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NEOPLASTIC AND NON-NEOPLASTIC TESTICULAR LESIONS IN DOGS: COLOR DOPPLER IMAGING, HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF THE CANINE TESTICULAR DISEASE

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Abstract

Testicular tumors are one of the most common neoplasms of male dogs, accounting for 90% of all tumors of the genitourinary tract. Interstitial Cell Tumors (ICT), Sertoli Cell Tumors (SCT) and seminomas are the most frequently detected. The risk factors implicated in the development of the neoplastic process involving testes are numerous. Their prevalence has been evaluated several times and in different time frame by authors. The outcomes vary widely. The reason behind the variability of the results is mainly related to the characteristics of the study group analysed. The prevalence of testicular cancer in the canine population is unclear, but the risk factors are commonly accepted by authors. Age, congenital conditions as cryptorchidism, breed and environment play a major role in the carcinogenesis of this type of tumors. Although many patients present clinical symptoms of the testicular disease, most canine primary tumors are incidental findings. The use of color Doppler ultrasound has become more and more important for the diagnosis of reproductive diseases in dogs. Color flow imaging has made ultrasound the modality of choice for the evaluation of the vascularization of the testes but its role in the characterization of the neoplastic lesions has not yet been defined. Intratesticular flow patterns change in course of pathological conditions of the testis. The ability to evaluate low blood flows can add valuable information to the diagnostic workup, improving the accuracy of ultrasound.

The aim of the present study is to assess the potential of color Doppler ultrasound to differentiate neoplastic and non-neoplastic testicular lesions in dogs through the evaluation of specific vascular flows parameters. Sixty-four male dogs of various breed, aged between 7 and 15 years affected by testicular disorders were selected. B-mode and color Doppler ultrasound examinations were carried out. Blood flow characteristics of the areas of interest were assessed through the evaluation of Resistive Index (RI), Pulsatility Index (PI) and Vascularity Index. All subjects underwent bilateral orchiectomy before submitting testes for histolopathological evaluations. Immunohistochemistry for CD31 on neoplastic nodules was carried out for the evaluation of the microvessel density within the lesion. The 78% of the lesions examined resulted to be neoplastic at the histological evaluation. Conventional B-mode US was able to detect the presence of all the lesions, but not accurate to assess margins in lesions at early stage. The sonographic appearance was not specific of the tumor type. At the color Doppler examination, all tumors showed increased vascularity into and around their bounds. Vascular flow signals were significantly intensified around and into the neoplastic lesions and significantly higher than non-neoplastic conditions, such as inflammatory and degenerative processes, fibrosis, necrosis and cysts. VI markedly increased in solid tumors. The variation of the RI in course of neoplastic, inflammatory and degenerative conditions was not significantly relevant. The sonographic evidences have been supported by immunohistochemistry.

Based on our results and on the understanding that the diagnosis of testicular tumors is dependent upon histological examination, the information on vascular patterns of

testicular lesions provided by color Doppler ultrasound may be a strong valuable tool for the assumption of the diagnostic opinion and may be considered one of the most reliable line of investigation of the canine testicular disease.

Chapter 1. Canine testicular tumors

1.1 Classification

Testicular tumors in dogs are classified in accordance with the *Histological Classification of the Tumors of the Genital System of Domestic Animals* (Kennedy et al, 1998). Tumors of the testis are grouped as follows:

1. Sex-cord stromal (gonadostromal) tumors
    1. Interstitial (Leydig) cell tumor
    2. Sertoli (sustentacular) cell tumor
2. Germ cell tumors
    1. Seminoma
    2. Teratoma
    3. Embryonal carcinoma
3. Mixed germ cell-sex cord stromal tumors

1.2 Epidemiology

Testicular tumors are the most common neoplasm affecting the genital system in male dogs, accounting for about 90% of all tumors of the genitourinary tract (Liao et al, 2009; Hayes & Pendergrass, 1976; Reifinger 1988; Dow 1962; Cotchin, 1960). Tumors of the testis range on the 4th place of all neoplasms in this species and testis is the second most

common sites for tumour growth in non-neutered male dogs (Fan & de Lorimer, 2007), second only to skin and skin adnexal cancer (Gamlem et al, 2008). Though the incidence of testicular tumors in dogs is high, as described by several studies (Nødtvedt et al, 2011; Liao et al, 2009; Grieco et al, 2008; Hayes & Pendergrass, 1976; Reifinger 1988; Dow 1962; Cotchin, 1960), some considerations should be done. In dogs, castration is less frequent in comparison with other species. A high percentage of male cats is neutered early to prevent sexual behaviour and this fact can affect the higher number of testicular tumors diagnosed in dogs. There are only isolated report of testicular tumors in cats (Miyoshi et al, 2001).

Several studies investigated the prevalence of testicular neoplastic diseases in canine populations. An outdated study conducted in Glasgow on a population of 580 dogs reported a prevalence of 16%. The dogs were submitted to routine necropsy (Dow, 1962). In a multi-year study of testicular tumors, based on data of the Canine Cancer Register collected from 1990 to 1998 in Norway, 7.1% tumors of male dogs were located in the testis (Nødtvedt et al, 2011). The prevalence was significantly higher in a case-series performed on a group submitted to routine necropsy in Milan in 2008. The prevalence was around 27% out of 232 dogs. As is evident from the data mentioned above, the results varied widely and the comparison between them is difficult to perform. The majority of the studies on prevalence of testicular cancer in dogs is based on samples obtained from necropsy or collected from routinely castration as well as from cryptorchid dog and patients with clear testicular tumors. Only few population-based publications are present in literature. Furthermore, selection bias may have influence the proportion of tumors in

the population selected: the prevalence increases when old dogs are included (Mosier, 1989). Despite the difficulties linked to data comparison, the increase of testicular cancer in the last 40 year in dogs is real and it goes along with the situation in man (Nødtvedt et al,2011; Koifman at al, 2002; Hardell et al, 2003). Although testicular tumors account only for 1,5% of male cancers in man, their incidence has been increasing for the last 50-years (Facchini et al, 2019, Nigam et al, 2015). The causes of this steady increase in recent years are not well understood but the role of environmental exposure has been strongly hypothesised (Facchini et al, 2019; Nødtvedt et al, 2011). In two epidemiologic studies conducted on military working dogs that had served in the Vietnam War, a strong correlation between the prevalence of testicular tumors and exposure to environmental carcinogens was demonstrated (Hayes at al, 1995; Hayes et al, 1990). Tumors were not the only entities found. Other pathologic changes of testes frequently observed in dogs exposed to military environment were: orchitis, epidydimitis parenchymal degeneration, haemorrhages and granulomas (Hayes at al, 1995; Hayes et al, 1990).

The three most common testicular tumors in dogs derive from specialized elements of the testis: the spermatic germinal epithelium, the interstitial cells of Leydig and the sustentacular Sertoli cells. The neoplastic proliferation of these cellular types give rise respectively to seminoma, interstitial testicular tumor (ICT) and Sertoli cell tumor (SCT). The forth type is far less common compared to the previous and it is called Mixed Germ Cell- Gonadal Stromal Tumor (MCG-SCT). MCG-SCT is characterized by the combination of germinal and stromal elements affected by neoplastic and proliferative processes. The three main types occur approximately with equal frequency in dogs.

According to some authors: 33, 9 % seminomas, 33% ICTs, 26,4 % SCTs, 5,2% Mixed Germ Cell-Stromal tumors (MGC-SCTs) (Nødtvedt et al, 2011; Hayes & Pendergrass, 1976). Other authors suggested that Sertoli cell tumors are less common: 34,4% seminomas, 26% ICTs, 22,9 % MGC-SCTs and 16% SCT in accordance with Liao et al. (2009); 50% ICT; 42% seminomas and 8% SCTs according to Grieco et al (2008). Tumors arising from other cell lineages are rare in dogs (Maxie, 2016; Fan & de Lorimier, 2007). Hemangiomas, granulosa cell tumors, sarcomas, teratomas, embryonal carcinoma, gonadoblastomas, lymphomas e rete testis mucinous carcinomas are rare but they have been reported (Fan & de Lorimier, 2007; Radi et al, 2004; Cooley & Waters, 2001).

The presence of more than one tumor type in the same is not rare and the presence of them within the same testis is described (Dzimira et al, 2017; Grieco et al. 2008). Approximately 40% dogs with testicular neoplasm have more than one primary tumors (Hayes & Pendergrass, 1976; Dow, 1962).

The clinical behaviour of testicular tumors in dogs is usually non-aggressive and tend to remain locally confined. Metastasis are uncommon but can occur.. When a primary testicular tumor shows high rate of malignancy, it may spread to the regional draining lymph nodes and ultimately to lungs, kidneys, spleen, adrenal glands, pancreas, skin, eyes and central nervous system (Lipowitz, 1973; HogenEsch et al, 1987). Sertoli cell tumor and seminoma have higher malignancy rate in dogs than Interstitial Cell Tumors (Fan & de Leorimier, 2007).

1.3 Risk factors for testicular cancer in dogs

Several factors may influence the development of neoplastic processes of the testis. Cryptorchidism, age, breed and exposure to environmental exposure are prime considerations (Liao et al, 2009; Fan & De Lorimier, 2007).

Cryptorchidism

Many report describe cryptorchidism as one of the most important risk factor for testicular cancer (Reif et al, 1976; Liao et al, 2009; Fan & De Lorimier, 2007; Reif &Brodey,1979; Reif et al, 1976). Cryptorchidism is the incomplete descent of the testicles, epididymis, and spermatic cord into the scrotum (Foster & Ladds, 2007). The risk of neoplasia increases several times in cryptorchid testes (Thonneau et al, 2003). Cryptorchid dogs are 10 up to 14.4 times more at risk to develop testicular cancer than normal dogs (Ortega-Pacheco et al, 2006; Sanpera et al, 2002; O'Keefe, 1995; Ladds, 1993). The position of the retained testis seems to influence the type of tumors (Ciaputa et al, 2012). Abdominal retained testes are predisposed to the development of supportive cells tumors, i.e Sertoli Cell Tumors (Meuten, 2002). The temperature of the abdominal cavity, higher than that of the scrotal sac, can damage most cells excepting of Sertoli cells. When retained testis has an inguinal location, the temperature is higher than that in the scrotal sac but lower than that of the abdominal cavity and the risk of developing germ cell tumors is higher (Chaganti & Houldsworth, 2000). Interstitial Cell Tumors seem not to be influenced by temperature (Thonnenau et al, 2003; Coupland et al, 1999). No relationship between retained testis and Leydig cell tumors has been described (Reif et al, 1979).

Age

Testicular tumors are typical of adult and mature dog. The mean reported age is 10 years (Nødtvedt et al, 2011). Dogs older than 10 years are more likely to develop tumors than younger dogs (Reif & Kennedy, 1979).

The mean age for the diagnosis of testicular tumors raising from abdominal retained testes is less than 10 years old. It has been hypothesized that carcinogenesis can be positively affected by the action of some factors and proteins, as HSP 70 (Heat Shock protein 70), superoxide dismutase and inhibin-α (Kawakami et al, 2007).

Carcinogenesis is a multistep process that takes time. As the life span of dogs increases, older animals provide that time that is necessary for the cancer to develop. Some considerations in this regard should be done about differences in life span of canine breeds. The very low morbidity of testicular tumors detected in some breeds, as Flatcoated Retriever and Rottweiler, could be explain as they do not reach the age at which testicular neoplasms usually occur (Nødtvedt et al, 2011).

Breed

Certain breeds appear to have higher risk to develop tumors of the male genital system and in particular cancer of the testis; this fact suggests that a genetic component to testicular cancer susceptibility may exists (Liao et al, 2009; Fan & de Lorimier, 2007). Boxer, German Shepherd, Afghan hound, Shetland sheepdog and Weimaraner are more likely predisposed to primary testicular tumors (Grieco et al, 2008; Lipowitz et al, 1973;

Reif & Brodey, 1969). Liao et al (2009) added Maltese dog and mongrel to the abovementioned list.

Other factors of risk

Other predisposing factors of this type of cancer are testicular dysgenesis and insensitivity to androgens caused by a gene mutation responsible for the structure of androgen receptors (Gorolla et al, 2005). Exposure to enviromental carcinogens, as phenoxy, herbicides, dioxin and tetracycline could play a role in the carcinogenesis of testicular tumors in dogs (Hayes at al, 1995; Hayes et al, 1990). Further studies are necessary to clarify their role.

1.4 Clinical presentation

Clinical signs associated with testicular tumors are not always present. They vary according to the tumor type and can be caused by the primary tumor, metastases or paraneoplastic syndromes produced by the functional neoplasm, i.e hyperestrogenism in presence of Sertoli Cell Tumors (Fan & de Lorimier, 2007).

Testicular tumors may results in enlarged testes, palpable or non-palpable testicular masses. Cryptorchid dogs may develop testicular masses with scrotal, abdominal or inguinal location. Paraneoplastic syndrome in course of testicular tumors are typical of Sertoli Cell Tumors (Lipowitz et al, 1973; Sherding et al, 1981). Approximately one-third of male dogs with SCT develop the hyperestrogenism syndrome (Kennedy et al, 1998;

MacLachlan & Kennedy, 2002). This pathological condition is less frequently reported in course of ICT and it is very rare in dogs with seminoma (Kim & Kim, 2005).

Chapter 2.
Anatomy of the canine testis

A deeper understanding of the anatomy and normal physiology of the testis is a prerequisite for the understanding of the proliferative process that may affect the different components of the testis as well as for the characterization of the histological types of primary tumors in dogs. The male reproductive system consists of organs, ducts and glands whose primary task is to produce and support the development of male gametes. The male genital system comprises testes, scrotum, epididymides, deferent ducts, spermatic cords, prostate gland, urethra and penis.

Testes are located within the scrotum in the inguinal region. The scrotum consists of a thin layer of skin overlying various fibroelastic and muscular sheets, divided in half by a median septum into two compartments. Each compartments contain testis, epididymis and distal part of the spermatic cord. The most prominent muscular component of the scrotum is the *tunica dartos* that runs on each testis and joins between them forming the intratesticular septum.

The left testis of the dog is usually caudal than the right, in this way the surfaces of both testes can glide on each other more easily and with lower pressure (Evans & Christensen, 2013).

Baumans et al (1981) stated that testes in dogs "pass through the inguinal canal on the third or fourth day of postpartum age and are located in the scrotum in the 35th postpartum day". The degree of testis descent differs among mammals. During their descent through the inguinal canal into the scrotum, testes cover theirself with two layers of peritoneum, parietal and visceral layer of vaginal tunic (*tunica vaginalis)*.The vaginal tunic is covered by the *spermatic fascia* of the abdominal wall. *Tunica vaginalis* together with the *spermatic fascia* form the double-walled extension of abdominal peritoneum. Accompanying this outpouching of the peritoneum is the cremaster muscle, a derivative of the internal abdominal oblique muscle. Cremaster insert on the spermatic fascia and parietal layer of the vaginal tunic. It has a protective function raising or lowering the testis in response to temperature or noxious stimuli.

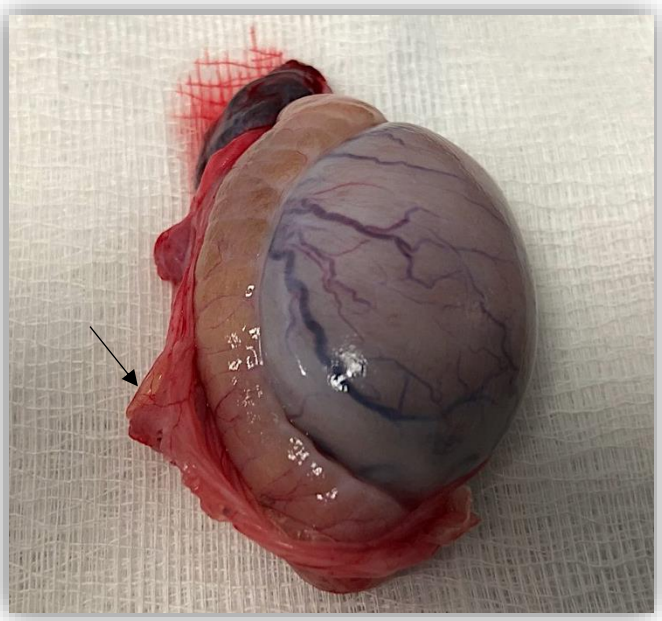

Figure 2.1*. Canine testis and epididymis. The vaginal tunic has been removed (arrow).*

The testis is the male gonad, pair and oval-shaped. The size varies among breeds and with body weight. It is approximately 3 cm length and 2 cm width in a 12-kg dog (Evans & Christensen, 2013). Adherent to its dorsolateral surface is the epididymis with its head located cranial and its tail at the caudal extremity of it. A dense, white, fibrous capsule, underlying the *tunica vaginalis*, called *tunica albuginea* covers the testis. The *tunica albuginea* gives off connective tissue septa that radiate the testicular parenchyma into lobules and converge centrally to join the *mediastinum testis*. The *mediastinum testis* is a cord of connective tissue oriented in a sagittal plane and running through the entire length of the testis. The functional unit of the testis composes the lobules: the seminiferous tubules. The seminiferous tubules build a collecting system. They communicate with the efferent ductules through confluent spaces and ducts of the rete testis, in the mediastinum of the testis.

