days after birth in ewe lambs (Savoie et al., 1979). This rise is likely to trigger follicular development. In our study, this may be the case of the High AFC group, were the increase of serum AMH levels after birth demonstrates postnatal proliferation of granulosa cells and activation of ovarian follicular development. In the low AFC groups, however, this pattern was not observed, at least in the first 9 weeks of age, so maybe it just never occurred or it was triggered later, as happens in preterm infant girls in which a delayed follicular development and increase in serum AMH levels was seen when compared with full term ones (Kuiri-Hanninen et al., 2011a). Unfortunately we could not detect FSH levels with the ELISA kit to determine if this pituitary activation occurs in the studied ewe lambs. In any case, the difference between the two groups in the AFC and AMH evolution points to a high individual variability in the occurrence of postnatal pituitary activation in sheep as it occurs in human. Nevertheless, the AMH gene dose-dependency observed in AMHKO mice (Durlinger et al., 2002b), with heterozygous mice having an intermediate phenotype between the wild-type and the AMHKO, hints that control of AMH production will not only be due to a feedback control of other hormones but to the intrinsic activity of the gene itself so that the observed differences could be inherent to the animal.

Folliculogenesis in adult sheep has been proved to take several months to reach the preovulatory stage (Campbell et al., 2000); approximately 130 days are needed for a primordial follicle to achieve 0.2 mm in diameter (Cahill, 1981). Folliculogenesis seems to be shorter in foetal life. At day 70 of gestation numerous ovigerous cords are still present in the

foetal ovary. First primordial follicles are seen at about day 75 of gestation (Sawyer et al., 2002) in sheep being more numerous at day 100 of gestation. Secondary follicles are already seen at day 120 of gestation (Bezard et al., 1987; McNatty et al., 1995), antral follicles at day 135 of gestation (McNatty et al., 1995). Thus, the difference observed in AFC between the studied groups might be already initiated during gestation.

Babies born small for gestational age have deceased ovarian volume, reduced ovulation rate and increased FSH levels when reaching puberty denoting that prenatal growth has endocrine effects that affects reproduction later in life (Chellakooty et al., 2003). The maternal environment plays an important role in the regulation of the high interindividual variation in the ovarian reserve (Ireland et al., 2011). Undernourishment affects differently the expression of apoptosis-regulating genes in the foetal ovary depending on the period in which the nutritional restriction has occurred (Lea et al., 2006). Previous experiments in cattle concluded that AFC decreases in calves whose mothers underwent a nutritional restriction receiving only the 60% of the maintenance requirements during the first third of gestation (Evans et al., 2010). Nutrition clearly influences folliculogenesis (Webb et al., 2004; Hernandez-Medrano et al., 2012), especially in sheep where follicle are very sensitive to nutritional inputs (Scaramuzzi et al., 2006), and maternal diet influences developmental programming of reproduction (Gardner et al., 2008). An interesting study in sheep worked out that foetal ovarian development was significantly retarded at 47 and 62 days of gestation in foetuses of undernourished ewes from the time of matting respect to overnourished ewes (Borwick et al., 1997). Similarly, an ulterior study confirms that underfeeding pregnant adult ewes before or during the time of folliculogenesis of their offspring delays foetal follicular development (Rae et al., 2001). Also, reduced pituitary activity was seen in 55 days-old ewe lambs whose mothers were undernourished from 30 days of gestation (Deligeorgis et al., 1996). In addition, maternal nutrition regulates cellular proliferation in ovine foetal ovaries (Lea et al., 2006). All this together leads to think that the diverse AFC observed at 40 days of age could be influenced by the nutritional status of the mother during gestation, this way animals with lowest AFC may came from mothers with a lower nutritional status than the

mothers of the ewe lambs with higher AFC. However genotype itself might affect ovarian development. In ewe lambs carrying the Booroola gene, that provides greater ovulation rates, development of the foetal ovary is delayed compared to ewe lambs not carrying the gene. So, differences in follicular development and pituitary function in neonatal and adult life may be a consequence of differences in the timing or organ development in foetal life (Smith et al., 1993).

In conclusion, we evidenced that AFC can predict oocyte quality in prepubertal ovaries reflecting an ovarian status suitable for follicular development, 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 follicle or the size of the primordial follicle pool in prepubertal ovaries. We also noted that during the first weeks after birth AMH levels are variable between individuals as occurs in adult life. However, in animals with high AFC, AMH does not remain constant for the same individual as happens in adulthood but increases during the first weeks to progressively drop suggesting that animals could be experiencing a kind of "minipuberty" during the early prepubertal period. Differences between groups are likely to be the result of changes initiated in foetal life.

4.3. EXPERIMENT 2: evolution from prepubertal period to adulthood

4.3. EXPERIMENT 2: evolution from prepubertal period to adulthood

4.3.1. Abstract

As it was evidenced in the preliminary experiment, AFC and AMH levels have a high inter-individual variability and higher levels were predictors of an enhanced response to exogenous FSH ovarian reserve test. Both AFC and AMH remain relatively constant for long-time periods within the same individual but it has been also described a distinct variation during the prepubertal period and a progressive decline with age in adulthood. Thus, we aimed to determine if the differences in AFC, AMH plasma levels and the response to exogenous FSH ovarian reserve test (EFORT) seen in Sarda ewes lambs at an early prepubertal age continue at a peripubertal age and a postpubertal age. From the results of the current experiment we inferred that obvious differences between groups at an early prepubertal age, both in the follicle population prior to the stimulation with FSH and after treatment, tend to disappear with age. As well, responsiveness to repeated stimulation with FSH decreases, with fewer follicles being recruited during the peripubertal and postpubertal stage, as compared with the prepubertal one. The number of follicles reaching large follicular sizes, equal or greater than 4 mm, increases once animals are approaching and reach puberty. Again, as in the preliminary experiment, during the early prepubertal period circulating AMH was positively correlated to AFC and to follicles developed in response to gonadotrophin administration, confirming that AMH is a good predictor of ovarian response to FSH stimulation. Its circulating levels, however, could not be detected in older sheep. Hence, selecting ewe lambs either for a high AFC or higher AMH levels did not guaranteed better responses to ovarian stimulation in adulthood but was still effective in the peripubertal period.

4.3.2. Specific objectives

To determine if the differences in AFC, AMH plasma levels and the response to exogenous FSH ovarian reserve test (EFORT) seen in Sarda ewes lambs at an early prepubertal age continue at a peripubertal age and a postpubertal age.

4.3.3. Material and Methods

4.3.3.1. Animals

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 from a bigger population (n=104) in which the ovarian phenotype was determined by characterizing the AFC 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 (rigid laparoscopic transducer UST-5526L-7.5, Aloka Co., Tokyo, Japan). Scanning was performed as described in preliminary experiment. Then 24 ewe lambs from the Low AFC group (≤15 follicles) and 24 more from the High AFC group (≥30 follicles) were chosen in order to determine differences between the two groups. 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 different ovarian phenotypes. Initial weight, when ewe lambs were classified into the two experimental groups and peripubertal weight were also recorded.

4.3.3.2. EFORT test: AFC and circulating AMH

An exogenous FSH ovarian reserve test (EFORT: Fanchin et al., 1994; Kwee et al., 2006) was made before puberty at an early prepubertal age (50 days of age, 7 weeks), at a peripubertal age (195 days of age, 28 weeks) and at a postpubertal age (496 days of age, 71 weeks). In all the lambs (n=48, 24 for High AFC group and 24 for Low AFC group), the ovarian phenotype was recorded by characterizing the AFC 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., Tokyo, Japan at 50 days of age and 82mm prostate transducer UST-660-7.5, Aloka Co., Tokyo, Japan at 195 and 496 days of age). Ultrasounds were carried out as previously described and validated by Gonzalez-Bulnes et al. (1994) as described in the preliminary experiment. At 195 and 496 days of age due to the larger size of the animals the observations were conducted with the sheep placed in dorsal recumbence on a metallic cradle as used for laparoscopy. The number of follicles for each category (total, 2 mm, 3 mm and ≥4 mm) was written down.

After recording the AFC, the responsiveness of the follicular population to the exogenous FSH ovarian reserve test was determined. First, blood samples were drawn 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 x *g* for 15 min at 4ºC and plasma was removed and stored at -20 ºC until assayed for AMH determination. Afterwards, in all the animals, one-shot intramuscular dose of 105 IU of porcine FSH (Folltropin®, Bioniche Animal Health, Minitub Ibérica, S.L., Reus, Spain) was administered. Twenty-four hours later, the growth of the antral follicles was assessed by a second ultrasonographic scanning and a second blood sample was drawn. For the EFORT test performed after puberty (496 days of age), the number of animals was reduced to 9 animals in the High AFC group and 15 animals in the Low AFC group. Also, in

order to accommodate to the larger size of the animals, the intramuscular FSH dose was increased to 175 IU.

Analysis of the EFORT included changes in follicular population and in plasma concentrations of AMH. 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.

4.3.3.3. Hormone assay in plasma samples

AMH concentration was measured by using the AMH Gen II ELISA kit (Beckman Coulter Inc., Brea, CA) as described in the previous experiments. Sensitivity of the assay was 0.08 ng/ml, whilst the inter- and intra-assay variation coefficients were 5.6% and 5.4%, respectively.

4.3.3.4. Statistical analysis

Differences between High and Low AFC groups in age, weight at birth and peripubertal weigh were determined by ANOVA. New follicles stimulated to growth after FSH administration were calculated by subtracting for each animal in each size category the follicles found on Day 0 from the follicles found on Day 1. Differences in follicle numbers (small, medium, large and total), new follicles and hormone concentrations between the experimental groups, between Days 0 and 1 of treatment for each EFORT, and among different EFORTs were assessed by ANOVA. When data were not normally distributed (Shapiro Wilk W test: $p<0.05$), the analysis of variance for non-parametric values (Kruskal-Wallis test) was used. In this case, between groups differences were analyzed by the post hoc paired comparison analysis (Wilcoxon's paired test). The method used to discriminate among

the means was Fisher's least significant difference (LSD) procedure. The probabilities obtained by the LSD test were corrected by the Bonferroni's correction for multiple comparisons. Possible correlations among follicle population and AMH levels were determined. Statistical analysis was performed using the statistical software program Statgraphic Centurion XV (version15.2.06 for Windows; StatPoint, Inc., Herndon, VA, USA) and a probability of $p \leq 0.05$ was considered to be the minimum level of significance. All results are expressed as mean ± S.E.M.

4.3.4. Results

4.3.4.1. Age and weight

There were no significant differences between animals from the High and Low AFC groups neither 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, nor 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).

4.3.4.2. Follicle population prior to EFORT

The evolution of the population of follicles prior to treatment with FSH was different between the two groups. The follicular population before gonadotrophin stimulation at 50 days of age differed significantly in most follicular categories to what found in the peripubertal (195 days of age) and postpubertal stage (496 days of age) for both groups (Figure 14). 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 to 50 days of age (Figure 14).

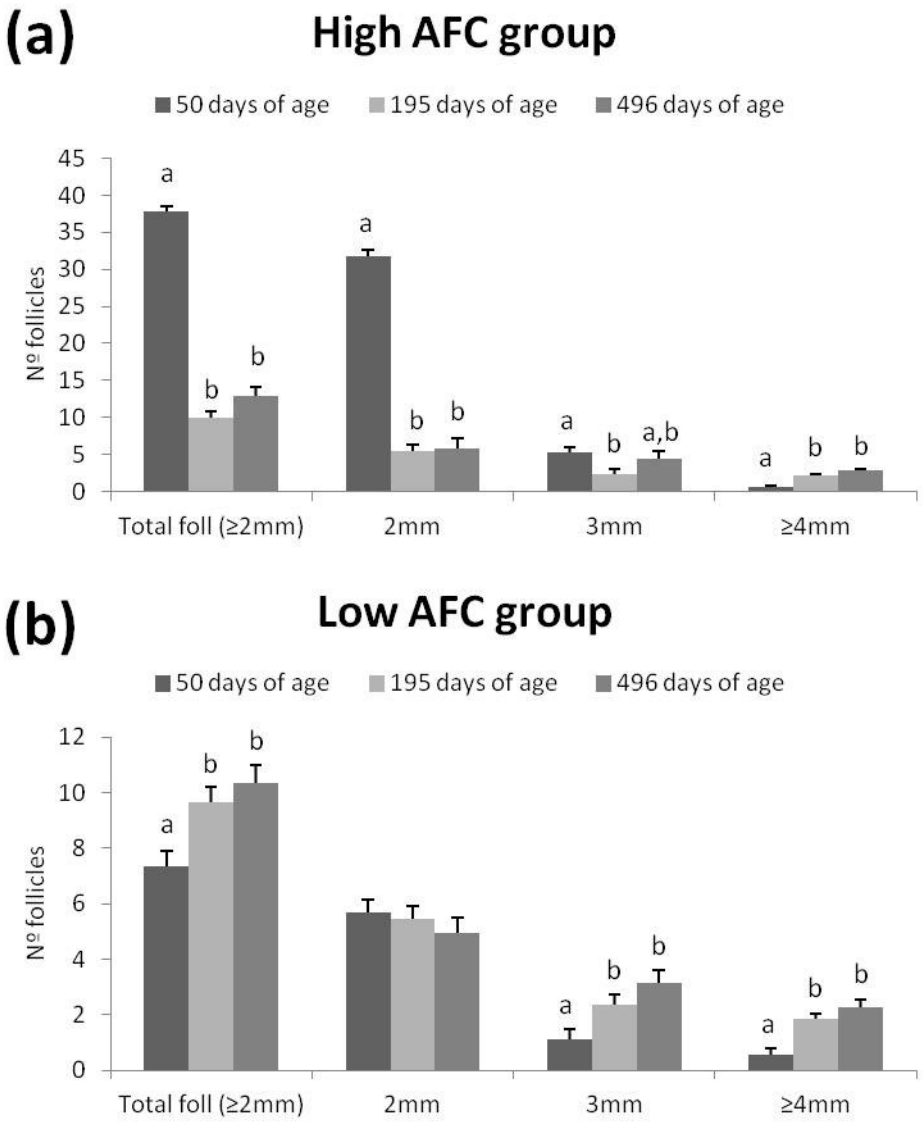

Figure 14: Initial follicle population at 50, 195 and 496 days of age prior to stimulation with FSH in ewes classified according to their AFC (High ≥30 follicles, Low ≤15 follicles) at an early prepubertal age. Mean initial number of follicles in each follicular category for High AFC group (a) and Low AFC group (b) are displayed above.

[a,b] Indicate a statistical difference in the same follicular category from the same group at various ages: $a \neq b$ $p<0.01$.

Similarly, 3 mm follicles seem to follow the same evolution as total follicles, increasing in the Low AFC group and decreasing in the High AFC group, although the decline in the High AFC group at 496 days of age is not as pronounced as what produced in total and 2 mm follicles with mean values of this category reaching halfway values between the observed ones at 50 and 195 days of age (Figure 14). In both groups ≥4 mm follicles became more numerous at 195 and 496 days of age compared to those found at 50 days of age.

4.3.4.3. EFORT: AFC and circulating AMH

At 50 days of age, animals were included into the High AFC group when 30 or more follicles ≥2 mm in diameter were identified in the ovaries, while were placed into the Low AFC group when only 15 or less follicles were seen. Therefore, the ovarian ultrasound scanning performed before exogenous FSH administration (Day 0) showed a significant difference in the mean number of total follicles (≥2 mm) in the ovaries between the two experimental groups (37.80 ± 1.03 vs. 7.36 ± 0.48 for High and Low AFC groups respectively, $p<0.0001$). This difference was mainly due to a significantly higher number of 2 mm follicles and, to a lesser extent, 3 mm follicles in the High AFC group (31.80 ± 1.14 and 5.36 ± 0.85, respectively) compared to the Low AFC group (5.68 ± 0.37 and 1.12 ± 0.27, $p<0.0001$; Figure 15, panel a). After exogenous FSH administration (Day 1), the number of total, 3 mm and ≥4 mm follicles increased both in the High and the Low AFC groups ($p<0.0001$); whereas the number of 2 mm follicles increases in the Low AFC group (5.68 ± 0.37 vs. 10.88 ± 1.19 for Day 0 and Day 1 respectively, $p<0.0001$) and decreases in the High AFC one (31.80 ± 1.14 vs. 23.72 ± 2.06 for Day 0 and Day 1 respectively, $p<0.0001$; Figure 15, panel b). Significant differences observed on Day 0 between the two experimental groups were maintained 24 hours after treatment (59.72 ± 4.22 vs. 22.68 ± 2.01, 23.72 ± 2.06 vs. 10.88 ±1.19, 26.36 ± 2.56 vs. 6.96 ± 1.01, for total, 2 mm and 3 mm follicles High AFC vs. Low AFC respectively, $p<0.0001$) and even extended to ≥4 mm

follicles which were lower in the Low AFC group (9.64 ± 1.19 vs. 4.84 ± 0.65, $p<0.0001$; Figure 15, panel b).

At a peripubertal age (195 days of age), the number of follicles ≥2 mm diminished in the High AFC group, while increased slightly in the Low AFC group compared to what previously observed at 49 days of age. Thus, the obvious differences in total follicle number between groups were no longer observed (10.04 ± 0.46 and 9.67 ± 0.52 for High vs. Low AFC groups). In untreated animals the only significant difference between groups was in the number of ≥4 mm follicles which were higher in the High AFC group (2.21 ± 0.18 vs. 1.83 ± 0.23 for High vs. Low AFC, $p<0.05$; Figure 15, panel c). On Day 1 the number of total, 3 mm and ≥4 mm follicles increased in the High AFC ($p<0.0001$), whereas the number of 2 mm follicles decreases compared to Day 0 ($p<0.04$; Figure 15, panel d). Also, if we bring into comparison Day 0 and Day 1 from the Low AFC group, the number of 2 mm follicles decreased while the number of 3 mm and ≥4 mm follicles increased ($p<0.005$); however the total number of follicles remained nearly unchanged suggesting the growth of follicles to larger sizes without a remarkable increase in follicular recruitment. When comparing both groups after FSH administration, High AFC group showed always higher follicle numbers per each follicular category (17.20 ± 1.01 vs. 10.46 ± 0.73 for total follicles, $p<0.04$; 3.88 ± 0.75 vs. 2.00 ± 0.43 for 2 mm follicles, 7.21 ± 0.63 vs. 4.42 ± 0.54 for 3 mm follicles, 6.13 ± 0.59 vs. 4.04 ± 0.44 for ≥4 mm follicles, $p<0.002$, for High AFC vs. Low AFC respectively; Figure 15, panel d).

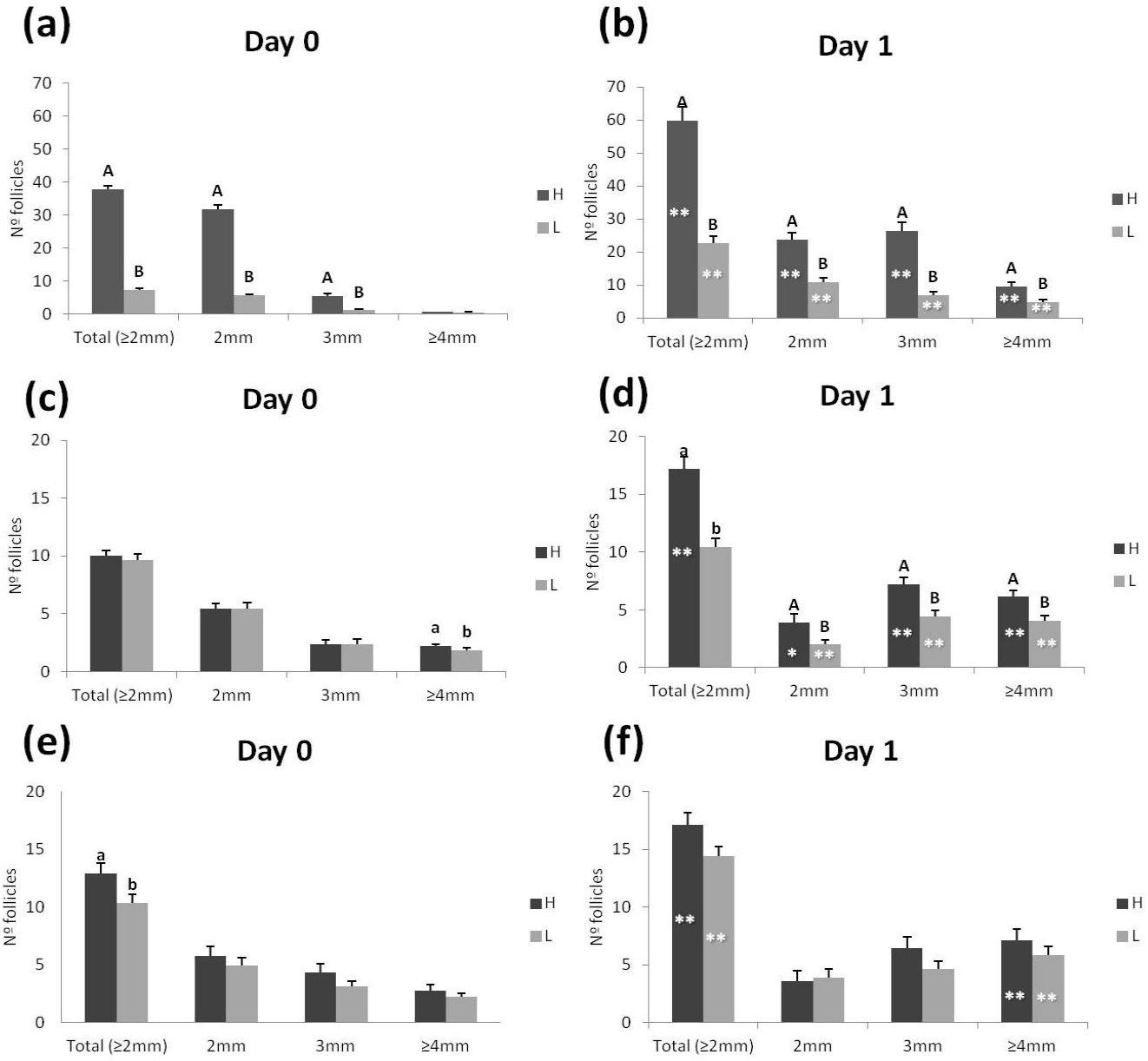

Figure 15: Follicle population before and after FSH administration in early prepubertal ewe lambs, peripubertal and postpubertal ewes classified according to their ovarian phenotypes at an early prepubertal age in High (H) and Low (L) AFC groups. Mean number of total, 2 mm, 3 mm, ≥4 mm in diameter before (left hand; Day 0) and 24 h after FSH treatment (right hand; Day 1) in 50 day-old ewe lambs (a, b), 195 day-old ewes (c, d) and 496 day-old ewes (e, f).

