Sveriges lantbruksuniversitet
Fakulteten för veterinärmedicin och husdjursvetenskap
Institutionen för kliniska vetenskaper

Immunohistochemical detection of thymidine kinase 1 in canine mammary tumors and lymphomas

Alexandra Leijon

Uppsala

2013

Examensarbete inom veterinärprogrammet

ISSN 1652-8697
Examensarbete 2013:71

SLU
Sveriges lantbruksuniversitet

Immunohistochemical detection of thymidine kinase 1 in canine mammary tumours and lymphomas

Immunohistokemisk detektion av thymidinkinas 1 i juvertumörer och lymfom hos hund

Alexandra Leijon

Handledare: Henrik von Euler, Institutionen för Kliniska Vetenskaper

Examinator: Staffan Eriksson, Institutionen för Anatomi, Fysiologi och Biomedicin

Examensarbete inom veterinärprogrammet, Uppsala 2013
Fakulteten för veterinärmedicin och husdjursvetenskap
Institutionen för Kliniska Vetenskaper
Kurskod: EX0736, Nivå A2E, 30hp

Key words: mammary tumor, lymphoma, dog, canine, thymidine kinase, immunohistochemistry
Nyckelord: juvertumör, lymfom, hund, thymidinkinas, immunohistokemi

Online publication of this work: http://epsilon.slu.se
ISSN 1652-8697
Examensarbete 2013:71

TABLE OF CONTENTS

SUMMARY

The dog has often served as a model animal for humans in scientific studies. This is due to the fact that dogs are susceptible to a wide range of diseases which also affect humans. One example is cancer, a condition that affects dogs as well as owners. Neoplastic diseases account for 18-23 % of all deaths in dogs (Bonnet *et al.,* 2005 and Jagielski *et al.,* 2002).

Mammary tumor, or tumor of the breast, is the most common tumor form in intact dogs and women (Im *et al.,* 2013). Mammary tumors originate in the udder, and are classified according to their histological characteristics. The dog has five pairs of mammary glands, and tumors are most commonly found in the most cranial and caudal of the glands. Lymphoma, or lymphosarcoma, is defined as a malignant tumor form originating from lymphocytes. It is the most common hematopoietic tumor in dogs. The incidence of lymphoma may be even higher in dogs than in humans (Jagielski *et al.,* 2002).

Thymidine kinase 1 (TK1) is a cytosolic enzyme expressed during the S-phase of the cell cycle. It catalyzes the phosphorylation of deoxythymidine into deoxythymidine monophosphate (dTMP). dTMP is then phosphorylated into deoxythymidine triphosphate (dTTP), which can be used as a substrate in the salvage pathway synthesis of DNA. The availability of dTTP is the rate limiting step in the DNA synthesis. Measurement of the activity of thymidine kinase in serum has been utilized to determine prognosis and result of treatment in animals and humans with hematopoietic malignancies. However, some patients with solid tumors do not express elevated levels of thymidine kinase in serum, and this has been a problem in past studies focused on serum assays.

The aim of this study is to investigate the expression of TK1 in tissue samples of lymphomas and mammary tumors immunohistochemically. Immunohistochemical detection of the proliferation marker TK1 may theoretically aid routine examination of histological samples postsurgically. Analysis of TK1 in tissue samples may be of use to establish minimal residual disease (MRD), and an elevated expression of TK1 may be indicative of a non-successful surgical treatment result.

20 mammary tumors (40% benign lesions) and 17 lymphoma cases were analyzed immunohistochemically at the National Veterinary Institute in Uppsala, Sweden. Out of those 17 lymphoma cases, 9 were of B-cell origin and 8 were of T-cell origin. 4 samples of normal lymph node tissue and 3 samples of normal udder tissue were also analyzed. The tissue samples used in this study had been previously fixed in formalin and were available in paraffinized sections. Tissue sections of 2-3 µm were deparaffinized and labeled with an anti-TK1 antibody (XPA 161, clone 528-2, Arocell). A labelled streptavidin-biotin system was used for detection (LSAB^{TM}, Dako) and 3,3´-diaminobenzidine (DAB) was used as chromogen. An appreciation of the percentage of positive cells was made manually by light microscopy and a mitotic index simultaneously counted. The expression of thymidine kinase was plotted against the mitotic index to see if a correlation existed.

The chosen method did not render positive results in mammary tumor cells. This was due to dissatisfactory signal intensity and background staining. Some staining was apparent in capsular lymphocytic infiltrations and in the stroma of two tumors. The median percentage of positive cells in lymphoma samples was 0,8% for B-cell lymphomas and 1,8% for T-cell lymphomas. The median value in normal lymph nodes was 1,3%. The expression of thymidine kinase was not correlated to mitotic index, and could not be statistically validated due to insufficient amount of samples.

In conclusion, there are indications that this method could be of use on tissue samples from patients with lymphoma. If the method is refined, detection of thymidine kinase may be of value to detect minimal residual disease. However, future studies will require trials of new antibodies and perhaps the use of an alkaline phosphatase based system. It would also be advisable to work with live patients, in order to analyze serum samples simultaneously.

SAMMANFATTNING (SUMMARY IN SWEDISH)

Hunden har fått tjäna som modelldjur för människan i många vetenskapliga studier. Detta beror på att hunden drabbas av samma typ av sjukdomar som människan. Ett exempel är cancer, som ofta drabbar såväl hundar som deras ägare. Neoplasier orsakar 18-23 % av dödsfallen hos hundar (Bonnett *et al.*, 2005 och Jagielski *et al.,* 2002).

Juvertumörer, eller tumörer i bröstet, är den vanligaste tumörformen hos intakta tikar och kvinnor (Im *et al.,* 2013). Juvertumörer har sitt ursprung i juvervävnaden och klassificeras enligt histologiska kriterier. Hunden har 10 juverdelar (fem par), och juvertumörer återfinns oftast i de mest kraniala och kaudala delarna. Lymfom, eller lymfosarcom, är en malign tumörform som har sitt ursprung i den lymfocytära cellpopulationen. Det är den vanligaste hematopoetiska tumörformen hos hundar. Incidensen av lymfom uppskattas vara högre hos hundar än hos människor (Jagielski *et al.,* 2002).

Thymidin kinas 1 (TK1) är ett cytosoliskt enzym som uttrycks under cellcykelns S-fas. Det katalyserar fosforyleringen av deoxythymidin till deoxythymidinmonofosfat (dTMP). dTMP fosforyleras sedan vidare till deoxythymidintrifosfat (dTTP), som i sin tur kan användas som substrat i salvage pathway syntesen av DNA. Tillgången på dTTP är det hastighetsbestämmande steget i DNA-syntesen. Mätningar av aktiviteten av TK1 med serum som provmaterial har använts för att bestämma prognos och undersöka behandlingsresultat hos djur och människor med hematopoetiska neoplasier. Många patienter med solida tumörer uppvisar dock ingen förhöjning av enzymaktiviteten i serum, och detta har varit ett problem i tidigare studier där serum har använts som provmaterial.

Syftet med denna studie är att undersöka uttrycket av TK1 immunohistokemiskt i vävnadsprover från hundar med juvertumörer och lymfom. Immunohistokemisk detektion av proliferationsenzymet thymidinkinas kan teoretiskt bli ett framtida hjälpmedel vid rutinmässig histologisk undersökning. Metoden skulle kunna ha en framtida applikation vid vävnadsundersökningar på extirperad vävnad efter kirurgiska ingrepp. Analys av TK1 i vävnadsprov skulle kunna vara av värde för att fastställa minimal residual disease (MRD) efter cytostatikabehandling, och ökat uttryck av TK1 i vävnad kan teoretiskt indikera att ett kirurgiskt ingrepp ej lyckats avlägsna en förändring med marginal.

20 juvertumörer (40 % benigna lesioner) och 17 lymfomfall analyserades immunohistokemiskt på Statens Veterinärmedicinska Anstalt (SVA) i Uppsala. Av de 17 lymfomfallen var 9 B-cellslymfom och 8 T-cellslymfom. 4 prov från normala lymfknutor och 3 prover från normal juvervävnad analyserades med samma metod. Vävnadsproven hade tidigare fixerats i formalin och fanns tillgängliga för studien i paraffinklossar. Vävnadssnitt om 2-3 µm avparaffinerades och märktes in med en anti-TK1 antikropp (XPA 161, klon 528-2, Arocell). Ett streptavidin-biotin system användes för detektion (LSAB^{TM}, Dako) och 3,3´-diaminobenzidin (DAB) användes som kromogen. En uppskattning av hur många procent av cellerna som var positiva för TK1 gjordes manuellt i ljusmikroskåp. Ett mitotiskt index räknades också manuellt. Uttrycket av TK1 plottades mot mitotiskt index för att se om en korrelation mellan parametrarna förelåg.

Något uttryck av TK1 kunde ej detekteras i juvertumörcellerna med den valda metoden. Detta berodde på otillräcklig signalstyrka i kombination med bakgrundsinfärgning. Dock kunde uttryck av TK1 ses i kapsulära lymfocytinfiltrat och stromat hos två tumörer. Medianuttrycket hos lymfomen var 0,8 % för B-cellslymfom och 1,8 % för T-cellslymfom. I normalvävnaden var medianuttrycket 1,3 %. Uttrycket av TK1 var ej korrelerat till mitotiskt index och kunde ej analyseras med statistisk signifikans på grund av otillräcklig provmängd.

Sammanfattningsvis finns indikationer på att denna metod kan vara användbar på vävnad från patienter med lymfom. Metoden kan ha framtida användning för att fastställa MRD. Dock kommer framtida studier att kräva validering av nya antikroppar och kanske även ett byte till ett alkalinfosfatasbaserat detektionssystem. Det kan också vara av värde att arbeta med levande patienter, så att det blir möjligt att analysera serumprov simultant.

ABBREVIATIONS

ABC	*avidin-biotin complex*
ADP	*adenosine diphosphate*
AgNOR	*argyrophilic nucleolar organizing region*
ATP	*adenosine triphosphate*
AZTMP	*3´-azido-deoxythymidine monophosphate*
CD	*cluster of differentiation/designation*
CHOP	*cyclophosphamide, hydroxydaunorubicin, oncovin, prednisolone*
CK	*cytokeratin*
CR	*complete remission*
CT	*computed tomography*
DAB	*3,3´-diaminobenzidine*
DLBCL	*diffuse large-cell B-cell lymphoma*
dT	*deoxythymidine*
dTMP	*deoxy-thymidine monophosphate*
dTTP	*deoxy-thymidine triphosphate*
ECL	*enhanced chemiluminescence*
EGFR	*epidermal growth factor receptor*
ER	*estrogen receptor*
FDG	*(18F)-fluoro-2-deoxy-D-glucose*
FLT	*(18F)-fluorothymidine*
HER2/neu	*human epidermal growth factor receptor*
HPF	*high power field*
IGH	*immunoglobulin heavy chain*
LDH	*lactate dehydrogenase*
kDa	*kiloDalton*
LSAB	*labelled streptavidin-biotin*
MALT	*mucosa-associated lymphoid tissue*
MDS	*myelodysplastic syndrome*
MRD	*minimal residual disease*
NK-cell	*natural killer cell*
PCNA	*proliferating cell nuclear antigen*
PCR	*polymerase chain reaction*
PET	*positron emission tomography*
PR	*progesterone receptor*
Rb	*retinoblastoma protein*
TBS	*tris-buffered saline*
TK	*thymidine kinase*
TK1	*thymidine kinase 1*
TK2	*thymidine kinase 2*
VEGF	*vascular endothelial growth factor*
WBC	*white blood cell count*

INTRODUCTION

Cancer prevalence in the canine population

Cancer is a form of disease that increasingly affects the dog population. The dog has often served as a model animal for humans in oncology studies. Dogs live together with their owners and are exposed to the same environmental factors as humans. It has been shown that the incidence of cancer in dogs in a specific geographical region is similar to that of the human population in the same region (*Jagielski et al.,* 2002). Like humans, dogs spontaneously develop age –related diseases. Furthermore, canines are also susceptible to a range of diseases which coincide with the pathology of humans. However, the clinical development of those illnesses occurs more rapidly in dogs than in humans, which also means that they are easier to study. Tumor of the breast, or mammary gland, is the most common tumor form in women and female dogs (*Im et al.,* 2013).

4 % of dogs with life insurance die each year before the age of 10, according to the Swedish insurance company Agria´s statistics. 68 % of insured dogs live beyond the age of 10 according to this register (Bonnett and Egenvall, 2010). The average age when cancer is diagnosed in dogs is 8,8 years for females and 7,9 years for males according to a Danish study (Brönden *et al.,* 2010). There is an increased risk of developing cancer with increasing age. The risk that a veterinary insurance case concerns a neoplastic disease increases from < 1 % for one year old dogs to 7 % in nine year olds (Bonnett and Egenvall, 2010). Neoplastic disease is the most common reason for euthanasia according to Agria´s statistics. In those cases where a cause for euthanasia was reported to the insurance company, neoplasia accounted for 18 % of the cases (Bonnet *et al.,* 2005). In another study, neoplasia was reported to account for 23 % of death causes in dogs (Jagielski *et al.,* 2002).

In human medicine, cancer registries have been implemented for many years. A cancer registry is defined as an organization that systematically collects information, reports and analyses related to various types of cancer (Nödtvedt *et al.,* 2012). Cancer registries for canine patients are growing in number, and are currently being implemented in countries such as the UK, the U.S, Canada and Italy. A report from the Norwegian dog cancer registry shows that more than 50 % of over 6000 reported tumor cases were of malignant decent or potentially malignant, the Boxer having the highest overall tumor risk according to the referenced study (Arnesen *et al.,* 1995).

There are population-based registries and hospital-based records. The hospital-based registries summarize only the cases treated in a specific hospital. This implicates that an incidence rate cannot be calculated based on numbers from a hospital-based registry. In Denmark, the registration of dogs with cancer is compulsory and a web-based registry is available since 2005 (Nödtvedt *et al.,* 2012). In the neighboring country of Norway, a canine cancer registry has been available since 1990. It is coordinated by the Veterinary School of Norway and collects information from four regions of Norway regarding the incidence of new cancer cases. The data is then compiled with information from the Norwegian Kennel Club for epidemiological purposes (Nödtvedt *et al.,* 2012). In Sweden, the registry of insured dogs in Agria has been used for epidemiological studies on cancer in dogs since 1995. Agria insures 40 % the dog population in Sweden (Bonnett and Egenvall, 2010). Using insurance records for epidemiological evaluation of a population is a useful tool, but has its limitations. It is therefore important to validate such records before they are used for research purposes (Egenvall *et al.,* 2009).

Definitions – mammary tumor and lymphoma

"Mammary tumor" is the collective name for tumors derived from the mammary gland or udder. Mammary tumors can be of benign or malignant character (Misdorp *et al.*, 1999). However, the disease "mammary cancer" (equal to breast cancer in humans) is caused by mammary tumors which are exclusively malignant. Tumors are classified according to their histological morphology. The histological evaluation of mammary tumors is discussed further in Appendix I.

The mammary gland is a modified sweat gland found only in mammals. It is derived embryonically from ectoderm (Pretzer, 2008). In the dog, the development is initiated by the formation of two parallel ventral thickenings of the ectoderm, or milk lines. This becomes visible at 25 days of gestation (Pretzer, 2008). Placodes are formed by migrating cells within the milk lines, and these eventually form the individual milk glands. The female canine usually develops 5 pairs of separate milk glands, although reports exist of dogs having 4 or 6 pairs (Silver, 1966). There are two thoracic (T1 and T2), two abdominal (A1 and A2) and one inguinal pair in the adult dog (Silver, 1966). Mammary tumors are more frequently found in T1 and the inguinal gland, the most cranial and caudal of the glands (Moulton *et al.*, 1970).

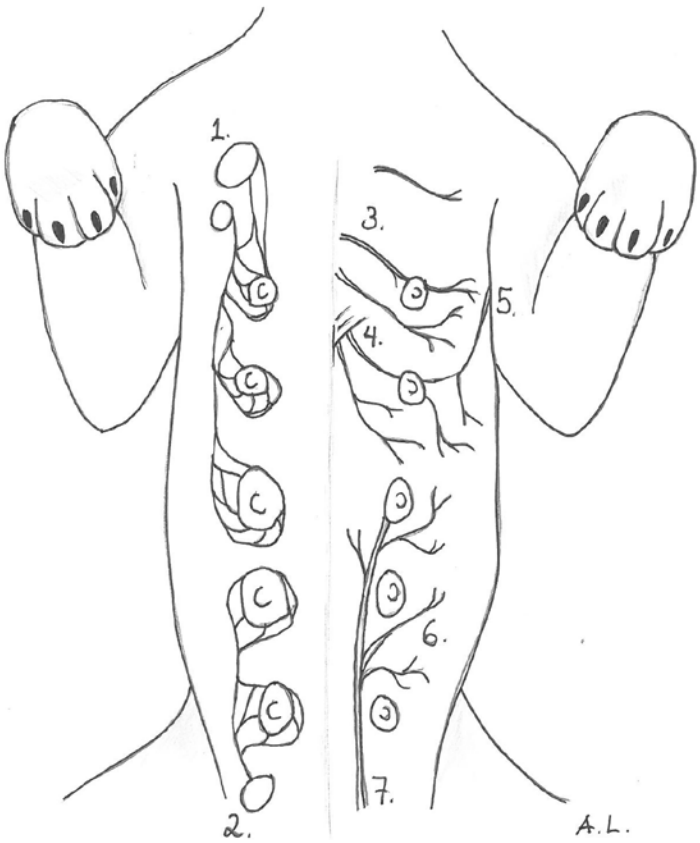


Fig 1. Schematic illustration of the lymphatic drainage (left side) and sources of major arterial blood supply (right side) of the normal canine udder. Done free hand after Slatter, Textbook of Small Animal Surgery, 3rd ed and Dyce, Sack, Wensing, Textbook of Veterinary Anatomy,4th ed. 1. Proper and accessory axillary lymph nodes; 2. Superficial inguinal lymph node; 3. Branches of A. thoracica interna; 4. Branches of A. epigastrica superficialis cranialis; 5. Branch of A. thoracica lateralis; 6. A. epigastrica superficialis caudalis; 7. A. pudenda externa. Arteries are not depiced in their entirety and their routes are purely schematic.

Malignant tumors in the udder have a tendency to metastasize to a variable extent. Carcinomas often metastasize through the lymphatic route, giving rise to daughter tumors in the lung, the liver, bone tissue and the urogenital system (Clemente *et al.*, 2010). In which direction the cancer cells spread within the udder is often determined by the lymphatic drainage of the udder. T1 and T2 (no 1-2) are connected to the axillary lymph centre (proper and accessory lymph nodes). In rarer cases, T1 may communicate with the sternal lymph node. However, the recruitment of the superficial cervical or ventral thoracic lymph centre may be observed in cases of mammary neoplasia (Pereira *et al.*, 2003). The abdominal glands (no 3-4) are drained by the axillary and inguinofemoral lymph nodes. Regarding A1, the axillary centre predominates. A2 is most commonly drained through the inguinal route, but it has been shown that the axillary and popliteal lymph node may be involved in dogs with neoplasia (Pereira *et al.*, 2003). The inguinal gland (mammary gland no 5) commonly connects to the superficial inguinal lymph node and the popliteal lymph node. (Pereira *et al.*, 2003). It has also been found that neoplastic mammary tumors more commonly communicate with the opposite side of the udder (50 % compared to 33 % in healty dogs). Moreover, neoplasias in the cranial abdominal gland are prone to forming lymphaticovenous communications, potentially enabling hematogenous spread (Pereira *et al.*, 2003).

Malignant lymphoma (lymphosarcoma) is a heterogeneous group of tumors derived from lymphocytes (B cells, T cells and natural killer cells). The prognosis differs depending on the histological characteristics of the lymphoma. Patients with T-cell lymphoma have a shorter median survival time compared to patients with B-cell lymphoma (Elliott *et al.,* 2013). Immunohistochemical detection of CD3 and CD79α may assist in determining whether the lymphoma is of B-cell- or T-cell decent (Valli *et al.,* 2013). CD18 can identify the presence of histiocytic lineages. Lymphomas can be roughly divided into high-grade or low-grade lymphomas. High grade lymphoma is the most common form in dogs and results in death within weeks after diagnosis if treatment is not initiated (Marconato *et al.,* 2011). The clinical implication of a low-grade lymphoma is not as severe as for high-grade lymphomas, and they grow slowly. Between 5,3-29 % of all malignant lymphomas in dogs are indolent and are defined as low-grade. Canine patients with low-grade lymphoma have an average survival rate of 4,4 years after diagnosis (Flood-Knapik *et al.,* 2012). In the study by Flood-Knapik *et al.,* samples from 75 dogs with indolent lymphoma were examined. In this study, 61,7 % were T-zone (T-cell lymphoma, average survival time 33,5 months) followed by marginalzone lymphoma with an average survival time of 21,2 months (Flood-Knapik *et al.,* 2012). Subclassification of lymphomas is performed microscopically, and will be further discussed in the section "Histological classification of mammary tumors and lymphoma" in Appendix I.

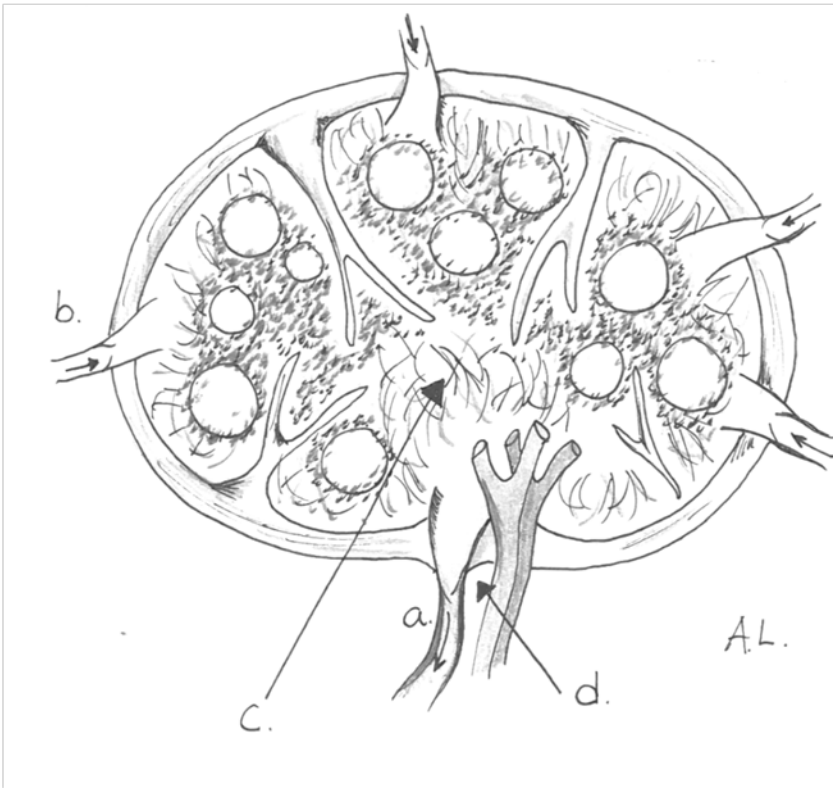


Fig 2. Schematic illustration of a normal lymph node. Done free hand after Schaller, Illustrated Veterinary Anatomy, 2nd ed. a. Efferent flow of lymphatics. B. afferent flow of lymphatics. c. Medullary region. d. Hilar region.

Epidemiology of mammary tumors and lymphoma in the canine population

Denmark has a national veterinary cancer registry established for the purpose of compiling case information regarding neoplastic diseases in the veterinary field. 1878 cases of neoplastic disease in dogs were reported to the registry during the time relapsed from the 15th of May 2005 to the 15th of April 2008 (Brönden *et al.,* 2010). The proportion of malignant tumors was 38 %. 45 % of tumor cases were classified as benign and 17 % remained unclassified. Lymphoma and mammary tumors make up a significant part of the total number of malignant tumors. Out of the 38 % malignant tumors in the Danish cancer registry, 6 % were classified as malignant mammary tumors and 6% were lymphoma cases (Brönden *et al.,* 2010).

Mammary tumor is the most common tumor form in intact bitches (Egenvall *et al.,* 2005). According to the Norwegian cancer registry, 59 % of reported tumors in dachshunds consisted of mammary tumors (Arnesen *et al.,* 1995). According to another Norwegian report, the incidence of malignant mammary tumors in female canines among any of the breeds included in the study is 53,3 % (Moe, 2001). Some studies have reported a higher proportion of mammary tumors, and in others they only make up around 40 % of the tumors in bitches (Sorenmo *et al.,* 2009). Tumors of the reproductive system (including the udder) constituted 28 % of the total number of reported neoplasias according to a Danish study (Brönden *et al.,* 2010). Similarly, the incidence of mammary tumors was 21,5 % according to a Polish study (Jagielski *et al.,* 2002). Some studies report a range in prevalence from 28 % to 91 % percent (Vos *et al.,* 1993). However, this range in prevalence could be due to inconclusive histological grading due to lack of consensus in the previous grading systems (Vos *et al.,* 1993).