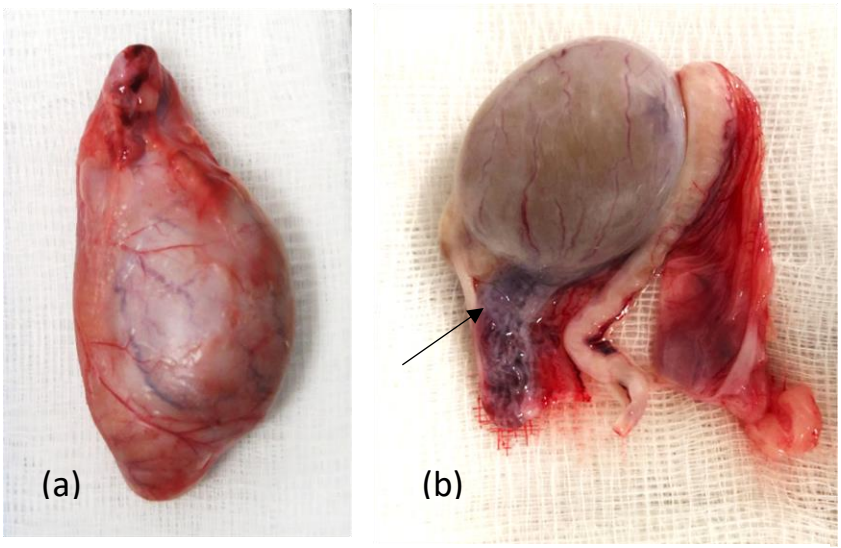

Figure 2.2. *Testis and epidydimis of the dog. (a) The vaginal tunic enfolds testis and epididymis. (b) After the removal of the vaginal tunic, the anatomical relationship of the scrotal organs is clearly visible. The testicular arteries runs along the posterior surface of the testis. The exit of the testicular veins exit from the mediastinum testis and drain into the pampiniform plexus (arrow).*

2.1 The functional elements of the testis: seminiferous tubules and its specialized cells

The seminiferous tubules are composed of spermatogenic (germinal) cells and Sertoli cells. They look like small-convoluted tubules lined by seminiferous epithelium and surrounded by fibroblasts and myoid cells. Germinal cells have spermatogenic functions: they divide and differentiate to form spermatozoa. The blood supply is limited to the basement membrane of the tubule and does not pass inside (Evans &Christensen, 2013). Germinal cell are connected with Sertoli cells in all stages of spermatogenesis, from spermatogonia to spermatozoa, extending from the basal membrane though the entire thickness of the seminiferous epithelium (Noakes et al, 2001).

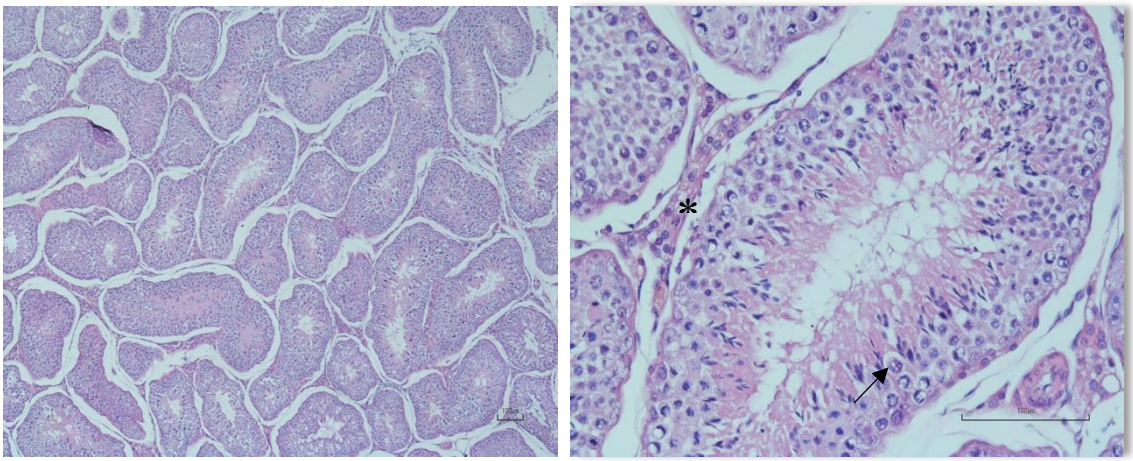

Figure 2.3. Microscopic appearance of the canine testis*. (a) Trasversal section of seminiferous tubules. (b) The seminiferous tubule is lined by the germinal epithelium. Between germinal cells population are Sertoli cells. Sertoli cells are large, columnar and follow the full thickness of the epithelium (arrow). Leydig cells are located within the connective tissue among tubules (asterisk). From the basement membrane the orderly maturation of germ cells is visible: spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa (H-E, 4X-20X).*

Between tubular elements, the interstitial compartment contains interstitial or Leydig cells, macrophages, lymphocytes, fibroblasts, mast cells, blood and lymphatic vessels. Leydig cells secrete testosterone acting through adenylate cyclase. Leydig cells are mainly influenced by LH secretion. Testosterone is required for the production of sperm and maturation in the epididymis, for the functionality of the prostate and for the development of sexual characteristics (Noakes et al, 2001). It is secreted into the tubule lumen where it is converted by 5-reduction into 5- dihydrotestosterone (DHT). Testosterone and DHT are bound by Androgen-Binding Proteins (ABP), producted by Sertoli cells. Their role is to keep high androgens concentrations into the lumen of seminiferous tubules and epididymis.

Sertoli cells are structurally and nutritionally supportive for the germinal epithelium. Their role in the development of functional testis is central. They drive sexual differentiation in fetal life and support spermatogenesis in adult life (Sharpe et al, 2003). Without the metabolic and physical support of Sertoli cells germ cell differentiation, meiosis and formation of spermatozoa would not occur (Sharpe, 1994). In addition to ABP secretion, Sertoli cells aromatize testosterone into oestrogens (Setchell et al., 1983) under the stimulus of pituitary FSH. Sertoli cell have also secretory functions; they secrete oestrogens, inhibin, GnRH-like peptide, proteins, lactate, pyruvate e tubular fluid. They form the blood-testis barrier being connected by tight-cell-like junctions (Noakes et al, 2001).

2.3 Vascularization of the testis

Testes and epididymis receive blood supply by testicular arteries and arteries of ductus deferens. The testicular artery derives from the ventral surface of the aorta at the level of the fourth lumbar vertebra; the right artery arises cranial to the left. The artery of the ductus deferens is a branch of the prostatic artery; it derives from the internal pudendal and follows the ductus deferens into the spermatic cord to the epididymis (Evans & Christensen, 2013). On approaching the testis the testicular artery forms numerous convolutions. Harrison (1949) conducted a comprehensive study on the distribution of vasal and cremasteric arteries to the testis. The testicular artery gives off branches first to the head and then to the of the epididymis before reaching the testis; after curving around the inferior pole of the testis, convolutes and passes near its superior pole. Several branches reach the testicular parenchyma in its course toward the inferior pole of the testis. The blood supply is limited to the basement membrane of the tubule and does not pass inside (Evans &Christensen, 2013).

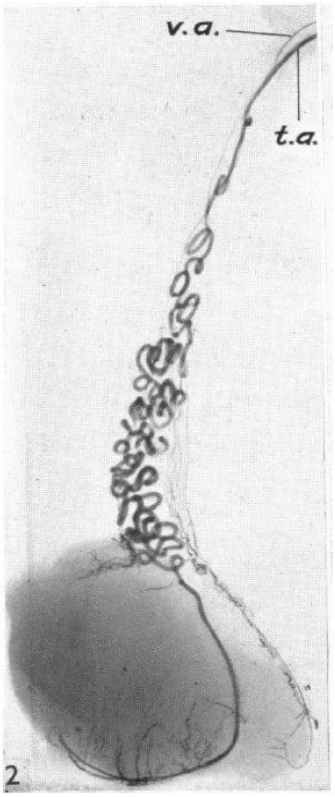

Figure 2.4. Arterial supply o the dog's testis visualized by radiography after the injection of 'Chlorbismol' through the testicular artery. *The testicular artery forms bundle of loosely packed irregular convolutions at about 4 cm from the superior pole of the testis. Before reaching the testis, its branch goes to the caudal epididymidis and anastomoses with the vasal artery. There are no vessels passing from the caudal epididymidis to the inferior pole of testis. At the superior pole, it penetrates through the tunica albuginea obliquely and passes to the caudal pole to curve and reach the inferior pole; then it passes up the front side. (t.a., testicular artery; v.a., vasal artery)(Harrison, 1949).*

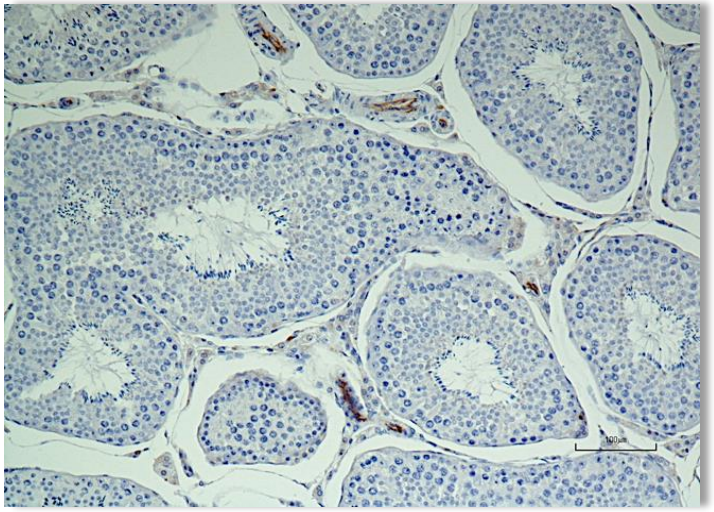

Figure 2.5. Microscopic section of the canine testis. *Microvessels are brown-stained and located in the stromal tissue among the seminiferous tubules. The blood supply is limited to the basement membrane of the tubules (IHC CD31; 10X).*

Cells of the interstitial compartment are more sensitive to vascular changes than germ cells (Harrison, 1949). When a iatrogenic vasal insult is applied, Leydig cells go into degeneration and some germ cells of the epithelium do not (Harrison, 1949). In addition, spermatogonia and Sertoli cells are less sensitive to vascular changes than mature germ cells, as described long years ago by Schinz & Slotopolsky (1924). The functional significance of the arterial convolution is related to thermo-regulatory properties of the testis. Scrotal temperature is lower than that of the abdominal cavity and the thermal gradient differs between different species. The association of convolutional complexity and small calibre of the blood vessels permit to slow down blood flows directed to the testis, giving them more time and larger surfaces for cooling (Harrison & Weiner, 1949). In addition, the venous plexus of the spermatic cord is in close contact with the artery and

it represents a wide area for heat exchange, since the venous blood leaving the testis is cooler than the blood leaving the abdominal cavity (Harrison & Weiner, 1949).

The venous supply is provided by the testicular vein, which forms an extensive plexus. The veins converge towards the caudal pole of the testis where they form the pampiniform plexus and then they pass in the spermatic cord surrounding artery and nerves (Harrison & Weiner, 1949). The right testicular vein empties into the caudal vena cava, the left veins drain into the left renal vein. Blood vessels and smooth muscle fibres are supplied by sympathetic nerves but the seminiferous epithelium and the interstitial tissue do not.

Lymphatic vessels anastomose into trunks and drain into the lumbar lymph nodes. Nerves of the testicular plexus derive from the abdominal aortic plexus, they accompany the testicular arteries and enter the testis with blood vessels (Evans & Christensen, 2013).

The normal physiological functions of the testis, spermatogenesis and hormone production are strictly related to the testicular vascularization (Ortiz-Rodriguez et al, 2017). Testicular flow transports oxygen, nutrients and hormones from and to the testis. The oxygen concentration is relatively low in the seminiferous tubules and the reduction of vascularization may bring to ischemic damage that impairs spermatogenesis (Setchell, 1990).

Chapter 3.
Primary testicular tumors

3.1 Interstitial Cell Tumor or Leydig cell Tumor (ICT)

Interstitial Cell Tumors (ICTs) are sex-cord stromal tumors (SCSTs) according to the World Health Organization (WHO) classification and the *Histological Classification of the Tumors of the Genital System of Domestic Animals* (Kennedy et al, 1998). ICT is typical of older dogs, it grows slowly and therefore it can be difficult to suspect its presence until it grows enough to modify the dimension of the testis causing surrounding compression and contralateral atrophy (Liao et al, 2009). ICT is not notably invasive and clinical signs can be absent.

Neoplastic cellular proliferation in course of interstitial cell tumor involves the interstitial cells among seminiferous tubules, closely with vessels of the interstitial compartment. The interstitial cells are well differentiated, round or polyhedral with abundant cytoplasm that often contains yellow-brown lypochrome pigments (Maxie, 2016). Interstitial cells are endocrine cell that produce androgens and in some cases, the excess of androgens can cause signs of hyperandrogenism in dog. Hormonal imbalances are reversible and can be corrected by surgical removal of the tumor. Neoplastic nodules are often multiple but they can be solitary, unilateral or bilateral; sometimes large tumors are multinodular. They are well demarcated on cut surface, yellow-coloured and measure from 1 mm to 2 cm in

diameter. They rarely cause the enlargement of the affected testis. Interstitial neoplastic nodules have the tendency to haemorrhage and this fact can lead to the formation of hematic lacunae, cysts and dark discoloration. Up to 15% of canine neoplastic interstitial cells are characterized by the presence of intranuclear cytoplasmic invagination, PAS-positive that consist in rough and smooth reticulum, vesicles, lipid vacuoles myelin figures. These evidences were not found in other type of tumors (Maxie et al, 2016).

Neoplastic interstitial nodules must be differentiated from the presence of nodular testicular hyperplasia. Nodular hyperplasia is a paraneoplastic condition that occur in mature and old dogs in course of senile atrophy of the genital organs. Nodules can be visible macroscopically. Usually they are small, nonencapsulated characterized by the proliferation of regular interstitial cells in the intertubular stroma (Maxie et al, 2016). Immunohistochemistry of canine interstitial tumors makes use of different antibodies: GATA-4 is consistently expressed by stromal cells.

3.2 Sertoli Cell Tumor (SCT)

Sertoli cell tumors belong to the group of sex cord- stromal tumors according with the World Health Organization (WHO) classification of urinary system and male genital tumors (Moch et al, 2016) and in line with the histological classification proposed by Kennedy et al (1998).

Sertoli cells are located within germinal cells of seminiferous tubules and play a crucial role for sperm production and spermatogenesis in mature testis (Berger, 2019). They support germ cells during their maturation process from spermatogenic to sperm cells through meiosis. Sertoli cells are columnar with lateral processes that line the tubules.

SCT is common in dogs but rare in other domestic species. In man sex-cord stromal tumors comprise less than 5 % of testicular cancer (Banerji, 2016). In retained testis, SCT is the most frequent (Grieco et al, 2008; Pendergrass & Hayes, 1975).

The correlation between SCT and cryptorchidism is thought to be related to high temperature, abnormal testicular development and dysgenesis of the testis (Hayes et al, 1985). SCT in retained testis can exhibit more aggressive behaviours compared to SCT of scrotal testis (Quartuccio et al, 2012). Sertoli Cell Tumors remained usually located to the testis and metastasis are rare (Dow, 1962). The evaluation of the degree of malignancy is based on morphology, involvement of draining lymph nodes and presence of distant metastases (Barrand & Scudamore, 2001). Typical metastasis involve the spermatic cord and regional lymph nodes or beyond (Foster, 2012).

