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Escola de Veterinária
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Pedro Antônio Bronhara Pimentel

CYTOLOGICAL SCORE OF CHEMOTHERAPEUTIC RESISTANCE, CLINICAL ASPECTS, AND EPIDEMIOLOGY OF CANINE TRANSMISSIBLE VENEREAL TUMOUR

Escore citológico de resistência quimioterápica, aspectos clínicos e epidemiológicos do tumor venéreo transmissível canino

Belo Horizonte
2024

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Advisor: Prof. Dr. Rodrigo dos Santos Horta

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Dr.(a). Rodrigo dos Santos Horta (Orientador)
Dr.(a). Renato de Lima Santos
Dr.(a). Elizabeth Proby Murchison
Dr.(a). Antônio Giuliano

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“Era um, era dois, era cem.
Mil tambores e as vozes do além (…)
E o futuro nas mãos do menino,
Batucando por fé e destino (…)
Os tambores de Minas soarão.”

Milton Nascimento

RESUMO

O tumor venéreo transmissível canino (TVTC), anteriormente denominado tumor de Sticker, destaca-se como a neoplasia com a mais notável capacidade adaptativa já documentada, envolvendo evasão do sistema imunológico, estabilidade genômica e transmissão por implantação tumoral. Este é o único tipo de neoplasia cuja linhagem somática perdura há milhares de anos e embora seja geralmente reconhecido por se manifestar na forma genital e se propagar através de contato sexual, o TVTC é notavelmente mais complexo, apresentando manifestações extragenitais e metástases. A morfologia celular compatível com tumor de células redondas, torna a citologia um método diagnóstico ideal, e as relações entre características prognósticas e preditivas com a citologia ainda carecem de estudo e são temas de discussão. Este estudo analisou os casos de TVTC registrados no Hospital Veterinário da Universidade Federal de Minas Gerais no período de 2012 a 2022, estabelecendo correlações clínicas, terapêuticas e epidemiológicas. Foram examinados 131 casos quanto a manifestações clínicas e dados epidemiológicos. Dentre esses, 40 casos apresentaram informações completas para a comparação da resposta terapêutica e características citológicas. As manifestações clínicas mais comuns foram as genitais (n= 114/131; 87%), seguidas das cutâneas (n= 14/131; 11,5%), nasais (n= 8/131; 6,1%) e orais (n= 6/131; 4,6%). Cães machos mostraram associação com o TVTC oronasal, apresentando um risco 5,2 vezes maior dessa manifestação em comparação com fêmeas. Além disso, cães de raça pura exibiram um risco significativamente aumentado para o TVTC nasal, com uma probabilidade 8,2 vezes maior. Uma escore citológico de quimiorresistência à vincristina no TVTC foi proposto considerando três critérios: anisocariose, contagem mitótica e células binucleadas. Todas as características foram padronizadas em análise em 5 hot spots, e os critérios de malignidade foram inversamente correlacionados com a quimiorresistência ($p = 0.0013$). O presente estudo sugere que a citologia pode ser utilizada como um método de detecção precoce de resistência quimioterápica ao sulfato de vincristina, estando indicado o uso do escore citológico de resistência para essa predição.

Palavras-chave: tumor de Sticker; vincristina; quimiorresistência; neoplasia; contágio.

ABSTRACT

Canine transmissible venereal tumour (CTVT), formerly known as Sticker's tumour, stands out as the neoplasm with the most remarkable adaptive capacity ever documented, involving evasion of the immune system, genomic stability, and transmission through tumour implantation. This is the only type of neoplasia whose somatic lineage has persisted for thousands of years, and although it is generally recognized for manifesting in genital form and spreading through sexual contact, CTVT is notably more complex, exhibiting extragenital manifestations and metastases. The cellular morphology consistent with a round cell tumour turns cytology into an ideal diagnostic method, and the relationships between prognostic and predictive features with cytology still lack study and are subjects of discussion. This study analysed cases of CTVT recorded at the Veterinary Hospital of the Universidade Federal de Minas Gerais from 2012 to 2022, establishing clinical, therapeutic, and epidemiological correlations. A total of 131 cases were examined for clinical manifestations and epidemiological data. Among these, 40 cases had complete information for the comparison of therapeutic response and cytological characteristics. The most common clinical manifestations were genital (n=114/131; 87%), followed by cutaneous (n=14/131; 11.5%), nasal (n=8/131; 6.1%), and oral (n=6/131; 4.6%). Male dogs showed an association with oronasal CTVT, presenting a 5.2 times higher risk of this manifestation compared to females. Additionally, purebred dogs exhibited a significantly increased risk for nasal CTVT, with an 8.2 times higher probability. A cytological score for vincristine chemoresistance in CTVT was proposed, considering three criteria: anisokaryosis, mitotic count, and binucleated cells. All characteristics were standardized in the analysis of 5 hotspots, and malignancy criteria were inversely correlated with chemoresistance ($p = 0.0013$). The present study suggests that cytology can be used as an early detection method for chemotherapy resistance to vincristine sulfate, with the use of the cytological resistance score indicated for this prediction.

Keywords: Sticker's tumor; vincristine; chemoresistance; neoplasia; contagion.

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ABREVIATIONS LIST

ABCB1	ATP-binding cassette sub-family B member 1
CR	Complete response
CTVT	Canine transmissible venereal tumour
DNA	Deoxyribonucleic acid
HDI	Human development index
MDR1	Multidrug resistance protein 1
MHC	Major histocompatibility complex
NOD/SCID	Nonobese diabetic severe combined immunodeficient
PCR	Polymerase chain reaction
PD	Progressive disease
PR	Partial response
PT	Progressor tumour
RNA	Ribonucleic acid
RT	Regressor tumour
SD	Stable disease
UFMG	Universidade Federal de Minas Gerais
VH	Veterinary Hospital

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1. INTRODUCTION

Canine transmissible venereal tumour (CTVT) is a contagious neoplasm, and its tumoral lineage has persisted for approximately 4,000 to 8,500 years through continuous transmission among canids (Baez-Ortega et al., 2019). The origin of this tumour can be traced back to a canid, possibly from Asian Siberia, with low heterozygosity. The population maintained the transmissible tumour for thousands of years until it began spreading to Europe and the Indian subcontinent approximately 1,000 to 2,000 years ago (Baez-Ortega et al., 2019; Strakova & Murchison, 2014). Around 500 years ago, concurrent with European colonialism, this neoplasm expanded to the Americas and subsequently to the rest of the world, with the dissemination of various tumour lineages facilitated by globalization and increased human migratory flows (Murchison et al., 2014; Strakova & Murchison, 2014). Currently, only a few countries are considered free from autochthonous cases of CTVT (Strakova & Murchison, 2014). However, modern migration flows, such as those from Eastern Europe to Western Europe and from Latin America to the United States, poses a significant potential risk for altering the disease status in these countries (Gibson et al., 2021; Hayes et al., 2023). CTVT is deemed endemic in low-income countries, typically linked to inadequate control of dog populations and unrestricted outdoor access (Gritzenco et al., 2022; Parikh et al., 2023; Tella et al., 2004).

The cytogenetic origin of the tumour is considered mesenchymal due to positive vimentin staining (Araújo et al., 2012), possibly indicating a histiocytic lineage, although this is a topic of much debate (Marchal et al., 1997; Mascarenhas et al., 2017). Positive lysozyme staining may suggest a histiocytic origin, but different studies have reported conflicting results, possibly due to confusion between tumour cells and macrophages (Mascarenhas et al., 2017). The positively marked ACM1 antigen, typical of macrophages, in CTVT cells is another indicator of this origin (Marchal et al., 1997), along with the presence of amastigote forms of Leishmania sp. identified in the tumour cells (Carreira et al., 2014).

This tumour commonly manifests in the genital region, affecting the vulva, vagina, penis, and prepuce of dogs (Araujo et al., 2016; Costa, Paiva, et al., 2023). Cutaneous, nasal, and oral manifestations, linked to contagion, also occur and are considered the most common extragenital manifestations (Ignatenko et al., 2020; Pimentel et al., 2021; Ramadinha et al., 2016). Genital presentations are well-documented, constituting 85-95%

of cases, while extragenital manifestations, more often reported in the last decade, vary in frequency across studies, but may be underreported (Huppes et al., 2014; Valençola et al., 2015). The primary site of metastasis is regional lymph nodes, occurring in less than 5% of cases (Araujo et al., 2016; Peixoto et al., 2016).

Cytological characterization of CTVT reveals round to oval cells with cytoplasm containing vacuoles and slightly basophilic staining, and dense nuclear chromatin (Amaral et al., 2007; Duzanski et al., 2022; Fêo et al., 2016). Previous studies have proposed a cytomorphological classification and attempted to predict chemotherapy resistance. The tumour was classified into three categories based on the shapes of plasmacytic and lymphocytic cells. However, different studies exhibit disparities in the prevalence of each type and its significance remains unclear (Chowdary et al., 2016; Paranzini et al., 2015; Setthawongsin et al., 2018).

Vincristine sulphate is widely acknowledged as a standard treatment for CTVT, usually given at a dose of 0.5-0.7 mg/m² weekly for up to six sessions (Bulhosa et al., 2020; Costa, Paiva, et al., 2023). While most dogs exhibit a complete response after its administration, some may require additional doses or a change in the treatment protocol. Despite various attempts involving different drugs, radiotherapy, and surgery, there is currently no consensus on the optimal approach for managing cases resistant to vincristine (Idowu, 1984; Parikh et al., 2023; Rogers et al., 1998).

2. LITERATURE REVIEW

2.1. Etiopathogenesis

As the oldest somatic lineage of cells in continuous transmission, the etiopathogenesis of CTVT is intriguing and represents a successful attempt of natural selection in tumour biology, a cancer that survived ages. Two centuries ago in England, Blaine characterized clinically the CTVT as a fungoid excrescence, a cureless cancer (Blaine, 1810). In the following century in Germany, Sticker described and transplanted CTVT and for this, literature turned the previously infective tumour/ infective sarcoma into the eponym Sticker's tumour/ Sticker's sarcoma (Barski & Cornefert-Jensen, 1966; Loeb, 1904; Smith & Washbourn, 1897).

A plethora of names has been used to describe the CTVT, as described in Table 1 below. In order to unify the nomenclature, BLOOM et al., 1951 suggested to standardize the term as transmissible venereal tumour. The addition of "canine" is gaining momentum in the last decades and clarify the population affected by this neoplasia.

Table 1. Alternative nomenclatures of canine transmissible venereal tumour in literature

Name	Year	Location	Reference
Canine transmissible venereal sarcoma	1981, 1987, 2000	India, United States	Das & Das, 2000; Palker & Thomas Yang, 1981; Yang et al., 1987
Canine transplantable lymphosarcoma	1904	Germany	Sticker, 1904
Canine venereal lymphoma	1973	Chile	Hernández-Jáurequi et al., 1973
Infectious lymphosarcoma of dogs	1906	United States	Beebe & Ewing, 1906
Infective sarcomata	1898	United Kingdom	Smith & Washbourn, 1898
Infective venereal tumour	1897	United Kingdom	Smith & Washbourn, 1897
Polyp	1963	Japan	Makino, 1963)
Sticker sarcoma	1970, 2012, 2020	Algeria, Germany, Hungary	Hithem et al., 2020; Sellyei et al., 1970; Wunderlich, 2012
Sticker's venereal sarcoma	1966	France	Barski & Cornefert-Jensen, 1966
Sticker's lymphossarcoma	1904, 1987, 2000	Brazil, United States	Degu et al., 1987; Loeb, 1904; Sousa et al., 2000)
Sticker's tumour	1968, 2006, 2016	Brazil, Cuba, Jamaica	Rocha et al., 2016; Herrera et al., 2006; Thorburn et al., 1968)

Currently, CTVT is classified as a round-cell tumour, like lymphomas, mast cell tumours, histiocytomas, and plasmacytomas (Araújo et al., 2012). It exhibits negativity for monoclonal CD117/tyrosine-kinase, a cluster of differentiation characteristic of mast cells (Araújo et al., 2012; Mascarenhas et al., 2017), as well as negativity for CD3 and CD79a, standard immunophenotyping markers for T and B cell lymphomas, respectively (Araújo et al., 2012; Flórez et al., 2016; Mascarenhas et al., 2017). Notably, it is positive for vimentin, a mesenchymal cell marker also positive in histiocytic sarcoma, but negative on histiocytomas (Araújo et al., 2012; Flórez et al., 2016; Mascarenhas et al., 2017). The question of what is the CTVT origin is still a subject of debate. Some authors defend a

hypothesis of lymphoid origin (Mascarenhas et al., 2014, 2017), but most believe it is derived from a cell of mononuclear phagocytic system, a histiocytic line (Ajayi et al., 2018; Bloom et al., 1951).

Throughout its history, neutral genetic drift appears to play a guiding role in the evolution of CTVT. This process stems from a basal trunk, with PTEN and CDKN2A identified as potential early driver genes (Baez-Ortega et al., 2019). The prevalence of cytosine to thymine substitutions characterizes the mutation pattern in CTVT (Murchison et al., 2014), suggesting a potential link to ultraviolet radiation exposure. This association is supported by Bayesian logarithmic regression analysis, which took into account latitude and mutations within distinct sublineages of the tumour (Baez-Ortega et al., 2019).

A stable genomic rearrangement present in CTVT somatic lineage occurs in the c-MYC oncogene from a long interspersed nuclear element (LINE), which is a useful method of diagnosis from quantitative PCR (Castro et al., 2017; Setthawongsin et al., 2016). CLL5 is protein coding gene also very important for CTVT research. It is a driver of regression after vincristine exposure, inducing anti-tumour immune response (Frampton et al., 2018).

The biological behaviour of CTVT categorizes it as a malignant neoplasm, specifically a transplantable cancer (Ke et al., 2022; Sticker, 1904). Despite some studies erroneously characterizing it as a benign neoplasm, such an attribution is only applicable to tumours lacking the capability of distant dissemination (Tompkins et al., 2020). In this context, two primary mechanisms of dissemination exist: metastasis and transmission through implantation.

Implantation through sexual transmission is clearly defined and it occurs during the friction of genital structures during canine coitus. This process detaches cells from the friable tumour and provides new sites for implantation. In the second stage of coitus, the copulatory tie, the penile bulb swells, connecting both dogs for around 25 minutes (Pal, 2011). This prolonged connection may facilitate the adequate implantation of cell groups. This process is typical of dogs and might have favoured the development and spreading of CTVT through generations in this specific species.

Other modes of tumour dissemination through implantation are not fully understood, but certain hypotheses are prevalent. The transmission may not be restricted solely to genital mucosa but could potentially occur in any mucosa, immunosuppressed or injured tissue with lower resistance to CTVT implantation (Cohen, 1973). In cases of cutaneous presentation, transmission may occur through activities such as licking,

sniffing, and biting from dogs with nasal or oral manifestations to dogs with skin lesions. In instances of oronasal manifestations, transmission is anticipated to take place through sniffing and licking behaviours, particularly from dogs with genital CTVT, with male dogs more commonly exhibiting this behaviour due to the prevalent nature of genital sniffing (Strakova et al., 2022).

Multiple studies have successfully achieved allogenic transplantation of CTVT cells in dogs. The majority utilized subcutaneous inoculation of cell samples (Hsiao et al., 2002; Pal, 2011). Moreover, successful intracranial transplantation has been reported for brain tumour ablation therapy testing (Ahrar et al., 2010; Schwartz et al., 2009), although ethical considerations may arise. Xenografts in murine models (NOD/SCID) have proven successful and should be considered as a viable alternative to studies involving non-natural implantation of CTVT in dogs (Harmelin et al., 2001; Ke et al., 2022).

Metastatic processes in CTVT remain inadequately elucidated. Metastasis is an intricate process wherein cells from a primary cancer disseminate to other tissues. It manifests in various forms, with cell dissemination occurring through monoclonal or polyclonal seeding and monophyletic or polyphyletic evolution (Turajlic & Swanton, 2016). Many types of neoplasms have distinct routes for metastasis. Generally, mesenchymal tumours are more inclined toward endothelial vascular metastasis to organs like the lungs, as observed in osteosarcomas (Wilk & Zabielska-Koczywas, 2021). Conversely, epithelial tumours tend to metastasize through lymphatic endothelial vessels to regional lymph nodes, a characteristic seen in mammary gland tumours (Collivignarelli et al., 2021). Transcoelomic spread, utilizing intracavitary routes for metastasis, is less common but represents a significant dissemination mechanism, particularly notable in ovarian cancer (Barbolina, 2018). On CTVT, it is postulated that metastasis may arise from a monoclonal lineage characterized by monophyletic seeding, given the genomic stability observed over millennia despite the existence of numerous distinct lineages (Murchison et al., 2014). These lineages are group-specific, displaying genomic distinctions across geographical regions and populations (Baez-Ortega et al., 2019). Consequently, each individual tends to exhibit only one specific monoclonal tumour lineage.

The metastasis of CTVT appears to be correlated with the immune conditions of the host. In experimental conditions, all dogs subjected to whole-body irradiation (200 rads, 220 kV, and 15 mA) developed the tumour within 17-24 days post-transplantation through subcutaneous injection. Additionally, 75% (6/8) of these dogs progressed to

metastasis within 100-240 days post-transplantation (Cohen, 1973). Following xenotransplantation in immunosuppressed mice and subsequent inoculation in dogs, the CTVT exhibited rapid growth and underwent transcriptome reprogramming (Ke et al., 2022). Infectious systemic diseases, such as visceral leishmaniasis and hemoparasitosis, may influence the antigenic recognition of CTVT, potentially impacting the effectiveness of the acquired immune response against the neoplasm (Marino et al., 2012).

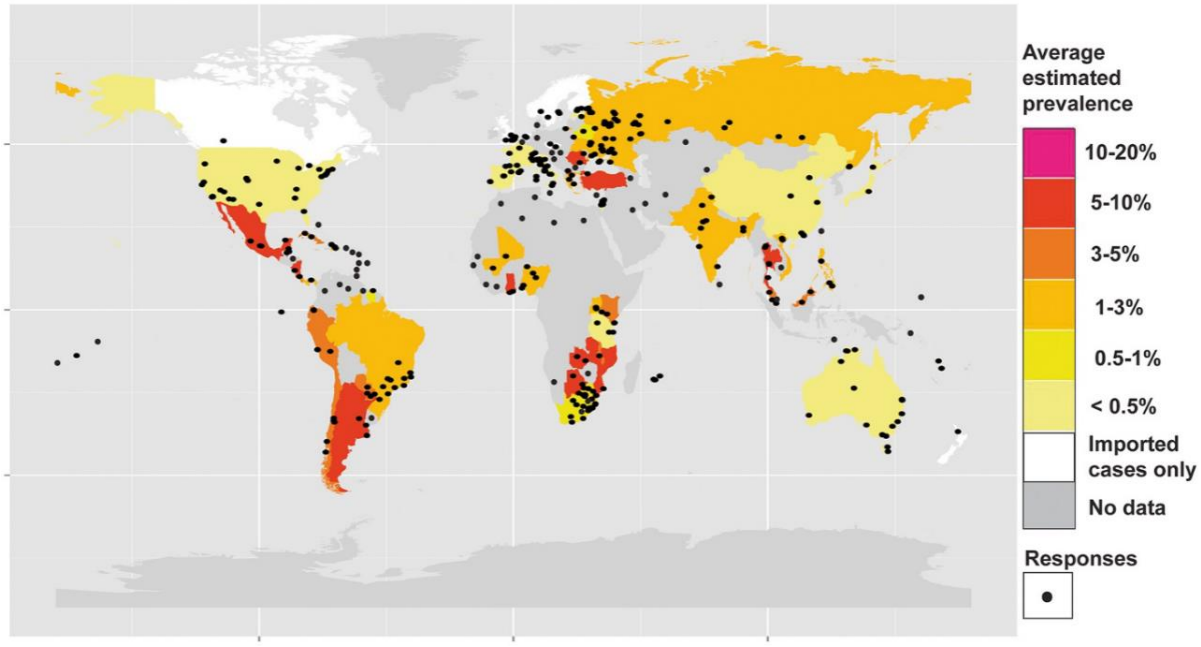
Tumour growth stage classifications are available in two tiers: progressor tumour (PT) and regressor tumour (RT) (Duzanski et al., 2022; Yang et al., 1987). Clinically, PT was defined as a constantly increasing in volume tumour and RT as a tumour decreasing in volume, rapidly or slowly (Yang et al., 1987). In histopathology, PT is characterized by high cellularity with low stromal density and RT as moderate to high stroma proliferation (Duzanski et al., 2022).

The absence of major histocompatibility complex (MHC) antigen expression in the tumour serves as a significant immunologic escape mechanism for CTVT. Consequently, this characteristic was analysed according to the growth stage of CTVT. PT lacks both Class I MHC and Class II MHC expression, whereas RT exhibit positive expression, with 31.5% for Class I MHC and 36.4% for Class II MHC (Yang et al., 1987). Recently, Duzanski et al. (2022) identified similar results; however, they observed that PT exhibited low expression of MHC, specifically 6.9% for Class I MHC and 4.1% for Class II MHC. In contrast, RT demonstrated a higher expression, with 18.6% for Class I MHC and 38.5% for Class II MHC.

2.2. Epidemiology

The worldwide distribution of CTVT has been reported in recent decades, as shown in Figure 1 (Strakova & Murchison, 2014). The risk factors for CTVT include low dog population control, including absence or ineffective laws, low rates of neutering, and high rates of dogs with unrestricted outdoor access (Costa, Paiva, et al., 2023; Ishengoma et al., 2018; Schectman et al., 2022).

Figure 1. Global distribution of CTVT by country, as reported by veterinarians from a clinical-epidemiological questionnaire.



Source: Strakova & Murchison, 2014.

In Western European countries, such as England, Scotland, North Ireland and Germany, autochthonous cases of the tumour are rare and often absent in surveys of canine neoplasia (Aupperle-Lellbach et al., 2022; Gibson et al., 2021). The eradication of local transmission of CTVT cases is closely linked to population control measures and reducing the population of dogs with unrestricted or partial access to the outdoor environment (Strakova & Murchison, 2014). However, human migration flows and international dog adoptions from endemic regions of the disease to non-transmission areas represent a risk for epidemics and the emergence of new clusters of cases. A retrospective study from the last decade (2010-2019) in the United Kingdom registered a recent flux of imported cases from Eastern European countries, mostly from Romania (81%), but also from Serbia (6%) and Greece (3%), Spain in Western Europe (3%), Gambia in West Africa (3%), and China in East Asia (3%) (Gibson et al., 2021). In a retrospective study, the single case identified in Germany involved a patient who had contact with a rescued Romanian dog that previously presented genital CTVT (Ignatenko et al., 2020a).

In most of North America, CTVT is either undetected or rare. Canada exhibits a similar disease pattern to Western Europe, with reported cases mostly of imported origin

(Mikaelian et al., 1998). In the United States, the majority of CTVT cases are found in states bordering Mexico, such as Arizona and Texas, and are often related to imported cases (Parker et al., 2021; Rogers et al., 1998). In contrast, CTVT is highly prevalent in Mexico, as in Toluca, a metropolitan area near the capital of Mexico, where it is the most frequent mesenchymal neoplasm, accounting for 26% of cases, and the most common reproductive tumour (García et al., 2019).

While data may be limited in certain countries, it seems that CTVT is effectively managed in Japan and Oceania. Once focus of analyses, there has been a significant decline in the number of articles reporting CTVT cases in Japan over recent decades (Hataya et al., 1958; Kok et al., 2019; Watanabe & Azuma, 1956). Australian cases of the tumour are rare as well and poorly described recently (LOCKE; YEH; HOOPER, 1975; Murchison et al., 2014).

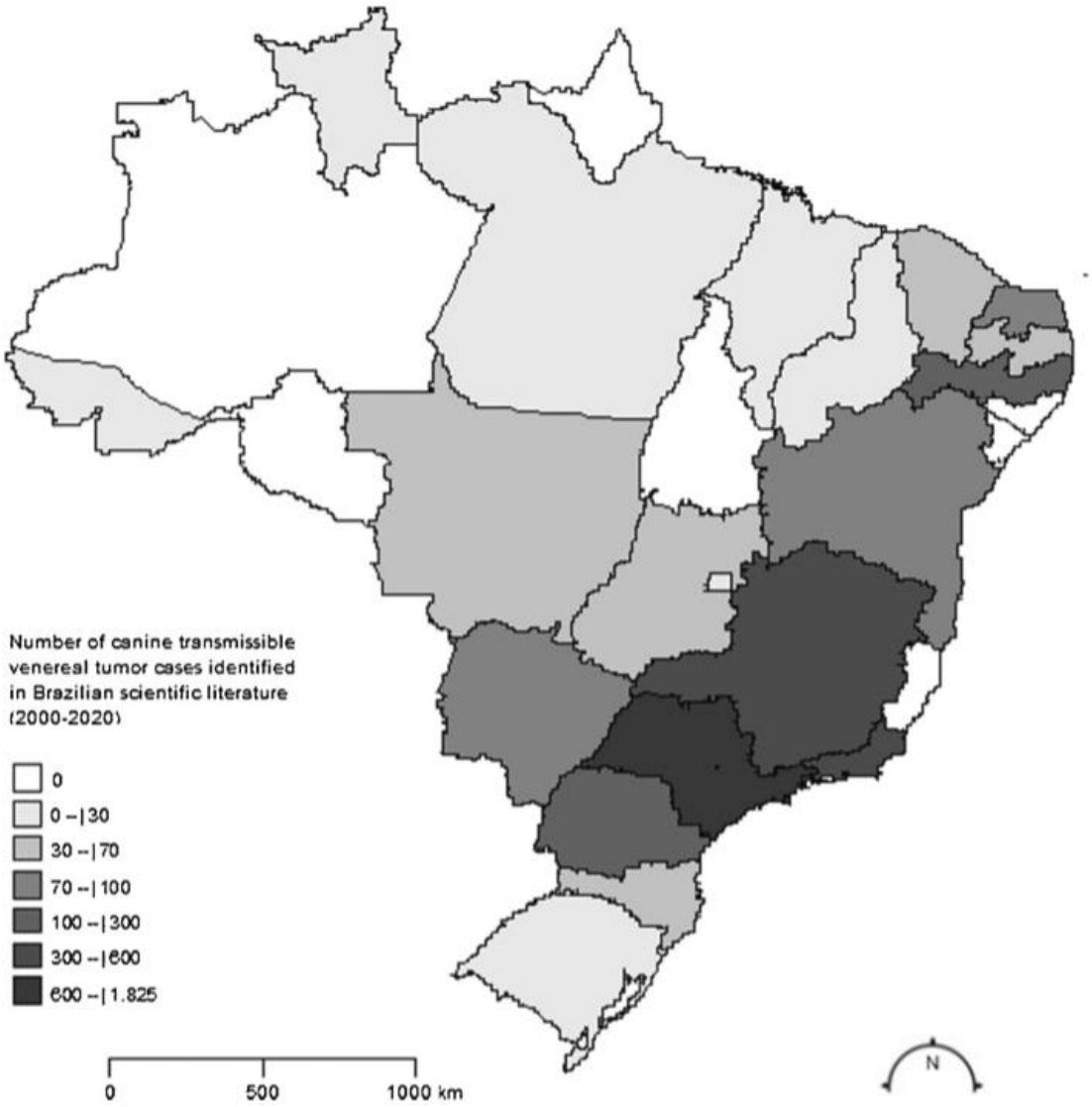
In countries across South and Central America, Africa, and the southern regions of Asia, there are risk factors for the spread and endemic maintenance of this disease. In Indian regions such as Gujarat, incidence of this neoplasia varied between 7.4% and 10.2% among all reproductive disorders in dogs (Parikh et al., 2022), and in the Morogoro region, Tanzania, 12% (36/300) of all examined dogs showed clinical manifestations of the tumour (Ishengoma et al., 2018). In Granada, Central America, the incidence of the tumour was lower, at 1.4% of the total dog population (Schectman et al., 2022).

Despite its high prevalence in low-income countries, it was even more widespread in domestic dogs decades ago. In 1968, Murray reported a frequency of 12.4% (20 out of 161) of CTVT in dogs in Kenya, which is similar to what is observed in Tanzania nowadays (Ishengoma et al., 2018; Murray, 1968). It was defined as the most frequent canine tumour in Jamaica in 1968 (Thorburn et al., 1968), Mexico in street dogs in 2010 (Pineda 2010), and still the most common tumour in Nigeria, exceeding in three times the sum of all other tumours in dogs (Raymond & Matondo, 2018).

Numerous studies describe CTVT in Brazilian metropolises, with higher frequency in the Southeast region, which may be biased due to larger number of scientific studies in that region (Pimentel et al., 2021). Research groups in São Paulo state have provided insights into the population of dogs with comprehensive retrospective studies (Amaral et al., 2004; Brandão et al., 2002; Fêo et al., 2016), shedding light on extragenital manifestations that might have been previously underdiagnosed. In Rio de Janeiro state, cases are frequently documented in areas such as Seropédica and the city of Rio de Janeiro (Araujo et al., 2016; Costa, Paiva, et al., 2023; Mascarenhas et al., 2014). In Minas Gerais

state, a single retrospective study provides information concerning cases in Uberaba (Huppes et al., 2014). While there are no reports of CTVT in every Brazilian state (e.g.: Amazonia and Espírito Santo), it is suggested that the disease is endemic nationwide, encompassing all regions and biomes, with over 3,500 cases reported between 2000 and 2020 (Figure 2) (Bulhosa et al., 2020; Kimura et al., 2012; Pimentel et al., 2021; Valençola et al., 2015; Valladão et al., 2010).

Figure 2. Map of the geographical distribution of documented cases of CTVT in Brazilian states, from 2000 to 2020



Source: Pimentel et al., 2021.

Although several Brazilian studies report CTVT, few studies have characterized its

incidence in Brazil. When examining dogs with various neoplasms, the frequency of CTVT varies from 0.9% to 17.1% according to the diagnostic method used (Amaral et al., 2004; Kimura et al., 2012; Rossetto et al., 2009; Viana et al., 2019). The diagnostic method is an important bias to consider in epidemiological studies because CTVT is commonly diagnosed through cytology (Noeme Sousa et al., 2014). Therefore, studies that focus on cytological examinations tend to overrepresent CTVT cases, while those that primarily analyse histopathology tend to underrepresent.

