UPDATE ON RADIOTHERAPY

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Introduction

Radiotherapy has developed to an important treatment modality in the field of veterinary oncology and therefore a basic understanding how this therapy can be integrated into the management of cancer patients is very important.

In small animal oncology radiotherapy is typically used as a local treatment modality either in cases that cannot be managed by surgery alone or that are inoperable. The aim of radiotherapy is to deliver a high tumour dose while sparing normal tissue as much as possible. Sparing of normal tissue can be achieved by modern equipment and optimal treatment protocols. Modern radiotherapy uses linear accelerators with computerized planning. Here conformal 3-D, IMRT (intensity-modulated radiotherapy) or even VMAT (volumetric-intensity modulated arc therapy) are available.

Radiation preferentially kills dividing cells exponentially. Due to the exponential cell killing, tumour size (= number of tumour cells) plays an important role for treatment outcome. Generally, the smaller the tumour the more likely a long term control can be achieved. Therefore radiotherapy is commonly combined with a surgical intervention.

Further, a combination with chemotherapy is always indicated in tumours with a high metastatic potential, as radiotherapy is not a systemic treatment. Radiotherapy in these cases helps to achieve local tumour control (often including the regional lymph node) while chemotherapy is aimed to delay/prevent systemic metastasis.

Radiotherapy can be used with a curative or a palliative treatment intend. Curative treatment is aimed to gain a long term tumour control, whereas palliative therapy is aimed to improve quality of life (pain relief, decrease of inflammation) and to achieve temporary tumour control. Palliative treatment is indicated in cases with advanced disease where a curative therapy is unlikely to achieve long term control.
Combination with surgical resection

Tumours that cannot be excised with the required surgical margins are indications for a curative intent radiotherapy. Waiting on a recurrent disease before recommending radiotherapy will decrease the chance of long term control and in some tumour types increase the risk for progression into a more aggressive tumour grade.

If radiotherapy is combined with surgery, a treatment plan should be discussed before to decide whether pre- or postoperative radiotherapy is indicated. Good communication helps with understanding the limitations of the other side (e.g. “wrong” orientation of the scar or very long scars might be a limitation for safe radiotherapy).

Radiotherapy can be provided pre- or postoperatively. Indications for a combined intervention are:

- Sufficient surgical margins cannot be achieved due to the location of the tumour (e.g. head-and neck and extremity)
- The pathology report identifies tumor cells at the surgical margin.
- Owner declines aggressive surgery (e.g. Amputation, mandibulectomy or maxillectomy) and prefers less aggressive resection in combination with radiotherapy.

Radiotherapy alone

Radiotherapy without surgery is indicated in cases where surgery can only be performed with a high risk of complications or where surgery is not amendable at all. In these cases curative intend radiotherapy is only indicated if the tumour is radiosensitive enough to achieve efficient control despite radiating larger tumour burdens. Otherwise, palliative radiotherapy would be recommended. Typical examples are brain tumours or fixed thyroid tumours.

Side effects

Prior the start of radiotherapy an informative discussion with the patient owner about potential side effects is important. Generally, acute and chronic side effects can occur with radiation therapy.
Acute side effects are commonly observed with curative protocols. Acute side effects are typically observed in dividing normal tissues such as mucosa and skin. They occur middle to end of therapy and can range from mild to severe mucositis and dermatitis. Acute side effects heal within 2-4 weeks and are typically not dose limiting. Supportive therapy depends on the severity of side effects and may include medications against itching and pain or antibiotics. After resolving of the acute reactions hairs in the radiated regions can regrow white and some degree of alopecia can occur.

Chronic side effects are seen in non-dividing tissues and can occur months to years after radiotherapy. In contrast to acute side effects they will not resolve and are dose limiting. The risk of chronic side effects increases with higher daily fractions and is the reason that curative protocols are split into many small single fractions. In bone tissue there is some risk to induce an osteosarcoma in the radiation field (< 3%) years after therapy. Examples for tissues where chronic side effects can occur are the ocular lens, the central nervous system, bone and muscle tissue, lungs, kidney or liver. A typical chronic side effect that can occur when radiating nasal tumors are a cataract or KCS.

Treatment planning
To avoid and reduce the risk of side effects, computerized planning is required in regions with complex anatomy (e.g. the head and neck region).

A so called planning CT scan is a CT-scan with the patient placed in the same position as under radiotherapy. Thus, a table top insert is used to achieve a flat table top comparable to the radiation treatment couch. Further, positioning devices for daily radiotherapy are used for the planning CT scan. Typical positioning devices are vacuum cushions which can be shaped individually for each patient. The CT images are transferred to a treatment planning computer and here a computerized 3D-plan can be established (or IMRT or VMAT plan).

Tumor entities commonly treated with radiotherapy
Radiotherapy can be used for a variety of different malignancies. As mentioned before, especially tumors in regions where aggressive surgery is difficult are commonly treated with
radiation. Therefore head-and neck tumors are frequently managed with a multimodality treatment approach.

In our first radiation cohort treated between 2006-2008 tumors from the skin and subcutaneous tissue were treated most frequently, followed by brain tumors and tumors of the oral cavity. Looking at location, in our cohort more than 50% of all radiated neoplasia belonged to head-and neck tumors.

**Examples**

*Tumors of the Skin and subcutaneous tissue*
- Soft tissue sarcomas including FISS (feline injection site sarcomas)
- Mast cell tumors (dog more important)

*Brain tumors*
- Meningioma, Glioma and others
- Pituitary macroadenoma

*Tumors of the oral cavity*
- Oral malignant melanoma
- Squamous cell carcinoma
- Oral soft tissue sarcomas

*Nasal tumors*
- Adenocarcinomas, squamous cell carcinoma, undiff. carcinoma
- Sarcomas, including chondrosarcoma, osteosarcoma and soft tissue Sa

*Thyroid carcinomas*

**Skin tumours treated with radiotherapy**

*Mast cell tumours*
Canine grade I mast cell tumours (Patnaik grading system) or low-grade MCTs (Kiupel grading system) frequently can be managed by surgical resection alone and have a low risk for recurrence or metastasis. However, grade II-III mast cell tumours (Patnaik grading) or high-grade tumours (Kiupel grading) can be more difficult to manage locally with surgery alone due to their more infiltrative behaviour.

Older literature reports control rates for grade II mast cell tumours of about 95% and 3-5 year control rates of about 85-93% with postoperative radiotherapy after incomplete resections. However, newer literature also describes relative low recurrence rates in grade II MCTs after marginal excisions and thus opens debate when to recommend adjuvant radiotherapy. Mitotic index or the Kiupel grading system can offer additional information about the biologic aggressiveness of grade II MCTs. Local grade III MCT or grade II MCTS in locations described with higher risk of metastasis (such as the muzzle, or inguinal or perineal region) can be managed with a combination of curative postoperative radiotherapy and additionally medical treatment (chemotherapy or tyrosine kinase inhibitors). A study in 31 dogs with grade III MCTs treated only with surgery and radiotherapy without adjuvant medical therapy achieved a progression free interval of 28 months.

Further, good outcome can also be obtained with hypofractionated radiotherapy (4 x 8 Gy) in inoperable MCTs treated with prednisone prior the start of RT to avoid degranulation. These patients showed a remission rate of 88.5% and a median progression free interval of 34 months.

**Soft tissue sarcomas**

Soft tissue sarcomas are a diverse group of tumours that frequently require a combination therapy of surgical resection and radiotherapy.

Especially, feline injection-site sarcomas have a high risk of local recurrence and require aggressive surgery and in cases of incomplete or close surgical margins adjuvant radiotherapy. In large tumours, preoperative radiotherapy might be required. Survival rates reported in the literature vary between 23-43 months in cats treated either with pre- or postoperative radiotherapy.
In dogs, soft tissue sarcomas that cannot be resected with clean margins can be treated with postoperative radiotherapy. The literature reports a median survival time of more than six years in non-oral sites. However, low-grade tumours are frequently managed by surgery alone. The recurrence risk and also metastatic potential increases in intermediate and high-grade tumours. Also hypofractionated radiotherapy (4 x 8-9 Gy) combined with intentional marginal resection can achieve long lasting tumour control in canine limb STS. In this study median disease free interval was not reached, despite only 33% of all tumours being low-grade STSs.

**Localized histiocytic sarcoma**

In dogs with periarticular histiocytic sarcoma (PAHS) as well as localized non-periarticular histiocytic sarcoma radiotherapy can be offered to control local disease. Histiocytic sarcomas are more radiosensitive than soft tissue sarcomas, but treatment decisions always must be carefully made in awareness of their high metastatic risk and systemic medical therapy is always recommended in localized histiocytic tumours. PAHS seem to have a more favourable outcome and if no metastatic disease is detected at the time of diagnosis long lasting tumour controls can be observed despite the overall guarded prognosis is this tumour entity.

**Tumors of the Oral Cavity treated with radiotherapy**

**Oral Melanoma (OMM)**

OMM is the most common oral tumor in dogs and can arise from the gingiva, lip, tongue, and hard palate. OMM is generally an aggressive and highly metastatic disease and has frequently spread locally to the regional lymphnodes at the time of diagnosis (approx. 50%). For a relative long time OMM was considered as radioresistant because this tumor requires a different fractionation scheme than many other tumors. With a more hypofractionated RT scheme using a higher dose per fraction response rates (CR and PR) are good and reach about 85%. Overall median survival times (MST) with radiotherapy are about 8 months. Without risk factors the MST is reported to be 21 months. Thus, careful staging is essential for appropriate treatment recommendations.
**Oral Squamous cell carcinoma (SCC)**

SCC is the second most common oral neoplasia in the dog. Buccal SCC arising in the gums has to be distinguished from the more aggressive tonsillar and lingual SCC. RT is indicated in cases where clean surgical margins cannot be achieved or those not amendable for surgery at all. Survival and control times are dependent on clinical stage and MST vary between 15-36 months.

In cats SCC is the most common tumor in older cats. Unfortunately, treatment success is unrewarding in this disease and most cats fail locally with recurrent disease. Survival rates from less than 6 months are commonly achieved and therefore only palliative radiotherapy is typically recommended.

**Oral FSA**

Canine FSA is the 3rd most common oral tumor in dogs. Mandible, maxilla and hard palate are classic tumor locations and bone invasion is also common. Overall metastatic rate ranges are higher than in SCC but lower than in OMM. Radiotherapy is best used as a combination approach with surgery but can be used as single modality in some cases. MST of about 12-26 months is reported and clinical stage is predictive for control times.

In cats oral FSA is the second most common oral neoplasia in the cat and surgery alone, surgery with radiotherapy or radiotherapy alone can be used. Progression free intervals of about 12-24 months are reported.
POSSIBILITIES FOR CANCER IMMUNOTHERAPY IN COMPANION ANIMALS

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**Introduction.** Activating the immune system for therapeutic benefit in cancer has long been a goal in immunology and oncology. A better understanding of molecular and cellular mechanisms governing the immune system formed the basis for development of a number of innovative and promising cancer therapies modulating non-specific and specific anti-cancer responses. Non-specific tumor killing is mediated by the innate immune system and is independent of T-cells, whereas tumor specific immunity is mediated primarily by both T and B cells. Both passive and active modalities have been used to generate therapeutic anti-tumor immune response. Passive immunotherapy involves the transfer of biological reagents, such as monoclonal antibodies or antigen-specific adaptive immune cells. Active immunotherapy tries to generate anti-tumor response of patient’s own immune system, typically through vaccination. It is becoming evident that the different strategies efficacy of immunotherapy are limited by multiple mechanisms of tumor –induced immunosuppression and active escape from anti-tumor attack.

**Non-specific immunotherapy.** Non-specific immunostimulators and immunomodulators have not found wide approval in routine clinical practice. Use of live attenuated _Mycobacterium bovis_ (strain Bacillus Calmette-Guerin; BCG) was conducted in dogs with osteosarcoma and significant increases in survival times were observed probably caused by activation of macrophage tumoricidal activity. Analog of Mycobacterial cell walls component
muramyl tripeptide phosphatidylethanolamine (MTP-PE) has been investigated in many tumors in human and veterinary patients, including osteosarcoma, hemangiosarcoma, and mammary carcinoma. The use of immunomodulator imiquimod in cats with multicentric squamous cell carcinoma in situ has been reported [Gill 2008]. The innate immune system can be activated by administration of cytokines, including IL-2, IL-12, IFN-γ, IFN-α, and TNF-α. While interferon-α has been used for immunotherapy of cancer in dogs and in cats, randomized clinical trials have not yet been conducted. IL-2 was investigated in dogs as a means of activating spontaneous NK cell activity. Inhalational administration of human IL-2 has also been shown to generate significant antitumor activity in dogs with lung metastases []. Gene therapy using recombinant canarypox virus expressing feline interleukin 2 (Oncept IL-2, Merial) has been examined for treatment of feline fibrosarcoma as adjuvant setting after surgery and simultaneously with radiotherapy. The treatment consisted of six consecutive doses administered subcutaneously at was administered subcutaneously around the tumor excision site. Treatment was well tolerated and resulted in a significant longer median time to relapse than in the reference treatment group (>730 v. 287 days), and a significant reduction of the risk of relapse by 56% at one year and 65% at two years [].

**Cancer vaccines.** Therapeutic cancer vaccines utilize a variety of approaches to active immune system, including the injection of whole cell or tumor cell lysates, peptide antigens, plasmid DNA, or activated immune cells primed with tumor antigens. The success of a cancer vaccine relies on its ability to overcome T cell tolerance to these antigens in order to induce a robust, durable immune response. The first therapeutic cancer vaccine licensed for use in canine oral melanoma was the xenogeneic DNA vaccine, Oncept (Merial). It contains a plasmid cDNA insert encoding human tyrosinase and breaking tolerance for canine
tyrosinase. The results of survival analysis for the outcome of death due to canine melanoma showed that there was a significant group difference with the vaccine group showing better survival [Bergman 2003, Liao 2006]. However, a more recent study found no improvement in survival times in Oncept vaccinated dogs [Ottnod 2013]. Thus, it is unclear at present exactly how effective the Oncept vaccine is in immunizing dogs against melanoma. Next cancer vaccine strategy is ex vivo production of dendritic cells which can generate strong T cell responses against cancer in patient [danull 2013].

In human oncology adoptive transfer of modified T cells is becoming more widely used in some cancers.

**Monoclonal antibodies.** Exciting results have been achieved in human cancer patient using engineered monoclonal antibodies that can directly bind to malignant cells and antagonize oncogenic pathways, act to block growth-promoting pathways in the tumor stroma (VEGF), and targeting T cell checkpoint molecules as PD-1 and CTLA-4. Recently monoclonal canine anti-CD20 and anti-CD52 antibody has been evaluated for lymphoma patients with very promising results.

**Conclusions.** It seems evident that immunotherapy will be the forth arm of the veterinary oncology, supporting surgery, radiotherapy and chemotherapy.

**References:**


METRONOMIC CHEMOTHERAPY

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Introduction
The conventional chemotherapy is based on administration of maximal tolerated dose (MTD) of cytostatic agents. Chemosensitive tumor usually contains a population of rapid dividing cells which compared to healthy cells are more sensitive to chemotherapy. Even so cells of nontumorous tissues are also affected (bone marrow, gastrointestinal epithelial cells) which is a reason to pulse application of MTD chemotherapy in different time intervals. The absence of those essentials breaks leads to unacceptable toxicity. Unfortunately pulse intervals of MTD of chemotherapy could influence recovery of surviving tumor cells and progression of tumor growth with less chemosensitive cells. A lot of mechanisms of chemoresistance are known. One of the most important is a genetic instability of tumor cells which enable the resistance development or lower cytostatic agent activity in adverse tumor microenvironment. The basic idea of metronomic chemotherapy (MC) is to continually administer cytostatic agents without neccessary breaks, which would also not allow the regeneration of tumor cells. To avoid the unacceptable cytotoxicity, the dose of the administered cytostatic agent in MC is drastically reduced.

Antiangiogensis
The growth of solid tumor depends on neoangiogenesis. Neoangiogenesis and vasculogenesis supply the neoplastic cells with oxygen and nutrients and are draining the waste products. This system intermediates the tumor growth. The tumor formation leads to increased expression of growth factors like vascular endothelial factor (VEGF), fibroblastic growth factor, platelets derived growth factor (PDGF). This process is also called angiogenic overlap, which is directly connected with further decrease of proangiogenic factors like thrombospondin – 1 (TSP – 1). In human oncology the elevated serum activity of VEGF and decreased activity of TSP – 1 correlates with decreased survival time in most solid tumors. Most of classic cystostatics show antiangiogenic activity. Most of endothelial tumor cells are rapidly dividing and therefore are sensitive to chemotherapy. Conventional MTD chemotherapy usually does not show clinical antiangiogenic effect because the pulse character
of application allows the regeneration of damaged endothelial cells. In a metronomic dosage it is the antiangiogenic effect that is relevant and shows simultaneously minimal adverse effects on healthy tissues. E.g. vinblastin and paclitaxel directly affect the endothelial dividing cells in an even lower cytotoxic dose. Cyclophosphamid and 5-fluorouracil blockade the mobilization of endothelial progenitor cells and are inducing the antiangiogenic inhibitors like e.g. TSP-1. Even the first few administered doses of MC leads to endothelial apoptosis followed by the vascular collapse and the tumor necrosis. The effect of the MC could be potentiated by use of tyrosin kinase inhibitors (TKI) COX-2 inhibitors.

Stimulation of cytotoxic immunity
One of the hallmark of cancer is the ability to escape from the surveillance of the immune system. Immunosuppression is caused by different exogenous and endogenous factors which facilitate the formation and growth of tumor. The tumor itself is able to avoid the surveillance due to the induction of immunosuppressive regulatory mechanisms (Treg) and myeloid suppressor cells (MDSC). Treg and MDSC play a key role in limitation of excessive inflammatory reaction. Unfortunately Treg and MDSC induced from a tumor growth lead to the immunosuppression. This is the reason for decreased tolerance of the immune system and further spreading of the neoplasia. Due to the immunophenotype switch any tumor-associated macrophages and dendritic cells lead to worsening of immunosuppression and immunotolerance in the tumor area. Most of cytostatic agents used by classic MTD chemotherapy induce the immunosupression. Vice versa the metronomic administration of some cytostatics is connected with immunomodulation contributing to tumor damage. Known effects of MC are an increase in a count and activity of Treg, dendritic cells and an increase in cytotoxic T-cell lymphocytes activity. The immunomodulatory effect of cyclophosphamide, chlorambucil, paclitaxel, doxorubicin, gemcitabin or metotrexat has been confirmed in human oncology. The combination of metronomic chemotherapy and TKI potentiates the cytotoxic activity of both agents.

Use of metronomic chemotherapy
MC is an alternative option of oncologic palliative therapy. MC offers low toxicity, easily administration (usually per os) and acceptable financial costs. The positive effect has been confirmed in STS, splenic hemangiosarcoma (survival comparable to conventional chemotherapy), apendicular osteosarcoma, transition cell carcinoma, oral squamous cell
carcinoma, feline injection site sarcoma and further carcinomas in dogs and cats (mammary gland carcinoma, lung carcinoma, thyroid gland carcinoma, paranal gland carcinoma, hepathocelular carcinoma, apocrine adenocarcinoma, nasal carcinoma), oral melanoma, oral fibrosarcoma, histiocytic sarcoma and mast cell tumor. The drug of choice is cyclophosphamide in a dose of 12,5-25mg/m$^2$ daily, the following choice is chlorambucil 2-4mg/m$^2$ SID or lomustine in a dosage of 2-3mg/m$^2$ SID.

**Toxicity of MC**

Clinical studies in human oncology confirmed mild to moderate toxicity of MC. Despite the lower toxicity of MC compared to conventional chemotherapy it is still necessary to be aware of possible myelosuppression and in the case of cyclophosphamide administering of sterile hemorrhagic cystitis. Adverse effects could be worsened by concurrent use of TKI. It is recommended to check the complete blood count, liver and kidney parameters two weeks after initiation of the therapy and then every 4-8 weeks. In cyclophosphamide administration, urine sediment analysis is also recommended. In case of the development of unacceptable side effects the reduction of dosage, supportive therapy or therapy termination should be considered. The risk of MC also includes contamination of the patient’s home environment. Every owner should be instructed about these facts. Critical times for contamination are administration of pills, vomiting of patient or contact with excrements. Long-term environmental contamination could increase the risk of cancer development in humans. Risks of MC should be carefully considered in families with small children, chronically ill or immundeficient family members.

**Evaluation of the therapeutic effect**

Therapeutic effect could be evaluated in the first 2-4 weeks after initiation of MC. Not only macroscopical reduction of the tumor size but also stabilization of the illness should be considered as a therapeutic success.

**Development of resistance and tumor malignisation**

As well as with conventional chemotherapy the MC also develops the resistance and further progression of the tumor. MC leads to endothelial apoptosis followed by collapse of tumor vascular structures and a tumor necrosis. Some of tumor stem cells are unfortunately capable
to survive despite of adverse environment. The origin of renewed tumor progression lies in these cell lineages. It has already been proved that resistant tumor cells are increasingly aggressive to the host and have higher metastatic potential. Because of that the use of MC can lead to malignisation of the ongoing illness in some patients.

**Conclusion**

MC represents one of revolutionary ideas in the philosophy of oncological treatment. Due to tempering of a tumor neoangiogenesis and immunomodulation, MC can be in some patients more effective and less toxic compared to conventional chemotherapy. On the other hand, also bring some potential of risks like environmental contamination and possible tumor malignisation.
SCINTIGRAPHY, HYBRID DIAGNOSTIC IMAGING, TARGETED THERAPY

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Functional diagnostic imaging (also called scintigraphy, nuclear medicine diagnostic) in veterinary and human medicine, is a method of detecting and/or measuring changes in biochemical characteristics of living organs, tissues eg.: metabolism, blood flow, regional chemical composition, absorption, specific receptor or immune mediated binding. As opposed to structural (also called morphological) diagnostic imaging, functional imaging laboratories on revealing biochemical and (patho)physiological activities within a certain tissue or organ by employing image modalities that use tracers (or carriers) to reflect spatial distribution of them within the living body. These tracers often are proportional (and competition) to some chemical compounds (like glucose, amino acids, transmitters …) normally also presented within the body. To achieve this, and let the biochemical processes “visible” radioisotopes are used because radiolabelled ligands have similar chemical and biological characteristics to the non-labelled ligands. By appropriate proportionality in radioligand (tracer) distribution nuclear medicine experts can determine the real intensity and distribution of certain substance within the body to evaluate the risk of developing pathological processes, diseases. Not only cancers but numerous other (endocrine-, orthopaedic-, neurological- …) diseases have their non-specific or specific tracers so that functional imaging is usable in almost all diseases.

Earlier competitive diagnostic imaging methods (radiology, ultrasonography, endoscopy, scintigraphy …) often rivaled against each other but nowadays imaging specialists used to collect the information provided by different methods and try to elaborate a more complex
diagnoses by the help of different images. In that way the earlier competitive methods transformed to be much more additive or companion-diagnostic imaging methods.

Image fusion (also called hybrid imaging) has more recently become a common term used in human and veterinary diagnostic imaging. The term is used when a patient morphological and functional images taken at the same (practically within several minutes) time in different data formats are fused (or hybridized). These different forms include the morphological images as computed tomography (CT), or more rarely magnetic resonance image (MRI), and the functional images single photon emission computer tomography, SPECT or positron emission tomography, PET). These fused (hybridized) images (SPECT/CT, PET/CT, PET/MRI) as they provide both the morphological and functional information to the clinicians and researchers could be extremely useful in detecting and specifying abnormalities within living organisms.

Detecting the functional alterations in our patients often open the way for novel therapeutical methods. It is known for decades that patients having multiple bone metastases diagnosed by bone scintigraphy ($^{99m}$Tc MDP) could be good applicants for radioisotope pain palliation ($^{153}$Sm-EDTMP, $^{186,188}$Re-HEDP) or "cold" (non-labelled) phosphonate treatments. Similarly thyroid patients showing high uptake of $^{99m}$Technetium pertechnetate or radioiodine nuclids will better respond to radioiodine treatment. The newer generation of more specific tracers (receptor-affin molecules, monoclonal antibodies and their fragments) allow us to use very sensitive and specific radiotracers to detect, stage …. and treat (!) …. then restage, evaluating patient-response and treatment efficacy for us. Performing this whole process we use radiopharmaceutical pairs: the diagnostic type of radiolabelled tracer, carrier allowing us SPECT or PET imaging, and the therapeutical form of the same or very similar molecule radiolabelled by beta or alpha emitter radioisotope resulting a higher, effective absorbed dose in target (usually tumor) tissue. All this is a more specific, more personalized way of medical thinking and acting for us veterinarians too.

**Figure 1. A-D** Photo (A), CT (B), SPECT (C) and fused SPECT/CT (D) images of a dog having a huge, invasive, inoperable primary tumor in left forearm with regional metastases. The high specific uptake of diagnostic radioligand ($^{99m}$Tc labelled folate receptor targetting nano particle) allow us the possibility of use a
novel therapeutic option (177Lu labelled folate receptor targetting nano particles) in that case.

A.) ________________________________ B.) ________________________________

C.) ________________________________ D.) ________________________________
CASE REPORT
SUCCESSFUL TREATMENT OF MULTICENTRIC LYMPHOMA REVERSED TO NEUROLOGICAL FORM IN 1,5 Y DOMESTIC CAT.