The mean age of diagnosis is 8,8 years for mammary tumors in all breeds, and 7,9 and 7,8 years in Boxers and Springer Spaniels respectively (Moe, 2001). The incidence of mammary tumors is very low among dogs younger than 2 years, and a subsequent increase is observed after 6-8 years of age. A peak is seen in the age-group 9-11 years, overall incidence being almost twice as high in pure bred dogs as it is for cross bred dogs of any age category (Dorn *et al.,* 1968). A home-cooked diet and obesity at 1 year of age and 1 year before diagnosis are factors associated with the development of mammary tumors in dogs (Pérez Alenza *et al.,* 1998). In the case-control study by Pérez Alenza *et al.,* the risk of developing mammary tumors was positively correlated to intake of red meat, and negatively correlated to intake of poultry and vitamin A. Odds ratio is 2,32 of developing mammary tumors for female dogs treated with progestins, suggesting hormone status as a risk factor (Stövring *et al.,* 1997).

Dachshunds, Scottish terriers and Golden retrievers have been suggested to have a higher incidence of mammary tumors than others (Moulton *et al.,* 1970). English Springer Spaniels, Cocker Spaniels, Poodles, Yorkshire Terriers, Dachshunds, Maltese dogs, Pulis, German Shepherds, English Setters and Pointers have been considered at higher risk of developing mammary tumors in numerous studies from 1978-2001 (Egenvall *et al.,* 2005). In a study on 80 000 insured dogs of 280 different breeds, Springer Spaniel, Dobermann and Boxer were found to be at the highest risk of developing mammary tumors when compared to other breeds (Egenvall *et al.,* 2005). In this study, Golden Retrievers were not found to be at higher risk of developing mammary tumors. It is important to note that neutering status was not taken into account in this study. Poodles are more commonly affected by benign tumors, according to a study from 1970 (Moulton *et al.,* 1970). It is important to note, that the validated WHO-system of classification by Misdorp et al dates back to 1999. Bitches are 62 times more likely than males to develop mammary tumors. Furthermore, mammary tumors in male dogs are usually benign and median age of onset is 11,5 years (Saba *et al.,* 2007). In intact dogs, mammary tumors account for 41,7 % of all tumors and 13,4 % in spayed dogs in a study conducted in California between 1963-1966. In this study, the overall cancer incidence in dogs was 381,2/100 000 dog years (Dorn *et al.,* 1968).

In order to keep statistics on spontaneously occurring tumors in the dog population, the Animal Tumor Registry was established in 1985 in Genoa, Italy. 6743 tumor biopsies from dogs were sent to the registry and included in an extensive histological study in the time period 1985-2002. 48,9 % of the material was classified as malignant. 27,1 % of the bitches with malignant tumors were > 11 years old at the time of diagnosis compared to 3,1% of the bitches in the age category < 3 years. The corresponding figures were 30 % and 5,7 % in males, indicating a predisposition for the development of cancer with increasing age. Mammary tumors comprised 70,5 % of all cancer cases in bitches in this study (Merlo *et al.,* 2008). 8,4 and 20,1 % of cancers were non-Hodgkin's lymphoma in females and in males respectively. The incidence rate for all types of canine cancers was 272,1/100 000 dog years at risk for females and 99,3/100 000 dog years at risk for males. This was calculated based upon data from the entire period. Why IR was almost 3 times higher for females than for dogs can mainly be explained by the high proportion of malignant mammary tumors in the female dog population (Merlo *et al.,* 2008). This is supported by other studies, in which the incidence rate for mammary tumors is 205/100,000 dog years at risk (Dobson *et al.,* 2002).

Malignant lymphoma is the most common hematopoietic cancer in dogs and there are reports saying that lymphoma cases make up 20 % of all cancer cases in dogs (Ito *et al.*, 2011). Other reports state that lymphomas only account for 5 % of all cancer cases, with an incidence of 25/100 000 dogs (Elliott *et al.*, 2013). The incidence of tumors of the lymphatic system in Poland is estimated at 4,4 %. However, the incidence of lymphoma in dogs exceeds the incidence of lymphoma in humans (Jagielski *et al.*, 2002). According to other studies, lymphoma cases make up 83% of all hematopoetic neoplasms, corresponding to 7-24 % of all neoplasms in canines (Sato *et al.*, 2012). In a French study from 2010, 7608 biopsies of canine lymphoma cases were studied and classified according to the Kiel-classification system. Out of the extranodal lymphomas (17,6 %), lymphoma was most often present in the skin (12,34 %), spleen (1,80 %), the digestive tract and the tonsils (1,48 % respectively). In this study, no difference was found in the distribution of age and sex in patients affected by B-and T-cell lymphoma. However, Boxers were overrepresented in the study. They accounted for 10,03 % of the lymphoma cases (compared with 2 % in France nationally). 2,32 % of B-cell lymphomas were found in the Boxer population, but they were clearly overrepresented in the number of T-cell lymphoma cases where Boxers accounted for 24,19 % (Ponce *et al.*, 2010).

In a study conducted by Sapierzynski et al from 2010, 100 fine needle aspirates from canine lymphoma patients were analyzed. In this study, 44 % and 56 % were females and males respectively. The median age was 7,5 years, with the spread between 1,5-15 years. According to a Brazilian study, the most affected breed was the mixed breed, constituting 43 % of the samples in the study. The Boxer and German Shepherd represented 14 % and 11 % respectively, and the mean age was 8,7 years with a spread of 5 months to 15 years. 85 % of dogs had T-cell lymphoma, and the rest had B-cell lymphoma (Kimura *et al.*, 2011). Other studies have reported a variation between 6,3-7,7 years (Teske, 1994), and that 60 % of lymphomas in canines are of B-cell origin (Dobson, 2004). Other studies have reported an increase in incidence of lymphoma with increasing age, peaking at 10 years (Edwards *et al.*, 2003). 29 % of the dogs in the study by Sapierzynski presented with lymphadenopathy alone, while 71% of the dogs also suffered extranodal symptoms (for example, weakness, weight loss, fatigue, recurrent episodes of fever). As a comparison, 37 % had lymphadenopathy in the study by Kimura et al; 23 % had extranodal lymphoma, 20 % cutaneous lymphoma, 18 % alimentary lymphoma and 2 % mediastinal lymphoma (Kimura *et al.*, 2011). In the study by Sapierzynski *et al*, 14 % of the lymphomas were classified as low-grade. 28 of the dogs in the study were mixed breed, and the remaining 72 dogs fell into 28 other breed categories. However, this study was not conducted using histopathological analysis; instead, cytopatological analysis was performed with the limitations this implies. Compared to histological analysis, the structural architecture of a tissue cannot be studied, and sample analysis is limited to cell morphology alone.

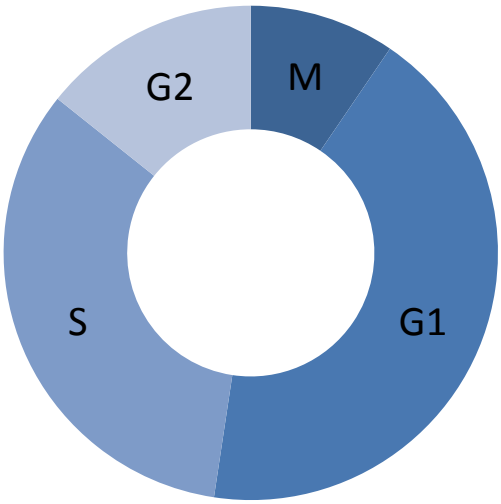
The estimated incidence of lymphoma is 13-33/100 000 dogs each year (Sapierzynski *et al.*, 2010). This author also refers to three French studies, where a breed predisposition has been reported in dogs suffering from lymphoma: the Boxer is overrepresented regarding T-cell lymphoma and German Shepherds more often suffer from B-cell lymphoma. There are studies indicating that the incidence rate for lymphoma is relatively high, 114/100 000 dog years at risk (Dobson *et al.*, 2002). Boxer, Bullmastiff and Bulldog have a higher incidence of lymphoma than other breeds (Edwards *et al.*, 2003). Airedale terrier, Scottish terrier and Bassett hound have an increased relative risk for developing lymphoma, while Pomeranians and Dachshund are considered to be at lower risk compared to other breeds (Teske, 1994). Some chemicals used for lawn care may increase the risk of lymphoma in dogs, however products used for flea control and tick control are not associated with an increased risk (Takashima-Uebelhoer *et al.*, 2012).

BACKGROUND

The concept of tumorigenesis

Normal cell division is strictly controlled. The event is mediated by a variety of factors acting in unison in a process called the cell cycle. A resting cell is set in the G0 phase, or resting phase. When a cell prepares for cell division, it enters the cell cycle in the G1 phase, the phase preceding replication (G stands for "gap"). This phase is also known as the growth phase, during which proteins needed for DNA synthesis are produced. Replication begins in the S phase, and ends when all chromosomes have been duplicated. The G2 phase succeeds the S phase, during which additional cell growth is promoted. Mitosis follows in the M phase, and is divided into a prophase, metaphase, anaphase and telophase. In the prophase, the chromatin becomes condensated and visible and the nuclear membrane disintegrates in prometaphase. This is followed by the metaphase when the chromosomes align for division. The anaphase is the stage when chromosomes are separated and the telophase is the phase when nuclear membranes are formed in the daughter cells. The process of the cell cycle is strictly controlled by molecules such as cyclins and cyclin dependent kinases (CDK), which are expressed during specific phases of the cell cycle. After finishing their purpose, the molecules are degradated in the proteasome, a process which is ubiquitin dependent. Growth factors promote the formation of cyclins, which then bind to CDK and initiate phosphorylation. One important check-point is the restriction point in the transition from G1-S. At this point, retinoblastoma protein (Rb) is hyperphosphorylated and factor E2F is activated. E2F in turn induces transcription of other factors needed for further progression in the cell cycle, such as DNA polymerase and thymidine kinase (Berenstein, 2004). The advancement in the cell cycle is inhibited by the retinoblastoma tumor suppressor protein (hypophosphorylated retinoblastoma protein) through direct binding of the E2F family and activation of other corepressors (MacDonald and Dick, 2012).

Fig 3. Schematic illustration of the cell cycle. Length of phase intervals may not reflect relapsed time of each phase proportionately.



In order for a tumor to form, the cell has to break away from its normal cell cycle control. Subsequent recruitment of abilities by the cell to undergo dedifferentiation (anaplasia), become locally invasive or even metastasize will lead to a tumorous development known as cancer. Oncogenes are genes which promote the formation of tumors. Oncogenes can be mutated, or simply expressed at elevated levels in tumor cells. Overexpression may occur secondarily through loss of regulatory activity. Chromosome translocation can also result in the activation of oncogenes. Oncogenes form proteins necessary for cell division or cell differentiation. Many receptors regulating the activity of growth factors are tyrosine kinases, and cancer drugs may target these receptors such as the tyrosine kinase inhibitor mastinib (Masivet®) used to treat dogs with mastocytoma. On the other hand, suppressor genes are genes which promote tumor growth when their normal function is lost. This is the case for a very famous suppressor gene called p53, which in turn activates the p21 pathway. p21 acts as a CDK inhibitor, halting the cell in G1 phase (Schmidt *et al.*, 2010).

The enzymatic function of thymidine kinase 1

Thymidine kinase (TK) is an enzyme expressed during S-phase of the cell cycle. The enzyme regulates the "salvage pathway" synthesis of DNA. There are two forms of TK; TK1 found in the cytoplasm and TK2 in the mitochondria. Previously, thymidine kinase 1 and 2 were thought to be of adult and fetal lineage respectively. In a study from 1971, thymidine kinase purified from human adult cells was considered more responsive to negative feedback than thymidine kinase from fetal cells. The biological behavior of fetal thymidine kinase thus resembled thymidine kinase from tumorous cell lines (Taylor *et al.,* 1972). TK1 catalyzes the phosphorylation of thymidine into thymidine monophosphate. In this reaction, deoxythymidine (dT) is phosphorylated into deoxythymidine monophosphate (dTMP) with adenosine triphosphate (ATP) as the phosphate donor. The reaction can be illustrated as follows:

dT + ATP → dTMP + ADP

dTMP is further phosphorylated into deoxythymidine triphosphate (dTTP) and can then be used as a substrate for the incorporation of pyrimidines in the growing DNA strand (Berenstein, 2004). New strands of DNA cannot be formed without the presence of dTTP, and dTTP is crucial for further replication and cell division. De novo synthesis of pyrimidines is a process that requires many more steps than what is required in DNA synthesis through salvage pathway. Availability of dTTP is the rate limiting step in DNA synthesis, and studies have shown that TK1-activity increases in serum in conditions such as lymphoma in dogs (von Euler *et al.,* 2009).

Fig 4. Simplified illustration of the de novo synthesis of dTMP

$$2\,ATP + CO_2 + Glutamine \xrightarrow[Carbamoyl\ phosphate\ synthetase\ II]{} Carbamoyl\ phosphate + 2\,ADP + P_i + Glutamate$$

$$Carbamoyl\ Phosphate + Aspartate \xrightarrow[Aspartate\ transcarbamoylase]{} Carbamoyl\ aspartate + P_i$$

$$Carbamoyl\ aspartate + H^+ \xrightarrow[Dihydroorotase]{} Dihydroorotate + H_2O$$

$$Dihydroorotate + NAD^+ \xrightarrow[Dihydroorotate\ dehydrogenase]{} Orotate + NADH + H^+$$

$$Orotate + PRPP \xrightarrow[Orotate\ phosphoribosyl\ transferase]{} Orotidine\ 5'\ monophosphate\ (OMP) + PP_i$$

$$OMP \xrightarrow[OMP\ decarboxylase]{} Uridine\ 5' - monophosphate\ (UMP) + CO_2$$

...

$$Deoxy\ uridine\ 5' - monophosphate\ (dUMP) + N^5, N^{10} Methylene\ tetra\ hydrofolate \xrightarrow[Thymidylate\ synthase]{} dTMP + Dihydrofolate$$

The sequence of human and ureaplasma urealyticum TK1 has previously been mapped, and the enzyme consists of a tetramer in its native form. Two domains are present: one αβ-domain and one zinc finger domain, the active site being situated between the two domains. A lasso-shaped structure of 70-88 amino acids makes up the active site. Two β-sheets are joined together via a zinc ion in this enzyme. The C-terminus is an ubiquitin ligase site, responsible for the cell cycle dependent degradation. Thymidine kinase is 89,3 % homologous between dogs and humans. The first 62 amino acids are identical, but the sequences are only 59,5 % identical in the last amino acids. Cellular thymidine kinase purified from canine MDCK is mainly active in a dimer or tetramer form. Western blot analysis confirmed bands of 26 kDa (compared to 25 kDa for human TK1) corresponding to active fractions in the range of 40-100 kDa as determined by radiochemical methods (Sharif *et al.,* 2012).

The role of thymidine kinase in diagnostics

TK-measurement in serum is available for use in human and veterinary medicine to aid diagnosis, prognostics and to predict relapse before the tumor becomes clinically manifest. However, these methods have mainly been of use on lymphoma and leukemia patients. TK-measurement can be used as a tool to predict the progression from myelodysplastic syndrome (MDS) in acute myeloid leukemia (AML) (Musto *et al.,* 1995). In this study, activity of TK1 was measured in serum by a radioenzymatic assay. Only a weak relationship could be found between the development of MDS and levels of lactate dehydrogenase (LDH).

Different assays are available today for measurement of TK1-concentration in serum. A radioenzymatic assay (TK-REA, Prolifigen) is available for use on canine serum samples. An enzyme-linked immunosorbent assay (ELISA) has also been evaluated and been proven to correlate with values obtained by TK-REA (von Euler *et al.,* 2006). Increasing TK-values in serum has been correlated with survival time and is indicative of relapse in dogs with lymphoma. A decline is observed when CR has been achieved, and values seen in dogs in remission do not differ from those obtained in healthy controls (von Euler *et al.,* 2004).

TK LIAISON (DiaSorin) is a non-radiometric immunosorbent assay that uses anti-AZTMP (3´-azido-deoxythymidine monophosphate) antibodies to measure TK1-activity. With this method, TK activity in serum was significantly lower in healthy dogs and dogs with inflammatory conditions, than in dogs with malignant lymphoma and leukemia. Activity was also significantly higher in dogs with leukemia and lymphoma that were not in remission, compared to dogs that were in remission (von Euler *et al.,* 2009). A dot-blot enhanced chemiluminescence (ECL) assay has also been tested on patients with gastric cancer. A decline in TK1 in serum (parallel to the enzymatic half-life of TK1) could be observed in cancer free patients postoperatively, and an increase by 173 % could be observed in patients with distant metastasis 35 days postoperatively. In contrast, enzymatic levels were reduced to 52,3 % in cancer-free individuals, and to 80 % in patients with metastatic disease (Zou *et al.,* 2002). The concentration of thymidine kinase determined by measurement by dot blot ECL is a biomarker for screening of different types of malignancies, as determined by a Chinese study on 35365 people (Chen *et al.,* 2011). This has also been determined in another study, where TK1 levels were related to progression of hyperplasia of the breast and prostate, and development of hepatic carcinoma (Huang *et al.,* 2011). Measurement of thymidine kinase concentration in serum by ECL dot blot is also related to prognosis of patients suffering from non-Hodgkins lymphoma (Pan *et al.,* 2010).

Measurement of TK1 in serum samples from human patients with chronic a lymphocytic leukemia (the most common haematological neoplasm of adults in the Western world) also correlates with survival and prognosis. Chronic Lymphocytic Leukemia (CCL) carries a variable prognosis, where some patients survive long after diagnosis and some patients progress to develop large-cell B-cell Lymphoma (Richter's syndrome). TK has been of diagnostic value in determining which patients are at risk of progressing to this stage. TK-activity is proportional to another prognostic marker, IGH (Immunoglobulin Heavy Chain), for which a mutation rate of > 2 % has been shown to correlate with an indolent clinical development. In the study concluding this, TK1 was measured by chemiluminescent assay (Konoplev *et al.,* 2010). TK-activity in serum as measured by the radioenzymatic Prolifigen assay can be used to assess prognosis independently of other prognostic factors in patients with CLL, such as lymph node size and WBC-count in human patients (Hallek *et al.,* 1999).

In an English study conducted on 73 dogs with lymphoma treated with chemotherapy (first time treatment), TK-levels (i.e. TK1-activity, U/L) fell when remission was achieved. This applied to the dogs that had elevated TK-levels at the beginning of treatment (Elliott *et al.,* 2013). There are applications in veterinary medicine for measuring TK activity in serum. This is done today at the Department of Clinical Chemistry, University of Agricultural Sciences, Uppsala, Sweden. In a study covering 20 dogs with lymphoma or leukemia (1 case of acute hematopoietic leukemia), TK activity in serum was significantly higher for all dogs. TK activity declined as the tumor burden was reduced after chemotherapy in 5 of the dogs (Nakamura *et al.,* 1997).

13 dogs were used as a control group and a blood sample was also drawn on 5 dogs with inflammatory conditions with resulting non-neoplastic leukocytosis. The TK-activity was slightly increased compared to the control group in 2 of these 5 sampled dogs. However, the TK-activity quickly fell in line with declining WBC. LDH activity and TK activity correlated with each other. Although the LDH activity increases in patients with lymphoma (also shown by Marconato *et al.,* 2010), LDH is not a proliferation marker and is also induced in other clinical conditions such as liver disease, myocardial lesions and hemolysis (Nakamura *et al.,* 1997).

TK activity can also be used to predict survival in dogs before initiation of treatment. 44 dogs then were treated with chemotherapy according to the ADRIA-plus-protocol (doxorubicin based). 50 % survival time was 9 months for dogs with TK values < 30 U/L in comparison with 1 month for dogs with TK > 30 U/L. The clinical response to treatment was reflected in the TK values and thus, TK-measurement in serum can be used to monitor the dogs with lymphoma and anticipate relapse before the disease becomes clinically evident (von Euler *et al.,* 2004).

Regarding solid tumors, measurements of the TK enzyme has mainly been studied in human patients with esophageal, cardial and lung carcinoma (Li *et al.,* 2010), thyroid and gastric carcinoma (Chen *et al.,* 2010) and renal cell carcinoma (Luo *et al.,* 2009). Activity of TK1 in serum is positively correlated to number and size of induced colorectal tumors in rats (Kuwa *et al.,* 1996). A study by He et al was conducted on material on material from human patients with non-Hodgkins lymphoma, lung cancer, esophageal cancer and breast cancer. Serum levels of TK1 as measured by ECL dot blot assay were found to correlate with pathological stages and clinical grades in patients with esophageal cancer and lung cancer respectively. In the breast cancer group, levels of TK1 in serum were able to predict relapse 3 months postoperatively (He *et al.,* 2010). As for those patients with lymphoma, TK1 levels were of use in determining 5 year survival before initiation of treatment and 28 days after.

Measurement of TK activity has been used clinically to a greater extent for breast cancer patients than for the other above mentioned tumors. Increased activity of TK among breast cancer patients is associated with worse prognosis for the patient and poorer survival postoperatively. High TK activity also indicates a poorer response to tamoxifen therapy (Foekens *et al.,* 2001). For breast cancer patients, a higher TK1-value in serum as measured by dot blot is related to a 6-7 times higher hazard ratio of developing recurrence (Huang *et al.,* 2012). The problem with measurements of TK1-activity in serum of dogs as well as people with solid tumors has been that some cancer patients do not exhibit elevated TK values compared to healthy individuals (von Euler *et al.,* 2004 and He *et al.,* 2005). It has been shown that the level of TK1 in serum does correlate with cellular level of TK1 within patient categories as divided by tumor volume, and serum TK1 in turn does correlate with the overall tumor burden in a study conducted on 89 human patients with non-Hodgkins lymphoma (Rehn *et al.,* 1995). Therefore, other TK assays such as FLT-PET (see next paragraph) can be evaluated in situ and might be of better use in reflecting any ongoing mitosis. It has been shown in dogs aswell that S-phase activity rates evaluated by DNA-histogram analysis is significantly related to survival in dogs suffering from malignant mammary tumors (Hellmén *et al.,* 1993).

TK has been used as a target enzyme in PET-scans in humans. The patient is injected with a substance tagged with positron emitting isotopes. In the decay of the isotope, a proton is converted into a neutron, a positron and a neutrino. The positron will eventually annihilate with an electron, resulting in the formation of photons. The photons are detected in a ring of scintillyzing crystals surrounding the patient, and an image is formed (Lawrence *et al.,* 2010). Two substances that have been used extensively in PET diagnostics are FDG and FLT. PET scan reflecting the uptake of the positron emitting isotope 3´-deoxy-3´ (F18) fluorothymidine (or FLT) is a more specific method for evaluation of the proliferative activity in a tumor than the traditionally used (18F)-fluoro-2-deoxy-D-glucose (FDG). FDG mirrors glucose uptake. The F-atom at C2 position prevents FDG from being a substrate in glycolysis; FDG "gets stuck" intracellularly (Lawrence *et al.,* 2010). Glucose is a substrate in many tissues. However FLT (substrate for TK) is more specific marker of proliferation (Kenny *et al.,* 2005). FLT is accumulated in primary tumors, lymph nodes and lung metastases of breast cancer patients. The accumulation of FLT is heterogeneous in both primary tumors that metastasis of breast cancer patients, reflecting an uneven distribution of cells in mitosis. The delivery and retention of FLT is higher in cancer tissue than in normal tissue.

Retention of FLT in breast tissues correlates with the proliferation marker Ki67, indicating that retention of FLT is correlated to increased proliferation. Retention of FLT stands in relation to the conversion of FLT to FLT-phosphate by TK1 (Kenny *et al.,* 2005). PET/CT examinations are increasing in veterinary medicine, but it has not been used extensively due to the cost of the analysis. CT mirrors the anatomical image, while a PET scan shows the metabolic profile. PET therefore has a potential area of use to more quickly find malignant processes. However, there is a need for closer study of FLT uptake in animals. FLT uptake is affected by chemotherapy in humans. Doxorubicin reduces absorption of FLT, while 5-fluorouracil, gemcitabine increases absorption of FLT (Lawrence *et al.,* 2010). Because the distribution, uptake and metabolism of FLT are less optimal than for FDG, FDG is still used in tumor diagnostics to a great extent. The uptake of FLT is generally lower than the uptake of FDG. FLT has a potential area of use as an indicator of how the tumor will respond to treatment (Herrmann *et al.,* 2007). A lower uptake of FLT in a tumor after indicates a positive response to the therapy and improved survival. PET-scan, however, is very expensive and is not readily accessible to canine patients as of date.

AIM AND HYPOTHESIS

Immunohistochemical methods have become routine in the diagnostics of tumors. Receptors that have been reported to be of importance for the development of mammary tumors are for example the receptors for estrogen and progesterone (ER/PR), BRCA1, p53, E-cadherin and HER2/neu and mitosis markers such as Ki67, AgNOR and PCNA (more on the pathogenesis can be read in "Appendix I"). As described in the "Background" section, analysis of thymidine kinase in serum samples has its limitations due to the heterogenecity of the activity of thymidine kinase in patients suffering from solid tumors compared to hematological malignancies. PET-scans are expensive and not widely used in clinical veterinary practice. However, it is common to evaluate neoplastic conditions through biopsies or extirpated tissue after surgery. Histological evaluation is the golden standard in oncological diagnostics in veterinary medicine today. Tissue specimens are most commonly fixed in formalin, although other modes of fixation are available. For these reasons, it is relevant to examine whether the detection of TK1 in tissue samples could potentially be used to assess tissue samples from dogs with neoplasia. Mammary tumors and lymphomas were chosen as models due to the widespread occurrence of these conditions in the canine population. Furthermore, these conditions are widespread among the human population as well, and studies on canines are therefore of comparative value. The purpose of this study is to act as a pilot in assessing the methodology for immunohistochemical detection of thymidine kinase in tissue samples from dogs. TK1 is an S-phase-specific enzyme, and therefore reflects active cell division. Such a marker is therefore potentially useful in cancer diagnostics as a complement to traditional histological assessment. If the expression of thymidine kinase is characterized, the method could have potential use in routine clinical pathology. Analysis of samples obtained from mastectomies could serve as a useful tool in determining the prognosis and treatment result after surgery of patients with mammary tumors. Immunohistochemical detection of thymidine kinase could serve as a marker in determining if surgical margins are sufficient, and if additional chemotherapy will be necessary. It could also have potential use in the establishment of minimal residual disease (MRD) after chemotherapy treatment. The current questions for this study are as follows:

- Can TK1 be detected in tissue samples from dogs using the chosen method?
- Is TK1 expressed in mammary tumors and lymphoma from dogs, and if yes, to an equal extent?
- Is there any difference between malignant and benign mammary tumors or B-and T-cell lymphoma in their expressions of TK1?
- Is there a difference in TK1 expression in neoplastic tissue compared to non-neoplastic tissue?
- Is the expression of TK1 related to the mitotic index?