The clinical presentation of SCT consists in a mass that can cause an enlargement of the affected testis. Even though SCTs do not cause clinical signs, some dogs develop extratesticular symptoms related to the feminization syndrome. Feminization in dogs is caused by high levels of endogenous oestrogens release by neoplastic Sertoli cells or by the excess of inhibin secretion that leads to a reduction of testosterone concentration. The feminization syndrome is a paraneoplastic condition characterized by gynecomastia,

attraction of other males, non-pruritic and bilateral and symmetrical alopecia, hyperpigmentation, prostatic dysfunctions and prostatic squamous metaplasia and pendulous penile sheath (Quartuccio et al, 2012). Other clinical signs comprise reduction of libido, female-like distribution of body fat, cutaneous and pilo-sebaceous atrophy, atrophy of testes and penis and swelling of the prepuce (Maxie et al, 2016). Around 20%-30% of dogs with SCT show signs of hyperestrogenism (Sanpera et al, 2002).

Feminization syndrome is typical of functional retained testis in dogs. Approximately 70% of dogs with SCT in retained testes shows signs of hyperestrogenism. Preputial cytology is considered sensitive and specific to detect oestrogen-producing testicular tumors (Dreimanis et al, 2012; Grellet e al, 2010). In dogs, serum oestradiol concentrations can range markedly within dogs frequently exceeding the reference interval, so they cannot be consider a reliable parameter for the diagnosis of oestrogen producing neoplasms. Some authors hypothesised that the inhibin secretion by neoplastic Sertoli cells inhibits testosterone production and alters the testosterone/estradiol normal ratio (Maxie, 2016). The hormonal contribution to the development of hyperestrogenic syndrome is not well understood (Foster & Ladds, 2007).

Oestrogens released by Sertoli cells can lead also to bone marrow toxicity, which results in leukopenia, thrombocytopenia, non-regenerative anaemia caused by blood loss and reduced erythropoiesis, infection and fever with neutropenia (Maxie et al, 2016; Sherding, 1981).

Macroscopically, Sertoli Cell Tumors appears as solid or cystic masses enclosed in tense testicular capsules. The larger tumors are multinodular. The microscopic evaluation of SCT show proliferative processes of supportive cells within the seminiferous tubules, which progressively penetrate the basement membrane building a mass. Neoplastic cells are arranged in a palisade manner (Maxie et al, 2016).

Extratesticular location of Sertoli cell tumors in dogs are described in the scrotal or prescrotal region and in the spermatic cord of neutered dogs (Maxie et al, 2016; Doxsee et al, 2006). The hypothesis that they are remnants implanted at the time of castration hormonally stimulated that change from cellular hyperplasia to neoplasia is reasonable.

3.3 Seminoma

Seminomas are common in canine testes, particularly in old dogs. The median age of dogs at diagnosis is 10 years (Grieco et al, 2004; MacLachlan & Kennedy, 2002, Dow, 1962). Seminoma is a germ cell tumor of the male genital system according to the World Health Organization classification. The prevalence of seminoma in dogs is slightly higher than SCT and ICT (Maioloino et al, 2002; Meuten, 2002). The behaviour of testicular seminoma in dogs differs widely from that of human. Human seminoma is often malignant and typical of young adults (Cheng, 2004; Nakanoma et al, 1992). The classification of seminoma in human patients is based on the positive or negative reaction with Period Acid Shiff (PAS) staining and on the expression of Placental Alkaline Phosphatase (PLAP) marker; Two types of seminoma have been described: Classical

Seminoma (SE) and Spermatocityc Seminoma (SS) (Mostofi & Sesterhenn, 1998). In man, the difference behaviour of these two forms is due to the different origin: SE is highly malignant, tipically found in young men and it origins from undifferentiated seminal cells, called gonocytes. Gonocytes are progenitor of seminal epithelial cells, positive to PAS staining for the presence of glycogen and they express the placental alkaline phosphatase (PLAP) (Grieco et al, 2007, Mostofi & Sesterhenn, 1998; Muller et al, 1987). The metastatic potential of SE in man is high (Kim et al, 2010; Hohšteter et al, 2009). SS is less malignant and rarely metastatizing, it derives from well-differentiated germ cells, mostly mature spermatocytes; SS develops in older men, it does not express PLAP and it is PAS-negative (Grieco, 2007). Spermatocytic seminomas have been recently classified as "spermatocytic tumor" by the World Health Organization 2016 (Moch et al, 2016). This change of nomenclature would enhance the non-aggressive behaviour of the tumor (Williamson, 2017).

In dogs, the classification of testicular seminoma is based exclusively on the histological aspect: intratubular seminoma and diffuse seminoma are described (MacLachlan and Kennedy 2002; Maiolino et al. 2004; Grieco et al. 2007). Any other classifications are not accepted (Kennedy et al, 1998). Intratubular seminoma is characterized by the complete filling of seminiferous tubules by cells with similar aspect and same immunohistochemical staining profile. Intratubular seminomas are considered the earliest development degree of the tumor. When the tubules get broken, neoplastic cells invade the adjacent regions becoming confluent. They produce interstitial infiltrations, microcystic or tubular structures. Some authors investigated the occurrence in the dog of

the two different types of seminoma described in man (Grieco et al, 2007; Hohšteter et al, 2009). Investigations conducted by Yu et al. (2009) and Grieco et al (2007) confirmed the existence of two different types, classical and spermatocytic, and they demonstrated that some type of seminomas express PLAP in dog and others do not (Yu et al, 2009; Grieco et al, 2007; Kim et al, 2010). Nonetheless, the biological behaviour and the time of presentation of SE and SS differ than in man; in dogs, SE tends to develop in older subject, as SS does. Recent studies (Bush et al, 2011; Thorvaldsen et al,2012) demonstrated that there are no real cases of pure classical seminoma in dogs, or if there are any cases, they are extremely rare. Canine seminoma is predominantly spermatocytic. (Bush et al, 2011; Thorvaldsen et al, 2012).

The clinical presentation of seminomas is a painless enlargement of the testis. Seminoma does not produce hormones and evidence of feminization are usually absent. Pain can develop because of the rapid growth of the tumor that can also cause bleeding of the mass (Ciaputa et al, 2012). If signs of hyperestrogenism are present, other concurrent pathologies must be carefully excluded. The biological behaviour of seminoma is mainly benign, although malignant histological features can be observed (Hohšteter et al, 2009); the metastatic potential is low (Kim et al, 2010) and when metastases are present, they involve the retroperitoneal lymph nodes (Restucci et al, 2003; Gawlik-Jakubczak & Krajka, 2005). Several markers are employed by immunohistochemistry for the differentiation of germ cells, e.g. E cadherin, calretinin, KIT, PGP 9.5 inhibin alpha and NSE. GATA 4 is a marker of Sertoli cell differentiation and can differentiate seminoma

from SCT and ICT through different staining pattern: germ cells are negative; sertoli and interstitial cells consistently expressed GATA-4.

3.4 Mixed germ cell-sex cord stromal tumor (MGT-SCT)

Mixed germ cell-sex cord stromal tumors (MGC-SCTs) are the fourth most common testicular tumors in dog. They consist of various combination of sex-cord stromal cells (Sertoli cells) and germ cells, intimately combined within a single tumor. They often contain entrapped seminiferous tubules in which germ cells are confined to the basal compartments (Roth et al, 2017). MGC-SCT has been recognized in human ovary and testis since 1953. MGC-SCT belong to the subgroup of sex-cord stromal tumors which comprises the admixture of stromal and sex-cord elements according to World Health Organization (WHO) classification of tumors of domestic animals (Kennedy et al, 1998). Gonadoblastoma is a synonym of MGC-SCT (Maxie, 2016). They are usually unilateral in dogs (Owston & Ramos-Vara, 2007). One case report of a bilateral gonadoblastoma has been described in a 10-year-old Collie dog (Reis-Filho et al, 2004).

3.5 Other primary testicular tumors

Other types of testicular tumors arising from different cellular lineages different from those previously described are rare in dogs (Liao et al, 2009). Haemangiomas, granulose cell tumors, sarcomas, embryonal carcinomas, lymphomas, teratomas and rete testis

mucinous adenocarcinomas are rarely reported in dogs (Radi et al,2004; Patnaik & Mostofi, 1993; Turk et al, 1981).

Chapter 4.
Non-neoplastic diseases of the testis

Pathological conditions of the male genital system are common in dogs. Despite this, knowledge about the incidence of testicular diseases, aside from those neoplastic and cryptorchidism, is limited (Khan et al, 2018; Ortega-Pacheco, 2006; Pendergrass & Hayes, 1975).

Cryptorchidism

Cryptorchidism occurs when one or both testes together with epididymis and spermatic cord fail to complete their descent into the scrotum (Foster & Ladds, 2007). This condition compromises the spermatogenesis leading to impair or absent function of the gonad. Cryptorchidism is associated to infertility if both testes are retained, due to the high intratesticular temperature that severely compromises spermatogenesis. Monolateral cryptorchidism often lead to lower sperm density, that results to be below expectation for the species considered. Despite that fact, testosterone production is not influenced by the ectopic position of the organ and cryptorchid dogs may show normal libido (Foster & Ladds, 2007). The heritability of the disease has been strongly suggested for several years by Cox et al (1978). The transmission involves more than one gene (Amann & Veeramachaneni, 2007; Romagnoli, 1991). Testis may be retained in the abdomen or the inguinal canal. Testes located in the inguinal canal can be identified by a careful palpation of the inguinal region, but their presence must be confirmed by ultrasound.

Retained testes have greater risk to develop testicular cancer, especially Sertoli cell tumors and seminomas. They are predisposed also to torsion. (Khan et al, 2018; Liao et al, 2009; Fan & De Lorimier, 2007; Romagnoli, 1991; Reif &Brodey,1979; Reif et al, 1976). Retained testes in dogs have similar high incidence of men to develop neoplasia (Pendergass and Hayes, 1975). Pendergrass & Hayes (1975) analysed 1266 cases of cryptotirchid dogs and they demonstrated that certain breeds had high risk to retain testes. These breeds are Chihuahua, Miniature Schnauzer, Pomeranian, poodle of miniature, standard and toy breeds, Siberian Husky, Shetland Sheepdog and Yorkshire Terrier. Kawakami (1988) studied the effect of removing retained testes by surgery on testosterone production in dogs that had lower concentration of testosterone than normal dogs. Testosterone level from the scrotal testes was lower prior to surgery but it returned to normal levels in 24 weeks after surgery (Kawakami, 1988)

Testicular degeneration

A wide variety of agents and etiologic factors, such as heat, toxins, infections and endocrine imbalance, may be involved in the damage of the functional units of the testis. The seminiferous epithelium is susceptible to thermic and physics insults (Noakes et al, 2001). Hormonal imbalances as cause of testicular degeneration have been widely described in dogs with primary lesions of the anterior pituitary gland, impaired gonadotrophin secretions, adrenal tumors and primary testicular tumors, in particular Sertoli cell tumors (McEntee, 1990). Testicular degeneration may be focal or diffuse, unilateral or bilateral depending on the etiology of pathologic process. Early lesions are

focal areas characterized by loss of primordial germ cells, individual or multinucleated giant cells. The loss of germ cells increases with the progression of the degenerative process. As the process progresses, sperm can accumulate in tubules near the rete testis and sperm stasis can be accompanied by calcium deposits. In very advanced testicular degeneration, the sustentacular cells get involved; the loss of interstitial cells follows the latter in severe degenerative lesions.

Histology of testicular degenerative lesions are nonspecific but areas of germ cell death are present in all cases of testicular degeneration.

Testicular degeneration related to age is a physiologic, progressive, irreversible and relentless mechanism that occurs in old dogs. It is characterized by the accumulation of senescent cells. Senescent cells are thought to drive age-related pathologies in aged animals, including cancer (Campisi & d'Adda di Fagagna.2007).

This type of cells has typical features (Merz et al, 2019):

- Altered morphology and enlarged shape
- Irreversible arrest of cell cycle and resistance to apoptosis
- Expression of secretory phonotype associated to senescence with paracrine activities and secretion of proinflammatory cytokines, chemokines, growth factors, proteases

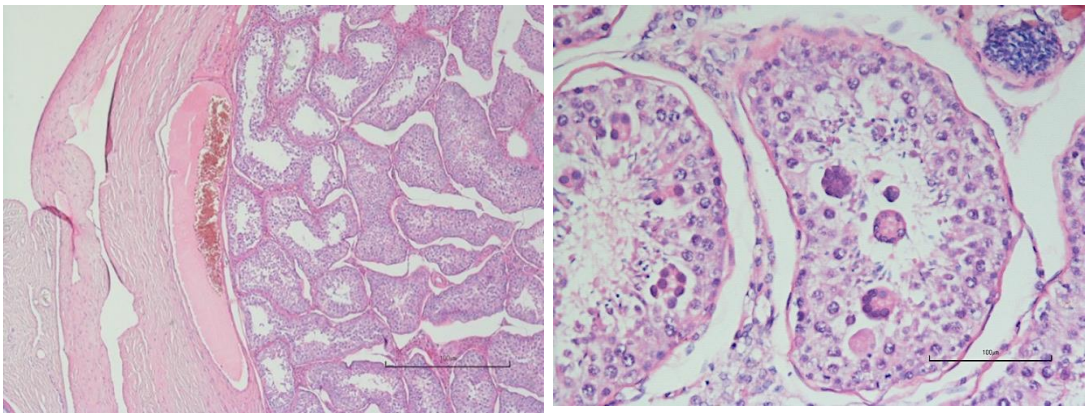

***Figure 4.1**. Microscopic pattern of testicular degeneration in a 10-years-old dog. Degenerative changes are present in multiple seminiferous tubules. Hypospermatogenesis with decreased numbers of spermatids and spermatocytes. Tubular vacuolation, multinucleated and apoptotic germ cell, disordered arrangement of the germ cell layers are visible (4X - 10X; H-E).*

Cellular senescence implies an irreversible cellular growth arrest and it develops in response to vary stress factors (Fridlyanskaya et al, 2015). Senescence of cells is not a sole prerogative of aging, but it exists also in young individuals where it prevents uncontrolled proliferations of cells after damaging insults to DNA (Merz et al, 2019).

Testicular degeneration related to age in dogs is characterized by a significant increase in number of Leydig cells, up to 2.95-fold and any significant decrease in epithelial area or seminiferous tubules diameters. In contrast to the situation in man where Leydig cells progressively decrease with aging (Merz et al, 2019).

Inflammatory lesions: Orchitis and Periorchitis

Orchitis is the clinical term that indicates the inflammatory state of the scrotal contents, even though in some cases only the epididymis is affected. In fact, true orchitis is much less common than epididymitis in domestic animals, probably due to the anti-inflammatory environment of the testis (Forster, 2012). When the process involve the *tunica vaginalis*, the results is called "periorchitis". Inflammatory lesions may occur together or separately. It can be unilateral or bilateral (McEntee, 1990).

The main causes of inflammation of the testis are bacterial infection, trauma and testicular torsion. Most are caused by Gram-negative bacteria, such as *Escherichia coli* (Forster, 2012). Bacteria can reach the testes via haematogenous spread, through retrograde migration from ductus deferens and epididymis or directly through scrotal skin lesions. Brucella species cause orchitis in many domestic species. Infection of the canine testis by haematogenous route can be due to B. abortus, B. suis and B. melitensis (NÖcler et al,2003). Inflammation of the testis caused by Brucella infection are often unilateral and associated with epididymitis, scrotal dermatitis and testicular atrophy. A case of orchitis caused by Leishmania Infantum infection in a dog has been described (Manna et al, 2012).

Four forms of orchitis have been described: intratubular, interstitial, necrotic and granulomatous. (Forster, 2012). Orchitis is intratubular when it is centred on seminiferous tubules. Intratubular ochitis appears grossly and poorly defined; foci of inflammation are yellow-coloured and they measured up to 1 cm; as the lesion becomes older, they become firm and white. At the beginning of the inflammatory process, the tubules loss their lining

maintaining their outlines. They can evolve in spermatic granuloma containing macrophages, lymphocytes and spermatozoa free within the tissue. At the edge of the lesion collagen forms. When the inflammation involves the interstitial compartment of the testis, it is called interstitial orchitis (Forster, 2012).

