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Clinical and cytogenetic studies in intersex ewes

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Abstract — Nine *Sarda* x *Lacaune* ewes with intersexual characteristics and an infertility condition at the reproductive anamnesis were analysed. In order to make a diagnosis, we have evaluated their behaviour and performed clinical and laparoscopic examination of the reproductive tract, as well as cytogenetic analysis. The ewes showed basically a female phenotype but a clinical examination revealed a different degree of masculinization in the morphology of external genital organs. A shorter vagina was observed in female-like ewes and a hypertrophic clitoris in male-like ewes. Laparoscopic analysis evidenced the presence of testis in seven individuals and, for two of them, the gonadal position was subcutaneous. Different male characteristics in the nine subjects, were also observed in their behaviour with a different degree of masculinization. Their blood samples were used for determining the percentage of male cells on lymphocytes chromosome spreads by using the C-banding technique. The haematopoietic chimeras (XX/XY) found in the lymphocytes confirmed the diagnosis of freemartinism for seven out of the nine subjects.

Key words: C-banding, intersex, sex chromosomes, sex determination, sheep

INTRODUCTION

Deviations from the normal chromosome constitution, which is homogametic (two X chromosomes) in females and heterogametic (XY) in males, can occur in several anomalies of the mammalian sex chromosomes. Eutherians (placental mammals) with a 45,X sex chromosomes karyotype show a female phenotype, which is known as Turner's syndrome in humans. Ninety percent of 45,X conceptuses result in spontaneous loss, generally of 28 weeks (powell 1999), while the surviving subjects present amenorrhoea and sterility. This syndrome has been rarely found in domestic animals (Gustavsson 1980; Iannuzzi *et al.* 2000; Makinen *et al.* 2001). In Klinefelter's syndrome (XXY), individuals are characterised by a male phenotype with hypogonadism, azoospermia and sterility (Polani *et al.* 1958; Jacobs and Strong 1959; Molteni *et al.* 1999).

These sex chromosome aberrations show that sex and male phenotype are strictly correlated to the presence of the Y chromosome, no matter how many copies of X are present (pask and graves 1999). The only rare exception is the sex reversal condition, where females are XY and males XX. Sex reversed animals are characterised by abnormal development of the genital tract with intersexual features (Pailhoux 2003; Vaughan *et al.* 2001) and infertility, (Boucekkine *et al.* 1994; Abdelmoula *et al.* 2003; Iannuzzi *et al.* 2001, 2004) which appears to be caused by deletions or mutations on genes of the sex determination pathway. Until recently, only transcription factor genes as *SRY, SOX9, DAX1* and *SF1* were known to be involved in the sex determination (Berta *et al.* 1990; Gubbay *et al.*, 1990; Koopman *et al.* 1990; Sinclair *et al.* 1990; Goster *et al.* 1992; Graves 1998; Meeks *et al.* 2003). However, Wnt-4 has been found to be a new sex determining signalling molecule, responsible for masculinization of XX mouse pups when deleted. On the other hand the over-expression of Wnt4 in XY mice results in a lower testosterone level and abnormal development of gonads (Vilain 2003).

The presence of a testis determining factor (TDF) on the Y chromosome allows testis determination and the development of male features. The SRY gene was isolated from this critical sex-determining region in both human (Gubbay *et al.* 1990; Sinclair *et al.* 1990) and several eutherian mammal species (Griffiths and Giwari 1993).

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SRY was found to be responsible for initiating testis development during mammalian embryogenesis (BERTA *et al*. 1990; KOOPMAN *et al.* 1990; SINCLAIR *et al*. 1990).

One of the most severe forms of sexual abnormalityies is freemartinism. Individuals affected by this anomaly, are XX/XY haemopoietic chimaeras and their development is the result of fusions in the placental circulation of at least one male and one female foetus. Females twins that can be influenced in their sexual development by the presence of males exhibit severe intersexuality presenting part of male and female sexual organs, and most of them are infertile. There is a dearth of information on freemartinism in *Ovine* for the low prevalence (1-7%) of this intersexuality condition in twins of different sex (DAIN 1971; SMITH *et al*. 2000, 2003). In contrast, it has been well studied in cattle where the anomaly affects 90% of the mixed sex twins (MARCUM 1974; KASTLI and HALL 1978; MCENTEE 1990). However, compared to cattle, freemartinism in sheep commonly occurs with an extreme masculinization of the reproductive tract (WILKIES *et al.* 1978).