The hypothesis is that the expression of TK1 will increase with increasing levels of mitotic activity (and malignancy in mammary tumors, since mitotic activity is a criterion in assessment of malignancy). Moreover, the expression of thymidine kinase will be evident to some extent in all of the lymphoma samples (and more apparent in T-cell lymphomas). The expression of thymidine kinase would also be positively correlated to the mitotic index.

MATERIALS AND METHODS

Tissue samples, previously fixed in formalin and embedded in paraffin, were retrieved at the archive of the Institution of Pathology and Wildlife Disease at the National Veterinary Institute, Uppsala, Sweden. 23 mammary tumors from 12 dogs and 16 lymphoma samples were originally included in the study. Out of those 23 tumor samples, 3 had to be excluded due to excessive cartilage formation. All of the mammary tumors were originally biopsy specimens sent to the National Veterinary Institute between the years 2010-2012. 3 of the lymphoma cases were necropsy specimens, the other were biopsy specimens sent in between 2010-2012. Samples of normal mammary tissue (3 samples) and normal lymph nodes (4 samples) were collected at necropsy at the Institution of Pathology, Swedish University of Agricultural Sciences. Samples were analyzed according to the method described below.

Paraffin embedded tissue samples were cut into 2-3 µm thick sections and deparaffinized in xylene, 2 x 10 minutes. Tissue sections where then passed through declining concentrations of ethanol: 99,5 % ethanol, 2 x 5 min, 95 % ethanol 1 x 5 min, 70 % ethanol, 1 x 5 min and finally distilled water 5 minutes. The sections were washed with TBS (Tris-Buffered Saline, 0.05 M, pH 7.6) in 5 minutes and incubated in Target Retrieval Solution in a microwave oven, 750 W, 8 min and 350 W, 14 minutes in order to break the protein – aldehyde bonds. Sections were cooled off in 20 minutes at room temperature, rinsed in tap water for 20 minutes and washed in the TBS buffer in 5 minutes. To block endogenous peroxidase, the samples were incubated in 0,3 % H_2O_2 diluted in TBS for 30 minutes, after which the sections were washed with TBS-buffer in 5 minutes. The sections were incubated in 2 % BSA (Bovine Serum Albumin) for 30 minutes in room temperature in a humidity chamber in order to block nonspecific binding sites. After this step, the sections were incubated with the primary antibody (XPA-161, clone 528-2, AroCell) diluted 1: 200 in 1 % BSA, and incubated in a humidity chamber at room temperature for 2 hours. Sections were then washed for 7 minutes in TBS after incubation. LSAB (labeled streptavidin-biotin system, Dako) was used for immunohistochemical detection. The sections were washed again in TBS-buffer for 5 minutes and then incubated in DAB (3,3´-diaminobenzidine) solution for 7 minutes (DAB solution consisted of 2 ml of DAB, 38 ml of TBS and 40 µl of H_2O_2 added as a catalyst). The sections were washed in tap water for 5 minutes, stained with Mayer's hematoxylin for no more than 2 minutes and then rinsed again in tap water until blue. Sections were passed through 95 % ethanol for 2 x 1 minute, 99,5 % ethanol in 2 x 1 minute and xylene in 2 x 10 minutes and mounted with Pertex. All samples were and examined using light microscopy (Zeiss 25 Standard). Tissue samples stained with hematoxylin – eosin were simultaneously examined to determine degree of malignancy and tumorous spread in the samples. Mammary tumors were divided into subtypes according to the WHO-classification system described by Misdorp *et al*, 1999 with proposed amendments by Goldschmidt et al from 2011. Lymphomas not previously immunophenotyped were immunohistochemically stained with an anti-CD79α antibody and a CD3-antibody to determine whether the tumor was of B-cell (positive to CD79α) or T-cell (positive to CD3) lineage.

Level of thymidine kinase expression was determined by examining 10 HPFs (40X). 100 cells in each HPF were counted and assessed regarding positivity for TK1. Results were added and divided by 10 to render a mean positive percentage of cells in the sample. Mitotic index was evaluated for the lymphoma samples by counting the number of cells in mitosis in 10 HPFs (40X). Mitotic index was plotted against expression of TK1 to determine if a correlation between the parameters could be established using the SDSS statistics program from IBM.

RESULTS

Mammary tumors

The distribution of breeds, ages and tumor types in the mammary tumor group is listed in table 1. Some dogs had multiple histological diagnoses, each diagnosis representing one sample. Age distribution was between 4-11 at time of diagnosis, with a mean age of 8,2 years. Two of the dogs (16,7%) were of mixed breed, the rest of the dogs were of eight different breeds.

Table 1. List of mammary tumors assessed in this study

Breed	Age	Diagnosis
Mixed Breed	10	Benign mixed tumor, complex carcinoma
Mixed Breed	8	Benign mixed tumor, complex carcinoma
German Shepherd	9	Tubular carcinoma, simple type
Papillion	10	Tubular carcinoma, simple type; solid carcinoma
German Shepherd	6	Benign mixed tumor, adenoma, simple type; complex carcinoma
Poodle	8	Benign mixed tumor, tubular carcinoma, simple type
Irish Setter	4	Benign mixed tumor, carcinoma complex
Irish Setter	8	Benign mixed tumor, complex carcinoma
Bichon Havanais	8	Complex carcinoma
Afghan Dog	11	Complex carcinoma
Poodle	Unknown	Tubular carcinoma, simple type
Schnauzer	8	Adenoma, simple type

Table 2. Distribution of mammary tumor samples

	Frequency	Percent
Adenoma, simple type	2	10 %
Benign mixed tumor	6	30 %
Complex carcinoma	7	35 %
Solid carcinoma	1	5 %
Tubular carcinoma, simple type	4	20 %

Due to excessive background staining and inadequate signal intensity in the epithelial components, samples could not be assessed immunohistochemically regarding TK1. The staining was too weak to be rendered positive, and the staining intensity was not stronger than that found in normal mammary epithelium. Normal mammary tissue samples came from one Dachshund (8 years), one Rottweiler (6 years), and one Bassett Griffon de Verden (8 years).

Thymidine kinase expression was found in capsular lymphocytic infiltrations in two of the samples. No statistically significant difference was found in normal samples compared to mammary tumors, since normal mammary gland epithelium expressed the same signal intensity and level of background staining.

Lymphomas

17 cases of lymphoma were examined. 3 cases were necropsy cases (17,6 %), the rest were biopsy specimens. Out of the 17 cases included in the study, 9 (53%) were of B-cell lineage and 8 (47 %) were of T-cell lineage. Ages of the dogs included in the study varied between 1-14 years, with a mean age of 7,6 years (dates of birth not known in the majority of the cases). One dog (5,9 %) was of mixed breed, for two of the dogs (11,8 %) the breed was not known, and the rest of the dogs came from ten different breeds. The expression of thymidine kinase in lymphoma samples is listed in table 6.

Table 3. Specification of breeds, ages and expression of thymidine kinase 1 in lymphoma samples

Breed	Age	Localization	Type	Biopsy/Necropsy specimen	Mitotic index	TK1-expression (mean % of tissue)
Bassett Hound	7	Lymph node	B	B	16	1,6 %
Rottweiler	8	Spleen	B	B	11	0,8 %
Golden Retriever	8	Spleen	B	N	4	0,4 %
Jack Russell Terrier	11	Lymph node	B	B	35	0 %
Nova Scotia Duck Tolling Retriever	4	Lymph node	B	B	12	2,6 %
Unknown	8	Subcutaneous	B	B	5	0,1 %
Rottweiler	5	Lymph node	B	B	17	0 %
Dachshund	9	Subcutaneous	B	B	3	1,7 %
Golden Retriever	8	Lymph node	B	B	2	0 %
Mixed Breed	7	Skin	T	B	12	2,9 %
Dachshund	1	Lymph node	T	B	2	0 %
Cavalier King Charles Spaniel	8	Skin	T	N	19	0 %
Unknown	8	Skin	T	B	13	14,9 %
Finnish Sptiz	10	Spleen	T	B	4	0,7 %
Flat Coated Retriever	Unknown	Alimentary	T	N	1	0 %
West Highland White Terrier	14	Skin	T	B	5	26,1 %
German Shepherd	5	Oral mucosa	T	B	6	0 %

4 samples from animals suffering from non-neoplastic conditions were also examined. All of these samples were collected at necropsy. Originally, 5 samples were collected but one lymph node was excluded due to fatty involution of the parenchyma. All of the animals showed low degree of decomposition.

Table 4. Expression of thymidine kinase in normal lymph nodes

Breed	Age	Cause of death	Expression of thymidine kinase 1 (mean % of tissue)
Rottweiler	6 years	Epilepsy	1,4 %
Samoyed	2 months	Aortic stenosis	0 %
Pug	9 years	Myelodegenerative disorder	2,6 %
Whippet	7 years	Traumatic injury to spine	1,1 %

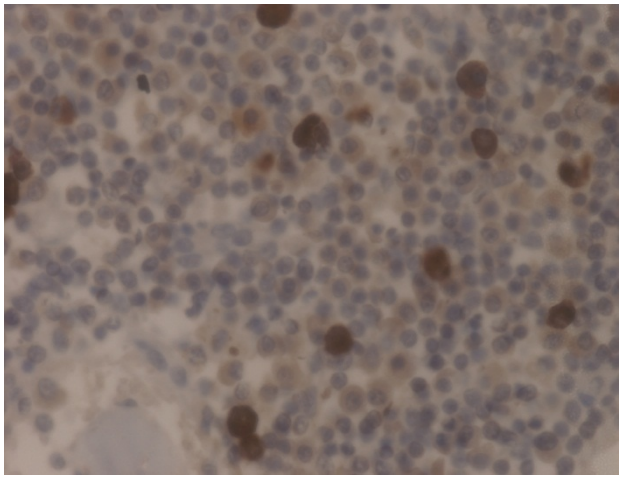
Differences in mean thymidine kinase expression could not be statistically assessed due to a low number of samples. A mean percentage of cells expressing thymidine kinase expression could not be calculated due to inadequate amount of samples and wide range in thymidine kinase expression. A median value was calculated as a comparison. Cells showed strong staining intensity for thymidine kinase.

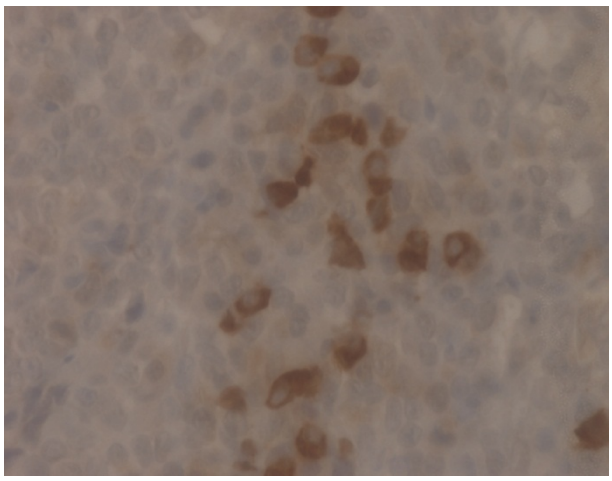
Table 5. Comparison of TK1 expression in B-cell lymphomas, T-cell lymphomas and normal lymph nodes

	Median thymidine kinase expression (% of tissue)	Range
B-cell lymphomas	0,8 %	0-26,1 %
T-cell lymphomas	1,8 %	0-2,6 %
Normal lymph nodes	1,3 %	0-2,6 %

Expression of thymidine kinase was not correlated to mitotic index.

Fig 5 (left) and 6 (right). Cells strongly positive to TK1 in a normal lymph node (fig 5) and in a T-cell lymphoma of the skin (fig 6), 50X.





Lymphatic tissue from animals suffering from neoplasia

Two lymph nodes from animals suffering from complex carcinoma (2) and lymphoma (1) without metastatic involvement were also examined. These lymph nodes expressed a high percentage of TK1 when compared to other samples. Median percentage of thymidine kinase expression was 3%.

Table 6. Expression of thymidine kinase 1 in lymph nodes from animals suffering from neoplastic disease without metastatic involvement

Breed	Age	Diagnosis	Expression of thymidine kinase 1 (mean % of tissue)
German Shepherd	6	Complex carcinoma	3 %
German Shepherd	Unknown	Complex carcinoma	0,4 %
Flatcoated Retriever	Unknown	Alimentary T-cell lymphoma	6 %

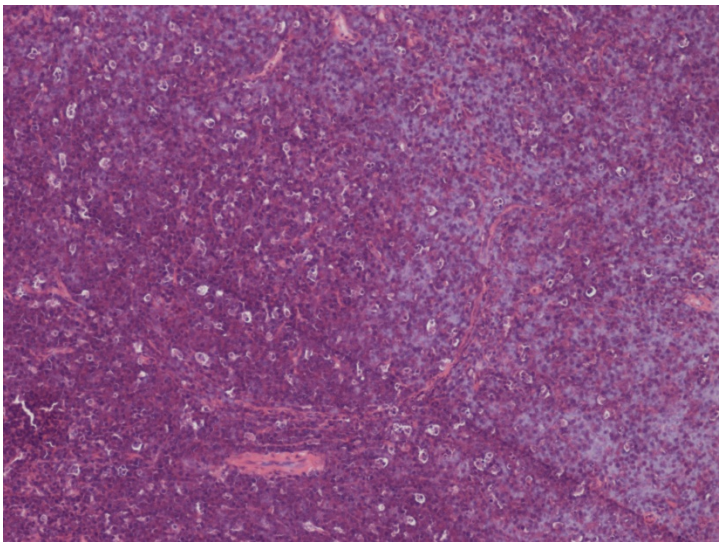
DISCUSSION

A statistically significant increase in the expression of thymidine kinase compared to normal tissue samples could not be determined in this study. The following discussion will regard possible causes for this, and improvement for future studies.

Preparation of samples

According to the records, 3 out of 17 cases of lymphoma were necropsy cases. As for the normal tissue samples, sampling was performed at necropsy. This has implications on morphological aspects of the tissue due to cadaverous change. Lymph node tissue samples in the "normal" group were taken directly from the site of necropsy into formalin. However, necropsy did not occur until the next day and cadaverous change was 1 on a scale from 0-3. 0 represents a specimen that has gone directly from euthanasia, and 3 represents significant cadaverous change. In the lymphoma group, cadaverous change was 3 for all specimens. This may have had an effect on the outcome of this study, since the cadaverous change in an organ can cause autolysis of cells, denaturation of proteins and further deteriorate the possibility of an accurate interpretation. Moreover, B-cells have been shown to undergo autolysis much faster than any other immunological cell, and within 3 days the cellular membranes disintegrate. Necrotic samples will appear amorphous, and intercellular boundaries will be hard, if not impossible, to distinguish. Even though the majority of the samples were originally biopsy specimens according to records, they could have been the subject of morphologic deterioration if they were not immediately quenched in formalin after sampling. This can lead to dehydration of the sample. Unfortunately, there were no means to control the sampling in this study since archived material was used. This bias has to be taken into account when samples are assessed. It is also important to ensure that the samples are quenched in adequate amounts of formalin (v/v ratio 90 % formalin and 10 % tissue). We can view an example of inadequate fixation here.

Fig 7. Inadequate fixation after sampling gives this specimen a stripy and washed-out appearance. This specimen is a B-cell lymphoma from a lymph node, hematoxylin – eosin stain, 10X.



However, formalin pigments may form if tissues are left in formalin for too long. All types of pigments may affect interpretation of results. There are other fixatives described, which may preserve the morphology of tissue specimens even more than formalin. One example is RCL-2, which has been described to be of use regarding immunohistochemistry with comparable results on detection of the estrogen- and progesterone receptor in breast tumor samples (Delfour *et al.*, 2006). One benefit of RCL-2, is the preservation of DNA integrity. However, formalin is the fixative of use in clinical veterinary medicine today. The reason archived and necropsy material was utilized in this study was due to its availability. This also implicated that procedures on live animals could be avoided for the purpose of the study and archived material was used in its place.

However, fresh biopsy specimens are preferable and it would be advisable to consider fresh biopsy specimens or cell cultures to be used for future assessment of this method. If live animals are used, it will also make it possible to draw blood samples for analysis of the activity of thymidine kinase in serum.

Morphological aspects

Morphology is a key element when it comes to interpretation of results. Problems arose with 3 of the mammary tumors, which simply slided of the glass during preparation. This applied to benign mixed tumors and tumors involving thin strips of skin and/or connective tissue, as can be found in the udder. Presence of cartilage in the samples made them slide of even more efficiently. In order to make the samples stick the samples to the glass, samples were placed in 60^{o}C for 10 minutes before they were placed in the first bath of xylene. Problems occurred after treatment in the microwave and simultaneous incubation with Target Retrieval Buffer. The Target Retrieval Buffer itself has a pH value of 6, compared to the Target Retrieval Buffer with pH 9 which is also available. The buffer is a weak citric acid solution and should not affect the morphology to a great extent. Previous studies on the expression of the androgen receptor has proven the method of antigen retrieval with citrate buffer (pH 6) and microwave pretreatment to be superior to enzymatic treatment with pepsin or trypsin followed by pronase (Shi *et al.*, 1993). The heat provided by the microwave is necessary to break the formalin induced protein-aldehyde bond. If this step is not done, then the tissue is still fixed and cannot be analyzed immunohistochemically. The water bath itself is very gentle, and samples were not placed directly under the faucet. To block endogenous peroxidase in the tissues, samples were incubated with methanol and hydrogen peroxide in a 0,3 % solution initially. However, the methanol was found to deteriorate morphology, and was therefore replaced by TBS for dilution of the 30 % hydrogen peroxide. Alcohol can also have a negative effect on morphology if samples are left to rehydrate for too long. However, it is important that the first steps in xylene and ethanol is properly done, otherwise samples will be rendered impossible to analyze due to the fact that the aqueous solutions used will not be able to penetrate the tissues since the presence of paraffin makes it hydrophobic.

In human medicine, freeze sections often replace formalin fixed sections. However, this is mainly used in human medicine intraoperatively. In freeze section preparation, acetone often replaces the use of alcoholic substances in preparation procedures and the Envision system has been implemented on use on freeze sections. Envision is also horse-raddish peroxidase based, with a dextran coupling for increased sensitivity (Kämmerer *et al.*, 2001). In human medicine, freeze sections have been proven superior to touch imprints in detecting macro- and micrometastases intraoperatively in axillary lymph nodes of patients with breast cancer (Krishnamurty *et al.*, 2009). The implementation of freeze sections in every-day diagnostics is not yet practiced in veterinary medicine and fixation in formalin is the “golden standard” in sample preparation for histological analysis. For this reason, it is advisable to continue developing the method on paraffinized material, since this is of greatest value to veterinary clinical pathology.

The antibody

Different antibodies and assays have been designed to detect TK1 in serum. These antibodies have been of monoclonal and polyclonal nature. One of those antibodies is XPA 210, which has been designated its name from the central amino acid sequence in the epitope (eXposedProliferationAntigen210). XPA210 has been shown to efficiently detect the presence of thymidine kinase in samples of renal carcinoma from human patients (Kruck *et al.*, 2012). In the referred study, there was a significant difference between samples with renal cell carcinoma and normal tissue (more on this is the section “A comparison to previous studies”). Trials have been made to produce polyclonal antibodies from rabbits, one of those antibodies having the ability to bind to recombinant canine TK1 as well as human TK1, with a 7 amino acid epitope between position 196 and 223 (Nyberg, 2007). It has been shown that intact TK1 is poorly immunogenic, and therefore immunization with peptide fragments has been necessary in order to produce antibodies against TK1.

The antibody used in this study is XPA161. A 23 peptide sequence corresponding to the lasso domain makes up XPA161 (SEQID01). The antibodiy XPA161 can be crosslinked to bovine serum albumin. The peptide is made by crosslinking by NeoMPS Inc in San Diego, producing a peptide of >96% purity consisting of amino acid sequence 161 to 183 of human TK1 (amino acid sequence AYTKRLGTEKEVEVIGGAD KYHS-NH2). The XPA161 antibody is manufactured according to the following procedure:

A cysteine residue is added to the N-terminus and a suspension is made with 100 µg peptide, 500 µg PBS pH 7,2 and mixed with equal amount of Freuds incomplete adjuvans. The antibody XPA161 is produced through immunization of female Blab/c mice, injected subcutaneously with the above-mentioned mixture and then reinjected at 2,4 and 6 weeks with boosters of 50-100 µg of antigen and Freuds incomplete adjuvans. Splenocytes are harvested from the mice and mixed with mouse myeloma cells SP2/0. Appropriate cell lines are selected through ELISA-analysis of cell cultures. The selected cells where cloned 3-4 cycles. Using the ELISA-method, the cell cultures with the highest amount purified antibody were selected, lyophilized and suspended in buffer (Gasparri *et al.*, 2010).

Immunohistochemical trials of the antibody have been performed on human tonsils and lymph node near a cancerous bladder. It has been shown that the antibodies XPA161 and XPA210 react with the same cells as Ki67/MIB-1-antibodies, although MIB-1-antibodies also show reactivity in the nucleus. The study included in the patent document EP 2164954 A2 used an avidin-biotin-peroxidase system (ABC) and the DAB chromogen. Dot blot assays have also been performed using the XPA161 as well as XPA210 on breast cancer samples and lung cancer samples from humans. XPA161 seems to react more with recombinant TK1 than wild type TK1. However XPA210 reacts similarly with both kinds of TK1. XPA210 is produced in a similar fashion to XPA161. However, to produce this antibody, American egg-laying hens are immunized intramuscularly and eggs harvested. The epitope used is the human amino acid sequence no 195-225 (GQPAGPDNKENCPVPGKPGEAVAARKLFAPQ-OH).

The canine TK1 protein is a 242 amino acid protein (human TK1 is 234 amino acids long) according to the NCBI protein database. Searches were made using the queries “thymidine kinase” and “homo sapiens” or “canis lupus familiaris”. The sequence of the canine equivalent is a predicted sequence according to the gene found in locus XP_003639284. The sequences are listed below, the red sequence being the canine version of the enzyme and the black sequence representing human TK1. Differing amino acids are highlighted in yellow and zinc-binding domains highlighted in red (adapted from Sharif *et al.*, 2012):

MSCINLPTVL PGSPSKTRGQ IQVILGPMFS GKSTELMRRV RRFQIAQYKC LVIKYAKDTR 60
MSCINLPTVL PGSPSKTRGQ IQVILGPMFS GKSTELMRRV RRFQIAQYKC LVIKYAKDTR 60

YSNSFSTHDR NTMEALPACL LRDVAQEALG VAVIGIDEGQ FFPDIVEFSE TMANAGKTVI 120
YSSSFCTHDR NTMEALPACL LRDVAQEALG VAVIGIDEGQ FFPDIMEFCE AMANAGKTVI 120

VAALDGTFQR KAFGTILNLV PLAESVVKLT AVCMECFREA AYTKRLGSEK EVEVIGGADK 180
VAALDGTFQR KPFGAILNLV PLAESVVKLT AVCMECFREA AYTKRLGTEK EVEVIGGADK 180

YHSVCRLCYF KKASGPPMGL DSRENKENVL VLVPGKPGEG KEATGVRKLF APQHVLQCSP 240
YHSVCRLCYF KKASGQPAGP DNKEN———— CPVPGKPGE– —AVAARKLF APQQILQCSP 240

AN 242
AN 232

Human TK1 has 11 cysteine residues in total, compared to 8 cysteine residues in canine TK1. However, 4 of these interact with zinc, rendering 7 and 4 free thiol groups respectively. The consequence of this may be formation of oligomers in wild type thymidine kinase, with the possibility of 3 more disulfide bonds in the human version of the enzyme (*Sharif et al.*, 2012). This may affect antibody sensitivity through different exposure of epitopes.

The predicted sequences of XPA161 in the dog and the human are:

Canine XPA161: YTKRLGSEK EVEVIGGADK YHS
Human XPA161: YTKRLGTEK EVEVIGGADK YHS

The presence of an S instead of T in position 167 of the enzyme could mean that the antibody XPA161 might not bind as efficiently to canine TK1 as human TK1. On the other hand, it is merely a conservative substitution from a serine to a threonine residue and should not affect sensitivity to a great extent. However, in the case of the sequence denoted XPA210 (defined as amino acid no 195-225), the sequences are not identical:

Canine XPA210: GPPMGL DSRENKENVL VLVPGKPGEG KEATG
Human XPA210: GQPAGP DNKENCPVPG KPGEAVAARK LFAPQ

For future trials, it would be advisable to try another antibody, for example a tailor made antibody of the XPA210 canine sequence. In order for an antibody to bind, the antigen must be exposed and this sequence must be readily accessible in the folded thymidine kinase protein in vivo. Perhaps it would be advisable to characterize wild type thymidine kinase in tumor samples, to determine if this sequence is expressed as expected. Any mutations or conformational changes to the protein could significantly deteriorate the sensitivity of this method.

