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Evaluation of environmental factors on canine reproduction and fertility: innovative strategies

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ABSTRACT

Fertility assessment is challenging both in human and canine species because it is defined by a combination of numerous genetic and environmental factors. In recent decades, a decline in humans fertility occurred and was related to environmental pollutants, such as endocrine disruptor chemicals (EDCs), that increased dramatically as result of technological development. Living close to their owners, dogs are exposed to similar risks whose impact on their reproduction is still unclear. Many widespread compounds may interfere with canine reproductive health from embryonic development up to conception.

Since environmental hazards in dogs are poorly understood, this PhD thesis comprises four studies devoted to exploring their consequences on canine reproduction and fertility.

The first two projects were centered on the exposure of breeding dogs to cigarette smoke. Cotinine was applied as marker of nicotine intake in male dogs and pregnant bitches belonging to smoking and non-smoking owners. Significant differences were highlighted between exposed and non-exposed dogs, however measurable levels of this metabolite were detected in serum, semen, hair, and amniotic fluid of all enrolled patients. Despite nicotine metabolites increase seminal oxidative stress, in our caseload the level of total antioxidant capacity (TAC) in ejaculate didn't correlate with male dogs' exposure to tobacco smoke. Albeit the practical effects of passive smoke should be investigated on a larger sample size, results obtained demonstrated a non-negligible exposure that could interfere with dogs' reproductive performance.

Apart from tobacco smoke consequences, within other two studies we focused on cryptorchidism. Through the application of immunohistochemical techniques and miRNomics, we found precancerous lesions and molecular dysregulation in both retained and scrotal gonads of affected patients. Interesting implications have emerged from this outcome especially concerning testicular neoplastic predisposition and the appropriate therapeutic approach in unilateral cryptorchids. In addition, molecular pathways suggested an abnormal expression of estrogen receptors that, as assumed in humans, may justify a greater sensitivity to EDCs even in cryptorchid dogs.

Regardless some limitations, the overall outcomes of the present doctoral dissertation pointed out the environmental role on canine reproduction. Although pollutants' effects can't be completely

eliminated, greater awareness should be placed on common bad habits like cigarette smoke which could impair fertility potential even in “man’s best friend”.

SCIENTIFIC BACKGROUND

CANINE REPRODUCTION AND FERTILITY

Investigating fertility is a hard work both in human and veterinary medicine partly because it results from the interaction between male and female spheres, and partly because it is affected by numerous genetic and environmental factors whose impact is difficult to weight.

Fertility impairment is an issue of great concern in modern society (Felgueiras et al., 2020). In humans, it is reported that almost 50% of infertility cases are due to women, and 20-30% of cases are due to men while the remaining 20-30% results from a combination of male and female factors, however these data are strongly influenced by World region, number of men included in statistical analysis and difference in spermiogram reference values (Agarwal et al., 2015, Esteves et al., 2012).

Similar statistics have not yet been conducted in canine species: thus, it was impossible to estimate if subfertility/infertility occurs more in male or female dogs and whether it is influenced by geographic region.

It is interesting to note that in human medicine female infertility is much more reported than male ones often for cultural and psychological reasons (Agarwal et al., 2015). In canine species, the situation is diametrically opposite, in fact, male fertility evaluation often precedes the one in female. The absence of the psychosocial factors found in humans together with the easy collection of semen sample allow a more frequent fertility assessment in stud dogs (Kolster, 2018, Barstow et al., 2018). Despite the only reliable proof of fertility is the birth of viable offspring, sperm evaluation provides information regarding the whole genital tract, from seminiferous tubules to the prostate, and the severity of the problem (Kolster, 2018, Freshman 1991, Meyers-Wallen, 1991). Indeed, not only infertility, but also subfertility is a reproductive health issue which is suspected when over 75% of breeding are unsuccessful (Freshman, 2001).

Over the last decades, in humans, total fertility rate (TFR), defined as the average number of live births per woman, has significantly declined in European Union (EU), Japan, and the United States (US) falling below replacement level. In parallel with TFR, human semen quality showed a relevant negative trend (Levine et al., 2017, Sengupta et al., 2018, Merzenich et al., 2010, Swan et al., 2000).

Lea and colleagues (2016) reported an analogous reduction of semen parameters even in breeding dogs over a period of 16 years (Figure 1). These downward trend in both human and canine species

was attributed to a common etiology related to endocrine disrupting compounds (Lea et al., 2016, Bay et al., 2006).

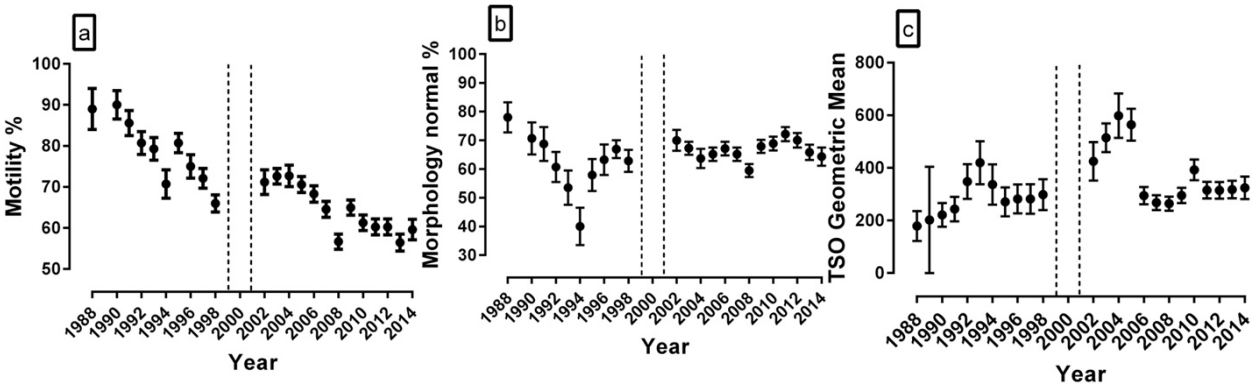

Figure 1. Changes in canine semen parameters over years. a) Percentage of motile spermatozoa, b) Percentage of normal spermatozoa, c) Total spermatozoa output. (Modified from Lea et al., 2016)

As mentioned above, assessing fertility in bitches is trickier mainly because they cycle infrequently and their genital tract is not easily accessible compared to other species (Wilborn & Maxwell, 2012). Performing a breeding soundness examination of female dog can be valuable, but diagnostic information is difficult to obtain and may result in substantial costs for breeders in spite of inconclusive answers (Barstow et al., 2018). Besides physical examination, ultrasonography and vaginoscopy allow to visualize all the entire female genital system, however mild signs of pathology may not be detected (Wilborn & Maxwell, 2012, Freshman, 1991). More invasive procedures such as cytology and biopsy are infrequently performed since they require general anaesthesia and surgery (Wilborn & Maxwell, 2012). All these reasons make it difficult to develop studies on female dog fertility evolution as the one previously described for the male counterpart.

ENVIRONMENTAL FACTORS AFFECTING FERTILITY

Both genes and environment exert substantial influence on fertility (Skakkebaek et al., 2016). In canine species, a strict breeding is carried out on purebred dogs. This genetic selection, mainly focused on peculiar morphological traits and working ability, too often doesn't consider the reproductive aspects thus leading to a reduction in fertility in both males and females (Marelli et al., 2019).

Despite the considerable relevance of selection made by humans, the role of the environment on canine fertility cannot be overlooked. Indeed, living close to their owners, dogs are exposed to the same environmental pollutants that have increased dramatically over the past few decades as a result of technological development (Bergman et al., 2013).

For the purposes of this thesis, some of the major factors affecting human and companion animals' health will be discussed within the following pages.

Endocrine disruptor chemicals

Endocrine disruptor chemicals (EDCs) are among the major environmental factors affecting fertility and have received increasing attention since 90s to date (Krzastek et al., 2020, Caserta et al. 2011). World Health Organization (WHO) defined these compounds as *"exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations"* (Bergman et al., 2013).

Endocrine system participates and coordinates major body functions through hormonal signalling pathways. Hormones are released in bloodstream allowing communication between endocrine glands such as pituitary gland, thyroid gland, adrenal glands and the gonads. The presence of specific cellular receptors is crucial, in fact hormones exert their effects targeting and binding these peculiar proteins (Klein, 2013).

Mimicking or impeding hormones, EDCs can act on membrane and nuclear receptors determining developmental, metabolic, and reproductive disorders (Yilmaz et al., 2020). This detrimental activity is insidious since it may occur even at very low doses and for a prolonged period of time (Vandenberg et al., 2012, Quilaqueo & Villegas, 2021). In 2020 an Experts Consensus defined the ten Key characteristics (KC) of EDCs that describe their mechanisms of action known to date (La Merrill et al.,

2020). These compounds could interact with hormone receptor either as agonists (KC1) or antagonists (KC2). In both cases they inappropriately facilitate or block the action of endogenous hormones. In addition, EDCs can interfere with hormonal signalling by modulating receptor expression (enhancing or degrading them) (KC3) or by altering intracellular signal transduction (KC4). A subtler feature is their ability to induce epigenetic changes in hormone-producing or hormone-responsive cells (KC5) and, similarly, they can stimulate or inhibit hormone synthesis (KC6). EDCs also act on hormonal transport through cell membrane (KC7) and in bloodstream (KC8) as well as on hormone metabolism and clearance (KC9) undermining their bioavailability. Finally, EDCs can impair cellular proliferation, differentiation and apoptosis of tissue involved in hormonal signalling (KC10). All of these key characteristics are illustrated in Figure 2.

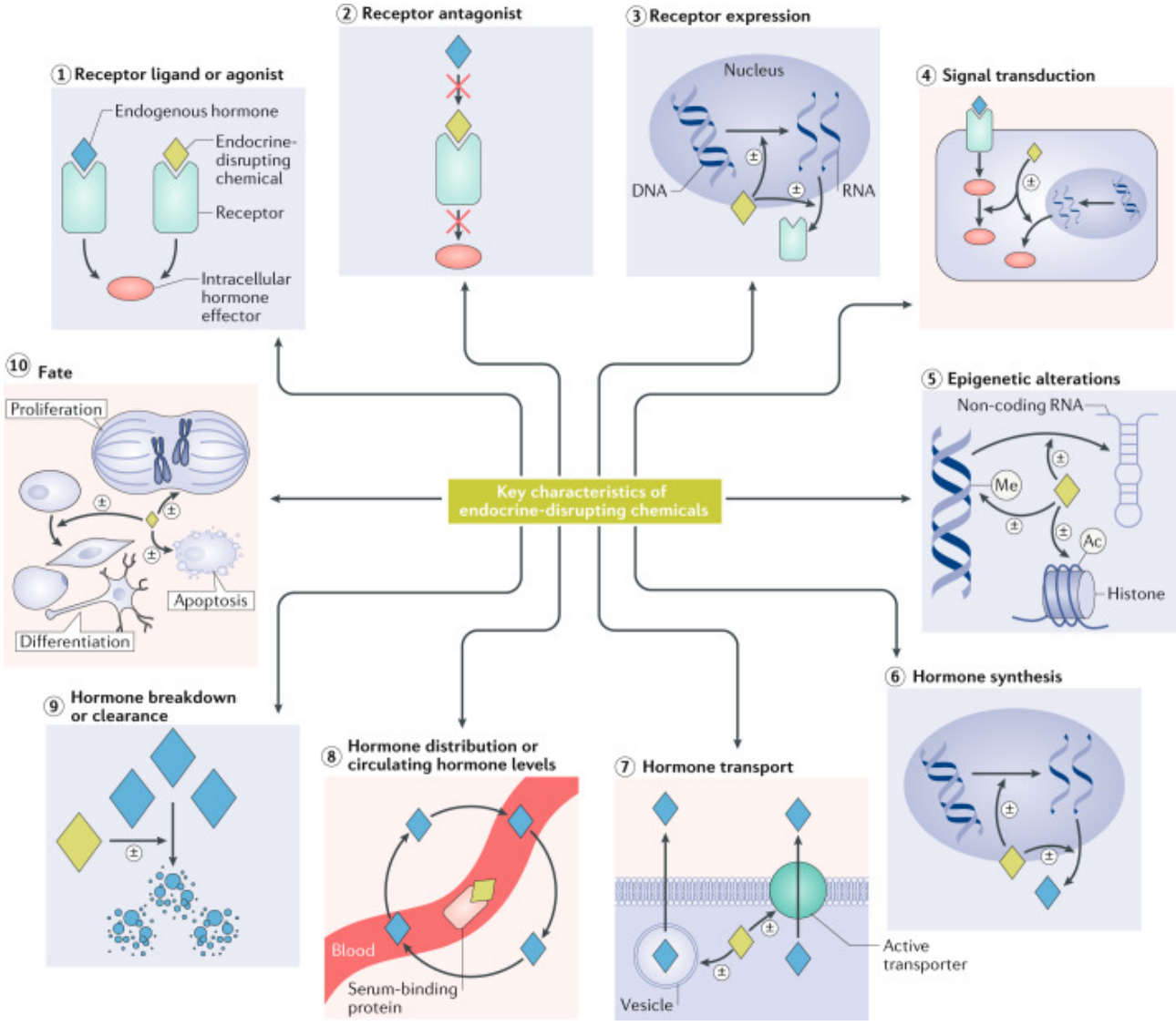

Figure 2. Mechanisms of action of EDC (La Merrill et al., 2020).

These toxic compounds can act throughout life; however, the form and magnitude of their effects varies depending on life stages. The most sensitive period ranges from embryonic phase to early childhood because, at this time, tissues and organs are still under development and are highly susceptible to hormonal and/or EDCs action (Newbold et al., 2007). Changes induced by EDCs in this phase may not be visible at birth, but they persist for the entire life and can emerge even decades later, for example in terms of disease sensitivity (Schug et al., 2011). Moreover, some chemicals seem to produce transgenerational effects leading to the transmission of the disease phenotype through future generations (Skinner et al., 2010). A vivid example of epigenetic transgenerational inheritance is given by diethylstilbestrol (DES). DES is a synthetic estrogen formerly used to prevent miscarriages and complications in pregnant women (Newbold et al., 2006). Subsequently, the exposure of gestating women to DES was linked to many reproductive disorders both in male and female progeny (Giusti et al., 1995). Finally, genital tract abnormalities and neoplastic predisposition were found even in second generation (Newbold et al., 2006) and hypothesized in third generation (Titus-Ernstoff et al., 2008).

In adulthood the negative consequences of EDCs are less intense and reduce when exposure ends (Bergman et al., 2013). The limited adverse effects in adults could be due to the more developed immune system and detoxifying metabolism, better mechanisms of DNA repair as well as effective blood/brain barrier which are immature in foetus and neonate (Schug et al, 2011).

Numerous substances with endocrine disrupting properties have already been identified and constituted a heterogenous groups of both natural substances, such as phytoestrogens, and artificial compounds like plasticizers, pesticides, industrial solvents, pharmaceutical agents and heavy metals (Kabir et al., 2015). The massive industrial and agricultural development during the last decades led to the spread of EDCs, many of which persist into environment and bioaccumulate reaching high concentrations in food chain. For this reason, exposure can occur through air, water, soil and food (Kidd et al., 2012).

Many health concerns were related to endocrine disruption both in humans and animals (Bergman et al., 2013, Hamlin & Guillette 2010, Diamanti-Kandarakis et al., 2009). Due to the severe impact of EDCs on reproductive functions, countless studies were conducted on male and female fertility at different life stages. As mentioned above, an increase in adverse effects were reported in man (Nordkap et al., 2012) in whom environmental hazard has been proposed as the underlying cause of Testicular Dysgenesis Syndrome (TDS) theory. It was first described by Skakkebaek (2001) who

recognized poor semen quality, cryptorchidism, hypospadias, and testicular cancer as symptoms of the same disorder with a univocal etiology based on environmental and lifestyle factors. Thereafter numerous authors have extensively studied TDS in humans (Virtanen & Adamsson 2012, Yeung et al. 2011, Veeramachaneni 2008; Martin et al., 2008).

The countless chemical compounds widespread into environment makes it difficult to assess their precise role on ejaculate. Nonetheless, some authors found that semen quality appeared significantly impaired by exposure to organochlorine pesticides which reduced ejaculate volume and increased spermatozoa morphological abnormalities (Yucra et al., 2006). Besides that, these chemicals can interfere with hypothalamic-pituitary-testes axis and steroid receptors (Mehrpouret al., 2014). Similarly, phthalates like BPA seemed to negatively affect androgens concentration, however their effect is still unclear (Mendiola et al., 2010, Meeker et al., 2010). Petersen and colleagues (2015) focused on PCB and demonstrated their correlation with different hormones while they found no effect on semen quality.

Although it is still debated (Lymperi & Giwercman, 2018), even the development of testicular germ-cell tumours in humans could be due to foetal and child exposure to endocrine disruptor chemicals (McGlynn et al, 2008). In fact, testicular cells differentiation is a strictly regulated process that is highly influenced by environmental conditions (Birnbaum & Fenton, 2003). EDCs could impair endocrine function both interrupting gonocyte development and producing precursors of carcinoma *in situ* (CIS) from which testicular germ-cell tumours originate (Rajpert-De Meyts & Hoei-Hansen, 2007, Figure 3).

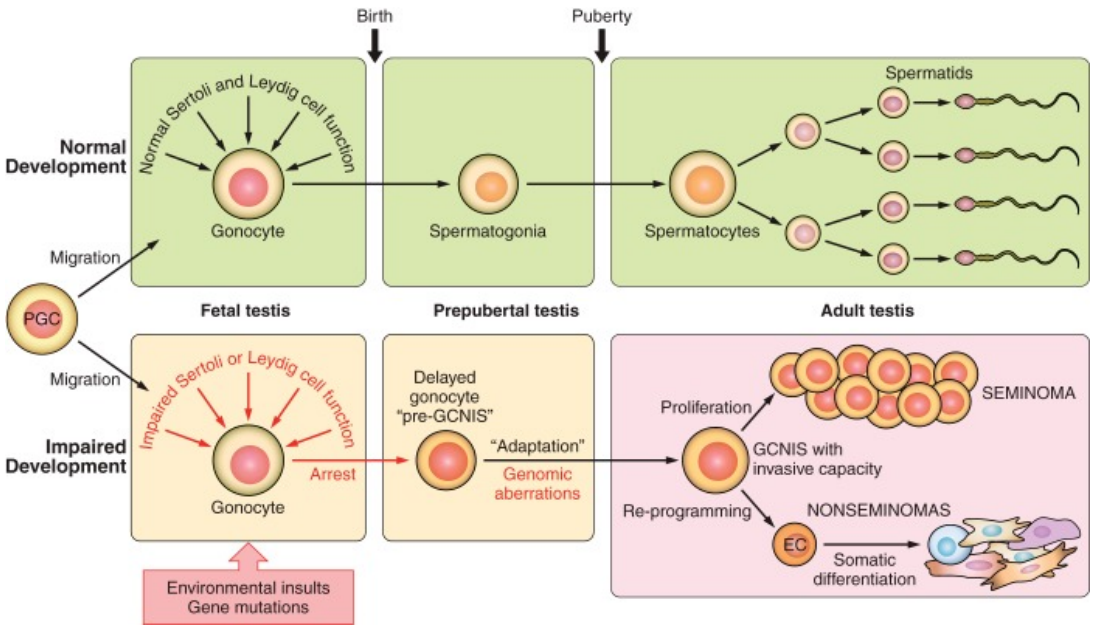

Figure 3. *Development of testicular germ cell tumor (Skakkebaek et al., 2016)*

A recent meta-analysis has collected all scientific evidence linking EDCs with cryptorchidism and hypospadias and found that there was a positive correlation between EDCs and hypospadias. Cryptorchidism risk, instead, increased only with prenatal exposure to phenol substances (Wu et al., 2022). Nonetheless, since EDC exposure is hard to measure and many of these compounds can act together at low doses, further studies could better elucidate the correlation between genital abnormalities and environmental toxicants.

Even in canine species, Testicular Dysgenesis Syndrome was guessed since dogs live close to the owners sharing the same environmental hazard and the same negative trend towards reproductive issues (Lea et al., 2016). So far histological and immunohistochemical clues of TDS were described in canine species (Pecile et al., 2021, Grieco et al., 2008), however literature is still poor, and a single study focused on the role of environmental chemical on canine semen viability, motility and DNA integrity (Lea et al., 2016). Analysing pet food and dog toys, several authors managed to find toxic compounds such as polybrominated diphenylethers (PBDEs), phytoestrogens, bisphenol A (BPA) and phthalates (Wooten & Smith 2013, Dye et al. 2007, Kang & Kondo 2002). Nevertheless, none of these studies deepened the relationship with poor semen quality, cryptorchidism, hypospadias, and testicular cancer.

