Diagnostic Implications of the Molecular Forms and Levels of Serum Thymidine Kinase 1 in Different Canine Malignancies

Kiran Kumar Jagarlamudi
Faculty of Veterinary Medicine and Animal science
Department of Anatomy, Physiology and Biochemistry
Uppsala

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Cover: Illustration of thymidine kinase 1 role in the salvage pathway of pyrimidine synthesis (Modified figure from von Euler et al., 2004).

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Abstract

Thymidine kinase 1 (ATP: thymidine 5'- phosphotransferase, EC 2.1.7. 21, TK1) is a cellular enzyme involved in salvage pathway for DNA precursor synthesis. TK1 activity fluctuates during cell cycle, it reaches peak in S phase and absent in M phase. Because of its tight regulation with cell cycle, TK1 has been used as proliferation marker for diagnosis and treatment monitoring of various malignancies in human and veterinary medicine. TK1 levels can be measured by activity based or antibody based assays.

The main aim of the research described in this thesis was to develop TK1 antibody based assays for determining serum TK1 protein levels in comparison with TK1 activity from dogs with different malignancies. Further analysis revealed a significant difference in the molecular forms of TK1 in sera from canine leukemia and mammary tumours.

In Study I, serum TK1 protein levels in dogs with different solid tumours were determined by using an antibody based assay i.e. immunoaffinity assay. TK1 protein levels were significantly higher in dogs with solid tumours than expected from the TK1 activity values. In contrast, the specific activity of TK1 in sera from healthy dogs was 2.5 fold higher than that of solid tumours. The molecular forms of recombinant, cellular and serum TK1 were investigated in study II. Dog recombinant and serum TK1 existed as oligomers with a molecular weight (MW) of 720-300 kDa. Cellular TK1 from both dogs and humans were mainly as tetramers. Human recombinant and serum TK1 activity eluted in two peaks: one at high and one at low MW, corresponding to 720-300 kDa and 200-50 kDa, respectively.

In study III, TK1 protein levels in sera from dogs with mammary tumours were determined by immunoaffinity assay. The TK1 protein assay differentiated mammary adenomas efficiently from healthy dogs, and adenomas from carcinomas, but this was not possible with the TK1 activity assay. In mammary tumour sera, active TK1 eluted as high MW oligomer similar to leukemia however, TK1 protein was detected not only as high MW form but also in the fractions where no TK1 activity was found. This indicates that serum TK1 exits in multiple forms in mammary tumours with a large fraction of inactive TK1 protein. Study IV describes the development of TK1-ELISA, based on a polyclonal and a monoclonal anti TK1 peptide antibodies, that was used to measure TK1 protein levels in dog sera. TK1 protein levels were significantly higher in sera from dogs with haematological tumours as well as solid tumours in comparison with healthy dogs. Overall, the results demonstrate that TK1 protein assays provide valuable diagnostic information in a variety of canine malignancies.

Keywords: Thymidine kinase 1, Immunoaffinity assay, anti TK1 antibodies, canine mammary tumours, TK1-ELISA.

Author's address: Kiran Kumar Jagarlamudi, Department of Anatomy, Physiology and Biochemistry, SLU, P.O. Box 7011, SE-75007, Uppsala, Sweden.
E-mail: KiranKumar.J@ slu.se.

Dedicated to my Family

"Take risks in your life"

If you win, you can lead!
If you lose, you can guide!

Swami Vivekananda

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Kiran Kumar J**., Sharif H., Westberg S., von Euler H., Eriksson S., 2013. High levels of inactive thymidine kinase 1 polypeptide detected in sera from dogs with solid tumours by immunoaffinity methods: Implications for in vitro diagnostics. *Vet J*. 197:854-860

II. Hanan S., **Jagarlamudi KK**., Liya W., Ellen H., Eriksson S., 2012. Quaternary structures of recombinant, cellular and serum forms of thymidine kinase 1 from dogs and humans. *BMC Biochem,* 13:12.

III. **Jagarlamudi KK**., Westberg S., Rönnberg H., Eriksson S., 2014. Properties of cellular and serum forms of thymidine kinase 1 (TK1) in dogs with acute lymphocytic leukemia (ALL) and canine mammary tumours (CMTs): Implications for TK1 as a proliferation biomarker. *BMC vet Res*. 10: 228.

IV. **Kiran Kumar J**., Laura M., Westberg S., Rönnberg H., Eriksson S., 2015. Development of a new ELISA for determining serum thymidine kinase 1 protein levels in canine malignancies and its clinical significance. (Manuscript).

Paper not included in this thesis:

I. **Jagarlamudi KK**., Hansson LO., Eriksson S. (2015). Breast and prostate cancer patients differ significantly in their serum Thymidine kinase 1 (TK1) specific activities compared with those hematological malignancies and blood donors: implications of using serum TK1 as a biomarker. *BMC cancer,* 15:66.

The contribution of Kiran Kumar to the papers included in this thesis was as follows:

I. Planned the studies, performed most laboratory work, analysed data and had main responsibility for writing and revising the manuscript.

II. Planned the studies with co-authors, performed part of laboratory work with minor contribution to writing part of manuscript.

III. Planned the studies with co-authors, performed most laboratory work, analysed data and had main responsibility for writing and revising manuscript.

IV. Planned the studies with co-authors, performed most laboratory work, data analysis and had main responsibility for writing and revising manuscript.

