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Canine Transmissible Venereal Tumor: An examination of tumor cytology and haematology in 9 affected dogs living in Namibia

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Abbreviations

CTVT	Canine transmissible venereal tumor
TVT	Transmissible venereal tumor
N:C ratio	Nucleus: Cytoplasm ratio
FNA	Fine needle aspiration
CBC	Complete blood count
WBC	White blood cell
TNM staging	Tumor, node and metastasis staging
BCS	Body condition score
HCT	Haematocrit
MCV	Mean corpuscular volume
RDW	Red cell distribution width
HGB	Haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCH	Mean corpuscular haemoglobin
RBC	Red blood cell
PLT	Platelets
MPV	Mean platelet volume
Ne	Neutrophils
Seg-ne	Segmented neutrophils
Eo-gran or Eo	Eosinophil granulocytes
Mono or Mon	Monocytes
Ly	Lymphocytes
Abs	Absolute (number)
Squam	Squamous epithelial cells
SD	Standard deviation

Introduction

Canine transmissible venereal tumor (CTVT) is a type of cancer in dogs which is spread by the transfer of living cancer cells between dogs. It is the oldest and most prolific known contagious cancer (Strakova and Murchison, 2014). Analogous to the spread of cancer within an individual, contagious cancers "metastasize" as allogenic grafts between individuals (Metzger and Goff, 2016). Transmission of CTVT usually happens via mating, but it can also occur through licking, sniffing and other direct contact (Cohen, 1985; Das and Das, 2000). The tumor is usually associated with the external genitalia, but it has been shown to occasionally metastasize to various other areas such as regional lymph nodes, skin (Varughese et al., 2012), parenchymal organs (Rogers et al., 1998), brain, eye (Ferreira et al., 2002), and internal reproductive organs (Bastan et al., 2006). The chemotherapeutic agent vincristine has been shown to be the most effective treatment method. Further treatment methods and combinations will be discussed in a later paragraph.

The tumor was first characterized by a Russian veterinarian, M.A. Novinsky, in 1876. He showed that TVT could be transmitted from one dog to another by the transfer of the tumor cells (Martins et al., 2005). Studies on CTVT phylogeny show that the lineage first arose in Asia between 4000 and 8500 years ago, and that the most recent common ancestor of the modern tumor occurred about 1900 years ago (Baez-Ortega et al., 2019).

A study conducted in 2014 determined that TVT is endemic in at least 90 countries worldwide. It was estimated to be present at a prevalence of at least 11 countries in Africa, 8 countries in Asia and 13 countries in South and Central America. In Australia and the United States, it was shown to be present only in remote indigenous communities, or in animals imported from endemic areas. It is present in some European countries, but has declined substantially in Northern Europe, possibly in response to the implementation of stricter dog control laws. The prevalence of TVT is undeniably linked to the presence of free-roaming dogs. The presence of the disease is negatively correlated with the socio-economic status of a country. There is no evidence of gender bias concerning TVT (Strakova and Murchison, 2014).

The aim of this paper is to analyze and compare tumor cytology and haematology data of nine dogs affected by CTVT. Emphasis will be placed on tumor cell morphology and white blood cell numbers.

Literature Review of Canine Transmissible Venereal Tumor

TVT over time

In a thorough and extensive study, Baez-Ortega et al. (2019) summarized the history, spread, diversity, mutational exposures and evolution of the CTVT clone. The team took advantage of the fact that the CTVT genome acts as a living biomarker and has recorded its surrounding changing mutagenic environments over time. The study involved an analysis of somatic mutations extracted from the protein-coding genomes (exomes) of 546 globally distributed CTVT tumors, and this allowed the construction of a time-resolved phylogenetic tree of the tumor. Thus, the spread of the tumor throughout the world could be traced, and certain mutations could be characterized in terms of location and time point.

As mentioned earlier, the lineage was found to have first arisen 4000-8500 years ago. In the last 500 years, in part due to the extreme increase of human maritime travel, CTVT was able to spread throughout the world quite rapidly. CTVT seems to have escaped from its founding population less than 2000 years ago, spreading within Asia and to Europe (Figure 1).

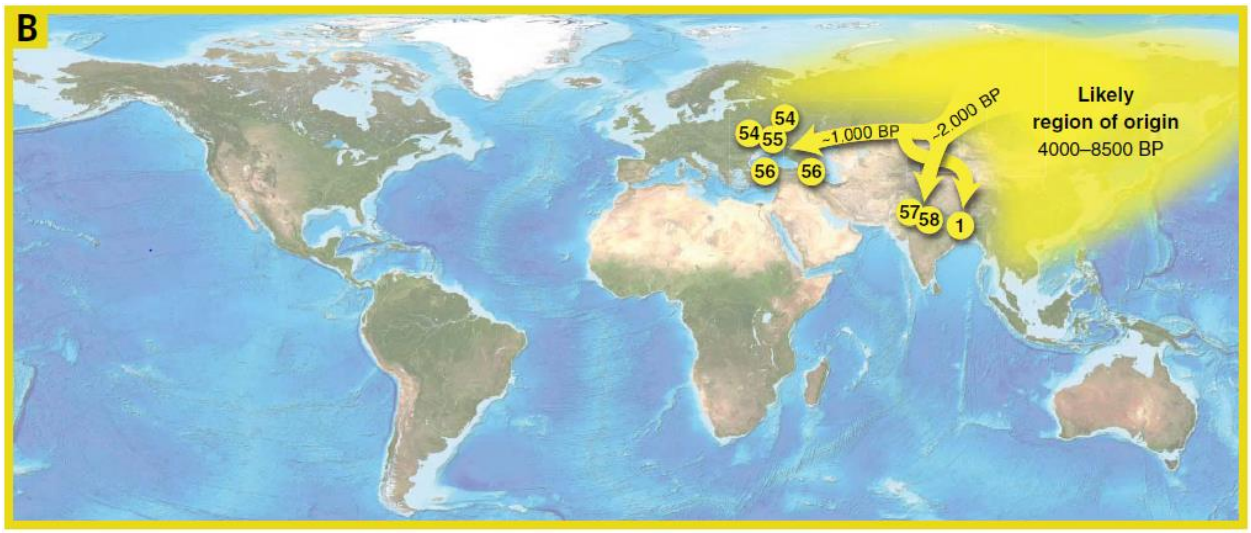


Figure 1: Early expansion (prior to ~500 BP); Rapid global expansion. (Baez-Ortega et al., 2019)

Expansion seen in the last 500 years can be seen in Figure 2 and 3, and can be divided into two sublineages. Figure 2 further divides the first sublineage. The one shown in red was introduced to the Americas with early colonial contact about 500 years ago. The black sublineage was brought into Africa about 300 years ago, and then was reintroduced to Europe and Asia.

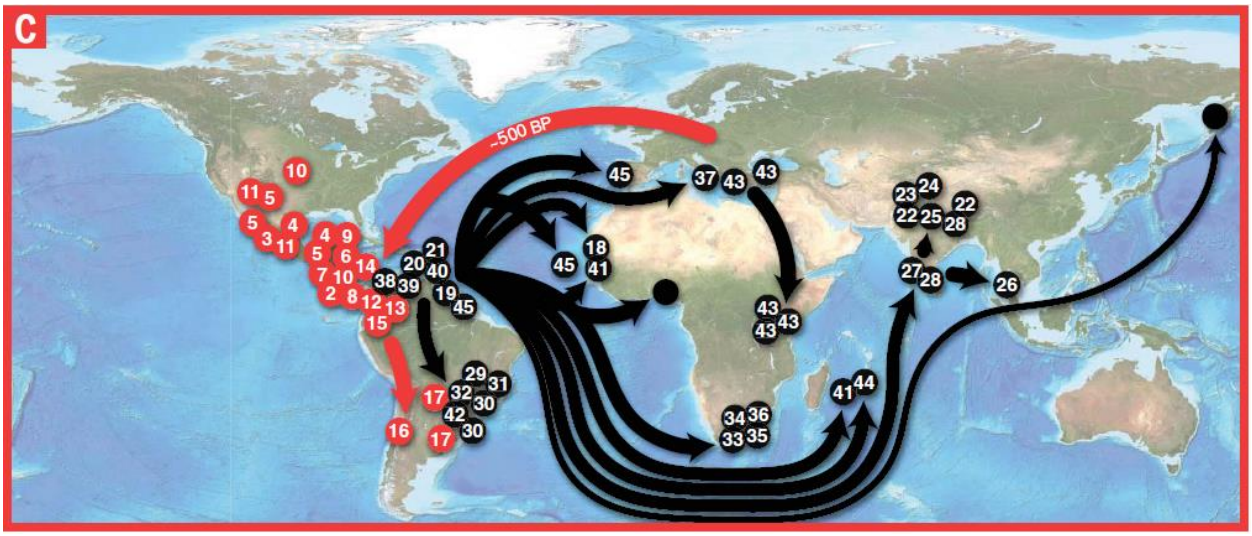


Figure 2: Late expansion (from ~500 BP); Sublineage 1 (Baez-Ortega et al, 2019)

The second sublineage that spread in the last 500 years is shown in Figure 3. It spread out of Asia and Europe into Australia and then the Pacific. It can be also be found in both North America and Africa. These findings suggest that starting in the 15th century, dogs were commonly transported vast distances by maritime travel.