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1,5 y old, domestic shorthair, castrated, tomcat were admitted to the clinic with the extremely dyspnoea. No flexible thorax cavity were found during clinical examination. On the x-ray – hydrothorax and tumor in the mediastinum were revealed. Diagnose of lymphoma were based on cytological examination of the fluid from the thorax cavity. Complete remission was achieved after 10 months of COP protocol. After 10 months neurological sings from CNU appeared – apathy; seizures; no menace, palpebral and corneal reflex at the right side were present. MRI examination and cerebro-spinal fluid examination was performed. There were elevation of the protein content and severe pleocytosis with abnormal lymphocytic cells in the CSF which confirmed diagnosis of the recurrence lymphoma. The cat was successfully treated by oral CCNU protocol.
UPDATE ON CANINE LYMPHOMA

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Introduction
Malignant lymphosarcoma comprises approximately 7-24% of canine neoplasms and is the third commonly encountered malignancy in canine patients. Lymphoma represents a diverse group of tumors with the common origin in lymphoid cells. That type of cancer is a clonal proliferation of malignant lymphocytes in solid tissues such as lymph nodes, bone marrow and all other organs. Treatment of this tumor is characterized by early and spectacular response very often followed by equally spectacular treatment failure. The sustainable long-term remission is one of the greatest clinical challenges.

Etiology
The etiology of canine lymphoma is largely unknown and is most likely multifactorial. The affected patients are usually middle-aged to older. A decreased risk of lymphoma is reported by female dogs. Differences in the prevalence of immunophenotypic subtypes of lymphoma among different breeds indicate kind of heritable risk. Some dog breeds are reported to have a higher incidence of lymphoma. The hypothesis that infectious factors may be involved in the pathogenesis of lymphoma in dogs has not been confirmed. However detection of Epstein–Barr virus infection linked to some forms of lymphoma in humans has been documented also in dogs and is currently further investigated. Involvement of environmental factors and alterations (endogenous and exogenous) of patient’s immune system is also one of possible triggers.

Classification of lymphoma
Classification of canine malignant lymphoma is based on anatomic location, histologic criteria and immunophenotypic characteristics. The most common anatomical type in a dog seems to be multicentric lymphoma, followed by gastrointestinal, mediastinal and cutaneous form. Atypical anatomic forms which are rarely seen include e.g. hepatosplenic lymphoma.
**WHO classification**

The World Health Organisation (WHO) published a classification scheme using the revised European American lymphoma (REAL) system. This system incorporates histologic, anatomic and immunophenotypic criteria (B- and T- cell immunophenotype) with the goal of reaching accurate and reproducible diagnosis. The system is used for better tailoring of treatment protocols and better correlation of prognosis. Various categories of the disease should correlate with biologic behavior, response to the treatment and prognosis.

The most common form of canine lymphoma is diffuse large- cell lymphoma – a high grade tumor mostly of B- cell origin (DLBCL). Only small percentage of canine lymphomas is considered to be low grade (e.g. T- zone lymphoma, follicular lymphoma, mantle cell lymphoma.).

Determining of the immunophenotype provides also very useful information.

**Diagnostics and staging**

Physical examination, complete blood count, blood biochemistry, urinalysis and basic to advanced imaging diagnostics should be done in every patient with the suspicious of lymphoma. Other laboratory methods to confirm the diagnosis and further differentiation of the tumor are also necessary.

**Blood analysis**

A lot of lymphoma-related hematologic abnormalities are observed. Most common finding is anemia - usually normochromic, normocytic (nonregenerative) consistent with anemia of chronic disease. However regenerative hemorrhagic and hemolytic anemias may also frequently occur. Due to myeloptysis could be pancytopenia presented. Thrombocytopenia is in some cases also immune mediated. Atypical lymphocytes circulating in peripheral blood may be indicative of bone marrow involvement and leukemia. Neutrophilia and eosinophilia can be also seen. Esosinofilia should be considered as a negative prognostic factor. Serum biochemical abnormalities often reflect the anatomic site involved. Paraneoplastic syndromes such as hypercalcemia are commonly seen. Hyperproteinemia with monoclonal gammopathy is observed in approximately 6% of dogs with lymphoma. Hypoproteinemia is frequently seen in animals with alimentary lymphoma. Renal infiltration with tumor can be shown by azotemia same as with liver enzyme elevation by liver infiltration. Several abnormalities in serum have been explored as biomarkers of lymphoma (e.g. alpha – fetoprotein, zinc,
chromium, iron, VEGF, lactate dehydrogenase, CRP, haptoglobin). The clinical, biologic and prognostic significance of these parameters is not yet clarified.

**Histologic and cytologic evaluation**

Morphologic examination that constitutes the illness is essential to the diagnosis of lymphoma. Prescapular and popliteal lymph nodes are preferable for biopsy sampling. In most cases the diagnosis of lymphoma can be made on evaluation of fine needle aspirates of affected tissues. Further classification into subcategories to make up the low-, intermediate-, and high – grade forms is performed most accurately on histologic sections and usually require also further molecular diagnostic methods. Histopathology is also a cornerstone for WHO classification which plays significant role in therapeutic decisions making. Whole node biopsy provides the maximal amount of information but needle core biopsies should be satisfactory. It is important to avoid crush artifacts or inadequate sample size.

**Immunophenotyping**

The immunophenotype of a lymphocyte is identified by determining the expression of molecules specific for B- cells (CD 79a, CD20, CD21) and T- cells (CD3, CD4, CD8). For accurate determination of immunophenotype, antibodies against lymphocyte markers are applied to tissue sections, cytology specimens or cells in a fluid medium. Small number of dogs can be positive for both T- and B- cells markers.

**Flow cytometry**

Flow cytometry (FC) is a technique for qualitative and quantitative assessment of multiple parameters of individual cells or particles in complex cell suspensions. Once a diagnosis of lymphoma has been established, samples aspirated from enlarged lymph nodes or other masses may be placed in a suitable buffer (typically, saline with a small amount of serum and EDTA or in PBS Medium), stained with fluorescent antibodies, and analyzed in a flow cytometer to classify the type of lymphoma. There are several antibodies available for the immunophenotyping of canine lymphocytes and malignant lymphomas. Anti-CD79 and anti-BLA36 are both widely used for the recognition of normal and tumor B cells. Other antibodies that recognize B cells are against CD18, CD21 and different classes of immunoglobulins, while anti- CD3, CD4, CD5, CD8, CD49d and PanT antibodies react specifically with T cells. CD18, CD45 and CD45RA are common lymphocyte antigens
expressed by both B and T cells. Non-B-non-T cell lymphomas are recognized by the expression of common lymphocyte antigens and a lack of B or T cell specific antigens.

**Clonality assay**
Molecular clonality assays assess rearranged lymphocyte antigen receptor gene diversity and can help differentiate reactive from neoplastic lymphoid proliferations as well as B and T cell lymphoma type. The sensitivity of this assay is approximately 70%-90%. False – negative rate is around 5%, especially in cases where the malignant cells are presented in too low frequency to be detected or if it goes about NK malignant cells. False positive results are observed in case of some infectious diseases (Ehrlichiosis, Lyme disease). Clonality assay can be very useful in a therapeutic monitoring even in minimal residual disease detection. Molecular remission rates can be detected by use of clonality assay in standard cytology specimens or in blood and bone marrow sample.

**Chemotherapy**
The different and individual chemotherapeutic approach is more than desirable. For example LBL is via WHO classification like acute lymphoblastic leukemia. From that point the same aggressive therapeutic approach should be considered. PTCL is often less chemosensitive but shows good response on CCNU. TZL/CLL is slowly progressive and with LCHOP protocol the complete remission is mostly not achieved but excellent long-term control is maintained with chlorambuciltherapy.

**Chemotherapy and MDR1 Defect**
MDR1 gene encodes a transmembrane P-glycoprotein (Pgp) that works as a transmembrane drug efflux pump that expels drugs from the tumor cell. This defect can be responsible not only for drug intolerance but also for the resistance to some chemotherapeutic efforts. Genetic testing seems to be crucial in predisposed dog breeds.

**Immunotherapy**
Using immunologic and biologic agents in a cancer treatment shows to be a new and very promising trend in veterinary oncology. There is a new canine anti-CD20 molecule being trialled with promising provisional results and excellent toxicity profile. Clinical trials of an anti-CD52 molecule for the treatment of T-cell lymphoma are also under way.
Some vaccine trials are in progress in the US and UK, including a DNA vaccine encoding CD20 molecule and also a heat shock protein adjuvant autologous tumor vaccine for dogs with B-cell lymphoma. Provisionally, evidence suggests they can improve outcome in these patients when given in addition to traditional chemotherapy or radiation therapy.

**Radiation therapy**

Lymphoma seems to be radiosensitive. It could be very useful in stage I lymph node and solitary extranodal disease or as a palliative for a local disease (e.g. mandibular lymphadenopathy, rectal lymphoma, mediastinal lymphoma, localized bone involvement, cutaneous lymphoma and spinal forms of lymphoma).

**Conclusion**

The accurate distinction of reactive and neoplastic cells can be challenging. Given the different prognoses and treatment strategies a correct diagnosis is crucial. All prognostic factors should be always considered before the treatment initiation. The tumor grading and staging seems to be very helpful in making therapeutic decisions and giving the prognostic options. The WHO classification should help the clinicians to reach the best therapeutic success. The proper diagnosis, staging, grading and evaluation of risk factors facilitate the adaptation the therapy to patient´s needs and owner´s wishes.
SURGICAL ONCOLOGY OF UROLOGIC TUMOURS IN SMALL ANIMALS

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Primary renal tumours are rare (2% of all neoplasia in small animals). Benign tumours (adenoma, interstitial nephroma, haemangioma, papilloma, lipoma, fibroma and neurofibroma) usually encounter sporadically in older dogs and cats. Tubular cell carcinoma is the most frequent malignancy in humans and dogs, which represents 85% of kidney’s primary malignant tumours, and is generally unilateral. Cystadenocarcinoma is frequently bilateral, and can be combined with generalised dermatofibrosis and uterine leiomyoma in German shepherds. Nephroblastoma is a congenital malignancy mostly found in young (< 4 years) dogs and cats. Further malignant tumours affecting kidney are TCC, SCC, anaplastic carcinoma HSA, FSA, LSA, and STS. Secondary (metastatic) renal tumours – mostly lymphoma in cats – may exceed the incidence of primary ones. Generalised alimentary lymphoma may affect kidneys in 40% of cases. Diagnosis of renal tumours is difficult because the 2/3 of cases are asymptomatic, and the remaining 1/3 usually show atypical physical signs (anorexia, apathy, weight loss, anaemia, neutrophilia, abdominal pain). Urologic symptoms are haematuria, pyuria, proteinuria, and isostenuria. Diagnostic imaging plays crucial role in confirming renal tumours. Although plain abdominal radiography might reveal enlarged kidney(s), positive contrast excretory urography or ultrasonography are necessary to make precise diagnosis, with 53%, 96% and 84% specificity, respectively. Computed tomography and PET-CT have already been published as useful imaging tools to describe renal tumours. Ultrasound-guided or laparoscopic core biopsy (grading) as well as clinical staging provide histopathologic categorisation of the neoplasia serving therapeutic planning. The most effective therapy of renal tumours is nephroureterectomy, which is the removal of the affected kidney along with the entire length of the ureter. In a study evaluating clinical data of 82 dogs operated with primary renal tumour, the median postoperative survival time after
carcinoma, sarcoma and nephroblastoma was 16, 9 and 6 months, respectively. In another study the survival after removing HSA was 278 days. Haemoperitoneum was a negative prognostic factor (62 vs. 186 days).

**Primary tumours of the ureter** (fibroepithelial polyp, leiomyoma, TCC, LSA, STS) are extremely rare in dogs. In cats there is only one case report about a bilateral ureteral TCC. *Locally metastatic tumours* originated from either the urinary bladder or the kidney are more frequent. In these cases the clinical signs are defined by both the organ affected by the tumour and the obstruction of the ureter. Ureter obstruction finally leads to hydroureter and hydronephros. **Diagnostic signs** are anorexia, pyrexia, haematuria, polyuria/polydipsia, and abdominal pain. Hydroureter and hydronephros are easily diagnosed by positive contrast excretory urography, ultrasonography and CT. **Therapy** is defined by the localisation of the tumour as well as the damage of the kidney and ureter. Accordingly, ureteral resection and anastomosis, or (in distally located neoplasia) resection and ureter re-implantation can be done. As salvage, nephroureterectomy is indicated in case of intact contralateral urinary organs. Inoperable malignancies affecting trigone area may be palliated via double “pigtail” catheterisation, which is able to make the obstructed ureteral lumen patent. However, the median survival time of “ureteral stenting” was just 57 days in a study evaluating 12 patients.

**Tumours of the urinary bladder** are the most common urologic neoplasia in dogs, representing just 1% of all tumours, however. In cats they are the second most frequent urinary tumours after renal lymphoma. **Primary bladder tumours** were 97% malignant, with 97% of epithelial origin in a study. The most common malignancy is TCC, followed by lymphoma, RSA, adenocarcinoma, SCC, HSA, FSA, and LMS. **Benign** primary bladder tumours (fibroma, papilloma, haemangioma, leiomyoma) are quite rare. **Metastatic neoplasia** are usually originated from the urethra or the prostate, via local invasion. The diagnosis is based on physical signs (dysuria, stranguria, haematuria and pollakiuria), which are similar to cystitis and cystolithiasis as differentials. Sediment of the urine sample may involve malignant transient cells. Diagnostic imaging tools such as positive contrast or double contrast cystography, as well as the combination of positive contrast excretory urography and pneumocystography have as high as 96% sensitivity. Nevertheless, 2D or 3D ultrasonography, and CT are also very informative tools about local invasiveness of the
tumour, and the involvement of urethra, ureters or prostate. **Therapy** depends on the type and the stage of the neoplasia as well as the state of the patient. Operable tumours are to be removed via *partial cystectomy or trigonal resection and ureter re-implantation*. Local recurrence is rather frequent because even wide margins may contain residual tumour cells in the 50% of cases. Inoperable neoplasia may be treated with *chemotherapy*. The combination of *piroxicam* and *mitoxantrone* showed a 291-day median survival. *Intracavitary cisplatin* or *carboplatin* are also promising options. Radiotherapy has not been used against bladder tumours due to low efficacy and severe side effects.

**Primary tumours of the urethra** are extremely rare, mostly affecting *beagles, older females*, and the *distal section* of the urethra. However, tumours originated from the *trigone area* or *prostate* frequently invade the proximal section of the urethra leading to dysuria due to partial obstruction. The most frequent urethral tumour is *TCC*, which extends into the trigone in 1/3 of cases. Otherwise it is growing slowly and metastasises into regional lymphonodes and the lungs. **Diagnosis** is based on *physical symptoms* (chronic dysuria, haematuria and urethral discharge). *Abdominal* and *rectal palpation* may reveal masses in advanced cases. *Difficulty catheterising* the urethra is the case in partial or total obstruction. *Positive contrast urethrography*, *vaginoscopy* (in females) and *urethroscopy* (in males) as well as *biopsies* are of high diagnostic value. **Therapy** can be *surgical* via *urethrectomy* and *proximal urethroscopy* in case of distally located tumors. *Chemotherapy* with *cisplatin*, local *interleukin-2*, *piroxicam* combined with *mitoxantrone* or *carboplatin* may be administered to enhance regression of TCC. Most recently *palliative urethral “stenting”* has been published to temporarily re-establish urethral patency. Another palliation is *tube-cystostomy* to divert urine.
CASE OF METASTATIC AAC IN BERNESE MOUNTAIN DOG

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**Introduction:** Apocrine sweat gland adenocarcinoma (AACs) account for 0.7 % to 2.2% of all skin-associated tumors in dogs. Approximately 70 % of canine apocrine tumors are benign in nature, but malignant ones tend to recur locally and metastasize to regional lymph nodes and the lung. Confirmed distant metastatic rate of AACs appears to be very low (2%) and it is associated with intravascular invasion.

This report describes AACs with distant metastasis in Bernese Mountain Dog, 7 years old. AAC was situated on left hind limb one year before first coming to our hospital. Incomplete excision was done and in time of first coming suspicious metastasis was in left inguinal lymph node (FNAB). Staging was performed, no other distant metastasis were found. Histological findings confirm metastasis of AAC. Adjuvant chemotherapy was proposed to the owner. It was rejected in this time. Oncological control after 3 months were done. This patient had concurrent problems – regurgitation of mitral and tricuspidal valve and Syndrom Cauda Equina (SCE). 18 months after excision of inguinal lymph node, metastasis in sublumbal lymph node was found. The following therapy toceranib in dose 2.75 mg/kg EOD. Result of therapy was SD for 3 month. After 3 months therapy was ended for financial reasons. After the end of therapy the disease progressed. The next therapy was MDT carboplatina 300 mg/m2 in four cycles. SD was achieved. One month after the end therapy disease started to progress. The second chemotherapy by carboplatina was given (five months after the first chemotherapy). SD for whole second chemotherapy by carboplatina was achieved. During the chemotherapy and after its end, symptoms of SCE worsened. Patient needed multimodal analgesia- movacoxib 2 mg/kg SID + tramadol 2 mg/kg SID + gabapentin 3 mg/kg SID. Quality of life was very good. Three years after first coming quality of patient’s life was reduced. The patient was painful and ataxic, the sublumbal lymph nodes were slowly growing. MRI was done. Results of MRI- extramedullary compression L7/S1, disc protrusion, degenerative changes. Static compression of 90% spinal canal in neutral position. More slight protrusions Th13/L1, L1/2/3. Euthanasia, three years after first coming, due to SCE.

**Results:** Presentation of rare case of metastatic sweat gland carcinoma in Bernese Mountain Dog. In this case, good control of AAC’s metastasis by using toceranib and carboplatina is presented.
MASITINIB ON FISS – CASE REPORT

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Patient: Micina, cat, mixed breed, born 2009, female neutered

09/2015 Owners came with Micina to their veterinary surgeon. They found little globular formation on the left side of thorax. She underwent surgery and histopathological examination.

Result – Felline injection side sarcoma, medium degree of malignancy, with infiltration to skeletal muscles. After that they were sent to us.

10/2015 Our first contact.

Staging: T0N0M0, clinical examination without pathological findings, blood analysis – leucopenia and neutropenia, biochemical profile without findings, chest Xrays – clear, abdominal USG - clear and we did second surgery – very agressive in the site of previous intervention – to cut out as much macroscopic healthy tissue as possible. No ribs involved.

Considering blood analysis findings we decided to not start adjuvant chemotherapy for next 4 weeks and after that we do again blood analysis and complete staging before start of adjuvant chemotherapy. Owners refused chemotherapy for their cat as next step. They went to another veterinarian, and get Vitamin C and Inositol hexaphosphate (Sanicel) for next 5 months and after that they had to do checking visit. They were very happy and were thinking about adding potato juice to Micina’s treatment... (no joke)

11/2015 Owners found again little globular formations in the area of previous surgery. They returned to us.

We did staging: T1N0M0 and second surgery – 3 cm of healthy tissue, 2 muscle layers. No histopathology was done. According to such quick recurence we supposed bad prognosis and we insist on adjuvant therapy. Owners still didn’t want chemotherapy for their cat. So we gave them option – off label use of masitinib on FISS. Molecular treatment sounded better then chemo, so they agreed.

Masitinib 50 mg 1 dose EOD, with supportive treatment – nespecific immunostimulative sirup (Plerasan), renal care additive (Ipakitine) and continue with Sanicel and Vitamin C.

Plan – clinical and blood checkup every 2w

12/2015 After 2 weeks of treatment – leucopenia, neutropenia.

Treatment paused for 1 week, atb cover – cefovecinum (Convenia). After 10 days without treatment we continued with lower dose.

Masitinib 25 mg 1 dose EOD with 2 weeks checkup interval. Cat was in complete remission.

03/2016 Treatment stopped because of relaps of leukopenia and neutropenia. Micina looked very well with no health problems. So we decided to not give her atb cover. Maybe bad decision. Pause in treatment lasts almost for three weeks. After that we continued with same dosage of masitinib and same interval of checkups.

05/2016 Patient is still in complete remision, oncological staging: T0N0M0. She is doing very well and owners are satisfied.
Endocrine Background and Consequences of Testicular Tumour in Dog

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Introduction: Primary testicular tumours and tumours due to cryptorchidism are the most common tumours affecting the male genital organs in dog. Major types of testicular tumours on basis of WHO classification are the sex-cord stromal tumours, namely Sertoli cell tumour, Leydig cell tumour and granulosa cell tumour. Seminoma as germ cell tumour occur with approximately probably equal frequency and tumours growing from interstitial tissue. Hormone secretion of tumour depends on the cell origin of tumorous transformation and enzymatic degradation of steroids, respectively. Metabolism of steroids is an enzyme dependent pathway which is moved from cholesterol through different steroids included testosterone to oestradiol as final molecule.

Materials and Methods: Thirty three dogs suffered from testicular tumour (21 cryptorchid, 12 scrotal localisation) and ten healthy male and eleven female as control were castrated and spayed. Serum sample was collected before induction of anaesthesia. Serum samples were kept at -20°C until measurement. Pregnenolone, corticosterone, cortisol, 17α-OHP, testosterone and 17β-oestradiol were measured by ELISA kit (DRG) (Diagnostic Systems Laboratories, Inc. Webster, USA) and progesterone (Quanticheck) (Veterinorg Ltd, Budapest, Hungary). A 1x1 cm size cubic tissue samples were fixed in 4% buffered formaldehyde solution after the removal of testicle. The histopathologic diagnosis was based on haematoxylin and eosin stain and the sections were immunostained using the EnVision System. Anti-PCNA antibody, anti-MMP9 antibody, anti-caspase 3 antibody, anti-estrogen receptor α antibody, anti-EGF antibody, anti-PGP antibody, anti-survivin antibody was used in immunohistochemical reaction, all of them is the product of Abcam (Cambridge, UK).

Results: Beside the normal testicles, seminoma, granulosa cell tumour, Sertoli cell tumour and Leydig cell tumour were diagnosed. Spermatogonia of normal testis were PCNA positive. In seminoma, where tumour cells, especially the larger size cells were positive in contrast with spermatogonia in convoluted seminiferous tubules around the tumour. Although Sertoli cell tumour and granulosa cell tumour were PCNA positive, primary spermatocytes in intact seminiferous tubules beside the tumours were positive exclusively at Sertoli cell tumour cases. Presence of ER was confirmed in spermatids of normal testicles, in Sertoli cell tumour and seminoma. Serum hormone concentrations of tumorous (mean±SD) were: pregnenolone 19.89±12.96 ng/mL; P4 2.23±1.33 ng/mL; corticosterone 94.35±65.58 nmol/L; 17α-OHP 0.96±0.66 ng/mL; cortisol 136.54±84.09 nmol/L; testosterone 9.15±5.54 ng/mL; 17β-oestradiol 69.17±48.37 pg/mL. Serum hormone concentrations of controls (mean±SD) were: pregnenolone 20.82±13.26 ng/mL; P4 5.38±5.35 ng/mL; corticosterone 88.78±67.91 nmol/L; 17α-OHP 0.98±0.65 ng/mL; cortisol 134.02±91.15 nmol/L; testosterone 8.73±4.70 ng/mL; 17β-oestradiol 56.66±40.88 pg/mL. Although the serum testosterone level differed in patients suffered from different testicular tumour, the serum oestrogen level was not different at the level of significance.

Conclusion. PCNA positivity was detected in cells with high proliferating activity, Sertoli cell tumour seems to have negative effect on cytogen function of identical non-tumorous testis tissue. ER expression confirm the complex role of sexual steroids.
HISTOPATHOLOGY AND IMMUNOHISTOLOGY IN SMALL ANIMAL ONCOLOGY

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A case report introduces an undifferentiated spindle cell tumour. A proliferative change has developed on rump area of a 12 year old dog. The aspiration cytology revealed inflammation. The large (approximately 10 cm in diameter), therapy resistant lesion was removed. The most conspicuous histology picture was the immature pleomorphic spindle cell proliferation embedded in the abundant collagenous matrix.

The tumour must be differentiated from the next tumours:
- Fibroma
- Schwannoma
- Leiomyoma
- Rhabdomyoma
- Fibrosarcoma
- Hemangiopericytoma
- Malignant peripheral nervesheath tumor
- Granular cell tumor
- Malignant fibrous histiocytoma
- Liposarcoma
- Leiomyosarcoma
- Rhabdomyosarcoma
- Melanoma
- Spindle cell carcinoma

Beyond special staining the immunohistochemistry (IHC) was the useful method for the correct diagnosis. The applied immunohistochemical panel will be demonstrated in the presentation.

IHC is an important tool in the surgical pathology, but the Achilles ankle of this method the fixation. The knowledge of the right fixation is important for the veterinary surgeon from this point of view.
SCLEROSING PERITONEAL MESOTHELIOMA IN A BERNESE MOUNTAIN DOG

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Brúno, 10 years old, male, Bernese mountain dog. He was presented at a veterinary clinic with enlarged abdomen due to severe ascites. There was no evidence of cardiological abnormality. During the abdominal ultrasound examination abdominal effusion sample was taken, and sent to cytology examination. Abdominal neoplasia was suspected. Brúnó was sent to our referral clinic for treatment options. The average condition of the dog was good. The abdominal ultrasound (US) revealed a huge amount of free fluid; the bowels were found in a big conglomerate; the liver lobes seemed normal, with slightly increased echogenicity; spleen, kidneys, prostate, urine bladder were normal. Exploratory laparotomy and the drainage of the fluid content was performed. Liver was rounded out the omentum and the intestines were in coherent conglomerates, the capsule of these conglomerates contained fibrotic and adipose tissue. Several samples were taken from these lesions for cytology and histopathology. The encapsulating fibrotic tissue was opened and the intestines were evolved. During median laparotomy incision we couldn’t find lesions the deepest zone which could have initiated the problem. Brúnó felt markedly better after the operation, but inappetence and vomiting occurred a few days later following surgery. MRI examination was ordered with the hope of finding a lesion deeper in the abdominal cavity but this examination failed to provide novelty compared the US. Then, we decided to perform a laparoscopy to scan the whole abdominal cavity again for every possible reason, with minimum harm to the dog. The bowels were freed from the capsule. This time the abdomen was filled up with sero-sanginous fluid, which contained floating particles. The peritoneum was permeated with a fibrous-like tissue forming ligament-like growths with, small vessels infiltrating it. The intestines were wrapped inside in this tissue and it formed several pouches around them. From the surface of this altered peritoneum small vessel clews emerged everywhere. Similar phenomenon was seen on the dorsal abdominal wall and on the surface of the gall bladder. The organs were partially covered with a firm, fibrous, pale pink coloured tissue, which was separable from the serosal membranes. After a few days of search, based on these findings, a diagnosis was made: sclerosing peritoneal mesothelioma. This is a very rare disease, which was reported only a few times in the literature and just in Bernese mountain dogs. The origin of the disease is likely autoimmune, but the exact cause has not been cleared yet. It responds to immunosuppressive agents temporarily, but the prognosis is very poor. After the start of the treatment of Brúnó’s, his condition improved for some weeks, but finally Brúnó was euthanased by the owner’s request. From our first examination until euthanasia 60 days had been spent.
UPDATE ON MAST CELL TUMORS

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Introduction
The clinical appearance of canine cutaneous mast cell tumours is extremely variable. They can be soft or firm, covered in hair or ulcerated. MCT can occur in the subcutaneous tissues and can feel exactly like a lipoma. In most cases MCT are single, but multiple tumours can be present (in around 9% of dogs).

