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# MicroRNA as epigenetic regulators of canine cryptorchidism

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ABSTRACT

Cryptorchidism, the failed descent of one or both testes into the scrotum, is a common developmental disorder in male dogs. Cryptorchidism may affect canine fertility, reducing the quality of the semen, and may promote spermatic cord torsion and onset of neoplasia. MicroRNAs (miRNAs) are epigenetic regulators of gene expression and their dysregulation is associated with disorders of spermatogenesis and testis neoplasia. The present study aimed at investigating the expression of miRNAs in formalin-fixed, paraffin-embedded (FFPE) canine retained testes and testes affected by seminoma, and at integrating miRNAs to their target genes. Forty testicular FFPE specimens from 30 dogs were included - 10 scrotal and 10 contralateral retained from 10 unilateral cryptorchid dogs; 10 tumoral testes affected by seminoma from non-cryptorchid dogs; 10 scrotal normal testes from non-cryptorchid dogs included as the control. The expression level of three miRNAs, namely miR-302c-3p, miR-302a-3p, and miR-371-3p, associated with testicular disorders, were quantified using RT-qPCR.

The comparative analysis demonstrated that the level of miR-302a-3p and miR-371a-3p were quantifiable exclusively in control testes. The expression level of miR-302c-3p was higher in the control than in the other groups; its expression decreased in retained testes compared to scrotal testes and testes with seminoma. Gene Ontology analysis pointed out that these miRNAs may be involved in the modulation of estrogen and thyroid hormone signaling pathways.

In conclusion, this study demonstrated that miRNAs are dysregulated in canine cryptorchid and seminoma-affected testes compared to control tissues, confirming the pivotal role of miRNAs in cryptorchidism.

## 1. Introduction

Cryptorchidism is a common developmental disorder in male dogs that results in the failed descent of one or both testes into the scrotum. It occurs more frequently in purebred than mongrel dogs either with a unilateral or bilateral presentation (Yates et al., 2003). The etiology of this congenital disease has a strong hereditary component and many genes are probably involved in its transmission (Khan et al., 2018). Therefore, to reduce the incidence of this pathology, affected dogs should be excluded from the breeding line (Romagnoli, 1991). Cryptorchidism may impact canine fertility resulting in poor semen quality (Kawakami et al., 1984). Moreover, the undescended testes are more susceptible to spermatic cord torsion (Pearson and Kelly, 1975) and develop neoplasia (Hayes et al., 1985) than scrotal ones. In the case of a unilateral condition, the actual impact on the contralateral scrotal testis in terms of cancer predisposition is not yet clear in the canine species. There are no guidelines on the appropriate surgical approach (unilateral or bilateral orchiectomy) for these specific patients (Romagnoli, 1991; Veronesi et al., 2009).

In the canine population, seminomas have the highest incidence among testicular tumors (Ciaputa et al., 2015) and also prevail over Sertoli cell and Leydig cell tumors in cryptorchid dogs (Liao et al., 2009; Pecile et al., 2021). Despite a less aggressive behavior in dogs than in humans (Grieco et al., 2007), distant metastases of seminoma occur in 10–15% of canine patients (Lucas et al., 2012; Dugat et al., 2015). Environmental factors have been suggested to be involved both in cryptorchidism occurrence via endocrine disruptor chemicals (Lea et al., 2016) and in the development of seminoma via its precursor, carcinoma in situ (Rajpert-De Meyts and Hoei-Hansen, 2007). A previous study focusing on exploring potential immunohistochemical markers showed suspected precancerous lesions in both gonads of unilateral cryptorchid dogs (Pecile et al., 2021).

MicroRNAs (miRNAs), comprising about 22 nucleotides, regulate gene expression at the post-transcriptional level and participate in

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several biological processes including cell proliferation and differentiation (Ambros, 2004). MiRNA dysregulation hesitates in molecular pathways modulation which could give rise to disease and neoplasia development (Condrat et al., 2020). In this respect, miRNAs are defined as suppressors or promoters of the tumorigenesis process (Peng and Croce, 2016) representing candidate biomarkers of diagnosis, staging, prognosis, and therapy. The miRNA epigenetic regulations of the scrotal testis in the unilateral form of canine cryptorchidism have never been explored.

After selecting three miRNAs associated with testis disorders in human medicine (Das et al., 2019; Badia et al., 2021; Nappi et al., 2019; Palmer et al., 2010; Piao et al., 2021; Dieckmann et al., 2017), namely miR-302c-3p, miR-302a-3p, and miR-371-3p, the present study focused on investigating their expression in canine retained testes and testes affected by seminoma using normal scrotal testes as control. Moreover, potential miRNAs targets and biological processes were examined through a gene functional analysis to identify molecular dysregulation that could promote the carcinogenesis process and increase neoplastic risk in both gonads of unilateral cryptorchid dogs.