The aim of this research was to study nine ewes twins of males with intersexual characteristics in order to improve our knowledge of freemartinism in sheep. Since the ewes were twins of males, we hypothesiszed that they could be freemartins. In order to make a conclusive diagnosis, we carried out both clinical (*including behavioural and laparoscopic examination of reproductive tract*) and cytogenetic analyses.

MATERIALS AND METHODS

The study was performed on nine 2-3 yrs old *Sarda* x *Lacaune* ewes, obtained from two different flocks in northern Sardinia during the breeding season. All the subjects presented an infertility condition.

Anatomical examination - We carried out clinical and gynaecological examinations in the nine animals. We also recorded abnormal characteristics in the shape and size of vulva and clitoris, in the vagina length, teat size, in the presence or absence and position of gonads.

Laparoscopic analysis - Laparoscopic examinations were performed on nine ewes after a 24 hour period of food deprivation, while water was withheld for 12 hours. They were kept standing. Paravertebral anaesthesia was induced in T13, L1, L2 and L3 nerves with 2% lidocaine®. The cannula-trocar for laparoscopy analysis was inserted on the right side. The reproductive tract was inspected after abdominal insufflation of CO_2 using a laparoscopy (Wolf 4939.31 lumina SL-telescope, 10mm Ø, 10° vision) with a light source (generator prox 5006, Wolf) and a video camera (Multi Endocam 5502, Wolf). Only one ewe (LS4641) was slaughtered, and the internal reproductive apparatus was examined.

Behavioural study - The behavioural patterns exhibited by the nine ewes interacting with both females and males were reported after monitoring the ewes every day for 30 minutes during the breeding season for a period of 40 days. The intersex ewes were individually presented to a ram of known libido to determine whether they would exhibit female behaviour. Normal ewes in oestrous were also used to evaluate male-like behaviour. The presence of approach, foreplay, mounting and thrusting was recorded.

Cytogenetic analysis - Peripheral blood was collected by jugular venipuncture from the nine intersexes in a Lithium Heparin treated tube. The whole blood was cultured at 38° C in an 5% CO_2 atmosphere for 72 hours in RPMI medium supplemented with 20% FCS, penicillin (0.6 mg/ml), streptomycin (0.5mg/ml), L-glutamine (0.1g/ml) and Concanavalin A (Sigma, 15 μg/ml) as mitogen. Cell were harvested after a Colcemid (1 μg/ml) treatment (2 h) and processed following standard procedures. Briefly, the cells were treated with KCl 0.075M hypotonic solution for 25 min and fixed three times in 3:1 methanol:acetic acid. Three drops of fixed cells were spread on wet and cold slides and air dried. Slides were aged one week at room temperature and treated for the CBA-banding technique (IANNUZZI 2003). At least 100 cells for each animal were studied to get the percentage of male and female cells.

RESULTS

Anatomical examination - At birth all the ewes showed a female phenotype. Afterwards, they began to manifest male characteristics with few differences among subjects. As shown in table 1, the external genitalia of the nine ewes were basically female with the presence of vestibule and vulva, of variable shape and size: development was normal in LS4309 and LS1910 ewes; hypoplasic/infantile-like external genitalia reported in subjects

Table 1 — Phenotypic sex and external genitalia description by anatomical examination in the nine ewes.

Ovines	Twins: n° and sex	Phenotypic sex	Esternal genitalia
LS4309	1 male	female	Normal Vulva
LS1910	1 male	female	Normal Vulva
LS4205	0	female	Hypoplasic Vulva
LS840	1 male	female	Hypoplasic Vulva
LS1171	1 male + 1 female	female	Hypoplasic Vulva
LS5790	1 female	female	Hypoplasic Vulva
LS590	2 male	female	Hypoplasic Vulva
LS4641	1 male	female	Vulva, Hypertrophic Clitoris
S197	1 male	female	Vulva, Hypertrophic Clitoris

LS4205, LS840, LS1171, LS5790, LS590; in the last two intersexes, LS4641 and LS197, juxtaposition of the *vulvae labia* was not possible due to the presence of a hypertrophic clitoris visible in its distal part (Figure 1). Mammary glands and nipples were smaller than in normal ewes of the same age.

