Drug resistance in canine multicentric lymphoma

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Drug resistance in canine multicentric lymphoma

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(met een samenvatting in het Nederlands)

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Opgedragen aan mijn vader
een blijvende bron van inspiratie
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Contents

1A

An introduction to canine lymphoma, multidrug resistance, and ABC-transporters

Canine Lymphoma: a review

Lymphoma is amongst the most commonly diagnosed malignancies[1] in the dog and represents the most commonly managed neoplasia in veterinary medical oncology. Although in the majority of cases (±80%) the disease is initially successfully managed with chemotherapy, only a minor proportion (±10%) of dogs with lymphoma is ultimately cured. This failure to cure canine lymphoma is thought to result from the development of resistance to (chemotherapeutic) treatment and is therefore referred to as drug resistance. Since in the later stages of the disease the observed resistance comprises multiple cytostatic agents, it is often referred to as multi-drug resistance or MDR.
The following chapter will provide an overview on canine lymphoma and (multi)drug resistance.

Epidemiology and etiology

Although canine lymphoma (cL) is in essence not a single disease but rather a collection of clinically and morphologically distinct forms of lymphoid cell neoplasia, it is the most common hematopoietic neoplasia in the dog with an estimated minimal annual incidence rate of 13-114 per 100,000 dogs[1-3]. The incidence of cL is rising, as in humans[4, 5], and a recent study suggested a correlation in the incidence of lymphoma in both species, potentially suggesting common risk factors[6]. Similarities in incidence, clinical presentation, molecular biology, treatment and treatment response between cL and human non-Hodgkin lymphoma (NHL)[3, 7], the fact that dog breeds represent closed gene pools and the dog's genome has been sequenced, combined with the willingness of pet owners to treat their dogs for this disease, makes cL a promising spontaneous large-animal model for human NHL.
Lymphoma affects dogs of any age, but is predominantly diagnosed in middle-aged to older dogs with the incidence rate increasing with age from 1.5 cases per 100.000 dogs for dogs <1 year of age to 84 per 100.000 for dogs >10 years[2]. There is no apparent sex predisposition, but intact female dogs appear to have a reduced risk[8] and early (<1 year) neutralization was shown to increase the risk of developing lymphoma (Golden retriever[9], Vizsla[10]). This finding is possibly similar to that in humans where pre-menopausal women have the lowest risk for developing NHL. Despite this potential effect of sex and the likely role of sex-steroids, progesterone- and estrogen-receptors are infrequently expressed on neoplastic lymphocytes[11].
Although cL can affect any dog breed, middle-sized to larger dog breeds are overrepresented (Table 1)[8, 12, 13]. This finding could not be related to growth hormone levels[14] and most likely reflects a genetic susceptibility in some of the larger dog breeds. A familial occurrence or clustering has been reported in a few breeds including the Bull mastiff[15], Rottweiler[12, 16] and Scottish terrier[12]. It was also demonstrated that some dog breeds are more prone to developing a specific immunophenotype of cL, which further supports a genetic background[6, 17].
Although no definitive cause for cL has been established, living in industrial areas and exposure to (household) chemicals[18, 19], living near waste incinerators, radioactive or a polluted sites[6, 20], and exposure to magnetic fields[21] were all shown to increase the risk of

Table 1. Breed with an increased and decreased risk for developing lymphoma[12-14].

Increased risk	Decreased risk
Basset hound, Bernese Mountain Dog, Bouvier des Flandres, Boxer, Bulldog, Bullmastiff, Cocker Spaniel, Dobermann Pinscher, German shepherd, Golden retriever, Irish Wolfhound, Labrador retriever, Rottweiler, Saint Bernard, Scottish terrier	Chihuahua, Dachshund, Pomeranian, Poodle (Miniature and Toy), Yorkshire terrier

developing cL. A possible role for the phenoxy-herbicide 2,4-dichlorophenoxyacetic acid[22, 23] was invalidated following re-analysis of the data[24]. Further evidence that environmental toxins might play a role in carcinogenesis comes from the observation that defective genotypes of the detoxifying enzyme glutathione-S-transferase (GST), and GST theta 1 (GSTT1) in particular, are over-represented in human cancers. In the dog 27 GSTT1 variants were identified of which one variant was significantly associated with cL. This genotype was found in 18% of all cL cases and the observed mutation was predicted to affect mRNA splicing and as a result enzyme expression and activity[25].

Failure to repair DNA-damage that arises for instance from oxidative stress or radiation, increases the risk for developing neoplastic disease. It was found that Golden retrievers with cL had a lower capacity for DNA damage repair compared to Golden Retrievers without cL and mixed-breed dogs[26].

Many animal species have a species-specific leukemia-virus and the existence of a canine "lymphoma" virus would therefore be likely. The detection of reverse-transcriptase activity in supernatants of lymph node cultures from dogs with cL[27] was the first suggestion of a possible viral cause and more recently a gamma-herpes (Epstein-Barr) virus was detected in a proportion of cL cases[28-30]. Nevertheless, there is no definitive proof of a viral etiology.

In humans *Helicobacter*-infections are associated with the development of gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Experimental infections in Beagles showed the formation of lymphoid follicles, but no progression to gastric lymphoma was noted[31].

A dysfunctional immune system might play a role in lymphomagenesis and in humans, immunosuppression (HIV-infection, immunosuppressive therapy for organ or stem cell transplantation), autoimmune disease or immunodeficiency disorders increase the risk of developing lymphoma. There are few data to support this theory in the dog, but auto-immune disease (most likely secondary) is common in dogs with cL[32] and there is a case-report of a dog with cL following cyclosporine treatment[33]. A case-control study in dogs with cutaneous lymphoma (*mycosis fungoides*) documented an increased risk for dogs that were diagnosed with atopic dermatitis[34]. A similar finding has been reported in human medicine and was suggested to result from immunodysregulation, either due to the disease itself, or concurrent immunosuppressive therapy.

Molecular changes in canine lymphoma

Whole genome and exome sequencing of human diffuse large B-cell lymphoma (DLBCL) cases, the most common form of human NHL, identified 322 recurrently mutated cancer genes that included pathways related to chromatin modification, NF-κB, PI3-kinase, B-cell

lineage and Wnt-signaling[35]. In dog with cL, some of these pathways have also been studied. Comparative genomic hybridization has demonstrated limited genomic instability in cL compared to human non-Hodgkin lymphoma with most copy number abnormalities in dog chromosomes 13 and 31 (human chromosomes 8 and 21) and no evidence of *CDKN2A/B* deletion in canine B-cell lymphoma[36, 37]. Although gain of dog chromosome 13 appears a consistent finding in cL, it was also found in other neoplasias[38, 39] suggesting a role in general tumor progression, rather than cL initiation. DNA methylation affects gene expression (transcription) and genomic hypomethylation (increased gene expression) is common in cL[40].

Increased Rb phosphorylation and subsequent activation of CDK4, is common in high-grade canine T-cell lymphoma and can result from deletion of p16/loss of dog chromosome 11[41], hypermethylation of the CpG island of the p16 gene[42] and possibly a deletion of the p15-p14-p-16 locus[43]. Increased Rb phosphorylation in high-grade canine B-cell lymphoma was associated with overexpression of c-Myc and trisomy of dog chromosome 13[41]. Canine and human c-Myc share a high degree of homology[44].

The canine and human p53 gene are similar in structure and function[45] and various p53 mutations (point mutations, deletions, insertions in one or both alleles) have been observed[46]. Nevertheless, p53 mutations are relatively rare in cL[45, 47]. Although the majority of cL cases show no to low-intensity p53 expression[48, 49], strong expression appears more common in older animals, high-grade, and possibly T-cell lymphomas[48].

Mutations in N-Ras are common in leukemia[50], but rare in cL[51, 52]. Activation of the MAP/ERK-pathway has been related to upregulation of P-gp and drug resistance in human B-cell lymphoma[53] and this was also observed in the side-population of a canine lymphoid leukemia cell line. MAP/ERK upregulation was associated with increased expression of P-gp, and lung-resistance protein (LRP) mRNA[54], while downregulation of this pathway coincided with a decreased Breast Cancer Resistance Protein (BCRP) expression[55].

Although PI3K mutations are rare in hematopoietic neoplasia, increased activation of PI3K-signaling is common. Interest in the PI3K-pathway recently increased due to the discovery of CAL-101, a selective inhibitor of the p110δ catalytic isoform of PI3K. CAL-101 decreases the constitutive expression of phospho-Akt and was successfully used in the treatment of indolent NHL[56]. Furthermore it was shown that the mTOR pathway plays a role in P-gp mediated drug resistance in B-cell lymphoma[57]. No data are available on the role of PI3K in cL.

NF-κB, a regulator of genes that control cell proliferation and apoptosis, is constitutively activated in human diffuse large B-cell lymphoma. Canonical activation of NF-κB and increased NF-κB target gene expression was demonstrated in a subset of dogs with B-cell lymphoma[58-60] and it was shown that the proteasome inhibitor bortezomib reduced NF-κB expression and inhibited cell proliferation in neoplastic canine lymphoid cell lines[61].

Loss-of-function mutations and deletions in the tumor suppressor gene PTEN, result in enhanced cell growth and decreased apoptosis and are commonly found in human cancers including lymphoma[62], but have not yet been studied in cL.

The Wnt/β-catenin pathway is involved in many types of cancer[63] and activation appears

common in leukemia[64] and diffuse large B-cell lymphoma[65]. The Wnt/β-catenin pathway has not yet been studied in canine hematopoietic neoplasia.

The proto-oncogen c-kit, a tyrosine protein kinase, is an important factor in the proliferation, survival and differentiation of hematopoietic stem cell including mast cells. The expression of c-kit in cL is typically very low, but was found to be increased in some high-grade T-cell lymphomas and acute leukemia[66, 67].

The Bcl-2 family consists of approximately 25 proteins that regulate apoptosis through controlling the Mitochondrial Outer Membrane Permeabilization Pore (MOMPP). The canine anti-apoptotic members Bcl-2 (B-cell lymphoma 2)[44], Mcl-1 (myeloid cell leukemia-1)[68], and Bcl-XL (B-cell lymphoma-extra large)[69] show a high degree of similarity to the human counter parts. Bcl-2 was not consistently upregulated in all B-cell cL cases and showed no relation with drug resistance or survival[47]. Expression of the pro-apoptotic protein Bad was demonstrated in some cL cases, particularly B-cell lymphomas, and not in non-neoplastic lymph nodes[70]. Survivin, a member of the inhibitor of apoptosis protein (IAP) family, inhibits caspases and thereby apoptosis. Survivin is commonly expressed in a variety of human and canine cancers including cL[71]. Increased survivin expression correlated with a shorter median disease-free period[72] and it was shown that the locked nucleic acid antisense oligonucleotide EZN-3042 was able to inhibit survivin transcription in *vitro*[73].

The high-mobility group of proteins (HMG) consists of three families, group A (*HMGA*), group B (*HMGB1*) and group N (*HMGN*) that regulate gene expression and genomic DNA conformation changes in embryonic cells. *HMGA1*, but not *HMGA2*, was overexpressed in cL and was higher in B-cell lymphoma[74].

The Bcl-6 gene encodes for a transcription factor that contributes to the development of human DLBCL. Bcl-6 mRNA and protein expression was respectively low or absent in canine high-grade B-cell lymphoma and failed to predict clinical outcome, which is in contrast to the situation in humans[75, 76].

Although angiogenesis and invasion seem less critical in the development of cL, dogs with cL have higher MMP-9 activity and VEGF levels than normal dogs[77]. MMP-9 and VEGF were higher in canine T-cell and stage V lymphoma and VEGF-expression correlated (positively) with grade in T-cell lymphomas, however, none of these parameters were useful in predicting prognosis[77-79]. The tumor suppressor gene tissue factor pathway inhibitor 2 (TFPI-2) is associated with inhibition of tumor invasion. Hypermethylation of the promotor for this gene and down-regulation of TFPI-2 expression was found in most canine high-grade B-cell lymphomas[80].

Cancer stem cells or tumor-initiating cells have been identified in many cancers and are promising therapeutic targets. There are currently two reports on possible stem cells in cL[81, 82]. These cells were characterized by high expression of Bmi-1 (regulates p16) and the ABC-transporters including abc1 (P-gp) and abcg2 (BCRP).

Clinical presentation

The most common clinical presentation of cL is the so-called multicentric form that affects the peripheral lymph nodes, but extra-nodal forms exist and include mediastinal,

abdominal (gastrointestinal, hepatic, hepato-splenic, splenic, renal), cutaneous, ocular, central nervous system and pulmonary lymphoma. The clinical presentation of cL can be further complicated by the presence of paraneoplastic syndromes.

Multicentric lymphoma

Multicentric cL (Fig. 1a) accounts for ±75% of all cL cases[83, 84] and is classified into five stages as defined by World Health Organization[85] (Table 2). Occasionally lymphoma is limited to a single lymph node (stage I) or several lymph nodes in a region of the body (stage II), but generalized, non-painful lymphadenopathy (stage III) with secondary involvement of liver and/or spleen (stage IV) or blood and/or bone marrow (stage V) are more common. A substage can be added to further characterize the clinical performance of the dog using the suffix a to indicate the absence of systemic signs, and b to indicate the presence of systemic signs such as fever, weight loss or hypercalcemia.

Table 2. The World Health Organization (WHO) stages for canine multicentric lymphoma[85].

Stage	
I	Single node or lymphoid tissue in single organ (excluding bone marrow)
II	Regional involvement of multiple lymph nodes (± tonsils)
III	Generalized lymph node involvement
IV	Stage I-III with involvement of liver and/or spleen
V	Stage I-IV with involvement of blood or bone marrow
Substage	
a	absence of systemic signs
b	presence of systemic signs (fever, >10% weight loss, hypercalcemia)

Mediastinal lymphoma

Thymic or cranial mediastinal lymphoma is more common in younger dogs[86] and is almost exclusively of T-cell origin[87]. Presenting clinical signs include dyspnea (due to the mass effect and/or pleural effusion), polyuria/polydipisia (due to hypercalcemia) or the so-called (cranial) vena cava syndrome (Fig. 1b). This syndrome is characterized by pitting edema of the head and neck resulting from a mediastinal mass that restricts venous return to the heart. While diagnostic imaging (radiographic, CT or ultrasonographic examination of the thorax/cranial mediastinum) will demonstrate the presence of a cranial mediastinal mass (Fig. 2a), only a cytological or histological biopsy of the mass will lead to a definitive diagnosis of a mediastinal lymphoma or thymoma. Although cytology is in most cases sufficient for obtaining a definitive diagnosis, immunophenotyping[88] or PARR (see section Diagnosis) are useful when cytology is inconclusive.

Gastrointestinal lymphoma

Gastrointestinal (GI) lymphoma has no age, sex or breed predisposition, but the Boxer and Sharpei are the breeds most commonly reported[89, 90]. GI lymphoma is typically of

T-cell origin[89] and can present as a focal, multifocal[91] or diffuse disease. Ultrasonographic examination of the GI-tract can be helpful in discriminating enteritis from intestinal neoplasia, but can be normal in up to 25% of dogs with GI lymphoma[92]. Loss of normal wall layering, but to a lesser extent increased wall thickness, and mesenterial lymphadenopathy are suggestive for neoplasia[93], but a definitive diagnosis will require a biopsy. In most

Figure 1. Clinical signs associated with canine lymphoma: lymphadenopathy (a), cranial vena cava syndrome (prior to chemotherapy b* and the same dog 2 days following chemotherapy b**), mucosal lymphoma (c), cutaneous lymphoma (d) and uveitis secondary to multicentric lymphoma (e).

cases endoscopic, mucosal biopsies will be sufficient for obtaining a diagnosis[94, 95], but occasionally full-thickness (transmural) biopsies[96] or a clonality assay (PARR)[97, 98] are required. A common paraneoplastic syndrome in canine GI T-cell lymphoma is local[99] or systemic (peripheral blood)[100] eosinophilia.

Hepatic lymphoma

Primary hepatic cL is relatively rare and the prognosis is poor compared to other anatomical forms of cL[101, 102]. Complete remission was obtained in close to half (8/18) of the dogs, resulting in a median survival of 63 days. Leukocytosis, neutrophilia, hypoalbuminemia, or hyperbilirubinemia reduced the likelihood of obtaining a complete response. Absence of complete response and hypoalbuminemia were associated with a shorter survival[101]. Two forms of primary hepatic T-cell lymphoma (hepatosplenic and hepatocytotropic) have been described and both had a very poor prognosis with almost all dogs dying within 24 days after the diagnosis[102].

Cutaneous lymphoma

Cutaneous lymphoma (Fig. 1d) is typically a T-cell lymphoma and more frequently epitheliotropic than non-epitheliotropic[103]. The WHO-classification recognizes three forms of cutaneous epitheliotropic T-cell lymphoma: *mycosis fungoides*, Sézary syndrome and pagetoid reticulosis[104]. There is no known cause for cutaneous lymphoma, but having been diagnosed with atopic dermatitis increases the risk for developing *mycosis fungoides*[34]. *Mycosis fungoides* is a CD8+ T-cel lymphoma that mainly affects older dogs (mean age 11 years) with no clear breed predilection[105] although case series report a relatively high number of Boxer dogs[106] and Bichon Frises[107]. Cutaneous epitheliotropic T-cell lymphoma typically presents as a chronic multifocal skin disease, but can also affect the mucous membranes (especially buccal) and mucocutaneus junctions[104] (Fig. 1c). Skin lesions are variable in appearance and include diffuse erythema, scaling, focal hypopigmentation, plaques and nodules[106, 107]. Initially the disease is limited to the skin, but later in the disease lymphadenopathy, leukemia and concurrent involvement of internal organs can occur, a stage that in humans is referred to as Sézary syndrome[106].

Ocular lymphoma

Primary (peri-)ocular cL is relatively rare (<0.5% of all lymphoma cases) and cL is more commonly associated with secondary uveitis[108, 109] (Fig. 1e). Ocular lymphoma is mostly of B-cell origin and can present as an intraocular mass or conjunctival disease[110-114], but can also affect extraocular structures like the palpebral conjunctiva and lymphoid tissue of the third eyelid[115, 116]. The prognosis for conjunctival lymphoma seems better than for intraocular lymphoma, with most intraocular cases progressing to central neurologic disease[117].

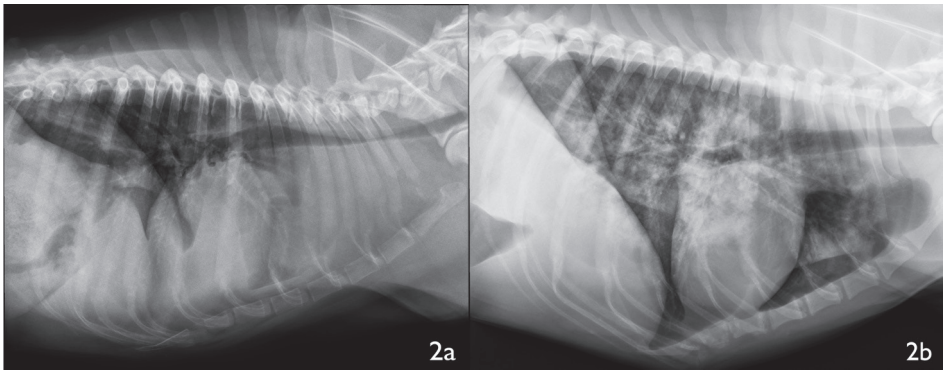

Figure 2. Left lateral thoracic radiographs of dogs with varying presentations of canine lymphoma, including a cranial mediastinal mass consistent with lymph node or thymic involvement (a) and diffuse mixed interstitial to alveolar pulmonary lung pattern in all lung lobes (b) (courtesy of the division of Diagnostic Imaging).

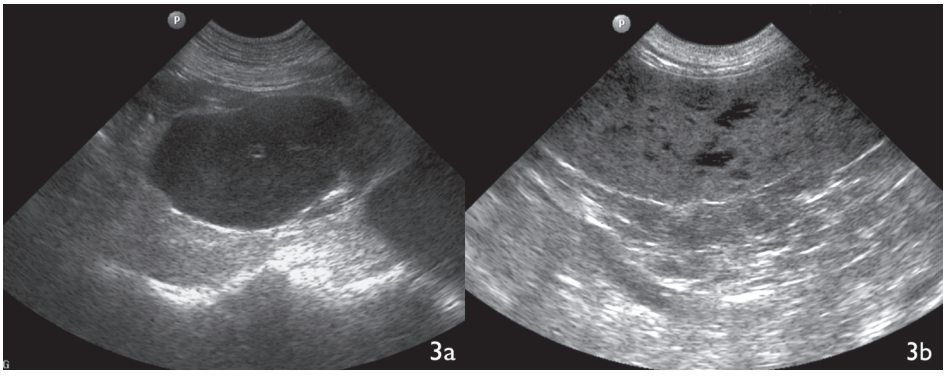

Figure 3. Abdominal ultrasonographic images showing typical findings in dogs with canine lymphoma including rounded, hypoechoic lymph nodes (a) and an enlarged spleen with multiple hypoechoic nodules, often referred to as a "Swiss-cheese" spleen (b) (courtesy of the division of Diagnostic Imaging).

Nervous system lymphoma

Nervous system lymphoma is relatively rare and can present as central (brain and/or spinal cord[118] including extraspinal structures[119, 120]) or peripheral nervous system disease. In case of primary brain lymphoma, cL affects only the brain and/or meninges[121, 122], while in case of secondary nervous system lymphoma both nervous and extra-nervous system locations are involved[123]. Central nervous system lymphoma typically presents as a multifocal disease and can lead to classic signs like seizures, changes in mental status, ataxia, and paresis/paralysis, but also central diabetes insipidus has been reported[124]. Although MRI-findings are not specific, a presumptive diagnosis is in most cases made by combining the findings

with clinical information[125]. A definitive diagnosis requires a cytological or histological biopsy of a mass lesion or analysis of cerebrospinal fluid that, in most cases, will show atypical lymphocytes[122]. In the case of secondary central nervous system lymphoma, the disease is not limited to the nervous system and a diagnosis is usually made following the biopsy of an extra-nervous system site.

Pulmonary lymphoma

Pulmonary involvement in cL is common and can be both primary, as well as secondary to any other form of cL[126]. Respiratory signs are rare[127] except when cL leads to pleural effusion and in most cases pulmonary involvement is only suggested on the basis of additional diagnostic tests (thoracic radiography, CT-scan) performed for screening or staging purposes. Pulmonary lymphoma can result in alveolar, bronchial and/or interstitial infiltrates, pleural effusion and lymphadenopathy[128] (Fig. 2b). Thoracic radiography and tracheal washes tend to underestimate pulmonary involvement compared to bronchioalveolar lavage[129]. Since pulmonary involvement does not affect the prognosis[127], thoracic radiographs are not recommended for routine staging.

Atypical forms of canine lymphoma

Canine lymphoma can affect any organ or location and a wide variety of these atypical form has been reported including oral[130], periapical[131], nasal[132, 133], choanal[134], vertebral[135, 136], skeletal[137, 138], skeletal muscle[139], synovial membrane (leading to ruptured cranial cruciate ligament)[140], adrenal (leading to hypoadrenocorticism)[141], renal[142, 143], urinary bladder[144], uterine[145], prostate[146, 147,] cardiac and pericardial[148, 149] involvement.

Paraneoplastic syndromes

Hypercalcemia, an uncommon but well-documented paraneoplastic syndrome in the dog, is most commonly associated with cL[150]. Hypercalcemia results from the production of PTH-related peptide (PTH-rP)[151] by CD4+ T-cell lymphoblasts[152]. Hypercalcemia reduces the collecting ducts' response to anti-diuretic hormone leading to renal diabetes insipidus and increased calcium levels in the pro-urine reduce sodium reabsorption in ascending loop of Henle, with both mechanisms contributing to polyuria. Furthermore hypercalcemia causes vasoconstriction of the afferent glomerular arteriole, thereby decreasing the glomerular filtration rate[153] and increasing the risk for hypoxia of the medulla (medullary thick ascending limb)[154] and potentially acute renal failure.

PTH-rP is not measured in the regular PTH-assay and requires a specific test (IRMA)[151]. However, the presence of PTH-rp can be suspected, since PTH-levels are typically (extremely) low in hypercalcemia of malignancy[155]. Although hypercalcemia is almost exclusively associated with T-cell lymphoma, it has occasionally been documented in B-cell lymphoma.

Other paraneoplastic syndromes include monoclonal gammopathy[156-158], hypoglycemia[159], polycythemia in renal cL[160], eosinophilia[99, 100], and immune-mediated diseases including immune-mediated hemolytic anemia[161], immune-mediated thrombocytopenia[32] and polymyositis[162].

Diagnosis
Clinical pathology

A complete hematological and clinical-chemistry profile is often routinely performed in cL cases and can show a wide range of non-specific abnormalities[163].

Most dogs will have a mild to moderate non-regenerative anemia, but anemia can also arise through blood-loss (GI lymphoma) or (secondary) immune-mediated hemolytic anemia[161]. Increased red blood cell counts (polycythemia) have occasionally been reported in renal lymphoma[142] and are thought to result from inappropriate erythropoietin secretion. Morphologic erythrocyte abnormalities can be observed and include schistocytes[164,] eccentrocytes[165] and acanthocytes[166].

Leukocyte counts in dogs with cL are typically normal, but both leukocytosis and leukopenia are described. In most cases leukocytosis represents inflammatory changes, rather than true leukemia, which accounts for ± 20% of the leukocytosis in cL[163].

Mild, asymptomatic thrombocytopenia is common[167], and in some instances thrombocytosis has been noted[164, 168]. Most dogs with cL will show mild abnormalities in their hemostatic profile that are consistent with hypercoagulability, which will often persist during chemotherapy[164, 169].

Changes in liver enzyme levels and kidney function can result from cL invading these organs, but are more often secondary to cL (reactive hepatopathy, dehydration anorexia). Serum LDH (lactate dehydrogenase) levels, and particularly an increase in the isoenzymes LDH2 and LDH3[170], are an important prognostic indicator in human NHL. Although one study showed that LDH levels and isoenzyme measurement were prognostic in cL[171], other large studies failed to show this relation[172, 173] and it is currently not recommended to include LDH measurement in the routine staging for cL patients. ALP-levels can increase with hepatic involvement and prior exposure to glucocorticoids, but were not predictive for treatment response[174].

Serum protein levels can decrease due to blood or protein loss in case of GI lymphoma, but also increase due to monoclonal gammopathy. Hypoglycemia is occasionally reported[159]. Hypercalcemia is documented in ±10-15% of cL cases and almost exclusively associated with T-cell lymphoma. Urinalysis is not routinely performed, but proteinuria is a common finding in cL. It is typically mild, independent of (sub)stage and has no impact on prognosis[175].

Bone marrow involvement is reported in up to 55% of dogs[176] and cannot be accurately predicted from peripheral blood counts[177]. Bone marrow core-biopsies or flow cytometry of bone marrow samples are not routinely performed in veterinary medicine, cytological examination of a single bone marrow aspiration sample is considered sufficient for identifying bone marrow involvement[178]. Since a bone marrow biopsy is considered an invasive procedure, and the outcome has limited effect on prognosis (unless there is massive bone marrow involvement) or treatment, it is presently not advised to routinely perform a bone marrow biopsy[179].

Diagnostic Imaging

Thoracic and abdominal radiographs from dogs with (multicentric) lymphoma will often show abnormalities, but these are typically non-specific and only suggest cL as a possible differential diagnosis[180]. For instance in ±70% of thoracic radiographs abnormalities were found and included thoracic lymphadenopathy, pulmonary infiltrates, and the presence of a cranial mediastinal mass[127] (Fig. 2).

Ultrasonography of peripheral lymph nodes and the abdomen is helpful in accurately assessing lymph node size and architecture[181, 182] (Fig. 3a) as well as hepatic and/or splenic involvement[183] (Fig. 3b), but not for diagnosing or excluding GI lymphoma, since findings are either non-specific or absent in up to 25% of dogs[92].

A CT-scan is superior for evaluating the extent of disease, but does not typically allow for the specific diagnosis of lymphoma. For instance in the case of a cranial mediastinal mass, a CT-scan is useful for staging purposes, but is not able to discriminate between a thymoma and a mediastinal lymphoma[184].

In human oncology the PET-CT scan is a routine staging procedure, but veterinary patients have limited access to this imaging modality and up-to-now only small case-series have been published[185-188]. The use of scintigraphy using radiolabeled peptide nucleic acid-peptide conjugate targeting Bcl-2 mRNA has been described in multicentric B-cell cL and was able to assess the extent of disease and monitor treatment response[189].

Cytology, Histology, Immunophenotyping

Cytological examination of a fine needle aspirate from a neoplastic lymph node is a quick, sensitive and minimally invasive technique for diagnosing high-grade cL[190, 191] and the diagnostic method of choice, but may be insufficient for diagnosing low-grade cL (Fig. 4).

Examination of a histological biopsy will improve the diagnosis of low-grade cL and allow for further sub-classification of cL. An excisional biopsy (removal of a complete lymph node) is preferred, but in many cases an incisional (thru-cut) biopsy is sufficient. The increased possibilities of flow cytometry to analyze fine needle aspirates from neoplastic lymph nodes might decrease the need for excisional biopsies[192, 193].

Histologically, cL is characterized based on a number of morphological criteria including growth pattern, nuclear size, nuclear morphology (chromatin and nucleoli), mitotic index and immunophenotype. Based on these characteristics, cL is classified using one of the classification schemes that have been developed over the past several decades, including the Rappaport[194, 195], Lukes-Collins (US)[195, 196], KIEL (Europe)[195, 197], Working Formulation[198, 199], updated Kiel[195, 200, 201], REAL[202] and WHO systems of classification[203-205] (Table 3).

The (updated) Kiel-classification can be applied to both histological[206] and cytological[190] samples and although it has some limitations in diagnosing certain low-grade subtypes, like marginal zone lymphoma[201], it is still the most commonly used classification scheme.

The WHO-system is based on the evaluation of histological biopsies and when the human classification scheme was applied to the dog, it was shown that close to 95% of 265 lymphoma cases were represented by only five subtypes: diffuse large B-cell lymphoma (54%), marginal zone (B-cell) lymphoma (4%), peripheral T-cell lymphoma not otherwise

Table 3. The current canine lymphoma WHO classification and corresponding categories in the Kiel classification and Working Formulation[85].

	WHO classification		Working Formulation	Kiel Classification
B	Precursor	B-ALL/B-LBL	ALL, LBL	Lb
	Mature	B-CLL/B-SLLL	B-CLL/B-SLLL	Lc
		LLI	-	-
		LPL	SLLP	Pl
	Follicular	MCL	DSCCL	Cc
		FCCL-I	FSCCL	Cc
		FCCL-II	FMCL	Cc-Cb
		FCCL-III	FLCL	Cc
		NMZ	CLL, FSCCL, DSCCL, DMCL	Lc, Cc, Cb
		SMZ		
		MALT-L		
	Plasmacytic	PCT Indolent	Extramedullary plasmacytoma	Extramedullary plasmacytoma
		PCT Anaplastic		
		Myeloma	Multiple myeloma	Multiple myeloma
	B-LCL	DLBCL, DLBBL	DMCL, DLCL, DLCCL	Cc, Cb
		LCIBL	LCIBL	Ib
		T-cell rich B-LCL	DMCL, DLCL	Cb
		Thymic B-LCL	DMCL, DLCL	Cb
	Butkitt-type, Burkitt-like		SNCCL	-
T	Precursor	T-ALL/T-LBL	ALL, LBL	Lb
	Mature	T-CLL/T-SLL	CL, SLL	Lc
		LGL	-	-
		NK-CLL	-	-
	Cutaneous	CEL	MF/SS or DLCL, DMCL, DSCCL	MF/SS
		CNEL	MF/SS-like or DLCL, DMCL, DSCCL	MF/SS-like
	PTCL		DSCCL, DMCL, DLCL, DLCCL	-
	ATL	ACL	DSCCL, DMCL, DLCL, DLCCL	-
		AIL		
	ITCL		DSCCL, DMCL, DLCL, DLCCL, LCIBL	-
	ALCL		LCIBL	-

B- or T-ALL, B- or T-acute lymphoblastic leukemia; B- or T-LBL, B- or T-lymphoblastic lymphoma; B- or T-CLL, B- or T-chronic lymphocytic leukemia; B- or T-SLL, B- or T-small lymphocytic lymphoma; LLI, B-cell lymphocytic lymphoma of intermediate type; LPL, lymphoplasmacytic lymphoma; LGL, large granular lymphocyte lymphoma or leukemia; NK-CLL, NK-cell chronic lymphocytic leukemia; MCL, mantle cell lymphoma; FCCL, follicle center cell lymphoma; MZL, marginal zone lymphoma; NMZ, nodal marginal zone lymphoma; SMZ, splenic marginal zone lymphoma; MALT-L, mucosa-associated lymphoid tissue lymphoma; PCT, plasmacytic tumours; B-LCL, large B-cell lymphoma; DLBCL or DLBCCL, diffuse large B-cell lymphoma, cleaved or not cleaved; LCIBL, large cell immunoblastic lymphoma; CEL, cutaneous epitheliotropic lymphoma; MF/SS, mycosis fungoides/ Sézary syndrome; CNEL, cutaneous non-epitheliotropic lymphoma; PTCL, extranodal or peripheral T-cell lymphoma; AIL, angioimmunoblastic lymphoma (also known as angioimmunoblastic lymphomatous disease); ATL, angiotropic lymphoma; ACL, angiocentric lymphoma; AIL, angioinvasive lymphoma; ITCL, intestinal T-cell lymphoma; ALCL, anaplastic large cell lymphoma; Lb, lymphoblastic; Lc, lymphocytic; Cc, centrocytic; Cb, centroblastic; Ib, immunoblastic; Pl, plasmacytic/plasmacytoid; F- or DSCCL, follicular or diffuse small cleaved cell lymphoma; F- or DMCL, follicular or diffuse mixed cell lymphoma; F- or DLCL, follicular or diffuse large cell lymphoma; DLCCL, diffuse large cleaved cell lymphoma; SLLP, small lymphocytic lymphoma plasmocitoid; SNCCL, small non-cleaved cell lymphoma; PCT, plasmacytoma.

specified (16%), nodal T zone lymphoma (14%) and T lymphoblastic lymphoma (5%) (Table 4)[204].

Although the majority of cL cases are of B-cell origin (±70%), with a smaller proportion of T-cell (±30%) or non-B/non-T cell lymphomas (<5%)[152, 195, 201, 207, 208], this distribution can vary significantly between canine breeds. For example the Irish Wolfhound, Shih Tzu, Airedale Terrier, Yorkshire Terrier, Cocker Spaniel and Siberian Husky are in >80% of cases of T-cell origin, while the Doberman Pinscher, Scottish Terrier, Border Collie, Cavalier King Charles Spaniel and Bassett Hound have in >80% of cases B-cell ML[17], but there might be geographical differences in B/T-distribution per breed[6].

Immunophenotyping can be slide-based on cytological[208] (Fig. 5) or histological [209] biopsy samples or performed with flow cytometry[192, 210] and were shown to have an excellent correlation[211]. The most commonly used antibodies include CD20, CD21, CD79α and PAX5 for B-cell lymphoma and CD3, CD4 and CD8 for T-cell lymphomas[208]. Although immunophenotyping with 2 antibodies (typically CD3 and CD79α or CD20) can result in up to 20% unclassified cL[212]. This is sufficient for routine patient management, but increasing the number of antibodies will result in more conclusive immunophenotyping and a lower percentage of non-B/non-T lymphomas.

More recently the use of the PARR (see *PCR-based techniques*) has been advocated as an alternative for immunohistochemistry or flow cytometry, but flow cytometry proved superior over the PARR (overall agreement between tests around 60%), although in the absence of fresh samples the PARR is an acceptable alternative[213].

PCR-based techniques

PCR-based techniques have been used for diagnosing, staging, immunophenotyping[213-215] and detecting minimal residual disease[216]. The most commonly used PCR technique is the PCR assay for antigen receptor rearrangement or PARR, which amplifies the variable regions of the immunoglobulin genes and the T-cell receptors. The presence of mono- or oligoclonal peak is highly suggestive of cL and although this test is very sensitive (70-90%), infections (like *E. canis*) or other neoplastic diseases (like AML) can lead to false-positive results[214]. Although the PARR has been used for staging, clinical stage proved a better prognostic indicator than PARR stage and is currently not recommended for this purpose[217]. The PARR can be used for immunophenotyping, but is less accurate and should be reserved for cases where there is no sample available for immunostaining or flow cytometry[213].

Biomarkers

Biomarkers are serum proteins that can be used to diagnose and/or monitor a specific disease. TK1, MCP-1, VEGF, MMP and endostatin have been evaluated in cL because of their use in human oncology, but electrophoresis techniques have also been used to identify potential specific canine biomarkers.

Serum Thymidine Kinase 1 (TK1) levels, a salvage enzyme involved in DNA precursor synthesis, are higher in dogs with cL than in healthy dogs or dogs with non-hematologic neoplasia and might correlate with stage and prognosis[218-220] and as a result appears the most promising

Table 4. Overview of most commonly recognized forms of canine lymphomas[204, 227, 228].

WHO	Updated Kiel	IPT	%	Gr	Clinical	Cytology	Histology	Therapy	Survival (months)
Diffuse large B-cell lymphoma	Centroblastic, immunoblatic, polymorphic	B	54	H	MCL, ± liver, ± spleen, ± blood/bone marrow	Large cells, scant cytoplasm, uniformly large nuclei, usually round, rarely cleaved or indented, centroblastic and/or immunoblastic, mitotic figures common	Diffuse, thinning of Ln capsule, compression of peripheral and medullary sinus, fading germinal centers, destruction of normal nodal structures, filling of the medullary cords with neoplastic cells, many tingible body macrophages, high MR	(L)CHOP	±9
Marginal zone lymphoma	Medium-sized macronucleated cells (MMC)	B	4	L	Splenic or nodal	Intermediate-sized nuclei, prominent single central nucleoli, abundant lightly stained cytoplasm, no mitotic figures	Coalescing aggregates of indolent B-cells surround fading remnants of germinal centers, resembling the marginal zone of a lymph node follicle	Splenic: surgery, no CHOP; Nodal: prednisolon + chlorambucil?, no CHOP	Splenic 13, Nodal 21
Peripheral T-cell lymphoma, NOS	Pleomorphic mxied	T	16	H	MCL, ± liver, ± spleen, ± blood/bone marrow	Large to variable cell size, frequently cleaved or oval nuclei, nucleoli inconsistent in number and size, pale cytoplasm, variable mitotic figures, few tingible body macrophages	Diffuse, thin Ln capsule, paracortical expansion, sinus compressed, focally obliterated, spread of neoplastic cells to perinodal tissue. The neoplastic cells are usually large, may be of variable cell size, frequently have cleaved or oval nuclei, nucleoli inconsistent in number and size, variable MR, may lack numerous tingible body macrophages.	(L)CHOP + CCNU?	±6
Nodal T zone lymphoma	Small clear cell	T	14	L	Regional - MCL	Small cells, round nucleus, can have irregular shallow nuclear indentation, extended pale cytoplasm (hand mirror/uropods)	Uniform population of small or intermediate T-cells expanding in paracortex and medullary cords, no effacement nodal architecture, nuclei no internal nuclear detail, shallow nuclear indentation, extended pale cytoplasm	Prednisolon + chlorambucil	20-33
T-lymphoblastic lymphoma	Lymphoblastic	T	5	H	MCL, ± liver, ± spleen, ± blood/bone marrow, ± mediastinal, ± hypercalcemia	Uniform population intermediate-sized cells, evenly dispersed chromatin, obscured nucleoli	Thin capsule, focal perinodal colonization, diffuse cortical and medullary filling, moderate anisokaryosis, nuclei range from round to oval or irregularly indented, densely stained cells (dispersed chromatin) that obscures nucleoli, no tingible body macrophages, high MR	(L)CHOP+ CCNU?	6-8

Gr: grade, H : High, IPT: immunophenotype, Ln: lymph node, L: Low, MCL: multicentric lymphadenopathy, MR: mitotic rate

biomarker. Monocyte chemotactic protein-1 (MCP-1) expression is higher in dogs with cL than healthy dogs and correlated with stage[221]. Vascular endothelial growth factor (VEGF) and matrix-metalloproteinase (MMP) 2 and 9 are under control of transforming growth factor beta (TGF-β). Dogs with cL had a higher MMP9 activity and VEGF levels combined with lower TGF-β levels than normal dogs[77]. Furthermore MMP9 and VEGF were higher in T-cell and stage V lymphoma and VEGF was higher in high-grade than low-grade T-cell cL. Endostatin prevents angiogenesis and tumor growth through inhibition of endothelial cell proliferation and migration and tended to be higher in dogs with cL, but was not useful as a biomarker[222]. Serum electrophoresis has identified multiple potential candidate proteins in dogs with cL including prolidase (proline dipeptidase), triosephosphate isomerase, glutathione-S-transferase and kininogen (all down-regulated), macrophage capping protein, haptoglobin, macroglobulin, α-antichymotrypsin and inter-α-trypsin inhibitor (all up-regulated)[223-226], but results on their clinical usefulness are not yet available.