Despite implications on semen quality and testes are easier to investigate, also many female reproductive disorders were related to EDCs even though more scientific evidence are needed (Caserta et al., 2011). Among them there are different ovarian disturbances regarding oocyte quality and aneuploidy, Polycystic Ovary Syndrome (POS), Premature Ovarian Failure (POF), and impaired cyclicity and fecundability (Crain et al., 2008). Not only ovaries, but also uterus can be altered by environmental toxic compounds. Animal studies showed that exposure to dioxin-like compounds promote endometriosis by interfering with hormonal regulation and immune system (Rier et al., 2001, Cummings et al., 1999). The development of leiomyoma was also associated with exposure to estrogenic compounds, in particular diethylstilbestrol (DES), during prenatal life (Baird & Newbold, 2005). Moreover, although few studies addressed this issue, placentation disfunctions resulting in pregnancy loss and reduced foetal growth were associated to EDCs exposure (Tachibana et al., 2007, Matsuura et al., 2004). Even in women, it was suggested that perturbations during developmental stages could interfere with female development, especially with folliculogenesis, and impair reproductive function in adult life. Taking a cue from man, this hypothesis was named Ovarian

Dysgenesis Syndrome (ODS) (Buck Louis et al., 2011). Unfortunately, in female dogs only few evidence of EDCs action can be found in literature mainly on ovaries removed during spaying and milk: thereby, an effect comparable to that observed in women can only be speculated (Sumner et al., 2020).

Tobacco smoke exposure

Tobacco smoke is a widespread vice in modern society (Ng et al, 2014). The harmful consequences of its consumption received great attention from scientific community due to the strong impact on Public Health (IARC, 2004). Nowadays, cigarettes smoke has been closely related to several type of cancer primarily affecting lungs and airways, digestive system including liver and pancreas, urinary and genital tract (IARC, 2004). In addition, it is a well-known risk factor even for chronic respiratory pathologies (Salvi, 2014), cardiovascular problems (Ambrose & Barua, 2004) and metabolic diseases such as type 2 diabetes (Śliwińska-Mossoń & Milnerowicz, 2017).

Thousands of compounds are produced and released in cigarette smoking process and many of them are toxic, mutagenic and carcinogenic. These chemicals, comprising nicotine and its metabolites, heavy metals and polycyclic aromatic hydrocarbons, can be found in mainstream as well as in sidestream smoke even though at different concentrations (Soleimani et al., 2022).

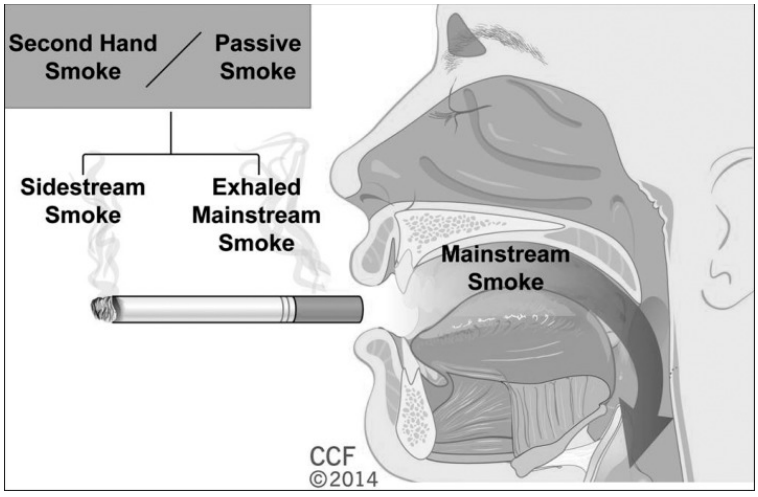

***Figure 4**. Different type of smoke produced (Harlev et al. 2015)*

The term mainstream smoke refers to the smoke actively inhaled and exhaled by smokers. Sidestream smoke, instead, is the one that remains in the air and is generated by cigarette's burning end. First-hand (or active) smokers assume both mainstream and sidestream smoke while second-

hand smokers are exposed to sidestream and exhaled mainstream smoke which comes from smoker's lungs (Harlev et al., 2015). Recently, third-hand smoke exposure has been described and included all tobacco residue that can be absorbed through dermal uptake or ingestion from contaminated surface (Jacob et al., 2017).

The consequences of first- and second-hand smoke have been investigated also in male and female reproductive field, sometimes providing conflicting results. Particularly in man, it was difficult to estimate the precise impact of smoking on fertility and semen quality because many confounding factors are present, like alcohol and drugs assumption, medical illness and occupational hazard, and different criteria were used to assess spermiogram (Sharma et al., 2016, Harlev et al., 2015). Nonetheless, many studies found a detrimental effect of smoking on semen parameters. In healthy men a significant reduction in ejaculate volume, sperm concentration and percentage of motile spermatozoa was found in smokers compared to non-smokers. Additionally, an inverse dose–based association between the number of cigarettes smoked and these semen parameters was suggested (Ramlau-Hansen et al., 2007). Studies conducted on patients attending infertility clinics highlighted similar alterations. Sperm density, total sperm count, and total motile sperm were significantly lower in smokers as well as the percentage of morphologically normal spermatozoa (Liu et al., 2010, Colagar et al., 2007, Kunzle et al., 2003, Zhang et al., 2000, Merino et al., 1998). Moreover, some authors noted a lower ejaculate volume in smokers especially when the number of cigarettes per day exceeded 16 (Zhang et al., 2000, Saaranen et al., 1987).

Nonetheless, it should be considered that spermiogram metrics may not be as meaningful to assess subtle structural and functional defects which can occur even with normal semen parameters (Harlev et al., 2015). For example, aberrations of axonemal microtubules that could alter flagellar movement appeared to be significantly higher in smokers (99%) compared to non-smokers (26%) (Yeung et al., 2009, Zavos et al., 1998). Additionally, other molecular factors can affect sperm motility in smokers such as creatine kinase and acrosin enzymatic function. The former participates in ATP metabolism and seems to be reduced in smokers' sperm (Ghaffari & Rostami, 2013, Miyaji et al., 2001); the latter is an essential protein for spermatozoa penetration of the zona pellucida and it displays lower activity in smokers even with normal semen parameters (El Mulla et al., 1995, Gerhard et al., 1989).

Another deleterious consequence of smoking is the accumulation of reactive oxygen species (ROS) in the sperm production site and the production of superoxide anions that damage spermatozoa membrane, proteins, enzymes, and DNA (Valavanidis et al., 2009). Oxidative stress is triggered by

nicotine metabolites that lead to inflammation and the infiltration of leukocytes into ejaculate further stimulating ROS production (Kao et al., 2008, Saleh et al., 2002).

Even though the consequences of active smoking on semen have already been established, few studies focused on the effect of environmental tobacco smoke (ETS) on male reproduction. Pacifici and colleagues (1995) were able to detect cotinine, a nicotine metabolite used as biomarker of smoke exposure, in seminal plasma of passive smokers. However, a single study on rhesus monkeys, non-human primates, investigated ETS consequences on sperm parameters and function, although it failed to find spermiogram abnormalities (Hung et al., 2009).

The noxious influence of tobacco smoke doesn't stop with the negative implications on male fertility since damages of major importance result from active or passive maternal smoking on foetal and neonatal health. Indeed, a significant increase in miscarriage, perinatal death and preterm birth risk was demonstrated in smoking mothers with a positive correlation to daily amount of cigarette consumed (Pineles et al., 2016, Pineles et al., 2014, Kyrklund-Blomberg et al., 2009). Intrauterine growth retardation (Abraham et al., 2017), behavioural and cognitive issues (Bublitz & Stroud, 2012, Weitzman et al., 2002) as well as developmental disorders like cryptorchidism (Yu et al., 2019) were also related to cigarette smoking during pregnancy.

While smoking is a conscious and dismissible action, exposure to environmental tobacco smoke (ETS) is more insidious and often inevitable, particularly in household and in the workplace. For this reason, numerous studies were conducted to estimate its impact on maternal and foetal/neonatal health and several consequences emerged. Women exposed to second-hand smoke during pregnancy had greater risk of preterm birth (Cui et al., 2016) while their babies seem to have lower weight and head dimension at birth (Salmasi et al., 2010). An abnormal placental development could underlie these findings and placental vascular impairment seemed to be confirmed by an increase of circulating red blood cells which is a marker of foetal hypoxia (Lindbohm et al., 2002, Dollberg et al., 2000). Additionally, congenital malformation such as orofacial clefts and neural tube defects occurred more frequently in babies whose mothers experienced passive smoking during pregnancy (Meng et al., 2018, Sabbagh et al., 2015).

Nicotine is the main tobacco alkaloid, accounting for 95% of the total content. Following inhalation, nicotine is rapidly absorbed through lungs and in less than 20 seconds can reach brain where the psychoactive action, responsible for tobacco addiction, is performed (Benowitz et al., 2009). Once entered the bloodstream, nicotine reaches many organs including kidney, spleen, skeletal muscle,

and liver where it is metabolized to cotinine, its major metabolite. Almost 70–80% of nicotine is metabolized to cotinine which is then excreted through urine (Benowitz et al., 2009).

In humans, cotinine has been extensively used as biomarker of ETS thanks to the clear dose-response relationship and the stability in non-invasive biological sample (Benowitz, 1996, Emmons et al., 1994).

In humans, cotinine can be found in different matrices like blood, hair, saliva, and urine. Cotinine accumulates in great concentration in urine which is the most sensitive matrix. Since it is ease and non-invasive to obtain urine sample, it is widely applied as marker of ETS (Torres et al., 2018). Even hair is a valuable non-invasive cotinine marker (Toraño & van Kan, 2003). Moreover, different from other matrices, hair cotinine proved to be a long-term marker of second-hand smoke in man reflecting the exposure during the previous three months (Bernert et al., 2011, Pichini et al., 1997).

In man, cotinine was also detected in the semen of smoking men, with a concentration comparable to that in blood (Vine et al., 1993), and in the seminal plasma of men exposed to second-hand smoke with levels of cotinine positively correlated to the degree of daily exposure (Pacifici et al., 1995).

In women, cotinine has been widely described in literature and, besides maternal serum, urine, and saliva (Baheiraei et al., 2012, Sachiyo et al., 2012, Eskenazi et al., 1995), it was found also in amniotic fluid and neonatal hair proving its capability to pass placental barrier (Eliopoulos et al., 1994, Jordanov, 1990). Since it pools both foetal excretions and maternal secretions, amniotic fluid appeared to be an excellent tool to detect intrauterine exposure. Moreover, neonatal hair proved to be an effective non-invasive matrix to estimate smoke exposure over the last trimester of pregnancy (Eliopoulos et al., 1994).

Dog as “man best friend” is often exposed to many health hazards related to owner’s habits and has been proposed as an effective model for investigating passive smoke consequences in human species. In this perspective, they are regarded as sentinel of environmental contamination because the shortest life span allows the early recognition of health implications, especially from an oncologic point of view (Bukowski & Wartenberg, 1997). Dogs live ever closer to humans and share the same domestic environment being potentially exposed to second- and third-hand smoke through air, dust surface and cigarettes butts (Jacob et al., 2017). Many authors faced the impact of passive smoke in exposed dogs mainly focusing on respiratory and neoplastic diseases. They showed that the risk for lung (Reif et al., 1992) and nasal (Reif et al., 1997) cancer, lymphoma (Pinello et al., 2017), respiratory disease (Lin et al., 2018, Yamaya et al., 2014, Roza & Viegas, 2007) and atopic dermatitis (Ka et al., 2014) appeared to be greater in dogs subjected to ETS.

Previous studies on canine species assessed smoke exposure looking for nicotine and cotinine in various matrices. In particular, they were found in blood, hair and urine (Bertone-Johnson et al., 2008, Knottenbelt et al., 2012, Bawazeer et al., 2011). However, to date, no study has addressed the detection of these ETS markers in other male and female matrices, such as ejaculate and amniotic fluid, nor it has evaluated the effect on canine reproductive health.

Oxidative stress

One of the most remarkable factors affecting semen quality is oxidative stress defined as an imbalance between reactive oxygen species (ROS) production and the activity of antioxidant system (Walczak–Jedrzejowska et al., 2012). As illustrated in Figure 5, several exogenous and endogenous factors can generate oxidative stress in semen. Among extrinsic sources there are bad lifestyle and habits, environmental pollution, and radiation exposure while intrinsic sources comprise inflammatory and infective diseases, increased scrotal temperature, aging and cancer (Alahmar, 2019, Sabeti et al., 2016).

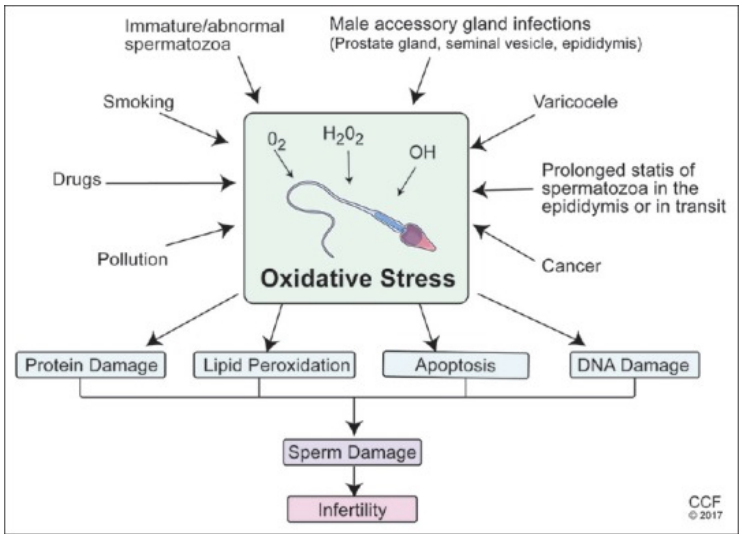

***Figure 5**. Cause and consequences of oxidative stress in ejaculate (Majzoub & Agarwal, 2017).*

When produced within specific limits, ROS are essential to physiological sperm function such as maturation, capacitation and acrosome reaction (Aitken, 2017, O'Flaherty, 2015). An excess of ROS exposes spermatozoa to oxidative stress-induced damages because of the high content of polyunsaturated fatty acids within their membranes together with the low concentration of

scavenging enzymes in the cytoplasm (Alvarez & Storey, 1995, de Lamirande & Gagnon, 1995). Despite the limited amount of cytoplasm, spermatozoa are protected by damages by both enzymatic and non-enzymatic antioxidant systems inside the cell and in seminal plasma (Aitken & Sawyer, 2003). In dogs, 95% of the seminal plasma is produced by the prostate which is the only accessory sex gland and produces the first and the third fractions of the ejaculate (Domoslawska et al., 2019). For this reason, in this species, prostatic fractions are the major source of antioxidant defences that include reduced glutathione (GSH), glutathione peroxidase (GPx), phospholipid hydroperoxide glutathione peroxidase (PHGPx) and superoxide dismutase (SOD) (Vieira et al., 2018, Angrimani et al., 2013, Strzeżek et al., 2009).

In humans, oxidative stress affects spermatozoa both at structural and functional level. Indeed, it negatively influences semen parameters such as motility, velocity, and morphology of male germ cells (Aitken et al., 2012), but also lead to apoptosis by interfering with lipid and protein structures (Wakamatsu et al., 2013). Furthermore, excessive ROS hesitate in impaired genomic and mitochondrial DNA integrity (Bui et al., 2018).

As described for man, elevated ROS levels were related to spermiogram abnormalities even in dogs. In particular, canine spermatozoa exposed to oxidative stress showed a reduction in motility and membrane function as well as a greater number of morphological defects (Tselkas et al., 2000). Nonetheless, in canine species, literature was mainly focused on ROS effects on frozen semen and post-thaw quality (Vieira et al., 2018, Prete et al., 2018, Lucio et al., 2016a, Lucio et al., 2016b). Cryopreservation allows ejaculate long-term storage as well as its worldwide shipping (Thomassen & Farstad, 2009). This process, however, generates high amount of ROS partly due to spermatozoa metabolism and partly caused by the removal of seminal plasma, sperm antioxidant reservoir, before freezing process (Lucio et al., 2016). To face this cryopreservation issue, several antioxidants were added to extenders in order to protect semen from oxidative stress injury (Bang et al., 2021, Schäfer-Somi et al., 2021, Shakouri et al., 2021, Kawasaki et al, 2020, Qamar et al., 2020, Strzeżek et al., 2012, Neagu et al., 2010, Monteiro et al., 2009, Beccaglia et al., 2009, Michael et al., 2007).

Unlike man, in canine species few studies examined the impact of oxidative stress on fresh semen quality. Since many environmental causes can alter ejaculate redox balance, further studies on this topic in dogs are desirable. In this respect, it should be considered harmful consequences seemed to be caused by elevated oxidants levels as well as by an unphysiological high exposure to reductants that culminates in the so-called “reductive stress” (RS) (Castagné et al. 1999) and the rupture of the

ideal redox balance towards both OS and RS had negative impact on semen parameters (Panner Selvam et al., 2020).

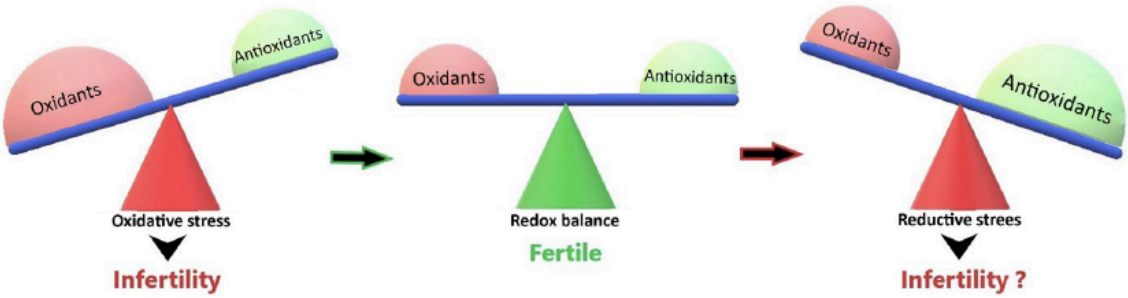

Figure 6. Variations of redox balance (Henkel et al.,2018)

miRNOMICS: OLD QUESTION, CUTTING-EDGE SOLUTIONS

Omics techniques are gaining more and more ground in scientific scene due to their ability to investigate biological processes in deep. The introduction of Next Generation Sequencing (NGS) allowed the acquisition of an unprecedented amount of information regarding genome leading to the spread of omics technologies. The term "-omics" refers to several disciplines which aim to describe and quantify different biological molecules in order to map out structure, function, and dynamics of an organism. Their application covers distinct areas from DNA (genomics), through mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics). Among them, a promising branch is miRNomics, a technique that analyzes miRNA and has been widely applied in medical field especially in oncology.

microRNAs

microRNAs (miRNAs) are small non-coding RNAs composed by a single-stranded RNA molecule of approximately 22 nucleotides (Ambros, 2004). Their maturation occurs through a series of steps. miRNAs originate in the nucleus, then undergo various modifications and finally are exported in the cytoplasm where their development ends (Huang et al., 2011). Representing approximately 3% of human genome (Bartel, 2004), miRNAs participate in numerous biological processes like cellular proliferation, differentiation and apoptosis, organ development, signalling pathways, metabolism, and homeostasis (Huang et al., 2010). In particular, they regulate gene expression at post-transcriptional level by pairing with a complementary portion of mRNA, the seed region (Brennecke et al, 2005). Binding to Argonaute proteins, miRNAs are able to inhibit protein synthesis using the following mechanisms:

A. prevention of translation onset
B. suppression of protein elongation
C. premature interruption of translation (ribosome drop-off)
D. protein degradation in parallel with its synthesis (Eulalio et al., 2008).

This regulatory system involves lot of the genome, in fact, in animals, each miRNA could target lots of different genes and a mRNA could be targeted by multiple miRNAs (Krek et al., 2005, Cai et al., 2009).

miRNAs and cancer

As described above, in physiological conditions miRNAs are fundamental to maintain the proper modulation of cellular processes, including proliferation, differentiation and apoptosis. In fact, their control on gene expression enables the body to deal with environmental stressors like hypoxia, oxidative stress and DNA damage that underlie various illnesses including cancer (Ali Syeda et al., 2020).

