Persistent Müllerian Duct Syndrome in a German Shepherd Dog

Lisa De Lorenzi\textsuperscript{a}  Silvana Arrighi\textsuperscript{b}  Debora Groppetti\textsuperscript{c}  Stefania Bonacina\textsuperscript{a}  Pietro Parma\textsuperscript{a}

Departments of \textsuperscript{a}Agricultural and Environmental Sciences, \textsuperscript{b}Health, Animal Science, and Food Safety, and \textsuperscript{c}Veterinary Medicine, Milano University, Milan, Italy

**Abstract**

In mammals, the regression of the müllerian ducts is regulated by the action of the AMH hormone which is produced by testes during embryonic development. The action of this hormone is mediated by the only known receptor AMHR2. Mutations occurring in the \textit{AMH} hormone and/or in the \textit{AMHR2} receptor gene cause the lack of regression of müllerian ducts, which may therefore persist even in male embryos carrying a XY chromosomal arrangement. This is known as the persistent müllerian duct syndrome (PMDS). A female German Shepherd dog was referred to the veterinary clinic because of urinary incontinence. She also showed an anatomical structure that protruded from and enlarged the vulvar labia. From the morphological appearance, one gonad resembled an ovary and the other a testicle. The histological examination instead showed that the gonads were both testes with an underdeveloped parenchyma and without signs of spermatogenesis. No alterations were found with regard to the uterus which showed a correctly developed body, cervix, and horns. Genetic analysis, performed on DNA extracted from blood, showed (i) the presence of both X and Y chromosomes, (ii) the absence of chromosome XX/XY chromosomerism, (iii) a normal \textit{SRY} gene coding sequence, (iv) a normal \textit{AMHR2} gene coding sequence, and (v) a normal \textit{AMH} gene coding sequence. In this study, we report and characterize a new case of PMDS in a dog excluding that the only mutation hitherto found in the \textit{AMHR2} gene is responsible for the observed phenotype.

In mammals, the process leading to the formation of organs and reproductive traits occurs after the determination of gonadic sex and strongly depends on the regression of 1 of the 2 primordial ducts (müllerian and wolffian ducts) and on the maintenance of the other one. In males, only the organs derived from the wolffian ducts are maintained as the consequence of the production of 2 testis-derived androgens: testosterone and anti-müllerian hormone (AMH, also called MIS). In females, the absence of androgens allows the development of the organs derived from the müllerian ducts and the regression of the wolffian ducts [Jost, 1947]. Moreover, a recent report states that the regression of the wolffian ducts is not a passive event as a result of lack of testosterone support but it is actively promoted by the COUP-TFII protein [Zhao et al., 2017].

**Keywords**

\textit{AMH} · \textit{AMHR2} · German Shepherd dog · Sequence analysis
The AMH hormone is a member of the TGF-β family which functions through a dimeric receptor composed of a specific receptor (type II receptor) and a receptor common to all members of the TGF family (type I receptor).

There are rare cases of XY embryos that, although they had functioning testes, did not follow a regular path of differentiation and retained derivatives of the müllerian ducts that did not regress. These subjects are then classified as having persistent müllerian duct syndrome (PMDS). In humans, the cause of the onset of this syndrome has been identified both in AMH gene mutations [Knebelmann et al., 1991] and in the presence of mutations involving the receptor linked to the androgen (AMHR2) [Imbeaud et al., 1995]. Approximately 90% of human PMDS cases have a mutation of either the AMH gene or the specific AMHR2 receptor. The remaining 10% of cases are idiopathic and the cause remains unknown [Picard et al., 2017].

In dogs, this syndrome was described for the first time in the 1980s in the Miniature Schnauzer [Marshallet et al., 1982] and then later identified in other breeds [Nickel et al., 1992; Kuiper et al., 2004]. More recently in dogs, PMDS has been related to an AMH receptor mutation [Wu et al., 2009]. This mutation has been identified in the Miniature Schnauzer, and therefore today there is a diagnostic test to identify carrier subjects belonging to this breed [Pujar and Meyers-Wallen, 2009]. To our knowledge, at the current time there are neither known PMDS cases related to this mutation in other breeds nor have other mutations been identified that lead to this syndrome.