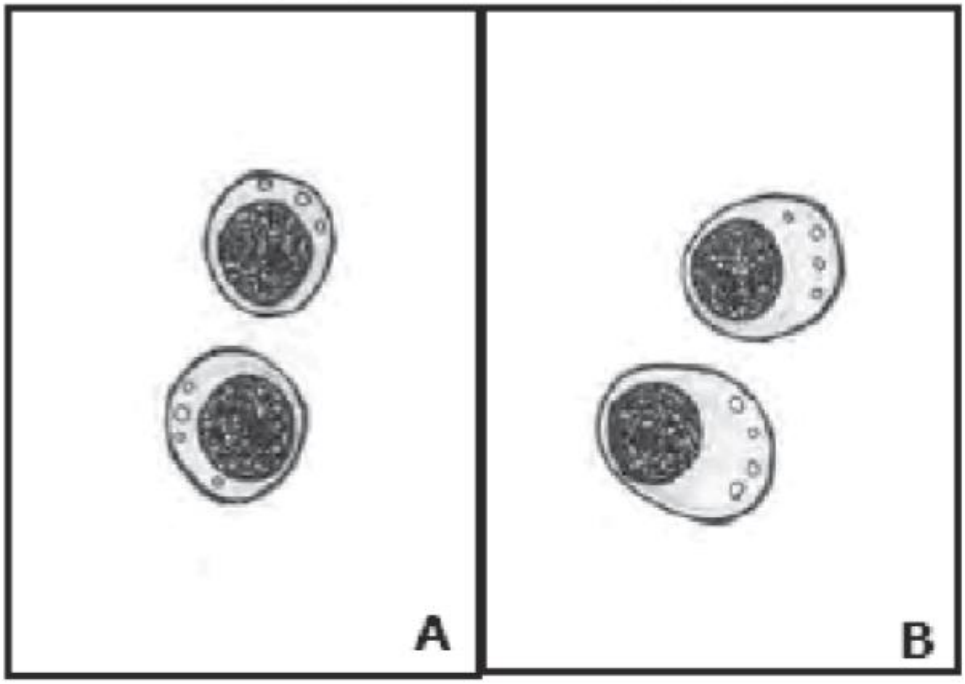
2.3. Diagnosis

The diagnosis of CTVT has evolved with increased recognition as a round-cell tumour. Historically, studies predominantly relied on histopathology for diagnosis until recent decades (Harmelin et al., 1995; Athar et al., 2001; Singh et al., 1996; Tella et al., 2004; Thrall, 1982) However, histologically it can be easily confused with lymphomas or histiocytic tumours. This confusion could be mitigated by analysing the cells in isolation, specifically through cytology, which is now acknowledged as the gold-standard method for CTVT diagnosis (Bulhosa et al., 2020; Costa, Paiva, et al., 2023; Sousa et al., 2014).

In a cytologic preparation, CTVT cells typically exhibit specific morphological features: they are predominantly round to oval in shape; have cytoplasm with distinct borders; round to ovoid-shaped nuclei that occasionally have abnormal features such as eccentric or paracentral disposed nuclei with coarse chromatin; one or no prominent nucleolus, uncommonly multiple; small to intermediate cytoplasmic vacuoles; lightly basophilic cytoplasm; and variable nucleus/cytoplasm ratio, according to the cytomorphology (Amaral et al., 2007; Costa et al., 2022; Reis-Filho et al., 2020). Atypia, including mitotic figures, binucleated cells, karyomegaly, anisocytosis, and anisokaryosis, are common (Valençola et al., 2015).

Previous studies proposed a three-type cytological categorization for canine CTVT based on the morphology of plasma cells and lymphocytes (Amaral et al., 2007; Fonseca et al., 2012). Plasmacytic cytomorphology is characterized by oval-shaped cells and a low nucleus/cytoplasm ratio, while lymphocytic cytomorphology is identified by round cells and a high nucleus/cytoplasm ratio. The mixed group includes tumour cell populations with relatively equal proportions of plasmacytic and lymphocytic cells, with neither type exceeding a 60% frequency, as demonstrated in Figure 3 (Amaral et al., 2007).

Figure 3. Cytomorphological subtypes of canine transmissible venereal tumour: Lymphocytic (A) and plasmacytic (B).



Source: Amaral et al., 2007.

A following study present the plasmacytic type as associated with vincristine sulphate chemoresistance (GASPAR et al., 2010), possibly related to increased expression of P-glycoprotein and increased drug efflux. Although, other studies have not demonstrated this association in other regions (Setthawongsin et al., 2018).

Cytological grading schemes have been validated for specific canine neoplasms, particularly mast cell tumours, utilizing mitotic figures and binucleation as criteria for malignancy (Camus et al., 2016). These serve as prognostic factors and are correlated with histopathological grading (Camus et al., 2016; Paes et al., 2022). In the context of CTVT, histology is unnecessary for diagnosis, and the primary treatment involves monotherapy with vincristine sulphate, eliminating the need for surgery (Costa, Paiva, et al., 2023). Therefore, choosing a cytological classification system would be preferable and not dependent on histopathological assessments.

Histopathology was a traditional diagnostic method for CTVT diagnosis, but cytology requires a less invasive, safer and cheaper approach, without need for surgery. The tumour is composed of cells arranged in mantles and sheets, interspersed with fibrovascular stroma. These cells typically exhibit well-defined cytoplasmic borders and round to oval nuclei with fine granular chromatin (Ayala-Díaz et al., 2019; Ignatenko et

al., 2020b). In a retrospective study, 3.5% (6/172) of round-cell tumours in dogs were misclassified as mast cell tumours (3), histiocytomas (2), and one remained unclassified. The definitive diagnosis through immunohistochemistry revealed CTVT, confirmed by positive staining for vimentin and negative staining for CD3, CD79, CD117, and cytokeratin AE1/AE3 (Araújo et al., 2012).

The polymerase chain reaction (PCR) is a relatively recent technique for CTVT diagnosis, demonstrating successful results (Setthawongsin et al., 2016). This test works from the identification of long interspersed elements (LINE-1) near c-myc oncogene (Setthawongsin et al., 2016; Vural et al., 2018). However, due to its higher cost and unavailability compared to cytology or histopathology, it should not be the sole option for diagnosis. This is particularly relevant given that many dogs with CTVT come from low-income areas, emphasizing the need for a cost-effective diagnosis to mitigate potential evasion of diagnosis.

Additional diagnostic methods include transmission studies, although they are scarcely used nowadays, and chromosome analysis. This tumour typically presents fewer chromosomes than a typical canine somatic cell, which has 78 chromosomes. For instance, in Japan, researchers identified 59 chromosomes in most CTVT cases (Makino, 1963).

2.4. Clinical aspects

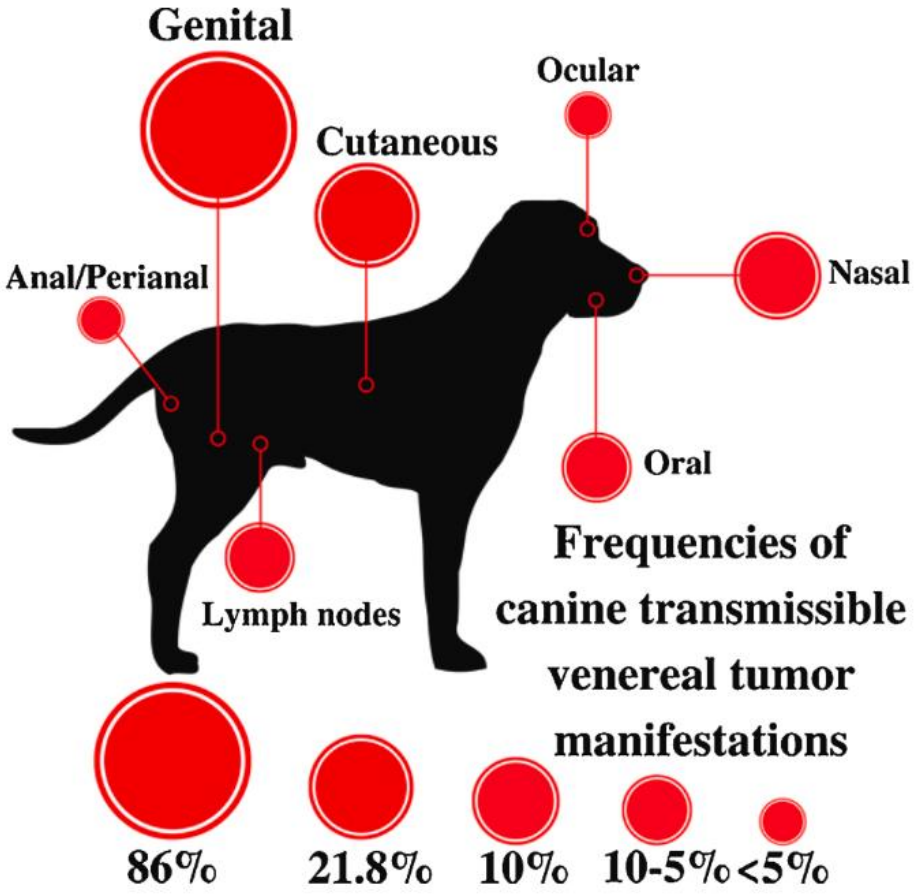
The main clinical manifestations of CTVT include genital presentations, affecting the vulva and vagina in females and the penis and prepuce in males, as well as cutaneous, nasal, and oral manifestations (Araujo et al., 2016; Huppes et al., 2014; Ignatenko et al., 2020a; Strakova et al., 2022). The genital presentation is thoroughly described and well-documented in the literature (Huppes et al., 2014). In contrast, atypical/extragenital manifestations are occasionally considered rare and have been detailed more properly only in the last decade (Fêo et al., 2016; Peixoto et al., 2016). The primary site of metastasis is regional lymph nodes, occurring in less than 5% of cases (Araujo et al., 2016; Peixoto et al., 2016).

The genital presentation characterizes this tumour as typically associated with coitus transmission and maintenance in genital organs, with reported frequencies commonly ranging between 85% and 90% (Araujo et al., 2016; Fêo et al., 2016; Ramadinha et al., 2016; Valençola et al., 2015). However, some studies identify this manifestation with a frequency of over 95% among all cases of the disease (Huppes et

al., 2014). This data may potentially be explained by cases that have gone undiagnosed, as this neoplasia is stigmatized as a venereal tumour by its name, and atypical presentations of the disease can represent a clinical challenge (Gritzenco et al., 2022; Parker et al., 2021).

Extragenital manifestations exhibit highly variable frequencies across studies, with some not mentioned in surveys, such as cutaneous and lymph nodes (Huppes et al., 2014; Ramadinha et al., 2016). The cutaneous presentation is usually the most frequently reported among the extragenital manifestations, ranging from 5.1% to 21.7% of all manifestations. Extragenital nasal and oral locations are the next most common, ahead of other sites and lymph node metastases, as illustrated in Figure 4 (Brandão et al., 2002; Costa et al., 2023; Peixoto et al., 2016; Pimentel et al., 2021).

Figure 4. Estimated frequencies of the main clinical manifestations of the canine transmissible venereal tumour (CTVT) according to the reported cases in Brazilian scientific literature.



Source: Pimentel et al., 2021

The occurrence of concurrent manifestations may complicate the diagnosis and has

been less frequently reported in Brazil. Recently, in Rio de Janeiro, Costa et al. (2023) demonstrated the occurrence of associated manifestations in 7.9% (20/252) of cases, which is significantly higher than the indicated by Peixoto et al. (2016), also in Rio de Janeiro, where combined genital and extragenital manifestations occurred in only 1.85% of cases (Costa, Paiva, et al., 2023; Peixoto et al., 2016). Conversely, most of the earlier studies do not mention this occurrence of two or more manifestations (Brandão et al., 2002; Huppes et al., 2014; Mascarenhas et al., 2014), possibly due to underdiagnosis. Abroad, this event is described more frequently: In Granada, around 11.5% (9/78) of CTVT cases presented two or more involved manifestations (Chikweto et al., 2013). A retrospective study involving dogs with ocular presentation of the tumour in Greece found that 12% (3/25) of patients had another concomitant manifestation, with 4% (1/25) as genital and 8% (2/25) oronasal (Komnenou et al., 2015). The most representative study of CTVT in the United States in recent decades indicated the presence of multiple manifestations in 6.9% (2/29) of dogs between 1984 and 1996 (Rogers et al., 1998). In dogs with concomitant visceral leishmaniasis and CTVT in Italy, 21% (4/19) of individuals developed a cutaneous manifestation concurrently with the genital manifestation, possibly indicating a relationship of immunosuppression mediated by the protozoan interfering with CTVT, although the sample size was limited (Marino et al., 2012).

Natural spontaneous complete remission is a rare occurrence in cancers, and for CTVT, it is not extensively documented, with reports limited to case studies (Costa et al., 2022; Perez et al., 1998) It is suggested a potential link to tumour immunogenicity and the recognition of major histocompatibility complex (MHC) in individuals who have experienced an immunosuppressed period. However, this phenomenon remains questionable, observed mostly in experimental studies of inoculated CTVT (Yang & Jones, 1973) and still insufficiently described in natural cases (Duzanski et al., 2022).

2.5. Treatment

Incorporation of multimodality approaches in cancer treatment is expected, although it is not the standard for CTVT. Following an era of testing treatments, marked by extensive surgeries with elevated recurrence rates (Batamuzi & Kessy, 1993; Idowu, 1984) and the introduction of radiotherapy (Hataya et al., 1958; Rogers et al., 1998), monochemotherapy with vincristine sulphate has been established as the preferred and first-line treatment for CTVT (Bulhosa et al., 2020; Ramadinha et al., 2016).

Vincristine sulphate, commonly known by its commercial name Oncovin® in chemotherapy protocols, is a vinca alkaloid with mitotic inhibitory properties, impeding mitotic fuse formation and chromosome separation during metaphase (Mee et al., 2022; Škubník et al., 2021). In contrast to lymphoma protocols in dogs that involve a combination of multiple drugs, CTVT treatment relies solely on vincristine, administered intravenously at a dose of 0.5 to 0.75 mg/m² on a weekly basis. Typically, four to six sessions are conducted until complete remission of the tumour is achieved (Komnenou et al., 2015; Ramírez-Ante et al., 2021; Setthawongsin et al., 2019).

Vincristine therapy for CTVT has an objective response rate exceeding 90%, resulting in complete remission in the majority of cases (Ayala-Díaz et al., 2019; Costa, Paiva, et al., 2023; Huppes et al., 2014). Despite the potential emergence of vincristine chemoresistance, its continued high response rate maintains it as the preferred first-line therapy for untreated CTVT cases. The development of chemoresistance to vincristine may be associated with increased drug efflux facilitated by the the ATP-binding cassette sub-family B member 1 (ABCB1), P-glycoprotein, as suggested by Gaspar et al. (2010) through elevated P-glycoprotein staining observed in cases with partial response. Although statistical significance was not achieved in the study, further investigations are needed to better comprehend this and other potential mechanisms (Dujon et al., 2020; GASPAR et al., 2010).

Doxorubicin (hydroxydaunorubicin) serves as a rescue therapy for CTVT recurrences or cases exhibiting partial remission following vincristine monotherapy (Nak et al., 2005; Reis-Filho et al., 2020). Its mechanism of action involves damaging tumoral cells by inhibiting the progression of topoisomerase II in DNA transcription (Mansoori et al., 2017). This chemotherapy, administered intravenously at a dose of 30mg/m² every 21 days for 2-4 cycles, is highly valuable as a second-line treatment (Duzanski et al., 2022; Huppes et al., 2014).

Another option for second-line therapy is chemotherapy with the alkylating nitrosourea lomustine, although its use is poorly described. Recent studies testing lomustine on untreated CTVT showed a complete response rate of 66.6% and partial response of 8.3%, which is lower than expected for vincristine (Costa, de Paiva, et al., 2023).

The utilization of surgery as the primary therapeutic approach results in recurrence rates that may exceed 50% when marginal removal is performed (Idowu, 1984; M. Athar et al., 2001). However, when surgery is associated with cauterization during the procedure

or utilized with vincristine as an adjuvant, it has been shown to improve the curative rate (Batamuzi & Kessy, 1993; Fathi et al., 2018). There is currently no guideline or study defining appropriate surgical margins for CTVT excision, as research groups and practitioners worldwide discourage surgery as a primary treatment for this tumour (Ganguly et al., 2016; Strakova & Murchison, 2014).

Surgery should still be considered only in exceptional cases, primarily when other treatments are locally unavailable. In post-chemotherapy cases where tissue differentiation from fibrosis is challenging, surgery can be useful for collecting samples for histopathology. However, alternative methods such as cytology can also be employed (Duzanski et al., 2022).

Radiotherapy stands out as one of the most effective therapies for CTVT treatment, with complete remission rates approaching 100% when the tumour is thoroughly irradiated (Hataya et al., 1958; Thrall, 1982). However, the high cost is a significant limitation. A notable response to radiotherapy was observed as early as 48 hours after the first fraction was administered, and complete responses were documented in all cases within one month in a retrospective study (Rogers et al., 1998). The total irradiation dose ranges from 10 to 18 Gy, delivered three times a week across three fractions, resulting in complete response without any recurrence during a 12-month follow-up period (Rogers et al., 1998; Thrall, 1982).

Electrochemotherapy is a locoregional cancer control method that involves systemic chemotherapy, particularly with bleomycin, followed by local electroporation of the tumour (Rangel et al., 2019). This treatment modality is gaining popularity, especially in low-income countries, representing a novel strategy for local control of tumours, once relied only in surgery, as radiotherapy is largely unavailable for veterinary patients in such countries. While only a few small series of cases and individual case reports have demonstrated its application in CTVT, the results have been remarkable (Rangel et al., 2019; Spugnini & Baldi, 2019). In three chemotherapy-resistant cases (two resistant to both vincristine and doxorubicin, and one resistant only to vincristine), successful treatment was achieved using intratumoral bleomycin at a concentration of 1.5 mg/ml. Five minutes after chemotherapy local infusion, trains of 8 biphasic electric pulses lasting 50 + 50 μs each, with 1 ms interpulse intervals, were administered. At 28 months post-treatment, none of the patients developed recurrences (Spugnini & Baldi, 2019).

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3. OBJECTIVES

3.1 General objective

Analyse retrospectively cytological criteria in samples from dogs with CTVT and their association with resistance to vincristine chemotherapy.

3.2 Specific objectives

Analyse the incidence of CTVT at the Veterinary Hospital of UFMG over the last ten years (2012-2022)

Identify the clinical manifestations of dogs diagnosed with CTVT at the Veterinary Hospital of UFMG in the last ten years.

Analyse cytological criteria in samples from dogs with CTVT and their association with resistance to vincristine chemotherapy and propose a resistance score system Propose a cytological grading system to predict vincristine chemoresistance.

Systematically review the literature about CTVT treatment and propose a guideline for treatment.

CHAPTER I[1]

CHEMORESISTANCE CYTOLOGICAL SCORE FOR CANINE TRANSMISSIBLE VENEREAL TUMOR: A SYSTEM PREDICTING VINCRISTINE SULFATE RESISTANCE

ABSTRACT

Canine transmissible venereal tumor (CTVT) is a prevalent diagnosis in many low-income countries, and managing its chemoresistant cases can be challenging within the conventional vincristine sulfate chemotherapy protocol. Also, a gap remains in identifying predictive markers of chemoresistance for this tumor. The objective of this investigation was to examine various cytological characteristics of CTVT in order to develop a cytological score system with a predictive value for vincristine sulfate resistance. For this, 40 cases were retrospectively studied according to their clinical aspects and response to vincristine chemotherapy. Cytological smears underwent a double-blind assessment for a modified cytomorphological classification. Subsequently, several cytological criteria were analyzed. A new classification for cytomorphology was proposed and tested, but there was no association with chemoresistance ($p = 0.083$). The novel cytology score classification allowed the identification of cases prone to chemoresistance, relying on three criteria: anisokaryosis, mitotic count, and the presence of binucleated cells. Malignancy criteria, evaluated in 5 hot spots, were inversely associated with chemoresistance ($p = 0.001$), predicted by low anisokaryosis, mitotic count of ≤ 6 in a 2.37 mm^2 area, and no binucleated cells. This study introduces the initial cytology score system for CTVTs, establishing a correlation with chemoresistance. It serves as a valuable predictor for vincristine treatment, potentially assisting practitioners in their clinical decision-making process.

KEYWORDS

Cancer, Sticker tumor, chemotherapy, cytology, dog, contagious.

[1] Chapter formatted according to the *Veterinary and Comparative Oncology*

1. INTRODUCTION

Canine transmissible venereal tumor (CTVT) is a globally distributed, contagious, non-infectious neoplasm distinguished by its genital manifestation, affecting mainly sexually intact and free-range dogs.[1,2] While atypical presentations may pose a diagnostic challenge, clinical cytology emerges as an effective approach for diagnosing most cases.[3,4] The distinctive cytomorphological features of CTVTs place them within the category of round cell tumors alongside lymphomas, mast cell tumors, plasma cell tumors, and histiocytomas.[5]

Despite its histiocytic origin, previous investigations have proposed a tripartite categorization scheme for CTVTs based on morphological characteristics resembling plasma cells or lymphocytes.[6,7] However, certain limitations appear to impact this classification: the absence of a hierarchy among the criteria and the presence of disparities across various studies regarding the prevalence of each type.[8–10] Furthermore, the clinical applicability of this classification, which segregates CTVT into plasmacytoid, lymphocytoid, and mixed, remains a subject of contention.[11] It is primarily attributable to insufficient survival data and an unclear correlation with chemotherapy response.

A cytological grading system was validated for mast cell tumors, employing mitotic figures and binucleation as discerning criteria, and subsequently collagen fibrils and fibroblasts were suggested to be included.[12,13] This grading system functions as a robust prognostic indicator and correlates with histopathological grading. In contrast, when dealing with CTVT, histological examination is not imperative for diagnostic purposes. The primary therapeutic approach typically requires monotherapy with vincristine sulfate, obviating the necessity for surgical interventions in over 90% of cases.[2,14] Thus, a cytological grading system is highly advantageous, rendering reliance on histopathological assessments less necessary.

The objective of this investigation is to perform a double-blind validation process for an adapted version of the cytomorphological classification system used for CTVT. Additionally, we aim to establish connections between cytological features and resistance to chemotherapy. The ultimate goal is to develop a novel cytological grading scheme as a predictor of chemoresistance.

2. MATERIALS AND METHODS

2.1 Sample collection and processing

From 2016 to 2022, all cases under consideration for this study received their diagnoses at the Veterinary Hospital of Universidade Federal de Minas Gerais, Brazil. The prescribed method for specimen collection involved fine needle aspiration puncture and imprinting. Subsequently, slides were promptly subjected to processing, employing the panoptic fast staining technique (Romanowsky). Our retrospective analysis focused exclusively on canine patients with definitive cytologic diagnoses, as delineated in the case selection diagram (Figure 1). The protocol and procedures utilized to analyze cytology samples underwent ethical review and approval by the Ethics Commission on Animal Use (CEUA), under CEUA-UFMG Protocol No. 292/2023.

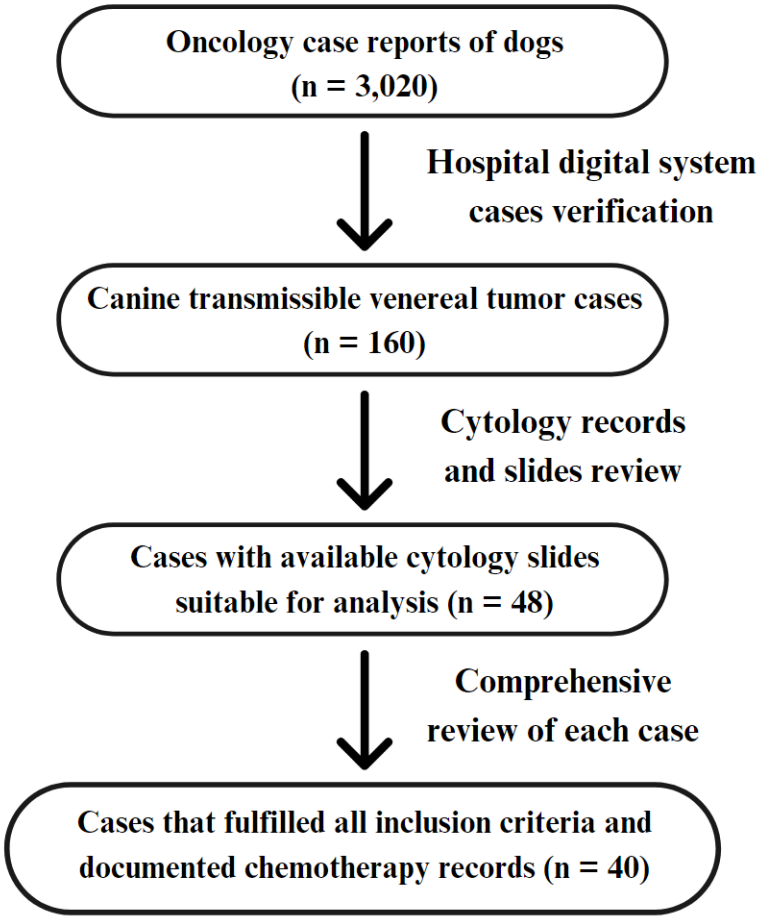

Figure 1. Flow diagram of CTVT cases eligible for the study.

The examination of CTVT slides was conducted using a Nikon® Eclipse E-200 optical microscope using all available objectives, and a 40x objective was employed for cytomorphology classification. Cytological observations were captured using a light

microscope (Olympus BX51). A minimum of 100 cells was predetermined as the requisite sample size for evaluation across all slides. Cases characterized by complete indistinct cytoplasmic definition and those exhibiting low slide quality were excluded.

Clinical data retrieval was conducted using the Veterinary Hospital system at Universidade Federal de Minas Gerais, Brazil. Notably, a separate researcher was responsible for gathering the clinical data, ensuring those who were analyzing the slides had no prior knowledge of the cytologic findings or the cases' clinical characteristics. The connection assessment between chemoresistance and cytological data was performed after the cytology analysis, maintaining the integrity of the analysis process.

2.2 Cytomorphology adaptation

Cytological slides from dogs with pre-existing diagnoses of CTVT were meticulously selected from the Clinical Pathology Laboratory at Universidade Federal de Minas Gerais. These chosen samples underwent a thorough evaluation, and subsequently, the cases were subjected to a comprehensive review.

Based on the investigations undertaken in the study by Amaral et al. (2007),[6] a simplified categorization method was employed to classify CTVTs into three subtypes, primarily considering two criteria: cell morphology (given greater significance) and nucleus-cytoplasm relationship (considered secondary), as shown in Figure 2. Due to the potential misidentification of CTVT origin from lymphocytes or plasma cell lineages due to the suffixes “cytic” in lymphocytic and plasmacytic, we preferred to use the terms plasmacytoid and lymphocytoid.

Cell shape played a central role in determining the cytological type. To elaborate further, plasmacytoid cytomorphology was characterized by the presence of oval-shaped cells and a low nucleus/cytoplasm ratio. Conversely, lymphocytoid cytomorphology was identified by cells exhibiting rounded shapes and a high nucleus/cytoplasm ratio. The mixed group encompassed tumor cell populations where plasmacytoid and lymphocytoid cells occurred in relatively equal proportions, with neither type exceeding 60% frequency. Furthermore, it is noteworthy that not all cells should be included in the cell count: cells with exposed nuclei, those with indistinct cytoplasmic borders, or those situated at the periphery of the microscopic field of view are avoidable during the counting process. This selective approach ensures an accurate and representative assessment of the cytological features of interest.

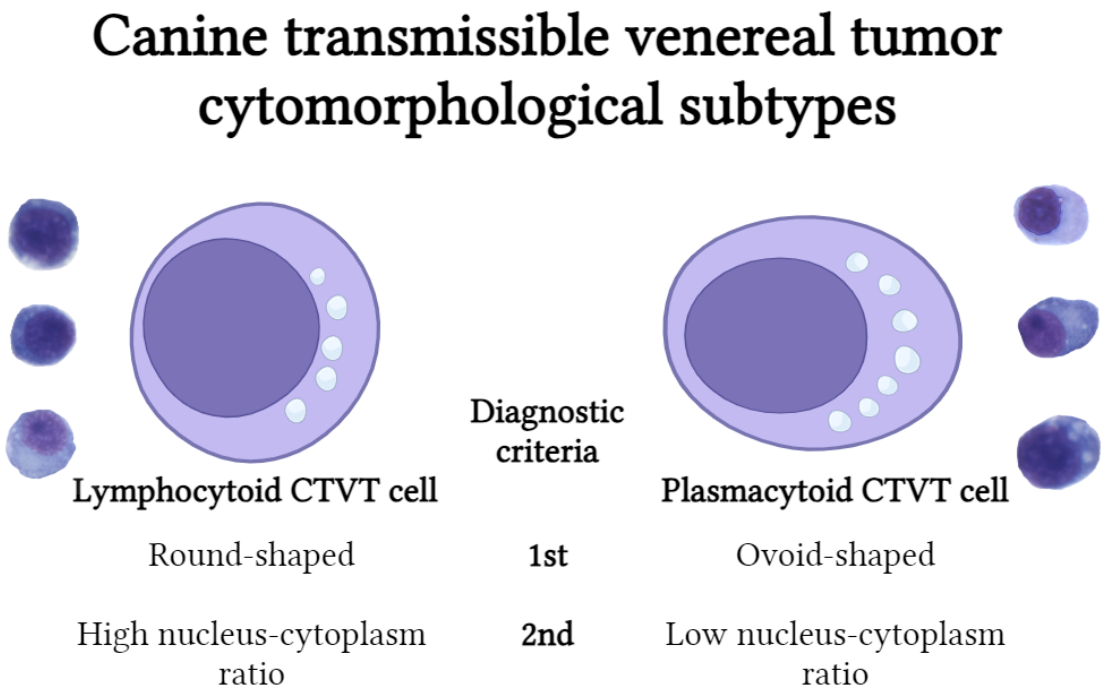

Figure 2. Plasmacytoid and lymphocytoid cytomorphology comparison. Plasmacytoid cytomorphology is distinguishable by oval-shaped cells with a low nucleus/cytoplasm ratio, while lymphocytoid cytomorphology features rounded cells with a high nucleus/cytoplasm ratio. Created with BioRender.com.

In a peer-blind system, each case underwent evaluation by two researchers with a cytopathology background. The cytomorphology results were examined after both researchers had established their final cell counts independently (100 cells). Any discrepancies or disagreements were subsequently subjected to a double-check process to arrive at a conclusive cytomorphological diagnosis. All the data about cytomorphology examination is in the patients' data (Appendix S1).

2.3 Cytological malignancy features

A total of eleven cytological features were assessed, some of which have been previously recognized as indicators of malignancy in canine neoplasms.[15–19] These features were considered as potential criteria for establishing a grading system. Morphologic parameters included: anisokaryosis; nuclear pleomorphism; mitotic count; binucleated cells; tadpole cells; nucleolus count; nucleus-cytoplasm ratio; presence of inflammatory cells; karyomegaly; cellular groupings; and cellularity. Additionally,

specific cytological aspects were assessed through both quantitative and qualitative methodologies, as further elucidated.

Anisokaryosis was evaluated based on its variation in nucleus size compared to the average nucleus diameter. It was categorized as follows: absent when anisokaryosis was insignificant; low, when anisokaryosis ranged from 1 to 1.5 times the average nucleus size; intermediate when anisokaryosis ranged from 1.5 to 2 times the average nucleus size; and high when anisokaryosis exceeds two times the average nucleus size. Quantitative analysis was performed to determine the percentage of cells displaying nucleus sizes different from the average. *Nuclear pleomorphism* was assessed for its presence or absence, and the intensity of pleomorphism was further categorized as absent, low, intermediate, or high. *Karyomegaly* was classified as either absent or present, if at least one cell displayed a nucleus size exceeding two times the average nucleus size.