## 2. Materials and methods

### 2.1. Inclusion criteria

In this retrospective study, 40 testicular formalin-fixed paraffin-embedded (FFPE) specimens from 30 dogs were selected from the archives of the Department of Veterinary Medicine and Animal Science of the Università degli Studi di Milano. The samples were collected for clinical-diagnostic purposes not specifically for this study which therefore requires no additional ethical approval beyond the informed consent obtained by the dogs' owners at the time of surgery.

The caseload consisted of 10 scrotal (S) and 10 contralateral retained testes (R) from 10 unilateral cryptorchid dogs, 10 tumoral testes affected by seminoma (T) from non-cryptorchid dogs, and 10 scrotal normal testes (H) from non-cryptorchid dogs, considered as control (Table 1).

All patients included in the study underwent routinary bilateral surgery for elective or therapeutic neutering and both testes were sent for histological examination.

**Table 1**
Summary of samples enrolled in the study.

| ID | Breed | Age (ys) | Testes position | Group |
|---|---|---|---|---|
| 1 | Mixed breed | 1,67 | Scrotal | H |
| 2 | Golden Retriever | 4 | Scrotal | H |
| 3 | Mixed breed | 1,58 | Scrotal | H |
| 4 | Chihuahua | 2 | Scrotal | H |
| 5 | Mixed breed | 1,5 | Scrotal | H |
| 6 | Mixed breed | 1,42 | Scrotal | H |
| 7 | Mixed breed | 1,08 | Scrotal | H |
| 8 | Mixed breed | 1,08 | Scrotal | H |
| 9 | Mixed breed | 0,75 | Scrotal | H |
| 10 | Mixed breed | 1 | Scrotal | H |
| 11 | Jack Russel | 0,67 | Abdominal | R |
| | | | Scrotal | S |
| 12 | Kurzhaar | 0,67 | Abdominal | R |
| | | | Scrotal | S |
| 13 | Mixed Breed | 0,75 | Abdominal | R |
| | | | Scrotal | S |
| 14 | Chihuahua | 0,83 | Inguinal | R |
| | | | Scrotal | S |
| 15 | Meticcio | 1 | Subcutaneous | R |
| | | | Scrotal | S |
| 16 | Cocker Spaniel | 1,3 | Scrotal | S |
| | | | Abdominal | R |
| 17 | Maltese | 1,33 | Subcutaneous | R |
| | | | Scrotal | S |
| 18 | Pinscher | 2 | Subcutaneous | R |
| | | | Scrotal | S |
| 19 | Whippet | 2 | Subcutaneous | R |
| | | | Scrotal | S |
| 20 | Bouledogue | 2,33 | Scrotal | S |
| | | | Abdominal | R |
| 21 | Rhodesian Ridgeback | 7 | Scrotal | T |
| 22 | German Shepherd | 13 | Scrotal | T |
| 23 | Mixed Breed | 14 | Scrotal | T |
| 24 | Mixed Breed | 15 | Scrotal | T |
| 25 | Mixed Breed | 12 | Scrotal | T |
| 26 | Pug | 9 | Scrotal | T |
| 27 | Mixed Breed | 6 | Scrotal | T |
| 28 | Mixed Breed | 12 | Scrotal | T |
| 29 | German Shepherd | 9 | Scrotal | T |
| 30 | Mixed Breed | 10 | Scrotal | T |

H: healthy testis, R: retained testis, S: scrotal testis and T: neoplastic testes.

### 2.2. Histology and sample collection

At the time of surgery, all testes were cut in half lengthwise in the midline and fixed in 10% neutral buffered formalin. Then, a complete longitudinal section was obtained which was routinely processed for histology. Sections (4 μm thick) were obtained from paraffin wax blocks and stained with hematoxylin and eosin (HE). A further 4 μm thick section was obtained from each sample for subsequent smallRNA extraction. Seminoma diagnosis was based on World Health Organization guidelines (Kennedy et al., 1998).

### 2.3. SmallRNAs extraction and quantification by RT-qPCR (qPCR)

SmallRNAs were extracted using the miRNeasy FFPE Kit (Qiagen, Cat. No. 217504) following the manufacturer's instructions. The RNA concentration was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The *Caenorhabditis elegans* miRNA cel-miR-39 (25 fmol final concentration) (Qiagen, Cat. No. 219610) was used as a synthetic spike-in for extraction and retrotranscription control. Reverse transcription was performed using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Cat. No. A28007), according to the manufacturer's instructions.