Distribution of TK1 in tissue samples and assessment of TK1 expression

One observation made in this study with regards to the expression of TK1, was that it was highly heterogenous. This implies that the method might be less suitable for cytology. In cytology specimens, the architecture cannot be examined, and there is a possibility that one might accidentally miss the area where TK1-activity is the highest, rendering a false negative result. Even if a neoplastic tissue is sampled through biopsy, there is a possibility that the area with the highest TK1-activity is missed in the sampling process. Generally, TK1 activity is highest in the periphery of the tumor, and the center is often subjected to vast necrosis due to lack of blood supply. The heterogenous expression made the assessment of the samples more uncertain. Moreover, assessment of the mitotic index is also a source of bias, since mitotic activity may vary greatly even between lymphoma subtypes. This has also been described in previous studies (Sokolowska *et al.*, 2012).

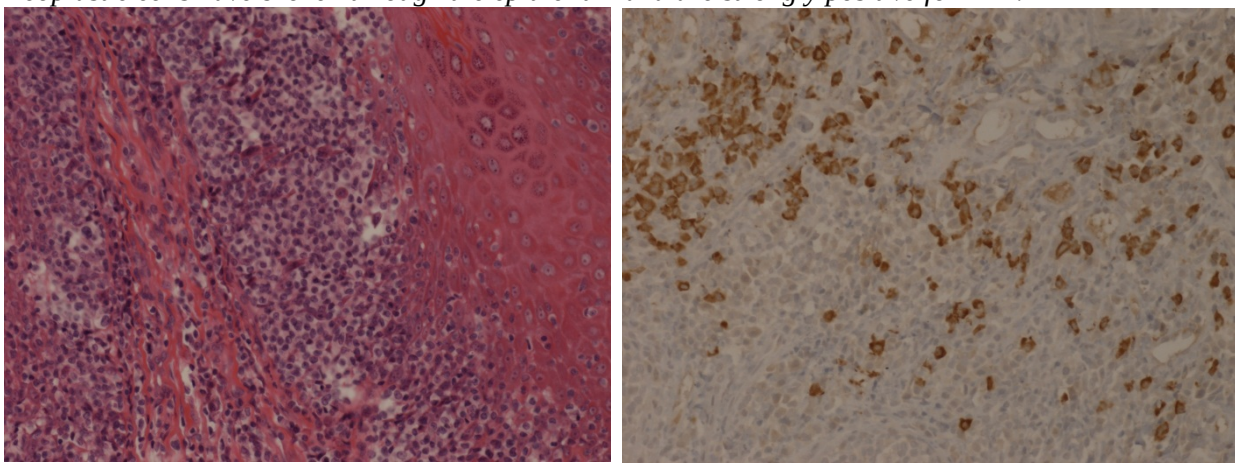
10 HPFs where examined and a mean value of expression was calculated. The fact that TK1-expressing cells seem to muster meant that some of the HPFs had no expression of TK1. The uneven distribution of TK1-expressing cell may render a misleading result. However, the dispersion of TK1-expressing cells may reveal the actual growth pattern of the tumor, and may still be of value in clinical pathology. This fact has to be taken into account in assessment of the samples.

The degree of staining with Mayer´s hematoxylin is also a matter of subjectivity: overstaining a sample might overshadow a weak expression of TK1, and too weak staining renders too much background. However, the cells showing expression of TK1 were strongly positive, and the dilution of the antibody could possibly be increased for lymphoma samples. Inadequate washing of samples and replacement of fluids used in the laboratory is also a source of bias. In this study, samples were stained in Mayer´s hematoxylin for a maximum of 1-2 minutes, and assessed with regards to degree of staining by microscopically during the process. Lymphatic tissue and mammary glands stain easily, and it might be necessary to work out a more standardized protocol for how long a section of 2-3 µm should be left in Mayer´s hematoxylin for the purpose in question.

Some of the samples showed strong expression of thymidine kinase. This became clear when examining samples from dogs with epitheliptropic (T-cell) lymphoma in particular. This is interesting, since other T-cell lymphomas were negative for thymidine kinase. T-cell lymphomas are a heterogenous group of tumors, and express different biological behavior as seen in this study. However, there was a tendency that T-cell lymphomas showed stronger expression than B-cell lymphomas, and this is of interest since patients with T-cell lymphomas often have lower activity of TK1 in serum (Elliott *et al.*, 2013).

One challenge is to quantify the expression of thymidine kinase when the barrier of skin has been invaded with neoplastic cells. When this occurs, particularly in conjunction with granulocytic inflammation, quantification of the expression in the samples becomes uncertain. However, this is indicative of an active lesion, and is not seen in normal skin samples. Before this method can be used clinically, it is vital to establish significant cut-off values, and to assess samples that are merely subject of inflammation, not neoplasia. In this pilot study, a simple approach was chosen to examine the samples. 10 HPFs where examined, and a mean value of TK1-expression was calculated. This might not be the best choice for analysis, because of the effect of the method morphologically on skin specimens in conjuncting with the uneven occurrence of neoplastic cells, particularly in cutaneous lymphoma. Moreover, neoplastic cells invade the skin in epitheliotropic lymphoma, as seen in one case in this study. It is important to note that necrotic debris can drastically make the distinction of cellular borders more difficult, and may also cause "leakage" of TK1 in the sample with additional background as a result.

Fig 8 and 9. Hematoxylin – eosin and TK1-stain (20X) of an epitheliotropic T-cell lymphoma. Neoplastic cells have broken through the epithelium and are strongly positive for TK1.

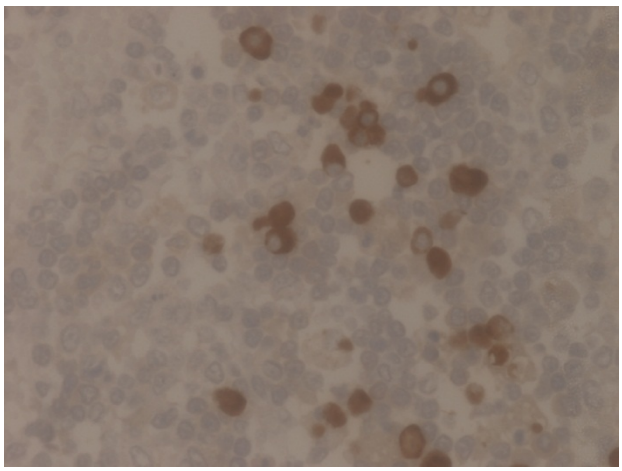


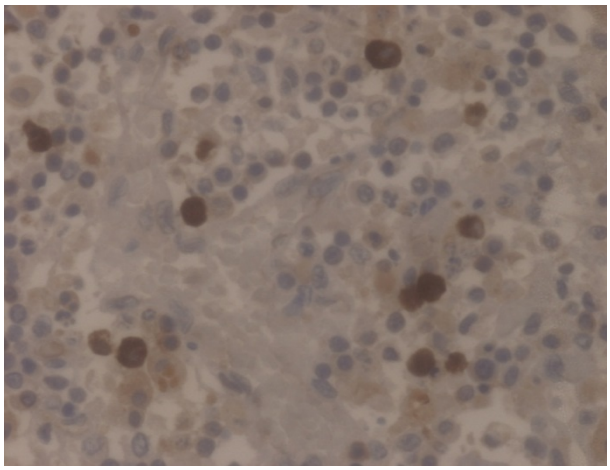
In this study, samples were assessed manually. For future studies, an automatic cell count might reduce the level of subjectivity. In immunohistochemical analysis of the estrogen receptor (ER), staining intensity and proportion of stained tissue is taken into account according to the Allred scoring system. A similar approach might be applicable to immunohistochemical analysis of thymidine kinase. However, the level of subjectivity is too high regarding manual reading at this point due to low staining intensity in mammary tissue. One important observation was the strong expression of TK1 in non-neoplastic samples taken from dogs suffering from known neoplastic conditions (two lymph nodes from dogs with mammary tumors and one sample from a regional lymph node from a dog with alimentary lymphoma). Those lymph nodes also expressed thymidine kinase, to a much higher extent than the primary tumors. This may indicate that the method could indicate cellular activity even when micrometastases are not evident morphologically.

One aspect worth noting is the occasional presence of nuclear staining. This was occasionally observed in non-neoplastic lymphatic tissue from patients with neoplasia (*fig 10*), and in tissue samples of normal lymphatic tissue from patients not suffering from neoplasia (*fig 11*). Nuclear thymidine kinase may reflect non-specific binding sites, or the presence of actual thymidine kinase in the nucleus. If the nuclear binding is specific, the presence of thymidine kinase in the nucleus might be an early indication of malignant progression. It is particularly interesting since nuclear staining was most prevalent in tissues deemed unaffected by neoplastic transformation. Further studies are necessary in order for this to be established. This may play a part in future clinical use of thymidine kinase in detecting minimal residual disease (MRD) after treatment with cytostatic agents.

Presence of nuclear thymidine kinase has also been observed in other studies. Nuclear staining has occurred in other studies using the XPA-210 antibody. Nuclear staining was observed in cases of clear cell renal carcinoma, and the authors concluded that this could be due to a lower epitope specificity of the XPA210 antibody (Kruck *et al.,* 2012). According to Brockenbrough *et al*, cytoplasmic staining was found to be positively correlated with nuclear staining, and the expression of TK1 was positively correlated to Ki67 labelling index in patients with non-small cell lung carcinoma (Brockenbrough *et al.,* 2009). In a recent study on human cervical neoplasia, tissue samples were assessed using immunohistochemical detection of thymidine kinase 1. The XPA-210 was used in this study as well. 216 cases of cervical intraepithelial neoplasia (CIN) and 84 cases of invasive cervical carcinoma were assessed. The study concluded that TK1 was expressed in the cytoplasm alone, or in the nucleus and the cytoplasm simultaneously. Staining for Ki67 was found exclusively in the nucleus. Labelling index for total TK1 (cytoplasmic and nuclear) increased with advancing stage in tissue samples from patients with CIN. The ratio between the nuclear and cytoplasmic TK1 expression increased for patients with CIN from stages I to III. Interestingly, the ratio did not appear to increase further for patients with invasive cervical carcinoma stages II-III. TK1 labeling index was significantly correlated to 5 year survival for patients with invasive cervical carcinoma. This applied especially to the expression of nuclear TK1. Moreover, expression of nuclear TK1 was an independent prognostic factor for patients with invasive carcinoma alone or CIN/invasive carcinoma. Nuclear TK1 could help identify patients with better survival rates, which cannot be achieved by staining for Ki67 (Chen *et al.,* 2013).

Fig 10 (left) and 11 (right), 50X. Staining for TK1 in a lymph node from a dog with lymphoma where the lymph node in question was not deemed affected by microscopy (left). Note the positive staining and also occasional nuclear staning. The right figure is of an architecturally normal lymph node, where positivity for TK1 was also found in the nucleus.





Alternatives approaches to detection with LSAB

In our samples, we detected a high degree of non-specific background staining. This can be attributed to either the antibody or the system used. Switching from LSAB to an ABC based system would probably not solve this issue since avidin, in constrast to streptavidin, is not isoelectrically neutral to tissue and might also bind to carboxylic acid groups (Diamandis and Christopoulos, 1991). The Envision system (Dako) is also a peroxidase based system, but is bound to a dextran backbone for increased sensitivity. The backbone can hold up to 100 enzyme molecules and 20 antibodies per backbone (Ramos-Vara and Miller, 2006). In comparison to LSAB, Envision does not implement the use of avidin-biotin, which may decrease level of background staining according to some studies (Ramos-Vara and Miller, 2006). However, the Envision system still implements detection with peroxidase, a substance which is apparently abundant in mammary tissue (see *fig 15*). The Envision system has been developed for use with antibodies derived from mouse, and could therefore be used with XPA161. The LSAB system is developed for use with antibodies from mouse, rabbit and goat according to the manufacturer. In this study, staining of positive cells was often very obvious and signal intensity in lymphoma samples was deemed satisfactory.

Another way to increase signal intensity in mammary tumor samples could be to decrease the degree of dilution of the primary antibody, or include the addition of biotinyl tyramide. According to one study, the addition of biotinyl tyramide may increase signal intensity by up to 10-100 times. However, the result obtained does depend largely on the primary antibody (Hunyady *et al.,* 1996).

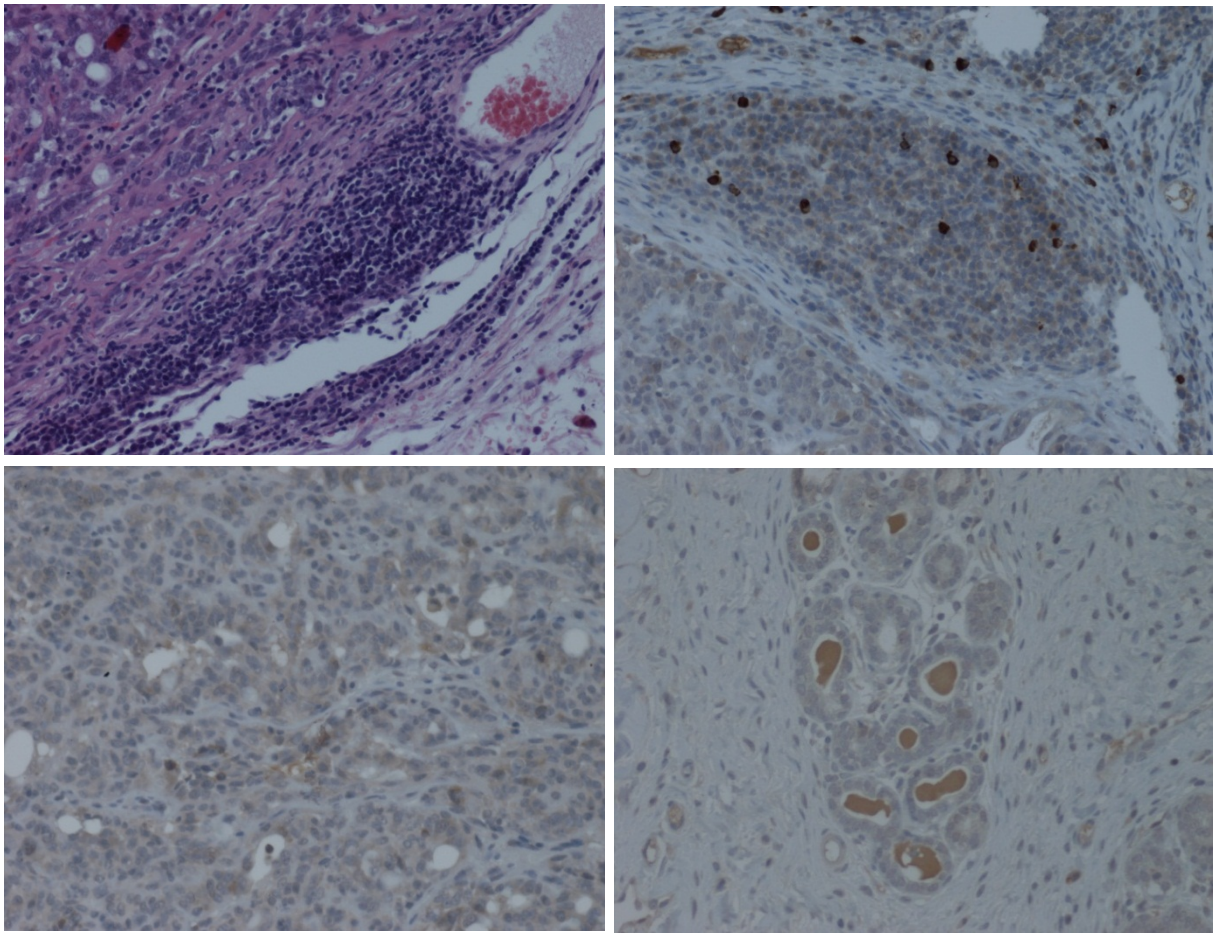
Due to high degree of background staining, an alkaline phosphatase based system might be better suited for this type of immunohistochemistry. This system is not routinely implemented in diagnostics at the National Veterinary Institute in Sweden today. Endogenous alkaline phosphatase is susceptible to blocking by levamisole (Cordell *et al.,* 1984), while calf intestinal alkaline phosphatase is not. The presence of endogenous peroxide and biotin may be a disturbing element in the interpretation of samples, and may be blocked by sodium azide in addition to H_2O_2 (Malorny *et al.,* 1988), and commercially available avidin-biotin blocking kits. One might also consider coating samples in avidin and biotin to rid samples of excess binding sites. Quenching samples in eggwhite, sodium azide and bovine serum albumin as a source of avidin and subsequent coating with skimmed milk as source of biotin has also been described as a blocking method (Miller *et al.,* 1999). In some of the samples, background staining was present in the positive controls but not the negative controls. This can be attributed to properties in the antibody or dehydration of specific samples, especially around the edges of the sections during preparation.

A comparison to previous studies

Although it has not been confirmed in this study, immunohistochemical studies on human tissue have shown that the expression of TK1 in the tissue of breast cancer patients is significantly higher than in normal breast tissue. Tissue histologically classified as normal may stain positively for TK1. This happens in up to 36% of cases, but this tissue stems from the "free margins" from patients with breast cancer, and may therefore have represented pre-cancerous state that was not yet microscopically detectable. 22% of adenosis samples were also positive for TK1, indicating that TK1-expression may be an early event in the development of breast cancer (Alegre *et al.,* 2012). In this study, a lymph node was analyzed from a lymphoma patient. This particular lymph node was rendered not to be involved by the lymphoma, but drained the particular area where the lymphoma was situated. This lymph node had high expression if thymidine kinase. This might indicate that activity can be detected earlier in the disease progression, and that TK might be an aid in the determination if prognosis if the method is refined. This could be in agreement with the findings by Alegre et al regarding the free margins of mammary tumors. Further assessment of thymidine kinase should involve the sampling of primary and secondary tumors as well as lymph nodes, to determine the distribution of the expression of thymidine kinase 1 more precisely.

Expression of TK1 was very weak in mammary tumor samples in this study and could not be assessed as positive without high levels of subjectivity. Some degree of staining was apparent, but not to a greater extent than that found in normal epithelium. However, staining became apparent in lymphocytic infiltrates found in the capsule of one mammary tumor in particular (images below). There was also presence of positive cells in the stroma. The method has to be further assessed for mammary tumors in particular and is not considered for clinical use as of date. The dilution of the antibody was 1:200, and a lower degree of dilution might be considered. However, it might be of value to try a new detection system and a new antibody first and foremost, to determine if detection of TK1 in mammary tumors could be of value. It appears that cells of lymphocytic origin express TK1 to a higher degree than cells of epithelial origin, even after neoplastic transformation. It is not uncommon to spot lymphocytes in the surrounding capsule enclosing a mammary tumor. These cells frequently expressed thymidine kinase. This may implicate that these tumors are an active part of the immune system against the tumor, but it may also suggest that they have a role in aiding the progression of the mammary tumor.

Fig 12 (upper left), 13 (upper right), 14 (lower left) and 15 (lower right). 20X. Example of the strongest staining intensity observed in mammary tumors in this study (a tubular carcinoma, simple type) This carcinoma has high degree of lymphocytic infiltration adjacent to the capsule. Note the expression of thymidine kinase in the lymphocytic infiltrates (fig 12 shows staining with hematoxylin – eosin, fig 13 shows immunohistochemical detection of TK1). In fig 14, we see the staining intensity in the parenchyma of the tumor, which is similar to that observed in normal epithelium (fig 15). The staining intensity of the tumorous parenchyma seen in this picture represents the strongest staining intensity obtained in the mammary tumor group in this study. The staining of the milk can be due to a reaction with the LSAB system and lactoperoxidase in the milk.



Whether or not thymidine kinase is an aid the detection of metastasis could not be determined in this study, and future studies are necessary. More research is required for TK-analysis in tissue samples to become clinically available, especially regarding solid tumors. In human samples, thymidine kinase expression as determined by immunohistochemistry is significantly related to survival in patients suffering from adenocarcinoma of the lung (Xu *et al.*, 2012). In the study by Xu *et al*, Envision and the monoclonal antibody from SSTK Ltd in Shenzhen, China, was used. Renal cells from humans have been studied using the XPA210 antibody. It has been established that expression of thymidine kinase in renal carcinomas accurately reflects the malignancy of the tumor, and that it is significantly different than the expression in normal renal tissue (Kruck *et al.*, 2012). However, results on human solid tumors have been conflicting. Other studies have found levels of TK1 to be naturally higher in normal tubular epithelium than in tissues affected by renal carcinoma or normal glomerular cells (Luo *et al.*, 2010). TK1 has been found to be expressed in squamous cell carcinomas of the uterus and esophagous, and adenocarcinomas of the gastrointestinal system. In this study, the F12 antibody by Abnova was used (Shintani *et al.*, 2010). In a study using the antibody clone F12 from Abnova, Taiwan, TK1-expression was significantly increased in cases of gastric and esophageal carcinoma. In this study, lung adenocarcinomas and renal carcinomas were often negative regarding TK1-expression (Shintani *et al.*, 2009). In this study, thymidine kinase was also expressed in the nucleus.

One problem is that immunohistochemical studies on TK1 have been performed with human tissue rather than canine tissue, making comparability to this study somewhat difficult. There are important similarities between dogs and women regarding the progression of cancer in the breast or udder. Those similarities include the array of receptors that are important in the carcinogenesis late in both dogs and humans. For example, there is a 25% overlap in the genetic profile of metastatic mammary carcinomas in canines and humans. The overlapping genes are mainly linked to proliferation, growth, transcriptional regulation, DNA repair, translation and kinase activity (Klopfleisch *et al.,* 2010). The van´t Ver 70 genetic profile is used to find human breast cancer patients at risk of developing metastases. 34 out of 70 genes in this panel have been identified in the dog, but 68% out of those 34 are of importance to the metastatic potential of canine carcinomas (Klopfleisch *et al.,* 2010). None of the mammary tumors showed a significant expression of thymidine kinase 1, despite the fact that this has been proven to be true in human tissues. This could be due to a species-specific difference, or a factor in the pathogenesis of mammary tumors as discussed below.

Aspects in the pathogenesis of mammary tumors influencing the outcome of the study

The extent to which hormones affect the development of mammary tumors has long been the subject of scientific studies. 80-100% of all intra epithelial lesions express the estrogen receptor. The expression of the estrogen receptor is downregulated in high grade changes such as atypical ductal hyperplasia or carcinoma in-situ (Antuofermo *et al.,* 2007). It has been shown that the estrogen receptor (ERa) and progesterone receptor (PR) is expressed to a greater extent in benign than malignant tumours (Chang *et al.,* 2009). Reduced expression of these receptors has been associated with increased tumor size, lymph node metastasis and other metastasis (Chang *et al.,* 2009). Expression of ER in humans with breast cancer is related to the mitotic index as measured by Ki67. In humans, PR is associated with lower frequency of metastasis, smaller tumor size and lower mitotic index. The lack of expression of ERa and PR is associated with poorer prognosis in both women and canines (Chang *et al.,* 2009). The expression of ER and PR was not investigated in this study, and it was therefore not possible to establish whether the expression of these receptors constituted a confounding bias. However, 40% of the mammary tumors in this study were benign. As previously mentioned, the expression of ER and PR has been found to correlate with the mitotic index as measured by Ki67. It has not been shown how the expression of female sex hormones is correlated to the expression of thymidine kinase.

One bias in this study is that we did not have access to any inflammatory carcinomas. It would be of interest to assess the expression of thymidine kinase in mammary tumors of higher degree of malignancy. Female sex hormones can induce synthesis of other factors which also contribute to cellular proliferation. Further studies are necessary to assess if this also applies to the expression of thymidine kinase. However, the susceptibility of mammary tumors to female sex hormones might be a confounding bias in the assessment of thymidine kinase expression. It would be advisable to also test the method on solid tumors of another origin.

Future studies

Micrometastases can be hard to detect, and this has been established in previous studies (Kohlberger e*t al.,* 2001). It is a possibility that thymidine kinase detection immunohistochemically is a more sensivite method for detection of cellular activity than ocular inspection. In this study, we could find cells that were clearly positive to thymidine kinase in tissue samples that were not neoplastically involved by ocular inspection. In the future, studies should also be focused on samples of peripheral lymph nodes from animals with neoplasia, and not just the primary tumors. Similarly, thymidine kinase might be a useful tool in differentiating cutaneous lymphoma from cutaneous inflammation. For this to be assessed, cutaneous biopsies from animals with non-neoplastic cutaneous disorders should be assessed as well. Also, it would be of interest to examine serum samples and biopsies from patients before and after chemotherapeutic treatment, and for this type of study, live animals have to be included. Perhaps thymidine kinase can be used to evaluate MRD if the method is refined. If so, this could be of use to determine treatment results, and an aid in selecting patients in need of chemotherapeutic treatment after surgery.

However, this requires control over the sampling process, to ensure that the peripheries of the tumors are included in the samples. We did not get clear signals from the mammary tumors in this study. However, they were 40% benign and many of them were of low degree of malignancy (35 % were diagnosed as complex carcinoma, 7/20 tumors). In future studies, tumors of higher degree of malignancy should be included, and inflammatory carcinomas be investigated.