[a, b, A, B] Indicate a statistical difference among both experimental groups: a ≠ b $p<0.05$; A ≠ B $p<0.01$.

* Asterisks in right hand panels indicate a statistical difference from values observed on Day 0 in the same experimental group: * $p<0.05$; ** $p<0.01$.

After puberty (496 days of age), during non-breeding season, the mean number of total follicles was lower in the Low AFC group before treatment (12.89 ± 0.93 vs. 10.33 ± 0.76 for High and Low AFC, $p<0.05$), while there were no significant differences in other follicular categories between the two experimental groups. Response to exogenous FSH within each group produced significant changes in total follicle number and ≥4 mm follicles (10.33 ± 0.76 vs. 14.40 ± 0.85, $p<0.002$ and 2.27 ± 0.30 vs. 5.87 ± 0.74, $p<0.0002$ for total and ≥4 mm follicles Day 0 vs. Day 1 in Low AFC group; 12.89 ± 0.93 vs. 17.11 ± 1.09, $p<0.01$ and 2.78 ± 0.49 vs. 7.11 ± 0.95, $p<0.001$ for total and ≥4 mm follicles Day 0 vs. Day 1 in High AFC group; Figure 15, panel f). On Day 1, no significant difference was seen between groups in the studied categories, as it is shown in Figure 15, panel f. Nevertheless, we still found a tendency in the High AFC group to have a higher number of total follicles compared to the Low one (17.11 ± 1.09 vs. 14.40 ± 0.85, $p=0.07$) and a significant higher number of 4 mm follicles (4.11 ± 0.48 vs. 2.80 ± 0.38, $p<0.05$), not reflected in the graphic since it shows not only 4 mm follicles but also superior sizes.

As follicular population prior to ovarian stimulation, the response to ovarian stimulation in terms of number of new follicles changed over time (Figure 16). 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 a peripubertal and a postpubertal age compared to the ones observed at an early prepubertal age. Fewer 2 mm follicles are lost in the High AFC group during the peripubertal (-1.58 ± 0.80) and postpubertal (-2.22 ± 1.27) EFORTs than in early prepubertal one (-8.08 ± 1.91), but also fewer follicles grew to larger categories. Furthermore, in the Low AFC new 2 mm follicles are recruited after exogenous

gonadotrophin stimulation at 50 days of age (5.2 ± 2.20) while at 195 and 496 days of age the lost of these follicles is greater than the recruitment (-3.46 ± 0.62 and -1.07 ±1.19).

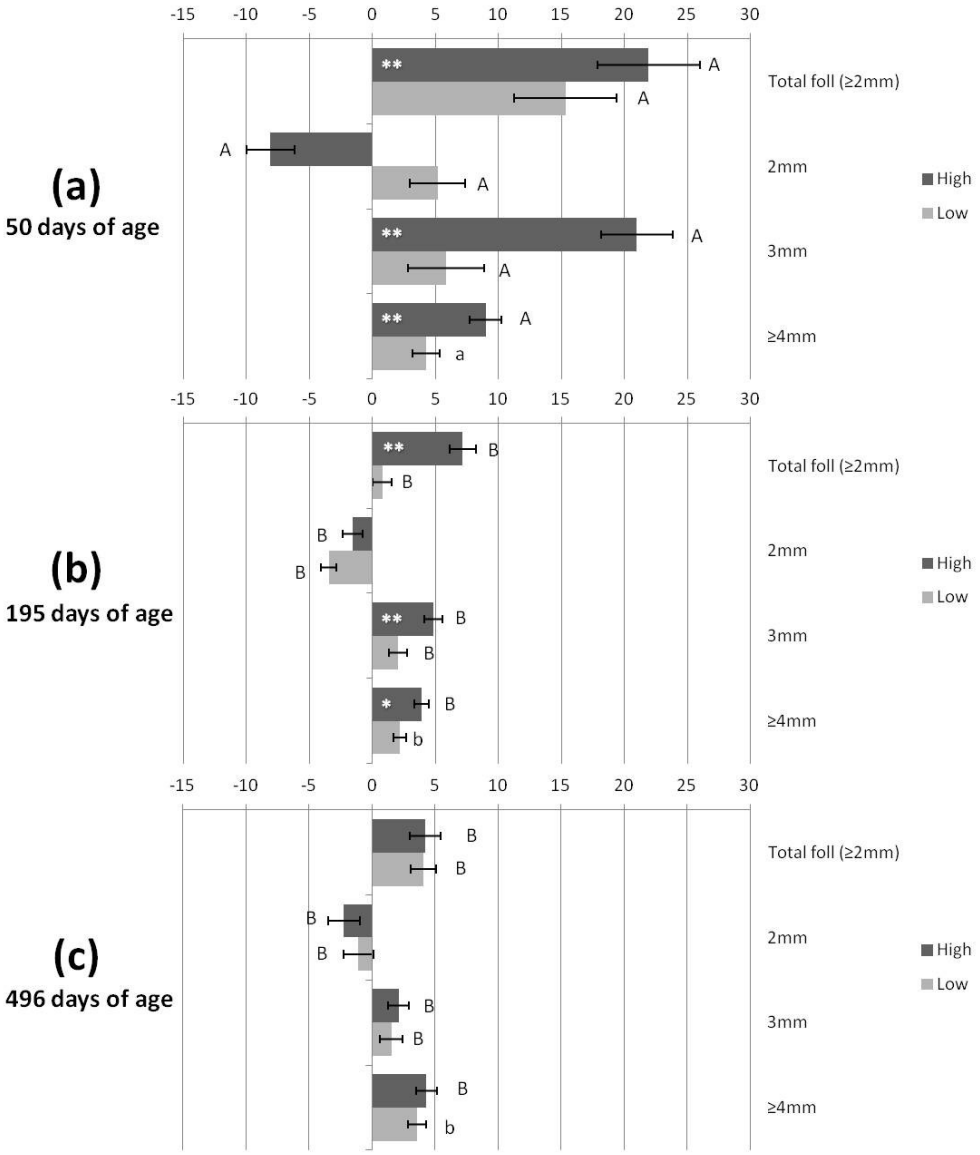

Figure 16: New follicles per follicular category 24 hours after an ovarian stimulation with FSH at 50 (a), 195 (b) and 496 (c) days of age in ewes classified according to their AFC (High ≥30 follicles, Low ≤15 follicles) at an early prepubertal age.

a, b, A, B Indicate a statistical difference in the same follicular category in the same group among different EFORTs: a ≠ b $p<0.05$; A ≠ B $p<0.01$.

* Asterisks indicate a statistical difference between groups in the same follicular category from the same EFORT: * $p<0.05$; ** $p<0.01$.

At 50 days of age the differences between groups in terms of new 2mm follicles (Figure 16) are marked, being negative for the High AFC group (-8.08 ± 1.91) and positive for the Low AFC group (5.2 ± 2.20; $p<0.0001$). Twenty four hours after FHS administration the number of new 3 mm and ≥4 mm follicles was higher in the High AFC group (21.00 ± 2.85 and 9.00 ± 1.24, for 3 mm and ≥4 mm follicles) compared to the Low AFC group (5.84 ± 3.02 and 4.28 ± 1.06, for 3 mm and ≥4 mm follicles; $p< 0.002$). On the contrary no significant difference was observed in the total number of new follicles between groups at an early prepubertal age. Subsequently, at 195 days of age the differences between groups continued, but, at this time, a greater number of new total follicles were recruited in the High AFC group (7.17 ± 1.06 vs. 0.79 ± 0.74 for High vs. Low AFC group; $p<0.0001$) and no significant difference was seen in the number of new 2 mm follicles. Lastly, at 496 days of age no significant differences were observed for either of the categories of follicles.

AMH could be only detected in early prepubertal lambs plasma samples since values from peri- and postpubertal samples probably reached levels below the detection limit of the ELISA kit. AMH was detected in all High AFC group samples while, in the Low AFC group, it could be only detected in the 70.83% (17/24) of the samples before treatment and the 54.17% (13/24) of the samples after treatment. At 50 days of age, AMH plasma levels were higher in High AFC lambs before (1.77 ± 0.21 ng/ml vs. 0.36 ± 0.08 ng/ml, $p<0.0001$) and after exogenous FSH administration (1.22 ± 0.17 ng/ml vs. 0.24 ± 0.06 ng/ml, $p<0.0001$; Figure 17). As depicted in Figure 17, only levels from the High AFC group significantly decreased after FSH treatment ($p<0.05$).

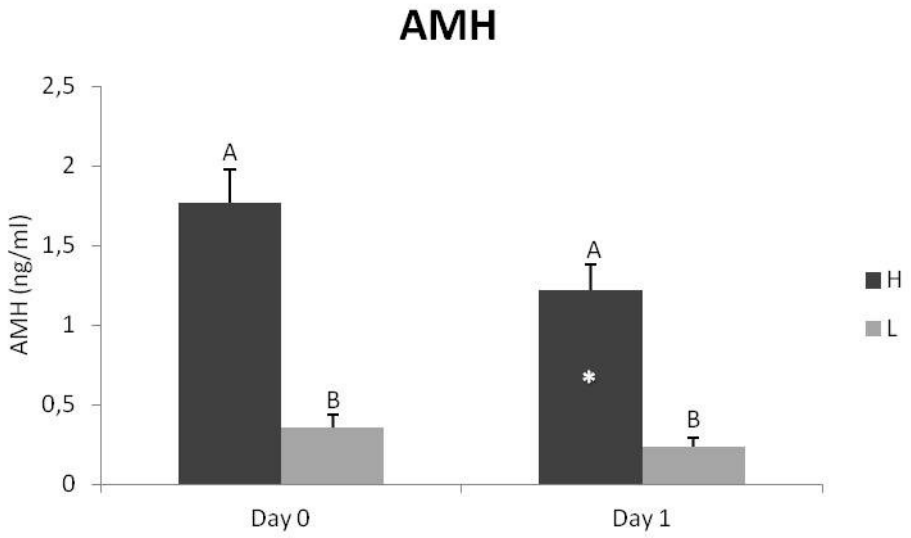

Figure 17: Anti-Müllerian hormone plasma levels before and after FSH administration in early prepubertal ewe lambs classified according to their ovarian phenotypes. Mean AMH plasma levels before (Day 0) and 24 h after FSH treatment (Day 1) in 50 day-old ewe lambs assigned to High (H) and Low group (L) according with the ovarian phenotype expressed at an early prepubertal age (High ≥30 follicles, Low ≤15 follicles).

A, B, Indicate a statistical difference among the two experimental groups: A ≠ B $p<0.01$.

* Asterisks indicate a statistical difference from values observed at Day 0 in the same experimental group: * $p<0.05$.

Correlations between AMH plasma levels and number of follicles per each follicular category in 50 days-old ewe lambs are displayed in Table 4. Briefly, AMH was positively correlated to 2 mm and total follicles on Day 0; with 2 mm, 3 mm, ≥4 mm and total follicles on Day 1 and with 2 mm, 3 mm and total follicles regardless of the day. Also AMH plasma levels on Day 0 were correlated with the mean number of follicles per each follicular category seen on Day 1.

Table 4: Correlations between plasma AMH levels and follicle number from ewe lambs at an early prepubertal age. Correlations between AMH plasma levels (ng/ml) and the number of follicles determined by transrectal ultrasound per each follicular category before a 105 IU exogenous FSH administration (Day 0), 24 hours after (Day 1) and between AMH plasma levels detected on Day 0 and the number of follicles found on Day 1.

	2 mm	3 mm	≥4 mm	Total follicles (≥2 mm)
AMH Day 0 – Follicles Day 0	r=0.692 p<0.0001	r=0.2278 p<0.112	r=-0.2523 p<0.077	r=0.6445 p<0.0001
AMH Day 1 – Follicles Day 1	r=0.762 p<0.0001	r=0.7566 p<0.0001	r=0.3398 p<0.016	r=0.8149 p<0.0001
AMH Day 0 – Follicles Day 1	r=0.7216 p<0.0001	r=0.8311 p<0.0001	r=0.3903 p<0.006	r=0.8498 p<0.0001

4.3.5. Discussion

From the results of the current experiment we inferred that obvious differences between groups in the follicle population prior to the stimulation with FSH observed at an early prepubertal age tend to disappear with age. In both groups responsiveness to repeated stimulation with FSH decreases with age, with fewer follicles being recruited during the peripubertal and postpubertal stage. However, differences in the response to the FSH administration, although not as apparent as in the early prepubertal period, could still be observed in the peripubertal and post-pubertal period. Again, as in the preliminary experiment, circulating AMH was positively correlated to AFC and to follicles developed in response to gonadotrophin administration during the early prepubertal period, confirming it as a good predictor of ovarian response to FSH stimulation.

In our research, 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, while in the Low AFC group increased compared to 50 days of age. The higher number of follicles seen in the High AFC

group could be the response to the occurrence of a minipuberty as we have discussed in the previous experiment, maybe due to a prepubertal increase in luteinizing hormone (LH) as it has been seen in heifers (Honaramooz et al., 1999).

A study in 8 Suffolk ewe lambs that followed AFC evolution by transrectal ovarian ultrasonography from 6 to 38 weeks of age described an increased in AFC from 24 weeks before puberty, then a decline in AFC started from 14 weeks before puberty although AFC remained still higher that that observed at 28 weeks before puberty (Bartlewski et al., 2006). This might match with what observed in the Low AFC group as the number of 2 mm or greater follicles increased at a peripubertal age compared to follicle number at approximately 7 weeks of age. As puberty and first ovulation approach, changes in LH pulse frequency and an increase in biological FSH activity associated with changes in the distribution pattern of FSH isoforms, may lead to the recruitment of a larger number of antral follicles (Bartlewski et al., 2002, 2006 ; Rawlings et al., 2003). On the other hand, the High AFC group and its decrease in the number of follicles may not be represented in this study due to the low number of animals followed. In fact the number of follicles greater than or equal to 2 mm in the initial weeks of monitoring differ greatly from the high values found in the High AFC group indicating that subjects with high number of antral follicles at an early prepubertal age were not enrolled in that study. Changes in the AFC in Suffolk ewe lambs appeared to be mainly due to variations in the number of 2 mm and 3 mm follicles, meanwhile no differences were found in the number of ≥4 mm follicles as puberty onset was approached. In our ewe lambs from the High AFC group the changes in total follicle population are mainly due to shifts in the number of 2 mm follicles. Contrary, in the Low group the increase in follicle number could not be justified by a significant increase in 2 mm follicles but to an increase in follicles of 3 mm and ≥4 mm. In any case, contrary to what is described by Bartlewski et al. (2006), the number of follicles ≥4 mm increased both in High AFC and Low AFC groups probably due to the hormonal influence of the hypothalamic-pituitary-gonadal axis beginning to operate cyclically. Another study in Ouled Djellel ewe lambs (Mahdi and Khallili, 2008) reported that the number of follicles ≥3 mm in diameter

rose gradually from birth to 14 weeks of age to peak again in the peripubertal period (24 weeks of age). This finding is more similar to what identified in our lambs in which ≥4 mm follicles, which are the most competent for embryo production in sheep (Veiga-Lopez et al., 2008), increased towards a peripubertal age.

Our data show that starting from 195 days of age, follicular population remains relatively constant to what found in the postpubertal period. These results are in agreement with previous studies which plead that AFC remains constant for long periods of time in adults (Burns et al., 2005; Ireland et al., 2007; Ireland et al., 2008). However, this could not be applied to the prepubertal period since the initial population of follicles was significantly different to both the peripubertal and the postpubertal follicle population for both studied groups. All this leads us to postulate that the factors affecting follicular development during the early prepubertal period are not maintained to the same extent in adult life.

Endogenous opioid peptides (EOP) may be involved in the phenomenon observed since they exert an inhibitory action on the secretion of GnRH/LH, controlling the amplitude and shape of the pulses (Goodman et al., 1995), and are involved in the inhibitory action of oestradiol on GnRH (reviewed by Goodman et al., 2002) and LH secretion (Honaramooz et al., 2000). Treatment with an opioid receptor antagonist (Naloxone) leads to an increase in LH levels in catheterized lamb foetuses (Cuttler et al., 1985) suggesting that their inhibitory function is already acting in foetal life. In fact, endogenous opioids make up an important inhibitory mechanism controlling pulsatile LH secretion in the prepubertal sheep (Ebling et al., 1989). Similarly oestradiol is extremely effective in inhibiting pulsatile GnRH secretion before puberty, but its inhibitory feedback action is reduced as the time of puberty is approached (Foster et al., 2006). Opioid inhibition may gradually decrease during development in favour of other non-opioid inhibitory mechanisms which would justify the lower response to treatment with Naloxone observed in 2 year-old sheep when compared with 1 year-old sheep during the non-breading season (Schall et al., 1991).

At 195 days of age the follicular population from both groups before FSH treatment differed only in the number of ≥4 mm follicle. The higher number of ≥4 mm follicles in animals from the High group could be the product of a more adequate hormonal milieu that allows further follicular growth as a result of higher gonadotrophin levels or differences in feedback sensitivities. It could also be reflecting an enhanced follicular development product of changes derived from the proximity of a more precocious onset of puberty since, as puberty approaches, the sensitivity to gonadal steroid negative feedback decreases while GnRH and LH pulse frequency increase (reviewed by Valasi et al., 2012). Also, the better response in animals from the High AFC group to treatment with FSH at 195 days of age, even with similar initial follicular populations, could be due to an increased ovarian maturity in these individuals. At 496 days of age differences between groups in the total number of follicles prior to FSH treatment correspond to a cumulative effect of a greater number of follicles in each follicular category but not significant within each follicular category.

As in the preliminary experiment, at an early prepubertal age the number of follicles grown after ovarian stimulation with exogenous FSH was higher in the High AFC group, thus animals with higher AFC respond better. In agreement with our results, ovaries from women containing more antral follicles respond better to stimulatory treatments than ovaries with a low number of antral follicles (Tomas et al., 1997; Chang et al., 1998; Bancsi et al., 2002; van Rooij et al., 2002) and the number of retrieved oocytes after exogenous stimulation is higher (Kawamata, 1994; Kwee et al., 2008; Broer et al., 2009; Melo et al., 2009; Hsu et al., 2011). Likewise, cattle with high number of antral follicles have greater number of medium and large follicles (≥5 mm in diameter) after a stimulation with gonadotrophins than cattle with low number of antral follicles (Singh et al., 2004), having a better quantitative (Rico et al., 2009) ovulatory response after the treatment.

The group with the highest number of 2 mm follicles, the High AFC, was the one that after treatment with FSH showed the greatest number of total follicles, medium-sized follicles (3 mm) and large follicles (≥4 mm) and in this experiment, differing from what observed in the preliminary experiment, the differences in the number of 2 mm follicles also

became significant between groups. Similarly, in cattle, the superstimulatory response can be predicted by the number of 2 mm follicles at wave emergence (Singh et al., 2004) as the population of small antral follicles is the main target of the superovulatory treatment being indicative of the population of gonadotrophin-responsive follicles able to develop after a stimulatory treatment (Rico et al., 2009). However, although between the two experimental groups no significant differences in the number of 2 mm follicles were detected neither at a peripubertal nor at a postpubertal age, the variance in the number of follicles, especially in the total number of follicles and the number of follicles ≥4 mm, were still evident between groups after treatment with FSH. We can speculate that some of the factors that fostered the variations between groups at a young age continue to exert some influence in the capacity of granulosa cells to respond to FSH stimulation. Furthermore, a study in Sarda ewe lambs receiving ovarian stimulation treatments several times during the prepubertal period (at 1, 3, 5 and 7 months of age) shows a gradual age-dependent fall in ovarian response, with major responses observed at 4 weeks of age (Ptak et al., 2003). These findings agree to what found in our experiment in which the response to ovarian stimulation was higher in ewe lambs at 50 days of age than at older ages.