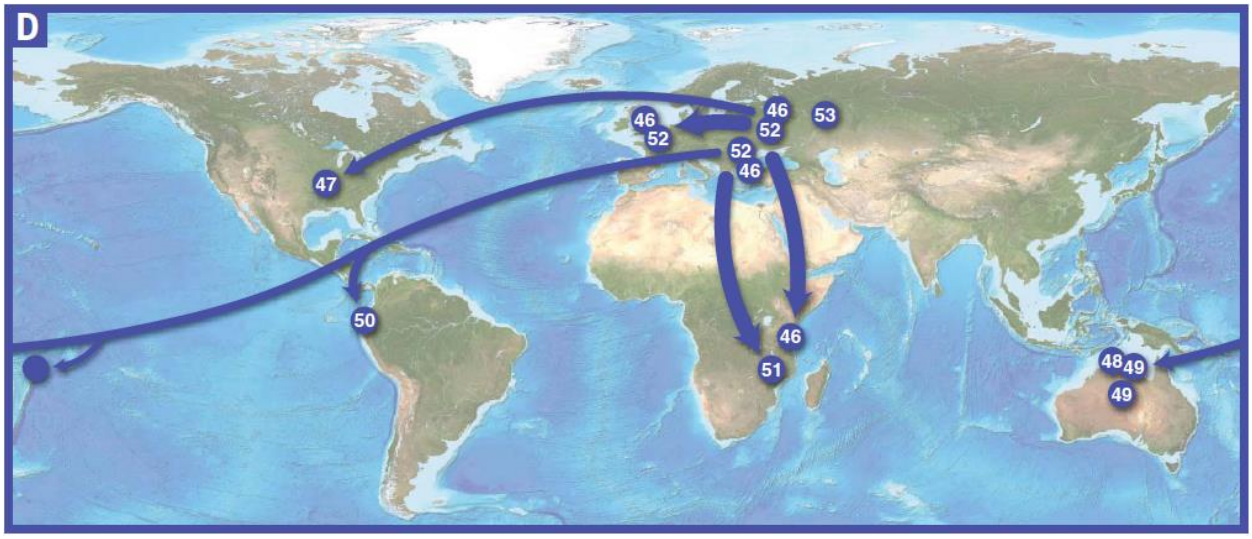


Figure 3: Late expansion (from ~500 BP); Sublineage 2 (Baez-Ortega et al., 2019)

In spite of all these findings, Wang et al. (2019) reported that the CTVT founder is likely an ancient American dog with introgression from populations carrying ancestry related to coyotes from the Monterey area, California, and Alabama.

Characteristics of the tumor

Cytogenetic origin

Considering the fact that CTVT originated from the canine genome, scientists conducted studies to determine the genetic distinction of the two. The dog has 78 diploid chromosomes in its somatic cell. 76 of these are autosomal chromosomes and are acrocentric, and two are metacentric sex chromosomes. The CTVT genome has only 59 chromosomes (Mukaratirwa and Gruys, 2003), with many of them being metacentric or submetacentric (Hasler and Weber, 2000).

Mutations and selection pressures

Baez-Ortega et al. (2019) determined that CTVT has a mutation burden which is so great that it exceeds that of even the most mutated human cancer types. However, it was discovered that its mutation *rate* is not very high, and thus the massive mutation burden owes to the lineage's vast age. Nowadays, there is very little indication that CTVT continues to adapt to its environment. It was found that negative selection may be an important factor in CTVT evolution, which contrasts the positive selection force often seen in human cancers. This notion is supported by the fact that the presence of inter-tumor competition would encourage the manifestation of negative selection in CTVT in the form of the extinction of less infective CTVT lineages. The results of the study actually suggest that the main evolutionary force in CTVT is neutral genetic drift, which indicates that neither positive nor negative selection is necessary to sustain the cancer over the long term.

Immunophenotype

Immunohistochemical studies of CTVT have tested it for various tumor markers (Table 1), and this enabled scientists to rule out some types of cancer. The tumor cells are negative for delta-light chains, immunoglobulins G and M, CD3, desmin, α-smooth muscle actin and keratins. These findings rule out smooth muscle, epithelial and T or B-cell origin. The cells were found positive for AAT, lysozyme and ACMI, which indicates a histiocytic origin (Mukaratirwa and Gruys, 2003).

Table 1: Antibodies and their cellular specificity used in immunohistochemical characterization of CTVT (Marchal et al., 1997; Mozos et al., 1996)

Keratin	Epithelial cells	-
Vimentin	Mesenchymal cells	+
Desmin	Muscle cells	-
α-smooth muscle actin	Smooth muscle cells	-
CD3	T-lymphocytes	-
IgG and IgM	B-lymphocytes	-
λ-light chains	B-lymphocytes	-
κ-light chains	B-lymphocytes	-
Lysozyme	Histiocytes	+
ACM1	Histiocytes	+
A-1-antitrypsin	Histiocytes	+

Although the origin of CTVT is uncertain, it was first described as histiocytic, lymphatic, or reticuloendothelial while the latest analyses suggest a macrophage or a myeloid origin (Újváry B et al., 2017).

Cytology and histopathology

Duncan and Prasse (1979) examined different canine round cells tumors and described their cytology. CTVT was classified as cellular, with round cells ranging from 14-30µm in diameter. Tumor cells occur discretely or in sheets. They sometimes resemble an epithelial tumor on cytology, but in general fit a round cell appearance. They contain round to oval nuclei with coarse chromatin arranged in a cord-like pattern. The N:C ratio is slightly less than 1:1, so quite high. In most cases, the nucleolus is large and visible. The finely granular cytoplasm is very characteristically vacuolated (see Figure 4, 5 and 6). Cytology is the preferred method of diagnosis, as it is such a morphologically characteristic tumor.

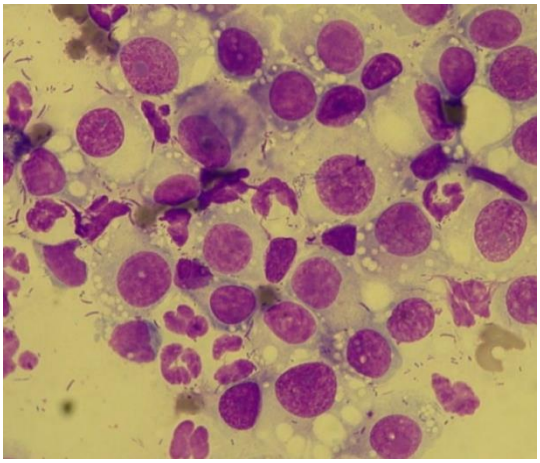


Figure 4: Bossy tumor impression smear (from this study)

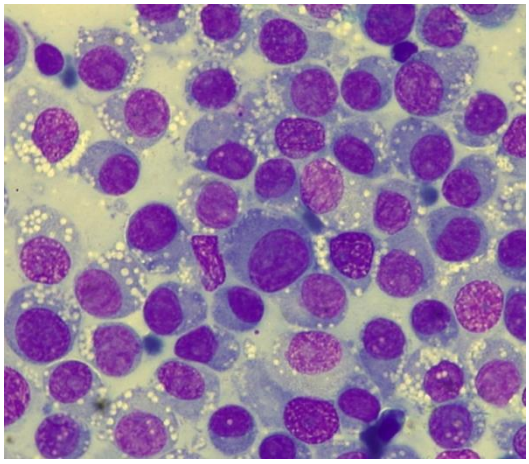


Figure 5: Chaka tumor impression smear (from this study)

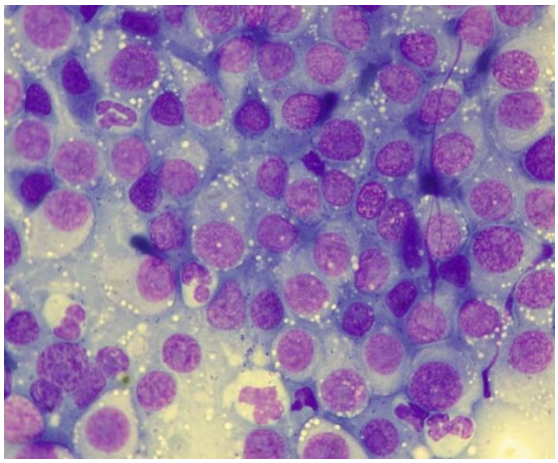


Figure 6: Leeu tumor impression smear (from this study)

Mohanty and Raya (1977) grew CTVT cells in vitro and concluded that there are two morphologically distinct types. In subsequent years this classification was refined into three

subtypes: plasmacytoid, lymphocytoid, and mixed. The lymphocytic subtype (Figure 7A) contains cells with 60% or more round morphology and a scarce and finely granular cytoplasm. Cytoplasmic granules occupy the cell periphery, and cells contain a round nucleus with rough chromatin and one or two prominent nucleoli. The plasmacytic subtype (Figure 7B) contains cells with 60% or more ovoid morphology. The N:C ratio is higher than in the lymphocytic subtype, and the nucleus is eccentrically located. The mixed subtype contains both cell morphologies, with neither surpassing 59% of total cells (Amaral et al., 2007).

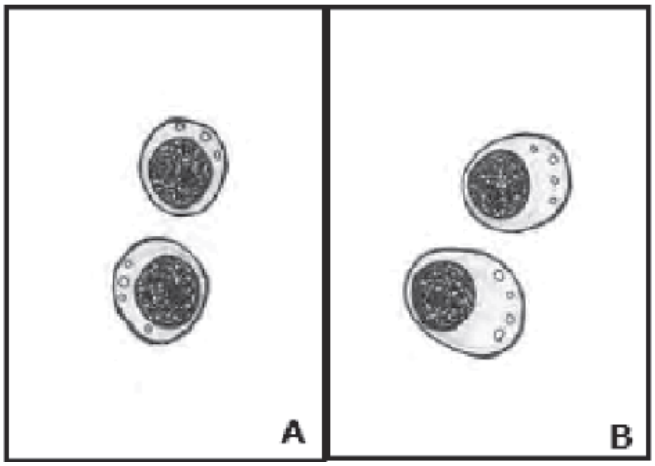


Figure 7: Cytomorphological scheme of CTVT types. Lymphocytic (A) and Plasmacytic (B) (Amaral et al., 2007)

Since 1994, the veterinary pathology service of FMVZ-UNESP, Botacatu, Brazil has employed this morphological classification (Bassani-Silva et al., 2003; Amaral et al., 2004). It was hypothesized that the different morphological subtypes represented different levels of malignancy, which was supported by the discovery that plasmacytic CTVTs had a higher incidence of metastasis as well as a higher frequency of nuclear abnormalities (Amaral et al., 2007). Furthermore, single-cell gel electrophoresis demonstrated that tumors with plasmacytoid morphology exhibited fewer DNA breaks, presumably helping them evade the immune system (Amaral et al., 2011).

In haematoxylin-eosin stained histological sections, CTVT characteristically presents as a group of compact masses of diffusely arranged cells which are supported by thin trabeculae of fibrovascular tissue. The cells are round or polyhedral with a centrally located, round nucleus containing a prominent nucleolus. The cytoplasm is slightly granular, vacuolated and eosinophilic and generally has indistinct borders (Mukaratirwa and Gruys, 2003).