The qPCR experiments were designed following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Small RNA TaqMan assays were performed according to the manufacturer's instructions. MicroRNAs were selected according to previous publications in which these miRNAs were found to be related to testicular neoplasia in humans (Das et al., 2019; Badia et al., 2021; Nappi et al., 2019; Palmer et al., 2010; Piao et al., 2021; Dieckmann et al., 2017). The selected probe assays (Thermo Fisher Scientific) included cel-miR-39-3p (assay ID 478293_mir), hsa-miR-371-3p (assay ID 478070_miR), hsa-miR-302a-3p (assay ID 478006_miR), hsa-miR-302c-3p (assay ID 478509_miR); hsa-miR-25-3p (assay ID 477994_miR) and hsa-miR-30b-5p (assay ID 478007_miR) were selected as reference miRNAs. Quantitative reactions were performed in duplicate in scaled-down (15 μl) reaction volumes in a CFX Connect Real-Time PCR Detection System (Bio-Rad), using 7.5 μl of 2× TaqMan Fast Advanced Master Mix (Cat. No. 4444557), 0.75 μl of miRNA specific TaqMan Advanced assay reagent (20×), 1 μl of cDNA, and water to make up the remaining volume. The thermal cycling profile was as follows: 50 °C for 2 min, 95 °C for 3 min and 40 cycles at 95 °C for 15 s and 60 °C for 40 s.

To evaluate the stability of reference miRNAs, namely hsa-miR-25-3p and has-miR-30b-5p, a geNorm analysis was performed using Biogazelle's qbase+ software (www.qbaseplus.com). The normalization factor was calculated as the mean of reference miRNAs. The relative expression was calculated using Bio-Rad CFX Maestro™ Software. MiRNA expression is presented in terms of fold change using the $2^{-\Delta\Delta Cq}$ formula.

### 2.4. MicroRNAs predicted target

The target genes of DE-miRNAs were predicted using MiRWalk 3.0 (Sticht et al., 2018), which includes 3 miRNA-target prediction

programs (miRDB (Wong and Wang, 2015), miRTarBase (Hsu et al., 2011) and Targetscan (Agarwal et al., 2015). The analysis was performed by targeting the entire gene sequence (including 5'UTR, CDS, and 3'UTR). The list of target genes predicted by the three tools was included in further analysis and functional mRNA enrichment was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatic resource (Huang et al., 2009) and biological pathways in the KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa et al., 2012) were examined for enrichment.

### 2.5. Statistical analysis

The statistical analysis was performed on XLStat software for Windows (Addinsoft, New York, USA). Due to not normal distribution of data, as assessed by Shapiro-Wilk test, the non-parametric.

Kruskal-Wallis test for multiple pairwise comparisons was used. Statistical significance was accepted for $p \leq 0{,}05$.

## 3. Results

### 3.1. Study population

Our caseload consisted of purebred dogs ($n = 14$) and mongrels ($n = 16$). Patients mean age was lower in non-cryptorchid control (1,61 ± 0,92 ys) and cryptorchid (1,29 ± 0,62 ys) dogs compared to dogs affected by seminoma (10,7 ± 2,98 ys).

The 10 retained testes (R) of unilateral cryptorchid dogs were mainly right-sided (80%) and were detected in the abdomen ($n = 5$), in the inguinal area ($n = 1$), and the pre-scrotal subcutaneous tissue ($n = 4$).

### 3.2. Histology

In both control (H) and scrotal (S) testes of cryptorchid dogs (Fig. 1), no lesions were observed and a normal seminiferous epithelium (Fig. 1a) and spermatozoa (Fig. 1b) were recognizable in the epididymis. In the retained testes (R), most seminal tubules were lined only by Sertoli cells (Sertoli cell only tubules – SCO) (Fig. 1c). Rare degenerated seminal cells were still detectable only in a 10-month-old dog with an inguinal retained testis.

In the 10 cases with seminoma (T), a variable-sized neoplasm effaced and replaced testicular parenchyma. Neoplastic cells were arranged in densely packed sheets, nests, and lobules sustained by a fine fibrovascular stroma. Neoplastic cells, round to polygonal, from 20 to 30 μm in diameter, had indistinct cell borders and moderate to high nuclear/cytoplasmic ratio. The cytoplasm was abundant, eosinophilic to amphophilic, and finely granular. Nuclei were centrally located, large, round to oval, vesiculate, and contain one prominent eosinophilic nucleolus and marginalized finely reticulated chromatin (Fig. 1d). Anisocytosis and anisokaryosis were present and mitosis ranged from 0 to 4 per HPF. Multinucleated neoplastic cells were occasionally visible. Multifocally within tumors, inflammatory aggregates composed of small mature lymphocytes were observed.