At 50 days of age in the High AFC group the increase in new follicles from medium and large sizes was again accompanied by a drop in the number of small follicles, as it was already evidenced in the preliminary experiment, suggesting that the growth to larger categories was higher than the recruitment of new follicles. The same patron was repeated in ulterior stimulations, losing 2 mm follicles but enlarging the number of new follicles in larger sizes even in the Low AFC group. However at 50 days of age the number of new 2 mm follicles in the Low AFC group increased, suggesting that there is a follicle gonadotrophin-responsive population with less than 2 mm in diameter capable of being recruited in response to exogenous gonadotrophin administration. A retarded follicular development in a more immature ovary as a result of the occurrence of a delayed "minipuberty" (Chellakooty et al., 2003; Lee, 2003) in the Low AFC group might justify the inferred existence of this

follicle-responsive population and the absence of further repetition of this phenomenon in stimulations at older ages.

Nutrition influences folliculogenesis (Webb et al., 2004; Hernandez-Medrano et al., 2012). Besides, food nutrients can modulate gene expression, including genes related to reproduction (Dawson, 2006). Maternal diet influences developmental programming of reproduction (Gardner et al., 2008) with foetal ovarian development being retarded in foetuses of undernourished ewes (Borwick et al., 1997; Rae et al., 2001). Nevertheless foetuses from undernourished mother have lower birth weight (Deligeorgis et al., 1996), while in our experiment no difference was revealed in weight neither at birth nor at the beginning of the study, that could justify undernourishment in the Low AFC group. Mothers were fed with the same diet during pregnancy, since they belong to the same research centre, although we cannot exclude differences in food intake between animals because they were not set individual portions and food was available to the entire group. However, there is a called "acute" effect of nutrition that unlike "dynamic" effect (flushing) or "static" effect improves reproduction characteristics without causing weight changes (Scaramuzzi et al., 2006). The "acute" effect is achieved with several energy-yielding sources in short-term administrations whose actions at the level of the ovary are more remarkable than the effects on the reproductive centres in the brain (Martin et al., 2004). Short-term glucogenic supplementation stimulates the growth of follicles <2mm in diameter and follicles 2-3mm in diameter, that grew to larger categories (Letelier et al., 2008; Berlinguer et al., 2012) and also improves oocyte quality with higher fertilisation and blastocyst rates (Berlinguer et al., 2012). Thus the acute effect of nutrition could be another possible explanation for the observed data in the group with high AFC.

AMH levels were detectable only in the first EFORT. The same way, in intact males AMH plasma concentration was not detectable in adults while it was at 4 weeks of age in lambs (Cazorla et al., 1998). No international assay standard for AMH measurement exists even in humans with AMH results being kit-dependent (reviewed by Broer et al., 2011). Two main ELISA kits for the determination of AMH levels manufactured by Diagnostic Systems

Laboratories, Inc. (DSL) and Immunotech (IOT) have been available (Li et al., 2012). Recently, a second generation anti-Müllerian hormone (AMH) ELISA kit was developed by Beckman Coulter using the same antibody as in the DSL kit but the standards of the IOT assay kit (Kumar et al., 2010). The Beckman Coulter ELISA kit, seems to be more useful than DSL method in women undergoing ART (Freour et al., 2007), although not suitable enough for detection of AMH plasma levels in adult Sarda sheep when strictly following manufacture instructions. AMH expression in follicles does not change with age (Kevenaar et al., 2006), this means that follicles from prepubertal animals could not have higher levels of AMH if not because there is a greater number of growing follicles. Thereby, the fact that AMH concentration could be detected in early prepubertal animals with reduced AFC and not in adult animals with a higher AFC could be explained by the existence of a larger population of ultrasonographically undetectable preantral and small antral follicles (<2 mm in diameter) that would contribute to raise AMH concentration at this age, as the AMH is produced in preantral and small antral follicles (sheep, Bezard et al., 1987; rat, Hirobe et al., 1994; mouse, Salmon et al., 2004; human, Weenen et al., 2004; cow, Monniaux et al., 2008, 2013; reviewed by Sarraj and Drummond, 2012). AMH levels should be relatively constant and therefore characteristic of each animal for extended periods in adults (Rico et al., 2009; Monniaux et al., 2011) although decreases with aging (Kevenaar et al., 2006). Unfortunately, we could not detected AMH levels to verity if different values at an early prepubertal are maintained later in life. Nevertheless a very recent study in Rasa Aragonesa ewes detected AMH levels in prepubertal and adult sheep with the same AMH GenII ELISA Kit from Beckman Coulter, discovering a decline in plasma AMH concentration at 6 months to reach similar levels to the observed ones in adulthood (Lahoz et al., 2013). This drop in plasma AMH levels probably occurred in our studied ewe lambs although we could not detect the AMH levels because of differences due to breed and protocol. Authors from this article modified the Beckman Coulter ELISA kit protocol increasing both the amount of sample used and the incubation time with primary and secondary antibodies. Also, in the aforementioned study, no significant relationship between prepubertal and postpubertal plasma AMH

concentrations could be established, similarly in our study the AMH prepubertal values might also be unrelated to adult levels reflecting the changes observed in the AFC.

Numerous studies have reported an excellent positive correlation of AMH with AFC in humans (r=0.71: de Vet et al., 2002; r=0.77: van Rooij et al., 2002; r=0.74: Fanchin et al., 2003) and animals (r=0.79: Rico et al., 2009). Our study has shown a similar positive correlation (r=0.64). Before treatment with FSH, AMH levels were highly correlated with the number of follicles 2 mm (r=0.69), but no significant correlation was found with larger follicles. This could be explained because major AMH-producing follicles are the preantral and small antral ones with AMH expression dropping drastically in follicles greater than 3.5 mm in diameter in sheep (Campbell et al., 2012). In a like manner, it has been described a fall in intrafollicular concentration in follicles greater than 3 mm in diameter in goat (Monniaux et al., 2011). After treatment with FSH, AMH levels were even more strongly correlated with AFC (r=0.81) and were positively correlated with all follicular categories size, probably due to the growth of the follicles that were participating in reaching those AMH levels but were not ultragraphically-detectable because of their size.

At Day 1 of treatment, in the High AFC group there is a significant drop in AMH that in the Low AFC group does not become significant. This finding might be explained considering that AMH decreases as follicles grow into larger categories near preovulatory sizes (Campbell et al., 2012; Jeppesen et al., 2013; Monniaux et al., 2013) and the oocyte matures (Kedem-Dickman et al., 2012). In the High AFC group there is a more pronounced growth of follicles to larger categories with a higher increment in new 3 mm and ≥4 mm follicles accompanied by a parallel decrease in 2 mm. Equally, a decrease in AMH levels was produced after treatment with gonadotrophins in women (La Marca et al., 2004) and goats (Monniaux et al., 2011). A less plausible explanation would be the decrease in AMH expression produced by high FSH concentrations as seen in cows (Scheetz et al., 2012; Monniaux et al., 2013), although this effect has not been seen in sheep (Monniaux et al., 2013).

High correlation detected between AMH levels before treatment and number of follicles in each of the categories studied after follicular gonadotrophin administration confirmed AMH as a good predictor of ovarian response to stimulation treatments. In agreement with our results several authors described that the number of oocytes obtained after ovarian stimulation treatments is highly correlated with AMH levels (Seifer et al., 2002; Gruijters et al., 2003; Fleming et al., 2006; Rico et al., 2009; Monniaux et al., 2011), and women with high AMH levels are likely to respond better to stimulation with exogenous gonadotrophins than women with low AMH levels (van Rooij et al., 2002; Ficicioglu et al., 2006; La Marca et al., 2007, 2010; Anderson et al., 2012).

For some authors AMH seems to inhibit follicles sensitivity to FSH (Durlinger et al., 2002b; Pellatt et al., 2010) while other authors point that AMH enhances the effect of FSH by inducing an increase in both follicle diameter and cell number in rat preantral follicles (McGee et al., 2001). In our experiment, at a prepubertal age animals with higher AMH levels developed a larger number of new follicles 24 hours after FSH administration than animals with low AMH plasma concentrations, suggesting that AMH should not be inhibiting sensitivity to FSH. However, AMH is not the only factor regulating sensitivity to FSH and other factors such as other TGF- β superfamily members (Richards, 2001) might be influencing the response to FSH.

4.4. EXPERIMENT 3: adulthood

4.4. EXPERIMENT 3: adulthood

4.4.1. Abstract

Several studies have associated the presence of relatively high numbers of antral follicles and AMH levels in adults with a wide variety of fertility indices in animals and women, such as higher number of retrieved oocytes after exogenous stimulation, higher oocyte and embryo quality, higher progesterone production, higher pregnancy rates and higher live-birth rates. Thus, the current experiment aimed to determine if the differences in AFC and AMH plasma levels seen at an early prepubertal age in Sarda ewe lambs with high or low AFC, entails differences in the oestrus cycle, fertility at first breeding season or the response to a multiple ovulation protocol in adult life. A greater number of animals from the High AFC group became pregnant during their first breeding season. Sheep were synchronized and pursuant oestrous cycle was monitorized. Ovulatory follicle growth dynamics, number of new and atretic follicles per day, number of double ovulation and oestradiol levels on heat day determined by RIA were evaluated during follicular phase. Corpora lutea growth dynamic and progesterone production measured by ELISA during luteal phase as well as cycle length were analyzed. No significant difference, except for a more progressive increment in the number of preovulatory size follicles in the High AFC group, could be established in the oestrus cycle between groups. Finally, ewes underwent a MOET treatment after which ovulation rate was determined and embryos were recovered, vitrified and transferred into synchronized recipients, again without any remarkable difference between animals from the two experimental groups. We concluded that selection of animals with high AFC at an early prepubertal age would allow the selection of the most precocious animals, as evidenced by the enhanced fertility at first breeding season increasing precocity, but could not predict further differences in other parameters related to reproductive functionality in the adulthood, such as follicular and corpora lutea dynamics during the oestrus cycle and the response to a standard MOET protocol.

4.4.2. Specific objectives

To determine if the differences in AFC and AMH plasma levels seen at an early prepubertal age in Sarda ewe lambs with high or low AFC, entails differences in the oestrus cycle, fertility at first breeding season or the response to a multiple ovulation and embryo transfer protocol in adult life.

4.4.3. Material and Methods

4.4.3.1. Animals

Animals from the previous experiment classified according to the number of ≥2 mm follicles in their ovaries at 50 days of age into High (≥30 follicles) or Low (≤15 follicles) AFC groups were used in the current experiment.

4.4.3.2. Fertility during the first breeding season

Fertility during the first breeding season in forty-four sheep (n=21 Low AFC group, n=23 High AFC group) was assessed by the number of animals that remained pregnant. 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 later, pregnancy diagnosis was performed using transrectal ultrasonography (Aloka SSD 500, fitted to 82mm prostate transducer UST-660-7.5, Aloka Co., Tokyo, Japan). Pregnant sheep displayed enlargement of the uterine horns, embryo heartbeat was evidenced and in more advanced stages of pregnancy placentomes were seen.

4.4.3.3. Monitoring of the oestrous cycle

The ewes, (n=14 for Low AFC group, n=9 for High AFC group) were synchronized to follow the pursuant oestrous cycle. In order to monitor growth dynamics of the follicles and corpora lutea, ovulation was synchronized with the insertion of one intravaginal progestagen-impregnated sponge (20 mg cronolone, Crono-gest sponge®, Intervet, Holland) for 7 days followed by one IM injection of 500 µg cloprostenol (Estrotek Iniet, ATI srl, Italy) at sponge withdrawal. Then, ovaries were daily scanned, as previously described, and draw in a map in order to record number, size and position of the follicles until oestrus detection. Oestrus detection was performed by introducing vasectomised rams with a colour marker in the womb. Data were normalized according to the day of heat detection. Growth dynamic of ovulatory follicle and number of double ovulations were analyzed; also number of follicles by size, number of new follicles and follicles undergoing atresia for each day were studied. A jugular blood sample with vacuum blood evacuation tubes containing lithium heparin (Vacutainer® Systems Europe, Becton Dickinson, Meylan Cedex, France) was taken from each sheep the day of heat detection. After recovery, blood samples were centrifuged at 1500 x g for 15 min at 4ºC and plasma was removed and stored at -20 °C until assayed for oestradiol.

The growth dynamic of corpora lutea was followed every other day and maximum diameter area was drawn in a map. When corpus luteum presented a cavity, the area from the cavity was subtracted from corpus luteum area. Simultaneously with ultrasounds, jugular blood samples were drawn 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 x g for 15 min at 4ºC and plasma was removed and stored at -20 °C until assayed for progesterone during luteal phase.

4.4.3.4. Hormone assay in plasma samples

Oestradiol was measured using the Spectria radioimmunoassay kit (Orion Diagnostic Corp, Espoo, Finland), as described by Romeu *et al.* (1995) and adapted for use in ovine plasma (Gonzalez-Bulnes *et al.* 2003). Sensitivity of the assay was 0.5 pg/ml, whilst the inter- and intra-assay variation coefficients were 6.1% and 3.5%, respectively.

Plasma progesterone concentration was measured using enzyme-linked immunosorbent assay kits (Progesterone ELISA; Demeditec Diagnostics GmbH, Kiel-Wellsee, Germany) based on the principle of competitive binding. Briefly, samples, calibrators and controls are added to the pre-coated with anti-progesterone antibody wells. Progesterone from the sample is co-incubated with a progesterone-horseradish peroxidase conjugate competing for binding to the anti-progesterone antibodies. After washing free progesterone is eliminated. Then substrate TMB is added to the wells and the reaction is stopped with an acidic stopping solution. Colour intensity developed is inversely proportional to the concentration of progesterone in the sample. Finally, absorbance is determined at 450 nm and each sample concentration is calculated from a standard curve. The assay sensitivity was 0.04 ng/ml, whilst the inter- and intra-assay variation coefficients were 9.9% and 5.4%, respectively.

4.4.3.5. Multiple ovulation and embryo transfer (MOET) treatment

4.4.3.5.1. Multiple ovulation treatment

Eighteen Sarda sheep classified according to their AFC at an early prepubertal age in previous experiments (n= 10 for the Low AFC group, n=8 for the High AFC group) were synchronized during non-breeding season with the insertion of one intravaginal progestagen-impregnated sponge (20 mg cronolone, Crono-gest sponge®, Intervet, Holland) for 14 days. Twelve days after sponge insertion animals started a treatment with

porcine FSH (Folltropin, Bioniche Animal Health, Minitub Ibérica, S.L., Reus, Spain). Each sheep received a total dose of 350 IU of FSH in 8 decreasing doses administered every 12 hours for a total of 4 days (140 IU the first day, 105 IU the second day, 70 IU the third day and 35 IU the last day, divided in two equal doses per day). Selected Sarda adult rams with proven fertility were introduced from 24 hours after sponge withdrawal. Eight days after sponge withdrawal sheep underwent an intervention to recover the embryos.

4.4.3.5.2. Recovery of embryos

Sheep were fasted for 24 hours before the operation and pre-anesthetized with 3 mg of acepromazine IV (Prequillan, FATRO-AI, Ozzano, Italy). Anaesthesia was induced with a dose of 10 mg/kg body weight and maintained with IV bolus of 25 mg/ml thiopental sodium (Pentothal Sodium®, Intervet Italia S.r.l., Milan, Italy). Through a laparoscopic incision uterus and ovaries were located. Then, the number of corpora lutea developed in response to multiple ovulation treatment was recorded (Figure 18, panel a). Each uterine horn was separately flushed. Briefly, a Foley catheter was inserted near the uterine bifurcation, while near the utero-tubal junction at the tip of the uterine horn a blunt needle was introduced into the uterine lumen (Figure 18, panel b). Each horn was flushed with 20 ml of medium TCM199 (with Earle's salts and bicarbonate) supplemented with 25 mmol HEPES, 0.1 g/l penicillin, 0.1 g/l streptomycin and 20% (v/v) FCS (Figure 18, panel c). Embryos were sought on the collected content. After surgery a prophylactic antibiotherapy of procaine penicillin and dihydrostreptomycin was administered to all sheep.

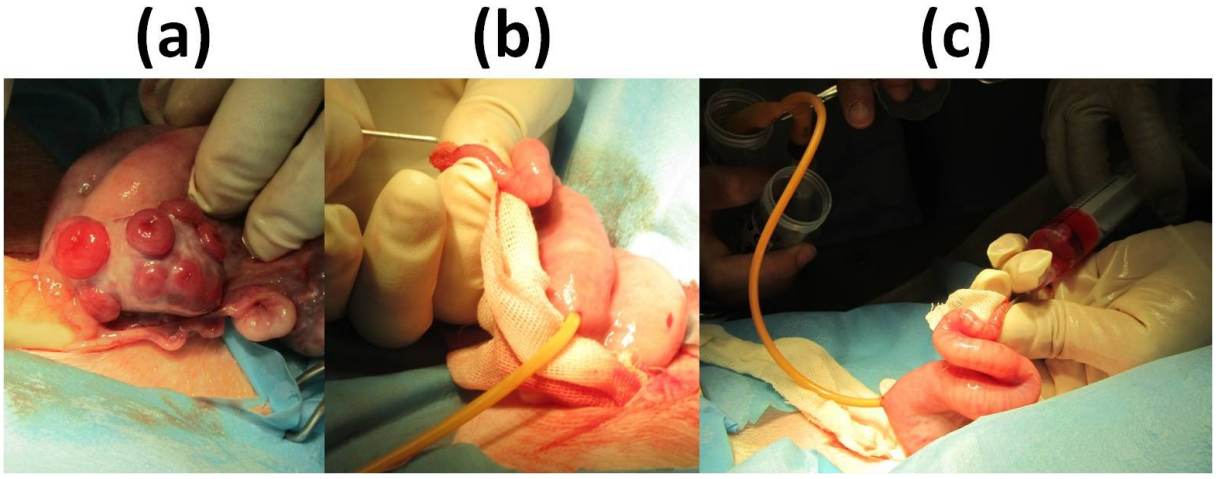

Figure 18: Example of a sheep ovary with numerous corpora lutea after a multiple ovulation treatment (a). Detail of Foley catheter insertion and blunt needle insertion points in a uterine flushing (b). Top view of the flushing circuit: the medium is introduced from the tip of the uterine horn dragging the embryos outside the uterus through the Foley catheter inserted at the level of the uterine bifurcation (c).

4.4.3.5.3. Vitrification of embryos

All vitrified embryos displayed an appropriate developmental stage at flushing and met the classification criteria of the International Embryo Transfer Society for quality 1 and 2 embryos:

-Quality 1 (excellent or good): symmetrical and spherical embryo mass with individual blastomeres that were uniform in size, colour, and density with at least 85% of the cellular material intact.

-Quality 2 (fair): moderate irregularities in overall shape of embryonic mass or in size, colour and density of individual cells with at least 50% of the cellular material intact.

Embryos were washed in new fresh medium. Couples of blastocyst, unless odd total number, were then transferred and maintained for 3 min to 150 µl drops containing the first

vitrification solution composed of PBS with 20% (v/v) FCS to which 10% (v/v) ethylene glycol and 10% (v/v) DMSO was added. Afterward, blastocyst were changed into 150 µl drops containing the second vitrification solution composed of PBS with 20% (v/v) FCS plus 0.5 M sucrose, 20% (v/v) ethylene glycol and 20% (v/v) DMSO. After 30 seconds in the second vitrification solution embryos were collected with an OPS and directly plunged into liquid nitrogen.

4.4.3.5.4. Embryo transfer

Recipient ewes with similar age and lambing experience were synchronized during breeding season with the insertion of one intravaginal progestagen-impregnated sponge (20 mg cronolone, Crono-gest sponge®, Intervet, Holland) for 14 days. On the day of sponge withdrawal animals received a 300 IU IM injection of PMSG (Folligon®, Intervet, Holland). Eight days after sponge withdrawal sheep underwent the embryo transfer operation after a 24-hour fast. Ewes were pre-anesthetized with 3 mg of acepromazine IV (Prequillan, FATRO-AI, Ozzano, Italy). Then, anaesthesia was induced with a dose of 10 mg/kg body weight (Pentothal Sodium®, Intervet Italia S.r.l., Milan, Italy). Through a laparoscopic incision uterus and ovaries were located. Once the existence of at least one corpus luteum was confirmed, pairs of embryos from the same group (donor ewes from High or Low AFC group) were transferred into the ipsilateral tip of the horn on which ovulation occurred in the recipient female (Figure 19).