The evaluation of *nucleoli* involved assessing the most common number of nucleoli per cell, categorized as either absent or one. Additionally, the percentage of cells displaying specific nucleoli numbers was recorded as absent, one, or multiple. The *nucleus-cytoplasm ratio (N/C)* was categorized by its intensity as follows: low (<0.8); intermediate (0.8-1.2); and high (>1.2). Furthermore, the variability of N/C ratios was analyzed based on variations among cells: low, as most cells exhibited a consistent N/C ratio; intermediate, as cells showed an intermediate degree of variation in their N/C ratio; High, as cells displayed significant heterogeneity in their N/C ratios.

Cellularity and the presence of *inflammatory cells*, predominantly neutrophils, lymphocytes, eosinophils, and macrophages, were analyzed based on their intensity in the context of tumor cells and inflammatory cells. Cellularity and inflammatory cells were categorized as low, intermediate, and high. Samples with low cellularity exhibited a scarcity of cells per 40x-magnification field, consistently surpassing the minimum requirement (>100 cells per slide). Conversely, high cellularity signified samples characterized by numerous fields replete with tumor cells. Intermediate cellularity denoted quantities falling between the aforementioned classifications. These aspects are elaborated in greater detail within the comprehensive cytology guide section of the study. This detailed evaluation provided a comprehensive understanding of the cytological characteristics observed in the samples.

Mitotic count was determined by calculating the sum of mitotic figures identified in five designated "hot spots", areas of higher cellularity. This calculation was conducted using a field number 22 eyepiece with a 430-micron field of view (2.37 mm^2) and a 40x

magnification. Mitotic counts were also categorized as absent or present. We applied the same quantitative and qualitative classifications to *binucleated cells* and *tadpole cells*. Both assessments were taken into account during the evaluation process. Tadpole cells display elongated cytoplasmic features, and these should not be mistaken for fibroblasts or overlooked during the cell counting process.

2.4 Criteria for chemoresistance classification

Patients were classified when additional therapies or six or more sessions of vincristine sulfate were necessary to achieve complete remission, exhibiting a delayed response (slow responders). Alternatively, resistant cases also included those necessitating additional therapies to attain complete remission. The determination of the chemotherapy session count for a slow response was based on the previous criteria outlined in Alzate et al., 2019, and Gaspar et al., 2010. The clinical response evaluation of the cases followed the classification established by the Veterinary Cooperative Oncology Group in 2013.[20]

2.5 Statistical analysis

Fisher's exact test was employed to examine the association between vincristine chemotherapy resistance and cytological features in CTVT. Additionally, a multivariate analysis was conducted to compare various cytological characteristics of CTVT. The ROC curve was employed to establish the cutoff for cytological criteria within the vincristine sulphate resistance score. Statistical analysis was performed with the software Microsoft Excel® and Prisma GraphPad Prism 9.5.1®. p-Values of ≤ 0.05 were considered statistically significant. The Cohen's kappa coefficient was employed to evaluate the reliability between evaluators, with values exceeding 0.8 deemed indicative of almost perfect agreement.

CELL LINE VALIDATION STATEMENT

A validation test of CTVT cell lines has not been conducted. All samples were exclusively from cytology.

3. RESULTS

3.1 Clinical data

Forty cases of CTVT were comprehensively reviewed and categorized. Among these cases, female dogs accounted for the majority at 60% (24/40), while male dogs represented 40% (16/40). Ten cases exhibited chemoresistance to vincristine sulfate, with 8 showing a delayed response and 2 necessitating a change in the treatment protocol. The average age of the dogs in the study was 4.4 ± 3.2 years (median of 3.2 years). Most dogs in the study were mixed-breed (33/40), comprising 82.5% of the cases. Purebred dogs made up the remaining 17.5% of cases, with each dog belonging to a distinct breed: English Pointer, German Pinscher, German Shepherd, Golden Retriever, Labrador Retriever, Miniature Poodle, and Shih-Tzu.

3.2 Cytomorphology adaptation

Among the cases examined, 45% (18/40) exhibited a predominant population of plasmacytoid tumor cells. Mixed-type CTVTs were the next most prevalent, comprising 27.5% (11/40) of the cases. Lymphocytoid CTVTs also corresponded to the same proportion as mixed-type cases, 27.5% (11/40). No cytomorphological types presented statistical significative association with chemoresistance.

In the double-blind evaluation process, there was a cytomorphology-type concordance of 90%, with 36 out of 40 cases showing agreement between the two researchers, free-marginal kappa of 0.85 (95% CI: 0.71-0.99), almost perfect agreement. Four cases presented disagreements between the researchers. Among the cases in which there was disagreement between researchers, after discussion and consent, two were classified as lymphocytoid/mixed, and two were classified as plasmacytoid/mixed. For further reference and analysis, detailed patient data, including the classification of each case and the complete cell count as assessed by each researcher, can be found in the supplementary material.

Figure 3 provides an illustrative example of the single-cell classification of a CTVT slide, highlighting various components such as cytomorphology, cells unsuitable for classification, and inflammatory cells.

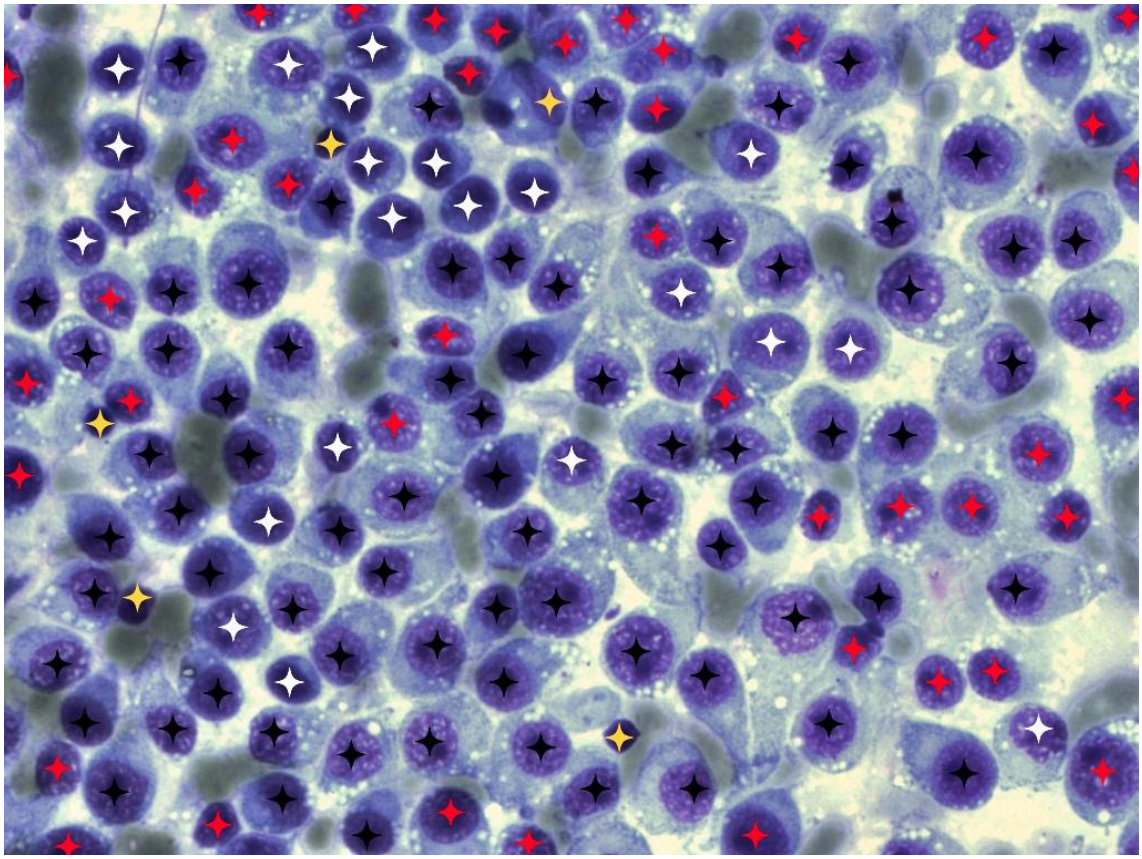

Figure 3. Canine transmissible venereal tumor, Romanosky stain. High cellularity of round to oval-shaped cells with basophilic nuclei, accompanied by intermediate anisokaryosis and anisocytosis (A). The cytomorphology is characterized by plasmacytoid tumor cells, denoted by black stars, and lymphocytoid tumor cells, indicated by white stars (B). Other cells such as lymphocytes and a macrophage, indicated by yellow stars, are also noticed. Some cells were excluded from the count due to inadequate cytoplasmic differentiation, cellular deterioration, or limited visibility at the edge of the objective, as indicated by red stars.

3.3 Cytological features and chemoresistance

Table 1 provides an overview of the frequency of various cytological features and malignancy criteria observed in CTVT cases. These features were analyzed in all 40 cases, investigating potential associations between these cytological characteristics and vincristine resistance. The cytological characteristics observed in CTVT cells are noteworthy. Anisokaryosis and nuclear pleomorphism, while individually distinct, are notably prevalent characteristics in 97.5% of instances (39/40). Mitotic figures and

binucleations are also quite common, with frequencies of 87.5% (35/40) and 75% (30/40), respectively. In terms of nuclear features, most CTVTs display a single nucleolus pattern (75%) and have an intermediate (52.5%) to high (42.5%) nucleus-cytoplasm ratio. Karyomegaly, observed in 52.5% of cases, and tadpole cells, present in 87.5% of cases, are also frequently encountered. Groupings of cells are relatively uncommon, occurring in only 20% of cases. Inflammatory cells and cellularity display variable patterns, as detailed in Table 1, reflecting the diverse cytological characteristics observed in CTVTs.

Table 1. Cytological features and malignancy features in canine transmissible venereal tumor cases

Cytological features	Classification	Cases	Frequency	Chemoresistant cases / Total (%)	p-Value
Anisokaryosis (intensity)[1]					
	Absent	1/40	2.5%	1/1 (100%)	.016*
	Low	7/40	27.5%	5/11 (45.5%)	
	Intermediate	27/40	57.5%	4/23 (17.4%)	
	High	5/40	12.5%	0/5 (0%)	
Anisokaryosis (% of cells)					
	≤ 20%	12/40	30%	6/12 (50%)	.016*
	> 20%	28/40	70%	4/28 (14.3%)	
Nuclear pleomorphism					
	Absent	1/40	2.5%	1/1 (100%)	.250
	Present	39/40	97.5%	9/40 (22.5%)	
Nuclear pleomorphism (intensity)[1]					
	Absent	1/40	2.5%	1/1 (100%)	.850
	Low	14/40	35%	3/14 (21.4%)	
	Intermediate	25/40	62.5%	6/25 (24%)	
Mitotic figures					
	Absent	5/40	12.5%	2/5 (40%)	.407

	Present	35/40	87.5%	8/35 (22.9%)	
Mitotic figures (per 5 HS)					
	≤ 6	21/40	52.5%	9/21(42.9%)	.006**
	> 6	19/40	47.5%	1/19 (5.3%)	
Binucleation					
	Absent	10/40	25%	5/10 (50%)	.035*
	Present	30/40	75%	5/30 (16.7%)	
Binucleated cells (per 5 HS)					
	≤ 1	21/40	52.5%	9/21(42.9%)	.006**
	> 1	19/40	47.5%	1/19 (5.3%)	
Tadpole cells					
	Absent	5/40	12.5%	1/5 (20%)	.782
	Present	35/40	87.5%	9/35 (25.7%)	
Tadpole cells (per 5 HS)					
	≤ 2	25/40	62.5%	7/25 (28%)	.571
	> 2	15/40	37.5%	3/15 (20%)	
No nucleolus (% of cells)					
	≤ 30%	24/40	60%	3/24 (12.5%)	.025*
	> 30%	16/40	40%	7/16 (43.75%)	

Single nucleolus (% of cells)					
	≤ 65%	21/40	52.5%	8/21 (40%)	.044*
	> 65%	19/40	47.5%	2/19 (10.5%)	
Multiple nucleolus (% of cells)					
	≤ 5%	25/40	62.5%	9/25 (36%)	.038*
	> 5%	15/40	37.5%	1/15 (4%)	
Most frequent Nucleus/cytoplasm ratio[2]					
	Low (≤ 0,8)	2/40	5%	0/2 (0%)	.579
	Intermediate (>0,8 and <1,2)	21/40	52.5%	5/21 (23.8%)	
	High (≥ 1,2)	17/40	42.5%	5/17 (29.4%)	
Nucleus/cytoplasm ratio (variation)[1]					
	Low	7/40	17.5%	3/7 (42.8%)	.229
	Intermediate	29/40	72.5%	6/29 (20.7%)	
	High	4/40	10%	1/4 (25%)	
Inflammatory cells (intensity)[1]					
	Absent	1/40	2.5%	0/1 (0%)	.456
	Low	15/40	37.5%	3/15 (20%)	
	Intermediate	17/40	42.5%	3/17 (17.7%)	

	High	7/40	17.5%	4/7 (57.1%)	
Karyomegaly					
	Present	19/40	47.5%	3/19 (15.8%)	.200
	Absent	21/40	52.5%	7/21 (33.3%)	
Groupings					
	Present	8/40	20%	1/8 (12.5%)	.361
	Absent	32/40	80%	9/32 (28.1%)	
Cellularity[1]					
	Low	5/40	12.2%	3/5 (60%)	
	Intermediate	15/40	36.6%	3/15 (20%)	.0533[3]
	High	20/40	50%	4/20 (20%)	
Vacuolation (in 100 cells)					
	≤ 90	17/40	42.5%	6/17 (35.3%)	
	> 90	23/40	57.5%	4/23 (17.4%)	.196
Mitotic count (in 100 cells)					
	≤ 1	11/40	27.5%	3/11 (27.3%)	.838
	> 1	29/40	72.5%	7/29 (24.1%)	
Karyomegaly (in 100 cells)					
	Absent	31/40	77.5%	9/31 (29%)	.274

	Present	9/40	22.5%	1/9 (11.1%)	
Binucleated cells (in 100 cells)					
	Absent	21/40	52.5%	6/21 (28.6%)	.583
	Present	19/40	47.5%	4/19 (21.1%)	
Tadpole cells (in 100 cells)					
	Absent	17/40	42.5%	5/17 (29%)	.579
	Present	23/40	57.5%	5/23 (11.1%)	

p-Values were deemed statistically significant by the Fisher's test if they were ≤ 0.05 (asterisk) and ≤ 0.01 (double asterisk). In these criteria ([1]), the groups combined for the statistical test were absent + low and intermediate + high. In this criterion ([2]), the groups combined for the statistical test were low + intermediate and high.

The three pivotal criteria employed to differentiate between chemoresistant and non-chemoresistant cases were the percentage of anisokaryosis ($p = 0.017$), the number of mitotic figures per 5 hot spots ($p = 0.006$), and the number of binucleations per 5 hot spots ($p = 0.006$). In chemoresistant cases, there were fewer indicators of malignancy compared to non-chemoresistant. In chemoresistant cases, reduced counts of mitosis and binucleated cells, and low anisokaryosis were observed.

Considering the chemoresistant CTVTs ($n = 10$), the distribution of cytomorphology types was as follows: mixed (4/10), lymphocytoid (4/10), and plasmacytoid type (2/10). The mean age of the dogs was 3.9 ± 2.4 years, with a median age of 3.4 years. Most cases (80%) occurred in mixed-breed dogs, consistent with the overall average for all cases and the other two cases occurred in a Labrador Retriever and in a Miniature Poodle. Male dogs represented a substantial proportion, accounting for 70% (7/10) of the chemoresistant cases ($p = 0.058$).

3.4 Chemoresistance score

Mitotic figures, binucleated cells, and anisokaryosis are negatively correlated to vincristine sulphate response in CTVT cases, as shown previously. So, Table 2 presents a cytology grading that considers three malignancy criteria for the definition of a chemoresistance score: Anisokaryosis, binucleations, and mitotic count. These three criteria should be assessed in 5 hot spots using the 40x objective in a FN 22 (equivalent to 2.37 mm^2), as stipulated by this study and recommended by the Veterinary Cancer Guidelines and Protocols.[16]

Table 2. Chemoresistance cytological score for canine transmissible venereal tumor

Criteria		Score
Anisokaryosis		
	Intermediate or high	0
	Absent or low	1
Binucleation		
	Present (5 hot spots)	0
	Absent (5 hot spots)	1
Mitotic count		
	High (>6/5 hot spots)	0
	Low (≤6/5 hot spots)	1

Sum of scores	Chemoresistance risk
0	Very low (0%)
1	Low (20%)
2	Intermediate (50%)
3	High (66.7%)

Chemoresistance was not observed in any cases with a score of 0 (0/15). Among those with a score of 1, only 2 out of 10 (20%) demonstrated resistance to vincristine sulfate. Cases with a score of 2 exhibited a 50% chemoresistance rate (6/12), while score 3 cases showed the highest likelihood of chemoresistance at 66.7% (2/3). A statistically significant difference was observed when comparing the combined groups of score 0 and score 1 with score 2 and score 3 ($p = 0.0013$). Tumors classified as high-score and intermediate-score exhibited 13.1 times higher likelihood of vincristine chemoresistance compared to tumors categorized as very low-score and low-score.

3.5 Comprehensive cytology guide

CTVT cells often present different morphological characteristics: Cells were round to oval-shaped and present distinct cytoplasmic borders in most cases; nuclei were predominantly round-shaped, but may vary to ovoid, and sometimes show abnormal presentations in few cells; nuclei are eccentric or paracentral, chromatin was coarse, and naked nuclei were present in few cases; usually, one or no prominent nucleolus was observed, but multiple nucleoli may be found; small to intermediate vacuoles were noticeable in the cytoplasm (“pearl necklace”

aspect), however not always detected in all cells; lightly basophilic cytoplasm; nucleus-cytoplasm relation was variable and cytoplasm tends to be intermediate; cell diameter varied from one to four times a dog red blood cell diameter (6-24 μm); atypia features were common, as mitotic figures, anisocytosis, and anisokaryosis. Therefore, the definition of CTVT as a round-cell tumor was uncontested. In all cases, both researchers defined the diagnosis of CTVT certainly by cytology, which was consider the gold standard test for diagnosis.

The background of most CTVT typically appeared basophilic and may contain marked red blood cells. In some instances, vacuoles from lysed cells may also be observed. Inflammatory cells were a common finding within CTVTs and often include neutrophils, macrophages, and small lymphocytes. Occasionally, eosinophils and plasma cells may be present as well. Furthermore, epithelial cells, represented by corneocytes and keratinocytes, were also commonly identified in CTVTs. These varied cellular components collectively contribute to the cytological characteristics and complexity of CTVT specimens. Figure 4 represents a slide of CTVT.

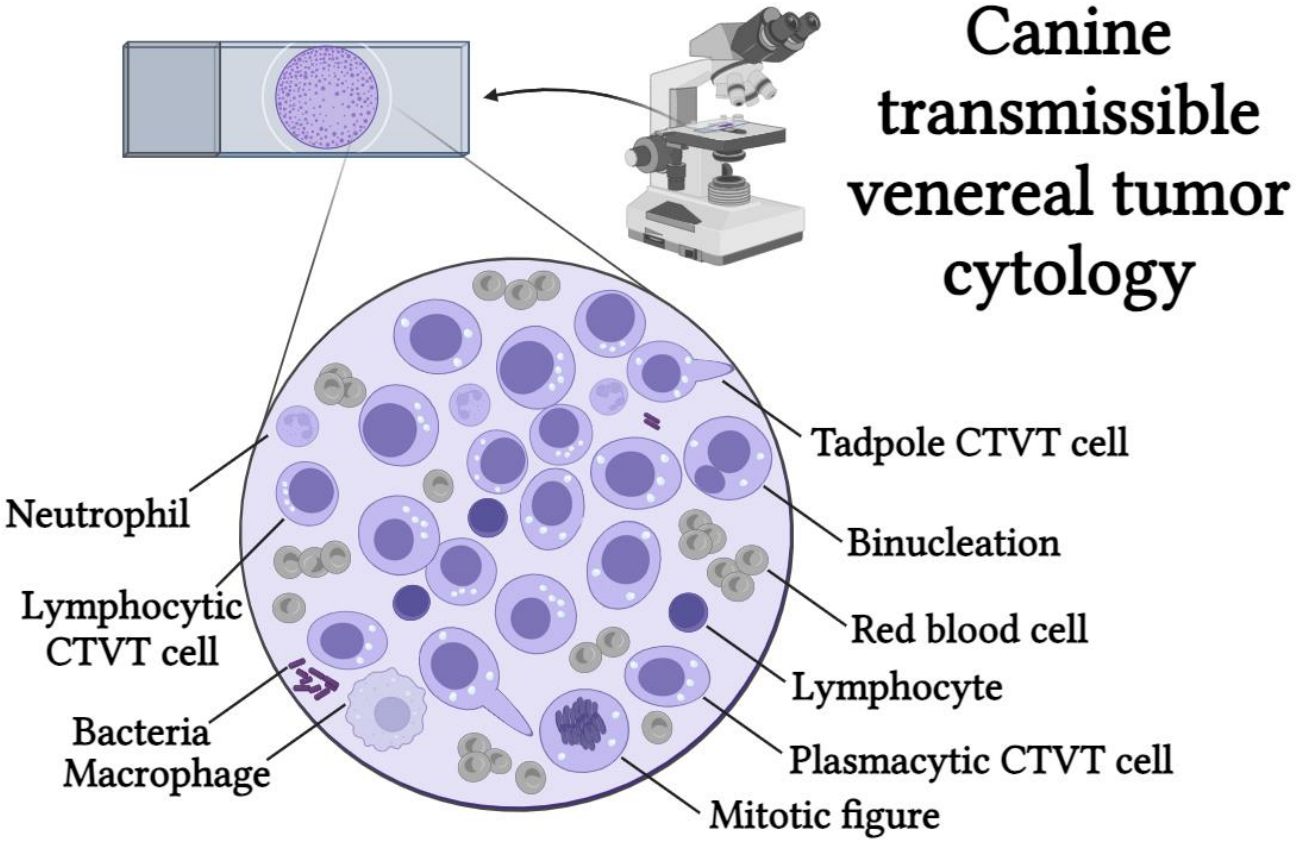

Figure 4. Illustration of a slide from a diagnosed case of canine transmissible venereal tumor, showcasing its distinctive attributes. The image presents common cell types in these cases,

notably lymphocytes, neutrophils, a macrophage, and red blood cells in the background. It exhibits distinct cytomorphology subtypes of CTVT: lymphocytoid and plasmacytoid. Additionally, tadpole CTVT cells, a binucleation, and a mitotic figure are also present. Created with BioRender.com.

Cytological malignancy features were frequent in CTVT cytology slides. Mitotic figures were expected and represent cells in the division, as shown in Figure 5. They may show different cell-cycle phases, typically prophase, metaphase, anaphase, and telophase. Prophase presents condensed chromosomes at the beginning of cellular division, with no spikes visible (5A). In metaphase, the chromosomes are arranged in dark aggregates and distributed in band/linear or ring-shaped forms (5A, 5C, 5D). Anaphase presents the chromosomes in distinct two aggregates in respective opposite poles of a single cell, sometimes with visible spikes (5B, 5D). Telophase is similar to anaphase, in which the chromosomes also display themselves in two aggregates, but a cleavage furrow is visible, at the end of mitosis (5A). Mitoses should be easy to identify, but sometimes it is hard to distinguish between typical and atypical due to their variety of shapes and diversity in cell-circle phases, as it occurs with ring forms of typical metaphase.

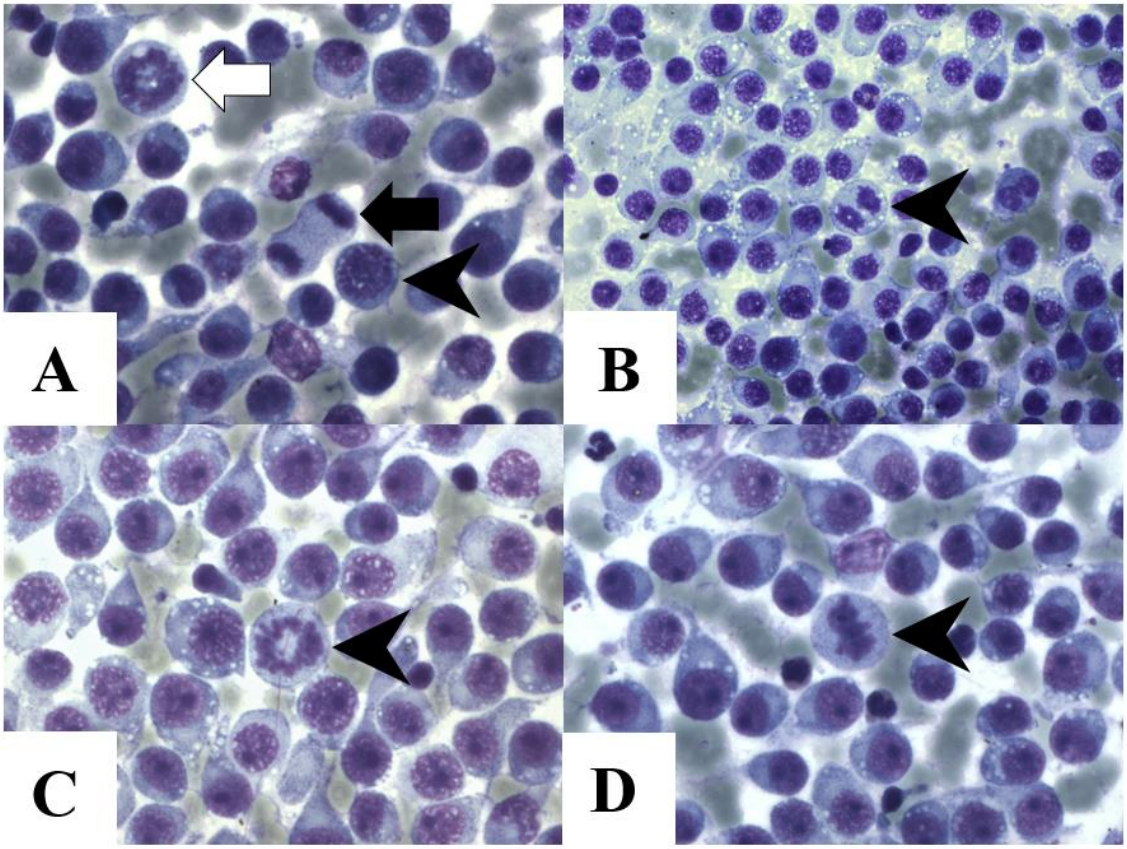

Figure 5. Typical mitoses in CTVT cytology. A) High cellularity. Cells are mostly round, but some oval and tadpole cells are also seen. Black arrowhead shows a typical mitosis, possibly in prophase with condensed chromosomes. White arrow shows another typical mitosis, possibly in prometaphase/metaphase with chromosomes disposed in a circle/ring form. The cell indicated by the black arrow shows possibly a typical mitosis in telophase, characterized by two dark bands of chromosomes on opposite sides of the cell, divided by a mild cleavage furrow. Romanowsky, magnification 200 x. B) Intermediate cellularity of round-shaped cells with basophilic background, interspersed for red blood cells and one eosinophil. Black arrowhead shows a typical mitosis, possibly in anaphase with chromosomes disposed in two aggregates. Romanowsky, 120 magnification 120 x. C) High cellularity of round to oval-shaped cells, including tadpole cells, with basophilic nuclei interspersed for red blood cells. Black arrowhead shows a typical mitosis, possibly in prometaphase/metaphase with chromosomes disposed in a circle/ring form. Romanowsky, magnification 200 x. D) Intermediate cellularity of mostly oval cells, some round-shaped and tadpole cells. Red blood cells, eosinophils, and neutrophils are also present. Black arrowhead shows a typical mitosis, possibly metaphase, denote by an aggregated dark band of rod chromosomes. Romanowsky, magnification 200 x.

Atypical mitotic figures were commonly observed in CTVT, often exhibiting unusual alterations in chromosome disposition. Figure 6 provides a visual representation of these atypical mitotic figures. Tripolar mitotic figures, as depicted in Figure 6A, are characterized by the presence of more than two bands of chromosomes with spikes, typically displaying an irregular distribution. In these figures, chromosomes are often more visible than in typical mitoses, as seen in Figure 5. Figure 6A may represent an anaphase with marked atypia in the distribution of chromosome spikes. In some cases, the cell-cycle phase cannot be clearly denoted due to the irregularity of the mitotic figure, underscoring the unique and complex cytological features of CTVTs.

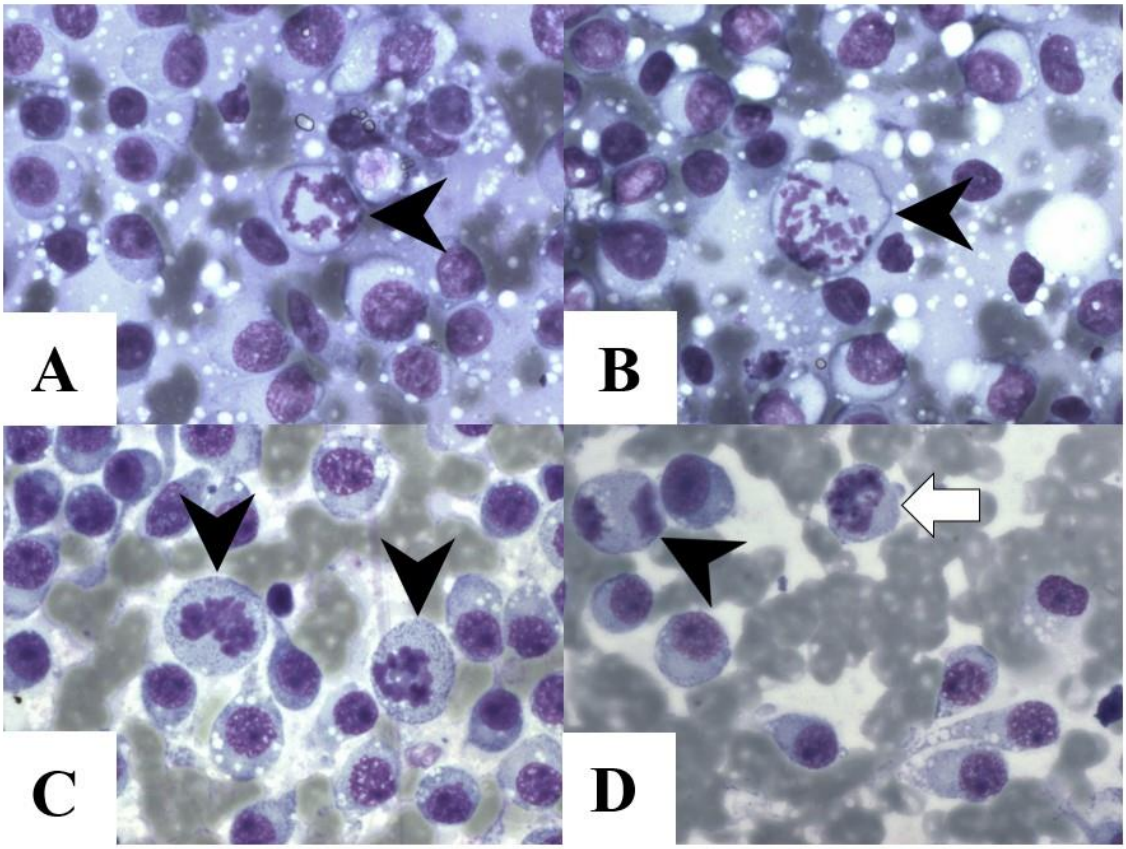

Figure 6. Atypical mitosis in CTVT cytology. A) High cellularity of round to oval-shaped cells with marked basophilic background. Few naked nuclei, red blood cells, and one macrophage are evident. Black arrowhead shows an atypical mitosis, possibly a multipolar prometaphase/metaphase with chromosomes disposed unequally. Romanowsky, magnification 200 x. B) High cellularity of round and oval cells with marked basophilic background. Red blood cells and naked nuclei are present. Black arrowhead shows an atypical mitosis with chromosomes unequally disposed in the cytoplasm. Romanowsky, magnification 200 x. C)

Intermediate cellularity of round to oval-shaped cells. Red blood cells are widely distributed. Two cells indicated by black arrowheads show atypical mitosis, with irregular disposition of chromosomes. Romanowsky, magnification 200 x. D) Intermediate cellularity of round, oval, and tadpole cells. Two mitotic figures are evident: Black arrowhead indicates a typical mitosis, possibly anaphase due to chromosomes disposition in two dark aggregates in opposite sides of the cell with some spikes visible; white arrow shows an atypical mitosis, condensed chromosomes with irregular distribution. Romanowsky, magnification 200 x.