### 3.3. miRNA expression

The miRNAs' relative abundance was quantified using RT-qPCR.

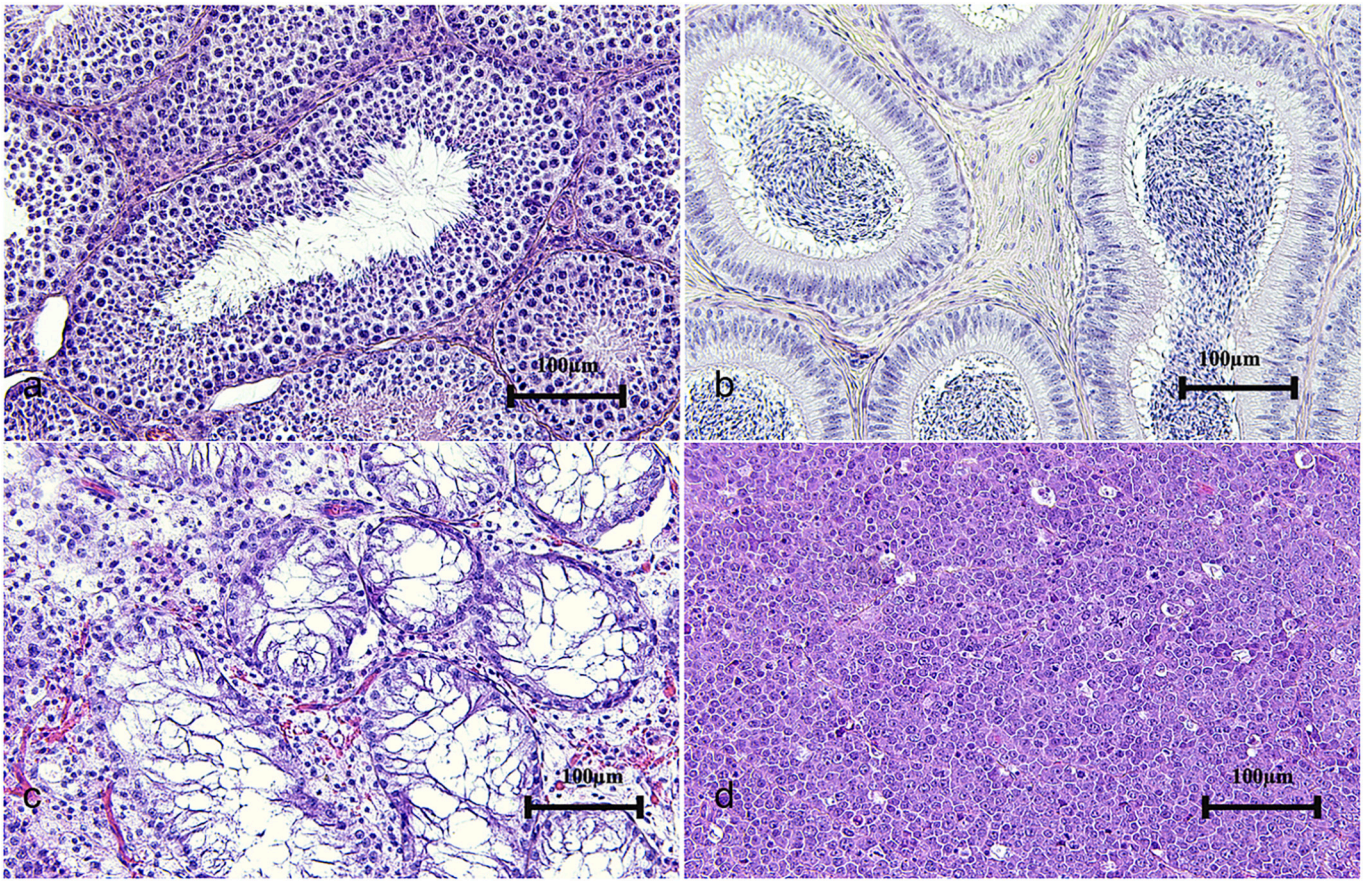


**Fig. 1.** Representative hematoxylin-eosin staining of a histologic section of (1a) a scrotal testes testis, seminiferous tubules are lined by normal seminiferous epithelium; (1b) the epididymis of a scrotal testes testis with intraluminal spermatozoa; (1c) a retained testis from cryprorchid dog, seminiferous tubules are lined only by Sertoli cells (Sertoli cell only tubules – SCO tubules); (1d) a canine seminoma, neoplastic cells with abundant cytoplasm are arranged in densely packed sheets, sustained by a fine fibrovascular stroma. Bar 100 μm.