Behaviour of the tumor

Transmission

After CTVT's infectious nature was first discovered by Novinsky in 1876, scientists wanted to determine what exactly the infectious agent was. Was the tumor triggered by a bacterial infection? And a few years later the possibility of a virus was also considered. A bacterial cause was presumably ruled out quite early due to the absence of bacteria during microscopic observation. However, a number of studies explored the possibility of a viral cause. In 1967, Lombard and Cabanie reported the discovery of cell-free transmission of the tumor. In 1985, Amber et al. were able to demonstrate, by electron microscopy, apparently virus-like particles within some CTVT cells. However, contrary to these findings, other researchers reported the absence of any viral-like particles within the CTVT cells (Cockril and Beasley, 1975). Nowadays, it is widely accepted in the scientific community that the infectious agent is the tumor cell itself. Mukaratirwa and Gruys (2003) presented six findings which strongly support the cellular transmission of CTVT, as well as the idea that the tumor originated from one cell type:

1. Tumors from different regions have the same number of chromosomes and a similar frequency of metacentric chromosomes.
2. The above characteristics are maintained after subsequent passages *in vitro* and *in vivo*.
3. The number of chromosomes in metastatic tumors is the same as that of the primary tumor.
4. Banding pattern analysis of CTVT chromosomes showed great structural rearrangements, and these rearrangements were identical in different tumors.
5. Using southern blot analysis, it was shown that CTVT from individual dogs from various regions contain identical rearranged gene (c-MYC oncogene).
6. In a xenograft model of CTVT in mice, the cells retained cytological, histological and karyotypical features.

It has been determined that the tumor can only be transmitted if the mucosa it comes in contact with is not intact; there must be some abrasion to allow the transplantation of exfoliated cells during coitus (Ganguly et al., 2013).

Cancer is not contagious in general, although transmissible cancer types exist. There are eight naturally occurring transmissible contagious cancers (one lineage in dogs, two lineages in Tasmanian devils, and five lineages in bivalves), with no underlying pathogen infections, that have been recorded in the wild (Metzger et al., 2016). Transmissible cancers have, however, been recorded under laboratory conditions and on rare occasions in humans (Újváry et al., 2017).

Growth phases

Both spontaneous and experimentally transplanted CTVT demonstrated a stage of rapid tumor growth initially, followed by a regressive stage (Mukaratirwa and Gruys, 2003).

Primary extra-genital CTVT

Although uncommon, there have been several cases of primary intranasal CTVT (Papazoglou et al., 2001; Ojeda et al., 2018). The transmission in these cases was presumably through the licking and sniffing of an affected dog. Primary cutaneous manifestations have also been documented (Albanese et al., 2002; Marcos et al., 2006), as well as primary ocular CTVT (Komnenou et al., 2015).

Metastasis

As mentioned earlier, this tumor is usually confined to the external genitalia. Metastasis is rare and tends to happen in ~5% of cases (Brown et al., 1980; Thrall, 1982). However, a Brazilian study of 132 dogs showed that metastasis happened in 25.2% of their cases (mostly to the skin), suggesting that the incidence of metastasis varies across geographically distinct tumor populations (Amaral et al., 2007).

CTVT most commonly spreads to the regional lymph nodes, but it has also been found to metastasize to other areas such as the skin (Varughese et al., 2012), muscle, eye (Komnenou et al., 2015), brain (Adams et al., 1970; Ferreira et al., 2000; Placke et al., 1987), internal reproductive organs (Bastan et al., 2006), and parenchymal organs (Rogers et al., 1998). A disseminated form also exists and usually manifests only in immune compromised patients (Albanese et al., 2006).

Effect on the animal

Clinical signs

Most TVT cases are primary genital tumors, resulting from sexual transmission of the tumor cells. In these cases, the clinical presentation has been found to include a decrease in appetite, weight loss, infertility, lethargy and anaemia (Hiblu et al., 2019). In addition, bleeding from the growth is often observed as the tumor tends to become ulcerated. Some cases, however, show very minimal clinical signs and may be limited to the mere presence of the cauliflower-like tumor.

In cases of primary intranasal tumors, the clinical signs were sneezing, snoring (Ojeda et al., 2018), epistaxis, serosanguineous or mucopurulent nasal discharge, facial swelling, and submandibular lymphadenopathy (Papazoglou et al., 2001). Dogs with primary ocular CTVT presented with conjunctival congestion, chemosis and ocular purulent, mucopurulent or haemorrhagic discharge (Komnenou et al., 2015). In some cases, the oral and nasal mucosa became involved, with respective clinical signs.

Immune reaction

The means by which CTVT is tolerated by the host's immune system despite being an allogenic graft are not completely understood. It is thought that it manages to evade the adaptive immune system during the initial rapid growth phase, in part through the down-regulation of major histocompatibility complex molecules (Siddle and Kaufman, 2014). The tumor tends to grow progressively for about three months before becoming subject to the host's anti-tumor immune response. CTVT triggers both the humoral and cellular immune system, and may result in transplantation immunity, thus preventing subsequent infection. Some tumors regress spontaneously (Mukaratirwa and Gruys, 2003).

During the progressive stage of the tumor, a certain tumor-associated antigen is shed into the host's circulation, the amount of which was shown to increase proportionately with tumor size (Palker and Yang, 1981). 48-72 hours after surgical removal of the tumor, this antigen could no longer be detected in the blood (Yang et al., 1991). This suggests that it plays a role in blocking the host's immune system (Black, 1980). To determine the effectiveness of post-regression immunity, a passive transfer of post-regression sera was performed in a number of dogs. This was found to inhibit further CTVT development in infected dogs, and prevent its

manifestation if administered simultaneously with tumor transplantation in healthy dogs (Powers, 1968). Moreover, the tumor was found to be inhibited in the offspring of dams immunized with CTVT before or during pregnancy (Yang et al., 1991).

Tumors were found to be frequently infiltrated by Natural Killer cells and T-lymphocytes (Burnet, 1970). It was also found that tumor regression of CTVT is associated with increased numbers of infiltrating lymphocytes, especially T-lymphocytes (Hsiao et al., 2002; Mizuno et al., 1989; Trial and Yang, 1985).

Treatment

Treatment of this tumor can be achieved through surgery, chemotherapy, radiotherapy, immunotherapy, or a combination of these. Excisional surgery was applied in the past, but the method showed a recurrence rate of up to 60 percent, with about half of these instances resulting from iatrogenic tumor cell transplantation during surgery. Several chemotherapeutic agents have been tested as treatment options, for example cyclophosphamide, methotrexate, doxorubicin, vincristine, and combinations of these with each other and with prednisone. Vincristine emerged as the most successful agent (Ganguly et al., 2013).

Thus, since the 1980s, the main treatment for CTVT has been vincristine, which is a cytotoxic microtubule inhibitor (Amber et al., 1990). The dose currently used is 0.025mg/10 kg body weight, once a week, for 2 weeks beyond resolution of the tumor (Ganguly et al., 2013). Despite this strong selection pressure, to date there has been no evidence of convergent evolution of vincristine resistance mechanisms in CTVT (Baez-Ortega et al., 2019). Other treatment methods have been investigated in a study by Amber et al. (1990). The team compared cyclophosphamide, methotrexate and vincristine in a group of 48 dogs. Neither cyclophosphamide nor methotrexate had any effect on dogs with CTVT when administered orally. When administered IV, however, cyclophosphamide was effective in two out of ten dogs over a six week treatment period. This was not comparable, however, to the great success seen with IV vincristine sulfate treatment, where complete remission was seen in all 20 patients, with only one relapse 12 months later. Furthermore, both methotrexate and cyclophosphamide caused adverse side effects in the patients, whereas there were no adverse side effects reported for vincristine in this study. Other studies, however, did report temporary mild side effects in less than 20% of cases. These included mild depression, partial anorexia, transient leukopenia and transient decrease in semen quality (Ganguly et al., 2013).

Radiotherapy has shown to be effective since the 1950s, at a dose of 1500-2500 rads, divided in bi-weekly sessions of 400-500 rads. However, vincristine has largely replaced this method as radiation therapy requires more specialized equipment and trained personnel and is therefore more expensive (Ganguly et al., 2013).

Several studies have tested immunotherapy as a treatment option for this tumor. For example, Bacillus Calmette-Guérin (BCG) and BCG/Vincristine combination therapy has been shown to cause remission of the tumor, with the combination having significantly shorter remission time (Mukaratirwa et al., 2009). Other successful methods of immunotherapy include the use of short-time generated dendritic cells loaded with CTVT-whole lysate (Fraco-Molina et al., 2018), IL-6 and IL-15 plasmids (Chou et al., 2009) and human interferon (Kanca et al., 2018). In recent years, the use of electrotherapy to deliver the chemotherapeutic agent bleomycin to an intranasal CTVT lesion was tested, and the results of this study confirmed the success of this method (Suzuki et al., 2016). Vilensky et al. (2005) tested the use of vascular photodynamic therapy (VPT) using Pd-bacteriopheophorbide (WST09) as an alternative treatment option. Following a single treatment session, tumors were shown to exhibit an 83 percent long-term cure rate.

Materials and methods

This investigation of Canine Transmissible Venereal Tumor was conducted in Swakopmund, a town in the Southern African country Namibia. CTVT is commonly present among dogs in the surrounding townships which house low or zero income people. Due to the poverty of the area, as well as a lack in education concerning these issues, many of the residents' dogs are intact. Despite spay and neuter efforts by local charities, many dogs reach sexual maturity and are able to maintain the tumor in the population.

The study was conducted with 9 dogs (5 female, 4 male). All cases included a complete blood count and a blood smear. All cases except one (Wagter) include a tumor FNA and/or impression smear. The blood smear and tumor cytology were both stained with REAG-QUICK PANOPTIC stain kit (Reagens Ltd, Budapest, Hungary). In addition, the history of each dog was noted and a general physical examination carried out. Each tumor was assessed using the TNM (Tumor-Node-Metastasis) system. If the dog's inguinal lymph nodes were enlarged, FNA samples were acquired from them, and if there was any indication of metastasis (e.g. ascites, enlarged liver, etc.), the affected areas were sampled for cytology as well.