Laparoscopic analysis - Subjects LS4309 and LS1910 presented normal female-like gonads by macroscopical examination. The former showed hypoplasic ovaries, without any luteal tissue, and some follicles in the right ovary were 2 mm in diameter, the rest of the genital tract including the vagina was absent. In the latter we observed ovaries apparently well-developed with follicles and *corpora luteum* on the left side. Besides, we also detected that the hypoplasic salpinges without an *infundibulum* were occluded in the ovarian extremity of the *ampulla* tract in ovine S4309. The uterus showed segmentary aplasia in the third medium of the left horn, causing a mucometra for the collection of gland secretion in the apical tract. Vagina and cervix were normal.

With the exception of LS4309 and LS1910, all subjects with male-like gonads were provided of epididymis, deferens and ampullae of deferens. Ewes LS4205, LS840 and LS1171 showed abdominal testis while in LS5790 and LS590 testis were in the canal inguinal.

As displayed in Figure 1, the gonads in the ovine LS4641 and also in the S197 appeared as subcutaneous testis. Confirmed by direct inspection (Figure 2b), the sex apparatus of ewe LS4641 showed vestibule and vulva and also marked male characteristics in the development of the testis due to the presence of epididymis, deferent and seminal vesicles.

Behavioural study - The appearance in all the nine ewes began to acquire masculine characteristics since the beginning of our study. This change was almost imperceptible in two of them (LS4309 and LS1910) with female-like ovaries that grew more similar to females; more evident in the other group of animal (LS4205, LS840, LS1171, LS5790 and LS590) that grew with few male features but it was strongly evident in the two intersexes (S197 and LS4641) with subcutaneous testicle, at adult age they acquired a typical male aspect in their head shape which began to be more flat and masculine as a typical ram profile (Figure 1a).

When each of the intersex observed for 40 days during the breading season was introduced into a flock, none exhibited any normal female responses in the presence of a fertile ram. In particular, some subjects (LS4205, LS840, LS1171, LS5790 and LS590) showed prevalently male behaviour in line with presence of testis in abdominal and inguinal position, respectively. The last two animals (S197 and LS4641) showed usually an aggressive and competitive behaviour in presence of a normal ram, according to the presence of subcutaneous testicle. In presence of an oestrus ewes, they also exhibited significant masculine actions as the mimicked of the approach of courtship and mounting.

Cytogenetic analysis - Cytogenetic analysis in all ewes revealed haematopoietic sex chromosome chimeras 54, XX/XY (Figure 3) with different percentages of male cells (Table 2).

DISCUSSION

The nine sheep studied, clearly displayed characteristics of intersexuality with different degree of masculinization. Two of them had basically a female phenotype, five ewes showed few male characteristics in both genital tract and behaviour, finally a marked male phenotype and behaviour occurred in the remaining two (table 1 and 2). These results were in accordance with the laparoscopic investigation, while the cytogenetic analysis supported the diagnosis of freemartinism for seven subjects with testicles out of the nine studied (Table 2). In the two ewes presenting female-like ovaries, the characteristics of the genital tract are different from what is commonly observed in freemartin sheep. In the sheep LS1910, the conformation of the genital tract could be compared to the morphological characters usually observed in bovine affected by “White heifer disease”. This