Analysis of the reference miRNAs expression stability by geNorm indicated that both reference miRNAs were suitable with the average M values of 0.787. Their mean was used for the normalization of the relative quantification data experiments. Cel-miR-39, an artificial spike-in, was used as an internal control of extraction and retrotranscription. The results are presented in Fig. 2. MiRNAs expression significantly varied among control (H), retained (R), and neoplastic (T) testes. The comparative analysis demonstrated that the level of miR-302a-3p and miR-371a-3p were quantifiable exclusively in control normal testes (H)· In detail, miR-302a-3p was found exclusively in control testes while it was almost undetectable in both scrotal ($p = 0.003$) and retained gonads of cryptorchid dogs ($p = 0.003$) and in neoplastic testes ($p = 0.003$) (Fig. 2a). Similarly, miR-371a-3p was almost undetectable in both scrotal ($p = 0.0003$) and retained testes ($p = 0.0009$) of cryptorchid dogs compared to control gonads. Additionally, it wasn't detectable also in gonads with seminoma ($p < 0.0001$) (Fig. 2c).

MiR-302c-3p was detected in all samples (Fig. 2b). In detail, miR-302c-3p was downregulated in both scrotal ($p = 0.04$), retained gonads ($p < 0.0001$) of cryptorchid dogs, and in neoplastic gonads ($p < 0.05$) compared to control testes. The amount of miR-302c-3p significantly decreased in retained compare to scrotal testes ($p = 0.03$) and to neoplastic gonads ($p = 0.029$) (Fig. 2b).

To test whether there were any significant effects of the testes condition, the miR-302a-3p, miR-302c-3p, and miR-371a-3p abundances were also analyzed together using Principal Component Analysis (PCA, correlation matrix, no rotation). Good suitability of data for PCA analysis was valued (KMO = 0.6498 and Barlett's test $p \leq 0.0001$). PCA revealed two main factors that together explain 97.97% of the variation. As shown in Fig. 3, the first factor (PC1-Component 1) explained variance = 70.53% (Eigenvalue = 2.116) and the second factor (PC2-Component 2) explained variance = 27.45% (Eigenvalue = 0.823) (Fig. 3). Based on the category, testes clustered homogeneously into two groups: the first one includes control samples, and the second one both scrotal and retained testes of cryptorchid dogs and testes with seminoma, on PC1 ($p \leq 0.001$).

### 3.4. *miRNA target prediction and pathway enrichment*

The predicted mRNA targets were 24 for miR-302c-3p, while miR-302a-3p and miR-371a-3p had 27 predicted genes.DAVID database was employed to perform Gene Ontology analysis for molecular function (MF), cellular components (CC), and biological process (BP). Molecular function items focused on proteins and nucleic acids binding and transcription factor activity, while cellular components terms concerned nucleus, cytoplasm, and cellular membrane and junction. Principal results for the biological process included transcription regulation at different levels, nervous system development, and regulation of the apoptotic process (Fig. 4).

Finally, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was carried out and showed that Estrogen and Thyroid hormone signaling along with regulation of calcium reabsorption was the most significant pathways.

## 4. Discussion

As one of the most common congenital defects in newborn dogs, cryptorchidism affects fertility increasing the risk of developing gonads neoplasm (Lea et al., 2016). Since the molecular mechanisms regulating cryptorchidism and the resulting cancer predisposition have not yet been fully elucidated, surgery is recommended for dogs with cryptorchidism occurring with unilateral and bilateral presentation (Yates et al., 2003). The present study investigated the relationship between miRNAs and retained and normally descended testes as well as the neoplastic predisposition of the scrotal gonad in unilateral cryptorchid dogs.

Two out of three investigated miRNAs, namely miR-302a-3p and miR-371a-3p, were not expressed in testes of scrotal and retained in cryptorchid dogs and testes affected by seminoma, while their amount in normal gonads was high. Compared to control gonads, some variances were expected in retained and neoplastic testes, where the normal structure and physiology were impaired. The incorrect location exposes the testis to high temperature, increasing oxidative stress and compromising Sertoli and germ cells' function (Kawakami et al., 2007); moreover, testis architecture is destroyed by an uncontrolled cellular proliferation (Mostofi and Sesterhenn, 1998). These abnormalities are both cause and consequence of molecular changes that reasonably can result in miRNA dysregulation (Ebrahimi et al., 2020).