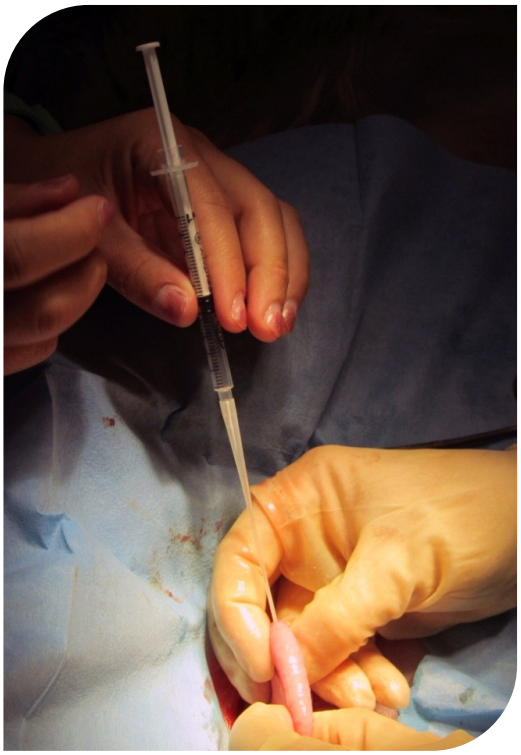

Figure 19: Inoculation of embryos into the uterine horn by a Tom Cat catheter connected to a 1 ml syringe.

For warming to a biological temperature, OPS were removed from LN_2 and holded for 6 s in the air. Then the tip of the straw was immersed in a 100 µl drop of sucrose 0.5 M solution (TCM199 with Earle's salts and bicarbonate supplemented with 25 mmol HEPES, 0.1 g/l penicillin, 0.1 g/l streptomycin, 20% (v/v) FCS and 0.5 mol/l sucrose) and content was released into the medium, gently mixed and maintained for 3 min to facilitate the exit of cryoprotectant from inside the embryos. Later, embryos were transferred into a 250 µl drop of medium without sucrose (TCM199 with Earle's salts and bicarbonate supplemented with 25 mmol HEPES, 0.1 g/l penicillin, 0.1 g/l streptomycin and 20% (v/v) FCS), stirred gently and maintained for at least 3 min. Then embryos were collected with a Tom Cat catheter and directly transferred into the recipient ewe (Figure 19). At day 45 of gestation the number of viable foetuses were determined by transabdoninal ultrasound scanning (Aloka SSD 500, fitted to linear reproductive transducer UST-588U-5, Aloka Co., Tokyo, Japan).

4.4.3.6. Statistical analysis

The Chi-square test was used to determine differences in pregnancy rates at the first breeding season between High and Low AFC groups, the number of vitrificable embryos from the total recovered from each group, the number of quality 1 and quality 2 embryos and the number of viable foetuses at 45 days in recipient ewes transferred with vitrified embryos from High and Low AFC donors. To analyze differences in ovulatory follicular growth, follicle number, new and atretic follicles, and CL functionality data were normalized with respect to the day in which heat was detected. First, ultrasonographic data during the induced oestrus cycle were summarized to characterize patterns of ovarian follicular growth and atresia and identify ovulatory follicles. All follicles detected by ultrasonography were classified by their largest diameter and, thereafter, were grouped as total ($\geq$2 mm), 2$\geq$x$<$3 (small), 3$\geq$x$<$5 (medium) or $\geq$5 mm (large). Second, ultrasonographic and plasma hormonal data during luteal phase were summarized to characterize patterns of growth dynamics and functionality of CL. Data of ovulatory follicles were used to characterize follicular growth of ovulatory follicles on the basis of (a) initial diameter; (b) maximum diameter reached before ovulation, (c) growth phase length: defined as the time taken by each ovulatory follicle to grow from their initial diameter to its maximum diameter; (c) growth rate: mm that the ovulatory follicle grew per day, it is obtained by dividing the difference between the maximum diameter and the initial diameter between the growth phase length. Ovulatory follicular growth dynamics, follicle number and size, new and atretic follicles, size of CL and hormone concentrations were analyzed by ANOVA, followed by a Kruskal–Wallis test, when Levene's test showed non-homogeneous variables. Ovulation rate, determined by the number of CL in the ovaries, and number of embryos recovered by ewe were analyzed by ANOVA. Statistical analysis was performed using the statistical software program Statgraphic Centurion XV (version15.2.06 for Windows; StatPoint, Inc., Herndon, VA, USA) and a probability of p$\leq$0.05 was considered to be the minimum level of significance. All results are expressed as mean $\pm$ S.E.M.

4.4.4. Results

4.4.4.1. Fertility in the first breeding season

Fifty per cent of the animals remained pregnant at first breading season (22/44); 68.18 % of pregnant ewes belonged to High AFC group (15/22) while the remaining 31.82 % belonged to Low AFC group (7/22). Absolute number of pregnant and non-pregnant ewes inside each group and percentage from the total of each group are summarized in Table 5.

Table 5: Fertility at first breeding season in ewes classified by their early ovarian phenotype. Comparison of absolute numbers of animals and proportion of ewes that remained pregnant or non-pregnant at first breading season from two Sarda ewe groups classified according to the number of follicles ≥2 mm in the ovaries at an early prepubertal age as High AFC group (≥30 follicles) and Low AFC group (≤15 follicles). Chi square test $p<0.05$.

	High AFC	Low AFC	Total
Pregnant/total group	15/23[a]	7/21[b]	21
(%/total group)	(65.22)	(33.33)	(47.73)

4.4.4.2. Monitoring of the oestrous cycle

Mean cycle length did not differ between groups (16.14 ± 0.25 days in the High AFC group and 16.14 ± 0.18 days in the Low AFC group). For the study of the follicular phase of each group, growth dynamic of the ovulatory follicle is resumed in Table 6, not presenting significant differences in any of the parameters outlined. Neither the percentage of animals with double ovulation within each group differed between groups: 22.22% (2/9) for the High AFC group and 21.43% (3/14) for the Low AFC group.

Table 6: Mean ovulatory follicle growth dynamics after synchronization of the oestrus cycle from two Sarda ewe groups classified according to the number of follicles ≥2 mm in the ovaries at an early prepubertal age as High AFC group (≥30 follicles) and Low AFC group (≤15 follicles).

	High AFC (n=11)	Low AFC (n=17)
Initial size (mm)	3.82 ± 0.53	4.35 ± 0.43
Maximum diameter (mm)	6.32 ± 0.27	6.03 ± 0.22
Growth phase length (days)	3.36 ± 0.25	3.35 ± 0.20
Growth rate (mm/day)	0.75 ± 0.14	0.50 ± 0.10

In the same way, there were no differences neither in the number of new follicles or follicles becoming atretic each day between both groups as displayed in Figure 20, nor in the total number of new and atretic follicles throughout the follicular phase (7.38 ± 1.08 vs. 6 ± 0.85 new follicles for High AFC group vs. Low AFC group, p=0.33; 7.5 ± 1.37 vs. 5.38 ± 1.08 atretic follicles for High AFC group vs. Low AFC group, p=0.09). Similarly, plasma oestradiol levels at heat were equal in both groups (4.05 ± 0.95 pg/ml and 4.15 ± 0.82 pg/ml for High and Low AFC groups respectively).

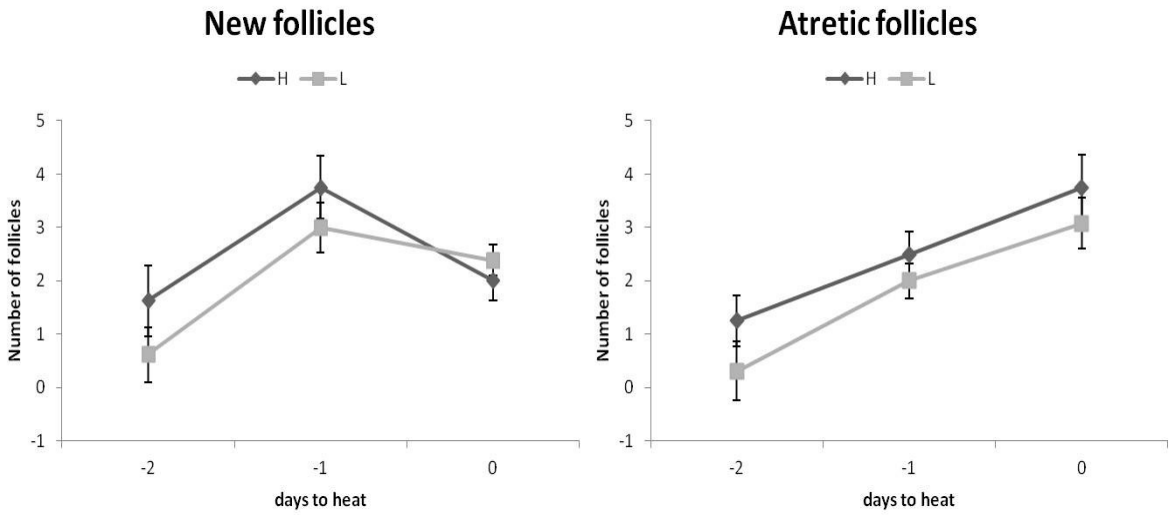

Figure 20: Mean number of new follicles (left hand) and follicles undergoing atresia (right hand) during the subsequent follicular phase after synchronization of the oestrus cycle in ewes classified according to the number of follicles ≥2 mm in the ovaries at an early prepubertal age as (H) High AFC group (≥30 follicles; n=9) and (L) Low AFC group (≤15 follicles; n=14). Data were normalized with respect to the day in which heat was detected.

Mean number of follicles per category size are displayed in Figure 21. There were no differences between groups in any of the follicular categories. Follicles remained constant for all follicular categories in the Low AFC group while follicles ≥5 mm increased significantly when approaching the onset of heat, simultaneously there is a decreasing tendency ($p<0.07$) in the $3 \geq x < 5$ mm group which probably grow to reach ≥5 mm.

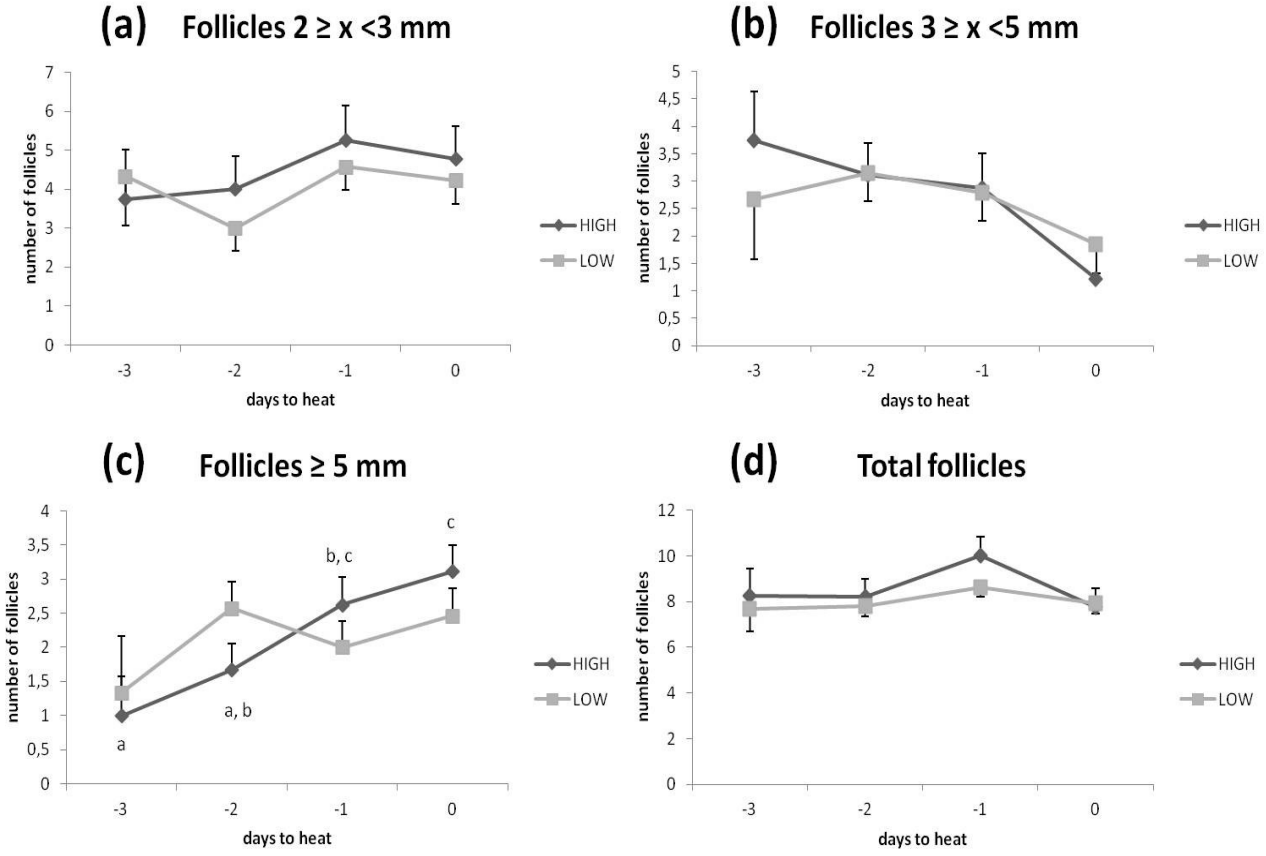

Figure 21: Number of follicles from 2 to less than 3 mm (a), from 3 to less than 5 mm (b), equal or greater than 5 mm (c) and total follicles ≥2 mm (d) determined by transrectal ultrasonography during the subsequent follicular phase after synchronization of the oestrus cycle in ewes classified according to the number of follicles ≥2 mm in the ovaries at an early prepubertal age as High AFC group (≥30 follicles) and Low AFC group (≤15 follicles). Data were normalized with respect to the day in which heat was detected.

a, b, c Indicate a statistical difference in values from the High AFC group: a ≠ b ≠ c $p<0.02$.

Results obtained from the study of the diameter of the CL and P4 production during the luteal phase are displayed in Figure 22. No differences were observed between High and Low AFC groups in corpora lutea growth and functionality.

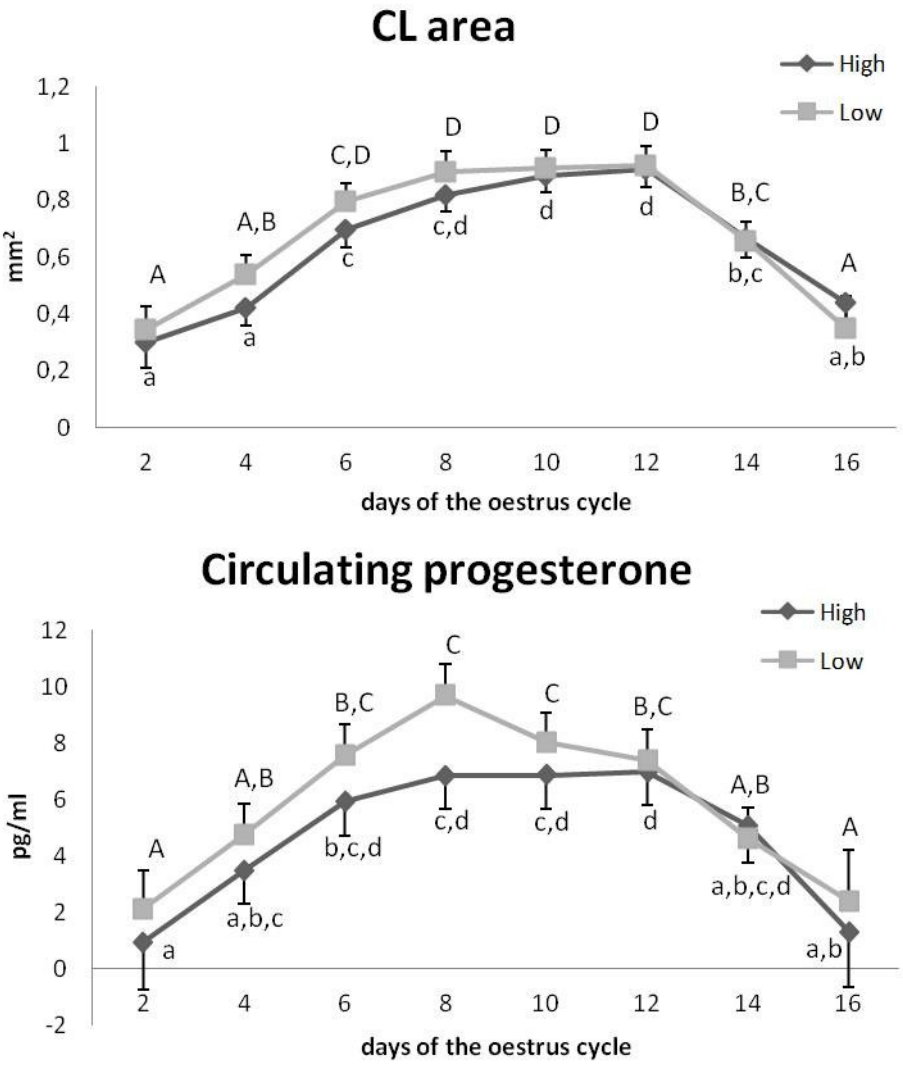

Figure 22: Evolution of the mean area of the maximum diameter in the corpus luteum (upper panel) and plasma progesterone concentrations (bottom panel) during the luteal phase of a synchronized oestrus cycle from ewes classified by the number of follicles ≥2 mm in their ovaries at an early prepubertal age into the High (≥30 follicles) or Low (≤15 follicles) AFC groups.

A, B, C, D Indicate a statistical difference in values from the Low AFC group: A ≠ B ≠ C ≠ D $p<0.0001$ for CL area; A ≠ B ≠ C $p<0.001$ for circulating progesterone.

a, b, c, d Indicate a statistical difference in values from the High AFC group: a ≠ b ≠ c ≠ d $p<0.0001$ for CL area; a ≠ b ≠ c ≠ d $p<0.03$ for circulating progesterone.

4.4.4.3. Multiple ovulation and embryo transfer (MOET) treatment

Two animals, one from each group, did not respond to the treatment and were eliminated from the experiment. No differences were established between the two groups in the ovulation rate in response to the superovulation treatment (number of corpora lutea, Figure 23), nor in the mean number of embryos recovered per each ewe (4.57 ± 1.55 vs. 4.67 ± 1.37 embryos/ewe in the High and Low AFC groups respectively, p=0.97) or in the number of embryos morphologically suitable for vitrification from the total of each group (20/32 and 31/42 for High and Low AFC group respectively, p= 0.3). There is a tendency to a higher embryo quality according to the morphological criteria values from the International Embryo Transfer Society (14/6 vs. 14/17, number of quality 1/quality 2 embryos vitrified from donor ewes from High vs. Low AFC group, p=0.08).

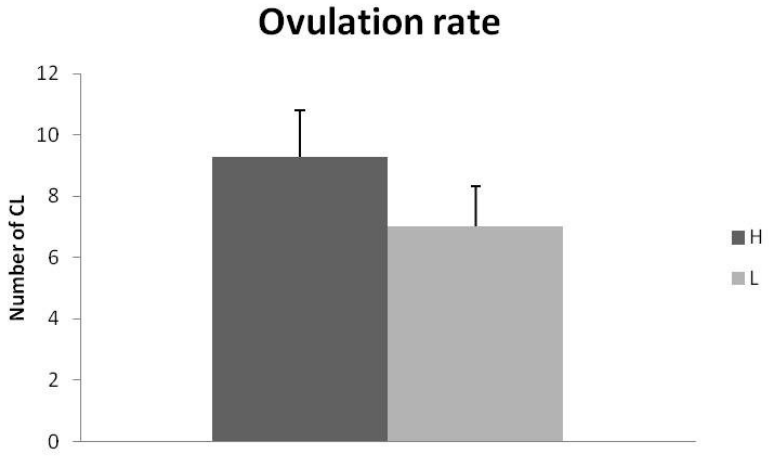

Figure 23: Ovulation rate after a multiple ovulation treatment with porcine FSH from ewes classified by the number of follicles ≥2 mm in their ovaries at an early prepubertal age into (H) High (≥30 follicles) or (L) Low (≤15 follicles) AFC groups.

At day 45 of gestational age there were not differences in the mean number of viable foetuses per recipient ewe between both groups (0.9 ± 0.25 vs. 0.89 ± 0.19 viable foetuses

per recipient ewe in ewe transferred with embryos obtained from donor of the High AFC group and Low AFC group respectively, p=0.98), neither in the absolute number of viable foetuses from the total number of transferred embryos from each group, as shown in Table 7 (p=0.65)

Table 7: Viable foetuses at day 45 of gestation from donor sheep of the High and Low AFC groups that underwent a MOET treatment

	High AFC donor	Low AFC donor
Viable foetuses/transferred embryos	9/20	16/31
(% Viable foetuses/transferred embryos per group)	(45.00)	(51.61)

4.4.5. Discussion

This study demonstrated that differences in follicular recruitment and growth during the early prepubertal period are accompanied by differences in the fertility rates in the first reproductive season. Ewe lambs belonging to the High AFC group on the basis of the number of antral follicles counted on their ovarian surface at 7 weeks of age showed higher pregnancy rates compared to the Low AFC group. This precocity was not, on the other hand, associated to differences in other parameters related to reproductive functionality in the adulthood, such as follicular and corpora lutea dynamics during the oestrus cycle and the response to a standard MOET protocol.