Nowadays, it has been widely proven that miRNAs are involved in all aspects of malignancies known as "hallmarks of cancer", namely: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion from apoptosis, limitless replicative potential, prolonged angiogenesis and tissue invasion and metastasis (Di Leva et al., 2014, Hanahan & Weinberg, 2000). Their dysregulation always occurs in neoplastic tissues compared to healthy ones leading to an abnormal growth and a lack of cellular differentiation (Lu et al., 2005). Moreover, comparing a variety of neoplasms, it was possible to characterize the miRNome for each kind of tumor and highlight the differential expression of miRNAs between them (Volinia et al., 2006). In this respect, the down-regulated miRNAs are considered as Tumor-suppressor miRs because their reduction promotes oncogenes translation and cancer progression. On the other hand, the up-regulated miRNAs are regarded as OncomiRs since they inhibit tumor suppressor genes (Mishra et al., 2016, Di Leva et al., 2014). This sort of miRNA-based identity card defines cancer pathological features such as aggressiveness, vascular invasion, mitotic index (Calin & Croce, 2006).

Circulating miRNAs: the novel biomarkers

Most of miRNAs fulfil their function at intracellular level, however some of these molecules can be found also in the extracellular space (Cui et al., 2019). In fact, miRNAs were detected in various body fluid including blood, urine, saliva, amniotic fluid and seminal plasma (Cortez et al., 2011, Weber et al., 2010). These cell-free miRNAs are termed "circulating miRNAs" and can be diffused within the body in both passive and active ways. In the first case, miRNAs are released as a result of cellular damage (Valihrach et al., 2020) while the active secretion of miRNAs occurs through mechanisms involving protein carriers and vesicles (Arroyo et al., 2011, Zhang et al., 2015). Among transport proteins, extracellular miRNAs can bind to low density (LDL) and high density (HDL) lipoproteins (Michell & Vickers, 2017), but also to Argonaute proteins (Arroyo et al., 2011) and nucleophosmin 1

(NPM1), a nucleolar protein (Wang et al., 2010). Vesicles transport, instead, is carried out via exosomes which are small vesicles that are actively loaded with miRNAs (Mittelbrunn et al., 2011) and then released after fusion with the cell membrane (Zhang et al., 2015). Protein-mediated transport seems to be complementary and independent from vesicles one since the profile of delivered miRNA is dissimilar. Nonetheless, all these kinds of transport guarantee miRNAs stability and protect them from degradation (Mori et al., 2019).

Circulating miRNAs represent a novel and intriguing method for intercellular communication that differs from common signalling pathways controlled by neurotransmitter, hormone, and cytokines (Cui et al., 2019). This is especially noteworthy in oncology because circulating miRNAs can reach the recipient cells allowing the invasion and metastasis of the primary tumor (Mei et al., 2011). The presence of extracellular miRNAs, however, find a powerful application for diagnostic, prognostic and therapeutic purposes. In fact, circulating miRNAs display many features required to a potential biomarker. They are sensitive and specific for different neoplasms (Lu et al., 2005), remain stable in body fluid thanks to protein binding and exosome that protect them from ribonucleases (Grasedieck et al., 2012; Mitchell et al., 2008) and can be collected in non-invasive ways (Mori et al., 2019). In addition, due to their small size (~22 nucleotides), miRNAs survive to formalin fixation and paraffin embedding allowing countless retrospective studies performed on long-term stored samples (Liu & Xu, 2011, Klopfleisch et al., 2011).

miRNAs and Testicular Germ Cell Tumor (TGCT)

Testicular germ cell tumors (TGCT) include two groups of neoplasms with different histological features: seminoma (SEM) and non-seminoma (NSEM). Despite their heterogeneity, TGCTs seem to arise from a common precursor, germ cell neoplasia in situ (GCNIS), that originates from a primordial germ cell (PGC) that failed to evolve to spermatogonium (Baroni et al., 2019, Berney et al., 2016).

The incidence of TGCT has substantially risen over the last decades in parallel with other reproductive abnormalities like cryptorchidism, hypospadias and testicular dysgenesis (Chieffi et al., 2012, Chia et al., 2010). Aside from genetic aspects, environmental factors seem to play a major role in TGCT onset interfering with primordial germ cells differentiation during pregnancy and favouring the development GCNIS (Rajpert-De Meyts & Hoei-Hansen, 2007).

Traditional biomarkers like alpha-fetoprotein (AFP), human-chorionic-gonadotropin (HCG) and lactate dehydrogenase (LDH) lack of sensitivity and specificity, being detectable in only 50% of patients. For this reason, they found poor application for diagnosis and follow-up of TGCTs. In recent years, searching for new biomarkers, many authors studied miRNA expression in TGCT and found two clusters that were up-regulated in these neoplasms irrespective of age, tumor site and histotype: miR-302/367 and miR-371-373 (Palmer et al., 2010). Sharing similar mRNA target (Gao et al., 2015), miR-302 and miR-367 speeds up the proliferation of embryonic and pluripotent germ cells by interacting with genes involved in cell cycle (Lie t al., 2016, Kuo et al., 2012). miR-371-373 cluster has been extensively studied in oncology because it is involved in cell proliferation (Lee et al., 2009) as well as in metastasis and invasion (Huang et al., 2008). Beside promoting proliferation and invasion (Zhou et al., 2012), its overexpression in TGCTs seems to produce the accumulation of DNA mutations (Wei et al., 2015, Voorhoeve et al., 2006). mir-371 group was than detected as circulating in blood of patients affected by TGCT thus it was proposed and subsequently confirmed as effective serum biomarkers for TGCT improving diagnosis and follow-up for these neoplasia (Dieckmann et al., 2019, Murray et al., 2011).

miRNA in the canine species

miRNomics is becoming more and more popular even in canine species, that is regarded as an animal model for humans. Many similarities in pathological, clinical and diagnostic features occur between man and dog diseases, especially in oncologic field. For this reason, in veterinary medicine, several studies investigated the role of miRNAs in different kinds of cancer (Sahabi et al., 2018).

The most explored malignancies include lymphoma (Craig et al., 2019, Uhl et al.,2011, Mortarino et al., 2010), mammary gland cancer (Chen et al., 2022, Fish et al., 2020, Osaki et al., 2016) and osteosarcoma (Leonardi et al., 2021, Gourbault & Llobat, 2020). In addition, miRNA dysregulation was studied in canine mast cell tumor (Zamarian et al., 2020), transitional cell carcinoma (Kent et al., 2017), melanoma (Zamarian et al., 2019) and hemangiosarcoma (Grimes et al., 2016).

To date, there is little information regarding miRNAs and canine genital system primarily about their expression in dogs' testes. In particular, Kasimanickam and colleagues focused on miRNAs expression in spermatogenesis (Kasimanickam et al., 2014) as well as in mature and immature gonads (Kasimanickam et al., 2015). In the first work, they found an up-regulation of 9 miRNA families (miR-200, Mirlet-7, miR-125, miR-146, miR-34, miR-23, cfa-miR-184, cfa-miR-214 and cfa-miR-141) and a

down-regulation of cfa-miR-19a, cfa-miR-29b, cfa-miR-29c, cfa-miR-101 and cfa-miR-137 following retinoic acid-induced spermatogenesis (Kasimanickam et al., 2014). The second study, instead, highlighted a different miRNA expression in gonads of adult dog compared with prepubertal ones showing that miRNA profile varies according to life stages (Kasimanickam et al., 2015). Ultimately, circulating miRNAs were evaluated as marker of testicular toxicity and cfa-miR-146b emerged as potential candidate (Shing et al., 2021).

In dogs, no studies were carried out on miRNAs and testicular tumors so far. Since miRNAs appear to be conserved among different species (Lee et al., 2007), the analysis of the aforementioned human markers could represent an interesting perspective even in canine TGCT.

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AIM OF THE STUDY

The definition of the environmental role on fertility is challenging both in humans and animals because countless factors are involved. Although it is difficult to clarify the cause-effect link and the mechanism of action, there is a growing body of evidence suggesting the detrimental effect of pollutants on gonadal development and reproductive potential. In human medicine, literature on this topic has increased greatly over years, conversely in canine species knowledge is limited despite dogs are often exposed to the same toxicants of man.

The overall aim of this PhD thesis was to deepen the impact of the environmental hazards on reproductive health both in male and female dogs.

For this purpose, the first couple of studies focused on the exposure of male dogs and pregnant bitches to cigarette smoke through cotinine, which is a marker of nicotine intake. In the former study we attempted to detect cotinine in serum, ejaculate and hair of dogs living with and without smoking owners. Then cotinine concentration was related to semen parameters and total antioxidant capacity (TAC), both in blood and seminal plasma, to estimate the consequences of oxidative stress due to smoking. Similarly, the succeeding study aimed to measure cotinine in maternal and foetal matrices like dams' serum, amniotic fluid and hair of pups and bitches. Even in these patients the relationship between cotinine levels and exposure degree was examined as well as pregnancy outcomes.

Within the two following projects, we examined cryptorchidism, a developmental disorder whose multifactorial etiology also includes the exposure to endocrine disruptor chemicals. Initially, we investigated the presence of precancerous testicular lesions, such as immaturity and atrophy, in scrotal and undescended testes of cryptorchid patients using immunohistochemistry markers. Subsequently, gonadal neoplastic predisposition of unilateral cryptorchid dogs was analysed by means of miRNomics, an innovative molecular technique that allowed us to evaluate changes in gene regulation occurring in this pathology.

RESEARCH PAPERS

Paper almost ready to be submitted to an international journal.

EFFECTS OF SMOKE EXPOSURE ON CANINE MALE REPRODUCTION

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Abstract

Tobacco smoke has numerous side-effects on human and animal health. In male reproduction, active smoke impairs seminal parameters, increases ejaculate oxidative stress, and causes DNA fragmentation in germ cells. The consequences of passive smoke on semen quality, instead, were poorly investigated and in canine species no study addressed this topic. For this reason, this study aimed to detect cotinine (a nicotine metabolite) in serum, ejaculate and hair of male dogs living with and without smoking owners and to weight its influence on semen parameters and total antioxidant capacity (TAC). Ten dogs were included in passive smokers group (PS) while other ten were regarded as not-exposed (NE). All of them were purebred dogs belonging to 13 different breeds; age ranged between 1,5 and 7,5 years (4,13 ± 1,75) and body weight varied between 16 and 77,7 kg (34,21 ± 12,82). Cotinine was detectable in all samples collected and its concentration was significantly higher in blood serum (P = 0,0002), semen (P = 0,0002) and hair (P = 0,0004) of dogs exposed to passive smoke compared with non-exposed patients. Nevertheless, no differences were found in spermiogram parameters between PS and NE groups and their total antioxidant capacity in plasma and sperm-rich fraction was comparable. These results showed little effect of second-hand smoke on semen parameters and oxidative stress level, however, to deepen functional abnormalities of spermatozoa further studies are needed. Despite this, cotinine was reconfirmed an effective biomarker of environmental tobacco smoke (ETS) exposure in dogs and, for the first time, it was found into canine ejaculate depicting a potential concern for male fertility.

Keywords: Dog, Cotinine, Semen, Oxidative stress

1. Introduction

Tobacco smoking is a widespread bad habit in modern society (Ng et al, 2014). The harmful consequences of its consumption received great attention from scientific community due to the strong impact on Public Health (IARC, 2004). Nowadays, cigarettes smoke has been closely related to several type of cancer primarily affecting lungs and airways (IARC, 2004). In addition, it is a well-known risk factor for chronic respiratory pathologies (Cheraghi & Salvi, 2009), cardiovascular problems (Ambrose & Barua, 2004) and metabolic diseases such as type 2 diabetes *(*Śliwińska-Mossoń & Milnerowicz, 2017*)*.

Despite a broad range of evidence has showed its negative impact, the role of tobacco smoke on male reproduction and fertility is still contradictory (Harlev et al., 2015). Nonetheless, many authors found detrimental effects of smoking on semen parameters both in healthy and infertile human patients. In the former, a significant reduction in ejaculate volume, sperm concentration and percentage of motile spermatozoa was reported (Taha et al., 2012, Ramlau-Hansen et al., 2007, Zavos et al., 1998). Infertile men, instead, showed lower levels of ejaculated volume, total sperm count, percentage of motile and morphologically normal spermatozoa (Liu et al., 2010, Kunzle et al., 2003, Zhang et al., 2000, Merino et al., 1998). The mechanisms through which cigarette smoke impair man fertility has not been fully understood. One of the most compelling hypotheses concerns oxidative stress (Aitken et al., 1989). The imbalance between reactive oxygen species (ROS) production and body antioxidant defences determines oxidative stress (Sharma et al., 1999). The high content of polyunsaturated fatty acids within the plasma membranes together with the low concentration of scavenging enzymes within the cytoplasm expose spermatozoa to oxidative stress-induced damages (Alvarez & Storey, 1995, de Lamirande & Gagnon, 1995). By determining leukocyte infiltration into ejaculate, cigarette smoke increases ROS levels and enhances seminal oxidative stress which could result in poor sperm function (Kao et al., 2008, Saleh et al., 2002).

Although the consequences of active smoking on semen have already been established in men, few studies focused on the effect of environmental tobacco smoke (ETS) that is, exposure to second-hand and third-hand smoke, on male reproduction. Pacifici et al. (1995) detected cotinine, a nicotine metabolite used as biomarker of smoke exposure, in seminal plasma of human passive smokers while no abnormalities in spermiogram and sperm function were found in rhesus monkeys, non-human primates, exposed to ETS (Hung et al., 2009).

Dog as “man best friend” is often exposed to many health hazards related to owners’ habits. In dogs exposed to second-hand smoke the risk for lung (Reif et al., 1992) and nasal (Reif et al., 1998) cancer, lymphoma (Pinello et al., 2017), respiratory disease (Lin et al., 2018) and atopic dermatitis (Ka et al., 2014) appeared to be greater. However, to date, no study has addressed environmental smoke effects on semen quality and male reproductive health in canine species. Trying to fill the gap, in the present study we aimed to evaluate the presence of cotinine within the serum, ejaculate and hair of dogs living with and without smoking owners. In addition, we related cotinine concentration with spermiogram parameters and total antioxidant capacity both in blood and seminal plasma of our patients.

2. Materials and methods

2.1. Animals

The study was carried out between 2020 and 2021 once the approval of the Ethical Committee was obtained (protocol OPBA_161_2019). Over this period, twenty purebred male dogs attending to the Reproduction Unit of the Veterinary Teaching Hospital (VTH) of the Università degli Studi di Milano were enrolled with the prior consent of the owners.

For each patient information regarding breed, age, body weight, diet, lifestyle, food supplementation and drugs assumption were recorded. Dogs were divided into two groups based on the smoking habits of their owners: passive smokers (PS) and not-exposed (NE). Patients were included in PS group if at least 10 cigarettes were smoked in domestic environment while they are considered as NE if nobody smoked near them.

Only dogs with no concomitant disease and general good health were included and underwent a complete andrological examination that involved semen analysis and ultrasonographic evaluation of genital tract. Both testes were measured with a caliper and volume established using the formula of an ellipsoid:

$$[Volume] = [Lenght] \times [Width] \times [Height] \times 0.5236$$ (Gouletsou et al., 2008).

Mean testicular volume was calculated as the arithmetic average between right and left testicular volume.

2.2. Semen collection and evaluation

Semen collection was conducted by manual stimulation in the presence of a bitch in oestrus or using swabs soaked in oestrus vaginal secretions. Using a soft plastic cone (Lane Manufacturing Company, Denver, USA) connected to pre-warmed 50 ml tubes (Falcon™), the ejaculate was divided into pre-sperm, sperm-rich and prostatic fluid fractions. Spermiogram was performed immediately after collection. At first, semen volume and colour were assessed, then 10 µl of sperm-rich fraction was microscopically observed to estimate the percentage of progressively motile spermatozoa. Movement quality was rated on a scale from 0 to 5 (0 = motionlessness, 1 – 3 = slow and non-linear movement, 4 = moderate and linear movement, 5 = fast, linear, and progressive movement) (Freshman, 2002, Johnston et al., 2001). Spermatozoa concentration was measured through a SDM1 spectrophotometer calibrated for the canine species (Minitube International AG, Germany). Viability and morphology were evaluated after staining smears with eosin-nigrosin and Rose Bengal-Victoria blue dye, respectively (Kolster, 2018, Galli et al., 1989). Two experienced clinicians independently analysed one hundred spermatozoa per slide under oil immersion (X100), then the percentage of viable and morphologically normal spermatozoa was defined as the mean score. Primary and secondary defects were also quantified as a percentage.

The overall quality of the spermiogram was defined based on features outlined in Table 1 which refers to previous literature (Johnston et al., 2001, Linde-Forsberg, 1991). Score 1 was associated to poor semen quality while score 2 indicated good to optimal semen parameters.

Once the spermiogram was concluded, prostatic fraction was centrifuged (10 minutes for 800g) and its supernatant as well as sperm-rich fraction were stored at -80°C for further analysis.

2.3. Blood and hair sample collection

Blood samples and hair from para-preputial area were collected before the ultrasound examination.

Blood samples were collected into K2EDTA and serum separator tubes from cephalic or saphenous vein. Subsequently, they were centrifuged (5 minutes for 3500 rpm) to get plasma and serum that were than stored at -80°C for further analysis. An aliquot of serum was assigned to cotinine assay while plasma was used for total antioxidant capacity measurement.

Hair was sheared close to the skin of the para-preputial area. Once obtained more than 100 mg of hair sample, it was stored into a paper envelope until analysis.

2.4. Genital ultrasound examination

Prostate and testes were evaluated using Esaote MyLab Five ultrasound system with multifrequency microconvex (5-9 MHz) and linear (9-13 MHz) probes.

After shaving, ultrasound was performed, and prostate size measured both on transversal and longitudinal axis recording any asymmetry of the lobes. Prostatic parenchyma was scanned for dishomogeneity, mineralization, cysts, abscesses, and focal lesions. Prostates with signs referable to Benign Prosatic Hyperplasia (BPH) were assigned with score 2 while that without ultrasonographic changes were considered healthy and assigned with score 1.

Similarly, both testes were measured and echostructure was examined for texture abnormalities and suspected neoplastic lesions (Mantziaras, 2020).

2.5. Cotinine assay

At the time of analysis, after thawing, blood serum and sperm rich fraction were centrifuged at 3500g for 15 minutes, then supernatant was collected and directly analysed. Hair cotinine assay required extraction that was performed following the procedure described by Bennett & Hayssen (2010). Calibration curves were developed following manufacturer instructions and are shown in Figure 1a and 1b. Then, cotinine concentration was determined in blood serum, sperm-rich fraction and para-preputial hair using an ELISA immunoassay kit specific for canine species and suitable for every matrix (Cotinine (Cot)ELISA kit, Cloud-Clone Corp.) (intra-assay CV < 10%; inter-assay CV < 12%).

2.6. Total antioxidant capacity (TAC) analysis

Total antioxidant capacity was measured in blood plasma and seminal plasma obtained after centrifugation of the prostatic fraction (10 minutes for 800g). After thawing, a 1:2 dilution in phosphate-buffered saline (PBS) was performed for each sample. Total antioxidant capacity was assessed using the ABTS radical cation assay as previously reported by Re et al. (1999).

2.7. Statistical Analysis

Data were analysed using IBM SPSS 26.0 statistical program (IBM, Armonk, U.S.A.) and descriptive statistics for quantitative variables were expressed as range, mean and standard deviation. The Mann-Withney non-parametric test was used to compare mean testicular volume, spermiogram parameters (except for movement quality), cotinine concentration in each matrix and total antioxidant capacity in plasma and semen between passive smokers (PS) and non-exposed (NE) groups. The differences in sperm movement quality, prostate condition, and global semen quality among the two groups were analysed using the Pearson Chi-Square test.

The Spearman's correlation coefficient was used to measure the association among serum, hair and seminal plasma cotinine concentration and age, body weight, spermiogram parameters as well as total antioxidant capacity. The same test was applied to study the correlation of semen parameters with each other and with total antioxidant capacity.

P value lower than 0.05 was considered statistically significant while $p < 0.001$ was considered highly significant.

3. Results

3.1. Study population

All the twenty dogs enrolled were purebred dogs belonging to 13 different breeds among which Kurzhaar (20%) and Labrador Retriever (15%) prevailed. Patients' age ranged between 1,5 and 7,5 years (4,13 ± 1,75) and body weight varied between 16 and 77,7 kg (34,21 ± 12,82). Based on their exposure to cigarette smoke, 10 dogs were regarded as passive smokers (PS) while the remaining 10 were considered non-exposed (NE). All dogs were fed with dry commercial food and none of them was administered with drugs or food supplementation. Except for three hunting dogs, other patients were regarded as companion animals.