Staging canine multicentric lymphoma

Staging multicentric cL is based on the WHO-staging scheme (Table 1) and requires a thorough patient history (substage a vs b), physical examination (stage I-IV) and evaluation of peripheral blood and bone marrow (stage V). Although additional laboratory tests and diagnostic imaging are recommended, it has to be appreciated that increasing the number of staging tests or choosing more sensitive staging techniques will only lead to more correct staging and likely stage migration, but not necessarily to a better prediction of prognosis[217].

Given the strong negative effect of hypercalcemia and the T-cell immunophenotype on prognosis, it is advised to include these two tests in the routine staging protocol. Although the results of thoracic radiography, unless the presence of cranial mediastinal mass is noted[127], and ultrasonography of the abdomen will improve correct staging[217], they do not affect prognosis in multicentric cL and are therefore not routinely recommended.

Despite the fact that bone marrow involvement has a significant effect on prognosis and cannot reliably be predicted from peripheral blood counts[177], a bone marrow biopsy is currently not routinely recommended[179].

Cytological examination of fine needle aspirates of extra-nodal sites (liver, spleen, blood and bone marrow) is currently the most commonly used staging technique, but the use of PARR[214, 215] and flow cytometry[227] have been reported. These results will often lead to stage-migration, but do not provide a better indication of prognosis.

Therapy

The majority of therapy-related studies focus on the chemotherapeutic treatment of intermediate to high-grade multicentric cL and information on the optimal treatment for low-grade and extranodal forms of cL is limited.

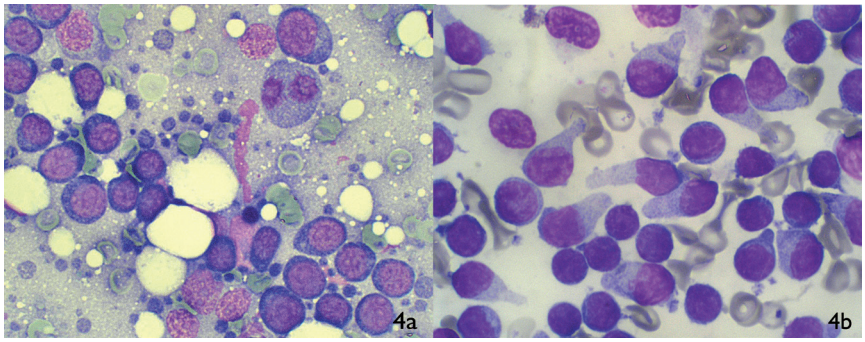

Figure 4. Microscopic pictures showing the typical cytological appearance of a high-grade lymphoma (a: diffuse large B-cell lymphoma, immunoblastic) and a low-grade lymphoma (b: lymphoplasmacytoid T-cell) (courtesy of dr. Erik Teske).

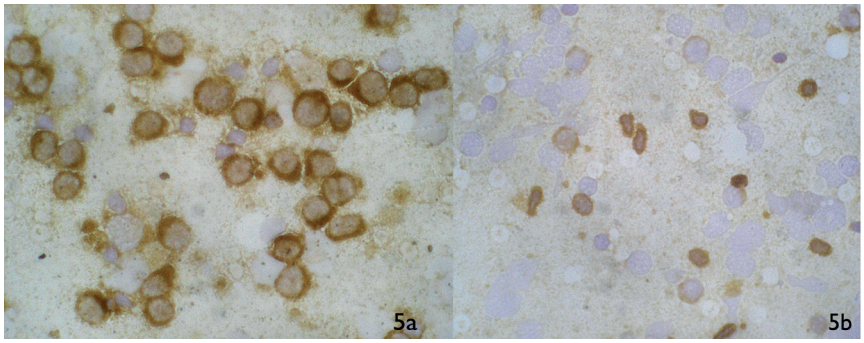

Figure 5. Microscopic pictures showing the cytological appearance of an immunocytochemical staining for CD79α (a) and CD3 (b) on a lymph node aspirate demonstrating CD79α-reactivity for the neoplastic B-cells and CD3-reactivity for the reactive (non-neoplastic) T-cells consistent with a high-grade B-cell lymphoma (courtesy of dr. Erik Teske).

Chemotherapy

Given the systemic nature of cL, chemotherapy is considered the therapy of choice. The goal is to obtain a maximum effect (high complete response-rate, long response-duration) with a minimum of consultations (reducing stress and discomfort for both animal and owner), drug administrations (reducing drug excretion in owner's environment) and toxicity. A comprehensive overview of the reported treatment protocols is provided in Tables 5 and 6.

Glucocorticoids

Glucocorticoids induce lymphocyte and lymphoblast apoptosis[230] and are routinely used in the treatment of cL. Most dogs will experience a good partial to complete response

Table 5. Comparison of different first-line therapy protocols reported for the treatment of canine multicentric lymphoma.

Protocol	Dogs (n)	Length protocol (weeks)	CRR (%)	Median DFP (days)	Median OST (days)	1-year OST (%)	2-year OST (%)	1-year CRR (%)	2-year CRR (%)
P[230]	49	As long as response	43	53*	NR	NR	NR	NR	NR
H (continuous)[240]	21	NR	76	206	266	NR	NR	NR	NR
H (continuous)[241]	27	15	52	309	322	20	0	77	NR
H (intermittent)[244]	18	H given up to maximum dose	78	81	171	NR	NR	NR	NR
CCNU-P[243]	17	15	35	40	111	NR	NR	NR	NR
(L)CVM[245]	147	As long as response	77	140	265	25	NR	NR	NR
COP[240]	20	As long as in CR	70	100	224	NR	NR	NR	NR
COP[246]	49	78	76	159	224	NR	NR	NR	NR
(L)CHP[247]	65	22	65	203	200	35	22	29	NR
(L)CHOP[171]	112	36, COP maintenance	73	238	344	NR	NR	42	28
L-CHOP[232]	138	10, L-asp maintenance	84	NR	NR	42	NR	NR	NR
LCHOP[248]	68	78	65	274	301	27	13	40	21
LVCAM**[249]	55	135	84	220	303	52	24	42	25
LVCA-S[250]	51	25	94	282	397	NR	NR	NR	NR
VELCAP-L[251]	98	75	69	385***	517***	NR	NR	53	25
VELCAP-S[252]	82	15	68	140	NR	NR	NR	NR	NR
VELCAP-SC[253]	94	21	70	168	302	44	13	17.4	15.5

P = prednisolon, H = hydroxydaunorubicine or adriamycine (=A), O = oncovin or vincristine (=V), C = cyclophosphamide or Endoxan (=E), L/EL = L-asparaginase, M = Methotrexate, NR = not reported. CR = complete response, CRR = complete response rate, DFP = disease-free period, OST = overall survival time. * mean,** LVCAM = University Winsconsin- Madison protocol, *** only reported for animals achieving CR.

Table 6. Comparison of different rescue chemotherapy protocols used for the treatment of canine multicentric malignant lymphoma.

Protocol	Dogs (n)	Overall response (%)	Median response duration (days)	Complete response (%)	Complete response duration (days)
Actinomycin D[260, 261]	25 49*	0 41	0 129	0 41	0 129
Mitoxantrone[242, 262]	15 44	 41	21 127	47 30	84 NR
Dacarbazine[263]	40	35	43	3	144
CCNU[264]	43	27	86	7	110
Dacarbazine- doxorubicine[265]	15	53	45**	33	105**
Dacarbazine - anthracycline or Temozolamide - anthracycline[266]	18 35	72 71	40 85	50 63	NR NR
MOPP[267]	117	65	61	31	63
DMAC[268]	54	72	61	44	112
LOPP[269]	44	52	106	27	112
BOPP[269]	14	50	130	29	130
LOPP-CFU[270]	33	61	98	36	NR
MOMP[271]	88	51	56	12	81
MPP[272]	41	34	56	17	238
L-Asparaginase, CCNU - prednisolone[273, 274]	31 48	87 77	63 70	52 65	111 90
CCNU - Dacarbazine[275]	57	35	62	23	83

MOPP: mechlorethamine, vincristine, procarbazine, prednisolone; DMAC: dexamethasone, melphalan, actinomycin D, cytosine arabinoside; LOPP: CCNU, vincristine, procarbazine, prednisolone; BOPP: carmustine/BCNU, vincristine, procarbazine, prednisolone; MOMP: mechlorethamine, vincristine, melphalan, prednisone; MPP: mechlorethamine, procarbazine, prednisolone.
* half the patients received prednisolone concurrently, ** survival.

that typically lasts for 60-90 days[231, 232] and should be considered a palliative treatment. Some studies have shown that pretreatment with glucocorticoids prior to starting chemotherapy results in lower response rates and shorter remission periods[163, 233-235] and the use of glucocorticoids should be withheld until the decision has been made to not pursue treatment with cytostatic drugs.
The absence of increased prostaglandin E_2 levels[236] combined with no[237] or little[238] COX-2 expression in neoplastic lymph node from dogs with multicentric lymphoma, makes a role for (selective) COX-2 inhibitors in the treatment of cL unlikely.

First-line protocols

Single-agent therapy

Various single-agent therapies have been described and include a mono-therapy with (PEG-) L-asparaginase[239, 240], doxorubicin[241, 242] mitoxantrone[243] and CCNU[244]. Of these protocols a monotherapy with doxorubicin, either as continuous (5x q3 weeks) or intermittent (induction followed by additional doses in case of progressive disease) protocol[245], appears the most effective therapy, but is still less effective than a doxorubicin-based multi-agent protocol and should be intended for palliative purposes.

Multi-agent therapy

Multi-agent therapy protocols are typically injection-protocols that combine Cyclophosphamide, vincristine (Oncovin), Prednisolone (COP) with doxorubicin (Hydroxydaunorubicin) (CHOP) and/or L-asparaginase (L-CHOP). Since doxorubicin-based multi-agent protocols result in the highest response rate and longest response durations, CHOP-protocols form the basis for most currently used treatment protocols for high-grade cL (Table 4).
Early protocols consisted of a more intensive initial protocol aimed at inducing a remission (induction phase) followed by a less intensive protocol aimed at maintaining the lymphoma in remission (maintenance phase). It was later shown that a continuous maintenance phase following successful induction, offered no treatment benefit and as a result protocol length he's gradually decreased. Although a 6-month protocol (induction phase followed by a short maintenance protocol) is considered the standard of care[248, 251, 255], shorter (12-week[256] and 15-week[257]) protocols have been reported and appear equally effective. Increasing treatment intensity, either by increasing the number of drugs, drug dosages or shortening dose intervals, has little to no effect on treatment results, but increases adverse events[258-260].

Rescue Protocols

Protocols used in case of failure to respond to a first-line protocol or following relapse are referred to as rescue protocols and include both single-agent and multi-agent protocols. The choice of treatment protocol varies depending on the moment of relapse with respect to the original (first-line) therapy, drugs used (e.g. cumulative cardiac toxicity of doxorubicin), and clinician's preferences. With respect to the moment of relapse, a distinction has to be made between a relapse during the primary protocol or after (successful) completion of

the primary protocol. A relapse during the primary protocol will often require the use of alternative drugs (i.e. drugs not included in the first protocol), while a relapse following completion of the primary protocol opens the possibility for reinstituting drugs from the original protocol. In general, rescue protocols tend to be less effective. Response rates vary, but are usually of shorter duration (2-3 months) than primary protocols and most rescue protocols tend to be more toxic.

Additional remarks on the chemotherapeutic treatment of cL

T-cell and stage V disease

Most studies show that T-cell and stage V lymphomas carry a poorer prognosis and as a result alternative protocols have been suggested. For T-cell lymphomas, non-CHOP-protocols like L-asparaginase-MOPP show promising results[277], although analysis of a large group of dogs with multicentric T-cell cL treated with a regular CHOP-protocol failed to show poorer treatment results[278].

For dogs with stage V disease a longer maintenance treatment or the use of additional drugs have been suggested and including cytosine-arabinoside appeared to improve treatment outcome[279].

Low-grade lymphomas

Since chemotherapeutic agents predominantly affect actively replicating cells, it could be argued that chemotherapy should be cautiously used in low-grade cL, especially in asymptomatic dogs, and that these dogs should merely be monitored similar to the situation in humans. Recently some cases series have demonstrated that dogs with indolent lymphomas have a good long-term prognosis and that the use CHOP-based protocols offers no survival benefit[228, 229, 280-282]. It is recommended that indolent nodal lymphomas should be monitored and not treated or treated with a low-intensity protocol (like chlorambucil and prednisolone), while for the splenic form only splenectomy is advised.

Tumor lysis syndrome

Chemotherapeutic treatment of dogs with neutropenia due to bone marrow involvement (stage V) or impaired liver or kidney function, remains challenging. On the one hand chemotherapy is the only way to restore organ function, while on the other hand these patients are at high risk for developing drug-related toxicity (sepsis) and tumor lysis syndrome[283-285] due to the reduced capability of metabolizing chemotherapeutic agents and excreting waste products. The risk of tumor lysis syndrome might be assessed through the measurement of serum uric acid, calcium, phosphate and potassium levels[285, 286]. For high-risk patients it is advised to combine intravenous fluid therapy with drugs that will not cause overwhelming cell death or whose metabolism is independent of liver and/or kidney function. Possible drugs to consider include pretreatment with glucocorticoids or start the protocol with L-asparaginase or vincristine. Alternative options include the use of allopurinol of rasburicase[287].

Central Nervous System lymphoma

Treating CNS lymphoma presents with the additional problem of the blood-brain barrier (BBB) that limits the penetration of cytostatic drugs into the CNS. There are two ways of circumventing this problem: either choose drugs that are able to pass the BBB like L-asparaginase, cytosine arabinoside[288] and nitrosurea compounds[289, 290] or administer the drug within the subarachnoid space (intrathecal cytosine arabinoside)[122].

Drug resistance

The efficacy of chemotherapy is limited by the onset of drug resistance and due to the limited alternative treatment options, the onset of drug resistance greatly impacts the prognosis. Drug resistance can have many causes, but drug transporters of the ATP-Binding Cassette superfamily[291], and P-gp (ABCB1) in particular, appear to play an important role in cL[292-298]. In case of the onset of drug resistance options are limited, but it can be tried to reverse drug resistance using P-gp inhibitors, switch to drugs that are not P-gp substrates including alkylating agents and L-asparaginase or consider radiotherapy.

Radiotherapy

Although lymphoid cells are very radiosensitive, lymphomas typically present as a systemic disease and as a result radiotherapy plays a limited role in the treatment of cL.

For localized cL, radiotherapy has been used in an adjuvant setting (mediastinal[299], urinary bladder[144]) and as a monotherapy in the treatment of oral mucocutaneous cL. In this specific form of cL radiotherapy led to a complete response in 5/12 dogs, with a median survival of 740 days and only mild toxicity[300].

Although radiotherapy has been used as a monotherapy for treating multicentric cL (2x half-body irradiation, 7 Gy, 4 weeks apart), treatment results were poor (objective response in 5/14 dogs; median duration of 102 and 54 days for respectively complete or partial response[301]) and adverse events were common and especially severe in dogs with an advanced stage of disease.

Radiotherapy is more often used in an adjuvant setting together with systemic chemotherapy. In this setting the whole body is irradiated, either in a single session of total body irradiation (TBI) or two sessions of half body irradiation (HBI). TBI carries the risk of severe, and potentially life-threatening, bone marrow depression, which requires the use of (autologous) bone marrow or peripheral blood stem cell transplantation. Bone marrow transplantation in the dog was already performed in 1979[302] and although initial reports showed promising results, treatment-related morbidity and mortality were high[303, 304]. A more recent report showed less treatment-related morbidity and mortality[305,] but nevertheless TBI has been gradually replaced by two sessions of HBI. The use of HBI has been evaluated following completion of or within a chemotherapy protocol and with a regular (high) or low-dose rate. Regular dose-rate HBI (2x 8 Gy in 2 days, 3-4 weeks apart) was administered during or following completion of a CHOP-based chemotherapy protocol and resulted in a modest increase in first (median 311 respectively 455 days) and overall (median 486 days respectively 560 days) remission period[306, 307]. An alternative approach

was the use of low-dose rate HBI (10 cGy/min; 2x 6 Gy, 2 weeks apart) within a CHOP-based chemotherapy protocol and this lead to prolonged remission (455-410 days) and survival (560-684 days) periods with acceptable toxicity[308-310].

Radiotherapy has also been used as a rescue therapy in dogs with drug-resistant multicentric cL and in these animals all peripheral lymph nodes were irradiated (6x 2Gy, 3x/week). All dogs obtained a complete remission resulting in a median survival of 143 days[311].

Immunotherapy

Immunotherapy has still been moderately applied in the treatment of cL, while it has become an important component in the treatment of a variety of human B-cell malignancies. In human B-cell lymphoma, the use of a monoclonal antibody targeting the CD20 receptor (Rituximab®) has significantly improved disease-free interval and overall survival. Although immunohistochemistry demonstrated the presence of CD20 in cL[312, 313], it failed to bind Rituximab®[314] and new anti-canine CD20 monclonal antibodies are being developed[315]. Other antibodies that have been reported are the murine anti-canine lymphoma monoclonal antibody (MAb-231) that showed both *in vitro*[316] and *in vivo*[317] activity in cL and the murine anti-human leukocyte antigen-DR monoclonal antibody (L243) that was able to temporarily stabilize disease progression in dogs with cL[318].

The use of vaccines for treating cL has been subject of many studies. In the earliest studies, killed lymphoma cell extracts were combined with Freund's adjuvans and although initial reports showed a treatment benefit[319], this was later attributed to the use of the Freund's adjuvans[320]. Intralymphatic administration of a killed autologous tumor vaccine following induction with chemotherapy was reported by Jeglum *et al*[321-323] but the reported results were inconsistent and survival benefit could only be demonstrated in subsets of patients.

A DNA-vaccine targeting canine telomerase reverse transcriptase was able to induce an immune response against telomerase in dogs with multicentric cL[324] and the combined use of the vaccine and chemotherapy (COP-protocol) resulted in both a lasting immune response, as well as an increase in survival without adverse events in dogs with B-cell lymphoma[325]. The use of an autologous vaccine consisting of hydroxylapatite ceramic powder with autologous heat shock proteins purified from a neoplastic lymph node also proved effective in prolonging disease control without increasing treatment toxicity[326].

The use of autologous CD40-activated B-cells loaded with total RNA from autologous lymphoma cells following induction of a complete response with chemotherapy resulted in a functional tumor-specific T-cell response *in vivo*, but there was no improvement in treatment results following the first-line treatment, but only improved rescue therapy results[327]. The use of adoptive immunotherapy using non-specific autologous T-cells was proven feasible and effective in increasing the length of first remission and overall survival in dogs with multicentric cL[328].

Miscellaneous and future treatments

Retinoic acid receptor (RAR) and retinoid X receptor (RXR) expression has been studied in cutaneous[329] and multicentric cL[330] and were commonly and exclusively expressed in neoplastic lymphoblasts in both forms of cL. Their role in cL is not understood, but offers potential applications for diagnosis and treatment. A small study in dogs with cutaneous cL showed a 42% (6/14) remission rate using isotretinoin and etretinate, but more studies are necessary before this treatment can be recommended[331].

Combining hyperthermia with chemotherapy failed to improve treatment results[332]. In 1962 Hatch[333] described a temporary halt in cL progression following a viral infection and more recent *in vitro* work demonstrated that canine distemper virus is capable of infecting lymphoid cells and inducing apoptosis[334] which might suggest a potential use for viral lympholytic therapy.

Targeted therapy will find its' place in cL as in other cancers and potential drug-able targets include the anaplastic lymphoma kinase[335] and NF-κB. NF-κB activity can be targeted using the NF-κB inhibitor Bortezomib®[61] or the NF-κB essential modulator (NEMO)-binding domain peptide[58]. NEMO-binding domain peptide was used in dogs with multicentric B-cell lymphoma and successfully inhibited NF-κB activity, reduced mitotic index and Cyclin D expression in most of the dogs with little associated toxicity[336]. Although cutaneous cL is typically treated with CCNU[337, 338], the tryrosine-kinase inhibitor masitinib[339] appears a suitable alternative.

Prognosis

Many prognostic factors have been evaluated in the dog and include clinical parameters, pretreatment clinical pathology results, histology, immunophenotype, grade, proliferation markers, molecular prognosticators, and biomarkers. In human high-grade NHL, disease prognosis is successfully stratified using the International Prognostic Index (IPI) that includes the factors age, stage, elevated serum LDH levels, performance status and involvement of extra-nodal sites, but a similar index has not yet been developed for high-grade cL.

Clinical parameters

Most literature on cL deals with medium- to high-grade multicentric lymphoma and reports that sex, weight, WHO-stage and substage are prognostic for remission and survival with female, small breed dogs in stage I-IV, substage a carrying a more favorable prognosis than male, large-breed, stage V and substage b dogs[172, 247, 250, 251, 340, 341].

Extranodal lymphomas, including GI[91, 342] hepatic[101, 102], mediastinal[127, 343], cutaneous[107] and renal lymphoma, show a poorer treatment outcome than multicentric lymphoma. Nevertheless, it should be realized that within these groups subsets of dogs can perform better. For example, while small intestinal lymphoma has a median survival of 77 days[342], most likely due to intrinsic drug resistance[91], rectal lymphomas have a survival of >1700 days[344].

Treatment with glucocorticoids prior to starting chemotherapy reduces the response rate to chemotherapy[233-235]. Failure to respond to therapy (no complete response) at the start of therapy or following relapse negatively affects treatment outcome. In all these situations,

treatment failure is thought to result from drug-resistance. Although pretreatment P-gp expression was found to be prognostic in older studies[292, 293], two recent studies[345, 346] failed to confirm these earlier reports.

Pretreatment clinical pathology results

In contrast to human NHL, absolute lymphocyte count and neutrophil/lymphocyte ratio (> 3.5) were not predictive for progression-free survival in the dog[347]. Increased neutrophil and mononcyte counts correlated with monocyte chemotactic protein-1 (MCP-1) overexpression and a shorter disease-free interval[221]. Hypercalcemia is a poor prognostic indicator[235], but also associated with the T-cell immunophenotype[172, 233] and it has been shown that the presence of hypercalcemia in T-cell lymphomas has no further effect on response to treatment or survival[278]. Serum LDH (lactate dehydrogenase)[172, 173] or ALP-levels[174] are not predictive for treatment response.

Histology, immunophenotype, grade and proliferation indices

The WHO-classification scheme uses histological subtype, grade and immunophenotype to further characterize cL into various subtypes and proved useful for predicting survival[205].
In general, T-cell lymphomas will have shorter remission and survival times[152, 172, 205, 206, 233], but exceptions occur. Most CD4+ T-cell lymphomas are histologically characterized as lymphoblastic or peripheral T-cell lymphomas and show an aggressive disease course (median survival 159 days). While most CD4+ cL are CD45+, low class II MHC expression, there is a minority characterized by CD45-, high class II MHC expression[348]. This CD4+ CD45- T-zone lymphoma is commonly found in older (median 10 years) Golden Retrievers that often present with lymphadenopathy and peripheral lymphocytosis and this subtype has a more indolent disease course (median survival 637 days)[282].
Proliferative indices include mitotic index, agryrophilic nucleolar organizer regions (AgNOR), PCNA and Ki-67 of which AgNOR and Ki-67 can be measured in histological and cytological samples[349]. AgNOR, Ki-67 and PCNA expression levels are higher in cL than in benign lymphoid proliferations[350, 351]. AgNOR and Ki-67 expression levels correlated with grade[352-354] and proved prognostic, but mitotic index and PCNA expression were of no added value[205, 353, 355].

Molecular prognosticators

With the use of DNA microarrays, DLBCL, the most common form of NHL in humans, could be subdivided into a germinal center B-cell-like (GCB) and a post-germinal center or activated B-cell-like (ABC) subtype, that each carry a different prognosis[356] and survival could be accurately predicted by using a subset of 17 genes[357]. A similar differentiation could be obtained with immunohistochemistry (Hans or Choi algorithm) using the expression of CD10, Bcl-6, MUM-1/RF4, FOXP1, Cyclin D2, Bcl-2, GCET1, and MTA3[358, 359]. Genome-wide gene expression analysis in canine lymphoma showed three distinct molecular, and prognostically significant, subgroups: high-grade T-cell, low-grade T-cell and B-cell lymphoma, and could be predicted based on the expression of four genes (*CD28, ABCA5, CCDC3* and *SMOC2*)[360]. In the dog, gene expression profiles appear

more useful than immunohistochemistry since dogs rarely express Bcl-6 and MUM-1/RF4[60]. Trisomy of dog chromosome 13[36], the combination of low class II MHC expression with large cell size (flow cytometry) and age[361], the absence of minimal residual disease (MRD) in peripheral blood[362, 363] and gene expression of VH1-44 (immunoglobulin heavy chain variable region)[364], were all associated with a better prognosis. p53 expression was associated with a shorter survival time, but not duration of first remission[345]. Although p16 (mRNA) expression is typically low in cL, overexpression correlated with a shorter survival time in high-grade multicentric B- and T-cell lymphoma.
Pre-treatment PARR results[215], expression of CD34, CD21 and DC5[361], expression of angiogenic factors (VEGF, VEGFR-1, VEGFR-2), micro-vessel density[79] were not prognostic.

Biomarkers

Of all reported biomarkers only serum TK1 levels[219] proved prognostic. Alpha 1-acid glycoprotein[365] and glutathione-S-transferase plasma levels[366] were not prognostic, but increased prior to relapse and could potentially be used as biomarkers. The results on alpha-fetoprotein (AFP) are conflicting[367, 368] and its' use is not recommended.

Future goals and challenges

Although our knowledge on the genetics, molecular biology, and diagnosis of cL has grown substantially over the past 25 years, this has had little effect on treatment and has only marginally improved prognosis.
Chemotherapy still remains the mainstay for the treatment of cL and it appears that we have reached a plateau in what this treatment modality has to offer. More elaborate and more intense chemotherapy protocols increase toxicity, but do not improve treatment outcome. This results partly from the onset of drug resistance, but in part also from the lack of new (classes of) chemotherapeutic agents. A potentially major step forward in the treatment of cL with chemotherapy would the possibility to prevent, delay or circumvent drug resistance, and in order to do so we need to improve our knowledge of drug resistance to cytostatic agents in general and cL in particular.
Since local therapies including surgery and radiotherapy remain to be of limited value, and new classes of cytostatic drugs are not available, we need to focus on other systemic treatment modalities including immunotherapy and targeted therapy. Especially for this last form of treatment, a detailed understanding of the molecular pathways involved in lymphomagenesis is adamant and requires a thorough characterization of each of the specific subtypes of cL.

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1B

An introduction to canine lymphoma, multidrug resistance, and ABC-transporters

Drug resistance in oncology and the role of ABC-transporters

Drug resistance versus treatment resistance

Chemotherapy is one of the major therapeutic modalities in human and veterinary oncology and the treatment of choice for systemic malignancies like hematopoietic tumors and metastatic cancers. Most cytostatic agents exert their effect through inhibiting mitosis (mitotic inhibitors) or interacting with DNA through inhibition of DNA synthesis (antimetabolites), causing DNA damage (including mutation, fragmentation, intercalation) or inhibition of DNA replication and transcription (alkylating agents, platinum-based drugs and topoisomerase inhibitors).

Drug resistance (DR) can be defined in various ways, but often starts with the clinical observation of treatment failure. Treatment failure in oncology is defined as failure to obtain complete disappearance or recurrence of tumor mass and/or associated paraneoplastic syndromes following an initial complete disappearance of the disease (relapse) with a specific treatment. The division between these two categories of treatment failure is to some extent artificial since this distinction depends on the test used to assess a (complete) response. Tests used vary in sensitivity and range from establishing absence of clinical signs based on patient history, physical exam and/or diagnostic imaging (clinical response) to disappearance of tumor cells in biological samples (cytological response), and in some types of neoplasia by demonstrating the disappearance of neoplastic DNA or specific tumor-associated DNA mutations using PCR-based techniques (cytogenetic or molecular response).

One of the reasons for treatment resistance is the difference between the expected effect (efficacy) of a certain therapy and the observed effect (effectiveness). This discrepancy is in part due to the fact that efficacy is based on results obtained from studies performed under ideal circumstances e.g. laboratory tests or highly controlled clinic trials, and that effectiveness is influenced by the effects of genetic variation and co-morbidities in patients, environmental effects, clinician, and in the veterinary situation, patient-owner's decisions that are encountered in clinical practice.

Another cause for treatment failure would be the resistance of tumor cells to drugs and this represents a common problem in medical oncology that limits the long-term successful use of chemotherapy. In most cases DR will develop over time under treatment (acquired DR), but it can be present from the onset of therapy (intrinsic DR) and although both situations represent two clinically distinct entities, the mechanisms underlying both of these situations are likely to be similar.

Drug resistance is not limited to classical cytostatic drugs and also limits the use of the newer targeted cancer drugs, like protein-kinase inhibitors, but these are beyond the scope of this overview.

Mechanisms of drug resistance

The phenotype of DR can develop through a variety of mechanisms, which can be subdivided into two main categories: either failure to reach sufficiently high drug levels at the tumor site or, despite sufficiently high drug concentrations at the tumor site, failure to achieve the appropriate (cellular) response.

Failure to reach therapeutic drug levels at the tumor site arises at the "supra-cellular" level

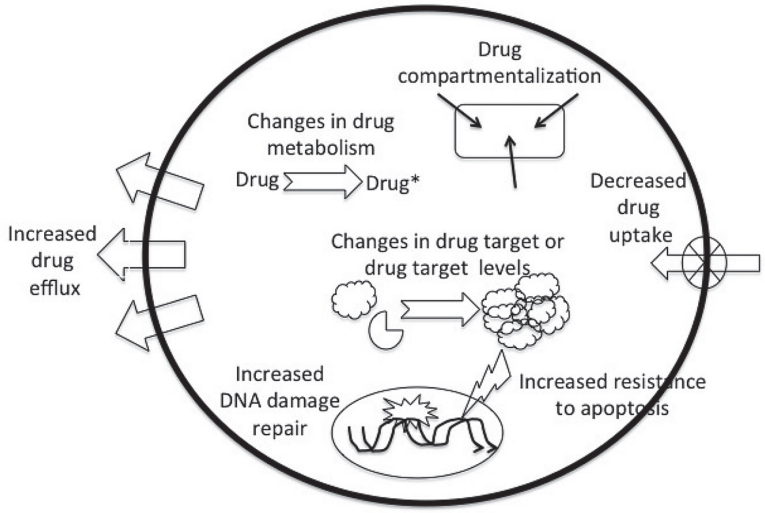

Figure 1. The major cellular drug resistance mechanisms.

Table 1. Major drug resistance mechanisms identified for the classical cytostatic agent drug classes.

Mechanism	Drugs
Decreased uptake	methotrexate, melphalan, nitrogen mustard, cisplatin, other antimetabolites
Changes in drug metabolism (changes in activation or inactivation)	many antimetabolites (e.g. 5-fluorouracil, cytosine arabinoside), alkylating agents, cisplatin
Increased efflux (including compartmentalization)	anthracyclines, vinca-alkaloids, etoposide, taxanes, methotrexate, melphalan
Modifications in target enzyme	methotrexate, other antimetabolites, topoisomerase inhibitors
Increased DNA repair	alkylating agents, cisplatin, anthracyclines, etoposide
Resistance to apoptosis	alkylating agents, cisplatin, anthracyclines, etoposide

and include iatrogenic and host factors. Iatrogenic causes for DR would include inappropriate choice of drug, drug dose or prolonged treatment intervals and create a false impression of DR. Host factors include poor drug absorption, changes in drug metabolism (reduced activation or increased inactivation), increased drug clearance, insufficient drug delivery due to insufficient perfusion or specific organ-barriers (e.g. the blood-brain barrier), tumor micro-environment (low pH, poor perfusion, poor oxygenation[1]) especially in solid tumors, and the degree of non-proliferating (quiescent) tumor cells[2].
Although pharmacokinetic causes, like specific organ-barriers or insufficient tumor perfusion, are common and clinically relevant, drug resistance more often results from pharmacodynamic causes that manifest at the cellular level and include decreased drug uptake, increased drug excretion, changes in drug metabolism (resulting in decreased activation or increased deactivation) and drug compartmentalization. Other mechanisms include increased repair of drug-associated (DNA) damage, increased resistance to apoptosis and changes in the drug target or drug target levels (Figure 1)[2].

The mechanisms underlying cellular DR can be specific for a given drug or drug class, but for most drugs multiple resistance mechanisms have been identified (Tables 1 and 2) that can be simultaneously present. In most cases drug resistance is not restricted to a single drug or drug class, but usually involves resistance to multiple, often structurally and chemically unrelated drugs, a situation referred to as multidrug resistance (MDR). MDR can result from the induction of a single resistance mechanism that is capable of handling multiple drugs, but it also possible that a single drug triggers the development of multiple resistance mechanisms enabling the cell to handle multiple drugs.
There is limited information available on DR in the dog, but in contrast to pharmacokinetic causes for DR that may vary due to species-specific differences in drug metabolism, cellular mechanisms are likely to be similar to those in humans due to the conserved nature of the pathways involved.

Development of drug resistance

DR is a stable or permanent characteristic for a given tumor and is generally assumed to have a genetic basis. The assumption of a genetic background is based on the observation that drug resistant clones generate spontaneously at a rate consistent with known rates of genetic mutation and that this rate can be further increased by exposing tumor cells to mutagenic compounds. Furthermore drug resistant cells retain their resistance in the absence of the initiating drug. The fact that drug resistant clones in a tumor are arise through spontaneous mutations forms the basis for the Goldie-Coldman hypothesis[3] that states that the probability of having at least one drug resistant cell within a tumor cell population depends on tumor size. The relation between tumor size and probability of cure (i.e. the absence of resistance) can be described by $P = e^{-\alpha N}$ (α: spontaneous mutation rate per cell division, N: number of tumor cells). The more cells (the bigger the tumor), the more likely there is a DR clone and with a mutation rate of $1x10^{-6}$, the chance of having a DR clone within 1 mm^3 tumor mass ($\pm 10^6$ cells) is >60%. This model predicts that in order to maximize treatment results,

chemotherapy should be administered when the tumor is at its smallest size, meaning early in the disease or in an adjuvant setting (chemotherapy following surgical removal of the tumor). The model also predicts that the efficacy of a multidrug protocol is higher than that of a monodrug protocol, in particular when a combination of drugs with different and independent resistance mechanisms is used.

There are two major theories as to how DR can develop. The first theory considers, similar to evolution, selection as the driving force, while the second assumes the presence of a drug resistant subpopulation of cancer stem cells.

In the case of selection, DR results from repeated exposure of tumor cells to cytostatic drugs that are intrinsically unable to induce a 100% cell kill. The intermittent exposure to cytostatic drugs combined with the genetic variation and genetic instability within the tumor, offers a selective advantage to the tumor clones that are less susceptible to the cytostatic effect of the drugs used. This selection will ultimately cause the emergence of a "new" resistant tumor cell population and acquired drug resistance.

The cancer stem cell hypothesis assumes the presence of a subpopulation of drug-resistant pluripotent cancer cells that retain the essential property of self-protection through the activity of multidrug resistance transporters[4]. These quiescent constitutively drug-resistant cancer stem cells remain at low frequency among a heterogeneous tumor mass and serve as a reservoir for drug resistant tumor relapses. Although DR may contribute to clinical drug resistance, according to this theory it is not the primary cause for cancer recurrence.

Recent data have shown that besides genetic (mutation, amplification) and epigenetic (DNA hypermethylation, histone deacetylation) changes, drug resistance might also be regulated through microRNAs[5].

Pharmacodynamic mechanisms for drug resistance

Decreased drug uptake

The uptake of drugs into the cells occurs through passive diffusion (e.g. doxorubicin, vinblastine), facilitated diffusion and active transport (e.g. nucleoside analogs). Cytostatic drugs can enter a cell along a concentration gradient in all three ways, but only active transport allows for transport against a concentration gradient. Decreased uptake can result from a decreased binding affinity of a drug to the transporter or a reduced number of transporters. Both mechanisms have been proven to exist, the former for melphalan (L- type amino acid transporters[6]) and the latter for methotrexate (reduced folate carriers[7]) and nucleoside analogs cytarabine, fludarabine and gemcitabine (reduced nucleoside-specific transport carriers[8]).

Changes in drug metabolism

Drug metabolizing enzymes are important determinants of systemic and intracellular drug concentrations. Although oxidation, reduction and hydrolysis (phase I reactions) and conjugation (phase II reactions) play a crucial role in protecting normal cells against toxins, they can also lead to drug resistance in cancer cells through decreased activation of prodrugs (decreased enzyme activity, reduced affinity to activating enzyme) or increased inactivation of drugs (increased enzyme activity)[9].

The cytochrome P450 (CYP) system has an important role in both inactivation of xenobiotics, including chemotherapeutic drugs, and activation of prodrugs (as for instance for most alkylating agents)[10]. High CYP3A4 expression correlates with increased drug inactivation and reduced efficacy as was proven in breast cancer where low CYP3A4 expression correlated with a better response to docetaxel[11].

Phase II reactions include conjugation of drugs to glucuronic acid, sulfate and glutathione (GSH) which improves drug excretion, reduces drug activity and detoxifies reactive electrophilic drugs[12]. An example of a phase II enzyme is uridine 5'-diphosphate-glucuronosyl transferase (UGT) that is associated with glucuronidation and inactivation of anthracyclines and topoisomerase I inhibitors[9].

Another important enzyme family is that of the glutathione transferases (GSTs), which conjugate GSH to toxic electrophilic compounds including endogenous metabolites resulting from oxidative stress and exogenous xenobiotics, including cytostatic agents[13]. Increased GSH- and GST-mediated detoxification plays a role in resistance to many alkylating agents[14], platinum drugs and doxorubicin[15]. One of the cytotoxic mechanisms of doxorubicin is the generation of peroxides and free radicals that are effectively inactivated with reduced GSH. Although these GSH-conjugated drugs are less toxic, they need to be excreted via MRP1 and MRP2 and intracellular accumulation can interfere with the regeneration of the parent components[16].

Other examples in altered drug metabolism include reduced intracellular methotrexate retention due to decreased polyglutamation (due to decreased of folylpolyglutamate synthase activity or increased γ-glutamatehydrolase activity)[17]), cytarabine resistance due to decreased kinase or increased deaminase activity[8], 5-FU resistance associated with changes in dihydropyrimidine dehydrogenase levels[18] and irinotecan resistance due to loss of activating carboxy-esterase-2 activity[19].

The heavy metal scavenger metallothionein can inactivate certain platinum complexes and reactive-oxygen species and elevated expression, for instance in response to hypoxia, is associated with a poor response to platinum drugs[20]. Metallothionein has been detected in canine primary lung carcinoma[21].

Increased drug efflux

Drug efflux is often referred to as the phase III system and an increased efflux capacity is potentially the most important DR mechanism given the wide range of cytostatic drugs and their metabolites that most of these transporters can handle. The first efflux pump discovered was P-glycoprotein (P-gp) with P standing for permeability. P-gp, also known as multidrug resistance protein 1 (MDR1) or ABCB1, turned out to be the first member of the ATP-Binding Cassette (ABC) superfamily, a group of proteins that uses ATP-hydrolysis to actively transport substances across biological membranes. Other important members of the ABC-superfamily associated with DR include multidrug resistance-associated proteins 1 (MRP1; ABCC1) and 2 (MRP2; ABCC2) and breast cancer resistance protein (BCRP; ABCG2). These ABC-transporters will be discussed in more detail in the following section.

Drug transport by ABC-transporters is not restricted to the cellular efflux, but also occurs at

a subcellular (organelle) level. Cytostatic drugs can, for instance, be transported from the cytosol into the endoplasmatic reticulum where they can no longer exert their cytotoxic effect, a process known as sequestration[22]. TAP (transporter associated with antigen processing or ABCB2) protein is one of the ABC-transporters whose subcellular expression is associated with sequestration and can result in a mild resistance to doxorubicin, vincristine and etoposide[23].

Although most transporters belong to the ABC superfamily, there are ATP-dependent non-ABC transporters associated with DR including lung-resistance protein (LRP) or Major Vault Protein and RLIP76. LRP is thought to confer drug resistance by regulating the nucleocytoplasmic transport and the cytoplasmic redistribution of drugs away from their cellular targets[24]. Although LRP has been detected in canine cancers[21, 25], there are no data on the relative contribution of LRP to canine DR. RLIP76 is capable of transporting a wide variety of cytostatic drugs, including doxorubicine and its glutathione-conjugates[26] in humans, but there are currently no data on the role of this transporter in the dog.

Changes in drug target

Changes in drug target are an important mechanism for DR to antimetabolites, taxanes and topoisomerase inhibitors. These drugs interact with intracellular protein targets and resistance is either due to an increase in target protein or a mutation of the target protein resulting in a reduced drug affinity, while at the same time maintaining the normal biologic activity. Examples include resistance to methotrexate (increased in dihydrodrofolate reductase (DFHR) expression and mutated DFHR leading to reduced affinity for methotrexate for[27]), and 5-fluorouracil (increased thymidylate synthase expression[18]. Microtubules are assembled from α-tubulin and β-tubulin heterodimers and overexpression or mutation of β-tubulin isotypes is associated with the primary or acquired DR to tubulin-binding agents like paclitaxel[28].

