Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado Integrado em Medicina Veterinária

Dissertação

Urothelial Carcinoma in Dogs: A Retrospective Epidemiological Study

Maria Margarida Ramos Bôto

Orientador(es) | Sandra Maria Branco

Ana Teresa Dias Machado

Maria Leonor Gonçalves Delgado Madureira

Évora 2024

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Presidente	|	Ricardo Jorge Romão (Universidade de Évora)
Vogais	|	Maria dos Anjos Pires (Universidade de Trás-os-Montes e Alto Douro) (Arguente) Sandra Maria Branco (Universidade de Évora) (Orientador)

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Abstract

Urothelial Carcinoma in Dogs: Retrospective Epidemiological Study

This dissertation includes a literature review on urothelial carcinomas (UC) or transitional cell carcinomas (TCC) in dogs and an epidemiological study on the incidence of these tumors in dogs in mainland Portugal. Between 2017 and 2021, information on animals with UC that had been identified by the INNO Laboratory's histopathology division was gathered and examined.

The purpose of this study was to determine if the incidence of these tumors correlates with the characteristics of the dogs and their environmental factors.

The results suggest that adding to the individual characteristics, exposure to environmental pollutants might play an important role in the carcinogenesis of urothelial carcinoma in dogs.

Keywords: Urothelial Carcinoma, Transitional Cell Carcinoma, Bladder Cancer, Epidemiology, Risk factors, Carcinogens, Diagnosis, Histological Grade

Resumo

Carcinoma Urotelial em cães: Estudo epidemiológico e retrospetivo

Esta dissertação inclui uma revisão bibliográfica sobre os carcinomas uroteliais ou carcinomas de células de transição em cães e um estudo epidemiológico sobre a incidência destes tumores em cães em Portugal continental. Assim, foram recolhidos e analisados dados entre 2017 e 2021, de animais diagnosticados com carcinoma urotelial pelo departamento de histopatologia do Laboratório INNO.

O objetivo deste estudo foi determinar se a incidência destes tumores está correlacionada com as características dos animais bem como os fatores ambientais.

Os resultados sugerem que, para além das características individuais, a exposição a poluentes ambientais pode desempenhar um papel importante na carcinogénese do carcinoma urotelial em cães.

Palavras-chave: Carcinoma Urotelial, Carcinoma das Células de Transição, Tumor da Bexiga, Epidemiologia, Fatores de risco, Carcinógenos, Diagnóstico, Grau histológico

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List of Symbols and Abbreviations

AQI - Air quality index

bFGF - Basic fibroblast growth factor

BRAF - B-isomer of RAF-kinases

BTA - Bladder Tumor-associated Antigen

CEUS - Contrast-enhanced ultrasound

CIS - Carcinoma in situ

CK - Cytokeratin

c-KIT - v-kit cellular homologue

COX - Cyclooxygenase

CT - Computed tomography

ddPCR - Droplet digital polymerase chain reaction

DNA - Deoxyribonucleic Acid

EGF - Epidermal growth factor

EGFR - Epidermal growth factor receptor

FGFR - Fibroblast growth factor receptors

FNA - Fine Needle Aspiration

GATA-3 - GATA binding protein 3

GI - Gastrointestinal

H&E - Hematoxylin-eosin

HER-2 - Human Epidermal Growth Factor Receptor 2

HPF - High-power field

IHC - Immunohistochemistry

IL-2 - Interleukin-2

InvUC - Muscle-invasive urothelial carcinoma

MAPK - Mitogen activated protein kinase

ERK - Extracellular regulated kinase

EU - European Union

MC - Mitotic count

MEK - MAPK/ERK kinase

NO_2 - Nitrogen dioxide

NOC - N-nitroso compounds

NSAID - Non steroid anti-inflammatory drug

O_3 - Ozone

PDGFR-β - Platelet-derived growth factor receptor beta

PM – Particulate matter

RAF - Rapidly accelerated fibrosarcoma

RAS - Rat sarcoma vírus

RECIST - Response evaluation criteria in solid tumors

RTK - Receptor tyrosine kinase

SO_2 - Sulphur dioxide

ST - Survival times

SD - Standard deviation

TCC - Transitional cell carcinoma

THM - Trihalomethane

TNM - Tumor/node/metastasis

TTHMs - Total trihalomethanes

UC – Urothelial carcinoma

UP - Uroplakin

UTI - Urinary tract infection

VEGF - Vascular endothelial growth factor

VEGFR2 - Vascular endothelial growth factor receptor 2

WHO - World Health Organization

PART I – LITERATURE REVIEW

1. Introduction

Urothelial Carcinoma (UC) or Transitional cell carcinoma (TCC) is the most common urinary bladder tumor. The importance of this neoplasms in veterinary medicine, is due to its high incidence in various domestic animal species and its life-threatening character. Some advances have recently been made, but the biopathology is complex and still poorly understood (Rasteiro, Sá E Lemos, Oliveira, & Gil da Costa, 2022).

The UC is a malignant tumor from the transitional epithelium of the urinary tract. It can occur anywhere from the renal pelvis, prostatic urethra to the distal urethra, but the most frequent location is the urinary bladder (Meuten, 2016).

The clinical presentation of canine UC is usually nonspecific and is comparable to several other urinary tract disorders. Subsequently, most UCs are not diagnosed in early stages, allowing the tumor to grow, infiltrate and eventually metastasize, and present a poor prognosis by the time of diagnosis (Rasteiro et al., 2022).

By the time they are clinically identified in dogs, 20% of them have lymph node metastases, 15% have lung metastases, and 6% have metastases to the lumbar or pelvic bones that may be seen on radiographs (Meuten, 2016).

2. Anatomy and Histology of the Bladder

The urinary bladder is a hollow, musculomembranous organ of the urinary system, that varies its form, size and position depending on the amount of urine it contains (Fig. 1) (Evans & de Lahunta, 2013).

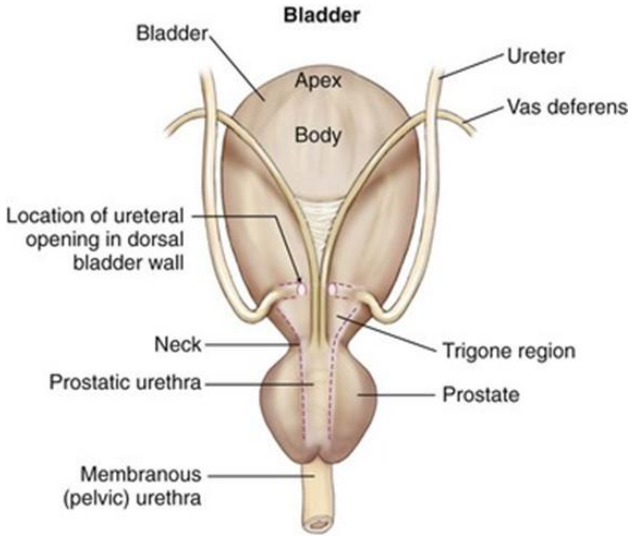

Figure 1 – Anatomy of the male canine urinary bladder (Source:Johnston & Tobias, 2018).

The bladder can be arbitrarily divided into three main regions: the neck connecting to the urethra, the body and cranially the apex. Internally a triangular area near the neck of the bladder is called trigone. The apex of the trigone region is the urethral orifice and the base is indicated by a line connecting the ureters openings (Evans & de Lahunta, 2013).

2.1. Histology of the Bladder

Structurally the mammalian urinary bladder comprises several layers (from outside to the inside): the serosa, *muscularis*, submucosa, *lamina muscularis* and *lamina propria* (Burcham, Thompson, & Henry, 2000; Evans & de Lahunta, 2013).

The muscularis consists of three layers of muscle fibers in the bladder wall, that are often referred to as the detrusor muscle of the bladder (Evans & de Lahunta, 2013).

A layer of transitional epithelium, also known as urothelium, lies on top of lamina propria and lines the inside of the urinary bladder. The urothelium is a unique, highly specialized epithelium and it has a variable number of cell layers, ranging from 2-3 in the distended bladder to 10-14 in the contracted state (Burcham et al., 2000; Cohen, 2013; Liebic, 2019).

The specializations of the epithelium serve to protect the underlying tissues against the hypertonic urine, while maintaining the urine composition similar to that delivered by the kidneys (Burcham et al., 2000; Cohen, 2013; Liebic, 2019).

There are different types of cells in the urothelium, with a basal cell layer composed of cuboidal cells resting on a basement membrane, intermediate cells and superficial umbrella cells (Fig. 2A).

The umbrella cells have unique morphological features that contribute to the urothelium function and integrity (Fig. 2B). These superficial cells are joined together by tight junctions and present extensive basolateral plasma membrane infoldings that interdigitate with the plasma membrane of the underlying transitional cells. Additionally, adjacent transitional cells are connected by numerous desmosomes and intermediate filaments that maintain the epithelial integrity during bladder distension (Evans & de Lahunta, 2013).

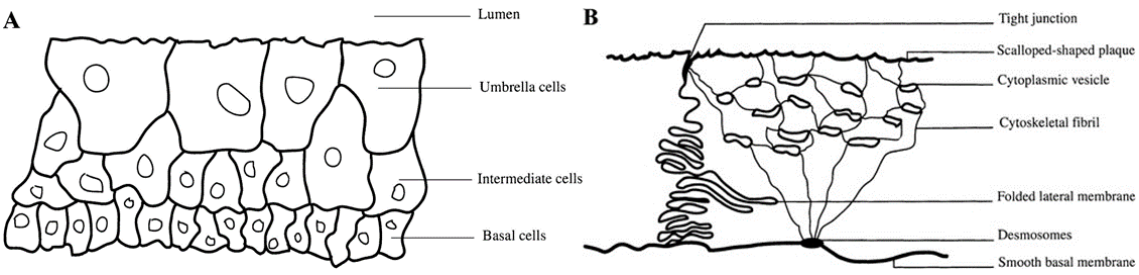

Figure 2 – A - Representation of urothelium layers. B - Representation of an umbrella cell (Adapted from Burcham, Thompson, and Henry 2000).

3. Epidemiology

Although the real incidence of canine UC is unknown, it is the most frequent kind of urinary tract neoplasia in dogs, accounting for 1.5 to 2% of all canine cancers (Deborah W Knapp et al., 2000; Meuten, 2016).

Despite bladder cancer being less common among canine patients than other types of cancer, veterinarians still identify a significant number of urothelial carcinoma cases every year, in addition it is acknowledged that many of these cases will go undiagnosed and untreated (Deborah W. Knapp et al., 2020; Meuten, 2016).

3.1. Risk Factors

Urothelial carcinoma's aetiology remains unclear, but it is known to be multifactorial and several risk factors have been identified such as: breed predisposition, sex predisposition, obesity, reproductive status, exposure to topical insecticides, exposure to environmental pollutants and cyclophosphamide treatment (Deborah W. Knapp et al., 2014; Deborah W Knapp et al., 2000; Meuten, 2016).

3.1.1. Breed

Urothelial Carcinoma is known to have a very strong breed associated risk, with Scottish Terriers being 16 to 20 times more likely to develop UC compared to mixed breeds (Deborah W. Knapp et al., 2020; Deborah W. Knapp & McMillan, 2012; Deborah W. Knapp et al., 2014).

Similarly other breeds such as Eskimo dog, West Highland White Terrier, Shetland Sheepdog, Beagle and Wire Hair Fox Terrier have been at increased risk compared to mixed breeds, however with lower odds ratio (Deborah W. Knapp et al., 2020; Deborah W. Knapp & McMillan, 2012; Deborah W. Knapp et al., 2014).

This breed-associated risk is most likely caused by a genetic predisposition to UC, due to differences in the biochemical pathways that activate and detoxify carcinogens (Deborah W. Knapp et al., 2014; Mutsaers, Widmer, & Knapp, 2003).

In previous studies no association has been observed between breed and the biological behavior of the tumor or its response to therapy, however a significant difference in the anatomical location of the tumor between the high-risk breeds and mixed-breed dogs was reported (Deborah W. Knapp et al., 2014; Mutsaers et al., 2003).

The location of the UC to the bladder or bladder and urethra alone was more common in the high-risk breeds than in mixed-breed dogs, compared to involvement of the prostate, ureter and kidney, more common in mixed breeds (Deborah W. Knapp et al., 2014).

3.1.2. Sex

Several studies confirmed that female dogs have an increased risk of bladder cancer with a female: male ratio between 1.71:1 to 1.95:1 (Deborah W. Knapp & McMillan, 2012; Deborah W. Knapp et al., 2014).

Curiously, the sex predisposition did not present or was much less prominent, with lower female/male ratios in several dogs of the high-risk breeds, such as Scottish terriers (0.8:1), Shetland sheepdogs (1.2:1), West highland terriers (1.3:1) and beagles (1.2:1) (Deborah W. Knapp et al., 2014).

It is believed that male dogs urinate more frequently than females, due to territorial marking, therefore are less exposed to carcinogens in urine (Deborah W. Knapp et al., 2014; Mutsaers et al., 2003).

Furthermore, females have increased body fat compared to male dogs, and consequently an increased storage of lipophilic environmental carcinogens (Mutsaers et al., 2003).

3.1.3. Reproductive Status

Various studies revealed that neutered dogs (of both sexes) were at increased risk, compared to sexually intact dogs of the same sex, this might be for the same reason of territorial marking, as well as hormonal predisposition (De Brot et al., 2018; Deborah W Knapp et al., 2000; Mutsaers et al., 2003).

Given that neutering affects levels of sex hormones and their receptors, these proteins are presumed to have a key role in the development of UC. Previous studies in humans have shown that reduced androgen receptor expression is associated with higher tumor stages and increased estrogen receptor-beta expression is associated with higher grade UC tumors (De Brot et al., 2018).

3.1.4. Age

UC usually affects older age dogs, with medium ages at diagnosis raging between 9-11 years. A minority of dogs might develop the tumor earlier, between 4-6 years. High risk breed dogs are significantly younger at time of diagnosis than non-risk breed dogs with UC (Deborah W. Knapp et al., 2020, 2014).

3.1.5. Obesity

Obesity was identified as a risk factor of UC in previous case control studies. Overweight animals exposed to carcinogenic substances were more likely to develop bladder cancer, than normal weight dogs (Glickman, Schofer, McKee, Reif, & Goldschmidt, 1989; Raghavan, Knapp, Dawson, Bonney, & Glickman, 2004a, 2004b).

This association can be explained by the lipophilic nature of carcinogenic substances, which are absorbed and more likely to be stored in body fat. Stored carcinogens would be gradually metabolized and excreted in the urine resulting in prolonged contact with the bladder epithelium. (Glickman et al., 1989; Raghavan et al., 2004a, 2004b).

3.1.6. Carcinogenic substances

3.1.6.1. *Cyclophosphamide Treatment*

Cyclophosphamide is an oxazaphosphorine alkylating agent used widely in the treatment of solid tumors and B-cells malignant diseases and in treatment of immune mediated diseases and before bone marrow transplants (Deborah W Knapp et al., 2000; Levine & Richie, 1989).

The major limiting factor in the use of this drug is its side effects in the urinary system. The side effects of urotoxicity in humans include irritative voiding symptoms, dysuria, strangury with microhematuria and in some cases life-threatening hemorrhagic cystitis. Hemorrhagic cystitis is considered dose related (Levine & Richie, 1989).

Studies concluded that the urotoxicity does not result of direct alkylating action of cyclophosphamide, but by the formation of acrolein, a liver metabolite of cyclophosphamide that when in contact with the urothelium resulted in vesical irritation and hemorrhage (Deborah W Knapp et al., 2000; Levine & Richie, 1989; Mutsaers et al., 2003).

It is believed that the carcinogenic effects of cyclophosphamide are related to chronic bladder irritation from exposure to acrolein (Mutsaers et al., 2003).

3.1.6.2. *Topical Insecticides*

A case control study by Glickman et al. 1989, described a significant association between the risk of urothelial carcinoma and the use of topic flea and tick treatment products such as shampoos, dips, powders, sprays, and collars, as well as the weight of the dogs exposed to such products.

The percentage of inert chemicals in these older flea and tick products has been estimated to be as high as 96%. Petroleum distillates and aromatic petroleum solvents like benzene, toluene, and xylene, which are known or suspected to be carcinogenic, are among these inert components (Raghavan et al., 2004b).