CTVT cells can exhibit marked anisokaryosis and anisocytosis, indicating variations in both nucleus size and cell size, respectively. Karyomegaly, as seen in Figure 7A, is characterized by nuclei with an abnormally enlarged diameter compared to typical cells of the same type. Binucleated cells, while less common than mitosis in CTVT cytology slides, can take various forms, such as oval-shaped cells (7B, 7D) or tadpole cells (7C). It is crucial to note that tadpole CTVT cells should not be mistaken for fibroblasts. Despite their fusiform appearance and variable cytoplasmic prolongation, the nuclei and cytoplasm of tadpole cells conform to the typical pattern of other CTVT cells concerning staining, vacuoles, chromatin characteristics, nucleoli, and nucleus shape (7C, 7D). These distinct morphological features contribute to the diversity of CTVT cytological characteristics.

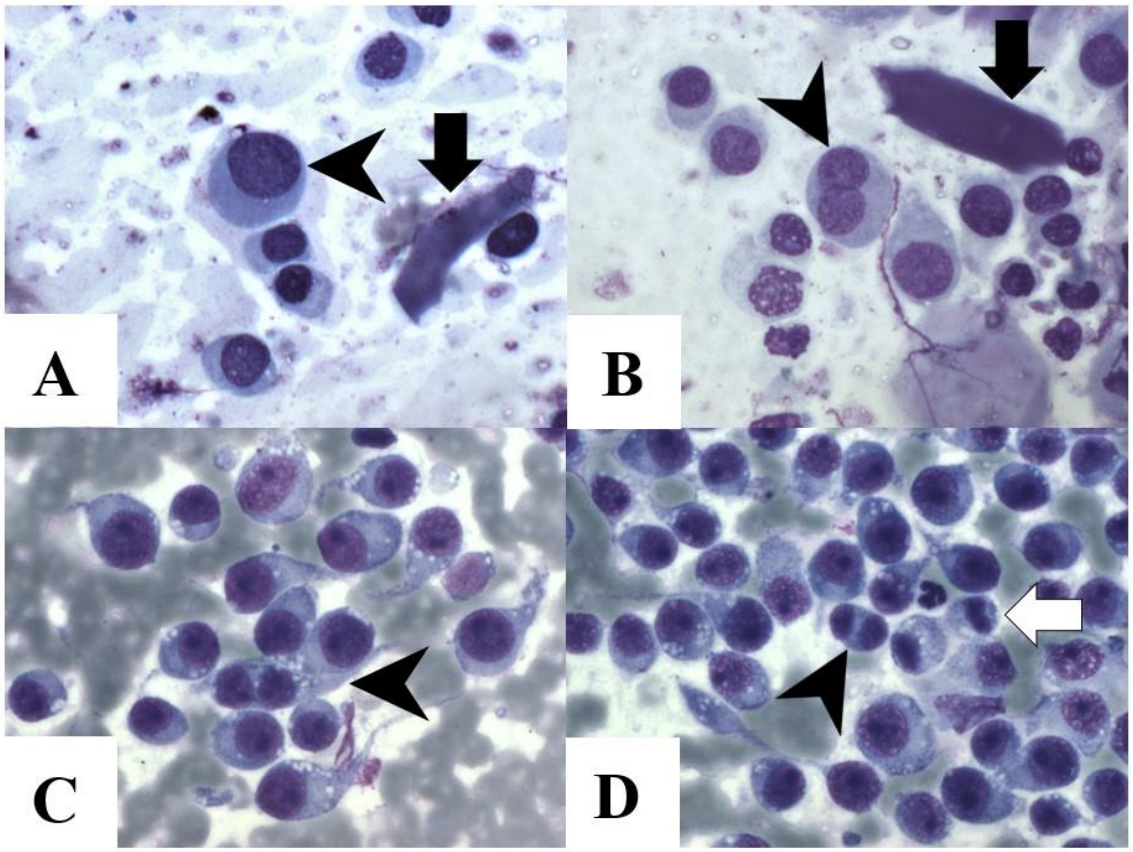

Figure 7. Binucleated cells and karyomegaly in CTVT cytology. A) Intermediate cellularity of round to oval-shaped cells with a lightly basophilic background. Black arrowhead shows an example of karyomegaly, a marked disparity between canine transmissible venereal tumor cells. A corneocyte is also visible (black arrow). Romanowsky, magnification 200 x. B) Intermediate cellularity of round to oval-shaped cells. A central binucleated cell with two round-shaped nuclei is evident in a canine transmissible venereal tumor cell (black arrowhead). Most cells present poor cytoplasmic definition and a corneocyte is also seen (black arrow). Romanowsky, magnification 200 x. C) Intermediate cellularity of mostly oval-shaped and tadpole cells. One binucleated cell is evident in a tadpole CTVT cell (black arrowhead). Red blood cells in high concentration. Romanowsky, magnification 200 x. D) High cellularity of predominantly oval-shaped cells. Black arrowhead indicates a binucleated cell with two oval-shaped nuclei. Some tadpole cells are also evident and a typical mitosis, probably at the end of telophase (white arrow). Red blood cells in intermediate concentration. Romanowsky, magnification 200 x.

Epithelial cells observed in CTVT cytology are typically composed of keratinocytes and corneocytes, and they are often associated with leukocytes, as illustrated in Figure 8. It is necessary to differentiate these epithelial cells from the large CTVT cells, as they can appear similar at first glance. Here are some key distinguishing aspects: keratinocytes have a lower

nucleus-to-cytoplasm ratio compared to CTVT cells (8A); Their cytoplasm is scarcer and larger than that of tumor cells; CTVT nuclei typically exhibit coarser chromatin than keratinocytes, which can aid in differentiation. Neutrophils are commonly evident in the cytoplasm of keratinocytes, providing an additional clue to distinguish them from CTVT cells. These differences in morphology and cellular characteristics can be helpful in accurately identifying and distinguishing between keratinocytes and CTVT cells in cytological slides.

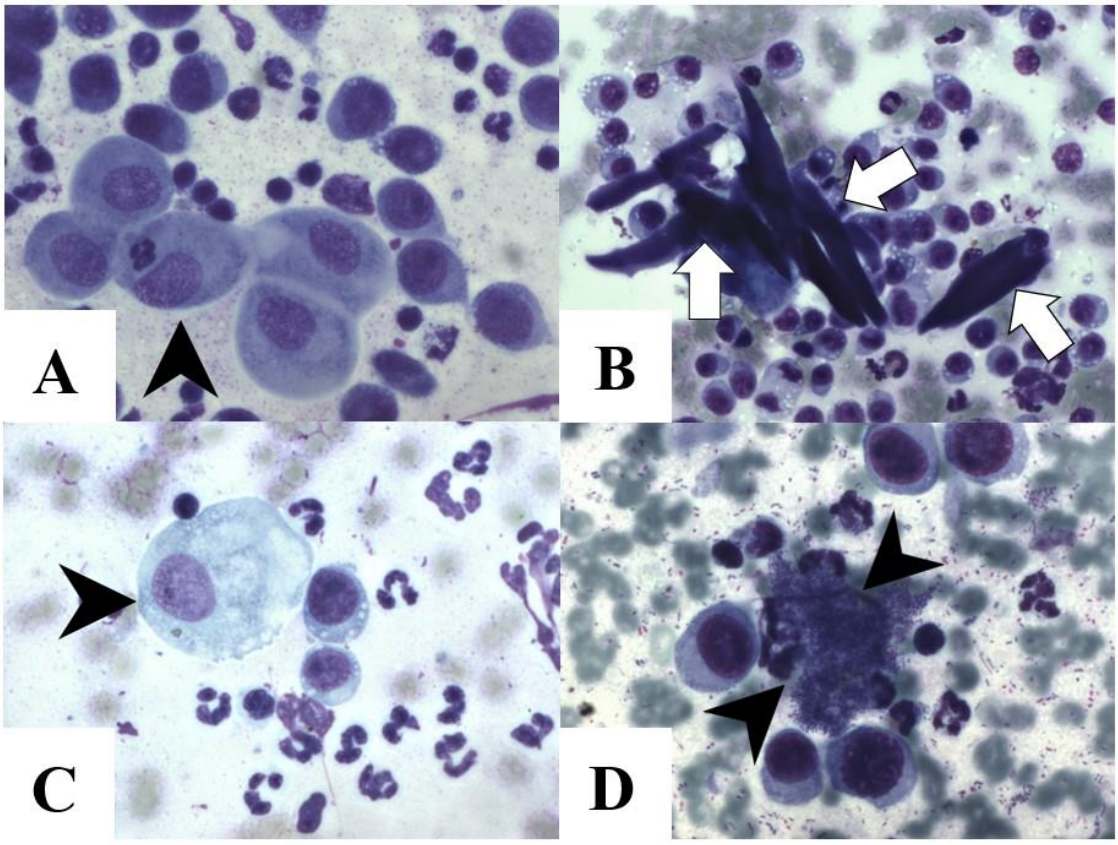

Figure 8. Epithelial cells and bacteria in CTVT cytology. A) Intermediate cellularity of CTVT round to oval cells and intermediate inflammatory pattern composed mostly of neutrophils and lymphocytes. Neutrophils often present naked nuclei and occasionally hypersegmentation. Note five large epithelial cells, characterized as keratinocytes, very larger than CTVT cells (black arrowhead). Romanowsky, magnification 200 x. B) Intermediate cellularity of mostly ovoid canine transmissible tumor cells interposed by red blood cells, neutrophils, and eosinophils. Highly basophilic papyriform structures, defined as corneocytes, are evident (white arrows). Romanowsky, magnification 120 x. C) A marked inflammatory pattern composed of neutrophils, mostly with naked nuclei, is evident. A central large epithelial cell, possibly a keratinocyte is visible (black arrowhead) along with two transmissible venereal tumor cells. Some red blood cells are also present. Romanowsky, magnification 200 x. D) A dense cluster

of bacteria, possibly Bacillus, is seen (Black arrowheads) along with canine transmissible venereal tumor cells. Red blood cells in high concentration and neutrophils are present. Romanowsky, magnification 200 x.

4. DISCUSSION

The previous classification for CTVT, as proposed by Amaral et al. (2007), outlines a comprehensive set of criteria for characterizing the tumor cells. These criteria encompass various aspects, including cell morphology, the relationship between the nucleus and cytoplasm, nuclear positioning, cytoplasmic granularity, the presence of vacuoles, and the number of nucleoli.[6] While this classification offers a detailed description, it does have certain limitations. One primary limitation is the absence of defined criteria of importance within the classification, which can lead to subjectivity in the analysis.[6,11,21] This subjectivity is evident when comparing studies, as there are significant variations in the prevalence of each cell type across different research efforts, as demonstrated in Table 3. [4,6,8–10,22–24]

Table 3. Literature comparison of cytomorphology in canine transmissible venereal tumor (CTVT)

Reference	Location	Years	Plasmacytoid	Mixed	Lymphocytoid
Amaral et al. (2007)*	São Paulo, Brazil	NR	52.5% (83/158)	29.1% (46/158)	18.4% (29/158)
Pimentel et al. (2024)[1]	Minas Gerais, Brazil	2016-2022	45.0% (18/40)	27.5% (11/40)	27.5% (11/40)
Faro et al. (2020)	Pará, Brazil	NR	11.1% (4/36)	13.9% (5/36)	75.0% (27/36)
Bulhosa et al. (2020)	Bahia, Brazil	2017-2018	61.8 % (21/34)	14.7 % (5/34)	23.5 % (8/34)
Setthawongsin et al. (2018)	Khon Kaen, Thailand	NR	68.2% (30/44)	18.2% (8/44)	13.6% (6/44)
Chowdary et al. (2016)	Andhra Pradesh, India	2013-2015	6.9% (2/29)	0.0% (0/29)	93.1% (27/29)
Valençola et al. (2015)	Mato Grosso do Sul, Brazil	2013-2014	81.8% (72/88)	11.4% (10/88)	6.8% (6/88)
Paranzini et al. (2015)	Paraná, Brazil	2012-2013	45.6% (21/46)	37.8% (16/46)	19.7% (9/46)
Lima et al. (2013)	Goiás, Brazil	2011	45.0% (9/20)	25.0% (5/20)	30.0% (6/20)

NR: Not reported. *Original study of the cytomorphological classification. [1]This study.

This study appears to have achieved results that closely align with the cytomorphology classification proposed by Amaral et al. (2007).[6] It may suggest a consistent and reliable application of the classification criteria, minimizing the discrepancies observed in other studies and strengthening the relevance of findings. However, there was no association with chemoresistance unlike what was previously suggested.[11]

Few reports in the literature indicate that plasmacytoid cases manifest themselves more aggressively and have greater chemoresistance, possibly due to a higher expression of P-glycoprotein, a chemoresistance mechanism of the tumor to vincristine sulfate, which is widely utilized in the treatment of this neoplasia.[11] Nonetheless, another study denotes no difference between the predominant cytomorphology in the tumor and the number of vincristine sessions required for complete remission.[8]

Despite anisokaryosis, binucleated cells, and mitotic count being established as criteria for the chemoresistance score, it is noteworthy that multinucleated cells also exhibited a statistically significant association with chemoresistance. However, for practicality in routine use, we opted to define a score with only three features for analysis, along with the best cut-offs tested in ROC curves for the score. The sole cytological feature examined by previous studies was the cytomorphological classification;[6,11] nonetheless, it was omitted from the score due to its lack of association with chemoresistance in the present study.

The mechanisms underlying chemotherapy resistance are classified as primary, on cancers not exposed to treatment, or acquired if it emerges after chemotherapy, with selection of more adapted clones.[25,26] Acquired chemoresistance has been reported in canine cancers, such as mammary gland tumors and lymphomas.[27,28]

As demonstrated in this study, CTVT remains highly responsive to vincristine sulfate treatment.[2,29] However, different forms of chemoresistance may occur. [11,30] Distinct sublineages and clades of CTVT are dispersed worldwide, originating from a common ancestor in Siberia.[31–33]. Regional patterns of mutations may confer chemoresistance to some populations and sensitivity to others, as potentially observed in Turkish and Romanian cases with a higher rate of chemoresistance to vincristine compared to Brazilian cases, which exhibit a higher rate of sensitivity to vincristine chemotherapy.[2,34,35] However, there remains a gap of studies systematically analyzing clinical data with consideration for CTVT clades and mutations that potentially contribute to tumor progression or confer chemoresistance.

While chemoresistance may be intrinsically linked to tumour cells, its proliferation rate, mutational burden or gene expression, it may also be related to external factors such as poor drug disposition and immune response.[25,36] During CTVT treatment, improper practices, with

lower dose or inappropriate drug interval, may elicit chemoresistance, while treatment failure may also occur in immunosuppressed patients, such as those with comorbidities or recently submitted to surgery or other invasive procedures.[37,38] This may result in emergence of resistant lineages which may spread to other dogs if patients under treatment are not segregated, especially in kennels or rescued populations. Therefore, it is essential to emphasize the importance of conducting regular studies and maintaining vigilance to monitor the effects of ongoing chemotherapy protocols for CTVT. Avoiding prolonged exposure to incomplete chemotherapy protocols is crucial, as this can potentially lead to the selection of resistant cell lineages.[25] All cases included in this study received appropriate doses, intervals, and the designated number of sessions, administered by the same practitioner.

Dysregulation in ABC transporters, particularly P-glycoprotein (ABCB1), has been linked to vincristine chemoresistance in people and dogs.[11,39,40] The overexpression of P-glycoprotein is mainly acquired after treatment exposure, but it may be primary, as reported in canine mammary gland tumors.[26] Nevertheless, it is inappropriate to solely attribute the chemoresistance risk in this study to increased drug efflux from MDR1 gene dysregulation.

In the present study, most resistant cases did not demand additional therapy beyond vincristine (8/10), which aligns with the findings of Costa et al. (2023).[2] It allowed us to classify these cases as slow responders, which might be related to the phase of the tumor development when treatment was initiated. A delayed response may be linked to various factors, including both primary mechanisms like distinct lineages of CTVT or the tumor's growth phase, and extrinsic factors associated with the tumor microenvironment and the immune response. The growth phases of CTVT are not fully understood, but samples can be broadly classified as progressive and regressive based on stroma and cellularity.[41–44] The progressive phase of CTVT is characterized by a high mitotic count, delicate stroma, and high cellularity, while regressive CTVT is associated with a low mitotic count, marked stroma, and low cellularity.[41,44] Progressive cases may exhibit a faster response to vincristine, whereas regressive cases may experience a delayed response due to the lower mitotic count. However, it is important to note that features such as cellularity and stroma are more appropriately evaluated in histopathology for a comprehensive understanding and thus, it was not evaluated in the cytological samples of this survey. Further studies should aim to categorize it more precisely.

The immune response may play a role in the natural progression of CTVT. In experimental conditions, 75% (6/8) of dogs subjected to whole-body irradiation (200 rads, 220 kV, and 15 mA) after CTVT subcutaneous injection developed metastasis.[37] Also, in experimental conditions, after xenotransplantation in immunosuppressed mice followed by

inoculation in dogs, the tumors demonstrated rapid growth and underwent transcriptome reprogramming.[36] In natural cases, however, the extent of the immune response's involvement remains unclear. Recently, the cellular immune response (CD3+, CD4+, CD8+, CD79+, monocytes and NK) of progressive and regressive CTVTs was analyzed, but no significant differences were identified.[41] In contrast, immunotherapy using dendritic cells (1.8×10^8) pulsed with exosomes has been proven to be effective in achieving complete remission of CTVT in seven weeks without the need for chemotherapy.[45]

MHC class I and II are more expressed in CTVT during the regression phase compared to the progression phase.[41,46] However, the mechanisms underlying the underregulation of MHC during spread and progression and its subsequent increase during regression remain unclear. Tumor infiltrating lymphocytes seem related to the increase of MHC in regression phase of inoculated cases,[46] but unclear in natural cases.[41] Experimentally, heat shock protein 60 demonstrates elevated levels during the clinical regression phase of CTVT.[43] This observation is significant, especially considering its absence in tissue samples from other canine tumors such as Sertoli cell tumor, mammary gland carcinomas, ovarian adenoma, squamous cell carcinoma, and melanoma. While testing in natural cases is required for further validation, this stress-induce protein holds promise as a potential diagnostic marker.

Complete spontaneous remission is exceedingly rare in cancer, and practitioners should not anticipate or rely on this outcome in their treatment strategies. It is important to emphasize that in the cases included in this study, none exhibited such a clinical outcome. The pathways leading to spontaneous remission in CTVT are not fully described and are reported in isolated case reports.[47] Predictors of complete remission without any treatments remain elusive — It is speculated that they may be related to tumor immunogenicity and the recognition of major histocompatibility complex class II (MHC II) in patients who have undergone an immunosuppressed period.[48] For now, spontaneous remission in CTVT remains a rare phenomenon, primarily observed in experimental settings and uncommon case reports.[41] Therefore, clinicians must continue relying on established treatment protocols and not assume or expect spontaneous remission as a common occurrence in their clinical practice.

This study reaffirms that cytology remains the best method for diagnosing CTVT. This is supported by our accurate re-diagnosis of all cases. Previous research consistently shows that cytology is superior to histopathology in distinguishing CTVT from other round-cell tumors.[3,22,49] When there is uncertainty about complete remission, additional cytology examination helps differentiate between residual tumor nodules and other conditions like inflammation or

fibrosis.[50] In summary, cytology is the primary diagnostic tool and is crucial for monitoring and assessing CTVT cases after treatment.

Neoplasms can exhibit various malignancy indicators in cytology, including a high mitotic count, atypical mitotic figures, anisocytosis, anisokaryosis, karyomegaly, and binucleation.[12,13,17,19] In a study by Valençola et al. (2015) that analyzed malignancy criteria in 88 cases of CTVT, atypical mitosis was observed in 87.5% of cases, binucleation in 68.2%, and karyomegaly in 63.6%.[9] Our results suggest a higher frequency of binucleation (73.2%), while karyomegaly was present in a similar frequency of cases (62.5%). Mitotic figures were nearly ubiquitous in our cases, so we categorized them into quantitative and qualitative groups. Multiple nucleoli were evident in 19.3% of Valençola et al. (2015), while we noticed it in 37.5% of cases considering >5% of multinucleated cells.

Grading systems for cancer evaluate malignant features as indicators of a poor prognosis and factors that reduce median survival time.[19,51] For cytologic grading of canine mast cell tumors, high-grade tumors are identified by criteria such as mitotic count and binucleation.[12,13] However, it is interesting to note that these characteristics play a different role in the CTVT score proposed. Unlike many other cancers, CTVTs rarely result in the death of affected dogs and typically respond well to vincristine chemotherapy. Therefore, the primary objective of our study was to establish a correlation between the response to vincristine treatment and cytological findings rather than focusing on survival times as a measure of prognosis.

This study presents some limitations. Firstly, collecting comprehensive clinical follow-up data from all patients was challenging, particularly given that many had continuous outdoor access, which also increase the risk of tumor transmission. Additionally, the variability in slide quality due to samples collected by professionals with different levels of experience posed a challenge and may have affected the standardization of the slides. Furthermore, the absence of a clear guideline for managing cases of chemoresistance to vincristine in patients with CTVT, as uncertainty regarding when to therapeutic change led to variations in clinical management. The decision to switch chemotherapy occurred at different times, typically after 4 to 6 vincristine sessions with no clinical improvement. These limitations underscore the complexity and variability encountered when managing CTVT cases in a real-world clinical setting.

Populations with distinct CTVT lineages may exhibit varying clinical behaviors, and the chemotherapeutic resistance score should undergo further evaluation in other populations. If validated, it could serve as a cost-effective method to aid in the clinical decision-making process. In risk-high cases for vincristine resistance, alternative treatments may be elected as a primary approach.

5. CONCLUSIONS

The identification of low mitotic count, no binucleated cells, and low anisokaryosis in CTVT cytology holds significant predictive value for assessing chemoresistance to vincristine sulfate. When organized into a scoring system, these characteristics effectively indicate the risk of chemoresistance. Counterintuitively, cytological malignancy features were inversely associated to CTVT vincristine chemoresistance.

The application of an adapted cytomorphology classification for CTVT demonstrated excellent concordance; however, it was not found to be correlated to vincristine chemoresistance.

DATA AVAILABILITY STATEMENT

The data for each case and cytology feature can be found in the Appendix. For any additional information related to this study, kindly reach out to the corresponding author upon request.

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CHAPTER II[2]

CANINE TRANSMISSIBLE VENEREAL TUMOUR: CLINICAL MANIFESTATIONS, CHEMOTHERAPY RESPONSE AND EPIDEMIOLOGICAL ASPECTS

ABSTRACT

Canine transmissible venereal tumour (CTVT) is a unique cancer known for its sexually transmitted implantation and low metastatic potential. Nevertheless, the tumour has never been thoroughly characterized in the Belo Horizonte region of Brazil. This retrospective study aimed to identify and describe the clinical manifestations, treatment efficacy and epidemiological aspects of the canine population affected by CTVT, diagnosed in a university veterinary hospital. A total of 131 dogs were diagnosed with CTVT through cytology or histopathology, comprising 80 females (61.1%) and 51 males (38.9%). The predominant clinical presentations included genital (n= 114/131; 87%), cutaneous (n= 14/131; 11.5%), nasal (n= 8/131; 6.1%), and oral (n= 6/131; 4.6%). The most commonly observed combinations of clinical manifestations were genital-cutaneous (n= 7/131; 5.3%) and oronasal (n= 4/131; 3.1%). Mixed-breed dogs are predisposed to CTVT ($p < 0.0001$). Male dogs are associated with nasal manifestation and show a risk 5.2 times higher of presenting nasal CTVT than females. Purebred dogs also exhibited a significantly increased risk for nasal CTVT, with odds 8.2 times higher. Trends show genital presentation occurring more frequently in females and cutaneous manifestations in male dogs. Clinical presentations, patient size, and breed were not associated with the number of chemotherapy sessions required. Regarding the epidemiological profile, mixed-breed dogs were the most affected (70.2%), with an average age at diagnosis of 4.5 years. This study underscores that CTVT exhibits a broad variety of clinical presentations extending beyond the genital region, emphasizing the importance of recognizing and diagnosing atypical manifestations for early intervention.

Keywords: Sticker tumour, contagious, neoplasm, vincristine, tumor.

[2] Chapter submitted to the *Research in Veterinary Science*

1. INTRODUCTION:

Canine transmissible venereal tumour (CTVT) is a malignant neoplasm whose origin date back thousands of years and has uniquely perpetuated itself through somatic cell lineage transmission in dogs (Baez-Ortega et al., 2019). Along past decades, it has been studied as a cancer research model, aiming to gain a better understanding of neoplastic progression and survival mechanisms (Dujon et al., 2020). Its contagious nature provides distinct prevention methods compared to most neoplasms in domestic animals, and autochthonous cases can cease through strict population control campaigns, as seen in Western European countries (Aupperle-Lellbach et al., 2022; Gibson et al., 2021; Strakova and Murchison, 2014). In contrast, CTVT is endemic in most low-income countries, particularly in Latin America, Africa, Eastern Europe and Asia (Akhtar et al., 2021; Arcila-Villa et al., 2018; Ayala-Díaz et al., 2019; Ignatenko et al., 2020; Ishengoma et al., 2018; Schectman et al., 2022).

The main clinical manifestations of CTVT includes genital presentation, involving the vulva and vagina in females, and the penis and prepuce in males. Cutaneous, nasal, and oral manifestations are also associated with implantation by contagion (Araujo et al., 2016; Valençola et al., 2015). Genital manifestations are widely described and mentioned in the literature for over a century (Beebe and Ewing, 1906; Hataya et al., 1958; Huppes et al., 2014). Conversely, atypical/extragenital manifestations are occasionally considered rare and have been described in more detail only in the last decades (Bandaranayaka et al., 2023; Ignatenko et al., 2020; Peixoto et al., 2016). Apart from its low metastatic potential, the main site is regional lymph nodes, although occurring in less than 5% of cases (Araujo et al., 2016; Peixoto et al., 2016).

In Brazil, several studies have characterized the presence of CTVT in urban centres, with emphasis on the states in the Southeast region (Pimentel et al., 2021). In the state of São Paulo, retrospective studies characterized the population of dogs with the neoplasm in cities such as Botucatu and São Paulo (Amaral et al., 2004; Kimura et al., 2012). In Rio de Janeiro, literature cases are concentrated in Seropédica and the city of Rio de Janeiro (Araujo et al., 2016; Ramadinha et al., 2016). In Minas Gerais, despite the largest urban centre being the metropolitan region of Belo Horizonte, most of the reported cases in the literature are concentrated in Uberaba (Huppes et al., 2014). In the city of Belo Horizonte, fewer than 15 cases have been reported in the last 10 years (Araújo et al., 2012; Martins et al., 2014; Santos et al., 2011), prompting the question of whether there are indeed few CTVT cases in the area or if few cases are reported in the literature.

The goal of this study was to characterize dogs with CTVT treated at a veterinary hospital, encompassing the identification of epidemiological risks, clinical presentations, and treatment efficacy. Additionally, the aim was to subclassify clinical manifestations based on their concurrent occurrence and analyse their relationship with epidemiological aspects.

2. METHODS AND MATERIAL

Data collection and clinical records

Medical records of cases of CTVT in the Universidade Federal de Minas Gerais Veterinary Hospital (VH) system were collected from 2012 to 2022. As exclusion criteria, individuals who were evaluated at the VH, but were not registered in the system or a conclusive diagnosis of the analysed tumour was not achieved through cytology or histopathology were not included. Diagnoses made at VH were exclusively performed by the veterinary pathology (histopathology) and clinical veterinary pathology (cytology) departments.

The collected patient information included age at diagnosis, year of diagnosis, sex, breed, clinical manifestations of the disease (genital, ocular, nasal, oral, cutaneous, perianal, in lymph nodes, and other less frequent sites), and treatment protocol, including chemotherapy and the number of sessions. Patient age was determined based on their age at the time of tumour diagnosis through morphological examination, cytology or histopathology. Since many CTVT-affected dogs had been rescued, determining their exact ages was challenging, and these cases were excluded from the age analysis and reported in the Supplementary material as “Undetermined”. Two categories were established to classify the age and size of the animals, following the criteria established by (Mila et al., 2015): Animals are categorized as either "young" (≤6 years of age) or "old" (>6 years of age), and they are further categorized as "small breed dogs" (<15 kg), "medium breed dogs" (15–25 kg), and "large breed dogs" (>25 kg).

Complete remission was defined as the absence of macroscopic identification of the neoplasm during follow-up appointments after the end of the chemotherapy protocol. Partial remission was considered as a reduction of over 30% in tumour volume after the chemotherapy protocol, but some of the tumour remained. Stable disease was defined as a decrease of less than 30% of the tumour volume or an increase of less than 20%. Progressive disease indicated more than a 20% increase in tumour volume. The objective response was defined as the combination of complete response and partial remission cases. If there was any uncertainty, a cytological examination of the suspected tissue was performed.

Data were retrieved from the VH’s clinical data storage software (SGV), stored in

Microsoft Excel® spreadsheets, and reviewed by peers before data analysis. Any incomplete data were cross-checked with the Oncology department and if indeterminate, they were not included in analyses. Ultimately, the collected information is organized and presented in the Supplementary Material.

Statistical analysis

The analysed variables included gender, size, weight, age, breed, mixed-breed, TVTC manifestations, TVTC therapy, and number of chemotherapy sessions. Data were organized using Microsoft Excel® software (version 2013), and descriptive analyses were presented in tables and graphs. The Fisher's exact test was employed to assess associations among the data sources and between each variable. Proportional tests were applied to identify differences in the proportions of the studied variables. The odds ratio (OR) was calculated as a measure of association, considering a 95% confidence interval (95% CI). Data analysis was performed using Stata software version 4.0, and a significance level of 5% was applied for all statistical analyses (Fávero and Belfiore, 2017).