Topoisomerases catalyze topological changes in the DNA structure, necessary for DNA duplication and RNA transcription, by causing a temporary single strand (topoisomerase I) or double-stranded (topoisomerase II) break in the DNA. Topoisomerase inhibitors inhibit either of these two enzymes, by forming stabilized DNA-topoisomerase complexes, preventing re-ligation of the nucleotide strands and inducing apoptosis. Resistance to topoisomerase I inhibitors (campothecin and topotecan) and topoisomerase II inhibitors (doxorubicin, mitoxantrone, etoposide), result from reduced topoisomerase expression or the development of a mutated topoisomerase with absent or reduced drug affinity[29].

Repair of drug-mediated DNA damage

Cells are constantly subjected to potential causes for DNA damage, including oxidative stress, radiation and chemical substances, resulting in ± $2x10^4$ DNA lesions per day. As a result cells have developed defense mechanism against reactive oxygen species and DNA repair mechanisms[30]. Loss of DNA repair mechanisms increases the susceptibility to mutation and cancer, while an increased repair capacity makes cells less susceptible to the growth inhibitory effects of cytotoxic drugs. DNA repair mechanisms include direct base repair, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR),

DNA double-strand break repair, interstrand crosslink repair[31, 32] and topoisomerase I and II-dependent DNA damage repair[33]. This type of DR has been identified to play a role in DR to alklyating agents, platinum drugs and topoisomerase inhibitors.
Increased expression of the NER enzyme ERCC1, is commonly identified in cisplatin-resistant cancer cells[34]. Alkylating agents cause a covalent modification (methylation or chlorethylation) at the O^6-position of guanine (chlorambucil, lomustine and temozolamide) or the N^7-position of purine bases (cyclophosphamide, iphosphamide), which are monofunctional (single-site adduction) or bifunctional (adduction of two adjacent sites)[35]. Repair mechanisms include direct base repair (MGMT), BER (DNA glycosylases) and NER. The major repair-mechanism for monofunctional alkylation of the O^6-guanine base is through the enzyme O^6-methyl-guanine DNA methyltransferase (O^6-MGMT), that removes the alkyl group from the O^6-guanine position and transfers it to a specific cysteine residue on the enzyme, thereby inactivating itself[36]. O^6-MGMT levels and methylation status of the gene's promotor region (epigenetic change) are useful predictors for the response to alkylating agents in gliomas[37] and B-cell lymphoma[38]. Bifunctional alkylating agents use MGMT for removing the primary adduct followed by NER (including ERCC1) and homologous recombination. Increased DNA-dependent protein kinase (essential for nonhomologous end-joining repair) and XRCC3 (homologous repair) expression have been associated with melphalan resistance in epithelial cells[39]. Double-stranded DNA breaks are repaired by homologous recombination (using undamaged DNA strand as template) and non-homologous end-joining (direct DNA religation without a template)[32].
DNA repair mechanisms have been studied in canine cancer including mammary tumors, mast cell tumors and B-cell lymphoma. Upregulation of DNA-repair enzymes Brca2 and RAD51 was demonstrated in canine mammary carcinomas[40, 41]. MMR enzymes (MLH1, MSH2, and MSH6) were demonstrated in mast cell tumors, but showed no difference in expression between grades[42]. No relation was found between O^6-MGMT mRNA levels (RT-qPCR) and treatment response to chemotherapy or (intrinsic) DR in canine B-cell lymphoma[25].

Resistance to apoptosis

Apoptosis results from activation of the death receptor-mediated (Fas/CD95 system) (extrinsic pathway) or the mitochondrial-mediated pathway (intrinsic pathway). Chemotherapy-induced damage, including DNA damage, radical formation and other forms of cellular stress, activates the intrinsic pathway resulting in the release of mitochondrial cytochrome c that combined with APAF-1 and caspase-9 forms the apoptosome. Although activation of the extrinsic pathway through upregulation of Fas ligand[43] has been demonstrated, dysregulation of the intrinsic pathway (Bcl-2, p53) seems more important[44]. The intrinsic pathway is regulated by proteins of the Bcl-2 family, proteases and amongst others reactive oxygen species, $Ca2^+$ and ceramide[45]. Mutations in the targets upstream of the mitochondria (p53, Akt, RAS), at the mitochondrial level (members of the Bcl-2 family) and those downstream of the mitochondria (heat shock proteins, inhibitors of the apoptosis like survivin), epigenetic silencing of APAF-1 (apoptotic peptidase-activating factor-1), caspase-3 deletion and caspase-independent factors, like apoptosis inducing

Table 2. Overview of reported drug resistance mechanisms per cytostatic drug class.

Drug class	General mechanism	Specific mechanisms	References
Alkylating agents	Decreased uptake	L-carrier (melphalan)	6
	Changes in drug metabolism	Cytochrome P450 polymorphisms (decreased activation cyclophosphamide), Increased GST-conjugation (increased detoxification)	14
	Increased drug efflux	Upregulation ABC-transporters (cyclophosphamide, ABCC2)	58
	Enhanced DNA-repair	Monofunctional agents (MGMT), Bifunctional agents (nucleotide excision repair, homologous recombination, DNA-dependent kinase, XRCC3)	35, 36, 39
Platinum drugs	Decreased uptake	Presumed active uptake	59
	Changes in drug metabolism	Increased inactivation (metallothionein, GSH, thiols)	15, 20
	Increased drug efflux	Upregulation ABC-transporters (cisplatin, MRP2, ATP7b)	60
	Enhanced DNA-repair	Nucleotide excision repair (ERCC1), Mismatch repair	34
	Resistance to apoptosis	p53 mutation, changes in apoptotic mediators (incl Bcl-2, AKT, Fas-L)	61
Mitotic inhibitors (Vinca-alkaloids, Taxanes)	Changes in drug metabolism	High cytochrome P450 expression (docetaxel)	11
	Increased drug efflux	Upregulation ABC-transporters	62
	Changes in drug target	Mutations/altered expression β-tubulin (paclitaxel)	28
Antimetabolites	Decreased uptake	Decreased expression of reduced folate carrier (methotrexate), Reduced nucleoside-specific transport carriers (cytsosine-arabinoside, gemcitabine)	7, 8
	Changes in drug metabolism	Reduced polyglutamation (methotraxate), dihydropyrimidinedehydrogenase (5-FU), Decreased deoxycytidinekinase (dCK) activity (reduced activation gemcitabine)	9, 17, 18
	Changes in drug target	Increased expression (DHFR, methotrexatethymidylate synthase 5-FU), mutated DHFR (methotrexate)	18, 27
Topoisomerase inhibitor I	Changes in drug metabolism	Reduced carboxyl-esterase-2 activity (reduced activation irinotecan), uridine 5'-diphosphate-glucuronosyltransferase (increased conjugation)	9, 19
	Increased drug efflux	ABC-transporters	62
	Changes in drug target	Reduced topoisomerase I expression	63
	Enhanced DNA-repair	tyrosyl-DNA-phosphodiesterase (TDP1) excision pathway, endonuclease pathways, homologous recombination, fork-regression pathway	33
Anthracylines	Changes in drug metabolism	Uridine 5'-diphosphate-glucuronosyltransferase, Increased GST-conjugation (increased detoxification)	9, 15
	Increased drug efflux	Changes in the intracellular distribution (ABCB2 doxorubicin), ABC-transporters (ABCB1, ABCC1, ABCG2)	23
	Changes in drug target	Reduced topoisomerase II expression, reduced affinity (mutation)	63, 64
	Enhanced DNA-repair	Topoisomerase II-DNA complex	62
	Resistance to apoptosis	Increased antioxidative capacity (GST and others)	15

factor (AIF), could potentially all reduce a tumor cell's sensitivity to cytostatic drugs[44]. Although modulating apoptosis appears a promising route in cancer treatment[46], there are conflicting results regarding the importance of resistance to apoptosis in DR[47, 48].

Cellular stress, including DNA-damage, leads to stabilization of p53 and cell cycle arrest allowing for either (DNA) repair or apoptosis. Loss-of-function mutations in the p53 gene are amongst the most commonly identified mutations in a wide variety of cancers in both humans and animals. Cell lines, including lymphoma cell lines, with p53 mutations show a poorer response to treatment with cytostatic agents and a DR phenotype[49].

p53 expression (immunohistochemistry) in canine lymphoma varied from low[50] to common[51], but was typically higher in T- than B-cell lymphomas. p53 mutations (cDNA or DNA sequencing) were demonstrated in approximately ¼ of the primary (9/38) and relapsed (2/8) lymphoma samples[25, 52]. Clinical drug resistance did not appear to be related to p53 mutation and it was argued that p53 mutations in relapsed lymphoma cases might have resulted from prior chemotherapeutic treatment.

The balance between pro- and anti-apoptotic members of the Bcl-2 family controls mitochondrial membrane integrity and overexpression of the anti-apoptotic Bcl-2/Bcl-xL proteins correlates with chemoresistance in leukemia[53], breast cancer[54] and chondrosarcoma[55]. Bcl-2 and survivin were studied in canine lymphoma and Bcl-2 expression (mRNA) in canine B-cell lymphoma could not be related to treatment response[25]. Increased survivin expression was associated with a shorter disease-free period, but failed to increase following relapse[56].

Besides resistance to apoptosis, another option for DR is the upregulation of pro-survival pathways and two of the key signaling pathways involved are the Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathway. Dysregulated signaling of these pathways often results from mutations in upstream growth factor receptors and/or genetic alterations in the kinases, all of which could potentially be inhibited with targeted agents and small molecule inhibitors like the tyrosine-kinase inhibitors[57].

ATP-binding cassette transporters

History

The ABC-transporter superfamily is one of the largest and most well conserved protein families in biology and its members are found in almost all life forms. Despite their importance, the first ABC-transporter was discovered as recently as 1976. The expression of Permeability glycoprotein or P-gp (or ABCB1) was first described in multidrug resistant Chinese hamster ovary cell line that had been selected for colchicine resistance[65]. From that moment, P-gp was considered the main cause for drug resistance in general, and cytostatic drugs in particular. Soon after its discovery research focused on identifying substances that were able to inhibit P-gp function in the hope of reversing drug resistance to cytostatic drugs. In the following years multiple other drug transporters were identified in drug resistant cancer cell lines[66].

It was almost 20 years later that by accident the important physiological role of P-gp became apparent. A colony of mdr1a (-/-) knock-out mice died following ivermectin treatment

for a skin mite infection and it was only then established that the absence of P-gp in the blood-brain barrier was responsible the observed ivermectin neurotoxicity[67]. A few years later history repeated itself, when a deletion mutation in the mdr1 gene was found to be responsible for the high susceptibility of the Scottish collie breed to ivermectin-induced neurotoxicosis[68].

ABC-transporters are located in the cell membrane and capable of exporting a wide variety of substrates, both exogenous substrates or xenobiotics that include drugs and toxins, as well as endogenous substrates including phospholipids, ions, peptides, steroids, polysaccharides, amino acids, organic anions, bile acids. ABC-transporters play an essential role in many physiological processes and it is therefore not surprising that ABC-transporters mutations can lead to a variety of genetic diseases including cystic fibrosis (chloride ion channel, ABCC7), adrenoleukodystrophy (very long chain fatty acids, ABCD1), progressive familial intrahepatic cholestasis (phospholipids and bile acids, ABCB4 and ABCB11), Dubin-Johnson syndrome (conjugated bilirubin, ABCC2), Stargardt disease (N-retinylidene-phosphatidyl-ethanolamine, ABCA4), Tangier disease (cholesterol, ABCA1), immune deficiencies (ABCB2 and ABCB3), Pseudoxanthoma elasticum (ABCC6), and persistent hyperinsulinemic hypoglycemia of infancy (ABCC8 and ABCC9)[69, 70].

ABC-transporters in pharmacology

In humans, 49 ABC-transporters have been identified and these are classified into seven subfamilies labeled A through G[71, 72] (for further information http://nutrigene.4t.com/humanabc.htm). ABC-transporter proteins are characterized based on the sequence and organization of their ATP-binding domain(s) or nucleotide-binding folds (NBF) that contains the for the ABC-transporters characteristic Walker A and B motifs. The functional protein contains as a minimum two NBFs located in the cytoplasm that hydrolyze ATP and transfer the energy (through a mechanism still not completely understood), and two transmembrane domains that enable transport of the substrate across the membrane.

ABC-transporters are located in the outer cell membrane, which enables the transport of substrates out of the cell, and in organelle membranes (endoplasmic reticulum, mitochondria, peroxisome), which allows for compartmentalize of substrates.

High expression levels of the various ABC-transporters are found in the intestine, liver, and kidney where they determine drug uptake and elimination, and in barrier tissues, like the blood-brain barrier and placenta, and stem cells where they determine drug distribution[73] (Table 3).

Another example of how ABC-transporters can affect a drug's pharmacological profile is through drug-drug interactions. The simultaneous use of two substances that are both substrates for the same ABC-transporter can interfere with the elimination of either of these two substances and lead to increased, and potentially toxic, levels of one of the co-administered substances. Although this might seem farfetched, both humans and animals often receive multi-drug therapies or treatments for multiple diseases, and furthermore it should be realized that also drugs used for routine preventative medical care[74] or herbal medicines[75] can have similar effects.

Over the last few years it has become clear that ABC-transporters polymorphisms can account for the variation in drug response observed within a population. Single-nucleotide polymorphisms (SNPs) have been demonstrated for P-gp, MRP1 and BCRP, of which some can result in changes in protein expression or function and affect drug absorption, excretion and distribution[76, 77].

ABC-transporters in drug resistance

Although fifteen of the 48 described ABC-transporters function as a drug-efflux pumps, DR is typically mediated by only three transporters: P-gp (MDR1/ABCB1), multidrug resistance-associated protein 1 (MRP1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2)[78]. *In vitro* studies have demonstrated that most ABC-transporters can transport a wide range of substrates, but there is a considerable degree of substrate overlap between the different transporters[79] (Figure 2, Table 3). Although (cancer) cells can express several ABC-transporters simultaneously, typically the expression of one transporter is dominant[80].

When studying the data on DR, results need to be viewed in light of the techniques used and the samples (cell line versus tumor-sample) studied. Transporter status can be quantified by mRNA expression (real time quantitative PCR), protein expression (immunohistochemistry, Western blot) and functional assays (dye-transport studies, cell viability assays) and these techniques can lead to inconsistent results. It has, for instance, been shown that mRNA levels in cell lines do not always accurately predict protein expression[81] or *in vitro* drug sensitivity[82] and ABC-transporter expression or function in tumor samples does not necessarily predict *in vivo* chemosensitivity[83, 84].
Studies on DR to cytostatic drugs in the dog, and the relevance of ABC-transporters there in, are limited and much of what we know is based on *in vitro* studies in canine cell lines. Nevertheless, there are some data on ABC-transporter expression (mRNA and immunohistochemistry) in canine neoplasia including lymphoma, mast cell tumors, and solid cancers (lung, hepatocellular and mammary carcinoma).

ABCB1 (MDR1/P-gp)

General

P-gp (ABCB1), also referred to as multi-drug resistance protein 1 or MDR1, is a 170 kDa transmembrane protein and was the first ABC-transporter identified. It was originally described in a multidrug resistant Chinese hamster ovary cell line selected for colchicine resistance[65].
In humans, P-gp is expressed at high levels in the apical membrane of epithelial cells including the small intestine, colon, liver and bile ducts, pancreatic ductules, kidney (proximal tubule), endothelial cells in the brain (luminal side), testes, inner ear, adrenal cortex, pregnant endometrium, placenta and hematopoietic stem cells[85, 86]. High pretreatment P-gp expression has been observed in both hematopoietic (leukemia, lymphoma and multiple myeloma) and solid tumors including kidney, colon, liver, adrenal, breast and ovarian carcinomas[85].

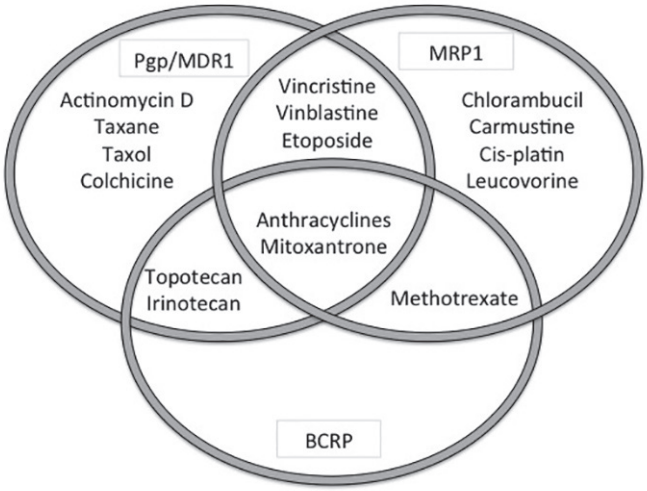

Figure 2. Overlap in cytostatic dugs as substrates for the major ABC-transporters ABCB1 (P-gp), ABCC1 (MRP1) and ABCG2 (BCRP).

Table 3. Overview of distribution of the ABC transporters P-gp, MRP1, MRP2 and BCRP in selected human barrier tissues[71, 73]

Tissue	P-gp	MRP1	MRP2	BCRP
Lung	apical	basolateral	not detected	apical
Intestine				
Duodenum	apical	basolateral	apical	apical
Jejunum	apical	basolateral	apical	apical
Ileum	apical	basolateral	apical	apical
Colon	apical	basolateral	apical	apical
Liver	apical	basolateral	not detected	apical
Kidney	apical	basolateral	apical	not detected
Brain				
BBB	apical	apical	apical	apical
BCSFB	apical	basolateral	-	-
Testis	apical	basolateral	not detected	not detected
Placenta	apical	basolateral	apical	apical

BBB = blood-brain barrier, BCSFB = blood-cerebrospinal fluid barrier

Although P-gp is capable of transporting a wide variety of structurally unrelated substrates (Table 5), they are typically neutral or cationic (at physiologic pH), amphipathic, lipid-soluble, organic compounds often with aromatic rings and a molecular weight within the 200 - 1.900 Da range. Endogenous substrates include steroid hormones (aldosterone, β-estradiol-17β-D- glucuronide), lipids (phospholipids, glycosphingolipids), peptides (β-amyloid peptides) and small cytokines (interleukin-2, -4 and IFN-γ)[70]. P-gp can transport a large number of drugs including cytostatic agents that are mainly natural product-derived drugs including the vinca-alkaloids, anthracyclines and taxanes.
Many P-gp polymorphisms have been described, as well as their effect on (cytostatic) drug therapy in a number of cancer types, but up to now no consistent pattern has emerged[87].

Dog

P-gp is highly conserved between species and canine P-gp shows ±90% homology with human P-gp[88]. In the healthy dog the distribution of P-gp has been characterized using both immunohistochemistry and mRNA expression[89, 90] and was found to be highly expressed in the liver (canalicular side of hepatocyte), bile ducts, kidney (mostly epithelium of proximal tubule), pancreatic ducts, adrenal cortex and brain (endothelial cells). Lower P-gp levels were detected in the stomach, small intestine and colon (both apical surface and diffuse cytoplasmatic staining), lung (apical margin alveolar and bronchiolar epithelium)[89, 90] and nodal B- and T-lymphocytes (membranous staining)[91]. P-gp was not detected in normal or hyperplastic mammary tissue[92, 93].
A deletion mutation in the *mdr1* gene was found to be the cause for ivermectin-associated neurotoxicosis in the Scottish collie[68]. This mutation was subsequently identified in a number of other dog breeds[94] (Table 6) and associated with various other drug toxicoses, with all of these drugs being known P-gp substrates[95-99].
The regulation of P-gp expression has been extensively studied and although not completely elucidated, many mechanisms have been identified[100]. In humans it has been shown that activation of Pregnane X receptor (PXR) by a variety of exogenous substances results in (amongst others) upregulation of P-gp expression[101], but this has as yet not been demonstrated in the dog. P-gp expression in the dog was shown to increase in response to exposure to endogenous substances, for instance increased bile acids[102], drugs, including prednisolone[103], rifampicin and ketoconazole[104], and certain diseases, e.g. epileptic seizures[105]. P-gp expression was associated with upregulation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) in the side-population of a canine 106 and a human lymphoid cell line[107] and the Akt inhibitor perifosin was able to reduce P-gp mRNA expression and DR in lymphoid cell lines[108].
It is well accepted that ABC-transporter substrates are able to induce ABC-transporter expression[104] and that this can have pronounced effects on the drug's pharmacokinetic and pharmacodynamic behavior and potentially even lead to DR. This theory has been suggested as the most likely cause for the poorer treatment results obtained with chemotherapy in dogs with multicentric lymphoma that were pretreated with glucocorticoids prior to starting chemotherapy[109-112].

Table 4. Overview of the relevant ABC transporters associated with drug resistance to cytostatic agents in humans (adapted from Dean M. The Human ATP-Binding Cassette (ABC) Transporter Superfamily [Internet]. Bethesda (MD): National Center for Biotechnology Information (US);2002 Nov 18).

Gene	Substrates	Inhibitors
ABCB1 (MDR1, P-gp)	colchicine, doxorubicin, vincristine, vinblastine, etoposide, digoxin, saquinivir, paclitaxel	verapamil, PSC833, GF120918 (GG918), V-104, Pluronic L61, LY335979, XR9576, OC144-093
ABCC1 (MRP1)	doxorubicin, daunorubicin, vincristine, vinblastine, etoposide, colchicine	Cyclosporin A, V-104, MK571
ABCC2 (MRP2, cMOAT)	vinblastine, sulfinpyrazone	PSC833, MK571
ABCC3	methotrexate, etoposide	
ABCC4	nucleoside monophosphates (thiopurines)	MK571
ABCC5	nucleoside monophosphates	
ABCG2 (BCRP)	mitoxantrone, topotecan, doxorubicin*, daunorubicin*, CPT-11	fumitremorgin C, Ko143, GF120918

* mutant BCRP

Table 5. A selection of clinically relevant drugs and compounds that are known P-gp substrates[71, 79].

Oncologic drugs	Non-oncological drugs
Cytostatic agents • Anthracyclines: doxorubicin, daunorubicin, epirubicin • Anthracenes: mitoxantrone, bisantrene • Antitumour antibiotics: actinomycin-D, mitomycin-C, plicamycin (mithramycin) • Taxanes: paclitaxel, docetaxel • Topoisomerase I inhibitors (campothecins): irinotecan, topotecan • Topoisomerase II inhibitors (epipodophyllotoxins): etoposide, teniposide, • Tyrosine-kinase Inhibitors: imatinib • Vinca-alkaloids: vincristine, vinblastine, vinorelbine, vindesine **Steroids** • Glucocorticoids: cortisol, hydrocortisone, dexamethasone, methylprednisolone, prednisolone, triamcinolone • Mineralocorticoids: aldosterone • Sex steroids: estradiol, progesterone **Tyrosine kinase inhibitors** • imatinib, gefitinib	**Antibiotics** • doxycycline, erythromycin, rifampin, tetracycline **Antifungals** • itraconazole, ketoconazole **Antiparasiticides** • ivermectin, moxidectin, selamectin **Antiemetics** • domperideone, ondansetron **Antidiarrheal agents** • loperamide **Anticonvulsant drugs** • phenobarbital, phenytoin, levetiracetam **Cardiac drugs** • digoxin, diltiazem, quinidine, verapamil, losartan **Antihistamines (H1/2-antagonsits)** • cimetidine, ranitidine, terfenadine **Immunosupppressants** • cyclosporine A, tacrolimus **Opioids** • butorphanol, morphine **Miscellaneous** • amitryptiline, colchicine, phenothiazines

P-gp expression has been demonstrated in both canine cancer cell lines, including lymphoid leukemia, mast cell tumors and osteosarcoma cell lines[113-115], and spontaneous canine tumors, including hepatic, adrenal, gastrointestinal, pulmonary and transitional cell carcinoma, mammary tumors, cutaneous mast cell tumors and malignant lymphoma[21, 89, 92, 93, 116-119]. In canine cell lines P-gp expression has been associated with resistance to vincristine and doxorubicin[88, 114], and although mRNA levels correlated with *in vitro* drug sensitivity, it failed to correlate with *in vivo* doxorubicin sensitivity[88].
P-gp expression in canine lymphoma is typically low, but varies between studies with 1 out of 31[120] to 5 out of 15[121] dogs testing positive for P-gp expression prior to chemotherapy. However, this frequency increases following tumor relapse[116, 120, 121] and high pretreatment P-gp expression appeared an independent (negative) predictor of survival[116, 121], although this could not be confirmed in a more recent study[122]. P-gp mRNA expression in canine lymphoma is generally low, but gastrointestinal lymphomas, well-known for their poor response to chemotherapy[123], showed higher expression levels compared to multicentric lymphoma cases[124]. Pre-treatment P-gp mRNA expression was not predictive for the 6-month treatment outcome in dogs with multicentric B-cell lymphoma[25]. P-gp mRNA expression in the peripheral blood of dogs with canine multincentric lymphoma was not predictive for disease outcome, but correlated with chemotherapy-related toxicity[125].

ABCC1 or MRP1

General

Multidrug resistance protein 1 (MRP1) or ABCC1 is a 190 kDa membrane-bound protein, first identified in a multidrug resistant lung cancer cell line in 1992[66]. MRP1 is typically found on the basolateral membrane of polarized epithelial cells in the intestinal mucosa, kidney (limb of Henle, collecting ducts), brain (choroid plexus), testes, and bone marrow[73]. MRP1 overexpression has been detected in drug-selected cell lines (including lung, leukemia, breast, bladder, prostate, and cervical cancer) and in both hematopoietic (leukemia) and solid tumors, including gastrointestinal tract, (non-small cell) lung, breast, ovarian and prostate carcinomas and melanoma[126, 127].
MRP1 is a transporter for both hydrophobic uncharged molecules and water-soluble anionic compounds including glutathione, glutathione-conjugates (leukotrienes, prostaglandins), glucuronide conjugates (β-estradiol-17β-D-glucuronide, glucuronosyl-bilirubin), sulfate conjugates (dehydroepiandrosterone-3-sulfate, sulfatolithocholyl-taurine) and heavy metal oxyanions including arsenite and trivalent antimonite[128]. MRP1 has been associated with resistance to natural anticancer drugs (vincristine, doxorubicin, epirubicin, etoposide, but not taxanes) and their conjugated metabolites, methotrexate, and GSH-conjugated metabolites of alkylating agents. More than fifty MRP1 polymorphisms have been described, but only a few are thought to potentially affect drug response[129].

Dog

Although MRP1 shows a high degree of homology between species[90], tissue expression can vary (significantly) between species. For instance MRP1 is present in canine and rat

Table 6. Dog breeds commonly diagnosed with an MDR-1 gene deletion[94].

Scottish Collie	Shetland sheepdog
Old English sheepdog	Australian shepherd
White German shepherd dog	Miniature Australian shepherd
English shepherd	Silken windhound
Longhaired whippet	McNab

Table 7. A selection of clinically relevant drugs and compounds that are known MRP1-substrates[71].

Drugs used in oncology	Non-oncological drugs
Cytostatic agents • Anthracyclines: doxorubicin, daunorubicin, epirubicin, idarubicin • Topoisomerase I inhibitors (campothecins): irinotecan, topotecan • Topoisomerase II inhibitors (epipodophyllotoxins): etoposide, teniposide, • Vinca-alkaloids: vincristine, vinblastine, vinorelbine, vindesine • Antifolates: methotrexate **Tyrosine kinase inhibitors** • imatinib, gefitinib	**Antibiotics** • difloaxcin, grepafloxicin **Others** • Metalloids • Peptides: glutathione • Glutathione conjugates: melphalan-SG, cyclophosphamide-SG, doxorubicin-SG • Sulfate conjugates: estrone-3-suphate, DHA-sulphate • Glucuronide conjugates: irinotecan-glucuronide, etoposide-glucuronide, estradiol-17-β-D-glucuronide • Folates: folic acid, L-leucovorin

hepatocytes, but not or only limited in human and monkey hepatocytes[130]. In the dog a high MRP1 expression is found in the brain, kidney, liver and testes, while lower levels are found in the lungs, intestines[90] and nodal lymphocytes[91].

Canine MRP1 is associated with resistance to vincristine and etoposide, but not anthracyclines[131]. In the dog MRP1 was detected in multicentric B-cell lymphoma[25] and solid tumors including pulmonary[21], hepatocellular[132] and transitional cell carcinomas[118], mammary tumors[133], and cutaneous mast cell tumors[117].

Pre-treatment MRP1 expression levels (mRNA) were not predictive for treatment outcome in dogs with multicentric B-cell lymphoma[25].

ABCG2 or BCRP

General

Breast Cancer Resistance Protein (BCRP/MXR/ABCG2) is a 72 kDa membrane-bound protein and was first described in 1998[134]. ABCG2 is expressed in the apical cell membrane of epithelial cells in the small and large intestine, kidney (renal tubular cells), liver (canalicular side of hepatocyte), brain (luminal side of capillary endothelial cells), retina, mammary gland (during pregnancy and lactation), placenta[135] and pluripotent hematopoietic stem cells[136].

BCRP can transport both positively and negatively charged drugs and their sulfate conjugates including mitoxantrone, methotrexate[137], campothecin, topotecan, etoposide

and tyrosine kinase inhibitors[138]. BCRP does not transport vinca-alkaloids, taxanes, doxorubicin (only mutant BCRP[139]) or cisplatin.
BCRP expression has been described in hematopoietic tumors (leukemia[140], lymphoma) and solid tumors (gastrointestinal tract, endometrium, lung, melanoma)[141]. BCRP expression appears to be more common in T-cell lymphomas[142]. *ABCG2* mRNA expression is commonly found in acute lymphoblastic leukemia, but failed to correlate with BCRP immunohistochemistry (BXP-21) or BCRP function. Despite this discrepancy positive BXP-21 straining correlated with disease-free survival[140].
A number of BCRP polymorphisms have been described of which some lead to reduced expression and/or function, but others result in changes in substrate specificity[139, 143].

Dog

Species differences in BCRP expression have been described and BCRP expression was for instance demonstrated in human and canine hepatocytes, but not in the mouse and monkey[130]. A study with cloned cDNA demonstrated that canine BCRP mediates resistance to doxorubicin, but not to methotrexate[133], which is opposite to the situation in humans where doxorubicin transport was only shown for a mutant form of human BCRP[139]. BCRP is expressed in canine mammary cancer cell lines [144], benign and malignant canine mammary tumors, and a higher expression in malignant tumors that increases with tumor grade[133, 145]. Pre-treatment BCRP expression levels (mRNA) were not predictive for the 6-month treatment outcome in dogs with multicentric B-cell lymphoma[25]. Regulation of *ABCG2* expression is not clear, but studies in canine lymphoid tumor cell lines suggest that the MAPK/ERK and JNK pathways are involved in downregulation of *ABCG2* expression[146].

Nuclear receptor superfamily and drug resistance

The nuclear receptor superfamily is a large family of receptors that includes the thyroid, glucocorticoid and estrogen receptor, but also pregnane X receptor (PXR) and constitutive androstane receptor (CAR)[147]. The orphan receptors PXR and CAR function as sensors for xenobiotics and regulate xenobiotic metabolism and clearance, and therefore potentially DR. PXR (NR1I2), first identified in 1998[148] is primarily activated by pregnanes, but recognizes a wide variety of ligands. These include dexamethasone, rifampicin, spironolactone, pregnenolone 16α-carbonitrile[149] and many anticancer drugs including vincristine, tamoxifen, vinblastine, docetaxel, cyclophosphamide, ifosfamide and paclitaxel[150]. Activation of PXR results in upregulation of target genes that include CYP3A4, CYP2B6, members of UDP-glucuronosyltransferases and sulfotransferases, MDR1, MRP3 and OATP2 transporters[101]. Increased PXR expression resulted in resistance to paclitaxel and cisplatin in HEC-1 cells[151] and to doxorubicin in human colon adenocarcinoma cells[150]. The constitutive androstane receptor (CAR, NR1I3) functions as a xenobiotic receptor that participates in the regulation of transcription of phase I and phase II drug-metabolizing enzymes and drug transporter genes such as MRPs (MRP2, MRP3 and MRP4), OATP2 and MDR1 facilitating detoxification and elimination[152]. The development of PXR inhibitors offers potentially promising new therapy, but designing a specific and non-toxic inhibitor is challenging[151].

Table 8. A selection of clinically relevant drugs and compounds that are known BCRP-substrates[71, 79].

Drugs used in oncology	Non-oncological drugs
Cytostatic agents • Anthracyclines: doxorubicin (mutant form), daunorubicin • Anthracenedione: mitoxantrone, bisantrene (mutant form) • Topoisomerase I inhibitors (campothecins): irinotecan, topotecan • Topoisomerase II inhibitors (epipodophyllotoxins): etoposide, teniposide, • Antifolates: methotrexate **Tyrosine kinase inhibitors** • imatinib, gefitinib, lapatinib	**Antibiotics** • ciprofloxacin, norfloxacin, nitrofurantoin **Anthelmintics** • albendazole, oxfendazole **Diuretics** • furosemide, hydrochloorthiazide **Porphyrins** • pheophorbide A, protoporphyrin IX, hematoporphyrin **Flavonoids** • genestein, quercetin **Fungal toxins** • alfatoxin B, fumitremorgin C, Ko143 **Drug & metabolite compounds** • acetaminophen-sulphate, estrone-3-sulphate, DHA sulphate, estradiol-17-β-D-glucuronide, dinitrophenyl-S-glutathione

Glucocorticoids and drug resistance

Glucocorticoids (GCs) are stress-induced steroid hormones and are in the blood predominantly bound to corticosteroid-binding globulin. Due to their lipohilic nature CBG-free GCs can easily cross membranes by passive diffusion and once in the cytoplasm they bind to GC-receptors. The activated receptor then translocates into the nucleus, where it binds to GC response elements in the promotor regions of the GC-responsive genes[153]. GC-responsive genes are numerous and diverse and affect metabolism, homeostasis and immune system. Most chemotherapy protocol use potent synthetic GCs, like prednisolone and dexamethasone, and their use is limited by their side effects (Cushing's syndrome) and the development of GC resistance.

For the treatment of lymphoid neoplasia, the effects on the immune system are most important and these can be summarized as induction of lymphoid cell apoptosis (intrinsic pathway), cell cycle arrest and inhibition of inflammation (repression of pro-inflammatory cytokines NF-κB, AP1). Apoptosis of lymphoid cells takes place at three levels: genomic signaling (induction of pro-apoptotic Bcl-2 family member Bim and GILZ or GC-induced leucine zipper), cytoplasmic signaling (increased cytosolic calcium, ceramide and reactive oxygen species levels and net potassium efflux) and the execution of apoptosis (activation of caspase 9 and subsequent apoptosis)[154]. Activation of these pathways has been demonstrated in human acute lymphoblastic leukemia[155].

GC resistance results predominantly from resistance to apoptosis and several pathways have been identified[156] and include insufficient ligand, mutations or insufficient expression of the GC receptor, deficiencies in the GC receptor-associated proteins, mutations in the apoptotic pathway, and activion of pro-survival kinases including RAS/RAF/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT[157]. Insufficient ligand can result from impaired GC

uptake, increased GC–binding proteins, reduced activation (prednisone), overexpression of efflux transporters (most commonly P-gp, MRP and LRP) and increased inactivation. GCs (dexamethasone) have been shown to induce resistance through upregulation of ABC-transporter expression in lymphoid cells[158] and GC (dexamethasone) resistance was associated with increased PI3K activity and decreased sensitivity to vinblastine and doxorubicin[159].

Strategies to prevent or modulate drug resistance

Prevention of drug resistance - Use of cytotoxic agents

Most tumors tend to become drug resistant and prevention or reversal of drug resistance would be a major improvement in the treatment of cancer patients with chemotherapy. Initial attempts to prevent drug resistance were based on the Goldie-Coldman hypothesis[3] and aimed at preventing drug resistance by using multidrug chemotherapy protocols[160] or high dose chemotherapy[161, 162]. The rationale behind this approach was to increase the overall cell killing and thereby prevent the development of drug resistant clones. Analysis of relevant clinical studies suggest that although tumor response is better in a multidrug protocol, most likely because of a greater reduction in tumor mass, and high-dose therapy results a higher killing of tumor cells, neither of these approaches prevents the emergence of DR. Furthermore this approach appears only effective in drug sensitive tumor classes, like lymphoma and leukemia.

In order to reduce the likelihood of chemotherapy resistance, it is advised to start treatment either as early as possible or following maximum surgical removal of the tumor, because a low tumor burden has the lowest chance of intrinsic DR. Unfortunately most tumors present at an advanced stage, which stresses the need for good screening programs.

Modulation of drug resistance - ABC-transporters modulators

An alternative approach has been to inhibit individual drug resistance mechanisms and since active drug efflux through ABC-transporters appears the most important mechanism, work has mainly focused on developing P-gp and other ABC-transporter inhibitors (Table 9). It has to be realized that ABC-transporters are not only present in cancer cells, but also in the liver, kidney, intestine and many tissue-barriers. Modulation of ABC-transporters (either induction or inhibition) can therefore significantly affect absorption, distribution and excretion of drugs, affecting the pharmacokinetics and pharmacodynamics and potentially leading to subtherapeutic or toxic drug levels.

Within 10 years following the discovery of P-gp, the first trials with P-gp inhibitors were started and these first generation P-gp inhibitors were drugs that were already used for other purposes (calcium-channel blockers, immunosuppressive agents). Although the in vitro studies with these inhibitors were promising, they proved generally ineffective due to their low inhibitory potency and significant (inhibitor-related) toxicity at the concentrations required for sufficient P-gp inhibition. This led to the development of second-generation P-gp inhibitors that were more potent, but also less selective and inhibited multiple ABC-transporters and some even the cytochrome P450 system (CYP3A4). The inhibitory effect

of the first- and second-generation P-gp inhbitors was demonstrated for a number of cytostatic agents by changes in the pharmacokinetics (increased area under the curve) and pharmacodynamics (resulting increased number of adverse events/toxicity)[163]. However, the lack of transporter specificity of these second-generation P-gp inhibitors caused pharmacokinetic interactions, reduced the metabolism and clearance of cytostatic drugs and led to unacceptable (cytostatic drug related) toxicity. The initial response was to lower the dose of the cytostatic agents, but since these inhibitory effects on metabolism and clearance proved unpredictable, some patients were still overdosed, while others were underdosed and as a result therapeutic benefit was not always apparent[62].
Third-generation P-gp inhibitors are both more potent and more specific and hold promise, but the results of large-scale clinical trials are as yet not available.
There are no specific MRP1-inhibitors, and although there are "specific" BCRP-inhibitors, none have been evaluated in clinical trials. The use of a combined P-gp/BCRP inhibitor is most interesting, but there are no data available on the clinical benefit.
There are concerns that the ultimate ABC-transporter inhibitor, a potent and specific, non-toxic drug that does not increase (cytostatic) drug-related side-effects, might not be realistic. Despite over 30 years of trying to modulate ABC-transporter function and a clear rationale for using ABC-transporter inhibitors, there is still no definite answer to the question whether or not they are (or will be) of clinical benefit in the treatment of drug-resistant cancers.

Since the dog is commonly used in preclinical drug testing, many potential P-gp inhibitors have been evaluated in the dog[164]. Pgp-mediated drug resistance was successfully reversed with the classical P-gp inhibitors verapamil and PSC833 in a variety of canine cell lines[113-115, 165]. PSC833 (Valspodar®) proved effective in overcoming Pgp-mediated doxorubicine

Table 9. Modulators of the ABC-transporters P-gp, MRP1, and BCRP[79].

Generation	P-gp	MRP1	BCRP
First	verapamil quinidine cyclosporine A		Ko143 Pantoprazole Gefitinib? Imatinib? Quercetin?
Second	PSC833 (valspodar) VX-710 (biricodar)	VX-710 (biricodar)	VX-710 (biricodar)
Third	GF120918 (elacridar) XR9576 (tariquidar) LY335979 (zosuquidar) ONT–093 (ontogen)		GF120918 (elacridar) XR9576 (tariquidar)

resistance in vitro (human osteosarcoma cell line) and was then used in dogs with appendicular osteosarcoma without causing toxicity[165].

Alternative drugs and therapies

Various other approaches including the use of small peptides designed to correspond to the transmembrane domain of P-gp[166], monoclonal antibodies or active immunization against P-gp[167, 168], down-regulating Pgp-expression[169] and gene silencing[170] have been described.

Over the past few years, the combined use of classis cytostatic agents with tyrosine-kinase inhibitors (TKIs) has gained interest as a potential new approach for reverting drug resistance. Tyrosine kinases play crucial roles in many of the pathways involved in cancer development, including cell proliferation, apoptosis, angiogenesis and metastasis and TKI's have been developed to specifically target these pathways. Resistance to TKI's is common and mediated by P-gp and BCRP[171], but TKI's are not only substrates, they are also inhibitors of these ABC-transporters and the combined use of TKIs with cytostatic agents has been successfully used in reverting drug resistance *in vitro*[172-174].

