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Immunohistochemical insights into a hidden pathology: canine cryptorchidism

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Abstract

Cryptorchidism is a common disorder in the canine population with some aspects still unclear. Although the bilateral condition is known to lead to fertility problems and predisposition to testicular cancer, the neoplastic risk for scrotal testis in unilateral cryptorchid dog is controversial. Therefore, the therapeutic approach to the canine unilateral cryptorchid is arbitrary so far.

This study aimed to investigate precancerous testicular lesions, such as immaturity and atrophy, and compare them in scrotal and undescended testes using an in-depth diagnostic analysis based on immunophenotypic patterns. With this purpose, 26 adult male dogs of different ages and breeds, affected by unilateral or bilateral cryptorchidism were enrolled. After surgical removal, testes were examined immunohistochemically to assess their positivity for specific markers of the canine foetal/neonatal period, that is vimentin (VIM), cytokeratin (CK), desmin (DES), inhibin-α (INH), and anti-Müllerian hormone (AMH) in Sertoli cells, and placental alkaline phosphatase (PLAP) in germ cells. Except for the ubiquitous VIM, all the markers were more expressed in neoplastic gonads compared to healthy ones ($P < 0.05$). Similarly, testes detected with Sertoli cell-only tubules as well as with Sertoli cells hyperplasia showed higher expression than gonads without such alterations for CK, DES, AMH and PLAP, and for CK and DES, respectively ($P < 0.05$). The same trend was observed in undescended respect to scrotal testes even though their positivity was significant only for DES, INH and AMH ($P < 0.05$). Immunohistochemical positivity found in scrotal testes of unilateral

cryptorchid dogs, even in absence of detectable anatomical abnormalities, was suggestive of precancerous lesions.

Despite the limited sample size, this study could help to clarify the predisposition to neoplastic development in normally descended testes. These markers expression in adult life could allow identifying the early stages of the testicular carcinogenesis process besides suggesting a precautionary bilateral surgical approach in unilateral cryptorchid dogs.

Keywords: Atrophy, Cryptorchidism, Dog, Immunohistochemistry, Testes

1. Introduction

Cryptorchidism defines a congenital developmental defect often detected in male dogs, that implies the failure of one or both testes to descend into the scrotum. Its incidence varies considerably among studies from 0.8 to 9.7% [1,2]. Increased risk of testicular neoplasia [2] and spermatic cord torsion [3] are the most relevant consequences of cryptorchidism, although fertility impairment was also reported [4]. Over the last decades, the number of men and dogs affected by cryptorchidism has critically increased, probably due to the greater exposure to environmental endocrine disruptor chemicals [5] which both share. Indeed, endocrine and environmental interferences during foetal life can cause several disorders, including abnormal gonadal development and altered testicular descent both in humans and domestic animals [6,7].

Cryptorchidism in humans has been associated with testicular dysgenesis syndrome (TDS) that primarily results in signs of testicular atrophy and immaturity [8,9]. The presence of Sertoli cell-only tubules (SCO), which are seminiferous tubules composed only of Sertoli cells without mature germ cells, is highly suggestive both of disorders affecting spermatogenesis [10] and testicular atrophy [8]. In cryptorchid men, SCO tubules were found both in undescended and scrotal contralateral testes [8,11,12]. In dogs, previous studies reported SCO tubules only in retained gonads and in atrophic testes of non-cryptorchid patients suspected of TDS [13,14]. Atrophic lesions such as SCO tubules were also described in testicular parenchyma of elderly man [15] and ageing dogs [16] related to a reduction of germ cells in seminiferous tubules [11]. In both cryptorchid and old patients, atrophic lesions are particularly important since they are considered a risk factor for testicular cancer development [17,18].

Despite its rising incidence, many aspects of the cryptorchidism in canine species remain unclear such as the assumptive predisposition of the scrotal contralateral testis to develop neoplasia [14,19]. Such unpredictability creates decision problems to clinicians regarding the appropriate

therapeutic management of the patients. To date, the best approach to canine cryptorchidism is surgery [20]. However, in case of a single retained testis, whether is better uni- or bi-lateral orchiectomy is still debated [14,20].