**Materials and Methods**

**Case Report**

A female German Shepherd dog was referred to the veterinary clinic because of urinary incontinence. She also showed an anatomical structure that protruded from and enlarged the vulvar labia (Fig. 1a) that was observed since its adoption at 60 days of age. The external genitalia might have looked like vulvar labia or a kind of prepuce that partially covered a hypospadiac penis (Fig. 1b). A total body computed tomography showed an anomalous course of both ureters forming a kneeling curvature at the bladder entrance, both dilated but not ectopic. Oval structures bilateral to the bladder, uterus, and a blind enlarged vagina were also recorded (Fig. 1c, d).

![Fig. 1. Macroscopic aspect of the reproductive organs of the dog. a Hypoplastic penis protruding from an ambiguous vulvar labia/prepuce. b Urethral opening defines the previous structure as a penis. c Testicular aspect of the right ovotestis gonad (ot) with pampiniform plexus and connected to the uterine horn (ut). d Excised genitalia. ot, bilateral ovotestis gonads; ut, uterus; p, penis.](image-url)
Fig. 2. Histological examination of the genital organs of the dog. 

a Both gonads share a parenchyma of testicular aspect, enveloped within a thick albuginea (asterisks) and made up by very small seminiferous tubules. b, c Within the seminiferous tubules no evidence of any spermatogenetic activity can be detected in a way to mimic the prepuberal testis. Interstitial Leydig cells are present (arrows). d Excurrent ducts. On one side, ductuli efferentes of usual aspect, lined by a normally developed ciliated epithelium, can be seen. e, f A well-developed, although inactive, epididymis is present. The epididymal epithelium lacked stereocilia (f). g Vas deferens (d) is accompanied by a uterine horn (ut). h, i Both the uterine wall (h) and the vas deferens (i) show an underdeveloped aspect. All tracts of the male excurrent duct were devoid of spermatozoa. j, k The normal aspect of the cervix can be seen.
Surgery was postponed to allow adequate body growth until around 7 months of age when a bloody-serous vulvar secretion due to bacterial cystitis was misinterpreted as the beginning of heat. At this time, exploratory laparotomy was performed and the reproductive tract removed by standard surgical methods for ovariohysterectomy and submitted for histologic examination. The penis was excised and the vaginal vestibule reconstructed as a female phenotype.

Histology

After ovariohysterectomy, genitalia were gently dissected from one another and processed for histology. Fragments of the specimens were fixed by immersion in 4% paraformaldehyde in 0.01 M PBS pH 7.4 for 24 h at 4°C, dehydrated in a graded series of ethanol, cleared with xylene, and embedded in paraffin. Microtome sections (4 μm thick) were routinely stained by hematoxylin/eosin (HE) technique for the microscopic evaluation of the detected genital organs. Observations were conducted under an Olympus BX51 microscope equipped with a digital camera and DP software (Olympus, Italy) for computer-assisted image acquirement and managing.

Genetic Analysis

As template for the PCR amplifications of the regions of interest, we used DNA extracted from blood using a commercial kit (UltraClean Blood Spin, MoBio). For SRY gene amplification we used the following primers: DogSRY2-F: 5′-gcgctgtaattttacgcttc-3′ and DogSRY2-R: 5′-agcaagtttccaacgctcat-3′. The presence of the sex chromosomes was verified by PCR analysis of the AMELX/Y genes as reported [Yan et al., 2003]. For amplifications of the coding region of AMH and AMHR2, the primers reported in Table 1 were used. All PCRs were done using the manufacturer’s instructions (AmpliTaq, Promega). Finally, the PCR products were purified with the Ultraclean PCR Clean-Up kit (MoBio), and both strands were directly sequenced.

Cytogenetic Analysis

Giemsa-stained metaphases, obtained from peripheral blood lymphocyte cultures, were prepared following standard methods [De Grouchy et al., 1964].

Results and Discussion

Histological examination of the gonads revealed paired testes, enveloped within a thick albuginea. The testicular parenchyma was made up by very small seminiferous tubules (Fig. 2a) with no evidence of any spermatogenetic activity (Fig. 2b, c) in a way to mimic the prepuberal testis. Interstitial Leydig cells could be detected (Fig. 2c), but neither of the 2 testes showed intra-testicular ducts, i.e., rete testis. The extra-testicular ducts looked differently on the 2 sides.