Several advantages have been found for ewe lambs successfully mating within their first year of age. The lifetime performance and productivity increase in these animals having a higher cumulative production of lambs (Hulet et al., 1969). In fact, a recent study describes

a lower culling risk in sheep younger than 395 day-old at first delivering which implies successful matting within the first 9 months of age (Kern et al., 2010). At the same time, in self-replacing flocks the cost of ewe replacement is reduced because the period during which the lambs are being fed without being productive is shorter (Marchant, 2004; Fogarty et al., 2007). A briefer generation interval derived from ewes lambing at a younger age allows also increasing the rate of genetic improvement (Marchant, 2004; Fogarty et al., 2007). Thus, the greater percentage of pregnant ewes in the High AFC group could be highly beneficial, so that choosing as ewe lambs replacement those with higher AFC and/or AMH plasma levels at an early prepubertal age might represent a useful tool in the selection programmes especially in self-replacing flocks.

Multiple factors could be affecting the onset of puberty and so successful matting at first breeding season. Age, body weight, sheep breed, nutrition and nutritional status, occurrence of a disease, season of the year and proximity of a male act on the onset of puberty (Vejlsted, 2010). However, our sheep were all pure Sarda breed and were kept under the same conditions, housed together and receiving the same diet after weaning, so sheep breed, nutrition and nutritional status, season of the year and proximity of a male should not be producing a higher precocity in ewe lambs from the High AFC group. Furthermore, animals used did not present any signs of disease. Weight at mating (Kenyon et al., 2004) an growth potential (Rosales Nieto et al., 2013) has also been positively related to higher reproductive performance in sheep. In the present study, however, no differences in peripubertal weight (the nearest weight to mating period) nor in weights at birth and weaning that could suggest an enhanced or retarded growth, could be found between animals from the High and Low AFC groups.

A higher AFC has been related to a higher oocyte (Mossa et al., 2008; Ireland et al., 2009) and embryo quality (Ireland et al., 2007) and higher pregnancy and live-birth rates (Chang et al., 1998; Maseelall et al., 2009; Holte et al., 2011). This could be related to the enhanced fertility at first breeding season in animals with a high AFC. However, total follicle population at a peripubertal age showed no difference between both groups in experiment

2. Therefore we can likely suppose that at the time of mating there were no differences in the AFC between the two groups. The different fertility rates between our groups may also be related to a greater quality of these or/and a higher precocity. In experiment 2 we evidenced that, despite having similar AFC at a peripubertal age, the number of follicles ≥4 mm was higher in the High AFC group compared to the Low AFC one. This follicular category has been proved to be the most competent for embryo production in sheep (Veiga-Lopez et al., 2008). Perhaps the higher oocyte developmental competence showed by the High AFC group in the in vitro culture experiment (experiment 1) is maintained until the peripubertal age. Differences established during the pre-and postnatal life in the maturation of the hypothalamic-pituitary-ovarian axis may account for the different oocyte and follicular competence observed in the two experimental groups. Further studies will be needed to elucidate this.

Not only AFC but also AMH levels seem to be strongly associated with higher pregnancy rates (Hazout et al., 2004), enhanced oocyte quality (Ebner et al., 2006; Silberstein et al., 2006; Brodin et al., 2013) and a greater number of high-quality embryos obtained after ovarian stimulation (Monniaux et al., 2011). Therefore AMH levels could be used as markers to identify animals that respond poorly to MOET or OPU protocols and discard them as donor females (Rico et al., 2012; Monniaux et al., 2013). In our study, pregnancy rate at first breeding season was superior in animals with higher AMH levels at 50 days of age. This is in agreement with a recent study made on Rasa Aragonesa ewe lambs that positively relates fertility at first mating with higher plasma AMH concentrations at 3.6 months of age (Lahoz et al., 2012), in which sheep becoming pregnant at first mating had greater levels of AMH than those becoming pregnant at second mating or not becoming pregnant.

The study of follicular waves dynamics and corpora lutea development during the oestrus cycle is a widespread tool to identify eventual alterations in ovarian function (Adams et al., 2012). Oestradiol is a good marker of follicular function in cattle (Campbell et al., 1995). Prolific sheep tend to have a greater number of smaller CL, coming from ovulatory

follicles with smaller diameters (Monget et al., 2002), with a lower total level of progesterone (Bartlewski et al., 2011) and in monovular ewes luteal tissue size correlates with concentrations in plasma progesterone (Gonzalez de Bulnes et al., 2000). In our study animals from both groups had similar mean CL areas that corresponded with similar progesterone concentrations; also, no difference on double ovulation rate that might indicate a higher prolificacy in any of the groups was detected. In cattle with low AFC despite having a higher LH secretion, lower circulating progesterone concentrations during luteal phase, due to a decreased capacity of progesterone production in luteal and granulosa cells, were detected (Jimenez-Krassel et al., 2009). In our study, no differences were observed in ovulatory follicle growth dynamics, number of new and atretic follicles each day, mean number of follicles per category size, oestradiol levels on heat day, diameter of the CL and P4 production during the luteal phase between High and Low AFC groups. The only difference found was a more linear increase in the number of ovulatory-sized follicles as days approach to heat day which, however, had no noticeable effect on the parameters examined in this study for ovulation rate or the quality of the subsequently formed corpora lutea. In addition, no differences between groups in follicle population from the sponge withdrawal till heat detection were found in the current experiment.

AMH shows a negative effect on oestradiol (Fanchin et al., 2007), inasmuch as it reduces the expression of the aromatase enzyme involved in converting androgens into oestrogens (Vigier et al., 1989; Eilso Nielsen et al., 2010; Monniaux et al., 2011; Chang et al., 2013). Thus, the expression of AMH is markedly reduced with increasing follicle size while expression of aromatase is enhanced (Campbell et al., 2012). In our experiment no differences were seen in oestradiol levels of ewes from the High or Low AFC group, but we were not able to determine AMH levels in adults. Probably, if we take into consideration the high correlation between AFC and AMH levels (de Vet et al., 2002; van Rooij et al., 2002; Gruijters et al., 2003; Kevenaar et al., 2006; Rico et al., 2009), no difference would be expected in AMH levels since there were no differences in the number of total and small follicles.

As happened in the oestrous cycle, we could not establish differences in the response to a multiple ovulation protocol. The success of superovulation treatments with gonadotrophins and the ovulatory response in sheep seem to be related to the number of small follicles present in the ovaries when the treatment is started (Gonzalez-Bulnes et al., 2000; Veiga-Lopez et al., 2005). Embryo outcome seems to be more related to medium follicles in sheep and goat (Gonzalez-Bulnes et al., 2004a; Veiga-Lopez et al., 2005). Since the number of small and medium follicles did not differ between animals from the High and Low AFC groups in the oestrus cycle studied nor in the follicular population prior to EFORT at 496 days of age (experiment 2), it might be assumed that again differences would not be present when the multiple ovulation treatment was initiated, leading to a lack of differences in the response between both groups. Thus, neither the AFC nor plasma AMH levels at an early prepubertal age could predict differences in the response to a multiple ovulation treatment with FSH as part of a standard MOET protocol in adulthood. Similarly, in a recent study (Lahoz et al., 2013), it was evidenced that prepubertal AMH were not related to the ulterior number of follicles that could be punctured in LOPU sessions after FSH treatment at an adult age.

Ovulation rate in our sheep, especially from the Low AFC group members (7 ± 1.33 CL and 9.29 ± 1.51 CL for the Low and High AFC groups, respectively), was similar to the ovulation rate described in Sarda ewes subjected to the same protocol (7 ± 3.2 CL: Mayorga et al., 2011). Contrary, our embryo recovery rate was lower than in the experiment of Mayorga et al. (2010), but recovery rates are related to operator expertise.

Vitrified ovine embryos need 9-12 h to resume DNA synthesis and 29-35 h to restore their full protein secretion capacity after thawing, to complete the resumption of their metabolic activity for further development (Leoni et al., 2003). However, embryos from both groups are not supposed to differ in these parameters since there was no difference in the number of viable foetuses at 45 days of gestation. The embryo transfer in our experiment again show similar results in both groups, which initially diminishes the possibility of finding differences in oocyte quality between groups, contrary to what observed in the early

prepubertal period. A tendency was found for the High AFC donors to have a higher number of quality 1 embryos, but this tendency was not accompanied by a higher rate of viable foetuses at day 45 of gestation. In fact the classification of embryos based on morphological criteria is subjective. Donor's response to MOET treatment in terms of ovulation rate and embryo recovery is repeatable within the same animals in ewes (Bari et al., 2001). Thus, nothing leads us to think that later in life differences between both groups will come up.

We were not able to observe differences in the parameters studied related to the reproductive functionality in the adulthood, such as follicular and corpora lutea dynamics during the oestrus cycle and the response to a standard MOET protocol. The most suitable explanation is that differences in AFC and AMH at an early prepubertal period, when our groups were established, disappear with age as AFC and AMH fluctuate throughout life (Kelsey et al., 2011; Fleming et al., 2012) being specially different in the prepubertal and peripubertal period compared to adulthood. Nonetheless, higher pregnancy rate was still sawn at first breeding season, but its physiological meaning and its impact on subsequent reproductive life-span still has to be determined.

5. GENERAL DISCUSSION

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The results of the experiments that compose this dissertation show a clear difference in follicular recruitment and growth between the prepubertal and postpubertal period. Both differences in the AFC and in the response to ovarian stimulation protocols changed with age. Differences in ovarian phenotype before treatment tend to disappear with age, to vanish altogether when we studied the oestrous cycle. This leads to the gradual weakening in the differences in the response to FSH, which were no longer visible in the EFORT at 496 days of age and the results from the MOET protocol. Probably due to the disappearance of differences in the number of small follicles which are the main target category in stimulation treatments with gonadotrophins (Gonzalez-Bulnes et al., 2000; Veiga-Lopez et al., 2005). Neither difference in the ovarian dynamics during the oestrus cycle was found that may justify differences in the reproductive outcome. However, ewe lambs with higher AFC and AMH in the early prepubertal age still had higher pregnancy rates at first breeding season. Therefore, the AFC and AMH levels may be useful tools in the precocious selection of ewes. AMH levels do not seem to be related to milk production in cattle (Rico et al., 2009), so this selection apparently will not affect milk production. Further research is needed to study possible changes that could affect other reproductive and productive aspects and to try to achieve reference values for the standardization of this selection criteria. Further studies are also needed to elucidate why and how differences observed at an early prepubertal age are produced, why disappear later in life and the reasons why and mechanisms by which an impact in the first breeding season fertility rate is still produced.

Throughout the dissertation we have speculate on several possible explanations. We believe that the most plausible explanation for our findings corresponds to an earlier and greater follicular activity (recruitment and development) in animals with high AFC. A follicular activity even exacerbated when compared to the number of follicles (around 8 follicles $\geq$2 mm) seen in adults of this breed (Mossa et al., 2008; Spezzigu, 2010; Berlinguer

et al., 2012). This is in agreement with the differences in AMH levels that were seen in the first 9 weeks of age which increased rapidly reaching higher values in ewe lambs from the High AFC group.

Although the number of antral follicles has been linked to the stock of primordial follicles from which they come from (Gougeon, 1996; Kevenaar et al., 2006), our research could not establish a larger population of primordial follicles in animals with high AFC. Therefore the development of a larger number of follicles is not justified by a larger population of primordial follicles, but might be related to the occurrence of a "minipuberty" whit changes in the hormonal milieu (Lee, 2003). Circulating FSH concentration transiently increases during postnatal pituitary activation in children (Beck-Peccoz et al., 1991; Kuiri-Hanninen et al., 2011a). A similar situation has been seen in cattle with a heightening in the follicular recruitment produced by a remarkable increase in FSH concentration that lately falls due to an increase in the secretion of steroid and inhibin from the numerous antral follicles that have grown during the previous weeks (reviewed by Hernandez-Medrano et al., 2012). FSH exhibits large variations between diverse individuals during the postnatal period (Chellakooty et al., 2003) perhaps differences between ewe lambs from our study are due to these variations. Thereby, 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.

Follicular development probably starts in late gestation in sheep since first primordial follicles are seen at day 75 of gestation (McNatty et al., 1995; Sawyer et al., 2002), primary follicles at day 100 of gestation (McNatty et al., 1995), while growing secondary follicles beginning to produce AMH are found at 120 days apparently triggered by a rise in FSH serum levels (Bezard et al., 1987). Similarly, the appearance of secondary follicles at day 120 is associated with an increase in serum FSH in bovine female foetuses (Tanaka et al., 2001). Maybe the maternal or the foetal hormonal milieu accelerated the recruitment and development of follicles and made it more mature and capable for development producing the enhanced developmental competence seen in oocytes from ovaries with high AFC in our

study. The number of growing follicles is determined by factors such as breed, season and nutrition (Armstrong and Webb, 1997). In our experiment the influence of these factors should be reduced because we used animals from a same breed, born in the same period (December-January), whose mothers received the same diet during both gestation and lactation. However, we cannot rule out differences in milk composition that could have promoted follicular development in the offspring since nutrition clearly influences folliculogenesis in sheep (Scaramuzzi et al., 2006). Other factors can affect follicle recruitment and development, such as the IGF and BMP systems (Monget and Monniaux, 1995; Monget et al., 2002). Even, some fecundity genes seem to affect organ differentiation or germ cell maturation development during foetal life without an evident occurrence of changes in gonadotrophin concentrations (McNatty et al., 1995). Thus, further studies are needed to elucidate the real causes of this presumed enhanced follicular development.

Differences in AFC at an early prepubertal period tend to disappear with age, and so does the responsiveness to ovarian stimulation protocols, because AFC fluctuate throughout life (Fleming et al., 2012) being specially different in the prepubertal and peripubertal period compared to adulthood. As some authors have suggested to explain the variances in follicular recruitment in the prepubertal period in sheep, changes in follicular population between the prepubertal and the peripubertal period could be plausibly due to changes in FSH release and clearance rates, and in biological FSH activity associated with changes in the distribution pattern of FSH isoforms as puberty approaches (see Padmanabhan et al., 1992; Rawlings et al., 2003).

Kisspeptin may also be involved in the results derived from the current dissertation. As in other species (reviewed by Smith, 2012), kisspeptin stimulate the secretory activity of GnRH neurons boosting the release of GnRH via GPR54 in the ewe (Messager et al., 2005; Okamura et al., 2013), resulting in an increase in FSH and LH secretion. Plasma kisspeptin concentrations are superior in children than in adults (Jayasena et al., 2013) and expression levels of KiSS-1 mRNA, which encodes kisspeptin, in female rat hypothalamus are high in the early prepubertal period, drop at the peripubertal period and peak coinciding with puberty

onset (Navarro et al., 2007), which might somehow impact the follicular development at an early age when compared with follicular development at and after puberty. Kisspeptin plays a key role in the pubertal maturation of the gonadotropic axis, being an essential gatekeeper in the control of puberty onset (Tena-Sempere, 2006; Sanchez-Garrido and Tena-Sempere, 2013). In fact, kisspeptin administration can activate the hypothalamic-pituitary-ovarian axis (Redmond et al., 2011) and trigger the neuroendocrine events that induce the onset of puberty (reviewed by Navarro et al., 2007). Also higher levels of kisspeptin have been observed in girls with central precocious puberty (de Vries et al., 2009). Therefore, higher levels of kisspeptin could have caused an advance in puberty onset in the High AFC group that could have increased the chances of getting pregnant at first breeding season during the time when ewes were in contact with rams.

Although not significantly, the response to ovarian stimulation treatments either composed of a single dose of FSH (EFORT) or multiple decreasing doses (multiple ovulation treatment) was higher in both cases in the animals with a high AFC at 50 days of age. Even, although not coincident with what seen in the monitoring of the oestrus cycle, at 496 days-old differences between groups in the total number of follicles prior to FSH treatment, corresponding to a cumulative effect of a greater number of follicles in each follicular categories but not significant within each follicular category, still exist. We suggest that differences in adulthood between animals with high and low AFC at an early prepubertal age might be revealed in certain situations, so we do not claim a complete disappearance of the differences in adulthood. All this, added to the fact that the animals present diverse fertility rates in their first breeding season, leads us to reaffirm that further studies are needed on the causes and implications in adult life of the differences seen in the prepubertal period, particularly targeting into hormonal profiles.

6. CONCLUSIONS

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1) The antral follicle count is highly variable between different ewe lambs in the early prepubertal period, being a higher antral follicle count and plasma anti-Müllerian hormone concentration predictive of an enhanced response to exogenous ovarian stimulation treatment.

2) A lower antral follicular count at an early prepubertal age is predictive of a poor oocyte in vitro developmental competence.

3) Differences in the antral follicle count in ewe lambs in the early prepubertal period are only indicative of differences in the number of antral follicles but not in the ovarian reserve.

4) The differences in the antral follicle count at an early prepubertal age in Sarda ewe lambs are accompanied by a different evolution of circulating anti-Müllerian hormone levels in the first weeks after birth suggesting a delayed follicle development in animals with low antral follicle count.

5) Differences in antral follicle count and ovarian response to exogenous stimulation in ewe lambs with diverse antral follicle count at an early prepubertal age tend to disappear with age.

6) Selecting ewe lambs with a high antral follicle count by a simple ultrasound at an early prepubertal age improves fertility rate at first breeding season.

7) No significant difference in the oestrus cycle of animals with diverse antral follicle count at an early prepubertal period can be established that might justify a different reproductive outcome.

8) Selection of ewes by their antral follicle count at an early prepubertal period does not change the results of multiple ovulation and embryo transfer treatment in adulthood.

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APPENDICES

Abbreviations

A

AFC	Antral follicle count
ALK	Activin receptor-like kinase
AMH	Anti-Müllerian hormone
AMHKO	AMH knockout
AMHRII	Anti-Müllerian hormone type II receptor
ANOVA	Analysis of variance
ART / ARTs	Assisted reproductive technology / technologies

B

BMP / BMPs	Bone morphogenetic protein / proteins
BSA	Bovine serum albumin

C

CL	Corpus luteum
CO_2	Carbon dioxide
COC / COCs	Cumulus-oocyte complex / complexes
COS	Controlled ovarian stimulation

D

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

E

E_2	17β-oestradiol, oestradiol
EDTA	Ethylenediaminetetraacetic acid
e.g.	Exempli gratia
EOP	Endogenous opioid peptides

F

FAO	Food and Agriculture Organization of the United Nations
FCS	Foetal calf serum
FOR	Functional ovarian reserve
FSH	Follicle-stimulating hormone

G

g	Earth's gravitational acceleration
GABA	Gamma-aminobutyric acid
GDFs	Growth and differentiation factors
GnRH	Gonadotrophin-releasing hormone

GPR54	G protein-coupled receptor 54
GSCs	Germline stem cells

H

h	Hours
H	High AFC group
H_2O	Water
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

I

IGF	Insulin-like growth factor
IM	Intramuscular
Istat	Istituto Nazionale di Statistica (Italy)
IU	International units
IV	Intravenous
IVC	In vitro culture
IVF	In vitro fertilization
IVM	In vitro maturation

K

KCl	Potassium chloride
kDa	Kilodaltons
kg	Kilograms
KH_2PO_4	Monobasic potassium phosphate

L

l	Litre / litres
L	Low AFC group
LH	Luteinizing hormone
LN_2	Liquid nitrogen
LOPU	Laparoscopic-ovum pick up
LSD	Least Significant Difference

M

M	Molar
mg	Milligrams
MIF	Müllerian inhibiting factor
MIH	Müllerian inhibiting hormone
min	Minutes
MIS	Müllerian inhibiting substance
ml	Millilitres
mM	Millimolar
mm	Millimetres
mmol	Millimoles
MOET	Multiple ovulation and embryo transfer
mOsm	Milliosmoles
mRNA	Messenger RNA

N

N_2	Nitrogen gas
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate
ng	Nanograms
NGFs	Non-growing follicles
nm	Nanometre

O

O_2	Dioxygen
OPS	Open Pulled Straw
OPU	Ovum pick-up
OR	Ovarian reserve

P

P_4	Progesterone
PBS	Phosphate buffered saline
PCOS	Polycystic ovary syndrome
pg	Picograms
PGCs	Primordial germ cells
$PGF_{2\alpha}$	Prostaglandin F2 alpha
PMDS	Persistent Müllerian duct syndrome
PMSG	Pregnant mare serum gonadotrophin
PVA	Polyvinylalcohol

R

RIA	Radioimmunoassay
RNA	Ribonucleic acid
R-Smad	Receptor-regulated Smad

S

s	Seconds
SEM	Standard error of mean
Smad	Sma- and Mad-related proteins
SOF	Synthetic oviductal fluid medium
SRY	Sex determining region of the Y chromosome

T

TCM	Tissue Culture Medium
TGF- β	Transforming growth factor β
TMB	Tetramethylbenzidine

V

v/v	Volume / volume ratio

W

w/v	Weight / volume ratio

Other

ºC	Degrees Celsius
µl	Microliters
µm	Micrometres, microns
%	Percentage
<	Less than
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
±	Plus-minus

Figure index

EXPERIMENT 2: evolution from prepubertal period to adulthood

EXPERIMENT 3: adulthood

Table index

Publications

Part of the results of this dissertation has already been released in various conferences and journals of national and international scientific interest, as cited below. It is currently being drafted the second manuscript related to this dissertation. Further researches related to the study of the potential reproductive differences of ewe lambs with diverse AFC at an early prepubertal age are currently taking place and will complete the here reported results in future scientific manuscripts.