One problem with immunohistochemistry is the aspect of sample preparation and morphology. It would therefore be of interest to evaluate the expression of thymidine kinase in tissue samples by other methods, such as Western blot or dot blot. Another aspect is to attempt to detect the expression of mRNA rather than protein immunohistochemically to eliminate the bias of postmortal proteindenaturation. Another approach is to perform protein extraction on tissue samples to lower the degree of subjectivity in the analysis. First step is to tailor an antibody from a canine epitope, to ensure specificity to canine TK1. Also, it would be advisable to try other detection systems to lower the degree of background staining. In this study, an AUC-curve could not be calculated since there is no golden standard protocol for this type of analysis as of date. The method needs further assessment for this to be established.

CONCLUSIONS

The method needs further evaluation regarding morphological aspects in the treatment of tissue samples, the antibody and the use of LSAB as detection system. It appears that lymphocytic tissue is more prone to express TK1, and that T-cell lymphomas express TK1 to a greater extent than B-cell lymphomas. This could be of future use in the detection of minimal residual disease. The expression of TK1 by mammary tumors in this study was inconclusive and weak due to background staining in combination with weak signal intensity. One bias was the proportion of benign lesions and the lack of inflammatory carcinomas. In future studies, it would be interesting to sample the periphery of the tumors to determine whether or not this technique is of use in postsurgical histological assessment. Regional lymph nodes from patients with neoplasia should also be evaluated in conjunction with the primary tumors. It would also be of interest to examine TK1 activity in serum samples simultaneously, to determine the level of agreement in the expression of thymidine kinase 1 in serum samples versus tissue samples.

APPENDIX I – PATHOGENESIS AND HISTOLOGY OF CANINE MAMMARY TUMORS AND LYMPHOMAS

The pathogenesis of mammary tumors and lymphoma

Although uncontrolled proliferation is essential for the development of cancer, the pathogenesis is far more complex and cannot be reviewed in its entirety here. However, it is important to recognize some specific factors that might influence the different biological behavior patterns of these canine mammary tumors and lymphoma.

The estrogen receptor, ER, is expressed in 65% of tumors of human breast cancer patients, and 50% of mammary tumors from dogs (Yang *et al.,* 2006). ER and PR are not expressed at all inflammatory carcinomas (Millanta *et al.,* 2010). 51.5% of patients expressing both ERa and PR respondend well to tamoxifen therapy, compared with 16.7% of the patients who only expressed ER in their tumors (Chang *et al.,* 2009).ER, is expressed more in benign than malignant tumors and has a positive correlation with the expression of BCL-2 in dogs with mammary tumors. This may imply that ER controls the expression of BCL-2, as is the case with human breast cancer patients (Yang *et al.,* 2006). BCL-2 is a protein that regulates apoptosis. It is also used to differ follicular lymphoma from hyperplasia in human patients. Expression of BCL-2 is related to better prognosis and reduced proliferative activity of the tumor.

One aspect that distinguishes dogs from humans regarding the pathogenesis of mammary tumors, is that canine mammary tumor tissue expresses PR to a greater extent than ER (about 70% and 50% respectively). The opposite is true for humans: malignant tumors more often express ERα than PR (between about 70-80% compared to between approximately 50-70% expressing the PR). Why this correlation has been observed is unclear. One explanation is analytical error, since the expression of PR is dependent on the expression of ER (Chang *et al.,* 2009). The expression of ER, PR and ErbB2 (epidermal growth factor receptor 2) has an impact on survival rates (overall survival rate) in dogs and women with breast cancer (Klopfleisch *et al.,* 2010). ErbB1 is also called EGFR and ErbB2 is also known as HER2/neu (Human Epidermal Growth Factor Receptor 2). 2/3 of all high grade carcinoma in-situ in dogs express HER2 (Antuofermo *et al.,* 2007), and no difference in the HER2 expression was observed in inflammatory carcinomas compared to non-inflammatory carcinomas (Millanta *et al.,* 2010). With regards to the ectopic cartilage formation observed in benign mixed tumors, bone morphogenetic protein 6 (BMP-6) and its corresponding receptors BMPR-IA, BMPR-IB and BMPR-II are thought to participate in the regulation of cartilage formation. The ligand and its receptors are expressed in myoepithelium and chondrocytes within the tumors (Akiyoshi *et al.,* 2004).

Factors influenced by female sex hormones include GH and IGF1 in an autocrine fashion through progesterone P4. In humans, it has been shown that GH induces the growth of breast cancer tumors. There is a higher concentration of GH and IGF-I in malignant tumors compared to benign tumors and dysplasias in dogs (Queiroga *et al.,* 2008). Autocrine production of GH in human tumors may lead to metastasis. It has been shown in human samples that expression of the sex hormones progesterone (P4) and estradiol-17B correlates with expression of GH and IGF-I. Progesterone seems to be able to induce expression of GH in breast cancer tumors in both dogs and humans. There is evidence that estradiol and IGF-I have a synergistic effect on tumor growth in both dogs and humans. One possible explanation is that estrogens sensitize cells to mitotic stimulus from IGF-I (Queiroga *et al.,* 2008).

There are studies suggesting that the insulin receptor may play a role in the carcinogenesis of breast cancer in humans. The expression of the insulin receptor might be downregulated in breast tissue in patients with carcinoma. Whether or not this correlation is significant has not yet been investigated in epidemiological studies. Studies on rats have shown that administration of insulin may cause induction of mammary tumors experimentally. The expression of the insulin receptor is strong in adenomas and normal mammary glands in dogs. The expression significantly reduces in mammary carcinoma and metastases, which can be visualized by detection of mRNa and protein immunohistochemically (Klopfleisch *et al.,* 2010).

Mammary tumors are solid tumors and are dependent of different oncogenes than lymphomas for their growth. Adhesion molecules are of importance to the development of mammary cancer. One bias in this study, is that we did not have the opportunity to examine any grade III tumors. Benign mammary lesions tend to express E-cadherin more frequently, and the decreased membranous expression of this adhesion molecule is associated with increasing degree of malignancy, metastasis and local invasion (Genelhu *et al.,* 2007). Eukaryotic Elongation Factor 1 delta (EEF1D) is also upregulated in dogs with mammary tumors. In humans, EEF1D is associated with oncogenic transformation and invasiveness of breast cancer tumors (Klopfleisch *et al.,* 2010). In addition to increasing the rate of proliferation in cells, EEFD1 may also inhibit E-cadherin dependent cell-cell interaction, and thereby increase the invasiveness of the tumor. Expression of E-cadherin and β-catenin is related to tumor invasiveness, but has not been significantly correlated to survival (Brunetti *et al.,* 2005). In humans, an elevated level of β-catenin is associated with poor survival in patients with adenocarcinoma of the breast (Méniel *et al.,* 2005). Moreover, expression of E-cadherin and B-catenin is not related to mitotic indices as measured by Ki67 (MIB1) or AgNOR (Brunetti *et al.,* 2005). Historically, immunohistochemistry directed at E-cadherin expression has been used to differentiate histiocytomas in the skin from other tumors of round cell origin. However, epitheliotropic lymphomas frequently express E-cadherin and this may not be as useful as previously assumed (Ramos-Vara and Miller, 2011).

It has been proven that genetic components are important for the development of mammary tumors. Breast Cancer Suseptibility Gene 1 (BRCA1) is a famous suppressor gene detected in people with breast cancer. BRCA1 is expressed in its non-mutated form in normal mammary tissue in dogs (Kim *et al.,* 2010). BRCA1 is a nuclear phosphoprotein. Reduced expression of BRCA1 in the nucleus, and increased expression of the protein in the cytosol is associated with increased malignancy, increased proliferation as measured by Ki67 and reduced expression of ERα (Nieto *et al.,* 2003). The normal function of BRCA1 is to repair oxidative damage on DNA strands that would otherwise cause stress in the cell and inhibit replication (Kim *et al.,* 2010). BRCA1 also has role in apoptotic control and cell cycle checkpoints (Im *et al.,* 2013). Suppressor genes associated with breast cancer such as BRCA1 and BRCA2 can be detected in 5-10% of human cases (Im *et al.,* 2013). BRCA1 is rarely expressed the mutant form in benign mammary tumors, and much more in malignant metastatic mammary tumors (Kim *et al.,* 2010). However, there are breed differences in the expression of the BRCA1 gene, the shih tzu breed often expressing more BRCA1 and also having mammary tumors with higher proportion of invading cells in the lymphatic system (Im *et al.,* 2013). The prevalence of mutant BRCA1 genes is higher in families affected by hereditary breast cancer than for the rest of the population (Im *et al.,* 2013).

In some models explaining carcinogenesis, Tumor Initiating Cells (TIC) are suggested to play a significant role. Cell populations expressing the progenitor markers CD34, CD117 and CD133 have been detected in canine B-cell lymphomas. These cells coexpress the B-cellsmarkers CD21/CD22. These B-cells also exhibit genetic deviations in the IgH region (Immuno-Globulin Heavy Chain), a mutation detected in the tumor cell population in general. The cells also had the ability to proliferate in tissues of immunosuppressed mice, indicating that the progenitor cells with lower degree of differentiation may have great importance for the development of lymphoma and other neoplastic conditions (Ito *et al.,* 2011). Regarding interleukin expression, human DLBCL-cells have been found to overexpress the IL-9 receptor. This interleukin is mainly secreted by T-helpercells, suggesting that this cytokine may act as a factor promoting tumor growth in addition to immune system signaling (Lv *et al.,* 2013).

T-helper cells in follicular lymphoma express IL-4 and CD40 ligand, leading to subsequent expression of CCL17 (a thymic and activation regulated chemokine) and CCL22 (chemokine derived from macrophages) from neoplastic cells (Rawal *et al.,* 2013). In cultures of follicular lymphoma cells ex vivo, CCL22 is spontaneously produced while CCL17 seems to depend on paracrine signals. CCL17 and CCL22 are also overexpressed in Burkitts lymphoma, Hodgkins lymphoma, CCLs, mantle cell lymphomas and DLBCLs. There is a synergism in the expression of IL4 and CD40L when it comes to production of CCL17 and CCL22. The chemokines induce further expression of T-cells producing IL-4 and not interferon producing cells, leading to immunosuppression in the microenvironment of the tumor and further promoting tumor growth (Rawal *et al.,* 2013). This research has been done on human samples.

In humans, the herpesvirus known as Epstein-Barr is thought to potentially cause epithelial and hematological neoplasms. Recently, an oncogenic herpesvirus has also been found in the B-cells of dogs with lymphoma through electron microscopy (Huang *et al.,* 2012).

Attempts have been made to tailor a vaccine using CD40+ cells, in an attempt to trigger CD4+ and CD9+ T-cells in the immune system in patients with lymphoma. Trials have shown initial promise in canine patients with lymphoma immunized with triggered CD40-activated B-cells (Sorenmo *et al.,* 2011). Immunization with adenovirus vectors carrying CD40-ligand has also been attempted in canine patients with lymphoma. CD40 is expressed on B-cells and receptor binding induces differentiation of these cells. However, integration of the vector requires expression of $\alpha_v\beta_{3/5}$ integrins on the surface of lymphoma cells, a molecule which canine lymphoma cells do not seem to express (O´Neill *et al.,* 2011).

One bodily defense mechanism is to force the cells into controlled cell death, apoptosis. Lymphoma cells may possess the ability to evade apoptosis through expression of survivin protein that prevents cells from apoptosis. Survivin is included in the IAP (Inhibitors of Apoptosis Proteins). Survivin can be detected immunohistochemically in up to 82% of lymphoma cells (Wimmershoff *et al.,* 2010). However, it is not yet known to what extent normal tissues express survivin and more studies are necessary to establish the significance of different oncogenes in the development of lymphoma.

A range of molecules have been investigated with regards to the expression found in dogs with intestinal lymphoma compared to dogs with inflammatory bowel disease (IBD), as these can be hard to distinguish histologically. One example is perinuclear antineutrophilic cytoplasmic antibodies (pANCA) in the neutrophils of humans affected by IBD. It has been proposed that lymphocytic-plasmacytic enteritis (IBD) may progress to lymphoma eventually (Mancho *et al.,* 2011). These antibodies are present in serum in dogs with both lymphoma and IBD. Perhaps this molecule is of value in the pathogenesis of both diseases. However, it is not of clinical use in the distinction between the diseases as of date (Mancho *et al.,* 2011).

The dog has served as a model animal in studies on lymphoma, and lymphoma specimens of canines and people do indeed share some similar traits when it comes to pathology. Contrary to previous studies in humans with DLBCL, it has been shown that the expression of BCL-6 is not related to prognosis and overall survival time in dogs with high grade lymphoma. (Sato *et al.,* 2012). BCL-6 is a transcriptional repressor gene suppressing the anti-apoptitic effects of NFκB, i.e. nuclear factor kappa beta. NFκB is an important transcriptional factor in the pathogenesis of lymphoma, particularly DLBCL in humans. Activation of NFκB drives genes regulating proliferation (cyclin D1 and D2) and inhibition of apoptosis (for example BCL-2). Inhibition of NFκB in in vitro cultures of malignant canine B-lymphocytes leads to apoptosis and inhibited tumor growth (Gaurnier-Hausser *et al.,* 2011).

Leukemia is a hematopoetic malignancy present in the blood stream. FMS-like tyrosine kinase 3 (FLT3) is often mutated in human patients with leukemia, which has recently been found to be the case for some dogs with acute lymphoblastic leukemia as well. It is expressed on lymphoid and myeloid progenitors and is involved in the regulation of normal differentiation and cell survival in the hematopoiesis (Suter *et al.,* 2011). Signaling pathways involved in the FLT3 activation cascade include MAP (mitogen activated protein) and STAT (signal transducers and activation of transcription). Lymphoma specimens of dogs and humans overexpress high-mobility group box protein 1 (HMGB1), a protein promoting inflammation, neovascularization and DNA-binding. HMGB1 signaling is conveyed via toll-like receptors and RAGE (activated glycation end-products), leading to activation of NFκB, cytokine expression and VEGF (vascular endothelial growth factor) activation. However, even though HMGB1 is upregulated in lymphoma samples from dogs, RAGE levels did not differ from the control groups in the referenced study, indicating that HMGB1 may play a different role in exerting its extracellular effects (Sterenzcak *et al.,* 2010). Another similarity between dogs and humans with hematopoetic neoplasms, is the fact that a low expression of major histocompatibility complex class II (MHCII) on lymphocytes of canine and human patients with B-cell lymphoma negatively affects the prognosis (Rao *et al.,* 2011). This may implicate that loss of MHCII reduces the immune response elicited by the tumor, allowing increased tumor progression to remain undetected by the immune system.

One famous suppressor gene mentioned in the section “The concept of tumorigenesis”, is the p53 gene. This gene is important to the development of both lymphoma and mammary neoplasia. In rats, 20% of female rats heterozygous for p53 knockout develop breast cancer. Out of those, 70% express the estrogen receptor. Homozygous knockout rats develop lymphoma and sarcoma with early onset, and are more readily prone to metastasis (Yan *et al.,* 2012). It has been shown that p53 and p21 are both up-regulated in areas of metaplasia in the mammary gland cells of mice with B-cathenin dysregulation, suggesting a protective effect of p53 against the actions of B-catenin. In mice with loss in the Apc-locus and p53, neoplastic development is clearly accelerated. Apc-loss on its own leads to metaplasia and not neoplastic development (Méniel *et al.,* 2005). Loss of p53 leads to activation of the NFκB signaling pathway in a mouse model expressing TAX, a human leukemia virus oncoprotein (Ohsugi *et al.,* 2013). However, cell cycle regulatory factors are also of importance to the development of lymphoma. In human studies, it has been proven that overexpression of cyclin D1 is of importance to the development of mantle cell lymphoma, whereas cyclin D2 is of greater importance to the development of DLBCL. Cyclin D2 is overexpressed in 98% of CD5+ DLBCLs, suggesting a role of cyclin expression not only in the overall development of lymphoma, but also in the differentiation into different subgroups of lymphoma (Igawa *et al.,* 2013).

T cells have been shown to have great significance in the development of breast cancer in women. B-lymphocytes are dominant only in the early stages of carcinogenesis. Lymphocytes migrate into mammary and breast tumors in both women and canines. In humans, it has been shown that presence of T-and B-cells is significant both in the defense against tumors, and to the progression of the tumor. These cells possess the ability to express cytokines (Tumor-Necrosis Factor alpha, Interleukin IL 4, 6, 10 and others). The tumor itself can also produce cytokines as way to promote its own growth (Kim *et al.,* 2010). Recently, regulatory T-cells were found to be increased in number in dogs with lymphoma, and CD4+ and CD8+ T-cells are less susceptible to mitotic stimulus than T-cells from healthy dogs (Mitchell *et al.,* 2012). It has been shown in lymphoma specimens of DLBCL of the central nervous system in humans, tumor cells may stimulate their own growth through expression of B-cell activating factor (BAFF) receptor and ligand in an autocrine fashion. BAFF belongs to the tumor necrosis factor family. Furthermore, proliferation inducing ligand (APRIL) and its corresponding receptor was also expressed in CD20+ cells (B-cells), indicating that the BAFF/APRIL signaling pathway may play an important role in the pathogenesis of these tumors (Birnbaum *et al.,* 2013). The expression of cytokines may assist the neovascularization process and paradoxically facilitate tumor growth. In dogs, it has been found that the lymphocyte population in the tumor mainly consists of T-cells rather than B cells (three times more T-cells than B-cells). Expression of IL1 and IL6 can be associated with metastasis and expression of both these cytokine can be seen in metastatic mammary tumors (Kim *et al.,* 2010). IL-1 may directly affect the proliferation, act as a chemokine for nearby leukocytes and increase neovascularization. It has been shown that malignant mammary tumors often express the inflammatory enzyme COX-2 (more information on this may be read in the section “Treatment options for mammary tumors and lymphoma and follow-up of treatment outcomes”). However, this has been proven not to be the case in epitheliotropic T-cell lymphoma, suggesting that this enzyme may not play a significant role in the ulceration and inflammation observed in these tumors (Bardagi *et al.,* 2012).

It has been demonstrated in humans, dogs, cats and horses that there is a shift in the lymphocyte population with increasing age. It is mainly the T-cell dependent immunity that becomes affected: the number of CD4+ decreases and CD8+ T-cells increase. Meanwhile, the total number of peripheral lymphocytes decreases. T-cells have an important role in the defense against neoplasias. The ratio of CD4 +/CD8 + cells was significantly higher in people with cancer dogs than in dogs that had simply gotten older. The CD4/CD8-ratio declined with increasing age, but increased in dogs suffering from cancer compared to healthy dogs of the same age (Watabe *et al.,* 2011). 45% of peripheral lymphocytes could neither be identified as B-or T-cells in dogs suffering from neoplasia, while all lymphocytes could be classified among the healthy dogs.

Histological classification of mammary tumors and lymphoma

Mammary tumors are considered to incur after the progression of preneoplastic lesions to invasive carcinoma. This progression has also been described for people with breast cancer, and the preneoplastic lesions are known as IELs (Intra Epithelial Lesions). Progression to cancer proposedly starts with adenosis, which then becomes sclerosing and develops into an intraductal papilloma, and further into a sclerosing papilloma. This is followed by ductal hyperplasia and finally a ductal carcinoma in situ has arisen (Antuofermo *et al.,* 2007). Carcinoma in situ is defined as the occurrence of malignant cells which have not yet penetrated the basal membrane. The progression eventually proceeds as a tumorous growth. Small tumors often consist of lobular hyperplasia, adenomas or complex adenomas. However, malignant lesions can arise from within benign tumors, which together with the increased incidence of multiple tumors in mammary cancer patients speak of a progressive development. Malignant tumors more frequently found in older individuals, and this applies to both canines and women (Sorenmo *et al.,* 2009).

Mammary tumors are classified on the basis of histological characteristics. Benign mammary tumors and lesions classified as cysts, gynecomastia, ductal hyperplasia, lobular hyperplasia or fibrosis usually carry a good prognosis. Benign tumors consist of adenomas (simplex, complex, basaloid), fibroadenoma, benign mixed tumors or ductal papilloma. Cytology and aspirates unfortunately have low importance for the determination of malignancy, due to the fact that the cells of the mammary gland can range from hyperplastic to apoptotic within the normal estrous cycle of the bitch. Moreover, malignant tumors may sometimes reside within a benign tumor, making the aspirate technique insufficient for diagnosis. However, a fine needle aspirate can help differentiate between mammary tissue and tumors of other cell lineages, such as mastocytoma and lymphoma, and other conditions such as inflammation (Dobson and Lascelles, 2011). The criteria on which malignancy is determined does not differ from any other tumor type: anisocytosis, anisokaryosis, basofilia, an increased number of nucleoli and cellatypia are all signs of malignancy. Mammary cancer tumors often tend to metastasize via the lymphatic route to mainly lung tissue (Clemente *et al.,* 2010), and there are even reports on skeletal metastasis to the ribs (Dobson and Lascelles, 2011). Carcinomas are classified according to cell lineage and the degree of differentiation, according to the WHO system adapted by Misdorp *et al* 1999:

- Adenocarcinoma complex: highly-differentiated tumors that still contain glandular structures and myoepithelium. This tumor type rarely metastasizes.
- Simple adenocarcinomas: these tumors do not contain neoplastic myoepithelium, and can be divided into alveolar, tubular, tubuloalveolar, papillary or papillary-cystic depending on growth pattern. Can give rise to metastases.
- Solid carcinomas: do not contain ductal structure. They carry a guarded prognosis and often metastasize.
- Anaplastic carcinoma: very poorly differentiated tumors with high propensity to metastasize via lymphatics and/or and the blood stream. This tumor type is clinically referred to as inflammatory carcinoma. This stems from the invasion of the local lymphatic vessels which gives the tumor an inflamed appearance. These tumors have very poor prognosis and often metastasize in of both dogs as humans (Clemente *et al.,* 2010).
- Specific types of carcinomas include spindle cell carcinoma, squamous cell carcinoma, mucinos carcinoma or lipid rich carcinomas.

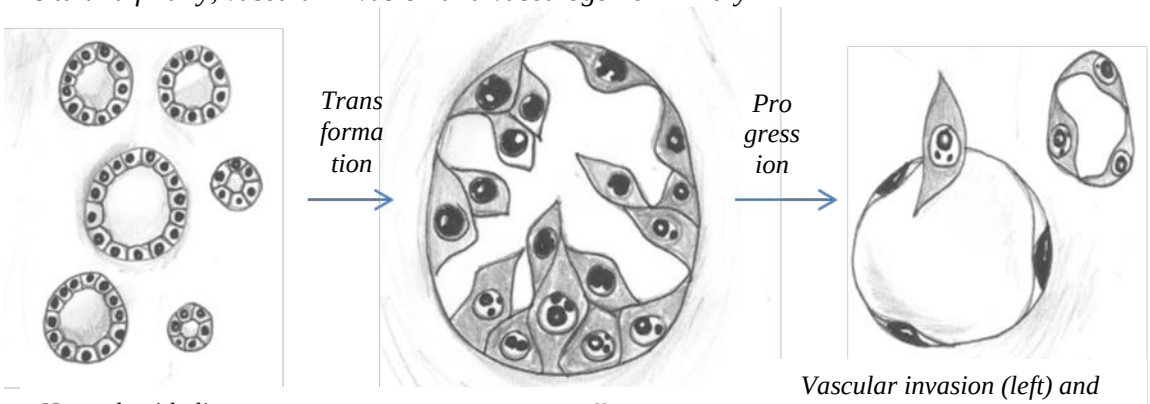
In addition to the validated method of analysis of canine mammary neoplasms by Misdorp *et al*, the Elston and Ellis grading method has been described for grading of canine mammary tumors (Karayannopoulou *et al.,* 2005).

It has been shown that inflammatory carcinoma of the breast tissue in dogs and humans exhibit "vasculogenic mimicry" (VM), i.e., neoplastic cells that form capillary-like channels which are not covered by normal epithelium. This has been proposed as a way for the tumors create neovascularization and be locally invasive into surrounding lymph vessels (Clemente *et al.,* 2010).

The frequency of vasculogenic mimicry is much higher in inflammatory carcinomas compared to non-inflammatory carcinomas in dogs (33% vs 5%, equivalent to 1 case of the total number surveyed according to Clemente *et al.*, 2010).

This is consistent with research in vivo and in vitro on inflammatory carcinoma in women, i.e., inflammatory carcinomas express vasculogenic mimicry to a greater extent than non-inflammatory carcinomas. This has been suggested to be a contributing factor to their proneness to metastasize and the clinical aggressiveness of the tumors. Co-expression of cytokeratin (CK), vimentin and vasculogenic mimicry has been observed in both dogs and women. In women, this co-expression is associated with a worse prognosis (Clemente *et al.*, 2010). It has not yet been proven that vasculogenic mimicry is associated significantly with invasion of lymphatic vessels and lymph nodes in dogs, but can be used as a marker of the anaplastic dedifferentiation of neoplastic cells (Rasotto *et al.*, 2012). VEGF is an important factor for the formation of lymphatic vessels, which are an essential part in the pathogenesis of inflammatory carcinomas since this condition is characterized by the local invasion of lymphatic vessels (Millanta *et al.*, 2010). VEGF expression is much higher in inflammatory carcinomas compared to non-inflammatory carcinomas in dogs. This is consistent with studies in humans, and suggests that VEGF plays a significant role in the angiogenesis and the increased vascular permeability in inflammatory carcinoma in dogs and women (Millanta *et al.*, 2010).