The complete blood count of each dog was analyzed using HESKA's HemaTrue® Veterinary Hematology Analyzer. The cytology of each tumor was evaluated using two different methods. One was a manual slide description and cell count. The other was the use of the ImageJ software to analyze microscope pictures taken of each tumor cytology slide.

Blood smear analysis:

In spite of the fact that there was possibility to perform complete blood count (CBC) by automatic cell counter, we evaluated the blood smears by microscopic analysis, too. White blood cell (WBC) count was determined by multiplying the average number of WBCs counted in 10, 40X high power fields by 2.0 $x10^9$/L (WBC count multiplication factor) (Jones KW, 2009.) The percent of different cell types were recorded and the absolute count of the different cell types were calculated by previously estimated WBC counts.

Absolute cell numbers were calculated by the following formula, similarly to the complete blood counts when we calculate the absolute cell counts from the white blood cell number and the percentage of the cell type.

Absolute counts of the cell types = (Percentage of cell type x Cells/view) / 100

The average number of the polychromatophilic red blood cells were counted in 10 oil immersion field (OIF).

Tumour lesion cytology smear analysis:

Non-TVT cells were counted in smears by manual microscopic analysis and the percent of the cell types were recorded together with the number of views analysed. TVT-cell count was analysed by using digital pictures of the smears using an image analysis programme (ImageJ).

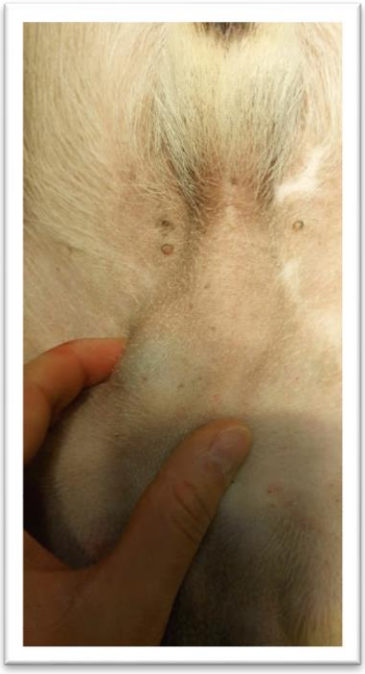
The nuclear area was determined by image analysis after calibration with 10 µm grid.

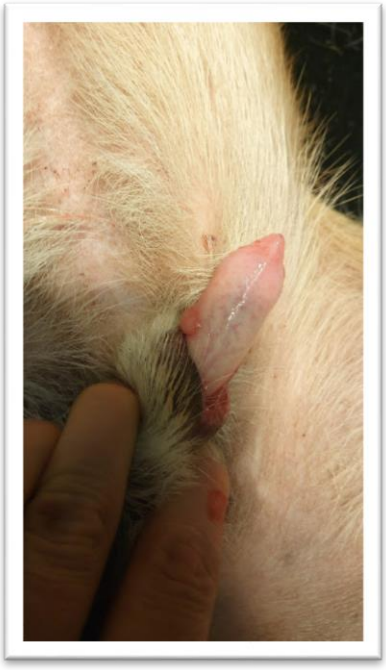
Results

Short description and pictures

1. Rocky:

Rocky was a 2 year old male intact Shepherd mix from the DRC Township. The owner was unaware of the tumor and the dog's appointment was for a simple neuter. The owner had another dog at home, but had not noticed any abnormalities with the other dog. Two tumors were noted on the glans penis, each about 1cm in diameter. Both inguinal lymph nodes were enlarged and hard on palpation, the right was bigger than the left. He was neutered and treated with Vincristine at this appointment.





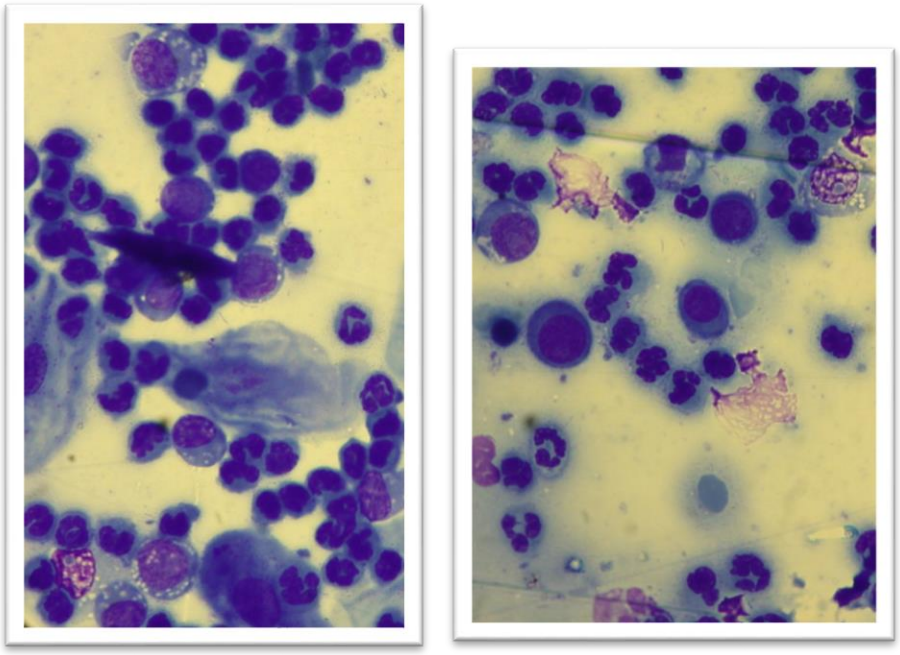


Figure 8: Rocky tumor impression smear

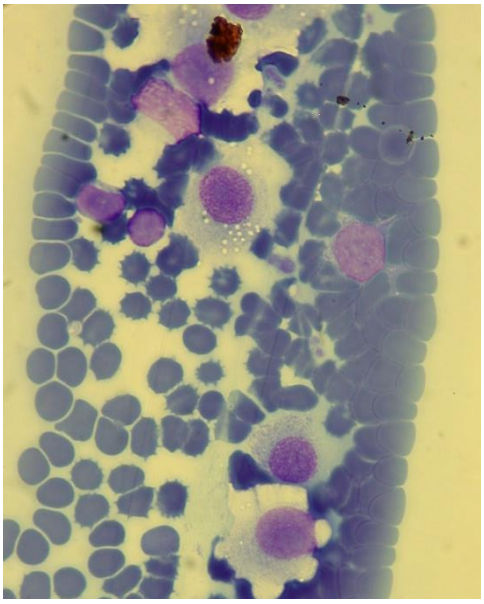


Figure 9: Rocky right inguinal lymph node aspirate

2. Chomi:

Chomi was a 2 year old female intact Pinscher mix from the Mondesa Township. She was spayed and also started on Vincristine treatment.

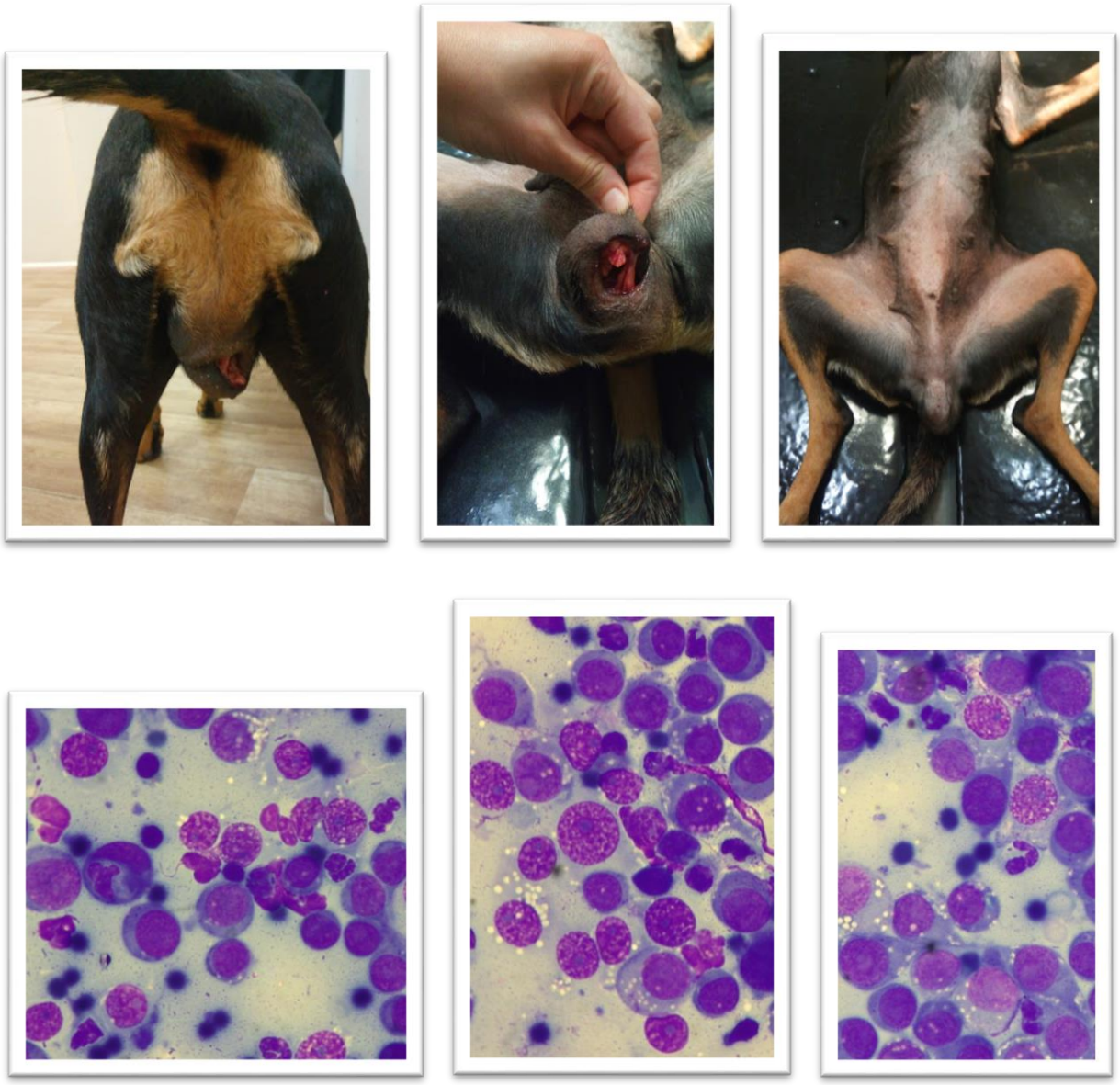


Figure 10: Chomi tumor impression smear

3. Chaka:

Chaka was a 10 year old male intact mixed breed dog from an unknown address. He was brought into the clinic due to neurological symptoms, and the owner was not aware of the tumor. The tumor on the glans was noted during a general physical examination. The neurological symptoms included trouble walking and lack of proprioception in the hind limbs, as well as facial hyperaesthesia. An advanced distemper infection was strongly suspected. The dog's coat showed symptoms of severe mange infestation. Chaka was euthanized at this time, and the relevant samples were taken before the euthanasia.