Other potential drugs to consider include proton-pump inhibitors[175] of which lansoprazole that has been used in veterinary cancer patients including canine lymphoma[176], the MDM2 (mouse double minute) inhibitor nutlin-3[177], and the *Akt* inhibitor Perifosin[108].

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Aims and scope of the thesis

The literature as presented in the two overviews in **Chapter 1** clearly demonstrated the relevance of canine lymphoma in veterinary oncology, the problem of drug resistance in the long-term management of this disease, and the potential role of ABC-transporters therein. Although there are a number of publications available on drug resistance in canine neoplasia, including lymphoma, as well as on ABC-transporters, these are often based on in vitro work in canine (normal and neoplastic) cell lines, or small-scale retrospective clinical studies. The number of prospective studies in clinical cases is limited and in order to improve our understanding on the role of ABC-transporters in canine lymphoma, we performed a prospective study in a larger cohort of dogs with multicentric lymphoma.

In order to be able to study the differences between a drug-sensitive and drug-resistant cell and in order to be able to validate tests for measuring canine ABC-transporter expression (mRNA and protein) and function, we created a canine doxorubicin-resistant lymphoid sub-cell line. Doxorubicin was purposefully chosen because of its broad mechanistic spectrum that often leads to multi-drug resistance, but also because doxorubicin is one of the most commonly used cytostatic agents in veterinary oncology. In **Chapter 2** we describe the changes in ABC-transporter expression and function associated with doxorubicin resistance, as well as the role of glucocorticoids therein.

When a tumor has become drug resistant, the only options are to switch to a new drug (class) or try to revert drug resistance. In veterinary medicine the choice of new drugs is limited and typically less effective and more toxic. As a result, reversal of drug resistance remains an interesting therapeutic option. In **Chapter 3** the drug resistant lymphoid cell model is used for testing the combined use of the tyrosine-kinase inhibitor masitinib with classical cytostatic agents for its potential to reverse drug resistance.

Several clinical studies on canine lymphoma have documented that the use of glucocorticoids prior to starting chemotherapy negatively affects treatment outcome with chemotherapy. This observation led us to the question whether the use of glucocorticoids is necessary within a multi-drug, doxorubicin-based chemotherapy protocol and whether glucocorticoid use might not contribute to a worse treatment response in case a rescue protocol would be initiated. In **Chapter 4** we describe the results from a randomized, prospective clinical trial on the benefits of the use of glucocorticoids.

All dogs from the clinical study reported in **Chapter 4** had lymph node samples taken at the start of treatment and throughout disease progression. In these tumor samples mRNA expression of the various ABC-transporters was measured and the results are presented in **Chapter 5**.

Since ABC-transporters are only one player in the clinical phenotype of drug resistance, other pathways need to be evaluated. Activation of the Wnt-pathway was demonstrated in hematopoietic neoplasia and has been associated with drug resistance in lymphoid neoplasia. Since there were no data available on the role of the Wnt pathway in canine lymphoma or drug resistance, it was chosen to start with this pathway and the initial results are shown in **Chapter 6**.

In **Chapter 7** the results of the review and the various studies, their contributions to our understanding of canine drug resistance and their implications on the management of canine lymphoma are discussed, as well as suggestions for future studies.

2

Multi-drug resistance in a canine lymphoid cell line due to increased P-glycoprotein expression, a potential model for drug-resistant canine lymphoma

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Abstract

Canine lymphoma is routinely treated with a doxorubicin-based multidrug chemotherapy protocol, and although treatment is initially successful, tumor recurrence is common and associated with therapy resistance. Active efflux of chemotherapeutic agents by transporter proteins of the ATP-Binding Cassette superfamily forms an effective cellular defense mechanism and a high expression of these transporters is frequently observed in chemotherapy-resistant tumors in both humans and dogs.

In this study we describe the ABC-transporter expression in a canine lymphoid cell line and a sub-cell line with acquired drug resistance following prolonged exposure to doxorubicin. This sub-cell line was more resistant to doxorubicin and vincristine, but not to prednisolone, and had a highly increased P-glycoprotein (P-gp/ABCB1) expression and transport capacity for the P-gp model-substrate rhodamine123. Both resistance to doxorubicin and vincristine, and rhodamine123 transport capacity were fully reversed by the P-gp inhibitor PSC833. No changes were observed in the expression and function of the ABC-transporters MRP-1 and BCRP.

It is concluded that GL-40 cells represent a useful model for studying P-gp dependent drug resistance in canine lymphoid neoplasia, and that this model can be used for screening substances as potential P-gp substrates and their capacity to modulate P-gp mediated drug resistance.

Keywords – canine lymphoma, *in vitro*, multidrug-resistance model, ABC-transporters, P-glycoprotein

Introduction

Canine lymphoma (cL), the most common hematopoietic neoplasia in the dog, is in many respects comparable to non-Hodgkin lymphoma in humans[1]. As in humans, treatment in the dog consists of a multidrug chemotherapy protocol that includes, as a minimum, cyclophosphamide, doxorubicin, vincristine and prednisolone (or CHOP-protocol)[2, 3] of which, as a single agent, doxorubicin appears most effective[4]. Despite a high initial response rate, tumor relapse is common and more often refractory to therapy, leading to treatment failure and ultimately the dog's death[5, 6]. The main cause for treatment failure is thought to be tumor drug resistance (DR) that can be present at the start of chemotherapy (intrinsic DR) or develop during or following chemotherapy (acquired DR). Several clinical studies on cL[7-10] have documented that treatment with glucocorticoids prior to starting chemotherapy lowers the overall response rate and shortens survival and this has been explained by assuming acquired, glucocorticoid-induced DR[11].

One of the mechanisms underlying DR is the active efflux of (cytotoxic) drugs by membrane bound transporter proteins of the ATP-Binding Cassette (ABC) superfamily[12, 13]. High expression of these ABC-transporters, and P-glycoprotein (P-gp; ABCB1) in particular, has been associated with both a decreased sensitivity to cytotoxic agents, as well as a poor prognosis in several types of cancer in humans[13-16] and dogs[17-20]. Other ABC-transporters associated with DR to cytotoxic agents in humans include multidrug resistance related protein 1 (MRP1; ABCC1) and breast cancer resistance protein (BCRP; ABCG2)[21], both of which have been studied in veterinary medicine[20, 22-24], but not in relation to cL.

Although DR represents a major obstacle in the successful management of cancer with chemotherapy in both humans and dogs, therapeutic measures to circumvent DR are still limited[25]. Studying the mechanisms responsible for DR will provide a better understanding of DR[26] and could potentially lead to the development of new therapies[16, 27]. For the dog both these goals would be greatly facilitated with a canine *in vitro* model, but a previously reported DR cell line[28] derived from the canine lymphoid GL-1 cell line[29] is no longer available.

The first goal of the current study was to re-establish a DR canine lymphoid cell line through selection for doxorubicin-resistance. Doxorubicin was purposefully chosen given its' efficacy in the treatment of cL, but also because resistance to doxorubicin is predictive for multidrug resistance in human neoplasia[16]. The second goal was to assess in both the original and the doxorubicin resistant sub-cell line, the antiproliferative effect of the drugs used in a CHOP-protocol, and the expression and function of the ABC-transporters P-gp, MRP1 and BCRP in both cell lines.

Material and Methods

Chemicals

PSC833 (Valspodar®) was a gift from Novartis Pharma AG (Basel, Switzerland) and Ko143 was kindly provided by Prof. Koomen (University of Amsterdam, the Netherlands). MK571 sodium salt was obtained from Alexis Biochemicals (Grünberg, Germany). Rhodamine 123 (Rh123), and 5(6)-carboxyfluorescein diacetate (CFDA) were purchased from Sigma-Aldrich

(St Louis, MO, USA). Pheophorbide A (PhA) was obtained from Frontier Scientific (Logan, USA). Doxorubicin hydrochloride and vincristine sulfate and prednisolone were purchased from Sigma-Aldrich (St Louis, MO, USA).

Cell lines, cell culture media and supplements

The canine lymphoid cell-line, GL-1 cells[29], was kindly provided by dr K. Ohno (University of Tokyo). The cells were confirmed to be of canine origin through DNA-sequencing (four regions, 900 base-pairs in total) and showed 100% homology with the canine reference genome. GL-1 cells grow in single cell suspension and showed strong immunoreactivity with CD34 and CD79α, a weak reaction with CD3 and CD4 and no reaction with CD21 and CD90, consistent with a precursor lymphoid cell of the B-lineage.

GL-1 cells were grown in suspension on RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 1% (v/v) L-glutamine (BioWhittaker, Maine, USA), 10% (v/v) fetal bovine serum (Gibco) and 100 U/mL penicillin and 100 µg/ml streptomycin (BioWhittaker) at 37°C, 5 % CO_2. The GL-1 cells were cultured with gradually increasing concentrations of doxorubicin and after 6 months a subline of the GL-1 cells was selected that could be cultured in the presence of 0.07 µM (40 ng/mL, hence GL-40) doxorubicin. Multiple batches of these GL-40 cells were stored in liquid nitrogen and were maintained in culture after thawing following a schedule of one passage with doxorubicin (20 ng/mL) and two passages without doxorubicin.

Proliferation Assay

Cell proliferation was assessed with the Cell Counting Kit-8 assay (CCK-8, Dojindo Molecular Technologies, Rockville, Maryland, USA) using the tetrazolium salt, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye following bioreduction in the presence of the electron carrier, 1-methoxy PMS. Cells were seeded in 96-well plates at a density of 2.10^4 cells per well in cell culture medium containing a concentration range of doxorubicin, vincristine or prednisolone and incubated for 24, 48, and 72 h at 37°C, 5 % CO_2. The tetrazolium solution was added to each well 2½ h before light absorbance analysis. The formation of the soluble formazan was measured by light absorbance at 450 nm in a microplate reader. Cell proliferation was calculated by dividing the light absorbance in treated cells by that in control cells after correction for background absorbance.

Concentration dependent effects were analyzed by non-linear regression after log transformation of the concentration and graphs were fitted according to a sigmoïd dose-response curve. A time-dependent effect was observed on GL-cell proliferation and results were reported after 72 h of incubation.

RNA isolation and synthesis of cDNA

Total RNA was isolated using the SV-total RNA isolation kit (Promega, Leiden, The Netherlands) according to the manufacturer's protocol including a DNAse treatment. The RNA was quantified spectrophotometrically at 260 nm (ND-1000, Nanodrop technologies) and stored at -70°C.

Table 1. Primer details.

Canine gene	GenBank	Forward primer	Reverse primer	Product size	Ta (°C)
***ABCB1* (P-gp)**	NM_001003215	CTATGCCAAAGCCAAAGTATC	GAGGGCTGTAGCTGTCAATC	80	57.5
***ABCC1* (MRP1)**	NM_001002971	CGTGACCGTCGACAAGAACA	CACGATGCTGATGACCA	118	60.9
***ABCG2* (BCRP)**	NM_001048021	GGTATCCATAGCAACTCTCCTCA	GCAAAGCCGCATAACCAT	146	60.0

T_a = annealing temperature

First strand cDNA from 1 µg total RNA was synthesized with the iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) containing both oligo (dT) and random hexamer primers in a final volume of 20 µL according the manufacture's recommendation. The cDNA was stored at -20°C.

Real time quantitative PCR analysis

Gene-specific primers were developed, commercially synthesized (Eurogentec, Maastricht, the Netherlands) and tested for efficiency by using a dilution series of cDNA. The efficiency of the primers was between 95-105% and only one product was formed as assessed by melting curve analysis. 50 ng reverse transcribed RNA, 7.5 pmol of each gene-specific primer (Table 1) and IQ™ SybrGreen Supermix (Biorad) in a final 25µL reaction volume was analyzed by quantitative PCR in a MyIQ single color real time PCR detection system (BioRad). Following an initial hot start for 3 minutes, 40 cycles were run with a denaturation step at 95°C for 20 seconds, an annealing step at 63°C for 30 seconds and an elongation step at 72°C for 30 seconds.

Immunocytochemistry

For the immunocytochemical detection of ABC-transporters in the GL-1 and GL-40 cells, cytospin smears were prepared by placing 5.10^5 cells in culture medium into a cytology funnel with pre-attached filtercard (Biomedical Polymers Inc, Gardner (MA), USA) that was fixed with a funnel clip onto a polysine microscope slide (Menzel-Gläser, Braunschweig, Germany) and centrifuged at 650 rpm for 10 minutes in a cytospin centrifuge (Thermo Scientific™ Cytospin™ 4 Cytocentrifuge, Thermo Shandon Limited, Runcorn, UK). The freshly prepared cytospin preparations (1.10^6 cells/slide) were air dried, followed by a 3 min fixation step in acetone. After rehydration in phosphate-buffered saline (PBS) and incubation with 10% swine serum in PBS (20 minutes), endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol (Cell Marque, Rocklin, CA, USA) for 10 min. Slides were then incubated with mouse antibodies directed against human P-gp[30] (C494, Alexis Switzerland), MRP1[31] (M_2III-6, kind gift by dr Scheffer, the Netherlands) and BCRP[32] (BXP-21, Abcam, UK) for 1 hour at room temperature. Antibody binding was detected by polyvalent biotinylated serum, HRP-labeled streptavidin and diaminobenzidine as a chromogen (Cell Marque, Rocklin, CA, USA). Following a 10-minute wash in *aqua dest*, counterstaining was performed with hematoxyline solution according to Mayer (Sigma, St Louis, MO, USA) for 3 minutes, followed by a 2-minute washing step in *aqua dest*.

Functional studies with fluorescent transporter substrates and selective inhibitors

Functional studies were performed using the typical fluorescent substrate-inhibitor combinations Rh123-PSC833 for assessing P-gp, CFDA-MK571 for MRP, and PhA-Ko143 for BCRP function[33-35]. All experiments with GL-40 cells were performed with cells that had been incubated for one passage without doxorubicin. Before the GL-1 and GL-40 cells were used in experiments with fluorescent dyes, cell viability was assessed with trypan blue exclusion and cell numbers were counted in a hematocytometer. A total of 5.10^5 viable cells per sample were plated into 96-well plates. In the transport studies, culture medium was replaced by Hanks Balanced Salt Solution (HBSS) without Ca^{2+}, Mg^{2+} and phenol red and with 1000 mg/L glucose (Gibco, Grand Island, NY, USA). All chemicals were dissolved in DMSO as 1.000x stock solutions resulting in a final concentration of DMSO of 0.1% for all samples.

P-gp and BCRP transporter activity were assessed in an efflux assay. A total of 5.10^5 viable cells per sample were plated into 96-well plates and after centrifugation (50 x g, 3 minutes) culture medium was replaced by HBSS containing Rh123 (4 µmol/L) or PhA (0.1 - 1 µmol/L) and cells were incubated for 30 minutes (37°C, 5% CO_2). Following this loading, cells were washed twice in PBS (Gibco, Grand Island, NY, USA) by centrifugation and the cell pellets were re-suspended in HBSS containing a concentration range of PSC833 or Ko143 as indicated in the results section. Incubation was continued for a further two hours, after which cell-suspensions were pelleted by centrifugation (4°C) and the medium was replaced by dye-free HBSS (4°C). These cell suspensions were collected and transferred into FACS-tubes and placed on ice until FACS analysis.

P-gp and BCRP transporter activity were also assessed in an uptake study. MRP activity was assessed only in a CFDA uptake study. In contrast to Rh123 and PhA, CFDA requires cleavage by intracellular esterases to generate CF, the fluorescent product measured in the uptake study. In order to demonstrate substrate and inhibitor specificity, a concentration series of the P-gp inhibitor PSC833 and the MRP inhibitor MK571 were tested in combination with Rh123 and CFDA. For the fluorescent dye accumulation assays, culture medium was replaced by HBSS containing the fluorescent transporter substrate dye and an inhibitor. After 30 minutes of incubation (37°C, 5 % CO_2), culture medium was replaced by PBS (4°C) and the cell suspensions were transferred into FACS-tubes and placed on ice until FACS analysis.

A flow cytometer (FACS Calibur, Biorad, The Netherlands) equipped with an argon 488 nm laser was used to analyze the samples. Cell-associated fluorescence of Rh123 and CF was measured using a 530-nm bandpass filter and cell-associated PhA fluorescence was measured using a 610-nm longpass filter. The samples were gated on forward scatter versus side scatter to exclude clumps and cell debris. Data were collected for a minimum of 10,000 gated events per sample.

FACS data analysis

FACS data represent the geometric mean cell-associated fluorescence intensity and are reported in Relative Fluorescence Units (RFU). Changes in the cellular accumulation (Rh123, CF or PhA) by the inhibitors were presented as RFU.

Efflux (Rh123, PhA) was calculated according to equation 1 and expressed as a percentage, with one hundred percent efflux meaning that after two hours of incubation no fluorescence was measured within the cells and zero percent efflux meaning that all fluorescence was retained within the cells due to efflux inhibition.

Efflux = $[(FL_0-FL_B) - (FL_{120}-FL_B)] / (FL_0-FL_B) \times 100\%$ (Equation 1)

FL_0: Fluorescence intensity at t=0 minutes

FL_B: Fluorescence intensity of the background

FL_{120}: Fluorescence intensity of the samples at t=120 minutes

Glucocorticoid receptor assay

The presence of a functional glucocorticoid receptor was assessed in both GL-1 and GL-40 cells using a luciferase assay as previously described by Gracanin *et al.*[36]. In short, GL-cells (8.105 cells/well) were seeded in 24-well plates and transfected using per well 2 µL Lipofectamine 2000 (Invitrogen, Bleiswijk, the Netherlands), 800 ng of Mouse Mammary Tumor Virus (MMTV)-luciferase containing a glucocorticoid receptor-responsive reporter, and 0.3 ng of human β-actin-promoter renilla as an internal control. Following transfection the cells were left to recover for 24 h and then incubated with prednisolone (0.1 µmol/L) for 24 h. Firefly and renilla luciferase activities were subsequently quantified using a Dual-Luciferase Assay System (Promega, Leiden, the Netherlands) in a Centro LB 960 luminometer (Berthold Technologies, Vilvoorde, Belgium). The canine mammary cell line CNMm was used as a positive control.

Effect of prednisolone on ABC-transporter expression

The cells were incubated with a concentration range of prednisolone for 3, 6 and 24 h in cell culture medium (RPMI 1640). At the time of sampling, the cells were harvested by centrifugation, medium was discarded and the cell pellet was lysed in Promega lysis buffer. RNA isolation, cDNA synthesis and quantitative PCR analysis were performed as described above. Results are reported as Relative Expression that was calculated according to equation 2.

Relative Expression = $2^{(Ct_{mean}-Ct_{sample})}$ (Equation 2)

Ct_{mean}: mean Ct value at t=0h for each ABC transporter for GL-1 or GL-40

Ct_{sample}: mean Ct value at t= 3, 6 or 24 h for each ABC transporter for GL-1 or GL-40

Statistical analysis

All experiments were repeated independently for three times. Differences in accumulation or efflux of fluorescent dyes in the absence or presence of a specific transporter inhibitor were analyzed for statistic significance using the One-Way ANOVA with Dunnett multiple comparison post hoc test. The level of significance was set at $P < 0.05$ and significant differences are mentioned in the text. All statistical analyses were performed using Graph Pad Prism software (San Diego, California, USA).

Results

GL-40 cells are resistant to doxorubicin and vincristine, but not prednisolone

The sub-cell line GL-40 was selected from GL-1 cells through intermittent incubation with gradually increasing concentrations of doxorubicin over a 6-month period and resulted with an increase in IC_{50}-doxorubicin from 17.2 nmol/L for GL-1 cells to 115 nmol/L for GL-40 cells. GL-40 cell showed resistance to vincristine with an IC50-vincristine 0.54 nmol/L for GL-1 cells and 21.4 nmol/L for GL-40 cells (Figure 1, Table 2). Prednisolone caused a mild (35%), but significant decrease in cell proliferation that was similar in both GL cell lines (Figure 2).

Immunocytochemistry of ABC-transporters P-gp, MRP1 and BCRP

P-gp expression was detected using the monoclonal antibody C494 and appeared more intense in GL-40 than GL-1 cells (Figure 3). Using the monoclonal antibody M2III-6, MRP1 expression was demonstrated in both cell lines, but appeared more intense in GL-1 cells (Figure 3). BCRP expression, using the monoclonal antibody BXP-21, was similar in both cell lines (Figure 3).

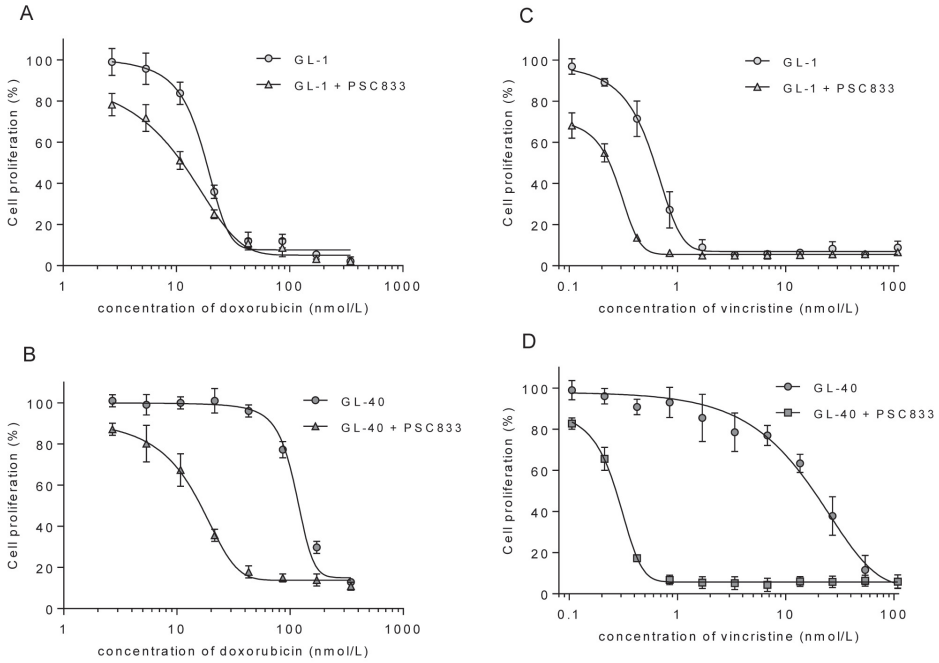

Figure 1. Inhibition of cell proliferation by doxorubicin (A, B) and vincristine (C, D) and the effect of PSC833 on drug sensitivity in GL-1 and GL-40 cells. Data are presented as mean ± SD of three independent experiments.

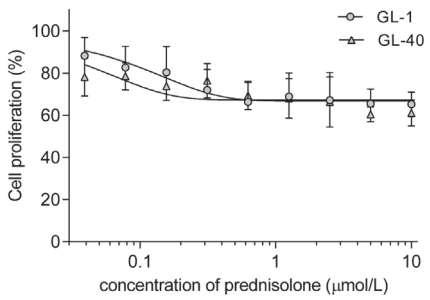

Figure 2. Inhibition of cell proliferation by prednisolone in GL-1 and GL-40 cells. Data are presented as mean ± SD of three independent experiments.

Table 2. Drug sensitivity represented by IC50-values (nmol/L; mean with 95% confidence intervals) in the GL-1 and GL-40 cells for doxorubicin and vincristine and the effect of PSC833 (1 µmol/L).

Drug	PSC833	GL-1	GL-40
Doxorubicin (nmol/L)	-	17.2 (15.0 - 19.5)	115 (109 - 120)
	+	13.3 (10.7 - 16.6)	15.8 (15.3 - 16.6)
Vincristine (nmol/L)	-	0.54 (0.52 - 0.57)	21.4 (16.9 – 27.2)
	+	0.32 (0.31 - 0.33)	0.31 (0.31 - 0.32)

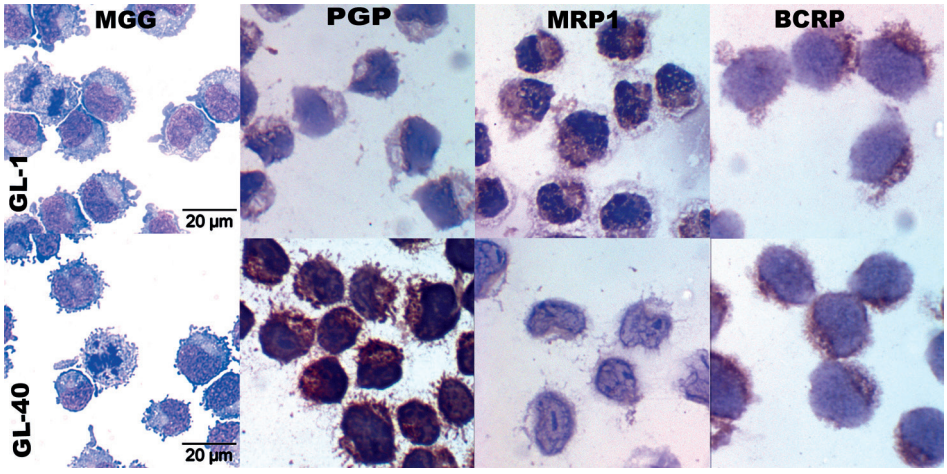

Figure 3. Microscopic photographs of GL-1 (top row) and GL-40 (lower row) cells stained with May-Grünwald Giemsa (MGG) and immunocytochemical staining for P-gp, MRP1 and BCRP (columns).

mRNA expression of the ABC-transporters P-gp, MRP1 and BCRP

P-gp mRNA expression was significantly lower in GL-1 cells (Ct ≥ 35) than in GL-40 cells (Ct = 22), indicating an approximately 7,500 times higher expression in the latter (Figure 4). MRP1 and BCRP expression were both detected (Ct values of 22 and 23 respectively), but similar in both GL-cell lines.

Functional studies for P-gp, MRP and BCRP

Rhodamine 123 as a probe dye for Pgp function

Rhodamine123 efflux from the GL-40 cells was approximately 4 times higher compared to the GL-1 cells (Figure 4) and the P-gp-inhibitor PSC833 decreased Rh123 efflux in a concentration dependent fashion in the GL-40 cells (IC_{50} 0.05 µmol/L), but not in the GL-1 cells (Figure 5).

Rhodamine 123 accumulation was significantly lower in GL-40 compared to GL-1 cells and PSC833 caused a concentration dependent increase in Rh123 accumulation in the GL-40, but not in the GL-1 cells (Figure 6). Only higher concentrations of MK571 (5 and 25 µmol/L) significantly increased Rh123 accumulation in the GL-40, but not the GL-1 cells (Figure 6).

CFDA as a probe dye for MRP-transporters

Cellular accumulation of CF following incubation with CFDA (1 µmol/L) was higher in the GL-40 cells compared to the GL-1 cells and MK571 increased CF accumulation in a concentration dependent way in both GL cell lines (Figure 7). The highest PSC833 concentration tested (1 µmol/L) resulted in a minor, but significant, increase in CF accumulation in the GL-1 cells (Figure 7).

Pheophorbide A as a probe dye for BCRP

PhA was tested at a concentration range from 0.1 to 1 µmol/L in combination with the BCRP inhibitor Ko143 at 1 µmol/L. PhA efflux (%) was similar in both GL-1 and GL-40 cells and decreased with increasing concentrations of PhA. Incubation with Ko143 had no effect on PhA efflux (figure 8). Cellular accumulation of PhA was comparable for both the GL-1 and GL-40 cell lines (figure 8), and Ko143 had no significant effect on the cellular PhA accumulation in either cell-line.

Doxorubicin and vincristine resistance in GL-40 cells is reversed by PSC833

PSC833 (1 µmol/L) restored GL-40's sensitivity to the antiproliferative effects of doxorubicin and vincristine to levels comparable with GL-1 cells (Table 2). PSC833 alone showed a mild, but equal, antiproliferative effect in both GL cell lines (Figure 1).

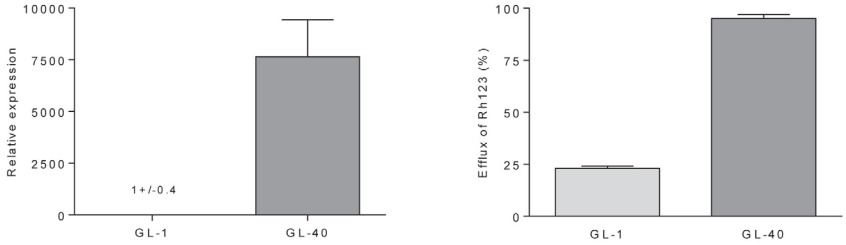

Figure 4. P-gp mRNA expression, measured by qPCR (left), and P-gp function, measured by rhodamine123 efflux (right), in the GL-1 and GL-40 cells, respectively. Data are presented as mean ± SD of three independent experiments.

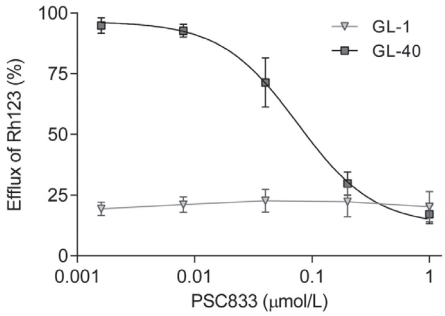

Figure 5. Efflux of the P-gp substrate rhodamine 123 by GL1 and GL-40 cells after 2 hours of incubation in dye-free medium with increasing concentrations of PSC833. Data are presented as mean ± SD of three independent experiments.

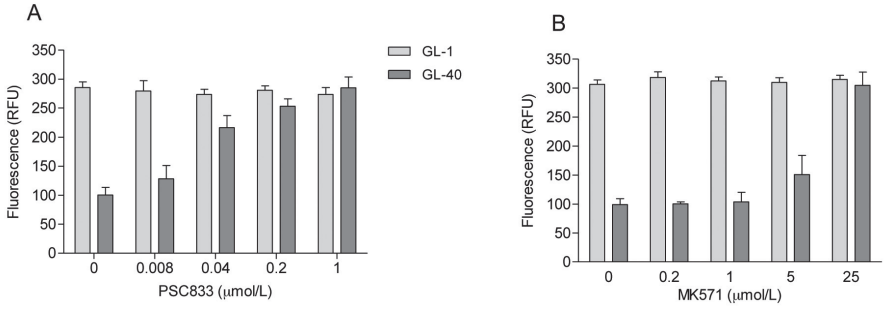

Figure 6. Cellular rhodamine 123 accumulation after 30 minutes of incubation with rhodamine 123 (4 μmol/L) in combination with increasing concentrations of PSC833 (A) or MK571 (B). Data are presented as mean ± SD of three independent experiments.

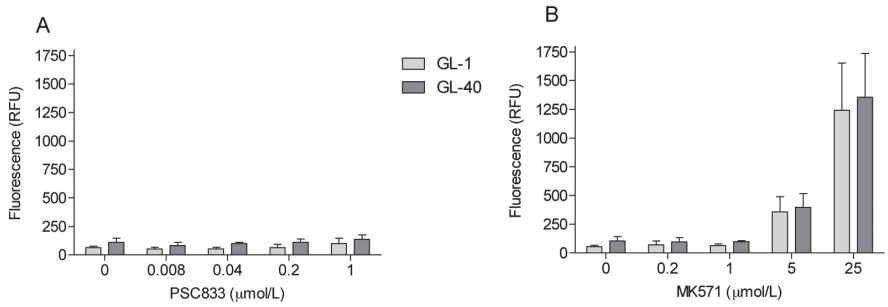

Figure 7. Cellular 5(6)-carboxyfluorescein accumulation after 30 minutes of incubation with 5(6)-carboxyfluorescein diacetate (1 μmol/L) and increasing concentrations of PSC833 (A) or MK571 (B). Data are presented as mean ± SD of three independent experiments.

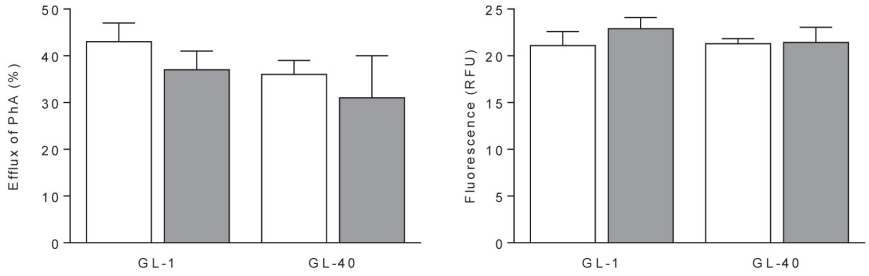

Figure 8. Efflux of Pheophorbide A (0.5 μmol/L) by GL1 and GL-40 cells after 30 minutes of incubation in dye-free medium in the absence or presence of Ko143 (A) and cellular Pheophorbide A accumulation in GL1 and GL-40 cells after 30 minutes of incubation with Pheophorbide A in the absence or presence of Ko143 (B). Data are from a representative experiment and presented as mean ± SD.

GL-cells have a functional glucocorticoid-receptor, but prednisolone fails to induce P-gp, MRP1 or BCRP expression

Following transfection of CNMm, GL-1, and GL-40 cells with MMTV-*luc*, prednisolone (0.1 μM) caused a, respectively, 6-, 4-, and 2.6-fold increase in luciferase-activity confirming the presence of active glucocorticoid receptors in both cell-lines.

Short-term incubation (3, 6, 24 h) of both GL-1 and GL-40 cells with prednisolone did not significantly change the mRNA expression of P-gp, MRP1, and BCRP (Figure 9). The low levels of P-gp mRNA expression in the GL-1 cells (Ct ≥ 35), resulted in a large variation in relative expression results.

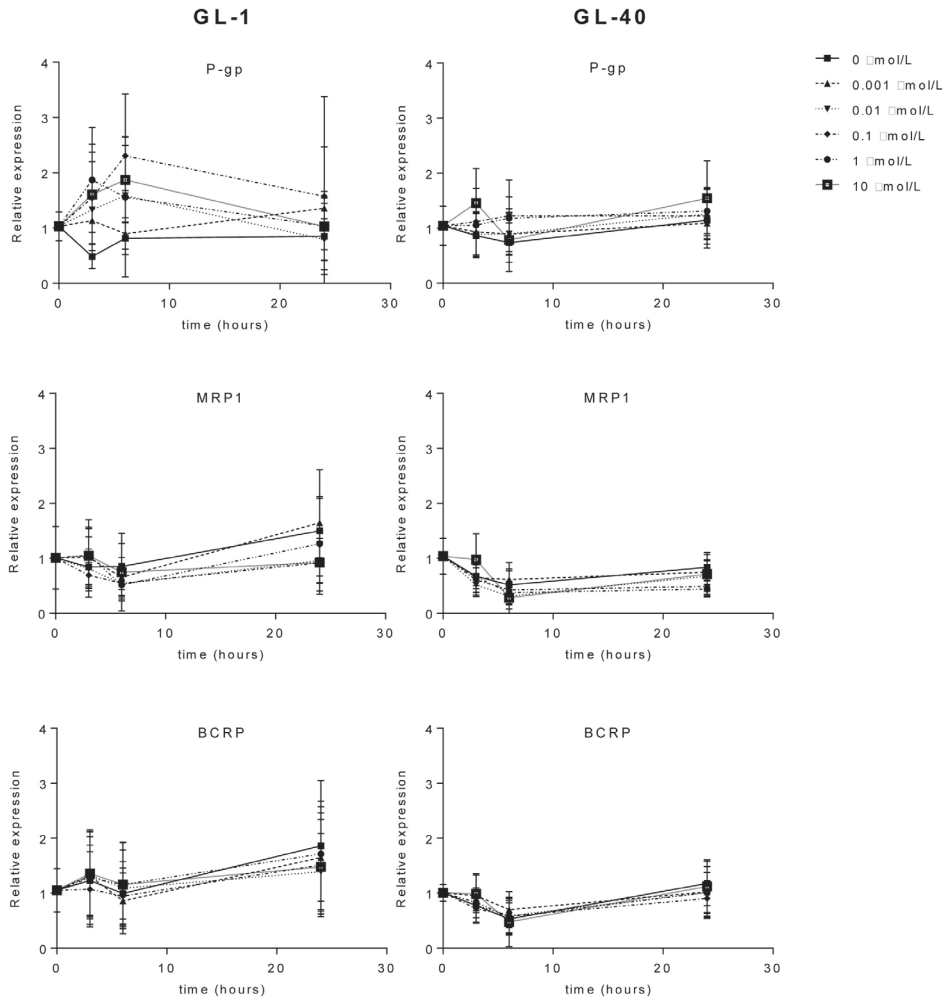

Figure 9. The relative mRNA expression of P-gp, MRP1 and BCRP in the GL-1 and GL-40 cells following incubation with prednisolone (μmol/L) for 0, 3, 6, and 24 hours. Data are presented as mean ± SD of three independent experiments.

Discussion

Selection for doxorubicin resistance by exposing the canine B-cell lymphoid leukemia cell line GL-1[29] to gradually increasing concentrations of doxorubicin led to the GL-40 sub-cell line that was six times more resistant to doxorubicin and showed cross-resistance to the structurally and mechanistically unrelated cytotoxic agent vincristine. The observed DR corresponded with an increased P-gp expression (mRNA and immunocytochemistry) and transport capacity for the P-gp model substrate Rh123. Both DR and Rh123-transport were completely reversed with the prototypical P-gp inhibitor PSC833. Therefore, it is concluded that P-gp causes multi-drug resistance in the GL-40 sub-cell line, which is in line with previous *in vitro* studies[28, 37]. Furthermore these results indicate that in the dog, as in humans[38], doxorubicin and vincristine are both P-gp substrates. Given the fact that P-gp expression is more prevalent in dogs with relapsed and DR cL[17, 18, 39], GL-40 cells represent a suitable *in vitro* model for studying DR in canine lymphoid neoplasia.

Induction of DR through incubation with increasing concentrations of a cytostatic agent, the method used in the current study, has a tendency to preferentially select for P-gp overexpression as the major DR-mechanism and might not necessarily reflect *in vivo* DR, which can be conveyed through both other efflux–transporters, as well as other mechanisms[40]. For example, besides increased P-gp expression, changes in cellular survival signaling cascades, resistance to apoptosis and upregulation of antioxidant defense enzymes[41, 42] have been shown to result in DR and these mechanisms might also have contributed to GL-40's DR phenotype.

Other ABC-transporters implicated in DR to cytostatic drugs in humans include MRP1 and BCRP. The canine orthologs of both these transporters have been evaluated in transfected cell lines and have associated MRP1 with resistance to vincristine, but not doxorubicin[43] and BCRP with resistance to doxorubicin[20]. In the GL-40 cells the level of MRP1 mRNA expression was similar to that of the parental GL-1 cells, although the less intense immunoreactivity and the higher CF retention in the GL-40 cells suggest a reduced MRP1 protein-expression and function. BCRP expression (mRNA, immunocytochemistry) and function (PhA transport) seemed low and equal for both GL cell-lines. Therefore, it can be assumed that neither MRP1, nor BCRP are a cause for GL-40's observed DR.

Prednisolone had a mild and equal antiproliferative effect on both GL-1 and GL-40 cells. The absence of prednisolone resistance in the GL-40 cells is not consistent with the assumption that prednisolone is a P-gp substrate and stands in contrast with human data[44] and a canine *in vivo* study[45]. The absence of a functional glucocorticoid receptor would explain this finding, but is rejected since a luciferase assay demonstrated activation of glucocorticoid receptor response elements following exposure to prednisolone in both GL cell lines. Alternative mechanisms for this unexpected glucocorticoid resistance would include down-stream effects like resistance to glucocorticoid-induced apoptosis[46]. This observation, as well as the cause for the lower luciferase-activity activity in the GL-40 cells, requires further investigation. Furthermore, prednisolone failed to induce expression of P-gp, MRP1 and BCRP mRNA in both GL-1 and GL-40 cells. Based on these data, prednisolone appears neither a substrate for, nor an inducer of P-gp in canine lymphoid cells, which

makes the assumption that prednisolone treatment leads to DR through induction of P-gp overexpression unlikely.

In this study PSC833 and MK571 were used as inhibitors for P-gp and MRPs respectively[34, 35]. Our data show that, in the concentration range tested, PSC833 is a selective inhibitor of canine P-gp with only a mild, and functionally not relevant, inhibition of MRP's at the highest concentration tested. MK571, however, has a comparable effect on Rh123 and CF accumulation in both cell lines suggesting comparable inhibitory potencies towards P-gp and MRP's, as was previously shown for the human homologue transporters[47]. The combination of PhA with Ko143 at 1 µmol/L, a concentration expected to fully block BCRP[33] was tested, but the differences in PhA accumulation between the two GL-cell lines and the effect of Ko143 on PhA accumulation were small and not statistically different. Further studies are needed to demonstrate their use for assessing canine BCRP function.

Because of the common observation of elevated levels of P-gp in multidrug resistant human cancers, significant effort has been spent on developing potent and selective compounds that can modulate P-gp function without undesirable toxicity like PSC833[48]. The highly increased Rh123 efflux and P-gp expression in the GL-40 cells makes these cells a good model for studying interactions of these compounds with canine P-gp. Next to P-gp inhibition, PSC833 is also capable of inducing apoptosis in human leukemia T-cells by increasing cellular ceramide levels[49]. An inhibitory effect of PSC833 on lymphoid cell proliferation was also observed in the current study, which appears independent of P-gp function since both GL-1 and GL-40 cells appeared equally sensitive.