Additionally, these earlier topical preparations also include a variety of active chemicals, such as organophosphates, pyrethrins, pyrethroids, carbamates, insect growth modulators, and silica gel, as well as a number of synergists, such as n-octyl bicycloheptene dicarboximide and piperonyl butoxide, which are absent from recent spot-on formulations (Raghavan et al., 2004b).

Newer, spot-on type flea control products appear safer. The findings of a case control study conducted in 2004 showed that the spot-on medications containing either fipronil or imidacloprid did not increase the risk of UC in Scottish Terriers (Deborah W. Knapp & McMillan, 2012; Raghavan et al., 2004b).

Furthermore, the percentage of owners who use older flea and tick products has reduced with time, minimizing exposure to this type of carcinogens (Raghavan et al., 2004b).

3.1.7. Exposure to Environmental Pollutants

Soil, water, and air-borne pollutants are a significant concern for the environment and health of animals and humans. Its harmful effects include an increased risk of developing certain types of cancer including urothelial carcinoma. The presence of these carcinogenic substances and its increasing number and accumulating doses in the environment consists one of the biggest challenges fighting cancers (Gatti, 2021).

There are many studies of the harmful effects of pollutants on human health, however the effect on pet dogs has only recently begun to attract more interest, which has been growing in recent years (Avila, Prieto, & Luna, 2023).

Dogs may be exposed to these pollutants through various means, including ingestion, inhalation, and contact with contaminated materials (Craun et al., 2021; N. Smith, Luethcke, Craun, & Trepanier, 2022).

A pilot study by Craun et al. 2021, compared the urinary exposures to chemicals potentially carcinogenic of dogs and owner in the same households. The findings revealed that dogs had much higher urine exposures of these chemicals. This might be a result of canine behaviors like sniffing, chewing, rolling, grooming, and grass-eating, as well as the fact that dogs spend more time in the household during the day.

3.1.7.1. *Soil Pollutants*

Some carcinogens found in soil, include heavy metals, industrial chemicals and mainly pesticides or agricultural products used in fertilization. Moreover, these substances can leach in aquifers and contaminate drinking water (Gatti, 2021).

Various studies have reported a link between exposure to herbicides, insecticides, and waste pollutants and an increased risk of urothelial carcinoma in dogs (Gatti, 2021; Glickman et al., 1989; Deborah W Knapp et al., 2013; Raghavan et al., 2004a; Reif, 2011).

A study, by Knapp et al. 2013, the concentrations of three carcinogenic chemicals commonly used in lawn care products were measured in the urine of the dogs and on the surface of the grass before application, and 24 and 48 hours after application. At least one of these chemicals was

detected in the urine of the vast majority of household dogs after lawn treatment. This suggests that the chemical carcinogens used on the lawn are easily internalized by dogs and excreted in the urine, exposing the urothelium to these harmful chemicals.

3.1.7.2. *Water pollutants*

According to several investigations, the main pollutants found in surface water include heavy metals, pesticides, and phenolics. The main sources of this pollutants are the contamination from agricultural products and drinking water disinfection by-products (N. Smith et al., 2022; Wasi, Tabrez, & Ahmad, 2013).

Water-borne pollutants have also been implicated in the development of urothelial carcinoma in humans as well as in dogs (Backer, Coss, Wolkin, Flanders, & Reif, 2008; Reif, 2011).

An earlier study in 2008, and a recent case-control study found that higher measured tap water concentrations of total trihalomethanes (TTHMs) was associated with higher risk of bladder cancer in dogs. TTHMs are reactive by-products of water disinfection. These chemicals were found to be mutagenic and have previously been linked to bladder cancer in humans (Backer et al., 2008; N. Smith et al., 2022).

Nitrate is an agriculturally-derived drinking water contaminant that serves as a precursor to N-nitroso compounds (NOC), which have the potential to cause bladder cancer in humans (Jones et al., 2016; N. Smith et al., 2022).

Arsenic is a naturally occurring metalloid that can be found in air, soil, and water in both organic and inorganic forms. Organic forms are non-toxic, but inorganic forms are harmful (Letaiová et al., 2012). In humans, there is strong evidence for a link between urothelial carcinoma and exposure to inorganic arsenic (A. H. Smith, Goycolea, Hanque, & Lou Biggs, 1998).

The recent case-control study by N. Smith et al. 2022, did not find a direct association between tap water concentration of nitrates or arsenic and UC in dogs, however due to the study limitations, the associations cannot be ruled out.

3.1.7.3. *Airborne pollutants*

Air pollution is a complex mixture of gaseous and particle components, making it challenging to establish a meaningful exposure metric when the biological processes are poorly understood (Liu et al., 2009).

The composition of pollutants in outdoor air varies greatly over time and space, reflecting the diversity of sources as well as the influence of atmospheric processes. The main atmospheric pollutants with acknowledged association to carcinogenic activity include ozone (O_3), nitrogen dioxide (NO_2), sulphur dioxide (SO_2) and particulate matter (PM) (Table 1) (IARC, 2016; Reif, 2011).

Table 1 – Major air pollutants with carcinogenic activity, physical state and source (Source: IARC 2016)

Air Pollutant	Physical State	Main Sources
Ozone (O_3)	Gas	Produced by photochemical reactions that occur when main precursor pollutants such as nitrogen oxides (NOX) and volatile organic compounds (VOC) from industrial operations or transportation, and sun radiation are present
Nitrogen Dioxide (NO_2)	Gas	Industrial, commercial, and residential combustion processes; Transport combustion engines; nitrogen-using manufacturing processes
Sulfur Dioxide (SO_2)	Gas	Combustion of sulfur-containing fuels during the production of electricity or during the support of industrial, commercial, and residential processes; Natural processes (volcanic activity)
Particulate Matter (PM_{10}, $PM_{2.5}$)	Particulate	Industrial, commercial, and residential combustion; Transport combustion engines; Natural sources such as volcanic eruptions, forest fires, and wind activity on land and water surfaces

In veterinary medicine, further studies are needed to investigate the carcinogenic effects of air pollutants and the risk of urothelial carcinoma, however, several studies throughout the years have found a geographic correlation of canine (and human UC) with high industrial activity areas, as a consequence of environmental pollution, primarily air pollution (Hayes, Hoover, & Tarone, 1981; Reif, 2011).

- ***Ozone (O_3)***

Ozone (O_3) is a highly oxidizing and very reactive gas consisting of three oxygen atoms. Ozone serves an important function in the stratosphere by filtering ultraviolet light from the sun, preserving life on Earth (APA I.P., 2021; IARC, 2016).

Tropospheric ozone is considered a secondary pollutant with an irritant effect that enters the airways and reduces lung function. It is known to induce eye, nose, and throat irritation, headaches, chest pains, and breathing difficulties in humans (APA I.P., 2021).

While no carcinogenic activity was detected, it is a sign of poor air quality. When O_3 levels are raised in metropolitan areas, levels of other photochemical oxidants such as aldehydes, organic acids, organonitrates, inorganic acids, hydrogen peroxide, and photochemically produced PMs are likely to be elevated as well (IARC, 2016; N. Smith et al., 2022).

N. Smith et al. 2022 observed that dogs with UC resided in regions with noticeably higher airborne levels of O_3 than older unaffected controls.

- ***Nitrogen Dioxide (NO2)***

Among the nitrogen oxides, NO_2 is the may be the most important pollutant and the only one that is regulated (APA I.P., 2021).

NO_2 is a brownish gas with a distinct odor that is highly corrosive and oxidizing. NO_2 has a number of adverse effects in humans, including damage to the central nervous system and tissue, irritation of the eyes and throat, lowered respiratory capacity, chest pain, and breathing issues (APA I.P., 2021).

An investigation in humans, published in 2009 provided evidence for the correlation between NO_2 and urothelial cancer (Liu et al., 2009).

To date there are no studies of the carcinogenic effect of this air pollutant and the risk of UC in dogs.

- ***Sulfur Dioxide (SO2)***

SO_2 is responsible for the formation of sulfuric acid, which is the main component of acid rain. Similarly to nitrogen oxides, it is involved in complex atmospheric reactions that result in the formation of secondary particles (APA I.P., 2021).

SO_2 has a number of negative effects on health and the environment, ranging from irritation of the eyes, nose, and throat to more serious respiratory problems such as lung damage, coughing, and bronchoconstriction (APA I.P., 2021).

When combined with the effects of other pollutants, SO_2 has a synergistic effect that can exacerbate a variety of disease conditions (APA I.P., 2021; IARC, 2016).

In 2009, a research revealed a link between this airborne pollutant and urothelial carcinoma in humans (Liu et al., 2009).

Similarly to NO_2, the carcinogenic potential of this air pollutant and the risk of UC in dogs have not yet been studied.

- ***Particulate Matter (PM10, PM2.5)***

Particulate matter consists of mineral and/or organic compounds present in the atmosphere in liquid or solid form. The particle size of particulate matter can vary greatly, and the smaller the particle size, the more probable it is to penetrate the respiratory system and create harmful consequences (APA I.P., 2021).

Particles having an aerodynamic diameter of less than 10 µm are the most dangerous to public health, because they can penetrate the pulmonary system. The proportion of PM_{10} with an

aerodynamic diameter of less than 2.5 µm, known as $PM_{2.5}$, or even smaller particles, can penetrate the respiratory system up to the alveolar level, interfering with the respiratory process and providing a serious public health concern (APA I.P., 2021; IARC, 2016).

Particulates can also bind to other pollutants, such as hydrocarbons and heavy metals, and carry them to the respiratory systems of both animals and humans, where they can be absorbed through the respiratory process and enter the circulation (APA I.P., 2021).

One recent study found a correlation between exposure to $PM_{2.5}$ and an increased risk of bladder cancer in humans (Coleman et al. 2020), nevertheless no correlation was found in dogs (N. Smith et al., 2022).

4. Carcinogenesis

Tumor cells are recognized by the abnormal growth patterns and by the fact that are no longer under the control of normal homeostatic growth mechanisms, including apoptosis. Malignant neoplasia, the malignant form of tumor, is the phenotypic end result of a whole series of changes, that may have taken a long period to develop (Deborah W. Knapp & McMillan, 2012).

Carcinogenesis is a multistep process, the different steps include initiation, promotion, and progression, under the influence of different substances such as cancer-producing agents, normal growth promoters and hormones. These steps reflect genetic alterations that drive the progression of a normal cell to a highly agressive cancer cell. This is supported by the fact that genomes of tumor cells are invariably altered in multiple sites (Deborah W. Knapp & McMillan, 2012).

Changes in genes can lead to either stimulatory or inhibitory effects on cell growth and proliferation. In one hand, the stimulatory effects are provided by proto-oncogenes. The mutations on these genes produce positive signals leading to uncontrolled growth. On the other hand, tumor formation can be a result of mutations on the tumor suppressor genes, and loss of its inhibitory functions (Deborah W. Knapp & McMillan, 2012).

The concept of "Hallmarks of Cancer" refers to a set of functional abilities that animal cells acquire as they transition from normal growth stages to neoplastic growth states. These abilities are essential for the development of malignant tumors (Hanahan, 2022; Hanahan & Weinberg, 2011).

There are many documented hallmarks of cancer, that include sustaining proliferative signaling, evading growth suppressors, resistance to cell death, inducing neovascularization or gaining access to vasculature, invasive behavior and metastasis, reprograming cellular metabolism, avoiding immune destruction, unlocking phenotypic plasticity, nonmutational epigenetic

reprogramming, polymorphic microbiomes, and senescent cells (Hanahan, 2022; Hanahan & Weinberg, 2011).

As seen previously, some individuals present strong genetic predisposition to develop bladder cancer, however there is a very well-marked association with the contact of urothelium with tumor inducing chemicals, also known as carcinogens (Deborah W Knapp et al., 2013).

Carcinogens ingested by one of multiple routes, inhaled, consumed, or absorbed through the skin, are concentrated in urine, and come in contact with the lining of the urinary tract. This exposure predisposes to the process of uroepithelial transformation (Theodorescu, 2003).

Chemical carcinogens can form directly or are metabolized to highly reactive electrophilic forms. These electron deficient species can attack many electron rich nucleophilic sites in molecules such as nucleic acids and induce mutagenesis. Damage to deoxyribonucleic acid (DNA), by these chemicals is believed lead to mutations in proto-oncogenes and tumor suppressor genes (Theodorescu, 2003).

Recent studies have identified some gene expression patterns in urothelial carcinoma, however its molecular characterization is still on early stages. Using cytogenetic, molecular genetic and immunohistochemical methods, numerous relevant molecular features were identified, as well as many similarities across species, in many cross species analyses (Farkhondeh, Kianmehr, Kazemi, Samarghandian, & Khazdair, 2020; Deborah W. Knapp et al., 2020; Theodorescu, 2003).

Some of the molecular features identified include overexpression of epidermal growth factor receptor (EGFR), under expression of p63, cyclooxygenase-2 (COX-2) overexpression, - B-isomer of RAF-kinases (BRAF) mutations, and many other gene mutations implicated in the development and progression of UC (Deborah W. Knapp et al., 2020).

5. Metastasis

UC as a high rate of metastasis. Metastatic cancers achieve a metastatic phenotype by expressing metastasis promoting genes and suppressing metastasis suppressor genes, that allows the malignant cells to successfully metastasize. Epidermal growth factor (EGF) and Vascular endothelial growth factor (VEGF) are known to have a key role in the proliferation and neovascularization at tumor site. The vascularity of the primary tumor is correlated with metastatic behavior and its outcome of several animal tumors, including UC (Deborah W. Knapp & McMillan, 2012; Theodorescu, 2003).

Bladder tumor most common metastatic sites include regional nodes (abdominal, pelvic, inguinal nodes) and lungs. Several other distant metastasis sites such as bone, liver, kidney, adrenal glands, skin, spleen, heart and brain have been reported (Deborah W. Knapp et al., 2020).

6. Diagnosis and Clinical Staging

The most common location of canine UC is in the trigone area of urinary bladder, other locations include fundus, ventral wall, and neck of the bladder. Prostatic and lower tract urinary urethra are other common sites of primary UC in dogs. (Meuten, 2016).

Most UC tumors are solitary but can be multiple and sometimes the tumor can cover nearly the entire mucosa. In the most infiltrative type of UC, the muscle layer is infiltrated, and the bladder wall is thickened (Fig. 3). In some advanced cases, transmural extension is present, and the tumors are found on the external surface of the bladder and in pelvic tissues (Meuten, 2016).

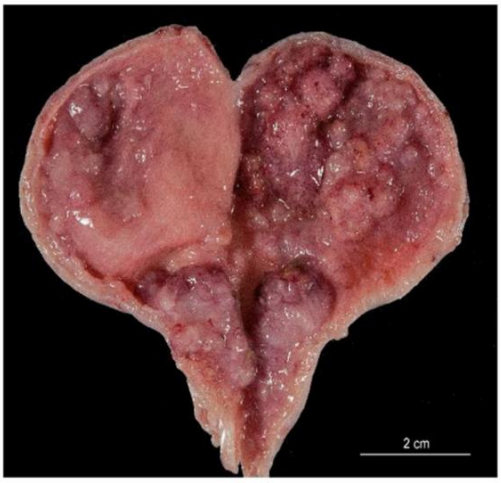

Figure 3 - Canine urothelial carcinoma; Post-mortem specimen of canine bladder with deep invasion of bladder wall (Source: Knapp et al. 2020).

Several conditions can mimic urothelial carcinoma regarding clinical signs, abnormal epithelial cells in urine and mass lesions within the urinary tract (Deborah W. Knapp & McMillan, 2012).

The importance of distinguishing UC from other conditions resides on the fact that the treatment and prognosis differ considerably and depend on the condition present.

Differential diagnosis of mass like lesions in the bladder include inflammatory or infectious conditions such as calculi, chronic cystitis, polypoid cystitis and granulomatous cystitis/urethritis; other benign neoplasias such as fibrous polyps, fibromas, rhabdomyomas/leiomyomas and inflammatory pseudotumors; and malignant neoplasias such as other urinary bladder carcinomas, prostatic adenocarcinoma, hemangiosarcoma, soft tissue sarcoma and lymphoma (Deborah W. Knapp & McMillan, 2012; Report, 2009; Serra, Hill, & Lawrence, 2016).