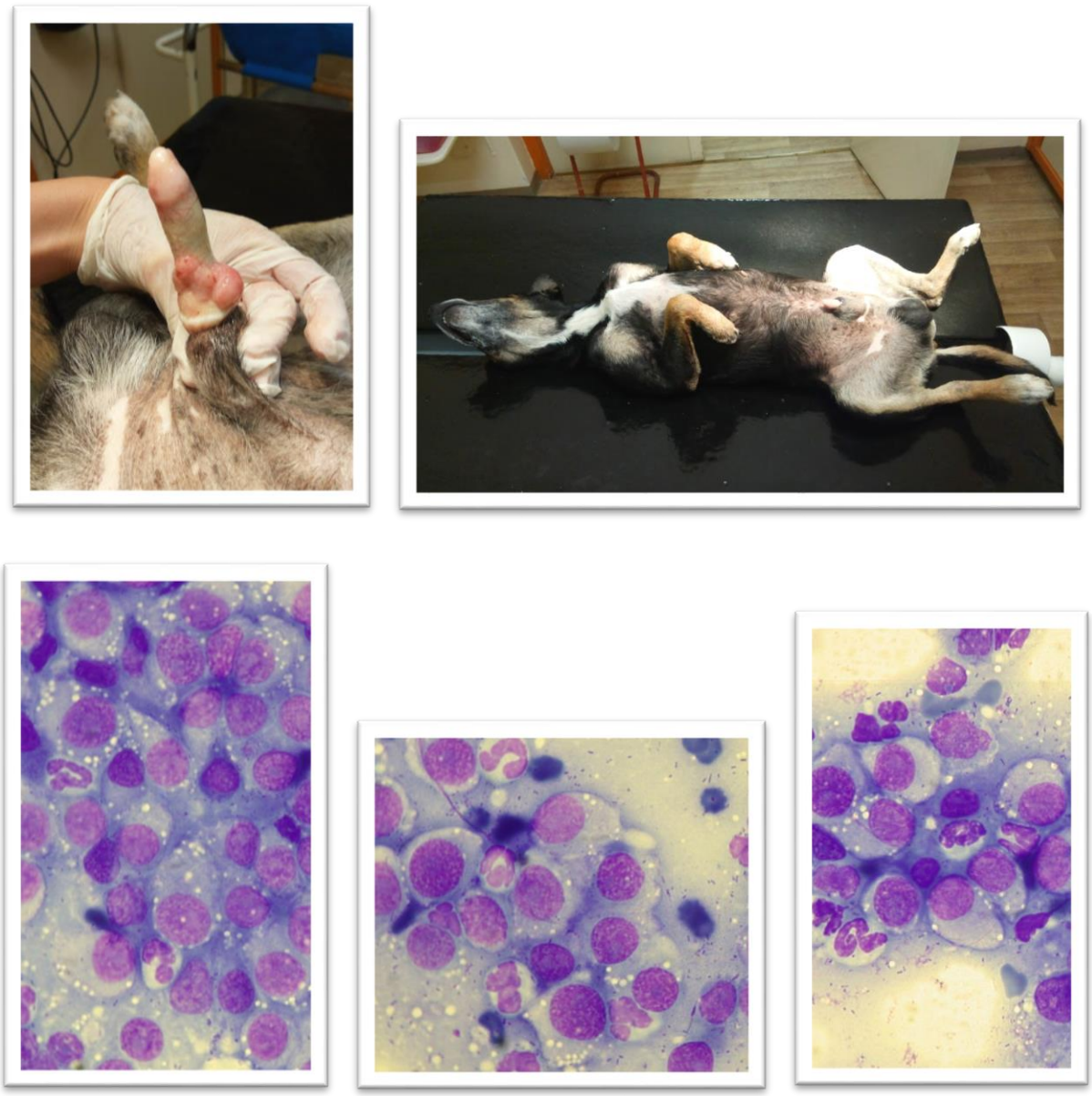


Figure 11: Chaka tumor impression smear

4. Whitey 2:

Whitey was a 1 year old female intact mixed breed dog from the Mondesa Township. The owner brought Whitey in due to the presence of blood on her tail. During the physical examination, the tumor was found easily by externalizing it out of the vulva. Whitey received her first Vincristine treatment at this time, and was spayed.

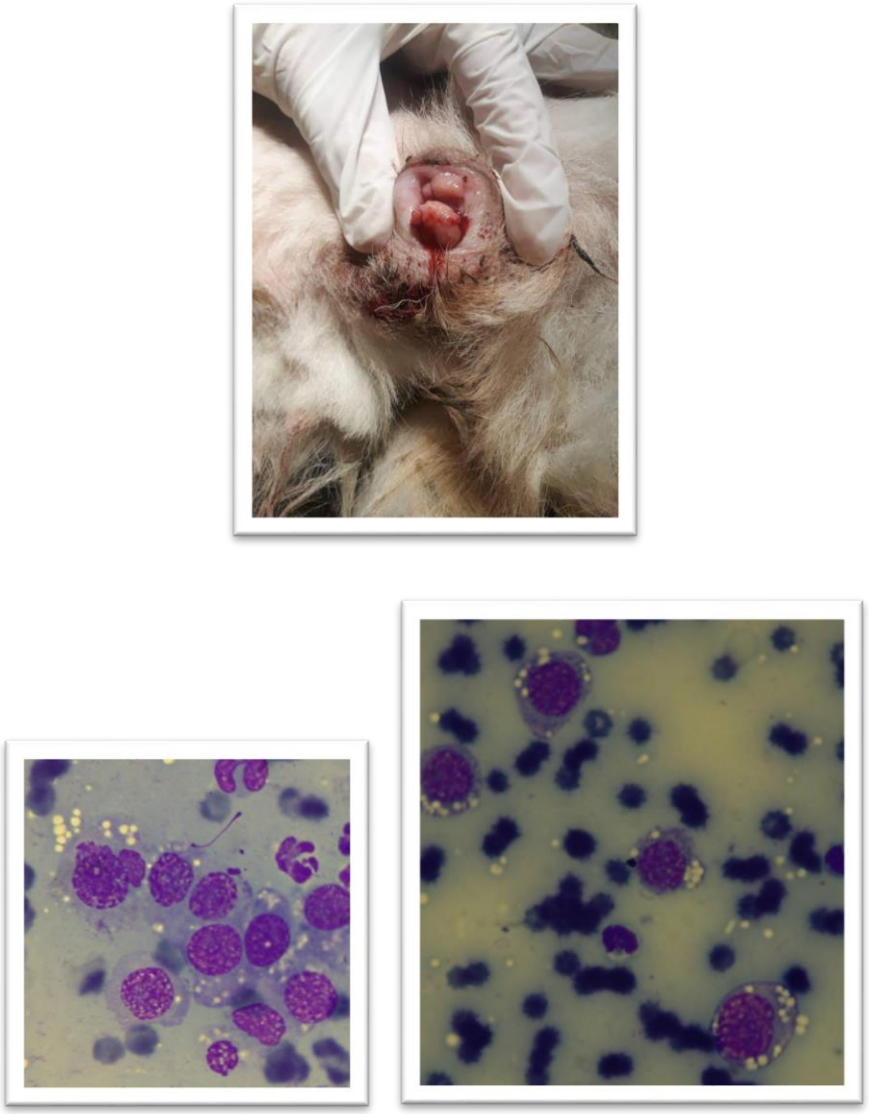


Figure 12: Whitey 2 tumor FNA

5. Bossy:

Bossy was a 4 year old female spayed mixed breed dog from Swakopmund. She was spayed about 6 months prior to the first vet visit concerning CTVT, therefore must have contracted the tumor before that point. The owner noticed vaginal bleeding over the past several months. She was kept with 2 other dogs – one male, one female. The vulva was bleeding during the visit. She was started on IV Vincristine treatment.