The third form of orchitis is characterized by necrosis. Necrotic orchitis is the most severe form. Brucella is responsible of this type of lesion. When the necrosis is extensive, the original structured are no longer recognizable within the caseous mass and grey-brown necrotic debris replace the testicular components. In dogs a forth form of orchitis has been described: a form of granulomatous orchitis caused by *Blastomyces dermatitidis* infection in endemic areas (Forster, 2012).

Few cases of orchitis with immune-mediated etiology in dogs with fertility disorders have been described in literature (Donnelly et al, 2016; Davidson et al, 2015).

Testicular cysts

Cysts of the testis are rare entity in dogs. Two cases of testicular cysts associated to neoplasms, monophasic teratoma and carcinoma, have been reported several years ago (Wakui et al, 1992; McEntee, 1990). Cysts are characterized by a thick, fibrous, connective wall lined by epithelial cells. The content is fluid. Normally, any inflammatory reaction around the formation is seen. The etiology has not yet been determined with certainty. In some case, the presence of cysts can be due to a form of cystic dysplasia of the testis (Wakui et al, 1997).

Testicular torsion

Testicular torsion is a twisting of the spermatic cord upon its axis that results in obstruction of blood vessels supplying testis and epididymis (Mostachio et al, 2007). In dogs, this condition is quite rare and often associated with cryptorchidism. The reason of that may be the greater mobility of retained testis in the abdomen than in the scrotal sac (Bartlett, 2002). This may implies that breeds at higher risk for cryptorchidims are more affected by testicular torsion than breeds at lower risk (Mostachio et al, 2007; Bartlett, 2002). The venous occlusion can lead to the necrosis of the testis.

The clinical presentation of testicular torsion includes acute abdominal pain, lethargy, anorexia, vomiting, locomotor difficulties, pyrexia and dysuria (Mostachio et al, 2007).

Several reports described the association between torsion and testicular neoplasia in dogs (Johnston et al., 2001; Bartlett, 2002). According to Johnston et al. (2001), the 36% of testicular torsions occur in neoplastic testes and commonly in cryptorchid dogs (Feldman and Nelson, 2004). The etiology of this pathological condition has not been well defined. Some authors hypothesized that testes located in the abdominal cavity affected by neoplastic alteration are allowed to do greater movements that can lead to the twisting of the spermatic cord (Johnston et al , 2001).

The torsion of scrotal canine testis is rare, but described by some authors (Hulse, 1973; Zymet, 1975; Young, 1979). In human medicine, the torsion of the testis is frequent in very young males and newborns (Pinto et al., 2001). When torsion of the spermatic cord is unilateral, the contralateral testis is not exempt from damage. In fact, some studies

conducted in humans with electromagnetism and radioisotopes revealed that blood flow circulation and reperfusion mechanisms were compromised in the contralateral testes with indication of hypoxia and oxidative stress phenomenon (Pinto et al., 2001; Heindel el al., 1999). Poor data are described in dogs.

Chapter 5.
Diagnostic tools for the detection of testicular abnormalities in dogs

The first step in the diagnostic process of testicular abnormalities is the physical examination. Male genitalia are accessible externally. The physical examination of the genital tract of male dogs, in association with other exams, e.g observation of copulation and evaluation of semen, have also the task to ascertain whether normal fertility can be expected from the animal or not (Noakes eta l, 2001). The careful palpation of the testis can reveal the presence of masses, warm and thigh scrotal areas, asymmetry or absence of one or both testes. Rectal examination can reveal hypertrophy of regional lymph nodes and abnormalities of the prostate gland. Many diagnostic techniques for the detection of testicular disorders have been proposed.

5.1 Ultrasonography

Ultrasound (US) is the modality of choice for the evaluation of testicular abnormalities and palpable lesions in dogs, as in human medicine (Huang & Sidhu, 2012; Hangiandreou, 2003). Ultrasound provides high-sensitivity for the detection of scrotal lesions and it is able to rapidly establish the intra- or extratesticular- site of the mass. The first veterinary report on the US application on reproductive organs was described by Palmer & Driancourt in 1980 in mares, approximately a century after the discovery of

piezoelectric effects of some crystals (Ginther, 2014). By this time, its popularity has been increasing year by year.

The principle underlying the functioning of ultrasound transducers is based on the piezoelectric influence. Some crystals can produce ultrasound signal waves, expanding or contracting in response to electric signals with alternating polarities. The application of crystals in transducers on tissues produces ultrasound waves and converts the echoes resulting from them in electric signals. The signals are then converted and displayed on a monitor as shades of grey representing the intensity of echoes and the location of tissue reflectors (Ginther, 2014).

At the basis of the image construction, there are sound waves with variable wavelengths and amplitude transmitted through a medium that can be solid, liquid or gas. Ultrasound waves travel through tissues and generate returning echoes. The echoes deriving from the propagation of the wave through the medium are displayed as reflection of sound travel through media with different acoustic impedance. The impedance is material or tissue dependent. The number of cycle repeated over a specific time interval determinates the frequency of the sound. Wavelength is shorter for high-frequency sound that stands for more cycles per second; in low- frequency sound wavelength are longer with fewer cycles per second. Frequency is measured in Hertz (Hz). Sounds used as diagnostic unit are "ultra" because beyond what the human ear can perceive. The human ear perceive sounds ranging from 20 to 20,000 cycle/s or up to 20 kHz; when the frequency is higher than 20 MHZ, the human ear can't perceive it. The frequencies used in ultrasound imaging range from 3 to 12 MHz or 3 to 12 million cycles per second (Kubale & Hetzel, 2005).

Ultrasound pulses are generated by piezoelectric elements assembled into a transducer that is both a transmitter and a receiver of sound. Traditional B-mode ultrasound, where "B" stands for "brightness modality", provides a two-dimensional image that consists in several adjacent ultrasound beams transmitted and received in the same plane (Kubale & Hetzel, 2005). Echoes are displayed at a brightness level proportional to their amplitude. Brightness signals are transmitted and temporary stored in a matrix and then the contents of the matrix is passed to a monitor at correlative sites and assembles into an ultrasound sectional image (Kubale & Hetzel, 2005).

Ultrasound probes vary for specific needs. Convex probes have rows of piezoelectric crystals aligned along a convex surface with different beams and tracks. The image produced by this probes is triangular because of the diverging lines of ultrasound waves generated. Their footprint is small and the scanning field is large. Linear probes have piezoelectric crystals aligned along a flat surface and the image produced is rectangular. They are used to evaluate superficial organs (Kubale & Hetzel, 2005).

Abdominal ultrasound is accurate and sensitive for the evaluation of male reproductive organs. It is widely accepted as first-line method for scrotal diseases and it is commonly used in male animals to evaluate breeding dogs, identify ectopic, non-palpable and retained testis, to assess the regional lymph nodes and to evaluate palpable or incidental testicular abnormalities (Fan & de Lorimier, 2007, Kubale & Hetzel, 2005).

Testes are examined and scanned in at least two planes, sagittal and transverse, with high-frequency probes of 7.5 MHz or more. Linear transducer with broad contact area and

good resolution are better than convex probes for the assessment of the scrotal region. The ultrasonographic appearance of the normal testis in dog is characterized by echogenic with homogeneous medium echo texture with an thin hyperechoic peripheral echo, representing the parietal and visceral tunics (Mattoon & Nyland, 2014). On the midsagittal plane the mediastinum testis is visible at the centre of the organ as a linear echogenic central structure that appears as a central focal echo on a midtransverse scan plane (Fig. 5.1)

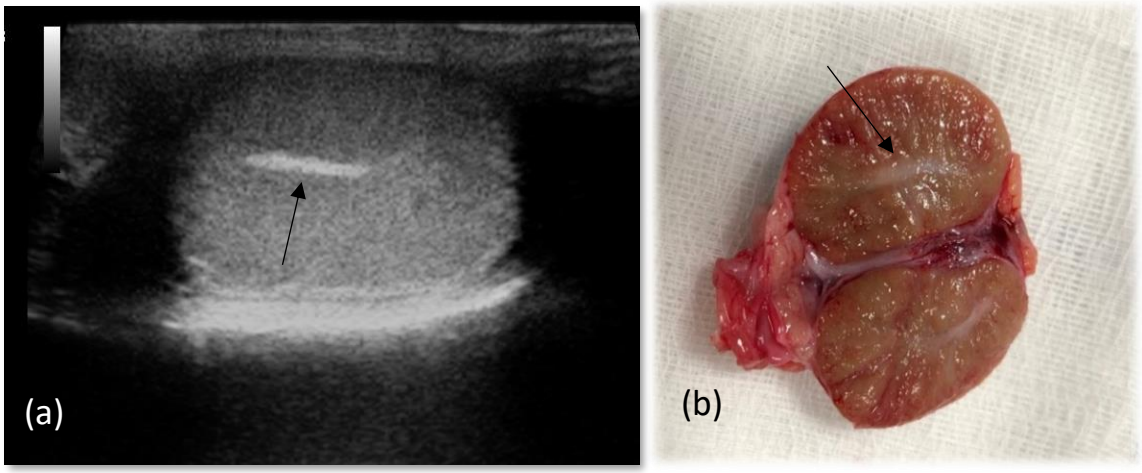

Figure 5.1. Sonographic and macroscopic aspect of the canine testis. *(a) Sagittal B-mode image of the testis. The echo pattern is homogeneous, fine and granular. The mediastinum testis is hyperechoic and clearly visible in the middle of the organ (arrow).* ***b****. Macroscopic aspect of the canine testis in sagittal section. The black arrow indicates the mediastinum testis.*

Color flow examination of the testes has been described first in human medicine (Horstman et al, 1991) and over a decade later it has been reported in dogs (Gumbsch et al, 2002). Nowadays, ultrasonography of the testes is performed to assess palpable and nonpalpable lesions in both human and veterinary medicine, as well as to localize retained testes and to detect scrotal and epididymal diseases (Mattoonn & Nyland, 2014).

Although the identification of testicular masses is supportive, the sonographic appearance does not give any further information regarding the nature of the lesion. Gross lesions are usually characterized by mixed parenchymal pattern than may include necrosis or haemorrhage (Mattoon & Nyland, 2014). The histopathological evaluation is essential for definitive diagnosis and for the classification the lesion type (Huang & Sidhu 2012).

Other clinical conditions, such as testicular torsion, orchitis and epididymitis can be detected by ultrasound (Mostachio et al, 2007).

5.2 Color Doppler Ultrasound (CDUS)

The Doppler technique has been used for many years as method of choice for the evaluation of blood flows in different organs and for the detection of vascular abnormalities. The Doppler Effect consists in sound waves reflected from moving targets, as blood cells. Signals are displayed in color as a function of their motion away and toward the transducer. Blood velocities are color-coded, calculated by the unit and displayed as amount of color saturation. Colors are super imposed on the two-dimension B-Mode images (Mattoon & Nyland, 2014).The frequency of the returned echoes is higher than that of the transmitted sound when the target is moving toward the transducer and this fact results in a positive shift. The positive shift is displayed with yellows, oranges and reds shadows. Yellow-white represents the highest velocity. On the contrary, when the motion is away from the transducer the frequency of the returned echoes is lower than that of the transmitted sound and the resulting shift is negative. The flow away from the transducer is displayed by shade of blue and green. The Doppler shift (F, Hz) is

the difference between the transmitted and the received frequencies. A greater velocity of moving targets means a greater Doppler shift (Mattoon & Nyland, 2014). Velocities recorded by color Doppler are mean flow velocities information over a large area obtained by many sample volumes, called gates, along a single line rather than a single sample in localized areas.

The first appliances of CDUS for the diagnosis of scrotal disorders have been described in human medicine. In the last decades it has been largely applied in the field of andrological and reproductive diseases of mammals, including men. The range of application of color Doppler US is wide. Color Doppler US is employed for the detection of testicular pathologies caused by altered blood flows, as varicocele, testicular infarction and torsion of the spermatic cord (Pavlica e Barozzi, 2001); it can reliably distinguish between early inflammatory disturbances of the scrotum, neoplastic changes, spermatic cord torsion and epididymo-orchitis (Gorecka-Szyld, 1999). Color Doppler US examinations can add important information where the physical examination is unreliable or limited by pain and pathological conditions, e.g. hydrocele (Pearl & Hill, 2007). Following its diffusion in veterinary practice, CDUS has become one of the most important imaging modality for the evaluation of the scrotal region and for the monitoring of reproductive health in male dogs (Günzel-Apel et al, 2001). Color Doppler US significantly increased the diagnostic capabilities of traditional B-mode scan adding physiological information from the organ. B-mode US gives information about the morphology of the organs. Information about direction, velocity, character and timing of blood flows improve the imaging accuracy. Furthermore, it allows repeating non-invasive

scanning assessment of the scrotal tissues in the same patients (Arteaga et al. 2005). In veterinary practice color Doppler ultrasound has been used for recent years to predict fertility disorders in companion animals (England et al, 2017; De Souza et al, 2015; Zelli et al, 2013) farm animals (Samir et al, 2018; Ginther, 2014; Kastelic & Brito, 2012; Ginther & Utt, 2004) and other mammals like camels, whereas it has been used for the comparison of blood flows in fertile and infertile males (Kutzler et al, 2011). In stallion for the evaluation of specific pathological conditions, such as varicocele (Pozor et al, 2004).

Flow can be detected even in very small vessels with the color Doppler ultrasound technique. Individual vessel flow perfusion is quantify by semi-quantitatively parameters that describe the resistance to blood flow in vessels peripheral to the vessel being examined. The features of flows are clearly defined by calculations performed by the US equipment. The main parameters are the Resistance Index (RI) and the Pulsatility Index (PI). They are angle-independent and allow to compare flow in systole and diastole phase, indicating the flow condition downstream of the vessel (Serine et al, 2010). RI and PI have negative correlation with the downstream tissue perfusion from the sample gate: when blood flows increases, RI and PI indices increase (Bollwein et al, 2016).

The normal spectral waveform of intratesticular arteries has been evaluated in men with a testicular volume higher than 4 cm^3. It has a low-resistance pattern and range from 0.48 to 0.75, with a mean value of 0.62 (Middleton et al, 1998).

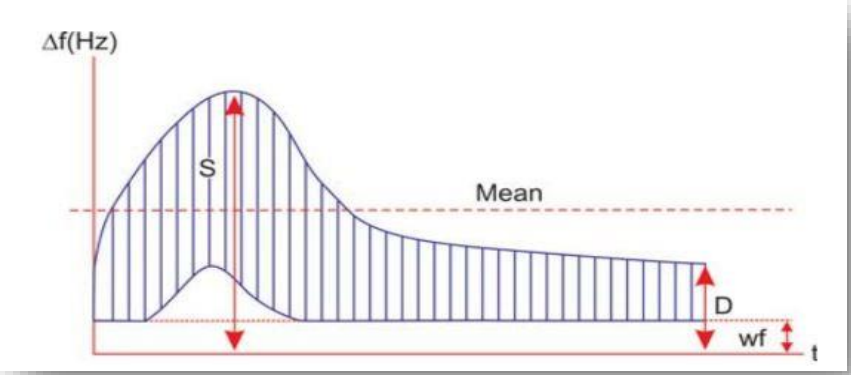

***Figure 5.2.**. The blood flow velocity waveform. The value S indicated Peak systolic Doppler shift and D is the maximal diastolic Doppler shift. S and D permit to define PI and RI (Modified from Kupesic & Kurjak, 2011).*

PI and RI are define by the following formulas:

$$\textbf{\textit{Resistance Index}} = \frac{S-D}{S}$$

$$\textbf{\textit{Pulsatility Index}} = \frac{S-D}{mean}$$

Testicular biopsy are not frequently used in veterinary practice. The main indication to perform biopsy of the testis is the evaluation of testicular lesions in valuable breeding animals, in which a certain diagnosis is needed before irreversible orchiectomy (Fan & de Lorimier, 2007).

Even if most of primary tumors of the canine testis are benign and remain locally confined, older dogs are have more risk to develop testicular tumors and neoplastic pathologies in general. For this reason, it is important to have a complete clinical state of play, as much as possible on staging of the tumor before planning the surgery.

5.3 Power Doppler Ultrasound

Power Doppler is a mode of operation that displays power, energy or strength of the Doppler signal using the color map overlaid on real-time B-mode images, as color Doppler does. It differs from Color Doppler since the power of the signal is determined by the concentration of red blood cells rather than flow velocities. Power Doppler can be consider a step backward that is very sensitive to detect slow flows in small and deep vessels and to display movements ; it is independent of the angle of insonation but more sensitive to artefactual motion related to the patient or to the transducer. The color in an area indicates that the precence of blood flow; the quantity of moving erythrocytes is represented by brightness.