Abbreviations

ALL	Acute lymphocytic leukemia
APC	Anaphase promoting complex
ATP	Adenosine triphosphate
AZT	3'-azidothymidine
AZTMP	3'-azidothymidine monophosphate
AUC	Area under curve
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
CMTs	Canine mammary tumours
CRP	C-reactive protein
dCK	Deoxycytidine kinase
dGK	Deoxyguanisine kinase
dNs	Deoxyribonucleosides
dNKs	Deoxyribonucleoside kinases
dT	Deoxythymidine
dTDP	Deoxythymidine diphosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
dUTP	Deoxyuridine triphosphate
DTE	Dithioerythritol
FLT	3′-deoxy-3′-fluorothymidine
GMP	Guanosine monophosphate
IMP	Inosine monophosphate
PSA	Prostate specific antigen
ROC	Receiver operating characteristic
STK1	Serum thymidine kinase 1
TK1	Thymidine kinase 1
TK2	Thymidine kinase 2
TS	Thymidylate synthase
UMP	Uridine monophosphate

1. Introduction

1.1 Cancer

Cancer is one of the most common diseases in humans and is a major cause of deaths. In 2012, it accounted for 14.6% of all human deaths according to *World Health Report*. Cancer development is a complex process that involves genetic abnormalities and loss of host defence. Only 5-10% of cancers are caused by genetic defects whereas a much larger proportion are due to environmental factors such as diet, infections, radiation, stress, alcohol and obesity. These factors may induce DNA alterations and loss of cell function that results in tumour growth (Anand et al., 2008).
Carcinogenesis is a process during which normal cells are transformed into cancer cells. This process leads to an imbalance between cell proliferation and cell death, mainly due to accumulation of genetic mutations.

In the process of carcinogenesis, a normal cell that becomes a tumour cell acquires six traits that are commonly observed in different cancers and are considered as the hallmarks of cancer:

1) Self-sufficiency in growth signals
2) Insensitivity to anti-growth signals
3) Evasion of programmed cell death (Apoptosis)
4) Limitless replicative potential
5) Sustained angiogenesis
6) Invasion and metastasis (Hanahan & Weinberg, 2000).
7) Emerging are abnormal metabolic pathways and evading immune system (Hanahan & Weinberg, 2011).

In normal physiological processes, cells require exogenous mitogenic signals for proliferation but tumour cells acquire the ability to generate growth factors or growth signals to which they are responsive via so called autocrine loops. The effect is not only due to autocrine secretions, but also sometimes due to

overexpression of receptors also makes cancer cells hyper-responsive to growth factors. Specific mutations in cancer cells can cause ligand independent signalling that switches the cell extra-cellular matrix (ECM) receptors in cancer cell to become more responsive to pro-growth signals, which leads to tumour self-sufficiency (Lukashev & Werb, 1998; Hanahan &Weinberg 2000). Loss of cell cycle regulation is also a common feature in many cancers. In general, cells divide in an orderly fashion according to the phases of the cell cycle. During G1 and G2 (gap phases), the cells are actively metabolizing but division cannot occur. Only in S (synthesis) phase, DNA replication result in chromosome duplication. During M (mitosis) phase, the chromosomes separate and the division of cytoplasm (cytokinesis) takes place. There are various checkpoints in the cell cycle at the end of G1 and G2 that can prevent the cell from entering S or M phases of the cycle. Cells that are not in the process of dividing are in the G0 stage, which includes most of adult cells (Fig 1A). The cell cycle is controlled by different check points that assess extra cellular signals, cell size and DNA integrity. Cyclins and cyclin dependent kinases (CDKs) are positive regulators which induce cell cycle progression and CDK inhibitors are negative regulators in the cycle. Deregulation of the cell cycle is a common phenomenon that occurs during tumour development (Hanahan & Weinberg, 2000).

Cancer cells evade anti-proliferative signals by inhibiting the expression of integrins and other adhesion molecules that carry growth regulator signals. Furthermore, cancer cells have high expression of pro-growth signals resulting in limitless proliferation (Hanahan & Weinberg, 2000). Tumour cells avoid apoptosis in order to expand the tumour cell population and a common strategy to avoid apoptosis is down regulation of the p53 tumour suppressor gene. This results in inhibition of key components, which plays an important role for inducing the apoptotic cascade (Hanahan & Weinberg, 2000; Harris, 1996). Furthermore, tumour cells lose the capacity for senescence, leading to the limitless replicative potential of cancer cells often occurring by telomere shortening. This in turn upregulates the expression of the telomerase enzyme that adds hexanucleotide repeats to the ends of telomeric DNA. Telomere lengths are maintained above the threshold results in unlimited proliferation of descendant cells (Bryan et al., 1995; Hanahan & Weinberg, 2000).
Another important feature of tumour cells is sustained angiogenesis. Initially tumour cells lack angiogenic ability and they acquire this ability via an angiogenic switch during tumour development. It occurs by changing the balance between angiogenesis inducers and countervailing inhibitors (Hanahan & Folkman, 1996). Many tumours had increased expression of angiogenesis inducers like vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs) compared to normal tissues. The down regulation of endogenous inhibitors such as thrombospondin-1 or β-interferon is also more frequent in tumour tissues compared to normal tissues (Hanahan & Weinberg, 2011).

Once the tumour cells acquire the ability to grow beyond limitations with sustained angiogenesis, results in invasion of primary tumour masses into the surrounding tissues. Through circulation of tumour cells to distant sites where they form new tumours, the metastatic process is established (Kawaguchi, 2005; Sporn, 1996). However, at cellular level, the development of cancer consists of three steps: the first step is tumour initiation due to genetic alteration that is fixed by the cell doubling rate of a certain tissue. This often leads to abnormal proliferation of a certain tumour stem cell clone. The second step is outgrowth into a population from this clonally derived tumour cell. In the last step, tumour progression, which continues as additional mutations, occurs within the tumour population. Once the variant cells with mutations are established within the tumour population, results in clonal selection. This process continues throughout tumour development and leads to tumour metastasis in various tissues or organs (Fig 1B) (Hanahan &Weinberg, 2000; 2011).