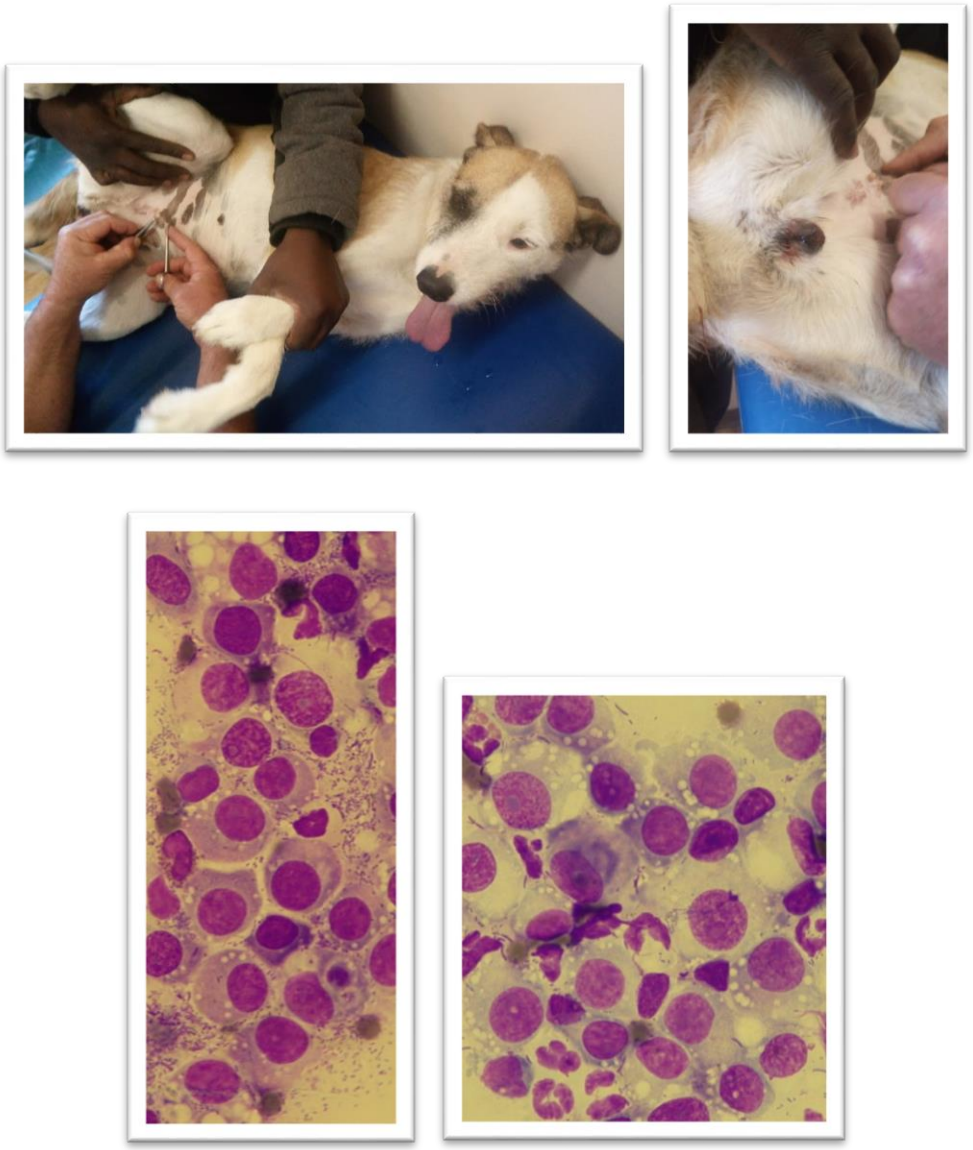


Figure 13: Bossy tumor impression smear

6. Whitey 1:

Whitey was a 5 year old female intact mixed breed dog from the DRC Township. The owner was unaware of the tumor; the appointment was for sterilization. The tumor was protruding out of the vulva. Whitey was spayed at this time, and received her first Vincristine treatment.

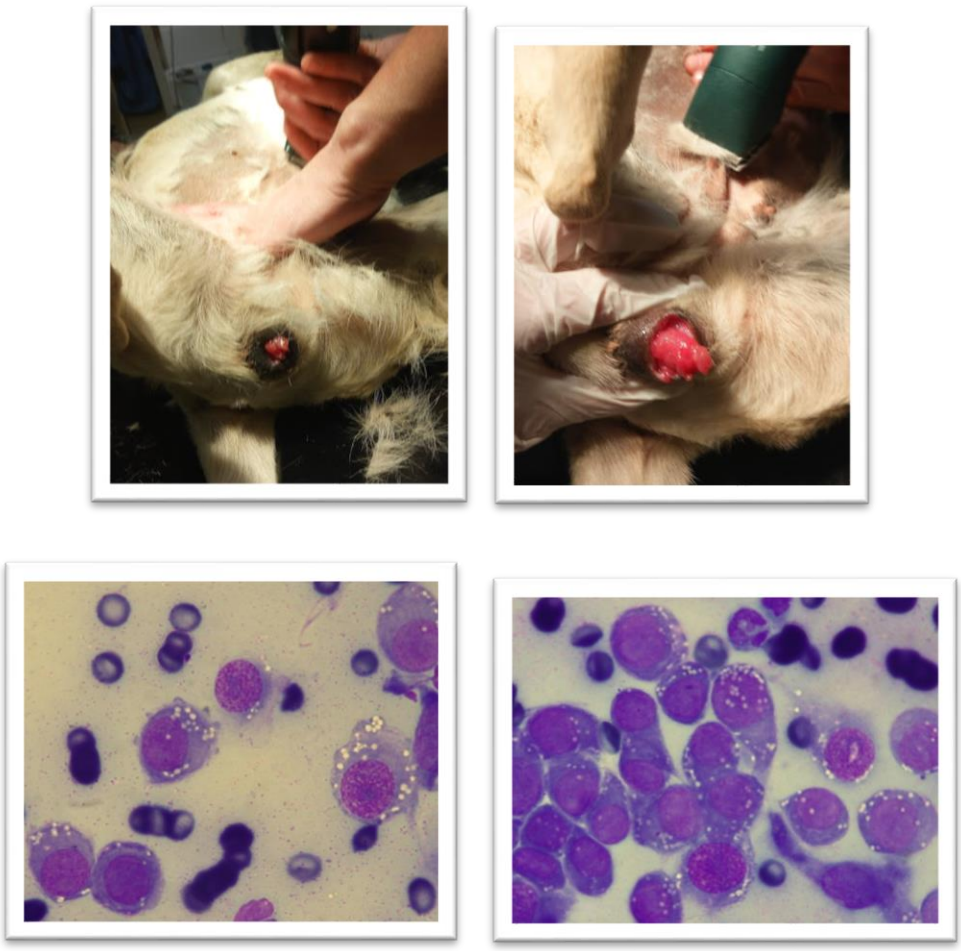


Figure 14: Whitey 1 tumor impression smear

7. Tracy:

Tracy was a 2 year old female intact mixed breed dog from the DRC Township. The tumor was large (>5cm), ulcerated and protruding out of the vulva. Tracy received her Vincristine treatment at this point, and was spayed a few days after the initial appointment.

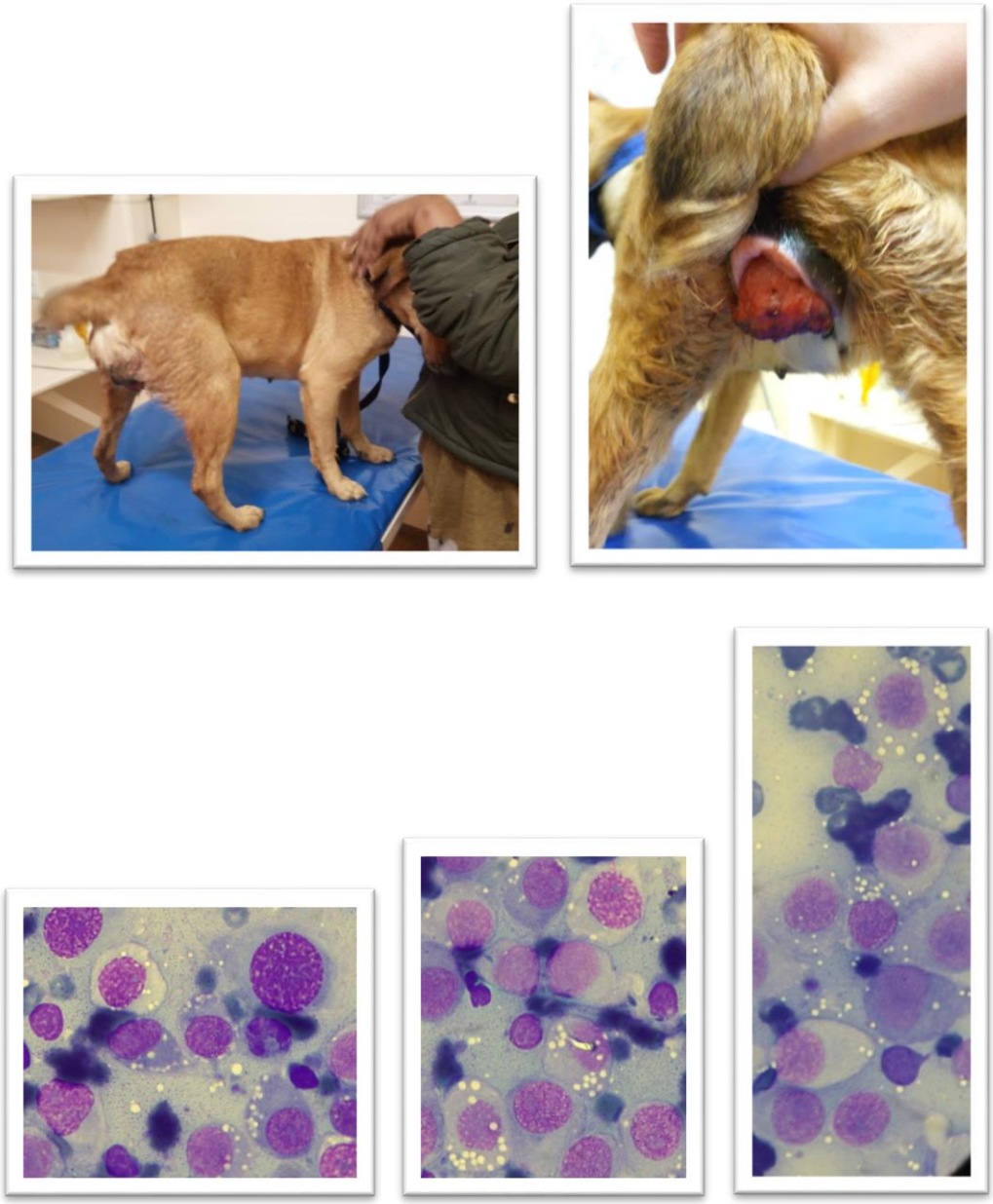


Figure 15: Tracy tumor FNA

8. Leeu:

Leeu was a 5 year old male intact mixed breed dog from the Mondesa Township. The dog presented with severe ascites and enlarged inguinal lymph nodes, and the liver was palpably enlarged. The tumor was large (>5cm) and appeared to be on the right side of the glans, but was difficult to externalize. FNA samples from both inguinal lymph nodes were taken. Samples were also taken from the abdominal fluid, the liver, and the urine. In addition, an abdominal ultrasound confirmed an enlarged liver with abdominal fluid. An X-ray was taken of both chest

and abdomen. The lungs looked clear, and the abdomen was obscured due to fluid. Urinalysis revealed a specific gravity of 1055, which indicates very concentrated urine. This could be due to dehydration, which fit with the clinical picture of the dog. A test strip revealed that protein was also elevated in the urine. The abdominal fluid was shown to contain TVT cells (see Figure 16), as were the lymph nodes aspirates. Leeu was neutered at this time, and received his first Vincristine treatment.