5.4 Contrast-enhanced Ultrasound (CEUS)

Contrast-enhanced ultrasound is a relative new technique that makes use of contrast agents as echo enhancer for the visualization of arterial supply, vascular bed and venous drainage of an organ or a system. The contrast agents consist in microbubbles measuring 7-10 μm coated with a galactose or lipid and filled with a high molecular weight inert gas. The gas is low soluble in water and this characteristic together with the low molecular weight provide resistance and persistence in blood (Nyman et al. 2005). The microbubbles are administered intravenously and they are not able to leave the intravascular space. The visualization is generally preformed with low frequency transducers (1MHZ to 3MHz). CEUS provides information on the vascularity of organs.

It is very sensitive to detect microvessels and particularly useful to detect and characterize lesions. In dogs, its use has been described for the detection of masses at the level of liver, spleen, kidney and prostate gland (Vignoli et al, 2011; Haers and Saunders, 2009; Ohlerth et al, 2007; Nyman et al, 2005). There are only few studies on the use of CEUS for testicular investigations in dogs (Haers et al, 2009; Volta et al, 2014) performed a study on 48 dogs for the evaluation of chronic pathologies affecting canine testes by mean of the contrast-enhanced ultrasonography. Among 60 pathologic testis analyzed, forty were neoplastic and 20 were affected by non-neoplastic pathologies, such as degenerations, chronic necrotizing orchitis, atrophy and interstitial hyperplasia. The neoplastic lesions were hyperenhanced to the surrounding tissue at the CEUS examination but these findings were not specific for different types of tumors. Most of benign lesions analyzed were hypoenhanced. CEUS is a valuable tool to describe focal testicular lesions in dogs and to show the lack of vascularization when testicular infarction occurs, but it is not exempt from limitations. Time is required to perform the scan, the cost of the adequate equipment is relative high as well as that of contrast media which moreover may cause adverse reactions (Volta et al, 2014). No less interesting, one of the huge limit of the application of CEUS in veterinary practice is the need for sedation of the patient. The dynamics of the vasal perfusion can be affected by the status of the vessels. It has been clearly demonstrated that the use of dexmedetomidine, alpha 2- adrenergic agonist drug used for sedation, reduce the organ blood flow in dogs and this fact can impact the perfusion parameters, e.g. arrival time and peak intensity of the medium, of the contrast-enhanced ultrasonography (Volta et al, 2014).

5.5 Real-time elastography (RTE)

Between the new techniques introduced to improve the accuracy of ultrasonography in the diagnosis of diseases of organs and tissues, elastography must be included. Real-time Elastography (RTE) has been described for the first time in 1987 by Krouskop et al (Ophir et al, 1991) and its first applications have been described for the diagnosis of breast lesions in women (Itoh et al, 2006). Subsequently, it has been applied for the diseases of different organs as liver, lymph nodes, thyroid, prostate, muscles in human medicine (Glinska-Suchocka et al, 2014; Dobruch-Sobczak & Sudoł-Szopińska, 2010). Its role has been recently investigated in deep for the detection of testicular lesions in men (Dikici et al, 2016; Goddi et al, 2012) and in dogs (Glinska-Suchocka et al, 2014). The technique is based on the assumption that different tissue have different degree of hardness and soft tissue become deformed more easily than hard tissues (Iton et al, 1991). The image of the tissue elasticity is displayed on the monitor in the form of colorful maps that completely superimpose the B-mode image. The measurement of the degree of elasticity is made in the real time by the operator. The color map ranges from red to blue colors, for most rigid to softest tissues, respectively (Glinska-Suchocka et al, 2013). Nowadays no specific color-coded elastography score is available for testicular masses. Many studies adopted the classification proposed by Ithoh et al (2006) for breast lesions, occasionally with modifications (Goddi et al, 2012). Only few report on the use of RTE for the characterization of canine testicular masses are available. In a recent study by Glinska-Suchocka et al (2014) conducted on nine dogs with testicular lesion the elastographic examination has revealed a 100% sensitivity, 81% specificity and 91%

accuracy in the detection on the masses. Authors recognized its safety and utility but more studies on greater numbers must be done to confirm the effectiveness of the modality in the diagnosis of the testicular disease in dogs (Glinska-Suchocka et al, 2014).

5.6 Histology and Immunohistochemistry

The diagnosis of testicular tumors along with the clinical stage is determined on the basis of the histologic evaluation of the testis. The immunohistochemistry is used to confirm the histogenesis and the subtype of the tumor. The selection of the appropriate immunohistochemical panel of antibodies is important to reach the correct diagnosis.

It is well known that angiogenesis play a crucial role in the tumor growth and progression. In several types of tumors the number of vessels within a mass have been evaluated using endothelial markers of the angiogenic process. The vessel concentration within a mass is called microvessel density (MVD) and it is considered one of the most prognostic factor in patients with different types of tumors in human medicine (Miyata et al, 2015). On the other hand, the role of angiogenesis in veterinary medicine and the relation between the proliferation of endothelial cells and the biological behavior of tumors is still poorly understood. (Restucci et al, 2000). Specific endothelial markers as CD34, CD31 and CD105 have been used for this purpose (Miyata et al, 2015). MVD in prostate cancer has investigate by some authors in past (Bettencourt al, 1998) as in recent times (Miyata et al, 2015). However, the literature regarding the significance of MDV in testicular cancer is relatively scarce. In our study, we used CD31 marker to evaluate the vascularization of the testicular lesions. CD31 is a transmembrane glycoprotein that play a role in different

physiologic and pathologic pathways like detachment of leucocytes, platelet and T-cell activation, atherosclerosis and angiogenesis.

CD31 is considered a very specific marker for endothelial cells and their tumors (Ramos-Vara et al, 2018).CD31 has been used for years for the evaluation of the microvessel density within a mass or a lesion. (Vleugels et al, 2019).

For human patients, many other markers have been used to characterize the neoplastic cellular population building the tumor, but only some of them have been tested in dogs (Hohšteter et al, 2014). c-KIT is used in humans to differentiate Classical Seminoma (CSEM) from Spermatocytic Seminoma (SSEM) (Emerson & Ulbright, 2005). C-KIT is also expressed in another type of tumor cancer, called Intratubular Germ cell Neoplasia of Undifferentiated origin (IGCNU) or *carcinoma in situ* that is considered very rare type of testicular cancer in dogs. The c-KIT expression in canine seminomas is not certain. Some reports described its expression in a high percentage of canine SEM (Grieco et al. 2010) and some authors affirmed that c-KIT is the most sensitive marker for canine SEM (Yu et al, 2009). Other authors strongly disagreed with this statement (Thorvaldsen et al, 2012). PLAP is the Placental Alkaline Phosphatase marker and it is widely used in human patients with classical seminomas, but PLAP immunostaining is very weak and heterogeneous in canine SCTs (Yu et al, 2009). Cytokeratins AE1/AE3 used to differentiate testicular germ cell tumors from embryonal carcinoma and yolk sac tumors in men; CD30 is positively expressed by neoplastically altered Sertoli cells and in canine embryonal carcinoma Hohšteter et al,2014), but it was not expressed in canine seminomas (Yu et al, 2009). Inhibin- alpha is secreted mainly from Sertoli cells and a

minor amount is secreted by Leydig cells (Debora et al, 2002). Its expression has been evaluated in human and canine tumors of the testis. Vimentin and inhibin-alpha are remarkable marker of SCT in dogs according with Yu et al (2009), but previous studies did not confirmed it (Taniyama et al, 2001). Only few data and sometimes discordant, are reported on the use of these markers for testicular tumors in dogs in literature.

Chapter 6.
Study of the canine testicular disease through color Doppler imaging, histology and immunohistochemistry

6.1 The aim of the study

My research project focused on the potential of Color Doppler imaging in assessing neoplastic and non-neoplastic lesions of the testis in dogs.

I was intent to establish if color Doppler technique can be considered a good tool for the *in-vivo* characterization of the pathologic findings of the canine testis. To the best of my knowledge, there are only few studies on the use of CDUS for the analysis of testicular abnormalities in dogs.

To achieve my goals, some important aspects have been developed. Sonographic features of neoplastic and non-neoplastic testicular lesions have been deeply examined; color and power Doppler parameters related to vascular flows have been analyzed with the purpose to determine if color Doppler sonograms can be used to differentiate between testicular tumors and benign lesions.

Histological and immunohistochemical evaluations were essential for the classification of the cancer types. Starting from the microscopic evidences it was possible to assess with confidence the match between color Doppler vascular signals and histopathology. The expression of CD31 immunohistochemical markers has been used for the characterization of the degree of vascularization of neoplastic lesions of the canine testis.

6.2 Material and Methods

Animals

The present study was duly approved by Ethic Committee of the University of Parma, OPBA institution (Organismo Preposto al Benessere degli Animali, DL n.24/14). The research has been conducted at the Department of Veterinary Medicine of the University of Parma.

Sixty-four male dogs aged between 7 and 15 years ranging from 15 to 43 kg were included in the study. The study group consisted of dogs of different breeds: 33 mixed breed, 10 Labrador Retriever, 9 German Shepherd, 7 Boxer and 5 Bracco Italiano.

The dogs were conducted by the owner to the Teaching Veterinary Hospital for the presence of symptoms related to testicular disorders; in four of them, the detection of the testicular disease was incidental, reported by the clinician during a routine physical examination. After the physical examination, the sonographic exam was performed. All dogs were enrolled in the study with the written consensus of their owners.

Ultrasonography

All subjects underwent the sonographic examination of the scrotal region. The dogs were placed in dorsal recumbence. Low abdomen and scrotal region were clipped and a layer of conductive gel was applied on the skin to provide a better transmission of ultrasound waves. All scans were made by the same operator. The testes were scanned using B-mode,

color and power Doppler US for both testis. By using B-mode US, surface, shape, contours and echotexture of the gonads were primarily defined.

The ultrasound equipment used was a MyLabTM30Gold (Esaote, Florence, Italy). The equipment had with two different high-frequency probes: 12 MHz linear transducer and 7.5 MHz convex transducer. The Pulse Repetition Frequency (PRF) was set between 0.7 and 1.4 KHz to optimize color and power Doppler parameters in detecting slow blood flows. The use of the highest frequency linear array transducer (7–14 MHz) allowed reaching adequate penetration of ultrasound through scrotal tissues. The examination was generally performed with transducer in direct contact with the patient; a stand-off pad has been used for assessment of superficial lesions where necessary. Both testes were examined along two planes, longitudinal and transverse axes. For each plane, multiple static images were recorded (Figure 6.1).

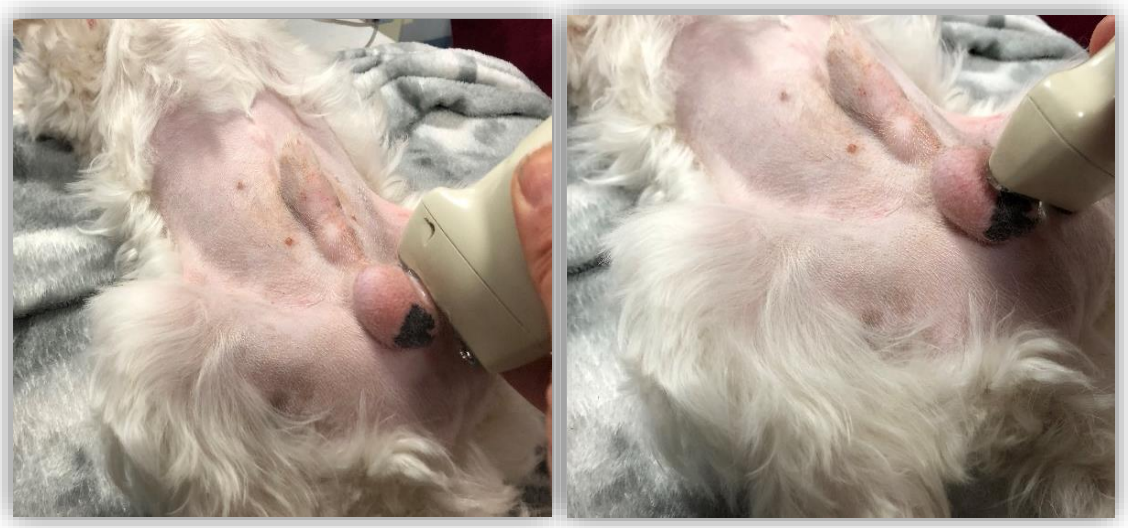

Figura 6.1.. Ultrasound examination technique. *The patient wass placed in dorsal recumbency. The testes were scanned in longitudinal (a), transverse (b) and dorsal planes to fully examine the scrotal organs. Linear array transducers were used.*

Color Doppler and spectral Doppler indices were optimized to demonstrate low flow velocities and blood flow into and surrounding scrotal structures (Kocakoc, Bhatt, & Dgra, 2007). Resistive index (RI), Pulsatility Index (PI), peak systolic velocity (PSV) and end diastolic velocity (EDV) were calculated to characterized resistance to flow in peripheral vascular beds. The minimum sample gate setting for this unit (1 mm) was used, and angle correction was between 30 and 60 degrees. The Resistive Index was calculated as following:

$$\mathrm{RI} = \frac{(\mathrm{PSV} - \mathrm{EDV})}{\mathrm{PSV}}$$

In according with Pozor & McDonnell (2004), the Pulsatility Index (PI) was calculated as follow:

$$\mathrm{PI} = \frac{(\text{maximum velocity} - \text{minimum velocity})}{\text{mean velocity}}$$

The measurement of the flow waveform was calculate three times in succession as shown in Figure 6.2. The mean value of them was calculated and recorded.

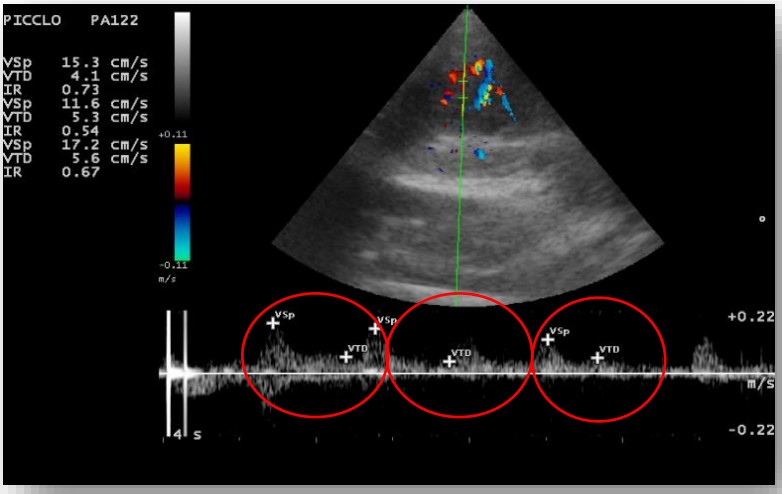

***Figure 6.2**. The waveform of blood flow shows three measurements for each parameters included in the RI formula. The flow information are represented in function of time. Velocity is plotted on the horizontal axis and time on the vertical axis. PSV: Peak Systolic Velocity; EDV: End Diastolic Velocity.*

Color Flow Mapping (CFM) was applied to visualize flows into and around the region of interest.

The analysis of the signals that allow the visualization of the hypo- and hypervascularization of the area has been performed by mean of ImageJ Software (NHI USA, on line version 1.42).

The Vascularity Index (VI) was determined as the ratio between the area occupied by vascular flow and the total area of the lesion (Bigliardi & Ferrari, 2011). In order to determine the surface of the lesion that was occupied by vascular flow signals, a threshold-based pixel count measurement of the image analysis software (ImageJ, NHI USA, on line version 1.42) was used, as shown in Figure 6.3.