6.1. Clinical Signs

Most of the clinical signs are nonspecific: anorexia, weight loss, weakness, dyspnea caused by lung metastasis and less commonly, lameness, caused by bone metastasis or hypertrophic osteopathy (Deborah W. Knapp & McMillan, 2012).

The urinary tract signs are indistinguishable from those observed with urolithiasis or lower urinary tract infection and may include hematuria, stranguria, dysuria, pollakiuria, abdominal pain, tenesmus and incontinence (Burgess & DeRegis, 2019; Meuten, 2016).

Urinary tract signs may be present for long periods of time and some dogs can have a transient response to empiric antibiotic therapy or anti-inflammatory medications, and this often leads to delayed diagnosis of UC (Burgess & DeRegis, 2019; Deborah W. Knapp & McMillan, 2012).

Through physical examination of dogs with UC, distended bladder and sometimes mass or thickening in the bladder or urethra can be detected, as well as internal lymph node enlargement. Males may present prostatomegaly (Burgess & DeRegis, 2019).

Physical exam should include a digital rectal examination, that might reveal thickening of the urethra and trigone region of the bladder. This part of the exam is critical in the diagnose of UC, because a portion of the urethra is shielded from other imaging techniques, such as ultrasound (Burgess & DeRegis, 2019; Deborah W. Knapp & McMillan, 2012).

In dogs with suspicion of bladder tumor, normal findings on physical examination do not rule out this differential, and further testing should be performed (Burgess & DeRegis, 2019).

6.2. Clinical Staging

After diagnosing UC, clinical staging is very important to anticipate the tumor progression and to implement the most effective therapy (Mutsaers et al., 2003).

Staging a tumor consists of evaluation of the tumor extent at the primary site and beyond that (Serra et al., 2016).

Assessment of the tumor includes physical exam, thoracic and abdominal radiography, abdominal ultrasonography and specific urinary tract imaging (double contrast cystography, ultrasonography or computed tomography) (Mutsaers et al., 2003; Rasteiro et al., 2022).

In 1980, the World Health Organization (WHO) has defined a Tumor/node/metastasis (TNM) classification scheme for canine urothelial carcinoma (Table 2). The TNM stage at diagnosis is strongly related to prognosis, as higher grade tumors present poor prognosis and shorter survival times (Deborah W. Knapp et al., 2020; Owen, 1980).

Table 2 – TNM Classification of Urothelial Carcinomas in Dogs (Source: Owen 1980)

T – Primary Tumor	
Tis	Carcinoma *in situ*
T0	No evidence of primary tumor
T1	Superficial papillary tumor
T2	Tumor invading the bladder wall, with induration
T3	Tumor invading neighboring organs (prostate, uterus, vagina, and pelvic canal)
N – Regional Lymph Node (Internal and External Iliac Lymph Node)	
N0	No regional lymph node involvement
N1	Regional lymph node involvement
N2	Regional lymph node and juxtaregional lymph node involvement
M – Distant Metastasis	
M0	No evidence of metastasis
M1	Distant metastasis present

Following a consistent protocol for clinical staging from visit to visit, is also useful to accurately track response to therapy (Deborah W. Knapp & McMillan, 2012).

6.3. Urinalysis

After physical exam, if UC is suspected urinalysis should be the next step on diagnosis. Fine needle aspiration of bladder tumors has been reported to cause intrabdominal and skin seeding, therefore urine should be collected by free catch or catheterization, which are less evasive (Deborah W. Knapp & McMillan, 2012; Serra et al., 2016).

Cystocentesis is not recommended in all dogs of predisposed breeds or dogs with a known bladder mass because of the risk of needle track implantation (Burgess and DeRegis, 2019).

Noninvasive diagnostic procedures can be performed with the urinary sample. These include urinalysis, urine culture, urine cytology and detection of biomarkers in the urine (Burgess & DeRegis, 2019; Rasteiro et al., 2022).

The urine of dogs with UC, will present abnormalities in 90% of the cases, usually nonspecific. The most common is hematuria, frequently the first detected. Hematuria is due to concurrent cystitis or disruption of blood vessels in the tumor or from contact and invasion into the adjacent parenchyma (Meuten, 2016).

Other frequent abnormalities are pyuria, proteinuria, and bacteriuria. Proteinuria may be misleading, as it is due to hematuria and pyuria. There are no reports that specify proteinuria of glomerular origin in animals with bladder cancer (Meuten, 2016).

6.4. Urine Cytology

Some urinary tract neoplasms, such as UC, may exfoliate into the urine, allowing the identification of the neoplastic cells during urine sediment examination (Wycislo & Piech, 2019).

Before examining the samples, urine effects on cellular details should be taken account. Cells immersed in urine will acquire artifacts that are amplified over time. To reduce the artifacts on the preparation, fresh urine is recommended (Meuten, 2016).

This method consists in collecting a fresh sample of urine, preparing a concentrated preparation, making a film of the sediment and staining it with Diff-Quik type stain (Meuten, 2016).

Cytological confirmation of tumor cells in the urine must be interpreted with caution, especially in cases where concurrent inflammation is present. In some cases, it is preferable to examine a second urine sample when there is no inflammation, or to recommend a different method of retrieving cells to establish the diagnosis (Meuten, 2016).

Inflammation stimulates hyperplasia of the urothelium. When inflammation of the urinary tract is present is difficult to distinguish between hyperplasia and dysplasia or neoplasia, it is important to recall that inflammation can also induce cellular atypia (Meuten, 2016; Wycislo & Piech, 2019).

UC diagnosis is based on the recovery of numerous, large, anaplastic epithelial cells. If inflammation is present anaplastic cytological changes must be marked (Meuten, 2016).

Looking for abnormal epithelial cells in urine is the least invasive and cheaper technique. Although this technique has its advantages, is the least effective, establishing an effective diagnosis in only 25-30% of cases. Positive findings with this technique can rule in the diagnosis of UC but negative ones do not rule out this differential (Meuten, 2016).

Diagnosing this type of tumors requires correlating cytological interpretation with other clinical data, especially imaging data (Meuten, 2016).

It is easier to make a definitive diagnosis of UC from fine needle aspiration (FNA) cytology that access the lesion site. These preparations are of higher quality and have fewer artifacts than samples prepared from urine (Meuten, 2016; Wycislo & Piech, 2019).

6.5. Urine Culture

In dogs with UC, the failure of host barriers, as a result of abnormal patterns of voiding, decreased mucosal defenses and decreased antibacterial properties by alterations of urine pH or host defense peptides may predispose to urinary tract infections (Budreckis et al., 2015).

In several studies, positive urine cultures were common in dogs with UC. The most common organisms cultured were *Staphylococcus spp.*, *E. coli* and *Streptococcus spp*.

As a result, dogs diagnosed with UC, should have a culture preformed before starting chemotherapy and cultures should be repeated regularly, during the course of treatment (Budreckis et al., 2015).

6.6. Clinical Pathology

Frequently the values of hematology and biochemistry of dogs with UC do not exhibit significant changes (Deborah W. Knapp & McMillan, 2012).

6.6.1. Hematology

When present, anemia is nonregenerative as a result of multiple mechanisms. The most relevant are blood loss in urine and chronic inflammatory disease. Neoplasms will cause anemia of chronic inflammatory disease equivalent to a chronic infectious disease. If anemia is unexplained and the patient is a Scottish terrier or a mass is identified in the bladder, the suspicion of UC is high (Meuten, 2016).

6.6.2. Serum Biochemistry

Hypercalcemia has been reported with a few tumors of the lower urinary tract, possibly as a result of paraneoplastic production of hormones or cytokines, however the cases related to bladder tumors are rare (Meuten, 2016).

Increased liver enzymes have been described in dogs with bladder and urethra tumors. The increases are mild and the mechanism is thought to be secondary to corticosteroid-induced stress or concurrent, and unrelated hepatic problems in older dogs (Meuten, 2016).

Azotemia is found in approximately 15% of dogs with bladder or urethral tumors, it might be a result of concurrent renal disease or dehydration, or post renal azotemia secondary to obstruction of the outflow urine (Meuten, 2016).

In rare cases, uroabdomen can occur, due to rupture of the bladder. In this situations the expected changes are azotemia, hyponatremia, hypochloremia, hyperkalemia, hyperphosphatemia and creatinine and urea nitrogen concentrations in the abdominal fluid and serum (Meuten, 2016).

6.7. Imaging Techniques

6.7.1. Ultrasonography and Contrast Enhanced Ultrasonography

Ultrasonography is used as a first line imaging modality, because it is not invasive, is widely available and does not require anesthesia. Ultrasonography is used to access the tumor location and its extent in the bladder, to evaluate the appearance and size of the regional lymph nodes and to screen other internal organs for metastasis. It can be used in addition to sample collection techniques and also helps in planning potential surgical intervention and to determine the response to medical therapy, assessing the evolution of the masses size (Deborah W. Knapp & McMillan, 2012; Serra et al., 2016).

The ultrasound findings consist of focal wall thickening with irregular mucosal surface and layering. The masses are broad-based and often have an heterogenous appearance and may present mineralization, occasionally they are diffuse and mimic cystitis (Fig.4). In severe cases the entire bladder lumen may be obliterated, and the bladder may have a solid appearance. Hydroureter/hydronephrosis may be seen, if there is obstruction by the mass (Wamsley & Alleman, 2007).

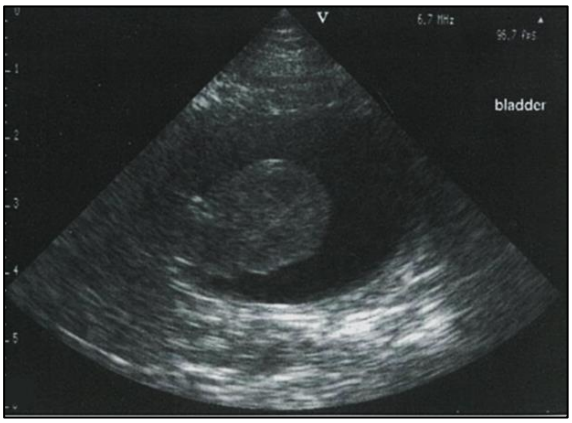

Figure 4 - A broad-based mass, extending from the lumen of the bladder, in transverse ultrasonographic image (Source: Wamsley and Alleman 2007).

Polypoid cystitis, granulomatous cystitis, and adherent blood clots all mimic bladder tumors. In addition to standard ultrasonography contrast-enhanced ultrasound (CEUS) has been used. This technique involves the injection of intravascular contrast agents, such as sulphur hexafluoride, to improve the traditional ultrasound in human and veterinary medicine (Macrì et al., 2018).This technique became a gold standard to fully study invasive bladder tumors in humans as well as in animals. It is very helpful in differentiating pathological entities, such as clots or surgical wall changes, obtaining a detailed image of the neovascularization within the tumoral lesion (Macrì et al., 2018).

6.7.2. Radiography

UC diagnosis might be difficult with plain abdominal radiography, because bladder silhouette is usually not distorted by the tumor, however a caudal abdominal mass, enlarged mineralized regional lymph nodes or lytic lesions in lumbar spine or pelvis might be detected (Burgess & DeRegis, 2019).

Contrast studies of the bladder are used to access the integrity and position of the bladder (Fig.5), as well as internal architecture and luminal contents, when these are not clear in the plain radiographs or when ultrasound is not available (Burgess & DeRegis, 2019; Wamsley & Alleman, 2007).

In the presence of UC, contrast medium adheres to ulcerated and inflamed surfaces of epithelial tumors highlighting bladder wall masses or thickening, which is more severe and focal than with cystitis (Wamsley & Alleman, 2007).

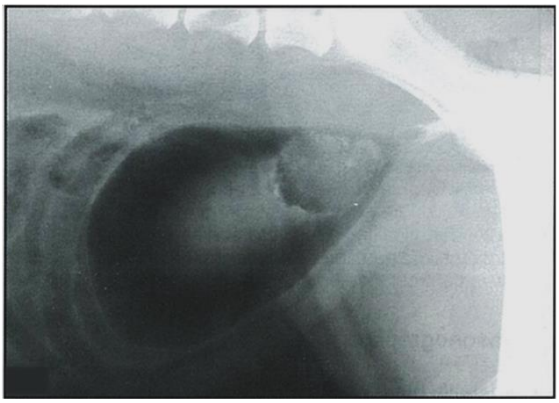

Figure 5 - Irregular mass located in the bladder neck, outlined by the contrast medium from a double-contrast cystography (Source: Wamsley & Alleman, 2007).

6.7.3. Computed Tomography

Other imaging techniques such as computed tomography (CT) are now increasingly used in the surveillance of dogs with bladder tumors.

CT as the capacity to accurately assess soft tissue structures in a cross-sectional dimension. The main role of CT in the diagnosis of urothelial tumors is to examine the regional lymph nodes for the presence of metastasis (Aminkov, 2021).

Contrast CT can accurately identify masses larger than 0.5 cm and can show mucosal abnormalities as small as 2 mm (Fig.6).

It can indicate appropriate areas for assessment and biopsy. It is minimally invasive and sometimes can be diagnostic, however the type of tumor can only be defined by histopathological evaluation (Aminkov, 2021).

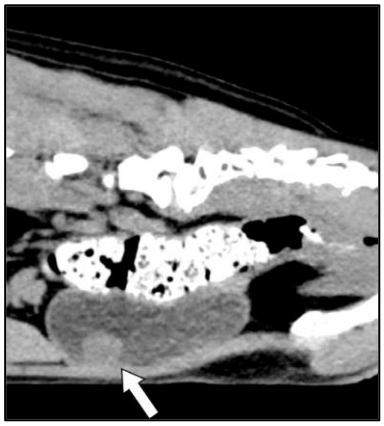

Figure 6 - Sagittal computed tomography of a dog with UC mass in the bladder (arrow); (Source: Iwasaki et al., 2019).

6.8. Cystoscopy

Cystoscopy allows visual examination of the entire urethral lining, trigone area, urethral orifices as well as bladder lining (Fig.7).

Nevertheless, the appearance of lesions is not diagnostic of UC, because benign tumors and benign urethritis/cystitis can present a similar appearance. This technique also allows biopsy collection for histologic evaluation (Burgess & DeRegis, 2019).

Like most of biopsies, the ability to access the lesion and obtain a sample of sufficient size and quality are as important as or more so than the microscopic assessment (Meuten, 2016).

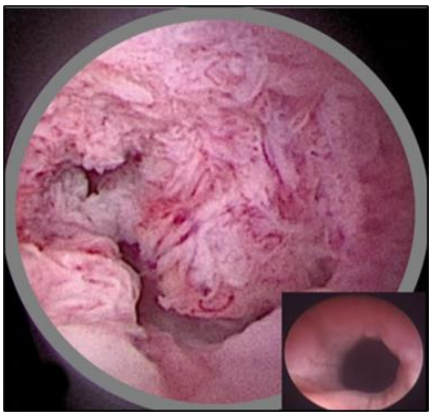

Figure 7 - Cystoscopic image of Canine Urothelial carcinoma, extending into the lumen of urethra, compared with a normal appearance of this region (insert) (Source: Knapp et al., 2020).

6.9. Fine Needle Aspiration

Fine needle aspiration (FNA) is an invasive method for obtaining tissue samples from lesions that cannot be obtained via traumatic catheterization. FNA is often preformed guided by ultrasound, to better access the lesion site, as an alternative to blind percutaneous aspiration. Multiple areas should be aspirated, including the lesion center and periphery to maximize the possibility of obtaining a diagnostic sample (Wycislo & Piech, 2019).

Percutaneous FNA of urethral or bladder masses is questionable. This debate arose as a result of several reports of UC seeding along the FNA needle tract (Klopfleisch, Sperling, Kershaw, & Gruber, 2011). Although the true incidence of needle tract metastasis due to this technique and the exact factors that contribute to its development are unknown, many clinicians prefer and recommend traumatic catheterization for bladder and urethral masses (Serra et al., 2016; Wycislo & Piech, 2019).

6.10. Traumatic Catheterization

Traumatic catheterization is a traumatic collection method that uses a sterile catheter to disrupt tissue fragments from tumorlike lesions located on the bladder or urethra. Tissue fragments are collected via negative pressure into the catheter and connected syringe (Wycislo & Piech, 2019).

This method is often successful in collecting cellular samples representative of the primary lesions, that can lead to diagnosis, when submitted to cytology examination. However, in some cases, the sample contains only superficial transitional epithelial cells from the periphery of the lesion, which may lead to inconclusive results (Wycislo & Piech, 2019).