In this uncertain context, immunohistochemistry could contribute to enhancing understanding of histopathological features and early changes occurring in cryptorchid dog testes, even before a full-blown neoplastic transformation. Immunohistochemistry is especially valuable for investigating testicular immaturity. Indeed, cellular markers that are distinctive of definite periods during testicular development have been explored both in humans [21-23] and dogs [24,25-28]. Since immature aspects can involve both seminiferous tubules structure and spermatogenesis, we used either vimentin (VIM), cytokeratins (CKs), desmin (DES), anti-Müllerian hormone (AMH) and Inhibin-α (INH) for studying Sertoli cells, and placental alkaline phosphatase (PLAP) to examine germ cells. All these markers have been previously demonstrated in dogs [13,24,25-28]. Except for VIM, which is still present in adult life [24], the other markers are specific to the foetal/neonatal period in dogs [24,28]. CK and DES are Sertoli cells cytoskeletal markers whose expression characterize foetal but not adult life [28]. AMH and INH are hormonal markers expressed by Sertoli cells up to 45 days post-natal period in dogs [25,26]. Afterwards, INH continues to be produced by Leydig cells [24,27] while AMH can't be detected over 120 days from birth [25]. CK, DES, AMH and INH re-expression in adult life is abnormal and indicates an immature phenotype of Sertoli cells [25,26] related both to atrophy [28] and Sertoli cell tumours development [24,29]. Concerning germ cells, in physiological condition PLAP is expressed in man [30] and dogs [28] only by gonocytes, that are precursors of germ cells evolving into spermatogonia before birth. After birth, PLAP positivity in both species is highly suggestive of spermatogenesis disorders that predispose to testicular germ cell cancer [13,31,32]. In fact, developmental interferences could result in gonocytes transformation into *carcinoma in situ* cells (CIS cells) that express PLAP and act as precursor of tumorigenesis process [32,33].

To the best of our knowledge, there are no references on this topic other than a single study on unilateral cryptorchid dogs. The latter found positivity to INH and PLAP in canine retained testes but failed to detect any marker of immaturity in contralateral descended gonads. The caseload at issue was representative of a few and young dogs, between 1 and 2 ys of age [14].

The present study aimed to insight into early testicular precancerous clues that we speculated may involve both cryptorchid and contralateral testes in dogs. With this purpose, gonads from uni- or bi-lateral cryptorchid dogs of different age were analysed through histology and immunohistochemistry.

2. Materials and methods

2.1. Animals

In the present study, cryptorchid dogs attending to the Reproduction Unit of the Veterinary Teaching Hospital (VTH) of the Università degli Studi di Milano were retrospectively considered. Data concerning breed, age and body weight were available in all dogs, as well as their clinical presentation, haematological examination, and ultrasound analysis for diagnosis of testes location. Only dogs submitted to bi-lateral orchiectomy and histologic exam of both testes were included.

2.2. Histology

From the archive of the Pathology Unit of VTH, histologic slides and paraffin blocks related to the cases enrolled in the study were retrieved. All testes had been submitted to the lab immediately after surgery, longitudinally sectioned on the midline and fixed in 10% neutral buffered formalin. From all testes a complete longitudinal section was obtained and then routinely processed for histology. Sections (4 µm) were cut from paraffin wax blocks and stained with haematoxylin and eosin (HE) for further histological examination particularly focusing on: seminiferous tubules lined only by Sertoli cells (SCO), precursors of spermatozoa, tubules with complete spermatogenesis, Sertoli and Leydig cells hyperplasia (SCH and LCH, respectively), and neoplastic lesions such as seminoma (SEM), Sertoli cell tumour (SCT), mixed seminoma/Sertoli cell tumour (SEM/SCT), and interstitial (Leydig) cell tumour (LCT). Histotype diagnosis was based on guidelines proposed by the World Health Organization [34].