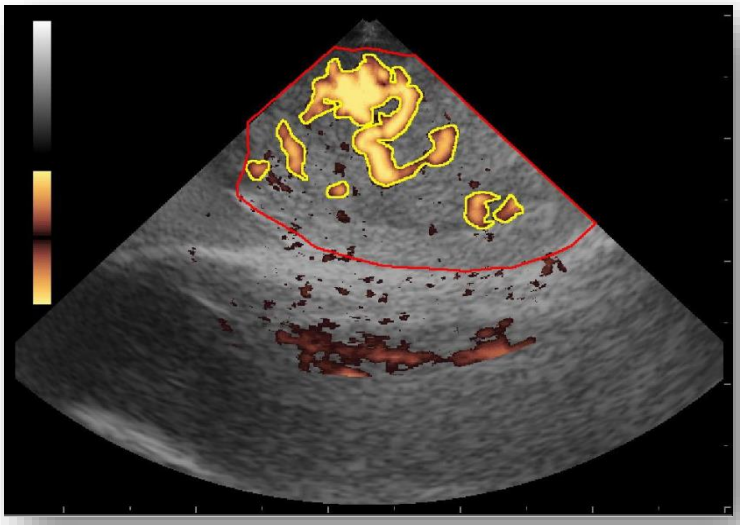

***Figure 6.3**. The power Doppler ultrasound mask was applied for the VI calculation. It overlays on B-mode real-time image. The red line outlines the contour of the lesion. The color spots marks the vascular signals and their intensity.*

Once ultrasound examination completed and data collected, all dogs underwent orchiectomy under general anesthesia.

Histology and immunohistochemistry

After the surgical removal, the testis were sent to the Anatomic Pathology division of the Department of Veterinary Medicine, University of Parma. Macroscopic evaluations of pathological testis were carried out.

The samples were fixed for 24 hr in 10% neutral buffered formalin, routinely processed through a series of alcohol-water solutions, from a alcohol concentration of 75% to 100%, xylene and then embedded in paraffin. 5 μm-sections were obtained from paraffin blocks with microtome (Leica®) and dried overnight at 37 °C.

Sections were stained with the standard Haematoxylin and Eosin (H&E) protocol for the histological examination (Fisher et al, 2008), as follow:

1. Deparaffinization of sections on xylene;
2. Rehydration of section passing through decreasing concentration of alcohol (100%, 90%, 70%) up to water;
3. Staining in Hematoxylin for 5 minutes;
4. Washing in running tap water for 5 minutes;
5. Staining in 1% Eosin for 1 minutes;
6. Washing in tap water for 5 minutes;
7. Dehydration in increasing concentration of alcohols and clearing in xylene;
8. Mounting on mounting media

After H&E staining, the histological sections were examined with Nikon Eclipse E800 microscope (Nikon Corporation, Japan) using Nikon PLAN APO lenses. Sections were photographed at 4x, 10x, 20x and 40x (Nikon PLAN APO lenses) with Camera DIGITAL SIGHT DS-Fi1 (Nikon Corporation, made in Japan); pictures were acquired with DS Camera Control Unit DS-L2 (Nikon Corporation, Japan) and stored in USB device.

The immunohistochemical staining was used to identify microvessels into and around lesions. The immunoreactivity of endothelial cells was tested by using CD31 marker.

CD31 was employed to highlight small, immature microvessels or single endothelial cells. The immunohistochemical analysis was performed with the ABC kit (Vectastain, Vector). Peroxidase blocking solution was used to deactivate endogenous peroxydase

(3% H_2O_2 in PBS). Antigen retrieval was performed in a microwave using 0,01 M citrate-buffer (pH 6.0). Sections were incubated with monoclonal mouse anti-CD31antibody (Clone JC7A0A, 1:20; Dako) for 1 hour at room temperature, followed by biotinylated goat-anti mouse secondary antibody (Vector Laboratories) and ABC peroxidase solution (ABC, Vectastain). For negative control, the primary antibody was omitted and the sections were incubated with antibody diluent, supplemented with a nonimmune immunoglobulin of the same isotype (Mouse IgG1 isotype, MAB 002, RD Systems), at the same concentration to the primary monoclonal antibody. After staining with DAB (Vector, SK9800), immunohistochemical sections were examined with Nikon Eclipse E800 microscope (Nikon Corporation, Japan) using Nikon PLAN APO lenses. Sections were photographed at 4x, 10x, 20x and 40x (Nikon PLAN APO lenses) with Camera DIGITAL SIGHT DS-Fi1 (Nikon Corporation, made in Japan); pictures were acquired with DS Camera Control Unit DS-L2 (Nikon Corporation, Japan) and stored in USB device. The positive reaction was characterized by the presence of brown staining in the cytoplasm of endothelial cells. A qualitative immunohistochemical evaluation of the positivity had been performed.

The microvessel density was determined to validate the data obtained with the color Doppler technique, rather than to quantify the angiogenesis of the tumors. Moreover, it was performed to understand if there is a correspondence with the intensity of the vascular signal showed by the lesion and if there were any differences in terms of vascularization between the different types of lesions analyzed.

For the evaluation of the density of vessels, the quantification method proposed by Koen et al. (2016) has been used. The sections were stained with monoclonal mouse anti-CD31 antibody (Clone JC7A0A, 1:20; Dako) and examined using Nikon Eclipse E800 microscope (Nikon Corporation, Tokyo, Japan) and Nikon PLAN APO lenses. Images were captured with a digital camera DS CCU DS-L2 (Nikon Corporation, Japan). To sample the areas for the vessel counting, the vascular hotspot method or Weidner's method was used (Koen et al, 2016). This method states that one to five areas with the greatest density of positively stained vessels at low magnification should be chosen. The areas called "hotspots" are counted at high magnification. In our study 3 tumors per type and 3 normal testes have been analyzed. For each tumors, 3 hotspots by means of the method previously described have been chosen.

For the MVD quantification every Cd31-positive stained vessels in the selected hotspot at 10X magnification were counted using a computer-aided software ImageJ 1.52q (Wayne Rasband, National Institutes of Health, USA).

Representative example of vessels positively stained with CD31 is shown in Figure 6.4.

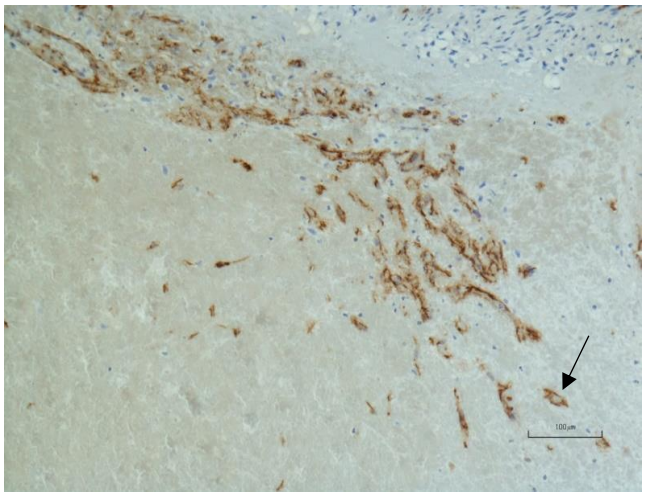

***Figura 6.4**. Interstitial cell tumor in a 11-years-old dog. Example of "hotspot" at 10X magnification for the MDV count. Vessels positively stained with CD31 marker are brown-colored (arrow).*

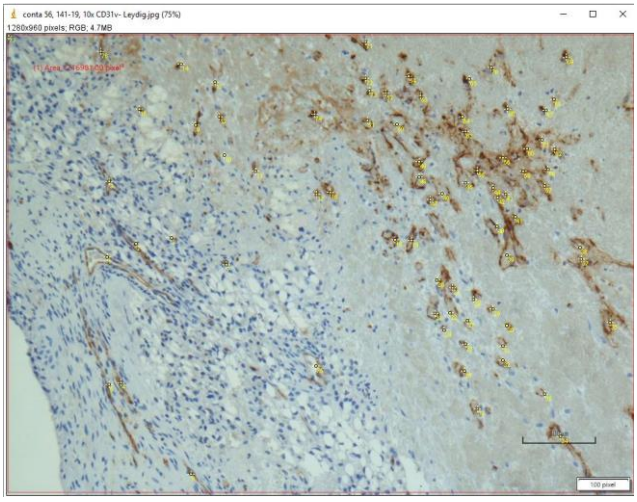

***Figura 6.5**. Example of a hotspot MDV count. CD31-stained vessels were counted using the image analysis software ImageJ pointing the counter on every vessels visualized.*

Statistics

The SPSS Software was used for statistical analysis of the data. Color Doppler imaging parameters were recorded and analyzed. Doppler indices (PSV, EDV, RI, PI) were calculated by algorithm package included in the ultrasound unit on the basis of spectral Doppler waveforms displayed. For the analysis of variance ANOVA test was employed.

Significant differences were determined at $P < 0.05$. Immunohistochemical data were express as the mean and the standard deviation SD for each types of tumors.

6.3 Results

At the clinical examination, predominant symptoms were:

- scrotal enlargement and discomfort (80%)
- testicular pain (10%),

while 10% of subjects did not report any clinical signs of genitourinary disorders. In those subjects testicular lesions were incidental findings (Figure 6.6).

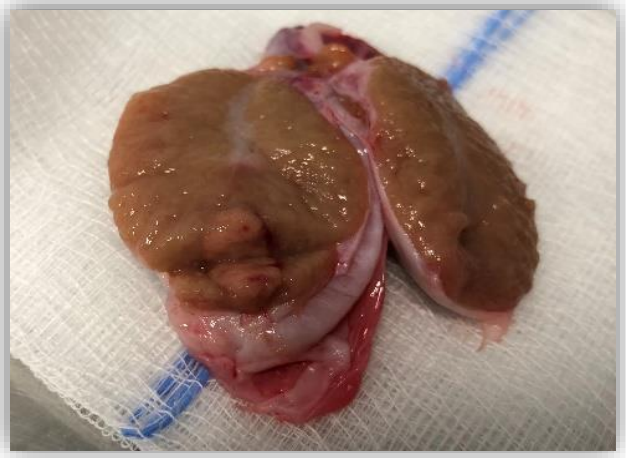

***Figure 6.6. Testicular nodule in a 8-years-old dog**. No clinical signs related to the presence of the mass were present. The pathological finding was incidental. The organs have been macroscopically and microscopically evaluated after surgery.*

The 78% (50/64) of lesions resulted to be neoplastic at the histological examination. Of fifty neoplasms, 10 (20%) affected both testes. Inflammatory, i.e orchitis and epididymitis, and degenerative lesions accounted for 13% (8/64) and 9% (6/64), respectively.

Conventional B-mode US was able to detect the presence of all the lesions within the normal parenchyma of the testis. B-mode US was not able to accurately assess margins in neoplastic lesions at the early stages of the disease. Neoplastic lesions appeared focal and markedly hypoechoic, clearly circumscribed by normal parenchyma (Figure 6.7).

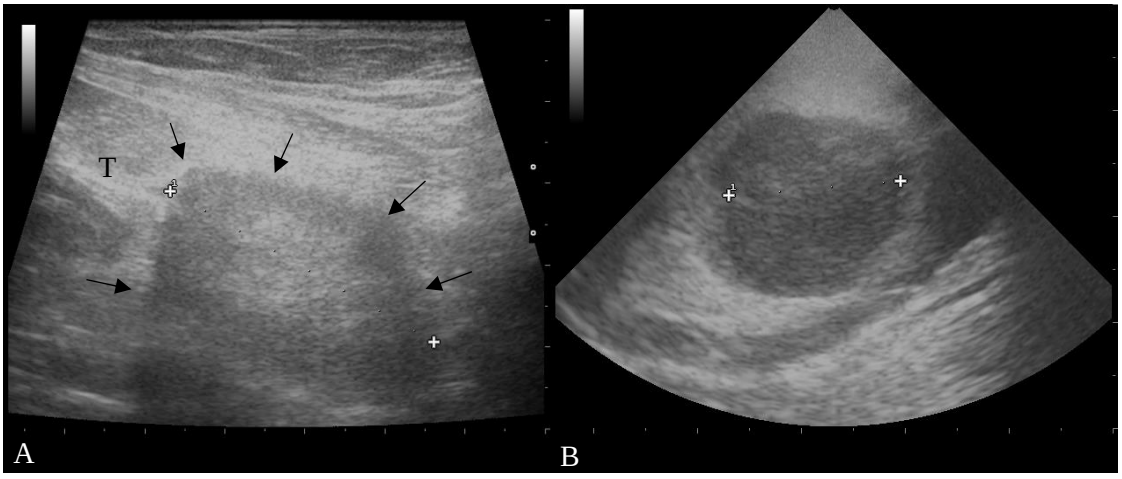

Figure 6.7. B-mode ultrasound of testicular masses in 10-years-old *(A) and 14 years-old-dogs (B). (A) The lesion (arrows) appears hypoechoic compared to the normal testicular parenchyma (T). The echostructure is heterogeneous and more intense echogenic areas are visible in the middle of the mass. Margins are not clearly defined. (B) The tumor is hypoechoic with clear lobulated margins measuring 1.5 cm at caliper markings. The histological evaluations of the lesions revealed their neoplastic origin: SCT (A); ICT (B).*

B-mode ultrasound aspect of lesions varies greatly. The echotexture was markedly heterogeneous in all neoplastic lesions analyzed. In 50% of the masses, margins were well-defined while twenty-five showed ill-defined margins (Figure 6.7).

Focal lesions were characterized by hypoechoic and slightly heterogeneous echotexture. The largest lesions had marked heterogeneous echostructure with complex parenchymal patterns. The sonographic appearance could not predict the tumor type.

At the color Doppler scanning, all lesions showed increased vascular flow signs into and around their boundaries and variable vascular patterns. Vascular signals were significantly intensified around and into the mass if compared to those of inflammatory and degenerative lesions ($P<0.05$) (Figure 6.7.1).

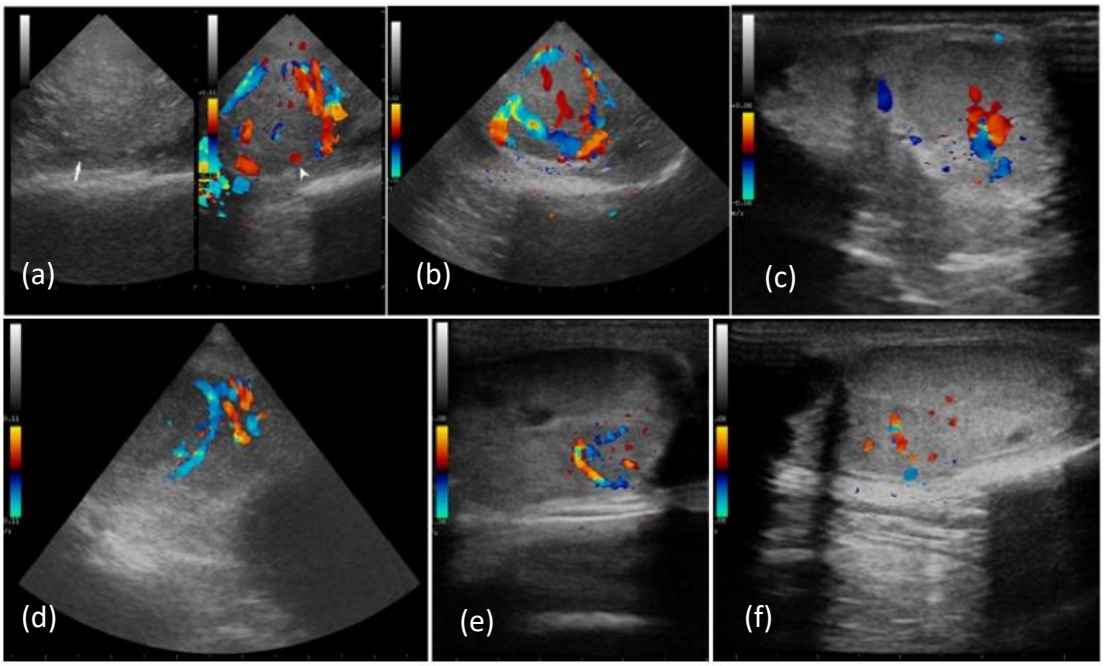

Figure 6.7.1. Neoplastic lesions of the canine testis. *The vascular flow signals were increased into and around the lesions. (a) A gross lesion with heterogenous echostructure was clearly detected by conventional B-mode ultrasound. On the right, color Doppler shows intense blood flows around the mass. (b) Color Doppler showed abnormal vascularity of a testicular mass. The histology confirmed its malignant nature: the lesions resulted to be diffuse seminoma. (c-f) Color Doppler ultrasound showed increased vascularity within all the lesions. The vascular architecture varies widely between the lesions.*

The tumor vascularization, i.e hyper- and the hypovascularity, was assessed by means of Vascularity Index (VI). The increase of vascular flow signs into the lesions and bounding the peripheral margins directly reflects the significant increase of the VI ($P<0.05$). An increase of 40% to 50% of the VI value was recorded in solid tumors ($P<0.05$), significantly higher than that recorded in presence of non-neoplastic conditions (Table 6.1). The VI alone was unable to characterize the nature of the tumor. No peculiar vascular features allowed to distinguish between different tumor types (ICT, SCT and seminoma).