Fig 16. Schematic and simplified illustration of the progression from normal epithelium to carcinoma in situ and finally, vascular invasion and vasculogenic mimicry



Sarcomas make up 5-10% of cases. Sarcomas consist mainly of extraskeletal osteosarcomas or fibrosarcomas. Gomoris reticulin stain can be used to differentiate fibrosarcomas from spindle cell carcinomas (Karayannopoulou *et al.*, 2005). The mammary gland is the most common localization of extraskeletal osteosarcoma, accounting for 64% of the tumors in this category (Langenbach *et al.*, 1998). The diagnosis of sarcoma is a risk factor for decreased survival postsurgically (Hellmén *et al.*, 1993). Sarcomas carry a poor prognosis and metastasize in 75% of cases (mainly via the hematogenous route). In rare cases, carcinosarcoma occurs in the mammary gland. Carcinosarcoma is a tumor of the mixed cellular lineage with both epithelial and mesenchymal origin. There are immunohistochemical tools available to distinguish different components within a tumor. Previous studies have shown that myoepithelial components exhibit five different patterns of growth within a tumor: spindle shaped interstitial cells, star shaped cells, cartilage, resting suprabasal cells and proliferative suprabasal cells. The transformation necessary for cartilage formation can be characterized by expression of a-actin, keratin components (no 19), S-100 protein and vimentin. Epithelial components are positive to keratin staining, connective tissue to staining for vimentin, and myofibroblasts to a-actin and vimentin respectively. Since myoepithelium makes up a significant part of a large number of carcinomas, this author suggests a new subdivision of pleomorphic carcinoma, including carcinomas except mucinous, squamous cell and comdeo carcinoma (Destexhe *et al.*, 1993).

A homologue to p53, namely p63, can be used with high specificity for detection of myoepithelial components, since this molecule is not expressed in stromal components of the tumor (Gama *et al.,* 2003).

Whether or not the myoepithelium has a significant role in the carcinogenes is still disputed. Highly differentiated myoepithelial cells have been shown to have a carcinostatic effect in vitro. Myoepitelioma in dogs is comparable compared to "basal-like" breast cancer in humans and have a worse prognosis than carcinoma complex. Differentiated myoepithelium express vimentin, and neoplastic myoepithelium expresses CD14 and p63. Myoepithelioma was not originally included in the WHO-classification system (Rasotto *et al.,* 2012). However, there is an additional classification system according to Clemente et al. This corresponds largely with Misdorp *et al*. Both systems evaluate the degree of tubuli formation, nuclear pleomorfism and degree of mitosis.

There are several classification systems for canine lymphomas, the most commonly accepted being the WHO-system, REAL-system and Kiel-system. The oldest systems include the Gall and Mallory system, introduced in 1942, and the Rappaport system, introduced in 1966. The Kiel-classification system was first established in 1974. It was based upon the suggestions that malignant lymphoma cells were in fact neoplastic versions of normal lymphocytes, and that substages of these cells would be represented as a progenitor lymphoid cell halted in their development (Parodi, 2001). Simultaneously in the seventies, other classification systems were proposed such as the Lukes and Collins classification system, the system by Dorfman and the WHO-classification system. For this reason, the Working Formulation was established in order to harmonize the different systems. Subsequently, the updated Kiel-classification system followed in 1990, the REAL-classification system in 1994, and the WHO-classification which was last updated in 2008 by Swerdlow (Parodi, 2001 and Swerdlow *et al.,* 2008). The Kiel-system has been utilized used by many authors for canine lymphoma. Scientific studies are ongoing to evaluate the comparability to the classification systems used in human medicine (Ponce *et al.,* 2010). The WHO established the classification criterion for human lymphoma, which has been claimed as the consensus regarding classification of human lymphoma since 2008 (Campo *et al.,* 2011). The ability to grade canine lymphoma according to subtype could constitute an opportunity to closely tailor the treatment of a specific patient. As in humans, different subtypes of lymphoma clearly exhibit different prognosis regarding therapy with cytostatic agents (Dobson, 2004 and Ponce *et al.,* 2004). In the referenced study, small clear-cell T-lymphoma (updated Kiel classification system) had the longest overall survival time (almost 27 months), and since the T-cell lymphoma group is heterogenous, purely immunotyping the neoplasm may be insufficient to indicate prognosis. Immunohistochemistry of CD79 (expressed in B-cells) and CD3 (expressed in T-cells) has been of great value in distinguishing the two groups. Mycosis fungoides (epiteliotropic lymphoma), is defined as a disease cause by the proliferation of cells of T-cell origin (Moore *et al.,* 1994).

In humans, the term “Hodgkins lymphoma” is often used. This form of lymphoma is less common than the more often referenced non-Hodgkins lymphoma (NHL), and there is only 1 case of Hodgkins lymphoma for every case of non-Hodgkins lymphoma in humans. This disease exhibits a biphasic peak in incidence, the first peak in the first third of life and the second occurring after 50 years of age. Apart from an inflammatory infiltrate of mainly lymphocytes, plasmacells and histiocytes, the disease is histologically characterized by the presence of malignant Reed-Sternberg cells. The former Rye classification proposing 4 types of Hodgkins lymphoma has now been replaced by the WHO classification system (Tsang *et al.,* 2006). In the pathogenesis of Hodgkins lymphoma, the expression of NFκB and infection of the Epstein-Barr virus have been associated with acquisition of the disease and disease progression.

Recently, it has been found that loss of the kelch-domain protein KLHDC8B may be linked to the chromosomal instability observed in Reed-Sternberg cells (Krem and Horwitz, 2013).

The dog has previously been used as a model animal for humans in lymphoma studies. Multicentric high-grade lymphoma in dogs is comparable with non-Hodgkins lymphoma in humans. Morphologically, one can find similarities between human lymphomas according to the WHO-classification system for humans and the updated Kiel-classification system for dogs. The centroblastic and immunoblastic subtypes of high grade large B-cell lymphoma are overrepresented among adult dogs and can be compared with DLBCL (Diffuse Large B-Cell Lymphoma) in adult humans. DLBCL-group is also the most common type of lymphoma in adults and accounts for 30-40% of all lymphomas in humans (Sato *et al.,* 2012). One difference, however, is that the dog's centroblastic and immunoblastic lymphoma histologically includes the presence of MMC (Macronucleolated Medium-sized Cells), which is missing in the corresponding form of lymphoma in humans. The significance of this for the use of the dog as a model animal for humans is unclear.

Burkitt-like B-cell-lymphoma has been observed in dogs and can be compared with Burkitt-type B-cell lymphoma in humans. This type of lymphoma is defined by a "starry sky" pattern, medium-sized cells, round nuclei with condensated cromatin and high mitotic index. 80% of the dogs were positive for the mitosis marker Ki-67, compared with almost 100% of human samples. It is suggested that plasmacytoid B-cell lymphoma in dogs can be a variant of Burkitt-type in humans, with the exception that this tumor in dog lacks central nucleoli and starry-sky designs, has less cells and more finely dispersed chromatin. The study also shows that small lymphocytic B-cell lymphoma in dogs may be more difficult to compare with human samples that are classified according to the WHO system. Low-grade B-cell lymphoma in dogs is classified as prolymphocytic, small lymphocytic and lymphoplasmacytic according to the Kiel classification system, while human classifications include chronic lymphocytic leukemia, mantle cell lymphoma, marginalzone lymphoma of the spleen, lymph nodes, MALT, lymfoplasmacytic and follicular lymphoma (Ponce *et al.,* 2010).

Histologically, a range of proliferation factors have been utilized to assess mitosis. Ki67 index is a way to determine the mitotic activity in tumors and Ki67 (MIB-1) is expressed in the nucleus. Ki67 is significantly related to the prognosis of human lymphoma patients with high-grade as well as low-grade lymphoma (Flood-Knapik *et al.,* 2012), and overall and disease free survival in human patients with breast cancer (Abboud *et al.,* 2008). However, this has not been shown to have significance for prognosis in dogs with high-grade lymphoma. Regarding low-grade lymphomas in dogs, the mitotic index is highest among follicular lymphomas, and lowest in T-zone lymphomas and marginalzone lymphomas. Expression of Ki67 is not correlated to the median survival time for dogs with low-grade lymphoma (Flood-Knapik *et al.,* 2012). In human patients with node-negative breast cancer, a mitotic index produced by combining percentage Ki67 (MIB1) positive cells and cells positive for another mitotic marker (AgNOR, argyrophilic nucleolar organizer region) is related to disease free survival and overall survival. AgNOR gives additional information regarding the length of the cell cycle, since the amount of AgNORs in interphase is directly related to the turnover of cells and speed of the individual cell cycle. Interphase AgNORs participate in the synthesis of ribosomal RNA and the size and number of AgNORs vary with the transcriptional activity within the cell (Abboud *et al.,* 2008). The AgNOR area and reduced number of AgNORs per nucleus has been associated with longer disease free survival in dogs with lymphoma aswell (Kiupel *et al.,* 1999).

Another example of a mitotic marker used in clinical pathology is PCNA (proliferating cell nuclear antigen). PCNA is expressed to a greater extent in metastatic mammary tumors than in non-metastatic mammary tumors (Klopfleisch *et al.,* 2010). PCNA increases replication by acting as a cofactor to

DNA polymerase delta. PCNA can also inhibit apoptosis by inhibiting tyrosine kinase-c-abl stability, as well as affect the RAD6-dependent DNA repair. RAN/TC4-binding protein (RANBP1) is upregulated in metastatic mammary tumors and in most human cancer conditions including breast cancer. It also has an influence on the proliferation of cells and prevents apoptosis. (Klopfleisch *et al.*, 2010). However, mitotic markers are not interchangeable in all circumstances. It has been previously described that the mitotic index as measured by Ki67 is indicative of the first relapse free interval in canine patients with lymphoma, but not the median overall survival time. The expression of PCNA was not indicative of neither. On multivariate analysis, it was shown that only a combination of the Ki67-index and apoptotic index (proliferation/apoptotic ratio, PAR) was indicative of the duration of first relapse free interval (Phillips *et al.*, 2000).

APPENDIX II - CLINICAL ASPECTS

Clinical staging of the canine cancer patient

Staging of the patient is performed according to the TNM system established by the WHO. T stands for tumor and is assessed based on tumor size. Palpation of regional lymph nodes is performed to reveal possible enlargement. Enlargement of a regional lymph node may indicate metastasis and worsens the prognosis. Since the 5th mammary gland may be connected to the popliteal lymph node, palpation of this lymph node should not be neglected. N stands for node and M stands for metastasis in the TNM system, and staging is performed regarding possible metastasis to the lymph node or distant anatomical locations. Malignant mammary tumors generally are larger than benign tumors, on average, 4.7 cm compared to 2.1 cm in a study of 90 cases (Sorenmo *et al.*, 2009). It has been shown that tumors measuring 5 cm or more are more likely to metastasize to local lymph nodes than smaller tumors (Chang *et al.*, 2005). Over 60% of the dogs have more than one tumor, and dogs with malignant mammary tumors are more likely to develop more primary tumors than dogs with benign tumors. In women with breast cancer are also at risk of developing new tumors in the chest on the opposite side if they have already been diagnosed with breast cancer (Sorenmo *et al.*, 2009). Palpation of the tumor reveals whether it is freely sliding against the underlying tissue, or if adherences have been formed. It has been shown that solid tumors (mammary tumors included) expressing adherence markers (for example galectin-1) may metastasize to lung tissue more efficiently (Ito and Ralph, 2012).

Table 7. Clinical TNM staging of mammary tumors, adapted from Cassali et al, 2011.

T	T0 No evidence of primary tumor	
	T1 Tumor size < 3 cm	a. Not attached
		b. Attached to the skin
		c. Attached to muscle
	T2 Tumor size 3-5 cm	a. Not attached
		b. Attached to the skin
		c. Attached to muscle
	T3 Tumor size > 5 cm	a. Not attached
		b. Attached to the skin
		c. Attached to muscle
	T4 Inflammatory carcinoma (tumor of any size)	
N	N0 No regional lymph node metastasis	
	N1 Ipsilateral lymph node involved	a. Not attached
		b. Attached
	N2 Bilateral lymph node involved	a. Not attached
		b. Attached
M	M0 No distant metastasis	
	M1 Distant metastasis present	

Table 8. Grouping of TNM stages into clinical stages, adapted from Cassali et al, 2011.

	T	N	M
Stage I	T1	N0	M0
Stage II	T0	N1a	
	T1	N1a	
	T2	N0 or N1a	M0
Stage III	T3	All N	
	All T	All Nb	M0
Stage IV	All T	All N	M1

Inflammatory carcinomas of the breast in dogs and humans have similar clinical characteristics. They are locally invasive and cause local inflammation with erythema, warmth and tenderness, and can thus be mistaken for dermatitis in both humans and animals. The tumors are generally very aggressive and treatment is usually only palliative (Clemente *et al.,* 2010). Survival at time of diagnosis of anaplastic carcinoma is only weeks long despite attempts of treating the condition. Therefore, it is the most aggressive mammary tumor in dogs and women (Clemente *et al.,* 2010). Inflammatory carcinomas often metastasize to other tissues than non-inflammatory carcinoma. Inflammatory carcinoma never metastasize to bone tissue, and more rarely to the lung, liver and kidney. However, inflammatory carcinoma metastasizes to the bladder and urogenital area (Clemente *et al.,* 2010).

Staging of the canine lymphoma patient differs slightly from the one practiced in patients with solid tumors. Lymphomas can be categorized according to anatomical location, where the multicentric form accounts for approximately 80% of cases. In addition to the multicentric form, there is also an alimentary form, cutaneous form, mediastinal form and extranodal form. As much as 82,4% of the dogs with lymphoma have lymphadenopathy, and 17.6% suffer from extranodal influence (Ponce *et al.,* 2010.) Clinical staging is performed according to the WHO system and takes into account the anatomic position of the lymphoma, as well as the patient's wellbeing.

Table 9. Clinical stages of canine lymphoma, adapted from Jagielski et al, 2002

Stage I	Single lymph node or lymphoid tissue in single organ involved (not including bone marrow)
Stage II	Involvement of several lymph nodes, with or without involvement of tonsills
Stage III	Generalized lymph node involvement
Stage IV	Liver and/or spleen involved, with or without lymph node involvement
Stage V	Involvement of bone marrow, blood or extranodal locations
	Substage a. without clinical signs of disease Substage b. with clinical signs of disease

Clinical aspects relevant to the assessment of the canine cancer patient

Investigation of a presumptive canine lymphoma or mammary tumor patient begins with the medical interview and physical examination. Once the diagnosis of mammary tumor or lymphoma has been established, further investigation is necessary to determine the prognosis of the patient. In patients with mammary tumors, increased production of GH (growth hormone) may occur. Treatment of female dogs with medroxyprogesterone acetate or proglistenone can cause local production of GH, IGF-I, IGF-II and IGF-binding proteins by the mammary gland, and the formation of benign mammary tumors (Mol *et al.,* 1996). However, it has been shown that the production of GH can increase in malign tumors negative for the progesterone receptor in both canines and women (Mol *et al.,* 1996). It has been shown that treatment with progestins of canines with pituitary dwarfism can significantly improve symptoms and increase production of IGF-I (Kooistra *et al.,* 1998). Expression of IGF-I in patients with mammary tumors is associated with increasing tumour size, fast-growing tumors, ulceration and the formation of adherences to underlying tissues in dogs (Queiroga *et al.,* 2008). GH-production might cause acromegaly in the patient (Mol *et al.,* 1996 and Queiroga *et al.,* 2008). In this case, it is not possible to analyze GH, but IGF-1-assays for measurement in serum are available including the enzyme-labelled chemiluminescent immunometric assay (Immulite 1000 IGF-1 assay, Diagnostic Products, Los Angeles, California, USA) which has been tested for use on canine serum (Tvarijonaviciute *et al.,* 2011). Measurement of IGF-1 is not readily available in Sweden today.

Hypercalcemia might occur as a paraneoplastic syndrome in lymphoma patients due to production of parathyroid hormone related peptide PTHrp. However, studies have failed to correlate the level of PTHrp with hypercalcemia in dogs with lymphoma, suggesting that another mechanism might also play a role in the pathogenesis. The connection between PTHrp levels and hypercalcemia could only be made for dogs with anal sac adenoarcinoma (Rosol *et al.,* 1992). Canine lymphoma patients with hypercalcemia at the time of diagnosis have a shorter median survival time than those who do not (Elliott *et al.,* 2013). A high level of calcium in the blood can cause polyuria/polydipsia, as described in case reports (Barthez *et al.,* 1995 and Bae *et al.,* 2007) and polyuria/polydipsia will be the result in 80% of patients with hypercalcemia (Teske, 1994). Neoplasia is the most common reason of hypercalcemia in dogs, lymphoma being the most common neoplasia causing hypercalcemia (Bergman, 2012). 88% and 38% of dogs with hypercalcemia will get anorexia or vomit, 87% and 68% will get elevated urea or creatinin levels as a result of reduced glomerular filtration. Hypercalcemia is more commonly found in patients suffering from T-cell lymphoma (Teske, 1994). To make matters worse, complex carcinoma in the mammary gland may also induce production of PTHrp (Bae *et al.,* 2007). In a study of Flood-Knapik *et al,* 25.3% of 75 dogs with low-grade lymphoma presented with lymphocytosis at the time of diagnosis (Flood-Knapik *et al.,* 2012). Measurement of ionized calcium gives the most accurate clinical assessment, since ionized calcium reflects the levels of PTH and vitamin D. Neoplasia, particularly lymphoma, is the most common cause of ionized hypercalcemia in dogs (Messinger *et al.,* 2009). Lymphocytosis, lymphopenia, anemia and thrombocytopenia may also be seen in blood samples (Teske, 1994). Myastenia gravis may be causally to cutaneous lymphoma, as described in a case report (Ribyard *et al.,* 2000). Hyperlactemia is associated with hematopoetic malignancies in humans. However, the neoplasm itself is not thought to be the sole cause of hyperlactemia in dogs with lymphoma. 70% of hyperlactemic dogs with lymphoma have an additional cause of hyperlactemia and factors such as corticosteroid administration have been found to be significantly associated with the presence of this condition in canine lymphoma patients (Touret *et al.,* 2012). Mild proteinuria is often observed in patients with lymphoma. However, the presence of mild proteinuria is not thought to be clinically significant (Di Bella *et al.,* 2013).

In addition to blood samples, histology can aid the clinician in further examination of the patient. If lymphadenopathy is present, further examination can include a fine needle aspirate (FNA) or a biopsy of the affected lymph node. This applies to both patients with mammary tumors as well as lymphoma patients. When a FNA is performed, cells are extracted via a syringe and the method is less invasive than a biopsy. The procedure can often be performed without sedation. One disadvantage of FNA is that it can be harder to diagnose certain subtypes of low grade lymphomas, in which case the architecture of the lymph node might be crucial to set a correct diagnosis. (Ponce *et al.,* 2010).

According to Sapierzynski *et al*, non-Hodgkin's lymphoma can be diagnosed based on aspirates in 80-90% of human patients, however the subtype of the lymphoma can only be determined in 67-86% of all aspirates. (Sapierzynski *et al.*, 2010). Change of diagnosis may occur in as much as 20.4% of after immunofenotyping with immunohistochemistry (Flood-Knapik *et al.*, 2012). There is also the possibility of performing a bone marrow aspirate. There are research groups who consider this to be a necessity to make accurate diagnosis and prognosis of the patient (Marconato *et al.*, 2008), and they are the research groups who believe that the procedure does not add anything regarding staging or treatment (Flory *et al.*, 2007).

Immunohistochemistry is a useful tool in the assessment of the canine cancer patient. Mantle cell lymphomas are aggressive tumors, and it is necessary to determine the mitotic index as a complement to the PAD to determine the prognosis since a small cell low-grade B-cell lymphoma can be transformed into high grade large B-cell lymphoma in some cases (Ponce *et al.*, 2010). The International Lymphoma Study group has shown that the diagnostic accuracy increases by 10-45% for the diagnosis of mantle cell lymphoma, T-zone lymphoma and DLBCL using immunohistochemistry. Immunohistochemistry can help differentiate T-zone lymphoma from marginal cell lymphoma by immunhistochemical detection of CD3 and CD79a (Flood-Knapik *et al.*, 2012).

Thoracic x-ray imaging might reveal possible metastasis. As mentioned earlier, mammary carcinoma often metastasize to lung tissue. The occurrence of subpleural osteoma should not be confused with metastasis in older dogs. Ultrasound of the abdomen allows visualization of the internal organs, in search of metastasis or primary foci of lymphoma. A problem that persists with respect to diagnostic imaging is the user dependent factor that cannot be eliminated. There is also the issue with artefacts, for example the accumulation of intestinal gas which obstructs the sound waves when performing an ultrasound (Wisner *et al.*, 2004). Flory et al argue for the ultrasound as a tool of diagnostic value for determining suspicious neoplastic changes in abdominal organs. (Flory *et al.*, 2007). To determine the nature of the lesion detected on an ultrasound, biopsy or FNA sampling is required. This can sometimes be performed with ultrasonic guidance and sedation (Wisner *et al.*, 2004). Despite this, it can be difficult to get an accurate diagnosis of the subtype of the lymphoma without genotyping as a complement (Ponce *et al.*, 2010).

Treatment options for mammary tumors and lymphoma and follow-up of treatment outcomes

Mammary tumors in dogs are treated surgically. However, the success of treatment is determined by a number of factors. Metastasis to adjacent lymph nodes is more likely to occur if the tumor is discovered more than 6 months before initiation of surgery (Chang *et al.*, 2005). In addition, the histological profile of the tumor plays a significant role. Spay status plays a more significant role regarding 2 year survival time postoperatively to dogs suffering from complex carcinoma than dogs suffering from simple carcinoma (Chang *et al.*, 2005). In other studies, obesity, age at diagnosis and spay-status has not been shown to affect the outcome postsurgically. Obesity has been related to the development of mammary tumors in other studies (Pérez Alenza *et al.*, 1998). Other studies have determined increasing age as a risk factor for decreased postsurgical survival (Hellmén *et al.*, 1993). However, histological diagnosis of anaplastic carcinoma, evidence of metastasis and tumor size are all factors affecting treatment outcome. Dogs dying of tumor-related causes postoperatively have shorter survival time than those who die of other causes, 14 months vs 23 months (Philibert *et al.*, 2003).

72% of vets in England always recommend castration of female dogs that are not going to be used for breeding (Beauvais *et al.*, 2012). When castration is performed has long been considered important for the dog's survival after treatment of a mammary tumor. Dogs castrated within a 2 year period starting from the date of surgery of their mammary tumors had a median survival time of 755 days compared with 301 and 286 for dogs castrated more than 2 years before the procedure, and intact dogs (Sorenmo *et al.*, 2000). This author suggests that castration associated with mastectomy can be an adjuvant therapy in order to increase the patient's survival. Another study has found that the risk of developing mammary tumors is significantly reduced in bitches castrated before 2.5 years of age (Sonnenschein *et al.*, 1991). Dogs treated with synthetic progestins in an experimental study had increased risk of

developing mammary tumors, but had no increased risk of developing cancer of the mammary gland (Misdorp, 1988). Treatment with medroxyprogesterone acetate (MPA) from 3 month to 1-15, years of age can induce the development of mammary tumors. However, these tumors were exclusively benign (Concannon *et al.,* 1981). Other studies have failed to establish a statistically significant connection between castration early in life and protection against mammary tumors (Spain *et al.,* 2004). Some authors claim that castration before first, second or third estrus reduces the risk of mammary tumors to 0,5%, 8% and 26% (Antuofermo *et al.,* 2007). Castration has been proposed as adjuvant treatment, because treatment with estrogen receptor blockers like tamoxifen in dogs increases the risk of pyometra, edema of the vulva and urinary tract infection (Chang *et al.,* 2009). The British Small Animal Veterinary Association has recently released a review discussing supposed bias in these studies (Beauvais *et al.,* 2012). Aspects such as the selection of study population, study design, confounders are considered. The role of sex hormones in the pathogenesis of mammary has been established in many scientific studies.

There are drugs available to treat cancer of the breast in humas. The amino acid sequence in canine ErbB1 is 91% homologous compared with human ErbB1, ErbB2 exhibit 92% homology (Singer *et al.,* 2012). ErbB1 and ErbB2 is overexpressed in 3 or 4 out of 10 surveyed mammary tumors according to Singer *et al,* 2012. The binding site of cetuximab (anti-ErbB1) and trastuzumab (anti-ErB2) differed from the human epitope in 4 or 1 amino acids respectively. These antibodies can also inhibit the growth of canine mammary tumor (Singer *et al.,* 2012).Those drugs are expensive and not widely used in dogs today. Treatment of mammary tumors with cytostatic agents such as 5-fluorouracil, doxorubicin and cyclophosphamide has been discussed (Sorenmo, 2003). However, mammary tumors in dogs may also express the enzyme cyklooxygenas2 (COX2). Unlike the isoenzyme COX1, COX2 is induced in the body. It catalyzes the conversion of arachidonic acid to prostaglandins, a family of inflammatory mediators (Queiroga *et al.,* 2005). COX2 is induced in breast cancer tumors, and the highest expression of the enzyme can be found in inflammatory carcinoma. This has also been demonstrated in human breast cancer patients. COX2 is induced in malignant neoplasms and expression increases with the degree of skin ulceration and tumor size (Queiroga *et al.,* 2005). Expression of COX2 is related to prognosis and this implicates that treatment with non-steroidal non-inflammatory drugs (NSAIDs) may be indicated (Queiroga *et al.,* 2005).