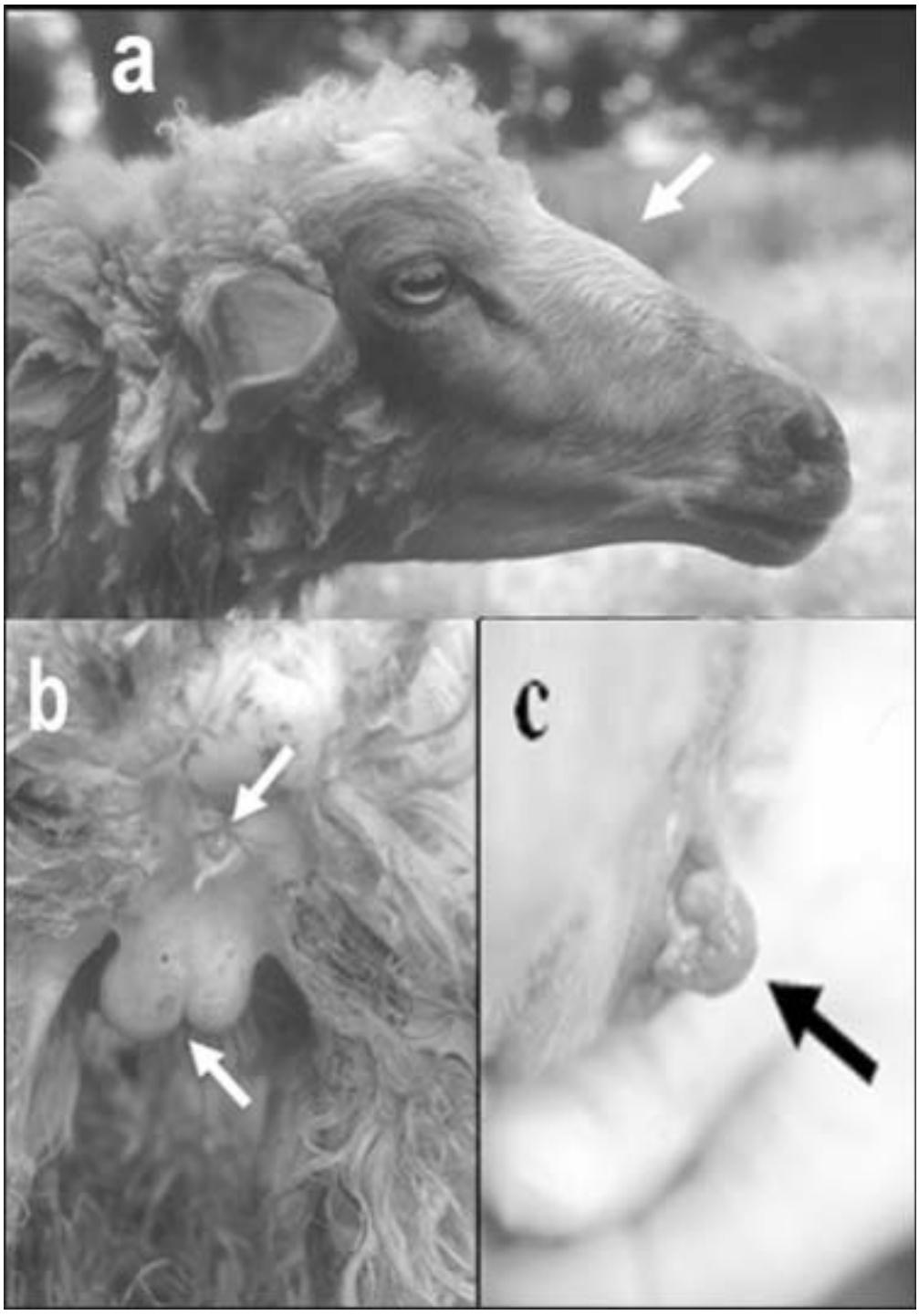

Fig. 1 — Phenotype of the ewe LS4641 Note the typical ram profile (**a**), the external genitalia showing subcutaneous gonads and hypertrophic clitoris (**b**), the latter shown in detail (**c**)

pathology is characterized by a prenatal ending in the development of Muller duct and can be distinguished from the freemartinism for the occurrence of normal ovaries, vestibule and vulva. The haematopoietic chimeras could be explained by the exchange of factors among the twins after the normal development of the gonads able to influence sexual differentiation. Being linked to a gene coding for the white coat, the disorder should be investigated at the genetic level. Indeed LS4309 showed features more similar to those usually observed in freemartin cattle.