To stage or not to stage?
The majority of canine mast cell tumours, although locally aggressive, are unlikely to metastasise and performing full staging on every patient may be unnecessary. Certain factors can be useful in guiding the clinician as to whether further staging is indicated.

• Histological grade: In the majority of cases, the histological grade of the MCT is not known prior to attempting excision – since it is not possible to predict grade based on FNA and cytology, the most common technique used for diagnosis. If for some reason, an incisional biopsy has been taken to grade the tumour (e.g. tumour located in a difficult site) then this could be useful. Grade III tumours and grade II tumours with a high mitotic index are much more likely to behave aggressively and metastasise - staging would be indicated in such patients.

• Rapid growth or tumour ulceration: This could indicate the tumour is likely to have an aggressive biological behaviour.

• Clinical stage: Lymph node metastasis would indicate screening for more distant metastasis.

• Location: Oral, mucocutaneous junction, nail bed and preputial / scrotal tumours have been reported to behave aggressively in some studies

• Recurrent tumours: These tend to have a poorer prognosis and staging is useful to plan further treatment

• Systemic signs: Signs relating to extensive histamine release, such as anorexia, vomiting, haematemesis, melaena or significant oedema, hypotension etc. could indicate disseminated disease

• Site not amenable to wide excision / site requiring expensive treatment

In dogs showing any of the above criteria, further staging is preferred prior to resection. In cases where none of the above occur, it may be appropriate to proceed to surgery, bearing in mind that further staging may be indicated once the histopathology has been reviewed.

Staging
Complete staging for canine MCT would involve FNA of the drainage lymph node(s), abdominal ultrasound + FNA of any enlarged lymph nodes, liver and spleen, thoracic radiographs (or CT). Following staging, appropriate treatment can be planned. If the disease is localised, wide excision of the primary tumour should be performed. If lymph node metastasis
is detected (or suspected), the node should be removed and sent for histopathology – additional therapy besides surgery is indicated if affected. If disseminated disease is present, systemic therapy is indicated. In some cases, surgery may be beneficial to reduce the disease burden, or to palliate clinical signs, for example if the primary tumour is ulcerated or bleeding.

**Using the pathology report to guide further treatment decisions**

**Histological grade and surgical margins**
MCT can be graded according to the Patnaik system, where grade I indicates a well-differentiated tumour, grade II indicates a tumour of intermediate differentiation and grade III is a poorly differentiated tumour. Grade is determined on a number of factors, such as cellular and nuclear pleomorphism, degree of differentiation, variation in granulation, mitotic figures and depth of invasion. For most grade I and most grade II tumours that are completely excised, no further therapy is indicated. The risk of metastasis is low (<10% of grade I tumours and <20% of grade II tumours). The patient should be monitored for recurrence, signs suggesting metastasis or for development of any new masses. For grade II tumours with a high mitotic index (>5 per 10 hpf), systemic therapy should be considered (see discussion of MI below). Grade I or grade II tumours with a low mitotic index with incomplete surgical margins will recur in only 1/3 of cases or less and they have a low risk of spread. Where possible, a revision surgery is indicated, (2-3cm margins around the scar and a fascial plane deep) with histopathological review of the excised tissue. If the margins are complete, monitor for recurrence. If the margins are incomplete, consider radiation or simply monitoring if mitotic index is <5. High grade MCT (i.e. grade III) with complete margins have a moderate risk of recurrence and a high risk of metastasis. Grade II with MI>5 per 10 hpf may behave similarly. Further staging of such patients may be indicated if not done initially. Systemic therapy is indicated to prevent or delay the growth of metastatic disease. High grade tumours with incomplete margins have a high chance of both recurrence and metastasis. Additional local therapy (additional surgery or radiation) and systemic therapy is indicated. The pathology report may require some interpretation with regard to margins. Mast cell tumours can secrete chemotactic factors for normal mast cells. A single mast cell at the periphery of excised tissue, discrete from the main tumour is not necessarily neoplastic, however sheets of tumour cells extending to the edge of the tissue is a more worrying situation, indicating further measures are necessary.

**Mitotic index**
Mitotic index is important in determining prognosis of MCT. It is particularly useful for grade II tumours, to try and predict whether they are likely to be “low-risk” or “high-risk” tumours. The pathologist assigns the MI by assessing the most aggressive part of the tumour microscopically and counting the total number of mitotic figures in 10 high power fields. The most common cut off for distinguishing low and high-risk canine MCT is 5 mitoses per hpf. In one study of dogs with MCT treated with surgery alone, the median survival time was significantly longer for those with a MI ≤5 (70 months) compared with those with a MI >5 (<2 months), regardless of grade. For grade II tumours, those with a MI ≤5 had a median survival time of 70 months, versus 5 months for a MI of >5. (Romansik, 2007). The Kiupel 2-
tier grading system for mast cell tumours uses a cut-off of MI ≥ 7 to indicate high grade tumours.

**What is the importance of other special stains / genetic tests?**

Proliferation markers, such as Ki-67 and AgNOR counts, which may give additional information above the MI, which can be useful, for example, in “borderline” cases.

**Ki-67 (MIB-1)** stains the nuclei of proliferating cells in the M&S phase of cell division. It can be measured in different ways. In one scheme, where the number of positive cells per 100 mast cells counted; a cut off of 1.8% of stained cells appears to have prognostic significance. For grade II tumours, 1 to 3 year survival probabilities were 0.95, 0.95, and 0.95 for Ki-67 ≤ 1.8%; but were 0.54, 0.45 and 0.33 for Ki-67 > 1.8% and these dogs were significantly more likely to die of their tumour (Maglennon *et al.* 2008). In another method of counting; a Ki-67 index of > 23 per grid area counted has a significantly increased risk of local recurrence, distant metastasis and MCT-related death. In a study by Berlato *et al.* 2013, MI and Ki-67 were compared for prognostication in canine MCT. Both mitotic index and Ki67 index were able to independently differentiate MCTs with a worse prognosis. The risk of dying due to MCT was similar in dogs with increased Ki67 or increased mitotic index. The dogs in this paper had been treated relatively homogeneously (i.e. no chemotherapy or radiation therapy after surgery). It has been noted, however, that discordant results sometimes occur with these tests, which can make interpretation difficult. A paper by van Lelyveld (2015) indicated discordant Ki-67 and MI results in 43% of MCT. This paper showed that Ki67 was highly sensitive (86.5%) at predicting MCT death but not very specific (57.9%); i.e. it tends to overestimate the number of MCT that are likely to be aggressive. MI > 5 was not very sensitive (32.4%) at predicting MCT death, but highly specific (96%); i.e. high MI accurately predicts death, but some tumours with low MI will still die from MCT. Low Ki-67 accurately predicted survival, but high Ki67 does not always mean a poor prognosis. The treatment given was not homogeneous in this study, which could have confounded survival times. Thus, information from the literature is somewhat controversial on this subject – It still can be difficult to predict the behaviour of some MCT accurately. Careful interpretation of all the information available (both clinical and laboratory reports) is important.

**AgNOR** is silver staining of nucleolar organising regions and is another proliferation marker. Increased AgNOR counts are associated with increased risk of local recurrence, distant metastasis and MCT-related death. Sometimes the Ag67 index is assessed (AgNOR count x Ki67 value, using grid area technique). A cut-off value of > 54 is indicative of poorer prognosis.

**KIT (CD117) mutation and KIT staining**

KIT (CD117) is a tyrosine kinase receptor for the haematopoietic growth factor stem cell factor (SCF), expressed on the surface of normal and neoplastic canine mast cells. Around 20-40% of canine MCT have mutations in the *KIT* gene, leading to constitutive activation of the receptor in the absence of bound SCF. In dogs, these mutations are usually in tandem duplications of a piece of DNA in exon 11, which encodes the juxtamembranous part of the receptor. Activating mutations in exons 8 and 9 have also been reported. Canine MCT possessing *KIT* gene mutations have been shown to have a poorer prognosis than those with normal *KIT*. Receptor tyrosine kinase inhibitors (e.g. masitinib and toceranib) are designed to
inhibit signalling through KIT and are particularly effective in MCT possessing mutated KIT (see section on drug treatment below). A PCR test is available to look for exon 8, 9 and 11 mutations. Such tests might be useful to direct therapy in favour of an RTKI first line in tumours possessing an activating mutation. Abnormal subcellular localization of KIT, as assessed by immunohistochemistry (i.e. a shift from the normal membranous location to a cytoplasmic location) is associated with poorer prognosis. Immunohistochemistry for KIT protein is available in the UK; however, KIT PCR or gene sequencing is not available as yet.

**Drug treatment for canine mast cell tumours**

The majority of mast cell tumours can be cured with appropriate surgery and or radiation therapy. Systemic drug therapy is indicated in a minority of cases: High grade mast cell tumours (grade III, or grade II with a high MI); metastatic disease beyond the regional LN; non-resectable MCT (also consider radiation); some cases with tumours at unfavourable location e.g. MCT arising from mucous membranes
MY INTERESTING CASES IN COMPARATIVE ONCOLOGY

Pradip Chaudhari

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Among various diseases of pet animals, cancer incidence and mortality has increased over several years. Managing cancer is a big challenge for veterinarians as it involves multidisciplinary approach, multiple specialities, several visits of patients to clinic and poor treatment outcome of advanced stage disease. The rising incidence of cancer among pet animals has created a need for specialised treatment, care, infrastructure and expertise. Our ten-year old comparative oncology program has initiated diagnosis and management of cancer along with further utilization of this information and biospecimens for cancer research in general. Under this program, more that 700 oncological cases have been registered. The increasing awareness of cancer among pet parents has attributed to early detection of cancer in these years. Cancer treatment cost and number of visits to clinic are major limiting factors for pet parents for noncompliance.

Our center provides cancer diagnosis and its management by surgery, anticancer drug therapy, radiation or adjunct therapy. The biospecimens obtained during the process of diagnosis and treatment are preserved for future reasearch utilization. The common cancers diagnosed at our center include mammary gland tumor, lymphoma, mast cell tumor, melanoma, soft tissue sarcoma and osteosarcoma. The common breeds that are diagnosed with cancer are Labrador, Pomeranian, German Shepherd, Boxer, Cocker Spaniel, Doberman, Golden Retriver, Rottweiler, Lhasa Apso, Great Dane,Pug etc. Some of these cases that posed great challenges for treatment in terms of uniqueness, criticality of the disease condition will be discussed in this lecture.

Keywords: veterinary oncology, Multidisciplinary, comparative oncology program, pet cancer
CASE REPORT: LOW-GRADE INTESTINAL LYMPHOMA IN 11-YEARS MIXED-BREED DOG

Jakub Pfeifr

Animed - small animal veterinary clinic, Brno, Czech Republic

Introduction: An 11 years old neutered female dog was presented because of gradual onset of intermitent diarrhea and weight loss from 8,1 kg to 6,5kg (in the time of final diagnosis). Other clinical signs included loss of appetite and random vomiting. During 5 months of patient´s clinical difficulties there were no abnormalities in blood work except for decreasing values of total protein (in time of diagnosis 31 g/l) and albumin (12 g/l in time of diagnosis) and vitamin B12 (47,2 pg/ml in time of diagnosis). Urine examination was repeated several times and showed normal UP/UC ratio. Complete ultrasound examination of abdomen showed no abnormalities - the intestinal wall was measured 0,35 up to 0,45 cm in diameter, abdominal lymph nodes were not enlarged, no fluid in abdomen was detected and hemoperikard was excluded. As the therapy (gastronistestinal diet, probiotics, famotidin, metoclopramid, metronidazol) had no effect and the patient´s condition worsened, probatotry laparotomy with incision biopsy of intestines was performed. Histopathologic and immunohistochemical examination did not reveal definitive diagnosis - the samples were evaluated as transient lesion between IBD and early stage of LGAL. Therefor PARR clonality examination was performed and monoclonal proliferation of the B cell population was identified. Final diagnosis was determined like a B-cell low-grade alimentary lymphoma.

Aims: patient’s good quality of life (no study or clinical research)

Results: Therapy with chlorambucil 2,8mg/m² + Prednisolon 40mg/m² SID was started (first week). The dose of prednisolon has been constantly decreased - second week 20mg/m² SID, third week 10mg/m² SID and fourth week and further 10mg/m² EOD. Supportive therapy consisted of vit B12 300IU two times per week SC, omeprazol, metoclopramid (only first ten days), omega 3 fatty acids, hydrolysed diet (later combined by the owner with home made food). After one month of therapy the patient gained weight from 6,5 kg to 7,3 kg, the clinical status has been stabilised as well as blood parameters including vitamin B12 (458,6 pg/ml). Up to twenty months of treatment the patient was doing fine (maximal weight 9kg). The dose of chlorambucil in that time varied depending on weight from 2,4- 2,9 mg/m². 20th month since the therapy was started the patient acutely died (25 months since the onset of clinical signs).
EVALUATION OF COX-2 EXPRESSION IN CANINE MAMMARY TUMORS AND ITS RELATION TO NSAID THERAPY

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Introduction: Mammary gland tumors are the most common neoplasm in intact female dogs. Therefore, it is very important to search for prognostic factors and new additional treatment possibilities besides the well-known therapies. Cyclo-oxygenase-2 enzyme (COX-2) is usually undetectable in normal tissues, but due to some cell activation processes, such as inflammatory cytokines, growth factors or oncogenes, the COX-2 receptors are overexpressed. The overexpression can be associated with some tumoral mechanisms, such as increased angiogenesis, inhibition of apoptosis, suppression of the immune response and greater invasive and metastatic capacities. The elevated expression of COX-2 has been reported in canine mammary neoplasms, suggesting a potential role for COX-2 in canine mammary tumorigenesis. Therefore, most probably, treatment modalities together with COX-2 inhibitors can be administered, in order to increase the survival of cancer patients. COX-2 inhibitors might decrease the proliferation and metastatic potential of the tumor cells together with their pain killer effect.

Aims: The aims of this study were to investigate COX-2 expression in canine mammary tumors using immunohistochemistry, and evaluate the effectiveness of NSAIDs (Non-steroidal anti-inflammatory drugs), such as piroxicam, firocoxib and meloxicam in patients with mammary gland neoplasm with respect to COX-2 expression.

Results: There were 42 dogs (40 females, 2 males) included in this study. All had mammary gland tumors. Cox-2 staining intensity was significantly higher in malignant types, than in benign tumors (p<0.05). The survival of the animals with different types of tumors was significantly higher in benign tumors (n=13) compared with malignant ones (n=29). Those patients which had higher COX-2 percent than 50% showed significantly lower survival time (ST=88,556) than those with COX-2 percent under 50% (ST=339.85). COX-2 expression negatively correlated with the survival time and the relapse, but positively correlated with the tumor volume (r=0,455). Those patients which received firocoxib (n=5) showed significantly higher survival (ST=338.3 days) than those which did not receive NSAIDs, or received other NSAIDs than firocoxib (n=21)(ST=195.1 days). Due to our study, COX-2 expression of mammary gland tumors might be used as a prognostic factor in diagnostics. Nevertheless, the use of NSAIDs raises the possibility of an additional therapy with other conventional cancer treatment.
CANINE MULTIPLE MAST CELL TUMOR AND ITS ASSOCIATION WITH ATOPIC DERMATITIS AND SEIZURES.

Hana Matušková, Veronika Šimerdová

Small animal referral clinic SIBRA, Bratislava, Slovakia

Introduction: This case report describes a patient with primary multiple cutaneous mast cell tumors which represent less common clinical presentation of cutaneous mast cell tumors. Similarly to solitary cutaneous mast cell tumor, their prognosis is based primarily on histopathological grade. For adequate therapy and prognosis it is important to differentiate primary multiple cutaneous mast cell tumors from poorly differentiated solitary cutaneous mast cell tumors with satellite lesions that are associated with aggressive biological behavior and worse prognosis.

Case description: A 4-year-old intact female Dogo Argentino was referred to our clinic for neurologic evaluation with an epileptic cluster of six seizures. The last seizure was seen at the time of the presentation as generalized tonic-clonic seizure lasting about one and half minute. The patient had no previous experience of seizures but the owner complained about various intermittent skin disorders such as pruritus, mild alopecia, hyperemia of pina, muzzle and both axillar and inguinal region. After neurological evaluation, which revealed only mild postictal neurologic deficits, multiple small nodular skin lesions were found during further examination. CBC, serum biochemistry, urinalysis were all normal as well as the results of MRI and CSF examination. Fine needle cytology of skin lesions revealed heterogenous population of mast cells with some criteria of malignity present (anisokaryosis, anisocytosis, uneven distribution of granules), fibroblasts, eosinophils and non-degenarate neutrophils in 5 from 6 samples. After completing the staging (abdominal ultrasound and thoracic radiographs) the supportive treatment with famotidin 1mg/kg PO SID, omeprazole 0,5mg/kg PO SID and loratadine 0,25mg/kg PO SID had started. Patient started with phenobarbital medication as well (3mg/kg PO BID). The surgery followed 3 weeks after initial presentation and during presurgical examination another 6 skin nodules were found. These were excised without cytological evaluation with the rest of the nodules, all with one centimeter margin. Twelve nodules were forwarded for histopathological evaluation. Nine of the lesions were diagnosed as low-grade (Kiupel) and grade I (Patnaik) mast cell tumor.

Results: In our case primary multiple cutaneous low-grade mast cell tumors were diagnosed. Based on recent discoveries, once completely excised no further adjuvant therapy is required as the risk of local recurrence and metastasis is very low. Atopic dermatitis may predispose to primary multiple low-grade cutaneous mast cell tumors as it leads to angiogenic accumulation of mast cells in reaction to chronic inflammation in affected tissues. The latest studies in humans revealed there might be a connection between MCT and seizures or other neurologic and psychiatric diseases. There is also a higher prevalence of epilepsy in human patients with atopic dermatitis. Although idiopatic epilepsy is suspected in our patient, relation between these diseases might be worth further evaluation.

References are available upon request.
MULTIDRUG RESISTANCE IN CANCER

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Effective treatment of metastatic cancers usually requires the use of toxic chemotherapy. In most cases, multiple drugs are used, as resistance to single agents occurs almost universally. For this reason, elucidation of mechanisms that confer simultaneous resistance to different drugs with different targets and chemical structures — multidrug resistance — has been a major goal of cancer biologists during the past 40 years. I will review the most common of these mechanisms, one that relies on drug efflux from cancer cells mediated by ATP-binding cassette (ABC) transporters. I will describe various approaches to combating multidrug-resistant cancer, including the development of drugs that engage, evade or exploit efflux by ABC transporters.
PARASITES AS ONCOGENES

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Concerning the aetiologies of cancer more than 100 oncogenes have been identified. Besides chemicals, radiation and hormones viruses, bacteria and some parasite species have been reported as the direct causes to specific cancers in the last few decades. No answer is known in the literature for the question how many parasite species can cause cancers. It has not been even known about the mechanism of the parasites as oncogenes.

There are much more information about the cancer of humans due to parasitic infections than animals. We have known that a number of parasite species, the major ones are the trematode parasite species such as Schistosoma haematobium and liver flukes such as Opisthorchis viverrini, Clonorchis sinensis can cause bladder and bile duct cancer in human. Schistosoma japonicum and Schistosoma mansoni have been linked to some colorectal cancers. Toxoplasma gondii infection causes ocular tumors, meningioma, leukemia and lymphoma. Trichomonas vaginalis is the major cause of prostate cancer.

After the introduction the lecture makes an attempt to summarize our current knowledge how the parasites can induce carcinogenesis resulting in specific cancers in animals. The following parasites species of animals as potential oncogenes will be discussed: Spirocerca lupi, Clonorchis sinensis, Echinococcus multilocularis Theileria annulata, Theileria parva. Finally the anticancer activity of some parasite species will be also mentioned.
ASPECTS OF SURGICAL ONCOLOGY

Pavol Valašek

VetPoint- small animal veterinary clinic, Bratislava

The oncological surgery is one of the oldest surgery divisions and thus has well defined rules. Recent training for surgical residents has a serious part in this, so it is more than wise follow these rules as much as possible.

Besides many, there are few, that you cannot break:

• **TALK** to your clients- explain them using “their language” what is the possible/definitive diagnosis, options of treatment, possible complications and above others- the **PROGNOSIS**

• **ALWAYS** know your „enemy“- the knowledge of the nature of the mass you are about to cut must be well recognized prior the surgery

• **PLAN** your surgery in the mean of:
  o TYPE of procedure (curative surgery vs. cytoreduction vs. palliative procedure!)
  o EXTENT of surgery needed to achieve the goals mentioned above
  o CLOSURE (simple vs. advanced closure techniques)

• **PLAN B**- always have it, if something goes wrong either during resection or closure

• **Always SUBMIT** samples from the final resection for histopathology

The oncological surgery is not a sole surgical act. It has to be always a team work. From the diagnostic part (biopsy, staging.....) thru surgery to adjuvant follow-up.

Oncological surgery is not an act of heroism. If you don’t feel comfortable with either diagnosis or surgical removal or closure, refer the patient to the colleague, who has more experience. You will remain on lower complication level and your client will be satisfied.

Instead of hazarding with your patient- work on building local network of clinics, which cooperate either in diagnostics, surgery treatment and following adjuvant therapy as well.

Although one of the oldest, the oncological surgery is still dynamically growing field of general surgery. Only the interdisciplinary approach is the most beneficial for our patient and we are obliged to be continuously educate our self.
WHAT’S NEW ABOUT TRANSITIONAL CELL CARCINOMAS

Katerina Stiborova

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Introduction: Transitional cell carcinoma (TCC) of the urinary system is a common tumor of the urinary bladder, urethra or a prostate. Unfortunately despite the progress in the veterinary medicine its treatment is usually frustrating due to early relapse and only short duration of a response.

Aims: The following abstract present a summary of a current diagnostic, clinical signs and treatment.

Results:
Urinary bladder cancer in dogs is a challenging disease to diagnose, stage, and treat. Fortunately, urinary bladder cancer is uncommon in the dog, comprising < 2% of all reported canine malignancies. Transitional cell carcinoma (TCC) is the most common neoplasm affecting the urinary bladder of dogs.

The etiology of canine TCC is most likely multifactorial. Risk factors that have been identified include exposure to topical insecticides for flea and tick control, exposure to marshes that have been sprayed for mosquito control, obesity, possibly cyclophosphamide administration, female sex, and specific breeds (eg, Scottish Terrier). Canine TCC is typically a disease of older dogs.

Common presenting signs included hematuria, stranguria, and other forms of dysuria and, less commonly, lameness, lethargy, and weight loss. A diagnosis of TCC requires histopathologic confirmation. Although neoplastic cells may be present in the urine of 30% of dogs with TCC, neoplastic cells are often indistinguishable from reactive epithelial cells associated with inflammation. Urine antigen tests for TCC have been found to be sensitive, however a high number of false-positive results limits the value of the tests. Methods for obtaining tissue for histopathologic diagnosis include cystotomy, cystoscopy and traumatic catheterization.

There are several treatment options for transitional cell carcinoma. Surgery may be indicated to obtain tissue for a diagnosis, to attempt to remove the TCC within the bladder if lesions are away from the trigone and to maintain or restore urine flow. Complete surgical excision of TCC is not usually possible because of the typical trigonal location, urethral involvement, and metastases. In a series of 67 dogs that underwent surgery in only 2 cases tumour was excised with free margins. Several surgical approaches have been performed (cystectomy, enterocystoplasty with cystectomy and subtotal intracapular prostatectomy, ureterocolonic anastomosis, vaginourethroplasty) however all are connected with some side effects and due to demanding postoperative care have not been broadly used.
Information on the use of radiation therapy in TCC is limited. There are only several reports which showed moderate response. The recent studies using IMRT showed response rate about 60% and median event free survival 311 days and survival 654 days.

The gold standard of an approach to TCC still remains chemotherapy\textsuperscript{3, 4, 5, 6, 7} (combination of various chemotherapy medications and nonsteroidal antiphlogistics). Various medical therapy has been used for a treatment of TCC. To the most commonly used chemotherapeutics belong mitoxantrone, carboplatin and vinblastine. Usually in case of further progression alkylating agents, gemcitabine or even chlorambucil could be used. However as already mentioned the outcome is usually poor and most dogs with TCC still die of the disease. The median survival time usually does not exceed more than 1 year.

Dogs with TCC are at high risk for secondary bacterial infections, therefore repeated bacterial cultures (due to development of multiresistant bacterias) and long term antibiotic treatment belong to very important part of a treatment.