In human medicine, several studies described miR-302a-3p and miR-371a-3p as oncogenes in testicular cancer in which they are upregulated controlling the expression of cell cycle regulating genes (Badia et al., 2021; Palmer et al., 2010; Piao et al., 2021). In particular, miR-302a-3p seemed to inhibit apoptosis increasing survivin protein (Das et al., 2019), and miR-371a-3p proved to be a sensitive and specific marker of testicular germ cell tumor (Nappi et al., 2019; Liu et al., 2021). In contrast with these findings, the levels of miR-302a-3p and miR-371a-3p were so low that they could not be quantified in our model, suggesting that in the canine species miR-302a-3p and miR-371a-3p may exert an opposite role acting as tumor suppressors at the testicular level. The same results characterized gonads of unilateral cryptorchid dogs even if no sign of malignancy was evident at histological examination. This outcome supports the hypothesis of a negative influence of the retained on the scrotal testis in unilateral cryptorchid dogs as previously suggested (Pecile et al., 2021). Although few data on this topic are reported, we may speculate that the dysregulation of miR-302a-3p and miR-371a-3p may influence molecular mechanisms leading to the development of seminoma. Therefore, on a molecular basis, predisposition for this specific histotype of testicular neoplasia in unilateral cryptorchid dogs might concern even the scrotal testis, regardless of its physiological position.

Another feature of miRNAs is their involvement in intercellular

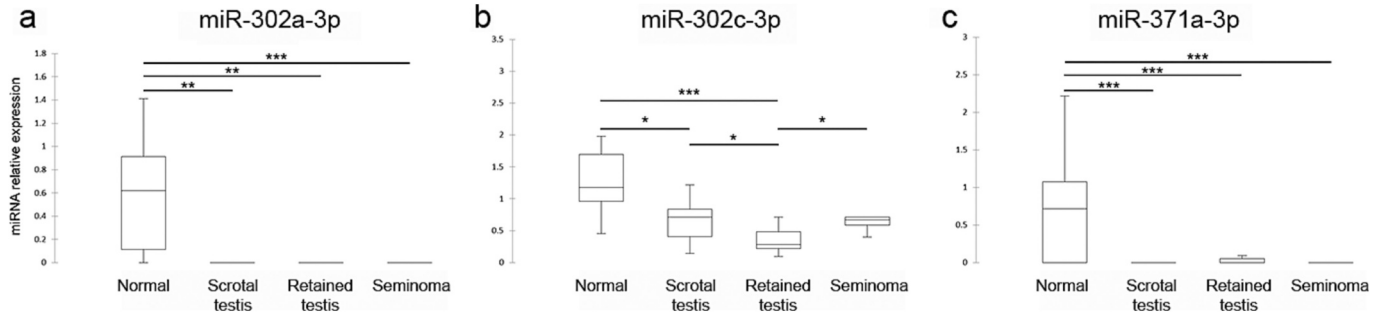


**Fig. 2.** Box Plot of miR-302a-3p (A), miR-302c-3p (B), and miR-371a-3p (C). Blackline inside the boxes marks the median. Significance was declared at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