2.3. Immunohistochemistry

Further serial sections 5 µm thick were obtained from paraffin blocks and immunohistochemically tested with the avidin-biotin-peroxidase complex (ABC) procedure [35] using a commercial immunoperoxidase kit (Vectastain Standard Elite; Vector Laboratories, Burlingame, CA, USA). Sections were dewaxed, treated with hydrogen peroxide 0.5% in methanol for 20 min, and rehydrated. Details of the primary antibodies used, target cells, source, antigen retrieval methods and dilutions are reported in Table 1. All the antibodies employed in the present study were already demonstrated as reactive on targeted canine tissues in previous studies [24,25,26,36,37].

After antigen retrieval, the sections were incubated for 30 min in normal horse serum (diluted 1:60). Primary antibodies, diluted in Tris buffer (pH 7.6, 1.0 M), were incubated at 4C overnight. Then, after washing in Tris buffer, sections were covered with the secondary biotinylated antibody (Vector Laboratories, Burlingame, CA, USA), diluted 1 in 200 and incubated at room temperature for 30 min. Secondary antibody was an anti-mouse IgG made in horse, except for the sections analysed for anti-Müllerian hormone that were covered with an anti-goat IgG made in horse. After washing, peroxidase-conjugated ABC (Vector Laboratories, Burlingame, CA, USA) 1:100 diluted, was allowed to react at room temperature for 30 min. The immunohistochemical reaction was developed with 3-amino-9-ethylcarbazole (Vector Laboratories). Sections were counterstained with Mayer's haematoxylin. As negative controls to evaluate the specificity of the markers, replicate sections were incubated with isotype-specific immunoglobulins [38].

As positive controls to confirm the immunohistochemical reaction, the gonads sections obtained from archive paraffin blocks samples of three further dogs were analysed. Namely, testis sections of the three healthy adult dogs were used for VIM (interstitial fibroblasts), CKs (pancytokeratin AE1/AE3) (rete testis epithelium), DES (vascular walls and peritubular myoid cells) and PLAP (peritubular myoid cells) detection [24]. Moreover, as in other previous studies, ovary sections of one healthy adult bitch were used for INH reaction (target cells: granulosa cells) [26,39]. For AMH according to the results of a previous study sections of immature testis of a pup were used as positive control [25].

Gonads of control dogs were deemed healthy based on clinical record and histological examination.

The percentage of immunolabelled cells was determined semi-quantitatively as in previous reports [24,25,26] and scored as follows: "-", none; "+", occasional (<10%); "++", low (11-40%); "+++", moderate (41-80%); "++++", high (81-100%).

Three blinded genital system experienced pathologists observed collegially histology and evaluated immunohistochemical sections (GV, GC, BB).

2.4. Statistical Analysis

Descriptive statistics were expressed as median and range (min, max) for clinical variables while as frequencies in contingency tables for histological variables. Data were analysed using IBM SPSS 26.0 (IBM, Armonk, U.S.A.). Dogs were stratified in groups based upon age (<2 ys; 2-6 ys; >6 ys), weight (<10 kg; 10-25 kg; >25 kg), testis location (scrotal or retained, this last was further divided into subcutaneous, inguinal, and abdominal), testicular atrophy (SCO presence or absence), Sertoli cell hyperplasia (SCH presence or absence), testicular tumours detection (healthy or tumoral),

tumoral histotype (seminoma; Sertoli cell tumour; Leydig cell tumour; mixed seminoma/Sertoli cell tumour), markers expression (presence or absence). Expression of each marker was related to clinical (age, weight, testis location) and histological (atrophy, Sertoli cell hyperplasia, tumour detection, histotype) variables. Moreover, clinical and histological outcomes were related to each other.

In case of unilateral cryptorchidism, tumours detection for each couple of testes was also evaluated and compared, that is both scrotal and retained testes healthy; scrotal healthy and retained testis neoplastic; both scrotal and retained testes neoplastic. The couple scrotal testis neoplastic and contralateral retained testis heathy was not detected.

All variables were analysed using Chi-Square test and were considered statistically significant for $P < 0.05$.