Table 6.1. Color Doppler parameters of the testicular lesions analyzed by mean of color Doppler ultrasound

TESTICULAR LESION	*TOTAL VASCULARITY AREA (NUMBER OF COLORED PIXEL)*	*VASCULARITY INDEX (VI)**	*RESISTIVE INDEX (RI)** means*	*PULSATILITY INDEX (PI) means*
NEOPLASIA	62341[a]	40%-50%	0.52[A]	0.61
INFLAMMATION	12247[b]	25%	0.45[B]	0.58
CYSTS	249[c]	0	-	-
FIBROSIS	347[d]	8%	0.57[C]	0.63
NECROSIS	662[e]	11%	0.52[D]	0.57

*Values with different subscripts *differ: (*a,b: $P<0.05$); (a,c: $P<0.05$); (a,d: $P<0.05$); (b,c: $P<0.05$); (A,B: $P<0.05$); (B,C: *$P<0.05$*); (a,e < 0.05); (C;D>0.05)

Peak Systolic Velocity (PSV) increased with the increase in size of the neoplastic nodule. Power Doppler scanning showed a low-resistance pattern of the intratesticular artery

blood flow (Table 6.1). The mean value of the Resistive Index was 0.54 and the mean value of the Pulsatility Index was 0.62.

The difference between neoplastic types was not statistically significant (P>0.05). The RI increase amounted to 40-50% if compared to RI values of non-neoplastic conditions, such as cysts, fibrosis and necrotic lesions.

One testicular cyst was detected. The cyst appeared anechoic, rounded to oval in shape with smooth and clear margins. At the color Doppler scanning, no detectable vascular signs were found into and at the boundaries of the cyst (Figure 6.8).

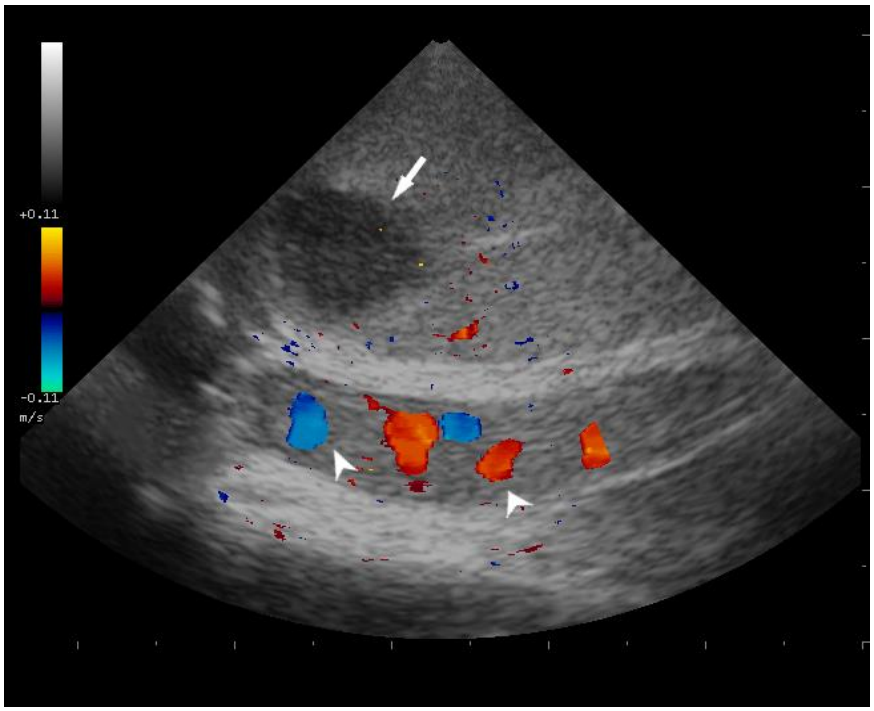

Figure 6.8. Single testicular cyst in a 7-years-old mixed breed dog. *Ultrasonographic sagittal image of the testis shows the presence of a cyst (arrow). The cyst is anechoic, round in shape and smooth bordered. Color Doppler US shows good parenchymal blood flows into the epididymis (arrowheads) but lack of blood flow signals involving the cyst.*

Orchitis was characterized by a severe enlargement of the testis. The scrotal surface was warm and painful. The portion of the testis affected by the inflammatory process showed irregular margin, diffuse and patchy hypoechogenity at the US scanning (Figure 6.9). Epididymitis was clinically characterized by scrotal enlargement and pain at the palpation of the scrotal region. At the US examination, the epididymis appeared enlarged and extratesticular fluid was present. The testicular parenchyma was not involved in the inflammatory process. The hydrocele appeared hypoechoic surrounding the testis. This condition indicates the reaction to the epididymal inflammation.

Color Doppler US showed diffuse hypervascularity of the region affected by the inflammation (Figure 6.10).

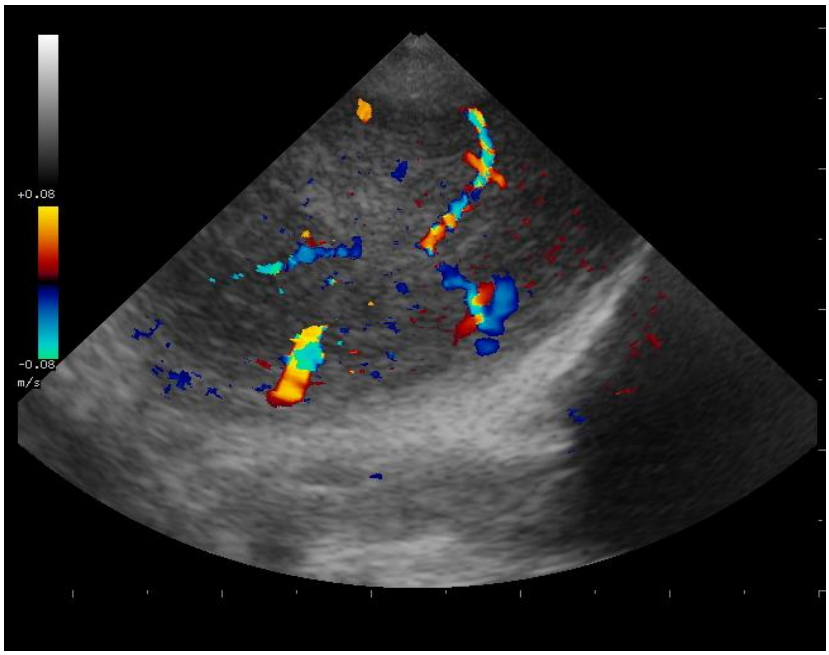

***Figure 6.9**. **Acute orchitis in a 10 years-old Labrador Retriever**. At the color Doppler examination a diffuse hypervascularity of the testicular parenchyma was noted. Absence of fluid and collections around the organ.*

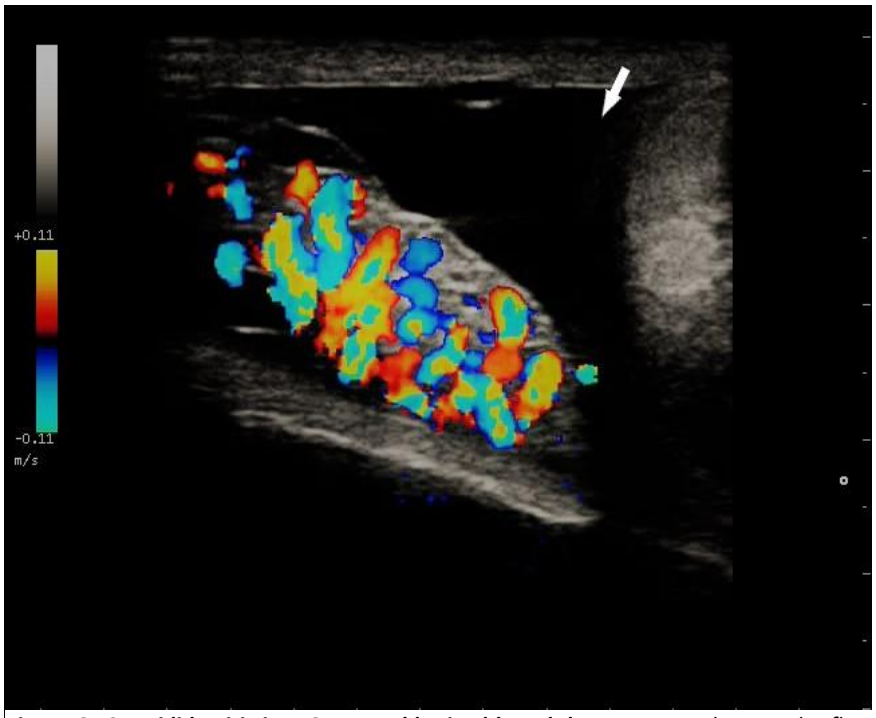

Figure 6.10. Epididymitis in a 6 years-old mixed breed dog*. Intense color Doppler flow signals interesting the entire epididymis, possibily due to the widening of the epidydimal blood vessels. Abnormal fluid collection located between the two layers of the tunica vaginalis into the scrotal sac.*

Of 6 degenerative lesions analysed, three of them were characterized by fibrosis; two consisted in necrotic foci and one was a cyst. Necrotic lesions (2/64) appeared hypoechoic with irregular and non-circumscribed margins. Necrotic lesions did not exhibit significant blood flows at the color Doppler scanning (Figure 6.11).

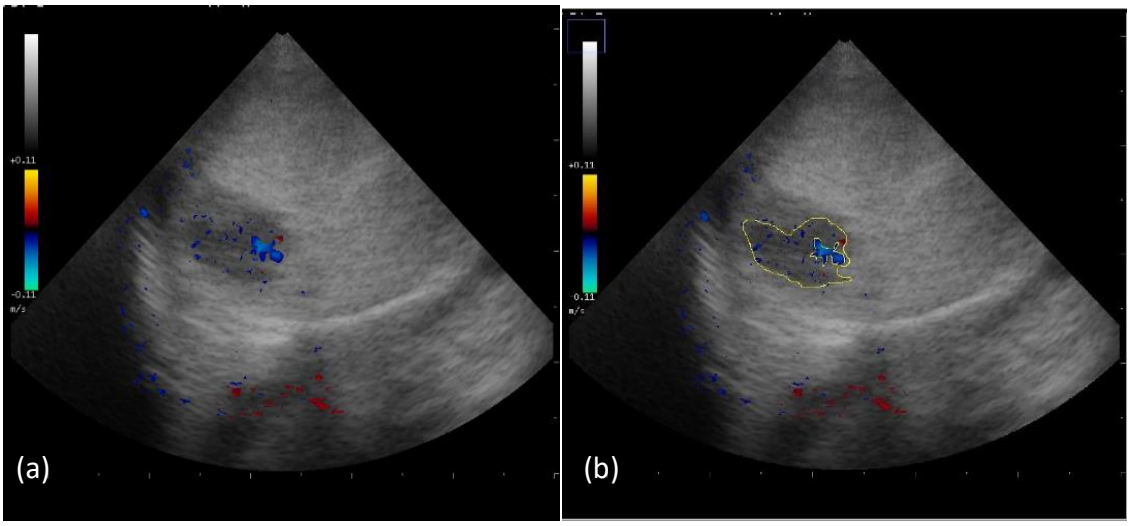

Figure 6.11. Necrotic lesion in a testis of a 12-years-old dog. *The lesion appear as a small hypoechoic area within the normal parenchyma of the testis. Margins were markedly irregular. At the color Doppler evaluation weak vascular signals were detected. The portion of the lesion occupied by vascular signals is small and the VI is low. (b) The areas used for the calculation of the Vascularity Index are visible. The histological evaluation of the lesion revealed a large area of necrosis.*

Fibrotic areas appeared hyperechoic with defined margin. No peripheral flows were detected at the color Doppler scan.

In fibrotic and degenerative conditions RI values was increased; RI was significantly lower in course of inflammatory processes probably due to the reactive hyperemia. The difference in PI values of inflammatory and degenerative conditions of the testis was not significant ($P>0.05$). In lesions characterized by necrosis, modifications of vascular flow signs were scarcely significant. At color Doppler US cysts and fibrosis showed minimal or no detectable vascular signals. The Vascularity Index was significantly higher in course of inflammation rather than in degenerative lesions ($P<0.05$; 25% vs 0-10%).

In addition to the examination of the pathological features of each lesions found within the testis, each contralateral testis of subjects enrolled in our study has been carefully examined. An example of the color Doppler aspect of a normal testis is showed in Figure 6.13.

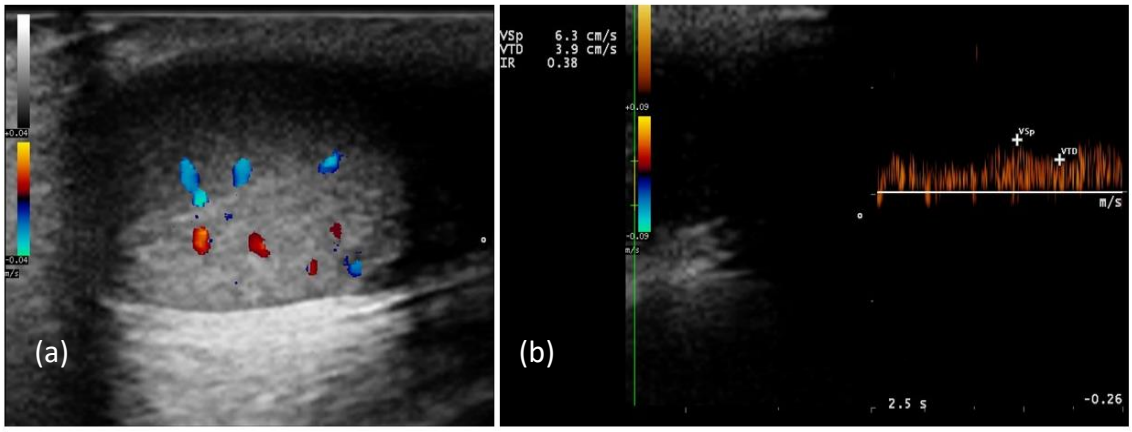

Figure 6.13. Color Doppler analysis of a normal testis. *(a) Multiple red and blue patches indicate the homogeneous distribuition of small vessels into the testicular parenchyma. (b) Color Doppler velocities and RI have been recorded. The RI is 0.38.*

The histologic type of each lesion was assessed by the microscopic evaluation.

Neoplastic lesions accounted for 78% (50/64) of all lesions analysed. Among them, 20 Interstitial Cell Tumors (ICT), 18 Seminomas and 12 Sertoli Cell Tumor (SCT) were identified.

The majority of primary testicular tumors were benign and the malignant forms are rare. Of eighteen seminomas encountered, 10 were classified as diffuse; two of them showed malignant histological appearance; the regional lymph nodes were normal at the ultrasonographic scan in all subject analysed.

At the histological evaluation, Sertoli cell tumors were characterized by the proliferation of Sertoli cells, arranged in solid trabeculae or tubular structures separated by abundant fibrous tissue. Dense and white connective bands subdivided the tumors (Figure 6.14).