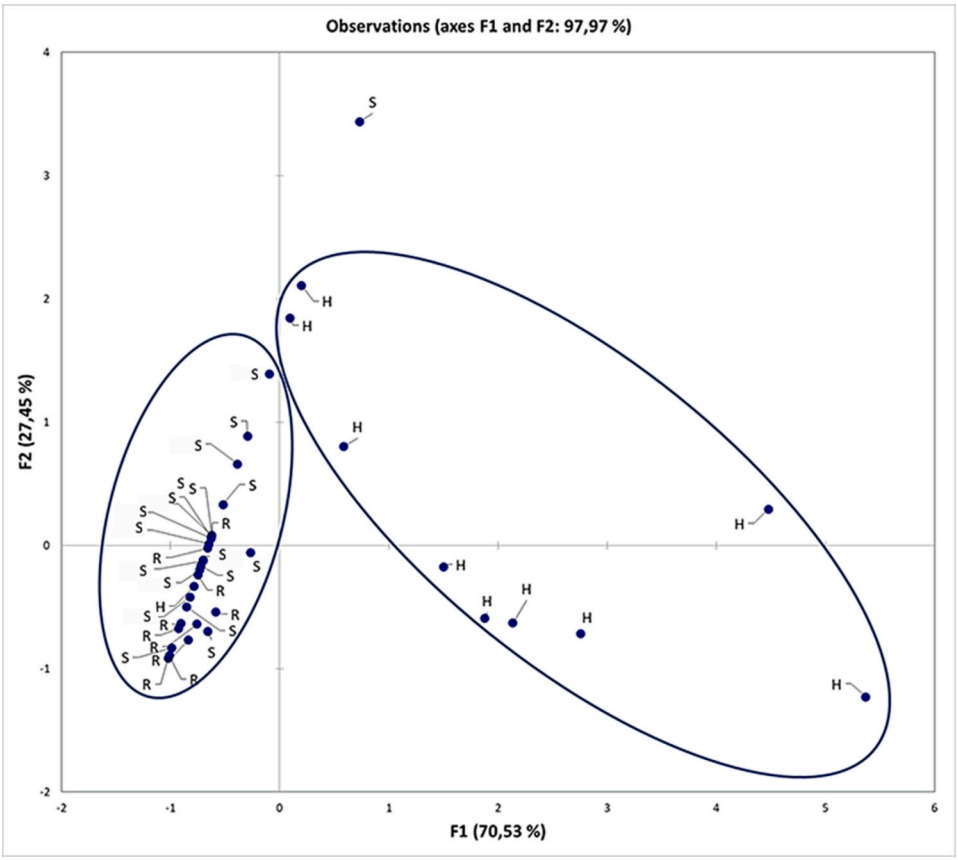


**Fig. 3.** Principal component analysis (PCA) of tested samples. Two-dimensional PCA was used to determine whether retained testes (R) and testes with seminoma (S) could be differentiated from healthy (H) samples.

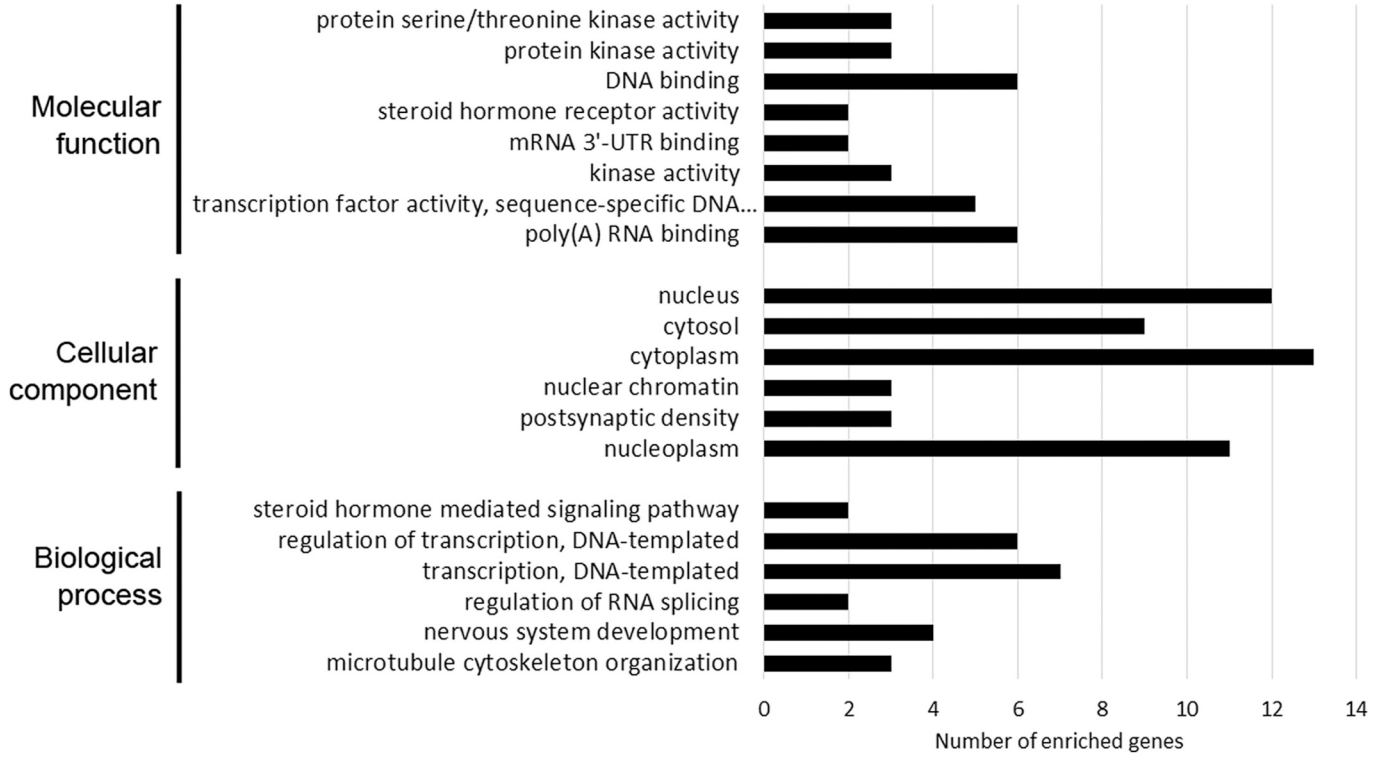


**Fig. 4.** Gene Ontology (GO) enrichment analysis of terms potentially regulated by DE-miRNAs. The target genes were annotated by DAVID in three categories: biological process, cellular component and molecular function.