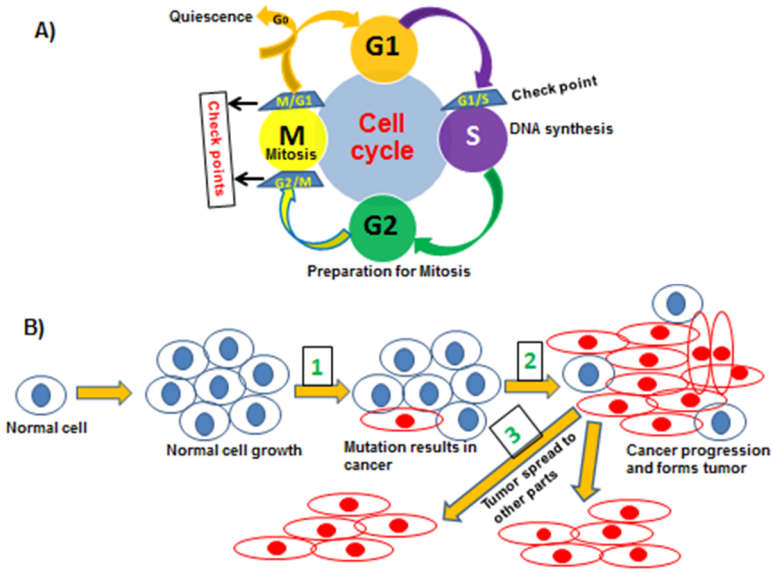

Figure 1: Cell cycle and carcinogenesis. A) Major events of cell cycle in different phases. B) The process of cancer development. Cancer development occurs in three different steps 1) initiation 2) promotion and 3) progression.

The subsequent chapters describe basic information about cancers in dogs and biomarkers for cancer detection, and the later chapters deal with thymidine kinase 1 (TK1) as a biomarker in more detail.

1.2 Cancers in dogs

Cancer is a major problem in dogs and one out of four dogs will develop cancer before the age of 10 years. The prevalence of cancer has increased in recent years and a number of factors may contribute to this such as feeding habits, hormone imbalance and infections. All dog breeds are affected by cancer but some breeds have a higher risk for a particular type of cancer, suggesting an underlying genetic predisposition to cancer. Many studies have shown that the aetiology and pathogenesis of canine tumours are similar to those of human tumours in several aspects. This indicates that dogs may serve as a good comparative model for human diseases. (MacEwen, 1990; Vail & MacEwen, 2000).

According to a Swedish insurance agency (AGRIA), 18% of dogs die each year from different types of malignancies (Bonnet et al., 2005). Another study showed that malignancies account for 23% of deaths in dogs (Jagielski et al., 2002). Yet another study found that the incidence of cancer increases with increasing age and the risk of developing cancer increases from <1% at the age of one year to 7% at the age of nine years (Bonnett & Egenvall, 2010). Dogs are affected by different types of cancers which are classified based on histopathological examination as benign or malignant that in turn indicates the extent of tumour progression. Tumours are further classified based on the origin of tissue or cell (Table 1). The most common cancers in dogs include lymphomas, osteosarcomas, hemangiosarcomas, mammary tumours and mast cell tumours. Cancer incidence depends on breed, age, sex and environmental factors. Detection of tumours at early stages will help to extend the life span of patients by earlier treatment.

Biomarkers are the substances found in the body when there is an alteration in normal body condition. According to the National Cancer Institute (NCI) a biomarker is defined as '*a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process or of a condition or disease*'. Tumour biomarkers are not only useful for early detection and to determine the effectiveness of anti-cancer therapy but also help to predict the recurrence of malignant diseases (Henry, 2010). Different studies have been done to determine the diagnostic and prognostic values of various proteins and enzymes as biomarkers in canine malignancies. Commonly used tumour biomarkers in canine oncology are described in later sections.

Table 1. Classification of tumours based on their origin (adopted from Withrow & McEwen's Small Animal Clinical Oncology, 2nd Edn).

	Benign	Malignant
A) Epithelial		
Squamous	Squamous papilloma	Squamous cell carcinoma
Transitional	transitional papilloma	Transitional cell carcinoma
Glandular	Adenoma, cystadenoma	Adenocarcinoma
Non glandular	Adenoma	Carcinoma
B) Mesenchymal		
Fibrous tissue	Fibroma	Fibrosarcoma
Fat	Lipoma	Liposarcoma
Cartilage	Chondroma	Chondrosarcoma
Bone	Osteoma	Osteosarcoma
Muscle (smooth)	Leiomyoma	Leiomyosarcoma
Muscle (skeletal)	Rhabdomyoma	Rhabdmyosarcoma
Endothelial cells	Hemangioma	Hemangiosarcoma
Synovium	-	Synovial cell sarcoma
Mesothelium	-	Mesothelioma
Melanocytes	Benign melanoma	Malignant melanoma
Peripheral nerve	Schwannoma	Malignant schwannoma
	Neurofibroma	neurofibosarcoma
C) Hematopoietic		
Lymphocytes	-	Lymphoma, Lymphosarcoma
Plasma cells	Cutaneous plasmacytoma	Multiple myeloma
Granulocytes	-	Myeloid leukemia
Red blood cells	-	Erythroid leukemia
Macrophages	Histocytoma	Malignant histiocytosis
Mast cells		Mast cell tumour
D) Brain		
Glial cells	Astrocytoma	Astrocytoma
	Oligodendroglioma	glioblastoma multiforme
Meninges	Meningioma	Malignant meningioma
E) Gonadal		
Germ cells	Seminoma, Dysgerminoma	Seminoma, Dysgerminoma
Supportive cells	Sertoli cell tumour	Sertoli cell tumour
	Granulosa cell tumour	Granulosa cell tumour
Interstitial cells	Leydig cell tumour, thecoma	

1.3 Biomarkers in Veterinary Medicine

Based on their function during tumour development, biomarkers can be classified as

I. Cell proliferation markers
II. Apoptosis
III. Matrix metalloproteinases (MMPs)
IV. Angiogenesis
V. Cell cycle regulators
VI. Serum biomarkers

I. Cell proliferation markers

One of the main characteristics of tumour cells is uncontrolled proliferation and the rate of proliferation determines the aggressiveness of the tumour. Different methods have been developed to detect cell proliferation in tumours including mitotic index (MI), incorporation techniques, flow cytometry, *in situ* hybridizations and immunohistochemical methods. Proliferation markers such as Ki-67, proliferating cell nuclear antigen (PCNA) and argyrophilic nucleolar organiser region (AgNOR) are used to determine the proliferation rate in different dog tumours by immunohistochemical methods.