6.11. Cytology

Cytological evaluation of the urinary tract is always recommended when the presence of mass lesions is detected within the kidneys, bladder or urethra (Wycislo & Piech, 2019).

Cytology is often diagnostic, except in cases of well differentiated UC displaying minimal pleomorphism or when the sampling only achieves collection of the surrounding hyperplastic urothelium. For this reason, the sampling methodology is very important for the diagnosis, as some collection methods may not lead to a conclusive result (Wycislo & Piech, 2019).

Concurrent inflammation can also difficult the diagnosis, because hyperplasic or dysplastic epithelium can mimic the appearance of neoplastic epithelium (Wycislo & Piech, 2019).

It is easier to reach a diagnosis from FNA samples, usually there is little to no inflammation present. In these cases, the more numerous the cytologic abnormalities, the more likely the cells

are neoplastic. If there are only a few cytologic abnormalities and there is inflammation, the cell atypia is more likely to be dysplasia or hyperplasia of urothelium (Meuten, 2016).

Cytology from urothelial tumors usually present exfoliated individual cells, or cohesive clusters with distinct cytoplasmic junctions. The neoplastic cells are usually highly pleomorphic and display marked cytologic criteria of malignancy, such as anisocytosis and anisokaryosis, as well as binucleation. The UC cells commonly contain single to multiple bright magenta cytoplasmic vacuoles, called Melamed-Wolinska bodies (Fig. 8), a specific feature of this neoplasm (Wycislo & Piech, 2019).

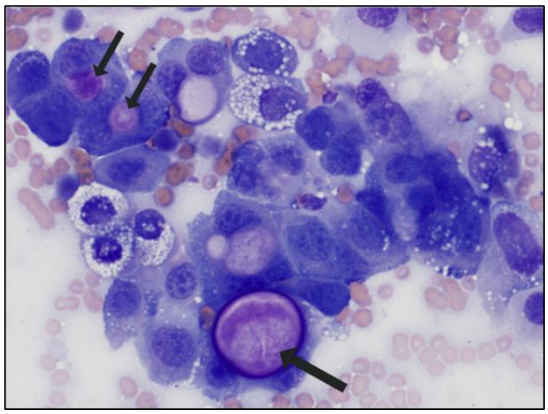

Figure 8 – Bladder mass aspirate (trigone region) from an adult dog with urothelial carcinoma. Melamed-Wolinska bodies (arrows) (Wright-Giemsa 500x) (Source: Wycislo & Piech, 2019).

In addition, identification of these specific Melamed-Wolinska bodies in carcinomas outside of the urinary tract, for example in lymph node, skin or abdominal or pleural fluid, can help to detect metastasis from a primary urothelial bladder tumor (Meuten, 2016; Wycislo & Piech, 2019).

6.12. Histopathology

Histopathologic evaluation of tumor biopsy is considered the gold standard for diagnosis of UC (Burgess & DeRegis, 2019).

Histologic diagnosis is usually straightforward, as the tumor is commonly well developed by the time of diagnosis. Problematic diagnosis come from small samples (from FNA or cystoscopy) and confined to the mucosa, or if it's a rare case of low grade tumor (Meuten, 2016).

In cases of biopsy or necropsy samples, the cellular features of UC are fully developed and easy to identify (Meuten, 2016).

The key features for diagnosis of urothelial carcinomas include, location on the bladder, large epithelial cells with multiple abnormalities, Melamed-Wolinska bodies and invasion to some degree of the histological section (Meuten, 2016).

Histologically, the tumor will often present itself as papillary, and the neoplastic cells have abundant eosinophilic cytoplasm, large nuclei, and multiple mitosis figures. Melamed-Wolinska bodies, seen in cytological and histologic preparations, appear as large cytoplasmic vacuoles, that can be empty or contain homogenous or stippled eosinophilic material (Meuten, 2016).

Neoplastic cells have multiple cellular and nuclear features of anaplasia, such as large and vesicular nuclei, prominent nucleoli, syncytial cells, and bizarre mitosis. Neoplastic cells can present various degrees of dysplasia and anaplasia, within the tumor. In some UC cases, squamous and glandular metaplasia can be found in the histological samples, but the predominant cellular proliferation is always transitional cells from urothelium (Meuten, 2016) (Fig.9).

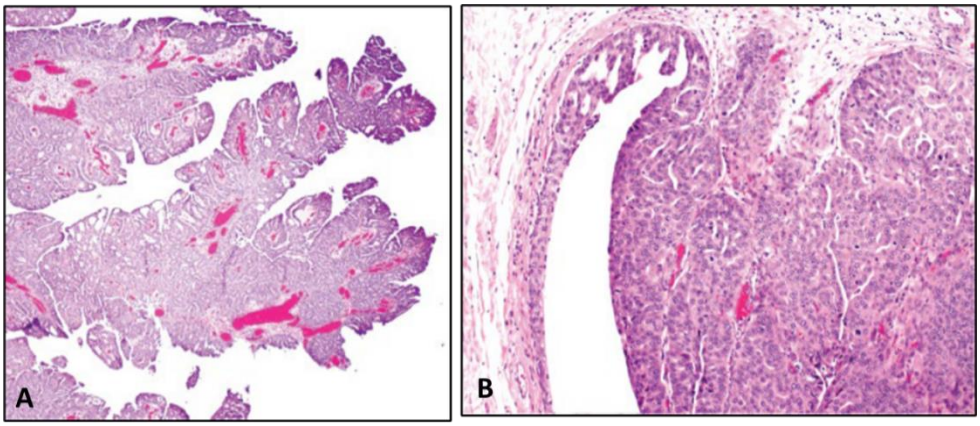

Figure 9 – A - Papillary UC growing from the bladder surface (Hematoxylin and Eosin (H&E)). B - Junction of normal mucosa (left) with UC (right). Neoplastic cells invaded the basement membrane and started to infiltrate the submucosa (H&E) (Source: Meuten, 2016).

UCs are divided into four growth patterns: papillary (50%) or nonpapillary (50%) tumors and infiltrating (≥90%) or noninfiltrating (≤10%) tumors (Meuten, 2016).

The papillary and infiltrating UC is the most frequent tumor type in canine species. These tumors exhibit papillary or cauliflower-like structures that project into the lumen (Brambilla et al., 2022).

The papillary and noninfiltrating UC type has an identical luminal growth pattern but does not penetrate the lamina propria or the stalk's stroma (Brambilla et al., 2022).

The second most common type of UCs are nonpapillary and infiltrating. These tumors might take the form of plaques, raised masses, or flat nodules. These tumors frequently have ulcerations and are more likely to penetrate the deeper muscle layers. The level of invasion determines the bladder wall's thickness. This UC type is the most probable to metastasize, and usually exhibit histological and cytological heterogeneity (Brambilla et al., 2022).

The least frequent type is nonpapillary and noninfiltrating urothelial carcinoma. It is synonymous with carcinoma *in situ* (CIS), which manifests as a flat lesion of cytologically malignant cells confined to the epithelium's surface (Brambilla et al., 2022).

Various degrees of lymphoid inflammation can be found in the primary lesion. Vascular invasion appears in approximately 40% of the cases and metastasis may occur with or without vascular invasion of the primary lesion site (Meuten, 2016).

5.12.1. Histological Grading

The UC grading scheme for the dog, has been based largely on the WHO histological criteria for human urothelial carcinomas. The use of the human grading system has some limitations, since the range of urothelial proliferative lesions is greater in humans than in domestic animals. (Avallone et al., 2021)

New grading criteria have been proposed for urothelial tumors in domestic animals, simplifying the classification scheme in two tumor types: Urothelial Papilloma and Urothelial carcinoma. This last group is then classified as low and high grade variants (Table 3), as suggested by Meuten in 2016 (Avallone et al., 2021; Meuten, 2016).

Dogs will develop high grade bladder tumors in almost 95% of cases. High Grade Urothelial Carcinomas are defined by several features of malignancy that include atypia, cellular and nuclear pleomorphism, mitotic activity, deeper invasion and lymphovascular invasion (Avallone et al., 2021; Meuten, 2016).

Despite being the most often used grading system for canine UCs, exact features or cutoffs (such as mitotic count) are not yet available (Avallone et al., 2021; Meuten, 2016).

Table 3 – Grades of Urothelial Tumors in animals (Source: Meuten 2016).

Urothelial Papilloma
Papillae, ≤7 cell layers, focal, no cellular or nuclear atypia, no mitoses or invasion. Inverted, papilloma projects below surface but does not invade, cells identical to papilloma, reported in cattle.
Urothelial Carcinoma Low Grade
Papillae or flat, orderly cell to cell; cellular atypia mild to moderate; nuclear abnormalities present: enlarged nuclei, nucleoli visible, rare to no mitoses; no invasion.
Urothelial Carcinoma High Grade
Papillae or flat, loss of cell polarity, disorganized growth; marked cellular atypia; marked nuclear pleomorphism: chromatin clumped, nucleoli prominent; numerous mitoses, some abnormal; invasion present, state depth of infiltration and if UC in blood vessels or lymphatics.

Further retrospective and prospective follow-up studies are required to determine the relationship between low- and high-grade features and the patient outcome, and establish which histological

grading has the most accurate prognostic value for patients or to finally propose a new acceptable grading system to better characterize canine UCs (Avallone et al., 2021; Brambilla et al., 2022; Burgess & DeRegis, 2019).

A recent study found that lymphatic invasion was associated with decreased survival rates when compared to animals who did not have it. According to the same study, histological subtype, histological grade, and the presence of muscle layer invasion were not significantly related to survival time. This study highlights the significance of assessing and reporting lymphatic infiltration as a predictive characteristic of UC tumors (Verônica M Govoni et al., 2021).

7. Biomarkers

Accurate, noninvasive methods are needed to detect the presence of this tumor in early stages, allowing early therapeutic intervention, and improve survival rates in affected dogs (Rasteiro et al., 2022).

These new methods consist of detection of tumor specific molecules (biomarkers) in urine or cytological samples, that can be easily collected through non-invasive techniques. This is possible because UC tumor cells and metabolites often shed into urine (Rasteiro et al., 2022).

The ideal biomarkers should have high sensitivity, high specificity, and high positive and negative predictive values. These biomarkers present high sensitivity when compared to healthy dogs, however other diseases that look similar (polypoid cystitis, urinary tract infection (UTI), urolithiasis) in the urinary tract may cause false positive results (Meuten, 2016).

Several potential biomarkers for canine UC have been developed and investigated for diagnostic or prognostic purposes. Many screening tests can be performed in urine samples include fibroblast growth factor, bladder tumor-associated antigen, fluorescence in situ hybridization, calgranulins and many others. Some of these tests are available for commercial use in dog (Meuten, 2016; Rasteiro et al., 2022).

7.1. Immunohistochemistry

Immunohistochemistry (IHC) is a method used to determine the expression of biomarkers in the tissue. Fundamentally the IHC test involves the recognition of antigens in the histologic sections by specific antibodies. The antigen–antibody binding is then visualized by light or fluorescent microscopy as a colored histochemical reaction (DAKO, 2013; Meuten, 2016).

Target antigens can be present in various organelles and locations inside or outside the cells. Its location inside the cells can be intranuclear, intracytoplasmic, or on nuclear and cell membranes.

Sometimes these antigens can be in specific locations in normal cells but in different locations in neoplastic or reactive cells. Additionally, antigen distribution can vary based on the location of tumor cells within the same tumor (DAKO, 2013; Meuten, 2016).

When selecting the IHC tests to diagnose a specific neoplasm, we should include markers that are expected to be positive for the suspected tumor type as well as markers that might be positive in the other tumor types in the differential diagnosis (DAKO, 2013; Meuten, 2016).

This method is a very powerful tool in oncology, however the results should always be interpreted as a complement of histopathologic evaluation of the morphologic changes, as well as the rest of the clinical data (DAKO, 2013; Meuten, 2016).

7.1.1. UP III, CK7 and CK 20

Immunohistochemical characterization of epithelial tumors of the urinary bladder has been achieved mainly with antibodies to Uroplakin III (UP III) and Cytokeratins (CKs).

UP III is the most common molecular marker of urothelium differentiation used in dogs. It belongs to the family of Uroplakins. Uroplakins consist of membrane-associated proteins expressed by urothelial cells that are important for cell-to cell adhesion. UPs are specific for transitional epithelium and its tumors. There are four transmembrane proteins: UP Ia, Ib, II and III. Uroplakin can identify the cells as urothelial, but it does not differentiate normal, hyperplastic or dysplastic cells from neoplastic cells (Meuten, 2016; Ramos-Vara, Miller, Boucher, Roudabush, & Johnson, 2003).

Cytokeratins comprise a family of at least 20 different polypeptides. These intermediate filament proteins can be found in epithelium and epithelial tumors. The determination of the CK profile of a particular carcinoma, is useful in the differential diagnosis. Bladder urothelium contains simple-epithelium type CKs (CK 7, 8,18,19,20) and stratified epithelium CKs (CK 13 and 17). The most common cytokeratins used to differentiate urothelial carcinomas from other origins carcinomas are CK 7 and CK 20 (Espinosa De Los Monteros et al., 1999; Ramos-Vara et al., 2003).

An IHC study compared the immunostaining of UP III, CK 7 and CK 20 in canine UCs. UP III was shown as a specific and sensitive marker for UC. CK 7 has higher sensitivity than UP III for UC, but it is expressed in non-urothelial tumors and in some normal tissues as is CK 20. For that reason, CK 7 should be used in tumors negative for UP III that are suspected of being UC (Ramos-Vara et al., 2003; Rasteiro et al., 2022).

7.1.2. Cyclooxygenase-2 (COX-2)

Cyclooxygenase (COX) enzymes catalyze the biosynthesis of prostaglandin, and are involved in inhibiting apoptosis, promoting cell proliferation, stimulating angiogenesis and decreasing immunity. COX-1 is generally present in normal tissues, while COX-2 is expressed in multiple neoplasms and inflammatory cells (Grassinger, Merz, Aupperle-Lellbach, Erhard, & Klopfleisch, 2019).

Some studies evaluated the expression of COX-2 in a variety of animal tumors, and its upregulation is evident in the neoplastic processes, promoting cell proliferation, angiogenesis, cell invasion and immune suppression. The COX-2 expression in humans is a marker of invasion, recurrence, and short survival times (Grassinger et al., 2019; Khan, Knapp, Denicola, & Harris, 2000; Meuten, 2016).

Regarding Canine UC, a study described regular expression of COX-1 in normal urinary bladder epithelium, and an intense COX-2 expression in UC (Khan et al., 2000).

A different study showed the difference of the location of COX-2 positive cells within the urothelium between nonneoplastic and neoplastic lesions. Urothelial polyps, polypoid cystitis and urothelial papillomas exhibited expression of COX-2 in the superficial layer of the urothelium, and rarely though the urothelium. Papillary urothelial carcinomas exhibited a random distribution of COX-2 expression (Sledge, Patrick, Fitzgerald, Xie, & Kiupel, 2015).

For many years therapies with nonselective COX and COX-2 specific inhibitors have been used for treating UC in dogs. Further studies are needed to evaluate the predictive value of this histochemical marker in treatment response (Rasteiro et al., 2022).

7.1.3. P63 and Ki67

P63 is a protein known to play an important role in cellular development of stratified epithelial cells in different organs. The loss of p63 has been associated with tumorigenesis and malignancy (K Hanazono et al., 2016).

Ki67 is a nuclear protein expressed by proliferating cells, that is widely used in human and veterinary medicine for assessment of cellular proliferation in tumors and established as a prognostic and predictive indicator, either alone or in association with other markers (Verônica Mollica Govoni et al., 2022; K Hanazono et al., 2016; Rasteiro et al., 2022).

Several studies in humans have shown that Ki67 is an important factor to consider in the prognosis and tumor behavior related to disease progression and histological grade (Verônica Mollica Govoni et al., 2022).

An IHC study in 2016, evaluated the expression of p63 and Ki67 in UC samples compared with polypoid cystitis and normal urothelium samples. The results showed staining scores of p63 significantly lower in UC, related with presence of vessel invasion and metastasis, and shorter survival times (K Hanazono et al., 2016).

In the same study, the ki67 staining scores in the UC sample were significantly higher than the other two. It was concluded that these markers could be used as diagnostic and predictive markers, however additional studies are needed to investigate its molecular features (K Hanazono et al., 2016).