signaling (Di Leva et al., 2014). This role is carried out by circulating miRNAs that through exosome or protein-bound work as messengers between cells in different parts of the body (Sun et al., 2018; Vickers et al., 2011). This peculiar property becomes extremely important in cancer since miRNAs allow communication among primary neoplasia and its metastases that can be exploited for diagnostic and therapeutic purposes (Ruivo et al., 2017). Circulating miR-371a-3p is a good serum biomarker to diagnose patients with primary testicular germ cell cancer (Mostofi and Sesterhenn, 1998). A similar molecular interaction may be hypothesized between retained and scrotal gonads in unilateral cryptorchid patients. However, no studies explored this issue so far.

Despite miR-302a-3p and miR-302c-3p belonging to the same miRNA cluster, a great discrepancy in their expression was pointed out in our model. The expression level of miR-302c-3p was different in the four tested groups. The most interesting result concerns the dysregulation of miR-302c-3p in scrotal testes of unilateral cryptorchid dogs, which emphasizes a certain degree of abnormality. In fact, despite the correct position of the gonad, there was a significant decrease compared to control normal testes ($p = 0.04$), but it was not as marked as in retained ones ($p < 0.0001$). This result led us to hypothesize that the epigenetic regulation of the scrotal testis may be influenced by miRNAs of the contralateral retained testis resulting in enhanced neoplastic predisposition and spermiogram variability (Kawakami et al., 1984). Circulating miRNAs produced by undescended testis may indeed epigenetically interfere with spermatogenesis in the contralateral scrotal testis leading to poor semen quality. This way of communication, an alternative to normal hormonal feedback, should be properly investigated. From this perspective, in future studies, it would be interesting to investigate the amount of circulating miR-302c-3p in the blood of cryptorchid dogs.

Gene ontology and analysis of KEGG pathways pointed out that dysregulated miRNAs may modulate estrogen signaling by targetting the expression of ESR1, the gene that encodes for estrogen receptors α (ERα). In humans, exposure to estrogenic substances has been identified as a plausible cause of impaired testicular descent (Thonneau et al., 2003). However, the dysgenic effect seems to be conditioned by the expression of ERα in Leydig cells (Cederroth et al., 2007). Previous studies detected these receptors also in healthy testes and both gonads of unilateral cryptorchid dogs (Nie et al., 2002; Jung et al., 2016). Therefore, a similar mechanism may be assumed also in canine species. In our model, the downregulation of miR-302c-3p in cryptorchid dogs suggested an increase in estrogen receptors α that may have exposed the gonads to estrogenic compounds even more and influenced their development. This outcome fits in perfectly with the testicular dysgenesis syndrome (TDS) theory described by Skakkebaek and coworkers (Skakkebæk et al., 2001) who recognized and proposed poor semen quality, cryptorchidism, hypospadias, and testicular cancer as symptoms of the same disorder based on a common environmental and lifestyle etiology. TDS has been firstly studied in humans (Martin et al., 2008) and then it was hypothesized that also dogs sharing the same environmental hazard with their owners may experience TDS (Pecile et al., 2021; Lea et al., 2016; Grieco et al., 2008). The present study supported these assumptions suggesting a plausible mechanism by which estrogenic compounds may exert their dysgenic activity.

## 5. Conclusions

To the best of the authors' knowledge, this is the first study investigating miRNAs expression in dogs affected by unilateral cryptorchidism and seminoma. Our findings suggested that the same epigenetic dysregulation affects retained and scrotal gonads of dogs with unilateral cryptorchidism, and neoplastic testis. Similar to what occurs in canine mammary tumors, not the single organ but the patient should be considered at risk.

Therefore, especially when the retained testis is already diagnosed with a testicular tumor, a conservative approach should be regarded with caution and a close follow-up of the preserved testes is recommended.

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## Authorship contribution statement

GP, CL, DG conceived and designed the experiments and provided the original idea of the study; GP, AP and DG enrolled patients and performed the surgery; VG and EB performed histology; GP and CL performed the RT-qPCR experiments; CL performed bioinformatics and statistical analysis. GP and CL wrote the first draft of the manuscript; all authors read and approved the final draft of the manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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