Posters

ANTIMULLERIAN HORMONE (AMH) AS AN AGE-RELATED PREDICTOR OF THE ANTRAL FOLLICLE COUNT (AFC) AND THE RESPONSE TO EXOGENOUS FSH OVARIAN RESERVE TEST (EFORT) IN SHEEP. Berlinguer F, Torres-Rovira L, Spezzigu A, Leoni GG, Succu S, Gallus M, Naitana S, Gonzalez-Bulnes A. 17th International Congress on Animal Reproduction (ICAR). Vancouver, British Columbia, Canada. July 29th- August 2nd, 2012.

Oral communications

SELECTION OF YOUNG EWE LAMBS ACCORDING TO THEIR ANTRAL FOLLICULAR COUNT: RESPONSE TO EXOGENOUS HORMONAL STIMULATION AND FERTILITY AT FIRST BREEDING SEASON. Torres-Rovira L, Manca ME, Gonzalez-Bulnes A, Spezzigu A, Piu P, Gallus M, Succu S, Chelucci S, Leoni G, Berlinguer F, Naitana S. X Congresso Nazionale SO.F.I.VET. (Italian Society of Veterinary Physiology). Taormina, Italy. July 8th – 9th, 2013. Award for best scientific communication.

Scientific International Journals

PREDICTIVE VALUE OF ANTRAL FOLLICLE COUNT AND ANTI-MÜLLERIAN HORMONE FOR FOLLICLE AND OOCYTE DEVELOPMENTAL COMPETENCE DURING THE EARLY PREPUBERTAL PERIOD IN A SHEEP MODEL. Torres-Rovira L, Gonzalez-Bulnes A, Succu S, Spezzigu A, Manca ME, Leoni GG, Sanna M, Pirino S, Gallus M, Naitana S, Berlinguer F. Reprod Fertil Dev. 2013 Sep 6. doi: 10.1071/RD13190.

Publications related to this Thesis

17th International Congress on Animal Reproduction (ICAR). Vancouver, British Columbia, Canada. July 29th– August 2nd, 2012. Electronic poster.

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Anti-Mullerian hormone as an age-related predictor of the antral follicle count and the response to exogenous FSH ovarian reserve test in sheep

F Berlinguer[1], L Torres-Rovira[1], A Spezzigu[1], G Leoni[1], S Succu[1], M Gallus[2], S Naitana[1], and A Gonzalez-Bulnes*[3]

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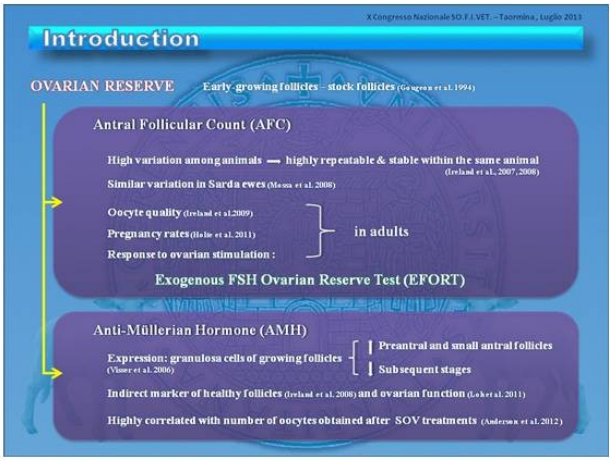
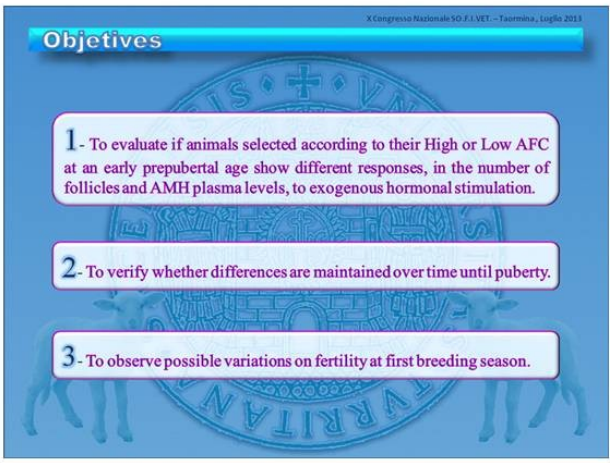
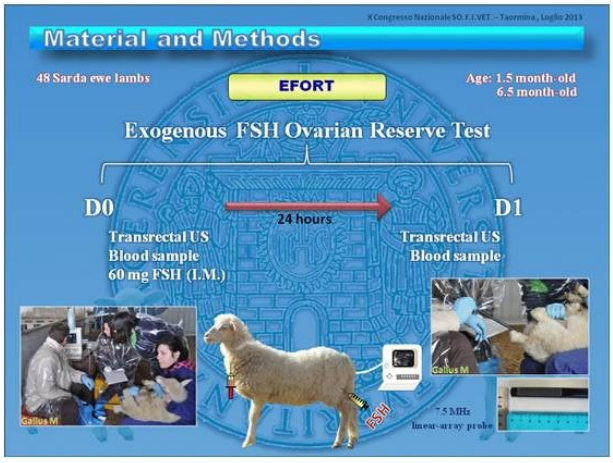
Inter-individual variability in the response to exogenous ovarian stimulation remains as the main limiting factor for embryo production in sheep, like in other species. Embryo outputs are related to the number of gonadotrophin-responsive follicles (i.e.: the ovarian reserve) and highly repeatable in consecutive stimulations of the same females. Thus, predictive tools for early selection of high-responder females are of critical importance in embryo programmes. Currently, anti-Mullerian hormone (AMH) is recognized, in human medicine, as a good marker of ovarian reserve status and represents a good predictor of ovarian response to ovarian hyperstimulation. The aim of this study was to assess age-related changes in AMH and its relationship with antral follicle count (AFC) as predictors of the response to an exogenous FSH ovarian reserve test (EFORT) in pre- and post-pubertal stages in sheep. Thus, plasma AMH concentrations and AFC evaluated by ovarian ultrasonography, prior and after the administration of one shot-dose of 60 mg of FSH (FolltropinTM; Bioniche Animal Health, Bio 98, Milano, Italy), were determined in a total of 15 Sarda sheep at 40, 110 and 210 days-old. The results indicate a significant decrease in AMH concentrations ($p < 0.05$) and the number of follicles with 1–2 mm in diameter with age ($p < 0.0005$), but a significant increase in the number of gonadotrophin-dependant follicles ≥4 mm ($p < 0.01$). Blood AMH levels were related to total AFC in pre-pubertal stages (40 and 110 days-old; $p < 0.01$) and, mainly, to the number of follicles with 1–2 mm in diameter ($p < 0.001$). Evaluation of AMH prior to EFORT was predictive of the growth of these follicles with 1–2 mm to larger sizes in response to the test at these pre-pubertal stages ($p < 0.005$). Thus, on the basis of the highly-repeatable response to exogenous gonadotrophin stimulation in the same individuals, the measurement of the hormone in pre-pubertal stages may be useful for selection of future donors in embryo programmes. Funded by Regione Sardegna progetti di ricerca di base legge regionale 7/2007 – bando 2008.

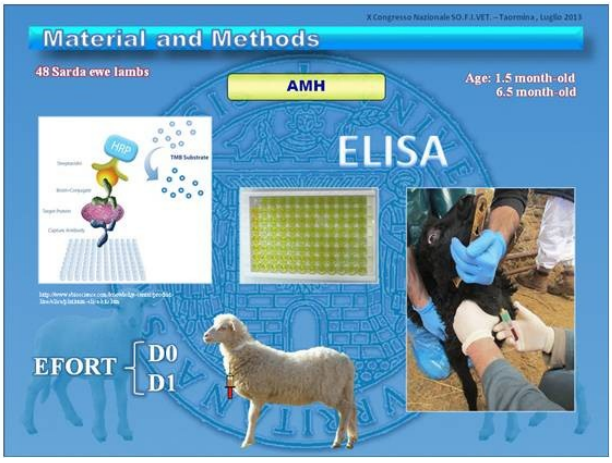
Key Words: Folliculogenesis, ovarian-stimulation, sheep, AMH

X Congresso Nazionale SO.F.I.VET. (Italian Society of Veterinary Physiology). Taormina, Italy. July 8th – 9th, 2013. Oral communication. Award for best scientific communication.



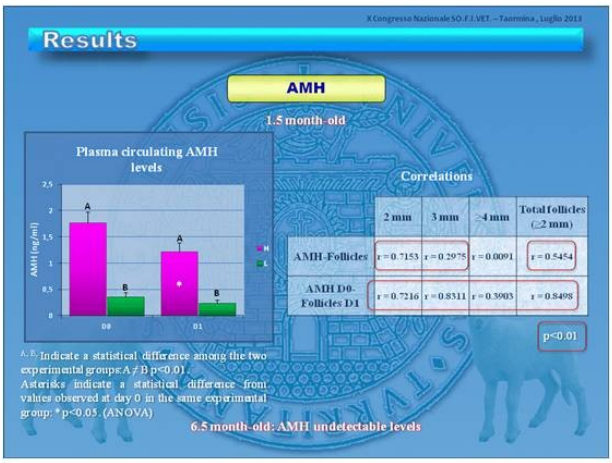




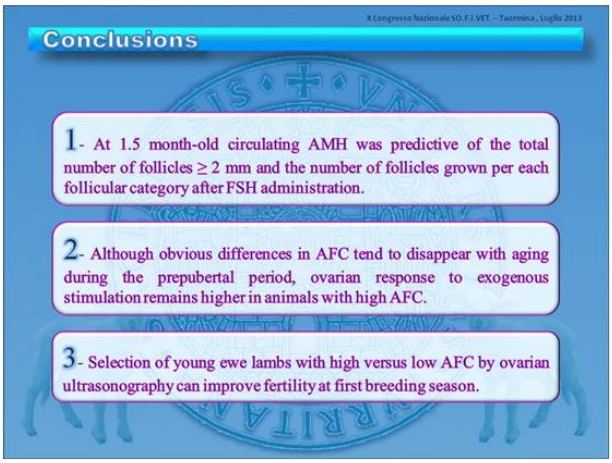



Material and Methods
Fertility
First breeding season:
August-September
44 Sarda ewe
23 High AFC
21 Low AFC


Results
EFORT
Age
1.5 month-old
(early
prepubertal)
6.5 month-old
(peripubertal)
Day 0
Day 1
Asterisks indicate a statistical difference from values observed at day 0 in the same experimental group: * p<0.05; ** p<0.01.
(ANOVA)


Results
AMH
1.5 month-old
Plasma circulating AMH levels
Correlations
2 mm
3 mm
≥4 mm
Total follicles (≥2 mm)
AMH-Follicles
AMH D0-Follicles D1
p<0.01
Asterisks indicate a statistical difference from values observed at day 0 in the same experimental group: * p<0.05. (ANOVA)
6.5 month-old: AMH undetectable levels


Results
Fertility
High AFC
Low AFC
Total
Pregnant
(% /total group)
Non pregnant
(% /total group)
Total
15
(65.22)
7
(33.33)
21
8
(34.78)
14
(66.67)
22
23
21
44
Chi-squared P= 0.03


Conclusions
1- At 1.5 month-old circulating AMH was predictive of the total number of follicles ≥ 2 mm and the number of follicles grown per each follicular category after FSH administration.
2- Although obvious differences in AFC tend to disappear with aging during the prepubertal period, ovarian response to exogenous stimulation remains higher in animals with high AFC.
3- Selection of young ewe lambs with high versus low AFC by ovarian ultrasonography can improve fertility at first breeding season.


Thank you
for your attention

SELECTION OF YOUNG EWE LAMBS ACCORDING TO THEIR ANTRAL FOLLICULAR COUNT: RESPONSE TO EXOGENOUS HORMONAL STIMULATION AND FERTILITY AT FIRST BREEDING SEASON.

Torres-Rovira L[1], Manca ME[1], Gonzalez-Bulnes A[2], Spezzigu A[3], Piu P[1], Gallus M[4], Succu S[1], Chelucci S[1], Leoni G[1], Berlinguer F[1], Naitana S[1].

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Key words: Anti-Mullerian Hormone, Antral Follicular Count, Ewe, Fertility

Introduction: Anti-Mullerian Hormone (AMH), Antral Follicular Count (AFC) and the response to exogenous hormonal stimulation have been used, in adults, as suitable markers to determine the ovarian reserve (1-4), to predict oocyte quality (5,6) and a wide variety of fertility indices (6-9).

This investigation aims to evaluate if animals selected according to their High or Low AFC at an early prepubertal age show different responses, in the number of follicles and AMH plasma levels, to exogenous hormonal stimulation; to verify whether differences are maintained over time until puberty; and to observe possible variations on fertility at first breeding season.

Material and Methods: Forty eight Sarda ewe lambs, with a mean age of 49.77 ± 1.15 days-old, were classified according to the number of follicles ≥2 mm in diameter present in their ovaries into Low AFC group (≤15 follicles) and High AFC group (≥30 follicles). The number of ≥2 mm follicles was determined by transrectal ultrasonography with a real-time B-mode scanner fitted with a 7.5 MHz rigid laparoscopic transducer (10). At 1.5 and 6.5 month-old, one-shot intramuscular dose of 105 IU FSH was administered to all animals and the number of ≥2 mm follicles was recorded before and 24 hours after treatment along with blood sample gathering. Plasma AMH concentration was analyzed with a commercial ELISA kit. Fertility at first reproductive season was assessed by the number of animals that remained pregnant. Rams were introduced into the ewe flock from August to the end of September, then, pregnancy diagnosis was performed using transrectal ultrasonography.

Results: At 1.5 month of age High AFC group had a significant higher number of 2mm, 3mm and total follicles (≥2 mm) than Low AFC group. This difference, remained after FSH administration and even became significant for ≥4 mm follicles. Follicles grew to larger sizes and follicular recruitment increased for both groups. At 6.5 month of age the High AFC group had a significantly higher number of ≥4 mm follicles. After exogenous FSH

stimulation, High AFC group had higher number of follicles in each category. AMH plasma levels at 1.5 month-old were significantly higher in the High AFC group before (1.766 ng/ml ± 0.143 for High AFC group vs 0.357 ± 0.143 for Low AFC group) and after ovarian stimulation (1.217 ng/ml ± 0.143 for High AFC group vs 0.239 ng/ml ± 0.143 for Low AFC group; $p<0.05$). Circulating AMH was positively correlated to total number of follicles ≥2 mm before ($r=0.65$) and after ($r=0.82$; $p<0.0001$) treatment. Also, initial AMH plasmatic levels were positively correlated to the number of follicles grown per each follicular category after FSH administration (2mm $r=0.72$, 3mm $r=0.83$, ≥4 mm $r=0.39$, total $r=0.85$; $p<0.01$). At 6.5 month of age animals had undetectable levels of AMH. Regarding fertility data, 65.22% of the High AFC ewes remained pregnant at first breading season versus 33.33% of the Low AFC ewes ($p<0.05$).

Conclusions: Selection of young ewe lambs with high versus low AFC by ovarian ultrasonography can improve fertility at first breeding season. Although obvious differences in AFC tend to disappear with aging during the prepubertal period, ovarian response to exogenous stimulation remains higher in animals with high AFC.

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CSIRO PUBLISHING

Reproduction, Fertility and Development
http://dx.doi.org/10.1071/RD13190

Predictive value of antral follicle count and anti-Müllerian hormone for follicle and oocyte developmental competence during the early prepubertal period in a sheep model

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Abstract. Circulating anti-Müllerian hormone (AMH) and antral follicle count (AFC) are addressed as suitable markers of oocyte quantity and quality during adulthood. To investigate whether AFC and circulating AMH could predict follicle development and oocyte quality during the prepubertal period we used 40-day-old ewe lambs with high, intermediate and low AFC (≥30, 16–29 and ≤15 follicles respectively). The analysis of the response to the exogenous FSH ovarian reserve test showed a positive correlation between AFC, AMH plasma levels, total follicle number and the number of large follicles (≥3 mm) grown after exogenous FSH administration. The incorporation of abattoir-derived oocytes collected from ovaries with different AFC in an *in vitro* embryo production system showed that a high AFC can predict oocyte quality in prepubertal ovaries, reflecting an ovarian status suitable for follicular development. The histological quantification of the ovarian reserve evidenced that AFC was not predictive of differences in either the number of healthy follicles or the size of the primordial follicle pool in prepubertal ovaries. Further studies are needed to investigate the implication on the reproductive performance of the significant inter-individual differences found in the present study in AFC and circulating AMH in the early prepubertal period.

Additional keywords: embryo, ovary, preantral follicle, ultrasound.

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Introduction

Several studies performed during the past decades have linked the quantity and quality of non-growing follicles in the ovary (the ovarian reserve) to reproductive features. The recognition that anti-Müllerian hormone (AMH) is produced by preantral and small antral follicles, with its serum concentrations reflecting both primordial follicle pool and the number of small growing follicles (human, Weenen *et al.* 2004; Visser *et al.* 2007; Hansen *et al.* 2011), has strengthened the study of the relationships between ovarian reserve, number of antral follicles and fertility.

The combination of different studies investigating models for determining functional ovarian reserve during childhood, adolescence and adult life (Hagen *et al.* 2010; Nelson *et al.* 2011*a*, 2011*b*) has provided a new perspective on follicular behaviour and challenged some previous hypotheses about follicular development (Fleming *et al.* 2012). In particular, it is now clear that the rate of follicle recruitment from the primordial follicle pool shows a steady increase during childhood and adolescence for reaching a maximum at 14 years old, which is coincidental with postpubertal establishment of adult ovarian function. Hence, both the number of primordial follicles recruited into the growing follicle pool and the rate of follicular growth are higher during the infant period than in adulthood (Hage *et al.* 1978). Similarly, a recent study indicated that in women the rate of follicle recruitment increases from birth to

14 years old and afterwards declines with age until menopause (Wallace and Kelsey 2010). On the other hand, although the number of antral follicles has been linked to ovarian reserve (Hansen *et al.* 2011) and oocyte quality (Holte *et al.* 2011) in adulthood, little is known about differences in antral follicle number during the prepubertal period and their eventual implication on ovarian function.

Similarly to follicular recruitment, serum AMH levels also changes across lifespan. There is an initial increase during childhood, then a distinct fluctuation around the time of puberty, followed by a secondary increase during the next decade to a maximum at around 25 years old (Kelsey *et al.* 2011). Subsequent to this peak, serum AMH undergoes a progressive decline to values below the levels of assay sensitivity between the ages 40 of and 45 years. From 25 years old it appears that the relationship between the decline in the size of the primordial follicle pool, the number of follicles undergoing recruitment and the serum AMH concentrations closely resemble each other (Hagen *et al.* 2010). Thus, AMH has emerged as a suitable marker for ovarian function (Loh and Maheshwari 2011).

On the other hand, there is a strong positive correlation between circulating AMH and follicular recruitment before the onset of puberty (human, Fleming *et al.* 2012), which contrasts with evidence indicating its inhibitory role on follicle recruitment at postpubertal stages (mice, Durlinger *et al.* 2002*a*; sheep, Campbell *et al.* 2012). Factors and mechanisms driving early prepubertal follicle development are indeed less understood. The prepubertal endocrine environment is markedly different from the adult with low and non-cyclical gonadotrophin and steroid levels. The full hierarchy of follicles within the ovary becomes established only after puberty, and it is likely that local autocrine and paracrine effects, reflecting the different stages of follicular development, may influence on-going follicular development (mice, Durlinger *et al.* 2002*a*; Da Silva-Buttkus *et al.* 2009). Theoretically, low pre- and peri-pubertal AMH levels may have a different clinical implication than later in life, and the interpretation of low serum AMH level as a marker of a lower ovarian reserve may not apply to the prepubertal period since mechanisms regulating follicle recruitment and ovarian function may differ from those acting in adulthood.

In view of these considerations, our study aimed to investigate whether inter-individual differences in circulating AMH and antral follicle count (AFC) could predict follicular development and oocyte quality in the early prepubertal period. It is well established that adult cattle can be reliably phenotyped based on robust individual differences in AFC during follicular waves (Evans *et al.* 2010). We have previously observed a similar high variation in the AFC in sheep (Mossa *et al.* 2008), even in the prepubertal period (F. Berlinguer, unpubl. observation). Our experimental model was, thus, based in ewe lambs expressing different ovarian phenotypes at 40 days of age. For the *in vivo* and *in vitro* trials, three groups of females were identified according to the total number of follicles ≥2 mm in diameter on the surface of the two ovaries (AFC): High (≥30 follicles), Intermediate (16–29 follicles) and Low AFC group (≤15 follicles).

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. All experimental procedures were carried out at the experimental facilities of the Department of Veterinary Medicine at the University of Sassari, Italy (latitude 40°43′ N). These facilities meet the requirements of the European Union for Scientific Procedure Establishments. The experimental procedures followed ethical guidelines for the care and use of agricultural animals for research (EC Directive 86/609/EEC for animal experiments).

All reagents and media were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified.

Experimental design

Three consecutive experiments were carried out to meet the objectives of this study.

Experiment 1 aimed to assess if circulating AMH reflects the AFC and therefore would predict the response to an exogenous FSH ovarian reserve test (EFORT, Kwee *et al.* 2006) in ewe lambs aged 40 days belonging to the three above-mentioned experimental groups (High, Intermediate and Low AFC).