3. Results

3.1. Clinical outcomes

After an initial enrolment of 41 cryptorchid dogs, 15 dogs were subsequently excluded from the study due to inadequate sample requirements caused by unilateral surgery, histology missing, or parenchymal abnormalities making it impossible to perform a complete analysis of both the testes. Therefore, 26 male dogs affected by unilateral (n = 21) and bilateral (n = 5) cryptorchidism represented our final caseload (Table 2). Dogs belonged to 13 different breeds (n = 17) and mongrel (n = 9) with Chihuahua (18%) the most represented. Dogs aged from 5 months to 13 ys (median 3 ys) and weighted from 1.3 to 43 kg (median 15 kg). The 31 undescended testes were detected in pre-scrotal subcutaneous tissue (n = 8), at inguinal level (n = 10), and in the abdomen (n = 13), and were evenly distributed between the right (n = 16) and the left (n = 15) side.

3.2. Histology

Histological findings are summarized in Table 2. The Sertoli cell-only (SCO) tubules (Fig. 1) were detected in 84.6% of dogs and in 56% of testes. SCO tubules were more frequently diagnosed in retained testes compared to scrotal ones ($P < 0.0001$) and prevailed in tumoral rather than healthy gonads ($P = 0.012$). Its detection was not influenced by age, weight, location of retained testes, and tumour histotype. However, in dogs younger than 11 ys this feature was only detected in retained testes. Sertoli cell hyperplasia (SCH) was always associated with SCO ($P = 0.025$). SCH was detected merely in undescended testes and prevailed in dogs heavier than 25 kg ($P = 0.036$). No significant

difference in SCH presence was found with respect to age, between healthy and tumoral testes, and among different tumour histotypes. No testes in this sample had signs of Leydig cell hyperplasia.

Testicular tumours (Fig. 2, 3 and 4) were diagnosed in 34.6% of dogs and 28.8% of testes. Animals suffering from tumour were both bilateral cryptorchids with all gonads involved (1 out of 5 dogs), and unilateral cryptorchids either bilaterally (5 out of 21 dogs) or unilaterally (3 out of 21 dogs) affected by neoplasm. Location of healthy and neoplastic testes is shown in Graph 1. The tumour development was influenced neither by clinical presentation (bilateral *vs.* unilateral cryptorchidism) and testis location. Age and weight of dogs with healthy testes and those affected by testicular tumours were 2 ys and 12.4 kg, and 10.5 ys and 28 kg, respectively. Dogs with tumours were older ($P = 0.001$) and heavier ($P = 0.047$) than healthy dogs. In particular, unilateral cryptorchid dogs with both testes healthy were younger (median 2 ys) than dogs with both scrotal and retained gonad neoplastic (median 11 ys, $P = 0.0001$). One dog had three tumour histotypes (SEM, SCT, and LCT) coexisting in the same gonad. Therefore, 17 tumours were detected in the 15 neoplastic testes. Seminoma (SEM) was diagnosed in 31% of dogs (52.94% of tumours), and Sertoli cell tumour in 15% of dogs (23.53% of tumours). One mixed seminoma/Sertoli cell tumour was found in only one testis (5.88% of tumours), and Leydig cell tumour in 8% of dogs (17.65% of tumours). Sertoli cells tumours were recorded only in undescended gonads. The overall distribution of histological features is shown in Graph 2.

3.3. *Immunohistochemistry*

All positive controls gave the expected results: strong positive immunolabelling was detectable in all the control target cells. As expected, Sertoli cells in control testes were highly positive for VIM and consistently negative for CK AE1/AE3, DES, INH, and AMH.

Immunolabelling for PLAP was not detected in the seminal cells compartment, indicating the absence of gonocytes in normal testes. However, in these testes myoid peritubular cells were always clearly and strongly immunolabelled, indicating the good reactivity of the samples.