In conclusion, P-gp appears to play an important role in multidrug resistance in canine lymphoid neoplasia, and we present a canine (lymphoid) cell model that allows for the study of multidrug resistance *in vitro* as well as the effect of MDR-modulators thereon.

Acknowledgements

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3

Masitinib reverses doxorubicin resistance in canine lymphoid cells by inhibiting the function of P-glycoprotein

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Abstract

Overexpression of ABC-transporters including P-gp, MRP1 and BCRP has been associated with multi-drug resistance (MDR) in both human and canine oncology. Therapeutic interventions to reverse MDR are limited, but include multi-drug protocols and the temporary concomitant use of inhibitors of ABC-transporters. Recently the use of tyrosine kinase inhibitors has been proposed to overcome MDR in human oncology. One of the tyrosine kinase inhibitors, masitinib, is licensed for veterinary use in the treatment of canine mast cell tumors. Therefore, the current study aimed to assess the potential of masitinib to revert MDR in canine malignant lymphoma using an *in vitro* model with canine lymphoid cell-lines. Masitinib had a mild antiproliferative effect on lymphoid cells, inhibited P-gp function at concentrations equal to or exceeding 1 µmol/L and was able to reverse doxorubicin resistance. The current findings provide the rationale for a combined use of masitinib with doxorubicin in the treatment of dogs with doxorubicin resistant malignant lymphoma, but await confirmation in clinical trials.

Keywords – canine lymphoma, multidrug-resistance, ABC-transporters, P-gp, masitinib

Introduction

Chronic myeloid leukemia (CML) in humans is strongly associated with a reciprocal translocation between chromosomes 9 and 22 creating the so-called Philadelphia chromosome resulting in the BCR-ABL fusion gene that codes for the BCR-Abl protein[1]. BCR-Abl is a tyrosine kinase that promotes cell division and blocks apoptosis thus leading to an unregulated growth of hematopoietic stem cells. In recognition of this pathway, the drug family of tyrosine kinase inhibitors (TKIs) has been successfully introduced in the therapy of CML patients. However, along with a wide clinical use, it became also evident that therapy resistance is regularly observed even against recent more potent TKIs. This has resulted in various attempts to combine conventional cytostatic drugs and TKIs to improve the therapeutic outcome. Recent studies demonstrated indeed a synergistic effect of the combined use of a TKI and conventional chemotherapeutic agents suggesting also that TKIs might have the potential to reverse drug resistance[2, 3].

The TKI masitinib (Masivet®, Kinavet®, AB Science, France) has been licensed for use in canine patients with mast cell tumors[4]. In a recent *in vitro* study Thamm et al.[5] demonstrated that masitinib sensitizes a variety of canine cancer to cytostatic drugs. These findings are based on the measurement of cell proliferation rates only and provided no details about the possible mechanism of action. However, they provided the first evidence that TKIs might be able to enhance the effect of conventional chemotherapeutic agents in veterinary medicine. Masitinib might be of therapeutic value in the treatment of canine lymphomas as well, although specific targets have not been identified yet.

As TKIs suppress BCR-Abl function by blocking its ability to use ATP, it was hypothesized that they might be also able to affect other proteins that have an ATP-binding site. Efflux transporter proteins using an ATP-binding site, known as ABC-transporters, are considered one of the most important mechanisms conveying multidrug resistance to chemotherapeutic agents, as they prevent intracellular distribution of chemotherapeutics, which thus fail to reach their target sites[6, 7]. Overexpression of ABC-transporter proteins, in particular P-glycoprotein (P-gp; ABCB1), as well as multidrug resistance related protein 1 (MRP1; ABCC1) and breast cancer resistance protein (BCRP; ABCG2), have been observed in a variety of human[8] and to a lesser extent in canine cancers, in particular hematopoietic neoplasia like lymphoma and leukemia [9-12].

Considering these findings that in human cell lines TKI's were shown to affect ABC-transporter function[2, 13, 14] we hypothesized that masitinib is capable of reverting drug resistance in canine malignant lymphoma cells. To support this hypothesis we tested the potential of masitinib to reverse doxorubicin resistance in a multidrug resistant canine lymphoid cell line and related this to the inhibition of P-gp function.

Materials and Methods

Chemicals

Mastinitib-mesylate was a kind gift of AB Science, S.A. (Paris, France). PSC833 (Valspodar®) was a generous gift of Novartis Pharma AG (Basel, Switzerland). MK-571 sodium salt was obtained from Alexis Biochemicals (Grünberg, Germany). Doxorubicin hydrochloride,

rhodamine 123 (Rh123), calcein-AM (CAM) and 5(6)-carboxyfluorescein diacetate (CFDA) were purchased from Sigma-Aldrich (St Louis, MO, USA). All stock solutions were prepared in DMSO, except for masitinib that was dissolved in milliQ water (pH 4). In the cell culture media the maximum concentration of DMSO did not exceed 0.1%.

Cell lines, cell culture media and supplements

From the canine lymphoid leukemia cell line GL-1, originally described by Nakaichi *et al.*[15], a sub-cell line GL-40 was created, which is resistant to doxorubicin and vincristine and shows P-gp overexpression. Cell lines were maintained in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 1% (v/v) L-glutamine (BioWhittaker, Maine, USA), 10% (V/V) fetal bovine serum (Gibco) and 100 U/ml penicillin and 100 µg/ml streptomycin (BioWhittaker) at 37°C in a humidified atmosphere of 5% CO_2. The GL-40 sub-cell line was intermittently cultured in the presence of 40 ng/ml (0.07 µmol/L) doxorubicin following the schedule of one passage with and two passages without doxorubicin to maintain drug resistance.

Proliferation assay

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8), a colorimetric assay using the tetrazolium salt, WST-8 (Dojindo molecular technologies, Rockville, Maryland, USA), according to the manufacturer's instructions. Cells were seeded in 96-well plates at a density of 2 x10^4 cells per well in cell culture medium containing a concentration range of masitinib as indicated in the figures and incubated for 24 or 48 hours, respectively, at 37°C, 5% CO_2. The tetrazolium solution was added to each well three hours prior to analysis and the soluble formazan was measured by light absorbance at 450nm in a microplate reader. Proliferation assays for the combined incubation of masitinib and doxorubicin used the same procedure except that the cells were incubated for 72 hours.

Fluorescent substrate accumulation assays

All experiments were performed with GL-40 cells that were incubated for 1 passage without doxorubicin. A total number of 0.5 x 10^6 viable cells were plated in 96-well plates and preincubated for 30 minutes at 37°C and 5 % CO_2. Thereafter cells were pelleted by centrifugation and the medium was discarded. The cells were re-suspended in Hanks balanced salt solution without Ca^{2+}, Mg^{2+} and phenol red (HBSS with 1000 mg/L glucose; Gibco) containing the specified fluorescent compound Rh123 (4 µmol/L), CAM (5 µmol/L), CFDA (1 µmol/L) or vehicle control and masitinib, a transporter-inhibitor (PSC833 or MK571) or vehicle control. After 30 minutes of incubation at 37°C, 5 % CO_2, the medium was replaced by cold PBS (4°C) and the cell suspensions were collected in FACS-tubes and placed on ice until FACS analysis.

FACS analysis

A flow cytometer (FACS Calibur, Biorad) equipped with an argon 488 nm laser was used for the detection of the cell-associated fluorescence of the samples. Cell-associated Rh123, CAM and CFDA fluorescence was measured with a 530nm bandpass filter. The samples

were gated on forward scatter versus side scatter to exclude clumps and cell debris and data were collected for a minimum of 10,000 gated events per sample.

Data analysis

The FACS data were collected as geometric mean fluorescence intensity for all samples obtained from 3 independent cell culture experiments per test protocol. Data are presented as Relative Fluorescence Units (RFU) (expressed as a percentage) calculated according to the following formula:

Uptake [%] = $(FL_S\text{-}FL_B) / (FL_C\text{-}FL_B) * 100\%$

FL_B: Fluorescence intensity of the background without fluorescent substrate

FL_S: Fluorescence intensity of the sample at t=30 minutes incubated with masitinib or the transporter inhibitor

FL_C: Fluorescence intensity of the sample incubated without masitinib or transporter inhibitor

Concentration dependent effects on cell proliferation were analyzed by non-linear regression after log transformation of the concentration (X-axis) and graphs were fitted according to a sigmoïdal dose-response curve. The 50% inhibitory concentration (IC_{50}) of a particular agent was defined as the drug concentration at which a 50% decline in effect was observed versus untreated control.

Differences in accumulation of fluorescent dyes in the absence or presence of masitinib or a specific transporter inhibitor were tested for significance using the One-Way ANOVA with Dunnett multiple comparison *post hoc* test. The level of significance was set at $P<0.05$. All statistical analyses were performed using Graph Pad Prism software (San Diego, California, USA).

Results

Cell proliferation assay with masitinib

The highest masitinib concentration (40 µmol/L) tested, was based on the solubility characteristics of the compound in the cell culture medium. Masitinib decreased cell proliferation equally in both GL-1 and GL-40 cells. The inhibitory effect appeared concentration dependent and a more than 80% reduction (compared to untreated controls) was observed after a 24 hour incubation with masitinib at a concentration of 40 µmol/L. After 48 hours of incubation with 10 µmol/L masitinib cell proliferation decreased to 83 ± 6% and 78 ± 7% of the controls for respectively the GL-1 and GL-40 cell lines (Figure 1).

Proliferation assay with the combination masitinib and doxorubicin

The doxorubicin resistant GL-40 cells were less sensitive to the antiproliferative effect of doxorubicin than the original GL-1 cell line as presented by a higher IC_{50} value (Table 1). Although masitinib at concentrations of 1 and 10 µmol/L had no additional effect on doxorubicin-induced cytotoxicity in the GL-1 cells, it enhanced the antiproliferative effect

of doxorubicin on GL-40 cells as demonstrated by a significant decrease in the IC_{50} value (Table 1).

Modulation of rhodamine 123, calcein-AM and CFDA uptake by masitinib

To assess the effect of masitinib on substrate accumulation, three different fluorescent compounds were used. Rhodamin 123 is a common substrate for the efflux transporter P-gp. Masitinib had no significant effect on Rh123 retention in the GL-1 cells, although a trend towards a decrease in Rh123 retention was observed at higher concentrations of masitinib. In contrast, masitinib increased Rh123 retention in the GL-40 cells at concentrations of ≥1 µmol/L, but to a lesser extent than prototypical P-gp transport inhibitor PSC833 (Figure 2). Calcein-AM was used as a probe for both, P-gp and MRP function in the GL-cells. Masitinib increased calcein retention in the GL-40 cells at concentrations of ≥1 µmol/L (Figure 3) and at 20 µmol/L masitinib calcein retention was similar to that obtained with PSC833. A slight increase in calcein retention was noted in the GL-1 cells at a masitinib concentration of 20 µmol/L, but this effect was less pronounced than the effect from the inhibitor MK571 (Figure 3).

CFDA was used as a probe for MRP1 function and masitinib had no effect on CF retention in the GL-1 or GL-40 cells, while the typical MRP-inhibitor MK571 caused a 20-fold increase in CF retention in the GL-1 cells (data not shown).

Table 1. IC_{50} values and 95%-confidence interval for doxorubicin (ng/ml) in combination with masitinib-mesylate (0, 1 and 10 µmol/L) in GL-1 and GL-40 cells.

Masitinib (µmol/L)	Doxorubicin IC50 (ng/mL)	
	GL-1	*GL-40*
0	8.1 (7.4-8.8)	92 (86-98)
1	9.5 (8.2-9.6)	48 (42-53)
10	8.9 (6.9-13.0)	15 (13-17)

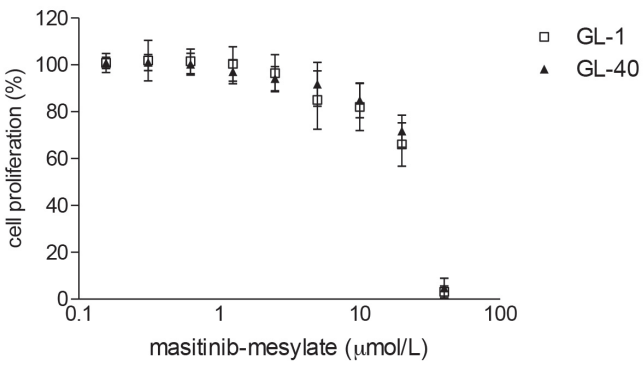

Figure 1. Cell proliferation of GL-1 and GL-40 cells following 48 hours of incubation with 10 μmol/L masitinib-mesylate as measured by CCK-8 conversion. Data are presented as a percentage of the untreated controls and as mean ± SD of three independent experiments.

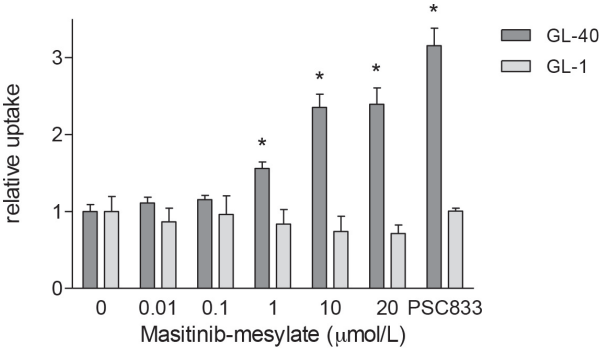

Figure 2. Uptake of rhodamine 123 (4 μmol/L) by GL-1 and GL-40 cells after 30 minutes incubation in the presence of masitinib-mesylate or PSC833 (1 μmol/L). Data are presented as mean ± SD of three independent experiments. Significant differences are marked * indicating P<0.05.

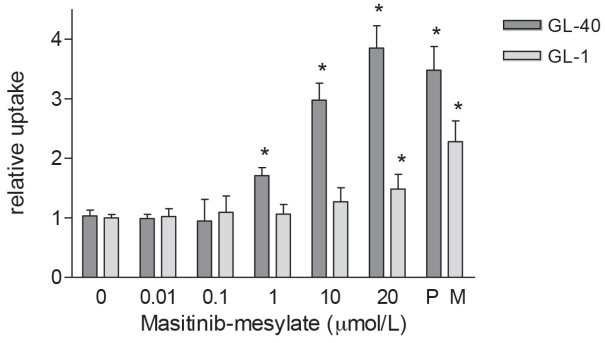

Figure 3. Uptake of calcein (5 μmol/L) by GL-1 and GL-40 cells after 30 minutes of incubation with masitinib-mesylate or 1 μmol/L PSC833 (P) or 25 μmol/L MK571 (M). Data are presented as mean ± SD of three independent experiments. Significant differences are marked * indicating P<0.05.

Discussion

The TKI masitinib has been licensed for the treatment of non-resectable mast cell tumors in dogs with a c-Kit mutation[16]. However, *in vitro* data in canine cell lines suggest also a potential role for masitinib in the treatment of other malignancies[5]. The current study shows that masitinib at low concentrations (1 and 10 µmol/L) has a mild antiproliferative effect on lymphoid leukemia cell lines GL-1 and GL-40. Moreover, it could be demonstrated that masitinib effectively reduced drug resistance in the doxorubicin resistant GL-40 cells. The sensitivity to doxorubicin, as indicated by IC_{50} values, increased 2- and 6-fold when GL-40 cells were co-incubated with 1 and 10 µmol/L masitinib, respectively.

Evidence for the involvement of the efflux transporter P-gp was already provided by these first experiments, as no effect of masitinib on doxorubicin-induced cytotoxicity was observed in the non-P-gp expressing GL-1 cells. Hence we aimed to confirm the inhibitory effect of masitinib on P-gp function by an uptake study using the typical P-gp substrate Rh123. Masitinib increased Rh123 uptake in the GL-40 cells confirming inhibition of P-gp function. Although a full concentration dose-effect curve could not be obtained due to the intrinsic cytotoxicity of masitinib and its limited solubility at higher concentrations, masitinib appears to be less potent than the prototypical P-gp inhibitor PSC833.

In the interpretation of the results obtained with masitinib it has also to be considered that although the GL-1 cells lack quantifiable levels of P-gp expression, masitinib slightly decreased Rh123 retention. A possible explanation for this effect is an impairment of the function of the organic cation transporter[17]. Inhibition of this uptake transporter embedded in the cellular membrane would result in a decreased Rh123 (re)uptake, which cannot be easily discriminated from the effects of an efflux transporter in the given experimental protocol.

At higher concentrations, masitinib caused a slight increase in calcein retention in the doxorubicin sensitive GL-1 cells. Calcein-AM is a substrate for ABC-transporters of the MRP-family (ABCC-family) and the increased calcein retention might result from inhibition of MRP function[18]. However. since masitinib had no inhibitory effect on the retention of CFDA, a typical MRP1 substrate[19], it seems likely that another ABCC-transporter, possibly MRP3 that is also expressed in GL-1 cells (data not shown), is inhibited by masitinib.

Although doxorubicin is a substrate for mutated (non-wildtype) BCRP in humans[20] and most likely for canine BCRP[10], doxorubicin resistance could not be associated with BCRP expression in the GL cell lines. Both the GL-1 and GL-40 cells have a similar expression level of BCRP and the BCRP inhibitor Ko143 did no effect pheophorbide A retention (data not shown). Although TKI's are typically BCRP substrates in humans, no comparable data are available from canine tissues or cells.

The clinical relevance of our in vitro data need to be confirmed in clinical trials, as the clinical efficacy of the combined therapy will be determined by the relative affinity to P-gp and the concentrations reached at the target site. In dogs masitinib at the licensed dose of 12.5 mg/kg bodyweight results in a maximum free plasma concentration of approximately 1 µmol/L *in vivo*[21]. Hence, it remains to be elucidated whether or not the masitinib concentration of 1-10 µmol/L, which was shown to effectively reverse doxorubicin resistance, can be

reached under *in vivo* conditions. Hence, clinical studies are needed to demonstrate the effects of masitinib on doxorubicin efficacy.

In conclusion, our *in vitro* data demonstrate that masitinib potentiates the antiproliferative effect of doxorubicin in doxorubicin-resistant canine lymphoid leukemia cells by inhibiting P-gp function. Although these findings need to be confirmed under *in vivo* conditions, they suggest new possibilities to improve the therapeutic protocols for canine patients with multi-drug resistance to cytostatic agents.

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4

Prednisolone inclusion in a first-line multidrug cytostatic protocol for the treatment of canine lymphoma does not affect therapy results

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Abstract

Chemotherapy protocols for canine lymphoma include the routine use of glucocorticoids for their lympholytic effect. However, glucocorticoids are associated with side-effects including polyphagia, polyuria, and weight gain, limit the use of NSAID's and are capable of inducing drug transporter expression that could lead to drug resistance. Despite these negative effects, there are no data to support the use of glucocorticoids as part of a multidrug chemotherapy protocol for the treatment of canine lymphoma.

A prospective, randomized clinical trial was performed in 81 dogs with multicentric lymphoma and no history of recent glucocorticoid use. All dogs were staged and treated with the same chemotherapy-protocol (L-asparaginase, cyclophosphamide, doxorubicin, vincristine, prednisolone) with half of the dogs receiving prednisolone. Both treatment groups were similar with respect to demographics, immunophenotype, and clinical stage except for a higher number of substage b patients in the prednisolone-group (5 versus 14; $P = 0.015$). Treatment results obtained with the initial (complete response rate 75%, disease-free period 176 days) and rescue (complete response rate 45%, disease-free period 133 days) treatment, overall survival (283 days) and adverse events (number and grade) were similar for both groups. Prednisolone, as part of a multidrug chemotherapy, protocol has no additional effect on treatment results and it is concluded that prednisolone can be omitted from first-line multidrug protocols used for the treatment of canine lymphoma.

Keywords – dog; chemotherapy; non-Hodgkin; lymphoma; glucocorticoids

Abbreviations

cL: canine lymphoma, CR: complete response, DFP: disease-free period, GC (s): glucocorticoid (s), MDR: multidrug resistance, OS: overall survival, PD: progressive disease, P-gp: P-glycoprotein, PR: partial response, RD: response duration, SC: stable disease, TTP: time to progression.

Introduction

Canine lymphoma (cL) is the most common hematopoietic neoplasia in the dog[1, 2] and typically presents as generalized lymphadenopathy[3]. Treatment for cL has evolved from a monotherapy with glucocorticoids (GCs)[4] to a multi-drug therapy with the GC prednisolone (P) and cytostatic agents; initially cyclophosphamide (C) and vincristine (O) (COP-protocol)[5], later also doxorubicin (H) (CHOP-protocol)[6-9]. CHOP-based protocols are the current standard of care for treating cL, a situation similar to that in human oncology prior to the introduction of Rituximab[10], and result in a 70-90% complete response rate and a disease-free period of 9-11 months[11]. Since only a small proportion of dogs is cured, most animals will experience tumor recurrence after which disease is more difficult to control[12].

The routine use of GCs in the treatment of lymphoid neoplasia, including cL, is based on the fact that GCs are capable of inducing apoptosis of lymphoid cells[13, 14]. However, GCs are associated with adverse effects including clinical signs like polydipsia, polyuria, polyphagia and weight gain, predispose to subclinical (urinary tract) infections[15] and diabetes mellitus[16], muscle atrophy and have negative effects on the quality of tendons and ligamentous structures[17]. Furthermore GCs limit the concurrent use of NSAID's and GC administration prior to starting chemotherapy has been associated with a poorer prognosis[18-21].

Failure of a tumor to respond to chemotherapy can result from tumor cell resistance to the cytotoxic agents used. Although drug resistance can arise through various mechanisms[22,] overexpression of drug transporters of the ATP-Binding Cassette superfamily, and P-glycoprotein (P-gp) in particular, appears to be the most important mechanism in human oncology[23]. In dogs, P-gp expression was demonstrated in therapy-resistant and relapsed cL cases and correlated with a poorer response to chemotherapy and shorter survival[24-26].

GCs are able to increase P-gp expression in human lymphocytes, both *in vitro*[27] and *in vivo*[28], as well as in the dog[29]. It could be hypothesized that GCs are able to induce P-gp expression in neoplastic lymphoblasts, leading to the development of drug resistance, which would explain the poorer therapy results in relapsed cL cases. On the other hand, removing the lympholytic effect of GCs from a chemotherapy protocol could lead to inferior therapy results.

To establish the effect of prednisolone within a multidrug chemotherapy protocol, a prospective, randomized study was performed. Dogs, newly diagnosed with multicentric lymphoma and no recent treatment with GCs, received the same doxorubicin-based multidrug chemotherapy protocol, but were randomly assigned to receive GCs or not. The authors' hypotheses were that prednisolone use would not affect treatment results in the first treatment round, but would prove a negative factor on treatment results following relapse.

Material and Methods

Patient population

All dogs were privately owned and presented to the Small Animal University Clinic at the Faculty of Veterinary Medicine, Utrecht University between September 2005 and January

2010 for the treatment of multicentric lymphoma. Dogs that had received steroids <3 months prior to diagnosis or required NSAID's were excluded from the study. Breed, sex, age and weight were recorded. Stage and substage were determined using the WHO-staging system for cL[30]. The minimum diagnostic dataset obtained for each dog included a hematological and clinical chemistry profile, bone marrow aspiration biopsy, and cytological examination of a lymph node aspirate to confirm the diagnosis of cL, establish grade (high versus low according to Updated Kiel[31, 32]) and immunophenotype using CD79a (pan–B marker) and CD3 (pan–T marker) (Dako, Glostrup, Denmark) [33]. Thoracic radiographs, abdominal ultrasound or other diagnostic tests were performed when indicated by clinical signs.

Following an informed consent from the owner, dogs were randomized to one of the treatment groups, chemotherapy "with" or "without" prednisolone. A randomization scheme was generated for 120 dogs (60 per group) and the randomization for each individual was enclosed in consecutively numbered, sealed envelopes. Dogs were numbered based on order of entry into the study after which randomization was performed by opening the corresponding envelope. Because the signs associated with glucocorticoid use (polydipsia, polyphagia) might prohibit a blinded study, no placebo was used for the dogs in the "without prednisolone"-group.

The study was evaluated and approved by the Research Scientific and Ethical Committee at Utrecht University.

Treatment

All dogs received the same doxorubicin-based multidrug protocol (Table 1) and some prednisolone (Table 2). A complete hematological analysis was performed immediately before each treatment with doxorubicin. Prior to doxorubicin treatment, dogs in the "prednisolone" group received an intravenous injection with dexamethasone, and dogs in the "without prednisolone"-group the antihistamine clemastine (2 mg/m^2 iv). In case of a clinically relevant chemotherapy-related adverse event, the scheduled treatment was either delayed or a 20% dose reduction was applied. Following relapse, further treatment was dependent on owner's wishes and individual clinician's preferences.

Laboratory results

Anemia was defined as a hematocrit < 0.42 L/L (mild anemia 0.35-0.42 L/L), leukopenia as a total white blood cell count <4.10^9/L, thrombocytopenia as thrombocyte count <144.10^9/L (mild thrombocytopenia 100-144.10^9/L), hypoalbuminemia as plasma albumin concentration <26 g/L and hypercalcemia as plasma calcium level > 3.0 mmol/L.

Response and toxicity

Response was assessed by palpation of peripheral lymph nodes. Objective response (CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease), disease-free period (DFP), response duration (RD), time to progression (TTP), and overall survival (OS) were recorded using the definitions proposed in the VCOG consensus document[34]. In

Table 1. Chemotherapy protocol

Day	1	8	22	33	43	57	60	71	80	92	113	127-148
L-asparaginase (10.000 IU/m^2 IM)	◆											
Doxorubicin (30 mg/m^2 IV)		◆	◆		◆			◆		◆		
Chlorambucil (25 mg/m^2 PO over 2 days)				◆					◆			
Vincristine (0.5-0.7 mg/m^2 IV)						◆						
Cyclophosphamide (200-250mg/m^2 PO)							◆				◆	
Chlorambucil (2 mg/m^2 1dd PO)												◆

Table 2. Prednisolone dose scheme

Day	8	15	22	29	43	57	71	85	99	113
Prednisolone (mg/m^2 once daily)	50	40	30	25	20	15	10	5	5 EOD	Stop

(EOD = every other day)

the first and second treatment round DFP, RD and TTP were calculated for those dogs that received treatment with cytostatic drugs. For dogs that received a monotherapy with GCs only TTP was calculated. All deaths during the study were considered disease or treatment related except for those cases where a clear cause of death was established that was not lymphoma- or chemotherapy-related. All dogs alive at the end of the study were censored at the time of analysis.

Toxicity was graded based on the VCOG criteria for adverse events[35].

Statistical analysis

Reported results were described per group as frequencies (categorical variables) or mean/median (continuous variables). Differences between groups were tested for significance with the Chi Square or Fisher Exact for categorical variables and either the Kruskal-Wallis test (non-parametric) or the unpaired *t*-test (parametric) for continuous variables after having tested for normality. The Kaplan-Meier product limit method was used for estimating DFP, RD, TTP and OS and tested for significance (Log-Rank or Breslow method). Prognostic variables for outcome (DFP first and second treatment round, OS) were individually assessed in the univariate Cox Proportional Hazard analysis and variables with $P<0.20$ were subsequently included in a multivariate analysis, with a backward elimination procedure. The level of significance was set at $P < 0.05$. Significant differences are indicated in the graphs and mentioned in the text, unless otherwise stated. All statistical analyses were performed using the software package IBM SPSS Statistics Version 19.

Results

Patient population

Eighty-eight dogs were entered into the study, but seven cases were excluded from the final analysis due to failure to return for further therapy after the first treatment (4x; 3x

owner's choice, 1 dog died from gastric dilatation volvulus), breach of therapy protocol (2x) and improper inclusion (1x).

The remaining 81 dogs were equally distributed over both treatment groups (41 no prednisolone versus 40 with prednisolone) and included 8 mixed-breed dogs and 73 purebred dogs representing 35 different breeds. The most commonly represented dog breeds were the German shepherd, Rottweiler (6 each), Bernese mountain dog, Boxer, Golden retriever (5 each), English Cocker Spaniel, Flatcoated retriever (4 each), dogue de Bordeaux, Bullmastiff (3 each), Border collie, Bouvier des Flandres, Great Dane, Rhodesian Ridgeback, Tibetan terrier, West Highland White terrier (2 each) and the other 20 breeds were represented by 1 dog each. There were 50 male (24 intact, 26 neutered) and 31 female (6 intact, 25 spayed) dogs with a mean age of 7.5 years (median 7.2, range 1.5 - 14.0) and a mean body weight of 35 kg (median 36, range 7 - 82) at the time of presentation. There were no differences between the two groups with regards to demographics (Table 3).

Clinical stage

Two (2%) dogs were classified as stage I, 27 (33%) as stage III, 26 (32%) as stage IV and 26 (32%) as stage V. Peripheral blood smears of all dogs were examined by an experienced hematologist and lymphoblasts were found in 12 cases (15%). Bone marrow aspiration was performed in 80 dogs and bone marrow involvement was cytologically demonstrated in 26 dogs (32%). Sixty-two (77%) dogs were classified as substage a and the remaining 19 (23%) as substage b. Seventy-three cases (90%) were designated high-grade lymphoma and the remaining eight (10%) as low-grade. There were no differences found between the two groups except for a higher percentage of substage b dogs in the "with prednisolone" group (12% versus 35%, P=0.015).

Laboratory results

Anemia was present in 35 (43%) dogs, but generally mild (25/35, 72%). Leukopenia was demonstrated in 3 (4%) dogs and thrombocytopenia in 11 (14%) dogs and usually mild (7/11). Twenty-six (32%) dogs were hypoalbuminemic and 7 (9%) hypercalcemic. There were no significance differences found between the two groups with regards to laboratory results.

Immunphenotyping

The immunophenotype was established in 70 dogs and showed 49 cases of B-cell (70%) and 21 cases of T-cell (30%) lymphoma and showed a similar distribution in both groups. In the remaining 11 cases immunophenotyping was either inconclusive (7) or not performed (4). Hypercalcemia was associated with the T-cell immunophenotype in 6 dogs and an inconclusive result in the other dog.

Treatment response

The initial treatment resulted in a 75% CR-rate and median DFP of 176 days and was similar for both groups (Table 4, Figure 1). CR-rate was higher for B- than for T-cell lymphomas (84% vs 43%, $P < 0.001$) and not affected by prednisolone use. Fifty-five (90%) dogs relapsed and

Table 3. Summary of demographic, clinical and relevant laboratory data summarized per treatment group and for all dogs.

		No prednisolone (n=41)	With prednisolone (n= 40)	All animals (n=81)
Sex	Male	26 (63%)	24 (60%)	50 (62%)
	Female	15 (37%)	16 (40%)	31 (38%)
Age (years)	Mean Range	7.7 (2.8-13.4)	7.3 (1.5–14.0)	7.5 (1.5-14.0)
Weight (kg)	Mean Range	37 (8-72)	33 (7-82)	35 (7-82)
Stage	I	1 (2%)	1 (3%)	2 (2%)
	III	14 (34%)	13 (33%)	27 (33%)
	IV	14 (34%)	12 (30%)	26 (32%)
	V	12 (29%)	14 (35%)	26 (32%)
Substage	a	36* (88%)	26* (65%)	62 (77%)
	b	5* (12%)	14* (35%)	19 (23%)
Lymphoblasts in	Blood	5 (12%)	7 (17%)	12 (15%)
	Bone marrow	12 (29%)	14/39 (36%)	26 (33%)
Immunophenotype	B	26 (63%)	23 (64%)	49 (64%)
	T	10 (24%)	11 (31%)	21 (27%)
	Inconclusive	5 (12%)	2 (6%)	7 (9%)
Grade	High	37 (90%)	36 (90%)	73 (90%)
	Low	4 (10%)	4 (10%)	8 (10%)
Anemia		18 (44%)	17 (43%)	35 (43%)
Leukopenia		1 (2%)	2 (5%)	3 (4%)
Hypercalcemia		4 (10%)	3 (8%)	7 (9%)

Significant differences are indicated with an *

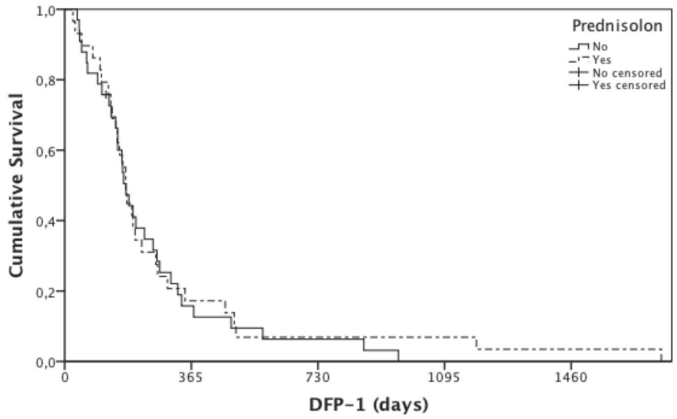
relapse-rate was similar for both groups. Six dogs were censored (4 alive at end of study, 2 died of lymphoma-unrelated causes).

Rescue treatment was initiated in 58 dogs, either with cytostatic agents (49), typically vincristine, cyclophosphamide, L-asparaginase and one additional dose of doxorubicine, or a monotherapy with GCs (9). Prednisolone was included in the rescue protocol for all 23 dogs from the "with prednisolone"-group and initially in six of 26 dogs from the "without prednisolone"-group. A rescue protocol with cytostatic agents resulted in a lower second CR-rate (45%, $P < 0.001$) and shorter second DFP (median 133 days, $P = 0.044$) than the initial

Table 4. Therapy results in objective response and median duration of response for the initial and rescue treatment summarized per treatment group and for all dogs.

	No prednisolone	WIth prednisolone	All animals
Response			
Initial treatment (n, %)			
CR	32 (78%)	29 (73%)	61 (75%)
PR	6 (15%)	3 (8%)	9 (11%)
SD	2 (5%)	5 (13%)	7 (9%)
PD	1 (2%)	3 (8%)	4 (5%)
Rescue treatment (n, %)			
CR	11 (42%)	11 (48%)	22 (45%)
PR	9 (35%)	4 (17%)	13 (27%)
SD	3 (12%)	4 (17%)	7 (14%)
PD	3 (12%)	4 (17%)	7 (14%)
Response duration (median in days; number of dogs)			
Initial treatment			
DFP	177 (32)	176 (29)	176 (61)
RD	153 (38)	166 (32)	165 (70)
TTP	153 (41)	137 (40)	148 (81)
Rescue Chemotherapy Treatment			
DFP	105 (11)	175 (11)	133 (22)
RD	83 (20)	133 (15)	119 (35)
TTP	72 (26)	75 (23)	74 (49)
Rescue Prednisolone Treatment			
TTP	58 (4)	45 (5)	58 (9)
Overall survival	286 (41)	225 (40)	283 (81)

CR = complete response, PR = partial response, SD = stable disease, PD = progressive disease, DFP = disease-free period, RD = response duration, TTP= time to progression



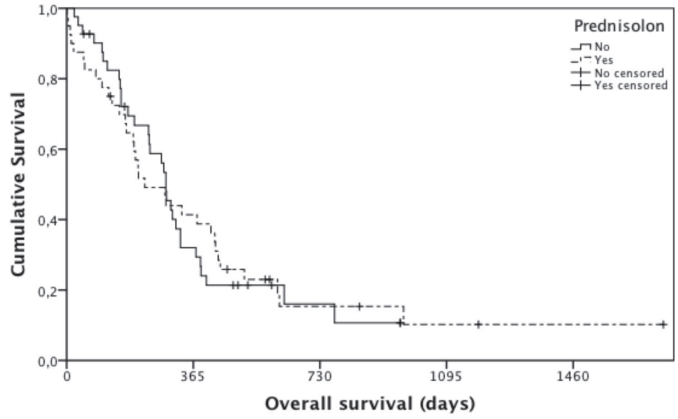


Figure 1. Kaplan-Meier survival curve for first disease-free period (DFP-1, top) and overall survival (bottom).

Table 5. The response (no CR versus CR) and duration of response (TTP vs DFP) following rescue treatment with either cytostatic drugs or monotherapy with glucocorticoids categorized by duration of first DFP.

Rescue therapy	Response	First DFP (days)		
		<180 (n=31)	180 – 365 (n=14)	>365 (n=4)
Chemotherapy	No CR (n, %) TTP (days)	22 (71)* 34**	5 (36)* 50**	0* -
	CR (n, %) DFP (days)	9 (29)* 122**	9 (64)* 175**	4 (100)* 135**
Glucocorticoids	TTP (n/days)	7/69	2/58	0/-

CR = complete response, TTP = time to treatment failure, DFP = disease free period, n = number of dogs, * significant difference between % No CR vs CR at each time period, ** significant difference between TTP vs DFP, not between time periods

treatment protocol. The use of prednisolone in the first treatment round had no effect on rescue chemotherapy results and response distribution (CR, PR, SD, PD) and duration of response (DFP, RD en TTP) were similar for both groups. The TTP following a rescue therapy with GC was similar for both groups. The overall median OS (283 days) was not different between the two groups (Figure 1).
Duration of first DFP correlated with the likelihood of obtaining a second CR ($P = 0.03$), but not duration of second DFP (Table 5). If relapse occurred during the initial treatment (DFP <180 days) 29% of the dogs had a second CR compared to 64% for dogs that had a relapse after having finished the protocol (DFP >180 days).

Prognostic factors for first and second remission duration and survival

A univariate analysis for duration of first DFP stratified for prednisolone use revealed that weight, age, WHO-stage, grade, immunophenotype, bone marrow involvement, leukopenia, leukemia, serum creatinine concentration, and hypercalcemia had a significant effect (Table 6). Univariate analysis for duration of OS stratified for prednisolone use demonstrated that weight, WHO-stage, grade, immunophenotype, bone marrow involvement, total leukocyte count, leukopenia, serum creatinine concentration, total serum calcium concentration and hypercalcemia had a significant effect (Table 6). Since bone marrow involvement and WHO-stage partially assess the same parameter, and the continuous parameters total leukocyte count and total serum calcium concentration gave lower *P*-values than their derived categorical variables leukopenia, leukemia and hypercalcemia, WHO-stage and the continuous variables were excluded from the subsequent multivariate analysis. A multivariate analysis stratified for prednisolone use (Table 6) demonstrated that weight, leukopenia, bone marrow involvement and hypercalcemia were the strongest predictors for both duration of first DFP and OS. Body weight was inversely correlated with both DFP and survival and the Cox regression hazard analysis showed that each kilogram increase in body weight increased the risk of relapse or death by respectively 3.1 and 1.6%. A univariate analysis for duration of second DFP demonstrated that prednisolone use during the first treatment round and duration of the first DFP had no significant effect on duration of the second DFP.

Toxicoses

The current protocol was well tolerated and mildly toxic. Of the 106 adverse events reported, 93% were classified as mild (grade 1 (82%) or 2 (12%)) and mostly of gastrointestinal origin, while the remaining 7% were classified as grade 3. No dose reductions or treatment delays were performed. Signs of (hemorrhagic) cystitis following administration of cyclophosphamide were reported in six dogs and necessitated a protocol change in four dogs (second dose of cyclophosphamide not given (2 dogs) or replaced by chlorambucil (2 dogs)) and in the remaining two dogs relapse occurred prior to the second dose of cyclophosphamide. Extravasation of doxorubicin resulted in grade 3 toxicity in two, dogs and contributed to the decision to perform euthanasia in one of these dogs. In one dog (great Dane) doxorubicin was replaced by mitoxantrone from the third dose onwards due

Table 6. Results of multivariate analysis for factors predicting relapse and death using the data from the disease-free period and survival stratified for prednisolone use.

Parameter	Relapse		Death	
	Hazard Ratio (95% CI)	*P*-value	Hazard Ratio (95% CI)	*P*-value
Weight	1.031 (1.008 – 1.054)	0.008	1.016 (0.997 – 1.035)	0.099
Leukopenia	29.16 (2.343 – 362.9)	0.009	6.365 (1.619 – 25.02)	0.008
Bone marrow involvement	1.622 (0.8447 – 3.116)	0.1461	2.205 (1.196 – 4.064)	0.113
Hypercalcemia	32.55 (6.638 – 159.7)	<0.001	3.77 (1.422 – 9.99)	0.008

Table 7. The reported adverse events (grade and frequency) differentiated for all dogs and per treatment group.

Adverse-events		Grade	Prednisolone		All dogs (n=81)
			No (n=41)	Yes (n=40)	
Gastrointestinal	Anorexia	1	7	6	13
	Vomiting	1	14	13	27
		2	1	2	3
		3	0	1	1
	Diarrhea	1	18	14	32
		2	2	4	6
		3	0	1	1
Hematologic	Anemia	3	0	1	1
	Neutropenia	1	0	1	1
	Thrombocytopenia	1	0	1	1
		3	0	1	1
Cutaneous	Alopecia	2	0	1	1
	Extravasation	3	0	2	2
Urinary	Polydipsia/ Polyuria	1	1	4	5
	Cystitis	1	2	1	3
		2	1	1	2
		3	1	0	1
Constitutional	Weight loss	1	2	1	3
		2	0	1	1
Cardiac	Left ventricular dysfunction	1	0	1	1
	Total		49	57	106

to a mild, but asymptomatic reduction in fractional shortening of the heart. There were no differences in number or degree of adverse events between both groups.