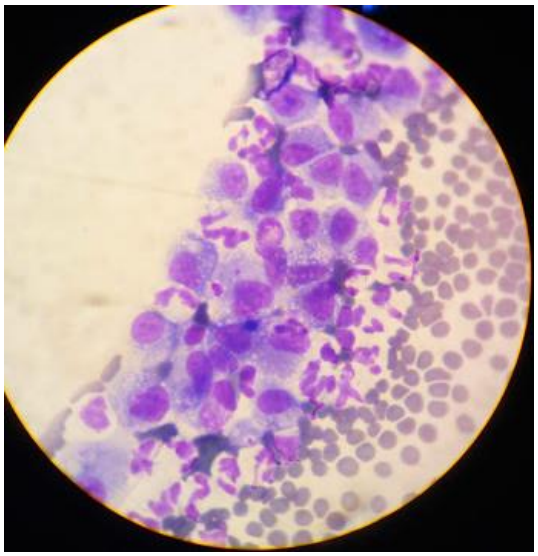


Figure 16: Abdominal fluid microscope picture

Figure 17: Leeu Abdominal fluid

Figure 18: Leeu Urine

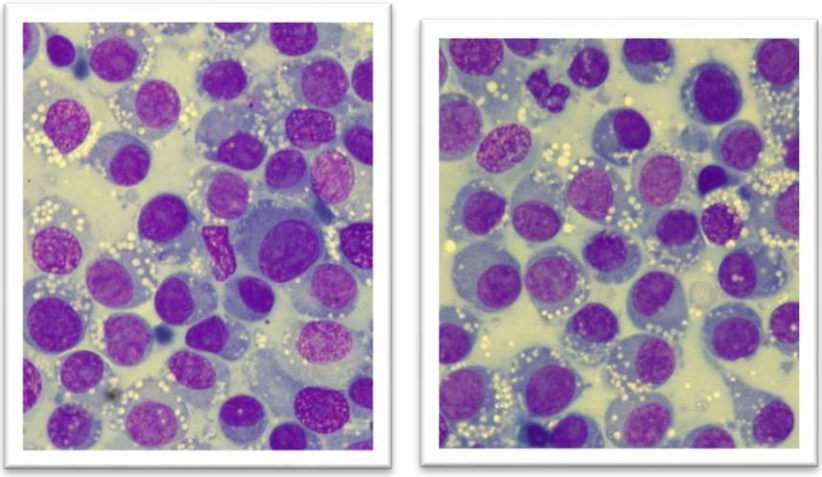


Figure 19: Leeu tumor FNA

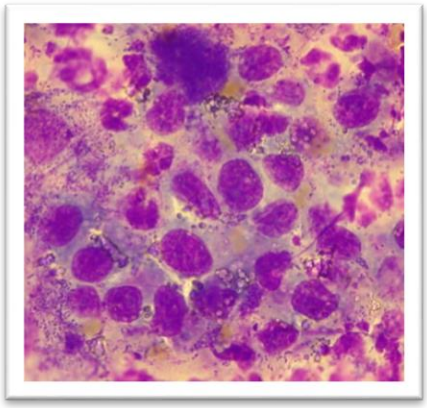


Figure 20: Leeu penis discharge

9. Wagter:

Wagter was a 1 year old male intact German Shepherd mix from a farm in Naibab. He was kept on the farm with an intact female dog. He received his first Vincristine treatment at this time, but the owner did not want to neuter the dog.

Figure 21: Wagter's examination

Figure 22. Wagter's penis lesion

Physical exam and tumor staging

Eight out of the nine dogs were in good general condition, and only two out of nine had symptoms of lethargy. The only one that was not in good general condition had a presumed advanced distemper infection. This shows that even dogs with high CTVT TNM stages do not necessarily show changes in general condition.

One of the nine dogs had evidence of organ metastasis. This number suggests a higher incidence of organ metastasis than found in previous literature (~5% of cases), but the total number of cases in this study (9) is too small to draw distinct conclusions concerning the incidence of metastasis.

Five of the nine cases had evidence of bacterial colonisation at the tumor site. As many of these dogs were free roaming in Namibian townships, it is not surprising that the genitals would have bacterial colonisation, even without a cancerous lesion.

Individual clinical data of the cases is summarized in Table 2.

Table 2: Clinical data of the dogs

	Age	Weight (kg)	Height (cm)	BCS (1-9)	Good general condition	Lethargic	TNM staging	Tumor substage (a=0, b=1)	Lymph node metastasis	Organ metastasis	Bacterial colonization
Rocky	2	23.5	55	4	1	0	2	0	1	0	0
Chomi	1	12.5	43	3	1	0	3	0	0	0	1
Chaka	10	17	35	4	0	1	3	1	1	0	1
Whitey 2	1	16	50	4	1	0	2	0	0	0	0
Bossy	4	17.8	40	5	1	0	1	0	0	0	1
Whitey 1	5	17	45	4	1	0	2	0	0	0	1
Tracy	2	16.5	43	4	1	0	3	0	0	0	0
Leeu	5	17.3	57	5	1	1	3	1	1	1	1
Wagter	1	25	60	4	1	0	3	0	0	0	_

The results of the laboratory analyses are summarized in Table 3, 4, 5, and 6.

Haematology

Table 3: WBC results derived by haematology analyzer

Patient	WBC total (10^9/L)	Lymphocytes (10^9/L)	Monocytes (10^9/L)	Granulocytes (10^9/L)	Lymphocytes %	Monocytes %	Granulocytes %
Rocky	5.4	1.9	0.8	2.7	36.7	13.3	50
Chomi	10.7	2.9	1.8	6	27.3	17.2	55.5
Chaka	5.6	0.6	0.7	4.3	10.9	12.8	76.3
Whitey 2	18.4	1.1	1.4	15.9	6.5	6.9	86.6
Bossy	15.3	6.4	1.4	7.5	41.9	9.3	48.8
Whitey 1	9.4	3.9	1.2	4.3	41.4	13.2	45.4
Tracy	21.0	2.5	0.8	17.7	11.8	4.1	84.1
Leeu	6.1	0.7	0.3	5.1	11.4	4.7	83.9
Wagter	10.7	2.9	1.8	6	27.3	17.2	55.5
Mean	11.4	2.5	1.1	7.7	23.9	10.9	65.1
SD	5.4	1.7	0.5	5.0	13.3	4.7	16.2
Normal range	6 – 17	0.9 – 5	0.3 – 1.5	3.5 – 12	17 – 29	0 – 5.4	52 – 77

Table 4: RBC results derived by haematology analyzer

Patient	HCT (L/L)	MCV (fL)	RDWa (fL)	RDW %	HGB (g/L)	MCHC (g/L)	MCH (pg)	RBC (10^{12}/L)	PLT (10^9/L)	MPV (fL)
Rocky	0.29	61	40.2	15.6	118	409	24.9	4.75	54	8.2
Chomi	0.161	56.8	36.1	15.6	65	403	22.9	2.84	170	7.8
Chaka	0.363	61.4	42.5	16.7	141	387	23.8	5.92	84	7.7
Whitey 2	0.165	54.2	34.7	16.4	67	407	22.1	3.05	101	9.1
Bossy	0.333	59.9	39.6	16	142	426	25.5	5.55	54	8.2
Whitey 1	0.182	60.5	36.8	14.3	79	436	26.4	3.01	66	8.2
Tracy	0.142	44.1	32.8	21.8	59	416	18.3	3.21	37	
Leeu	0.285	51	35.2	18.1	112	395	20.1	5.58	29	
Wagter	0.298	57.7	39.1	16.8	121	406	23.5	5.16	154	8.9
Mean	0.2	56.3	37.4	16.8	100.4	409.4	23.1	4.3	83.2	8.3
SD	0.1	5.4	3.0	2.0	31.2	14.1	2.4	1.2	46.9	0.5
Normal range	0.37 – 0.55	60 – 72	35 – 36	12 – 17.5	120 – 180	320 – 385	19.5 – 25.5	5.5 – 8.5	200 – 500	5.5 – 10.5

Blood smear analysis

Table 5: Results of the microscopic blood smear analysis

Patient	WBC	% Band Ne	% Eo-gran	% Lympho-blasts	% Mono	% Seg-Ne	% Small Ly	No/OIF Poly-chrom. RBC	Abs Band Ne	Abs Eo-gran	Abs Lympho-blasts	Abs Mono	Abs Seg-Ne
Rocky	3.10	0.00	0.00	17.02	2.13	59.57	21.28	21.06	0.00	0.00	0.53	0.07	1.85
Chomi	5.20	0.00	2.00	6.00	2.00	66.00	24.00	21.06	0.00	0.10	0.31	0.10	3.43
Chaka	18.30	0.00	8.77	3.51	7.02	71.93	8.77	22.55	0.00	1.60	0.64	1.28	13.16
Whitey 2	4.80	0.00	0.00	4.55	4.55	84.09	6.81	22.14	0.00	0.00	0.22	0.22	4.04
Bossy	8.20	0.00	4.48	5.97	0.00	34.33	55.22	21.60	0.00	0.37	0.49	0.00	2.82
Whitey 1	7.40	0.00	4.00	6.00	6.00	48.00	36.00	19.31	0.00	0.30	0.44	0.44	3.55
Tracy	0.20	0.00	0.00	0.00	0.00	83.33	16.67	29.43	0.00	0.00	0.00	0.00	0.17
Leeu	1.40	3.03	0.00	12.12	3.03	63.64	18.18	24.44	0.04	0.00	0.17	0.04	0.89
Wagter	6.90	1.61	6.45	8.06	6.45	66.14	11.29	23.50	0.11	0.45	0.56	0.45	4.56
Mean	6.17	0.52	2.86	7.03	3.46	64.11	22.02	22.79	0.02	0.31	0.37	0.29	3.83
SD	4.99	1.02	3.08	4.69	2.52	14.92	14.41	2.74	0.04	0.49	0.20	0.39	3.57