Mitotic index (MI)

The Mitotic index (MI) is an indirect measure of cell proliferation based on quantification of the fraction of mitotic cells as percentage of the total number of cells. Studies showed that MI can significantly differentiate benign from malignant canine mammary tumours (Preziosi et al., 1995) and MI is also useful to separate tumours with similar histological appearance, like canine basal cell carcinomas from squamous cell carcinomas (De Vico et al., 1994). In veterinary oncology, MI has been shown as a prognostic indicator for different tumours, including mast cell tumours, mammary tumours and melanomas (Bostock et al., 1989; Bostock, 1979; Sarli et al., 2002). A major concern with this method is variations in cell size and the difficulty in discriminating mitosis from necrosis by hematoxylin and eosin staining.

Incorporation techniques (thymidine and bromodeoxyuridine)

Thymidine incorporation is one of the earliest techniques used for determining cell proliferation. Later, thymidine was replaced by the analogue bromodeoxyuridine (BrDU) for measuring cell proliferation. BrDU is incorporated into DNA of S phase cells and it can be detected by immunohistochemistry or flow cytometry using BrDU antibodies. Some studies were done by using this method to measure the rate of cell proliferation

in dog tumours (Sakai et al., 2002; Yoshida et al., 1999). Furthermore, BrDU incorporation can be used to grade mast cell tumours in dogs based on aggressiveness (Sakai et al., 2002). The need to administer BrDU into living animals before analysis is a major drawback with this method.

Ki-67

Ki-67 is a non-histone nuclear protein which is present in all phases of cell cycle except in G0. However, during interphase Ki-67 is detectable only in the nucleus and it is relocated to the surface of chromosomes during mitosis. Even though it is detectable in all phases of the cell cycle, the highest level is found in G2 and M phase cells (Gerdes et al., 1983). The prognostic and diagnostic values of Ki-67 expression have been studied in different canine tumours. The Ki-67 index correlated with the histological grade of canine lymphomas and suggests that Ki-67 can be used for classification of lymphomas based on tumour aggressiveness (Fournel- Fenry et al., 1997). Furthermore, the Ki-67 index could predict the duration of the relapse free interval after chemotherapy (Phillips et al., 2000). It is also a prognostic marker for dogs with mast cell tumour (Abadie et al., 1999). In canine mammary tumours, it was shown that a high Ki-67 index positively correlated with increased risk of metastasis, short disease free survival and overall low survival rates (Pena et al., 1998; Sarli et al., 2002; Zuccari et al., 2004). Even though Ki-67 has prognostic value, its clinical use in canine oncology has been limited due to sampling inconsistency, lack of standardized techniques and differences in immunostaining procedures.

Proliferating cell nuclear antigen (PCNA)

PCNA is a nuclear protein acting as a co-factor for DNA polymerase delta and plays an important role in DNA replication (Bravo et al., 1987). PCNA is produced in mid G1 phase and throughout S phase but it is detectable in all phases of cell cycle because of its long half-life. Higher concentrations of PCNA were observed throughout S phase, which correlates with DNA synthesis (Bravo et al., 1985). The prognostic value of PCNA was evaluated in different dog tumours. In case of mast cell tumours, disease free survival and overall survival time was longer in patients with low PCNA index (Simoes et al., 1994). Moreover, PCNA index is useful in grading mast cell tumours according to their aggression (Hung et al., 2000). For mammary tumours, the PCNA index did not relate significantly with metastasis, disease free survival and overall survival rates (Pena et al., 1998). In addition, the presence of PCNA during all cell cycle phases and its long half-life may give false positive results.

Argyrophilic nucleolar organiser region (AgNORs)

AgNORs are a set of nucleolar proteins that are coded by ribosomal genes. In proliferating cells, the quantity of AgNORs progressively increases from early G1, reaches a peak in S phase and remains constant until the end of G2 (Sirri et al., 1999). Unlike other biomarkers described above, AgNORs does not measure the percentage of proliferating cells, but gives information about the rate of cell proliferation. AgNORs could provide valuable information in grading of canine lymphomas (Kiupel et al., 1998) and in the case of mast cell tumours, dogs with high levels of AgNOR had higher chance of tumour reoccurrence after surgical excision compared to dogs with low AgNOR. In addition, the overall survival rate was lower for dogs with high level of AgNOR (Abadie et al., 1999; Simoes et al., 1994). Similar results were also found in dogs with mammary tumours where high AgNORs was associated with short survival (Sarli et al., 2002).

II. Apoptosis

Another common feature of tumour growth is imbalance between tumour cell proliferation and cell death. Programmed cell death i.e. apoptosis plays an important role in tumour growth and aggressiveness because this process is deregulated during tumour progression. There are some studies published on the role of apoptosis for diagnosis and prognosis of canine tumours. Dogs with malignant mammary tumours had higher apoptotic indices compared to those with benign tumours and no correlation was observed between apoptotic indices and metastasis or tumour diameter (Funakoshi et al., 2000). Even though apoptosis plays a substantial role in several steps of carcinogenesis but its clinical value in canine oncology is still unclear.

III. Matrix metalloproteinases (MMPs)

Degradation of the extra cellular matrix (ECM) is crucial during progression of tumour diseases. Matrix metalloproteinases (MMPs) are zinc-dependent neutral endopeptidases, which hydrolyze protein and proteoglycan components of the ECM. Under physiological conditions, MMPs are expressed by a variety of cells and tissues. The MMPs are involved in a number of pathological processes that are responsible for accelerated ECM breakdown, which strongly correlates with tumour invasion and metastasis. The levels of MMPs have apparent clinical significance in malignant diseases. In different dog malignancies like mast cell tumours and mammary tumours, studies have shown higher levels of MMPs in neoplastic tissues compared to normal tissues (Leibman et al., 2000; Loukopoulos et al., 2003; Aresu et al., 2011). However, prognostic significance of MMPs in canine tumours is not clear.