Experiment 2 studied the influence of the ovarian phenotype expressed during the early prepubertal period on oocyte quality, in terms of *in vitro* developmental competence. Pairs of ovaries from 40-day-old ewe lambs were collected from a commercial slaughterhouse and classified into the three mentioned AFC groups. COCs recovered from the three experimental groups were cultured *in vitro* up to the blastocyst stage and the quality of the embryos in the three categories was assessed by evaluating their cryotolerance and total cell number.

Experiment 3 determined whether differences in oocyte quality and the EFORT between ovaries expressing different phenotypes during the postnatal period were predictive of differences in the ovarian reserve. Total number of primordial, primary, secondary, antral, polyovular and atretic follicles was determined in histological sections of the ovaries classified as belonging to the High, Intermediate and Low AFC groups.

Experiment 1: relationship between circulating AMH, AFC and response to EFORT

A group of 39 Sarda ewe lambs, around 35–45 days old and 8–10 kg bodyweight was used. Animals were maintained under the same conditions throughout the experimental procedure, being housed outdoors with indoor access. In all the ewe lambs, the ovarian phenotype was determined by characterising the AFC by transrectal ultrasonography, with a real-time B-mode scanner (Aloka SSD 500; Aloka Co., Tokyo, Japan) fitted to a 7.5-MHz linear-array probe (laparoscopic rigid transducer UST-5526 L-7.5; Aloka). Scanning was performed as previously described and validated in our laboratory (Gonzalez-Bulnes *et al.* 1994). In brief, observations were conducted with the sheep placed in dorsal recumbence on a metallic cradle as used for laparoscopy. After introducing a hydrosoluble contact gel into the rectum, the probe was introduced so that the transducer was perpendicular to the abdomen wall. When the urinary bladder was surpassed and the uterine horns were located, the

probe was rotated laterally 90° clockwise and 180° counter-clockwise to observe both ovaries and their structures. Each ovary was scanned several times from different angles in order to determine number and size of all follicles ≥2 mm. Animals were divided, at first ultrasound observation, into three experimental groups accordingly to the number of follicles ≥2 mm (Fig. 1): Low AFC group (≤15 follicles; $n = 13$), Intermediate AFC group (16–29 follicles; $n = 15$) and High AFC group (≥30 follicles; $n = 11$).

Immediately after determining AFC, the responsiveness of the follicular population to the exogenous FSH ovarian reserve test (EFORT, Kwee *et al.* 2006) was determined. First, blood samples were drawn 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 until assayed for AMH and 17β-oestradiol. Afterwards, all the animals were administered one-shot intramuscular dose of 60 mg FSH (Folltropin; Bioniche Animal Health, Minitub Ibérica S.L., Reus, Spain). Twenty-four hours later, the growth of the antral follicles was assessed by a second ultrasonographic scan and a second blood sample was drawn. Analysis of the EFORT included changes in follicular population and in plasma concentrations of AMH and 17β-oestradiol.

AMH concentration was measured by using the AMH Gen II ELISA kit (Beckman Coulter Inc., Brea, CA, USA). The kit was validated for use in ovine plasma by determining the parallelism of different dilutions of plasma from lambs, ewes with low and high AFC and ovariectomised sheep. Sensitivity of the assay was 0.08 ng mL^{-1}, whilst the inter- and intra-assay variation coefficients were 5.6% and 5.4%, respectively. Oestradiol was measured using the Spectria radioimmunoassay kit (Orion Diagnostic Corp, Espoo, Finland), as described by Romeu *et al.* (1995) and adapted for use in ovine plasma (González-Bulnes *et al.* 2003). Sensitivity of the assay was 0.5 pg mL^{-1}, whilst the inter- and intra-assay variation coefficients were 6.1% and 3.5%, respectively.

Experiment 2: effects of ovarian phenotype on in vitro *developmental competence of oocytes and quality of blastocysts*

Collection and classification of ovaries

Complete reproductive tracts (uterus plus ovaries) from Sarda ewe lambs of similar age and weight (40 days old, bodyweight 8–10 kg) were obtained from a commercial slaughterhouse and transported to the laboratory, within 1 h, in Dulbecco's phosphate-buffered saline (PBS; 2.7 mM KCl, 1.5 mM KH_2PO_4, 136.9 mM NaCl, 8.9 mM Na_2HPO_4-$7H_2O$) supplemented with penicillin and streptomycin (50 mg mL^{-1}) at 30–35°C. The pairs of ovaries were classified into the three above-mentioned groups according to the total number of follicles ≥2 mm in diameter on their surface (Fig. 1). Ovaries assigned to the three experimental groups were used for evaluation of *in vitro* oocyte developmental competence ($n = 658$) and for histological quantification of the ovarian reserve (Experiment 3; $n = 22$). Before being incorporated into the two experimental trails, ovaries were separated from the rest of the reproductive tract, then weighed and measured for length and height.

In vitro *embryo production*

The procedure for *in vitro* embryo production was repeated 11 times. Ovaries assigned to the three experimental groups were processed separately. Within each group, oocytes selected for *in vitro* maturation were pooled, cultured together and considered as one batch. After being washed in fresh PBS, the ovaries

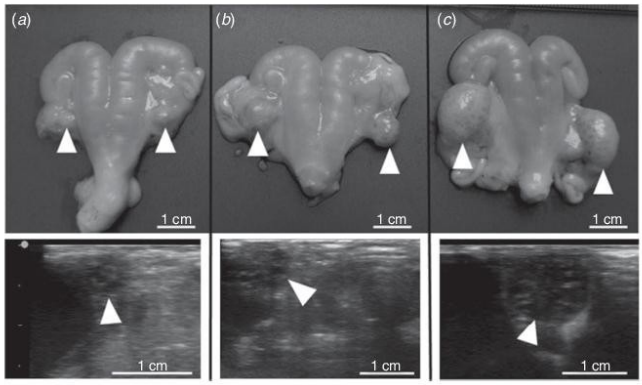

Fig. 1. Reproductive tracts from ewe lambs aged 40 days and ultrasound images obtained *in vivo*. Ewe lambs were classified according to the total number of follicles ≥2 mm in diameter on the surface of the ovaries into (*a*) Low group, ≤15 follicles, (*b*) Intermediate group, between 16 and 29 follicles or (*c*) High group, ≥30 follicles. The upper panel shows the ovaries from the three experimental groups and the lower part the respective ultrasound images obtained *in vivo* by transrectal ultrasonography. Ovaries are indicated with arrowheads.

were sliced using a micro-blade and the follicle content was released into medium TCM199 (with Earle's salts and bicarbonate) supplemented with 25 mmol HEPES, 0.1 g L^{-1} penicillin, 0.1 g L^{-1} streptomycin and 0.1% (w/v) polyvinylalcohol (PVA). Cumulus–oocyte complexes (COCs) with 4–10 layers of granulosa cells, oocytes with a uniform cytoplasm, homogenous distribution of lipid droplets in the cytoplasm and outer diameter of ~90 μm (mean) were selected for these experiments. The selected COCs, after three washes in the same fresh medium, were matured *in vitro* in TCM199 supplemented with 10% heat-treated fetal calf serum (FCS), 1 IU mL^{-1} ovine FSH, 1 IU mL^{-1} ovine LH and 100 μM cysteamine. Thirty to 35 COCs were put in 600 μL of the maturation medium in a four-well Petri dish (Nunclon, NalgeNunc International, Roskilde, Denmark), layered with 300 μL mineral oil and cultured for 24 h in 5% CO_2 in air at 39°C.

After maturation, the COCs were stripped of the granulosa cells and fertilised *in vitro* at 39°C and 5% CO_2, 5% O_2 and 90% N_2 atmosphere in four-well Petri dishes (Nunclon). Frozen–thawed spermatozoa from the same ejaculate of one ram were used across all experimental procedures. The fertilisation system was composed of 300 μL of synthetic oviductal fluid (SOF) medium supplemented with 2% oestrus ovine serum and swim-up-derived motile spermatozoa at 1×10^6 spermatozoa mL^{-1} layered with mineral oil. Finally, after 22 h, presumptive zygotes were mechanically denuded of their cumulus cells and cultured to blastocyst stage in four-well Petri dishes containing SOF plus essential and non-essential amino acids at oviduct concentration (Walker *et al.* 1996) and 0.4% bovine serum albumin (BSA) under mineral oil in maximum humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2. The number of cleaved oocytes showing two distinct blastomeres was recorded at 26 h after insemination. Culture dishes were observed daily starting from the sixth day of culture and newly formed blastocysts were recorded.

Vitrification and warming procedures

Vitrification and warming media were prepared using PBS supplemented with 20% (v/v) FCS as base medium. Embryos were vitrified according to a simple method (Leoni *et al.* 2002). Briefly, blastocysts were put into 200-μL drops of 1.4 M glycerol for 5 min, then into 200-μL drops of 1.4 M glycerol and 3.6 M ethylene glycol for 5 min before being transferred into a 15-μL column of 3.4 M glycerol and 4.6 M ethylene glycol and loaded into the centre of 0.25-mL plastic insemination straws using a fine glass capillary pipette. In the straw, the embryos in vitrification medium were separated by four air bubbles (60 μL) from two columns (30 μL) of vitrification medium and two columns (120 μL) of 0.5 M sucrose solution. After sealing, the straws were transferred directly into liquid N_2 (LN_2) and stored therein.

For warming to a biological temperature, the straws were transferred from LN_2 into a water bath at 35°C for 10 s. The content of each straw was expelled into a Petri dish and stirred gently to facilitate the mixture of the two solutions. The embryos were retrieved and transferred into 200-μL drops of 0.25 M sucrose solution supplemented with 20% FCS or 0.1% PVA for 3 min to allow the removal of intracellular cryoprotectants. Embryos were held for 10 min in corresponding media of PBS containing 20% FCS or 0.1% PVA for re-hydration and equilibration.

Assessment of embryo quality

We determined blastocyst cryotolerance after vitrification and warming to assess differences in embryo quality between the experimental groups, in terms of *in vitro* re-expansion and hatching rates during 72 h post-warming culture. The embryos that re-expanded the blastocoelic cavity were considered to be viable (Leoni *et al.* 2006), as this parameter represents a reliable indicator of the quality of *in vitro*-produced blastocysts (Rizos *et al.* 2003; Leoni *et al.* 2008).

After warming, blastocysts were cultured in TCM199 supplemented with 10% FCS in a humidified atmosphere with 5% CO_2 in air at 39°C and examined every 12 h for 72 h to assess re-expansion and hatching rates. Blastocysts were considered to be completely re-expanded when their diameter was recovered to the original value before vitrification. Hatched blastocysts were fixed in a solution of methanol in PBS (40% v/v) and stained with propidium iodide (10 μg mL^{-1}). After washing in PBS–PVA, embryos were placed on glass slides, covered with coverslips and observed under a fluorescent microscope to count the total cell number.

Experiment 3: Histological quantification of the ovarian reserve in ovaries of different phenotypes

Histological processing of the ovaries

Pairs of ovaries from 40-day-old ewe lambs were assigned to the High, Intermediate and Low AFC groups according to the total number of follicles, as described above. One of each pair of ovaries was then selected for histological analysis making a total of six ovaries in the Low AFC group, eight ovaries in the Intermediate AFC group and eight ovaries in the High AFC group. Before fixation, in order to allow an adequate penetration of the fixative, the ovaries from the Intermediate and High AFC groups were half-sectioned along the major diameter of the ovary, while, for the Low group whole ovaries were used. Thereafter, ovaries were fixed overnight in Dietrich fixative (30% v/v ethanol 95%, 10% v/v formalin (37% formaldehyde solution containing 10–15% methanol), 2% v/v glacial acetic acid, 58% v/v distilled water) and transferred into PBS till paraffin embedded.

Paraffin blocks were serially sectioned at 5-μm intervals using a rotary microtome (Leica RM2245; Leica Microsystems Srl, Milano, Italy) and every 20th section was placed on a glass microscope slide. Slides were stained with haematoxylin and eosin and observed under a microscope to determine the number of morphologically healthy primordial, primary, secondary, early antral and late antral follicles, as well as polyovular and atretic follicles. For the Intermediate and High groups only sections from one of the ovary halves were analysed.

Classification of follicles and follicle count

Only follicles of each 20th section with a cross-section of the oocyte nucleus were counted. Follicles were classified as morphologically healthy follicles when presenting an intact basal membrane, well-organised granulosa cell layers with only occasional pyknotic nuclei, none or few atretic granulosa cells in

the follicular antrum and intact oocyte and nucleus. The type of follicles is described in Fig. 2. Due to the advanced stage of atresia of some follicles it was difficult to classify the atretic follicles into each follicle type; therefore, atretic follicles were classified only as atretic and were counted even when there was no nucleus. Follicles having more than one oocyte were classified as polyovular independently of the atretic or non-atretic stage of the oocytes inside.

In order to estimate the total number of follicles in the ovaries, as previously described by Ireland *et al.* (2008) in cattle ovaries, a correction factor was applied. For ovaries in the Low group the number of counted follicles for each type was multiplied by the correction factor 40 (20×2; 20 corrects for counting follicles in every 20th section and 2 corrects for counting follicles in a single ovary), while for ovaries in the Intermediate and High groups the correction factor used was 80 ($20 \times 2 \times 2$; 20 corrects for counting follicles in every 20th section, 2 corrects for counting follicles in only one-half of the ovary and 2 corrects for counting follicles in a single ovary).

Statistical analysis

Differences in follicle numbers and hormone concentrations between the three experimental groups and between Days 0 and 1 of treatment were assessed by general lineal model

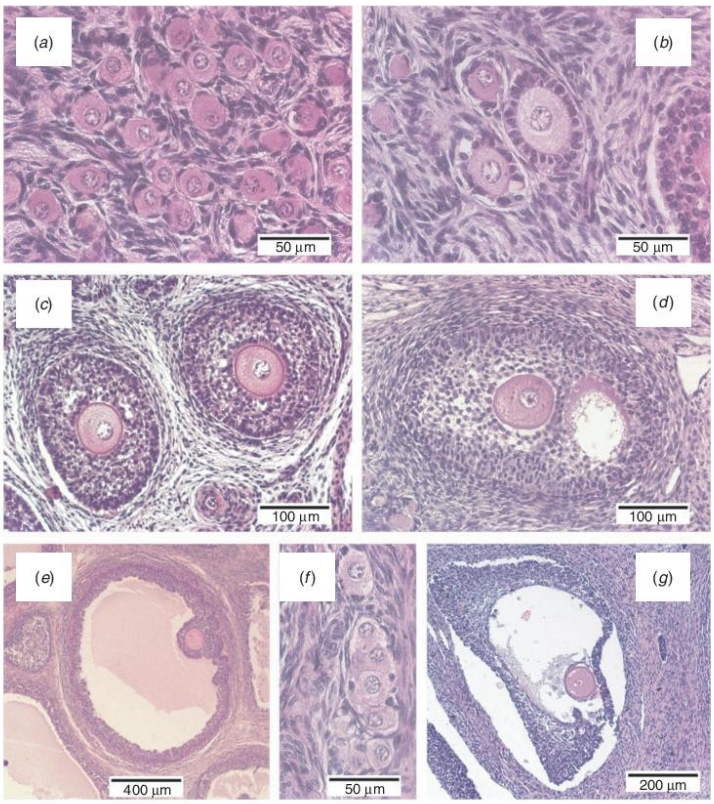

Fig. 2. Histological sections of ovaries from 40-day-old ewe lambs. (*a*) Primordial follicles showed an oocyte surrounded by a flat single layer of follicular cells. (*b*) Primary follicles had an oocyte surrounded by a cuboidal epithelium of one to less than two layers of granulosa cells. (*c*) Secondary follicles presented the oocyte and zona pellucida surrounded by a variable number of concentric layers, two or more, of cuboidal granulosa cells without a discernible antrum. (*d*) Once a small developing antrum was identified follicles were recorded as early antral follicles. (*e*) Late antral follicles exhibited a clearly formed antral cavity with an almost isolated oocyte surrounded by the cumulus oophorus. (*f*) Follicles with two or more oocytes inside were classified as polyovular follicles. (*g*) Example of atretic follicle with detachment of cells in the antrum and absence of cumulus oophorus.

where: $Y = \mu + \text{day} + \text{group} + \text{day} \times \text{group} + \text{ewe lambs}$. Day 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. Differences in oocyte developmental competence between the three experimental groups were assessed by ANOVA of the following ratios: oocytes selected for IVM/ovaries collected; fertilised oocytes/oocytes selected for IVM; cleaved oocytes/fertilised oocytes; blastocysts developed at Day 7 of culture/cleaved oocytes; blastocysts developed at Day 8 of culture/cleaved oocytes; blastocysts developed at Day 9 of culture/cleaved oocytes; total blastocysts/cleaved oocytes. Differences between the three groups in ovary wet-weight, the number of collected ovaries belonging to the three experimental groups and total cell number in the blastocysts were assessed by ANOVA. When data were not normally distributed (Shapiro Wilk W test: $P < 0.05$), the analysis of variance for non-parametric values (Kruskal–Wallis test) was used. In this case, between-group differences were analysed by the post hoc paired-comparison analysis (Wilcoxon's paired test). Fisher's l.s.d. test was used for the mean separation. The chi-square test, or Fisher exact test when appropriate, was used to determine differences in blastocyst re-expansion and hatching rates. Possible correlations between the following variables were determined: (1) size of the ovaries and *in vitro* developmental competence of the oocyte, as evaluated in terms of fertilised and cleaved oocytes and subsequent embryo output (we considered, for each replicate, the *in vitro* developmental competence of each batch of oocytes and the sum of the wet-weights of all the ovaries assigned to each experimental group), (2) size of the ovaries and numbers of growing and primordial follicles and (3) follicle population and AMH levels. 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 $P \leq 0.05$ was considered to be the minimum level of significance. All results are expressed as mean ± s.e.m.

Results

Experiment 1: relationship between circulating AMH, AFC and response to EFORT

Ovarian ultrasound scanning performed before exogenous FSH administration indicated that the initial follicular population in ewe lambs aged 40 days was mostly represented by follicles 2 mm in diameter (Fig. 3*a*). The highest number of total and 2-mm follicles was observed in the High AFC group (40.8 ± 1.5

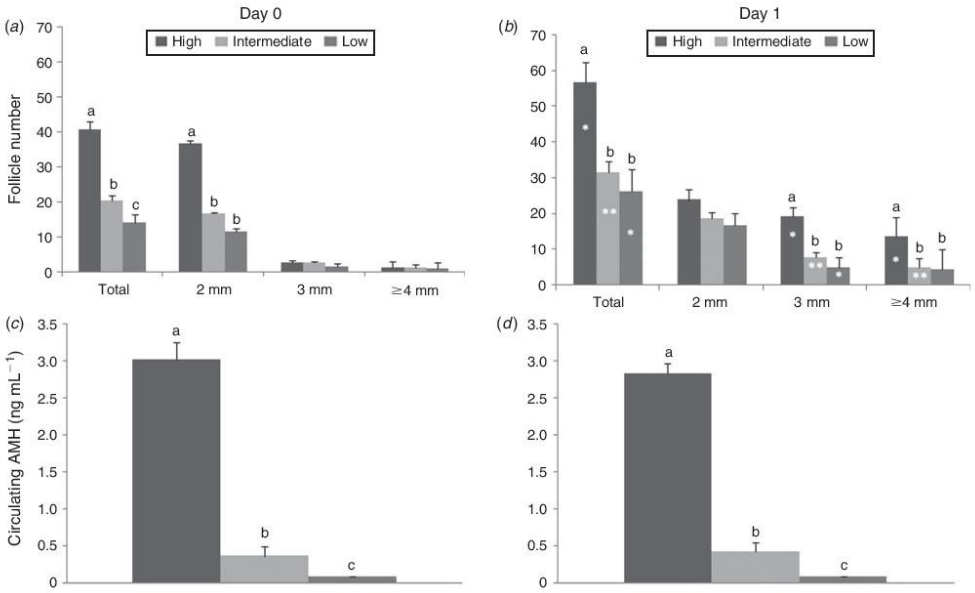

Fig. 3. Follicle population and anti-Müllerian hormone plasma levels in 40-day-old ewe lambs with different ovarian phenotypes before and after FSH administration. (*a*, *b*) Mean number of total follicles and follicles of 2 mm, 3 mm and ≥4 mm in diameter and (*c*, *d*) mean circulating AMH concentrations (*c*) before (Day 0) and (*d*) 24 h after FSH treatment (Day 1) in 40-day-old ewe lambs assigned to High (≥30 follicles), Intermediate (16–29 follicles) or Low group (≤15 follicles) according with the ovarian phenotype expressed. [a,b,c] Indicate a statistical difference between the three experimental groups: a ≠ b ≠ c, $P < 0.0001$; a ≠ b $P < 0.05$. (*b*) Asterisks indicate a statistical difference from values observed at Day 0 in the same experimental group: *$P < 0.05$; **$P < 0.01$.

and 36.6 ± 2.2, respectively), followed by the Intermediate (20.5 ± 0.9 and 16.6 ± 1.2) and then by the Low AFC group (14 ± 1.8 and 11.5 ± 2.5; $P < 0.0001$). The mean number of follicles of 3 mm and ≥4 mm in diameter did not differ between the three groups.