Tumour lesion cytology smear analysis

Table 6: Cell analysis of the tumour smears

	Cell/ view	Ne %	Ly %	Mon %	Eo %	Nuclear area x100 µm2	Squam. %.	Mitotic figures %	Ne Abs	Ly Abs	Mon Abs	Eo Abs	Squam. Abs.	Mito Abs
Rocky	159.67	89.65	1.67	0.32	0.00	9.42	4.18	4.18	143.14	2.67	0.51	0.00	6.67	6.67
Chomi	41.00	76.00	21.00	2.00	0.00	7.87	0.00	1.00	31.16	8.61	0.82	0.00	0.00	0.41
Chaka	66.70	90.70	4.95	1.20	0.00	4.96	2.40	0.75	60.50	3.30	0.80	0.00	1.60	0.50
Whitey 2	1.60	56.71	5.97	28.36	0.00	4.96	0.00	8.96	0.90	0.10	0.45	0.00	0.00	0.14
Bossy	8.33	82.00	7.60	10.40	0.00	7.53	0.00	0.00	6.83	0.63	0.87	0.00	0.00	0.00
Whitey 1	73.50	62.55	35.88	0.21	0.00	8.59	0.00	1.36	45.97	26.37	0.15	0.00	0.00	1.00
Tracy	41.50	66.97	18.07	14.16	0.00	10.35	0.00	0.80	27.79	7.50	5.88	0.00	0.00	0.33
Leeu	5.23	1.90	93.08	1.90	0.64	7.64	0.00	2.48	0.10	4.87	0.10	0.03	0.00	0.13
Average	**49.7**	**65.8**	**23.5**	**7.3**	**0.1**	**7.7**	**0.8**	**2.4**	**39.6**	**6.8**	**1.2**	**0.0**	**1.0**	**1.1**
SD	±48.85	±26.77	±28.31	±9.31	±0.21	±1.8	±1.49	±2.74	±44.07	±7.93	±1.79	±0.01	±2.20	±2.11

The average of views was 17.4 (±13.50), and the average of cells recorded was 358.4 (±198.23). The average cell number per view (cell density) was 49.7 (±48.85). The average TVT cells /view was 12.33 (±5.47).

There was difference in nuclear area among the dogs. Some were statistically also different from each other (Figure 23, Table 7 and 8).

Figure 23: Result of the nuclear area measurements in different dogs with TVT

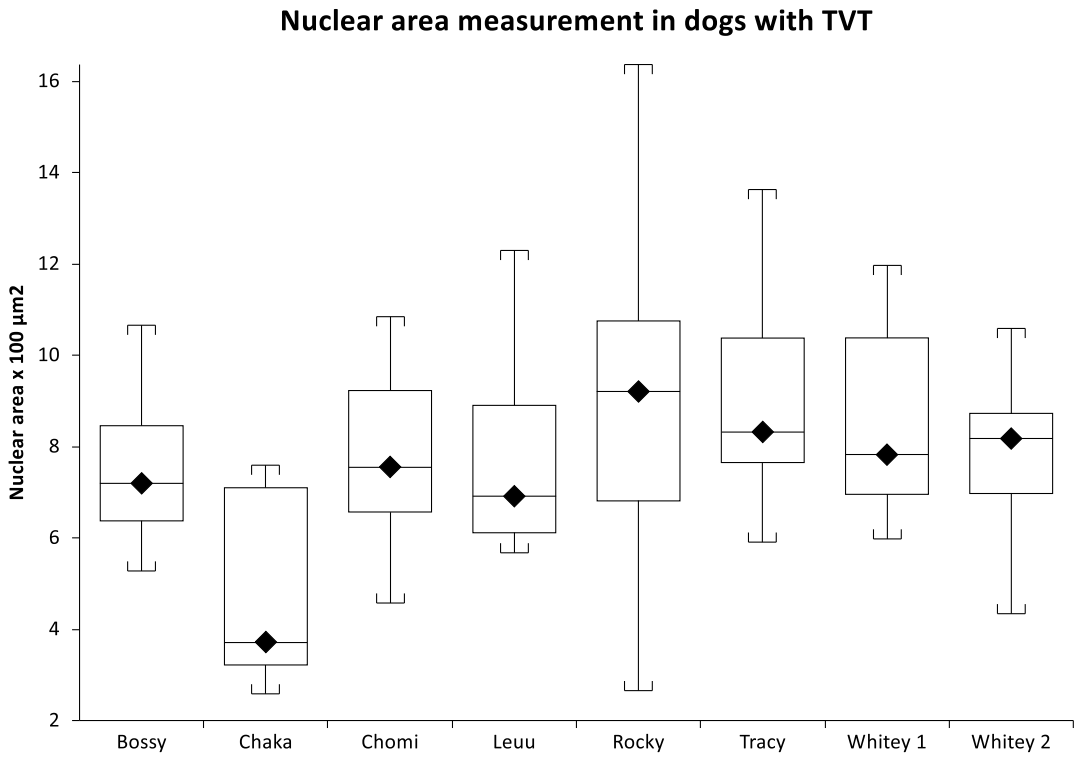


Table 7: Result of the nuclear area measurements in different dogs with TVT

	Bossy	Chaka	Chomi	Leuu	Rocky	Tracy	Whitey 1	Whitey 2
Mean	7.53	4.96	7.87	7.64	9.42	10.35	8.59	8.64
SD	±1.48	±1.99	±1.75	±1.94	±3.03	±6.65	±2.03	±2.79

Significant difference in nuclear size of TVT cells was found among the different dogs.

Table 8: Statistically significant results of the nuclear area measurements in different dogs with TVT

Chomi vs. Rocky	p= 0.042
Leeu vs. Rocky	p= 0.0184
Leeu vs Tracy	p= 0.0598 (two sided) and 0.0299 (low sided)
Chaka vs. Whitey 1	p= 0.0042
Chaka vs. Whitey 2	p= 0.0074

Mann-Whitney U test (non-parametric)

Nuclear area of TVT nuclei is inversely correlated significantly with the absolute count of segmented neutrophil granulocytes (r= -0.7217, p= 0.0283) in the blood and almost significantly with white blood cell count (r= -0.655, p=0.056), relative and absolute monocyte count (r=-0.620, p=0.075; r=-0.6001, p=0.0876, respectively).

Absolute count of mitoses of TVT cells in smears correlated significantly with relative count of lymphoblasts in the blood (r=0.7491, p=0.0201), cell density of the tumour smears (r=0.9070, p=0.0007), the neutrophil granulocyte absolute count (r=0.9286, p=0.0003), the squamous cell absolute and relative counts of the tumour smears (r=0.855, p= 0.00325; r=0.9681, p= 0.000019, respectively).

Although white blood cell count analyzed by automatic cell counter or estimated by microscopic analysis of the blood cells did not show good correlation at all (r=-0.3635, p= 0.337), there was a good correlation between lymphocyte absolute and relative count (r=0.8426, p= 0.0043; r=0.8413, p= 0.004, respectively) and the neutrophil granulocyte absolute count (r= 0.813, p= 0.0078).

Discussion

In spite of the advanced state of these dogs with CTVT (especially Leeu), only one out of the nine dogs was euthanized. Chaka was euthanized due to a suspected advanced distemper infection presenting with distinct neurological symptoms, and not due to the CTVT infection. All the dogs except for Chaka received vincristine at the appropriate dose (0.025mg/10 kg). All dogs were anaemic and only two showed leukocytosis.

All dogs in this study had HCT values below the reference range, and MCHC values above the reference range. This suggests chronic anaemia, which may have been caused by a combination of factors including the tumor itself (often ulcerated and bleeding), concurrent *Ehrlichia canis* infection (which was very common in that area), and blood-sucking parasites such as fleas and ticks. Although, we did not find the parasites in blood smears of the dogs.

Six out of the nine dogs had low RBC count, low MCV, low HGB and a high RDWa, which again suggests a chronic anaemia.

Blood loss as a cause of anaemia is highly suggestive due to the fact that the dogs were microcytic (mean: 56.3 fL, ±5.4) and the platelet counts were also low (mean: $83.2x10^9$/L, ±46.9). Anaemia was observed as a clinical sign in several other studies (Aprea et al., 1994; Valladão et al., 2010; Antonov, 2015). The regenerative type is suggested as the percentage of the polychromatophilic red blood cells is slightly higher than 20 / immersion oil field (mean: 22.79, ±2.74). Marked anemia is not common according to the case reviews. One of them reports that the CBC revealed a mild non-regenerative anemia [hematocrit (HCT) 34%; reference interval (RI): 37% to 55%, reticulocyte count 1.5%)] and marked thrombocytopenia (36 × 109/L; RI: 175 to 500 × 109/L) (Milo and Snead, 2015). Although, considerable hemorrhagic vulvar discharge may occur and can cause anemia if it persists (Martins et al., 2005), contributing factors such as parasitic infestation are considered in our cases.

We have elaborated the nuclear area measurement by image analysis and stated the average area of TVT-cell (mean: 7.7, ±1.8). It seems to be interesting that some dogs have significant alteration from each other's nuclear size.

Nuclear area which can be a marker of cell proliferation might be supressed by the inflammatory cells (neutrophils and monocytes). We assumed it by the fact that nuclear area is

inversely correlating with the absolute count of segmented neutrophil granulocytes in the blood and almost significantly with white blood cell count, relative and absolute monocyte count. This suggests a possible defence effect on tumour cell multiplication by the inflammatory process.

The proliferation of TVT cells is expressed by the number of mitotic figures. The absolute count of mitoses of TVT cells in smears is correlating with relative count of lymphoblast in the blood. This can be because proliferative (mitotic) TVT cells might activate the lymphoid subsets in the blood which try to fight against the neoplasm. The correlation of mitotic TVT cells with cell density of the tumour smears, the neutrophil granulocyte absolute count and the squamous cell absolute and relative counts can be explained by the simple situation that the higher number of different cells within one smear includes higher number of mitotic figures.

Despite other findings (Jones KW, 2009), we realized that in our cases the estimated WBC did not correlate well with the automatic cell counting, although the individual cell percentages and absolute counts showed reasonably good correlation. The discrepancy of this can be the cellular inhomogeneity of the blood smears, and the low number of nucleated cells within the smears.

Summary

Apart from the detailed description of the nine dog cases with TVT the major novelty of this study is the nuclear area measurement and its negative correlation with the inflammatory cells in the blood, moreover the correlation of the mitotic figures with the large lymphocytes in the blood. Furthermore, we found that our cases were markedly more anaemic than the dogs in other studies, although the low platelet count was corresponding.

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