Treatment of lymphoma is not curative, but rather focuses on extending and improving the quality of life of the patient. There are a number of different chemotherapy protocols, all of which must be carefully considered before they are applied to current patient to get the best results and minimize toxicity (Simon *et al.,* 2006). Protocols are named with abbreviations based on the first letter of the constituent medications. A protocol used extensively is treat T-cell lymphoma is the CHOP Protocol, where CHOP stands for cyclophosphamide, hydroxydaunorubicin (doxorubicin), oncovin (vincristin) and prednisone. L-asparginase can also be added, an enzyme that is injected subcutaneously and is not a cytostatic agent. B-cell lymphomas are generally more chemosensitive than T-cell lymphomas, and the treatment outcome for such protocols varies between 60-90% for CR (Complete Remission) with a median survival of 6-12 months (Marconato *et al.,* 2011). The addition of cytosine arabinoside enhances chances for remission and survival regardless of tumor phenotype in dogs with lymphoma affected by bone marrow infiltration (Marconato *et al.,* 2008). Overall, remission rates vary from 75-90% with overall survival times of 6-15 months. However, protocols containing doxorubicin generally produce a better response. One problem is identifying patients available for treatment. Although 60% of lymphomas are of B-cell origin, there is substantial variation in treatment response within the B-cell lymphoma group and immunophenotype alone is not sufficient to select which patient should be available for treatment (Dobson, 2004).

Similarly to bacteria and parasites, tumors can become resistant to the medicines we use to treat cancer. This is called MDR (Multi Drug Resistance) and signifies that different patients with the same tumor type, the same history and the same clinical conditions respond differently to the same treatment protocols. This is due to the ability of tumor cells to continually transform genetically and epigenetically (Savage *et al.,* 2009). The risk of MDR can be reduced by adding for example fludarabidine to the protocol, a drug that is not a substrate for P170-glycoprotein. Tumors that have grown refractory to CHOP protocol, or in case of relapse, a new treatment strategy might become necessary. One example is MOP Protocol, where mechlorethamin and procarbizine are added

(Northrup *et al.*, 2009). Another example is the DMAC Protocol, containing dexamethasone, actinomycin D, cytosine arabinosid and melphalan (Alvarez *et al.*, 2006). The goal is mainly to supply drugs that are not substrates for P 170. L-asparginas and lomustine (CCNU) are drugs used in combination with prednisolone to achieve improved therapeutic efficacy and survival (Saba *et al.*, 2007). Remission is obtained in 20-50% of cases in 2-3 months, and regardless of the protocol used to treat the patient, the risk of relapse and resistance to drugs remains a major problem (Saba *et al.*, 2007). In a cohort study with 127 dogs with lymphoma, 13% of patients lived > 2 years after their diagnosis. The patients who participated in the study had all been treated with chemotherapy in animal hospitals, where 12 of the 13 long-term survivors were treated according to the widely used CHOP-protocol. One dog was treated with doxorubicin alone (Marconato *et al.*, 2011). All 13 dogs had B-cell lymphoma. Sensitivity and specificity for this was 85% and 77% to identify dogs that survive > 2 years after his diagnosis, however, was the negative predictive value is high (98%). Parameters assessed were the prevalence of hypercalcemia, bone marrow infiltration, weight, stage and hematocrit before initiation of treatment.

In addition to chemotherapy treatment, radiation has also been suggested as an alternative (Vaughan *et al.*, 2007). Bone marrow transplant has also been discussed (Frimberger *et al.*, 2006). In these patients, it is important to establish the MRD (Minimal Residual Disease), but the methods and consensus on protocols for how this should be done have not been established. Protocols are being evaluated and a polymerase chain reaction (PCR) method for this is being developed. In a Japanese study, 29 dogs with high-grade B-cell lymphoma according to the Kiel classification system and lymphadenopathy were treated with chemotherapeutic agents according to the University of Wisconsin-Madison chemotherapy protocol (UW25, vincristine, doxorubicine and cyclophosphamide). 27 of the dogs were in CR, 2 of the dogs reached partial remission. Neoplastic lymphocytic cells in peripheral blood was determined by PCR-analysis. The PCR-product is an amplification of the rearranged immunoglobulin heavy chain (IgH) gene. The product was inserted into a vector and subjected to sequence analysis. Through real time PCR analysis, the number of neoplastic peripheral lymphocytes could be assessed. The cytoreductive effect of the three cytostatic agents included in the protocol was assessed. This analysis could demonstrate that the cytoreductive efficacy of cyclophosphamide was less than the other agents, and that dogs who did not respond to cyclophosphamide had shorter first remission than dogs who responded to treatment (Sato *et al.*, 2011).

In addition to chemotherapy, treatment with anti-tumor antibodies is also available. However, rituximab had no inhibitory effect on cancer cells in-vitro (Impellizeri *et al.*, 2006). Vaccination against cancer has been discussed and experiments are on-going, but this will not be discussed further in this text.

It can be clinically difficult to determine if CR has been achieved on cellular and molecular level. PCR techniques and genotypic detection methods have been developed for use on humans to investigate if CR has been reached (Dölken *et al.*, 2001). In veterinary medicine, these techniques are little investigated, but the use of thymidine kinase has in recent years improved the ability to detect and monitor patients with lymphoma by serum measurements (von Euler *et al.*, 2004). LDH (lactate dehydrogenase) has also been used to monitor patients with an established diagnosis of lymphoma after the end of treatment (Marconato *et al.*, 2010).

ACKNOWLEDGEMENTS

I wish to especially thank Henrik von Euler, Ewa Westergren, Staffan Eriksson and Fredrik Södersten for their participation in this project. Assistance provided by the staff at the Institution of Pathology at the National Veterinary Institute and the Swedish University of Agricultural Sciences was also greatly appreciated; thank you for lending me your invaluable expertise and your facilities. I would also like to extend my sincerest appreciations to Linpac Plastics and Gulli Strålfeldts fund for sponsoring the project and enabling its completion.

REFERENCES

1. Abboud P, Lorenzato M, Joly D, Quereux C, Birembaut P, Ploton D (2008) Prognostic value of a proliferation index including MIB1 and argyrophilic nucleolar organizer regions proteins in node-negative breast cancer. *American Journal of Obstetrics and Gynecology 199(2):146.e1-7.*
2. Akiyoshi T, Uchida K, Tateyama S. (2004) Expression of bone morphogenetic protein-6 and bone morphogenetic protein receptors in myoepithelial cells of canine mammary gland tumors. *Veterinary Pathology 41(2):154-163.*
3. Alegre MM, Robison RA, O'Neill KL. (2012) Thymidine kinase 1 upregulation is an early event in breast tumor formation. *Journal of Oncology 2012;2012:575647.*
4. Alvarez FJ, Kisseberth WC, Gallant SL, Couto GC. (2006) Dexamethasone, melphalan, actinomycin D, cytosine arabinoside (DMAC) protocol for dogs with relapsed lymphoma. *Journal of Veterinary Internal Medicine 20(5):1178–1183*
5. Antuofermo E, Miller MA, Pirino S, Xie J, Badve S, Mohammed SI. (2007) Spontaneous mammary intraepithelial lesions in dogs--a model of breast cancer. *Cancer Epidemiology, Biomarkers & Prevention 16(11):2247-2256.*
6. Arnesen K, Gamlem H, Glattre E, Moe L, Nordstoga K. (1995) Registration of canine cancer. *Tidsskrift for den Norske lægeforening : Tidsskrift for Praktisk Medicin 115(6):714-717.*
7. Bae BK, Kim CW, Choi US, Choi EW, Jee H, Kim DY, Lee CW. (2007) Hypercalcemia and high parathyroid hormone-related peptide concentration in a dog with a complex mammary carcinoma. *Veterinary Clinical Pathology 36(4):376-378.*
8. Bardagí M, Fondevila D, Ferrer L. (2012) Cyclooxygenase-2 is not expressed by canine cutaneous epitheliotropic T-cell lymphoma. *Veterinary Dermatology 23(5): 460–461*
9. Barthez PY, Davis CR, Pool RR, Hornof WJ, Morgan JP. (1995) Multiple metaphyseal involvement of a thymic lymphoma associated with hypercalcemia in a puppy. *Journal of the American Animal Hospital Association 31(1):82-85.*
10. Beauvais W, Cardwell JM, Brodbelt DC. (2012) The effect of neutering on the risk of mammary tumours in dogs -a systematic review. *Journal of Small Animal Practice 53(6):314-322.*
11. Bergman PJ. (2012) Paraneoplastic hypercalcemia. *Topics in Companion Animal Medicine, 27(4):156-158.*
12. Berenstein D. (2004) Structure-function studies of human cytosolic thymidine kinase. *PhD Thesis at the Department of Life Sciences and Chemistry, Roskilde University, Denmark.*
13. Birnbaum T, Langer S, Roeber S, von Baumgarten L, Straube A (2013) Expression of B-cell activating factor, a proliferating inducing ligand and its receptors in primary central nervous system lymphoma. *Neurology International 21;5(1):e4.*
14. Bonnett BN, Egenvall A, Hedhammar A, Olson P. (2005) Mortality in over 350,000 insured Swedish dogs from 1995-2000: I. Breed-, gender-, age- and cause-specific rates. *Acta Veterinaria Scandinavica 46(3):105-120.*
15. Bonnett BN, Egenvall A. (2010) Age patterns of disease and death in insured Swedish dogs, cats and horses. *Journal of Comparative Pathology 142 Suppl 1:S33-38.*
16. Brockenbrough JS, Morihara JK, Hawes SE, Stern JE, Rasey JS, Wiens LW, Feng Q, Vesselle H. (2009) Thymidine kinase 1 and thymidine phosphorylase expression in non-small-cell lung carcinoma in relation to angiogenesis and proliferation. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society 57(11):1087-1097.*

17. Brunetti B, Sarli G, Preziosi R, Monari I, Benazzi C. (2005) E-cadherin and beta-catenin reduction influence invasion but not proliferation and survival in canine malignant mammary tumors. *Veterinary Pathology 42(6):781-787.*
18. Brønden LB, Nielsen SS, Toft N, Kristensen AT. (2010) Data from the Danish veterinary cancer registry on the occurrence and distribution of neoplasms in dogs in Denmark. *Veterinary Records 166(19):586-590.*
19. Campo E, Swerdlow SH, Harris NL, Pileri S, Stein H, Jaffe ES (2011) The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. *Blood 117(19): 5019-5032.*
20. Cassali, GD, Lavalle GE, de Nardi AB, Ferreira E, Bertagnolli AC, Estrela-Lima A, Alessi AC, Daleck CR, Salgado BS, Fernandes CG, Sobral RA, Amorim RL, Gamba CO, Damasceno KA, Auler PA, Magalhães GM, Silva JO, Raposo JB, Ferreira AMR, Oliveira LO, Malm C, Zuccari DAPC, Tanaka NM, Ribeiro LR, Campos LC, Souza CM. (2011) Consensus for the Diagnosis, Prognosis and Treatment of Canine Mammary Tumors. *Brazilian Journal of Veterinary Pathology 4(2):153-180.*
21. Chang SC, Chang CC, Chang TJ, Wong ML.(2005) Prognostic factors associated with survival two years after surgery in dogs with malignant mammary tumors: 79 cases (1998-2002). *Journal of the American Veterinary Medical Association 227(10):1625-1629.*
22. Chang CC, Tsai MH, Liao JW, Chan JP, Wong ML, Chang SC. (2009) Evaluation of hormone receptor expression for use in predicting survival of female dogs with malignant mammary gland tumors. *Journal of the American Veterinary Medical Association 235(4):391-396.*
23. Chen ZH, Huang SQ, Wang Y, Yang AZ, Wen J, Xu XH, Chen Y, Chen QB, Wang YH, He E, Zhou J, Skog S. (2011) Serological thymidine kinase 1 is a biomarker for early detection of tumours--a health screening study on 35,365 people, using a sensitive chemiluminescent dot blot assay. *Sensors (Basel) 11(12):11064-11080*
24. Clemente M, Pérez-Alenza MD, Illera JC, Peña L. (2010) Histological, immunohistological, and ultrastructural description of vasculogenic mimicry in canine mammary cancer. *Veterinary Pathology 47(2):265-274.*
25. Concannon PW, Spraker TR, Casey HW, Hansel W. (1981) Gross and histopathologic effects of medroxyprogesterone acetate and progesterone on the mammary glands of adult beagle bitches. *Fertil Steril 36(3):373-387.*
26. Cordell JL, Falini B, Erber WN, Ghosh AK, Abdulaziz Z, MacDonald S, Pulford KA, Stein H, Mason DY. (1984) Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *Journal of Histochemistry and Cytochemistry 32(2):219-229.*
27. Delfour C, Roger P, Bret C, Berthe ML, Rochaix P, Kalfa N, Raynaud P, Bibeau F, Maudelonde T, Boulle N. (2006) RCL2, a new fixative, preserves morphology and nucleic acid integrity in paraffin-embedded breast carcinoma and microdissected breast tumor cells. *Journal of Molecular Diagnostics 8(2):157-169.*
28. Destexhe E, Lespagnard L, Egeyter MD, Heymann R, Coignul F. (1993) Immunohistochemical Identification of myoepithelial, epithelial, and connective tissue cells in canine mammary tumors. *Veterinary Pathology 30(2):146-154.*
29. Di Bella A, Maurella C, Cauvin A, Schmidt JM, Tapia BB, North SM. (2013) Proteinuria in canine patients with lymphoma. *Journal of Small Animal Practice 54(1):28-32.*

30. Diamandis EP, Christopoulos TK. (1991) 1. The biotin-(strept)avidin system: principles and applications in biotechnology. *Clinical Chemistry 37(5):625-636.*
31. Dobson JM, Samuel S, Milstein H, Rogers K, Wood JL. (2002) Canine neoplasia in the UK: estimates of incidence rates from a population of insured dogs. *Journal of Small Animal Practice 43(6):240-246.*
32. Dobson J. (2004) Classification of canine lymphoma: a step forward. *Veterinary Journal 167(2):125-126.*
33. Dobson JM, Lascelles BDX. (2011) BSAVA Manual of Canine and Feline Oncology, 3rd ed., ISBN 978 1 905319 21 3, pages 237-240. *Published by the British Small Animal Veterinary Association.*
34. Dorn CR, Taylor DO, Schneider R, Hibbard HH, Klauber MR (1968) Survey of animal neoplasms in Alameda and Contra Costa Counties, California. II. Cancer morbidity in dogs and cats from Alameda County. *Journal of the National Cancer Institute 40(2):307-318.*
35. Dölken G. (2001) Detection of minimal residual disease. *Advances in Cancer Research 82: 133–185.*
36. Edwards DS, Henley WE, Harding EF, Dobson JM, Wood JL. (2003) Breed incidence of lymphoma in a UK population of insured dogs. *Veterinary Comparative Oncology 1(4):200-206.*
37. Egenvall A, Nødtvedt A, Penell J, Gunnarsson L, Bonnett BN.(2009) Insurance data for research in companion animals: benefits and limitations. *Acta Veterinaria Scandinavica doi: 10.1186/1751-0147-51-42.*
38. Elliott JW, Cripps P, Blackwood L. (2013) Thymidine kinase assay in canine lymphoma. *Veterinary Comparative Oncology 11(1):1-13*
39. Feliciano M.A., Vicente W.R., Silva M.A. (2012) Conventional and Doppler ultrasound for the differentiation of benign and malignant canine mammary tumours. *Journal of Small Animal Practice 53(6):332-337*
40. Flood-Knapik KE, Durham AC, Gregor TP, Sánchez MD, Durney ME, Sorenmo KU. (2012) Clinical, histopathological and immunohistochemical characterization of canine indolent lymphoma. *Veterinary Comparative Oncology doi: 10.1111/j.1476-5829.2011.00317.x.*
41. Flory AB, Rassnick KM, Stokol T, Scrivani PV, Erb HN. (2007) Stage migration in dogs with lymphoma. *Journal of Veterinary Internal Medicine 21(5):1041–1047.*
42. Foekens JA, Romain S, Look MP, Martin PM, Klijn J G M, (2001) Thymidine Kinase and Thymidylate Synthase in Advanced Breast Cancer: Response to Tamoxifen and Chemotherapy. *Cancer Research 61(4):1421-1425.*
43. Frimberger, AE, Moore, AS, Rassnick, KM, Cotter, SM, O'Sullivan J.L, Quesenberry, PJ. (2006) A combination chemotherapy protocol with dose intensification and autologous bone marrow transplant (VELCAP-HDC) for canine lymphoma. *Journal of Veterinary Internal Medicine 20(2):355–364.*
44. Gama A, Alves A, Gartner F, Schmitt F. (2003) p63: a novel myoepithelial cell marker in canine mammary tissues. *Veterinary Pathology 40(4):412-420.*
45. Gaurnier-Hausser A, Patel R, Baldwin AS, May MJ, Mason NJ. (2011) NEMO-binding domain peptide inhibits constitutive NF-κB activity and reduces tumor burden in a canine model of relapsed, refractory diffuse large B-cell lymphoma. *Clinical Cancer Research 17(14):4661-4671.*
46. Genelhu MC, Cardoso SV, Gobbi H, Cassali GD (2007) A comparative study between mixed-type tumours from human salivary and canine mammary glands. *BMC Cancer 7:218.*

47. Goldschmidt M, Peña L, Rasotto R, Zappulli V. (2011) Classification and grading of canine mammary tumors. *Veterinary Pathology 48(1):117-131.*
48. Hallek M, Langenmayer I, Nerl C, Knauf W, Dietzfelbinger H, Adorf D, Ostwald M, Busch R, Kuhn-Hallek I, Thiel E, Emmerich B (1999) Elevated serum thymidine kinase levels identify a subgroup at high risk of disease progression in early, nonsmoldering chronic lymphocytic leukemia. *Blood 93(5):1732-1737.*
49. He Q, Zhang P, Zou L, Li H, Wang X, Zhou S, Fornander T, Skog S. (2005) Concentration of thymidine kinase 1 in serum (S-TK1) is a more sensitive proliferation marker in human solid tumors than its activity. *Oncology Reports 14(4):1013-1019.*
50. He E, Xu XH, Guan H, Chen Y, Chen ZH, Pan ZL, Tang LL, Hu GZ, Li Y, Zhang M, Zhou J, Eriksson S, Fornander T, Skog S. (2010) Thymidine kinase 1 is a potential marker for prognosis and monitoring the response to treatment of patients with breast, lung, and esophageal cancer and non-Hodgkin's lymphoma. *Nucleosides, Nucleotides & Nucleic Acids. 29(4-6):352-358.*
51. Herrmann K, Wieder HA, Buck AK, Schöffel M, Krause BJ, Fend F, Schuster T, Meyer zum Büschenfelde C, Wester HJ, Duyster J, Peschel C, Schwaiger M, Dechow T. (2007) Early response assessment using 3'-deoxy-3'-[18F]fluorothymidine-positron emission tomography in high-grade non-Hodgkin's lymphoma. *Clinical Cancer Research 13(12):3552-3558.*
52. Hellmén E, Bergström R, Holmberg L, Spångberg I.B., Hansson K, Lindgren A (1993) Prognostic Factors in Canine Mammary Tumors: A Multivariate Study of 202 Consecutive Cases. *Veterinary Pathology 30(1):20-27.*
53. Huang S, Lin J, Guo N, Zhang M, Yun X, Liu S, Zhou J, He E, Skog S. (2011) Elevated serum thymidine kinase 1 predicts risk of pre/early cancerous progression. *Asian Pacific Journal of Cancer Prevention 12(2):497-505.*
54. Huang SH, Kozak PJ, Kim J, Habineza-Ndikuyeze G, Meade C, Gaurnier-Hausser A, Patel R, Robertson E, Mason NJ.(2012) Evidence of an oncogenic gammaherpesvirus in domestic dogs. *Virology 427(2):107-117.*
55. Huang ZH, Tian XS, Li R, (2012) Elevated thymidine kinase 1 in serum following neoadjuvant chemotherapy predicts poor outcome for patients with locally advanced breast cancer. *Experimental and Therapeutic Medicine 3(2):331-335.*
56. Hunyady B, Krempels K, Harta G, Mezey E. (1996) Immunohistochemical signal amplification by catalyzed reporter deposition and its application in double immunostaining. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society 44(12):1353-1362.*
57. Igawa T, Sato Y, Takata K, Iwaki N, Tanaka T, Asano N, Maeda Y, Orita Y, Nakamura N, Nakamura S, Yoshino T. (2013) De novo CD5-positive diffuse large B-cell lymphomas show high specificity for cyclin D2 expression. *Diagnostic Pathology doi: 10.1186/1746-1596-8-81.*
58. Im KS, Kim IH, Kim NH, Lim HY, Kim JH, Sur JH. (2013) Breed-related differences in altered BRCA1 expression, phenotype and subtype in malignant canine mammary tumors. *Veterinary Journal 195(3):366-372.*
59. Impellizeri, J.A., Howell, K., McKeever, K.P., Crow, S.E. (2006) The role of rituximab in the treatment of canine lymphoma: an ex vivo evaluation. *The Veterinary Journal 171(3):556–558.*
60. Ito D, Endicott MM, Jubala CM, Helm KM, Burnett RC, Husbands BD, Borgatti A, Henson MS, Burgess KE, Bell JS, Kisseberth WC, Valli VE, Cutter GR, Avery AC, Hahn KA, O'Brien TD, Modiano JF. (2011) A tumor-related lymphoid progenitor population supports hierarchical tumor organization in canine B-cell lymphoma. *Journal of Veterinary Internal Medicine 25(4):890-896.*

61. Ito K, Ralph SJ (2012) Inhibiting galectin-1 reduces murine lung metastasis with increased CD4(+) and CD8 (+) T cells and reduced cancer cell adherence. *Clinical and Experimental Metastasis 29(6):561-572.*
62. Jagielski D, Lechowski R, Hoffmann-Jagielska M, Winiarczyk S. (2002) A retrospective study of the incidence and prognostic factors of multicentric lymphoma in dogs (1998-2000). *Journal of Veterinary Medicine. A, Physiology, pathology, clinical medicine 49(8):419-424.*
63. Karayannopoulou M, Kaldrymidou E, Constantinidis TC, Dessiris A (2005) Histological Grading and Prognosis in Dogs with Mammary Carcinomas: Application of a Human Grading Method. *Journal of Comparative Pathology 133(4):246–252.*
64. Kenny LM, Vigushin DM, Al-Nahhas A, Osman S, Luthra SK, Shousha S, Coombes RC, Aboagye EO. (2005) Quantification of cellular proliferation in tumor and normal tissues of patients with breast cancer by [18F]fluorothymidine-positron emission tomography imaging: evaluation of analytical methods. *Cancer Research 65(21):10104-10112.*
65. Kim J-H, Yu C-H, Yhee J-Y, Im K-S, Sur J-H. (2010) Lymphocyte infiltration, expression of interleukin (IL) -1, IL-6 and expression of mutated breast cancer susceptibility gene-1 correlate with malignancy of canine mammary tumours. *Journal of Comparative Pathology 142(2-3):177-186.*
66. Kimura KC, Zanini DA, Nishiya AT, Dias RA, Dagli MLZ. (2001) Morphology and Immunophenotypes of Canine Lymphomas: a Survey from the Service of Animal Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo. *Brazilian Journal of Veterinary Pathology 4(3):199-206.*
67. Kiupel M, Teske E, Bostock D. (1999) Prognostic factors for treated canine malignant lymphoma. *Veterinary Pathology 36(4):292-300.*
68. Klopfleisch R, Lenze D, Hummel M, Gruber AD. (2010) Metastatic canine mammary carcinomas can be identified by a gene expression profile that partly overlaps with human breast cancer profiles. *BMC Cancer doi: 10.1186/1471-2407-10-618.*
69. Klopfleisch R, Hvid H, Klose P, Da Costa A, Gruber AD. (2010) Insulin receptor is expressed in normal canine mammary gland and benign adenomas but decreased in metastatic canine mammary carcinomas similar to human breast cancer. *Veterinary Comparative Oncology 8(4):293-301.*
70. Klopfleisch R, Klose P, Weise C, Bondzio A, Multhaup G, Einspanier R, Gruber AD. (2010) Proteome of metastatic canine mammary carcinomas: similarities to and differences from human breast cancer. *Journal of Proteome Research 9(12):6380-6391.*
71. Kohlberger P, Gantert M, Volk-Orlowska T, Kieback DG, Gitsch G. (2001) Immunohistochemical detection of lymph node metastases in node-negative breast cancer patients. *Anticancer Research 21(1B):697-699.*
72. Konoplev SN, Fritsche HA, O'Brien S, Wierda WG, Keating MJ, Gornet TG, St Romain S, Wang X, Inamdar K, Johnson MR, Medeiros LJ, Bueso-Ramos CE. (2010) High serum thymidine kinase 1 level predicts poorer survival in patients with chronic lymphocytic leukemia. *American Journal of Clinical Pathology 134(3):472-477.*
73. Kooistra HS, Voorhout G, Selman PJ, Rijnberk A. (1998) Progestin-induced growth hormone (GH) production in the treatment of dogs with congenital GH deficiency. *Domestic Animal Endocrinology 15(2):93-102.*
74. Krem MM, Horwitz MS (2013) Mitotic errors, aneuploidy and micronuclei in Hodgkin lymphoma pathogenesis. *Communicative and Integrative Biology 6(3):e23544.*
75. Krishnamurthy S, Meric-Bernstam F, Lucci A, Hwang RF, Kuerer HM, Babiera G, Ames FC, Feig BW, Ross MI, Singletary E, Hunt KK, Bedrosian I. (2009) A

Prospective Study Comparing Touch Imprint Cytology, Frozen Section Analysis, and Rapid Cytokeratin Immunostain for Intraoperative Evaluation of Axillary Sentinel Lymph Nodes in Breast Cancer. *Cancer 115(7):1555-1562.*

76. Kruck S, Hennenlotter J, Vogel U, Schilling D, Gakis G, Hevler J, Kuehs U, Stenzl A, Schwentner C. (2012) Exposed proliferation antigen 210 (XPA-210) in renal cell carcinoma (RCC) and oncocytoma: clinical utility and biological implications. *BJU International 109(4):634-638.*

77. Kurzrock R, Ravandi F. (2006) Purine analogues in advanced T-cell lymphoid malignancies. Seminars in Hematology 43, 27–34.