References:
1. Withrow and MacEwen’s Small animal oncology, fifth edition, Elsevier 2013
6. Allstadt et col. „Randomized Phase III Trial of Piroxicam in Combination with Mitoxantrone or Carboplatin for First-Line Treatment of Urogenital Tract Transitional Cell Carcinoma in Dogs.” \textit{Journal of Veterinary Internal Medicine} ( 2015);29:261–267
FREE RADICALS IN CANCER THERAPY

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Introduction

Free radicals and reactive species are considered mainly as harmful side products of biochemical reactions because of their cytotoxic effect when produced in large amounts locally. Cancer cells normally produce more reactive oxygen species than do normal cells, but diverse cancer chemotherapeutic agents may be selectively toxic to tumor cells because they augment oxidant stress and push these already stressed cells beyond their limit. Therefore, certain therapies take advantage of the toxic effect of free radicals and use oxidant stress-mediated strategies to kill malignant cells.

Aims

The lecture gives a short overview of free radical production induced by some chemotherapeutic agents, radiation and photodynamic therapies (PDT), and talks in more details on PDT in veterinary sciences. For PDT to cause tissue damage, three elements must be present simultaneously: (1) a non-toxic photosensitizing agent, (2) visible light of a specific wavelength, and (3) molecular oxygen. After the photosensitizer has accumulated more or less selectively in the malignant tissue, the tumor, and only the tumor is illuminated. The sensitizer absorbs the light and is converted to an excited triplet state which readily reacts with molecular oxygen producing a burst of toxic oxygen-centered free radicals and reactive species, inducing preferably tumor cell apoptosis.

Materials and Methods

We have treated several cases of feline SCC and sarcomas using 5-aminolevulinic acid as a photosensitizer precursor and irradiating the tumor with orange light at a wavelength around 630 nm for 10 min.

Results

PDT has been a successful treatment, especially in the case of Stage 1 tumors.

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INTRANASAL SCC IN TWO DOGS

Dušan Král

RegiaVet Praha, Czech Republic

The case report describes different results of the nasal SCC treatment in two dogs 10.5 and 13.5 years old. These clinically similar cases of nasal SCC in a German Pinscher and a Labrador Retriever were solved by radiotherapy (12 fractions of 4Gy), COX2 inhibitors and chemotherapy or the application of toceranib (Palladia).
Introduction:
Hepatocellular carcinoma (HCC) is the most common primary liver tumor in dogs. The morphological presentation can be either massive, nodular or diffuse. When there is a solitary mass confined to a single liver lobe, this is referred to as a massive HCC. This type is most common and has been associated with a lower metastatic rate compared with the nodular and diffuse forms. In nodular HCC multiple discrete masses affect one or more liver lobes, while the diffuse form affects the entire liver. There is no significant breed or sex predisposition. The mean age of affected dogs is 7-11 years. Clinical presentation is often vague; lethargy, anorexia and weight loss are often the only clinical signs. Only 35% - 45% of the cases have a palpable mass in the cranial abdomen. Haematology can be normal or show anaemia and leukocytosis. Biochemistry often reveals increased liver enzymes. Abdominal ultrasound is useful to identify a focal hepatic mass, however it does not provide information about its nature. Cytology diagnosis obtained from ultrasound guided fine needle aspirates (FNAs) has a high positive predictive value (87.7%), but cannot entirely rule out the presence of neoplasia when no malignant cells are seen. Advanced imaging such as Computer Tomography (CT) or Magnetic Resonance Imaging (MRI) delivers greater detail which allows for comprehensive surgical planning and concurrent staging. Liver lobectomy is the gold standard treatment for massive HCC: the prognosis is usually good, as local recurrence and distant metastasis are uncommon. In cases where surgical resection is not feasible, metronomic chemotherapy or embolization can be considered.

Case history:
This case is an 11 year old female spayed Border collie 19.5kg of weight who was presented to the Oncology Service at Willows Veterinary Centre and Referral Service with 2 weeks history of intermittent diarrhoea and anorexia. No abnormalities were detected on physical examination. Serum biochemistry revealed a markedly increased alanine aminotransferase (ALT). Haematology was unremarkable. A solitary hepatic mass was identified on abdominal ultrasound. A CT scan confirmed the presence of a large heterogenous hepatic mass localised to the right lateral lobe as well as multiple smaller hepatic nodules. Ultrasound guided fine needle aspirates (FNAs) were taken from the mass and nodules. Cytology confirmed the mass was a HCC, while the nodules appeared to be cytologically benign. Surgical resection of the hepatic mass was performed. Biopsies were also taken from the other nodules. The dog was discharged from the hospital the subsequent day with the following post-operative medical treatment: amoxicillin/clavulanic acid 20mg/kg Q12h for 5 days, meloxicam 0.1mg/kg Q24h for 5 days and tramadol 2.5mg/kg Q8h. Histopathology of the large mass confirmed HCC. The biopsies of the liver nodules also demonstrated malignancy, revealing them to be intrahepatic metastasis. The dog started adjuvant metronomic chemotherapy with cyclophosphamide 10mg/m² Q24h and firocoxib 5mg/kg Q24h, in order to inhibit progression of the remaining lesions. Five months post-surgery the patient is still receiving chemotherapy and clinically asymptomatic.
References:
RECENT ADVANCES ON HEREDITARY DISEASES IN DOGS FOR THE CLINICIAN AND PATHOLOGIST

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Many of the characteristic breed traits and common and rare diseases and predispositions seen in veterinary practice have a heritable basis. Recent exciting advances in our current knowledge of the completed dog genome sequences offer the opportunity to clinicians to use these emerging tools in clinical practice and have a positive impact on the health of dogs and in particular the diagnosis, management, and control of hereditary diseases.

There are many unique traits of canine breeds and many hereditary disorders and genetic predispositions to disease have been identified. While clinical and routine laboratory and imaging tests are helpful, specific biochemical and DNA tests have become available for >174 single gene defects through various laboratories in dogs. Moreover, with DNA tests it is now possible to determine the ancestry of mixed breed and purebred dogs, a first example of a complex trait. As it is difficult to keep track of all the diseases, tests and treatments, a web-based database for available DNA tests on hereditary diseases in companion animals for clinicians is being introduced (http://research.vet.upenn.edu/WSAVA-LabSearch).

Because of the increased awareness of breeders, pet owners, and veterinarians of genetic defects and the improved diagnostic abilities in clinical practice, the number of reported hereditary diseases in small animals is rapidly growing. At present, >900 hereditary diseases in dogs have been adequately documented. For the small animal practitioner, it can be a daunting, nearly impossible task to remember all these diseases and be aware of the many novel tests and their appropriate management and control.

Databases on hereditary diseases: It is difficult for a clinician to keep up with the rapidly accumulating information on clinical genetics and the large spectrum of disorders and genetic predispositions. Thus, comprehensive update resources are needed. There are several website that provide some information on many different diseases in companion animals such as “Inherited Diseases in Dogs” (http://www.vet.cam.ac.uk/idid/); Mendelian Inheritance in Animals, http://www.angis.org.au/Databases/BIRX/omia; Canine Inherited Disease Database http://www.upei.ca/~cidd/intro.htm; and the FAB list of feline hereditary disorders www.fabcats.org/breeders/inherited_disorders. The WSAVA Committee on Hereditary Diseases has set up a database on genetic tests for hereditary diseases (http://research.vet.upenn.edu/WSAVA-LabSearch; www.wsava.org and www.VIN.com) with pertinent practical information on clinical features, genetic diagnostics, and management specifically for the clinician.

Beyond physical examination and imaging tools, genetic, metabolic, and other laboratory techniques are used to diagnose hereditary disorders in companion animals. Most genetic defects cause clinical signs early in life. The term congenital does only imply that the disease
is present at birth, and does not necessarily mean it is inherited. A common presentation is failure-to-thrive compared to littermates. They are poor doers, often fade (hence the term fading puppy syndrome), and finally die. Failure-to-thrive should not be confused with growth retardation, dwarfism. In addition to these relatively unspecific clinical signs, some defects may cause specific clinical manifestations. Easy to recognize are malformations that involve any part of the skeleton and lead to disproportionate dwarfism, gait abnormalities, and/or facial dysmorphia. A large number of hereditary eye diseases have been described in dogs, some of which are not recognized until adulthood. Neuromuscular signs may vary from exercise intolerance to ataxia and seizures. Defects of many other internal organs are associated with unspecific clinical signs.

Diagnostic tests are generally required to further support a genetic disorder in a diseased animal. Radiology and other imaging techniques may reveal skeletal malformations or cardiac anomalies, and an ophthalmologic examination may further define an inherited eye disease, although some are not recognized until several years of age. Routine tests such as complete blood cell count, chemistry screen, and urinalysis may suggest some specific hematological or metabolic disorders or rule out many acquired disorders. Furthermore, clinical function studies may more clearly define a gastrointestinal, liver, kidney, or endocrine problem. Histopathology and/or electron microscopy of a tissue biopsy from an affected animal or from the necropsy of a littermate or relative may give the first clue to a genetic defect.

A few laboratories provide special diagnostic tests that allow a specific diagnosis of an inborn error of metabolism. Inborn errors of metabolism include all biochemical disorders due to a genetically determined, specific defect in the structure and/or function of a protein molecule. Disorders of intermediary metabolism typically produce a metabolic block in a biochemical pathway leading to product deficiency, accumulation of substrates, and production of substances via alternative pathways. The most useful specimen to detect biochemical derangements is urine because abnormal metabolites in the blood will be filtered through the glomeruli, but fail to be reabsorbed, as no specific renal transport system exist for most abnormal metabolites. The Metabolic Genetic Disease Laboratory at the University of Pennsylvania offers such tests [http://research.vet.upenn.edu/penngen](http://research.vet.upenn.edu/penngen). Similarly Cornell’s Comparative Coagulation Laboratory offers functional testing for many bleeding disorders ([http://ahdc.vet.cornell.edu](http://ahdc.vet.cornell.edu)) and the Comparative Neuromuscular Laboratory makes some functional and mostly histological analysis available for muscle and nerve disorders ([http://vetneuromuscular.ucsd.edu](http://vetneuromuscular.ucsd.edu)). Once the failing system has been identified, the defect can be determined at the protein level. Homozygously affected animals have very low protein activity and/or quantities (0-10%). These tests may also be used to detect carriers (heterozygotes), who typically have intermediate quantities at the protein level (30-70%), but no clinical signs. Unfortunately, protein assays require submission of appropriate tissue or fluid under special conditions to specialized laboratories along with a control sample, and are labor intensive.

Many DNA screening tests have been developed. These tests are mutation or DNA marker specific and can, therefore, only be used in animals suspected to have the exact same gene defect. Small animals within the same or a closely related breed will likely have the same
disease-causing mutation for a particular disease. However, dogs and cats as well as unrelated breeds of a species with the same disorder will likely have different mutations. On the other hand, a few mutations have been found in a few breeds or may be widespread within the canine population. For instances different mutations have been found to cause anemia due to pyruvate kinase deficiency in the different breeds, while a single mutation in the phosphofructokinase gene has been found to cause hemolytic anemia in English Springer Spaniels, Cocker Spaniels, Whippets, and mixed breed dogs. For many inherited disorders, the defective gene remains unknown; however, for a few, a polymorphic DNA marker that is linked to the mutant allele has been discovered. Some mutation and linkage tests have to be further defined such as renal dysplasia in several terrier breeds. At present, mutation-specific and some linkage tests are available only for single gene defects in small animals; however, complex genetic traits may also soon be approached by these methods. Many predispositions such as inflammatory, immune-mediated, malignant disorders have a genetic basis. While many more single gene defects are being studied from clinical signs to the molecular defect, current investigations are shifting toward complex genetic traits. The many breed predispositions for various complex genetic traits are particularly attractive to further define their molecular bases.

DNA tests have several advantages over other biochemical tests. The test results are independent of the age of the animals, thus, the tests can be performed at birth or at least long before an animal is placed in a new home as well as before clinical signs become apparent. DNA is very stable and only the smallest quantities are needed; hence, there are no special shipping requirements as long as one follows the specific mailing instructions for biological products. DNA can be extracted from any nucleated cells, e.g., blood, buccal mucosa (using cheek swabs), hair follicle, semen, and even formalinized tissue. For instance, blood can be sent in an EDTA tube or a drop of blood can be applied to a special filter paper; buccal swabs can be obtained with special cytobrushes – the cheek cells and not the saliva is needed and swabs need to be completely dried. The DNA segment of interest, which is surrounding the mutation, is amplified with appropriate DNA primers utilizing the polymerase chain reaction (PCR). The mutant and/or normal alleles are identified by DNA fragment size or base pair differences. These tests are generally simple, robust, and accurate as long as appropriate techniques and controls are used. Furthermore, they can be used not only for the detection of affected animals, but also for carriers from birth on. All currently available DNA tests for hereditary diseases in dogs and cats and associated laboratories worldwide can be found at http://research.vet.upenn.edu/WSAVA-LabSearch. Furthermore panel screening for all reported mutations has been reported and may be most cost effective as long as one is assuring that the mutation found also causes disease in another breed and genetic counseling is provided.

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RED BLOOD CELL MORPHOLOGICAL ABNORMALITIES IN PERIPHERAL BLOOD

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Systematic evaluation of red blood cell (RBC) morphology in a blood smear includes assessment of abnormalities in pattern, size, color and shape, identification of inclusions and quantification of nucleated red blood cells (NRBC). Although some information is available from automatic analysis, morphological smear examination cannot be replaced by any other test.

**Abnormalities in pattern:**
Agglutination - three-dimensional aggregations of RBC resembling grapelike clusters that are formed due to the binding of antibodies to RBC surface (immune-mediated hemolytic anemia (IMHA), EDTA pseudo-agglutination in cats).
Rouleaux formation - RBC aggregations forming linear arrangements resembling stacks of coins indicating hyperglobulinemia and/or increased fibrinogen concentrations (under reactive conditions or in lymphoproliferative disorders).

**Abnormalities in size (anisocytosis, macrocytosis, microcytosis):**
Abnormalities in RBC size in a blood smear correspond to changes in diameter and not in volume as measured by hematologic analysers. Cells can appear smaller but be of normal volume whereas other cells appear bigger because they are thinner and more spread. Low numbers of larger or smaller RBC do not increase, resp. decrease the mean cell volume (MCV) out of reference interval. Beside sideropenic anemias, microcytosis is a common laboratory finding (85% of dogs) in patients with portosystemic shunt (Niles et al. 2001). Macrocytosis without reticulocytosis is a rare finding.

**Abnormalities in color (polychromasia, hypochromasia, ghost cells):**
Hypochromasia - RBC with enlarged central pallor due to decreased hemoglobin (Hb) content caused by defective Hb production or inhibition of Hb production. The iron deficiency anemia in dogs is accompanied by RBC shape abnormalities suggesting fragmentation as a result of diminished deformability. Hypochromic RBC must be distinguished from torocytes, an artifactual RBC change that mimics hypochromasia but has no diagnostic relevance.
Polychromasia - immature RBC (reticulocytes) with diffusely basophilic cytoplasm due to high amounts of ribosomes corresponding to active erythropoiesis.
Ghosts cells - hemolysed RBC with the remaining membranes seen as “ghosts” in intravascular hemolysis due to oxidant injury, hypophosphatemia, erythroparasites, bacteria (Clostridia, Leptospira) or snake envenomation (Masserdotti 2009).
Abnormalities in shape (poikilocytes, echinocytes, acanthocytes, schistocytes, keratocytes, eccentrocytes, pyknocytes, spherocytes, target cells, stomatocytes, elliptocytes, drepanocytes):

Poikilocytes - general term for RBC with abnormal shape. Low numbers of misshapen RBC can be seen in blood from clinically healthy dog or cat.

Echinocytes - spiculated RBC with numerous short, sharp or blunt projections of uniform length and evenly spaced around the cell membrane. Echinocytes are often formed as artefacts (resulting from cell aging or exposure to excessive concentrations of EDTA). Echinocytosis is expected when increased pH or cell dehydration (low intracellular potassium), in inherited red blood cell disorders (decreased ATP production), in renal diseases, under drugs expanding the outer leaflet of the membrane (salicylates, phenylbutazone, furosemide, doxorubicin) or after snake envenomation (resulting from ATP depletion by ATPase enzymes and alteration of membrane by phospholipases) where echinocytes with 92% positive dogs were a consistent finding (Hackett et al. 2002).

Acanthocytes - RBC with irregularly sized spicules characterized by 2-20 membrane projections unevenly distributed over the cell surface. Acanthocytes occur through fragmentation injury (disruption due to abnormal vasculature) or alterations in lipid composition of the RBC membrane (abundant membrane due to high cholesterol to phospholipid ratio). Due to their rigidity, they are less deformable and may have a shorter life span resulting in hemolytic type of anemia. Acanthocytes were observed in dogs with hemangiosarcoma (HSA), lymphoma, osteosarcoma, gastrointestinal, musculoskeletal, renal, immune-mediated and different other diseases (Warry et al. 2013). Mean number of acanthocytes in HSA with 54% positive dogs was significantly higher than in other disorders (Tant et al. 2004).

Schistocytes - red blood cell fragments reflecting mechanical injury to erythrocytes associated with conditions in which the normally smooth endothelium is irregular, the vascular lumen is crossed by fibrin strands or the blood flow is turbulent. Schistocytes suggesting evidence of microangiopathic hemolysis are seen in disseminated intravascular coagulation (DIC), vasculitis, vascular neoplasm, glomerular diseases, portosystemic shunts, iron deficiency. In dogs with HSA they occurred in 46% of patients, in which severe schistocytosis was presented by primary spleen or liver neoplasia (Hammer et al. 1991). Schistocytes are rarely seen in cats with DIC, more common they occur in liver diseases.

Keratocytes - RBC with one or two “horn-like” projections or with a “bite-shaped” defect in the cell outline. Keratocytes can indicate fragmentation injury associated with microangiopathic hemolysis (DIC, vasculitis, HSA) or mechanical fragility (iron deficiency anemia), oxidant injury or liver disease.

Eccentrocytes - RBC with dense Hb contracted to one side of the cell leaving a pale eccentric area formed as a result of direct oxidative damage to the RBC membrane and cytoskeleton. The prevalence of eccentricytosis in diseased dogs is generally low (1.4%) and is associated with drug administration, onion/garlic ingestion, vitamin K antagonist intoxication, diabetes mellitus, T-cell lymphoma and severe infections with young dogs and whippets more likely to be affected (Caldin et al. 2005).

When the thin membrane of the eccentricyte is removed or ruptures, a small cell lacking central pallor is formed. These “pyknocytes” can be mistaken for spherocytes.

Spherocytes - erythrocytes of a spheric form that appear smaller in diameter and more dense with reduced area of central pallor. They are produced when antibody coating RBC bind to
the Fc portion of macrophages resulting in partial phagocytosis of the RBC. The remnant has a reduced surface area to volume ratio and assumes a sphere shape. Moderate to marked spherocytosis is characteristic of immune-mediated hemolytic anemia. Although low numbers can be seen in some dogs and others may have no spherocytes at all. In dogs with regenerative IMHA spherocytosis is seen in 89% but there are not as frequently seen in non-regenerative forms of IMHA or the precursor-directed IMHA (Weinkle et al. 2005). Low numbers of spherocytes can be seen in many other acquired diseases as fragmentation anemias, oxidative injury, coral snake envenomation, bee stings, hemophagocytic syndrome.

*Target cells* (codocytes or leptocytes) - RBC having a condensed Hb within the area of normal central pallor, resembling a “bullseye” and reflecting an increased surface to volume ratio. Their deformability is normal but they are more resistant to osmotic lysis. Increased numbers of target cells can be an indicator of a balanced increase in cholesterol and phospholipids in the RBC membrane occurring in liver disorders or hypothyroidism (Goodfellow et al. 2008).

*Stomatocytes* - RBC with an elongated “mouth-like” or “slit-like” area of central pallor. An occasional stomatocyte might be seen in several pathological conditions (liver disease, lead poisoning) or with higher percentage (up to 19%) in hereditary stomatocytosis (Bonfanti et al. 2004). Stomatocytes are usually macrocytic and hypochromic, osmotically and mechanically fragile because of the increased volume to surface area ratio.

*Elliptocytes* - elongated RBC. Elliptocytosis can indicate liver disease (lipidosis in cats), myelofibrosis (non-regenerative forms of IMHA) or inherited/congenital red blood cell membrane abnormality.

*Drepanocytes* - sickle-shaped RBC. The sickling is thought to be an in vitro phenomenon due to variants of hemoglobin forming insoluble, elongated polymers when blood exposed to oxygen or due to alkalosis.

**Inclusions:**

*Howell-Jolly bodies* - small nuclear remnants persisting usually as a single basophilic perfectly round inclusion within the cytoplasm. In canine blood they are not routinely observed because they are effectively removed in the spleen. Occasionally they can be seen in healthy cats because of the non-sinusoidal spleen which is less efficient at removing such RBC. In both species Howell-Jolly bodies are commonly observed in regenerative anemias, compromised or absent splenic function (corticosteroids, splenectomy) or erythroid dysplasia (myelodysplastic syndrome).

*Basophilic stippling* - small aggregations of ribosomal RNA in the cytoplasm that may occur in regenerative anemias, lead poisoning or dyserythropoiesis (Williams and Williams 1990, Lukaszewska and Lewandowski 2008).

*Cabot rings* - rarely observed ellipsoid or figure 8 structures originating from mitotic spindles described in association with severe dyserythropoiesis (Lukaszewska and Lewandowski 2008).

*Heinz bodies* - precipitation of hemoglobin as a result of oxidative injury in form of a round inclusion(s) attached to the inner erythrocyte membrane usually extending above the surface. Small Heinz bodies can be observed in healthy cats (in amount ≤10%), but not in blood from healthy dogs. They stain prominently with new methylene blue or brilliant cresyl blue. RBC containing Heinz bodies distorting the membrane have reduced lifespan. Increased numbers of Heinz bodies may be seen in cats with diabetes mellitus, lymphoma or hyperthyroidism. In dogs Heinz body formation is associated with onion and garlic ingestion, zink, copper and
skunk mask toxicity as well as propofol, vitamin K₃, benzocaine, phenylhydrazine or acetaminophen administration (Yamato et al. 2005, Zaks et al. 2005, Bexfield et al. 2007).

**Nucleated RBC:**
Nucleated RBC are most commonly identified in the context of regenerative (mainly hemolytic) anemia. However they can be seen in many other conditions without anemia as altered splenic function, bone marrow disorders, heat stroke, septicemia/endotoxemia, lead poisoning and different organ diseases. Their finding reflects increased erythropoiesis, decreased removal from circulation or bone marrow injury. Erythroblastosis occurring in almost 42% of dogs with systemic inflammatory response syndrome is associated with significantly higher mortality (Müller et al. 2014). With 90% positive dogs evidence of NRBC was the most prevalent abnormality in patients with heat stroke with significantly higher NRBC and proportion of rubricytes found in non survivals (Aroch et al. 2009).
SEPSIS AND COAGULATION DISORDERS

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Sepsis is a clinical syndrome defined by clinical and laboratory findings and characterized as overwhelmed systemic response of the body to the injury caused by pathogens. Definition of the sepsis in human medicine has been established in 1991 at an AACCP / SCCM meeting and consequently has been undertaken to the veterinary medicine. Sepsis has been defined as systemic inflammatory response syndrome (SIRS) with confirmed infection. SIRS is present, if two or more of following criteria are fulfilled: tachycardia, tachypnoe, hyper/hypothermia, leukocytosis/leukopenia or increased number of neutrophil bands. Concrete cutoff values in dogs and cats vary among the studies, but one of the most common are shown in table 1.

During SIRS, activation of immune and other cells leads to the release of pro-inflammatory cytokines. Cytokines are mediators of immune response: they stimulate endothelial cells to expression of adhesion molecules for leukocyte priming, stimulate production of reactive oxygen species in neutrophils, activate acute phase protein synthesis in the liver and, last but not least, they are able to change thrombotic properties of endothelial cells. Under normal circumstances, endothelial cells have anti-thrombotic properties – they produce prostaglandin I which inhibits activation, aggregation, adhesion and secretion of platelets and cause relaxation of the vessel wall, thrombomodulin which activates (anticoagulant) protein C or release heparin-like molecules, which stimulate antithromin. After activation, endothelial cells are pro-coagulant: they release von Willebrand factor (molecule essential for platelet adhesion), tissue factor (activator of coagulation cascade) and decrease the amount of plasminogen activator inhibitor. This leads to activation of factor X, XII, prekallikrein and kininogen. During sepsis, macrophages, monocytes and neutrophils are sources of tissue factor, initiator of coagulation cascade. Expression of tissue factor is there controlled by activated factor X and FXa seems to be a direct modulator of coagulation activity. Tissue factor binds to FVII and activates FX, which generates thrombin. Thus, in bacterial sepsis, thrombin can be generated in absence of endothelial injury. Thrombin amplifies its own production, activates platelets and promote clot formation. Activated platelet and monocytes shed PS bearing microparticles which accelerate and disseminate trombin formation. Exception.

<table>
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<th>SIRS if ≥2 are present</th>
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<td>pulse</td>
<td>respiratory rate</td>
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this, pattern- and damage-associated molecular patterns (eg. lipopolysacharide) trigger tissue-factor expression on monocytes and neutrophil extracellular trap release by neutrophils promoting immunothrombosis. Although immunothrombosis plays a role in host defence against bacterial dissemination, uncontrolled immunothrombosis may also lead to disseminated intravascular coagulation (DIC). Furthermore, studies on coagulation and sepsis revealed depletion of tissue factor pathway inhibitor (TFPI) in early sepsis worsening pro-coagulant state. Lack of inhibition facilitates dissemination and progression. Moreover, if the severity of the infectious disease is the same, coagulopathy of infectious disease in surgically patients is increased by addition of the coagulation disorder due to surgical stress. In treatment of basic disease, the surgeons and intensivists must take that coagulopathy of the surgical stress deteriorates DIC temporarily into consideration.