Mean testicular volume differ greatly among dogs ranging from 4,45 cm^3 of an Entlebucher Mountain Dog to 34,87 cm^3 of a Bracco Italiano (17,38 ± 8,47). Prostate with pathological signs were found in 6 out of 20 patients.

Table 2 illustrates the characteristics of dogs belonging to PS and NE group. No statistical differences in these parameters were found between two groups.

3.2. Spermiogram outcomes

Spermiogram of each patient is presented in Table 3. Total concentration of spermatozoa ranged from 86 x 10^6 to 1370 x 10^6. The percentage of progressive motile spermatozoa was 40 to 90 and the quality of their movement 1 to 5. Sperm viability was high in all patients while morphologically normal spermatozoa differed greatly among patients, ranging from 9 to 92%. Similarly, the percentage of primary defects varied from 0 to 56 and secondary defects from 7 to 39.

The percentage of progressive motile spermatozoa was positively correlated with both viability (P = 0,0003) and morphologically normal spermatozoa (P = 0,005) while it was negatively related to the presence of primary defects (P = 0,001).

The evaluation of spermiogram parameters revealed no significant difference between PS and NE groups. However, the average total sperm count was slightly lower in dogs exposed to passive smoke (620,7 x 10^6) compared to non-exposed (751,8 x 10^6). Moreover, primary defects were higher in non-exposed dogs (11,2%) compared to passive smokers (7,2%). The other semen parameters, namely percentage of progressive motile spermatozoa, movement quality, viability, and secondary defects were similar in two groups. The overall quality of spermiogram didn't change significantly between PS and NE groups.

3.3. Ultrasonographic findings

No suspected neoplastic lesions were found in prostate and testes of patients included in the study. Benign Prostatic Hyperplasia (BPH) was diagnosed in 6 patients (n = 5 in NE group, n = 1 in PS group) with a significant prevalence in NE compared to PS (P = 0,019).

3.4. Cotinine detection

Cotinine was detectable in all blood serum, sperm-rich fraction and hair samples of both PS and NE groups. The highest cotinine concentration was recorded in serum with an average of 4,7 ± 3,9 ng/ml

and was positively correlated to that in hair (1,9 ± 1,07 ng/m; P < 0,0001) and semen (0,9 ± 0,6 ng/ml; P < 0,0001) (Figure 2).

Cotinine concentration was significantly higher in blood serum (P = 0,0002), semen (P = 0,0002) and hair (P = 0,0004) of dogs exposed to passive smoke compared with non-exposed patients (Figure 3).

No differences were found between cotinine levels in all matrices and spermiogram parameters.

3.5. Total antioxidant capacity

Total antioxidant capacity was almost equal in the two matrices with slightly higher value in plasma (9,11 ± 0,67 µmol Trolox equivalent/L) than in sperm-rich fraction (8,00 ± 1,41 µmol Trolox equivalent/L).

No statistical difference in total antioxidant capacity was found between Passive Smokers and Non-Exposed groups both in serum and in ejaculate (Figure 4). Seminal parameters were not correlated to total antioxidant capacity in sperm, except for the percentage of progressive motile spermatozoa which was negatively (P = 0,028).

4. Discussion

Due to its long half-life, in humans cotinine is a reliable marker of nicotine intake both in active and passive smokers (Benowitz, 1996) and has been detected even in non-invasive matrices such as saliva and hair (Toraño & van Kan, 2003). In particular, hair nicotine and cotinine have proven to be long-term markers of second-hand smoke in man reflecting the exposure during the previous three months (Bernert et al., 2011, Pichini et al., 1997). The presence of these two toxicants in hair was previously reported as strongly associated with ETS exposure even in dogs (Knottenbelt et al., 2012, Bawazeer et al., 2012). As expected, based on previous literature (Knottenbelt et al., 2012, Bawazeer et al., 2012), we succeeded in quantifying cotinine in hair sample finding a significantly higher concentration in dogs exposed to ETS compared to non-exposed ones (P = 0,0004). This outcome pointed out the effectiveness of hair cotinine as long-term marker of ETS in dog (Knottenbelt et al., 2012, Bawazeer et al., 2012)

Due to its metabolism, in man cotinine can be found in high concentration in urine (Torres et al., 2018). Although far below urinary levels, cotinine was also detected in the semen of smoking men,

with a concentration comparable to that in blood (Vine et al., 1993), and in the seminal plasma of men exposed to second-hand smoke with levels of cotinine positively correlated to the degree of daily exposure (Pacifici et al., 1995). Similarly, in canine species, cotinine was identified in serum and urine with a concentration proportional to the number of cigarettes smoked by the owners. In moderate smokers mean urinary concentration in dog was 10,4 ng/ml that rose to 22,5 ng/ml with heavy smoker owners (Bertone-Johnson et al., 2008). No previous data exist on canine cotinine in semen. Despite the concentration in dogs was always lower compared to serum and hair ones, seminal cotinine was significantly higher in dogs with smoking owners than in not exposed dogs (P = 0,0002) proving once again its effectiveness as biomarker of exposure.

Pacifici et al. (1993) also reported the adverse effect of nicotine metabolites on semen parameters especially concerning sperm motility both as percentage of motile spermatozoa and as forward motility. In line with the previous study on Rhesus macaques (*Macaca mulatta*) which didn't find spermiogram abnormalities in primates exposed to ETS (Hung et al., 2009), in our caseload no significant deterioration of semen parameters was highlighted in dogs exposed to cigarettes smoke albeit a reduction in total sperm count was noted. Nevertheless, it is important to consider that spermiogram metrics may not be as meaningful to assess subtle structural and functional defects which can occur even with normal semen parameters (Harlev et al., 2015). Among them, for example, axonemal microtubules aberrations and reduction of creatine kinase and acrosin activity, that can impact on male fertility and whose incidence seemed to be greater in smokers (Ghaffari et al., 2013, Zavos et al., 1998, Gerhard et al., 1989).

Cotinine can cross the blood-testis barrier and interfere with germ cells development mainly at DNA levels (Pereira et al. 2014, Linschooten et al. 2013, Marchetti et al. 2011). Some DNA damages, such as DNA fragmentation, occur more frequently in smokers (Sepaniak et al., 2006) and are caused by oxidative stress (Dai et al., 2015). In our sample, total antioxidant capacity was similar in passive smokers and non-exposed dogs both in plasma and in sperm-rich fraction. Moreover, unlike a previous study (Zhang et al, 2013), we didn't find leukocytes in seminal smears which can justify an increase in reactive oxygen species in dogs exposed to ETS. Since total antioxidant capacity comprises the activity of many antioxidant compounds (Bartosz, 2003), our result could be biased by confounding factors like patients' different diet, age, and other environmental risk apart from tobacco smoke. The enrollment of patients belonging to the same facility and the application of ROS - TAC score (Sharma et al., 1999), may help to elucidate the real effect of passive smoke on seminal oxidative stress and its harmful effect on sperm function.

5. Conclusions

In spite of the small sample size, cotinine was confirmed as a robust biomarker of smoke exposure even in canine hair and seminal plasma. Little effect of second-hand smoke on semen parameters were found, therefore, further studies are needed to deepen functional abnormalities of spermatozoa.

Authorship contribution statement

Giulia Pizzi: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. Silvia Michela Mazzola: Methodology, Formal analysis, Investigation, Data curation. Eleonora Fusi: Methodology, Formal analysis, Investigation, Data curation. Valerio Bronzo: Formal analysis. Debora Groppetti: Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing.

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Legends

Table 1. Metrics used to assess semen quality

	Score	
	1	2
Total sperm count	< 300 x10^6	≥ 300 x 10^6
Progressive motile spermatozoa (%)	< 70	≥ 70
Movement quality	< 4	≥ 4
Normal morphology of spermatozoa (%)	< 70	≥ 70

Table 2. Differences between "Passive Smokers" dogs and "Non-Exposed" dogs

	Passive Smokers	Non-Exposed
Age (ys)	3,6 ± 1,3	4,6 ± 2,1
Body weight (kg)	36,08 ± 15,9	32,3 ± 9,2
Mean testicular volume (cm^3)	15,2 ± 5,8	19,8 ± 9,4
n° of pathologic prostates	1	5

Table 3. Spermiogram outcomes

ID	Breed	Smoke Exposure	Total Sperm Count	Motility (%)	Movement Quality	Viability (%)	Morphology (%)		
							N	1	2
1	Kurzhaar	NE	702	90	4	88	74	1	25
2	Rhodesian Ridgeback	NE	1080	60	4	87	79	6	16
3	German Shepherd	NE	912	90	5	95	86	4	10
4	Kurzhaar	NE	1085	90	5	98	73	5	22
5	Bracco Italiano	NE	1370	90	5	96	87	5	8
6	German Shepherd	NE	468	80	4	88	64	14	22
7	Bernese Mountain Dog	NE	116	40	2	86	9	56	35
8	Kurzhaar	NE	119	90	5	90	92	1	7
9	Hovawart	NE	670	80	4	94	67	16	17
10	English Setter	NE	996	90	5	96	83	4	13
11	Entlebucher Mountain Dog	PS	715,5	50	4	87	17	44	39
12	Kurzhaar	PS	86	90	4	97	78	0	22
13	Labrador Retriever	PS	1358	90	5	95	74	6	20
14	Labrador Retriever	PS	1148	90	5	95	82	3	15
15	Labrador Retriever	PS	936	90	4	96	68	6	26
16	Bassethound	PS	400	90	4	95	85	5	10
17	Great Dane	PS	130,5	70	4	92	75	4	21
18	Afghan Hound	PS	747	90	5	93	90	1	19
19	Pharaoh Hound	PS	293,6	90	5	95	90	0	10
20	Bassethound	PS	392	90	5	97	75	3	22
Mean			686,2	82,0	4,4	93,0	72,4	9,2	18,9
Standard deviation			419,7	15,1	0,8	3,9	21,8	14,7	8,4

N: morphologically normal spermatozoa, 1: primary defects, 2: secondary defects

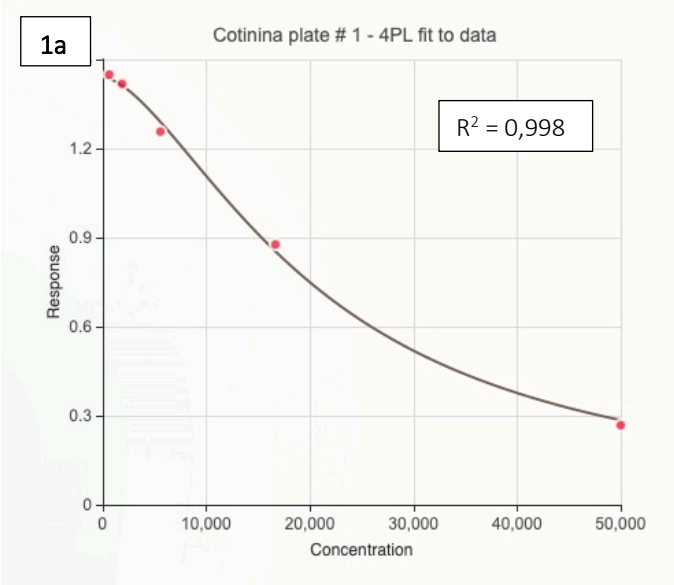

***Figure 1a**. Calibration curve (Plate 1)*

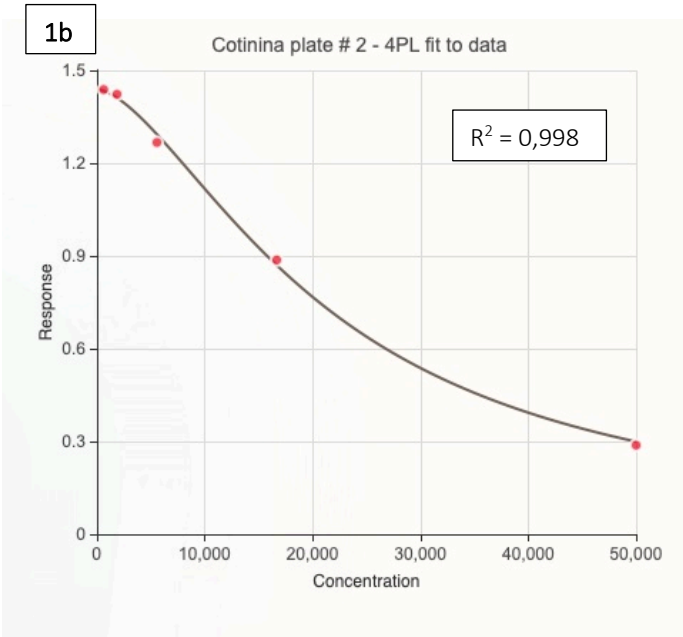

***Figure 1b**. Calibration curve (Plate 2)*

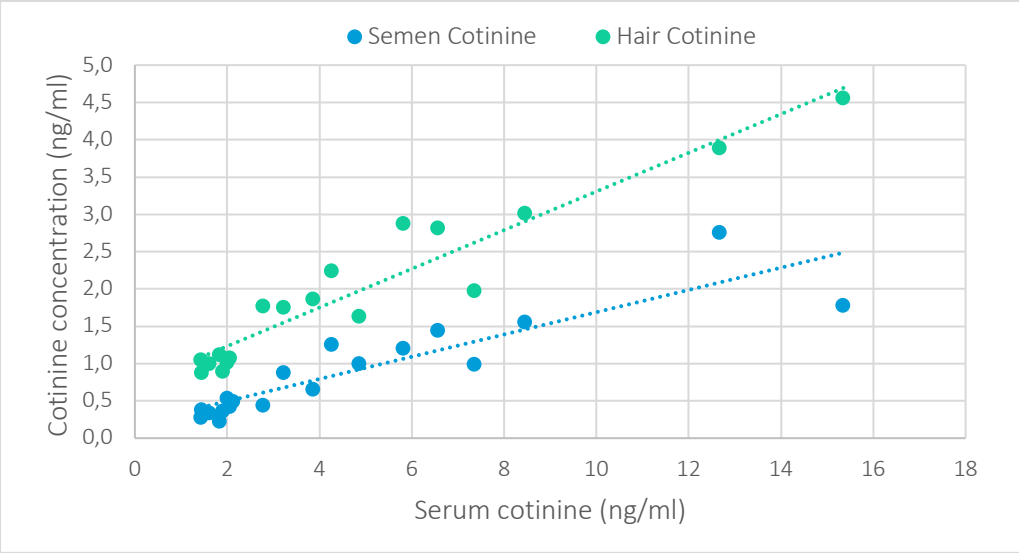

***Figure 2**. Correlation between serum cotinine concentration and its concentration in semen and hair*

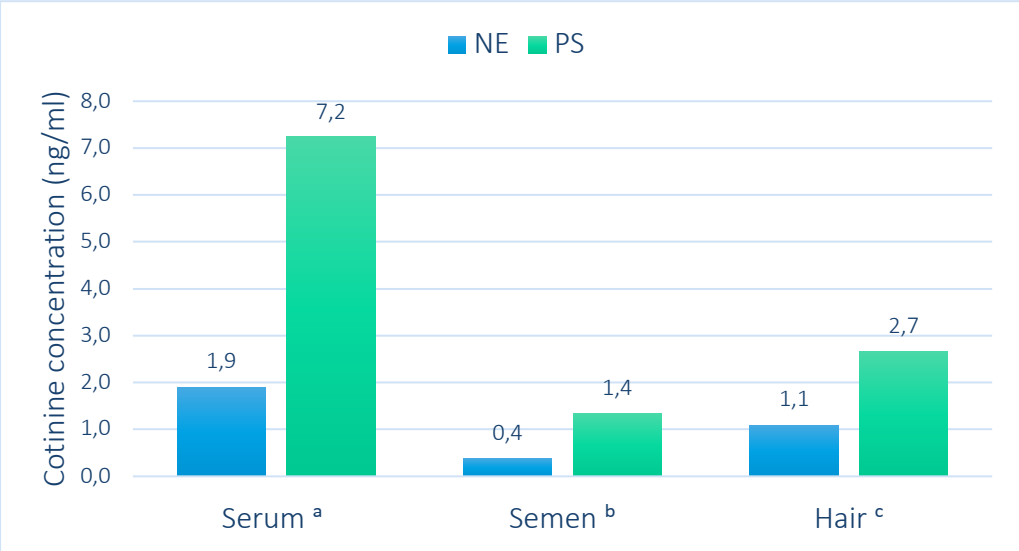

***Figure 3**. Comparison of cotinine concentration in passive smokers and non-exposed dogs among different matrices.*

NE: non-exposed dogs, PS: passive smoker dogs, ª indicates P =0,0002, ᵇ indicates P =0,0002, ᶜ indicates P =0,0004

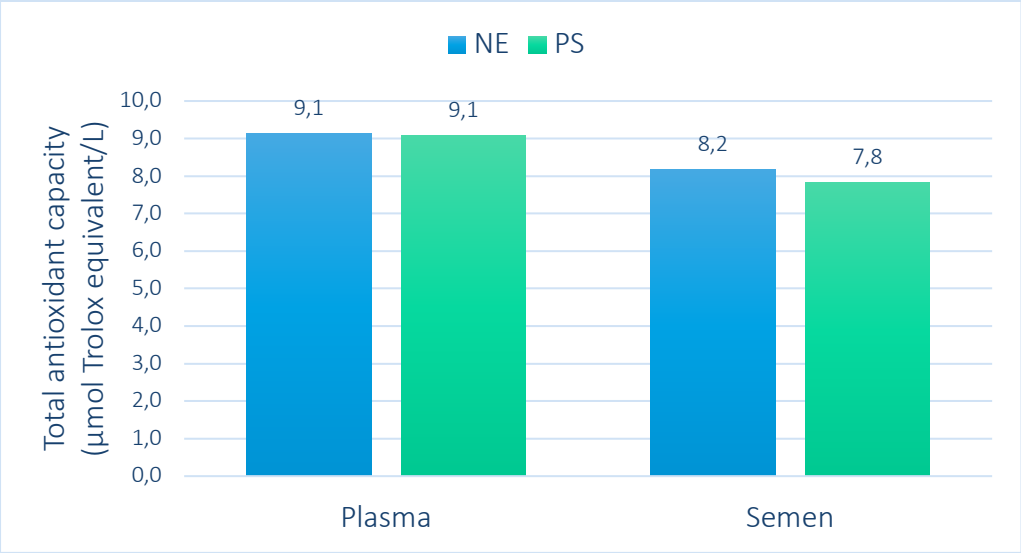

***Figure 4**. Total antioxidant capacity in plasma and semen.*

NE: non-exposed dogs, PS: passive smoker dogs

Paper almost ready to be submitted to an international journal.

TOBACCO SMOKE EXPOSURE IN PREGNANT DOGS: A PILOT STUDY

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Abstract

Maternal active and passive smoking has well-known harmful consequences on foetal and neonatal health with greater risk of preterm birth, low birthweight, and congenital malformations. In humans, cotinine (a nicotine metabolite) was found in neonatal hair as well as in amniotic fluid showing an intrauterine exposure to tobacco smoke. No such information is available in the canine species and the effect of environmental smoke exposure on dams and puppies is unknown. The present study aimed to fill this gap trying to detect and quantify cotinine in maternal (serum and hair) and neonatal (amniotic fluid and hair) matrices exploring dams' exposure to cigarette smoke and its effect on pregnancy outcomes. Twelve purebred bitches were enrolled in the study: 6 exposed to passive smoke (EX) and 6 non-exposed (NE). Dams aged between 2 and 8 years (3,58 ± 1,83), weighted from 14 and 63,6 kg (35,58 ± 14,7) and delivered a total of 61 pups. Cotinine was detectable in all samples. Its concentration in maternal serum was positively correlated with that in maternal ($P < 0,0001$) and neonatal ($P < 0,0001$) hair and in amniotic fluid ($P < 0,0001$). Cotinine level was significantly greater in all maternal and neonatal matrices of EX group compared to NE ones ($P = 0,004$), but no correlation with litter size was found. Present results provide the first evidence of cotinine passage in canine amniotic fluid although with concentration lower than in humans. The clinical effects on dams and pups deserve wider examination, however passive smoke exposure seemed to constitute an often-neglected risk factors even in pregnant dogs.