Immunohistochemistry outcomes are summarized in Table 2. Sertoli cells were always positive for VIM both in scrotal and cryptic testes (Fig. 5, 6 and 7), except for one un-reactive inguinal gonad. The other immunohistochemical markers for Sertoli cells were more expressed in undescended than scrotal gonads, although this correlation was significant only for DES, INH and AMH ($P = 0.05$), as shown in Graph 3a. Even in germ cells, PLAP detection prevailed in retained testes compared to scrotal ones (Fig. 8 and 9) but without a statistical significance. In particular, considering only the retained testes ($n = 31$), CK and DES (Fig.10 and 11) were both detected in 33.3% of gonads with

Sertoli cells positivity ranging from occasional to low. Occasional positivity was also recorded for INH in 25.8% of undescended gonads while 80.6% of retained testes were occasionally to highly immunoreactive for AMH (Fig. 12). Seminal cells positive for PLAP were detected in 48.4% of undescended gonads with percentage of labelled cells varying from occasional to low. Markers expression was not influenced by the location of the retained testes.

In scrotal testes, Sertoli cells exhibited from occasional to low positivity for CK and DES in 14.3% and 9.5% of gonads, respectively. INH was found in a single scrotal testis with a low percentage of labelled cells. AMH was observed in 28.6% of descended gonads with immunoreactivity ranging from occasional to high (Fig.7) . Germ cells in scrotal testes showed PLAP in 28.6% of gonads with occasional to low positivity.

All testes affected by SCO or SCH showed higher markers expression than testes without such alterations. In testes diagnosed with SCO, a significant different positivity was recorded for CK, DES, AMH and PLAP ($P = 0.034$, $P < 0.0001$, $P = 0.001$, and $P = 0.013$, respectively). Testes affected by SCH showed higher positivity for CK and DES compared to gonads without hyperplasia ($P < 0.0001$ and $P = 0.006$, respectively).

As shown in Graph 3b, markers expression prevailed in neoplastic testes over healthy ones ($P < 0.05$). Moreover, unilateral cryptorchid dogs with both neoplastic testes showed a higher expression of DES in Sertoli cells ($P = 0.012$) when compared to dogs with both healthy gonads and with only the retained testis affected by tumour. In the 75% of testes with seminoma, Sertoli cells expressed occasional to low CK and occasional to high AMH. Both DES (Sertoli cells) and PLAP (gonocytes) were occasionally to lowly detected in the testicular parenchyma of 55.5% of testes with SEM, and INH had occasional positivity in 22.2% of these testes. All the Sertoli cells tumours showed occasional to high positivity for AMH while DES were both occasionally to rarely expressed in 50% of SCT. CK and INH were both occasionally expressed in 25% of testes with SCTs. One retained testis affected by Sertoli cell tumour was un-reactive to VIM and DES, and the PLAP expression was not valuable. The single testis affected by mixed SEM/SCT was located in the inguinal region and showed occasional to high positivity for all markers except INH. AMH and PLAP were always expressed in the testicular parenchyma harbouring Leydig cell tumours with low to high positivity and occasional to low positivity, respectively. Occasional to low immunolabelling for DES was also observed in Sertoli cells of the 66.7% of testes with LCT. Occasional Sertoli cells positivity for CK and INH was detected only in one testis affected by LCT that was retained and expressed all immunohistochemical markers. However, it should be noted that this gonad was also diagnosed with seminoma and Sertoli cell tumour.

Except for DES (P = 0.019), AMH (P = 0.001) and PLAP (P = 0.016), that prevailed in patients older than six ys, the expression of the other markers was not affected by the patients age.

4. Discussion

Even though canine cryptorchidism is a common disorder, mainly reported in toy breeds [40], there are still unsolved issues that make patients management challenging. In accordance with previous results [19], in this caseload unilateral condition (81%) prevailed over bilateral cryptorchidism (19%). This outcome further emphasizes the importance of setting a univocal therapeutic path in dogs with only one retained testis. Guidelines for the management of cryptorchid patients have already been defined in humans [41] in which diagnosis of undescended testes should be confirmed between 3 and 6 months after birth [42] in order not to delay orchidopexy [43]. Indeed, early surgery can preserve man fertility potential [44]. The risk for testicular malignancy reduces when orchidopexy is performed before puberty but persists despite surgical treatment [45]. In dogs, orchidopexy is discouraged since spermatogenesis recovery allows genetic transmission of cryptorchidism to the offspring [20,46]. Besides this reason, in the past bilateral orchiectomy was recommended also to prevent the development of testicular neoplasia and spermatic cord torsion [20]. It should be noted that unlike man, in canine species the therapeutic approach is not influenced by psychosocial implications. However, in recent years, concerns raised about risks and benefits of surgical sterilization both in male and female dogs. Beyond surgical and anaesthetic complications, several studies pointed out a relationship between neutering and oncological, endocrine, orthopaedic and behavioural disorders [47]. On this basis, one study recommended a conservative approach to unilateral cryptorchidism by surgically removing only the undescended gonad and carefully monitoring the contralateral scrotal one left *in situ* [14]. Nevertheless, the small sample size together with the young age (1 and 2 ys) of dogs enrolled in the aforementioned study involves caution in generalizing its conclusions.