Clinical signs of DIC vary. Its presentation depends on the phase of the DIC development. The gold standard test is evidence of thrombi formation in vessels during biopsy or necropsy, however, fibrin thrombi lyse rapidly after death. Clinically, DIC can be divided into „non-overt“ and „overt“ DIC. During non-overt DIC, coagulation system is activated, but not overwhelmed, generally with signs of hypercoagulability. Diagnosis in this phase is challenging – thrombi detection is sometime possible using Doppler ultrasonography, or in the case of pulmonary thromboembolism there can be an alteration in the blood gas results. Laboratory diagnostics in this phase is not very helpful. Sometimes, there can be already elevation of D-dimers. Viscoelastic methods (thromboelastography) might be useful in some patients, but one must be careful in interpreting when the concurrent hypercoaguable disorder is present (e.g. anemia). Repeated coagulation tests might reveal kinetic pattern of DIC and are highly recommended.

Over DIC is characterized by clinical manifestations with evident thrombosis and/or hemorrhage. Thrombosis is much more dangerous state than hemorrhage what should be reflected in the therapy. Widespread thrombosis leads to the hypoperfusion, organ failure, MODS and death. Hemorrhage as a clinical sign of DIC can be present in dogs, but rarely in horses and cats. The cause of the different phenotype of the DIC is suggested to be associated with different levels of plasminogen activators and inhibitors. Laboratory diagnosis is based on the magnitude and kinetics of laboratory changes. Routine (PT, aPTT, TCT, platelet count, FDP) and special (AT activity, TEG) coagulation test are recommended and the DIC positive patient is supposed to have at least 2 abnormal tests together with confirmed underlying disease associated with DIC. Contrary to the human medicine, fragmented red blood cells (schistocytes) are not routine finding in DIC.

The goal of DIC treatment is to keep stable perfusion of the organs thus anticoagulant therapy was thought to be therapy of the choice. However, clinical studies in human and veterinary medicine are not providing consistent successful results with heparin therapy. Clopidogrel is not helpful because platelet function is already impaired due to fibrin degradation products. Inhibitors of coagulation, e.g. protein C, are not available in veterinary medicine. Beside anticoagulant therapy, anti-inflammatory therapy is crucial in the patients with sepsis.

References upon request.
HEMOLYTIC ANEMIA: IS IT IMMUNE-MEDIATED?

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Introduction
Immune-mediated hemolytic anemia (IMHA) is one of the most common and serious hemolytic anemias in dogs, but occurs rarely in other animal species. In IMHA an immune response, including anti-erythrocytic antibodies, complement and macrophages, targets directly or indirectly erythrocytes and a hemolytic anemia ensues. There are many triggers for IMHA such as infections, drugs and other agents, and cancer leading to secondary IMHA, but in many dogs no cause is identified (so-called idiopathic, autoimmune or primary IMHA) or a genetic predisposition has been proposed (Cocker spaniels). Furthermore, alloimmune hemolytic anemias, such as hemolytic transfusion reactions, both acute and delayed, and neonatal isoerythrolysis (only litters from transfused bitches), are caused by specific anti-erythrocytic alloantibodies. In contrast to other species, dogs with IMHA also develop an often overwhelming inflammatory response resulting in thrombosis and necrosis of various organs. And while the anemia can be corrected with transfusions, these complications in dogs are causing severe morbidity and mortality despite aggressive immunosuppression and antithrombotic interventions.

This session will review the diagnostic approach to IMHA with special emphasis on the Coombs’ test including the authors’ comparative prospective study on different in-clinic and laboratory Coombs’ tests. Other hemolytic anemias such as infectious, inherited and toxic causes need to be carefully excluded.

Immune Destruction of Erythrocytes
Regardless of the underlying cause, IMHA results from a breakdown in immune self-tolerance or from a deficit in the control mechanism that regulates B and T lymphocyte activity as well as macrophage reactivity. Immune destruction of erythrocytes is initiated by the binding of IgG or IgM antibodies to the surface of erythrocytes. Under most clinical circumstances, immune destruction is an extravascular process that depends on recognition of erythrocytes opsonized with IgG, IgM and/or complement by specific receptors on reticuloendothelial cells. Macrophages with engulfed erythrocytes may be noted on cytological examination of blood and tissue aspirates as erythrophagocytosis, but this is not definitive proof of an immune-mediated process. Antibody-coated erythrocytes may also be lysed by complement fixation and the membrane attack complex, which is clinically noted as intravascular hemolysis.

A diagnosis of IMHA must demonstrate accelerated immune destruction of erythrocytes. Evidence of a hemolytic anemia is suggested clinically by icterus and a regenerative anemia with hyperbilirubinuria, and hemoglobinemia and hemoglobinuria refers to an intravascular process. However, the erythroid response in the bone marrow may be blunted by the immune and inflammatory process or the underlying disease thereby leading to non-regenerative anemias. Besides documenting a hemolytic anemia, one or more of the following three hallmarks must be present to support a diagnosis of immune-mediated hemolysis: persistent
autoagglutination, marked spherocytosis and a positive direct Coombs’ test result. As in human medicine, the Coombs’ test should be considered the best test to definitively diagnose IMHA, although marked spherocytosis and persistent/true autoagglutination (after 3x washing of EDTA blood with saline) are other important parameters indicating immune-destruction of erythrocytes.

**Autoagglutination**

Anti-erythrocytic IgM and in large quantities IgG antibodies may cause direct erythrocyte autoagglutination. The autoagglutination may be seen by naked eye in an EDTA tube or on a glass slide or may become apparent as small clumps of erythrocytes on blood smears. For yet unexplained reasons, canine erythrocytes have a tendency to unspecifically agglutinate in the presence of plasma and colder temperatures as well as possibly with excessive EDTA anticoagulant. Mixing blood with one drop of saline may break up rouleaux formation but not other forms of unspecific red cell agglutination. It is, therefore, important to determine whether the agglutination persists after “saline washing”, which has been coined persistent or true autoagglutination. This is accomplished by adding physiologic saline to the tube containing a small amount of EDTA-anticoagulated blood, mixing, centrifuging and removing the supernatant including the plasma and repeating this saline washing 3 times. True or persistent autoagglutination is indicative of an immune process, but precludes the performance of Coombs’ test or blood typing and crossmatching procedures which are based upon an agglutination reaction as result. Those based upon chromatographic techniques do not seem to be affected by autoagglutination as free red cells can move along the strip. If the agglutination breaks up after washing, the Coombs’ test is expected to be positive, if it is a case of IMHA. There is no evidence for washing away red cell bound antibodies in dogs.

**Spherocytosis**

If erythrocytes are only partially phagocytized or lysed by complement in circulation, erythrocytes with reduced surface area to volume ratio, known as spherocytes, are formed. They appear spherical and microcytic with no central pallor and are considered fragile. Note proper areas on the blood smear needs to be reviewed to find spherocytes in between single regular discoid red cells. Large numbers of spherocytes (>20/microscopic high power field) are nearly diagnostic for IMHA, whereas small numbers may be seen with other conditions including DIC, endotoxemia and zinc intoxication. In our experience all dogs with marked spherocytosis and suspected to have IMHA also had a positive Coombs’ test. However, only 60-80% of dogs with a positive Coombs’ test or clinically diagnosed with IMHA had marked spherocytosis. Hereditary spherocytosis due to genetic membrane defects has rarely been seen in dogs, but should be considered as a differential diagnosis in dogs with negative Coombs’ test results.

Because of the difficulties with the Coombs’ test (see below), Slappendale had proposed to use the erythrocytic osmotic fragility test at specific saline concentrations as a mean to diagnose IMHA and this test is currently used in various clinics in Europe. However, there are many other reasons for increased fragility of erythrocytes beside IMHA including hereditary red cell defects. This test is not used in human medicine and has not been shown to be superior to determination of marked spherocytosis and a positive Coombs’ test in dogs with IMHA. The osmotic fragility test is also a cumbersome and not well standardized technique.
Positive Direct Coombs’ Test Result

The direct Coombs’ test is also known as direct antiglobulin test (DAT) and is used to detect antibodies and complement on the surface of erythrocytes when the anti-erythrocyte antibody strength or concentration is too low to cause spontaneous agglutination (subagglutinating titer). Separate canine-specific IgG, IgM, and C3b antibodies as well as polyvalent antiglobulin reagents are available. They are added at various concentrations after washing the patient’s erythrocytes free of plasma (3x as shown above) and mixtures are generally incubated at room temperature or 37°C (cold agglutinins appear to be rarely of clinical importance and rarely cause hemolysis). The strength of the Coombs’ reaction does not necessarily predict the severity of hemolysis, but reaction changes are useful in monitoring the disease.

Typically tube or microtiter methods have been used exclusively in the reference or teaching laboratory setting, but a flow cytometric method has also been introduced in a couple of places. A standardized, sensitive, and simple gel column method was available by DiaMed (Switzerland), but unfortunately the company was sold to another company which decided to not pursue the veterinary market. A novel standardized antiglobulin test method has just been developed by Alvedia (France) similar to the immunochromatographic strip technique for blood typing of dogs and cats (see updates on blood typing and crossmatching). Although many commercial laboratories offer Coombs’ testing for dogs, clinicians have questioned the tests sensitivity and specificity and often forgo the test and/or use response to therapy as a diagnostic. However, negative Coombs’ test results may be seen because of technical reasons, insufficient quantities of bound antibodies, the presence of weakly bound antibodies, or the disease in remission. The Coombs’ test stays positive for days to months after initiating treatment. A few days of immunosuppressive therapy will likely not reverse the Coombs’ test result, as unlikely a transfusion would cause a positive Coombs’ test result. Thus, dogs with negative Coombs’ test results should be reevaluated for other causes of hemolytic anemia.

In a recent prospective study of anemic and non-anemic dogs we compared various direct Coombs’ test methods including microtiter plate assays, gel column, capillary, and immunochromatographic techniques using polyvalent antiglobulins in a laboratory setting and found excellent correlations between tests and with spherocytosis and without noticeable interference by immunosuppressive or transfusion therapy in anemic dogs.

In conclusion, a diagnosis of IMHA requires the documentation of red blood cell destruction and an immune process. While regenerative anemia, icterus, and hyperbilirubinuria are suggesting a hemolytic anemia, evidence of true autoagglutination, spherocytosis, and/or a positive direct Coombs’ test are required to document immune destruction. The authors also recommend monitoring IMHA patients for the disappearance of these immunological parameters to adjust and taper therapy.

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**BONE MARROW CYTOLOGY**

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**Introduction:**
Cytological examination of bone marrow aspirate is still not used as often as could be and sometimes is falsely indicated. Besides that, veterinarians are not providing it, the owners are reluctant because of it is supposed to be much invasive. In fact, the bone marrow cytology is irreplaceable in some clinical instances. The sampling is technically easy and need just some practice, and could be done in dogs in local anesthesia only. However the cytological examination needs experienced cytologist and the interpretation then close cooperation with clinician. Core biopsy needs general anesthesia and histological examination of core biopsy gives more information about the architecture of the tissue, quantification of the cell numbers and iron deposits, but is less informative in the morphology of the individual cells.

**Possible clinical indications**
1. Non-regenerative or semi-regenerative anemia
2. Thrombocytopenia, even severe with signs of bleeding
3. Persistent neutropenia
4. Pancytopenia
5. „true“ polycythemia, without dehydration
6. Unexplainable severe thrombocytosis
7. Unexplainable persistent leukocytosis esp. lymphocytosis
8. Morphological abnormalities of the blood cells (e.g. nucleated red blood cells without associated anemia)
9. Fever of unknown origin
10. Diagnostic and/or monitoring of leishmaniosis
11. Unexplainable hypercalcemia (suspected lymphoma/leukemia)
12. Unexplainable hyperglobulinemia (suspected multiple myeloma)
13. Part of the staging of some tumors, esp. lymphoma or mast cell tumors.

**Contraindications**
Aggressive patient, which could not be put in general anesthesia

**Sampling technique**
1. Location of the aspiration: the most practical iliac crest, proximal humerus
2. Local anesthesia: usually lidocain 2%, topically on the periosteum.
3. Aspiration: needle 16 - 18 G needle with stylet. With clockwise - counterclockwise movement is needle insert deep in the marrow, then aspirated with force into the 20ml syringe.
4. Slide preparation: immediately after collection the drop of the sample must be put on the glass and with smear technique are produces at least two slides.
5. Staining: after drying the slides are stained precisely with some advanced staining type, I use the May-Grunwald-Giemsa staining.

**Systematic approach to evaluate the aspirate**
Describing the bone marrow slide needs certain amount of theoretical and practical experience. According to my experience when starting with bone marrow cytology there is always supervision by advanced cytologist requisite.

1. Quality of the sample, blood contamination, presence of the marrow particles (flecks – non-cohesive clusters of the marrow cells), quality of the staining.
2. Cellularity must be evaluated on particles only. Normal proportion is about 50% hematopoietic cells and 50% fat. More than 75% of the cell is considered hyper- and less than 25% as hypocellular sample. Influence of the age must be taken into account.
3. Number of megakaryocytes. The average is about 5 cells on one mean marrow particle.
4. Description of the erythroid series: presence of all types in the appropriate relation and morphology. The rule is that the majority of the cells are matured (rubicytes a and metarubricytes) and the “young” (rubriblasts) are rare – orderly maturation. Mild increase in immature cells is called left shift. Irregular maturation is called maturation arrest.
5. Description of the myeloid series: presence of all types in the appropriate relation. Bands and segs should be about 80%. Total predominance of blasts is suggestive for leukemia.
6. Myeloid/erythroid ratio. Should be counted or at least estimated on 10 fields. The normal range is wide (0,75/1 to 2/1).
7. Presence of plasma cells; more than 3% is suggesting of immune mediated process, more than 20% with signs of malignancy indicate multiple myeloma
8. Presence of lymphocytes/lymphoblasts. The count should not exceed 5-10%.
9. Presence of other cells; mast cells, histiocytes, metastatic neoplastic cells.
10. Parasites and infection, e.g. Leishmania, Ehrlichia/Anaplasma, Mycobacterium.
11. Summary of the findings – cytological diagnosis, e.g hyperplasia or dysplasia
12. Clinical interpretation, recommendation of further diagnostic steps or therapy

Frequent diseases diagnosed by the bone marrow aspirate (after study by D. Weiss, 2006)

- Secondary bone marrow diseases (26%) – hyperplasia because of bleeding, infectious or immune mediated diseases or hypoplasia because of renal failure, chemotherapy or sepsis
- Non-neoplastic, non-dysplastic primary marrow diseases (24%) – immune mediated anemia (maturation arrest, pure red cell aplasia) and thrombopenia, SLE, hemophagocytic syndrome, necrosis of the marrow (drug associated, idiopathic), iron deficiency anemia
- Malignant tumors (18%) – leukemia, lymphoma, multiple myeloma, malignant histiocytosis. Others (carcinoma, mast cell tumor and sarcoma) are rare.
- Myelodysplastic syndrome (MDS) and dysmyelopoiesis (9%) – secondary are more common than primary MDS
- No pathology or not diagnostic 23%

Literature: by author
IMMUNOPHENOTYPIC CHARACTERIZATION OF CANINE MALIGNANT LYMPHOMA IN POLAND

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Introduction: Lymphomas are a heterogeneous group of cancers of the lymphoid system. The main method of treating lymphoma is chemotherapy but the effectiveness of the treatment depends on the type and individual characteristics of cancer cells. These may include the ability to excessive proliferation or resistance to apoptotic signals. Availability of well-defined, canine specific antibodies for flow cytometry offers the possibility to characterize canine leukocyte antigens and lymphoid population and this is very important in lymphoma/leukemia diagnosis. As clonality is the hallmark of malignancy, molecular methods such PCR that determine the presence of clonal gene rearrangements of B- or T-cell receptors are very helpful. As the ability of anticancer drugs to induce apoptosis depends on the expression of pro- and anti-apoptotic proteins in cancer cells, western blotting could be used to evaluate the expression of caspases, Bcl-2 family proteins, nuclear receptors or various kinases and transcription factors. Due to substantial heterogeneity within each group of cancers and different patient response to treatment, the therapeutic effects are expected to be improved by using ex vivo drug sensitivity testing that enables a clinician to select drugs with the highest anti-tumor activity or to eliminate the less effective ones.

Aims: The aim of our study was to determine the incidence of high-grade B- and T-cell lymphomas in the population of examined dogs in Poland, accurate characterization of malignant lymphocytes, and an attempt at finding the most common changes in the expression levels of proteins involved in apoptosis. In vitro tests for assessing chemosensitivity of canine high grade primary lymphoma cells to various cytostatic drugs were also evaluated.

Results: From among 109 collected samples, 86 lymph node FNA samples were included in this study. The frequency of different types of canine high grade lymphomas was 71% for B-cell type, 17% for T-cell type and 12% were classified as mixed or null cell type (cells not expressing CD3 and CD79a) lymphomas. PARR assay was performed for a total number of 45 samples. Ten cases were finally classified as mixed or null cell type and 14 were excluded from the study. In the remaining 19 samples, PARR assay results corresponded to the cytometric diagnosis. Clonal gene rearrangements of both B- and T-cell receptors were found in two cases.
All cases of B-cell lymphomas were positive for CD79α, CD45, and CD45R. Fifty-seven of these cases (93%) were positive for CD21 and MHC class II. Eight cases (13%) were positive for CD5. Among B-cell lymphomas, in eight (13%) cases the expression of hematopoietic precursor antigen CD34 was detected. Unusual phenotypes were identified in 26 (39%) cases of B-cell lymphoma. The most common findings, beyond the expression of CD34, included diminished or absent expression of MHC class II (6%) and positive expression of CD8 (8%). T-cell lymphomas were diagnosed in 15 (17%) dogs and all these cells were positive for CD45 and CD3. CD45R was expressed in 13 (87%) of the examined samples. Twelve samples (80%) were positive for CD4 and nine (60%) for CD5. No lymphomas derived from cytotoxic T-cells (CD8+) were found. Aberrant phenotypes were reported more often in T-cell lymphomas than in B-cell lymphomas, accounting for 65%. The lack of CD5 expression, found in six of the examined cases (40%), constituted the predominant aberrancy. In two samples, a co-expression of T- and B-cell markers (13% of T-cell lymphomas expressed also CD79α) were found. Considering acute, severe clinical course of the disease and poor response to treatment typical for T-cell lymphomas, both cases were classified as T-cell lymphoma.

Expression of several pro- and anti-apoptotic proteins in canine lymphoma cells affecting the efficacy of chemotherapy was analyzed by western blotting. Results were highly variable but revealed differences between B and T cell types. Seventy four percent of B type hyperplasia cases were characterized by elevated levels of Bcl-2, decreased expression of procaspase-3 and activation of NF-kB. Constitutive activation of NFkB is associated with tumor development and progression and it is proposed that high expression of Bcl-2 proteins may be a consequence of NFkB activation. At the same time, more than 90% of malignant T lymphocytes showed increased levels of Bcl-2, Bcl-xl, Mcl-1, procaspase 3 and c-Abl. In general, T-type cells are more resistant to chemotherapy and in some cases the resistance to chemotherapy was noticeably associated with much lower expression of caspase-3.

In in vitro chemosensitivity tests, T-cells exhibited significantly lower sensitivity to the majority of tested substances than B-cells. These results correlate with a clinical investigation showing poorer prognosis for high grade T-cell canine lymphoma, and with the results of similar studies in established canine lymphoma and leukemia cell lines.

Research focusing on detailed characteristics of canine lymphoma/leukemia cells by using additional diagnostic techniques such as flow cytometry, PCR, western blotting or in vitro chemosensitivity test may be an important tool in the diagnostic process as well as in the development of new therapeutic strategies for the treatment of hematopoietic cancers in dogs. As a dog is a suitable model for the study of human NHL, such studies may also be useful in human medicine.
EXPERIENCE WITH PELGER-HÜET ANOMALY

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Introduction: Pelger-Hüet anomaly is an inherited disorder of maturation of myeloid cells, in particular granulocytes and monocytes. Nucleus hyposegmentation, respectively hypolobulation, of these cells is the hallmark of this anomaly. Pelger-Hüet anomaly is often misinterpreted as inflammatory disease or preleukemic syndrome, which can lead to unnecessary diagnostics and treatment. Predisposition to Pelger-Hüet anomaly has been described in Australian Shepherd, Australian Cattle Dog, Basenji, Border Collie, Cocker Spaniel, German Shepherd and Samoyed.

Aims: The principal aim of our study was to estimate the prevalence of Pelger-Hüet anomaly in Australian Shepherds in the Czech Republic. The second aim was to perform grading according to Bowles in Pelger-Hüet positive and negative Australian Shepherds and to compare mean nuclear score between these two groups.

Results: In total, blood smears of 79 Australian Shepherds were evaluated. In 18 dogs (22.8 %), Pelger-Hüet anomaly was detected, 61 dogs (77.2 %) were Pelger-Hüet negative. In the USA, Pelger-Hüet anomaly was found in 9.8 % Australian Shepherds. Our results suggest that the prevalence of Pelger-Hüet anomaly in Australian Shepherds in some regions may be much higher and affect almost one quarter of dogs. In Pelger-Hüet positive dogs, mean nuclear score according was 2.56±0.26 in neutrophils and 2.58±0.29 in eosinophils. In 10 Pelger-Hüet positive dogs (55.6 %), monocyte hypolobulation was observed. In Pelger-Hüet negative dogs, mean nuclear score was 5.90±0.42 in neutrophils and 4.66±0.27 in eosinophils. Monocyte hypolobulation was not noted in any of these dogs. Calculation of neutrophil and eosinophil mean nuclear score revealed clear separation between Pelger-Hüet positive and negative dogs. Monocyte hypolobulation was less obvious than granulocyte hypossegmentation. Mean nuclear score values are in agreement with data obtained in Foxhounds and a Danish/Swedish Farmdog. So far, mean nuclear score have not been determined in Australian Shepherds. Our results suggest that especially neutrophil mean nuclear score can realibly distinguish Pelger-Hüet positive and negative dogs. Awareness of Pelger-Hüet anomaly is essential to avoid excessive diagnostics and treatment in Pelger-Hüet positive dogs. Pelger-Hüet anomaly has also consequences for dog breeders, as mating two Pelger-Hüet positive dogs can lead to mortality in up to 25 % puppies.

References available upon request.
COAG VET CCRP: EVALUATION OF A NEAR PATIENT CANINE SPECIFIC CRP MEASURING SYSTEM

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Introduction: In dogs, C-reactive Protein (CRP) is a major Acute Phase Protein (APP) with rapid and characteristic response for inflammatory stimuli making CRP a useful marker of ongoing inflammatory activity. CRP level changes mirror different origins of systemic inflammation (infections, oncological or postoperative complications etc.) and the effects of treatments. Thus, CRP is an important element of the inflammatory panel measurements in veterinary clinical laboratories. Recently laboratories almost exclusively use CRP tests utilizing antibodies raised against human CRP molecules, which can cause false results with canine serum. Moreover, since the CRP response and normalization of its level are rapid processes and following up of treatments requires multiple measurements, tests based on canine specific antibodies with quick response time and easy repeatability, that is canine specific near patient systems, has growing importance.

Aims: Clinical evaluation of Coag VET system with Coag VET cCRP test in comparison with a widely used laboratory CRP test and measurement of white blood count (WBC) as reference method.

Results: Analytical and diagnostic performance were evaluated with 70 dogs diagnosed with inflammation (1 result was excluded due to data loss) compared with 20 clinically healthy, non inflammatory patients. Determination of analytical performance of Coag VET cCRP included repeatability and reproducibility measurements of a serum sample of a dog with systemic inflammation. Repeatability was characterized with 8.77 % CV from 10 intraday results, while reproducibility was 8.69 % CV from 2 parallel results measured on 5 consecutive days. Diagnostic performance of both Coag VET cCRP and the laboratory test was characterized statistically with binary classifiers (sensitivity, specificity, accuracy, positive predictive values/PPV and negative predictive value/NPV), with different plots (scatter, difference and box plots) and with Cohen’s kappa compared to WBC count as reference. Cut-off level of Coag VET cCRP was 20 µg/ml while the laboratory test used 10 µg/ml cut-off level characteristic for human reagents. Sensitivity of Coag VET and the laboratory method was 63 and 74 %, respectively, specificities were 84 and 71 %, PPVs were 83 and 76 % while NPVs were 65 and 69 %, respectively. Accuracy was 72 % for both systems. Cohen’s kappa of the two methods was 0.48 signaling a moderately good equivalence. Difference plot showed no significant and systemic difference between the two methods. Box plots also indicate equivalence although Coag VET system showed normal distribution of the results while normality of the results of the laboratory method is weaker.

Conclusions: Coag VET cCRP test measured on Coag VET is a reliable alternative of laboratory canine CRP measurements making possible of near patient CRP determination that is monitoring the ongoing inflammatory process and the effects of treatments.
COMPARATIVE EVALUATION OF CLONALITY ASSAYS AND IMMUNOPHENOTYPING IN CANINE LYMPHOMA

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Introduction: Clonality assessment by PCR for antigen receptor gene rearrangements is an advanced diagnostic technique being recommended in lymphoproliferative diseases of companion animals, particularly in ambiguous cases. Clonality testing allows distinction between clonal and polyclonal lymphocyte populations by the assessment of diversity within the complementarity determining region 3 (CDR3) of the immunoglobulin (Ig) and T-cell receptor (TCR) genes.

Aims: The aim of this study was to compare the results of clonality testing with data obtained by flow cytometric immunophenotyping in dogs with lymphoma.

Materials and Methods: Dogs with lymphoma diagnosed by cytology and classified using flow cytometry were retrospectively included in this study. DNA was extracted from fine needle aspirates obtained previously for immunophenotyping. Clonality was evaluated using published primer sequences by amplification of the immunoglobulin heavy chain (IgH) gene rearrangements for B-cell clonality and T-cell receptor gamma chain (TCRG) gene rearrangements for T-cell clonality. PCR products were separated by agarose gel electrophoresis and visualized under UV light. Results were compared with the results of immunophenotyping.