Keywords: Dog, Cotinine, Amnios, Pregnancy

1. Introduction

The noxious influence of maternal smoking on foetal and neonatal health is well recognized in humans. Tobacco smoke was demonstrated to increase the risk of miscarriage, perinatal death and preterm birth, proportionally to daily amount of cigarette smoked (Pineles et al., 2016, Pineles et al., 2014, Kyrklund-Blomberg et al., 2009). Intrauterine growth retardation (Abraham et al., 2017), behavioural and cognitive issues (Bublitz & Stroud, 2012, Weitzman et al., 2002) as well as developmental disorder like cryptorchidism (Yu et al., 2019) were also related to cigarette smoking during pregnancy.

While smoking is a conscious and dismissible action, exposure to environmental tobacco smoke (ETS) is more insidious and often inevitable, particularly in domestic setting. Countless studies were conducted to estimate the passive smoke impact on maternal and foetal/neonatal health and several consequences have been highlighted in humans. Women exposed to second-hand smoke during pregnancy are at greater risk of preterm birth (Cui et al., 2016) and their babies seem to have lower weight and head dimension at birth (Salmasi et al., 2010). Additionally, congenital malformations such as orofacial clefts and neural tube defects occurred more frequently in babies whose mothers experienced passive smoking during pregnancy (Meng et al., 2018, Sabbagh et al., 2015).

Nicotine and its metabolites were described as effective markers of both direct and indirect smoke exposure (Benowitz, 1996). In particular, cotinine has been widely described in literature and, besides maternal serum, urine and saliva (Baheiraei et al., 2012, Sachiyo et al., 2012, Eskenazi et al., 1995), it was also found in amniotic fluid and neonatal hair in humans (Eliopoulos et al., 1994, Jordanov, 1990). Since it pools both foetal excretions and maternal secretions, amniotic fluid appeared to be an excellent tool to detect intrauterine exposure. Moreover, neonatal hair proved to be an effective non-invasive matrix to estimate smoke exposure over the last trimester of pregnancy (Eliopoulos et al., 1994).

On the contrary, the effect of environmental smoke exposure on canine pregnancy is poorly investigated and no information regarding dams and puppies is available. The close lifestyle sharing between pets and their owners make them prospective sufferers of negative impact of second- and third-hand smoke. However, owners/breeders do not seem to perceive or be aware of the risk of smoke exposure to their pets as well.

The present study aimed to detect and quantify cotinine in maternal and neonatal matrices. In particular, the concentration of cotinine in maternal serum and hair along with neonatal hair and amniotic fluid collected at birth was measured and related to dams' exposure to cigarette smoking and some pregnancy outcomes.

2. Materials and methods

2.1. Animals

This study was conducted between 2020 and 2021 after approval of the Ethical Committee (protocol OPBA_77_2017). Bitches referred to the Reproduction Unit of the Veterinary Teaching Hospital (VTH) of the Università degli Studi di Milano to undergo elective C-section were enrolled once owners' consent was obtained. Maternal age and body weight were recorded. Based on exposure to passive smoke, bitches were divided into two groups: exposed (EX) and not exposed (NE). Patients were included in EX group if at least 10 cigarettes were smoked in domestic environment while they are considered as NE if nobody smoked near them.

Bitches were monitored from proestrus to C-section through vaginal cytology and plasma progesterone measurement (ELFA method, miniVIDAS, Biomerieux) to identify LH surge and predict the date of delivery (Linde-Forsberg, 1991). After mating, pregnancy was diagnosed and checked by ultrasound examination (Esaote MyLab Five with multifrequency microconvex (5-9 MHz) and linear (9-13 MHz) probes) (Johnson, 2008). Caesarean section was performed following the common anaesthetic (Groppetti et al., 2019) and surgical procedure (Johnston et al., 2001).

After delivery, puppies underwent neonatal care (Johnston et al., 2001) and Apgar score (Groppetti et al., 2010) was assessed for all pups within 5 min from birth (Table 1). Once ligation of the umbilical cord was performed, pups were weighted and rectal temperature was measured. Emergency cares were provided to pups in critical condition.

2.2. Maternal and neonatal sample collection

Maternal blood and hair samples were collected before anaesthesia induction.

Hair was clipped from the forelimb area to place the IV catheter in the dams' cephalic vein, then it was stored into a paper envelope until analysis.

Blood samples were collected into serum separator tubes and centrifuged (5 minutes for 3500 rpm) to get serum stored at -80°C for further analysis.

Amniotic fluid was taken at extraction time from each pup using a sterile syringe. For each litter, amniotic fluid samples from all pups were pooled and stored at -80°C until processing. Pups' hair samples were clipped from the ventral surface of the tail base. Again, for each litter the littermates' hair was pooled together and stored into a paper envelope until analysis.

2.3. Cotinine assay

After thawing, blood serum and amniotic fluid samples were centrifuged at 3500g for 15 minutes then supernatant was collected and analysed. Cotinine extraction from maternal e neonatal hair was performed following the procedure reported in literature by Bennett & Hayssen (2010). Calibration curves were developed following manufacturer instructions and are shown in Figure 1a and 1b. Finally, cotinine concentration was determined in blood serum, amniotic fluid, maternal and neonatal hair using an ELISA immunoassay kit specific for canine species and suitable for every matrix (Cotinine (Cot)ELISA kit, Cloud-Clone Corp.) (intra-assay CV < 10%; inter-assay CV < 12%).

2.4. Statistical Analysis

Data analysis was performed using IBM SPSS 26.0 statistical program (IBM, Armonk, U.S.A.). Descriptive statistics for quantitative variables were expressed as range, mean and standard deviation.

In exposed and non-exposed dogs, the comparison between litter size and cotinine concentration in dams' serum and hair, in amniotic fluid and in neonatal hair was assessed using the Mann-Withney nonparametric test. Litter size was also considered as a categorical variable and stratified (≤ 3 puppies; > 3 puppies) and Pearson Chi-Square test was applied to compare the two groups.

The correlation between maternal serum and hair cotinine concentrations and clinical data such as age, bodyweight and litter size were evaluated using Spearman's correlation coefficient.

Statistical significance was set at $p < 0,05$ and level of $p < 0,001$ were regarded as highly significant.

3. Results

3.1. Clinical outcome

Twelve bitches were enrolled in the study: six exposed to passive smoke (EX) in household and six non-exposed (NE). As shown in Table 2, all dogs were purebred and belonged to 8 different breeds with Bernese Mountain Dog (33%) the most represented. Dams aged from 2 to 8 years (3,58 ± 1,83) and weighted 14 to 63,6 kg (35,58 ± 14,7).

A total of 61 puppies were delivered. Litter size ranged from 2 to 8 pups (5,08 ± 2,39) and was negatively correlated with maternal age (P = 0,008; Figure 2).

3.2. Cotinine detection

Cotinine was detectable in all maternal and neonatal samples that is, in blood, hair and amniotic fluid. The highest concentration of cotinine was measured in maternal serum (8,6 ± 8,8 ng/ml) followed by maternal hair (5,3 ± 5,4 ng/ml) and amniotic fluid (5,2 ± 5,2 ng/ml) that showed similar values, lastly by neonatal hair (4,04 ± 3,6 ng/ml) with the lowest levels.

Cotinine levels in maternal serum were positively correlated with that in maternal (P < 0,0001) and neonatal (P < 0,0001) hair and in amniotic fluid (P < 0,0001; Figure 3).

Bitches exposed to cigarette smoke had cotinine levels significantly greater than non-exposed dogs in all the matrices as well as their neonates (P = 0,004; Figure 4).

Cotinine concentration was not correlated with maternal age and body weight and litter size. Moreover, no differences in maternal age, body weight and litter size were evidenced between exposed and non-exposed groups.

4. Discussion

To the best of the author knowledge, despite the several studies addressed in humans, no data on canine cotinine concentration in dams and pups exposed to smoke during pregnancy have been previously published. Moreover, cotinine was never measured in canine amniotic fluid while only few authors refer to its concentration in blood and hair of dogs (Bertone-Johnson et al., 2008, Bawazeer et al., 2012).

First, cotinine can be measured in various biological substrates such as amniotic fluid, as well as blood and hair, even in canine species. Cotinine was detectable in all maternal and neonatal matrices and all were significantly correlated with each other, as reported in humans (Jauniaux et al, 1999). In particular, the maternal serum cotinine concentration was positively correlated with maternal hair ($P < 0,0001$), neonatal hair ($P < 0,0001$) and amniotic fluid ($P < 0,0001$). This makes hair and amniotic fluid two effective alternatives to blood since they are less invasive.

As expected, due to the intimate sharing of life habits with their owner, our results agreed with previous studies in humans reporting higher value of cotinine in women exposed to ETS (Eskenazi et al., 1995, Luck et al., 1985). Indeed, dams with smoking owners had the highest blood concentrations of cotinine ($P = 0,004$) that is, about 7,8-fold greater than not exposed bitches. This is the first time that cotinine measurement is performed in pregnant dog, therefore no reference values exist. Similarly, maternal hair cotinine was 8,2 times higher in exposed compared to non-exposed dogs of our caseload ($P = 0,004$). As previously described in dogs (Knottenbelt et al., 2012, Bawazeer et al., 2012), this outcome reflected the prolonged exposure to nicotine of dams in smoking household. Indeed, cotinine can accumulate during hair growth thus being regarded as long-term marker of cigarette smoke exposure (Al-Delaimy, 2002, Eliopoulos et al., 1994).

During pregnancy, babies could incorporate cotinine derived from foetal blood into hair fibres proving that intrauterine exposure occurs with both active- and passive-smoking mothers (Llaquet et al., 2010). A similar process may be speculated in puppies since hair cotinine level was significantly higher in pups of exposed bitches compared to non-exposed ones ($P = 0,004$) in which concentration was about 9-fold lower.

Cotinine level measured in our serum and hair samples (both maternal and neonatal) appeared to be greater in dams and pups exposed to passive smoke compared to human reports in pregnant women (Florescu et al., 2007, Jauniaux et al, 1999). Reasonably, this difference between dams and pregnant women is justified by differences in nicotine metabolism. It is noteworthy that pregnant women displayed an opposite trend with an acceleration in both nicotine and cotinine clearance (up to 140%) which is more pronounced at 18 – 22 and 32 – 36 weeks of gestation (Bowker et al., 2015, Dempsey et al., 2002). To explain this discrepancy, deeper information on nicotine metabolism in canine species is needed.

Nicotine, together with its metabolites, has been shown to cross human placental barrier and reach the fetus (Luck et al., 1985). Acting as a collector of maternal and foetal secretions, amniotic fluid can

contain nicotine and cotinine whose concentrations are proportional to the degree of mother's smoke exposure (Jauniaux et al, 1999). Moreover, human reports found that amniotic cotinine prevailed over that in maternal serum proving an accumulation in foetal fluid (Jauniaux et al, 1999, Jordanov, 1990). Our results provide the first evidence of cotinine passage in canine amniotic fluid. In line with the abovementioned authors (Jauniaux et al, 1999, Jordanov, 1990), amniotic cotinine was significantly higher in dams exposed than not exposed to ETS (P = 0,004). However, amniotic concentration of cotinine was lower than maternal serum one. It should be noted that women had a haemochorial placenta that implies a strong relationship among maternal vessels and the trophoblast (Dockery et al., 2000). Canine placenta, instead, is endotheliochorial, that is a greater number of tissue layers separates maternal from foetal circulation (Kowalewski et al., 2021). These structural differences can explain disagreement between woman and dog results, suggesting canine placenta as a more effective barrier against this toxic substance compared to human one. However, as already noted, limited sample size prevented us from estimation of the effects of smoke exposure on dams and puppies' health.

Regard to clinical effects of smoke exposure on neonatal outcomes, the little sample size of this caseload limited the generalization of results. Litter size was negatively correlated to maternal age (P = 0,008) while was not influenced by ETS. In women, passive smoke exposure during pregnancy can increase the risk of preterm birth (Cui et al., 2016) causing a reduction in local immunity that favours micro-organism ascent and hesitates in the rupture of membranes (Dempsey & Benowitz, 2001). It was not possible to evaluate this effect in our canine patients since all pups were born at term and mature with elective C-section. Additional studies conducted on dogs delivering at term are needed to approach these issues.

5. Conclusions

Amniotic fluid as well as blood and hair are reliable matrices for assessing canine exposure to smoke.

Besides easy storage (RT) and sample analysis, hair would be preferred for measuring cotinine concentration due to simplicity of collection and non-invasiveness, mainly in newborn pups.

Although preliminary, new, and interesting cues emerged from this study on cotinine detection in biological matrices of dams and offspring exposed to tobacco smoke during pregnancy. A greater

awareness of dog breeders and owner on the risks related to smoke exposure in pets is recommendable.

Authorship contribution statement

Giulia Pizzi: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. Silvia Michela Mazzola: Methodology, Formal analysis, Investigation, Data curation. Alessandro Pecile: Rosources. Valerio Bronzo: Formal analysis. Debora Groppetti: Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing.

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Legends

***Table 1**. Apgar score parameters*

Parameters	Score		
	0	1	2
Mucous colour	Cyanotic, pale	Pink	Reddish
Heart rate (BPM)	<120	120–180	>180
Respiratory rate (bpm)	<15	15–30	>30
Reflex irritability	None	Feeble reaction	Active reaction
Mobility	None	Hypo-mobility	Active mobility
Suckling	None	Weak	Energetic
Vocalization	None	Mild	Vigorous

BPM: beats per minute, bpm: breaths per minute

Table 2. *Clinical characteristics of "Exposed" and "Non-Exposed" dams*

ID	Breed	Age (ys)	Weight (kg)	Smoke Exposure	Litter size
1	Ambully	2	28,3	NE	5
2	Bouledogue	2	17,65	NE	8
3	Bernese Mountain Dog	4,5	63,6	NE	3
4	German Shepherd	8	30,5	NE	4
5	Kurzhaar	5	33	NE	2
6	Staffordshire Bull Terrier	2	14	NE	6
7	Bernese Mountain Dog	2	45,9	EX	8
8	Bassethound	2	31,5	EX	8
9	Bassethound	4,5	32,3	EX	3
10	Entlebucher Mountain Dog	4,5	27,5	EX	3
11	Bernese Mountain Dog	3,5	48	EX	8
12	Bernese Mountain Dog	3	54,7	EX	3

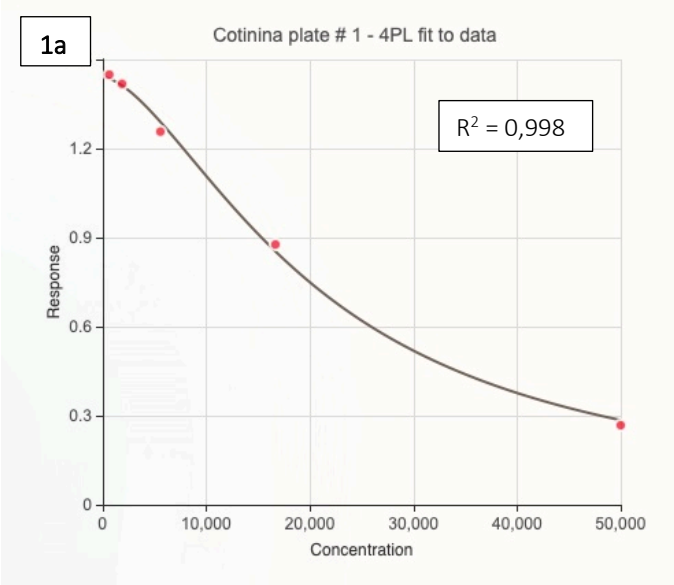

***Figure 1a**. Calibration curve (Plate 1)*

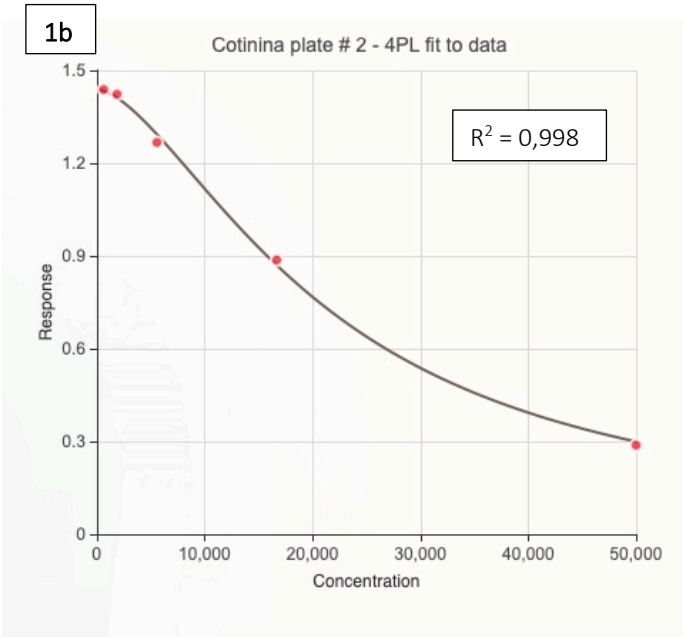

***Figure 1b**. Calibration curve (Plate 2)*

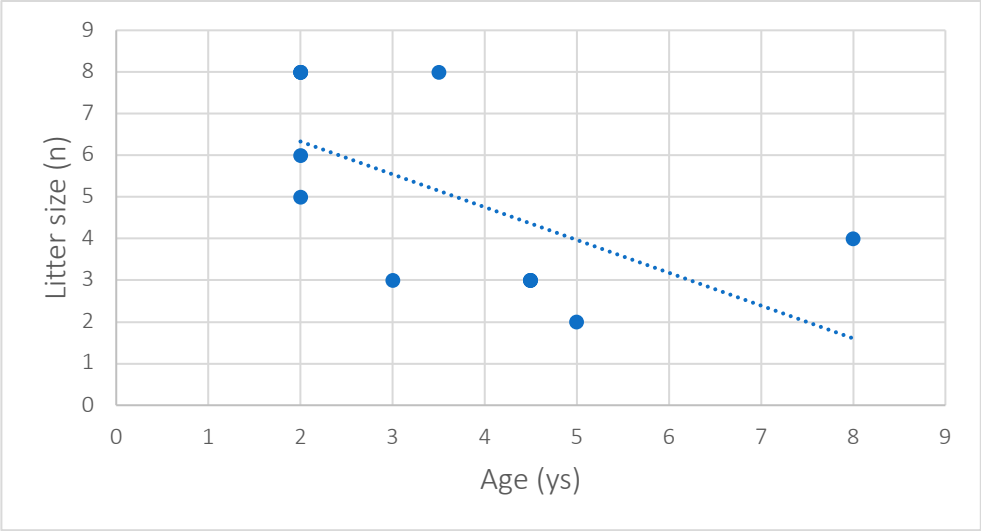

***Figure 2**. Correlation between dams age and litter size.*

n: number of pups.