7.1.4. Caveolin-1 and GATA-3

Caveolins are plasma membrane proteins known to regulate complex intracellular signaling pathways related to cancer progression and resistance to therapies. In humans Caveolin-1 expression has been correlated with clinicopathological factors and tumor progression, with an important role in several biological processes such as endocytosis, vesicular transport and signaling pathways (Verônica Mollica Govoni et al., 2022).

GATA binding protein 3 (GATA-3) is a zinc finger transcription factor involved in the differentiation and cell specification processes of several tissues, including urothelium. In humans is one of the most important markers for urothelial differentiation, however its expression in canine UC studies are still on early stages (Verônica Mollica Govoni et al., 2022).

In 2022, a study evaluated the immunostaining of these markers and the histopathological features of the UC tumor and found a correlation between the markers and the mitotic count (MC) of the samples analyzed. The correlation between GATA-3 and MC was low and positive, indicating that tumors with higher expression values of the marker tend to have higher MC. Caveolin-1 expression showed a low negative correlation, indicating that tumors with higher expression values of the marker tend to have lower MC (Verônica Mollica Govoni et al., 2022).

7.1.5. Epidermal Growth Factor Receptor (EGFR)

Epidermal growth factor receptor (EGFR) has been evaluated by IHC as a marker for UC in dogs. EGFR is a receptor tyrosine kinase of the ErbB family. The overexpression of this receptor was reported in several human and canine tumors. A study that compared UC samples with normal urothelium and polypoid cystitis samples, reported high levels of EGFR associated with UC, however no association was found between the over expression of EGFR and the malignant behavior of the tumor or the survival times of the dogs (Kiwamu Hanazono et al., 2015).

This study concluded that, EGFR expression can be used as a sensitive diagnostic marker rather that a prognostic marker in canine UC. EGFR immunostaining could be helpful for urine cytology diagnostic, due to its high specificity, when a provisional diagnosis is needed (Kiwamu Hanazono et al., 2015).

Further research is needed for understanding the expression of EGFR in canine UCs (Kiwamu Hanazono et al., 2015; Rasteiro et al., 2022)

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7.1.6. Human Epidermal Growth Factor Receptor 2 (HER-2)

Human Epidermal Growth Factor Receptor 2 (HER-2) is another member of the ErB family, it is a defective transmembrane tyrosine kinase receptor, that has an important role in the control of epithelial cells growth and differentiation. In humans, the overexpression of this protein is considered as a poor prognostic factor (Millanta et al., 2018; Rasteiro et al., 2022).

In human urothelial carcinomas HER-2 is overexpressed frequently in higher grade tumors and in more advanced stages. A study in canine urothelial tumors found HER-2 to be significantly overexpressed in UC samples compared to non-neoplastic ones (Millanta et al., 2018).

Additional studies are needed, in order to investigate the potential of HER-2 as a prognostic/malignancy marker as well as a possible therapeutic target (Rasteiro et al., 2022).

7.1.7. Receptor Tyrosine Kinases (RTKs)

There have been numerous reports of the expression of the receptor tyrosine kinases (RTKs) vascular endothelial growth factor receptor 2 (VEGFR2), platelet-derived growth factor receptor beta (PDGFR-β), and v-kit cellular homologue (c-KIT) in canine and human urothelial carcinomas (Korec et al., 2021; Setyo, Donahoe, Shearer, Wang, & Krockenberger, 2023; Walters, Martin, Price, & Sula, 2018).

In recent investigations, the staining results of UC, inflammatory non-neoplastic (cystitis), and normal urine bladder samples were compared. The most promising biomarker in both investigations was PDGFR-β, with UC, cystitis, and normal bladder groups showing a significant difference in PDGFR-ß staining intensity (Setyo et al., 2023; Walters et al., 2018).

More research is required to determine whether these receptors have any related mutations and to define the function of these RTKs in tumorigenesis, therapeutic response, and clinical outcome in dogs with UC (Setyo et al., 2023).

7.2. Other Biomarkers

7.2.1. BRAF mutation

The family of rapidly accelerated fibrosarcoma (RAF) kinases are known for their role in the mitogen activated protein kinase/extracellular regulated kinase (MAPK/ERK)-pathway, an evolutionary conserved molecular pathway, which controls cellular growth, proliferation, differentiation and cell survival. (Gedon, Kehl, Aupperle-Lellbach, von Bomhard, & Schmidt, 2021; Hiroyuki Mochizuki & Breen, 2015)

Mitogen activated protein kinase (MAPK) pathway signaling can be initiated by many different extracellular signals such as growth factors and mitogens (Fig. 10).

Ligand binding to receptor tyrosine kinases triggers phosphorylation and activation of rat sarcoma vírus (RAS) family proteins, which in turn activates RAF proteins.

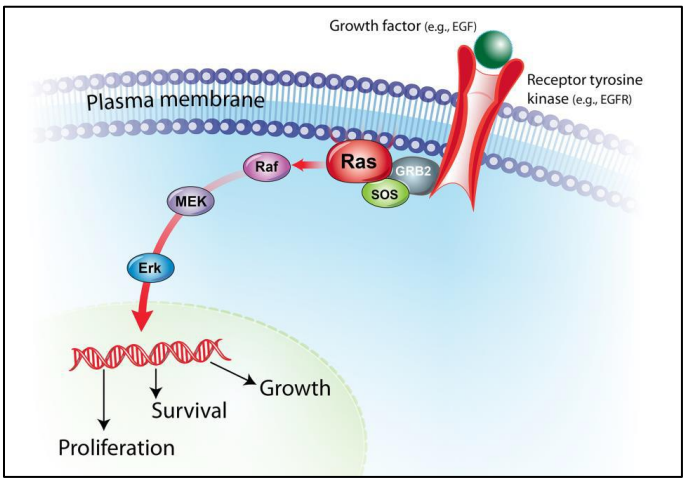


Figure 10 - Mitogen activated protein kinase/extracellular regulated kinase (MAPK/ERK)-pathway (Source: Hiroyuki Mochizuki, Kennedy, Shapiro, & Breen, 2015).

Activation of RAF leads to subsequent activation of MAPK/ERK kinase (MEK), initiating the signal transduction of several genes involved in different cellular processes (H. Mochizuki & Breen, 2017; Hiroyuki Mochizuki & Breen, 2015).

The MAPK pathway can be activated in many cancers through different molecular mechanisms, enabling neoplastic cells to grow independently of extracellular proliferation signals (Hiroyuki Mochizuki & Breen, 2015). Activation of MAPK signaling through mutations in the B-isomer of RAF-kinases (BRAF) is a common event in human tumor progression and metastasis (Gedon et al., 2021).

The presence of BRAF mutations in a wide variety of human cancers, led to studies to detect the presence of this mutations in various canine tumors. In this studies, UC and prostatic carcinoma, showed the highest frequencies of the BRAF mutations. This suggests the dysregulation of MAPK

pathway may play an important role in the pathogenesis of the disease (Gedon et al., 2021; Hiroyuki Mochizuki & Breen, 2015; Hiroyuki Mochizuki et al., 2015).

Detection of BRAF mutations can be used as a non-invasive technique to diagnose and monitor UC tumors in liquid samples, such as blood or urine, without biopsy of the tissues. In addition to this, the identification of BRAF mutations rises the possibilities to apply therapies targeting constitutively-activated MAPK in UC patients, improving clinical outcomes (Hiroyuki Mochizuki & Breen, 2015; Hiroyuki Mochizuki et al., 2015).

A different study, that correlated breed, BRAF mutation status, histological grade and Cox-2 expression, concluded that both Terrier and Non-terrier breeds, with BRAF mutation, a high grade tumor with increased Cox-2 expression is most likely (Grassinger et al., 2019).

In 2021, a retrospective study, showed that in general BRAF mutation status does not appear to be prognostic of overall survival in UC patients (Gedon et al., 2021).

A test is currently commercially available to detect the BRAF mutation in urine samples. This test based on droplet digital polymerase chain reaction (ddPCR) and could be particularly useful as a noninvasive screening test (Rasteiro et al., 2022).

7.2.2. Bladder Tumor-associated Antigen (BTA)

This test was originally developed for humans, and then a veterinary version was developed to assess canine patients. The test uses antibodies to detect a urinary bladder tumor-associated glycoprotein complex, that is found in urine in the presence of urinary tract invasion or tissue damage (Billett, Moore, & Holt, 2002; Rasteiro et al., 2022).

Studies performed evaluating the specificity and sensitivity of the test for diagnosis of UC in dogs, showed high sensitivity but low specificity. This test is not recommended for a definitive diagnosis; however, it can be useful as a screening test to rule out UC (Billett et al., 2002; Rasteiro et al., 2022).

7.2.3. Basic Fibroblast Growth Factor (bFGF)

Basic fibroblast growth factor (bFGF) is a proangiogenic peptide associated with tumor progression and has been detected at high levels in the urine of patients with UC tumors compared with healthy ones. The detection and quantification of bFGF is accomplished with ELISA test kits (Allen, Waters, Knapp, & Kuczek, 1996; Mohammed et al., 2003; Rasteiro et al., 2022).

An early study in 1996 concluded that bFGF can be used as a useful test to distinguish between dogs with UTI and dogs with UC or as an indicator of treatment response. Additional studies are required to assess de sensitivity and specificity of this test on a larger dog population (Allen et al., 1996; Rasteiro et al., 2022).

8. Treatment

The treatment of this tumor include surgery, radiation therapy, chemotherapy and other drugs, or combinations of these.

8.1. Surgery and Interventional Urology Procedures

Surgery can be used to collect tissue samples for diagnosis, tumor removal, when the lesion is located outside de trigone area, or to restore the urine outflow (Deborah W. Knapp & McMillan, 2012).

In dogs, surgical excision isn't often preformed, because most UC tumors are not in a location where complete surgical excision is possible (trigone area). Other reasons why this approach isn't the first choice, include the multifocal distribution in the bladder, frequent presence of metastasis in other locations beyond the bladder (urethra, prostate and other organs), the morbidity of the procedure and the expense involved (Fulkerson & Knapp, 2015; Deborah W. Knapp & McMillan, 2012).

In cases where excision is possible, partial cystectomy, systemic chemotherapics and daily COX inhibitors have improved outcomes and overall survival time (Bradbury et al., 2021).

When the trigone area is affected, total cystectomy can be performed. This can be achieved through uretercolonic anastomosis, anastomosing the ureters to the colon after the complete excision of the bladder and other affected structures. Recently other techniques such as ureteropreputial and ureterovaginal anastomosis have been studied in dogs with UC. However high morbidity and complication rates are observed (Fulkerson & Knapp, 2015; Liptak et al., 2004).

Although surgical procedures are rarely curative, they can be carried out for the management of urethral obstruction secondary to the tumor mass, in order to maintain or restore the urine flow. Cystostomy tubes, or catheters can be placed to bypass the urethral obstruction. Laparoscopic removal of urethral tumors and electrosurgical resection have also been reported as optional techniques, however further studies are necessary to evaluate their potential benefits (Fulkerson & Knapp, 2015; Deborah W. Knapp & McMillan, 2012).

8.2. Radiation Therapy

The use of radiation therapy has been reported in a few studies, occasionally combined with surgery and medical therapies, however it was associated with several complications and therefore not routinely used in the management of dogs with UC (Choy & Fidel, 2016; Nieset, Harmon, Johnson, & Larue, 2014; Rasteiro et al., 2022).

The fundamental difficulty with radiation therapy is the variability in the size, position, and shape of the bladder as well as the irradiation of the surrounding tissues. Radiation therapy can be used intraoperatively or externally. More research is required to fully comprehend this modality's benefits in comparison to medical therapy (Choy & Fidel, 2016; Nieset et al., 2014).

8.3. Medical Therapy

Systemic medical therapy represents the mainstay of UC treatment. Although is not usually curative, several different drugs can lead to remission or stabilization of the tumor. Systemic treatment usually comprises chemotherapy, COX inhibitors and combinations of these (Fulkerson & Knapp, 2015; Deborah W. Knapp & McMillan, 2012; Deborah W. Knapp et al., 2014; Rossman et al., 2021).

Most therapies are well tolerated but sometimes resistance to drugs may occur. Therefore, revising the medical protocol regularly is very important. Some cases of canine UC, where multiple treatment protocols were applied over the course of the disease showed the best results (Deborah W. Knapp & McMillan, 2012; Rasteiro et al., 2022).

Before starting a chemotherapy protocol, renal and liver assessment should be performed. During the course of treatment these parameters should be regularly monitored, as well as gastrointestinal (GI) signs (Rasteiro et al., 2022).

Many chemotherapeutic drugs and protocols have been studied in canine UC management. These genotoxic agents can be used either as single agents or in combination therapies. The most used drugs include carboplatin, cisplatin, doxorubicin, gemcitabine, mitoxantrone, vinblastine, vinorelbine and metronomic chlorambucil (Fulkerson & Knapp, 2015; Rasteiro et al., 2022; Schrempp et al., 2013).

Due to its antitumor activity, nonselective COX inhibitors and COX-2 specific inhibitors have been extensively used as a first line approach for treatment and palliative management of UC in dogs. Piroxicam is usually the first choice non steroid anti-inflammatory drug (NSAID), often used as a single agent with excellent results, including total and partial remission of the tumor and stabilization of the disease in most of the cases (Mohammed et al., 2003). Deracoxib, firocoxib have also been studied for the treatment of UC. The most used chemotherapy protocol consist of a combination of mitoxantrone and piroxicam (Fulkerson & Knapp, 2015; Deborah W. Knapp & McMillan, 2012; Rasteiro et al., 2022).

The risk and intensity of the side effects to the cytotoxic drugs vary with each drug, and include GI signs a suppression of bone marrow, requiring constant monitoring of overall health parameters. Adverse effects to NSAID are mainly due to its GI and nephrotoxicity. The vast

majority of the dogs tolerate these treatments with great results and a good quality of life (Fulkerson & Knapp, 2015; Rasteiro et al., 2022).

8.4. Localized Therapies

Some localized intravesical treatments have been investigated in dogs with UC, as well as in humans.

Intravesical administration of mitomycin C a chemotherapeutic agent as shown a promising antitumor activity, however adverse effects observed require a further investigation (Abbo, Jones, Stewatr, Fourez, & Knapp, 2010; Rasteiro et al., 2022).

Some research on photodynamic therapy have applied this treatment to dogs with bladder carcinoma. Photodynamic therapy typically involves administering tumor localizer photosensitizer, followed by activation with light of appropriate wavelength, that causes lethal phototoxic effects in tumor cells. Further studies are demanded to access the safety and efficacy of this treatment (Rasteiro et al., 2022; Ridgway & Lucroy, 2003).

Other localized therapy technique consists in the local application of Interleukin-2 (IL-2) cytokine. In recent years, it has been used in human and veterinary immunotherapy protocols. This protein is secreted by different types of immune cells and can bind to IL-2 receptors. The main advantage of this protein is its ability of recruiting cytotoxic T- lymphocytes selectively to tumors. Because IL-2 is usually locally applied, it is more effective and causes fewer side effects compared with the systemic route. It has been considered as a safe and minimally invasive palliative treatment for cases where the surgical approach is not possible (Konietschke, Teske, Jurina, & Stockhaus, 2012; Rasteiro et al., 2022).

8.5. Emerging Targeted Therapies

Targeting therapy is becoming essential in the cancer treatment. Its greatest antitumor effects, minimal risk of toxicity, and the more individualized treatment protocols are very promising (Fulkerson & Knapp, 2015; Deborah W. Knapp et al., 2014).

Recently, several targeted therapy approaches have been investigated. One of the approaches involve targeting folate (vitamin B9) receptors, due to the high uptake of folate and folate drug conjugates into certain neoplastic cell compared with normal ones. In a study with canine UC by Knapp et al. 2014, folate receptors were detected in the most primary lesions, as well as nodal a lung metastases and folate uptake was detected in both primary and metastatic lesions. Initial studies with folate-targeted vinblastine in dogs positive to folate-receptor marking, showed great

tumor responses, such as partial remission and stabilization of the disease. Further studies are ongoing (Rasteiro et al. 2022).

Therapies targeted at epigenetic changes that promote tumorigenesis are also being developed. Two main example of the epigenetic events that can lead to cancer development and progression are: aberrant DNA methylation in cancer-related genes, causing gene silencing and histone acetylation, that causes downregulation of the expression of tumor suppressor genes (Eto et al., 2019; Fulkerson & Knapp, 2015; Deborah W. Knapp et al., 2014).