IV. Angiogenesis

Development of new blood vessels from the existing vasculature is known as angiogenesis. It plays an important role in invasion and metastasis of solid tumours. Without angiogenesis, solid tumours cannot grow larger than 1-2 mm because tumour proliferation is limited by nutrient supply. Therefore, angiogenesis can be a prognostic indicator for tumour progression. The degree of angiogenesis is assessed by the intra-tumoural micro vessel density (IMD), which is a measure of the number of new intra-tumoural vessels in a neoplasm. IMD is significantly higher in malignant canine tumours, i.e. tumours with metastasis compared to benign tumours. IMD is also an independent prognostic factor for canine mast cell tumours (Preziosi et al., 2004).

V. Cell cycle regulators

The uncontrolled cell proliferation of cancer cells is achieved by different alterations and mutations in cell cycle regulators. p53 is one of the most studied tumour suppressors in human and dog malignancies. Mutation in the p53 gene occurs commonly in different tumours and mutant p53 protein is more stable compared to wild p53 protein, as a sign of disrupted regulation of p53. Immunohistochemical studies of the p53 protein in canine tumours have shown over expression of p53 in malignant lymphomas and mast cell tumours (Jaffe et al., 2000; Sokolowska et al., 2005). Furthermore, p53 over-expression in dogs with mammary tumours correlated with short survival rates (Wakui et al., 2001).

Most of these tumour biomarkers described above are measured in tissues by immunohistochemistry, which limits their clinical applicability. Some proteins and enzymes are present in the blood of patients with malignant diseases at elevated levels and thus can serve as convenient biomarkers. Basic facts about some of these markers were described below.

VI. Serum biomarkers

Lactate dehydrogenase (LDH) levels and C-reactive protein levels (CRP) have been studied in relation with canine malignancies.

LDH is an enzyme found in most body tissues and it plays an important role in cellular intermediary metabolism. Tissue damage results in release of LDH into the blood stream. Several investigations have been done to determine the prognostic capacity of serum LDH in different canine malignancies. However, serum LDH levels were elevated not only in malignancies but also in several other conditions such as infections, which significantly limits its clinical value as a tumour biomarker (Nakamura et al., 1997; Zanatta et al., 2003; von Euler et al., 2006).

Acute phase proteins (APPs) are serum proteins released from the liver in response to infection, inflammation or trauma. APPs include C-reactive protein (CRP), haptoglobin and serum amyloid A (SAA). CRP is one of the APPs produced predominantly in the liver in response to pro-inflammatory cytokines. Serum CRP levels are used as a biomarker of systemic inflammation and its levels correlate with severity and duration of the inflammatory stimuli. Serum CRP levels are elevated in different malignancies but they are also elevated in variety of diseases with systemic inflammation (Nakamura et al., 2008). Recent study showed an alternative way to use serum CRP as a marker in combination with thymidine kinase 1 (TK1, see below) by creating a neoplastic index (NI). Evaluation of NI in dogs with different tumours apparently differentiates those with malignancy from healthy dogs more effectively than either of the two markers alone (Selting et al., 2013).

2. Overview of DNA precursor synthesis

Nucleic acids are the intracellular molecules that store the genetic information in the form of DNA or RNA. Nucleotides are the building blocks of DNA or RNA, which are involved in various functions such as DNA replication, transcription, and protein synthesis, as well as DNA repair. Nucleotides are composed of a nitrogenous base, a five-carbon sugar (ribose/deoxyribose), and one to three phosphate groups. The nucleotides are subdivided into two families: the purine bases including adenine (A) and guanine (G) and the pyrimidine bases include cytosine (C) thymine (T) and uracil (U). Thymine is only found in DNA whereas uracil is found in RNA. The five-carbon sugar is either a ribose in RNA, or a 2-deoxy ribose in DNA. The attachment of one phosphate group to the 5-hydroxyl group of pentose sugar forms a nucleoside monophosphate (NMP/dNMP), further addition of a second and a third phosphoryl groups to NMP results in nucleoside diphosphate (NDP/dNDP) and triphosphate (NTP/dNTP), respectively. DNA precursors are synthesized by two pathways, either the *de novo* or the salvage pathway.

2.1 The *de novo* pathway

In the *de novo* pathway, both purines and pyrimidines are synthesized from small molecules such as amino acids, phosphoribosyl pyrophosphate (PRPP) and CO_2, through reactions that involve many enzymes. The initial product during purine biosynthesis is IMP (inosine 5-monophosphate), which is later converted into AMP (adenosine 5-monophosphate) or GMP (guanosine 5-monophosphate). These ribonucleoside phosphates (NMPs) are further phosphorylated to di phosphates (NDPs) and tri phosphates (NTPs) for RNA synthesis. NDPs are reduced to deoxyribonucleoside diphosphates (dNDPs) by ribonucleoside reductase (RNR). The dNDPs undergo phosphorylation forming the dNTPs (Fig 2) (Nordlund & Reichard, 2006; Reichard, 1988: Thelander & Reichard, 1979). In a special section of the *de novo* pathway, dTMP is synthesized from dUMP via thymidylate synthase (TS). dTMP is further

phosphorylated into dTTP, which serves as DNA precursor (Montfort & Weichsel, 1997).