Discussion

The current study's patient population was comparable to previous reports[6-8, 20] and both treatment groups were similar with respect to all relevant parameters except for a higher percentage of substage b dogs in the "with prednisolone"-group. Prednisolone use had no effect on CR-rate (78% in "without"-, 73% in "with prednisolone"-group), median DFP (177 days in "without"-, 176 days in "with prednisolone"-group) or adverse events in the first treatment round. Rescue treatment with cytostatic agents was started in 49 dogs and showed a lower CR-rate (45 versus 75%) and shorter median DFP (133 versus 176 days) than the initial treatment, but similar for both groups, as was median OS (286 days in "without"-, 225 days in "with prednisolone"-group). Although the study has limitations including the number of dogs and drugs used, the absence of histological subclassification and limited staging, it is concluded that prednisolone use is not necessary in a first-line multidrug chemotherapy protocol and could be reserved for rescue treatment.

Although the current study failed to demonstrate a difference in DFP or OS, a power calculation ($\alpha = 0.05$, power 80%) had shown that 120 dogs would be required to establish a 50% change in 1-year survival. An interim analysis with 80 dogs failed to demonstrate a difference and since it was considered unlikely that the accrual of 40 additional dogs would change this outcome, the study was discontinued.

In human oncology it has been demonstrated that resistance to doxorubicin, most likely due to its multifactorial mode of action, is the best predictor for classic MDR[36]. Given the authors' interest in MDR, its potential role in treatment failure in cL, as well as the role of GCs therein, it was chosen to predominantly use doxorubicin and prednisolone and limit the use of other cytostatic agents. The current results suggest that it is unlikely that GC, when used concurrently with cytostatic drugs, induce MDR in the dog, but further *in vitro* and in vivo studies are necessary to characterize MDR in cL and the role of GC therein.

As previously reported, weight, leukopenia, bone marrow involvement and hypercalcemia were strong predictors for duration of DFP and OS[6-9]. Although bone marrow involvement correlates with WHO-stage and hypercalcemia with immunophenotype, bone marrow involvement and hypercalcemia were stronger predictors than WHO-stage and immunophenotype. This study failed to confirm the previously reported variables age, sex, substage, anemia, thrombocytopenia and monocytosis[6-8, 37] as prognostic indicators. Since bone marrow involvement appeared to be a strong prognostic indicator, and less than half the stage V dogs had lymphoblasts in the peripheral blood, and presence or absence of cytopenias did not consistently correlate with bone marrow involvement, a bone marrow biopsy should be considered part of the routine staging protocol. This is in contrast to earlier recommendations by the VCOG-consensus group[34].

To the authors' knowledge only one study has looked into factors determining the likelihood of a second CR and duration of a second DFP[12] and identified both duration of first DFP and time off treatment as the only significant factors. The current study confirmed the effect of

duration of first DFP on the likelihood of obtaining a second CR, but failed to demonstrate an effect on duration of second DFP.

Based on the current results it is concluded that there is no proof to support the use of prednisolone in a first-line multidrug-protocol. Furthermore the study provides no indication that prednisolone use within a chemotherapy protocol contributes to the development of MDR.

Conflict of interest statement

The authors disclose no conflict of interest.

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5

A longitudinal study on ABC-transporter expression in canine multicentric lymphoma

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Abstract

Canine lymphoma is typically treated with a doxorubicin-based multidrug chemotherapy protocol and although initially successful, tumor recurrence is common and often refractory to treatment. Failure to respond to chemotherapy is thought to represent drug resistance and has been associated with active efflux of cytostatic drugs by transporter proteins of the ATP-Binding Cassette (ABC) family, including P-gp (ABCB1), MRP1 (ABCC1) and BCRP (ABCG2).

ABC-transporter mRNA expression was assessed in 63 dogs diagnosed with multicentric lymphoma that were treated with a doxorubicin-based chemotherapy protocol. Expression of *ABCB1, ABCB5, ABCB8, ABCC1, ABCC3, ABCC5*, and *ABCG2* mRNA was quantified in tumor samples (n = 107) obtained at the time of diagnosis, at first tumor relapse and when the tumor was no longer responsive to cytostatic drugs while receiving chemotherapy and related to patient demographics, staging, treatment response and drug resistance (absent, intrinsic, acquired).

ABC-transporter expression was independent of sex, weight, age, stage or substage, but T-cell lymphoma and hypercalcemia were associated with increased *ABCB5, ABCC5*, and decreased *ABCC1* expression. Drug resistance occurred in 35 of 63 dogs and was associated with increased *ABCB1* expression in a subset of dogs with B-cell lymphoma and in T-cell lymphomas with increased *ABCG2* and decreased *ABCB8, ABCC1*, and *ABCC3* expression. ABC-transporter expression in the pre-treatment sample was not predictive for the length of the first disease-free period or overall survival. Glucocorticoid use had no effect on ABC-transporter expression.

Drug resistance in canine multicentric lymphoma is an important cause for treatment failure and associated with *ABCB1* and *ABCG2* overexpression.

Keywords: ABC-transporters; dog; multidrug resistance; non-Hodgkin lymphoma; P-glycoprotein

Abbreviations

cML: canine multicentric lymphoma, CR: complete response, DFP: disease-free period, GC(s): glucocorticoid(s), DR: drug resistance, OST: overall survival time, PD: progressive disease, PFS: progression-free survival, P-gp: P-glycoprotein, PR: partial response, SD: stable disease.

Introduction

Canine multicentric lymphoma (cML), although in essence not a single disease but rather a collection of histologically distinct subtypes of lymphoid neoplasia[1], is the most common hematopoietic malignancy in the dog. Canine lymphoma is comparable to non-Hodgkin lymphoma in humans[2] and routinely treated with a doxorubicin-based multidrug chemotherapy protocol[3, 4]. Despite a high initial response rate, tumor recurrence (relapse) is common and associated with a less favorable treatment response thought to result from drug resistance (DR).

Resistance to anticancer drugs is associated with multiple genetic and epigenetic changes, but active efflux of drugs by transporter proteins of the ATP-Binding Cassette (ABC) superfamily is an important mechanism[5]. In humans, expression of P-glycoprotein (P-gp/ABCB1), multidrug resistance related protein 1 (MRP1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2) in tumor cells is associated with decreased sensitivity to cytotoxic agents *in vitro*, the clinical phenotype of DR and poor treatment outcome in multiple types of cancer[6]. Recently other ABC-transporters including ABCB5[7], ABCB8[8], ABCC3[9] and ABCC5[10, 11]) have also been implicated in human DR. In canine neoplasia P-gp[12-18] , MRP1[13, 14, 19] and BCRP[14, 20] have been studied, but there are no data on ABCB5, -B8, -C3 and -C5 expression.

Several studies in dogs with cML have documented that glucocorticoid use prior to starting cytotoxic chemotherapy has a negative effect on treatment outcome[21-24]. This finding was explained by assuming glucocorticoid-induced ABC-transporter expression, and although dexamethasone is able to induce P-gp expression in human hepatocytes, enterocytes[25] and lymphocytes[26], there are no data that support this hypothesis in the dog[27].

The goal of the current study was to measure ABC-transporter mRNA-expression in cML throughout disease progression and correlate this to DR, prognosis, and glucocorticoid use.

Materials and methods

Dog population

Tumor samples were obtained from privately owned dogs presented to the University Clinic for Companion Animals, Faculty of Veterinary Medicine, Utrecht University for treatment of cML. Dogs in this study represent a subset of dogs previously reported[28] and included all high-grade lymphoma cases for which a pre-treatment and, when applicable, at least one follow-up sample was available for PCR analysis.

Breed, sex, age and weight at time of diagnosis were recorded for each dog. The WHO-staging system for cL[29] was used to determine stage and substage. The minimum diagnostic dataset obtained for each dog included a hematological and clinical chemistry profile, a cytologic bone marrow aspirate, cytological examination of a lymph node aspirate and immunocytochemistry (CD79a and CD3 (Dako)). The study was evaluated and approved by the Research Scientific and Ethical Committee at Utrecht University and prior to entering the study an informed consent was obtained from all owners.

Treatment

None of the 63 dogs that entered the study had received any form of prior treatment for cML and they were all treated with the same doxorubicin-based chemotherapy protocol, with half of them being randomised to receive additional treatment with prednisolone resulting in 31 dogs that received prednisolone and 32 dogs that did not (Table 1). Following relapse, rescue treatment was offered, but not standardized and included treatment with doxorubicin, high-dose COP, mitoxantrone, L-asparaginase, CCNU, and prednisolone.

Response criteria

Treatment response was based on physical examination and measurement of the peripheral lymph nodes (palpation). Relapse was confirmed by cytological examination of a lymph node aspirate. Complete response (CR), partial response, stable disease, progressive disease, disease-free period (DFP), progression-free survival (PFS), and overall survival time (OST) were defined as proposed in the VCOG consensus document[30]. All deaths during the study were considered disease- or treatment-related, unless a clear cause of death was established and not lymphoma- or chemotherapy-related.

All dogs alive at the end of the study were censored at the time of analysis. Intrinsic DR was defined as failure to obtain a first CR while under treatment with chemotherapy and acquired DR was defined as failure to re-induce CR while receiving treatment with any type of cytostatic agent (excluding glucocorticoids) following a previous CR.

Collection of tumour samples

Tumour samples were obtained from a neoplastic lymph node through fine needle aspiration (FNA) and collected into an RNAse-free container that was frozen in liquid nitrogen and stored at -70 °C until further processing. Samples were collected prior to receiving chemotherapy (sample 1), at first tumour relapse (sample 2) and when cML was no longer responsive to cytostatic drug therapy following more than two CRs (sample 3). In total one hundred and seven (n = 107) tumour samples were collected, including 63 pre-treatment samples (sample 1), 32 first relapse samples (sample 2) and 12 end of treatment samples (sample 3) (supplementary Fig. 1).

RNA isolation and cDNA synthesis

Total RNA was isolated using the SV-total RNA isolation kit (Promega) according to the manufacturer's protocol including a DNAse treatment and eluted in milli-Q water. The amount of RNA was quantified spectrophotometrically at 260 nm (ND-1000, Nanodrop technologies). Purity of the samples was assessed by absorbance at 230 and 280 nm, and samples with A260/230 >1.8 and A260/280 >2 were included in the study. First strand cDNA was synthesized from 1 µg total RNA using the iScript cDNA Synthesis kit (Bio-Rad) containing both oligo (dT) and random hexamer primers in a final volume of 20 µL. cDNA was stored at -20°C until analysis.

Table 1. Chemotherapy and prednisolone dose protocol.

Day	1	8	22	33	43	57	60	71	80	92	113	127-148
L-asparaginase (10.000 IU/m^2 IM)	◆											
Doxorubicin (30 mg/m^2 IV)		◆	◆		◆			◆		◆		
Chlorambucil (25 mg/m^2 PO over 2 days)				◆					◆			
Vincristine (0.5-0.7 mg/m^2 IV)						◆						
Cyclophosphamide (200-250mg/m^2 PO)							◆				◆	
Chlorambucil (2 mg/m^2 1dd PO)												◆

Day	8	15	22	29	43	57	71	85	99	113
Prednisolone (mg/m^2 once daily)	50	40	30	25	20	15	10	5	5 EOD	Stop

(EOD, every other day; IM, intramuscular; IV, intravenous; PO, per os)

Table 2. Oligonucleotide primers (5′→3′).

Gene	GenBank	Forward primer	Reverse primer	T_a (°C)
ABCB1	NM_001003215	CTATGCCAAAGCCAAAGTATC	GAGGGCTGTAGCTGTCAATC	57.5
ABCB5	XM_539461	TTGGTAGGAGAAAAAGGAGCAC	CTAAGATCAGGATTTTTGGTTTTC	59.5
ABCB8	XM_539916	CCTGCTTGAGCGCTTCTATGA	GACTGGCTCCTGGCTGATGA	61.0
ABCC1	NM_001002971	CGTGACCGTCGACAAGAACA	CACGATGCTGCTGATGACCA	60.9
ABCC3	DQ225112	CATCCTGGCGATCTACTTCC	CAGGATCTCACTCATCAGCTTG	62.0
ABCC5	NM_001128100	TGTACGCCTGCCATTCC	CAGCGATCATTCCAACACA	57.0
ABCG2	NM_001048021	GGTATCCATAGCAACTCTCCTCA	GCAAAGCCGCATAACCAT	60.0
B2M	AB745507	TCCTCATCCTCCTCGCT	TTCTCTGCTGGGTGTCG	61.2
GAPDH	NM_001003142	TGTCCCCACCCCCAATGTATC	CTCCGATGCCTGCTTCACTACCTT	58.0
SDHA	DQ402985	GCCTTGGATCTCTTGATGGA	TTCTTGGCTCTTATGCGATG	61.0
TBP	XM_849432	CTATTTCTTGGTGTGCATGAGG	CCTCGGCATTCAGTCTTTTC	57.0
YWAZ	XM_843951	CGAAGTTGCTGCTGGTGA	TTGCATTTCCTTTTTGCTGA	58.0

T_a = annealing temperature

Quantitative real-time PCR

Intron spanning forward and reverse primers were designed for canine *ABCB1, ABCB5, ABCB8, ABCC1, ABCC3, ABCC5* and *ABCG2* (Table 2) and tested for efficiency by using a dilution series of cDNA. The efficiency of the primers was between 95-105% and based on melting curve analysis only one PCR product was generated.

Quantitative real-time PCR was performed in duplicate using 50 ng cDNA, 7.5 pmol of each gene-specific primer and IQSybrGreen Supermix (Bio-Rad) in a final 25µL reaction volume was analyzed by quantitative PCR in a MyIQ single color real time PCR detection system (Bio-Rad). Following an initial hot start (95°C) for 3 min, 40 cycles were run with a denaturation step at 95°C for 20 s, an annealing step at the specific annealing temperature for 30 s and an elongation step at 72°C for 30 s.

The reference genes *GAPDH, TBP, YWAZ, SDHA* and *B2M* were selected based on a previous report on canine reference genes[31] and primers were tested for efficiency and specificity. RNA transcription of target genes was normalized against the geometric mean of the five reference genes. Gene expression analysis was performed using the *ΔΔ*CT method[32].

Statistical methods

All parameters were described as frequencies or median with range. Differences between groups were tested for significance with the χ^2 or Fisher's exact test and Kruskal-Wallis test or unpaired Student's *t*-test after having tested for normality. The Kaplan-Meier product limit method was used for estimating DFP, PFS and OST. Differences in gene expression between groups were calculated using REST 2009 software[33, 34]. Cluster analysis, using Pearson's correlation coefficient for genes and Spearman rank for samples, was performed using statistical package R version 2.15.3 (http://www.r-project.org). All other statistical analyses were performed using the software package IBM SPSS Statistics Version 22. The level of significance was set at $P < 0.05$.

Results

Clinical data

Sixty-three dogs were enrolled in the study including seven mixed-breed dogs and 56 purebred dogs representing 30 different breeds. Patient characteristics are summarized in Table 3. Prednisolone was included in the initial treatment protocol in 31/63 (49.2%) dogs. The initial treatment failed to induce a CR in 10 dogs; 27 dogs had a CR and relapsed within 150 days; 18 dogs had a CR and relapsed after more than 150 days and eight dogs had no relapse for the duration of the study (median follow-up 463 days; range 49-1720 days). CR following initial treatment was 53/63 (84%) and resulted in a first median DFP of 185 days (95% confidence interval, CI, 157-213 days) and median PFS of 176 days (95% CI 154-198 days). The 10 dogs with intrinsic DR were all treated with glucocorticoids and five received additional rescue chemotherapy, but none obtained a CR.

Relapse occurred in 45/53 (84.9%) dogs that had an initial CR and treatment was continued in 43/45 (95.6%) with monotherapy using glucocorticoids ($n = 9$) or chemotherapy ($n = 34$). Rescue chemotherapy had a second CR rate of 18/34 (53%) and a median second DFP of 122 days (95% CI 105-139). Rescue therapy resulted in a lower CR rate (53% vs 84%; $P = 0.024$) and shorter median DFP (185 vs 122 days; $P = 0.016$) than the initial treatment.

For analysis of OST, 10 dogs were censored (three were alive without relapse, seven died in CR from cML-unrelated causes), resulting in a median OST of 273 days (95% CI 215-331 days).

Drug resistance

Thirty-five of the 107 samples were designated as DR (10 intrinsic, 25 acquired). Intrinsic DR was more frequent amongst T cell (6/15) than B cell lymphomas (4/40; $P = 0.010$) (Supplemental Fig. 1).

Table 3. Clinical data of dogs ($n = 63$) included in the study. Continuous variables are reported as median (range), discrete variables as absolute (n) and relative (%) values.

Variable		
Dogs	Mixed breed ($n = 7$), German shepherd ($n = 5$), Bernese mountain dog ($n = 4$), Boxer ($n = 4$), English Cocker Spaniel ($n = 4$), Rottweiler ($n = 4$), Bordeaux dog ($n = 3$), Flatcoated retriever ($n = 3$), Golden retriever ($n = 3$), Border collie ($n = 2$), Bullmastiff ($n = 2$), Great Dane ($n = 2$), Tibetan terrier ($n = 2$)	
Age (years)	6.6 (1.5 - 14.0)	
Weight (kg)	36.0 (7 - 82)	
Sex (M : F)	38 : 25 (60 : 40)	
WHO-stage		
I	1 (2)	
II	0 (0)	
III	21 (33)	
IV	18 (29)	
V	23 (37)	
Substage	a = 48 (76)	b = 15 (24)
Immunophenotype*	B = 40 (73)	T = 15 (27)
Hypercalcemia	7 (11)	
Neoplastic cells in		
Peripheral blood	12 (19)	
Bone marrow	23 (37)	
Prednisolone included	yes = 31	no = 32
Treatment outcome		
Treatment response	First ($n = 63$)	Rescue ($n = 34$)
Progressive disease	3 (5)	5 (15)
Stable disease	5 (8)	4 (12)
Partial response	2 (3)	7 (21)
Complete response	53 (84)	18 (53)
Disease-free period (days)	185 (157 - 213)¶	122 (105 - 139)¶
Overall survival time (days)		273 (215 - 331)¶

* In eight cases not performed or inconclusive, ¶ 95% Confidence interval, WHO, World Health Organization.

ABC-transporter mRNA expression

Expression of ABC-transporter mRNA was detected for all seven ABC-transporters in all 107 samples, except for *ABCB5*-expression that was detected in 69/107 (64%) samples and was typically low (mean Ct = 32.3, median Ct = 32.9).

ABC-transporters expression and patient characteristics

ABC-transporters expression in pre-treatment samples (sample 1) was independent of sex, age, weight, WHO stage or substage. T cell immunophenotype and hypercalcaemia were associated respectively with a 5.9-fold ($P = 0.021$) and 11.3-fold ($P = 0.022$) higher *ABCB5*, 3.7-fold ($P < 0.001$) and 9.2-fold ($P = 0.016$) higher *ABCC5*, and 0.3-fold ($P < 0.001$) and 0.4-

fold ($P < 0.001$) lower *ABCC1* expression compared to B cell lymphomas and normocalcaemic dogs.

ABC-transporter expression and drug resistance

Comparing no DR ($n = 72$) to DR (intrinsic and acquired; $n = 35$) showed no significant differences in ABC-transporter expression, although *ABCB1* (1.7-fold higher; $P = 0.05$) and *ABCC1* (0.6-fold lower; $P = 0.05$) expression approached significance. No significant changes in ABC transporter expression were found between sampling times and categories of DR (Fig. 1).

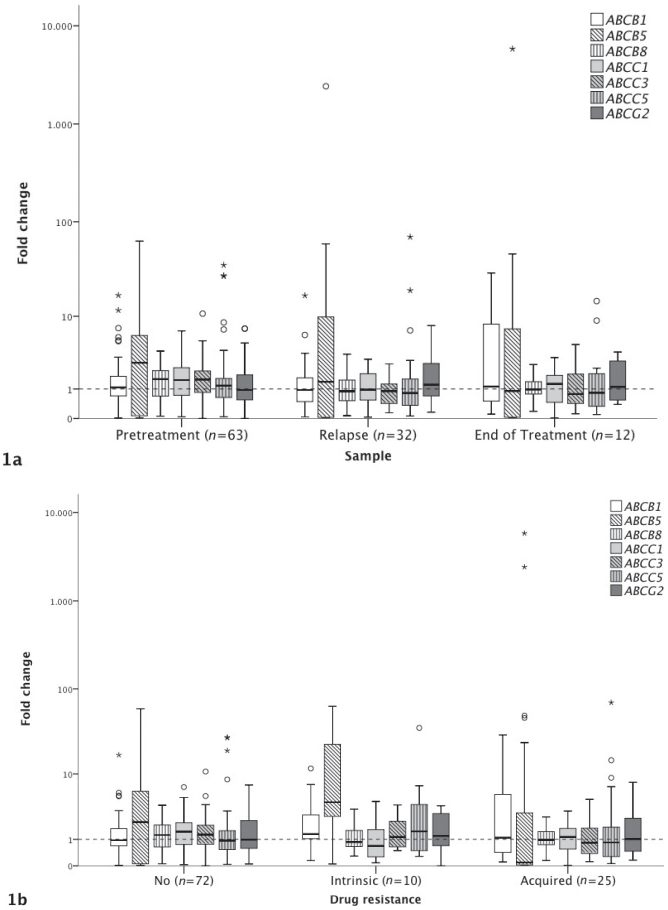
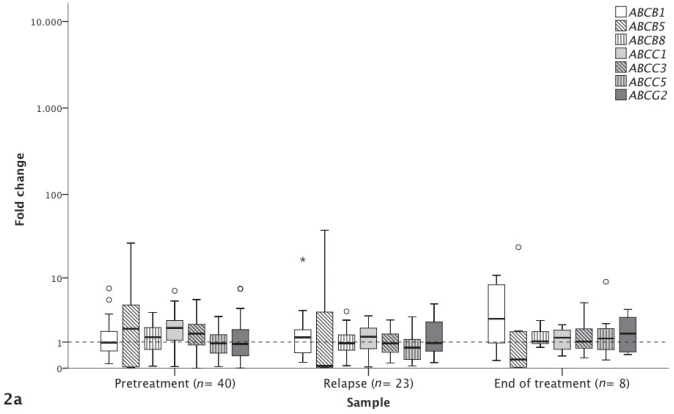


Figure 1. Box and whisker plots representing fold-changes in mRNA expression for the ABC transporters *ABCB1*, *ABCB5*, *ABCB8*, *ABCC1*, *ABCC3*, *ABCC5* and *ABCG2* in 63 dogs with multicentric lymphoma throughout disease progression (a) and categorised by drug resistance (b).

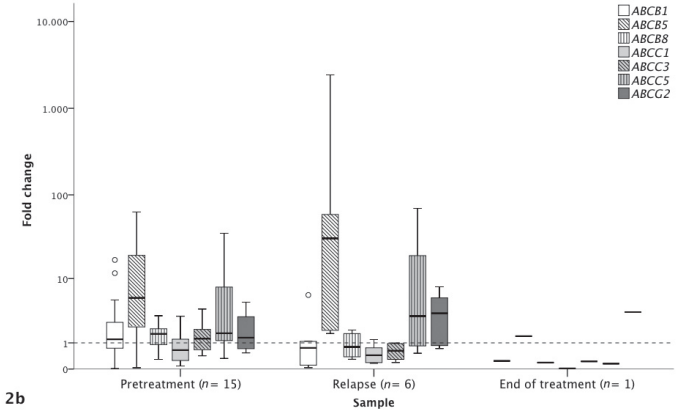
ABC-transporter expression, drug resistance and immunophenotype

B cell lymphomas had a 2.5-fold higher ($P = 0.02$) *ABCB1* expression in sample 3 compared to sample 1, as well as a 0.2-fold lower ($P = 0.03$) *ABCG2* expression in intrinsic DR compared to no DR (Figs. 2 and 3; supplementary Tables 2 and 3). For T cell lymphomas, sample 2 had a 0.4-fold ($P = 0.01$) lower *ABCC3* expression compared to sample 1, whilst acquired DR was

associated with a 3.4-fold higher ($P = 0.02$) *ABCG2*, a 0.3-fold lower ($P = 0.02$) ABCB8, a 0.2-fold lower ($P = 0.04$) *ABCC1* and a 0.3-fold lower ($P = 0.04$) *ABCC3* expression compared to no DR (Figs. 2 and 3; supplementary Tables 2 and 3). T cell lymphomas had a 11.3-fold higher ($P = 0.03$) *ABCG2* expression in intrinsic DR, as well as a 350-fold higher ($P < 0.001$) *ABCB5*, a 4.9-fold higher ($P = 0.009$) *ABCG2* and a 0.09-fold ($P = 0.001$) lower *ABCC1* expression in acquired DR compared to B cell lymphomas (Fig. 3; supplementary Tables 2 and 3).

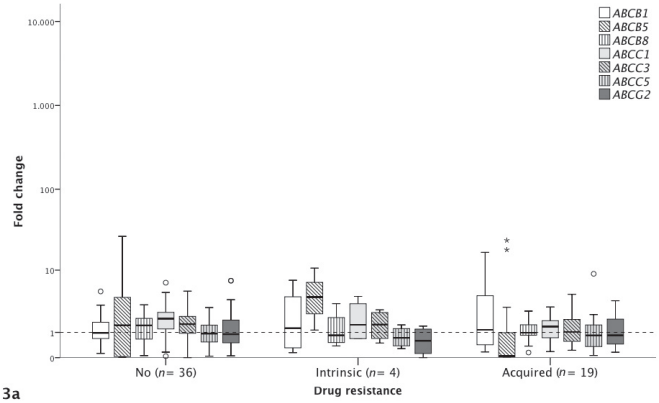


2a

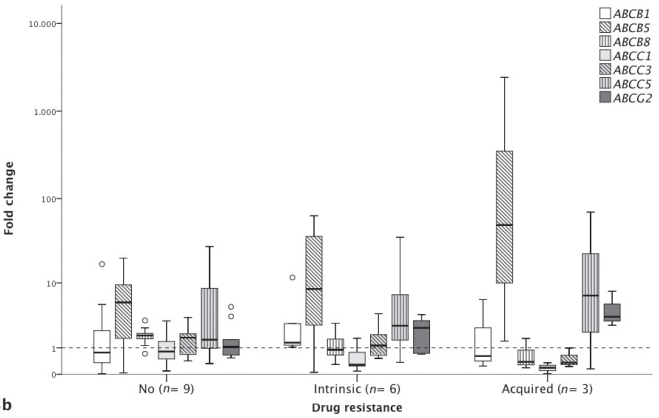


2b

Figure 2. Box and whisker plots representing fold-changes in mRNA expression for the ABC transporters *ABCB1*, *ABCB5*, *ABCB8*, *ABCC1*, *ABCC3*, *ABCC5* and *ABCG2* in 40 dogs with B cell (a) and 15 dogs with T cell lymphoma (b) throughout disease progression.



3a



3b

Figure 3. Box and whisker plots representing fold-changes in mRNA expression for the ABC transporters *ABCB1*, *ABCB5*, *ABCB8*, *ABCC1*, *ABCC3*, *ABCC5* and *ABCG2* in 40 dogs with B cell (a) and 15 dogs with T cell lymphoma (b) categorised by drug resistance.

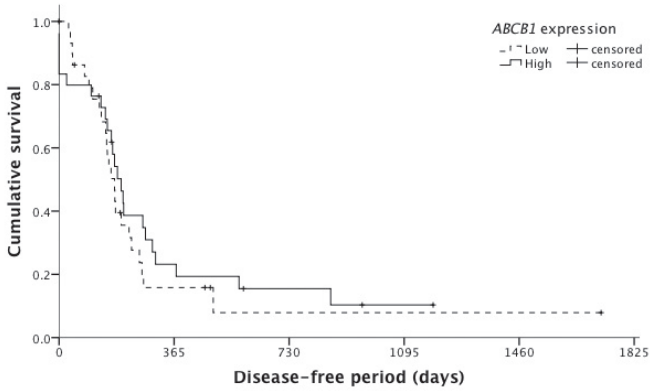


Figure 4. Kaplan-Meier survival curve for the progression-free survival in 63 dogs with multicentric lymphoma categorised by lower or higher than median *ABCB1* expression.

ABC-transporter expression and prognosis

Pre-treatment mRNA expression levels for all ABC transporters, including ABCB1, were not significantly correlated with length of the first DFP or OST (Fig. 4). ABC transporter expression in the relapse sample (sample 2) was not correlated with the length of the second DFP or PFS.

ABC-transporter expression and prednisolone use

The effect of prednisolone on ABC-transporter expression was analysed in those dogs that relapsed following an initial CR, during the initial chemotherapy protocol (sample 2 for all dogs with a DFP < 150 days). Sixteen cases (seven with and nine without prednisolone in the first protocol) fulfilled these criteria and no significant changes were found in ABC-transporter mRNA expression between these two groups.

Cluster analysis of ABC-transporter expression

Cluster analysis of ABC transporter gene expression in pre-treatment samples (sample 1) showed two ABC transporter clusters (*ABCB1/ABCB5/ABCC5, ABCB8/ABCC1/ABCC3/ABCG2*) and two main groups of samples (Fig. 5a). Intrinsic DR samples clustered in one main group, but not in either of the two subgroups. In follow-up samples (samples 2 and 3), the ABC transporters clusters were identical, but no clustering of DR samples was noted (Fig. 5b).

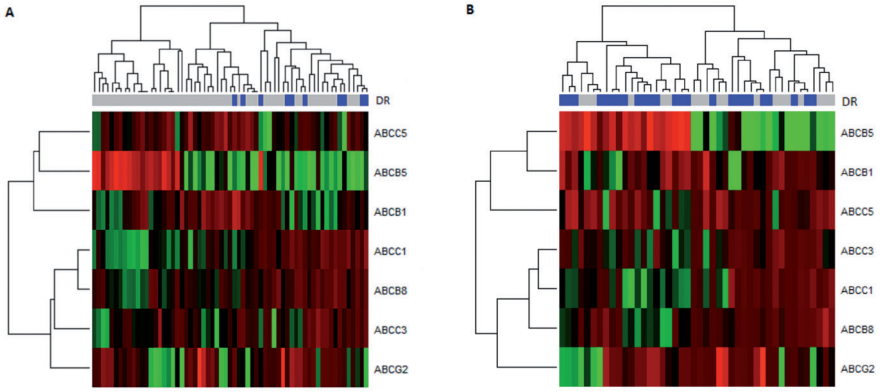


Figure 5. Heat map generated by unsupervised hierarchical clustering of the ABC transporter genes *ABCB1, ABCB5, ABCB8, ABCC1, ABCC3* and *ABCG2* for sample 1 (a, $n = 63$) and samples 2 and 3 (b, $n = 44$). Each column represents a single tumour sample; each row represents the expression of a specific ABC transporter (green, increased expression; red, decreased expression). The top row depicts the presence of drug resistance (present, blue; absent, grey).

Discussion

The current study is the first large-scale prospective study on ABC-transporter expression in cML and the first veterinary report on *ABCB5, -B8, -C3* and *-C5* expression. Although immunohistochemistry has been described for assessing *ABCB1*-transporter expression and DR in the dog[12, 15, 17] and remains the gold standard, it has two limitations: it requires a histological biopsy and specific antibodies that are for the non-classical ABC-transporters are either not available or not validated in the dog.

Quantitative real-time PCR is a very sensitive technique that requires a small sample size and is not limited by the availability of specific antibodies. Fine-needle aspiration of a lymph node is noninvasive, routinely performed in dogs with cML for diagnostic and prognostic purposes, and expression profiling on fine-needle aspirates from dogs with cML showed excellent correlation with the results obtained from tissue samples[35]. Interpreting quantative real-time PCR results can be challenging, because results are affected by the choice of reference genes and reference samples. Furthermore, the resulting protein expression and function is affected by a variety of other factors. Nevertheless, mRNA expression has been found predictive for CR-rate, DFP, OST and the risk of chemotherapy-related adverse events in a number of human neoplasias[36-39].

Cytology is an excellent technique for diagnosing cML, but has its limitations in diagnosing low-grade lymphomas, as well as in further subtyping lymphomas. Although the WHO-classification scheme recognizes many histologic entities, only 5 histological subtypes account for almost 95% of all canine lymphomas[1]. Since all cases in the current study were medium- to high-grade lymphomas (according to the Updated Kiel classification), it can be assumed that most cases were 'diffuse large B-cell lymphoma' and 'peripheral T-cell lymphoma not otherwise specified'.

In the current study DR, either innate or acquired, was the cause for treatment failure in at least 56% (35/63) of dogs and not associated with increased *ABCB1* expression (1.7 fold, *P*=0.051), but with decreased (0.59 fold) *ABCC1* expression (*P*= 0.049). Although an increased P-gp expression was linked to DR[12, 15, 17] , the current, as well as previous studies[40, 41], failed to associate increased *ABCB1* mRNA expression with clinical DR.

Acquired DR was associated with increased *ABCB1* expression in a subset of B-cell lymphomas and increased *ABCG2* expression in T-cell lymphomas. Both *ABCB1* and *ABCG2* are capable of transporting doxorubicin and play a role in cellular sensitivity to doxorubicin in humans[42]. However, *ABCG2*-mediated doxorubicin resistance in humans results from a mutated *ABCG2*[43], while in the dog in vitro doxorubicin resistance was observed with cloned wild type BCRP[14].

Hypercalcemia is typically associated with T-cell lymphomas, and both factors are associated with a more guarded prognosis[44-46]. The current study included 15 T-cell lymphomas of which seven were hypercalcemic, and both T-cell lymphoma and hypercalcemia were associated with increased *ABCB5* and *-C5* expression. Although *ABCC5* expression is associated with resistance to some nucleoside analog drugs[10, 11], these were not included in the current study protocol. However, *ABCB5* expression has been linked to doxorubicin resistance *in vitro*[47] and DR in human leukemias[7] and could explain a higher risk for treatment failure in T-cell lymphomas treated with a doxorubicin-based chemotherapy protocol.

For a number of ABC-transporters decreased expression levels were observed and this might be due to compensatory down-regulation of transporters with overlapping substrate spectrum, as was previously described for *ABCB1* and *ABCC1*[48] . An alternative explanation would be that these ABC-transporters do not play a role in canine DR and as a result there is no selection for increased expression.

High expression levels of *ABCB1, -B5, -C5* or *-G2* in cML samples were associated with DR, but failed to predict treatment response. In the current study pre-treatment ABC-transporter mRNA-expression was not predictive for treatment response, which is consistent with a recent prospective study on *ABCB1* expression in cML[49]. Relapsed lymphomas are often refractory to therapy, as was confirmed by the lower second CR-rate, but no significant correlations were found between ABC-transporter expression in the relapse sample (Sample 2) and second treatment response. The authors conclude that, besides ABC-transporters, other DR-mechanisms, including increased cell growth and proliferation, resistance to apoptosis and enhanced DNA-repair[5], must play a role in DR.

The use of prednisolone within a multidrug chemotherapy protocol had no effect on treatment outcome or ABC-transporter expression and it seems unlikely that prednisolone induces DR through ABC-transporter expression. This stands in contrast with the situation in humans[26, 50], but was previously documented in a canine lymphoid leukemia cell line[27]. Although the current study does not explain why pretreatment with glucocorticoids prior to starting chemotherapy leads a poorer prognosis, it is unlikely to result from glucocorticoid-induced ABC-transporter expression.

Cluster analysis confirmed two clusters of ABC-transporters (*ABCB1/ABCB5/ABCC5* and *ABCB8/ABCC1/ABCC3/ABCG2*) and clustering by sample showed the clearest correlation with *ABCB5* expression, but was not associated with DR. So far we have not found a biological explanation for the results of the cluster analysis.

Conclusions

DR in canine cML was associated with increased mRNA expression of *ABCB1* in a subset of B cell lymphomas and of *ABCG2* in T cell lymphomas. Glucocorticoids did not increase ABC transporter mRNA expression. Pre-treatment ABC transporter expression was not prognostic for treatment outcome in cML and other mechanisms leading to DR need to be evaluated.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Addendum. Supplemental data

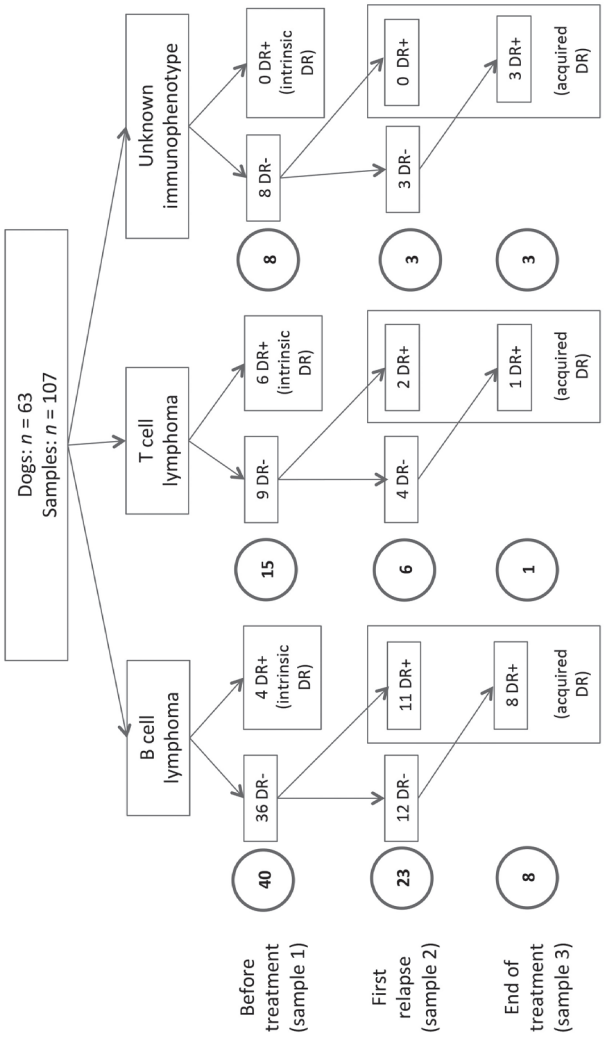

Figure 1. Flow-chart showing the distribution of samples per time point and immunophenotype (number in circles) and absence (-) or presence (+) of drug resistance (DR = drug resistance).

Table 1. Expression of ABC-transporter mRNA per sample (compared to Sample 1) or drug resistance category (compared to No drug resistance).

Transporter		Sample Time (compared to Sample 1)				Drug Resistance Category (compared to No drug resistance)					
		2	*P*	3	*P*	DR*	*P*	Intrinsic	*P*	Acquired	*P*
All (n=63)	*ABCB1*	0.95	0.84	1.82	0.16	**1.69**	**0.05**	1.79	0.19	1.55	0.19
	ABCB5	0.66	0.48	0.77	0.76	1.15	0.83	5.10	0.05	0.64	0.54
	ABCB8	0.69	0.06	0.77	0.30	0.74	0.10	0.79	0.43	0.73	0.11
	ABCC1	0.69	0.10	0.64	0.18	**0.59**	**0.05**	**0.48**	**0.04**	**0.62**	**0.05**
	ABCC3	0.64	0.06	0.71	0.35	0.80	0.40	1.05	0.89	0.71	0.26
	ABCC5	0.76	0.32	0.84	0.65	1.06	0.84	1.63	0.23	0.90	0.72
	ABCG2	1.41	0.16	1.44	0.32	1.02	0.95	0.76	0.53	1.14	0.58
B (n=40)	*ABCB1*	1.16	0.56	**2.49**	**0.02**	1.43	0.23	1.13	0.81	1.51	0.18
	ABCB5	0.39	0.16	0.40	0.35	0.51	0.31	7.00	0.16	0.29	0.09
	ABCB8	0.76	0.24	1.11	0.74	0.89	0.60	0.86	0.74	0.90	0.64
	ABCC1	0.64	0.07	0.69	0.27	0.75	0.21	0.97	0.94	0.71	0.16
	ABCC3	0.76	0.44	1.03	0.95	0.96	0.93	1.18	0.77	0.92	0.86
	ABCC5	0.71	0.17	1.53	0.21	0.90	0.70	0.83	0.68	0.92	0.78
	ABCG2	1.39	0.34	1.77	0.27	0.75	0.40	**0.19**	**0.03**	1.00	1.00
T (n=15)	*ABCB1*	0.52	0.50	0.26**	0.32	2.70	0.29	3.45	0.27	1.65	0.77
	ABCB5	6.94	0.11	0.38**	0.68	3.64	0.30	1.66	0.69	17.55	0.13
	ABCB8	0.58	0.09	**0.15****	**0.04**	**0.46**	**0.03**	0.57	0.08	**0.29**	**0.02**
	ABCC1	0.80	0.65	**0.03****	**0.01**	0.36	0.09	0.55	0.27	**0.16**	**0.04**
	ABCC3	**0.40**	**0.01**	**0.19****	**<0.01**	0.66	0.28	0.93	0.86	**0.33**	**0.04**
	ABCC5	1.42	0.67	0.06**	0.10	1.29	0.79	1.16	0.85	1.57	0.73
	ABCG2	1.80	0.16	2.61**	0.37	1.97	0.08	1.49	0.33	**3.43**	**0.02**

* Drug resistance (DR) represented the combined intrinsic and acquired drug resistance samples; ** included for completeness, but for T-cell lymphoma, sample 3 n=1

Table 2. Expression of ABC-transporter mRNA in T-cell lymphoma compared to B-cell lymphoma per sample.