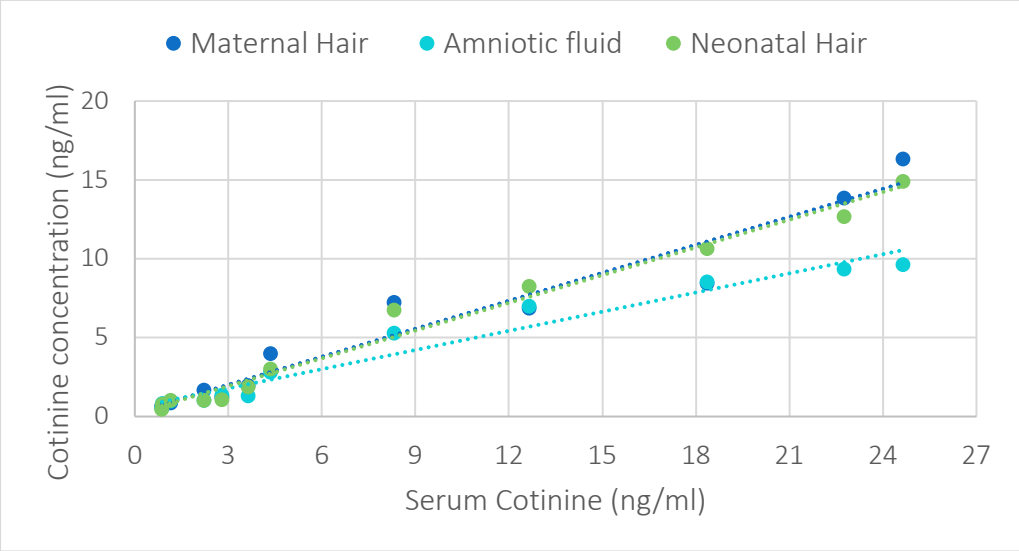

***Figure 3.** Correlation between maternal cotinine concentration in serum and other matrices*

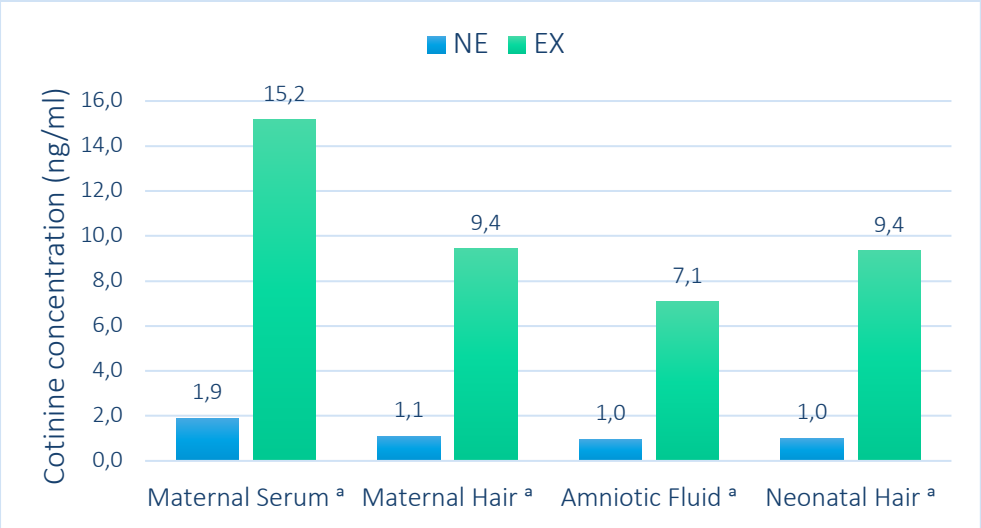

***Figure 2**, Comparison of cotinine concentration in exposed and non-exposed dogs in the different matrices.*

NE: non-exposed dogs, EX: exposed dogs, ª indicates P =0,004

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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 characterizes 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

Table 1*. Primary antibodies used for the immunohistochemical tests: source, dilution, unmasking technique performed and target cells*

Antibody	Target cells	Source	Unmasking tecnique	Dilution
Placental Alkaline Phosphatase, mouse - monoclonal	Gonocytes	DAKO Corporation, Carpinteria, CA, USA	MW, 750 W in pH8 EDTA buffer, 8’	1:25
Vimentin clone 3B4, mouse – monoclonal	Mature Sertoli cells	DAKO Corporation, Carpinteria, CA, USA	Microwave 650 Watt in pH 6.0 citrate buffer, 10’	1:1000
CK AE1/AE3, mouse - monoclonal	Immature Sertoli cells	Zymed San Fancisco, CA, USAControllare	Pepsin, 37°C, 14 min	1:3000
Anti-Muellerian hormone, Goat – polyclonal	Immature Sertoli cells	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	Microwave 650 Watt in pH 6.0 citrate buffer, 10’	1: 30.000
Inhibin-α, mouse – Monoclonal	Immature Sertoli cells	Serotec Corporation, Oxford, UK	Microwave 650 Watt in pH 6.0 citrate buffer, 10’	1:40
Desmin, mouse - Monoclonal	Immature Sertoli cells	Novocastra, Newcastle, UK	Pepsin, 37°C, 14’	1:300

Table 2. Histological and immunohistochemical findings in cryptorchid dogs

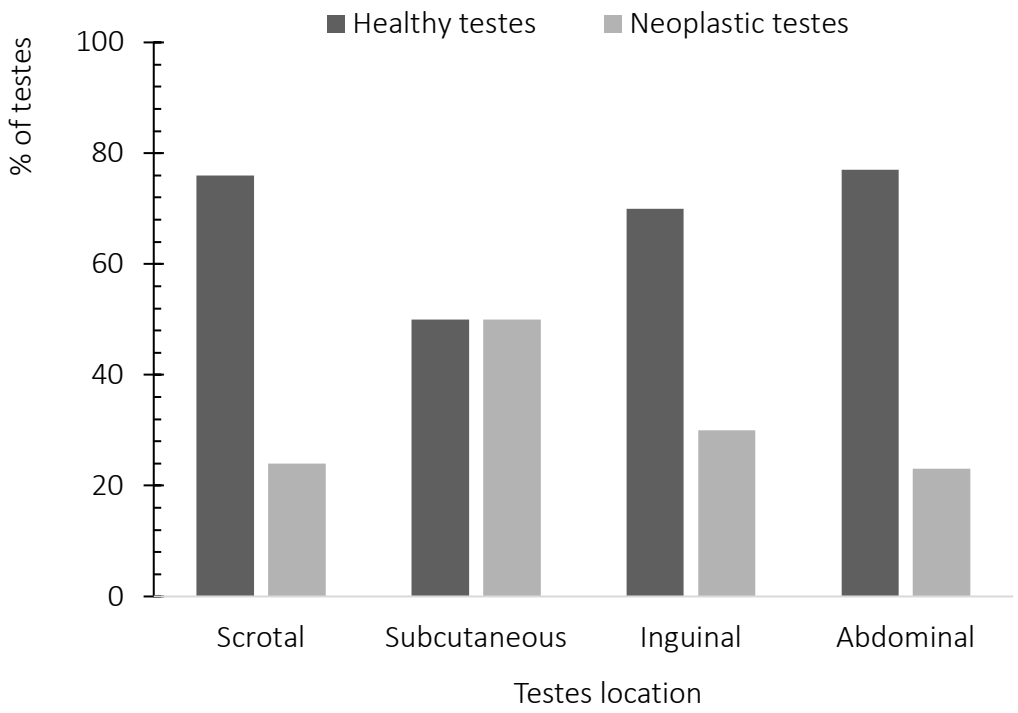
ID.	Breed	Age (ys)	BW (Kg)	Cryptorchid	Testes position	Histological findings	Immunohistochemistry					
							VIM	CK	DES	INH	AMH	PLAP
1	Shiba Inu	0,42	10	Bilateral	Inguinal	SCO	++++	-	Un-reactive	-	++++	+
					Inguinal	SCO	++++	-	Un-reactive	-	++++	+
2	Poodle	0,67	4,5	Bilateral	Inguinal	SCO	++++	-	-	-	++++	-
					Inguinal	SCO	++++	-	-	-	++++	-
3	Jack Russel	0,67	4,5	Unilateral	Abdominal	H	++++	-	-	+	++++	-
					Scrotal	H	++++	-	-	++	++++	-
4	Kurzhaar	0,67	22	Unilateral	Abdominal	H	++++	++	-	+	++++	+
					Scrotal	H	++++	+	-	-	-	+
5	Mixed Breed	0,75	28	Unilateral	Abdominal	SCO, SCH	++++	+	+	-	++++	-
					Scrotal	H	++++	-	-	-	-	-
6	Chihuahua	0,83	3,2	Unilateral	Inguinal	SCO	++++	-	-	-	+++	+
					Scrotal	H	++++	-	-	-	-	-
7	Cocker Spaniel	1,3	15	Unilateral	Scrotal	H	++++	-	-	-	-	-
					Abdominal	SCO	++++	-	-	-	++++	+
8	Cocker	1,58	11	Unilateral	Subcutaneous	H	++++	-	-	-	++++	-
					Scrotal	H	++++	-	-	-	++++	-
9	Pinscher	2	3,9	Unilateral	Subcutaneous	H	++++	-	-	-	-	-
					Scrotal	H	++++	-	-	-	-	-
10	Golden Retriever	2,67	35,5	Bilateral	Abdominal	SCO, SCH, SEM	++++	++	+	+	+	-
					Abdominal	SCO, SCH, SEM	++++	++	+	+	+	+
11	Bouledogue	2,33	15	Unilateral	Scrotal	H	++++	-	-	-	-	-
					Abdominal	SCO, SCH	++++	+	+	-	-	-
12	Mixed Breed	2,5	26	Unilateral	Scrotal	H	++++	-	-	-	-	-

13	Mixed Breed	3	11	Unilateral	Abdominal	SCO, SCH	++++	+	-	-	+	-
					Scrotal	H	++++	-	-	-	-	+
14	Boxer	3	32,5	Bilateral	Abdominal	SCO	++++	+	+	-	-	+
					Abdominal	SCO	++++	-	-	-	++	-
					Inguinal	SCO	++++	-	-	-	-	-
15	Beagle	4	13	Unilateral	Scrotal	H	++++	-	-	-	-	-
					Abdominal	SCO, SEM	++++	-	-	+	+++	-
16	Chihuahua	6	1,3	Unilateral	Subcutaneous	SCO, SCT	++++	-	-	-	++++	+
					Scrotal	H	++++	-	-	-	-	-
17	Mixed Breed	6	28	Unilateral	Scrotal	H	+++	-	-	-	-	-
					Subcutaneous	SCO, SEM	++++	-	-	+	+++	-
18	Mixed Breed	8	33	Unilateral	Scrotal	H	++++	-	-	-	-	-
					Inguinal	SCO	++++	+	+	-	-	+
19	Mixed Breed	8	12,4	Bilateral	Subcutaneous	SCO	++++	-	-	-	-	+
					Abdominal	SCO	++++	-	+	-	+++	+
20	Mixed Breed	9	14	Unilateral	Scrotal	H	++++	-	-	-	-	-
					Subcutaneous	SCO	+++	-	-	-	+	+
21	Chihuahua	10	3	Unilateral	Abdominal	SCO	++++	-	-	+	+++	-
					Scrotal	H	++++	-	-	-	-	-
22	Mixed Breed	10,5	38	Unilateral	Subcutaneous	SCO, SEM	++++	+	++	-	+++	+
					Scrotal	LCT	++++	-	-	-	++	+
23	German Shepherd	11	36,8	Unilateral	Scrotal	SCO, SEM	+++	-	-	-	+	-
					Inguinal	SCT	Un-reactive	-	Un-reactive	-	++++	Un-valuable
24	German Shepherd	11	43	Unilateral	Inguinal	SCO, SCH, SEM/SCT	++++	++	+	-	+++	+
					Scrotal	SCO, SEM	++++	++	++	-	-	+

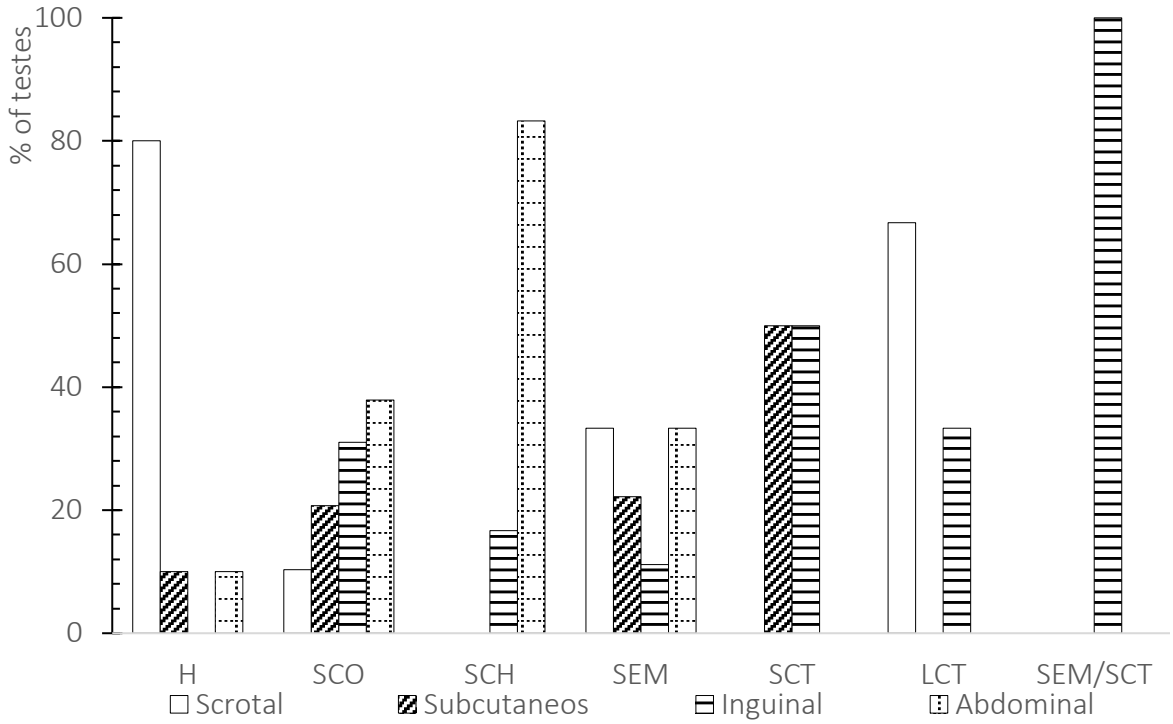
					Scrotal	SCO, LCT	++++	-	+	-	+++	++
25	Mixed Breed	13	15,2	Unilateral	Inguinal	SCO; LCT, SEM, SCT	++++	+	++	+	++++	++
26	English Setter	13	21	Unilateral	Subcutaneous	SCO, SCT	++++	-	+	-	+	-
					Scrotal	SEM	++++	+	-	-	++++	+

H: healthy testis, SCO: Sertoli cell-only tubules, SCH: Sertoli cell hyperplasia, SEM: Seminoma, SCT: Sertoli cell tumor, LCT: Leydig cell tumor, SEM/SCT: mixed Seminoma/Sertoli cell tumor, VIM: vimentin, CK: cytokeratin, DES: desmin, INH: inhibin-α, AMH: anti-Müllerian hormone, PLAP: placental alkaline phosphatase.

***Graph 1**. Incidence of healthy and neoplastic testes based on their location*

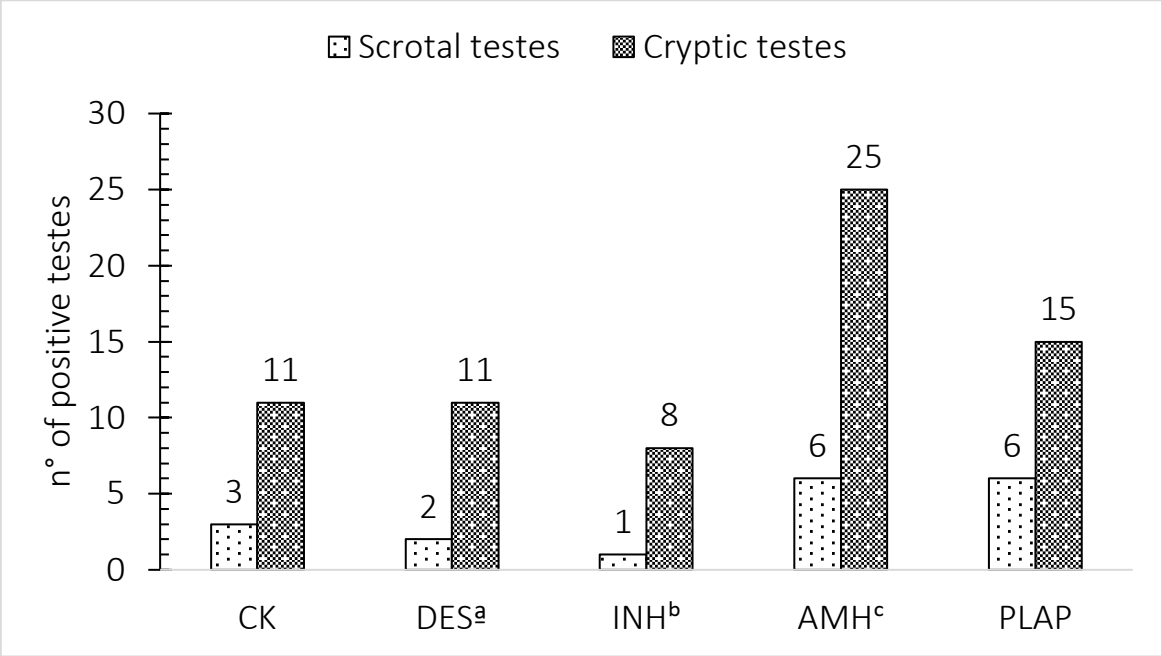


***Graph 2**. Histological features of scrotal and cryptic testes*



H: healthy testes, SCO: Sertoli cell only tubules, SCH: Sertoli cell hyperplasia, SEM: Seminoma, SCT: Sertoli cell tumor, LCT: Leydig cell tumor, SEM/SCT: mixed Seminoma/Sertoli cell tumor.

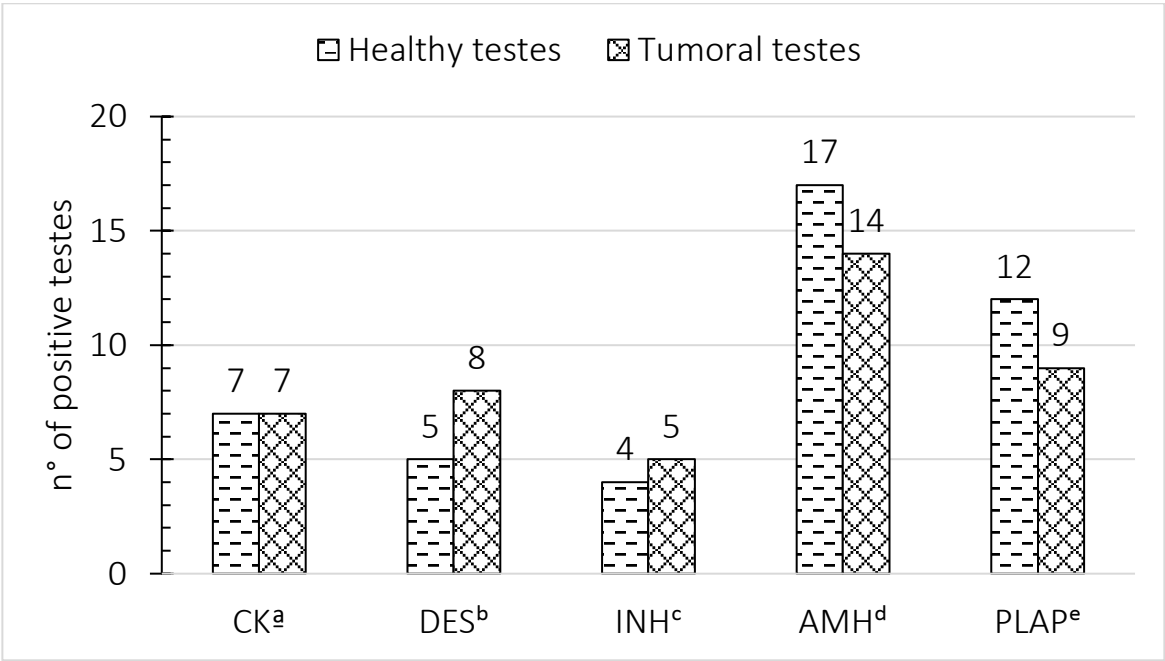
***Graph 3a**. Markers expression in scrotal and cryptic testes*



[a] indicates P = 0.012, [b] indicates P = 0.036, [c] indicates P < 0.0001.

Positivity refers to markers' expression regardless of their intensity (+, ++, +++, ++++)

***Graph 3b**. Markers expression in healthy and neoplastic testes*



[a] means P = 0.022, [b] means P = 0.001, [c] P = 0.016, [d] means P = 0.001, [e] means P = 0.019.