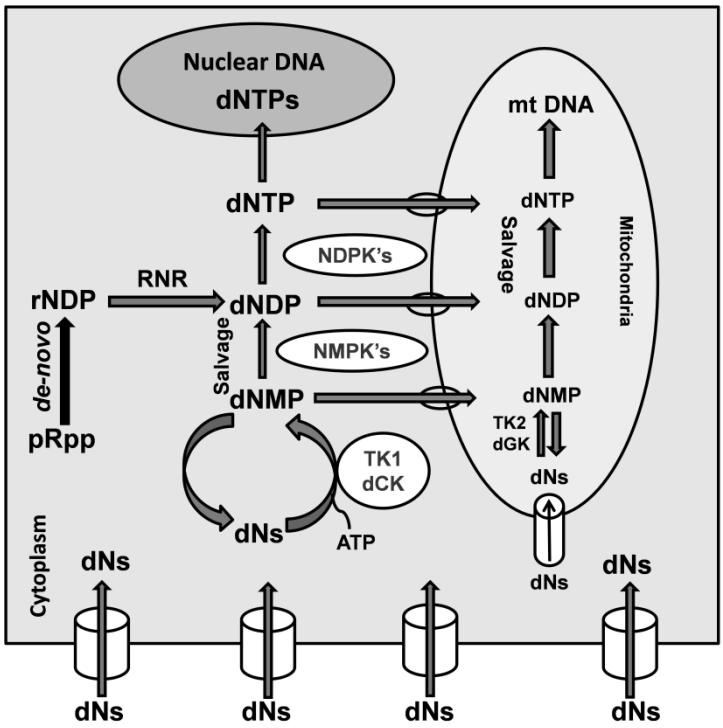

Figure 2: Synthesis of DNA precursors in the cytosol and mitochondria. The synthesis of dNTPs occurs by two pathways, ribonucleotide reductase (RNR) dependent *de novo* synthesis in the cytosol and salvage of deoxyribonucleosides, performed by kinases in the cytosol and mitochondria (Adopted from Saada, 2009).

2.2 The salvage pathway

This is an alternative pathway in which deoxyribonucleosides (dNs) from degraded DNA or from food are used as precursors. It is a complement to the *de novo* synthesis in all animal cells. The dNs are phosphorylated to dNMPs by deoxyribonucleoside kinases resulting in trapping of the nucleotides inside the cell (Fig 2). This step is often rate limiting in the salvage pathway (Arner & Eriksson, 1995). Similar to the *de novo* pathway, dNs are subsequently phosphorylated by NMPKs and NDPKs, forming dNTPs, which are used for DNA replication and repair (Fig 2) (Arner & Eriksson, 1995).

3. Deoxyribonucleoside kinases (dNKs)

dNKs are rate determining enzymes during nucleotide salvage by catalyzing the phosphorylation of deoxyribonucleoside to deoxyribonucleoside monophosphate (dNMPs). Nucleoside triphosphates act as phosphate donor. In mammals, there are four dNKs with overlapping substrate specificities. The dNKs include thymidine kinase 1(TK1), deoxycytidine kinase (dCK), thymidine kinase 2 (TK2) and deoxyguansine kinase (dGK). TK2 and dGK are mitochondrial enzymes whereas TK1 and dCK are cytosolic. Based on the amino acid sequence the dNKs are divided into two groups: the TK1 group and dCK, dGK and TK2 group (Eriksson et al., 2002). dNKs are of medical interest because they are involved in several human diseases. For example mitochondrial dNKs are needed for maintenance of mitochondrial DNA function and deficiency of either TK2 or dGK results in tissue specific mitochondrial DNA depletion syndrome (MDS) leading to severe myopathy, hepato/neuropathy and progressive external opthalmoplegia (Ronchi et al., 2012; Tyynismaa et al., 2012; Wang, 2010; Eriksson et al., 2002).
dNKs also plays important role in anti-viral and anti-cancer therapy since many of the anti-viral drugs are nucleoside analogs (NAs). These NAs are activated by dNKs to nucleoside analogue monophosphates (NAMP), and after further phosphorylation to their triphosphate forms they can be incorporated into viral or cellular DNA and interfere with viral DNA synthesis leading to blocked viral infection (Leoni et al., 1998). Moreover, dNKs are used in suicide gene therapy, in which a nucleoside kinase gene is inserted and expressed in target cells, and after treatment with NA and formation of NATP, which interferes with DNA replication resulting in cell death. Several experimental studies have been done using this concept for cancer treatment, based on herpes simplex virus (HSV) type 1-TK gene (Sterman et al., 2005; Ural et al., 2000).

In the next section basic properties of the four mammalian dNKs are described with focus on thymidine kinase 1 (TK1). Based on the subcellular location, dNKs are classified as mitochondrial dNKs and cytosolic dNKs.

3.1 Mitochondrial deoxyribonucleoside kinases (dNKs)

Thymidine kinase 2 (TK2, E.C. 2.7.1.21)

Thymidine kinase 2 (ATP: thymidine 5'-phosphotransferase) is a mitochondrial thymidine kinase that catalyzes the phosphorylation of deoxythymidine (dT), the same substrate for TK1. However, TK2 differs from TK1 in substrate specificity by phosphorylating dCyd (deoxycytidine) and its analogs to the corresponding 5'-monophosphates using ATP or CTP as phosphate donors (Eriksson et al., 2002; Munch-Petersen et al., 1991). The TK2 gene is 45 kb with 10 exons varying in size from 32 bp to 1304 bp and it is located on chromosome 16q22 (Eriksson et al., 2002). TK2 is predominantly localized in mitochondria and expressed in all tissues (Munch-Petersen, 2010; Wang & Eriksson, 2000). TK2 levels are low in proliferating cells compared to TK1 but in non-proliferating cells TK2 is the only deoxy ribonucleoside phosphorylating enzyme (Eriksson et al., 2002). TK2 plays an important role in continuous and balanced supply of dNTPs for mitochondrial DNA replication and possibly nuclear DNA repair in resting cells (Wang et al., 2003).