Transporter	Sample 1	*P*	Sample 2	*P*	Sample 3*	*P*
ABCB1	1.03	0.93	0.46	0.17	0.11	0.12
ABCB5	**5.89**	**0.02**	**105.72**	**<0.001**	5.58	0.88
ABCB8	1.21	0.44	0.92	0.84	**0.16**	**0.04**
ABCC1	**0.34**	**<0.001**	0.43	0.06	**0.02**	**0.04**
ABCC3	1.18	0.71	0.62	0.16	0.21	0.18
ABCC5	**3.65**	**<0.001**	**7.28**	**<0.001**	0.13	0.15
ABCG2	1.97	0.08	**2.56**	**0.03**	2.89	0.30

* included for completeness, but for T-cell lymphoma, sample 3 n=1

Table 3. Expression of ABC-transporter mRNA in T-cell lymphoma compared to B-cell lymphoma per drug resistance category.

Transporter	no DR	P	DR*	P	Intrinsic	P	Acquired	P
ABCB1	0.64	0.34	1.20	0.73	1.95	0.44	0.70	0.69
ABCB5	5.85	0.06	**41.80**	**<0.01**	1.39	0.84	**349.75**	**< 0.01**
ABCB8	1.48	0.18	0.76	0.37	0.99	0.96	0.48	0.10
ABCC1	**0.43**	**0.02**	**0.21**	**<0.001**	0.24	0.07	**0.09**	**<0.001**
ABCC3	1.23	0.67	0.84	0.58	0.97	0.93	0.44	0.13
ABCC5	**3.37**	**<0.01**	**4.81**	**0.01**	4.73	0.12	5.77	0.07
ABCG2	1.42	0.37	**3.73**	**0.02**	**11.34**	**0.03**	**4.85**	**0.01**

* Drug resistance (DR) represented the combined intrinsic and acquired drug resistance samples

6

Activation of the Wnt-pathway in canine multicentric lymphoma

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In preparation

Abstract

Background – Multicentric lymphoma is the most common canine hematopoietic neoplasia and typically treated with a doxorubicin-based multidrug chemotherapy protocol. Although initially successful, tumor recurrence is common and often refractory to treatment. Failure to respond to chemotherapy results from drug resistance and has been associated with increased expression the ABC-transporters ABCB1 and ABCG2, but other factors, including activation of the Wnt-pathway, might also play a role.

Materials and methods - mRNA expression of *Axin2, LEF1, Survivin, Cyclin-D1,* and *BCL9* was measured in 55 dogs diagnosed with multicentric lymphoma. Tumor samples (n = 96) were taken prior (n = 55) and during (n = 41) treatment with a doxorubicin-based chemotherapy protocol and related to patient demographics, staging, treatment response and clinical drug resistance.

Results – mRNA expression of *Axin2, LEF1, Survivin, Cyclin-D1*, and *BCL9* was detected in almost all tumor samples and demonstrated increased *Axin2* and *LEF1* expression in T-cell lymphomas that support activation of the Wnt-pathway in this subgroup. Age, sex, WHO-stage, substage, treatment response and drug resistance could not be associated with activation of the Wnt-pathway.

Conclusions – Activation of the Wnt-pathway appears to play a role in T-cell lymphoma and given the poorer prognosis of this subgroup with chemotherapy, further investigations into targeted therapeutic interventions in this pathway are warranted.

Keywords non-Hodgkin lymphoma, *LEF1, Axin2, BCL9, Survivin*

Abbreviations

cML: canine multicentric lymphoma, CR: complete response, DFP: disease-free period, DR: drug resistance, OST: overall survival time, PD: progressive disease, PFS: progression-free survival, PR: partial response, SD: stable disease.

Introduction

The Wnt-pathway is a well-conserved signaling pathway in animals and plays a crucial role in the embryonic development and stem cell maintenance in the intestine and skin as well as hematopoiesis[1]. Dysregulation of this pathway has been reported in human[1, 2] and canine neoplasia[3-7].

Following the binding of Wnt ligands to Frizzled and low-density lipoprotein receptors (LRP5 and LRP6), the Wnt-pathway is activated through inhibition of the APC (Adenomatous Polyposis Coli)/GSK3β (Glycogen Synthase Kinase-3)/Axin-destruction complex. This inhibition increases cytoplasmic levels of β-catenin that can now translocate to the cell nucleus, where together with the T-cell factor/lymphoid enhancer binding factor/(*TCF/LEF*) and coactivator B cell lymphoma[9] (*BCL9*)[8], β-catenin increases transcription of a number of genes, including *c-myc, Axin2/conductin 9, Cyclin-D1,* and *Survivin/BIRC5*[10], which result in enhanced cell proliferation, survival, migration and invasion.

Canine multicentric lymphoma (cML), the most common hematopoietic neoplasia in the dog, is comparable to non-Hodgkin lymphoma in humans[11] and routinely treated with a doxorubicin-based multidrug chemotherapy protocol[12, 13]. Upregulation of the Wnt-pathway has been described in a number of human hematopioetic malignancies including acute lymphoblastic leukemia[14], mantle cell lymphoma[15] and non-Hodgkin lymphoma (Diffuse Large B-cell Lymphoma)[16]. Furthermore activation of the Wnt pathway has been associated with drug resistance in lymphoid neoplasia[17, 18]. Due to the absence of information on the activation of the Wnt-pathway in canine lymphoma or a possible relation to drug resistance, mRNA expression of *Axin2, LEF1, Cyclin-D1, Survivin* and *BCL9* were measured in tumor samples from dogs diagnosed with cML.

Materials and Methods

Patient population

Tumor samples were obtained from privately owned dogs presented to the University Clinic for Companion Animals, Faculty of Veterinary Medicine, Utrecht University for treatment of cML. Dogs in this study represent a subset of dogs previously reported[19] and were included if a pre-treatment and, when applicable, a minimum of 1 follow-up sample was available for PCR analysis.

Breed, sex, age, and weight at time of diagnosis were recorded for each dog. The WHO-staging system for cL[20] was used to determine stage and substage. A minimum diagnostic dataset obtained for each dog included a hematological and clinical chemistry profile, bone marrow aspiration biopsy, cytological examination of a lymph node aspirate and immunophenotyping using CD79a and CD3 (Dako, Glostrup. Denmark).

The study was evaluated and approved by the Research Scientific and Ethical Committee at Utrecht University and prior to entering the study an informed consent was obtained from all owners.

Treatment

The dogs had not been treated for cML prior to entering the study and received the same doxorubicin-based chemotherapy protocol[19]. Following relapse, rescue treatment was offered, but not standardized.

Response criteria

Treatment response was based on physical examination and measurement of the peripheral lymph nodes (palpation). Relapse was confirmed by cytological examination of a lymph node aspirate. Complete response (CR), partial response, stable disease, progressive disease, disease-free period (DFP), progression-free survival (PFS), and overall survival time (OST) were defined as proposed in the VCOG consensus document[21]. All deaths during the study were considered disease- or treatment-related unless a clear cause of death was established and not lymphoma- or chemotherapy-related. All dogs alive at the end of the study were censored at the time of analysis.

Tumor sample collection

Tumor samples were obtained from a neoplastic lymph node through fine-needle aspiration and collected into a RNAse-free container that was snap-frozen in liquid nitrogen and stored at -70°C until further processing. Samples were collected prior to receiving chemotherapy (sample 1), at first tumor relapse (sample 2), and when cML was no longer responsive to cytostatic drug therapy following more than 2 CRs (sample 3).

RNA isolation and cDNA synthesis

Total RNA was isolated using the SV-total RNA isolation kit (Promega, Leiden, the Netherlands) according to the manufacturer's protocol including a DNAse treatment and eluted in milli-Q water. The amount of RNA was quantified spectrophotometrically at 260 nm (ND-1000, Nanodrop technologies, Wilmington, DE, USA). Purity of the samples was assessed by absorbance at 230 and 280 nm, and samples with A260/230 >1.8 and A260/280 >2 were included in the study. First strand cDNA was synthesized from 1 µg total RNA using the iScript™ cDNA Synthesis kit (Bio-Rad. Hercules. CA. USA) containing both oligo (dT) and random hexamer primers in a final volume of 20 µL. cDNA was stored at -20°C until analysis.

Quantitative real-time PCR

Intron spanning forward and reverse primers were designed for canine *Axin2, LEF1, Cyclin-D1, Survivin* and *BCL9* (Table 1) and tested for efficiency by using a dilution series of cDNA. The efficiency of the primers was between 95-105% and based on melting curve analysis only one PCR product was formed.

Quantitative real-time PCR was performed in duplicate using 50 ng cDNA, 7.5 pmol of each gene-specific primer, and IQ™ SybrGreen Supermix (Bio-Rad. Hercules. CA. USA) in a final 25µL reaction volume was analyzed by quantitative PCR in a MyIQ single color real time PCR detection system (Bio-Rad, Hercules, CA, USA). Following an initial hot start (95°C) for 3 minutes, 40 cycles were run with a denaturation step at 95°C for 20 seconds, an annealing

Table 1. Primer details.

Gene	GenBank	Forward primer	Reverse primer	Ta (°C)
LEF1	XP_863334.2	AGACATCCTCCAGCTCCTGA	GATGGATAGGGTTGCCTGAA	60.0
AXIN2	XM_548025	GGACAAATGCGTGGATACCT	TGCTTGGAGACAATGCTGTT	60.0
SURVIVIN	AY741504	CCTGGCAGCTCTACCTCAAG	TCAGTGGGACAGTGGATGAA	58.0
CYCLIN D1	NM_001005757.1	GCCTCGAAGATGAAGGAGAC	CAGTTTGTTCACCAGGAGCA	60.0
BCL9	XM_005630706	AAGGGGAAAAGGGAGCGAAGTATT	CATGGGAGGGGGTGGAAGA	62.0
B2M	AB745507	TCCTCATCCTCCTCGCT	TTCTCTGCTGGGTGTCG	61.2
GAPDH	NM_001003142	TGTCCCCACCCCCAATGTATC	CTCCGATGCCTGCTTCACTACCTT	58.0
SADH	DQ402985	GCCTTGGATCTCTTGATGGA	TTCTTGGCTCTTATGCGATG	61.0
TBP	XM_849432	CTATTTCTTGGTGTGCATGAGG	CCTCGGCATTCAGTCTTTTC	57.0
YWAZ	XM_843951	CGAAGTTGCTGCTGGTGA	TTGCATTTCCTTTTTGCTGA	58.0

T_a = annealing temperature

step at the specific annealing temperature for 30 seconds, and an elongation step at 72°C for 30 seconds.

The reference genes *GAPDH, TBP, YWAZ, SADH*, and *B2M* were selected based on a previous report on canine reference genes[22] and primers were tested for efficiency and specificity. RNA transcription of target genes was normalized against the geometric mean of the five reference genes. Gene expression analysis was performed using the ΔΔCT method[23].

Statistical methods

All parameters were described as frequencies (categorical variables) or median with range (continuous variables). Differences between groups were tested for significance with the chi-square or Fisher's exact test for categorical variables and either Kruskal-Wallis test or unpaired Student's *t*-test for continuous variables after having tested for normality. Correlation was analyzed using either Pearson's or Spearman's rho correlation coefficient. The Kaplan-Meier product limit method was used for calculating DFP, and OST.

Differences in gene expression between groups were calculated using REST 2009 software[24, 25].

Statistical analyses were performed using the software package IBM SPSS Statistics Version 22. The level of significance was set at $P < 0.05$.

Results

Clinical data

Fifty-five dogs were enrolled in the current study including seven mixed-breed dogs and 48 purebred dogs representing 26 different breeds. The most common breeds included the German shepherd ($n = 5$), Boxer, English Cocker Spaniel, Rottweiler (n=4 each), Bernese mountain dog, Flatcoated retriever, Golden retriever (n=3 each), Bordeaux dog, Bullmastiff and the Great Dane (n=2 each). Further patient characteristics are summarized in Table 2.

Table 2. Clinical data of dogs (*n*=55) included in the study. Continuous variables are reported as median (range), discrete variables as absolute (*n*) and relative (%) values.

Variable		
Age (years)	6.6 (1.5 - 14.0)	
Weight (kg)	36.0 (7 - 82)	
Sex (M : F)	38 : 25 (60 : 40)	
WHO-stage		
I	1 (2)	
II	0 (0)	
III	21 (33)	
IV	18 (29)	
V	23 (37)	
Substage	a = 42 (76)	b = 13 (24)
Immunophenotype*	B = 40 (73)	T = 15 (27)
Hypercalcemia	6 (11)	
Lymphoblasts in		
Peripheral blood	9 (16)	
Bone marrow	19 (35)	
Prednisolone included	yes = 28	no = 27
Treatment outcome		
Complete response	45 (82)	
Disease-free period	176 (149 - 203)*	
Overall survival time (days)	240 (152 - 328)*	

* 95%-confidence interval

Table 3. Pearson correlation coefficient (r) for AXIN2, LEF1, SURVIVIN, CYCLIN D1, and BCL9 mRNA expression in tumor samples from 55 dogs with multicentric lymphoma.

		LEF1 (n=96)	**SURVIVIN (n=96)**	**CYCLIN D1 (n=96)**	**BCL9 (n=83)**
AXIN2	*r*	0.026	-0.024	0.084	-0.067
	P	0.80	0.82	0.42	0.55
LEF1	*r*		0.34	0.040	0.45
	P		0.001	0.7	<0.001
SURVIVIN	*r*			0.15	0.17
	P			0.15	0.13
CYCLIN D1	*r*				-0.088
	P				0.43

LEF1, Axin2, Cyclin-D1, Survivin and BCL9 mRNA expression

LEF1, Axin2, Cyclin-D1, and *Survivin* mRNA expression was detected in all 96 samples, while *BCL9* was detected in 83 (86%) samples. Significant correlations between mRNA expression were found for *LEF1* and *Survivin* ($r = 0.34$, $P = 0.001$) and *LEF1* and *BCL9* ($r = 0.45$, $P < 0.001$) (Table 3).

Expression of *Axin2, LEF1, Cyclin-D1, Survivin*, and *BCL9* in the pretreatment sample (sample 1) was independent of sex, age, WHO-stage, and substage and only a mild correlation ($r = -0.28$, $P = 0.039$) was observed between *Axin2* expression and body weight (Tables 4 and 5).

Table 4. Pearson (r) correlation coefficient for *AXIN2, LEF1, SURVIVIN, CYCLIN D1*, and *BCL9* mRNA expression and patient characteristics (body weight, age) and treatment response (disease-free period, overall survival time) in 55 dogs with multicentric lymphoma.

		AXIN2 (n = 55)	LEF1 (n = 55)	SURVIVIN (n = 55)	CYCLIN D1 (n = 55)	BCL9 (n = 52)
Body weight	*r*	-0.28	0.15	-0.19	0.11	-0.17
	P	0.039	0.27	0.17	0.44	0.22
Age	*r*	-0.20	-0.12	-0.19	-0.057	0.14
	P	0.15	0.39	0.17	0.68	0.34
DFP	*r*	0.17	-0.11	0.61	-0.008	-0.17
	P	0.22	0.42	<0.001	0.95	0.24
Overall survival time	*r*	0.22	-0.14	0.54	-0.047	-0.15
	P	0.11	0.30	<0.001	0.73	0.30

Table 5. Fold changes in *AXIN2, LEF1, SURVIVIN, CYCLIN D1*, and *BCL9* mRNA expression for sex, immunophenotype, WHO-stage, -substage and drug resistance in 55 dogs with multicentric lymphoma.

			AXIN2	LEF 1	SURVIVIN	CYCLIN D1	BCL9
Sex	F vs M (*n* = 18 vs 37)	FC	1.22	0.72	0.92	0.91	0.78
		P	0.69	0.37	0.57	0.66	0.60
WHO-stage	leukemic vs non-leukemic (*n* = 19 vs 36)	FC	0.86	1.03	1.03	1.27	1.04
		P	0.77	0.93	0.88	0.25	0.94
Substage	b vs a (*n* = 12 vs 43)	FC	1.02	0.99	1.35	1.12	0.61
		P	0.97	0.98	0.066	0.60	0.32
Immuno-phenotype	T vs B (*n* = 15 vs 40)	FC	18.53	4.12	1.06	1.27	1.60
		P	<0.0001	<0.0001	0.71	0.27	0.33
Drug resistance	yes vs no (*n* = 35 vs 61)	FC	0.88	0.95	0.88	1.06	0.48
		P	0.74	0.85	0.26	0.72	0.049
	acquired vs intrinsic (*n* = 22 vs 13)	FC	0.26	0.29	0.59	0.54	0.29
		P	0.064	0.012	0.001	0.016	0.082

The T-cell immunophenotype was associated with a higher *LEF1* (18.5 fold. $P < 0.0001$) and *Axin2* (4.12 fold $P < 0.0001$) expression compared to B-cell lymphomas (Table 5, Fig 1). High (>2x median) *Axin2* and *LEF1* expression was significantly more common in T- (8/15 and 13/15) than in B-cell lymphomas (3/30 and 4/40) ($P = 0.0002$ and $P < 0.0001$).

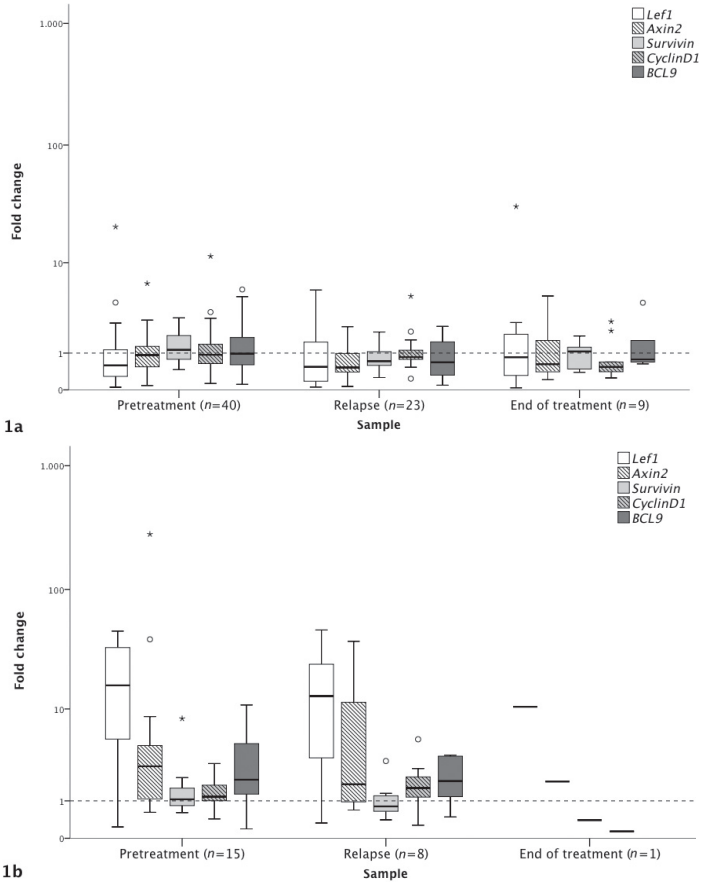

Figure 1. Box-whisker plot representing fold-changes in mRNA expression for *LEF1, AXIN2, SURVIVIN, CYCLIN D1*, and *BCL9* in 40 dogs with B-cell (top) and 15 dogs with T-cell lymphoma (bottom) throughout disease progression.

Pretreatment expression of *AXIN2, LEF1, CYCLIN D1*, and *BCL9* was not significantly correlated with length of the first DFP or OST (Table 4). Although a mild effect of *SURVIVIN* expression on DFP and OST was found, this was due to the presence of a single outlier. Following removal of this single outlier, this significant effect was gone. Using Kaplan-Meier analysis, higher or lower than median expression of none of the genes had a significant effect on DFP or OST.

For all samples ($n = 96$) DR was associated with a 0.48 fold decreased *BCL9* expression ($P = 0.049$) and comparison of intrinsic versus acquired drug resistance showed a decreased expression of *Axin2* (fold change 0.29, $P = 0.012$), *Survivin* (fold change 0.59, $P = 0.001$) and *Cyclin-D1* (fold change 0.54, $P = 0.016$) in the acquired expression.

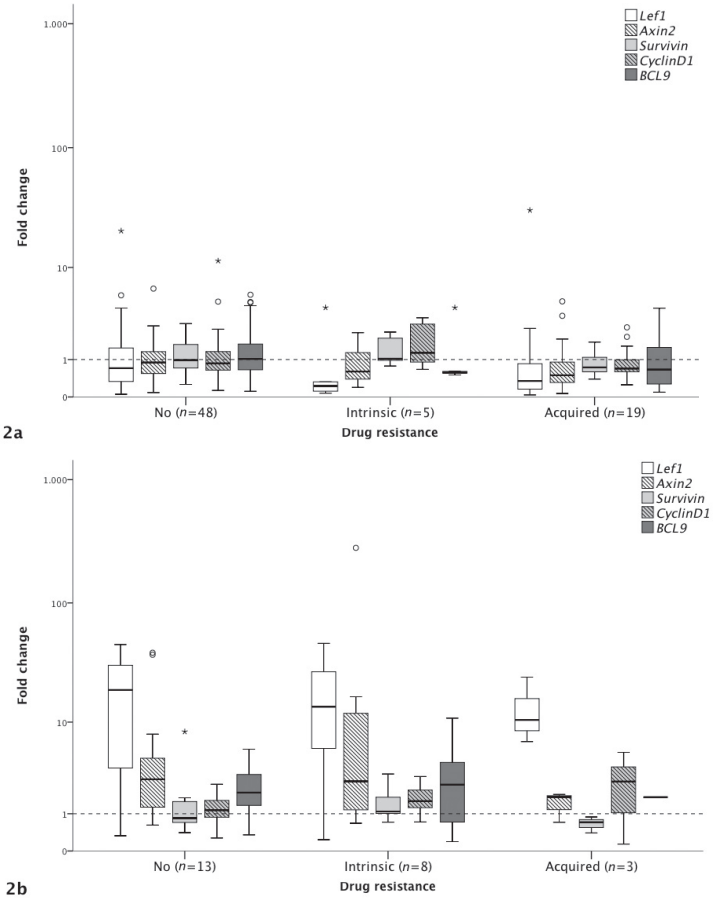

Figure 2. Box-whisker plot representing fold-changes in mRNA expression for *LEF1, AXIN2, SURVIVIN, CYCLIN D1*, and *BCL9* in 40 dogs with B-cell (top) and 15 dogs with T-cell lymphoma (bottom) categorized by drug resistance.

DR, absent, intrinsic and acquired, in T-cell lymphoma were respectively associated with a higher *LEF1* (12.6 fold, P <0.0001; 31.3 fold, P = 0.004; 29.0 fold. P < 0.002) and *AXIN2* (3.83 fold P < 0.0001, 7.25 fold, P = 0.05; 2.26 fold. P = 0.205) expression compared to B-cell lymphomas (Fig 2). Since there is no significant increase in *LEF1* and *AXIN2* expression between no DR and DR (either intrinsic or acquired), the difference in expression appears related to immunophenotype rather than DR.

Discussion

The current study is the first large-scale prospective study on activation of the Wnt-pathway in cML and supports activation of the Wnt-pathway in a subset of cML, in particular T-cell lymphomas, as could be expected based on the role of the Wnt pathway in T-lymphocyte proliferation[26].

Although both *Axin2* and *LEF1* expression were increased in T-cell lymphomas, no significant correlation was found between the mRNA expression of these two genes. It has been previously demonstrated that increased *LEF1* expression can lead to β-catenin independent

activation of the Wnt pathway in human lymphoma[27] whereas in canine mammary cancer cell lines *LEF1* expression is associated with Wnt-ligand independent activation of the canonical Wnt pathway[5] explaining why the expression of these two genes do not necessarily have to correlate. As *AXIN2* expression is a direct target of the canonical Wnt pathway it might prove to be a better reporter for Wnt activation.

Following activation of the Wnt-pathway, *AXIN2* expression is upregulated and, together with the tumor suppressor APC and the serine/threonine kinase GSK3β, *AXIN2* inactivates cytoplasmatic β-catenin thereby inhibiting the Wnt-pathway and effectively creating a negative feedback loop[9]. In the current study *AXIN2* expression showed a mild correlation with body weight, which was independent of the (T-cell) immunophenotype. Although *AXIN2* is involved in skeletal growth[28], this is unlikely to explain the correlation between *AXIN2* expression and body weight in skeletally mature dogs that develop cML.

Proteins of the TCF/LEF-family are thought to function as transcriptional repressors of Wnt-pathway. *LEF1* is an important factor in normal hematopoiesis including both B- and T-lymphocytes[29] and neutrophilic granulocytes[30] and is commonly identified in a number of hematologic malignancies including T-cell lymphoma and T-cell lymphoblastic leukemia[14, 31] and acute myeloid leukemia[32]. A high expression is associated with a poor prognosis in B-cell acute[33] and chronic[34] lymphoid leukemia. Although *LEF1* expression was high in a number of dogs with cML, it failed to correlate with the length of DFP and OST.

SURVIVIN expression did not correlate with DFP and OST, which is in contrast to previous publications in canine lymphoma[35] and osteosarcoma[36], but in line with other studies in a variety of canine tumors including mast cell tumors[37], osteosarcoma[38] and mammary carcinoma[39] that failed to demonstrate a negative correlation between *Survivin* expression and survival.

Overexpression of *Cyclin-D1*, an important regulator of the G1/S transition of the cell cycle, is uncommon in human diffuse large B-cell lymphoma[40] with the exception of mantle cell lymphoma where it is associated with a specific translocation t(11;14) (q13;q32)[41]. In the current study no effect was found between *Cyclin-D1* expression and immunophenotype, disease progression or drug resistance.

B-cell lymphoma[9] (*BCL9*) forms together with the other coactivators Pygopus and *TCF/LEF* a complex that allows for β-catenin-mediated Wnt-transcription[8]. Immunohistochemical *BCL9* expression was shown to correlate with tumor grade in human colorectal neoplasia[42], mammary carcinoma[43], and hepatocellular carcinoma[44], but appeared only predictive for survival in hepatocellular carcinoma. Up to now there were no data available on *BCL9* expression in the dog, but the current study shows that although *BCL9* expression is detectable in the majority of cML cases with a trend towards higher *BCL9* expression in T-cell lymphomas, but expression was not predictive for prognosis.

Previous studies have shown a relation between activation of the Wnt-pathway and drug resistance in acute lymphoid leukemia[17, 18], but this finding could not confirmed in the present study. Drug resistance was associated with a down-regulated *BCL9* expression in B-cell lymphoma and a trend to increased expression in T-cell lymphomas. *BCL9* overexpression in relation to drug resistance has recently been reported in human myeloma cell lines[45].

In conclusion, the data from the current support activation of the Wnt-pathway in T-cell lymphomas, but its role in DR appears limited. Given the poorer prognosis of dogs with T-cell lymphoma treated with chemotherapy compared to those with B-cell lymphoma, these results provide a basis for using modulators of the Wnt-pathway in this subgroup of dogs.

Competing interests

The authors declare that they have no competing interests.

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7

Summarizing Discussion

Canine lymphoma and drug resistance: what can we conclude?

Introduction

Canine lymphoma (cL) is the most common hematopoietic neoplasia in the dog and the neoplastic disease most frequently treated with chemotherapy in veterinary oncology. Based on similarities in epidemiology, clinical presentation, cytological and histological morphology, treatment and treatment response, cL is comparable to human non-Hodgkin lymphoma[1, 2].

Although cL affects all dog breeds, the relative risk varies between breeds, which implies a genetic background. Dog breeds represent (more or less) closed gene pools with a high degree of familial relationship, and recently the canine genome has been sequenced[3, 4]. For these reasons and the fact that dogs and humans share the same environment[5], the dog is a valuable animal for studying lymphomagenesis[6]. Furthermore, these similarities combined with the willingness of pet owners to treat their animals for this disease, makes the dog a promising spontaneous large-animal model for human NHL.

Canine lymphoma is an important disease in veterinary medical oncology and the success of treating this disease appears mostly limited by the development of drug resistance. Drug resistance can have multiple causes, but drug transporters appear particularly important. A number of studies[7-10] have reported that pretreatment with glucocorticoids reduces the chance that a dog with cL will respond to chemotherapy and this was thought to result from the induction of drug resistance by glucocorticoids. But if this is the case and almost all dogs treated with a multidrug protocol including prednisolone will experience a relapse, this would imply that all relapsed tumors have been (pre)-treated with glucocorticoids and that this might negatively affect further treatment results. This led to several questions: (i) what causes drug resistance in canine lymphoma, (ii) do glucocorticoids induce dug transporters in dogs as in humans, (iii) are glucocorticoids necessary in a canine multidrug chemotherapy protocol, and finally (iv) are there options to reverse drug resistance.

Canine lymphoma: are glucocorticoids necessary?

In this thesis (**Chapter 4**) we describe a cohort of dogs presented for treatment of multicentric lymphoma. This cohort was in accordance with the reported literature showing a slight male predominance and a mean age of 7.5 years, and included previously reported predisposed breeds[11-13] like the German shepherd, Rottweiler, Bernese mountain dog, Boxer, English Cocker spaniel, the Golden and Flatcoated retriever, dogue de Bordeaux and Bullmastiff. The dogs in the current study were staged according to the WHO-staging system resulting in a equal distribution over stages III, IV, V, with 75% of the dogs being classified as substage a. All lymphomas were diagnosed with cytology and classified and graded according to the updated Kiel classification, showing 49 (70%) high-grade B-cell, 15 (21%) high-grade T-cell and 6 (9%) low-grade T-cell lymphomas. Clinical pathology results showed a mild anemia in 35 (43%) dogs, mild thrombocytopenia in 11 (14%), hypercalcemia in 7 (9%) dogs, all of which were diagnosed with T-cell lymphoma, and leukemia in 12 (15%). A further 14 (18%) dogs were identified as stage V based on bone marrow aspirates. These results match earlier reports from European studies[14, 15].

All dogs were treated in a prospective randomized controlled clinical trial, with a doxorubicin-based multidrug chemotherapy protocol, with half of the dogs being randomized to receive prednisolone in addition to their protocol. This resulted in an overall complete response rate of 75%, a median first disease-free period of 176 days, an overall survival time 283 days, and 1- and 2- year survival rate of respectively 40 and 17% and these results are comparable to those reported in the literature[16-19].

The prognosis of canine lymphoma was negatively affected by body weight (1 kg increase in body weight reduces the disease-free period with 3%), T-cell immunophenotype, hypercalcemia, leukemia/stage V, and failure to obtain a complete response. Following relapse, chances for obtaining a second complete response with a rescue protocol were overall lower (43%), but strongly affected by the moment of relapse in relation to the first treatment protocol. Relapse during the initial protocol gave a 30% chance for a second complete response, but increased to 60% in case relapse occurred in the half year following completion of the protocol and was 100% in case the relapse occurred after having been out of treatment for more than half a year. The length of the second remission was independent of the moment of relapse, but in general slightly shorter than the first remission. Although our results on second response rate concur with a previous study, we were not able to demonstrate a similar effect on the duration of second remission[20].

Excluding prednisolone from the multidrug chemotherapy protocol did not affect treatment outcome of the initial or subsequent rescue treatment, but neither could the concurrent use of prednisolone be identified as a risk factor. These results, combined with the obvious side effects of prednisolone use, led us to conclude that prednisolone is not necessary in future first-line multidrug protocols and that prednisolone should be reserved for use in drug-resistant or relapsed lymphomas[21].

Drug resistance

As reviewed in **Chapter 1** drug resistance, defined as failure to respond to chemotherapy, is an almost inevitable problem in medical oncology. There are currently two major theories as to how drug resistance would arise: selection of drug resistant tumor cell clones or selection of tumor stem cells. Drug resistant tumor clones arise through spontaneous mutation, a process that can be accelerated further by the use of mutagenic agents including many cytostatic drugs, and the Goldie-Coldman hypothesis[22] predicts the presence of a drug resistant tumor cell clone in any tumor of sufficient size. The further use of chemotherapy then drives selection towards the more drug-resistant clones. The tumor stem cell hypothesis predicts the presence of a low number tumor stem cells that are intrinsically multi-drug resistant within the tumor[23]. Chemotherapy eliminates the majority of the sensitive tumor cells, leaving the small number of intrinsic resistant tumor stem cells Multiple mechanisms leading to drug resistance have been described, including failure of the cytostatic drug to reach the tumor due to changes in uptake, metabolism, or elimination of the drug; presence of specific tissue barriers, like the blood-brain barrier; or insufficient tumor perfusion. However, cellular mechanisms appear more important and these include the inability to obtain sufficiently high intracellular drug levels through reducing cellular

uptake or increasing drug efflux, compartmentalization of the drug, changes in drug target, or reduced effect of the drug due to resistance in apoptosis or enhanced DNA-damage repair[24].

In human oncology expression of transporter proteins of the ABC (ATP-binding Cassette) superfamily has been studied in a wide variety of malignancies including lymphomas[25, 26] and increased expression of these proteins, and P-gp (ABCB1), MRP1 (ABCC1) and BCRP (ABCG2) in particular, has oftentimes been associated with drug resistance[27, 28]. Although inhibition of ABC-transporters sounds as promising option to reduce drug resistance, it should be realized that ABC-transporter expression is not restricted to tumor cells. They are found throughout the entire body and ABC-transporters affect systemic drug levels through drug clearance via liver, kidney and intestinal tract, regional concentrations due to their presence in tissue barriers (e.g. blood-brain barrier), and intracellular levels through drug efflux and drug compartmentalization. As a result, modulation of ABC-transporters can have significant effects on a drug's pharmacokinetic profile and could potentially lead to increased toxicity.

Drug resistance in canine lymphoma: in vitro

Establishing a possible role for the ABC-transporters P-gp (ABCB1), MRP1 (ABCC1) and BCRP (ABCG2) in drug resistance in canine lymphoma required a suitable cell model. Since high-grade B-cell lymphoma represents the most common form of cL and as a single agent, doxorubicin appears to be the most effective drug in treating cL, a canine B-cell lymphoid leukemia cell line, was exposed to increasing doses of doxorubicin as described in **Chapter 2** resulting in a doxorubicin resistant cell line. The ABC-transporters were then assessed in both the original (drug sensitive) and the drug resistant cell line using three different assays: mRNA expression, immunocytochemistry (protein expression), and transporter function. Primers, antibodies and function tests (dye uptake or efflux assays) were validated for assessing canine P-gp, MRP1 and BCRP[29].

It was found that doxorubicin resistance correlated with increased P-gp expression (mRNA, protein) and function (transport capacity), and that this resistance could be completely reversed with the classic P-gp inhibitor PSC833. The presence of MRP1 and BCRP was also demonstrated, but was found equal in both cell line suggesting that these transporters did not have a causative role in the observed drug resistance.

It was also demonstrated that increased P-gp expression coincided with resistance to the structurally and mechanistically unrelated drug vincristine, and this resistance could also completely reversed with PSC833, confirming that P-gp has an important role in canine multi-drug resistance.

Although it was demonstrated that prednisolone had a direct mild lympholytic effect, this effect was equal for both cell lines and did not fit with the assumption that prednisolone is a P-gp substrate. It was also demonstrated that prednisolone was not able to induce ABC-transporter expression in either of the two cell lines. This was an unexpected finding since dexamethasone has been shown to be both a P-gp substrate, as well as a strong inductor of P-gp in a variety of human cells including lymphocytes[30, 31] and induction of P-gp expression

in the enterocytes significantly reduced prednisolone uptake in healthy dogs[32]. Although this seems counterintuitive, it should be noted that the capacity of glucocorticoids to induce P-gp may vary between glucocorticoids (prednisolone vs dexamethasone), species[33, 34] and tissues, and as a result observations cannot be generalized. It is therefore important to assess whether or not prednisolone is capable of increasing P-gp expression in lymphocytes of healthy dogs and in lymphoblasts of dogs with cL. These results do, however, fit with the findings in the clinical trial that the prior use of prednisolone in the initial chemotherapy protocol was not associated with a poorer response to chemotherapy in relapsed patients.

Drug resistance in canine lymphoma: in vivo

Failure to respond to chemotherapy was the most common cause for treatment failure in the cohort of dogs studied and was further characterized in **Chapter 5**. Therapy resistance or tumor relapse were the two most common reasons for euthanasia in dogs with multicentric lymphoma. In order to establish a relation between drug resistance, either intrinsic or acquired, and ABC-transporter expression in cL, mRNA expression of *ABCB1, -B5, -B8, -C1, -C3, –C5* and *G2* were measured prior to the start of chemotherapy, at first relapse, and when the tumor was no longer (clinically) responsive to chemotherapy. Intrinsic drug resistance was found to be more common in T-cell (6/15) than in B-cell lymphomas (4/40) and was the main reason for euthanasia in this T-cell lymphoma group. In B-cell lymphoma, acquired drug resistance appeared more important, with 19/36 dogs developing clinical drug resistance following one or more complete responses.

Although almost all ABC-transporters were detected in almost all samples, significant differences in expression were noted. Overall, T-cell lymphomas showed a higher *ABCB5* and *-C5* expression than B-cell lymphomas. Intrinsic drug resistance in B- and T-cell lymphomas was not associated with significant changes in ABC-transporter expression when levels were compared to their respective drug sensitive tumor samples, but a significantly higher *ABCG2* expression was found when intrinsic drug resistant T-cell lymphomas were compared to B-cell lymphomas. Acquired drug resistance showed an increased *abcb1* expression in B-cell lymphomas, while in T-cell lymphomas an increased *ABCB5* and *-G2* expression was noted. Our data suggest that B- and T-cell lymphomas, intrinsic and acquired drug resistance are associated with differences in ABC-transporter expression. These findings have not been previously reported and require further confirmation in larger cohorts of dogs. However increased expression of *ABCB5* has previously been associated with drug resistance in human hematological malignancies[35] and *ABCG2* expression proved the most commonly expressed ABC-transporter in human T-cell lymphoma[36]. These findings might also imply that T-cell lymphomas might benefit more from the use of cytostatic drugs that are not transported by *ABCG2* and these would include alkylating agents like CCNU. This is in concordance with the results of a preliminary study in dogs with T-cell lymphoma that showed that the addition of CCNU to a regular (L)CHOP-protocol significantly prolonged survival[37].

Pre-treatment expression of none of the ABC-transporters tested, proved predictive for complete response rate, length of disease free period of overall survival. This is in contrast

to early studies on P-gp expression in canine lymphoma[38-40], but mirrors the results from more recent studies[41, 42]. As was expected, not every relapsed lymphoma proved drug resistant, but also here drug resistance could not be predicted from ABC-transporter gene expression in the relapse sample. The fact that ABC-transporter expression failed to predict prognosis, can have several reasons. Firstly, it remains difficult to relate mRNA expression to protein expression and even more importantly protein function. It was shown that even low levels of mRNA expression, can be associated with significant changes in drug resistance[43]. Secondly most of our knowledge on drug resistance is derived from cell line experiments that typically develop ABC-transporter overexpression as a major drug resistance mechanism, but do not represent the whole spectrum of drug resistance mechanisms found in clinical oncology[44, 45]. Although ABC-transporters play a major role in drug resistance, it seems likely that other drug resistance mechanisms including changes in cellular survival signaling cascades, resistance to apoptosis, enhanced DNA-repair mechanism or upregulation of antioxidant defense enzymes are also involved[46-50].
Although induction of P-gp expression by prednisolone was not tested in peripheral blood lymphocytes, it was assessed in tumor samples from a subset of dogs and failed to show changes in expression of the various ABC-transporters. This is in line with our in vitro observations and suggests that prednisolone is not an inducer of P-gp mediated drug resistance in the dog.

Canine lymphoma: implications for drug therapy

In veterinary oncology CHOP (cyclophosphamide-doxorubicin-vincristine-prednisolone)-based protocols are considered the gold standard for treating canine lymphoma, and of these drugs a monotherapy with doxorubicin provides the longest disease-free period, suggesting that it is the most effective drug in the treatment of canine lymphoma. However, both doxorubicin and vincristine are P-gp substrates[29, 51, 52] and doxorubicin potentially also for canine BCRP[53], which would suggest that CHOP-based protocols may not be the protocols of choice for drug resistant lymphomas and possibly T-cell lymphomas.
In case of (clinical) drug resistant lymphoma, one option would be to choose a protocol that is based on drugs that are not transported by P-gp or BCRP and potential drugs would include alkylating agents, L-asparaginase and glucocorticoids in both B- and T-cell lymphomas, and possibly mitoxantrone in B-cell lymphomas. The identified drug classes are already used in the currently advised rescue protocols and this approach seems only moderately effective.
An alternative option would be to reverse drug resistance by inhibiting ABC-transporter function and it was shown that *in vitro* that both the classical P-gp inhibitor PSC833 (**Chapter 2**) and the tyrosine-kinase inhibitor masitinib (**Chapter 3**) were capable of reversing P-gp mediated drug resistance in canine lymphoid cells[29, 54].
Although blocking P-gp appears sufficient for drug resistant B-cell lymphomas, it might prove necessary to also block BCRP in drug resistant T-cell lymphomas and from this perspective the dual P-gp/ BCRP inhibitors elacridar and tariquidar are of particular interest.
Although the use of ABC-transporter inhibitors in humans with (drug resistant) cancer has

Table 1. ABC-transporters associated with drug resistance for the major cytostatic drug classes.