Several studies are evidencing the usefulness of marking key enzymes to these epigenetic changes, for the diagnosis, prognosis, and treatment of cancer in human and veterinary medicine. Molecules that inhibit the activity of DNA methylation associated enzymes and histone acetylation associated enzymes have been evaluated as a possible antitumor therapy (Eto et al., 2019; Fulkerson & Knapp, 2015; Deborah W. Knapp et al., 2014).

As previously observed, the identification of the BRAF mutation in UC and its high prevalence raised the possibility of targeting the BRAF/MAPK pathway as a viable therapeutic approach in tumors with the BRAF mutation. In human medicine, several drugs have been developed to target selectively the BRAF mutation in different tumors, with good results. Studies in canine UC revealed resistance to BRAF inhibitors, in some BRAF-positive cancers. As a result, the presence of the mutation does not always correlate with clinical response to BRAF inhibitors (Rossman et al., 2021).

9. Monitoring Tumor Response to Treatment

As seen previously, surgical excision of the UC tumor might not always be possible, and as a result, the treatment often consists of non-surgical therapies. These therapies are aimed to reduce the tumor volume and to mitigate clinical signs, thus improving the quality of life (Leffler et al., 2018).

In order to evaluate and categorize the effectiveness of chemotherapeutic drugs and the response of an individual patient to treatment, continuous reassessment of the patient should be performed as well as monitoring tumor staging (Fulkerson & Knapp, 2015; Leffler et al., 2018).

Urothelial carcinomas can present different sizes, shapes, and distributions that can vary throughout the course of the disease. These changes are usually marked in an individual dog, in response to treatment (Leffler et al., 2018).

Response evaluation criteria in solid tumors (RECIST) guidelines provided objective measures to evaluate the progression of the disease. Linear measurements in the longest dimension and percentage changes in length reflect the response to treatment or the tumor progression:

Progressive disease when there is a size increase of 20%; Partial response when there is a 30% decrease of size; Stable is an increase of less than 20% and/or a decrease of less than 30% (Leffler et al., 2018).

The most used method for monitoring tumor response is ultrasonography due to its accessibility and cost-effectiveness. For the results to be reliable it is essential to have a standardized protocol, that includes having the same operator preform each examination, standardize dog's position and to have a similar level of bladder distention for each examination (Fulkerson & Knapp, 2015).

10. Prognosis

High grade invasive UCs are among the most malignant neoplasms seen in veterinary medicine, ranking alongside osteosarcomas, oral melanomas, lymphoblastic lymphomas (Meuten, 2016).

The prognosis for dogs with urothelial carcinoma in the bladder or urethra is generally poor, with less than 20% of treated dogs surviving for more than one year. Although UC is not curable, it can be controlled with a variety of treatment options. In recent years, advances in medical, surgical, chemotherapeutic, targeted delivery systems and molecular therapies have helped extend the lives of the affected dogs for several months and excellent quality of life, however survival times (ST) remain relatively short (Deborah W. Knapp & McMillan, 2012; Meuten, 2016).

TNM stage at diagnosis is the most consistent prognostic factor of UC. The advanced TNM stage has been significantly associated with shorter ST. Factors associated with a more advanced TNM stage include younger age (associated with increased risk of nodal metastasis), prostate involvement (associated with increased risk of distant metastasis), and higher T stage (associated with increased risk of nodal and distant metastasis) (Deborah W. Knapp & McMillan, 2012; Deborah W Knapp et al., 2000).

Primary location of the tumor is also important, when the primary tumor is not controlled, the urinary tract obstruction can cause death prior to the development of lethal metastasis. When the primary tumor can be controlled, metastatic disease occurs more frequently (Deborah W. Knapp & McMillan, 2012; Deborah W Knapp et al., 2000).

Previous studies have also identified tumor location within the bladder, prostate involvement, and vascular and lymphatic invasion as poor prognostic indicators (Deborah W. Knapp & McMillan, 2012; Mutsaers et al., 2003).

A recent study in 2019, found a correlation of several other prognostic factors, such as primary urethral localization, sternal lymphadenomegaly and bone metastasis with shorter ST. In this study, dogs with urethral UC had higher metastasis rates at diagnosis and shorter ST than dogs with bladder UC. Sternal lymphadenomegaly was also identified as a poor prognosis indicator

with short ST, reflecting a progressive stage of the tumor. Bone metastasis was also found to be related to shorter ST, similar to lung metastasis (Iwasaki et al., 2019).

Another study in 2021, evaluated the correlation of the BRAF mutation on canine UC and the survival times, but no significant association was found (Gedon et al., 2021).

Regarding treatment protocols, a different study compared the clinical outcomes of different treatment approaches in dogs with UC. This study reported median overall survival times of 335 days for dogs treated with medical therapy (systemic chemotherapy/COX inhibitors) and 498 days for dogs with partial cystectomy and adjuvant systemic chemotherapy/COX inhibitors (Bradbury et al., 2021).

Persistent or recurrent urinary tract infections should also be a concern when evaluating UC. These infections can enhance tumor progression and diminish treatment response, as the bacterial infection leads to marked inflammation that plays an important role promoting tumor development including initiation, promotion, malignant transformation, invasion and metastasis (Fulkerson & Knapp, 2015; Hanahan, 2022).

11. Comparative Oncology

Comparative oncology studies the occurrence of cancers in companion animals and determine its translational relevance to human cancers. Many features of naturally occurring tumors in dogs, including UC, make them an attractive model for human cancer research with many advantages over induced cancer models. Some of the advantages of dog models include, similar body size, a shared home environment, an intact immune system, spontaneous tumor formation and natural co-evolution of tumor and host stroma (LeBlanc & Mazcko, 2020).

Urothelial carcinoma in dogs is known to present key features with translational relevance to develop new treatment strategies for high-grade muscle-invasive urothelial carcinoma (InvUC) in humans, including similar histopathology, gene expression, clinical presentation, aggressive behavior, frequent distant metastasis, and response to chemotherapy (Dhawan et al., 2022; LeBlanc & Mazcko, 2020).

Despite the remarkable similarities, there are some differences between the species. Regarding its frequency and clinical presentation, the sex predilection between dogs and humans with UC is very different. In humans the ratio man versus women is 2:1, whilst in dogs is more frequent in females, with a ratio 2:1 in favor of female dogs.

Smoking is a relevant risk factor of bladder cancer in humans (Letaiová et al., 2012), a correlation with exposure to sidestream smoke and UC in dogs was recently found (D. W. Knapp et al., 2024).

The distribution of the tumor lesions within the bladder is also distinct between dogs and humans. In dogs, the most common region affected is the trigonal area and is frequent its extension to the urethra. In humans there is a more homogeneous distribution of the tumor across the bladder (Deborah W. Knapp et al., 2014).

Another difference is the histological grade. Although great similarity is noted in the microscopic features, the vast majority of human UCs are superficial and low-grade type, whilst in dogs this presentation is very uncommon. In dogs, the majority of tumors consist of intermediate to high grade UC (Deborah W. Knapp et al., 2014; Meuten, 2016).

Over the years, several grading systems have been described for this type of tumor, in human and veterinary medicine, to support the assessment of tumor behavior and patient prognosis, however, some of the created grading systems are unwieldy, unreliable and not always reproducible (Brambilla et al., 2022).

The most recent grading human system proposed by the WHO in 2016, and the similar veterinary one proposed by Meuten in 2017, (Table 4) are organized into only two grades.

Table 4 – Scheme of UC grading systems in human and veterinary medicine (Source: Brambilla et al., 2022).

Histological Grade	**Human UC** (WHO in 2016)	**Canine UC** (Meuten in 2017)
Low Grade	Slender, papillary stalks with branching and minimal fusion. Orderly appearance with easily recognizable variations in architectural and cytologic features. Variations in nuclear polarity, size, shape, and chromatin pattern. Nuclei uniformly enlarged with mild differences in shape, contour, and chromatin distribution. Nucleoli present but inconspicuous. Mitoses infrequent at any level, more frequent basally.	Papillae or flat, orderly cell to cell. Mild-to-moderate cellular atypia. Nuclear abnormalities present: enlarged nuclei, nucleoli visible, with limited to no mitoses. No invasion.
High Grade	Papillary architecture with fused papillae and branching. Predominant pattern of disorder with easily recognizable variations in architectural and cytologic features. Marked variations in nuclear polarity, size, shape, and chromatin pattern. Nuclei often pleomorphic with moderate-to-marked variation in size and irregular chromatin distribution. Nucleoli are prominent. Frequent mitoses that may be atypical and occur at any level. The thickness of the urothelium may vary, often with cell dyscohesion. May be invasive.	Papillae or flat, loss of cell polarity, disorganized growth. Marked cellular atypia. Marked nuclear pleomorphism: chromatin clumped, nucleoli prominent. Mitoses numerous, some abnormal. Invasion present, state depth of infiltration, and if UC in blood vessels or lymphatics.

While a continuous comprehensive molecular investigation and characterization of human urothelial carcinomas was able to identify several important molecular features of InvUC, the molecular characterization of canine UC is still in the early stages, especially in regard to mutation signatures and epigenetic events (Deborah W. Knapp et al., 2020).

Some similarities found were the mutation of the p53 in humans and its homolog p63 related to tumor suppression inactivation pathway, the overexpression of molecular markers such as EGFR and Cox-2. One interesting difference was the BRAF mutation status, which is common in dogs and rare in humans (Deborah W. Knapp et al., 2020).

In addition to the presence of similar biomarkers, the compressed life span of dogs, the motivation of the tutors to have cancer detected early in their dog and the availability of non-invasive screening tests offer an excellent opportunity for screening and early detection of UC in dog models (Deborah W. Knapp et al., 2020).

One of the most important areas in which dog studies can provide translational value is in treatment trials, comprising cox inhibitor trials, targeted therapy studies, epigenetic based therapy and metronomic chemotherapy (Deborah W. Knapp et al., 2020, 2014).
Some of the key characteristics concerning the treatment options presently available in humans and dogs are summarized in Table 5.

Table 5 - Treatment options for urothelial carcinoma in humans and dogs (Adapted from Knapp et al. 2020).

Type of Therapy	Human InvUC	Canine UC
Cystectomy	Cystectomy is the frontline treatment of choice in eligible patients with bladder-confined cancer. It is typically combined with neoadjuvant chemotherapy.	Cystectomy is not usually performed in dogs due to the morbidity and cost of the procedure.
Radiotherapy	Radiotherapy is used in trimodal therapies (transurethral resection, radiotherapy, and chemotherapy)	Studies to determine the efficacy of radiotherapy in dogs are limited. Trimodal therapy has not been investigated in dogs
Chemotherapy	Chemotherapy is most often used in the neoadjuvant setting and in the treatment of emergent metastasis.	Since cystectomy is rarely performed in dogs, chemotherapy is used to treat the primary cancer in the urinary tract, as well as to treat metastasis.

	Chemotherapy protocols can include methotrexate, vinblastine, doxorubicin and cisplatin.	Chemotherapy protocols in dogs include cisplatin, carboplatin, vinblastine, mitoxantrone, and others.
Cyclooxygenase (Cox) inhibitors	Cox inhibitors are not routinely used as anticancer agents in human bladder cancer.	Cox inhibitors are a mainstay of canine bladder cancer treatment, with great results. Cox inhibitors are also used to improve remission rates with chemotherapy.
Targeted agents	A fibroblast growth factor receptors (FGFR) inhibitor (Erdafitinib) is approved for use in human bladder cancer.	FGFR mutations are less common in canine bladder cancer, and agents targeting FGFR have not been tested in dogs. Other targeted therapies have been used (i.e. folate targeted therapy)
Immunotherapy	Several Immune checkpoint inhibitors have been approved for use in humans.	Immune checkpoint inhibitors are not yet available for use in dogs.

The advances on the research in dog models offers an opportunity to test new strategies in primary prevention, early detection and intervention. The results will help other dogs and ultimately humans with UC (Deborah W. Knapp et al., 2020, 2014).

PART II - Retrospective Epidemiological Study in Portugal

1. Introduction

Urothelial carcinoma is the most frequent malignant canine urinary bladder cancer. Although advances have been made in recent years, regarding the identification of the tumor and the treatment strategies, the expected outcome of this disease is still very poor. The etiology is multifactorial, and several risk factors have been identified, such as age, sex, breed and exposure to environmental pollutants (Dhawan et al., 2022; Deborah W. Knapp et al., 2020; Meuten, 2016; Mutsaers et al., 2003). By identifying the potential risk factors and closely monitoring animals at risk of developing UC, we can either prevent or manage the disease in earlier stages, which leads to better prognosis and outcomes.

2. Objectives

This epidemiological study had three main purposes:

- Find a correlation between the incidence of UCs with the clinical data of the dogs considered for this study.
- Estimate the incidence and geographical distribution of UCs in dogs diagnosed by histopathology.
- Identify potential risk factors for the carcinogenesis of UCs, related to the individual characteristics of the animals and the exposure to environmental pollutants according to their geographical area.

Complementary objectives:

- Collect data on environmental pollutants and their geographical distribution in Portugal, in order to characterize the different regions of Portugal and the risk of exposure to environmental pollutants.

3. Materials and Methods

In this study, medical records of histological diagnosis of urothelial carcinoma in dogs, diagnosed at the INNO Veterinary Laboratory, Braga, Portugal between 2017 and 2021 were retrospectively reviewed.

Histological diagnoses were made on formalin-fixed tissue samples, received by INNO Laboratory from veterinary clinics and hospitals from all of Portuguese territory including the autonomous regions of Madeira and Azores.

The histological preparation and evaluation were performed as described in Histopathological Processing and Evaluation.

From all the UC diagnosed cases, during the period between 2017 and 2021, only 26 were selected. Only dogs with diagnosed bladder UC were included in this study.

All the cases of canine UC located outside the bladder (ureters and urethra) were excluded.

The information about each dog is gathered through the histopathological form, filled in by the sending clinic or hospital. These forms have information regarding breed, age, sex, tumor location, tumor size or sample size. Some dogs were excluded from this study because most of the information was lacking.

The geographical information regarding this population was based on the location of the veterinary clinic the tutor attended to. The different locations were then grouped by its regions according to NUTTS II (Northern Portugal, Central Portugal, Lisbon Metropolitan Area, Alentejo and Algarve).

Research was conducted to find and review articles published between 2010 and 2023 on the topic of environmental pollutants in Portugal, specifically air pollutants, trihalomethanes and nitrates, to better understand the risk of exposure to these contaminants, in the various regions of Portugal.

The results were statistically analyzed using SPSS Statistics version 28.0 software and Microsoft Excel (Microsoft Office 2021, Microsoft Corporation, Washington, EUA). For categorical variables, the results were shown as absolute and relative frequency, and for continuous variables, as mean and standard deviation (SD).
The chi-square test was used to evaluate statistical associations. A result was considered significant when the P-value was less than 0.05.

Some of the variables were rearranged into new subcategories in order to minimize the results' dispersion. Pure breed and undetermined breed were the two categories used to classify the variable breed. In terms of variable age, two categories have been established: ≤11 and >11 years. The geographical area variable was split into two categories: Southern Portugal (regions of the Algarve, Alentejo, and Lisboa Metropolitan Area), and Northern Portugal (regions of the Central and Northern Portugal). The mitotic count was grouped into two categories: ≤3 and >3.

3.1. Histopathological Processing and Evaluation

The biopsies and exeresis samples, arrive at INNO laboratory within a container with a liquid fixation agent (formalin solution).

Regarding the size, the formalin-fixated samples were divided in two groups. The Biopsies group, with samples varying between 0.2cm and 0.8cm, and the Exeresis group, with samples varying between 1 cm wide and 5 x 4 x 1cm. From all the samples received at the laboratory, 13 were biopsies (50%) and the other 13 were exeresis samples (50%).

Once the tissues are properly fixated, the macroscopic examination is performed, as well as margin trimming in larger samples, to collect the most information possible from the cases.

The formalin-fixed samples are placed in suitably labeled cassettes and then processed in a tissue processor (Thermo Scientific™ Shandon Citadel 2000). Tissue processing includes a sequence of steps where the samples are dehydrated through a graded series of ethanols (up to 100%) and infiltrated with histological wax.