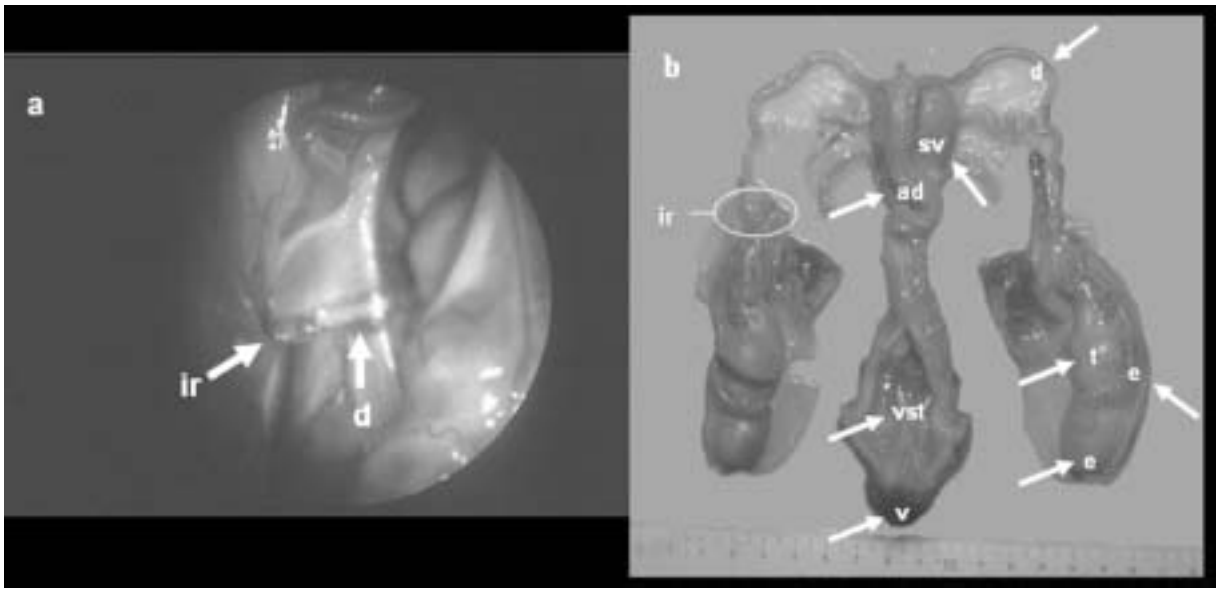

Fig. 2 — Details of the intersex LS 4641 A Laparoscopy view of the deferent (**d**) that enters the internal inguinal ring (**ir**) B Genital apparatus with visible testicle (**t**), epididymis (**e**), deferent (**d**), seminal vesicles (**sv**), ampoules of deferent (**ad**), vestibule (**vst**), vulva (**v**) and internal inguinal ring (**ir**)

Our results also confirmed the extreme masculinization of the reproductive tract in freemartin sheep compared to that observed in cattle (Wilkies *et al.* 1978). Despite the fact that the reported incidence of freemartinism in sheep twins with different sex is less than 7% (Dain 1971; Long 1980; Matejka *et al.* 1987; Hanrahan *et al.* 1990; Gill and Davies 1991), this sexual congenital pathology is nevertheless the cause of infertility.

Behavioural analysis is in line with the degree of inter-sex condition. None of the freemartin ewes exhibited a normal female response in the presence of a fertile ram, including the two intersexes with female-like phenotype and ovaries. The presence of a mucometra with the occurrence of a *corpus luteum*, apparently well formed could interfere on the prostaglandin secretion, hence the inhibitition of the normal oestrus. This could explain the lack of interaction with a ram. Nevertheless, the absence of interest by a normal male for the sheep LS4309 could be explained by the presence of hypoplasic and consequently not functional gonads. Moreover, those ewes with testis (table 2) manifested male behaviour, especially the two freemartin ewes with subcutaneous testicles which exhibited a significantly masculine behaviour in the presence of oestrus ewes, as well as aggressive and competitive action in the presence of a ram. These data are in accordance with the presence of a male-like internal genital tract, as displayed in Figure 2b for subject LS4641.

Cytogenetic analysis was helpful in establishing a definitive diagnosis for most of the subjects (Figure 3, Table 2) for the presence of a haematopoietic sex chromosome chimera 2n=54, XX/XY. Like most mammals, in sheep the Y chromosome is the smallest one and heterochromatic, although it shows a stronger C-positive band on the

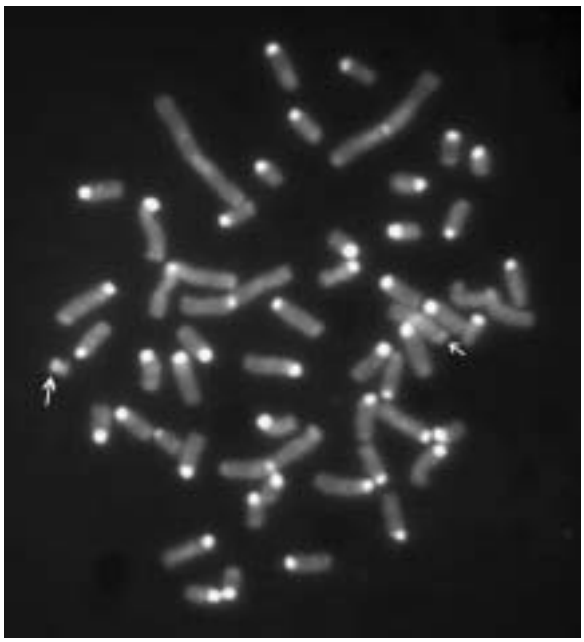

Fig. 3 — CBA-banded metaphase plate in a male cell of ewe LS840. X (small arrow) and Y (large arrow) chromosomes are indicated.