3. RESULTS

Epidemiology

During the analysed decade (2012-2022), the VH database contained information on 60,256 dogs, out of which 3,020 were oncology cases. A total of 160 cases received a conclusive diagnosis of CTVT, but only 131 of them had a comprehensive clinical description available. This resulted in an absolute incidence of 21.7 cases per 10,000 dogs, or approximately 1 case for every 460 dogs treated at the VH (Fig. 1). At the Oncology Service, 3,020 patients were attended, constituting 5.0% of the cases seen at the VH, with CTVT accounting for 4.3% of the cases attended.

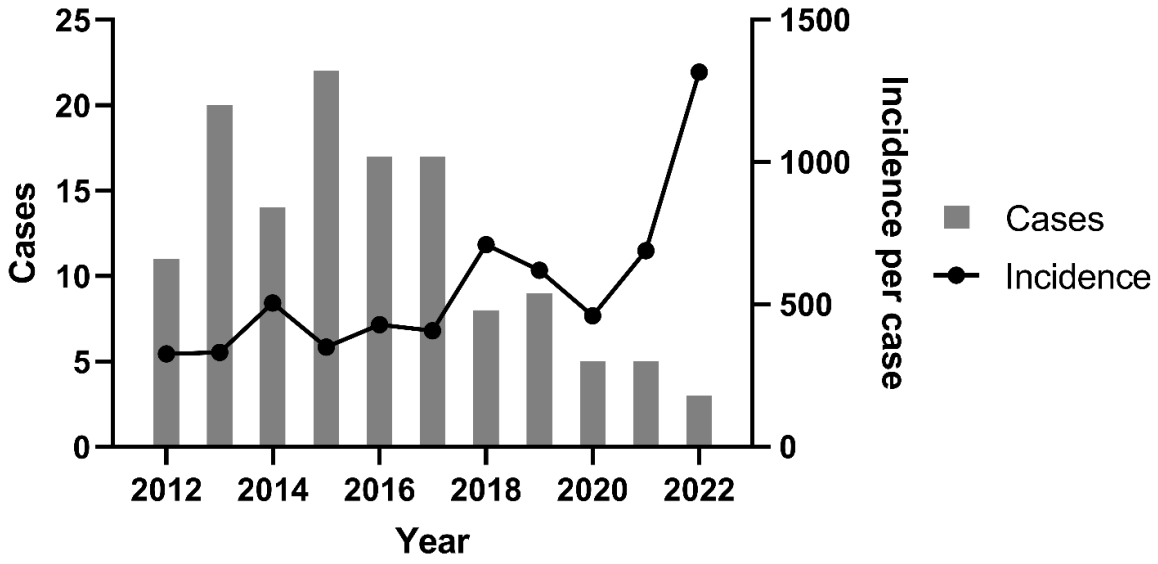

Fig. 1. Cases of canine transmissible venereal tumour (CTVT) per year from 2012 to 2022 in Belo Horizonte, Brazil.

A significant decline in incidence is observed over two distinct time periods: 2012-2017 and 2018-2022 (p = 0.004). The highest incidence rate was observed in the first year of the study (2012), with 30.6 cases per 10,000 dogs, which corresponds to 1 case of the disease in every 327 individuals, while the lowest incidence rate occurred in the last year of the study (2022), with 7.6 cases per 10,000 dogs, corresponding to 1 case per 1,317 dogs attended at the hospital.

Mixed-breed dogs represented 70% (92/131) of the cases, while the most common pure breeds were: Poodle (n = 9/131, 6.9%), Labrador Retriever (n = 5/131, 3.8%), Boxer (n = 4/131, 3.1%), Golden Retriever (n = 3/131, 2.3%), German Shepherd (n = 3/131, 2.3%), Shih Tzu (n = 3/131, 2.3%), Miniature Pinscher (n = 3/131, 2.3%), and Yorkshire Terrier (n = 3/131, 2.3%). Individuals of other breeds accounted for 4.6% (6/131) of the cases, including one of each of the following breeds: Basset Hound, Beagle, Chow-Chow, Belgian Shepherd, American Pitbull, and English Pointer. Table 1 displays the relationship between CTVT manifestations and breed status, whereas Fig. 2 illustrates the odds ratio of breed in a graphical plot. Eighty female dogs (61.1%) presented CTVT, while males accounted for 51 cases (38.9%). There was no association between the number of chemotherapy sessions and gender (p>0.05).

The most significant association was observed in nasal manifestations related to purebred dogs (p=0.009). The calculated odds ratio was significant, with an OR of 8.2 (95% CI= 1.9 – 40.7), indicating that purebred dogs are 8.2 times more likely to develop nasal manifestations in cases of CTVT compared to mixed-breed dogs. Conversely, in the case of genital

manifestations, it was more likely to occur in mixed-breed dogs (p=0.04). The odds ratio, using the same reference, was 0.3 (95% CI: 0.1 – 0.9), indicating that mixed-breed dogs are 3.2 times more likely to exhibit genital manifestations than purebred dogs (95% CI: 1.1 – 9.2). Other manifestations did not present significant odds ratios or p-values by Fisher's exact test (lymph node, ocular, perianal, cutaneous, and oral).

Table 1

Associations between dog breed and canine transmissible venereal tumour manifestations in Belo Horizonte, Brazil (2012-2022).

CTVT Manifestations	**Purebred**		**Mixed-breed**		**Total**	**p-value**	**Odds ratio (95% CI)[1]**
	n	Incidence	n	Incidence			
Genital	30	1:1,525.0	84	1:181.8	114	0.043*	0.3 (0.1 – 0.9)
Lymph nodes	0	–	4	1:3,817.3	4	0.317	–[2]
Ocular	0	–	3	1:5,089.7	3	0.554	–[2]
Perianal	2	1:2,2875.5	2	1:7,634.5	4	0.582	2.4 (0.4 – 15.9)
Cutaneous	5	1:9,150.2	10	1:1,526.9	15	0.768	1.2 (0.4 – 3.7)
Nasal	6	1:7,625.2	2	1:7,634.5	8	0.009*	8.2 (1.9 – 40.7)
Oral	2	1:2,2875.5	4	1:3,817.3	6	1.0	1.2 (0.2 – 5.3)
Total[3]	39	1:1,147.5	92	1:165.9	131	<0.0001*	0.1 (0.1 – 0.2)

*Significative association in Fisher's exact test ($p<0.05$). [1]Odds ratio considered purebred as referent category (exposed). [2]In these binary outcomes in which the Purebred group had no occurrences of the outcome, calculating the odds ratio was not appropriate. [3]The sum of cases does not correspond to the sum of cases for each manifestation due to cases with concomitant manifestations.

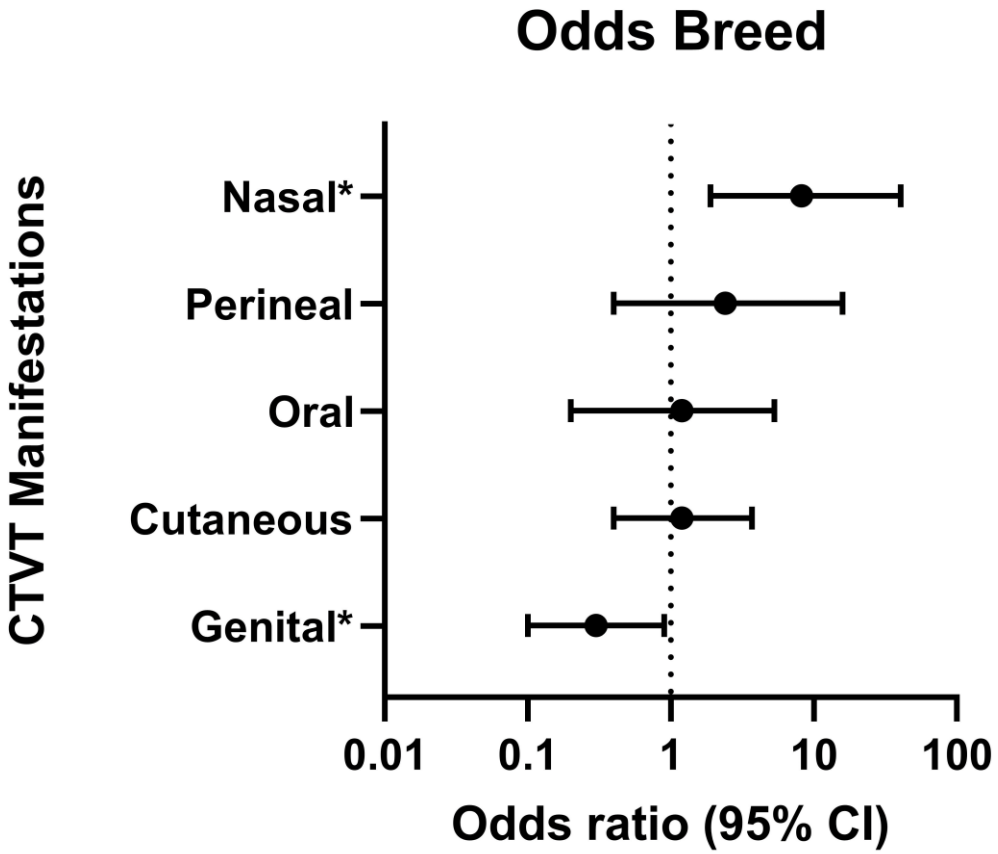

Fig. 2. Odds ratio plot depicting the association between breed and the manifestations of canine transmissible venereal tumour. The purebred category was used as the reference category in comparison to mixed-breed dogs. CI represents the confidence interval. *The odds ratio is considered statistically significant with a 95% CI.

The average age of the dogs was 4.5 years, with a standard deviation of 3.1 years. The age range was extensive, with the youngest dog aged 6 months and the oldest 14 years. Out of the cases, only seven (7.1%) were aged over ten years, while twenty-five cases (25.3%) were less than two years old. The majority of the dogs fell within the categories of young and adult (≤ 6 years old), accounting for 77.2% of the cases, with the most common age group being 1.1 to 3 years old (38.6%).

Clinical manifestations

One hundred thirty-one dogs met the inclusion criteria. Among the clinical manifestations of the neoplasia, genital manifestation was the most frequent, occurring in 87% of cases (114/131). Exclusively genital presentation corresponded to 77.9% (102/131) of cases, considering the vulva and vagina of females and the penis and prepuce of males. Cases

with concurrent extragenital and genital manifestations accounted for 9.2% (12/131) of affected individuals. Fig. 3 represents a CTVT case treated with vincristine sulphate.

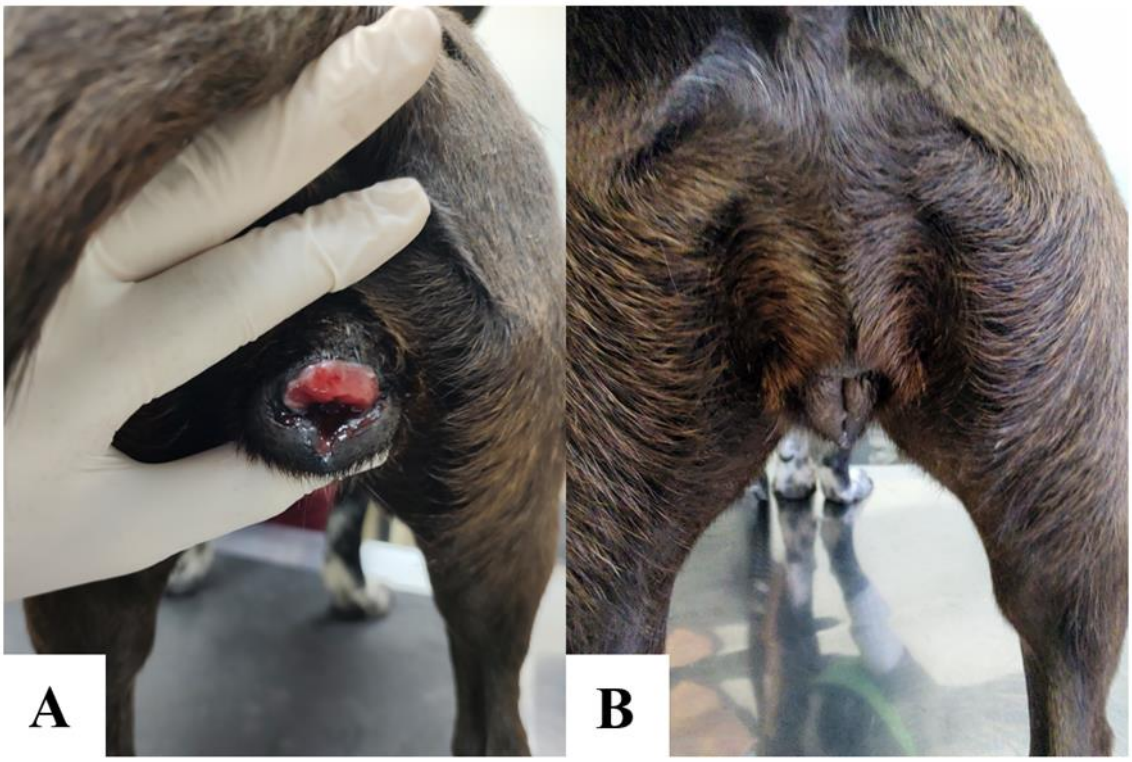

Fig. 3. 1-year old bitch presenting canine transmissible venereal tumour in the vulva. The tumour without any treatment, pre-chemotherapy (A). The tumour area after chemotherapy with vincristine sulphate: 4 sessions, intravenously, once a week, dose of 0.7 mg/m² (B). The patient present complete remission.

Extragenital manifestations constituted 22.1% (29/131) of cases, with 13% (17/131) as exclusively extragenital, including cutaneous, nasal, oral, or other non-genital manifestations. Cutaneous manifestations were the most common, representing 11.5% (15/131) of cases, followed by nasal (6.1%, 8/131) and oral (4.6%, 6/131) presentations. Perianal presentation and metastasis in lymph nodes each represented 3.1% (4/131) of cases. All other identified manifestations combined accounted for 3.1% (4/131) of cases, ocular represented 2.3% (3/131), with one case each of the following presentations: mammary gland, urethral, and vesical manifestations. One case of CTVT presented with suggestive involvement of the central nervous system (prosencephalon). The patient exhibited remission of the right cerebral lobe nodule after lomustine and vincristine treatment, as confirmed by two computed tomography scans. In this case, neurological clinical signs significantly reduced after chemotherapy, and the

cytological diagnosis of CTVT had been previously confirmed in the eye.

Single presentations of the disease were observed in 84% (110/131) of dogs, while 12.9% (17/131) had two concurrent manifestations. Only three individuals had three concurrent presentations (2.3%), and one was diagnosed with five presentations (0.8%). The most frequent associations of manifestations were genital-cutaneous (5.3%) and oronasal (3.1%). No associations were observed between the size of dogs and CTVT manifestations.

Table 2 describes the differences between males and females concerning the combination of clinical manifestations that affect them. The only statistically significant association of manifestations in relation to gender disparity was oronasal ($p= 0.021$). Other manifestations showed trends, with genital manifestations occurring more in females and exclusive cutaneous manifestation occurring more in males, but none presented a significant odds ratio.

Table 2

Clinical exclusive or associated manifestations of canine transmissible venereal tumour in Belo Horizonte, Brazil (2012-2022).

Clinical manifestations		**Cases in male dogs**	**Cases in female dogs**	**Total of cases**	**Frequency (%) in male dogs**	**Frequency (%) in female dogs**	**Total frequency (%)**	**p-value**	**Odds ratio**
Manifestations per dog									
	1	40/51	70/80	110/131	78.4%	87.5%	83.9%	0.222	0.5 (0.2 – 1.3)
	2	8/51	9/80	17/131	15.7%	11.3%	12.9%	0.594	1.5 (0.5 – 3.9)
	3	3/51	0/80	3/131	5.9%	0%	2.3%	0.057	–[2]
	5	1/51	1/80	1/131	0%	1.3%	0.8%	1	–[2]
Type of manifestation									
Exclusive	Genital	34/51	64/80	98/131	66.7%	80%	74.8%	0.101	0.5 (0.2 – 1.1)
Association	Genital-cutaneous	3/51	4/80	7/131	5.9%	5%	5.34%	1	1.2 (0.3 – 4.6)
Association	Oronasal	4/51	0/80	4/131	7.8%	0%	3.1%	0.021*	–[2]
Exclusive	Cutaneous	3/51	1/80	4/131	5.9%	1.3%	3.1%	0.299	4.9 (0.7 – 64.8)
Exclusive	Nasal	2/51	1/80	3/131	3.9%	1.3%	2.3%	0.559	3.2 (0.4 – 47.2)
Exclusive	Perineal	1/51	2/80	3/131	2%	2.5%	2.3%	1	0.8 (0.1 – 6.9)
Exclusive	Oral	0/51	1/80	1/131	0%	1.3%	0.8%	1	–[2]
Exclusive	Bladder	0/51	1/80	1/131	0%	1.3%	0.8%	1	–[2]
Association	Other	10/51	6/80	10/131	7.8%	7.5%	7.6%	0.322	1.7 (0.6 – 4.4)

*Significative association in Fisher's exact test ($p<0.05$). [1]Odds ratio considered Male as referent category (exposed). [2]In these binary outcomes in which the Male or Female groups had no occurrences of the outcome, calculating the odds ratio was not appropriate.

The odds ratio of manifestations analysed alone is describe in Table 3 and illustrated in a forest plot in Fig. 4, considering male dog as referent category relative to CTVT manifestations. Males accounted for 75% (6/8) and 66.6% (4/6) of dogs affected by nasal and oral manifestations, respectively. A similar pattern was observed with the cutaneous manifestation, in which males presented a higher frequency compared to females, representing 60% (9/15) of the cases (p= 0.094). The nasal manifestation was the only one with a significant odds ratio of 5.2 (95% CI= 1.2 – 25.9), occurring in 6/51 males and 2/80 bitches (p=0.055).

Table 3

Clinical manifestations of canine transmissible venereal tumour in Belo Horizonte, Brazil (2012-2022).

Clinical manifestations	**Cases in male dogs**	**Cases in female dogs**	**Total of cases**	**p-value**	**Odds ratio[1]**
Genital	41/51	73/80	114/131	0.108	0.4 (0.2 – 1.0)
Cutaneous	9/51	6/80	15/131	0.094	2.6 (0.9 – 8.1)
Nasal	6/51	2/80	8/131	0.055	5.2 (1.2 – 25.9)
Oral	4/51	2/80	6/131	0.207	3.3 (0.7 – 17.8)
Lymph node	3/51	1/80	4/131	0.299	4.9 (0.7 – 64.8)
Perianal	2/51	2/80	4/131	0.642	1.6 (0.2 – 10.4)
Ocular	1/51	2/80	3/131	1	0.8 (0.1 – 6.9)

[1]Odds ratio considered Male as referent category (exposed).

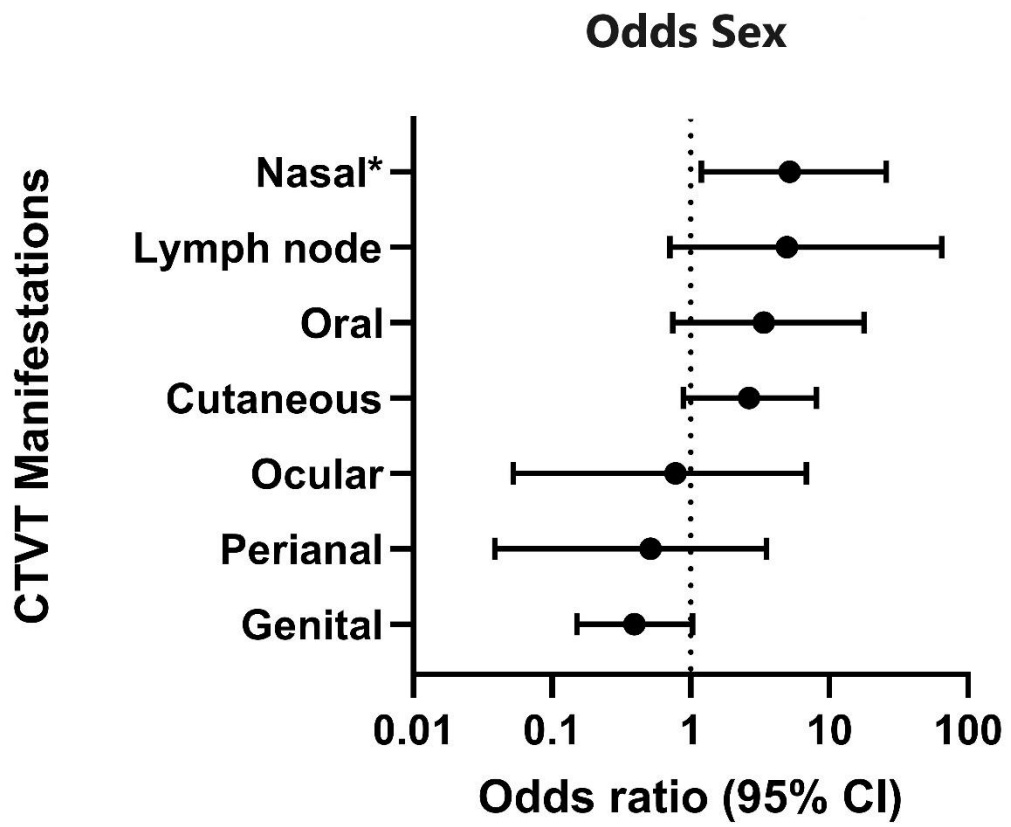

Fig. 4. Odds ratio plot depicting the relationship between gender and the manifestations of canine transmissible venereal tumour. The male category was utilized as the reference category in comparison to female dogs. CI stands for confidence interval. *The odds ratio is considered statistically significant with a 95% CI.

Diagnosis:

The diagnosis description was available in all cases, with 3/131 (2.3%) diagnosed by histopathology and 128/131 (97.7%) by cytology, which is the gold-standard method for definitively diagnosing CTVT at the VH. The three cases diagnosed by histopathology were extragenital presentations, including two nasal and one ocular manifestation. Notably, the primary clinical suspicion in these cases was not CTVT. All definitive treatments were carried out following the conclusive diagnosis of the tumour.

In cytology, CTVT presented typically round to ovoid-shaped cells, round, well-defined nucleus with coarse chromatin, variable nucleus-cytoplasm proportion, high in lymphocytic cases and moderate to low in plasmacytic cases (Fig. 5). Common findings include mitotic figures (typical and atypical), tadpole cells (tumour cells with cytoplasmic extensions, resembling mesenchymal cells), bacteria in imprinting samples, neutrophils, eosinophils,

macrophages and lymphocytes.

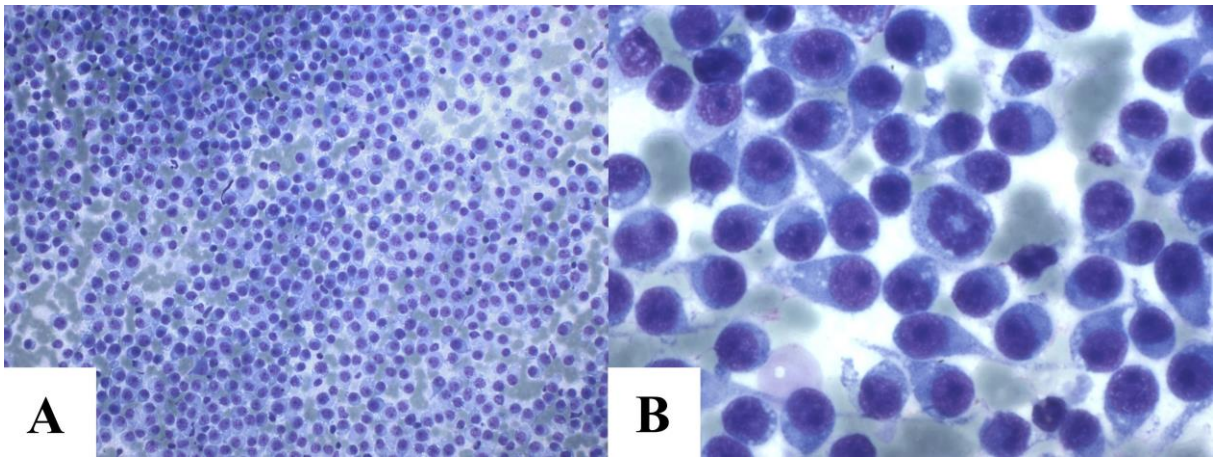

Fig. 5. Cytology of canine transmissible venereal tumour. Intense cellularity of CTVT cells. Romanowsky, 20 x 2 magnification (A). Intense cellularity of CTVT cells with a mitosis and various tadpole cells. Cells present mostly plasmacytic cytomorphology. Romanowsky, 100 x 2 magnification (B).

Treatment:

The main reported treatment modality was chemotherapy using the first-choice drug vincristine sulphate, in 97.7% (85/87). There was 97.6% (83/85) of objective response, including 91.8% (78/85) of complete response and partial remission achieved in 5.8% (5/85) of the cases. Stable disease was observed in 2.4% (2/85) of cases. The protocol employed consisted of intravenous administration of vincristine sulphate at a dose of 0.7 mg/m², administered weekly for 3 to 7 sessions. If the patient showed a high sensitivity to the treatment, such as experiencing grade II-IV neutropenia, the dose was reduced to 0.5 mg/m². The mean number of sessions involving vincristine was 4.5, with a median of 4 and a standard deviation of 0.97 (Table 4).

Table 4

Associations between gender and number of vincristine chemotherapy sessions in dogs with canine transmissible venereal tumour in Belo Horizonte, Brazil (2012-2022).

Gender	Parameters	Number of Vincristine Chemotherapy Sessions					Mean	Median	Standard Deviation	Total	p-value
		3	4	5	6	7					
Female	n	7	22	9	7	1	4.41	4	1.00	46	0.743
	%	9.6%	30.1%	12.3%	9.6%	1.4%				63%	
Male	n	2	11	7	6	1	4.74	5	1.02	27	
	%	2.7%	15.1%	96%	8.2%	1.4%				37%	
Total	n	9	33	16	13	2	4.53	4	0.97	73	
	%	12.3%	45.2%	21.9%	17.8%	2.7%				100	

In cases of partial remission following the vincristine sulphate protocol, the secondary treatment choices included surgery in three cases, monotherapy with lomustine (orally, 50 mg/m²) for three sessions in one case, and monotherapy with doxorubicin (intravenously, 30 mg/m²) for one session in another case. All patients achieved complete remission after these secondary treatments. However, the patient treated with vincristine + lomustine experienced a recurrence after eight months but was successfully treated again with the same protocol, ultimately achieving complete remission.

Two cases that had stable disease after the vincristine sulphate protocol also underwent successful surgery and did not experience recurrences. Additionally, two cases initially received surgery as their primary treatment option, although CTVT was not initially considered as the diagnosis in either case. Nevertheless, both cases did not experience recurrences as well.

Fig. 6 illustrates the main aspects of canine transmissible venereal tumour in dogs in Belo Horizonte, Brazil. Noticeable clinical-therapeutical points were the high sensibility to vincristine monotherapy and clinical manifestations, mostly genital, but with >25% cases presented genital concomitant with another manifestation or any extragenital without genital association. Most dogs were mixed-breed, female and young adults (1.1-3 years old). Additional information regarding each case is available in the Supplementary Material.

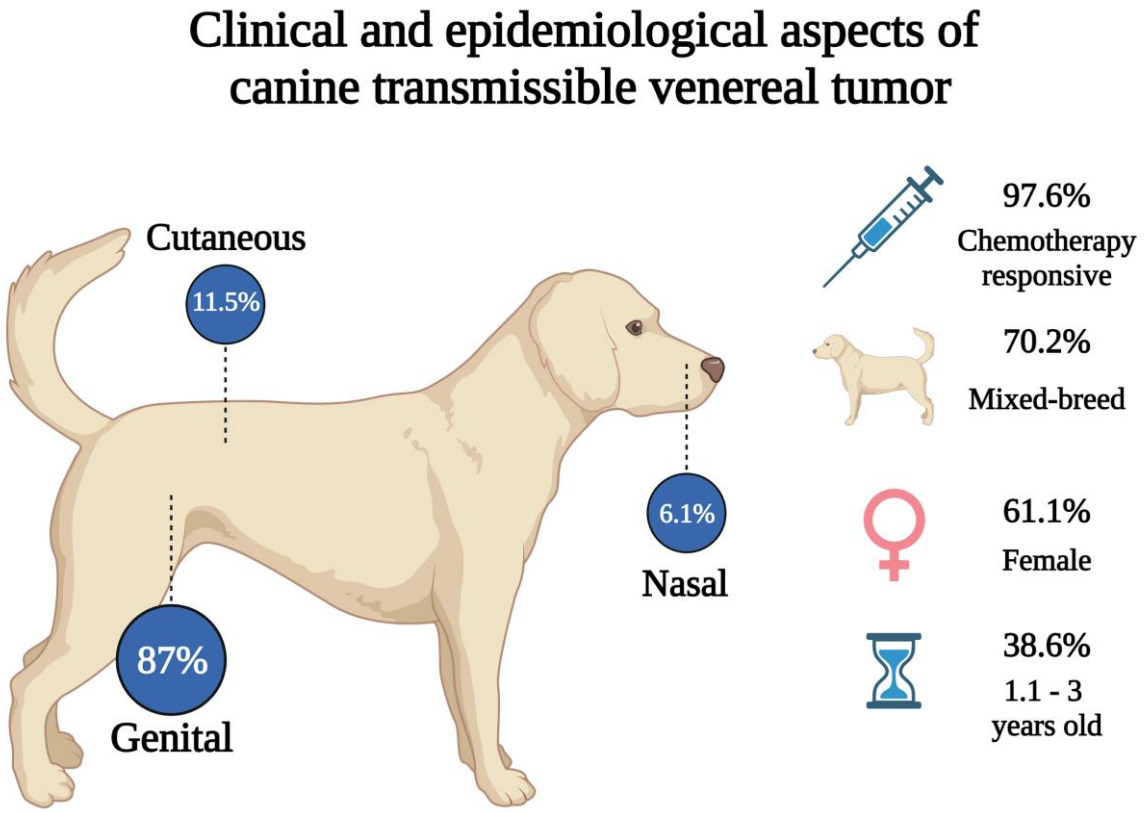

Fig 5. Main clinical and epidemiological aspects of canine transmissible venereal tumour (CTVT) in Belo Horizonte, Brazil.

4. DISCUSSION

The canine transmissible venereal tumour (CTVT) is widely documented as one of the most prevalent neoplasms in dogs across Latin America. From Argentina to Mexico, this neoplasm has maintained its endemic status over the course of several decades, and the associated risk factors persist (Ayala-Díaz et al., 2019; García et al., 2019; Guevara Manuel Alejandro et al., 2017). Our study contributes valuable data to reinforce this knowledge within the context of Minas Gerais, marking the first investigation to analyse its prevalence over a 10-year period in this region.