TK2 exits in variable forms from monomers to hexamers. Early studies described TK2 as a dimer with a molecular weight (MW) of 70 kDa (Lee & Cheng, 1976). Later studies with full-length recombinant mouse TK2 showed that it was an active dimer of about 60 kDa (Wang & Eriksson, 2000). However, more recent gel filtration analysis of human recombinant TK2 demonstrated that it was a dimer, which oligomerized to form high MW complexes. However, it is still unclear which form of TK2 is present *in vivo* (Sun et al., 2010). TK2 is also able to phosphorylate several nucleoside analogs used in anti-viral and anti-tumour therapies, such as 3'-azido-2'-deoxythymidine (AZT), arabinofuranosyl thymidine and 3'-fluoro- 2', 3'-dideoxythymidine but at varying and often very low rates. The kinetics of TK2 differs depending on the substrate, i.e. TK2 phosphorylates deoxythymidine with negative cooperativity but with deoxycytidine (dCyd) and deoxyuridine (dUrd) it follows Michaelis-Menten kinetics (Wang et al., 2003; Munch-Petersen et al., 1991). Genetic mutations in TK2 is associated with heterogeneous myopathic form of MDS, especially two missense mutations His90Asn and Ile181Asn result in skeletal myopathy, mitochondrial DNA (mt DNA) depletion and death at early age (Moreas et al., 1991). Later, TK2 knock out mice was generated to understand the physiological role of TK2 in MDS. Immediately after birth mice showed progressive reduction of mt DNA, especially in skeletal muscles, brain, heart and liver. They died within 2-4 weeks, most likely due to unbalanced mitochondrial dNTP pools those result in MDS (Akman et al., 2008).

Deoxyguanosine kinase (dGK, E.C. 2.7.1.113)

dGK (NTP: deoxyguanosine 5'-phosphotransferase) is another mitochondrial enzyme involved in phosphorylation of purine deoxyribonucleosides using NTP as phosphate donor. Thus, via the combined action of TK2 and dGK all four deoxyribonucleosides can be phosphorylated in order to supply building blocks to mtDNA synthesis. Similar to TK2, dGK is constitutively expressed in most tissues, and the amount of dGK generally correlates to the amount of mitochondria (Wang & Eriksson, 2003; Eriksson *et al.*, 2002; Arner & Eriksson, 1995). Human dGK is encoded by a 32 kb gene with 7 exons located on chromosome 2q13. The active enzyme is homodimer with MW of 60 kDa (Wang et al., 1996; Wang et al., 1993). Normally dGK is located in mitochondria but it relocates to the cytosol during apoptosis in an apparently similar process as cytochrome C. The role of dGK in apoptosis is still unclear; it may be involved in activation of apoptotic nucleotide co-factors after re-localization to the cytosol (Jullig & Eriksson, 2001). Like other dNKs, dGK is able to phosphorylate several purine analogs efficiently (Sjoberg et al., 1998). dGK and dCK show similarity in the amino acid sequence and have overlapping substrate specificity for analogs such as 2-chlorodeoxyadenosine, arabinosyl guanine, and 2', 2'- difluorodeoxyguanosine (Munch-Petersen & Piskur, 2007). dGK most likely plays an important role in activation of certain analogs in tissues, where deoxycytidine kinase (dCK, see below) activity is low. dGK deficiency is associated with another form of MDS i.e. the hepato-cerebral form which leads to severe liver failure with or without neurological impairment along with rotary nystagmus and hypotonia (El-Hattab & Scaglia, 2013; Suomalainen & Isohanni, 2010; Poulton *et al.*, 2009).

3.2 Cytosolic deoxyribonucleoside kinases (dNKs)

Deoxycytidine kinase (dCK, E.C. 2.7.1.74)

Deoxycytidine kinase (dCK, NTP: deoxycytidine 5'-phosphotransferase) is a cytoplasmic enzyme that phosphorylates purine and pyrimidine deoxyribonucleosides to their corresponding monophosphates by using nucleoside triphosphates such as ATP or UTP as phosphate donors (Eriksson et al., 2002). dCK expression varies in different tissues, i.e. it is high in lymphoid tissues, intermediate in proliferating cells of colon and mucosa, and low in differentiated tissues such as liver, muscle and kidneys. Human dCK is encoded by single copy gene of 34 kb located on chromosome 4 band q13.3-21.1 with 7 exons that varies in size from 90 to 1544 bp. dCK is a homo dimer with an identical polypeptide of 261 amino acids and MW of 30 kDa of each (Song et al., 1993; Bohman & Eriksson, 1988). dCK expression is

regulated most likely by both post-transcriptional and post-translational mechanisms (Hazra et al., 2011; Eriksson et al., 2002; Arner & Eriksson, 1995).
dCK phosphorylates deoxycytidine more efficiently than deoxyadenosine (dA) and deoxyguansine (dG). The enzyme does not follow Michaelis-Menton kinetics but shows negative co-operativity. It has broad range of substrate specificity and phosphorylates several nucleoside analogs that are important in anti-viral and anti-cancer therapy such as β-L-2´-3´- dideoxy-3´-thiacytidine (lamivudine), 2- chlorordeoxyadenosine (cladribine) and diflurodeoxycytidine (gemcitabine), 2-fluoro-arabinofuranosyl adenine (fludarabine), 2´-3´-dideoxycytidine (zalcitabine) and arabinosyl adenine (vidarabine) (Sabani et al., 2003; Eriksson et al., 2002; Eriksson et al., 1991).
In order to understand the biological function of this enzyme, *Toy et al*. created dCK knock out mice and the mice exhibited impaired T and B lymphocytes production but other organs and tissues were not affected (Toy et al., 2010). Another similar study by *Austin et al* who reported that dCK knock out mice showed replication stress i.e. DNA replication was arrested in early S phase of T and B lymphoid cells. This provides a connection to the high dCK activity in lymphoid tissues, correlating with the symptoms in the knock out mice model (Austin et al., 2012).