Table 2 — Type of gonads and localisation; Mullerian duct (*vagina present for maximum 1-2 cm) and Wolffian duct derived; percentage of lymphocyte XY in the nine intersexes.

Ovines	Gonad localisation	Developed from		
		Mullerian duct	Wolffian duct	Lymphocyte XY %
LS4309	Hypoplasic Ovaries-like (abdominal)	Vagina absent, Aplasic uterus		28
LS1910	Ovaries-like (abdominal)	Vagina, Segmentar aplasic uterus		63
LS4205	Testicles (abdominal)	Vagina absent*	Epididymis, Deferens, Ampullae	65
LS840	Testicles (abdominal)	Vagina absent*	Epididymis, Deferens, Ampullae	57
LS1171	Testicles (abdominal)	Vagina absent*	Epididymis, Deferens, Ampullae	17
LS5790	Testicles (inguinal)	Vagina absent*	Epididymis, Deferens, Ampullae	52
LS590	Testicles (inguinal)	Vagina absent*	Epididymis, Deferens, Ampullae	76
LS4641	Testicles (subcutaneous)	Vagina absent*	Epididymis, Deferens, Ampullae, Seminal Vesicles	40
S197	Testicles (subcutaneous)	Vagina absent*	Epididymis, Deferens, Ampullae Seminal Vesicles	55

p-arm (Figure 3). The X chromosome is an acrocentric chromosome with evident p-arm and C-band negative (Figure 3). The C-banding technique is normally applied in all suspected sex chromosome abnormalities since it allows sex chromosomes (especially acrocentric) to be easily distinguished from the autosomes (IANNUZZI 2003; IANNUZZI *et al.* 2000, 2001, 2004, 2005).

As we expected, all nine subjects showed haematopoietic chimeras with different percentages of male lymphocytes. In subject LS4309, with female-like ovaries, we found a lower percentage of male cells than those observed in the other subject with female phenotype (LS1910). Furthermore in LS4641 ovine, we found the presence of 40% of male cells that was not in accordance with the male-like phenotype with subcutaneous testicles and male behaviour. We observed a discrepancy among anatomical, laparoscopic and behavioural data compared to cytogenetic analysis. As in sheep, in bovine freemartin the degree of masculinization is not related to the level of XX/XY chimaerism (MARCUM 1974; VIGIER *et al.* 1972). Different percentages of male cells and different degrees of internal sex adducts have also been described in river buffalo freemartin females which were found sterile even when the percentages of male cells were very low (IANNUZZI *et al.* 2005). It is possible that the different percentages of male cells are due to different times of placental anastomosis formation. Furthermore, gonad differentiation seems to start earlier (one week) in males than in females (RUVINSKY and SPICER 1999).

It has been indicated that in eutherian mammals the development of scrotum and mammary glands is hormone-dependent (JACOBS and STRONG 1959; FORD *et al.* 1959). However, in less evoluted mammals such as the monotremes, genital organs like the pouch, mammary glands and scrotum develop from abdominal skin and are regulated by the sex gene pathway only (FOSTER *et al.* 1992; WAI-SUM *et al.* 1988). Marsupial intersexes offer a model to study sexual differentiation (SHARMAN *et al.* 1990; COOPER *et al.* 1993; HUGHES *et al.* 1993; WATSON *et al.* 2000; SANTUCCIU *et al.* 2003) and can be very useful for the study of intersex eutherian mammals.

Abnormal patterns of steroid secretion on freemartin ewes were recently reported. Several masculinised freemartins show a testosterone circulating concentration significantly higher than in normal ewes (PARKINSON *et al.* 2001). Most reports suggest that bovine freemartins have low circulating testosterone levels (RANDEL *et al.* 1971; SABA *et al.* 1975). Further histological investigations are needed to get a conclusive diagnosis.

In conclusion, our work show that all the nine sheep showing intersexual characteristics presenting a different degree of masculinization. However, no evident correlation was found between the haematopoietic chimeras and the intersexual phenotype.

Future investigation on hormone profiles and -in particular- the post-mortem histological examination of ovarian parenchyma structure of the two subjects presenting female-like gonads will contribute to clarify and confirm our hypothesis.

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