78. Kuwa K, Kudo H, Sakamoto S. (1996) Serum thymidine kinase as a tumor marker of colorectal carcinogenesis induced with 1,2-dimethylhydrazine in rats. *Oncology Reports 3(1):15-16.*

79. Kämmerer U, Kapp M, Gassel AM, Richter T, Tank C, Dietl J, Ruck P. (2001) A New Rapid Immunohistochemical Staining Technique Using the EnVision Antibody Complex. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society 49(5): 623–630.*

80. Lawrence J, Rohren E, Provenzale J. (2010) PET/CT today and tomorrow in veterinary cancer diagnosis and monitoring: fundamentals, early results and future perspectives. *Veterinary Comparative Oncology 8(3):163-187.*

81. Li Z, Wang Y, He J, Ma J, Zhao L, Chen H, Li N, Zou J, He E, Skog S. (2010) Serological thymidine kinase is a prognostic factor in oesophageal, cardial and lung carcinomas. *European Journal of Cancer Prevention 19(4):313-318.*

82. Luo P, He E, Eriksson S, Zhou J, Hu G, Zhang J, Skog S. (2009) Thymidine kinase activity in serum of renal cell carcinoma patients is a useful prognostic marker. *European Journal of Cancer Prevention : the Official Journal of the European Cancer Prevention Organisation (ECP) 18(3):220-224.*

83. Luo P, Wang N, He E, Eriksson S, Zhou J, Hu G, Zhang J, Skog S. (2010) The proliferation marker thymidine kinase 1 level is high in normal kidney tubule cells compared to other normal and malignant renal cells. *Pathology Oncology Research 16(2):277-283.*

84. Lv X, Feng L, Fang X, Jiang Y, Wang X. (2013) Overexpression of IL-9 receptor in diffuse large B-cell lymphoma. *International Journal of Clinical and Experimental Pathology 6(5):911-916.*

85. Macdonald JI, Dick FA.(2012) Posttranslational modifications of the retinoblastoma tumor suppressor protein as determinants of function. *Genes and Cancer 3(11-12):619-633.*

86. Malorny U, Bildau H, Sorg C. (1988) Efficient inhibition of endogenous peroxidase without antigen denaturation in immunohistochemistry. *Journal of Immunological Methods 111(1):101-107.*

87. Mancho C, Sainz Á, García-Sancho M, Villaescusa A, Rodríguez-Franco F. (2011) Evaluation of perinuclear antineutrophilic cytoplasmic antibodies in sera from dogs with inflammatory bowel disease or intestinal lymphoma. *American Journal of Veterinary Research 72(10):1333-1337.*

88. Marconato L, Bonfanti U, Stefanello D, Lorenzo MR, Romanelli G, Comazzi S, Zini E. (2008) Cytosine arabinoside in addition to VCAA-based protocols for the treatment of canine lymphoma with bone marrow involvement: does it make the difference? *Veterinary and Comparative Oncology 6(2):80–89.*

89. Marconato L, Crispino G, Finotello R, Mazzotti S, Zini E. (2010) Clinical relevance of serial determinations of lactate dehydrogenase activity used to predict recurrence in dogs with lymphoma. *Journal of the American Veterinary Medical Association 236(9):969–974.*

90. Marconato L, Damiano S, Valenti P, Bonfanti U, Comazzi S, Roccabianca P, Caniatti M, Romanelli Gi, Massari F, Zini E. (2011) Predictors of long-term survival in dogs with high-grade multicentric lymphoma. *Journal of the American Veterinary Medical Association 238(4):480-485.*
91. Méniel V, Hay T, Douglas-Jones A, Sansom OJ, Clarke AR. (2005) Mutations in Apc and p53 synergize to promote mammary neoplasia. *Cancer Research 65(2):410-416.*
92. Merlo D F, Rossi L, Rossi L, Pellegrino C, Ceppi M, Cardellino U, Capurro C, Ratto A, Sambucco P L, Sestito V, Tanara G, Bocchini V (2008) Cancer incidence in pet dogs: findings of the Animal Tumor Registry of Genoa, Italy. *Journal of Veterinary Internal Medicine 22(4):976-984.*
93. Messinger JS, Windham WR, Ward CR. (2009) Ionized hypercalcemia in dogs: a retrospective study of 109 cases (1998-2003). *Journal of Veterinary Internal Medicine 23(3):514-519.*
94. Millanta F, Caneschi V, Ressel L, Citi S, Poli A (2010) Expression of Vascular Endothelial Growth Factor in Canine Inflammatory and Non-inflammatory Mammary Carcinoma. *Journal of Comparative Pathology 142(1):36-42.*
95. Miller RT, Kubier P, Reynolds B, Henry T, Turnbow H. (1999) Blocking of Endogenous Avidin-Binding Activity in Immunohistochemistry: The Use of Skim Milk as an Economical and Effective Substitute for Commercial Biotin Solutions. *Applied Immunohistochemistry & Molecular Morphology 7(1):63-65.*
96. Misdorp W. (1988) Canine mammary tumours: protective effect of late ovariectomy and stimulating effect of progestins. *The Veterinary Quarterly 10(1):26-33.*
97. Misdorp W., Else R.W., Hellmén E., Lipscomb T.P. (1999) Histological Classification of Mammary Tumors of the Dog and Cat. *Armed Forces Institute of Pathology in collaboration with the American Registry of Pathology and the World Health Organisation Collaborating Center, Washington D.C. 2nd volume.*
98. Mitchell L, Dow SW, Slansky JE, Biller BJ (2012) Induction of remission results in spontaneous enhancement of anti-tumor cytotoxic T-lymphocyte activity in dogs with B cell lymphoma. *Veterinary Immunology and Immunopathology 145(3-4):597-603.*
99. Moe L. (2001) Population-based incidence of mammary tumours in some dog breeds. *Journal of Reprododuction and Fertility 57:439-43.*
100. Mol JA, van Garderen E, Rutteman GR, Rijnberk A. (1996) New insights in the molecular mechanism of progestin-induced proliferation of mammary epithelium: induction of the local biosynthesis of growth hormone (GH) in the mammary glands of dogs, cats and humans. *The Journal of Steroid Biochemistry and Molecular Biology 57(1-2):67-71.*
101. Moore PF, Olivry T, Naydan D. (1994) Canine cutaneous epitheliotropic lymphoma (mycosis fungoides) is a proliferative disorder of CD8+ T cells. *American Journal of Pathology 144(2):421-429.*
102. Moulton JE, Taylor DO, Dorn CR, Andersen AC. (1970) Canine mammary tumors. *Veterinary Pathology 7(4):289-320.*
103. Musto P, Bodenizza C, Falcone A, D'Arena G, Scalzulli P, Perla G, Modoni S, Parlatore L, Valvano MR, Carotenuto M. (1995) Prognostic relevance of serum thymidine kinase in primary myelodysplastic syndromes: relationship to development of acute myeloid leukaemia. *Brittish Journal of Haematology 90(1):125-130.*
104. Nakamura N., Momoi Y., Watari T., Yoshino T., Tsujimoto H., Hasegawa A., (1997)Plasma thymidine kinase activity in dogs with lymphoma and leukemia. *The*

Journal of veterinary medical science / the Japanese Society of Veterinary Science 59(10):957-60.

105. Nieto A, Pérez-Alenza MD, Del Castillo N, Tabanera E, Castaño M, Peña L (2003) BRCA1 Expression in Canine Mammary Dysplasias and Tumours: Relationship with Prognostic Variables. *Journal of Comparative Pathology 128(4):260-268.*

106. Northrup NC, Gieger TL, Kosarek CE, Saba CF, LeRoy BE, Wall TM, Hume KR, Childress MO, Keys DA. (2009) Mechlorethamine, procarbazine and prednisone for the treatment of resistant lymphoma in dogs. *Veterinary and Comparative Oncology 7(1):38–44.*

107. Nødtvedt A, Berke O, Bonnett BN, Brønden L. (2012) Current status of canine cancer registration - report from an international workshop. *Veterinary and Comparative Oncology 10(2):95-101.*

108. Nyberg P. (2007) Thymidinkinas 1 som Tumörmarkör vid Juvertumörer hos Tik. *Master thesis, University of Agricultural Sciences, Uppsala, Sweden.*

109. Ohsugi T, Ishida T, Shimasaki T, Okada S, Umezawa K. (2013) p53 Dysfunction Precedes the Activation of Nuclear Factor-κB During Disease Progression in Mice Expressing Tax, a Human T-cell Leukemia Virus Type 1 Oncoprotein. *Carcinogenesis. [Epub ahead of print]*

110. O'Neill AM, Smith AN, Spangler EA, Whitley EM, Schleis SE, Bird RC, Curiel DT, Thacker EE, Smith BF.(2011) Resistance of canine lymphoma cells to adenoviral infection due to reduced cell surface RGD binding integrins. *Cancer Biology and Therapy 11(7):651-658.*

111. Pan ZL, Ji XY, Shi YM, Zhou J, He E, Skog S. (2010) Serum thymidine kinase 1 concentration as a prognostic factor of chemotherapy-treated non-Hodgkin's lymphoma patients. *Journal of Cancer Research and Clinical Oncology 136(8):1193-1199.*

112. Parodi AL. (2001) Classification of Malignant Lymphoma in Domestic Animals: History and Conceptual Evolution. *European Journal of Veterinary Pathology 7(2):43-50.*

113. Pereira CT, Rahal SC, de Carvalho Balieiro JC, Ribeiro AA. (2003) Lymphatic drainage on healthy and neoplasic mammary glands in female dogs: can it really be altered? *Anatomy, Histology, Embryology, 32(5):282-290.*

114. Pérez Alenza D, Rutteman GR, Peña L, Beynen AC, Cuesta P. (1998) Relation between habitual diet and canine mammary tumors in a case-control study. *Journal of Veterinary Internal Medicine 12(3):132-139.*

115. Phillips BS, Kass PH, Naydan DK, Winthrop MD, Griffey SM, Madewell BR (2000) Apoptotic and proliferation indexes in canine lymphoma. *Journal of Veterinary Diagnostic Investigation 12(2):111-117.*

116. Ponce F, Magnol JP, Ledieu D, Marchal T, Turinelli V, Chalvet-Monfray K, Fournel-Fleury C. (2004) Prognostic significance of morphological subtypes in canine malignant lymphomas during chemotherapy. *The Veterinary Journal 167(2):158–166.*

117. Ponce F, Marchal T, Magnol JP, Turinelli V, Ledieu D, Bonnefont C, Pastor M, Delignette ML, Fournel-Fleury C. (2010) A morphological study of 608 cases of canine malignant lymphoma in France with a focus on comparative similarities between canine and human lymphoma morphology. *Veterinary Pathology 47(3):414-433.*

118. Pretzer SD. (2008) Canine embryonic and fetal development: a review. *Theriogenology 70(3):300-303.*

119. Queiroga FL, Perez-Alenza MD, Silvan G, Peña L, Lopes C, Illera JC. (2005) Cox-2 levels in canine mammary tumors, including inflammatory mammary carcinoma:

clinicopathological features and prognostic significance. *Anticancer Research 25(6B):4269-4275.*

120. Queiroga FL, Pérez-Alenza MD, Silvan G, Peña L, Lopes CS, Illera JC. (2008) Crosstalk between GH/IGF-I axis and steroid hormones (progesterone, 17beta-estradiol) in canine mammary tumours. *Journal of Steroid Biochemistry and Molecular Biology 110(1-2):76-82.*

121. Ramos-Vara JA, Miller MA (2006) Comparison of two polymer-based immunohistochemical detection systems: ENVISION+ and ImmPRESS. *Journal of Microscopy 224(2):135-139.*

122. Ramos-Vara JA, Miller MA (2011) Immunohistochemical expression of E-cadherin does not distinguish canine cutaneous histiocytoma from other canine round cell tumors. *Veterinary Pathology 48(3):758-763.*

123. Rao S, Lana S, Eickhoff J, Marcus E, Avery PR, Morley PS, Avery AC. (2011) Class II major histocompatibility complex expression and cell size independently predict survival in canine B-cell lymphoma. *Journal of Veterinary Internal Medicine 25(5):1097-1105.*

124. Rasotto R, Zappulli V, Castagnaro M, Goldschmidt MH. (2012) A Retrospective Study of Those Histopathologic Parameters Predictive of Invasion of the Lymphatic System by Canine Mammary Carcinomas. *Veterinary Pathology 49(2):330-340.*

125. Rawal S, Chu F, Zhang M, Park HJ, Nattamai D, Kannan S, Sharma R, Delgado D, Chou T, Lin HY, Baladandayuthapani V, Luong A, Vega F, Fowler N, Dong C, Davis RE, Neelapu SS (2013) Cross Talk between Follicular Th Cells and Tumor Cells in Human Follicular Lymphoma Promotes Immune Evasion in the Tumor Microenvironment. *Journal of Immunology [Epub ahead of print]*.

126. Rehn S, Gronowitz JS, Killander C, Sundström C, Glimelius B. (1995) Deoxythymidine kinase in the tumour cells and serum of patients with non-Hodgkin lymphomas. *British Journal of Cancer 71(5):1099-1105.*

127. Ridyard AE, Rhind SM, French AT, Munro EA, Hill PB. (2000) Myasthenia gravis associated with cutaneous lymphoma in a dog. *Journal of Small Animal Practice 41(8):348-51.*

128. Rosol TJ, Nagode LA, Couto CG, Hammer AS, Chew DJ, Peterson JL, Ayl RD, Steinmeyer CL, Capen CC. (1992) Parathyroid hormone (PTH)-related protein, PTH, and 1,25-dihydroxyvitamin D in dogs with cancer-associated hypercalcemia. *Endocrinology 131(3):1157-1164.*

129. Saba CF, Thamm DH, Vail DM. (2007) Combination chemotherapy with Lasparaginase, lomustine, and prednisone for relapsed or refractory canine lymphoma. *Journal of Veterinary Internal Medicine 21(1):127–132.*

130. Sapierzyński R., Micuń J., Jagielski D., Jurka P. (2010) Cytopathology of canine lymphomas (100 cases). *Polish Journal of Veterinary Sciences 13(4):653-659.*

131. Sato M, Kanemoto H, Kagawa Y, Kobayashi T, Goto-Koshino Y, Mochizuki H, Takahashi M, Fujino Y, Ohno K, Tsujimoto H. (2011) Evaluation of the prognostic significance of BCL6 gene expression in canine high-grade B-cell lymphoma. *Veterinary Journal 191(1):108-114.*

132. Sato M, Kanemoto H, Kagawa Y, Kobayashi T, Goto-Koshino Y, Mochizuki H, Takahashi M, Fujino Y, Ohno K, Tsujimoto H. (2012) Evaluation of the prognostic significance of BCL6 gene expression in canine high-grade B-cell lymphoma. *Veterinary Journal 191(1):108-114.*

133. Savage P, Stebbing J, Bower M, et al. (2009) Why does chemotherapy cure only some cancers? *Nature Clinical Practice, Oncology 6(1):43–52*

134. Schmidt S, Essmann F, Cirstea IC, Kuck F, Thakur HC, Singh M, Kletke A, Jänicke RU, Wiek C, Hanenberg H, Ahmadian MR, Schulze-Osthoff K, Nürnberg B, Piekorz RP. (2010) The centrosome and mitotic spindle apparatus in cancer and senescence. *Cell Cycle 9(22):4469-4473.*
135. Sharif H, Jagarlamudi KK, Liya W, Ellen H, Staffan E. (2012) Quaternary structures of recombinant, cellular, and serum forms of thymidine kinase 1 from dogs and humans. *BMC Biochemistry doi: 10.1186/1471-2091-13-12.*
136. Shi SR, Chaiwun B, Young L, Cote RJ, Taylor CR. (1993) Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society 41(11):1599-1604.*
137. Shintani M, Urano M, Takakuwa Y, Kuroda M, Kamoshida S. (2010) Immunohistochemical characterization of pyrimidine synthetic enzymes, thymidine kinase-1 and thymidylate synthase, in various types of cancer. *Oncology Reports 23(5):1345-1350.*
138. Silver IA. (1966) The anatomy of the mammary gland of the dog and cat. *Journal of Small Animal Practice 7(11):689-696.*
139. Simon D, Nolte I, Eberle N, Abbrederis N, Killich M, Hirschberger J. (2006) Treatment of dogs with lymphoma using a 12-week, maintenance-free combination chemotherapy protocol. *Journal of Veterinary Internal Medicine 20(5):948–954.*
140. Singer J, Weichselbaumer M, Stockner T, Mechtcheriakova D, Sobanov Y, Bajna E, Wrba F, Horvat R, Thalhammer JG, Willmann M, Jensen-Jarolim E. (2012) Comparative oncology: ErbB-1 and ErbB-2 homologues in canine cancer are susceptible to cetuximab and trastuzumab targeting. *Molecular Immunology 50(4):200-209.*
141. Sokołowska J, Micun J, Malicka E, Lechowski R. (2012) Proliferation activity in canine lymphomas. *Polish Journal of Veterinary Sciences 15(4):727-734.*
142. Sonnenschein EG, Glickman LT, Goldschmidt MH, McKee LJ. (1991) Body conformation, diet, and risk of breast cancer in pet dogs: a case-control study. *American Journal of Epidemiology 133(7):694-703.*
143. Sorenmo KU, Shofer FS, Goldschmidt MH. (2000) Effect of spaying and timing of spaying on survival of dogs with mammary carcinoma. *Journal of Veterinary Internal Medicine 14(3):266-70.*
144. Sorenmo K. (2003) Canine mammary gland tumors. *The Veterinary Clinics of North America: Small Animal Practice 33(3):573-596.*
145. Sorenmo KU, Kristiansen VM, Cofone MA, Shofer FS, Breen AM, Langeland M, Mongil CM, Grondahl AM, Teige J, Goldschmidt MH. (2009) Canine mammary gland tumours; a histological continuum from benign to malignant; clinical and histopathological evidence. *Veterinary and Comparative Oncology 7(3):162-172.*
146. Sorenmo KU, Krick E, Coughlin CM, Overley B, Gregor TP, Vonderheide RH, Mason NJ. (2011) CD40-activated B cell cancer vaccine improves second clinical remission and survival in privately owned dogs with non-Hodgkin's lymphoma. *PLoS One. 6(8):e24167.*
147. Spain CV, Scarlett JM, Houpt KA. (2004) Long-term risks and benefits of early-age gonadectomy in dogs. *Journal of the American Veterinary Medical Association 224(3):380-7.*
148. Sterenczak KA, Joetzke AE, Willenbrock S, Eberle N, Lange S, Junghanss C, Nolte I, Bullerdiek J, Simon D, Murua Escobar H. (2010) High-mobility group B1 (HMGB1)

and receptor for advanced glycation end-products (RAGE) expression in canine lymphoma. *Anticancer Research 30(12):5043-5048.*

149. Støvring M, Moe L, Glattre E. (1997) A population-based case-control study of canine mammary tumours and clinical use of medroxyprogesterone acetate. *Acta Pathologica, Microbiologica et Immunologica Scandinavica 105(8):590-596.*

150. Suter SE, Small GW, Seiser EL, Thomas R, Breen M, Richards KL. (2011) FLT3 mutations in canine acute lymphocytic leukemia. *BMC Cancer doi: 10.1186/1471-2407-11-38.*

151. Swerdlow, S.H., Campo, E., Harris, N.L., Jaffe, E.S., Pileri, S.A., Stein, H., Thiele, J., Vardiman, J.W. (2008) WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, 4th ed. *WHO Press, Lyon, France.*

152. Takashima-Uebelhoer BB, Barber LG, Zagarins SE, Procter-Gray E, Gollenberg AL, Moore AS, Bertone-Johnson ER. (2012) Household chemical exposures and the risk of canine malignant lymphoma, a model for human non-Hodgkin's lymphoma. *Environmental Research doi: 10.1016/j.envres.2011.12.003.*

153. Taylor AT, Stafford MA, Jones OW. (1972) Properties of thymidine kinase partially purified from human fetal and adult tissue. *The Journal of Biological Chemistry 247(6):1930-1935.*

154. Teske E. (1994) Canine malignant lymphoma: a review and comparison with human non-Hodgkin's lymphoma. *Veterinary Quarterly 16(4):209-219.*

155. Touret M, Boysen SR, Nadeau ME.(2012) Retrospective evaluation of potential causes associated with clinically relevant hyperlactatemia in dogs with lymphoma. The Canadian veterinary journal. *La Revue Vétérinaire Canadienne 53(5):511-517.*

156. Tsang, RW, Hodgson DC, Crump M. (2006) Hodgkin's Lymphoma. *Current Problems in Cancer 30(3):107-152.*

157. Tvarijonaviciute A, Tecles F, Carillo JM, Rubio M, Ceron JJ. (2011) Serum insulin-like growth factor-1 measurements in dogs: performance characteristics of an automated assay and study of some sources of variation. *Canadian Journal of Veterinary Research 75(4):312-316.*

158. Valli VE, Kass PH, Myint MS, Scott F (2013) Canine Lymphomas: Association of Classification Type, Disease Stage, Tumor Subtype, Mitotic Rate, and Treatment With Survival. *Veterinary Pathology [Epub ahead of print]*.

159. Vaughan, A, Johnson, JL, Williams, LE. (2007) Impact of chemotherapeutic dose intensity on first remission duration in dogs with lymphoma treated with a chemoradiotherapy protocol. *Journal of Veterinary Internal Medicine 21(6):1332–1339.*

160. von Euler H, Einarsson R, Olsson U, Lagerstedt AS, Eriksson S. (2004) Serum thymidine kinase activity in dogs with malignant lymphoma: a potent marker for prognosis and monitoring the disease. *Journal of Veterinary Internal Medicine 18(5):696-702.*

161. von Euler H, Ohrvik AB, Eriksson SK (2006) A non-radiometric method for measuring serum thymidine kinase activity in malignant lymphoma in dogs. *Research in Veterinary Science 80(1):17-24.*

162. von Euler H, Rivera P, Aronsson AC, Bengtsson C, Hansson LO, Eriksson SK (2009) Monitoring therapy in canine malignant lymphoma and leukemia with serum thymidine kinase 1 activity--evaluation of a new, fully automated non-radiometric assay. *International Journal of Oncology 34(2):505-510.*

163. Vos JH, van den Ingh TS, Misdorp W, Molenbeek RF, van Mil FN, Rutteman GR, Ivanyi D, Ramaekers FC. (1993) Immunohistochemistry with keratin, vimentin,

desmin, and alpha-smooth muscle actin monoclonal antibodies in canine mammary gland: malignant mammary tumours. *Veterinary Quarterly 15(3):96-102.*

164. Watabe A, Fukumoto S, Komatsu T, Endo Y, Kadosawa T. (2011) Alterations of lymphocyte subpopulations in healthy dogs with aging and in dogs with cancer. *Veterinary Immunology and Immunopathology 142(3-4):189-200.*

165. Wimmershoff J., Polkinghorne A., Grest P., Schade B., Marchal T., Keller S.M., Guscetti F. (2010) Immunohistochemical detection of survivin in canine lymphoma. *Journal of Comparative Pathology 142(4):311-322.*

166. Wisner ER., Pollard RE, (2004) Trends in veterinary cancer imaging. *Veterinary and Comparative Oncology 2(2):49–74*

167. Xu Y, Shi QL, Ma H, Zhou H, Lu Z, Yu B, Zhou X, Eriksson S, He E, Skog S. (2012) High thymidine kinase 1 (TK1) expression is a predictor of poor survival in patients with pT1 of lung adenocarcinoma. *Tumour Biology 33(2):475-83.*

168. Yan HX, Wu HP, Ashton C, Tong C, Ying QL. (2012) Rats deficient for p53 are susceptible to spontaneous and carcinogen-induced tumorigenesis. *Carcinogenesis 33(10):2001-2005.*

169. Yang W-Y., Liua C-H., Changd C-J., Leec C-C., Changd K-J., Lina C-T. (2006) Proliferative Activity, Apoptosis and Expression of Oestrogen Receptor and Bcl-2 Oncoprotein in Canine Mammary Gland Tumours. *Journal of Comparative Pathology 134(1):70–79.*

170. Zou L, Zhang PG, Zou S, Li Y, He Q. (2002) The half-life of thymidine kinase 1 in serum measured by ECL dot blot: a potential marker for monitoring the response to surgery of patients with gastric cancer. *International Journal of Biological Markers 17(2):135-140.*