After processing, the samples are positioned and embedded in paraffin using an embedding center (Thermo ScientificTM HistoStar), in order to form a block that can be sectioned into 3 µm slices.

Lastly, the microtome (Thermo Scientific™ HM 325 Rotary Microtome) is used to achieve thin slices to properly evaluate the samples. Slices are then mounted on glass slides and stained with a standard hematoxylin-eosin stain (H&E).

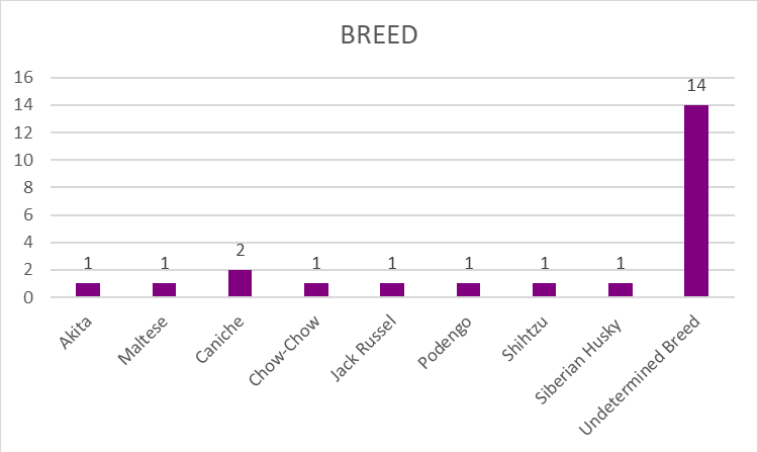
The histopathology slides are then assessed by a veterinary pathologist that evaluates the cellular atypia, mitotic count, growth pattern and invasive behavior.

Canine urothelial carcinomas are diagnosed routinely and graded, whenever is possible, as Low-grade or High-grade tumors, according to Meuten's classification scheme (Meuten 2016).

4. Results

4.1. Breed, gender, and age

From the 26 dogs included in this study, only 23 had information regarding the breed. From these 23 dogs, nine were pure breeds (39%) and fourteen were undetermined breed (61%). Within the



pure breed dogs, there were two *Caniche-Poodle* and one of the following breeds: *Akita, Maltese, Chow-chow, Jack Russel, Podengo, Shihtzu* and *Siberian Husky* (Fig. 11).

From the 26 dogs enrolled in this study, nine were males while the other 17 were females. The incidence was higher in females (65%) than in males (35%). The female to male ratio was 1.89:1. (Fig.12)

Figur n=23)

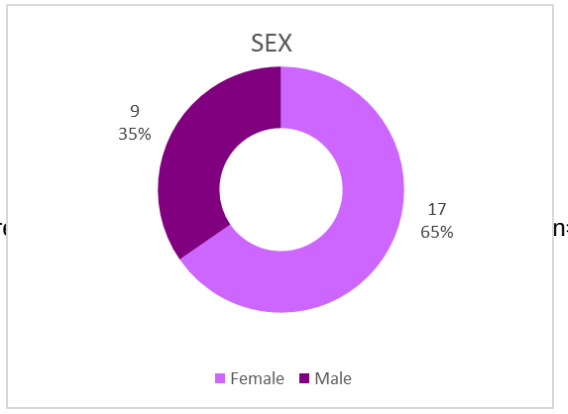


Figure 12 – Frequency distribution of the variable sex (n=26)

Considering the age, from the 26 dogs englobed in this study, only 23 had information about the age by the time of diagnosis. In these 23 dogs, the age values varied between seven and fifteen years, and the most frequent age was 11 years (Fig.13).

Half of the 23 dogs with known age, was aged between nine and thirteen years old, and the average age at the time of diagnosis was 10,8 years, with a standard deviation of 2,39 (n=23).

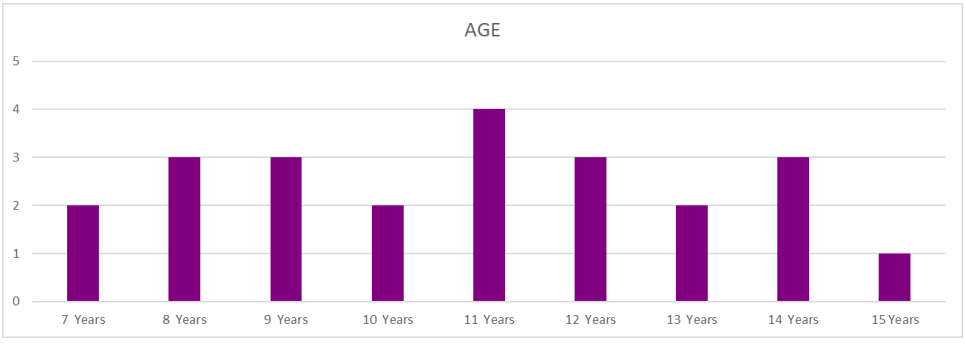


Figure 13 – Frequency distribution of the variable age at time of diagnosis. (n=23)

4.2. Histopathological Results

Histological Classification:

In this study, concerning growth pattern, seven were not possible to evaluate, due to the small sample size. The diagnosis was achieved based on the cellular morphology, but it was not possible to access the cellular arrangement and the papillary formation patterns. From the 19 cases where the growth pattern was accessed, six cases had papillary formations (32%), and which could be grouped under the papillary subtype, 13 cases didn't have papillary formations (68%), and were classified under nonpapillary subtype (n=19) (Fig.14).

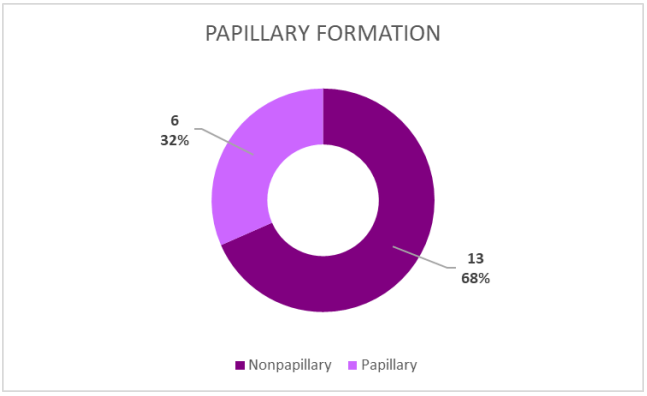


Figure 14 – Distribution of papillary formation in evaluated samples (n=19)

From the 26 samples enrolled in this study it was only possible to evaluate the infiltration in 18 samples with sufficient size (n=18). From the 18 samples with sufficient size, two of them showed noninfiltrative behavior (11%), while 16 revealed infiltration of the surrounding tissues (89%) (n=18) (Fig.15).

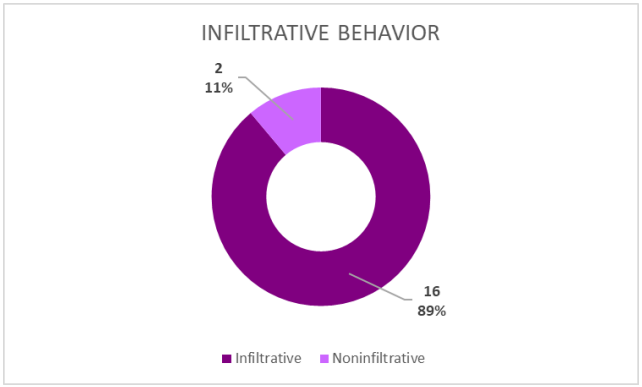


Figure 15 – Distribution of infiltrative behavior in evaluated samples (n=18)

It was only possible to evaluate simultaneously the growth pattern and infiltration in 17 of the 26 slides (n=17). The most common histological type found in 11 samples was nonpapillary and infiltrating (65%). The second most common type was papillary and infiltrating, which was found

in four samples (23%). Papillary noninfiltrating and nonpapillary noninfiltrating were detected in only one sample (6%) each (Fig. 16/17).

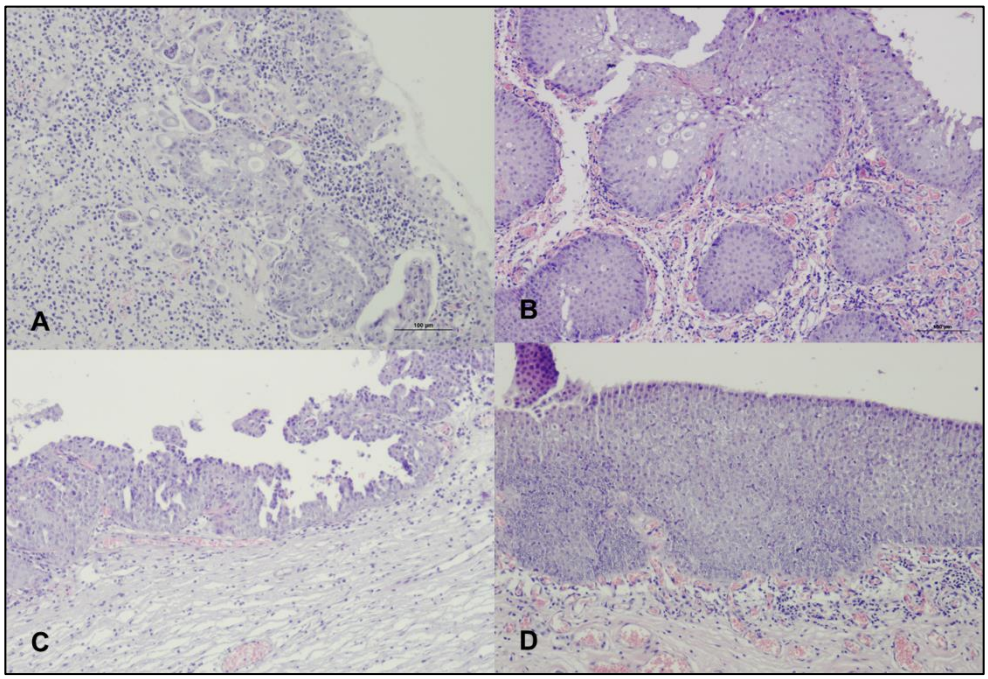


Figure 16 – Histological Types. A - Papillary and infiltrating; B - Nonpapillary and infiltrating; C - Papillary noninfiltrating; D - Nonpapillary noninfiltrating (H&E, 100x)

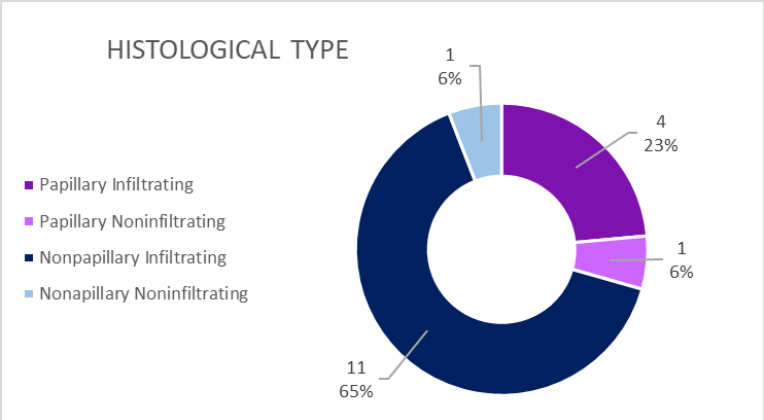


Figure 17 - Distribution of histological type in evaluated samples (n=17)

Only 15 of the 26 samples could be evaluated for the presence of mitotic figures. The samples were too small to count the mitotic figures in 10 high-power fields (HPF) (Fig.18).

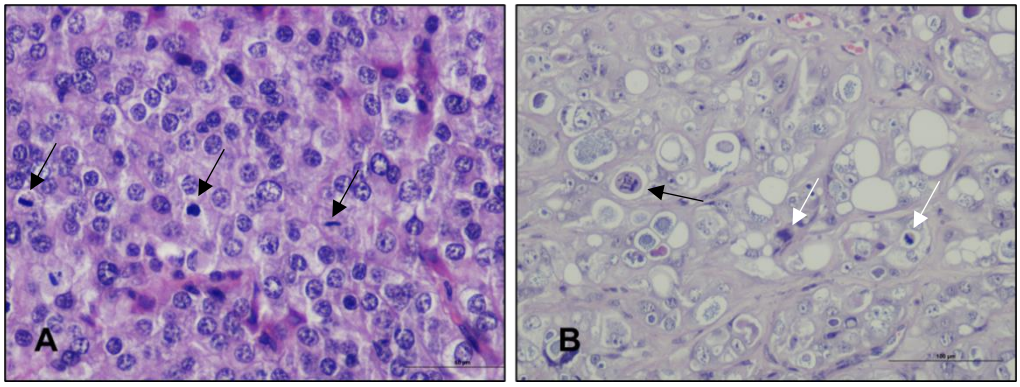


Figure 18 – A - Mitotic figures in a HPF (Arrows) (H&E, 400x); B – Atypical mitotic figure (black arrow); Mitotic figures (White arrows) (H&E, 400x)

From the 15 samples where the MC could be determined, only six (40%) presented low MC values (≤3), while the other nine (60%) showed higher MC (>3) (Fig. 19). The highest MC value was 24, and the lowest was one. The most frequent MC values were two and six. MC values varied between two and seven in half of the samples.

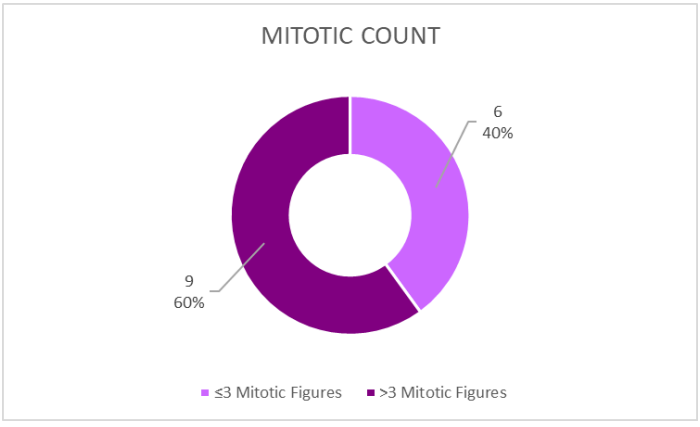


Figure 19 - Mitotic count in evaluated samples (n=15)

The histological classification was based mainly on two features of neoplastic cells: growth pattern (papillary or non-papillary) and behavior (invasive or non-invasive). When available, MC was also considered.

The small sample size of some cases, resulted in limited information regarding papillary formation, infiltrative behavior and MC, which are essential features to consider for histological grading. As a result, histological grading was only possible to determine in 19 out of the 26 analyzed slides. The histological grading resulted in four low-grade UCs (16%) and 16 high-grade UC (84%) (n=19) (Fig.20).

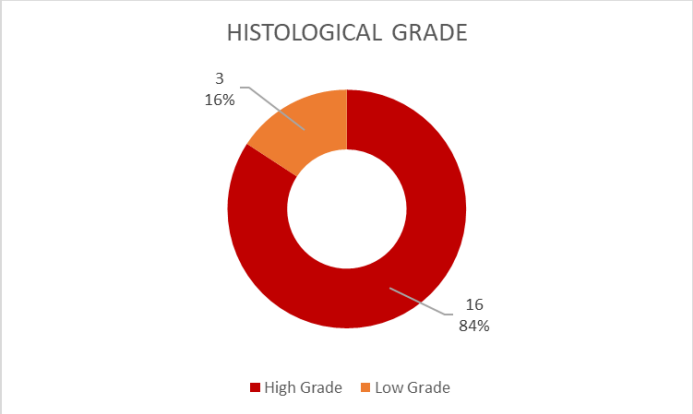


Figure 20 - Distribution of histological grade in evaluated samples (n=19)

The correlation between the individual features of the dogs included in this study (breed, sex age, the geographical location) and the histological features of the UC (growth patterns, infiltrative behavior, MC and histological grade) was analyzed.

The results are summarized in Table 6.