Attempting to overcome this debate, we applied immunohistochemistry to the study of specific markers expression in retained and scrotal testes focusing on testicular degenerative processes that could lead to neoplastic transformation. Indeed, expression of CK, DES, INH, AMH and PLAP in testes of dogs over four months of age is suggestive of immaturity and atrophy [25,26,28] that can predispose to carcinogenesis process [24,28]. As expected, based on literature [22], all Sertoli cells in the gonads of this caseload were diffusely labelled for VIM, regardless of testes location, except for one un-reactive retained testis. Immunostaining was repeated twice on that sample with no positive results. The same sample was unreactive also for DES and PLAP. VIM also

failed to stain fibrous stroma, therefore the immunohistochemical result was interpreted as due to un-optimal sample fixation. The expression of markers of immaturity such as CK, DES, INH, AMH and PLAP observed in retained gonads, even significant only for DES, INH and AMH, could reflect abnormalities due to their altered development coming from an incorrect location.

In previous studies on canine cryptic gonads, gross examination showed a reduced size of retained testes compared to normally descended ones [3] and histology pointed out the absence of spermatogenesis in seminiferous tubules and abnormalities of both Sertoli and Leydig cells in cryptic testes [46]. The presence of Sertoli cell-only tubules is a common finding in human and canine undescended testes [11,14] that is generally related to gonadal atrophy [8]. In our sample, a significantly higher occurrence of SCO tubules in retained than scrotal testes was recorded. In dogs younger than 11 years this feature was only detected in retained testes thus stressing the negative effect of their pathological location on gonads development leading to atrophy appearance [8]. In patients older than 11 ys SCO was also detected in scrotal testes probably associated with the atrophic degenerative process of senescence [16]. In accordance with the anatomical finding, immunohistochemistry showed a significant higher expression of CK, DES, AMH and PLAP in atrophic testes.

Another frequent histological alteration in canine retained testes is Sertoli cell hyperplasia [48]. Actually, we detected SCH only in undescended testes, mostly abdominally located (83%). SCH could result from thermal stress related to gonads incorrect location that increases specific heat shock proteins with an anti-apoptotic effect on Sertoli cells [49]. All the aforementioned alterations could play a role into carcinogenesis process [28,49] justifying the increased risk for testicular cancer which is up to 13.6 times higher in retained testes compared to normally descended ones in dogs [50].

The incidence of testicular tumour in cryptorchid dogs is described between 3% and 19% [2,51]. In the present study, testicular neoplasia affected 35% of dogs. This higher percentage could be attributed to an increase in oncological pathologies along with extended life expectancy in dogs [52]. Moreover, an increased risk of developing testicular tumours has been reported based on breed, including German Shepherd dog [40], as also observed in this cohort. Canine seminomas were contradictorily reported both to prevail in scrotal testes compared to retained ones [53,54] and to be higher in cryptic testis [40]. In the latter SEMs seem mostly to affect abdominally located testes rather than inguinal or subcutaneous ones [54]. In our study, SEMs were mostly detected in cryptic gonads (69%), namely at abdominal location (50%). In contrast with some studies referring Sertoli cell tumour as the prevailing histotype in cryptorchid dogs [2,50,53,54], but in line with Liao (2009), we recorded a higher rate of SEMs (53% of tumours) compared to SCTs (24%) and Leydig cell tumour (18%)[40]. A similar trend with an increase in germ-cell tumours has been observed in cryptorchid