Positivity refers to markers' expression regardless of their intensity (+, ++, +++, ++++)

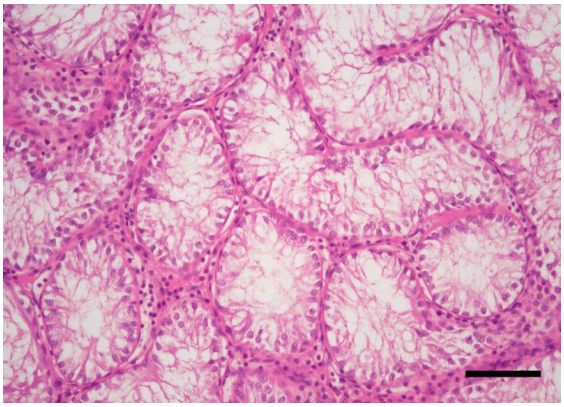


***Figure 1**. Histologic section of canine cryptic testis. Seminiferous tubules lined only by Sertoli cells (Sertoli cell only tubules – SCO tubules) (HE stain. BAR 150 micron).*

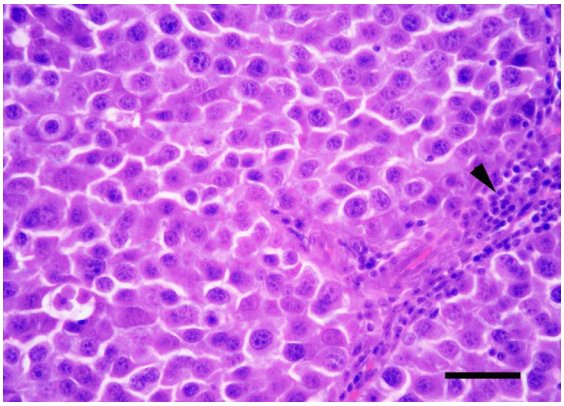


***Figure 2**. Histologic section of a canine cryptic testis. Seminoma. Sheets of round to oval neoplastic cells, accompanied by small lymphocytic aggregates (arrowhead) (HE stain. BAR 50 micron).*

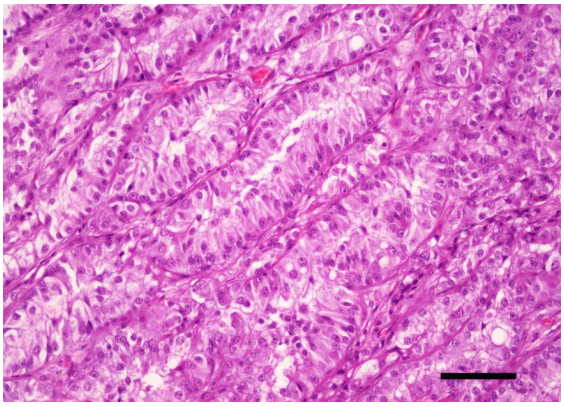


***Figure 3**. Histologic section of a canine cryptic testis. Sertoli cell tumor. The tumor is composed by tubules lined exclusively by neoplastic Sertoli cells (HE stain. BAR 150 micron).*

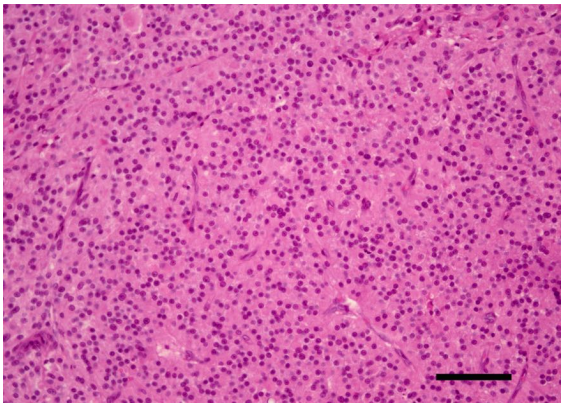


Figure 4. *Histologic section of a canine cryptic testis. Leydig cell tumor. Neoplastic polygonal cells arranged in cords separated by scant fibrovascular stroma (HE stain. BAR 200 micron).*

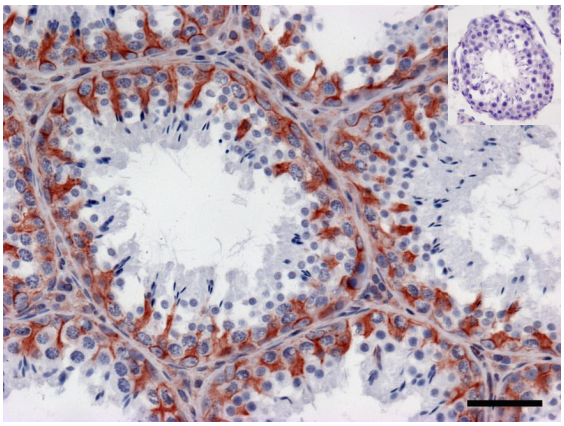


Figure 5. *Canine scrotal testis immunohistochemically stained for vimentin. Sertoli cells are strongly vimentin-positive (some indicated with arrowheads) while germ cells are negative (immunohistochemistry, hematoxylin counterstained. BAR 50 micron). Top right: immunoglobulin isotype control.*

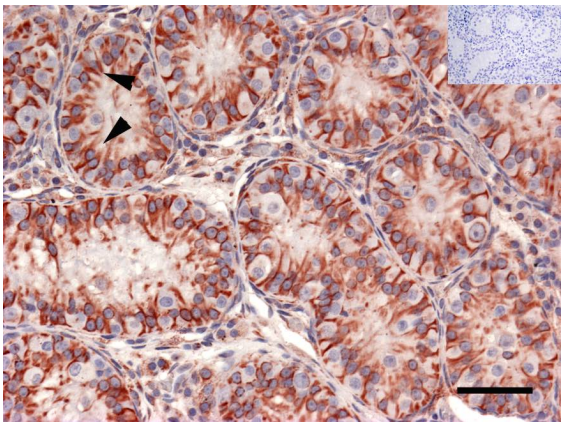


Figure 6. *Canine cryptic testis immunohistochemically stained for vimentin. Sertoli cells are strongly vimentin-positive (some indicated with arrowheads) while germ cells are negative (immunohistochemistry, hematoxylin counterstained. BAR 50 micron). Top right: immunoglobulin isotype control.*

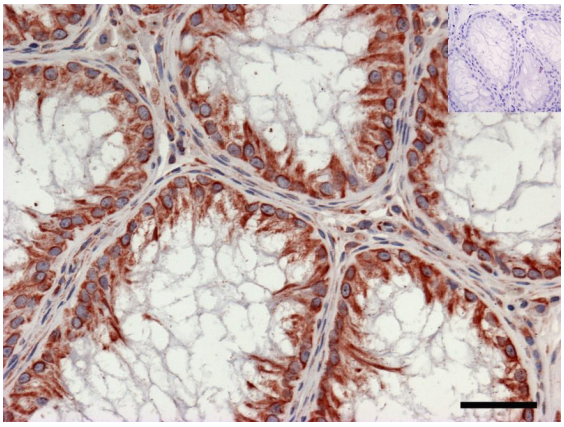


Figure 7*. Canine cryptic testis immunohistochemically stained for vimentin. Sertoli cell only tubules. Sertoli cells are strongly vimentin-positive (immunohistochemistry, hematoxylin counterstained. BAR 50 micron). Top right: immunoglobulin isotype control.*

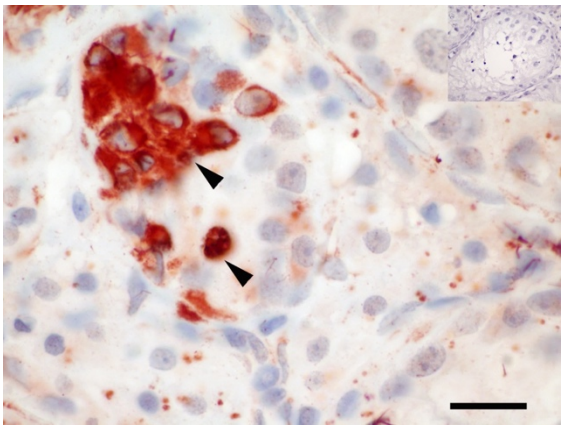


Figure 8*. Canine cryptic testis immunohistochemically stained for PLAP. Note a group of PLAP-positive gonocytes (arrowheads) in a seminiferous tubule (immunohistochemistry, hematoxylin counterstained. BAR 50 micron). Top right: immunoglobulin isotype control.*

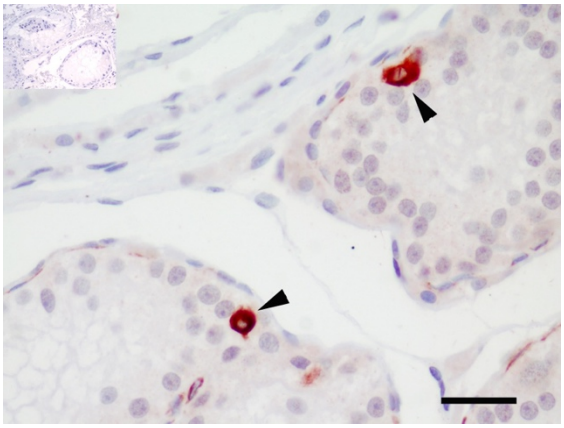


Figure 9*. Canine scrotal testis immunohistochemically stained for PLAP. Note occasional PLAP-positive gonocytes (arrowheads) in a seminiferous tubule (immunohistochemistry, hematoxylin counterstained. BAR 50 micron). Top left: immunoglobulin isotype control.*

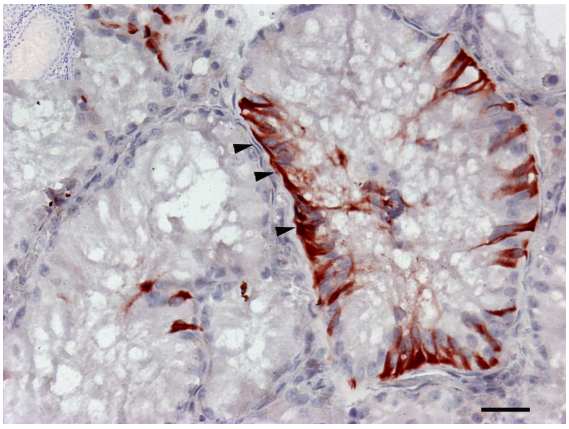


***Figure 10**. Canine cryptic testis immunohistochemically stained for cytokeratins. Scattered cytokeratins-positive Sertoli cells (some indicated with arrowheads) are recognizable into seminiferous tubules (immunohistochemistry, hematoxylin counterstained; 200X magn magnification. Top left: immunoglobulin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

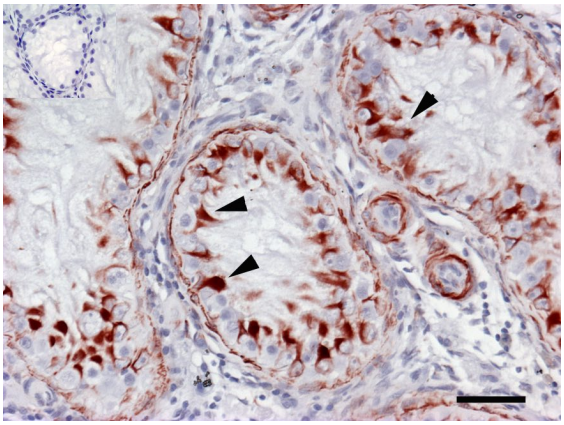


***Figure 11**. Canine cryptic testis immunohistochemically stained for desmin. Desmin-positive Sertoli cells (some indicated with arrowheads) are recognizable into scattered seminiferous tubules (immunohistochemistry, hematoxylin counterstained. BAR 150 micron). Top left: immunoglobulin isotype control.*

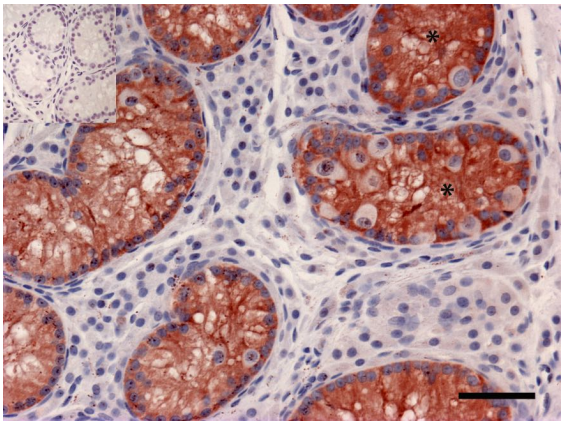


***Figure 12**. Canine cryptic testis immunohistochemically stained for anti-Muellerian-hormone (AMH). Note AMH-positive Sertoli (some indicated with asterisks), intermixed with negative early germ cells into seminiferous tubules (immunohistochemistry, hematoxylin counterstained. BAR 150 micron). Top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

Supplementary Material

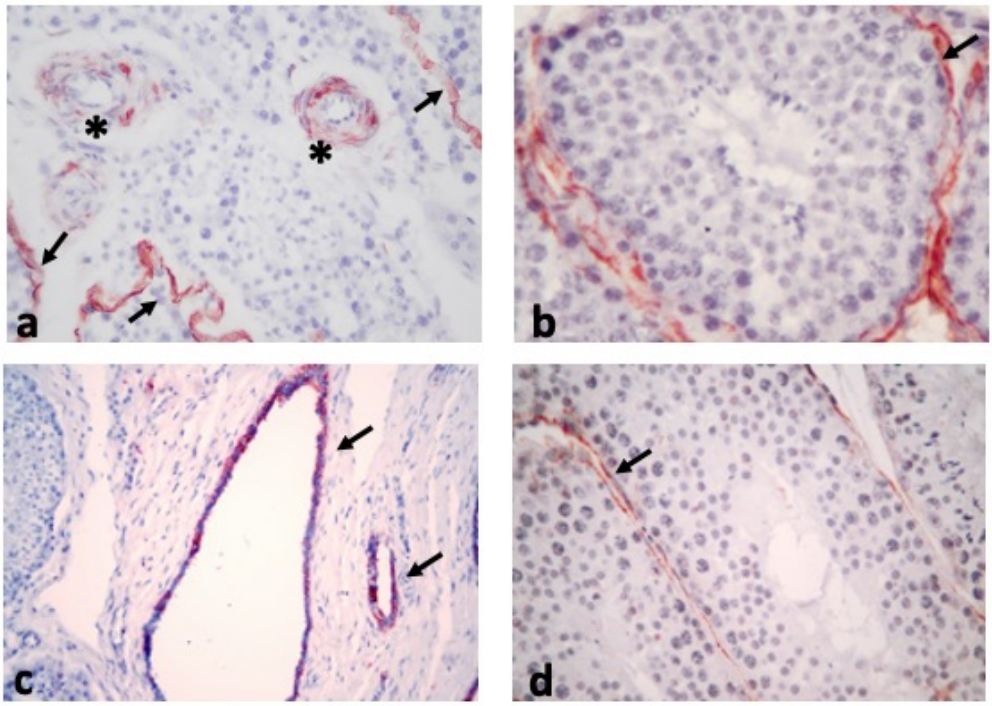


Figure 13. *(A) Canine normal testis. Immunohistochemistry for desmin. Positive control structures are vessels walls (asterisks) and peritubular myoid cells (arrows). Seminal cells are consistently negative. (B) Canine normal testis. Immunohistochemistry for desmin. Positive control peritubular myoid 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 cytokeratins. Positive control structures are rete testis tubules (arrows). Seminal cells, visible on the left, are consistently negative.*

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MOLECULAR STRATEGIES APPLIED TO THE STUDY 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 control. The expression level of three miRNAs, namely miR-302c-3p, miR-302a-3p, and miR-371-3p, associated to 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 oestrogen and thyroid hormone signalling 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.

Keywords: Dog, Cryptorchidism, MicroRNA, Seminoma

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 aetiology of this congenital disease has a strong hereditary component and many genes are probably involved in its transmission (Khan et al., 2018). Cryptorchidism impacts greatly on canine fertility resulting in poor semen quality (Kawakami et al., 1984), moreover, to reduce the incidence of this pathology, affected dogs should be excluded from breeding line (Romagnoli, 1991). In addition, the undescended testes are more susceptible to spermatic cord torsion (Pearson & Kelly, 1975) and 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. In fact, there is a lack of guidelines for an appropriate surgical approach (unilateral or bilateral orchiectomy) to these specific patients (Veronesi et al., 2009, Romagnoli, 1991).

Our previous study on exploring potential immunohistochemical markers showed suspected precancerous lesions in both gonads from unilateral cryptorchid dogs (Pecile et al., 2021). To clarify the involvement of the scrotal testis in unilateral form of canine cryptorchidism and in support of a correct and conscious therapeutic plan, innovative 'omics' technologies were applied. In particular, analysis of miRNAs, that are small non-coding RNAs preserved after formalin fixation (Klopfleisch et al., 2011), has been performed. These molecules, comprising about 22 nucleotides, regulate genes expression at the post-transcriptional level and participate in several biological processes including cellular proliferation and differentiation (Ambros, 2004). miRNA dysregulation hesitates in molecular pathways disfunction that could give rise to disease and neoplasia development (Condrat et al., 2020). In this respect, miRNAs could be regarded as suppressor or promoter of tumorigenesis process

(Zhao et al., 2016) thus representing advanced biomarkers of diagnosis, staging, prognosis and therapy.

In canine population, seminomas have the highest incidence among testicular tumors (Ciaputa et al., 2012) and also prevail over Sertoli cell and Leydig cell tumors in cryptorchid dogs (Pecile et al., 2021, Liao et al., 2009). Despite a less aggressive behaviour in humans than in dogs (Grieco et al., 2007), distant metastases of seminoma occur in 10-15% of canine patients (Lucas et al., 2011, Dugat et al., 2015). Environmental factors have been suggested to be involved both in cryptorchidism via endocrine disruptor chemicals (Lea et al., 2016) and in the development of seminoma via its precursor, carcinoma in situ (Rajpert-De Meyts & Hoei-Hansen, 2007).

The present study focused on investigating miRNAs expression in both retained and testes affected by seminoma compared to healthy scrotal ones in dogs. Three miRNAs, related to testicular germ cell tumor (TGCT) in humans, were selected to be detected and measured in dogs. In particular, miR-302c-3p and miR-302a-3p that seemed to act as oncogenes in testicular tumors where they are upregulated (Das et al., 2019), and miR-371-3p that has proved to be a great biomarker for TGCT in man (Dieckman et al., 2017, Nappi et al., 2019), were examined.

Lastly, a gene functional analysis was performed in attempt to understand biological targets and pathways of the selected miRNAs and to identify molecular dysregulation that could promote carcinogenesis process and increase neoplastic risk in both gonads of unilateral cryptorchid dogs.

2. Materials and methods

2.1. Inclusion criteria

For 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 of the Università degli Studi di Milano. As detailed in Table 1, caseload consisted of 10 healthy non-cryptorchid testes (H), 10 scrotal (S) and 10 contralateral retained testes (R) from unilateral cryptorchid dogs, and 10 testes affected by Seminoma of non-cryptorchid dogs (T).

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

2.2. Histology And sample collection

All testes were longitudinally sectioned on the midline and fixed in 10% neutral buffered formalin, then a complete longitudinal section was obtained and routinely processed for histology. Sections (4µm) were cut from paraffin wax blocks, stained with hematoxylin and eosin (HE) and reviewed. Seminoma diagnosis was based on World Health Organization guidelines (Kennedy et al., 1998).

After bright field microscopy observation, a 4 µm slide was cut from paraffin blocks of healthy and cryptorchid patients, while a biopsy punch was used to get a portion of Seminoma from neoplastic paraffin blocks. All samples were then placed into Eppendorf tubes and submitted for miRNA extraction.

2.3. MicroRNAs extraction and quantification by RT-qPCR (qPCR)

MiRNAs were extracted using a miRNeasy FFPE Kit (Qiagen, Cat. No. 217504) following the manufacturer's instructions. The RNA concentration was determined using spectrophotometry (NanoDrop; Thermo Scientific). Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (AppliedBiosystems; Thermo Fisher Scientific), Monza, Milan, Italy, Cat. No. 4366596) and using miRNA-specific stem-loop reverse transcriptase (RT) primers, according to manufacturer's instructions. Reverse transcription reactions were performed in 15 µl volume reactions containing 1.5 µl 10 X miRNA RT buffer, 1 µl MultiScribe reverse transcriptase (50 U/µl), 0.30 µl 100mM dNTP mix, 0.19 µl RNase Inhibitor (20 U/µl), 6 µl of custom RT primer pool and 3.01 µl of nuclease-free water. The custom RT primer pool was prepared combining 10 µl of each individual 5 X RT primer in a final volume of 1000 µl; the final concentration of each primer in the RT primer pool was 0.05 X each. Then 3 µl RNA was added to each RT reaction. Every RT reaction mixture was incubated on ice for 5 min, at 16°C for 30 min, at 42°C for 30 min and then at 85°C for 5 min.

The qPCR experiments were designed following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. Small RNA TaqMan assays were performed according to manufacturer's instruction. MicroRNAs were selected according to previous publications where they were related to testicular neoplasia in humans (Das et al., 2019, Dieckmann et al., 2017, Nappi et al., 2019). The selected primer/probe assays (Life Technologies; Thermo Fisher Scientific, Monza, Milan, Italy) included hsa-miR-25-3p, hsa-miR-371-3p, hsa-miR-302a-3p, hsa-miR-302c-3p and hsa-miR-30b-5p. 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 2X TaqMan Fast Advanced Master Mix (Cat. No. 4444557), 0.75 µl of miRNA specific TaqMan Advance assay reagent (20X), 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 15s and 60°C for 40s.