The retrospective survey uncovered a CTVT prevalence of 0.2% between 2012 and 2022, with this specific data lacking in numerous Brazilian studies. The frequency of CTVT cases, when expressed as a proportion of the total number of tumours in dogs, ranges from 0.9% to 17.1% according to previous research (Amaral et al., 2004; Andrade et al., 2012; Kimura et al., 2012; Rossetto et al., 2009; Viana et al., 2019). It's worth noting that the diagnostic method used can significantly impact these reported frequencies. For instance, Kimura et al. (2012)

identified a frequency of 2% in São Paulo city based solely on histopathological data, whereas Rossetto et al. (2009) found an 11.9% frequency in Londrina using data exclusively from cytology. It's important to highlight that both cities have similar human development indexes (HDIs), with respective values of 0.805 and 0.778 (Instituto Brasileiro de Geografia e Estatística, 2010).

Despite its prevalent occurrence in low-income countries, CTVT was even more widespread worldwide decades ago. It was defined as the most frequent canine tumour in Jamaica in 1968 (Thorburn et al., 1968) and in Kenya, when Murray reported a frequency of 12.4% (20 out of 161) of CTVT (Murray, 1968). In stray dogs, it remains very common and is considered the most common tumour in some countries, such as Mexico (Cruz et al., 2010). The population bias is significant because the maintenance mechanisms of the disease are intrinsic to stray dogs. In case series from veterinary hospitals that exclusively handle referral cases or offer high-cost treatments, there might be a significant reduction in the number of cases.

A pronounced reduction in cases has been strikingly demonstrated in the United Kingdom, following the implementation of comprehensive public dog population control policies (Gibson et al., 2021; Strakova and Murchison, 2014). In these countries, CTVT has become rare over several decades. While a decrease in cases has been observed in Belo Horizonte, it is essential to note that for a thorough evaluation, a larger and more representative sample encompassing the entire dog population of the city should be taken into account.

The genital presentation was the most frequently reported, in 87% of cases, in line with the literature characterizing CTVT as a neoplasm typically transmitted and maintained in genital organs (Araujo et al., 2016; Fêo et al., 2016), identifying frequencies close to those found in this study, 85.7% (Valençola et al., 2015) and 87% (Araujo et al., 2016). However, some studies identify this manifestation with a > 95% frequency among all cases of the disease (Huppes et al., 2014). This data may potentially be explained by underdiagnosed cases, as this neoplasm is stigmatized as a venereal tumour by its very name, and atypical forms of the disease can represent a clinical challenge (Gritzenco et al., 2022; Parker et al., 2021).

Extragenital manifestations vary widely in frequency among studies, and some are not mentioned in surveys, such as cutaneous and lymph node involvement (Huppes et al., 2014; Mascarenhas et al., 2014; Peixoto et al., 2016). Cutaneous presentation was the most frequently extragenital manifestation reported in this study, as previously demonstrated by (Peixoto et al., 2016), with 21.7% of cases affecting the skin. Extragenital locations, including nasal and oral, tend to be more frequent after cutaneous involvement, as suggested by the literature, and they

are more common than involvement of other sites and lymph node metastases (Araujo et al., 2016; Peixoto et al., 2016).

Although it is noticed that the majority of dogs diagnosed with CTVT are females (61.1%), there is an inversion of this proportion when analysing the oral and nasal manifestations of the disease. In this context, it was observed that males accounted for 75% (6/8) and 66.6% (4/6) of dogs affected by nasal and oral manifestations, respectively. Nasal manifestation was the only one significantly associated with gender in CTVT, as demonstrated. Male dogs appear to be predisposed to nasal CTVT due to their habit of licking and sniffing more than females (Scandurra et al., 2018; Strakova et al., 2022). A similar pattern was observed with the cutaneous manifestation, in which males presented a higher frequency compared to females, representing 60% (9/15) of the cases, while bitches accounted for 40% (6/15) of the cases. However, discussions on this topic are still lacking in studies.

While there wasn't a clear sexual predisposition indicated, there were more females affected than males in this survey, a trend also reported in most studies evaluating this gender ratio (Pimentel et al., 2021) and very close to what was identified by (Araujo et al., 2016). If age, gender, and other risk factors were segregated in order to see clinical patterns of the disease, this could be better understood.

The average age of dogs with this neoplasm appeared to be higher than what was reported in the literature from over a decade ago (Amaral et al., 2004; Silva et al., 2007), but it follows a similar pattern as identified in studies from the last decade, with an increase in adult to old dogs (Costa et al., 2019; Fêo et al., 2016), possibly due to the increased life expectancy of companion animals. Mixed-breed dogs are the most commonly affected by the neoplasm in recent studies (Fêo et al., 2016; Mascarenhas et al., 2014), which can be explained by their contact with the outdoor environment, either before adoption or even while under the care of owners.

The occurrence of CTVT concurrent manifestations might complicate the diagnosis and is poorly mentioned in Brazil. Recently, in Rio de Janeiro, (Costa et al., 2023) demonstrated the occurrence of associated manifestations in 7.9% (20/252) of cases, which is much higher than the figure reported by (Peixoto et al., 2016), also in Rio de Janeiro, where concurrent genital and extragenital manifestations occurred in only 1.9% of cases. On the other hand, most previous studies did not mention this occurrence of two or more manifestations (Brandão et al., 2002; Huppes et al., 2014; Mascarenhas et al., 2017). Abroad, this event is described more frequently. In Granada, about 11.5% (9/78) of CTVT cases occurred with two or more manifestations involved (Chikweto et al., 2013). A retrospective study involving dogs with

ocular presentation of the tumor in Greece found that 12% (3/25) of patients had another concurrent manifestation, with 4% (1/25) being genital and 8% (2/25) oronasal (Komnenou et al., 2015). The most representative study of CTVT in the United States in recent decades indicated the presence of multiple manifestations in 6.9% (2/29) of dogs between 1984 and 1996 (Rogers et al., 1998). In dogs with canine visceral leishmaniasis in Italy, 21% (4/19) of individuals developed cutaneous manifestations concurrently with genital ones, possibly indicating a relationship with protozoan-mediated immunosuppression affecting CTVT, although the sample was limited (Marino et al., 2012). Our study showed a high frequency of concurrent presentations (14.5%), higher than in Brazilian studies and similar to international studies (Chikweto et al., 2013; Costa et al., 2023; Huppes et al., 2014; Mascarenhas et al., 2017).

Cytology was the primary method for diagnosing CTVT, consistent with previous studies on this neoplasm (Fêo et al., 2016; Rossetto et al., 2009). It is a faster and more cost-effective method compared to histopathology and is commonly used for diagnosing round cell tumours (Araújo et al., 2012). Histopathology, on the other hand, is primarily employed for morphological diagnosis in necropsies, especially for tumours with atypical manifestations or for examination following surgical excision (Arias et al., 2016; Kimura et al., 2012). This was the case in our study, where CTVT was diagnosed by histopathology post-surgical excision when it was not the primary clinical suspicion.

The standard treatment with vincristine sulphate showed high efficacy, as expected and previously identified in Brazil (Bulhosa et al., 2020). CTVT is a chemosensitive neoplasm, and spontaneous regression, even if partial and not long-lasting, can occur more frequently compared to other neoplasms in dogs (Costa et al., 2022; Duzanski et al., 2022; Yang and Jones, 1973), primarily due to the potential immune recognition of tumour antigens after therapy, as demonstrated through the use of dendritic cells pulsed with tumour exosomes (Ramos-Zayas et al., 2019). The use of other chemotherapeutic agents, such as lomustine, is reserved for resistant cases to vincristine (Costa et al., 2023). Surgery as a treatment typically results in high recurrence rates (Dameski et al., 2018; Hithem et al., 2020). However, in this study, there were no instances of recurrence among the patients who underwent surgery. Therefore, it was only recommended when the tumour was chemoresistant or when there was another surgical indication, such as enucleation or resolution of peripheral neuropathy.

The limitations of this study include the dropout of patients after complete remission of the neoplasm, which hinders medium and long-term oncological follow-up. However, recurrences after conventional vincristine therapy are uncommon. Additionally, this neoplasm more frequently affects stray dogs, which are associated with owners in socio-economic

vulnerability, which can make treatment costs unfeasible favouring post-diagnosis evasion.

5. CONCLUSIONS

Our findings indicate a predisposition of mixed-breed dogs to CTVT, while male and purebred dogs are at higher risk for nasal presentation, contrasting with the predisposition of mixed-breed dogs towards genital presentation. Notably, a significant chemosensitivity to vincristine sulphate was observed in the cases, supporting its continued use as a first-line treatment. This study establishes, for the first time, the endemic nature of CTVT in Belo Horizonte, Brazil, although its incidence has decreased over the past decade. Furthermore, given the limited research in many countries, it is imperative to validate the identified risks in diverse geographic regions beyond our regional cohort.

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CHAPTER III[3]

GUIDELINES FOR CANINE TRANSMISSIBLE VENEREAL TUMOR TREATMENT: A SYSTEMATIC REVIEW

ABSTRACT

The canine transmissible venereal tumor (CTVT) treatment has undergone substantial evolution since it first description in 1810 and first successful chemotherapy treatment due to advancing insights about the origin and genetics of this cancer. Chemotherapy, notably vincristine, has been extensively used with favorable outcomes. However, the emergence of chemoresistance poses challenges, prompting the exploration of alternative therapies. This systematic review aims to address existing gaps in the literature, offering a comprehensive understanding of CTVT treatment practices over the decades. The ultimate goal is to formulate a guideline that gather the evolving strategies to effectively manage CTVT treatment. Among 3,633 initially identified studies, only 42 met rigorous inclusion/exclusion criteria. Vincristine sulfate monotherapy is currently the recommended first-line treatment for CTVT. When administered intravenously on a weekly basis, 4-6 sessions of vincristine at 0.5-0.75 mg/m² resulted in a notable 93.1% complete response in dogs. Extending the number of sessions to 8 achieved an impressive 98.9% complete response rate. Second-line choices encompass radiation therapy and doxorubicin, with surgery considered as a third-line option. Notably, after second- and third-line therapies, based on the comprehensive analysis of all studies, complete remission is anticipated in virtually all cases. Despite potential future challenges with chemoresistance, the current guide offers a thorough approach to CTVT treatment, synthesized from meticulous data collection in a systematic review.

Keywords: Oncology, chemotherapy, vincristine, Sticker tumor, chemoresistance.

[3] Chapter formatted according to the *Veterinary and Comparative Oncology*

1. INTRODUCTION

Although vincristine monotherapy is currently used for treatment of canine transmissible venereal tumor (CTVT), there is no consensus about the most effective treatment or second-line options.[1,2] Since its first description, by Blaine (1810)[3] and throughout the past century, growing insights into the disease origin and genetics, its patterns of spread, and geographical distribution have played a crucial role in shaping the decisions regarding its treatment.[4–6] Initially, back in 1906, CTVT was still regarded as an infectious and incurable tumor.[7,8] The reclassification of this cancer as a non-infectious round-cell tumor in the first half of the 20th century marked a significant milestone, leading to an enhanced understanding of its etiopathogenesis.[5,8–10] Until 1970, chemotherapy was infrequently mentioned as a treatment for CTVT. However, in the subsequent decades, there was a notable shift towards extensive chemotherapy use, supplanting traditional approaches such as radiation therapy and surgery.[10–12] This shift in perspective has led to high rates of complete response and, more importantly, cure.[6,13]

Vincristine sulfate, a vinca alkaloid, is globally acknowledged by small animal oncologists as a standard treatment for CTVT.[1,14,15] However, what course of action should be pursued when vincristine proves ineffective? It raises intriguing questions about chemoresistance in this tumor, a potentially emerging problem. The slow accumulation of mutations over thousands of years in the evolution of CTVT may be significantly altered by incomplete treatments, particularly when there is transmission of chemotherapy-exposed cells in shelters and places with large numbers of dogs.[16,17] Discussions persist regarding the optimal dosage and duration of vincristine sulfate treatment, including when to modify the treatment and the subsequent choice of drugs.[18,19] A systematic compilation of treatments and guidelines for managing cases of chemoresistance is still lacking in the literature.

Radiation therapy is considered an effective treatment for CTVT and has been used in high-income countries for decades;[10,20] however, it remains a distant reality in Sub-Saharan, Latin American, Middle Eastern, and South Asian countries—areas with high frequency of tumor,[1,21,22] which can be attributed to epidemiological risk factors such as a high rate of outdoor access, ineffective spay/neuter campaigns, and challenges in implementing positive dogs' treatment.[23–25]

The objective of this study was to characterize and analyze the treatments for CTVT over the past decades through a systematic review. Subsequently, we aimed to develop guidelines in collaboration with a group of small animal oncologists on how to proceed

in the treatment of CTVT.

2. METHODS

Review topic

Therapeutic options for CTVT treatment and how to proceed in case of chemoresistance to first-line conventional treatment, vincristine sulfate.

Search strategy and databases

Systematic search was conducted from October 2023 to November 2023 in the databases Google Scholar (https://scholar.google.com) and PubMed (https://pubmed.ncbi.nlm.nih.gov). The descriptors and Boolean operators were used in both databases as follows: "treatment" OR "therapy" OR "chemotherapy" OR "surgery" OR "treatments" OR "therapies" AND "transmissible venereal tumor" OR "transmissible venereal tumour" OR "Sticker tumor" OR "Sticker tumour". The time frame used for the search was 1950-2023, and no other criteria were applied in this initial identification of studies.

Inclusion and exclusion criteria

For inclusion, the study should present natural CTVT cases treated with at least one specific and well-described protocol (e.g.: if chemotherapy, it should include dose, number of sessions and interval between sessions). Cases presented in experimental studies conducted in laboratory that tested the therapies on dogs after CTVT idiopathic implantation were not considered as natural cases, so the studies were not included. Data concerning the efficacy of the treatment must be available for inclusion, as objective response, complete response, partial response and/or no clinical response. The languages of studies analyzed were English, Portuguese and Spanish. No restrictions were applied regarding the country of origin of the article.

Non-original studies, book chapters, congress abstracts, dissertations, thesis, narrative reviews, integrative reviews, other systematic reviews, and articles in which ethical approval was unclear were excluded. Duplicates were also removed. The study types included interventional studies, observational studies, and controlled trials. However, single case reports did not meet our inclusion criteria. No restrictions were applied to publications that involved more than one type of therapy.

The diagnosis of CTVT should include at least one morphological exam, either

cytology (imprinting, swab impression or fine-needle aspirate) or histopathology (incisional or excisional biopsy). History, anamnesis, imaging exams and clinical examination solely are not conclusive methods of diagnosis, so studies which relied exclusively on these aspects were excluded from final selection.

Stages of systematic analysis

The instructions in the Cochrane Handbook for Systematic Reviews of Interventions by Higgins et al. (2023)[26] were followed for the multi-stage review. All studies included in each stage were recorded in Excel® sheets and stored in Mendeley® bibliographic management software files.

After the search with the specific descriptors in each electronic database, the initial screening was performed including titles and abstracts reading. The following information was recorded from each included article: first author, publication year, article title, and the source link of the article. Duplicates identified in the same or different databases were removed.

All studies approved in screening phase underwent the full-text review, analyzing Objective(s), Methods and Materials, Results, Discussion, Conclusion(s), and Supplementary material, when available. If the study met the eligibility criteria, information of interest was extracted: article type, number of cases, treatment protocol, and country of the study. In instances of unclear or missing data, the respective corresponding author of each study was contacted. To seek clarification on data from 20 studies, all corresponding authors were contacted. Out of these, five authors responded and actively contributed to the process.[15,18,27–29]

3. RESULTS

Selected studies

A total of 3,633 studies were identified in the initial search—3,410 in Google Scholar and 223 in PubMed. Screening of abstracts and titles narrowed down the articles to 258, with the removal of 12 duplicates. Following full-text screening and data collection, the selection process further reduced to 42 articles for sequential analysis. Detailed data are available and in Supplementary Material.

Most studies (32) assessed the treatment for canine transmissible venereal tumor with vincristine sulfate in a single-drug protocol and 7 analyzed vincristine sulfate therapy associated with other treatments: Bacillus Calmette–Guérin (2), surgery (1), interleukin-

2 (1), L-asparaginase (1), IFNa-2a (1), and ivermectin (1). Radiation therapy was assessed in 3 studies, all evaluated untreated cases, as first-line therapy, and 2 included chemoresistant cases. Surgery was evaluated in 5 articles, 4 analyzing untreated cases and 1 used surgery as third-line therapy in vincristine sulfate and doxorubicin resistant cases.

Other chemotherapies included doxorubicin, cyclophosphamide, vinblastine and lomustine. Doxorubicin was the second chemotherapy more studied, after vincristine sulfate, in 6 studies, while only 1 tested it as first-line therapy in untreated cases and 5 used this drug only in vincristine resistant cases. Vinblastine was studied in 2 articles. Cyclophosphamide and lomustine were analyzed only in untreated cases, each in 1 different study.

The Immunomodulation of CTVT without chemotherapies was also tested in distinct forms. Bacillus Calmette–Guérin was tested as single therapy in 2 studies, while ns1 gene, vp3 gene, and ns1 + vp3 gene combination was assessed in 1 study, and dexamethasone therapy in another 1 article.

Twenty countries presented natural CTVT cases from selected studies analyzed by our review. In Americas, 21 studies were included, mostly from Brazil, 10 studies. United States, Mexico, Colombia, and Chile had 2 studies each included. Curaçao and Cuba had 1 study each included. In Asia, 13 studies were included, 5 from Turkey, 3 from India, 2 from Thailand, 1 from Israel, 1 from Pakistan, and 1 from Japan. Since the Turkish cities were located in the Asian part of the country (Ankara, Bursa, and Konya), the continent of origin considered was Asia. In Africa, 6 studies were selected, 3 from Nigeria, and 1 each from the following countries: Egypt, Tanzania, and Zimbabwe. Europe had only 2 studies included, 1 from Greece and 1 from Ukraine.

The cases' compilation included 1068 dogs properly diagnosed with CTVT in the following countries: Brazil (408 cases), Nigeria (139 cases), Turkey (85 cases), Tanzania (70 cases), India (68 cases), Colombia (45 cases), United States (42 cases), Mexico (29 cases), Thailand (29 cases), Chile (26 cases), Greece (25 cases), Pakistan (18 cases), Zimbabwe (15 cases), Cuba (14 cases), Egypt (13 cases), Curaçao (12 cases), Ukraine (11 cases), Israel (10 cases), Japan (8 cases), and Germany (1 case). Figure 1 presents a map showcasing all the countries with eligible studies on the treatment of natural CTVT cases.

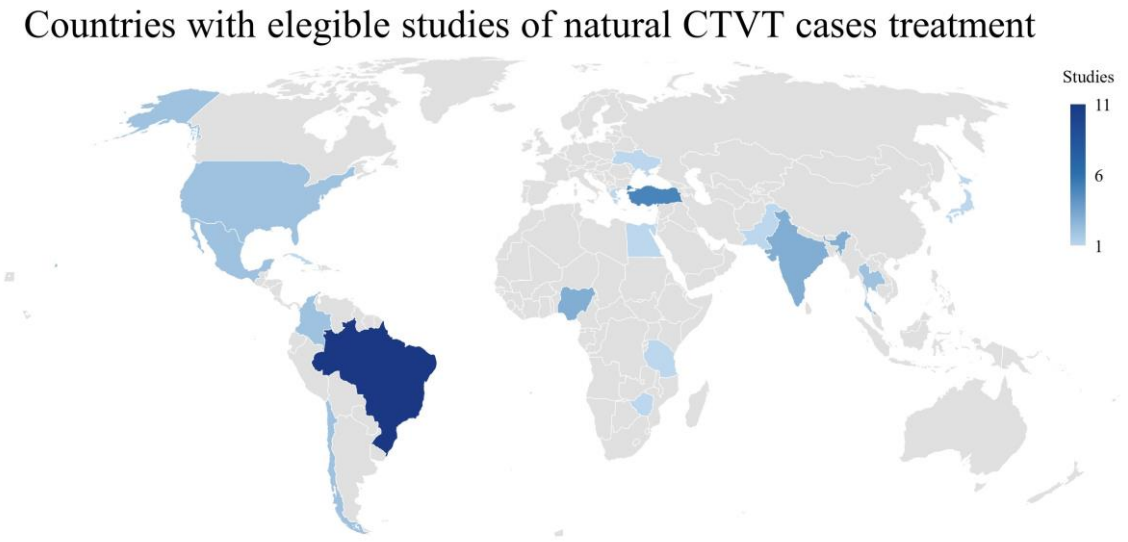

Figure 1. Global map illustrating the count of eligible studies for this systematic review in each country, exclusively encompassing the only countries with natural CTVT cases.

Vincristine sulfate in monotherapy

Chemotherapy with vincristine sulfate is the conventional protocol for CTVT. Thirty-two studies analyzed the efficacy of this single-drug treatment (Table 1), with doses ranging from 0.5 to 0.75 mg/m², considering body surface, or 0.023 to 0.03 mg/kg, considering weight. All studies employed weekly intervals, but there was a wide variation in doses until complete remission, ranging from 1 to 12. The route of administration is standardized as intravenous, and most included studies adhered to this route. Due to the vesicant potential of vinca alkaloids, the subcutaneous route should never be used.

Most studies did not provide the exact number of sessions required for complete remission for each case but instead reported intervals. The most common interval for vincristine sessions leading to complete remission was 4-6 sessions (82.5% of cases), while 10.6% of cases required 3 or fewer sessions, and 6.9% needed 7 or more sessions. Up to 6 sessions of this single-drug protocol resulted in complete remission in 93.1% of dogs, and up to 8 sessions led to complete remission in at least 98.9% of cases. Only three studies reported cases treated with more than 8 doses of vincristine sulfate that achieved complete remission.[18,30,31] Two studies specified the number of doses: one case received 9 doses,[30] and another case received 12 doses,[31] accounting for 0.3% (2/660) of all cases. The last study presented only an interval of 7-10 doses, including 6 dogs.[18]

The response to vincristine sulfate in the last decades was incredibly high, 94.2% (622/660) of analyzed cases presented complete remission and 4.7% (31/660) presented

partial remission, resulting in an objective response of 98.9% (653/660). The remaining 7 cases without objective response presented stable disease, in 5 cases (0.8%), and progressive disease, in 2 cases (0.3%). Although important, several studies have not specified the criteria for clinical response evaluation, but we recommend following Veterinary Cooperative Oncology Group (VCOG) criteria.

Follow-up time, from the end of the treatment to the last contact, varied from 1 to 49 months, but most studies did not provide adequate information. Considering a minimum follow-up time of 3 months, recurrence was observed in only 0.8% (2/253) of dogs who achieved complete remission with vincristine sulfate. In these cases, recurrence occurred at 8 and 10 months after the end of the chemotherapy protocol.[20,32] One study reported two possible recurrences. The first was suggested to be cutaneous when the previous tumor was genital, so it was considered in this review as a new independent lesion from a new contagious source, instead of a recurrence; the second case occurred in the superficial inguinal lymph node, so it was considered as a recurrence/metastasis.[32] Another study reported a recurrence of a nasal CTVT, which was not treated after local relapse.[20] Interestingly, since 1998, no analyzed studies have reported recurrences.

Table 1. Summary of data from selected studies: Vincristine sulfate monotherapy for canine transmissible venereal tumor treatment

Year	Reference	Origin of cases	No of cases	Protocol	CR[1]	PR	SD	PD	Recurrence
1990	Amber et al.[32]	Nigeria (Zaria)	20	0.5 mg/m², IV, weekly	20/20 (100%)	-	-	-	1/20 (5%)
1995	Harmelin et al.[31]	Israel (Jerusalem)	10	0.6 mg/m², IV, weekly	8/10 (80%)	-	-	2/10 (20%)	NR
1996	Singh et al.[33]	India (Punjab)	12	0.025 mg/kg, IV, weekly	11/12 (91.7%)	-	1/12 (8.3%)	-	NR
1998	Rogers et al.[20]	United States (Texas)	5	0.5 mg/m², IV, weekly	5/5 (100%)	-	-	-	1/5 (20%)
2000	Erünal-Maral et al.[34]	Turkey (Ankara)	12	0.025mg/kg, IV, weekly	12/12 (100%)	-	-	-	0/12 (0%)
2000	González et al.[35]	Chile (Santiago)	19	0.03mg/kg, IV, weekly	19/19 (100%)	-	-	-	0/19 (0%)
2001	Athar et al.[36]	Pakistan (Faisalabad)	6	0.025 mg/kg, IV, weekly	5/6 (83.3%)	1/6 (16.7%)[2]	-	-	0/5 (0%)
2004	Tella et al.[37]	Nigeria (Ibadan)	10	0.025 mg/kg, IV, weekly	10/10 (100%)	-	-	-	NR
2005	Nak et al.[6]	Turkey (Bursa)	37	0.025 mg/kg, IV, weekly	31/37 (83.8%)	6/37 (16.2%)	-	-	0/31 (0%)
2006	Herrera et al.[38]	Cuba (Sancti Spíritus)	9	0.025 mg/kg, IV, weekly	9/9 (100%)	-	-	-	0/9 (0%)
2009	Mukaratirwa et al.[39]	Zimbabwe (Harare)	5	0.025 mg/kg, IV, weekly	5/5 (100%)	-	-	-	0/5 (0%)
2013	Lima et al.[40]	Brazil (Goiás)	13	0.025 mg/kg, IV, weekly	13/13 (100%)	-	-	-	NR
2014	Hantrakul et al.[41]	Thailand (Ratchaburi)	6	0.7 mg/m², IV, weekly	5/6 (83.3%)	1/6 (16.7%)	-	-	NR
2014	Huppes et al.[25]	Brazil (Uberaba)	39	0.075 mg/kg, IV, weekly	36/39 (92.3%)	3/39 (7.7%)	-	-	NR
2015	Komnenou et al.[42]	Greece (Thessaloniki)	25	0.6 mg/m², IV, weekly	24/25 (96%)	-	1/25 (4%)	-	0/24 (0%)
2015	Paranzini et al.[43]	Brazil (Paraná)	46	0.5 mg/m², IV, weekly	31/46 (67.4%)	14/46 (38.9%)	1/46 (2.2%)	-	NR
2016	Ramadinha et al.[18]	Brazil (Rio de Janeiro)	65[3]	0.025 mg/kg, IV, weekly	64/65 (98.5%)	-	1/65 (1.5%)	-	0/64 (0%)
2018	Kanca et al.[44]	Turkey (Ankara)	9	0.025 mg/kg, IV, weekly	9/9 (100%)	-	-	-	0/9 (0%)
2018	Vural et al.[45]	Turkey (Ankara)	18	0.025 mg/kg, IV, weekly	18/18 (100%)	-	-	-	NR
2019	Setthawongsin et al.[14]	Thailand (Bangkok)	11	0.025 mg/kg, IV, weekly	9/11 (81.8%)	1/11 (9.1%)	1/11 (9.1%)	-	NR
2019	Ayala-Díaz et al.[46]	Mexico (Oaxaca)	21	0.023 mg/kg, IV, weekly	21/21 (100%)	-	-	-	0/21 (0%)
2019	Alzate et al.[47]	Colombia (Caldas)	21	0.5 mg/m², IV, weekly	21/21 (100%)	-	-	-	0/21 (0%)
2020	Reis-Filho et al.[48]	Brazil (Paraná)	22	0.75 mg/m², IV, weekly	18/21 (85.7%)	3/21 (14.3%)	-	-	NR
2020	Ignatenko et al.[30]	Ukraine (Kyiv) and Germany (Munich)	12	0.7 mg/m², IV, weekly	12/12 (100%)	-	-	-	0/12 (0%)
2020	Bulhosa et al.[15]	Brazil (Bahia)	10	0.5 mg/m², IV, weekly	10/10 (100%)	-	-	-	NR

2021	Abdelnaby et al.[49]	Egypt (Cairo)	13	0.025 mg/kg, IV, weekly	13/13 (100%)	-	-	-	0/13 (0%)
2021	Ramírez-Ante et al.[28]	Colombia (Caldas)	24	0.5 mg/m², IV, weekly	24/24 (100%)	-	-	-	NR
2022	Souza et al.[50]	Brazil (Alagoas)	12	0.025 mg/kg, IV, weekly	12/12 (100%)	-	-	-	0/12 (0%)
2022	Duzanski et al.[51]	Brazil (São Paulo)	17	0.75 mg/m², IV, weekly	15/17 (88.2%)	2/17 (11.8%)	-	-	0/15 (0%)
2023	Ferreira et al.[27]	Brazil (Pernambuco)	10	0.75 mg/m², IV, weekly	10/10 (100%)	-	-	-	0/10 (0%)
2023	Costa et al.[13]	Brazil (Rio de Janeiro)	116	0.7-0.75 mg/m², IV, weekly	116/116 (100%)	-	-	-	NR
2023	Parikh et al.[52]	India (Gujarate)	6	0.025 mg/kg, IV, weekly	6/6 (100%)	-	-	-	NR

CR: Complete response. PR: Partial response. SD: Stable disease. PD: Progressive disease. NR: Not reported. [1]Some studies were carried out before the release of the VCOG guidelines (Nguyen et al., 2013), but it is possible that the criteria were equivalent. When the study regarded a 100% reduction in tumor size as complete remission, and in all cases met this criterion, the implementation of the VCOG guideline is acknowledged. Other studies considered partial remission of >50% of tumoral volume reduction (PR >50%). [2]Reported partial remission as an incomplete regression. [3]Certain patients discontinued the treatment (39). We were unable to ascertain whether there would be a change in clinical response and the reasons for treatment abandonment.

Doxorubicin

Chemotherapy with doxorubicin, an alternative to vincristine monotherapy for CTVT, has gained momentum in the last decade. The first selected study dates back to 2012, while the most recent study is from 2022, encompassing research from both Turkish and Brazilian studies.

Five studies employed doxorubicin as a second-line therapy exclusively for vincristine-resistant cases, totaling 15 cases. The majority of these studies used a similar protocol: 30 mg/m² administered intravenously, with a 21-day interval between doses.[25,43,48,51] One study deviated by using a dose of 1 mg/kg, also administered intravenously with a 21-day interval.[6] In all studies, one to three sessions were applied, leading to a complete response rate of 66.7% in dogs previously exposed to vincristine. Four cases exhibited stable disease (26.7%), and one case achieved partial remission (6.7%). No studies reported recurrences (Table 2).

Table 2. Doxorubicin as second line therapy for canine transmissible venereal tumor treatment

Year	First author	Cases origin country (state/city)	No of cases	Dose	CR	PR	SD	PD	Recurrence
2005	Nak et al.[6]	Turkey (Bursa)	6	1mg/kg, IV, weekly	2/6 (33.3%)	-	4/6 (66.7%)	-	0/6 (0%)
2014	Huppes et al.[25]	Brazil (Uberaba)	3	30mg/m², IV, each 3 weeks	3/3 (100%)	-	-	-	NR
2015	Paranzini et al.[43]	Brazil (Paraná)	1	30mg/m², IV, each 3 weeks	1/1 (100%)	-	-	-	NR
2020	Reis-Filho et al.[48]	Brazil (Paraná)	3	30mg/m², IV, each 3 weeks	2/3 (66.7%)	1/3 (33.3%)	-	-	NR
2022	Duzanski et al.[51]	Brazil (São Paulo)	2	30mg/m², IV, each 3 weeks	2/2 (100%)	-	-	-	0/2 (0%)

CR: Complete response. PR: Partial response. SD: Stable disease. PD: Progressive disease. NR: Not reported.