men [55]. Although still debating [6], foetal and child exposure to endocrine disruptor chemicals (EDCs) seems to play an important role in the development of testicular germ-cell tumours in humans [56]. In fact, testicular cells differentiation is a strictly regulated process that is highly influenced by environmental condition [57]. EDCs could impair endocrine function both interrupting gonocyte development and producing precursors of carcinoma *in situ* (CIS) from which testicular germ-cell tumours originate [33]. Moreover, EDCs effect have been imputed as a plausible cause of testicular dysgenesis syndrome (TDS) that is, a male reproductive disorder characterized by cryptorchidism, hypospadias, poor semen quality and testicular germ cell cancer [58]. Due to the intimate sharing of life habits between humans and dogs with the same pollutants environmental exposure, it is consistent to speculate the same can occur in canine species. To date, a single study described histological signs of TDS in canine testes, assuming the existence of this pathology even in dogs [13].

Sertoli cell tumours were commonly reported in abdominal and inguinal undescended testes [59]. In our sample, SCTs affected only retained testes and were equally located among inguinal and subcutaneous areas. The prevalence of SEMs and SCTs in undescended testes is ascribed to the effect of body temperature on testicular functions [2,20], as it occurs for SCH. Many studies conducted in different species investigated the effect of heat on testes [60]. Undescended testes are exposed to body temperature that is higher than at the scrotal area [61]. This thermal stress inhibits the differentiation of spermatogonia resulting in an arrest of spermatogenesis, reduced seminiferous tubule size, germ cell depletion, and fibrosis [62], but also enhances the production of reactive oxygen species and specific heat shock proteins involved in SCT proliferation [48,49].

In agreement with literature [20], we detected Leydig cell tumours always in scrotal testes (67%), except for one inguinal retained testis. Interestingly the scrotal gonad of this latter patient was also affected by LCT.

Markers expression significantly prevailed in tumoral gonads and positivity recorded in scrotal contralateral testes of unilateral cryptorchid dogs is an un-precedent result emerging from the present study. In old dogs this expression could be related to senescence or testicular neoplasia whose risk increases with age [63] regardless cryptorchid concomitant condition. In young dogs their expression combined with no histological lesions is suggestive of an early stage of carcinogenesis process. In particular, supposedly healthy scrotal testes of adult dogs were not expected to show PLAP positivity that is expressed during foetal life from gonocytes but not from germ cells after birth [28]. Since PLAP positivity has been reported in CIS cells and in testicular germ-cell tumours such as seminoma [64], we speculate on prospective role of this marker in early detection of testicular precancerous lesions even before histological recognition. In humans, scrotal testis of unilateral cryptorchid patients has been reported with an increased risk to develop testicular tumour [65] due to

the early appearance of histological defects [8,66]. In cryptorchid dogs, a similar predisposition in contralateral descended testis is still controversial [14]. Furthermore, canine diagnosis is frequently delayed compared to humans or concealed even for fraudulent reasons, making a precise dating of testicular descent in proper scrotal position tricky. In our sample, immunohistochemical positivity in scrotal testes, especially when apparently healthy, seemed to imply a greater neoplastic risk even in canine species.

5. Conclusions

Albeit with some limitations related both to the small sample size and few middle-aged dogs, our results suggest a potential risk of neoplastic transformation in the contralateral scrotal testis of cryptorchid dogs, as already reported in humans [65,67]. Immunohistochemistry provided prognostic evidence for malignancy development that is important in the decisional process and surgical approach. Failure to remove the scrotal testis, even in the absence of clinically appreciable alterations, could constitute a hazard for the dog health.

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CRediT authorship contribution statement

Alessandro Pecile: Conceptualization, Methodology, Investigation, Data curation, Writing – review & editing. **Debora Groppetti:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. **Giulia Pizzi**: Data curation, Writing – review & editing. **Chiara Giudice**: Methodology, Data curation**. Barbara Banco**: Methodology, Data curation. **Valerio Bronzo**: Formal analysis, Writing – review & editing. **Valeria Grieco:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no competing interests.