Data were normalized relative to the expression of hsa-miR-25-3p and has-miR-30b-5p. MicroRNAs expression levels are presented in terms of fold change normalized to hsa-miR-25-3p and has-miR-30b-5p expression using the formula $2^{-\Delta\Delta Cq}$.

2.4. Statistical Analysis

The statistical analysis was performed on XLStat software for Windows (Addinsoft, New York, USA). The data were tested for normality using the Shapiro-Wilk test, and as the data were not normally distributed the Kruskal-Wallis for multiple pairwise comparisons was applied, as a non-parametric test. Statistical significance was accepted for $p \leq 0,05$.

3. Results

3.1. Study population

Aside from the mongrels (n = 16), in this study 12 breeds were represented (n =14) with a majority of Chihuahua and German Shepherd dogs. Patients mean age was lower in healthy (1,61 ± 0,92 ys) and cryptorchid dog (1,29 ± 0,62 ys) compared to dogs affected by Seminoma (10,7 ± 2,98 ys).

The 10 undescended testes were mainly right sided (80%) and were detected in the abdomen (n=5), in the inguinal area (n=1) and in the pre-scrotal subcutaneous tissue (n=4).

3.2. miRNAs expression

miRNAs expression significantly varied among healthy, retained, and neoplastic testes (Fig. 1). Indeed, while miR-302c-3p was detected in all samples, miR-371a-3p and miR-302a-3p were almost absent in retained testes and in testes affected by seminoma.

As shown in Fig. 1b, miR-302c-3p was downregulated in both scrotal (p=0,04) and retained gonads (p<0,0001) of cryptorchid dogs compared to healthy testes. A similar trend was found also in gonads

affected by seminoma even without a statistical significance (p=0,06). Moreover, in cryptorchid patients, a considerable difference was highlighted between scrotal and undescended testes with miR-302c-3p upregulated in normally descended ones (p=0,03). Although without statistical significance, mir-302c-3p expression in tumoral gonads was higher than in retained ones and comparable with scrotal testes of cryptorchid dogs.

miR-302a-3p was found exclusively in healthy testes while it was absent in scrotal (p=0,003), retained (p=0,003) and neoplastic ones (p=0,003) (Fig 1a).

Like miR-302a-3p, miR-371a-3p had no or negligible expression in both scrotal (p=0,0003) and undescended testes (p=0,0009) of cryptorchid dog compared to healthy gonads. Additionally, it wasn't detectable also in gonads affected by seminoma (p<0,0001) (Fig. 1c).

3.3. miRNA Target Prediction and Pathway Enrichment

Using MiRWalk 3.0, predicted target genes of 3 selected miRNAs were identified. Shared by 2 databases, namely miRDB and miRTarBase, 24 targets were found for miR-302c and 27 predicted genes for miR-302a and miR-371a. Candidate genes are illustrated in Table 2.

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 biological process included transcription regulation at different levels, nervous system development and regulation of apoptotic process (Fig. 2).

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

4. Discussion

Due to its rising incidence, cryptorchidism is a relevant pathology in the canine species (Lea et al., 2016), however various etiopathogenetic and therapeutic uncertainties persist. The present miRNA investigation aimed to face some of these issues.

In our caseload a significant difference in miRNAs expression was detected in healthy testes which were upregulated compared to retained and testes affected by seminoma. This difference was especially noticeable for miR-302a-3p and miR-371a-3p that were almost absent in pathologic patients (cryptorchid or neoplastic). Compared to normal gonads, some variances were expected in retained and neoplastic testes in which the normal structure and physiology of the gonad was impaired. In the former, the incorrect location exposes the testis to high temperature, increasing oxidative stress and compromising Sertoli and germ cells function (Kawakami et al., 2007); in the latter, testis architecture is destroyed by an uncontrolled cellular proliferation (Mostofi & Sesterhenn, 1998). These abnormalities are both cause and consequence of molecular changes that reasonably can result in miRNA dysregulation (Ebrahimi et al.,2020).

In humans, several studies described miR-302a-3p and miR-371a-3p as oncogenes in testicular cancer in which they appear to be upregulated (Palmer et al., 2010, Murray et al., 2011). In particular, miR-302a-3p seemed to inhibit apoptosis increasing survivin protein (Das et al. 2019) while miR-371a-3p proved to be a sensitive and specific marker of testicular germ cell tumor (Liu et al 2021). In our sample, both these miRNAs were not expressed in neoplastic testes. This downregulation suggested that, in contrast with human findings, in the canine species miR-302a-3p and miR-371a-3p could have an opposite role acting as tumor suppressors at testicular level. Similarly, we detected the absence of these two miRNAs in both gonads of unilateral cryptorchid dogs even if no sign of malignancy was evident at histological examination. This outcome confirmed the suspicion of a negative influence of the retained testis on the scrotal one in unilateral cryptorchid dogs as suggested by previous immunohistochemical analysis (Pecile et al., 2021). Although literature is lacking on this topic and further studies are needed to clarify this aspect, it can be assumed that miR-302a-3p and miR-371a-3p dysregulation could be ground for cellular changes that can evolve into seminoma. Therefore, on 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 signalling (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). A similar molecular interaction could be hypothesized between retained and scrotal gonads in unilateral cryptorchid patients. However, to date, no study has ever evaluated its presence.

Despite miR-302a-3p and miR-302c-3p belong to the same miRNA cluster, in our sample there was great discrepancy in their detection. We found a differential expression of miR-302c-3p among all four groups of testes. The most interesting result concerns dysregulation of miR-302c-3p in contralateral scrotal testes of unilateral cryptorchid dogs, that emphasizes a certain degree of abnormality. In fact, despite the correct position of the gonad, there was a significant downregulation compared to healthy testes ($p=0,04$), but it was not as marked as in retained ones ($p<0,0001$). Observing this downward trend, we speculated on a possible miRNA-mediated influence of the cryptic gonads on the contralateral scrotal ones. This alleged link between testes could explain not only the aforementioned neoplastic predisposition, but also the spermiogram variability often seen in these patients (Kawakami et al., 1984). Circulating miRNAs produced by undescended testis, could indeed interfere with spermatogenesis leading to poor semen quality. This way of communication, alternative to normal hormonal feedback, should be properly investigated. In this perspective, in future studies it would be interesting to search for miR-302c-3p in blood and compare its expression among healthy and unilateral cryptorchid patients.

Gene ontology and analysis of KEGG pathways for miR-302c-3p showed its involvement in Estrogen signalling by regulating the expression of ESR1, the gene that encode for estrogen receptors α (ERα) (https://david.ncifcrf.gov/). In human species, exposure to estrogenic substances has been identified as a plausible cause of impaired testicular descent (Thonneau et al., 2013). 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 could be assumed also in canine species. In our caseload, the downregulation of miR-302c-3p found in cryptorchid dogs suggests an increase in estrogen receptor 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. Indeed, TDS was first described by Skakkebaek (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.

Thereafter numerous authors have extensively studied TDS in humans (Martin et al., 2008) and its existence was guessed even in dogs since they live close to the owners sharing the same environmental hazard (Lea et al., 2016). So far histological and immunohistochemical clues of TDS were described in canine species (Pecile et al., 2021, Grieco et al., 2008). The present study supported these speculations suggesting a plausible mechanism by which estrogenic compounds could exert their dysgenic activity.

5. Conclusions

To our knowledge, this is the first study that investigate miRNAs expression in dogs affected by unilateral cryptorchidism and seminoma.

As in dogs with mammary tumor, in which the subject and not the single gland has a neoplastic predisposition, even in dogs affected by unilateral cryptorchidism it can be assumed that cancer susceptibility is shared by both gonads. Therefore, especially when retained testis is already diagnosed with testicular tumor, a conservative approach should be regarded with caution.

Authorship contribution statement

Giulia Pizzi: Conceptualization, Methodology, Investigation (RT-qPCR experiments), Resources, Data curation, Writing – original draft. Debora Groppetti: Conceptualization, Methodology, Resources, Writing – review & editing. Eleonora Brambilla: Investigation (histology), Resources. Alessandro Pecile: Resources. Valeria Grieco: Investigation (histology), Resources. Cristina Lecchi: Conceptualization, Methodology, Formal analysis, Investigation (RT-qPCR experiments), Resources, Data curation, Writing – original draft, Visualization, Supervision.

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Legends

Table 1. Characteristic of canine patients

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 testes, R: retained testes, S: scrotal testes, T: testes affected by Seminoma

Table 2. Predicted target genes

miR-302c-3p	EIF2S1, ELK4, ESR1, GABPB1, GDF11, HABP4, HIP1, HOOK3, INO80D, IPO9, KCNB1, KIF13A, KREMEN1, MAP1B, MBNL3, MIXL1, NFIB, PRRG4, PSD3, PTGDR2, SAMD12, SH3GLB1, VPS53, ZNFX1
miR-302a-3p miR-371a-3p	DSTYK, EIF2S1, ELAVL2, ELK4, ESR1, FKBP14, GABPB1, GDF11, HABP4, IPO9, IRAK4, KCNJ8, LRRC58, MAP1B, MBD2, MBNL3, NR2C2, PLCB4, PRRG4, PSD3, RASGEF1A, SERF1B, SON, TAOK1, WEE1, ZNF385A, ZNFX1

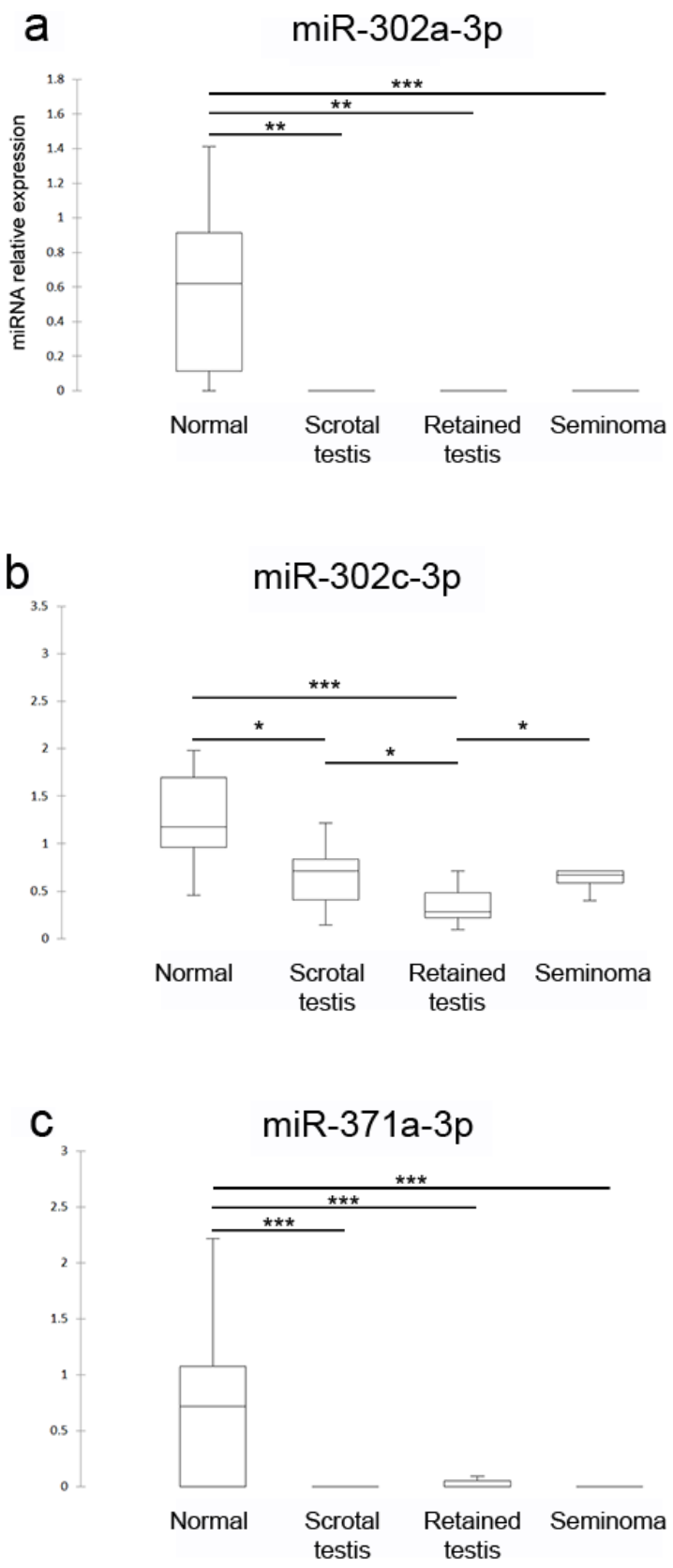

Figure 1. miRNAs expression in healthy, unilateral cryptorchid and oncologic patients.

(a) miR-302a-3p, (b) miR-302c-3p and (c) miR-371a-3p.

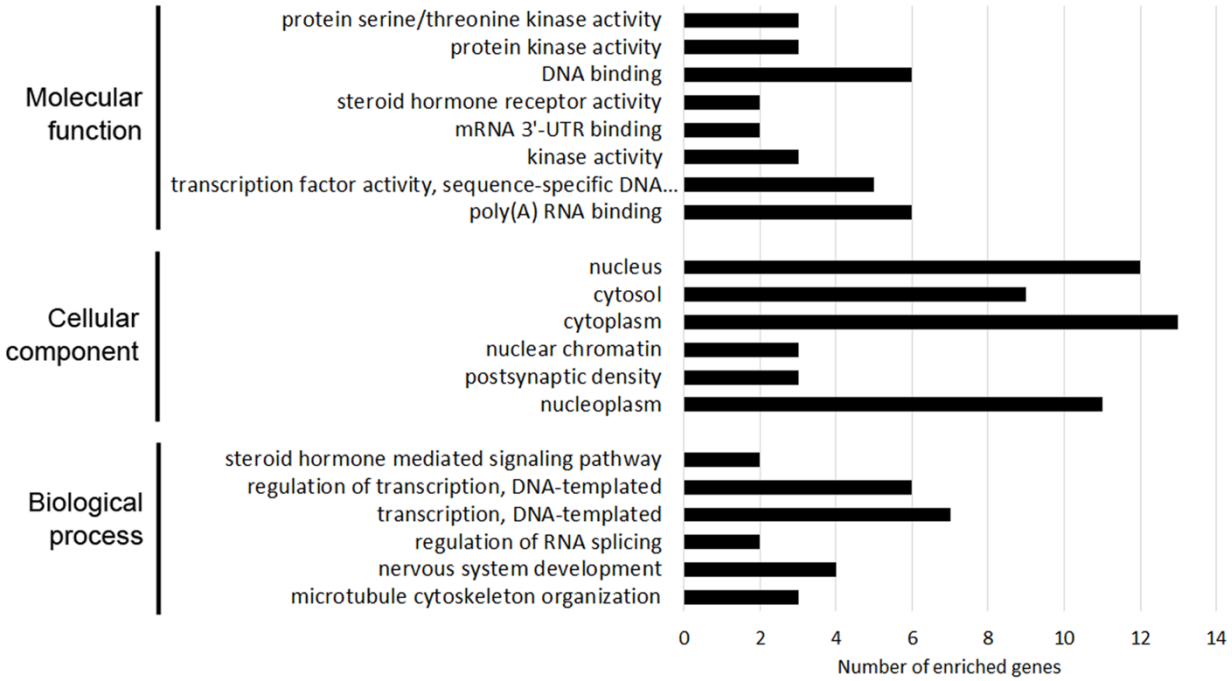

Figure 2. *Gene Ontology outcome given for molecular function, cellular components, and biological process*

GENERAL DISCUSSION

Fertility is defined as the ability to conceive and deliver viable offspring. Its evaluation is extremely challenging both in human and canine species since it results from the combination of male and female aspects that are defined by genetic and environmental determinants.

There is limited awareness regarding the impact of environmental risk factors on dogs' reproduction. Data reported in human literature suggest that these elements can influence fertility at many different levels beginning with embryonic development up to conception. In canine species, however, knowledge is fragmented and incomplete, despite dog has often been proposed as a model for man since they share the same exposure hazard.

Through this PhD thesis an attempt was made to add new pieces to canine fertility complex puzzle and to provide some matter for reflection on the management of breeding dogs.

The first two studies analysed the effects of a common pollutant, namely cigarette smoke, both on male dog and pregnant bitches. Regardless the spread of this deleterious habit, its consequences in canine species still remain poorly understood notwithstanding dogs' high exposure caused by its cohabitation with the smoking owners. Literature on this topic was limited, therefore the first step was to measure cotinine level in serum, semen, hair and amniotic fluid of patients enrolled. Its detection in all patients of our caseload, including those not subjected to passive smoke, made it clear that environmental contamination due to second/third-hand smoke is much more widespread than expected and persists in all places frequented by dogs. As in humans, cotinine concentration seemed to be proportional to exposure degree stressing the effectiveness of this molecule as cigarette smoke marker even in canine species.

In the former study on male dogs, some differences emerged in terms of seminal parameters between passive smokers and non-exposed dogs. These discrepancies in total sperm count and morphological defects deserve further attention as well as the molecular damages produced by smoke on spermatozoa. Unfortunately, a major limitation was the inability to perform an ejaculate evaluation through the CASA system. This computerized analysis would have allowed to objectively define spermatozoa motility whose changes can escape observation even by an experienced operator.

Albeit oxidative stress (OS) is a known detrimental agent for semen quality whether it originates from endogenous (e.g. infections) or exogenous (e.g. passive smoke) sources, we didn't manage to find measurable differences in total antioxidant capacity (TAC) of dogs exposed to tobacco smoke. This result differed from what expected based on human literature, therefore we hypothesized that TAC, which include many different molecules, could be a too generic parameter to evaluate smoke effect on seminal oxidative stress. Moreover, the variable household condition along with the different lifestyle of patients included in our caseload can be a limiting factor into oxidative stress evaluation. Unpredictable stressors, in fact, may have occurred before the andrological examination interfering with TAC level of each dog. To reduce this bias, in a theoretical ideal condition, patients enrolled should be of the same size, body weight, breed, be housed in the same facility and fed with the same diet. In addition, a distinction should be made between companion and working dogs since the dissimilar activity levels could produce variable degree of oxidative stress impacting on semen quality regardless of their ETS exposure.

The small sample size of both studies didn't allow us to precisely define the consequences of passive smoke on breeding animals. In particular, in pregnant bitches, large-scale studies can be far more interesting allowing to check whether exposure to passive smoke increases the risk of preterm birth and malformations (such as cleft palate) or leads to low conception rate and pups' birthweight as happens in humans.

Taken together, these data highlight that environmental smoke shows implications even on canine genital tract, however owners and breeders are not aware of this easily disposable risk factor which can undermine dog reproduction.

Moving away from tobacco smoke, other two studies were conducted on developmental disorders focusing in particular on cryptorchidism which is a very widespread and challenging disease whose multifactorial etiology also includes exposure to endocrine disruptor chemicals (EDCs).

Initially through immunohistochemistry and later with miRNomics, we deepened this pathology from a molecular point of view. Obtained results showed that one of the underlying factors of this disease could be the dysregulation of the estrogen receptors alpha that would expose both gonads to the deleterious action of environmental disruptor compounds. Therefore, in this respect, the altered development would always affect both gonads even in the case of unilateral presentation predisposing both testes to a greater neoplastic risk.

This overall perspective of the patient led to a reflection on the best therapeutic approach in dogs affected by unilateral cryptorchidism in which the removal of one or both testicles should be weighed also considering malignancies predisposition of the scrotal testis. The identification of miRNAs, that act as markers of testicular tumours in canine species, could allow better monitoring of patients in which a conservative surgery was performed. Indeed, circulating miRNAs could favour an early recognition of carcinogenic changes allowing a rapid intervention when needed.

CONCLUSIONS

The comprehension of fertility remains an ambitious target in all species since many endogenous and exogenous factors may interact. The role played by the environment on this reproductive aspect has yet to be precisely defined and little is known on companion animals.

The insights provided in the present PhD thesis underlined the environmental impact on dogs' reproductive life from embryonic stage until adulthood. Despite some pollutants such as EDCs can't be completely eliminated, greater awareness of risks associated to bad habits like cigarette smoke should be encouraged in veterinarians as well as in breeders and owners.

Cues disclosed in this dissertation should be regarded as starting points for further studies, however we hope that our contribution could help other clinician in dealing with this tough issue in canine species.