As a first-line treatment, a single study employed the conventional doxorubicin protocol (30 mg/m², intravenously, every 21 days) in only 9 dogs.[53] Five sessions were administered, leading to complete remission in all patients. Follow-up and recurrence information for these cases was unavailable.

Other chemotherapies

Alternative chemotherapies, distinct from vincristine and doxorubicin, were infrequently utilized for CTVT. Two studies examined vinblastine, with two using it as a single drug and one in combination with methotrexate.[18,33] Lomustine and cyclophosphamide were each investigated in only one study.[54,55]

Vinblastine sulfate, another vinca alkaloid akin to vincristine, exhibits similar properties. In the two studies that tested it in monotherapy, high responsiveness was identified, with 97.9% (47/48) of dogs with CTVT achieving complete remission and 2.1% (1/48) experiencing stable disease. The protocols employed in these studies varied; one utilized a dose of 2.5 mg/m² administered intravenously on a weekly basis, primarily for 4 to 6 sessions,[18] while the other employed a dose of 0.15 mg/kg administered intravenously on a weekly basis, averaging 2.8 ± 0.3 sessions.[33] However, the follow-up period was short (4 months) in one study and lacking in the other, making it challenging to properly address information regarding recurrences.[18,33]

In combination with methotrexate, vinblastine sulfate at 0.1mg/kg exhibited a poorer response compared to its use as a single drug at 0.15 mg/kg.[33] Both were administered intravenously on a weekly basis and methotrexate was used at a dosage of 0.35 mg/kg. Complete response occurred in 75% of dogs (6/8), with 25% (2/8) experiencing stable disease. The average number of sessions was 3.4 ± 0.2.[33]

Cyclophosphamide, an alkylating drug, was evaluated in a single study involving 8 dogs with CTVT in Mexico.[54] The protocol consisted of two cycles with a dose of 5 mg/kg administered intravenously daily for 10 days in the first cycle and for 7 days in the second cycle, with a 5-day interval between the cycles. Complete remission was achieved in only 50% (4/8) of dogs, while an unspecified incomplete reduction occurred in the remaining 50% (4/8) of dogs.[54] Notably, half (2/4) of the dogs that achieved complete remission experienced recurrence after two months.

Lomustine, also an alkylating agent, was administered to 12 dogs with CTVT, resulting in a complete response rate of 66.7% (8/12), a partial response in 8.3% (1/12), and stable disease in 25% (3/12).[55] Notably, the definition of partial response, distinct from that proposed by VCOG, considered it as more or equal to a 50% reduction in tumor volume, which could

potentially increase cases categorized as stable disease. The protocol involved the oral administration of 70-85 mg/m² every 3 weeks, with 1 or 2 sessions. The limited number of sessions may have influenced the poor response compared to the conventional vincristine protocol.[55]

Surgery and electrocauterization

Surgical treatment, associated or not with electrocauterization, commonly employed for CTVT until 1980, has lost prominence. The most recent selected study dates back to 2006. As the first intervention, 4 studies analyzed its efficacy in four low-income countries: Cuba, Nigeria, Pakistan, and Tanzania.[36,38,56,57]

The recurrence rate of surgery as a single therapy for CTVT is significantly higher compared to conventional vincristine chemotherapy, with a relapse rate of 74.6%. Most cases (52.5%) experience relapse before 6 months, but there is still a notable rate after 12 months (6.8%).[36,56] When combined with cauterization, the recurrence rate decreases to 31.3%, although it remains notably high.[38,56,57]

No studies provided specific information on surgical margins or compared various local approaches for CTVT removal. Although most studies commented on it, there is a significant lack of criteria for its use. In a unique instance, surgery was utilized as a third-line therapy for both vincristine and doxorubicin in two chemoresistant cases, with no recurrence identified in any case during the follow-up period (7-49 months).[6]

Radiation therapy

Similar to the surgical approach, studies employing radiation therapy for CTVT tend to be older, with the most recent selected study dating back to 1998. Unlike surgery, radiation therapy was evaluated for CTVT in high-income countries such as Japan and the United States, when natural cases were more prevalent. Only three studies met the selection criteria.[10,20,58]

Various protocols were employed to achieve complete remission through radiation therapy. In Japan, a regimen of twice-weekly orthovoltage radiation over three weeks (six exposures) resulted in a total dosage of 12 to 24 Gy, using equipment with specifications of 150KVp, 3mA, a filter of 0.5mm of Cu + 1.0 mm of Al, and an anode-skin distance of 30 cm.[10] In Pennsylvania, United States, the protocol exhibited considerable variation, with time between fractions ranging from 2 to 21 days, targeting a total orthovoltage radiation dose of 10 to 30 Gy. The equipment specifications included 250 kVp, 20 mA, and a filter of 1.5 or 3.0 mm of Cu.[58] In Texas, United States, cobalt radiation therapy was administered in three fractions

(Monday-Wednesday-Friday), resulting in a total dosage of 10 to 18 Gy.[20] Field size information was available in one study, with variations of 4×6 cm, 6×8 cm, or 8×10 cm, depending on the tumor's size.[10] In all 33 cases, radiation therapy achieved complete remission. The cases were monitored for at least 12 months, with no reported recurrences.

The same studies from the United States that tested radiation therapy as a first-line therapy also evaluated its effectiveness in vincristine-resistant cases.[20,58] A total of 12 cases were subjected to the previously discussed protocols, achieving complete remission in all cases, and there was no reported recurrence during a 6-month follow-up period.

Vincristine associations

A wide variety of protocols were tested in conjunction with vincristine to enhance the cure for CTVT and reduce the necessary vincristine chemotherapy sessions. Seven studies were identified and selected. Four articles associated vincristine with a kind of immunotherapy or potential immunomodulator (Interleukin-2, recombinant human interferon alpha-2a, and Bacillus Calmette–Guérin).[39,44,52,59] From the remaining three, one of each also combined vincristine with debulking surgery, an antiparasitic drug (ivermectin), and L-asparaginase chemotherapy.[14,15,36]

Despite a low case count (12), the LAP-VCR (L-asparaginase and vincristine sulfate) protocol appears promising.[14] All CTVT cases that completed the treatment achieved complete remission in a shorter period than the group receiving vincristine as a single drug, although without statistical significance ($p > 0.05$). This protocol involves a dose of 5,000 IU/m² of L-asparaginase administered subcutaneously in weeks 1 and 3, while vincristine is administered at a dose of 0.025 mg/kg intravenously in weeks 2 and 4, for at least 1 cycle (4 weeks).[14]

Immunotherapy for CTVT exhibits promise; however, substantial progress is still needed. A protocol combining intratumoral application of vincristine (0.5-0.7 mg/m², weekly) and IL-2 (2×10^6, weekly) without well-defined intervals between doses (1 to 4) demonstrated a limited clinical response.[59] After 3 months post-treatment, only 25% achieved complete response. Another approach involving intratumoral recombinant human interferon alpha-2a (IFNa-2a) at a dose of 1.5 million IU (5.55 mcg/0.25 mL), administered weekly, in combination with vincristine (0.025 mg/kg, intravenously, weekly), appears to reduce treatment by two weeks compared to dogs treated solely with vincristine.[44] However, it's noteworthy that only 6 animals were treated in the combined group. Bacillus Calmette–Guérin (BCG) was assessed in conjunction with vincristine by two studies employing the same chemotherapy protocol of 0.025 mg/kg, intravenously, weekly. One study administered 5 mL of BCG, approximately 2.5

x 10^8 Colony Forming Units (CFU), intratumorally, daily for 5 days, while the other study used 5 mL of BCG, 2 to 8 x 10^6 CFU, intratumorally, weekly for 3-4 sessions.[39,52] Both studies achieved complete remission in all cases of CTVT. However, the limited number of cases (11) raises uncertainty about whether vincristine alone could have led to complete remission without BCG. In one study, the follow-up time was less than three months,[39] and in the other, it was unavailable.[52]

Ivermectin, an antiparasitic drug, was evaluated in a single selected study.[15] In a brief cohort of 20 dogs with CTVT, this study compared vincristine monotherapy (0.5 mg/m², IV, weekly) with vincristine, administered in the same dosage, route, and interval, combined with ivermectin at 0.5 mg/kg, 1%, subcutaneously, weekly, administered 24 hours before the chemotherapy. The study found no significant differences between the groups in terms of efficacy or the duration of the protocol (p= 0.8).[15] Surgical debulking was evaluated in a single study involving only 6 dogs, following 2-3 sessions of vincristine (0.025 mg/kg, IV, weekly). The study reported no recurrences.[36]

Immunotherapy in monotherapy

The previously discussed protocols of BCG immunotherapy in association with vincristine were also tested in monotherapy by the same two studies.[39,52] In Zimbabwe, using a higher CFU of 2.5 x 10^8 in a daily protocol, all dogs achieved complete remission (5),[39] while in India, with a lower CFU of 2 to 8 x 10^6 in a weekly protocol, the patients only exhibited stable disease (6).[52] The short cohort prejudices analyses, and the follow-up was poor. Nevertheless, BCG may play an interesting role in CTVT microenvironment immunomodulation.

A novel therapy for CTVT tested three protocols for immunomodulation: Canine Parvovirus ns1 gene, Chicken Anemia vp3 gene, and a combination of ns1 + vp3 genes.[60] The results suggest oncolytic potential caused by the Canine Parvovirus ns1 gene protocol, but clinical descriptions were scarce, and therapy response poorly characterized. Monotherapy immunotherapies are not currently a viable option for CTVT treatment based on current data.

Treatment guideline

1st line therapy:

Vincristine sulfate is recommended as the standard monotherapy protocol for untreated cases of CTVT, including both genital and extragenital forms. The recommended dose is 0.5 to 0.75 mg/m², administered intravenously, in bolus, on a weekly basis.[6,13,15,30,32,41,47] We

recommend utilizing a body surface-based dose in chemotherapy for better individualization of each patient's treatment. The initial recommendation is 4 to 6 sessions of vincristine,[25,30,42,43,45] although some patients may achieve complete remission before completing this course.

Prior to each chemotherapy session, it is essential to collect and analyze blood exams. The weight-based dose (0.025 mg/kg) becomes equivalent to the maximum body surface-based dose (0.75 mg/m²) for dogs weighing approximately 30 kg. Beyond this weight, it deviates from the body surface-based dose, increasing the final volume of chemotherapy. Below this weight, it deviates from the other dose with a smaller final volume of chemotherapy.

This protocol is well-defined for genital forms and some more common extragenital manifestations, such as cutaneous, oral, nasal, ocular and lymph node metastasis.[13,18,30,42] However, it does not cover central nervous system involvement or other rare presentations. Data on these specific presentations is still limited, and conclusions regarding their treatment remain uncertain.

The protocol should be extended to up to 8 sessions total if complete response to chemotherapy is not achieved but the dog shows partial remission,[15,28,47,48,51] defined as at least a 30% reduction in the total tumor volume according to VCOG.[61] In case of stable disease or progressive disease, the protocol should not be extended, but changed to a second line therapy.

Dogs with complete response after vincristine chemotherapy should be monitored at least every 4 months until one year after the end of treatment. In case of doubt regarding whether a post-chemotherapy lesion is fibroblastic (stroma) or a tumor, cytology should always be considered.

2nd line therapy:

Radiation therapy is undoubtedly an excellent treatment for curing CTVT. However, its high cost is a significant impediment. Therefore, two second-line therapies are suggested here: radiation therapy and monotherapy with doxorubicin. If available, radiation therapy should be preferred over doxorubicin therapy due to higher complete response rate.

A minimum cumulative radiation dose of 10 Gy should be administered for effective treatment. A cumulative dose of 10 to 30 Gy is recommended in a hypofractionated protocol conducted on a Monday-Wednesday-Friday schedule with 6 fractions.[10,20,58] It is suggested that more recent studies employing updated protocols and modern equipment should be conducted. To achieve a cure, all visible lesions should be irradiated; otherwise, the tumor may not relapse.

Doxorubicin, administered as a monotherapy, is recommended for cases of chemoresistant CTVT that do not respond to vincristine. The suggested dosage is 30 mg/m²,

with a three-week interval (21 days) between doses.[25,48,51] For dogs weighing less than 15 kg it is suggested to use a lower dose, of 1mg/kg, in order to mitigate serious adverse events.[6] The standard dose of 30 mg/m² is relatively high for small dogs; however, it is important to note that no studies have specifically utilized this lower dose only for small dogs with CTVT. With either dosage it is recommended to use the intravenous route, once this agent is highly vesicant, with an infusion period lasting 10 to 20 minutes. Up to three sessions may be necessary in order to achieve complete response.[6,25,48,62] Prior to each session, it is advisable to conduct a blood count, and before the first session, an echocardiographic exam is suggested due to the potential cardiotoxicity of this drug in dogs.

Dogs that initially exhibited chemoresistant tumors to vincristine but achieved complete remission after doxorubicin chemotherapy or radiation therapy should undergo close monitoring, with assessments scheduled at least every 2 months until one year after the completion of treatment.

3rd line therapy:

Surgery, once extensively condemned as an option for CTVT treatment, becomes a viable choice after resistance to chemotherapy and radiation therapy, aiming to impede further tumor progression. While well-established surgical margins are lacking, we recommend a minimum of 2 cm for lateral margins and one anatomic plane as the deep margin, if achievable.

Debulking should not be an option, but electrocautery or electrochemotherapy might contribute to reducing local recurrence, although data is still scarce, and new studies are required.[36,56,57] Any dog that presents such a degree of tumor resistance requiring a surgical approach needs to be cautiously monitored, at least every 2 months, until completing a year of follow-up. Figure 2 illustrates, through a flow diagram, the suggested conduct of CTVT treatment.

As of now, there is no existing study with a substantial number of cases employing electrochemotherapy for the treatment of macroscopic CTVT. Consequently, it cannot be recommended at this time.

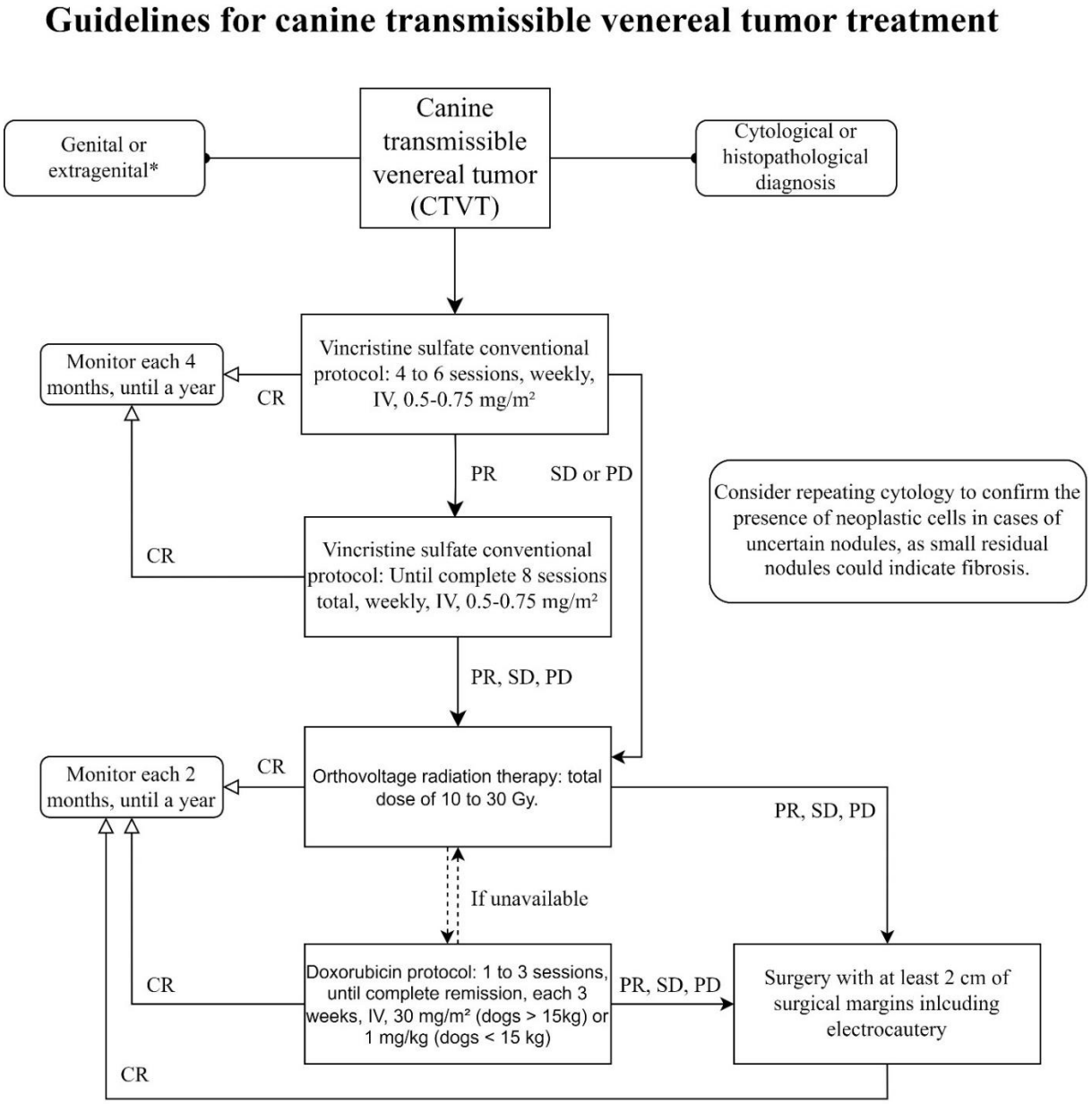

Figure 2: Treatment flow diagram for canine transmissible venereal tumor: Guidelines. *Not all extragenital presentations have recognized responsiveness to vincristine as first-line therapy or other treatments.

4. DISCUSSION

The canine transmissible venereal tumor (CTVT) is a complex tumor, often underestimated. The endeavor to formulate treatment guidelines necessitates a comprehensive consideration of its etiology and pathogenesis. While our recommendations imply that the majority of cases would respond favorably to the suggested therapies, instances involving new mutations and originating from sublineages underrepresented in the 42 selected studies of this

systematic review may pose greater challenges in treatment.

As anticipated, vincristine sulfate exhibited a significant curative potential for CTVT.[15,63] The analysis of over six hundred cases revealed that unresponsiveness (stable disease and progressive disease) or a suboptimal chemotherapy response (partial response) occurred in less than 6% of cases. Such occurrences may be attributed to treatment-related factors, encompassing drug administration, procedural aspects, or practitioner-related aspects. [25,64] Additionally, the potential emergence of chemoresistance mechanisms within the tumor, possibly developed extrinsically due to prior exposure to chemotherapy, could contribute to such outcomes.

The intravenous administration of vincristine in dogs is widely acknowledged. Although intraperitoneal administration has been deemed safe, studies on this route have been relatively recent and have primarily focused on treatments in cats.[65,66] While some studies mention subcutaneous administration of vincristine,[64] it is strongly discouraged due to its cytotoxic vesicant potential. This method has the potential to cause necrosis in the skin and deep tissues.[67] The drug's dose is also a crucial aspect of treatment, as underdoses might prolong the treatment and increase the likelihood of evasion, further exacerbating current problems due to financial issues.[25]

The determination of the vincristine dose format requires careful consideration. The weight-based dose (typically 0.025 mg/kg) can vary significantly from the body surface-based dose (typically 0.7 mg/m²),[14,38,50] depending on the dog's weight. In a small dog (5 kg), the doses may diverge by up to 60% in the final volume. While doses for a medium-sized dog (20 kg) become closer, in a giant dog (50 kg), deviations reappear, leading to up to a 30% difference in the final volume.

Certain dog breeds, like Collie, Australian Shepherd, German Shepherd, and Border Collie, are predisposed to a deficiency in the membrane transporter p-glycoprotein.[68] Therefore, it should be investigated before initiating a vincristine protocol in dogs with CTVT and the dose adjusted according to the result.

Chemoresistant mechanisms in CTVT might include the overexpression of p-glycoprotein, encoded by the ATP-binding cassette sub-family B member 1 (ABCB1) gene.[69,70] If mutated, it could enhance the efflux of vincristine to the extracellular environment, diminishing the cytotoxic potential of chemotherapy.[70] Successive incomplete exposures to vincristine could progressively select the most adapted cancer clones over time. Minimal differences between cytomorphological subgroups of the tumor have been demonstrated,[69] but further research, especially multicentric, prospective studies, are required.

Ivermectin association with vincristine could potentialize its action through the inhibition of P-glycoprotein.[15] Ivermectin results in the decreased transcription of the p-glycoprotein due to the inhibition of the NF-κB transcriptional factor, which may be effective in vincristine-resistant cases due to the overexpression of P-glycoprotein.[71] Similar to vincristine, ivermectin is also a substrate for P-glycoprotein, potentially leading to competition between the two molecules and resulting in increased intracellular concentration. L-asparaginase is not affected by this multidrug resistance mechanism of increased efflux caused by the ABCB1 gene mutation, also known as multidrug resistance protein 1 (MDR1 gene), so it could be a good combination with vincristine, also for chemoresistant cases.[14,72] In further studies, both ivermectin and L-asparaginase should undergo testing for cases resistant to vincristine chemotherapy.

In most countries where CTVT is endemic, vincristine chemoresistance appears to be very rare, as observed in most of Brazil, Colombia, Greece, Mexico, and certain areas of Turkey.[13,27,42,45–47] However, contrasting studies suggest a potentially higher chemoresistance rate in Romania, the United Kingdom, and other areas of Turkey (Bursa) and Brazil (Paraná).[6,43,73,74] Much remains to be studied and correlated between CTVT sublineages, their geographical distribution, and the occurrence of chemoresistance.

The decision for a second-line therapy considered two aspects: high efficacy and applicability. Doxorubicin presented a lower efficacy but a higher cost benefit, while radiation therapy is much more expensive but has a higher efficacy.[6,20,25] Therefore, the decision for a second-line therapy must be balanced by the practitioner.

Doxorubicin, as a second-line therapy with the conventional and suggested protocol (30 mg/m², intravenously, each 3 weeks), has a complete response rate of 88.9%,[25,43,48,51] while the protocol used by Nak et al. (2005) demonstrated only a 33.3% complete response (1 mg/kg, intravenously, weekly).[6] The weekly interval for doxorubicin is considered unusual, and the 1 mg/kg dosage is recommended for dogs below 15 kg. The higher dose of 30 mg/m² might be more effective, and it still does not reach the cumulative dose of 240 mg/m² if used for just three sessions.

Experimentally, preliminary laser ablation studies have repeatedly shown the effective local potential of this therapy.[75,76] Whole-body irradiation in dogs experimentally inoculated with CTVT, as demonstrated by Cohen in 1973, revealed that the tumor in these dogs may exhibit accelerated growth and increased aggressiveness.[4] This phenomenon could be attributed to the impact on the tumor microenvironment, coupled with the immunosuppression induced by irradiation, potentially influencing the clinical response of CTVT.[51] In vivo, considering

only natural cases, all radiation therapy studies showed a very high complete response rate, reaching 100% when only the irradiated tumors are considered.[10,20,58] However, for disseminated CTVT, chemotherapy might be a better option due to its systemic action, not just locoregional.

Electrochemotherapy is a promising locoregional therapy that is gaining momentum in developing countries. However, for CTVT, no studies met the inclusion criteria of this review, as none reported this treatment in 5 or more cases. In a group of 3 cases, all achieved complete remission with a long-term follow-up without recurrence (> 24 months).[77,78] Yet, new studies are required with a larger cohort, but it may be a good option in the future as a second-line therapy, cheaper than radiation therapy and possibly more effective than doxorubicin.

Surgery is not primarily recommended for CTVT due to its high responsiveness to vincristine and the high rates of recurrence, as demonstrated by the compilation in this review.[18,56,57] Studies have shown its efficacy in small cohorts as a second- or third-line therapy in chemoresistant cases, with low rates of recurrence.[6] Nevertheless, criteria in these studies are unclear regarding lateral and deep surgical margins. Therefore, a better definition in future studies could help determine how surgery may assist in treating CTVT with the lowest rate of recurrence.

Other chemotherapies lack evidence of efficacy or show lower complete response rates compared to those mentioned above. In this context, we included studies involving vinblastine, l-asparaginase, lomustine, and cyclophosphamide.[14,18,54,55] However, none of these studies provided sufficient data for inclusion in the guidelines. The l-asparaginase protocol combined with vincristine might have a higher efficacy than vincristine in monotherapy, but the single study that used this protocol must be replicated, potentially involving different populations of dogs with CTVT.[14] The other chemotherapies show poorer clinical response.

Non-selected studies due to low cohort, non-natural cases, lacking data or other exclusion criteria, tested chemotherapies such as 5-Fluorouracil[73] and vinorelbine,[79] and other modalities of treatment, such as autohemotherapy,[80] IL-6 and IL-15 plasmids immunotherapy,[81] and dendritic cells pulsed with exosomes.[82]

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to disclose.

5. CONCLUSION

The treatments for canine transmissible venereal tumor have evolved significantly over the last century. Currently, it is possible to formulate a comprehensive guideline based on extensive literature from various countries and encompassing distinct therapeutic approaches. Vincristine sulfate monotherapy is recommended as the first-line therapy for most cases. Radiation therapy and doxorubicin also provide valuable options supported by literature for use as second-line therapy. While chemoresistance may pose a challenge in the coming decades, we have currently established a comprehensive treatment guide for CTVT through meticulous data collection in a systematic review.

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8. CONCLUSIONS

This study signifies a notable advancement in deepening our understanding of CTVT, accentuating its diverse clinical presentations in Brazil, offering a comprehensive treatment guideline, and establishing a purposeful predictive marker for vincristine chemoresistance through cytology.

By examining CTVT cytomorphology, our study reveals intriguing associations between specific features and chemoresistance. These findings emphasize the potential of cytological examinations in predicting and comprehending the tumour's response to chemotherapy. They contribute valuable information for refining treatment strategies and predicting therapeutic outcomes.

This study highlights its local characteristics, emphasizing predominant genital manifestations in young mixed-breed dogs. The neoplasms exhibit high responsiveness to vincristine sulphate chemotherapy. Recognizing this clinical-epidemiological profile is vital for early diagnosis and proper treatment of CTVT in dogs.

The treatments for canine transmissible venereal tumour (CTVT) have witnessed significant evolution over the past century. Our systematic review has illuminated the optimal approaches for both first- and second-line therapies. Vincristine sulphate monotherapy emerges as the recommended first-line treatment for the majority of cases, with radiation therapy and doxorubicin providing well-supported alternatives for second-line therapy. The potential challenge of chemoresistance underscores the importance of ongoing research.

Promisingly, our findings suggest effective CTVT control in Brazil through initiatives like neuter centres and collaborations within zoonosis control centres. The data from our veterinary school hospital indicates a decline in CTVT incidence. However, further research is needed to fully comprehend the correlation between implemented measures and the observed reduction, both in our locality and other metropolitan areas across Brazil.