On one side, a well-developed, although inactive, epididymis was present. It started with ductuli efferentes of usual aspect, lined by a normally developed ciliated epithelium (Fig. 2d). The different regions of the epididymis followed (Fig. 2e), but the epididymal epithelium lacked stereocilia (Fig. 2f). More distally, the vas deferens was accompanied by one uterine horn (Fig. 2g), and no uterine tube was detected. Both the uterine wall and the vas deferens had an underdeveloped aspect (Fig. 2h, i). All tracts of the male excurrent duct were devoid of spermatozoa.
On the contralateral side, neither epididymis nor uterine tube was present, and the excurrent duct was represented only by 1 uterine horn. Where the 2 uterine horns gathered together to form the uterine corpus, on one side it was still possible to notice the vas deferens close to the uterus. The cervix was macro- and microscopically normally conformed (Fig. 2 j, k).

PCR analyses performed on blood-derived DNA showed the presence of the full coding sequence of the SRY gene and the presence of X and Y chromosomes (Fig. 3 a, b). Moreover, SRY gene sequencing revealed no differences from the reference sequence (AF107021). The subject under investigation was genetically male. The analysis of more than 100 metaphase (Fig. 3 c) highlighted the presence of cells all with a XY karyotype. The presence of more than 3% of XX cells is excluded with a probability of 95% [Hook, 1997].

The action of the AMH gene is mediated through its only known receptor AMHR2. For dog, there is only one predicted sequence, XM_543632, in the databases. This sequence contains the entire AMHR2 coding region, but a comparison with the 7 ESTs available (CX85782, DN52582, CO627226, CO596251, CO602819, CO597408, CO601090) points out a significant difference in the 3’ region of the coding region. The XM_543632 sequence contains 2 unknown bases (NN), while in all ESTs available, these 2 bases are not present. The consequence is a different termination of the coding sequence (Fig. 4a) which is reflected in a different protein sequence at the NH2 end. The difficulty to define the terminal part of the coding sequence of this gene is also demonstrated by another predicted sequence, ENSCAFT00000011129. In this case, the translation of another protein is expected (Fig. 4b). Finally, the available sequence (XM_543632) involves an ATG codon that appears to be used prior to the start codon in other species (Fig. 4c). Therefore, all predicted regions were sequenced using the primers reported in Table 1.

All hypothetically coding regions were sequenced and 2 variants were noticed: a heterozygous C>T change in exon 7 and a heterozygous C>A change in exon 6. In both cases there is no amino acid change, and the 2 variants are now reported in the SNP database with the codes rs23409994 and rs851957017.

The available GenBank sequence (NM_001314127) derives from mRNA KP223256 and contains the whole coding region. However, sequencing is complicated by the presence of a genomic gap positioned between exons 4 and 5 that highly interferes in the choice of primers for PCR amplifications. Primer selection is further complicated by the presence of GC-rich sequences. For these reasons we were able to sequence the first 831 bp of the coding region, but the last 888 bp (whole exon 5 and
21 bp at 3′ end of exon 4) have not been sequenced. The results showed no differences with the reference genomic sequences.

The PMDS syndrome is well described in humans in which a genotype/phenotype relationship has been verified for mutations of the AMH gene (PMDS type I) [Knebelmann et al., 1991] and of the AMHR2 receptor (PMDS type II) [Imbeaud et al., 1995]. This syndrome has also been identified in goat [Haibel and Rojko, 1990], cat [Schulman and Levine, 1989], cattle [Panasiewicz et al., 2015], and dog [Wu et al., 2009]. However, in the latter species only one mutation has been identified in AMHR2 to be causative; this mutation, however, seems to be related only to dogs belonging to the Miniature Schnauzer breed.

This study confirms that the dog represents an excellent animal model for the study of pathologies linked to sex differentiation and/or sex determination as it spontaneously presents a considerable number of abnormal subjects belonging to different breeds. In addition, the ever-increasing availability of genetic information allows very detailed and precise analyses.

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Statement of Ethics


Disclosure Statement

The authors have no conflicts of interest to declare.
References