Results: 15 dogs with lymphoma were included in the study. For B-cell lymphoma, a strong B-cell clonal result was present in 7/10 dogs; weak B-cell clonality was identified in 2/10 dogs. Clonality was not detected in one dog with a B-cell lymphoma. Strong T-cell clonal results were seen in all 5 dogs with T-cell lymphoma. Clonality for both T and B-cells was detected in one dog with a T-cell thymic lymphoma.

Conclusion: The results of this study indicate that clonality testing in comparison with flow cytometry is a sensitive (14/15 – 93%) diagnostic test for canine lymphoma. However, it cannot supersede flow cytometry for the assessment of the lymphocyte cell lineage as cross-lineage rearrangement can occur.
ALVEOLAR ECHINOCOCCOsis AS A DIFFERENTIAL DIAGNOSIs OF LIVER TUMORS IN A DOG

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Introduction

The casuistics of the Alveolar echinococcosis in a dog as the intermediate host is presented. This very rare finding was the first described case in the Czech Republic. In the westbohemian region an increased incidence of the tape worm Echinococcus multilocularis in foxes is currently detected, which represents the potential risk of infection to people and dogs. The number of positive findings of E. multilocularis in foxes detected by the Regional Veterinary Administration in Pilsen through the period of years 2008-2011 in individual districts of our region is increasing. The average prevalence is about 33%. In the Czech Republic this zoonosis is until this time very rarely found in people, but an increasing risk is assumed all over Europe.

The definitive hosts of Echinococi are Carnivores, mainly the fox, but also other canine and feline beasts. Infection is by ingestion of larvocysts in tissues of intermediate hosts. Tape worms then sexually mature in intestines of the definitive host and exclude extremely durable and resistant eggs. The Intermediate hosts are mainly Rodents, but potentially any warm-blooded creature, including people. Infection is by eggs from contaminated environment and in their tissues then create cysts causing alveolar echinococcosis.

Alveolar echinococcosis arised in an intermediate host is initially asymptomatic. In people symptoms arise about 10-25 years after the infection and can resemble tumors. On the contrary to the cystic echinococcosis, alveolar cysts arise in 99% only in the liver. Cysts are not well demarked to the surrounding tissue and due to the exogenous budding can spread also to other organs.

Aim: Case report

The female of the Labrador retriever, 4,5 years old, only 15,5 kg of the weight was referred with the suspicious abdominal mass. Main symptoms were the weight loss, progressive abdominal distention with a non differentiated formation in the epigastrium, repeatedly detected increased transaminases. Clinically there was a poor condition, no icterus, abdominal distention with a large palpable resistance in the epigastrium without pain.

Ultrasonographically a large cystic formation in a full contact with hepatic tissue with irregularly thick wall and heteroechogenic content was detected. The liver was generally small, without congestion, the gall bladder was filled without dilation of the ductus. The following exploratory laparotomy revealed a large cystic mass about 20 cm in diameter coming from the left liver lobe. A vigorous surgery including the partial lobectomy, cholecystodudonostomy and extirpation of multiple enlarged hepatic, gastric, duodenal and pancreatic lymph nodes was performed.
Results:
Macroscopically the cyst had a thick irregular wall about 1.5 cm in size and proliferating nodules into the lumen, the content of the cyst was watery yellowish fluid with fibrin. Histologic diagnosis was the alveococcal cyst. After the diagnosis, specific antibodies were detected using serology through the National Reference Laboratory for Tissue helminthoses of the 1st Faculty of Medicine of Charles University in Prague) and the Echinococcus multilocularis was confirmed.
In the clinical findings alveolar echinococcosis can imitate the liver tumor, so it is necessary to mention even this rare possibility in the differential diagnosis. The incidence of the Echinococcus multilocularis in Europe is increasing.
PITFALLS AND DIFFICULTIES IN THE INTERPRETATION OF GENETIC TEST RESULTS OF DOGS AND CATS: THE EMERGING NEED OF CLINICAL GENETIC COUNSELLING IN VETERINARY MEDICINE HIGHLIGHTED BY SELECTED CASE STUDIES

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Genetics and genomics are hot topics in the biomedical research of the 21\textsuperscript{th} century. Since the first draft sequence of the entire human genome was published in the year 2001, more and more species have been sequenced with more and more advanced technologies resulting in databases with different resolution and accuracy. Though we are far from understanding the complex function of the genome, there are high expectancies from genetic data, based on the belief that DNA can exactly predict future health issues. The only problem is that our knowledge is still limited and growing evidence suggests a more complex regulatory system in which information encoded in the DNA sequence is only a part of the whole. Therefore it should be used in disease prediction with caution and circumspection. This is already well understood in human medicine.

In the past few years, a rapidly growing popularity of genetic testing of purebred dogs and cats could be observed worldwide. The number of genetic tests available on the market exceeds 100 and this number is growing continuously. Genetic testing has a high market potential. The popularity of genetic testing among breeders and owners originates in the false illusion that normal results can predict and warrant a healthy animal. In contrast to that, an increasing number of ambiguities can be found related to interpretation of test results both in case of health issues of individual animals and in breeding decisions. To resolve these problems, veterinarians should became familiar with the biological and technical basics of genetic testing as well as their limitations. The problems and difficulties related to DNA tests in purebred dogs and cats will be shortly summarised below and highlighted by selected case studies in frame of the presentation.

Disease-related mutations can be grouped into well known and proven disease causing mutations (e.g. VWF-1 mutation in von Willebrand disease type-I in many dog breeds) or disease-associated mutations which segregates together with the trait/disease but they don’t have a causative role (e.g. because they are intronic polymorphisms). There are combined examples which may also play an either not well-known role or a not exclusive one in the pathogenesis (e.g. the STRN mutation in arrhythmogenic right ventricular cardiomyopathy of boxers, or PDK4 mutation in dilated cardiomyopathy in some breed lines of doberman pinschers). Another important information is the suggested mode of inheritance of a particular disease. This can be a classical mendelian monogenic pattern which may be handled quite simply. Genetic test providers often simplify the question to this model illustrated by well known Punnett-tables. Results are then given as clear (homozygous wild type), carrier (heterozygous mutant) and affected (homozygous mutant). In reality, however, penetrance is often incomplete, age-related, and expression is variable, both of which make the clinical picture more complex (e.g. in case of polycystic kidney disease of cats, progressive retinal atrophy in dogs). Considering polygenic or even more complex traits, where also epigenetic and environmental factors play a role, only a risk-estimation can be given. From a technical point of view, commonly used genetic testing methods usually detect
single nucleotid changes (point mutations) or larger deletions or insertions in a targeted gene which originate in a scientific source. However, average gene sizes are variable; they may range from 10,000 to 50,000 base pairs. This means that tested mutations can be the cause of a disease but other mutations which are not tested but may also be present can also play a causative role. In terms of this, a clear result does not warrant a healthy animal. For this reason, the entire sequencing of the target gene/exon is common in the human medicine, but more expensive. On the other hand an “affected” result does not always mean automatically that the animal will develop the disease-phenotype. In addition to that the onset of symptoms and clinical severity can often not be predicted.

The DNA testing laboratory should be selected carefully. The minimal requirements are that tested mutation and the analytical methods used should be found on the result as well as some general interpretation. The preferred laboratory should participate in at least one independent, international quality control program. The proper sampling method for DNA testing has also a crucial role. Whole peripheral blood sample anticoagulated by EDTA is always preferable to saliva or cheek swab.

Information about heritable defects with causative roles are often derived from relatively small inbred research populations. They are either conserved ancient mutations which are valid in a larger population or they may be not valid in an independent population. Other information come from larger population based association studies which are usually not strongly causative. All of them may have high limitation potency as far as they are not validated in other independent and especially in the local population. Ideally allelic and genotype frequencies should be known for the local populations as well reliable disease frequency registers based on proven clinical presence of a disease in breedlines of purebred animals. Until they are not available, genetic testing can be advised in individual cases based on case history together with a functional diagnostic testing, or for collecting information related to the local population. Interpretation of results should be made very carefully. Even if the above mentioned ideal situation about validity of genetic tests are given, genetic test results should always be supported by functional tests, symptoms and other diagnostic methods. Unfortunately, this approach is often missed and also the terms ‘genetically affected’ and ‘affected by the disease’ are used sometimes erroneously as synonyms. Purebred dogs and cats have a unique, artificial genomic structure. This typically shows a low genetic heterogeneity together with high phenotype variability compared to other species. This is mainly due to the strong artificial selection steps known as main bottleneck effects. As a consequence, inherited diseases are often breed-related and expectations of the ‘disease-free purebred animal’ are unrealistic. Instead of that a careful compromise should be targeted in breeding strategies. In case of health issues of individual animals where inherited disease is suspected, complex clinical diagnostics should be made besides genetic testing.

Genetic tests are undoubtedly very useful tools of diagnostics and breeding, if they are selected and interpreted with proper knowledge. If it is not so, they can have a detrimental effect on breed health, and may provoke unnecessary fear and worry in the owner for a high cost. For that reason, clinical genetic counselling is going to gain a high importance in the veterinary medicine in the near future. Such counselling should be provided by a trained veterinarian having both clinical experience and deep knowledge in clinical genetics as well as in molecular biological methods.
IMPORTANCE OF COAGLUOPATHIES IN COMPANION ANIMALS

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Practical Tools to Approach Bleeding Patients

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Bleeding disorders are a common presentation in dogs and less commonly in cats and may be inherited or acquired. Furthermore, thrombotic conditions are being increasingly recognized. This lecture will focus on the clinical diagnostic approach to a bleeding animal. There are several point-of-care and reference laboratory tests permitting the separation between primary and secondary hemostatic defects as well as a specific diagnosis. Particularly challenging is the diagnosis of Disseminated Intravascular Coagulation (DIC), a syndrome observed with a variety of disorders. Also note the lecture on sepsis and coagulation.

Bleeding diatheses are generally separated into primary and secondary hemostatic disorders and in some cases both systems are affected, such as in disseminated intravascular coagulation (DIC). Primary hemostatic disorders include not only the common thrombocytopenias but also thrombopathias, vasculopathies, and von Willebrand disease. Secondary hemostatic disorders include all coagulation factor deficiencies involved in fibrin formation and are strictly speaking the coagulopathies. Platelet and vascular problems often present with surface hemorrhage, while coagulopathies generally cause hematomas and cavity bleeds. Excessive hemorrhage at an injury or surgery site and bleeding from multiple places are suggestive of bleeding disorder, and there are a several breed predilections for specific hereditary defects.
Hemostatic tests are indicated whenever an animal is bleeding excessively, prior to surgery when an increased bleeding tendency is suspected, to monitor therapeutic interventions, and for genetic screening in certain breeds or families with a known bleeding disorder. Hemostatic abnormalities should be assessed prior to instituting therapy whenever possible or at least appropriate blood samples should be collected pretreatment. Excellent venipuncture with discarding of the first few drops of blood (to avoid platelet activation and tissue factor) and extended compression over jugular, saphenous or femoral vein is required. The cuticle bleeding time crudely assesses overall hemostasis, but is not standardized and painful and is, therefore, not recommended. A minimal database includes a packed cell volume and total protein evaluation, and evaluation of a blood smear can provide a platelet estimate and identify platelet size and clumping as well as schistocytes. The results can also provide some measure of the extent of blood loss and red blood cell transfusion requirement.

**Tools for Primary Hemostastic Defects**

Platelet counts can be estimated on a blood smear or specifically counted by a hematology instrument. Since 8-15 platelets (1 platelet equals 20,000/µl) are normally found per high power oil emersion microscopic field, an absence to low number of platelets suggests a severe thrombocytopenia. Various modern impedance and laser hematology instruments have the ability to count platelets and measure their mean size including platelet size distribution and platelet crit; they may have been validated, but some have difficulties in differentiating large platelets from erythrocytes (particularly in cats). Furthermore, platelets can readily be activated which results in platelet aggregation, hence, platelet counts need to be confirmed by a careful review of a blood smear including the feather edge for platelet clumps (preferably on fresh non-anticoagulated blood). Hemorrhage is generally not observed unless the platelet count is <40,000/µl (normal 150-500,000/µl) or there is also a coagulopathy like DIC.
Thrombocytopenia, a common cause of surface hemorrhage in dogs, can result from impaired thrombopoiesis, increased platelet destruction and consumption, and sequestration of platelets (splenomegaly). Reduced platelet production may be isolated or associated with an overall decreased hematopoiesis due to many drug reactions (estrogens, chemotherapeutics, azathioprine), infections (Ehrlichia spp.), and myelophthisis (leukemia, myeloma, myelofibrosis), but remains often idiopathic (immune-mediated?). Accelerated platelet destruction is commonly associated with immune-mediated thrombocytopenia (IMT, including idiopathic thrombocytopenia purura [ITP]), but enhanced platelet consumption may also be observed with neoplasia, vasculitis and disseminated intravascular coagulation (DIC). IMT can be divided into primary, also known as idiopathic thrombocytopenia purpura (ITP), and secondary forms triggered by infections (Ehrlichia, Rickettsia, and Babesia spp., Anaplasma spp., vaccines), drugs, and cancer. Anticoagulant rodenticide poisoning can also be associated with mild to moderate thrombocytopenia. However, acute and chronic blood loss is not resulting in any significant consumptive thrombocytopenia unless there is concomitantly a vasculopathy or DIC present. Thrombocytopenia occurs rarely in cats and is generally associated with drug exposure (griseofulvin, methimazole), viral infections, or malignant diseases.

A diagnosis of thrombocytopenia is made by a platelet estimate on a blood smear or complete blood cell count, but any thrombocytopenia must be verified by a review of a blood smear. Spurious thrombocytopenia may due to instrument limitations; e.g. megaplatelets in Cavalier King Charles and few other breeds, and platelet aggregates with many illnesses and collection techniques; also Greyhounds have generally a mild thrombocytopenia. Classic signs of thrombocytopenia include petechiation, ecchymosis, epistaxis, and gastrointestinal blood loss. The most severe thrombocytopenias, seen with IMT/ITP, often cause only mild hemorrhage. Following a careful history, a search for an underlying cause is warranted to identify an infection (blood smear, serology, PCR) or cancer (also involving lymphnodes and spleen). Bone marrow examination is safe, but may rarely reveal a specific cause on initial presentation. A diagnosis of ITP is mostly based upon excluding other causes of thrombocytopenia, but platelet-associated antibodies can also be determined to support an immune mechanism for thrombocytopenia. Detection of platelet-associated antibodies further supports an immune-mediated thrombocytopenia, but this test is rarely available. Serum titer, antigen and PCR tests for tick-born (ehrlichiosis, babesiosis, leishmaniasis, Rocky mountain spotted fever) and other infectious diseases are indicated in certain countries or areas. The presence of schistocytes and thrombocytopenia suggests intravascular disseminated coagulation, where intravascular fibrin strands fragment erythrocytes. Because von Willebrand disease is such a common mild primary hemostatic defect in dogs, plasma vWF measurements by ELISA through a commercial laboratory are indicated. For breeding purposes, DNA testing is also available for some canine breeds.

Finally, in light of normal platelet count and plasma vWF values, a prolonged buccal mucosal bleeding time (BMBT) indicates a thrombopathia. Disposable devices are available that facilitate making 1-2 standard 1 mm deep mucosal incisions. The platelet function analyzer (PFA100) is a simple tool to functionally assess primary hemostasis. Electron microscopic and platelet aggregation and nucleotide studies allow further characterization of platelet dysfunctions in specialized laboratories. For a couple of hereditary thrombopathias even a
DNA test is now available such as for Glanzmann thrombasthenia in Great Pyrenees and Otterhound, and thrombopathia in the Spitz, Basset, Landseer and Swiss Mountain dog, and macrothrombocytopenia in Cavalier King Charles (http://www.vetmed.auburn.edu/faculty/pathobiology-faculty/boudreaux).

**Coagulation Tests**

Whereas the whole blood clotting time test is insensitive and mostly inaccurate, there are several standardized coagulation screening tests that are useful to define coagulopathies in clinical practice. Nearly all coagulation tests assess the function of certain parts of the coagulation system in fresh whole blood or fresh (or frozen) plasma to generate fibrin in a fibrometer; recalcified citrated plasma is used and many tests are comparing a patient sample directly with a simultaneously obtained control or pooled plasma (plasma from 10 animals). Generally coagulation times, which is measuring the time to clotting (fibrin formation), are much shorter in small animals than in humans; thus, every coagulation test needs to be run on an instrument for animals and validated for the animal species.

The intrinsic and common pathways are assessed by either the activated coagulation time (ACT) or activated partial thromboplastin time (aPTT or PTT). Factor XII of the intrinsic cascade is activated by diatomaceous earth (celite) in the ACT test and by kaolin or other contact phase substrates in the aPTT test. The extrinsic and common pathways can be assessed by the prothrombin time (PT) test. In these two assays different tissue factors (thromboplastins) are activating factor VII, which in turn will lead to fibrin formation.

Until recently the ACT tube test was the only point-of-care test available for clinical practice, whereas PTT and PT tests were performed in commercial laboratories. There are now new point-of-care coagulation instruments (e.g. IDEXX Coag DX and the Abaxis VetScan VSpro) introduced that are capable of determining without delay on small amounts (50 µl) of fresh citrated whole blood the aPTT and PT, thereby making separation of citrated plasma and shipment of frozen plasma to the laboratory for initial coagulation screening unnecessary. In practice, a reasonable and simple approach for a bleeding animal to be screened for a coagulopathy would be to measure the ACT or PTT first as either test detects all coagulopathies (except for hereditary factor VII deficiency in Beagles, Scottish Deerhounds and Alaskan Klee Kais), but the aPTT is more standardized and the ACT can only be run on fresh whole blood. If the aPTT (or ACT) is prolonged, a PT test would be indicated to differentiate between an intrinsic and common pathway defect or a combined coagulopathy involving several coagulation factors.
Hemostatic screening tests and groups of bleeding disorders

<table>
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<tr>
<th>Disorder</th>
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<td>Thrombocytopenia &amp; vWD</td>
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<td>Intrinsic coagulopathy</td>
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<td>Extrinsic coagulopathy (FVII)</td>
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<td>Combined coagulopathies (DIC, liver, rodenticide)</td>
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N = normal; I = increased (prolonged) time; D = decreased

Although hereditary coagulopathies can be suspected based upon the pattern of coagulation test abnormalities, specific factor analyses are needed to confirm a diagnosis. A young male animal who is bleeding and has a mildly prolonged aPTT but normal PT likely has hemophilia A or B (factor VIII or IX deficiency), an X-chromosomal recessive disorder. However, factor XI deficiency is associated with the same test abnormalities and is inherited by an autosomal recessive trait (e.g. Kerry blue terriers). For several hereditary coagulopathies DNA tests are already available ([http://research.vet.upenn.edu/penngen](http://research.vet.upenn.edu/penngen)), while for others the specific plasma factor deficiency can be determined through the Comparative Hemostasis Laboratory at Cornell University. Finally, factor XII deficiency, particularly common in domestic shorthair cats, and prekallikrein deficiency causes marked aPTT prolongations but no excessive bleeding tendency. Rodenticide poisoned animals that are bleeding or are at risk for bleeding will have severe prolongations in all of the above coagulation tests, but would have a normal thrombin time (TT). The thrombin time is independent of vitamin K-dependent coagulation factors and is a functional assay for fibrinogen to form fibrin. The protein induced by vitamin K antagonism or absence (PIVKA) test is a modified PT test and not diagnostic for rodenticide poisoning, but a toxicological investigation (product identification, blood toxicology analysis) may confirm the rodenticide poisoning. Moderate thrombocytopenia may be associated with rodenticide poisoning. All liver diseases may result in varied coagulopathies due to impaired coagulation factor synthesis and vitamin K malabsorption.

Similarly, disseminated intravascular coagulopathies due to many different disorders is associated with variably prolonged coagulation times. More helpful to the diagnosis of DIC are the recognition of schistocytes, thrombocytopenia, low fibrinogen and antithrombin III levels, and increased D-dimers and fibrin split (degradation) products. Finally, thromboelastography (TEG or ROTEM) techniques can now be used in the emergency room, intensive care units, and referral centers to assess overall hemostasis and particularly thrombotic/fibrinolytic tendencies of citrated whole blood.
Supported in part by a grant from the NIH (OD 010939). The author’s laboratory PennGen is offering some advanced hemostatic testing.

ANAPLASMOSIS, AN UPDATE

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Introduction: Anaplasmosis is an umbrella term for clinical diseases, caused by Anaplasma sp. organisms. Anaplasma sp. are gram-negative bacterial organisms in the order rickettsiales. They live obligatory intracellular, lack a cell wall and are susceptible to some antibacterial drugs. They were separated from the genus Ehrlichia in 2001 as own family. The Anaplasma species are A. phagocytophilum, the causative agent of the granulocytotropic anaplasmosis dogs, cats, horses (formally A. equi), cattle and humans (formally HGE) and A. platys, the causative agent of the thrombocytotropic anaplasmosis in dogs. Both have a worldwide distribution. In their target cells Anaplasma sp. are present as 0.2-0.6 μm large elementary bodies. At a later stage these form morulae with a diameter of up to 6 μm and are seen as typical inclusion bodies in granulocytes or thrombocytes.

A number of different strains have been identified by culture or PCR (diversity in the groESL sequence). These differ by host preference, geographic distribution, or pathogenicity.

The main vectors of A. phagocytophilum are ticks from the genus Ixodes (I. persulcatus complex) with different species. They are widely spread throughout the Northern Hemisphere. In Europe the main vectors are I. ricinus and I. persulcatus with partly overlapping habitats. The reservoir are different rodents and red deer. The transmission by infected ticks is the predominant way of infection, but also other ways have been reported, such as iatrogenic infections (blood transfusion) or transplacental infections have been reported.

The worldwide distribution of A. phagocytophilum follows the distribution of the primary tick vector Ixodes sp. Since Ixodes sp. is widely spread in Europe from Scandinavia to the Southern Mediterranean, the anaplasmosis is found in most European countries. A minimum feeding time of 24 to 48 hrs is required for Ixodes sp. to transmit A. phagocytophilum. The incubation period is 4-18 days, antibodies can be detected at the earliest after morulae are formed. The antibody titre increases rapidly.

Aims: The aim of the lecture is to present an overview of Anaplasma species, their vectors, and hosts. We also show some recent epidemiological data from Austria. Finally, diagnostic options are discussed.

Results:
Seroprevalence:
The seroprevalence depends on the geographic location, ranging from 3.2% in Spain to >30.5% in Bulgaria. The median prevalence from different studies was 18.2%.

Geographical and seasonal occurrence
There is general agreement that *I. ricinus* and *I. persulcatus* ticks have expanded in Europe in the last decades, probably due to climate change and potentially other factors. Therefore, the prevalence of *Anaplasma* should increase as well. This, however, could not be confirmed by our preliminary data showing the percentage of seropositive dogs from 2005 to 2015 (Fig. 1). This may be caused by a better tick prophylaxis or by a more specific anamnesis prior to testing.

In central Europe (data from Austria) dogs with antibody titres and clinical disease or detection of the infectious agent (morulae, PCR) peak in May and June (Fig. 2).

**Fig. 1**: decline of the seroprevalence of *A. phagocytophilum* in dogs.  
**Fig. 2**: seroprevalence, detection of morulae, or PCR in dogs by month.

Clinical symptoms
Symptoms include fever up to 41°C for 2-5 days with anorexia during bacteraemia, further diarrhoea, and vomiting. Rare symptoms are lameness, polyarthritis, epistaxis or neurologic symptoms suspicious for meningitis.

Laboratory tests
Testing for *Anaplasma* antibodies is done by IFAT, IC (fast tests), where cross reactions in different serologic test may occur between both species, *A. phagocytophilum* and *A. platys*. Tests for Anaplasma organisms include PCR and the detection of the typical morulae in granulocytes or thrombocytes. Only the later test is usually connected to clinical disease, but morale are only present for 4 to 8 days, mining the detection fairly insensitive.

Neither antibodies nor positive PCR results are a direct indication of disease. They always have to be regarded together with clinical symptoms.

Haematological changes include normocytic, normochromic, non-regenerative anaemia, a marked thrombocytopenia and a leukopenia or - less frequently - leukocytosis.

Conclusion
Anaplasmosis is a wide-spread disease with a broad range of species infected, caused by *A. phagocytophilum* or *A. platys*. Despite of the high seroprevalence (overt) clinical disease is rare in dogs and other species.
Literature can be requested from the authors
UPDATE ON VETERINARY HEMATOLOGY

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Hematologic evaluation, the complete blood cell count (CBC) including a white blood cell differential (DIFF) are an indispensable part of diagnostic workup and health check panels. Assessment of hematologic data is not only helpful detecting hematologic diseases. Diseases of other organ systems modulate the CBC results as well, so that CBC evaluation contains information regarding grade and nature of the underlying disease.

Technologic advancements within the last decades have resulted in a wide variety of instruments available for veterinary office labs, all of them bear advantages but also some drawbacks which will be briefly reviewed here. High quality laboratory test results can only be obtained when appropriate preanalytic conditions are maintained. Even the most sophisticated instrumentation cannot generate accurate results low quality samples. Last but not least microscopic slide evaluation is still an indispensable tool in clinical practice.