After exogenous FSH administration (Day 1), the mean number of total follicles rose within each group ($P < 0.05$), mainly due to an increase in the number of 3-mm and ≥4-mm follicles ($P < 0.05$). The number of 2-mm follicles did not vary significantly from the initial values within any of the groups (Fig. 3*b*). Analysing the difference in the follicular population between the three experimental groups after exogenous FSH administration, a higher number of total, 3- and ≥4-mm follicles was found in the High group compared with the Intermediate and Low ones.

In addition, the growth dynamics of the follicle differed between the three experimental groups (Fig. 4). By comparing the new follicles grown after exogenous FSH administration (whose number was obtained by subtracting follicles recorded on Day 1 from those recorded on Day 0), we found that the increase in the number of large follicles (≥3 mm) was accompanied by a decrease in the number of 2-mm ones only in the High AFC group.

Before exogenous FSH administration, the mean plasma concentration of AMH in all the ewe lambs was 0.93 ± 0.42 ng mL^{-1}. Plasma AMH levels were significantly related to the number of total follicles ($r = 0.967$; $P < 0.001$), due to a high correlation with the number of follicles 2 mm in size ($r = 0.943$; $P < 0.0001$). Thus, AMH concentrations were higher in the High AFC group (3.03 ± 0.22 ng mL^{-1}) than in the Intermediate (0.36 ± 0.13 ng mL^{-1}; $P < 0.01$) and Low AFC groups (0.08 ± 0.01 ng mL^{-1}; $P < 0.05$; Fig. 3*c*). After exogenous FSH administration, the High AFC group maintained the higher AMH plasma levels (2.84 ± 0.13 ng mL^{-1}) compared with the Intermediate (0.42 ± 0.13 ng mL^{-1}; $P < 0.05$) and the Low AFC groups (0.08 ± 0.01 ng mL^{-1}; $P < 0.05$; Fig. 3*d*). Within each experimental group, no significant variation was found in the AMH plasma levels after FSH treatment. On Day 1, AMH plasma levels were again significantly related to the number of total follicles ($r = 0.794$; $P < 0.005$) but, in this case, due to a high correlation with the number of follicles 3 mm in size ($r = 0.854$; $P < 0.001$). A positive correlation was also found between AMH plasma levels on Day 0 and the number of large follicles (3 and ≥4 mm in diameter) grown after exogenous FSH administration at Day 1 ($r = 0.572$, $P < 0.005$).

Plasma oestradiol levels were undetectable in all ewe lambs both before and after FSH administration.

Experiment 2: effects of ovarian phenotype on oocyte in vitro developmental competence

Marked inter-individual differences in ovarian follicle number were evident in the collected ovaries. In addition, the proportion of ovaries among the three groups varied significantly ($P < 0.0001$), with the lowest number of ovaries (8.5%) being

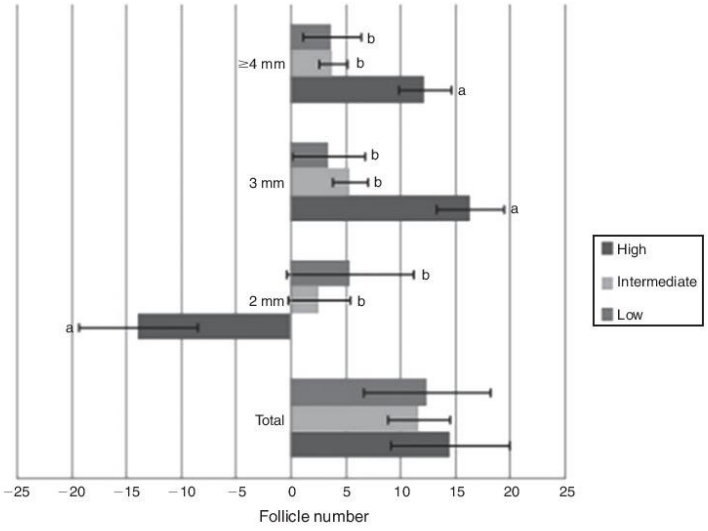

Fig. 4. New follicles stimulated to growth after FSH administration in 40-day-old ewe lambs with different ovarian phenotypes. Ewe lambs were assigned to High (≥30 follicles), Intermediate (16–29 follicles) or Low group (≤15 follicles) according to the ovarian phenotype expressed and the number of new follicles was recorded by ovarian ultrasound scanning 24 h after FSH administration. [a,b] Indicate statistical difference: a ≠ b, $P < 0.05$ (ANOVA).

Table 1. Comparison of the proportion and sizes of ovaries assigned to the High (≥30 follicles), Intermediate (16–29 follicles) or Low (≤15 follicles) groups according to the ovarian phenotype expressed, and number and *in vitro* developmental competence of oocytes collected from ewe lamb (40 days of age) ovaries

Different superscripts indicate statistical difference between the experimental groups: $\alpha \neq \beta \neq \gamma$, $P < 0.0001$ (Chi-square test); $a \neq b \neq c$, $P < 0.0001$; $a \neq b$, $P < 0.05$ (ANOVA)

Parameter		Group		
		High	Intermediate	Low
Ovaries	Ovaries (%)	56^{α} (8.5)	222^{β} (33.7)	380^{γ} (57.8)
	Ovary wet weight (mg)	609.9 ± 21^{a}	264.9 ± 21^{b}	54.8 ± 24.3^{c}
	Ovary height (mm)	1.3 ± 0.04^{a}	0.95 ± 0.04^{b}	0.65 ± 0.05^{c}
	Ovary length (mm)	1.01 ± 0.05^{a}	0.78 ± 0.05^{b}	0.5 ± 0.05^{c}
Oocytes	IVM (per ovary; mean ± s.e.m.)	1832^{a} (36.4 ± 2.9)	1928^{b} (9.9 ± 2.7)	343^{c} (1.1 ± 2.8)
	IVF (%)	1411 (79.6 ± 4.3)	1355 (70.2 ± 3.9)	274 (81.6 ± 4.1)
Embryos (%)	Cleaved[A]	1087 (74.1 ± 5.7)	1082 (81.3 ± 5.3)	187 (71.3 ± 5.5)
	Day 7[B]	99 (6.3 ± 2.3)	68 (5.6 ± 2.2)	14 (3.9 ± 2.2)
	Day 8[B]	99^{a} (10 ± 2)	104^{a} (9.7 ± 1.9)	10^{b} (4.1 ± 1.9)
	Day 9[B]	9 (1.1 ± 0.5)	17 (1.3 ± 0.5)	3 (0.4 ± 0.5)
	Total[B]	207^{a} (17.4 ± 2.8)	189^{a} (16.6 ± 2.6)	27^{b} (8.3 ± 2.7)

[A]Percentages are calculated on fertilised oocytes.
[B]Percentages are calculated on cleaved oocytes.

Table 2. *In vitro* re-expansion, hatching rates and total cell number of vitrified–warmed blastocysts obtained from ewe lamb ovaries (40 days of age) with different follicle numbers (High AFC ≥30; Intermediate AFC 16–29; Low AFC ≤15)

Group (*n*)	Re-expansion (%)	Hatching (%)	Total cell number[A] (mean ± s.e.m.)
High (130)	66 (50.7)	44 (33.8)	92 ± 4.3^{a}
Intermediate (134)	58 (43.2)	36 (26.9)	75.1 ± 4.6^{b}
Low (27)	17 (62.9)	7 (26)	72 ± 7.6^{b}

[A]Total cell number was determined on hatched blastocysts. Different superscripts indicate statistical difference: $a \neq b$, $P < 0.05$ (ANOVA).

assigned to the High group followed by the Intermediate (33.7%) and Low groups (57.8%; Table 1). The number of COCs selected for IVM per ovary varied significantly between the groups. In particular, more COCs were obtained from the High group than from the Intermediate and Low ones ($P < 0.0001$). Differences in oocyte developmental competence between groups are summarised in Table 1. A higher embryo output was obtained from oocytes collected from High and Intermediate groups when compared with the Low group ($P < 0.05$). In addition, in the High and Intermediate groups a higher number of blastocysts developed on Day 8 of culture compared with the Low one ($P < 0.05$).

Ovary wet-weight proved to be strongly correlated with the number of collected oocytes per gram of ovarian tissue (correlation coefficient, 0.90; $P < 0.0001$); a positive correlation was also found with the number of oocytes fertilised (0.53; $P < 0.001$) and cleaved (0.49; $P < 0.01$), with the number of blastocysts obtained at Day 8 of culture (0.45; $P < 0.01$) and the total number of blastocysts obtained without regards of the day (0.48; $P < 0.01$).

Blastocyst cryotolerance, as evaluated by *in vitro* re-expansion and hatching rates, did not differ between the experimental groups (Table 2). Total cell number was higher in blastocysts obtained from oocytes belonging to ovaries with high follicle number compared with the other groups ($P < 0.05$).

Experiment 3: determination of ovarian reserve in ewe lambs expressing different ovarian phenotypes

The largest ovaries, as determined by their wet weight, height and length, belonged to the High group, followed by the Intermediate and then by the Low group ($P < 0.0001$; Table 1). However, the total number of morphologically healthy follicles and the number of morphologically healthy primordial, primary and secondary follicles did not vary in relation with the ovarian phenotype expressed, as ovaries with the smallest size bore the highest number of healthy follicles per mg of tissue ($P < 0.0001$; Table 3). Ovarian phenotype reflected the number of morphologically healthy early and late antral follicles, which reached the highest absolute value and proportion over healthy follicles in the High group, followed by the Intermediate and then by the Low group. No correlation was found between ovarian sizes (weight, height and length) and primordial follicle number, nor between the number of growing follicles (primary, secondary and antral follicles) and the number of primordial follicles, nor between the number of antral follicles (early antral plus late antral) and the total number of morphologically healthy follicles.

Discussion

The results of the present experiments extend our knowledge of the relationships between AMH, follicular development and the ovarian reserve during the early prepubertal period. Circulating AMH was positively correlated with AFC and to the development of large follicles (≥3 mm in diameter) in response to FSH stimulation. A low AFC, although not accompanied by a

Table 3. Total number of follicles in ovaries of ewe lambs (40 days of age) assigned to the High (≥30 follicles), Intermediate (16–29 follicles) or Low (≤15 follicles) groups according to the ovarian phenotype expressed

[a,b,c] Different letters indicate statistical difference between the experimental groups: a ≠ b ≠ c, $P < 0.0001$ (ANOVA). Differences between the experimental groups in early and late antral follicles account for both their total number and their proportion of healthy follicles

Follicle parameters	High ($n = 8$)	Intermediate ($n = 8$)	Low ($n = 6$)
Total no. follicles in ovaries			
Healthy + atretic	237 850 ± 43 593	250 270 ± 43 593	206 753 ± 50 337
Healthy (%)	219 430 ± 40 570 (93.9 ± 0.8)	231 685 ± 40 570 (93.2 ± 0.7)	194 587 ± 46 846 (92.1 ± 0.7)
Healthy per mg ovaries	190 ± 119[a]	437 ± 119[a]	1922 ± 138[b]
Primordial (%)[A]	207 020 ± 39 975 (92.8 ± 1.2)	221 915 ± 39 975 (95.3 ± 1.2)	184 060 ± 46 159 (93.8 ± 1.4)
Primary (%)[A]	8920 ± 1052 (4.9 ± 0.7)	6280 ± 1052 (2.9 ± 0.7)	7547 ± 1215 (4.5 ± 0.8)
Secondary (%)[A]	2490 ± 512 (1.6 ± 0.4)	3170 ± 512 (1.6 ± 0.4)	2940 ± 591 (1.7 ± 0.5)
Early antral (%)[A]	1310 ± 107[a] (0.8 ± 0.1)	680 ± 107[b] (0.4 ± 0.1)	333 ± 124[c] (0.2 ± 0.1)
Late antral (%)[A]	1000 ± 81[a] (0.7 ± 0.2)	320 ± 81[b] (0.2 ± 0.2)	40 ± 93[c] (0 ± 0.2)
Polyovular (%)[B]	1260 ± 316 (0.5 ± 0.1)	1390 ± 316 (0.5 ± 0.1)	767 ± 365 (0.33 ± 0.1)

[A]Percentages are calculated on healthy follicles.
[B]Percentages are calculated on total follicles.

reduction in the size of the primordial follicle pool, was predictive of poor oocyte *in vitro* developmental competence.

In our study, mean plasma AMH levels in 40-day-old ewe lambs were higher than values reported from other authors in older ewe lambs (range from 0.043 ± 0.015 to 0.163 ± 0.04 at 3.6 months of age; Lahoz *et al.* 2012). In heifers, plasma AMH concentrations were found to increase markedly between 1 and 3 months of age, remaining high at 6 months, then declining again until puberty (Monniaux *et al.* 2013). Since no previous study has examined AMH across the lifespan in healthy ewes, this variation may be linked to several factors other than age (see also Kelsey *et al.* 2011).

Results of the *in vivo* trial evidenced a strong positive correlation between AFC and circulating AMH levels in the early prepubertal period. In this way, ovarian phenotypes expressing the highest number of 2-mm follicles also had the highest levels of AMH. Previous studies in adults have shown a close relationship between antral follicles and serum AMH (human, de Vet *et al.* 2002; Laven *et al.* 2004; Pigny *et al.* 2006; Jayaprakasan *et al.* 2010), confirming that AMH reflects the size of the growing follicle pool. In sheep, as in other species (mice, Durlinger *et al.* 2002*a*; human, Weenen *et al.* 2004), AMH is strongly expressed by the granulosa cells of preantral and small antral follicles (sheep, Campbell *et al.* 2012). These stages of follicle development are gonadotrophin-responsive in sheep (Campbell *et al.* 2004) and other species (primates, Gougeon 1996; cattle, Webb *et al.* 1999).

In our study, the analysis of the response to the EFORT in 40-day-old ewe lambs expressing different ovarian phenotypes revealed that in individuals with a high AFC a higher number of large follicles developed in response to FSH stimulation compared with individuals with intermediate and low AFC. In addition, in ewe lambs with high AFC the increase in the number of large follicles was accompanied by a decrease in the number of 2-mm ones. These results suggest that in individuals with a high AFC small follicles were more sensitive to grow in response to exogenous FSH stimulation than those from ewe lambs with lower AFC. The number of antral follicles counted by ultrasonography correlated positively with AMH plasma levels both before and after FSH administration. In addition, the evaluation of AMH plasma levels before the EFORT was positively correlated with the number of large follicles grown after exogenous FSH administration.

It is hypothesised that AMH attenuates antral follicle responsiveness to FSH in adults. Experiments conducted in mice have suggested that AMH not only inhibits growth initiation of primordial follicles (Durlinger *et al.* 1999, 2002*a*, 2002*b*), but also participates in regulation of the growth of preantral and small antral follicles by inhibiting their sensitivity to FSH (Durlinger *et al.* 2001). Both *in vitro* and *in vivo* studies seem to suggest that follicles are more sensitive to FSH in the absence of AMH (mice, Visser and Themmen 2005). In addition, a recent study evidenced that antral follicle responsiveness to FSH, as far as it takes into account the available pool of FSH-sensitive follicles, is negatively correlated with circulating AMH levels in normo-cycling women (Genro *et al.* 2011). These data apparently stand in contradiction to the evaluation of AMH as a reliable marker of ovarian response to hormonal stimulation in women (La Marca *et al.* 2007), cows (Ireland *et al.* 2011; Rico *et al.* 2012) and goats (Monniaux *et al.* 2011). These findings may result from the putative positive correlation between peripheral AMH levels and the pre-treatment number of small antral follicles (Genro *et al.* 2011). In small ruminants variations in ovarian responses to FSH are known to reflect the follicular population present during the initiation of treatment (Gonzalez-Bulnes *et al.* 2004; Veiga-Lopez *et al.* 2005; Menchaca *et al.* 2010). At prepubertal stages, when the pituitary–ovarian axis is generally regarded as being quiescent and circulating sex steroids are minimal, the relationship between follicle recruitment and circulating AMH is less clear. In a previous study, an increased rate of recruitment from the primordial pool was observed in AMH-null prepubertal mice (Durlinger *et al.* 1999). On the other hand, a recent study in humans indicates a strong positive correlation between circulating AMH and follicular recruitment before the onset of puberty (from birth to 9 years

old, Fleming *et al.* 2012). The possibility that AMH may play a permissive or even a stimulatory role in follicle recruitment and survival of early growing follicles has been also suggested by *in vitro* studies (human, Schmidt *et al.* 2005). These findings are in agreement with the results of the present study, where the highest levels of AMH were found in individuals showing the highest number of 2-mm follicles and exhibiting the best response to the EFORT in terms of follicle competence to grow in response to gonadotrophin stimulation.

Our study also showed that the ovarian phenotype expressed during the early prepubertal period can predict oocyte quality, as evaluated by *in vitro* developmental competence. Oocytes collected from ovaries with the highest AFC proved to be the most competent to develop into a blastocyst after incorporation into the *in vitro* production system. In addition, ovary weight was positively correlated with subsequent *in vitro* embryo output. These results confirm previous findings in adult ewes (Mossa *et al.* 2008). Follicle numbers have been positively associated with a variety of measures of fertility in single-ovulating species, like humans (Kupesic *et al.* 2003; Scheffer *et al.* 2003), cattle (Cushman *et al.* 2009) and sheep (Mossa *et al.* 2008). In young adult cattle, antral follicle number has been positively related to AMH levels, the number of healthy oocytes and follicles (Burns *et al.* 2005), corpus luteum functionality (Jimenez-Krassel *et al.* 2009), responsiveness to superovulation, oocyte quality and *in vitro* blastocyst development (Ireland *et al.* 2008). Cows with a high AFC had higher pregnancy rates, shorter calving-to-conception intervals and received fewer services during the breeding season, when compared with cows with low AFC (Ireland *et al.* 2011). It has been suggested that a low number of antral follicles would create a hormonal milieu that negatively affects ovarian function and oocyte quality (Jimenez-Krassel *et al.* 2009). Individual follicles from cattle with low AFC have higher follicular-fluid concentrations and produce more oestradiol than follicles from animals with high AFC (Ireland *et al.* 2009) and it has been speculated that this difference may be a consequence of higher circulating FSH concentrations in the low versus the high AFC groups. In addition, circulating androgen and progesterone concentrations are indeed higher in cattle with high compared with low AFC (Jimenez-Krassel *et al.* 2009).

It is noteworthy to stress that during the prepubertal period the hormonal milieu is markedly different from during adult life, and steroid production is minimal. In this study plasma oestradiol levels were undetectable in all the ewe lambs used for the *in vivo* trial. The full hierarchy of follicles within the ovary (i.e. large antral and dominant follicles) becomes established only after puberty, and follicles acquire their full (pituitary-controlled) steroidogenic competence only at the end of development. The ovarian scanning performed in this study revealed that, as expected, in the early prepubertal ovary the follicular population is mostly represented by follicles 2 mm in diameter.

The mechanisms of inter-follicular regulation are poorly understood (mice, Durlinger *et al.* 2002*a*; Da Silva-Buttkus *et al.* 2009), but it is likely that local autocrine and paracrine products, reflecting the different stages of follicular development, may influence the development of other follicles and thus oocyte quality. Further research is needed to elucidate the role of these putative paracrine factors during the very first stages of follicle development and how they can influence the ovarian milieu and the acquisition of oocyte developmental competence in a virtually steroid-free environment.

AFC is currently regarded as a reliable marker of the ovarian reserve both in adult women (Hansen *et al.* 2011) and in animal models (cattle, Ireland *et al.* 2008). In our study in prepubertal ovaries, AFC was not predictive of differences in either the number of healthy follicles or the size of the primordial follicle pool and no correlation was found between numbers of growing (from primary to late antral) and primordial follicles. Previous studies have shown that the number of growing follicles is correlated with the size of the primordial follicle stock (Gougeon 1996, Scheffer *et al.* 1999). In addition, a striking correlation has been found between ovarian volume and non-growing follicle population (Wallace and Kelsey 2004). The most likely explanation for our findings is 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. In infant boys and girls a postnatal pituitary activation has been described. In infant boys, it 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.* 2011), and this period is therefore considered to be an important phase in reproductive development in males. In infant girls, circulating FSH concentration transiently increases during postnatal pituitary activation (Beck-Peccoz *et al.* 1991), while AMH levels increase soon after birth, peaking a few months after birth (Hagen *et al.* 2010). However, the possible importance of this activation as regards female reproductive development is not understood.

We can thus speculate that during the prepubertal period follicular recruitment and development reflect the changes in the endocrine milieu and are preparatory to the subsequent development of reproductive function. A recent study showed that ewe lambs with the highest AMH plasma levels at 3.6 months were also the most precocious in terms of early puberty, resulting in the improved fertility observed when testing fertility of different ewes at the same age (Lahoz *et al.* 2012). Thus, AFC could represent a sign of follicular maturation in the prepubertal ovaries, and its close relationship with the non-growing follicle pool could develop only after the full establishment of reproductive activity.

In conclusion, our data show that AMH plasma levels are positively related to AFC and to the number of large follicles grown after exogenous FSH administration during the early prepubertal period. In addition, we showed that AFC can predict oocyte quality in prepubertal ovaries, reflecting an ovarian status suitable for follicular development. 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. Further studies are needed to investigate the implications on the reproductive performance of the significant inter-individual differences found in the present study in AFC and circulating AMH in the early prepubertal period.

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