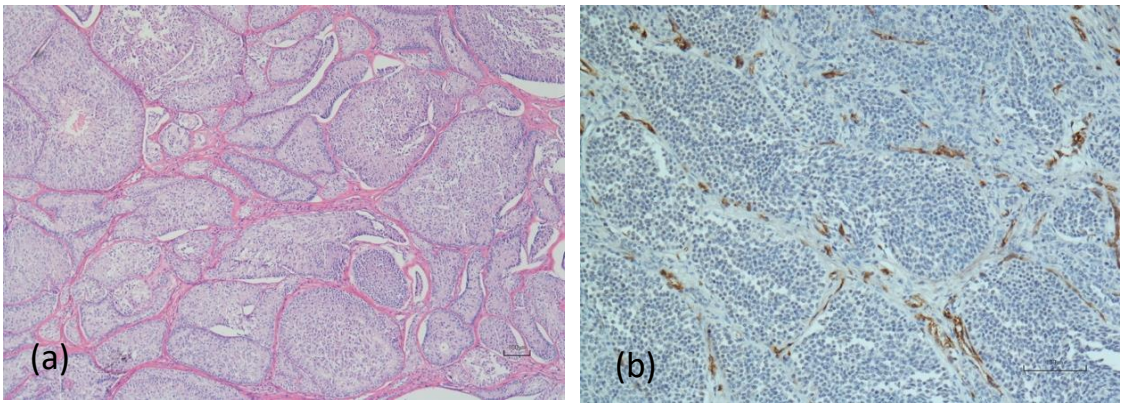

Figure 6.14. Malignat Sertoli Cell Tumor in a 10 years-old dog*. a) Irregular neoplastic tubules are separated by fibrovascular stroma (4x, H-E) b) Immunohistochemical reaction for CD31 shows microvessel density (4x, IHC)*

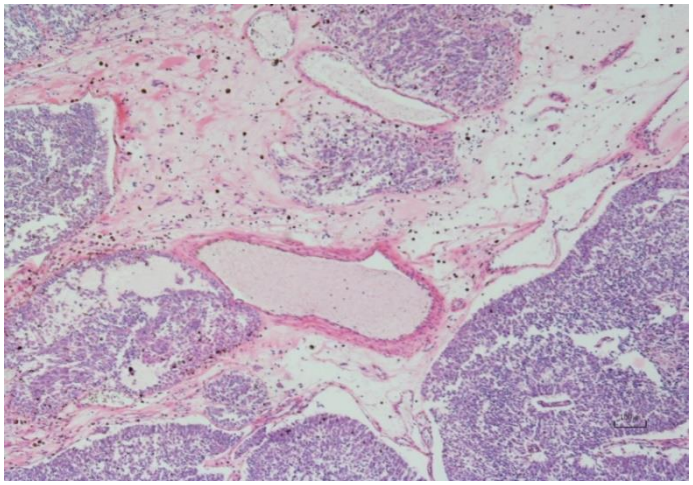

Figure 6.15. Sertoli Cell Tumor in a 7-years-old dog*. The neoplasia is characterized by large sheets of uniform, elongated (or fusiform) cells separated by blood vessels and fibrous septa (10X, H-E).*

ICT were composed by modified interstitial cells with eosinophilic cytoplasm, finely granular and occasionally macrovacuolated. Neoplastic cells were arranged in cords surrounding cystic structures and lake of fluid normally containing erythrocytes (Figure 6.16).

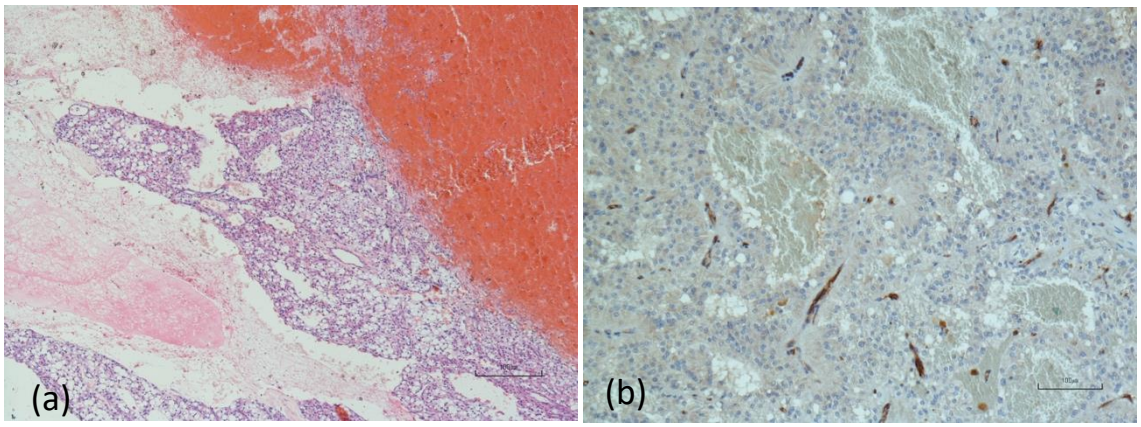

Figure 6.16. Interstitial Cell Tumor of cystic-vascular type in a 13 years-old dog *(4X;H-E, IHC-CD31). Sheets (or cords) of polyhedral cells surrounding spaces filled with erythrocytes (4x, H-E) (a) CD 31 immunostaining highlights numerous blood vessels. (10x, IHC). (b) C*D31 immunostaining highlights numerous blood vessels (10x, IHC).

Two types of seminoma were identified: intratubular and diffuse pattern of proliferation of large polyhedral germ-cells proliferation were founded. Seminomas were characterized by the presence of numerous mitotic figures within neoplastic cells.. Small to large lymphocytes aggregates were common. The diffuse pattern was the most frequent, characterized by modified germinal cells extending from tubules to the interstitium. (Figure 6.17). Neoplastic cells were large, round to oval in shape.

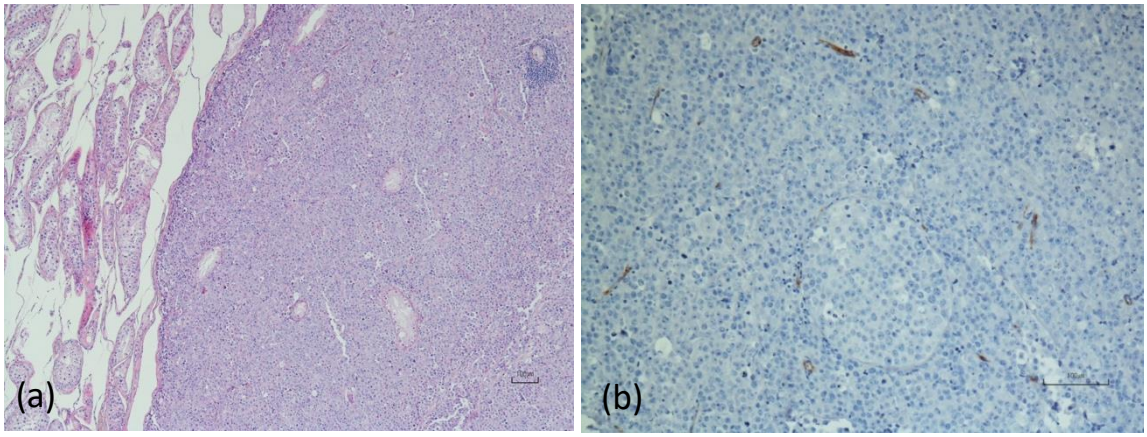

Figure 6.17. Seminoma in a 7-year-old dog. *(a) Histology showed neoplastic proliferation of germ cells invading the interstitium and leading to the obliteration of seminiferous tubules. Cells are round and polyhedral with large nuclei extending from tubules to interstitium. Single or multiple nucleoli characterize the germinal cell proliferation. Mitoses, nodular or diffuse infiltrates of lymphocytes are frequent. (4x, H-E). (b) CD31 shows peritumour and intratumour vessels (10x, H-E).*

The immunohistochemical evaluation showed the positive expression of CD31 by endothelial cells in both pathological and normal testicular tissues. The positive reaction was identified with the brown staining of the endothelial cellular membrane. The increase in density of the microvascularization was detected for each lesion of neoplastic nature analysed.

The number of microvessels in 10X selected hotspots ranged widely between tumors, even with same origins and nature. Therefore, MVD was not specific of the neoplastic type. Results of the counts are describe in Table 6.2.

Table 6.2. Results of vessels counting in different types of primary testicular cancers in dogs. Three samples of each type have been counted.

Tumor type	Samples	MVD*
SEM	134-19	14,67 ± 4,04
	131-19	13 ± 2,00
	148-19	13,67 ± 1,15
ICT	102-19	17,33 ± 1,65
	141-19	72,33 ± 4,51
	412-19	27,33 ± 2,52
SCT	296-17	12 ± 1,15
	143-19	14 ± 3,33
	161-18	13,24 ± 4,44

*Mean value of MVD are calculated on 3 hotspot at 10X magnification. Values are shown following the model Mean ± Standard Deviation. MVD: Microvessel Density. SEM: Seminoma; ICT: Interstitial Cell Tumor; SCT: Sertoli Cell Tumor

The histological characteristics of non-neoplastic conditions of the testis were less variables than those characterizing the neoplastic types. Intratubular, granulocytic cellular infiltrations with active hyperemia of interstitial vessels characterized inflammatory lesions.

Histological changes of degenerative lesions (fibrosis, cysts and necrosis) results in vacuolation of Sertoli cells, disorganization and exfoliation of germ cells and presence of multinuclear giant cells in the lumen of degenerated seminiferous tubules.

6.4 Discussion

Ultrasound play a major role in the detection of testicular diseases in dogs and it has been widely considered the first modality for scrotal imaging for many years in dogs, as in humans (Huang & Sidhu, 2012). The merits of its use as elective tool for the evaluation of the testis are many. Its repeatability and the affordable cost of the equipment, together with the lack of contraindications for the patient make ultrasound of primary importance in veterinary practice. Traditional grey-scale US is highly sensitive to identify testicular masses and to locate extratesticular and intrastesticular abnormalities, but its ability to provide information on the nature of the lesion is poor (Huang & Sidhu, 2012).

Over the years, there has been great improvements of diagnostic techniques that exploits ultrasounds to visualize organs and tissues. Color Doppler ultrasound, contrast-enhanced ultrasound (CEUS) and elastography are example of this upgrading. The introduction of second-generation contrast media for CEUS after the year 2000 has provided a specific tool for detailed characterization of intratesticular vascular flows in human and veterinary medicine (Volta et al., 2014; Bigliardi & Ferrari, 2011; Nyman et al., 2005). The exposure of contrast agents to intermittent pulses with low mechanical index generates harmonics echoes from nonlinear microbubble oscillation making CEUS a highly qualitative examination for the identification of vascular phases, i.e arterial and venous. However, its use is not free from complications. Using second-generation media requires expert ultrasonographers; the success of the procedure may be affected of many factors such as degree of contrast enhancement, type of the contrast media, imaging unit model, injection

protocol, dosages, mechanical index and site of the focal zone (O'Brien et al., 2004). Patients that undergo contrast-enhanced US exam require to be anaesthetized before starting the procedure; moreover, data takes time to be processed and the software equipment must be dedicated: its cost is high. For these reasons, even if the contrast-enhanced characterization of lesions within the testis can provide fine structural details on vascular flows nourishing neoplastic and non-neoplastic masses, CEUS might not be the first-line imaging technique for the evaluation of testicular diseases in clinical practice. The possibility for the clinician to reach valuable information at lower-risk for the patient in order to plan the best therapeutic approach may be very appealing. In some instances the color Doppler demonstration of the avascularity of a lesion, for example in course of cysts or fibrosis, would allow to avoid bilateral orchiectomy in favour of the "watchful waiting" of the mass with ultrasound. This might be very important in breeding dogs in whom the reproductive activity is relevant. In our study, the potential of color and power Doppler have been carefully investigated. Even if the predictive value of color Doppler is not sufficient to obviate the need for the histological examination, color Doppler information can be interpreted along with other ultrasonographic characteristics to increase the diagnostic accuracy of the sonographic exam, as well as the confidence of the clinician in arriving at the correct diagnosis. For this purpose, one of the most valuable index of color Doppler sonography is the Vascularity Index. The Vascularity Index results markedly increased when a network of new blood vessels bounds and nourish a newly formed mass within the testis. The great increase of the VI, higher than 40% to 50%, can strongly suggest the presence of a neoplastic process. Nevertheless, the VI increase is not

a sole feature of tumors, but other pathological conditions of the testis can alter it. In course of inflammation, the index appeared slightly increased, even though to a much lesser extent if compared to neoplastic masses.

In our study, we found that increase of vascular signs in neoplastic masses were not only due to the neovascularization of the mass. Some tumors had large and distended vessels bounded the margins of the mass; the increase in diameters of the vasal lumen may significantly contribute to the increase of vascular signals at the color Doppler scan. Most of them were Interstitial Cell Tumors.

Benign conditions of the testis and non-neoplastic lesions, i.e necrotic areas, fibrosis and cysts, typically do not show an increased vascularity. In some instances, conventional B-mode ultrasound alone could raise confusion because non- neoplastic lesions can sometimes resemble hypoechoic nodules, if visualized in cross-section. The VI of such lesions is not relevant and color Doppler allow to hypothesize with good confidence their non-neoplastic nature. VI is not the only parameter used for the evaluation of testicular abnormalities with the color Doppler tecnique. The Resistive Index is used as well. RI is a diagnostic tool for the characterization of scrotal inflammatory diseases in man (Carillo et al., 2012) and in infertile or dyspermic patients. RI is markedly increases in patients with abnormal sperm counts and dyspermia (Carillo et al., 2012). From our study, the RI was not specific for the type of tumor and it was similar for the different types, along with the PI values.

The use of color and power Doppler allow quickly excluding the malignant nature of these type of lesions, showing the absence of vascular signals within a lesion. The

increasing blood flow perfusion into and around nodules that is already present at the early stages of the pathologic process, supplies the clinician useful information on the possible nature of the pathology. In accordance with other authors (Horstman et al., 1992), we found that Peak Systolic Velocity (PSV) increased with increasing size of neoplastic nodules.

The usefulness of color and power Doppler in the diagnosis of testicular neoplasms and scrotal abnormalities has been strongly confirmed by many studies in human medicine (Horstman et al., 1992; Shartz et al., 2005), as well as the correlation between tumor size and degree of vascularity (Forster et al, 2017). Some authors described the enhancement of vascularization in 95% primary testicular tumors larger than 1.6 cm in diameter in human patients, but they also concluded that no correlations existed between degree of vascularity, nature of the lesion and grading of tumor. Therefore, the increase of vascularization in a mass is not a reliable indicator for the malignant potential of the disease (Kocakoc et al., 2007). From our study, we found that the degree of vascularization cannot be correlated to specific type of tumors (SCT, ICT, Seminoma); but all primary tumors (ICT, SCT and seminoma) showed an increase of vascular proliferation within the tumoral mass. Immunohistochemistry strongly confirmed color Doppler findings.

A network of microvessels supplied every lesions of neoplastic nature. In normal testes, vessels were regular in number and in shape. They were located in the interstitial space among the tubules; normally the number of microvessels range from 18 to 36/mm^2 (Restucci et al, 2003). The relation between angiogenesis and biological behavior of the

tumor is still poorly understood in veterinary oncology (Restucci et al, 2000). Even though, in this study, the immunohistochemistry revealed the rise in microvessel density in every neoplastic lesion examined if compared to those of normal testes, the results obtained by the MVD counts ranged widely in tumors of the same type and the observations were not statistically significant.

Further studies with a larger sample size are necessary to establish the possible association between intensity of the color Doppler signal and number of microvessels in a mass, as well as the MVD value of primary tumors. The studies on testicular cancer conducted in human medicine are focused mainly on the malignant potential of the tumors. Neoplastic masses in men have higher likelihood of malignancy than in dogs (Manecksha & Fitzpatrick, 2009). The recognitions of typical imaging features of vascular flow may allow for confident suspect of active proliferative nature of the lesion and may allow the exclusion of benign conditions, such as fibrosis, necrosis and cysts.

In course of inflammatory processes, the increase of blood flow to the testis detected by color Doppler ultrasound is significantly lower compared to that of neoplastic conditions. When spermatic cord torsion occurs, color Doppler flows are absent.

The benefits of using CDUS to evaluate parenchymal dysfunctions or malignant processes of the testis with scrotal disorders are many. Its safety, reliability and repeatability, together with the limited cost of the procedure make this imaging technique a valuable tool to differentiate between neoplastic and non-neoplastic testicular lesions in dogs. CDUS enables the clinicians to overcome considerable hurdles related to sedation, costs of equipment and high specialization requirements that characterize other diagnostic

procedures (Volta et al, 2014). Vascular patterns and impedance of blood flows in peripheral vascular beds are relevant elements for the generation of diagnostic hypothesis (Kocakoc et al., 2007). Despite the characterization of histologic subtypes of primary testicular basing is not possible with imaging alone, color Doppler ultrasound may improve the confidence of the clinician in arriving at the correct diagnosis and it can be strongly considered an important line of investigation for the examination of the canine testicular disease.

Appendix A. Publications from the present thesis.

Preliminary results of the present doctoral thesis have been published in:

Bigliardi E, Denti L, De Cesaris V, Bertocchi M, Di Ianni F, Parmigiani E, Bresciani C, Cantoni AM. 2018. Color doppler ultrasound imaging of blood flows variations in neoplastic and non- neoplastic testicular lesions in dogs. Reproduction in Domestic Animals, 1-9. *doi:10.1111/rda.13310*

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