Prenanalytic considerations

Anticoagulants
Hematologic analyses require anticoagulated whole blood. K$_2$- or K$_3$-EDTA is the recommended anticoagulant of choice for mammalian species because cell morphology is ideally preserved. In some avian (e.g. crows, jays, ravens, magpies, cranes) and reptilian species heparin is preferred. Vacutainer® tubes should be used whenever the size of the patient allows it, because they help to obtain the optimal anticoagulant-blood volume ratio. Underfilling of test tubes is less problematic, because an abundance of anticoagulant induces only slight shrinking of RBCs, whereas overfilling causes clot formation so that no accurate cell counts can be generated. As EDTA binds bivalent cations such as Ca$^{++}$, EDTA-plasma is inappropriate for many biochemical routine tests. If in non-compliant patients, where only one sample vial can be harvested, heparin should be chosen as an anticoagulant. Heparin anticoagulated samples can also be used for hematologic analyses. However the acid pH of heparin causes mild bluish discoloration when Romanowsky-type quick stains are used, so that subtle morphologic changes might be difficult to detect. The other CBC results remain unaffected.

Stability and storage
As a rule of thumb, hematologic analyses should be performed as soon as possible after sampling. If some delay is unavoidable as in ambulatory practice at least a blood smear should be prepared on site. Most hematologic measurands are considered stable for about 24 hours. Platelets disintegrate first. Prolonged storage at room temperature or refrigerated (4-6°C) lead to RBC swelling and loss of cellular hemoglobin. In horses neutrophils deteriorate rapidly and show nuclear crenation and cytoplasmic vacuolization very soon, so that preparation of a blood smear immediately after blood sampling is recommended for morphological assessment. Abnormal cells such as blasts indicating hematologic malignancies also deteriorate very rapidly ex vivo. In suspect cases the preparation of blood smears immediately
after sampling is recommended. The air dried slides should be submitted to the reference laboratory for morphologic evaluation. When samples are shipped refrigerated, make sure that the cooling elements do not freeze the hematologic samples, because that will destroy the sample due to cell lysis.

Sample handling
Before removing an aliquot of the whole blood samples for any analyses such as preparing a blood smear or analyzing the sample with an automated system, the sample must be thoroughly mixed to ensure homogeneous distribution of cellular elements! Gravity induces cell sedimentation, so that heavy cells (mature RBCs) sink to the bottom WBC form a layer above topped by PLT; If a sampling nozzle of an analyzer takes a sample in out of a sample, where blood sedimentation has already started, a variety of erroneous results can be artificially produced: If the bottom layer is sampled, a high RBC count and hematocrit with low TWBC and low PLT will be generated, whereas sampling of top layers will produce anemia, leukocytosis and thrombocytosis!

Hematologic Technologies
Four different types of hematologic technologies are available:
1. Manual methods
   a. estimation of cell numbers by microscopic evaluation of blood smears;
   b. microscopic evaluation of blood smears
   c. hemacytometer
2. Centrifugal systems
   a. Spin-hematocrit (PCV)
   b. Quantitative buffy coat analysis (QBC)
3. Impedance hematology counters
4. Laser- flow-cytometry

1. Manual Methods
   a. Preparation of a blood smear and estimation of TWBC and PLT
Microscopic evaluation of a well prepared blood smear allows an estimation of TWBC and PLT. Automated counts can be validated by this technique.

Technique: An EDTA-anticoagulated blood sample is thoroughly mixed. The drop and drag technique is not difficult to perform: a drop in the size of a glass pinhead is placed on the proximal edge of a clean glass slide, which is laid flat on a desktop. A second slide with polished edges is placed in front of the drop. With a swift movement, the spreader slide is drawn backwards so that the blood spreads along the slides edges and then the drop is dragged across the slide. Proceed swiftly to ensure homogenous dispersion of cells and to avoid artifacts. The ideal smear shows 3 areas, the dense corpus, the monolayer and the feathered edge. In the monolayer area, the approximately 50% of the RBCs touch each other. This is the area, where TWBC and PLT estimates and differentials are performed. The smears are allowed to dry – completely dried smears show a matt appearance. Drying is a very effective first fixation step. Dried blood smears, when stored in a dark, dry environment (cardboard-box) can be stained weeks and month later. Dried blood smears should never be
stored in the refrigerator, because condensed water droplets will spoil the smear by lysing the blood cells.

**Staining:** Commercially available Romanowsky type dip stains, such as DiffQuick®, Hemafix®, Hemacolor® usually provide excellent results. Be careful to change dyes and fixative on a regular base. Make sure, that the lid on the staining jar has a tight fit, so that the alcohol does not evaporate leaving many stain precipitates.

**TWBC estimation**

Screen slide at 400x magnification to select appropriate magnification for counting and the appropriate multiplier. Count cells in 10 better 20 view fields; Calculate the average cell number/ view field.

Proceed as follows:

<table>
<thead>
<tr>
<th>Cell number/400x field</th>
<th>Appropriate magnification</th>
<th>Appropriate multiplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1</td>
<td>100x</td>
<td>100</td>
</tr>
<tr>
<td>2-20</td>
<td>400x</td>
<td>1400</td>
</tr>
<tr>
<td>&gt;1000</td>
<td>1000x</td>
<td>8000</td>
</tr>
</tbody>
</table>

The result represents an approximation of TWBC/ µl

**PLT estimation**

Prepare a blood smear as described above; Screen slide at 1000x magnification (oil immersion). A PLT within reference intervals (WRI) is expected, if you see 10-30 PLT/1000x field of view.

**b. Microscopic evaluation of blood smears**

A systematic approach for microscopic slide evaluation is highly recommended. All three cell lines the erythrocytes, leukocytes and platelets must be evaluated.

Start with scanning the slide on low (100x) magnification:

- Look for PLT aggregates in the feathered edge when automated PLT count is low
- Look for WBC aggregates in the feathered edge
- Look for microfilaria in the feathered edge in endemic areas
- Look for RBC aggregates (indicator of immune mediated anemia)
- Estimate TWBC
- Get an impression of the DIFF

- Screen for abnormal cells
  - nucleated red blood cells (n-RBC)
  - atypical lymphocytes
  - blast cells
Nucleated red blood cells (n-RBC): in former times n-RBC have been called normoblasts – this term should be avoided, because of the negative associations the term “blast” elicits among hematologists and patients! These cells are characterized by dense nuclei with mature chromatin. Mature chromatin has the same structure as the chromatin of a normal segmented neutrophilic granulocyte. The nucleus, very dense and centrally located, the cytoplasmic rim is wider than in lymphocytes. According to the hemoglobin content and loss of cell organelles the color of the cytoplasm changes from deep dark blue to polychromatic (violet) and red. The maturation pyramid should be preserved otherwise an erythorid dysplasia is present.

Atypical lymphocytes: These cells are larger, have bigger nuclei sometimes with less condensed chromatin pattern and/or also a broader cytoplasmatic rim. The cytoplasm can be light to dark blue. They can be easily recognized in low magnification. Screening the entire slide on low magnification gives a better impression of their frequency than identifying some of them by oil immersion – the latter might lead to overinterpretation of their significance.

Interpretation: atypical lymphocytes can be reactive or indicating lymphoproliferative disease. Sometimes differentiation between these two quite different entities is not possible at the first morphologic evaluation. Age, history and clinical features have to be considered. Repeated evaluation is indicated – in cases of persistence or increase in numbers more sophisticated analyses such as immunophenotyping might be helpful.

Blast cells: Blast cells are immature cells, which are usually not present in the peripheral blood. They usually indicate leukemia. They are characterized by round nuclei, with immature (that means finely granular) chromatin pattern and nucleoli. Nucleoli are the morphologic hallmark for blast cells. However results of immunophenotyping which allows a more accurate identification of blast cells by a positive reaction to CD 34, which is only available for dogs, has shown, that not all CD 34+ cells show nucleoli. But if nucleoli, that is light blue round to slightly angular spots within the nucleus, are present, the cell is addressed as a blast cell.

Search of blood parasites such as Microfilaria, Babesia, Anaplasma, Ehrlichia or hemotrophic mycoplasma ssp. is best accomplished in the feathered edge area, albeit artifacts are also more frequent. However searching for these smaller elements requires the 400x magnification for searching and 1000x (oil immersion) for finalizing the diagnosis. 1000x is necessary for detecting hemortrophic mycoplasma, which is even then quite a challenge and requires confirmation by molecular techniques.

At 400x magnification RBC morphology and PLT number and morphology can be screened in more detail. The DIFF can be made at 400x or 600x or 1000x magnification.

Evaluation of RBC morphology
Erythrocytes are evaluated in terms of their arrangement (agglutination, rouleaux formation, size (normocytic, macrocytic, microcytic, anisocytosis = variation in size), color (normochromic, hypochromic, polychromasia), shape (spherocytosis, echinocytes, acanthocytes, schistocytes, keratocytes; poikilocytosis = variability in shapes) and inclusions (Howell Jolly bodies, which are nuclear remnants; Heinz bodies representing oxidative hemoglobin damage; Basophilic stippeling which might indicate lead toxicity; parasites such as Babesia ssp. or hemotrophic mycoplasma ssp.).
Polychromatophilic are RBC, which stain bluish red, because they are reticuloocytes and the RNA remnants give their cytoplasm a bluish stain, the already present hemoglobin stains red. Polychromasemia indicates regenerative anemia; n-RBC see above;

**Evaluation of WBC morphology**
Screening at low magnification 100x or 200x allows the identification and quantitative assessment of a left shift and toxic changes can also be observed.

**Toxic changes in leukocyte morphology**
These changes are induced by a severe systemic inflammation with a high demand for neutrophilic granulocytes in peripheral tissues. The term is actually an unfortunate misnomer, depicting changes observed in man during endotoxemia. Then it was assumed, that endotoxins change neutrophil morphology in peripheral blood. Today it is understood, that toxic changes occur within the bone marrow and are due to accelerated production incited by an increased demand in the peripheral tissues. There is not time for maturation so that certain cell organelles being normal in early stages of cell development remain present within the cytoplasm, such as aggregated endoplasmatic reticulum (ER) in the form of Doehle bodies or ribosomes (cytoplasmic basophilia). Cell divisions are also omitted leading to giant forms. Abnormal nuclear lobulation, hydropic degeneration of nuclear chromatin and nuclear ring forms are other features summarized under “toxic changes”. Toxic changes often appear prior to changes in cell concentrations and they disappear before the leukogram returns to normal. Hence they are considered a highly sensitive indicator of accelerated leukopoiesis. However, the changes resemble storage induced changes, so that blood smears prepared from fresh samples are needed for reliable categorization.

**Differential blood cell count (DIFF)**
For a microscopic differential 100 better 200 cells are microscopically evaluated and allocated to the respective leukocyte population.
A free android app for DIFF counting is available on the internet and is very useful for performing a differential cell count: [https://play.google.com/store/apps/details?id=appinventor.ai_korobiya.WBC_Counter](https://play.google.com/store/apps/details?id=appinventor.ai_korobiya.WBC_Counter)
Impedance counters and to some extent also laser-counters do classify n-RBCs as lymphocytes so that erroneously increased TWBC are generated. For diagnostic purposes it is advisable to correct the TWBC for n-RBCs. To do so, 100 nucleated cells are classified as erythroid or leukocytic; The n-RBC concentration is calculated and subtracted from the automated TWBC.

c. **Hemacytometer**
Manual counting of white blood cells (TWBC) and platelets (PLT) are still considered reference techniques for the evaluation of hematology systems. Single use hemacytometers (C-chip©) and pipetting systems (Leuco-TIC©) facilitate the procedures and reduce hemacytometer-adjustment and pipetting errors. The Leuco-TIC© system replaces the former Unopette ©.
**Technique:** The Leuco-TIC© system consists of an end-to-end micro-capillary and a stained counting solution to deliver a 1:20 dilution and chamber filling capillaries. The end to end capillary is allowed to fill with a drop of a well mixed EDTA-anticoagulated blood sample by
capillary forces. Remove eventual remnants of blood from the outside of the capillary. The capillary is put into a single use vial with a defined volume of counting solution. The vial is closed and must be shaken very well to rinse the blood sample out of the capillary. The capillary stays! After a short incubation of 30 sec to complete hemolysis and staining, a drop of the mixture is picked up by a filling capillary and the hemocytometer chamber or C-chip® can be loaded. The disposable chip provides a ready to use counting area. However time and a considerable amount of practice are still required to produce accurate results.

2. **Centrifugal systems**
   a. **Spin-hematocrit ; Packed cell volume (PCV)**

Preparation of a spin-hematocrit to evaluate the packed cell volume (PCV) is a useful, simple and accurate technique to collect a lot of information.

**Method**: estimation of red blood cell volume compared to total blood volume by centrifugal sedimentation.

**Technique**: a heparinized or EDTA coated 70 mm capillary is filled at a maximum of ¾ of its length with well mixed EDTA-anticoagulated blood. Let the capillary forces help to fill the capillary. Clean the outside of the capillary from blood remnants. One end is closed either by sealant. Then the capillary is placed into a hematocrit centrifuge, the sealed end pointing to the periphery and centrifuged at 13,000 G for 5 minutes. Do not forget to put a counterbalance into the centrifuge – this enhances the life expectancy of the equipment. Then the red blood cell (RBC) column is either measured by a nomogram or a ruler; if a ruler is used, the PCV is calculated according to the following formula:

\[
PCV \% = \frac{\text{mm length of the RBC column} \times 100}{\text{mm length of entire blood sample}}
\]

Be careful not to include the sealant into the measurement. When the 70 mm capillaries are used some additional information can be gained:

Inspect the plasma for hemolysis (red), icterus (yellow) or lipidemia (creamy layer or turbidity). The leukocytes sediment on top of the red blood cells, forming the so called buffy coat which appears in healthy animals as a narrow white band of approximately 1 mm length.

An enlarged buffy coat over 1 mm indicates either leukocytosis (more common) or thrombocytosis (less common) and microscopic inspection of a blood smear should follow.

**Buffy coat preparation**: the buffy coat cells can be harvested from the capillary by breaking the capillary slightly within the upper quarter of the red blood cell column. The buffy coat with the top layer of the RBCs is dropped onto a slide and spread like a blood smear. This technique can be used to screen for blood parasites such as Babesia ssp., Anaplasma, Ehrlichia or Microfilariae or even mast cells. It is supposed to increase the sensitivity of the microscopic technique – drawbacks are the increased number of artefacts.

**Interpretation of results**: An increase in PCV might be relative or absolute. Relative increases are due to loss of fluids, that means dehydration, whereas absolute increases indicate polycythemia vera, which can be primary or secondary. Secondary polycythemia vera is caused by erythropoietin (EPO) producing tumors or hypoxia induced increased production of EPO in severe cases of cardiac disease. Primary polycythemia is a neoplastic disorder arising from the bone marrow, where
the production of mature red blood cells is tremendously increased producing PCVs consistently over 75%.

b. Quantitative buffy coat analysis (QBC)

This system is a highly economic rapid and simple screening method for low throughput in house laboratories. It requires only minimal maintenance steps and is as easily performed as a spin-hematocrit. Data are generated in about 10 minutes after blood sampling. It uses the differing densities of the different cell types in peripheral blood, to separate them by centrifugation. PCV, the total white blood cell count (TWBC), a two part differential (polymorphonuclear white blood cells (neutrophils + eosinophils) and mononuclear cells (lymphocytes + monocytes), platelet concentration (PLT) and reticulocytes (RETIC) can be measured. This was the first system delivering an automated two part differential in veterinary medicine.

**Method:** The blood cell populations are separated by centrifugation in capillary which is coated with acridin orange and oxalate. Acridin orange stains the nucleic acids and lipids in WBC and PLT. Oxalate optimizes stacking of the different cell layers. Mature RBC sink to the bottom, immature RBC that is reticulocytes are on top of the red blood cell segment. Polymorph-nuclear WBC, that is neutrophils and eosinophile, are next followed by mononuclear cells such as lymphocytes and monocytes, which are topped by PLT. Above the PLT is plasma. Under UV or laser light, the cell layers show a characteristic fluorescence so that they can be identified. WBC and PLT are stacked in the buffy coat layer, which is magnified by the insertion of a plastic cylinder, which blocks 90% of the capillary diameter and has a similar density as WBC so that it settles in the buffy coat area during centrifugation. The length of the different cell layers is measured by a laser beams so that the volume of each cell layer can be calculated. The number of each cell population can be calculated by the assumption of a standardized cell volume.

**Technique:** The acridin-orange+oxalate coated capillary is inserted into a pipette, to ensure accurate filling. Then a plastic stopper is put on the free end. Afterwards the capillary is removed from the pipette and the acryl glass cylinder is threaded into it. The cylinders must not be touched, so that no lipids or dust interferes with their sedimentation capabilities. Therefore the cylinders are delivered in a device, from which they can be inserted into the capillary without touching. Then the capillary is centrifuged for 5 minutes. Then the capillary is inserted into the reading device. The appropriate animal species has to be selected for the reading. The laser beam in the reading device performs different scans for DNA and RNA/Lipid fluorescence and creates a diagram. Changes or differences between the layers are recognized by changes in the DNA or RNA measuring curve. Results are displayed as graphs and numerical results. It is extremely important to familiarize with the characteristics of the graphs, because inspection of curve patterns gives valuable additional information which goes beyond numerical results.

**Limitations:** The system is an excellent screening tool for health checks, where no high grade abnormalities are expected. Accuracy of results declines in highly pathologic samples – but they are recognized as such, so that appropriate steps for further investigations can be made. The system does not provide an entire 5-part differential. It cannot detect left-shifts and the detection of eosinophilia is also not possible in each case.

3. Impedance Hematology Analyzers
Impedance based particle counters have been standard in veterinary hematology for decades. The Coulter principle, which was developed for particle counting and sizing was developed in the fifties of the last century.

Method: Cells dispersed in a dilute electrolyte solution induce changes in the impedance of an electric field created by two electrodes framing a small aperture. Cells being sucked in a linear flow through the aperture create characteristic pulses. The frequency of pulses determines the cell concentration and their amplitude is correlated with cell volume. As a known volume of a blood dilution is drawn through the aperture an accurate measurement of cell concentration is possible. The different cell populations are separated by their size: PLT are smaller than red blood cells (RBC) and RBCs are smaller than leukocytes. For establishing the TWBC, RBCs are hemolyzed. An aliquot of the WBC dilution is used for photometric hemoglobin measurement. The nucleated cells remain as particles and can be detected by the analyzer. They can be differentiated by size into lymphocytes (L), neutrophils (N) and macrophages. Cell differentiation by size alone is limited so that no reliable 5-part differentials can be created.

Technique: Before a sample can be measured, the appropriate animal species has to be selected. The sample must be mixed thoroughly before being introduced into the analyzer. Depending on the system results are available within a couple of minutes. Results are displayed as numerical results and histograms of cell size and frequency. Familiarization with histogram interpretation gives a variety of valuable additional information regarding accuracy of the measurement and interpretation. Aberrant graphs indicate either inaccurate or abnormal results, so that a microscopic slide evaluation is warranted.

The red blood cell distribution width (RDW) is the calculated area under the RBC-size histogram and is measure of anisocytosis, which changes much earlier than the MCV. When the red blood cell size histogram shows a “shoulder” towards an increased cell volume – this means regenerative anemia. When the peak of the RBC histogram is shifted towards small cell volumes, microcytosis is present.

The normal WBC size distribution histogram shows 2 peaks (Bactrian-camel-peak), in cases of toxic neutrophils or left shift only one peak is observed (dromedary-peak).

Limitations: no reliable 5-part differential can be generated. Eosinophils, left shifts and blast cells cannot be identified. Nucleated red blood cells (n-RBC) cannot be differentiated from other nucleated cells, so that n-RBCs create erroneously high TWBC.

Differentiation of RBC and PLT might be difficult in species or situations when RBC and PLT size are too similar. In those cases inaccurate concentrations are measured. Sometimes PLT clumps form particles in the size of WBCs, so that they induce falsely increased TWBC counts. This can be recognized as a vertical break-line of the PLT- and WBC size distribution histogram respectively.

4. Laser- flow-cytometry

Laser based flow cytometry is the latest advancement in hematologic techniques. At the moment it is considered the gold standard technique in hematology. Flowcytometric systems have been used in high throughput laboratories in academia and in reference labs for about
two decades. Some of the systems use combinations of impedance and flow cytometry, others only optical systems. Nowadays smaller systems for in house laboratories are available, albeit a certain throughput is still necessary to ensure economic balance.

**Method:** Cells exhibit characteristic light absorbance and scattering patterns when passing a laser beam in a linear flow. Special dyes support this technology. Data on cell size, cell shape, granularity, nuclear size and shape are accumulated and allocated to the different cell population by sophisticated algorithms. RBC, reticulocytes and PLT can be differentiated by hemoglobin content and internal structures. A full 5-part differential can be generated by this technology.

**Technique:** A well mixed EDTA-anticoagulated whole blood sample is introduced into the system such as into an impedance analyzer. Before that the appropriate animal species has to be selected. Each species has a unique TWBC dot plot because of morphologic differences in leukocytes, especially the eosinophils. Graphical display of results is also generated by this technology. Scattergrams or dot-plots provide valuable additional information on accuracy of the measurement and aid interpretation. For best practice use, familiarization with dot-plot interpretation is highly recommended. If the cell populations are not clearly separated and straight lines appear between adjacent populations a microscopic inspection of a slide is indicated as special morphologic characteristics hampered the automatic differentiation. So called flags (alarms) are automatically generated to induce additional morphologic assessment by the operator.

**Limitations:** Even the most refined technology has its limitations as it is designed to identify normal cells. Abnormal cells such as blasts, severely toxic or immature cells can be only recognized as such, but their final identification remains in the hands/eyes of a well trained hematologist. So the art of microscopic slide evaluation is still not outdated (yet).

Additional reading:


EXPERIENCES WITH C-REACTIVE PROTEIN AND SERUM AMYLOID A MEASUREMENTS

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**Introduction:** C-reactive protein (CRP) and Serum Amyloid A (SAA) are part of the acute phase protein family. They are widely used as markers of acute systemic inflammation both in human and veterinary medicine. We have been using CRP as such in dogs since 2009 and CRP measurement became part of every canine routine profile since 2015. SAA has been in use since 2012 for cats and horses. It is incorporated in our general equine profile but not in the feline ones.

**Aims:** Retrospective study to analyse diagnostic power of CRP and SAA in different disease states and compare with other analytes measured concomitantly.

**Conclusion:** Both CRP and SAA were found to be more sensitive in detecting inflammation than automated or microscopic hematology including evaluation of left shift. Data from different pathologic states will be presented in the oral presentation to illustrate the value of these parameters in a commercial laboratory setting.
CYTOLOGY OF PERICARDIAL EFFUSIONS IN DOGS WITH HEART TUMOURS

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We performed cytology analysis of pericardial effusion in 70 dogs with symptoms of cardiac tamponade between 2001 - 2015. Sixty nine samples were apparently bloody appearance (specific gravity of the supernatant over 1020 g/cm3, the number of nucleated cells from 0.5 to 97.2 G/l), 1 pink water sample (specific gravity of the supernatant 1010 g/cm3, the number of nucleated cells of 0.2 G/l).

An echocardiogram was performed in all cases. Abnormal mass were identified in 31 cases (15 x cardiac base, 12 x other area of the heart, 2 x indistinguishable origin, 2 x mediastinum), primary cardiac failure were found in 3 cases (echocardiographic and ECG abnormalities). Primary problem was not detected in 36 patients.

No cardiac mass group of pericardial effusion had specific gravity of the supernatant 1020-1045 g/cm3 (mean 1029) and the total nucleated cells count 0.5 - 80 G/l (mean 14.6 G/l); Differentiation nucleated cell demonstrated neutrophil dominance in 13 sample, macrophages dominance in 6 cases, equal count neutrophils and macrophages in 6 cases, activated mesotelias in 2 cases, blast cell dominated in 1 case (round cell neoplasia).

Evaluation of pericardial effusion showed specific gravity of supernatant 1020-1047 g/cm3 (mean 1032), total nucleated cells count 1.9 to 97.2 G/l (mean 17.4) in the group with ultrasoud demonstrated cardiac mass. Differentation nucleated cell identified neutrophil dominance in 19 cases, macrophages in 5 cases, equal count neutrophils and macrophages in 8 cases, lymphocyte dominance in 2 cases, blastic round cell neoplasia in 1 case. The targeted cytological screening for malignant cells was perfomed in group with cardiac mass. There were identified abnormal mesenchymal cells in 3 cases and anomalies of epitelioglandular cell type in 5 cases.

Sensory pink sample was taken from 9 year male old yorkshire terrier with echocardiographically identifiable cardiac mass on cardiac base (cytology with lymphocytic dominance).

Only nine cases were monitored more than four months in no cardiac mass group. Surgical perikardiektomia were performed in 2 refractory cases and neoplasia had been discovered (hemagiosarkom and mesothelioma).
DIROFILARIOSIS IN HUNGARY, EXPERIENCES FROM MOSQUITO SURVEILLANCE STUDIES IN HUNGARY AND SERBIA

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Introduction: Dirofilariosis is a common and widespread veterinary health issue in several European countries with notable zoonotic potential. The causative agents are \textit{Dirofilaria immitis} and \textit{Dirofilaria repens} nematoda species which are transmitted by different mosquito vectors. Similar to other mosquito-borne infections, the knowledge about mosquito species involved in disease transmission is crucial for the complex understanding of local transmission cycles. Mosquitoes are one of the main pillars of local disease transmission and even without understanding their vector capability they serve as perfect indicators to assess risk of infection.

Aims: The main goal of the study was to assess the risk of infection in abundant mosquito breeding territories in multiple localities in Serbia and Hungary. Examining the mosquito populations can reveal the potential vector species, which regarding different breeding preferences may also lead to potentially better understanding on the risk of infection.