Drug	P-gp	MRP1	BCRP	Others
Doxorubicin, and other anthracyclines	+	+	+?*	ABCB2?, ABCB5?
Vincristine, and other vinca-alkaloids	+	+	-	-
Alkylating agents, including CCNU	-	-	-	ABCC2?
Glucocorticoids				
Prednisolone	-	-	-	
Dexamethasone	-	-	-	
L-asparaginase	-	-	-	-
Antimetabolites (methotrexate)	-	+	+	ABCC3
Anthracenedione (mitoxantrone)	-	-	+	
Topoisomerase I/II-inhibitors (topotecan, etoposide)**	+	+		ABCC3
Platinum-drugs***	-	-	-	ABCC2

* mutant BCRP in humans, possibly wild-type canine BCRP, ** not used in veterinary medicine, *** not suitable for treating lymphoma

been troublesome, the third generation inhibitors are more potent and more specific and are being evaluated in clinical trials[55]. These inhibitors could therefore potentially be used for treating dogs with drug resistant lymphoma. On the other hand, it is also important to note that there is a potential role for the dog with drug resistant lymphoma in the preclinical testing of new transporter inhibitors.

The simultaneous use of the tyrosine-kinase inhibitor masitinib and cytostatic agents was shown to have a synergistic effect on growth inhibition and increased the sensitivity to cytostatic drugs in a variety of canine tumor cell lines[56]. Since the combined use of a tyrosine-kinase inhibitor and a cytostatic agent was reported to revert drug resistance in human cell lines[57-59], testing masitinib in the drug resistant canine lymphoid cell line was a logical next step (**Chapter 3**). Since masitinib effectively inhibited P-gp and was able to reverse doxorubicin resistance, these results provide a basis for initiating a prospective clinical trial on the use of masitinib in dogs with (P-gp dependent) drug resistant lymphoma.

ABC-transporter expression can be upregulated through activation of the orphan nuclear receptors PXR and CAR, and blocking these receptors appears a promising therapy and has recently become possible with the development of the PXR-inhibitor FLB-12[60]. Another option for inhibiting ABC-transporter function would be through targeting ABC-transporter mRNA with antisense oligonucleotides and siRNA45. And lastly the role of other (non-ABC-transporter dependent) drug resistance mechanisms needs to be critically evaluated and is likely to result in new therapeutic options.

Canine lymphoma: the future

Although looking at alternative chemotherapeutic drug protocols, exploring the use of transporter inhibitors or trying to inhibit other resistance mechanisms sounds attractive, these solutions will most likely be temporary and other therapeutic options for treating canine lymphoma need to be explored. The two most attractive options include targeted therapy and immunotherapy.

Targeted therapy involves the use of drugs that target specific pathways in cancer cells and include small molecule inhibitors (e.g. tyrosine-kinase inhibitors) and monoclonal antibodies. This approach has already been successfully used in the treatment of a number of human and canine cancers. For these types of treatment, it is of the utmost importance to have a thorough understanding of the driving pathways and molecular mechanisms involved in a specific type of neoplasia, but this information is currently still scarcely available for canine lymphoma. However, activation of the NF-κB, Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR, some of which are also linked to drug resistance[61], appears common in human non-Hodgkin B-cell lymphoma and might prove potentially useful drug targets in the dog as well. In T-cell lymphomas, we have demonstrated upregulation of *LEF1* and *AXIN2*, which is consistent with activation of the Wnt-pathway (**Chapter 6**). Inhibition of the Wnt-pathway might prove a potential new target for treating this subset of dogs that already has an intrinsically poorer prognosis.

Immunotherapy aimed at CD20+ B-cells (Rituximab®) has dramatically improved the prognosis of human B-cell neoplasia and research to identify further B- and T-cell epitopes[62] is ongoing. The use of therapeutic antibodies and tumor vaccines is therefore a promising treatment option and should remain a focus of further studies in he dog as well.

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8

Summary

This thesis can be divided in three parts, and following an initial introduction to canine lymphoma and drug resistance in **Chapter 1**, initial hypotheses and assays were validated in a drug resistant lymphoid cell line in **Chapter 2** and this model was then applied to assess a potential therapy for circumventing drug resistance in **Chapter 3**. The conclusions of our *in vitro* work where then verified in clinical samples obtained in a clinical trial described in **Chapter 4, Chapter 5** and **Chapter 6**. In **Chapter 7** the conclusions from the various studies are combined and as a whole put in perspective to our current knowledge on drug resistance in the dog.

Canine lymphoma is an important neoplastic disease in the dog and can, as yet, only be managed with chemotherapy. Although chemotherapy is initially successful in controlling the disease, this control is temporary and cancer will recur in in ±75% of dogs within 2 years of the diagnosis and is then often more difficult to control. Failure to respond to chemotherapy (treatment failure) either from the start, during, or after finishing the treatment protocol, implies therapy resistance which can have a number of underlying causes of which the increased drug efflux through upregulation of transporters proteins of the ATP-Binding Cassette (ABC) family has been studied most extensively in human oncology. Our understanding on the role of ABC-transporters in canine lymphoma and drug resistance (DR) is still limited, but there are data to support a similar role of these transporters in canine lymphoma as well as some other forms of cancer.

In order to study DR, a lymphoid cell model had to be developed and for this purpose doxorubicin was chosen. Since doxorubicin exerts its therapeutic effect through a wide variety of mechanisms, it is a highly effective drug and is used in the treatment of a variety of veterinary cancers including canine lymphoma. However, for the same reason that doxorubicin is so effective, resistance to doxorubicin is also highly predictive for the onset of multi-drug resistance or MDR.

As was suspected based on the available literature, it was shown in **Chapter 2** that the doxorubicin resistant canine lymphoid cell line, in comparison to its doxorubicin-sensitive parental cell line, has a highly upregulated expression of abcb1, that results in increased expression of P-gp as well as an increase in transport capacity of the prototypical P-gp substrate Rhodamine123. Furthermore reversing P-gp function with the classical P-gp inhibitor PSC833 (Valspodar®) reversed resistance to doxorubicin. In line with our hypotheses, resistance to doxorubicin coincided with resistance to vincristine, another cytostatic drug commonly used in the treatment of canine lymphoma, which was also fully reversed with PSC833 and supports the important role of P-gp in MDR as described in human oncology. The other ABC-transporters associated with MDR in human oncology, MRP1 and BCRP, were not upregulated in this specific drug resistant cell line, which might be anticipated since once the cell has become resistant to the drug used for selecting DR, there is no stimulus driving selection for further resistance mechanism, other than potentially a survival benefit for those cells that have higher basal expression of P-gp.

It is generally assumed that ABC-transporter substrates are also capable of inducing ABC-transporter expression, as we were able to confirm for doxorubicin, but this could not be demonstrated for prednisolone. Prednisolone has been documented a P-gp substrate

in dogs and treatment with glucocorticoids in dogs with lymphoma prior to starting chemotherapy is associated with a lower treatment response that was explained by assuming the induction of ABC-transporters, coinciding with induction of DR to cytostatic agents. Although we were able to demonstrate a mild cytotoxic effect on canine lymphoid cells, as well as the presence of a functional glucocorticoid receptor, there was no proof that prednisolone was capable of inhibiting P-gp function (based on inability to reduce Rhodamine123-transport) or inducing abcb1 mRNA expression.

Based on the important role of P-gp in MDR in both human and canine oncology as described in literature, and further supported by the observation that P-gp overexpression caused MDR in the canine lymphoid cell model, approaches for inhibiting P-gp mediated MDR were evaluated *in vitro* as described in Chapter 3. Therapeutic interventions to reverse MDR are limited, but include multidrug protocols and the temporary concomitant use of ABC-transporter inhibitors. The use of PSC833 at concentrations of 1 µmol/L, levels achievable *in vivo*, was shown in **Chapter 2** to effectively inhibit P-gp and revert DR. The routine use of PSC833 in humans with DR neoplasia is still hindered by the unpredictable effects of PSC833 on cytostatic drug levels, oftentimes resulting in either subtherapeutic, ineffective or supratherapeutic, and toxic drug levels and the development of newer inhibitors and other strategies continues.

Recently, the concomitant use of tyrosine kinase inhibitors (TKI) with cytostatic agents was proposed as an alternative method for overcoming MDR in human oncology. The combined use of masitinib, a TKI licensed for veterinary use in the treatment of canine mast cell tumors, with the cytostatic drugs doxorubicin and vincristine was evaluated *in vitro* for its capacity to reduce DR to these cytostatic agents in the MDR lymphoid cell model. Although masitinib had a mild antiproliferative effect on lymphoid cells, the true strength of masitinib lies in the inhibition of P-gp, but not MRP function, and reverting DR to doxorubicin starting at concentrations equal to or exceeding 1 µmol/L, which are levels that can be achieved *in vivo*. These findings provide a rationale for the combined use of PSC833 or masitinib with P-gp substrates, including doxorubicin and vincristine, in the treatment of dogs with DR lymphoma, but will require confirmation in clinical trials.

The use of glucocorticoids prior to starting chemotherapy has been associated with a higher chance for treatment failure and its use leads to adverse events including lethargy, panting, polyphagia, polyuria, and weight gain. Furthermore glucocorticoids prohibit the use of non-steroidal anti-inflammatory drugs, and are capable of inducing ABC-transporter expression *in vitro* and therefore potentially induce DR. Especially the capacity of prednisolone to induce DR combined with the fact that most dogs will have a relapse of their lymphoma and would then have been pretreated with glucocorticoids, led us to the question whether the use of prednisolone would be detrimental. Despite all these potentially negative effects, there are no data to support the use of glucocorticoids as part of a multidrug chemotherapy protocol for the treatment of canine lymphoma and for this purpose a clinical trial was initiated of which the results are presented in **Chapter 4**.

A prospective, randomized clinical trial was conducted in 81 treatment-naïve dogs with multicentric lymphoma and no history of recent glucocorticoid use. All dogs were staged

and treated with the same chemotherapy protocol (L-asparaginase, cyclophosphamide, doxorubicin, vincristine, prednisolone or L-CHOP protocol) with half of the dogs receiving prednisolone. Both treatment groups were similar with respect to demographics, immunophenotype, and clinical stage, except for a higher number of substage b patients in the prednisolone group (5 vs. 14; $P = 0.015$). Treatment results obtained with the initial treatment (complete response rate 75%, disease-free period 176 days) and rescue treatment (complete response rate 45%, disease-free period 133 days), overall survival (283 days) and adverse events (number and grade) were similar for both groups. It was found that next to failure to obtain a complete remission, hypercalcemia, leukopenia, T-cell immunophenotype, a higher body weight and bone marrow involvement were the strongest (negative) prognostic factors for disease-free period and survival. The use of prednisolone had no negative effect on the outcome of a follow-up treatment following relapse, and only the moment of relapse in relation to the end of the first chemotherapy protocol proved significant. In general, the longer it takes for a relapse to occur, the higher the likelihood for a second complete response, while the duration of a second disease-free period was similar to that of the first. It is concluded that the use of prednisolone within a doxorubicin-based multidrug chemotherapy protocol has no additional effect on treatment results and can be safely omitted from first-line multidrug protocols, but contrary to what was assumed, also has no negative effect on treatment outcome.

The treatment of canine lymphoma is initially successful, but tumor recurrence is common and often refractory to treatment, ultimately leading to a prematurely death. Failure to respond to chemotherapy, either from the start, during, or after completion of the chemotherapy protocol is classified as DR. DR has been associated with the active efflux of cytostatic drugs by transporter proteins of the ATP-Binding Cassette (ABC) family of which P-gp (ABCB1), MRP1 (ABCC1) and BCRP (ABCG2) seem to be the most important. In order to confirm a role for ABC-transporter in DR, lymph node samples were collected dogs with multicentric lymphoma prior to the start of chemotherapy, at first relapse, and when the tumor no longer responded to chemotherapy.

In **Chapter 5** we report the ABC-transporter mRNA expression in 63 dogs diagnosed with multicentric lymphoma treated with the same doxorubicin-based chemotherapy protocol and half of the dogs receiving prednisolone. Expression of *ABCB1, -B5, -B8, -C1, -C3, -C5*, and *-G2* mRNA was quantified in tumor samples obtained and related to patient demographics, staging, treatment response and DR (absent, intrinsic, acquired). ABC-transporter expression in the pretreatment sample was independent of sex, weight, age, stage or substage, but T-cell lymphoma and hypercalcemia showed an increased abcb5 and –c5 expression compared to B-cell lymphoma and normocalcemia. Of the 63 dogs in the study 10 failed to obtain a complete response, and 45 had a relapse and 8 did no relapse during the duration of the study. Relapse does not imply DR, although the complete response rate in the second treatment round was significantly lower than in the first round (53% vs 84%). Clinical DR was diagnosed in 35 (10 intrinsic DR, 25 acquired DR) of 63 dogs and was associated with an increased *ABCB1* expression in B-cell lymphoma and an increased *ABCG2* expression in T-cell lymphoma. ABC-transporter expression in the pretreatment sample was

not predictive for the length of the first disease-free period or survival. Contrary to what is suggested in the literature, but in accordance with our *in vitro* experiments, glucocorticoid use failed to induce ABC-transporter expression. It is concluded that DR is a major problem in canine multicentric lymphoma, and the cause for treatment failure in more than half of the dogs and is only followed by the owner's choice to discontinue chemotherapy following 1 or more relapses (20 dogs). Furthermore there appears to be a difference in ACB-transporter expression between drug-resistant B- and T-cell lymphomas. This underlines the need to think of B- and T-cell lymphoma as two separate entities, stresses the need to routinely perform immunophenotyping, consider different treatment protocols for these two diseases, and different ABC-transporter inhibitors in case of the onset of DR.

Although ABC-transporter expression occurs in dogs with multicentric lymphoma, it failed to accurately predict prognosis and suggest the existence of other DR mechanisms. One of these mechanisms is activation of the Wnt-pathway that has been associated with DR in human hematopoietic malignancies. In order to confirm a potential role of the Wnt-pathway in DR in canine lymphoma we measured mRNA expression of *AXIN2, LEF1, SURVIVIN, CYCLIN D1*, and *BCL9* in dogs with multicentric lymphoma of which the results are presented in **Chapter 6.** Tumor samples were collected from 55 dogs diagnosed with multicentric lymphoma prior (n = 55) and during (n = 41) treatment with a standardized doxorubicin-based chemotherapy protocol and related to patient demographics, staging, treatment response and clinical DR. The mRNA expression of *AXIN2, LEF1, SURVIVIN, CYCLIN D1*, and *BCL9* was detected in almost all tumor samples and demonstrated increased *AXIN2* and *LEF1* expression in T-cell lymphomas compared to B-cell lymphomas and support activation of the Wnt-pathway in this subgroup. Age, sex, WHO-stage, substage, treatment response and DR could not be associated with activation of the Wnt-pathway. It is concluded that activation of the Wnt-pathway is common in canine lymphoma and appears to play a role in T-cell lymphoma, but not in DR. Given the poorer prognosis of this subgroup of dogs when treated with chemotherapy, further investigations into the use of modulators of the Wnt-pathway, as well as other pathways, is warranted.

In **Chapter 7** the results of the current studies are summarized and reviewed, as well as limitations to our current knowledge identified, and suggestions for further follow-up studies made.

Samenvatting in het Nederlands voor niet-ingewijden

Dit proefschrift kan worden onderverdeeld in drie delen. Na een overzicht over lymfeklierkanker bij de hond en ongevoeligheid (resistentie) voor chemotherapie (cytostatica) in zijn algemeenheid en de rol van ABC-transporters in het bijzonder in **Hoofdstuk 1**, eindigt dit hoofdstuk met het benoemen van de doelen van en de hypotheses voor het uitgevoerde onderzoek. In **Hoofdstuk 2** beschreven we hoe de initiële hypothesen zijn getest als ook de hiervoor benodigde testen zijn ontwikkeld in een voor dit specifieke doel gecreëerde chemotherapie-resistente hondencellijn. Vervolgens wordt in **Hoofdstuk 3** beschreven hoe dit model gebruikt is om een potentiële therapie voor het omkeren van deze resistentie tegen chemotherapie te bestuderen. De conclusies van al dit laboratorium werk werden vervolgens gecontroleerd in tumormonsters verkregen uit honden met lymfeklierkanker en deze studies zijn beschreven in de **Hoofdstukken 4, 5 en 6**. Tenslotte worden in **Hoofdstuk 7** de conclusies van de verschillende onderzoeken gecombineerd en in het perspectief geplaatst van onze huidige kennis over chemotherapieresistentie bij de hond.

In **Hoofdstuk 1** wordt begonnen met een uitgebreid overzicht te geven over het maligne lymfoom, ofwel lymfeklierkanker, bij de hond. Lymfeklierkanker is een van de meest belangrijke kwaadaardige, tumoreuze aandoeningen bij de hond en laat zich goed vergelijken met de ziekte van non-Hodgkin bij de mens. De ziekte wordt bij de hond (vooralsnog) alleen behandeld met chemotherapie en hoewel deze bij het merendeel van de honden aanvankelijk succesvol is, is dit effect in de regel tijdelijk en bij ±75% van de honden zal de tumor binnen 2 jaar na het stellen van de diagnose weer terugkeren. Vaak blijkt de tumor bij terugkeer moeilijker te behandelen en dit toont zich als het niet opnieuw volledig verdwijnen van de tumor met de ingestelde chemotherapie. De afwezigheid van een reactie op chemotherapie, suggereert een ongevoeligheid voor de therapie die ook wel chemotherapie-resistentie (CR) wordt genoemd. Deze resistentie kan meerdere oorzaken hebben, maar een toegenomen capaciteit om de chemotherapie versneld uit de tumorcel te pompen door het maken van extra transporteiwitten (de "pompen") van de ATP-Binding cassette(ABC)-familie is een bekende en veel bestudeerde oorzaak voor CR bij kanker bij de mens. Onze kennis over de rol van deze ABC-transporters in het lymfoom bij de hond en hun eventuele rol in de resistentie tegen chemotherapie is nog beperkt, maar er zijn zeker goede aanwijzingen dat ook bij de hond deze transporteiwitten een soortgelijke rol spelen bij CR bij het maligne lymfoom, als ook een aantal andere vormen van kanker.

Om CR bij lymfeklierkanker te kunnen bestuderen werd als eerste doel gesteld om een chemotherapie-resistente honden lymfeklierkankercellijn te maken zodat deze als laboratorium-model kan fungeren. Dit celmodel, beschreven in **Hoofdstuk 2**, is verkregen door een honden (lymfoïde) leukemie-cellijn langdurig bloot te stellen aan een toenemende dosering van het chemotherapie-middel doxorubicine. Er is bewust gekozen voor dit specifieke middel omdat doxorubicine zijn anti-tumor effect via meerdere, verschillende mechanismen uitoefent en daarom zeer effectief is. Daarnaast is het ook een veel gebruikt cytostaticum, niet alleen voor de behandeling van maligne lymfoom, maar ook voor verschillende andere vormen van kanker bij de hond. Om dezelfde reden dat doxorubicine zo effectief is, is het ook te verwachten dat bij ongevoeligheid

voor doxorubicine, de kans ook zeer groot is dat er ook resistentie tegen diverse andere chemotherapie-middelen speelt en dit verschijnsel wordt ook wel multidrug resistentie of MDR genoemd. Zoals vermoed werd op basis van de beschikbare literatuur (**Hoofdstuk 1**), werd met het celmodel bevestigd dat de doxorubicine-resistente cellijn een sterk toename van de ABC-transporter P-gp (P-glycoproteine of ABCB1) liet zien in vergelijking met de oorspronkelijke doxorubicine-gevoelige cellijn. Deze toename van het P-gp eiwit ging ook gepaard met een toename van de transportcapaciteit voor het prototypische P-gp substraat Rhodamine123. Verder bleek de klassieke P-gp remmer PSC833, ook wel bekend als Valspodar®, in staat om de P-gp functie te blokkeren wat de doxorubicine-resistente cellen weer net gevoelig maakte voor doxorubicine als de oorspronkelijke gevoelige cellijn. Zoals we van tevoren al verwacht hadden, bleek de ongevoeligheid voor doxorubicine samen te vallen met resistentie tegen vincristine, een ander veel gebruikt cytostaticum bij de behandeling van lymfeklierkanker bij de hond. Ook deze ongevoeligheid kon volledig ongedaan worden gemaakt met behulp van PSC833. Deze bevindingen bevestigen de belangrijke rol van P-gp in MDR bij de hond en zijn volledig in overeenstemming met de gegevens zoals die beschreven zijn bij de mens. Bij de mens zijn naast P-gp ook andere ABC-transporters geassocieerd met MDR, zoals MRP1 (Multidrug Resistance Protein-1) en BCRP (Breast Cancer Resistance Protein). Hoewel beiden werden aangetoond in zowel de doxorubicine-gevoelige als de doxorubicine-resistente cellijn, werd er van beiden geen toename gezien in de resistente cellijn. Dit laatste is niet geheel onverwacht, want op het moment dat een tumorcel resistent is geworden voor het geneesmiddel waarmee voor resistentie is geselecteerd, is er geen prikkel meer voor verdere selectie op andere resistentiemechanismen.

Algemeen wordt aangenomen dat de stoffen die door ABC-transporters worden uitgepompt, ook in staat zijn om de functie van deze transporters te vergroten door het stimuleren van extra aanmaak van deze transporters of pompen, een proces wat ook wel bekend staat als inductie. We weten dat doxorubicine een substraat is voor P-gp bij de mens en met het opheffen van de ongevoeligheid voor doxorubicine door de P-gp remmer PSC833 en de ontwikkeling van resistentie tegen doxorubicine door een toename van P-gp expressie, bevestigt deze studie dat dit ook bij de hond het geval is.

Prednisolon is vaak tijdelijk (enkele weken) een effectief middel in de behandeling van lymfeklierkanker en wordt om deze reden dan ook vaak gebruikt na het stellen van deze diagnose. Meerdere studies die de behandeling van lymfeklierkanker bij de hond beschrijven, hebben gevonden dat een behandeling met prednisolon of een andere glucocorticoïd voorafgaande aan het starten van de chemotherapie tot een minder goede reactie op chemotherapie leidt. Een eenduidige verklaring is er niet, maar er wordt vanuit gegaan dat prednisolon een P-gp substraat is bij de hond en dat deze verminderde reactie van de tumor op chemotherapie zou kunnen komen doordat prednisolon leidt tot een toename van de ABC-transporters welke dan samenvalt met het opwekken van resistentie voor chemotherapie. In de huidige studie konden wij een (mild) remmend effect van prednisolon op de celgroei van de leukemiecellen aantonen, maar dit effect bleek gelijk in de beide cellijnen, wat niet direct passend is bij het feit dat prednisolon door P-gp wordt

uitgepompt. Verder werd er ook geen toename van P-gp mRNA (deze geeft de opdracht voor het maken van het P-gp transporteiwit) gevonden als we de beide cellijnen bloot stelden aan prednisolon. Een mogelijke verklaring hiervoor zou kunnen zijn dat de beide cellijnen ofwel geen glucocorticoïd-receptor zouden hebben, ofwel niet in staat zouden zijn om deze stimulatie om te zetten in een effect/reactie. Beide mogelijke verklaringen konden we echter uitsluiten met een zogenaamd reporter systeem en onze resultaten suggereren dan ook eerder dat prednisolon geen substraat is voor het P-gp in deze cellijnen, en misschien dus zelfs niet voor het honden P-gp in zijn algemeenheid.

Enerzijds gebaseerd op de beschikbare literatuur waar de belangrijke rol van P-gp in MDR bij de mens, maar anderzijds ook gesteund door de toename van P-gp in de chemotherapie-resistente honden leukemie cellijn, was de logische vervolgstap om op zoek te gaan naar mogelijkheden om deze door P-gp veroorzaakte CR ongedaan te maken. Een eerste gedachte zou dan zijn om op zoek te gaan naar stoffen die de P-gp pompfunctie remmen of blokkeren. De eerste P-gp remmers gaven helaas veel bijwerkingen, maar een veelbelovende P-gp remmer is PSC833. Het gebruik van PSC833 in concentraties van 1 µmol/L, niveaus welke in de levende hond haalbaar blijken, was in staat P-gp volledig te remmen en CR op te heffen zoals beschreven in **Hoofdstuk 2**. Het routinematig gebruik van PSC833 bij mensen met CR tumoren wordt nog steeds bemoeilijkt door het onvoorspelbare effect van PSC833 op de bloedspiegels van de diverse cytostatica en dit kan resulteren in zowel te lage (dus ineffectieve), als te hoge (dus gevaarlijke of toxische) plasmaspiegels en de ontwikkeling van nieuwere remmers en andere strategieën blijft dan ook een focus van verder onderzoek.

Recent is het gelijktijdig gebruik van tyrosine kinase remmers (TKRs) met cytostatica beschreven als een mogelijkheid om CR tegen te gaan bij de mens. In de diergeneeskunde is de TKR masitinib geregistreerd voor de behandeling van mastceltumoren bij de hond. Het was dan ook een logische stap om te kijken of het gelijktijdig gebruik van masitinib met het cytostaticum doxorubicine in staat was om de CR tegen doxorubicine in het honden celmodel te verminderen of idealiter volledig tegen te gaan. De resultaten hiervan worden gepresenteerd in **Hoofdstuk 3** en tonen, dat hoewel masitinib op zichzelf een mild groeiremmend effect heeft op lymfoide cellen, maar de ware winst van masitinib zit in het remmen van de ABC-transporter P-gp. Dit zien we doordat de capaciteit om het Rhodamine123 uit te scheiden afneemt door masitinib toe te voegen in concentraties van 1 µmol/L en hoger en dit zijn spiegels die ook in de hond kunnen worden bereikt. Masitinib blijkt in deze concentraties ook in staat om de CR voor doxorubicine in deze cellijn vrijwel volledig ongedaan te maken en dit is gezien het effect op de Rhodamine-uitscheiding zeer aannemelijk het gevolg van P-gp remming zodat doxorubicine minder goed de cel uitgepompt kan worden. Deze bevindingen zijn veelbelovend en bieden een basis voor het gecombineerd inzetten van P-gp remmers, als masitinib of PSC833, met chemotherapie bij de behandeling van honden met CR lymfeklierkanker, op voorwaarde dat de gebruikte chemotherapiemiddelen een P-gp substraat zijn, zoals onder andere doxorubicine en vincristine. Voor dat deze therapie echter als effectief kan worden bestempeld, moeten deze resultaten worden bevestigd in klinische studies.

Het gebruik van glucocorticoïden voorafgaand aan het starten van chemotherapie is, zoals hierboven al benoemd, geassocieerd met een hogere kans op het falen van de chemotherapeutische behandeling. Niet onbelangrijk om te vermelden is dat het gebruik van glucocorticoïden als prednisolon daarnaast tot een aantal ongewenste bijwerkingen leidt zoals bv gedragsveranderingen (mat/tam worden van de hond), toename van hijgen, toegenomen eet- en drinklust, veel plassen en gewichtstoename. Verder zal het gebruik van glucocorticoïden een beperking opleggen aan het gebruik van bepaalde type pijnstillers (de zogenaamde NSAID's) wat met name bij kreupele honden onwenselijk kan zijn, en tenslotte zijn zij dus mogelijk in staat om de aanmaak van ABC-transporters, en dus potentieel ook CR, te stimuleren.
Alle momenteel gangbare chemotherapie-protocollen bevatten glucocorticoïden, meestal prednisolon, als onderdeel van de behandeling. Als prednisolon CR zou kunnen induceren en we weten dat vrijwel alle honden een relapse van hun lymfoom zullen ontwikkelen, zou dat betekenen dat bij relapse uiteindelijk alle honden zijn voor behandeld met glucocorticoïden. Mogelijk draagt dit dan ook bij aan de slechtere reactie op behandeling na het terugkeren van de lymfeklierkanker. Dit leidde bij ons tot de vraag of het gebruik van prednisolon binnen een chemotherapie protocol noodzakelijk is en of het misschien zelfs niet eerder potentieel een ongunstige factor zou kunnen zijn bij eventuele vervolgbehandeling. Ondanks al deze negatieve effecten van glucocorticoiden zijn er geen gegevens die een positief effect van het gebruik van glucocorticoïden binnen een multidrug chemotherapie protocol bij de behandeling van honden met lymfeklierkanker ondersteunen en met deze vraag als doel werd een klinisch studie gestart waarvan de resultaten zijn weergegeven in **Hoofdstuk 4**.
Voor deze, een zogenaamde prospectieve, gerandomiseerde, studie werd bij 81 honden met lymfeklierkanker die nog niet behandeld waren voor hun lymfeklierkanker *én* geen geschiedenis van recent gebruik van glucocorticoïden hadden. Alle honden werden op dezelfde wijze onderzocht (gestageerd) en behandeld met een chemotherapie protocol bestaande uit L-asparaginase, cyclofosfamide, doxorubicine, vincristine, prednisolon of L-CHOP protocol, maar slechts de helft van deze honden kreeg prednisolon. Beide behandelgroepen waren volledig vergelijkbaar voor wat betreft zaken als ras, geslacht, leeftijd, gewicht, klinisch stadium, tumorgraad, en immunophenotype met uitzondering van een en groter aantal substadium b patiënten in de groep die prednisolon kreeg (5 versus 14; $P = 0.015$). De behandelresultaten van het eerste chemotherapieschema (bij 75% van de honden volledige verdwijnen van de tumor voor een periode van 176 dagen), die van de eventuele tweede (ofwel rescue) behandeling (bij 45% van de honden volledige verdwijnen van de tumor voor een periode van 133 dagen), de totale overleving (283 dagen) en de bijwerkingen (aantal en graad) waren identiek voor beide groepen. Analyse van de gegevens toonde dat het niet volledige verdwijnen van de tumor, het hebben van een hoog calciumgehalte in het bloed, een te laag aantal witte bloedcellen, T-cel lymfomen, een hoger lichaamsgewicht en het hebben van tumorcellen in bloed of beenmerg een negatief leffect op de ziektevrije periode en overleving hadden. Het niet gebruiken van prednisolon in de eerste behandeling had geen effect op de resultaten van

de eerste behandeling, maar gaf ook geen verbetering van de resultaten van een eventuele vervolgbehandeling na relapse. Het moment van terugval in relatie tot het einde van de eerste chemotherapie behandeling bleek wel van belang. In het algemeen kan gesteld worden dat hoe langer het duurt voor een terugval optreedt, hoe groter de kans dat een vervolgbehandeling tot een tweede volledige response leidt. Daarnaast blijkt de lengte van een tweede ziektevrije periode gelijk aan die van de eerste. Geconcludeerd wordt dat het gebruik van prednisolon in een op doxorubicine-gebaseerde multidrug-chemotherapie protocol geen meerwaarde heeft voor de behandelresultaten en het veilig achterwege kan worden gelaten. In tegenstelling tot wat werd verwacht, kon er echter ook geen negatief effect van een behandeling met prednisolon worden aangetoond.
Hoewel de behandeling van lymfeklierkanker bij de hond initieel succesvol is, zal de tumor bij het merendeel van de honden terugkomen (de zogenaamde relapse) en blijkt dan vaak minder gevoelig voor verdere chemotherapeutische behandeling zoals ook in de studie beschreven in **Hoofdstuk 4** werd gezien. Het niet reageren op chemotherapie maakt de tumor, door gebrek aan andere therapieën, onbehandelbaar en leidt tot het voortijdig overlijden van de hond. De afwezigheid van een response op chemotherapie, vanaf het begin (intrinsieke CR) van de behandeling als ook later na een initieel goede reactie (verkregen CR), wordt gezien als CR en onder andere in verband gebracht met het actieve uitpompen van de medicatie door transporteiwitten van de ATP-binding cassette(ABC)-familie en dan met name P-gp (ABCB1), MRP1 (ABCC1) en BCRP (ABCG2).
Om een eventuele rol van deze ABC-transporters bij CR bij honden met lymfeklierkanker te bevestigen, werden lymfekliermonsters verzameld van honden met multicentrische maligne lymfoom voorafgaand aan de start van de behandeling met chemotherapie, bij de eerste relapse, en op het moment dat de tumor niet meer reageerde op chemotherapie. In **Hoofdstuk 5** beschrijven we de mRNA expressie van de ABC-transporters *ABCB1, -B5, -B8, -C1, C3, C5,* en *-G2* in 63 honden lymfeklierkanker die allen worden behandeld met hetzelfde op doxorubicine gebaseerde chemotherapie protocol waarvan de helft van de honden ook prednisolon kreeg. Expressie van deze ABC-transporters werd gekwantificeerd in deze tumor monsters en gecorreleerd aan signalement, tumor (sub-)stadium, reactie op behandeling en het type CR (afwezig, intrinsiek, verkregen). Hoewel ABC-transporter expressie in het monster afgenomen voorafgaande aan de behandeling onafhankelijk bleek van geslacht, gewicht en leeftijd van de hond, als ook het WHO-stadium en substadium van de ziekte, werd bij T-cellymfomen en bij aanwezigheid van verhoogd calciumgehalte in het bloed (wat gekoppeld is aan T-cel lymfomen) een toegenomen *ABCB5* en *-C5* expressie te zien in vergelijking met B-cel lymfoom en een normaal bloedcalciumgehalte. Van de 63 honden in de studie toonden 10 geen volledige response en 45 kregen een recidief en 8 kregen geen terugval tijdens de duur van de studie. Een relapse betekent niet automatisch CR, want hoewel in een tweede behandelronde de tumor minder vaak volledig verdween (53% vs 84%), was bijna de helft van de relapse monster was dus niet CR. Klinische werd CR bij 35 (10 intrinsieke CR, 25 verkregen CR) van de 63 honden vastgesteld en was slechts in een deel van de honden met B-cel lymfoom geassocieerd met een verhoogd ABCB1 expressie en met een verhoogde *ABCG2* expressie in de T-cel lymfomen. ABC-

transporter expressie in het monster voorafgaande aan de behandeling, bleek niet in staat te voorspellen wat de kans op volledige verdwijnen van de tumor was, de duur van het verdwijnen van de tumor of duur van de overleving. Anders dan dat in de literatuur wordt gesuggereerd, maar in overeenstemming met onze experimenten in het celmodel, bleek prednisolon niet in staat om de ABC-transporter expressie op te wekken. Er wordt dan ook geconcludeerd dat CR een groot probleem is bij honden met lymfeklierkanker en de oorzaak voor het falen van de behandeling in meer dan de helft van de honden gevolgd door de keuze van de eigenaar om de chemotherapie na 1 of meer recidieven (20 honden) te stoppen. Verder zijn er aanwijzingen voor een verschil in ACB-transporter expressie tussen resistente B- en T-cel lymfomen. Dit onderstreept de noodzaak om B- en T-cel lymfomen als twee aparte entiteiten te zien en benadrukt de noodzaak om routinematig de B-/T-cel bepaling uit te voeren. Vanuit behandeloogpunt moet overwogen worden verschillende behandelprotocollen voor deze twee ziekten te creëren en bij CR mogelijk ook de voorkeur te geven aan verschillenden ABC-transporter-remmers.

Hoewel toegenomen expressie van ABC-transporters dus voorkomt bij honden met lymfeklierkanker, blijkt het niet mogelijk om op basis van deze toegenomen expressie de prognose te voorspellen en dit suggereert het bestaan van andere CR mechanismen. Een van deze mechanismen zou activatie van het zogenaamde *Wnt*-pathway kunnen zijn en activatie van dit pathway blijkt bij de mens een rol te spelen bij CR bij diverse vormen van leukemie en lymfeklierkanker. Om een mogelijke rol van het *Wnt*-pathway in CR bij het maligne lymfoom van de hond te kunnen inventariseren, hebben we de mRNA expressie van een aantal genen betrokken bij dit pathway (*AXIN2, LEF1, SURVIVIN, CYCLIN D1* en *BCL9*) gemeten in tumormonsters van honden met lymfeklierkanker. Deze honden waren allemaal behandeld met hetzelfde op doxorubicine gebaseerde chemotherapie protocol en er werden tumormonsters genomen voorafgaand aan de behandeling met chemotherapie, bij terugkeer van de tumor en op het moment dat de tumor volledige chemotherapie-resistente vertoonde. De resultaten hiervan worden gepresenteerd in **Hoofdstuk 6** en tonen de aanwezigheid van *AXIN2, LEF1, SURVIVIN, CYCLIND1* en *BCL9* mRNA in vrijwel alle tumormonsters, maar laten ook zien dat de expressie van *AXIN2* als *LEF1* in T-cel lymfomen hoger is dan B-cel lymfomen. Dit suggereert een mogelijke activatie van het *Wnt*-pathway in deze subgroep van lymfomen. Leeftijd, geslacht, WHO-stadium, substadium de reactie op chemotherapie-behandeling en het optreden van CR bleken niet geassocieerd met activatie van het *Wnt*-pathway. Geconcludeerd wordt dat de expressie van *Wnt*-pathway genen gebruikelijk is bij honden met lymfeklierkanker en dat activatie van dit pathway mogelijk een rol speelt bij T-cel lymfomen, maar niet bij het ontstaan van CR. Omdat honden met T-cel lymfoom, ongeacht hun behandeling met chemotherapie een slechtere prognose hebben dan B-cel lymfomen, bieden de uitkomsten van deze studie een theoretische basis om verder onderzoek te doen naar de mogelijkheden van het inzetten van modulatoren van het *Wnt*-pathway bij T-cel lymfomen.

In **Hoofdstuk 7** worden de resultaten van de huidige onderzoeken samengevat en aangegeven wat deze hebben toegevoegd aan onze kennis over CR, welke hiaten er zijn, en wordt geëindigd met het doen van suggesties voor verdere vervolgstudies.

9

Dankwoord, CV, Publicaties

Dankwoord

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Promoveren laat zich misschien wel het beste vergelijken met reizen. Het doel van reizen is niet alleen het bereiken van de eindbestemming, maar minstens zo belangrijk is ook de weg er naartoe, het reizen zelf. Reizen maakt je rijker en is *de* manier om nieuwe mensen (en jezelf), ideeën en gebruiken te leren kennen. Hoewel bij reizen het einddoel en de te volgen route in de regel duidelijk voor ogen staan, blijkt dat ondanks alle zorgvuldige planning vooraf de reis toch vaak de nodige onverwachte verrassingen met zich meebrengt. En dan komt het op de vindingrijkheid en flexibiliteit van de reiziger aan om alsnog het beoogde einddoel te bereiken.

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Bilthoven, oktober 2014

Curriculum vitae

Maurice Zandvliet was born in Tilburg on February 1st 1973 and after having finished secondary school (Gymnasium-beta) at the Theresia Lyceum in Tilburg in 1991, he started his training in veterinary medicine at the RUCA in Antwerp, Belgium. Although he successfully completed his first year in Antwerp, he moved to Utrecht in 1992 and continued his training there. During his studies he was an active member of a number of social and professional student organizations including the International Veterinary Student's Association (IVSA), student member of the Faculty board, (co-)organized several symposia and conferences and completed his Honours Program (Master in Veterinary Research) under the supervision of Professor Dr. G. Dorrestein on "Lung fibrosis in Amazon parrots".

After having received his DVM from the University of Utrecht (2000), an Internship and a Residency in Internal Medicine were completed at the Department of Clinical Sciences of Companion Animals of the same faculty. The European board exams were successfully passed in 2007. During this period he qualified for his BKO (junior teaching qualification) and was a board member of the KNMvD (Royal Dutch Veterinary Association) group representing the Dutch Veterinary Specialization and Specialists. Since 2006 he works part-time in private practice in referral center "de Wagenrenk".

In 2008 he changed his focus to medical and radiation oncology and for this purpose additional training was received from the departments of Radiotherapy and Oncology at Utrecht University Medical Centre, the Oncology-Radiotherapy unit at the faculty of Veterinary Medicine (VUW) in Vienna, Austria and the departments of Internal Medicine and Oncology at Colorado State University, USA, as well as additional courses in Radiation Hygiene and Radiobiology in the Netherlands. During 2011-12 an alternative residency program in Medical Oncology was completed at Department of Department of Clinical Sciences of Companion Animals, Utrecht University under supervision of prof dr. E. Teske and he plans to take the European board exams for Medical Oncology in 2015.

In 2007 the first steps were taken towards a PhD-project aimed at characterizing multidrug resistance in canine lymphoma, which has resulted in his thesis that he plans to defend on December 11th 2014.

He is a board member of the Dutch Veterinary Cancer Society (NKFD) since 2009 and of FelCan since 2013.

He is an active speaker on regional, national and international post-graduate training and conferences on both Medicine and Oncology.

Maurice lives in Bilthoven, with his partner Stefanie and their beautiful little girl, Emily (2013). The little spare time left he likes to fill with sports, reading, travelling, and he enjoys drinking a glass of good Belgian beer in the company of friends.

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