Table 6 – Correlation between UC histological features and the individual features of the dog

	Growth Pattern		**Infiltrative Behavior**		**Mitotic Count**		**Histological Grade**	
Individual Features	**X^2**	***p***	**X^2**	***p***	**X^2**	***p***	**X^2**	***p***
Breed	3,864	0,49	0,036	0,849	1,250	0,264	0,093	0,761
Sex	0,046	0,829	0,865	0,352	2,861	0,091	0,005	0,943
Age	0,032	0,858	0,830	0,362	0,008	0,928	1,587	0,208
Geographical area	0,277	0,599	0,117	0,732	2,861	0,091	0,882	0,348

Although there was no statistically significant correlation, it was noted that there was a tendency for higher MC in males (p=0,091) and in northern region (p=0,091).

As the histological grading relies mainly in the growth patterns and infiltrative behavior, and MC, the relationship between these histological features and the histological grading was investigated (Table 7).

Table 7 – Correlation between UC histological features and the histological grade

	Histological Grade	
Histological Features	**X^2**	***p***
Growth Pattern	1,800	0,180
Infiltrative Behavior	18,000	<0,001
Mitotic Count	1,397	0,237

The infiltrative behavior and histological grade showed a strong correlation (p<0,001). No statistically significant correlation was found between growth pattern (p= 0,180) or mitotic count (p=0,237) and the histological grade.

Concurrent primary neoplasias:

One of the 26 samples in this study was accompanied by mammary and splenic samples, which were later diagnosed as ductal carcinoma and splenic hemangiosarcoma.

4.3. Geographic Distribution

The district with the higher incidence of UC was Setúbal, with eight dogs registered (30,77%), followed by Lisboa and Coimbra with three cases each (11,54%). Beja, Braga, Faro, and Porto registered two cases each (7,69%) and Viseu, Leiria, Castelo Branco and Santarém registered only one (3,85%) (n=26).

The NUTS II distribution of Portugal's different regions revealed that the Lisbon metropolitan area had the highest incidence, with 11 cases (42%). With seven cases, the Central region of Portugal came in second (27%), and four cases were reported in the Northern region (15%). With only two cases (8%) each, Algarve and Alentejo had the lowest incidence (n=26) (Fig. 21).

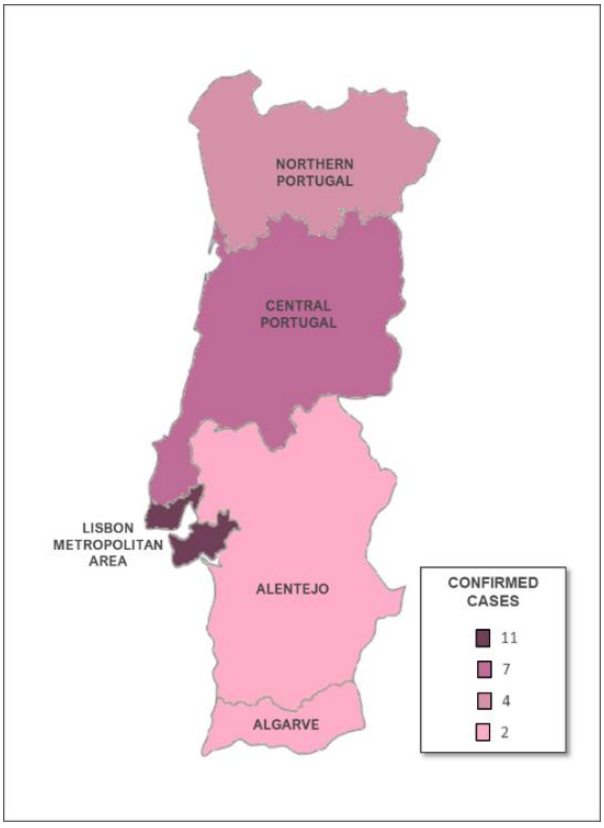


Figure 21 – Spatial distribution of incidence of UC in dogs, per Portuguese region (NUTS II) between 2017 and 2021 (n=26)

4.4. Geographical Results of Environmental Pollutants in Portugal

Air Pollutants

It is challenging to define an exposure measure that is valuable because air pollution is a complex mixture of various gaseous and particulate components (Liu et al., 2009).

The air quality index (AQI) aims to inform the public about air quality in Portugal. The index, which divides a scale of colors into five categories ranging from "Very Good" to "Bad," is a classification based on the concentrations of pollutants recorded at monitoring stations and represents the worst classification ever obtained (Table 8) (APA I.P., 2021; Monteiro, Vieira, Gama, & Miranda, 2017).

Table 8 - Air Quality Index (AQI) (µg/m^3) (Source: APA I.P. 2021)

Classification	PM_{10}	$PM_{2.5}$	NO_2	O_3	SO_2
Very Good	0-20	0-10	0-40	0-80	0-100
Good	21-35	11-20	41-100	81-100	101-200
Medium	36-50	21-25	101-200	101-180	201-350
Poor	51-100	26-50	201-400	181-240	351-500
Bad	101-1200	51-800	401-1000	241-600	501-1250

The index is calculated by taking into account the arithmetic averages of the measured values of the pollutants ozone (O_3), nitrogen dioxide (NO_2), particulate matter less than or equal to 10 m (PM_{10}), and less than or equal to 2.5 m ($PM_{2.5}$), including sulfur dioxide (SO_2) when available (APA I.P., 2021; Monteiro et al., 2017).

A study in 2017, measured the emissions of various airborne pollutants between 2009 and 2015 in the Portuguese territory. This study demonstrated that the distribution of air pollutants differs greatly from region to region (Torres et al., 2017, 2018).

Although the actual amount of pollutant gas emissions has been decreasing over the last few years, there are still quite high values in industrial areas (Fig.22)(Torres et al., 2018).

In addition to having high pollution emissions, industrial areas with dense populations like Setúbal, Lisbon, and Porto also had high air pollution concentrations (Torres et al., 2017, 2018).

According to NUTS II distribution of Portuguese territory, Lisbon metropolitan area presents the highest values of various air pollutants, followed by the Northern and Central Portugal. The regions that have maintained the lowest values of air pollutants over time are the Algarve and Alentejo (Torres et al., 2018).

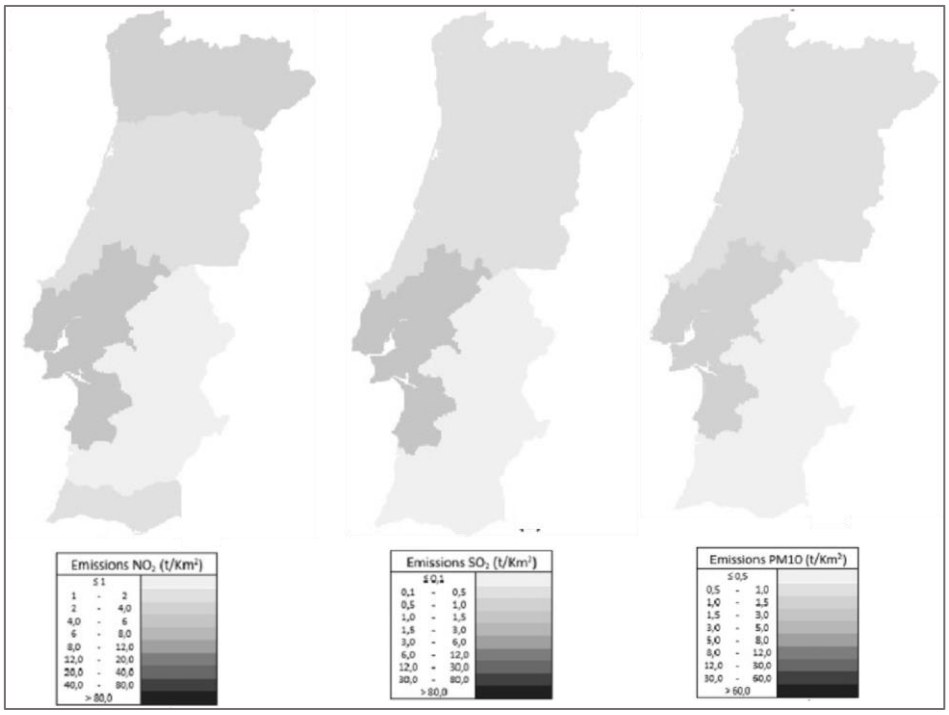


Figure 22 – Spatial distribution of emissions of several air pollutants in 2015 (t/Km^2) (Source: Torres et al. 2018)

Other recent studies have been correlating these high values of air pollutants and public health problems in certain areas of the country, mainly in metropolitan areas, even generating concern among the inhabitants of these areas (Brito, Bernardo, Zagalo, & Gonçalves, 2021; Canha, Justino, Gamelas, & Almeida, 2022; Torres et al., 2018).

Soil and Water Pollutants

- **Trihalomethanes**

The presence of trihalomethanes in the tap water of 26 European nations was examined in a recent study published in 2020 (Evlampidou et al., 2020).

According to this study, the average annual THM concentration in Portugal is 23.8 micrograms per liter. The same research showed that Portugal is one of the countries where the peak concentration of trihalomethane (THM) compounds exceeds the limit of 100 micrograms per liter permitted in the European Union (EU) (Evlampidou et al., 2020).

- **Nitrates**

There are currently nine designated nitrate vulnerable zones of agricultural origin in groundwater in Portugal (Fig.23). The nitrate concentration monitoring networks include inland surface water stations (rivers and reservoirs), groundwater stations, transitional waters, and coastal waters. This monitoring network has been kept up over the years because it focuses on areas with agricultural

activity (agriculture and livestock) in order to assess the impact of agricultural activity on the groundwater environment (DGADR & APA, 2016).

According to the 2012-2015 Report - Nitrates directive (DGADR & APA, 2016), from the total 441 groundwater monitoring stations, 148 (34%) stations are part of the vulnerable zones with concentrations over the nitrates directive's threshold (50 mg NO_3/L) (GPP, 2021). The use of groundwater for human consumption can represent a public health problem in these regions.

Figure 23 - Regions vulnerable to nitrates from agricultural sources (Source: GPP 2020)

5. Discussion

The findings of this study corroborate the majority of the previously published bibliographic information on the subject, particularly with some of specific features of the affected canine population.

Regarding the breed, there is a tendency for the undetermined breed dogs to be more affected. None of the high-risk breeds mentioned in the literature (Scottish Terrier, West Highland White Terrier, Shetland Sheepdog, Beagle, and Wire Hair Fox Terrier) were reported in this research. These findings contradict the literature (Deborah W. Knapp et al., 2020; Deborah W. Knapp & McMillan, 2012; Deborah W. Knapp et al., 2014), which can be explained by the small number of cases studied or by the more common breeds found in Portugal.

As for the sex, the females were significantly more affected than males. In our study, the female to male ratio was 1.89:1, wich corroborates with the published research that identified the females at increased risk of developing UC, with a female to male ratio between 1,71:1 to 1,95:1. (Deborah W. Knapp et al., 2014; Mutsaers et al., 2003)

The study population was between seven and fifteen years old, the average age was 10,8 years. This value is within the age range referred to in the literature as the most frequent for the appearance of this type of cancer, which is between nine and eleven years. (Deborah W. Knapp et al., 2020)

In terms of the histological patterns, papillary growth was more common (68%) than nonpapillary growth (32%). A more equitable distribution between papillary (50%) and nonpapillary (50%) types is stated in the literature, which is different from the current findings. The insufficient sample size of several slides, as well as the small population examined, might explain these results (Meuten, 2016).

As for the infiltrative behavior, infiltrative UC was predominant (89%) over noninfiltrative UC (11%), which corroborates with the literature that describes a frequency ≥90% of infiltrative tumors and ≤10% noninfiltrating tumors (Meuten, 2016).

The most common histological type was nonpapillary and infiltrating (65%), followed by papillary and infiltrating (23%), papillary noninfiltrating (6%) and nonpapillary noninfiltrating (6%). These results differ slightly from the literature that describes papillary and infiltrating type as the most common, followed by nonpapillary and infiltrating. The nonpapillary and noninfiltrating is very uncommon, according to the literature. Similarly to the other discrepancies between this study and the literature, these findings may be explained by the small population studied and the insufficient sample size of some slides (Meuten, 2016).

We considered for this study ≤3 mitotic figures as low MC and >3 mitotic figures as high MC, considering other morphological features in the slides, however in the literature exact features or cutoffs are not yet available to grade the urothelial carcinomas. The biggest obstacle to effectively assessing MC, which is expressed as the number of mitoses per HPF or 10 HPF, is the sample size (Avallone et al., 2021; Brambilla et al., 2022).

In this study, high-grade UC accounted for 84% of the cases, while low-grade UC accounted for only 16% of the cases. This results are slightly lower than literature which show a predominance of high-grade carcinomas (95%) over low-grade ones (5%) (Meuten, 2016).

No correlation between the individual features of the dogs englobed in this study and the histological features of the UC was found. This can be a result of the study's small sample size. For that reason, a correlation between these variables should not be ruled out. Further studies are needed to study the association between the individual features and the biological behavior of the tumor.

As expected, the infiltrative behavior and histological grade showed a strong correlation ($p<0,001$), however no statistically significant correlation was found between growth pattern or mitotic count and the histological grade. These findings might be explained by the current grading systems for UC, as the main malignancy criteria is infiltration of the surrounding tissues, and therefore classified as high grade (Avallone et al., 2021; Brambilla et al., 2022).

In this study, one of the cases had two secondary primary tumors (hemangiosarcoma and ductal carcinoma) which is consistent with the literature. A recent study by Knapp et al. 2020, reported that 13% of the dogs with UC presented secondary primary tumors including hemangiosarcoma, lymphoma, and other carcinomas.

In terms of geographical distribution, the metropolitan region of Lisbon had the highest incidence of UC, followed by the central and northern regions, and finally the Alentejo and Algarve, which had the fewest cases. These results corroborate with the consulted bibliography and can be attributed to areas of higher density and greater industrialization, consequently areas with higher concentrations of environmental pollutants (Hayes et al., 1981; Reif, 2011).

Despite the fact that there is no simple correlation between environmental factors (air quality index, tap water trihalomethane concentrations, and high nitrate concentrations in groundwater sources) and increased incidence of bladder tumor in dogs, these findings support the hypothesis that environmental pollutants may contribute to the occurrence of urothelial carcinoma in dogs in certain Portuguese regions.

During the development of this study, several limitations were encountered, one of the main ones was the low number of diagnosed cases. Given the wide variety of individual characteristics found in companion animals, a larger number of cases is necessary to draw conclusions that are representative of the population.

Other limitation found was the lack of relevant information provided by the veterinary practices regarding the animal's history and clinical exams, as well as the lack of follow-up information after the diagnosis in order to identify prognosis factors.

The insufficient sample size was also identified as a limitation in this study, as some of the histological features and the histological grading could not be assessed. Due to the invasiveness of the UC, exeresis is not often possible. Biopsy samples can be diagnostic, however the results are limited since some features, including infiltrative behavior, are not always assessed, impacting histological grading. Further studies are needed to establish optimal criteria for grading these small samples.

Despite the limitations discovered, most of the conclusions are consistent with the subject's published literature. It was possible to recognize the existence of specific individual characteristics leading to the development of these neoplasms as well as the most common histological types.

In the future, the goal would be to create a longer-term study, with collection of an extensive clinical data, and follow up information, minimizing/eliminating the limiting points identified in this small research.

6. Conclusion

Individual characteristics such as breed, sex, age, obesity and reproductive status, as well as exposure to certain chemical substances have been extensively described in the literature as risk factors in the development of UC in dogs as well as humans (Glickman et al., 1989; Deborah W. Knapp et al., 2020; Deborah W. Knapp & McMillan, 2012; Deborah W. Knapp et al., 2014; Deborah W Knapp et al., 2000, 2013; Meuten, 2016; Mutsaers et al., 2003; Raghavan et al., 2004a).

This study intended to analyze the risk factors for the carcinogenesis of UCs, related to the individual characteristics of the animals and the exposure to environmental pollutants in Portugal. The results suggest that adding to the individual characteristics, environmental pollutants might play an important role in the carcinogenesis of urothelial carcinoma in dogs.

Further studies are required to clearly identify risk factors and better understand carcinogenesis mechanisms of UC in dogs. It is becoming clear that addressing the issue of soil, water, and air-borne pollutants, is critical to reduce the risk of developing urothelial carcinoma in dogs.

Insights from these studies are needed to develop new preventive strategies, explore early detection and intervention approaches and more effective treatments, improving the prognosis and outcomes.

There is great potential for expanding this research with a significant translational value for human health, helping other dogs and ultimately humans with urothelial carcinoma.

7. References

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