Results: During our study, several mosquito species were listed as potential vector species for \textit{D. immitis} and \textit{D. repens} parasites. The Southwestern part of Hungary is mainly affected by \textit{D. repens}, while \textit{D. immitis} was not detected from this region. In contrast, the northern territory of Serbia, is mainly infected with \textit{D. immitis} parasites, which can further raise the risk of infection in surrounding territories of Hungary.
CHALLENGING LEUKAEMIA CASES

Viktória Kunos, Nándor Balogh

Praxislab Kft Hungary

Introduction: Acute megakaryoblastic leukemia (AMLM7) is a rare form of acute myeloid leukaemia. The diagnosis is challenging, because no clear criteria are established for the diagnosis, and specific megakaryoblastic markers are usually not included in the antibody panels utilized for flow cytometric or immunocytochemical examinations. In previous studies blast cells were identified as megakaryocytic precursors by positivity to megakaryocyte-derived cell-specific markers mostly including CD61, von Willebrand factor (vWF), CD41. Other markers as CD9, CD43, CD62P were also occasionally used for the diagnosis according to veterinary literature. Where specific diagnostic modalities or markers are not available, the diagnosis should be based on specific cytomorphology of the blast cells in the blood, bone marrow, spleen or liver.

Case presentation: Two dogs, a 10 year old, female golden retriever and a seven year old mixed breed female dog were presented to the referring veterinarians with general symptoms, weakness and lethargy. On the physical examination pale mucous membranes, enlarged spleen and liver were detected in both dogs. Blood samples were sent to our laboratory, where severe non regenerative, normocytic, normochromic anemia, severe thrombocytopenia and leukopenia were measured by ADVIA 120 hematology analyzer. Both blood smear microscopic evaluation revealed large numbers of nucleated red blood cell population and atypical blast cells. These cells were medium-large sized (up to four of the red blood cells’ size), the cytoplasms were scarced to moderately abundant, basophilic, often with cytoplasmic projections and small, clear vacuoles. The nuclei were usually round and centrally located, but several binucleated large cells were also seen. Biochemical analysis of the bloods showed same alterations, elevated alanine-aminotransferase, aspartate-aminotransferase, alkaline phosphatase enzymes activity and C-reactive protein.

Fine needle aspiration cytology samples from the liver and spleen were also evaluated. The hepatocytes had abundant, moderately basophilic cytoplasm and one or two small round nucleolus, and green pigments in the cytoplasm and among the cells, consistent with regeneration and cholestasis. Among the hepatocytes large numbers of atypical blast cells were seen resemble the ones of detected in the blood. The same cells were observed in the spleen samples.

The histopathological examinations of both spleens showed necrotic stroma tissue surrounded by diffuse undifferentiated round cells with frequent mitosis and multinucleation. The cytoplasms were narrow, nucleoli were rarely seen. In the livers of the dogs the same cells were detected among the dilated sinuses. In the golden retriever extremely high number of multinucleated giant megakaryocytes and precursors were also visible.

Blood, cytology and histopathology result confirmed the diagnosis of an acute form of leukemia. Based on the cytomorphology, megakaryoblastic leukemia was suspected in both cases. Definitive diagnosis was not yet possible to establish, because the lack of specific antigen available. A blood sample from one of the dogs was sent to University of Milan,
where the diagnosis of acute megakaryoblastic leukemia was confirmed by experienced clinicopathologist. Immunohistochemistry is in progress.

**Conclusion:** Although acute megakaryoblastic leukemia is a rare form of leukemia, the incidence of the disease is probably higher, then the number of diagnosis. According to the literature special attention should be taken in case of specific cytomorphology of the blast cells, like central round nucleus, cytoplasmic projections, and vacuoles, bi- or multinucleated cells, and large cytoplasmic fragments/macroplatelets. It is also suggested to increase the number of the markers of the immunologic panel if all lymphoid and myeloid markers are negative.
NEW DIAGNOSTIC TOOLD FOR CANINE LYMPHOMA

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Canine lymphoma is the most common hematopoietic neoplasia in dogs. The multicentric presentation and, more precisely, the diffuse large B-cell lymphoma, is the most frequently found histomorphologic variant in this species. Classical diagnostic tools, such as cytology and histopathology, are still essential, and the use of immunological techniques have improved substantially their diagnostic usefulness. Flow cytometry and PCR for antigen receptor rearrangement (PARR) are becoming more and more available, and they are used not only for diagnostic purposes, but also for better characterization and prognosis establishment in patients with lymphoma.

Cytology:
Cytology is a non-invasive, fast and inexpensive technique. The diagnosis of malignant lymphoma is readily reached when the normal population of lymphocytes have been replaced by large and atypical lymphocytes in the lymph nodes or when these cell populations are found in other organs. The diagnosis becomes more difficult in early stages of the diseases, when the neoplastic cells are small lymphocytes or when reactive hyperplasia is concomitant with neoplasia. Immunocytochemistry allows immunophenotyping and techniques for transformation of lymphocytic effusions into histologic specimens for immunohistochemistry has been described.

Histopathology:
Histopathological examination remains being the gold standard for the diagnosis of lymphoma. The combination of cell morphology, architectural pattern, mitotic activity and immunophenotype of neoplastic cells (by means of immunohistochemistry), allows the classification of lymphoma into one of the several categories of the updated Kiel classification.
Tissue sections and paraffin-embedded tissues are available for the use of further techniques if necessary, such as immunohistochemistry and PARR.
Lymphoma subtypes classified by clinical and histopathological features, have been recently associated with prognosis and therapeutic recommendations have been made for the different lymphoma entities.

Flow cytometry:
Flow cytometry is based on the use of lymphocytes’ light scatter properties and the detection of surface and intracellular antigens by means of specific antibodies. The challenges for this procedure are the limited number of antibodies available in veterinary medicine in comparison to human medicine and the need for a minimum number of living cells in suspension to run the analysis.
Flow cytometric analysis not only detect the size and type of cells involve in the lymphoproliferative process, but also their level of differentiation and the under- or over-expression of some antigens or aberrant expression in others. In Table 1 there is a list with representative antibodies used with diagnostic purposes in canine lymphoma.
### TABLE 1.
**CELL MARKERS COMMONLY DETECTED BY FLOW CYTOMETRY IN DOG LYMPHOCYTES**

<table>
<thead>
<tr>
<th>MARKER</th>
<th>MAIN CELL TYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>All leukocytes</td>
</tr>
<tr>
<td>MHC II</td>
<td>Most lymphocytes</td>
</tr>
<tr>
<td>CD34</td>
<td>Hematopoietic stem cells</td>
</tr>
<tr>
<td>CD3</td>
<td>T cells</td>
</tr>
<tr>
<td>CD5</td>
<td>T cells</td>
</tr>
<tr>
<td>CD4</td>
<td>Helper T cells (also neutrophils)</td>
</tr>
<tr>
<td>CD8</td>
<td>Cytotoxic T cells</td>
</tr>
<tr>
<td>CD21, CD22</td>
<td>Mature B cells</td>
</tr>
<tr>
<td>CD79A, CD79B</td>
<td>B cells of all stages</td>
</tr>
<tr>
<td>SURFACE IGM</td>
<td>Immature B cells</td>
</tr>
<tr>
<td>CD1</td>
<td>Dendritic cells (some B cells)</td>
</tr>
</tbody>
</table>

Specific flow cytometric patterns has been recognized for some lymphoma subtypes (e.g. diffuse-large B cell lymphoma, marginal-zone lymphoma and T-zone lymphoma). In addition, prognostic patterns has been identified for B-cell lymphoma, with loss of MHC II associated with poorer prognosis. In dogs with high-grade B cell lymphomas, the detection and quantification of proliferation-associated nuclear proteins (Ki67) also helped in prognosis prediction.

**PARR:**

Identification of T-cell or B-cell clonality requires detection of clonal receptor gene rearrangement. As part of normal development, rearrangement of T-cell receptor (TCR) for T-cells and immunoglobulin (Ig) receptor for B-cells, results in nearly every lymphocyte having slightly different DNA sequences for receptors. However, small stretches in the DNA coding for each receptor are similar in all lymphocytes, allowing the use of primers that amplify most receptors and yield PCR products that vary slightly in size and composition. Neoplastic transformation of lymphocytes occurs after the cells have undergone receptor rearrangement and, therefore, malignant daughter cells have the same antigen receptor gene. The results of clonality assessment in this cases is the detection of a single PCR product that represents a monoclonal population.

This test can be performed from cell suspensions, fixed and stained smears or paraffin-embedded tissues, allowing morphologic assessment of cells and evaluation for cellularity before the test is performed.

False positive and false negative results are also possible. When a clonal population is detected, a neoplastic process is more likely, but the presence of a benign clone due to antigenic stimulation or unspecific amplification should be considered. When a polyclonal population of lymphocytes is detected, a reactive process is more likely, but primer site mutation or insufficient primer coverage, as well as the presence of a clone obscured by a surrounding polyclonal background are also possible.

In conclusion, although the use of novel diagnostic techniques has greatly improved the possibilities for the diagnosis and characterization of canine lymphoma, it is of great importance the integration of the result from those tests with clinical history, physical...
examination and morphological data to minimize misdiagnoses.
TRANSFUSION MEDICINE AND BLOOD COMPATIBILITY TESTING IN DOGS

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Introduction

Veterinary clinicians and clinical pathologists play a key role in providing safe and effective transfusion therapy. Blood typing is clinically important to ensure blood compatibility and therefore is recommended for any dog in need of a transfusion or considered to become a blood donor. Moreover, previously transfused dogs also should be crossmatched. Unless blood typing is performed regularly in practice, blood may be sent to a clinical pathology laboratory for typing. Different viewpoints exist regarding the extent and methods used for compatibility testing, and various techniques for laboratory and point-of-care use have been applied or are being developed. This presentation will present the current knowledge of the canine blood types and their clinical importance, review the different typing and crossmatching techniques for the in-practice and clinical pathology laboratory, and illustrate this with case examples.

Canine Blood Types

Blood types are genetic markers on erythrocyte surfaces that are antigenic and species specific. A set of blood types of two or more alleles makes up a blood group system. Dogs have likely more than a dozen blood group systems mostly known as dog erythrocyte antigens (DEA). However, there is no DEA 2 blood group and some may be rather labeled high frequency or common red blood cell (RBC) antigens (e.g. DEA 4) and some have not yet received a DEA designation (e.g. Dal). Canine erythrocytes are either positive or negative for a blood type (e.g., DEA 4+ or DEA 4-), and these blood types are likely codominantly inherited. The DEA 1 system was thought to be an exception with DEA 1.1 (A1), DEA 1.2 (A2) and potentially DEA 1.3 (A3) being allelic. Thus, a dog could apparently be DEA 1.1+ or DEA 1.1- and DEA 1.2+ or DEA 1.2-. However, these studies were based upon weak polyclonal antibodies (DEA 1.1 and 1.X) requiring Coombs’ reagents. Recent studies with a monoclonal antibody showed that the DEA 1 blood group is a continuum from DEA 1- to weakly to strongly DEA 1+; hence DEA 1.2 typing is no longer offered. The degree of DEA 1 expression is constant and DEA 1+ appears to be dominantly inherited. A recent survey in North America indicates that most dogs are either DEA 1- or strongly DEA 1+ with fewer dogs being weakly to moderately DEA 1+. The biochemical structure of the DEA 1 remains still unknown, but a genome wide association study has identified a likely single locus.

Recent surveys revealed that the Dal-type is not restricted to Dalmatians but is also seen in Beagles, Doberman Pinschers, and Lhasa Apsos and thus typing for this blood type is becoming more important particularly for those requiring multiple transfusions. In a related study dogs from North America were screened for two new blood types, preliminarily called Kai 1 and Kai 2. Most dogs were Kai 1+ and only few dogs were Kai 2+ or Kai 1-/Kai2-. The
clinical importance is yet to be determined albeit anecdotally dogs can develop anti-Kai 1 alloantibodies. The PennGen Laboratory currently offers Dal and Kai 1 and Kai 2 typing.

The clinically most important canine blood type is DEA 1, which elicits a strong alloantibody response after sensitization of a DEA 1- dog by a transfusion and thus can be responsible for a transfusion reaction in a DEA 1- dog previously transfused with DEA 1+ blood. It is currently unknown if DEA 1- dogs are equally sensitized by weakly to strongly DEA 1+ blood, or if weakly DEA 1+ dogs are sensitized by strongly DEA 1+ blood. Furthermore, transfusion reactions against other blood types or common antigens have rarely been observed and reported. They include reactions against the DEA 4, Dal, Kai 1 and other common RBC antigens; other clinically important blood types may be found in the future. No reagents currently are available against several antigens or are only available on a limited basis, and additional blood types continue to be recognized. Only limited surveys on the frequency of these blood types have been reported, which suggest possible geographic and breed-associated differences.

Strongly antigenic blood types are of great clinical importance because they can elicit a potent alloantibody response. These alloantibodies may be of the immunoglobulin G (IgG) or IgM class and may be hemagglutinins or hemolysins. Based upon experimental and clinical data, dogs can become sensitized after receiving a mismatched transfusion (i.e., a blood unit positive for one or more blood types not found on the recipient’s RBCs). There are no clinically important, naturally occurring alloantibodies (also known as isoantibodies) present before sensitization of a dog with a transfusion. Sensitizing dogs in experimental studies in the 1950s led to the documentation of some transfusion reactions caused by blood group incompatibilities and to the characterization of new blood types.

Clinically the most antigenic blood type in dogs is the DEA 1. Transfusion of DEA 1+ RBCs to a DEA 1- dog invariably elicits a strong alloantibody response. Following a first transfusion, anti-DEA 1 antibodies develop after more than 4 days and may cause a delayed transfusion reaction (rarely clinically documented). However, a previously sensitized DEA 1- dog can develop an acute hemolytic reaction after a second transfusion of DEA 1+ blood. Transfusion reactions also may occur after a sensitized dog receives blood that is mismatched for a RBC antigen other than DEA 1 (e.g., DEA 4 and Dal). However, in most cases the incompatible blood type has not been determined. Because administration of a small (<1 ml) amount of incompatible blood can result in life-threatening reactions, the practice of giving small “test volumes” of donor blood to assess blood-type compatibilities is unacceptable. In contrast, pregnancy does not cause sensitization in dogs, because of a complete placenta, and does not induce alloantibody production; thus dogs with prior pregnancies can be used safely as blood donors.

**Canine Blood-Typing Procedures**

Because of the strong antigenicity of DEA 1, typing of donors for DEA 1 is recommended. Whenever possible, the recipient also should be typed to allow the use of DEA 1+ blood for DEA 1+ recipients. Canine blood typing tests are generally based on serologic identification by agglutination reactions but chromatographic strip methods are also offered. Originally serum from sensitized dogs has been used for typing, but such polyvalent alloantibodies vary from batch to batch, may require Coombs’ reagent to enhance agglutination, and may not be always available and are therefore not optimal. Two monoclonal antibodies against DEA 1 have been developed. The gel column technology,
widely used in human blood banking, was found to be an excellent standardized laboratory method (DiaMed), but is unfortunately no longer commercially available. A blood typing card has been available with modifications since the mid-1990s as a simple in-practice kit to classify dogs as DEA 1- or DEA 1+ (degree of reaction can vary). A standardized simple immunochromatographic technique became available in the mid-2000s from Alvedia. Another cartridge with a similar strip technique was introduced by DMS/AgroLabo, but has not been evaluated. Moreover, a third cartridge method in which blood flows through the cartridge is also available (DMS/Abaxis) but seems to produce inconsistent results.

Polyclonal reagents against other DEA types are currently only available on a limited bases for DEA 3, 4 and 7 from Animal Blood Resource International (prior Michigan state University and Midwest Blood Services). And only limited anti-Dal reagents from sensitized dogs are currently available in a couple of laboratories like Montreal University and PennGen, monoclonal anti-Kai 1 and anti-Kai 2 alloantibodies have been developed in South Korea. DEA 1 typed and matched patients in need of a transfusion may be typed for DEA 4, Dal and Kai 1/2, which may then permit the localization of a type-matched donor dog.

Caution should be exercised whenever the patient’s blood is autoagglutinating or has a low hematocrit (<10%). If autoagglutination is not too severe, it does not appear to affect the Alvedia strip technique because only free RBCs are moving up the strip. Clinicians and technicians should check for autoagglutination of blood with buffer/saline on a slide or the card. Autoagglutinating blood may be first washed three times with ample physiological saline to overcome the apparent autoagglutination similar to what is done for the Coombs’ and crossmatch testing. However, if autoagglutination after three washes persists at more than 1+, it is considered to reflect true autoagglutination, which may preclude typing (as well as Coombs’ testing and crossmatching), because it always looks like DEA 1+ blood. In such circumstances, DEA 1- blood should be used, until the patient does not agglutinate anymore and can be retyped. DEA 1+ blood from severely anemic animals may not agglutinate when exposed to the anti-DEA 1 or other reagents because of a prozone effect. In these cases, some of the patient’s plasma may be discarded before applying a drop of blood onto the card. Finally, recently transfused dogs may display a mixed field reaction, with only the transfused or recipient cells agglutinating if they were DEA 1 mismatched.

Blood Crossmatching Test

Whereas blood typing tests reveal the blood group antigens on the red blood cell surface, blood crossmatching tests assess the serologic compatibility or incompatibility between donor and recipient. Thus the crossmatch test checks for the presence or absence of naturally occurring and induced alloantibodies in serum (or plasma) without determining the blood type and thus does not replace blood typing. These antibodies may be hemagglutinins and/or hemolysins and can be directed against known blood groups or other RBC surface antigens. Many laboratories commonly use a standardized tube crossmatching procedure, but the interpretation of the agglutination reaction is highly variable. The crossmatching test requires some technical expertise, may be accomplished through a veterinary laboratory along with blood typing, and is done with washed EDTA-anticoagulated blood from recipient and potential donor(s). The DiaMed gel column technique and more recently the in-clinic DMS gel tube assay have been evaluated and were found to be simple, sensitive, and standardized methods to crossmatch dogs and cats. In addition, Alvedia introduced a simple strip crossmatch test with a Coombs’ phase.
The major crossmatch tests search for alloantibodies in the recipient’s plasma against donor cells, whereas the minor crossmatch test looks for alloantibodies in the donor’s plasma against the recipient’s RBCs. Generally tube segments from collection bags are used for this purpose in dogs. The presence of autoagglutination or severe hemolysis may preclude the crossmatch testing. A major crossmatch incompatibility is of greatest importance, because it predicts that the transfused donor cells will be attacked by the patient’s plasma, thereby causing a potentially life-threatening acute hemolytic transfusion reaction. Because fatal reactions may occur with less than 1 ml of incompatible blood, compatibility testing by administering a small amount of blood is not appropriate; this has been shown in experimental studies to potentially result in fatal reactions. A minor crossmatch incompatibility should not occur in dogs if canine donors have not been transfused previously and is of lesser concern because donor’s plasma volume is small, particularly with packed red cell products, and is diluted markedly in the patient. Do not use previously used dogs as donors.

The initial blood crossmatch between two dogs that have never before received a transfusion should be compatible, because dogs do not have naturally occurring alloantibodies. Therefore, a crossmatch may be omitted before the first transfusion in clinical practice for dogs. Because the crossmatch does not determine the blood type of the patient and donor, a compatible crossmatch does not prevent sensitization of the patient against donor cells within 1 to 2 weeks. Thus, previously transfused dogs should always be crossmatched, even when receiving again blood from the same donor. The time span between the initial transfusion and incompatibility reactions may be as short as 4 days and the induced alloantibody can last for many months to years (i.e., years after the last transfusion alloantibodies may be present). Again, a blood donor never should have received a blood transfusion to avoid sensitization. The practice of transfusing patients with the least compatible unit does not have any scientific basis. Nevertheless, some minor agglutination results in crossmatching a patient may be unrelated to alloantibodies and unspecific (e.g., patient’s RBC damage by uremia and other illnesses, donor cells after extended storage of unit in the refrigerator). Of course, any patient with true/persistent autoagglutination may not be matched to any donor.

Although transfusion of blood and its components is usually a safe and temporarily effective form of therapy, there is always a risk for potential hazards. Adverse reactions usually occur during or shortly after the transfusion and can be due to any component of whole blood. Most transfusion reactions can be avoided by carefully selecting only healthy donors; using appropriate collection, storage, and administration techniques; performing blood typing and crossmatching; and administering only the needed blood components.

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ROLE OF EPIGENETIC REGULATION IN CANCER DRUG RESISTANCE

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Despite the rapid development of new antitumor strategies and the identification of novel drug targets, drug resistance is still a serious problem both in the human and in the canine cancer clinic. Cancer cells may evade toxic chemotherapy by reducing the amount of drug targets, modifying apoptotic pathways, increasing the enzymatic breakdown of drug molecules, upregulating DNA-repair proteins or overexpressing drug efflux transporters. The increased expression of ABCB1, a prominent member of the ABC transporter superfamily, provide an efficient mechanism to reduce intracellular concentrations of several widely used anticancer drugs. Using in vitro models, our aim was to characterize regulatory mechanisms that underlie the “phenotype-switch” of cancer. We find that epigenetic changes are responsible for the rapid emergence of drug resistance, and also for the paradoxical resensitization of tumors following “drug holidays”. Our studies on primary tumor cultures isolated from a clinically relevant mouse model of breast cancer point to new possibilities to overcome drug resistance.
SIGNIFICANCE OF THE EPIGENETIC REGULATION TO INHIBIT THE DEVELOPMENT OF CHEMOTHERAPY RESISTANCE

Edina Karai¹, András Füredi², Kornélia Szebényi², Péter Vajdovich¹, Gergely Szakács²

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Although, recent advances in tumor therapies have revealed significant impact on patient survival, treatment of malignancies is still a major challenge in human and canine cancer. The efficacy of new, promising drugs (signaling pathway and angiogenesis inhibitors) is often hampered by the emergence of multidrug resistance (MDR). As a result of MDR, the initial hypersensitivity of cancer is lost as the cells evolve to survive in the highly toxic environment. One of the main contributors of the mechanisms responsible for MDR is the overexpression of one of the members of the ABC (ATP-binding cassette) transporter family, namely P-glycoprotein (Pgp, ABCB1, MDR1). Pgp extrudes a wide spectrum of cytotoxic compounds (from chemotherapeutics to anti-HIV drugs) out of the cells, resulting in the decreased intracellular concentration of the drugs. Recently, an alternative mechanism has been proposed with an emphasis on epigenetic regulation.

First, in this study we’ve used a fluorescence-based FACS assay to measure Pgp-activity of canine B cell lymphoma samples (n=70) collected before and during the chemotherapy treatment. As expected, higher Pgp activity (MAF>0.2) at diagnosis proved to be an adverse prognostic factor for survival (p<0.05). Longitudinal follow up of canine patients evidenced the frequent increase of Pgp activity during the course of chemotherapy.

Secondly, to study the mechanisms underlying the rapid emergence of drug resistance in dogs, we performed in vitro experiments, too using P388 B mouse lymphoblastic leukemia cell line model. We’ve examined the effect of epigenetic inhibitors (Temozolomide, SAHA) and COX-2 inhibition on Pgp expression by combining specific inhibitors with long term (over 100 days) doxorubicin treatment of P388 cells. In 26 days a rapid increase of Pgp expression was also observed in P388 cells treated only doxorubicin (13nM), which could be specifically prevented by the COX-2 inhibitor Celecoxib.

Our data suggest that combination therapy using Doxorubicin and epigenetic inhibitors e.g. Temozolomide, SAHA or COX-2 inhibitor (Celecoxib) may be an effective means to prevent the emergence of MDR.
DIAGNOSIS OF CANINE AND FELINE LEUKAEMIA BY
HAEMATOLOGY ANALYZERS

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The aim of the study. We’ve compared three hematology devices (one impedance and two laser types), to find differences in blood analyses of patients with haemopoietic malignancies, compared to the manual smear analysis.

Material and method. Blood samples from the tested individuals were collected in K3-EDTA tubes, and measured by Abacus Junior 5 Vet, Siemens Advia 120, and Sysmex Xt-2000iv hematology analyzers within 24 hours after sampling, blood smear analysis and immunophenotyping were also performed, the latter by FACS analysis. The smears were examined by counting 250 cells of all samples.

Results: Six dogs and one cat were examined in the study; three dogs suffered by chronic small lymphocytic leukemia (CLL); one dog with CLL and higher numbers of large lymphocytes; two dogs with acute lymphoblastic leukemia (ALL); and one cat with systemic mast cell tumor.

Based on the results of impedance technique machine (Abacus Junior 5 Vet), we suspected CLL in all CLL cases, although the percentage results showed significant difference compared to the smear analysis (deviation of small lymphocyte count was 17 % compared to smear analysis). In ALL cases the impedance device mixed the large lymphocytes with neutrophil granulocytes (deviation of neutrophil count was 34,1% compared to smear analysis).

Advia 120 had almost the same results in cases of CLL, compared to the smear analysis, while in patients with ALL, lot of large lymphocytes were detected as small lymphocytes (deviation of small lymphocyte count was 34,3% compared to smear analysis). LUC value was the one third of the total large lymphocyte count (LUC was 10% compared to 35,3% large lymphocyte in blood smear).

Sysmex Xt 2000iv has given almost the same percentage of smear analysis for CLL cases. Large lymphocytes, in ALL cases were detected as monocytes (deviation in monocyte count was 43.4% compared to smear analysis). This machine provides a possibility to re-identify cells of which populations are hanging out of the edges of the fixed gates. This technique could improve the accuracy of the measurement, in patients with higher populations of large lymphocytes (38,3% suspected large lymphocytes by manual analysis of Sysmex, compared to 35,3% of large lymphocytes in smear analysis).

The aforementioned details were more observable in the cat with systemic mast cell tumor. Impedance machine detected them as neutrophils (deviation of neutrophils: 56.3%), while two laser cell counters detected them as monocytes (deviation of monocytes compared to the smear analysis measured by Sysmex and Advia was 25.8% and 23.4, respectively) The subsequent manual analysis with Sysmex could determine mast cells more accurately. It identified them as lysis resistant cells (LRR). LRR by Sysmex and mast cell percentage by smear analysis was 39.6% and 38,6%, respectively.

Conclusion: CLL cases were measured reliably by the three automated hematology analyzers. The impedance device could not recognize large lymphocytes. The laser machines determined some of them as monocytes, but the manual analysis of Sysmex proved to be the most accurate.