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Legends

Fig.1. Histologic section of canine cryptic testis. Seminiferous tubules lined only by Sertoli cells (Sertoli cell only tubules – SCO tubules) (HE stain. BAR 150 micron).

Fig 2. Histologic section of a canine cryptic testis. Seminoma. Sheets of round to oval neoplastic cells, accompanied by small lymphocytic aggregates (arrowhead) (HE stain. BAR 50 micron).

Fig.3. Histologic section of a canine cryptic testis. Sertoli cell tumor. The tumor is composed by tubules lined exclusively by neoplastic Sertoli cells (HE stain. BAR 150 micron).

Fig 4. Histologic section of a canine cryptic testis. Leydig cell tumor. Neoplastic polygonal cells arranged in cords separated by scant fibrovascular stroma (HE stain. BAR 200 micron).

Fig.5. Canine scrotal testis immunohistochemically stained for vimentin. Sertoli cells are strongly vimentin-positive (some indicated with arrowheads) while germ cells are negative (immunohistochemistry, hematoxylin counterstained. BAR 50 micron). Top right: immunoglobulin isotype control.

Fig.6. Canine cryptic testis immunohistochemically stained for vimentin. Sertoli cells are strongly vimentin-positive (some indicated with arrowheads) while germ cells are negative (immunohistochemistry, hematoxylin counterstained. BAR 50 micron). Top right: immunoglobulin isotype control.

Fig.7. Canine cryptic testis immunohistochemically stained for vimentin. Sertoli cell only tubules. Sertoli cells are strongly vimentin-positive (immunohistochemistry, hematoxylin counterstained. BAR 50 micron). Top right: immunoglobulin isotype control.

Fig.8. Canine cryptic testis immunohistochemically stained for PLAP. Note a group of PLAP-positive gonocytes (arrowheads) in a seminiferous tubule (immunohistochemistry, hematoxylin counterstained. BAR 50 micron). Top right: immunoglobulin isotype control.

Fig 9. Canine scrotal testis immunohistochemically stained for PLAP. Note occasional PLAP-positive gonocytes (arrowheads) in a seminiferous tubule (immunohistochemistry, hematoxylin counterstained. BAR 50 micron). Top left: immunoglobulin isotype control.

Fig.10. Canine cryptic testis immunohistochemically stained for cytokeratins. Scattered cytokeratins-positive Sertoli cells (some indicated with arrowheads) are recognizable into seminiferous tubules (immunohistochemistry, hematoxylin counterstained; 200X magnification. Top left: immunoglobulin isotype control.

Fig.11. Canine cryptic testis immunohistochemically stained for desmin. Desmin-positive Sertoli cells (some indicated with arrowheads) are recognizable into scattered seminiferous tubules (immunohistochemistry, hematoxylin counterstained. BAR 150 micron). Top left: immunoglobulin isotype control.

Fig.12. Canine cryptic testis immunohistochemically stained for anti-Muellerian-hormone (AMH). Note AMH-positive Sertoli (some indicated with asterisks), intermixed with negative early germ cells into seminiferous tubules (immunohistochemistry, hematoxylin counterstained. BAR 150 micron). Top left: immunoglobulin isotype control.

Supplementary Material

A Power Point file is provided showing images of normal testis positive control structures. Four images, indicated as “a”, “b”, “c” and “d” are provided in the ppt slide:

a) Canine normal testis. Immunohistochemistry for desmin. Positive control structures are vessels walls (asterisks) and peritubular myoid cells (arrows). Seminal cells are consistently negative.
b) Canine normal testis. Immunohistochemistry for desmin. Positive control peritubular myoid cells (arrow) at higher magnification. Seminal cells are consistently negative.

c) Canine normal testis. Immunohistochemistry for cytokeratins. Positive control structures are rete testis tubules (arrows). Seminal cells, visible on the left, are consistently negative.

d) Canine normal testis. Immunohistochemistry for PLAP. Positive control are peritubular myoid cells (arrow). No positive gonocytes were present in the seminal epithelium.