APPENDICES

SUPPLEMENTARY MATERIAL - CHAPTER II

Identification									
Case number	Hospital identification	Date of birth	Diagnosis	Species	Breed	Weight (Kg)	Diagnostic date (day/month/year)	Age (years)	Sex
1	759	01/02/2007	CTVT	Canine	Boxer	22,50	11/07/2012	5,44	F
2	997	19/07/2010	CTVT	Canine	Belgian Sheperd	30,00	19/07/2012	2,00	F
3	1028	30/07/2010	CTVT	Canine	American Pit Bull Terrier	23,10	07/09/2012	2,11	F
4	1072	21/07/2002	CTVT	Canine	Mixed-breed	23,55	21/07/2012	10,01	F
5	1111	23/05/2012	CTVT	Canine	Boxer	23,00	27/02/2014	1,77	F
6	1499	01/01/2009	CTVT	Canine	Labrador Retriever	28,00	06/08/2012	3,60	F
7	1553	08/08/2010	CTVT	Canine	Mixed-breed	10,50	08/08/2012	2,00	M
8	1735	16/08/2009	CTVT	Canine	Mixed-breed	06,00	16/08/2012	3,00	F
9	1924	02/02/2010	CTVT	Canine	Mixed-breed	09,80	24/08/2012	2,56	F
10	1930	01/02/2010	CTVT	Canine	Mixed-breed	24,50	24/08/2012	2,56	F
11	2052	02/02/2006	CTVT	Canine	Mixed-breed	06,95	29/08/2012	6,58	F
12	2131	02/02/2009	CTVT	Canine	Mixed-breed	08,20	01/09/2012	3,58	F
13	2283	03/10/2003	CTVT	Canine	German Sheperd	35,00	08/09/2012	8,94	M
14	6219	12/04/2011	CTVT	Canine	Mixed-breed	10,00	16/03/2016	4,93	F

15	6886	04/03/2004	CTVT	Canine	German Sheperd	31,00	04/03/2013	9,01	F
16	7106	01/02/2005	CTVT	Canine	Labrador Retriever	20,00	13/03/2013	8,12	F
17	7370	02/01/2008	CTVT	Canine	Miniature Poodle	07,00	22/03/2013	5,22	M
18	7924	15/04/2003	CTVT	Canine	Mixed-breed	19,70	15/04/2013	10,01	F
19	8049	02/07/2006	CTVT	Canine	Yorkshire Terrier	05,85	14/06/2013	6,96	M
20	8210	02/03/2008	CTVT	Canine	Mixed-breed	05,00	26/04/2013	5,15	M
21	8887	Undetermined*	CTVT	Canine	Mixed-breed	20,35	29/05/2013	Undetermined*	M
22	9963	02/08/2009	CTVT	Canine	Mixed-breed	15,00	12/07/2013	3,95	M
23	10006	19/04/2004	CTVT	Canine	Shih-tzu	02,95	19/07/2013	9,25	F
24	10110	18/07/2008	CTVT	Canine	Boxer	30,50	19/07/2013	5,01	F
25	10365	16/08/2010	CTVT	Canine	Miniature Poodle	06,15	31/07/2013	2,96	M
26	10777	29/03/2010	CTVT	Canine	Mixed-breed	23,00	19/08/2013	3,39	M
27	11031	01/02/2008	CTVT	Canine	Yorkshire Terrier	03,90	19/09/2013	5,64	M
28	11072	11/09/2011	CTVT	Canine	Mixed-breed	11,00	31/08/2013	1,97	F
29	12150	Undetermined*	CTVT	Canine	Mixed-breed	04,10	12/10/2013	Undetermined*	F
30	12644	01/11/2009	CTVT	Canine	Miniature Poodle	06,95	01/11/2013	4,00	F
31	12791	12/03/2013	CTVT	Canine	Yorkshire Terrier	03,70	20/02/2014	0,95	M
32	12807	13/11/2012	CTVT	Canine	Mixed-breed	07,50	16/04/2015	2,42	F
33	13309	05/06/2012	CTVT	Canine	Mixed-breed	17,00	27/11/2013	1,48	F
34	13613	05/02/2003	CTVT	Canine	Miniature Poodle	05,15	09/12/2013	10,85	M
35	13694	Undetermined*	CTVT	Canine	MIniature Poodle	10,00	13/12/2013	Undetermined*	F
36	13932	22/12/2007	CTVT	Canine	MIniature Poodle	00,01	31/12/2013	6,03	M
37	14252	28/10/2012	CTVT	Canine	Mixed-breed	12,00	08/01/2014	1,20	F

38	14482	29/01/2012	CTVT	Canine	Mixed-breed	15,70	18/01/2014	1,97	F
39	14596	Undetermined*	CTVT	Canine	Golden Retriever	19,80	23/01/2014	Undetermined*	M
40	16332	Undetermined*	CTVT	Canine	Mixed-breed	22,50	07/04/2014	Undetermined*	M
41	17873	09/07/2010	CTVT	Canine	Mixed-breed	13,00	26/06/2014	3,97	M
42	18312	11/08/2008	CTVT	Canine	Mixed-breed	30,00	11/07/2014	5,92	F
43	18370	Undetermined*	CTVT	Canine	Mixed-breed	06,00	22/07/2014	Undetermined*	F
44	18509	Undetermined*	CTVT	Canine	Mixed-breed	17,40	22/7/2014	Undetermined*	F
45	19112	08/09/2010	CTVT	Canine	English Pointer	36,65	18/8/2014	3,95	F
46	19851	02/10/2011	CTVT	Canine	Mixed-breed	06,45	16/09/2014	2,96	F
47	20733	26/12/2002	CTVT	Canine	Mixed-breed	20,15	06/11/2014	11,87	M
48	21896	Undetermined*	CTVT	Canine	Mixed-breed	11,40	11/12/2014	Undetermined*	M
49	22073	28/12/2012	CTVT	Canine	Beagle	15,00	26/03/2015	2,24	F
50	22391	09/07/2008	CTVT	Canine	Mixed-breed	NR	14/08/2016	8,10	M
51	22809	24/01/2010	CTVT	Canine	German Pinscher	02,70	21/01/2015	4,99	F
52	23072	09/02/2014	CTVT	Canine	Mixed-breed	09,50	16/05/2015	1,26	F
53	24216	05/06/2001	CTVT	Canine	Miniature Poodle	07,00	03/11/2015	14,42	F
54	24346	11/04/2012	CTVT	Canine	Boxer	22,50	27/03/2015	2,96	M
55	24509	Undetermined*	CTVT	Canine	Mixed-breed	11,30	08/04/2015	Undetermined*	M
56	24648	06/10/2013	CTVT	Canine	Mixed-breed	00,01	13/04/2015	1,52	M
57	25145	Undetermined*	CTVT	Canine	Mixed-breed	03,20	29/04/2015	Undetermined*	F
58	25858	01/06/2014	CTVT	Canine	Mixed-breed	11,10	27/05/2015	0,99	F
59	26020	Undetermined*	CTVT	Canine	Mixed-breed	07,30	02/06/2015	Undetermined*	F
60	26023	08/06/2014	CTVT	Canine	Mixed-breed	NR	03/06/2015	0,99	M

61	26793	09/08/2008	CTVT	Canine	Mixed-breed	20,00	04/07/2015	6,90	M
62	27006	18/08/2008	CTVT	Canine	Mixed-breed	19,00	13/07/2015	6,90	M
63	27594	01/10/2005	CTVT	Canine	MIniature Poodle	10,00	10/08/2015	9,86	F
64	27942	27/08/2014	CTVT	Canine	Mixed-breed	07,70	22/08/2015	0,99	F
65	28659	02/10/2013	CTVT	Canine	Mixed-breed	14,00	22/09/2015	1,97	M
66	29228	07/02/2013	CTVT	Canine	German Pinscher	08,00	13/10/2015	2,68	M
67	29345	12/11/2010	CTVT	Canine	Mixed-breed	20,00	17/10/2015	4,93	F
68	29759	14/04/2014	CTVT	Canine	Mixed-breed	12,00	05/11/2015	1,56	F
69	30545	10/02/2003	CTVT	Canine	Mixed-breed	06,60	04/12/2015	12,82	F
70	31466	17/02/2011	CTVT	Canine	Mixed-breed	35,00	22/01/2016	4,93	M
71	31580	05/03/2009	CTVT	Canine	Mixed-breed	23,00	28/01/2016	6,90	M
72	31653	07/02/2015	CTVT	Canine	Mixed-breed	15,00	02/02/2016	0,99	F
73	32162	24/06/2011	CTVT	Canine	Mixed-breed	NR	26/02/2016	4,68	F
74	32223	12/03/2014	CTVT	Canine	Mixed-breed	20,00	16/02/2016	1,93	F
75	32410	04/04/2011	CTVT	Canine	Mixed-breed	12,00	08/03/2016	4,93	F
76	32684	Undetermined*	CTVT	Canine	Mixed-breed	23,00	21/03/2016	Undetermined*	F
77	33038	05/03/2015	CTVT	Canine	Mixed-breed	17,00	01/04/2016	1,08	M
78	33921	17/05/2015	CTVT	Canine	Mixed-breed	17,00	13/05/2016	0,99	M
79	34631	29/06/2013	CTVT	Canine	Golden Retriever	35,00	14/06/2016	2,96	F
80	35533	16/08/2011	CTVT	Canine	Mixed-breed	30,00	20/07/2016	4,93	M
81	35677	Undetermined*	CTVT	Canine	Mixed-breed	15,50	28/07/2016	Undetermined*	F
82	36089	24/02/2016	CTVT	Canine	Mixed-breed	11,60	28/08/2016	0,51	M
83	36581	22/09/2014	CTVT	Canine	Mixed-breed	09,65	28/08/2016	1,93	F

84	37589	30/10/2014	CTVT	Canine	Mixed-breed	14,00	19/10/2016	1,97	F
85	38969	Undetermined*	CTVT	Canine	Mixed-breed	17,00	13/02/2017	Undetermined*	F
86	40505	25/03/2014	CTVT	Canine	Mixed-breed	12,10	09/03/2017	2,96	F
87	40522	30/03/2013	CTVT	Canine	Mixed-breed	07,90	28/01/2017	3,84	F
88	40756	Undetermined*	CTVT	Canine	Mixed-breed	NR	30/03/2017	Undetermined*	F
89	40863	09/04/2014	CTVT	Canine	MIniature Poodle	19,00	24/03/2017	2,96	M
90	41661	18/05/2014	CTVT	Canine	Mixed-breed	26,70	02/05/2017	2,96	F
91	42626	05/07/2013	CTVT	Canine	Mixed-breed	12,00	30/08/2017	4,16	F
92	42970	25/07/2012	CTVT	Canine	Mixed-breed	17,10	29/06/2017	4,93	M
93	42977	Undetermined*	CTVT	Canine	Mixed-breed	15,00	30/06/2017	Undetermined*	F
94	43213	27/07/2014	CTVT	Canine	Mixed-breed	17,00	11/07/2017	2,96	F
95	44300	21/08/2008	CTVT	Canine	Mixed-breed	11,00	04/09/2017	9,04	F
96	44706	21/09/2017	CTVT	Canine	German pinscher	04,00	21/09/2017	Undetermined*	F
97	45473	03/07/2016	CTVT	Canine	Mixed-breed	07,90	01/12/2017	1,41	F
98	45589	21/06/2014	CTVT	Canine	Shih-tzu	09,90	14/11/2017	3,40	F
99	46054	14/12/2013	CTVT	Canine	Labrador Retriever	27,00	28/11/2017	3,96	M
100	46608	10/01/2014	CTVT	Canine	Golden Retriever	30,00	20/12/2017	3,95	F
101	46718	Undetermined*	CTVT	Canine	Mixed-breed	11,60	27/12/2017	Undetermined*	F
102	47421	Undetermined*	CTVT	Canine	Mixed-breed	09,10	02/02/2018	Undetermined*	F
103	50565	22/09/2008	CTVT	Canine	Mixed-breed	10,00	01/08/2018	9,86	M
104	50669	Undetermined*	CTVT	Canine	Mixed-breed	11,80	08/08/2018	Undetermined*	F
105	50890	21/09/2012	CTVT	Canine	Labrador Retriever	21,00	21/08/2018	5,92	M
106	50898	Undetermined*	CTVT	Canine	Mixed-breed	16,80	22/08/2018	Undetermined*	M

107	51742	Undetermined*	CTVT	Canine	Mixed-breed	17,00	08/10/2018	Undetermined*	F
108	52634	04/06/2016	CTVT	Canine	Mixed-breed	10,00	21/11/2018	2,47	M
109	53062	19/01/2012	CTVT	Canine	Mixed-breed	10,30	13/12/2018	6,90	M
110	54306	11/03/2017	CTVT	Canine	Mixed-breed	NR	01/03/2019	1,97	M
111	54454	28/03/2016	CTVT	Canine	Mixed-breed	13,25	15/03/2019	2,96	F
112	54460	13/03/2011	CTVT	Canine	Mixed-breed	06,00	13/03/2019	8,01	M
113	55514	Undetermined*	CTVT	Canine	Mixed-breed	10,00	02/05/2019	Undetermined*	M
114	55658	19/11/2016	CTVT	Canine	Chow-chow	16,00	08/05/2019	2,47	F
115	55975	02/06/2017	CTVT	Canine	Mixed-breed	09,00	23/05/2019	1,97	F
116	57492	Undetermined*	CTVT	Canine	Mixed-breed	11,00	09/08/2019	Undetermined*	F
117	59703	Undetermined*	CTVT	Canine	Mixed-breed	11,60	05/12/2019	Undetermined*	F
118	60425	16/12/2017	CTVT	Canine	Mixed-breed	10,10	06/12/2019	1,97	M
119	60850	09/01/2018	CTVT	Canine	Mixed-breed	14,65	03/02/2020	2,07	F
120	61786	21/02/2017	CTVT	Canine	Mixed-breed	09,10	21/02/2020	3,00	M
121	61788	08/03/2017	CTVT	Canine	Basset Hound	14,25	21/02/2020	2,96	M
122	61790	Undetermined*	CTVT	Canine	Mixed-breed	13,50	21/02/2020	Undetermined*	M
123	63766	15/12/2014	CTVT	Canine	Mixed-breed	13,90	13/11/2020	5,92	F
124	64626	10/02/2018	CTVT	Canine	Mixed-breed	21,50	25/01/2021	2,96	M
125	65024	11/03/2018	CTVT	Canine	Labrador Retriever	28,50	23/02/2021	2,96	F
126	65277	13/04/2015	CTVT	Canine	German Sheperd	25,00	06/04/2021	5,99	F
127	66548	11/06/2014	CTVT	Canine	Mixed-breed	06,30	06/07/2021	7,07	F
128	69127	13/12/2020	CTVT	Canine	Shih-tzu	06,40	14/12/2021	1,00	M
129	71690	28/07/2010	CTVT	Canine	Mixed-breed	16,65	16/05/2022	11,81	F

130	75092	01/10/2020	CTVT	Canine	Mixed-breed	20,50	19/12/2022	2,22	F
131	73560	07/10/2018	CTVT	Canine	Mixed-breed	23,00	16/09/2022	3,95	M

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Tumour Manifestations								
Case number	Genital	Lymph nodes	Ocular	Perianal	Cutaneous	Nasal	Oral	Others
1	X							
2	X							
3	X					X		
4	X							
5					X			
6	X				X			
7	X							
8	X							
9	X							
10	X							
11	X							
12	X							
13				X				
14	X							
15	X							
16	X							
17	X							

18	X							
19						X		
20	X							
21	X							
22	X							
23	X							
24	X							
25	X							
26	X	X						
27						X		
28				X				
29	X							
30	X				X			
31					X			
32							X	
33	X							
34	X							
35	X							
36	X							
37	X							
38	X							
39	X							
40	X							

41	X							
42	X							
43	X							
44	X							
45	X							
46	X							
47	X							
48	X							
49	X							
50	X				X			
51	X							
52	X							
53				X				
54						X	X	
55					X			
56	X							
57	X							
58	X							
59	X							
60	X							
61	X							
62	X							
63	X							

64	X							
65	X							
66	X							
67	X							
68	X							
69	X				X			
70	X							
71	X							
72	X							
73	X							
74	X							Mammary gland
75	X							
76	X							
77	X							
78						X	X	
79	X							
80	X							
81	X							
82	X	X			X			
83	X							
84	X	X	X		X		X	
85	X							
86	X			X				

87	X							Uretra
88	X							
89	X							
90	X							
91	X							
92	X							
93	X							
94	X							
95	X							
96	X				X			
97								Bladder
98						X		
99						X	X	
100	X							
101	X							
102	X							
103	X	X			X			
104	X							
105	X							
106	X							
107	X							
108	X							
109	X							

110	X				X			
111			X					Central nervous sistem (forebrain)
112	X							
113	X							
114	X							
115	X							
116	X							
117	X							
118	X		X		X			
119	X							
120						X	X	
121	X							
122					X			
123	X							
124	X							
125	X							
126	X							
127	X							
128	X							
129	X							
130	X							
131	X				X			

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Treatment and diagnosis					
Case number	1st Therapy	Chemotherapy sessions (number)	Clinical response to vincristine	Adjuvant therapy	Diagnostic method
1	NR	NR	NR		Citology
2	NR	NR	NR		Citology
3	Vincristine sulphate	5	Complete remission		Citology
4	Vincristine sulphate	3	Complete remission		Citology
5	Surgery	NR	NR		Citology
6	Vincristine sulphate	4	Complete remission		Citology
7	Vincristine sulphate	NR	Complete remission		Citology
8	NR	NR	NR		Citology
9	Vincristine sulphate	6	Complete remission		Citology
10	NR	NR	NR		Citology
11	Vincristine sulphate	4	Complete remission		Citology
12	Vincristine sulphate	4	Complete remission		Citology
13	NR	NR	NR		Citology
14	Vincristine sulphate	NR	Complete remission		Citology
15	Surgery	NR	NR		Citology
16	NR	NR	NR		Citology
17	Vincristine sulphate	5	Complete remission		Citology
18	NR	NR	NR		Citology
19	Vincristine sulphate	5	Complete remission		Citology
20	NR	NR	NR		Citology
21	Vincristine sulphate	3	Complete remission		Citology

22	NR	NR	NR		Citology
23	NR	NR	NR		Citology
24	NR	NR	NR		Citology
25	Vincristine sulphate	5	Complete remission		Citology
26	Vincristine sulphate	NR	Complete remission		Citology
27	NR	NR	NR		Histopathology
28	Vincristine sulphate	4	Complete remission		Citology
29	NR	NR	NR		Citology
30	Vincristine sulphate	NR	Complete remission		Citology
31	NR	NR	NR		Citology
32	NR	NR	NR		Citology
33	Vincristine sulphate	4	Complete remission		Citology
34	NR	NR	NR		Citology
35	NR	NR	NR		Citology
36	NR	NR	NR		Citology
37	Vincristine sulphate	5	Complete remission		Citology
38	Vincristine sulphate	4	Complete remission		Citology
39	Vincristine sulphate	NR	Complete remission		Citology
40	Vincristine sulphate	7	Complete remission		Citology
41	Vincristine sulphate	4	Complete remission		Citology
42	NR	NR	NR		Citology
43	Vincristine sulphate	4	Complete remission		Citology
44	Vincristine sulphate	4	Complete remission		Citology
45	Vincristine sulphate	5	Complete remission		Citology
46	Vincristine sulphate	4	Complete remission		Citology

47	Vincristine sulphate	4	Complete remission		Citology
48	Vincristine sulphate	4	Complete remission		Citology
49	NR	NR	NR		Citology
50	Vincristine sulphate	5	Stable disease	Surgery	Citology
51	Vincristine sulphate	NR	Complete remission		Citology
52	NR	NR	NR		Citology
53	Vincristine sulphate	NR	Complete remission		Citology
54	Vincristine sulphate	5	Complete remission		Citology
55	Vincristine sulphate	NR	Complete remission		Citology
56	NR	NR	NR		Citology
57	Vincristine sulphate	4	Complete remission		Citology
58	Vincristine sulphate	4	Complete remission		Citology
59	NR	NR	NR		Citology
60	NR	NR	NR		Citology
61	NR	NR	NR		Citology
62	NR	NR	NR		Citology
63	Vincristine sulphate	4	Complete remission		Citology
64	NR	NR	NR		Citology
65	Vincristine sulphate	4	Complete remission		Citology
66	Vincristine sulphate	NR	Complete remission		Citology
67	NR	NR	NR		Citology
68	Vincristine sulphate	3	Complete remission		Citology
69	Vincristine sulphate	4	Complete remission		Citology
70	Vincristine sulphate	4	Complete remission		Citology
71	Vincristine sulphate	6	Complete remission		Citology

72	Vincristine sulphate	5	Complete remission		Citology
73	Vincristine sulphate	3	Complete remission		Citology
74	Vincristine sulphate	3	Complete remission		Citology
75	Vincristine sulphate	3	Complete remission		Citology
76	Vincristine sulphate	5	Complete remission		Citology
77	NR	NR	NR		Citology
78	Vincristine sulphate	4	Complete remission		Citology
79	Vincristine sulphate	4	Complete remission		Citology
80	Vincristine sulphate	6	Partial remission	Surgery	Citology
81	Vincristine sulphate	4	Complete remission		Citology
82	Vincristine sulphate	6	Partial remission	Doxorubicin	Citology
83	Vincristine sulphate	3	Complete remission		Citology
84	NR	NR	NR		Citology
85	Vincristine sulphate	6	Partial remission	Surgery	Citology
86	NR	NR	NR		Citology
87	Vincristine sulphate	6	Partial remission	Lomustine	Citology
88	NR	NR	NR		Citology
89	Vincristine sulphate	4	Stable disease	Surgery	Citology
90	Vincristine sulphate	4	Complete remission		Citology
91	Vincristine sulphate	6	Complete remission		Citology
92	Vincristine sulphate	4	Complete remission		Citology
93	Vincristine sulphate	6	Complete remission		Citology
94	Vincristine sulphate	4	Complete remission		Citology
95	NR	NR	NR		Citology
96	Vincristine sulphate	5	Complete remission		Citology

97	Vincristine sulphate	7	Complete remission		Citology
98	Vincristine sulphate	5	Complete remission		Histopathology
99	Vincristine sulphate	6	Complete remission		Citology
100	NR	NR	NR		Citology
101	NR	NR	NR		Citology
102	NR	NR	NR		Citology
103	Vincristine sulphate	5	Complete remission		Citology
104	Vincristine sulphate	4	Complete remission		Citology
105	Vincristine sulphate	4	Complete remission		Citology
106	NR	NR	NR		Citology
107	Vincristine sulphate	NR	Complete remission		Citology
108	Vincristine sulphate	4	Complete remission		Citology
109	NR	NR	NR		Citology
110	Vincristine sulphate	6	Complete remission		Citology
111	Vincristine sulphate	6	Complete remission		Histopathology
112	NR	NR	NR		Citology
113	NR	NR	NR		Citology
114	NR	NR	NR		Citology
115	Vincristine sulphate	6	Partial remission	Surgery	Citology
116	Vincristine sulphate	4	Complete remission		Citology
117	NR	NR	NR		Citology
118	Vincristine sulphate	3	Complete remission		Citology
119	Vincristine sulphate	4	Complete remission		Citology
120	Vincristine sulphate	NR	Complete remission		Citology
121	NR	NR	NR		Citology

122	Vincristine sulphate	4	Complete remission		Citology
123	Vincristine sulphate	4	Complete remission		Citology
124	Vincristine sulphate	6	Complete remission		Citology
125	Vincristine sulphate	NR	Complete remission		Citology
126	Vincristine sulphate	4	Complete remission		Citology
127	Vincristine sulphate	5	Complete remission		Citology
128	NR	NR	NR		Citology
129	Vincristine sulphate	5	Complete remission		Citology
130	Vincristine sulphate	3	Complete remission		Citology
131	Vincristine sulphate	5	Complete remission		Citology

CTVT: Canine transmissible venereal tumour; NR: Not reported; Undetermined*: Rescued dog; F: Female; M: Male; X: represents positive for the respective manifestation.

SUPPLEMENTARY MATERIAL - CHAPTER III

Summary of data from selected studies: Vincristine sulfate monotherapy for canine transmissible venereal tumor treatment

Year	First author	Origin of cases	Type of study	No of cases	Protocol	No of sessions (patients)	Response evaluation criteria equivalency[1]
1990	Amber, E. I.	Nigeria (Zaria)	Prospective	20	0.5 mg/m², IV, weekly	4 (4), 4-5 (9), and 6 (7)	VCOG guideline
1995	Harmelin, A.	Israel (Jerusalem)	Prospective	10	0.6 mg/m², IV, weekly	2-6 (9) and 12 (1)	Unclear
1996	Singh, J.	India (Punjab)	Prospective	12	0.025 mg/kg, IV, weekly	2.4 ± 0.2	Unclear
1998	Rogers, K. S.	United States (Texas)	Retrospective	5	0.5 mg/m², IV, weekly	4-8 (5)	VCOG guideline
2000	Erünal-Maral, N.	Turkey (Ankara)	Prospective	12	0.025mg/kg, IV, weekly	3 (2), 4 (6), 5 (4)	VCOG guideline
2000	González, C.	Chile (Santiago)	Prospective	19	0.03mg/kg, IV, weekly	2-6 (19)	VCOG guideline
2001	Athar, M.	Pakistan (Faisalabad)	Prospective	6	0.025 mg/kg, IV, weekly	3 (1), 4(3), 5(2)	Unclear
2004	Tella, M. A.	Nigeria (Ibadan)	Prospective	10	0.025 mg/kg, IV, weekly	4 (10)	VCOG guideline
2005	Nak, D.	Turkey (Bursa)	Prospective	37	0.025 mg/kg, IV, weekly	2-7 (37)	Unclear
2006	Herrera, I. C.	Cuba (Sancti Spíritus)	Prospective	9	0.025 mg/kg, IV, weekly	3-4 (9)	VCOG guideline
2009	Mukaratirwa, S.	Zimbabwe (Harare)	Prospective	5	0.025 mg/kg, IV, weekly	5 (5)	VCOG guideline
2013	Lima, C. R. O.	Brazil (Goiás)	Prospective	13	0.025 mg/kg, IV, weekly	4-5 (12), 7 (1)	VCOG guideline
2014	Hantrakul, S.	Thailand (Ratchaburi)	Prospective	6	0.7 mg/m², IV, weekly	3-8 (6)	PR as >50%
2014	Huppes, R. R.	Brazil (Uberaba)	Retrospective	39	0.075 mg/kg, IV, weekly	4-6 (36)	Unclear
2015	Komnenou, A. T.	Greece (Thessaloniki)	Retrospective	25	0.6 mg/m², IV, weekly	3-4 (25)	VCOG guideline
2015	Paranzini, C. S.	Brazil (Paraná)	Retrospective	46	0.5 mg/m², IV, weekly	Up to 6 (46)	Unclear
2016	Ramadinha, R. R.	Brazil (Rio de Janeiro)	Retrospective	65[3]	0.025 mg/kg, IV, weekly	1-3 (15), 4-6 (43), 7-10 (6)	VCOG guideline
2018	Kanca, H.	Turkey (Ankara)	Prospective	9	0.025 mg/kg, IV, weekly	3 (1), 4 (1), 5 (3), 6 (4)	VCOG guideline
2018	Vural, S. A.	Turkey (Ankara)	Prospective	18	0.025 mg/kg, IV, weekly	3-6 (18)	VCOG guideline
2019	Setthawongsin, C.	Thailand (Bangkok)	Prospective	11	0.025 mg/kg, IV, weekly	8 (11)	Unclear
2019	Ayala-Díaz, S.	Mexico (Oaxaca)	Prospective	21	0.023 mg/kg, IV, weekly	5 (13), 6 (8)	VCOG guideline
2019	Alzate, J. M.	Colombia (Caldas)	Prospective	21	0.5 mg/m², IV, weekly	3-4 (9), 5-8 (12)	VCOG guideline
2020	Reis-Filho, N. P.	Brazil (Paraná)	Prospective	22	0.75 mg/m², IV, weekly	2 (1), 3 (4), 4 (4), 5 (1), 6 (12), 7 (1)	Unclear

2020	Ignatenko, N.	Ukraine (Kyiv) and Germany (Munich)	Retrospective	12	0.7 mg/m², IV, weekly	4 (3), 5 (5), 6 (3), 9 (1)	VCOG guideline
2020	Bulhosa, L. F.	Brazil (Bahia)	Prospective	10	0.5 mg/m², IV, weekly	1 (1), 3 (2), 4 (5), 7 (2)	VCOG guideline
2021	Abdelnaby, E. A.	Egypt (Cairo)	Prospective	13	0.025 mg/kg, IV, weekly	4 (13)	VCOG guideline
2021	Ramírez-Ante, J. C.	Colombia (Caldas)	Prospective	24	0.5 mg/m², IV, weekly	3 (1), 4 (6), 5 (7), 6 (2), 7 (4), 8 (3)	VCOG guideline
2022	Souza, J. V. A.	Brazil (Alagoas)	Prospective	12	0.025 mg/kg, IV, weekly	4 (12)	VCOG guideline
2022	Duzanski, A. P.	Brazil (São Paulo)	Prospective	17	0.75 mg/m², IV, weekly	3-8 (17)	PR as >50%
2023	Ferreira, M. A. Q. B.	Brazil (Pernambuco)	Prospective	10	0.75 mg/m², IV, weekly	6 (10)	VCOG guideline
2023	Costa, T. S.	Brazil (Rio de Janeiro)	Retrospective	116	0.7-0.75 mg/m², IV, weekly	Up to 4 (62), 5-6 (51), >6 (3)	VCOG guideline
2023	Parikh, N. P.	India (Gujarate)	Prospective	6	0.025 mg/kg, IV, weekly	4 (6)	VCOG guideline

VS: Vincristine sulfate. CR: Complete response. PR: Partial response. SD: Stable disease. PD: Progressive disease. NR: Not reported. [1]Some studies were carried out before the release of the VCOG guidelines (Nguyen et al., 2013), but it is possible that the criteria were equivalent. When the study regarded a 100% reduction in tumor size as complete remission, and in all cases met this criterion, the implementation of the VCOG guideline is acknowledged. Other studies considered partial remission of >50% of tumoral volume reduction (PR >50%). [2]Reported partial remission as an incomplete regression. [3]Certain patients discontinued the treatment (39). We were unable to ascertain whether there would be a change in clinical response and the reasons for treatment abandonment.

Summary of data from selected studies: Vincristine sulfate monotherapy for canine transmissible venereal tumor treatment

Year	First author	CR	PR	SD	PD	Follow-up time	Recurrence	Time to recurrence
1990	Amber, E. I.	20/20 (100%)	-	-	-	12 months	1/20 (5%)	1:10 months
1995	Harmelin, A.	8/10 (80%)	-	-	2/10 (20%)	NR	NR	NR
1996	Singh, J.	11/12 (91.7%)	-	1/12 (8.3%)	-	NR	NR	NR
1998	Rogers, K. S.	5/5 (100%)	-	-	-	> 12 months	1/5 (20%)	1:8 months
2000	Erünal-Maral, N.	12/12 (100%)	-	-	-	12 months	0/12 (0%)	No recurrence
2000	González, C.	19/19 (100%)	-	-	-	NR	0/19 (0%)	No recurrence
2001	Athar, M.	5/6 (83.3%)	1/6 (16.7%)[2]	-	-	6 months	0/5 (0%)	No recurrence
2004	Tella, M. A.	10/10 (100%)	-	-	-	NR	NR	NR
2005	Nak, D.	31/37 (83.8%)	6/37 (16.2%)	-	-	7–49 months	0/31 (0%)	No recurrence
2006	Herrera, I. C.	9/9 (100%)	-	-	-	NR	0/9 (0%)	No recurrence
2009	Mukaratirwa, S.	5/5 (100%)	-	-	-	84 days	0/5 (0%)	No recurrence
2013	Lima, C. R. O.	13/13 (100%)	-	-	-	NR	NR	NR
2014	Hantrakul, S.	5/6 (83.3%)	1/6 (16.7%)	-	-	NR	NR	NR
2014	Huppes, R. R.	36/39 (92.3%)	3/39 (7.7%)	-	-	NR	NR	NR
2015	Komnenou, A. T.	24/25 (96%)	-	1/25 (4%)	-	12 months	0/24 (0%)	No recurrence
2015	Paranzini, C. S.	31/46 (67.4%)	14/46 (38.9%)	1/46 (2.2%)	-	NR	NR	NR
2016	Ramadinha, R. R.	64/65 (98.5%)	-	1/65 (1.5%)	-	> 4 months	0/64 (0%)	No recurrence
2018	Kanca, H.	9/9 (100%)	-	-	-	2–18 months	0/9 (0%)	No recurrence
2018	Vural, S. A.	18/18 (100%)	-	-	-	NR	NR	NR
2019	Setthawongsin, C.	9/11 (81.8%)	1/11 (9.1%)	1/11 (9.1%)	-	NR	NR	NR
2019	Ayala-Díaz, S.	21/21 (100%)	-	-	-	3 months	0/21 (0%)	No recurrence
2019	Alzate, J. M.	21/21 (100%)	-	-	-	3 years	0/21 (0%)	No recurrence
2020	Reis-Filho, N. P.	18/21 (85.7%)	3/21 (14.3%)	-	-	NR	NR	NR

2020	Ignatenko, N.	12/12 (100%)	-	-	-	≥12 months	0/12 (0%)	No recurrence
2020	Bulhosa, L. F.	10/10 (100%)	-	-	-	NR	NR	NR
2021	Abdelnaby, E. A.	13/13 (100%)	-	-	-	6 months	0/13 (0%)	No recurrence
2021	Ramírez-Ante, J. C.	24/24 (100%)	-	-	-	NR	NR	NR
2022	Souza, J. V. A.	12/12 (100%)	-	-	-	1 month	0/12 (0%)	No recurrence
2022	Duzanski, A. P.	15/17 (88.2%)	2/17 (11.8%)	-	-	12 months	0/15 (0%)	No recurrence
2023	Ferreira, M. A. Q. B.	10/10 (100%)	-	-	-	6 months	0/10 (0%)	No recurrence
2023	Costa, T. S.	116/116 (100%)	-	-	-	NR	NR	NR
2023	Parikh, N. P.	6/6 (100%)	-	-	-	NR	NR	NR

VS: Vincristine sulfate. CR: Complete response. PR: Partial response. SD: Stable disease. PD: Progressive disease. NR: Not reported. [1]Some studies were carried out before the release of the VCOG guidelines (Nguyen et al., 2013), but it is possible that the criteria were equivalent. When the study regarded a 100% reduction in tumor size as complete remission, and in all cases met this criterion, the implementation of the VCOG guideline is acknowledged. Other studies considered partial remission of >50% of tumoral volume reduction (PR >50%). [2]Reported partial remission as an incomplete regression. [3]Certain patients discontinued the treatment (39). We were unable to ascertain whether there would be a change in clinical response and the reasons for treatment abandonment.