Thymidine kinase 1 (TK1, E.C. 2.7.1.21)

Thymidine kinase 1 (ATP: thymidine 5'- phosphotransferase, EC 2.1.7. 21) is a salvage pathway enzyme that converts deoxythymidine (dT) to deoxy thymidine monophosphate (dTMP) by transferring the γ- phosphate group from ATP. dTMP subsequently phosphorylates into deoxythymidine diphosphate (dTDP) and deoxythymidine triphosphate (dTTP), which further incorporates into DNA. The dTTP in turn regulates TK1 action by negative feedback and this may be a rate-limiting step in DNA precursor synthesis (Eriksson et al., 2002; He et al., 2002; Munch-Petersen, 2009). TK1 has narrow substrate specificity since it phosphorylates only dT and dUrd, whereas other dNKs have broader substrate specificities.
TK1 activity is absent in resting cells but in proliferating cells, TK1 is cell cycle regulated. It reaches a peak at late G1, early S phase and is drastically reduced to undetectable levels at the end of mitosis (Sherely & Kelly, 1988). Because of its association with DNA synthesis and the S phase of the cell cycle, TK1 has been used as proliferation marker since the very early days of cell biology. TK1 activity measurements have also been used as a biomarker for prognosis and monitoring of treatment for cancer diseases in humans and dogs. TK1 has been studied extensively in many aspects i.e. enzyme structure, cell cycle regulation, substrate kinetics and as a biomarker in different malignant diseases which will be described in detail below.

4. Thymidine kinase 1 (TK1)

4.1 TK1 sequence

The first human thymidine kinase cDNA was cloned and characterized 30 years ago (Flemington *et al.*, 1987; Bradshaw & Deininger, 1984). TK1 is located on chromosome 17 and the cDNA is 1241 bp, with an open reading frame of 702 bp coding for a protein of 234 amino acids.

TK1 exits as homodimer or homotetramer with a sub-unit size of 25.5 kDa (Munch-Peterson et al., 1993). Human TK1 shares higher sequence identity with TK1 from different mammals where most of the N-terminal is conserved and diversity confined to the C-terminal region. When the human TK1 sequence is compared to the dog TK1 sequence, the primary amino acid sequences are highly homologous in the N-terminal with an identity of 89.5% and the diversity (about 10%) occurs in the C-terminal 40 amino acids. Furthermore, human TK1 contains 11 cysteines whereas canine TK1 contains 8 cysteines. The extra cysteines in human TK1 that are absent in dog TK1 are shown in Fig 3 as blue circles.

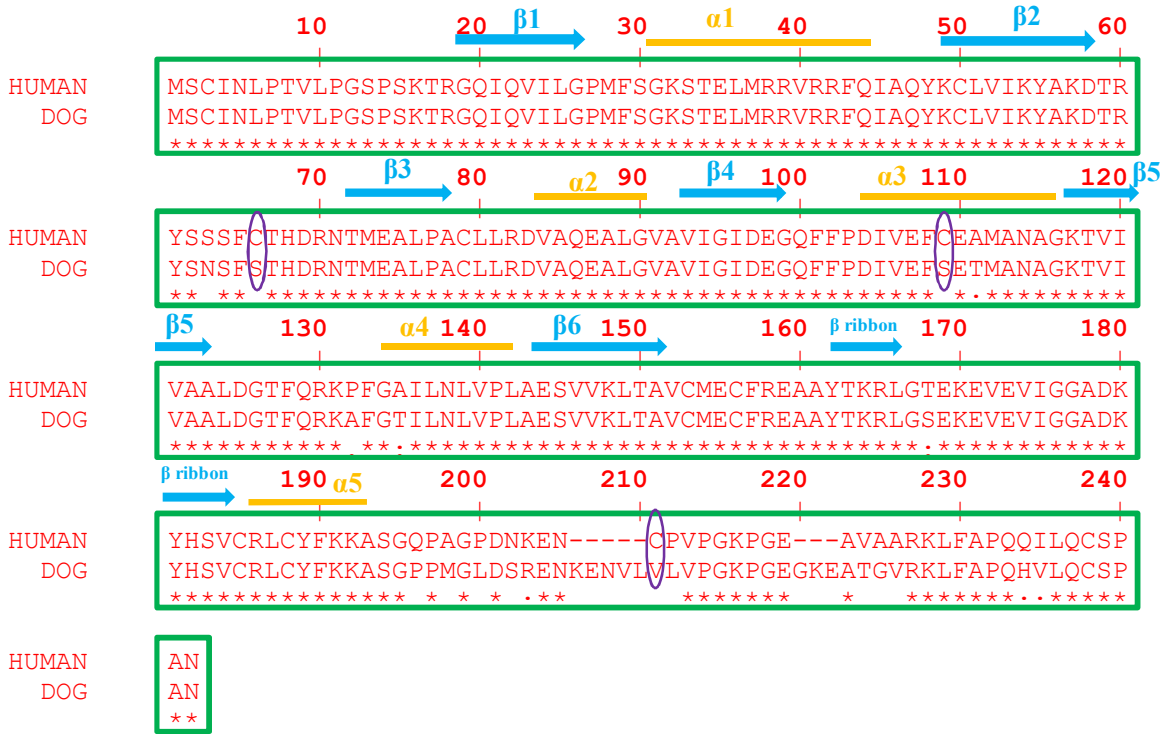

Figure 3. Alignment of the sequences from human TK1 (PO4183) and dog TK1 (XM_540462). The secondary structures of human TK1 are marked above in the alignment are from Welin et al, 2004 (see also Fig 4). Blue circles show the cysteines difference between human and dog, (*) identical residues, (.) semi conserved residues. The alignment was done with the Clustal Omega program.

4.2 TK1 structure

The crystal structure of Human and *Ureaplasma urealyticum* TK1 was resolved by *Welin et al* in 2004. Several earlier attempts to solve the crystal structure of human TK1 were unsuccessful. The difficulty in solving the TK1 structure was most likely due to the flexible ends of enzyme and when the C-terminal 41 amino acids were truncated it was possible to obtain suitable crystals (Bringer et al., 2005; Welin et al., 2004). The truncated enzyme retains its enzyme activity as shown (Kauffman & Kelly., 1991). Furthermore, truncated TK1 could be stabilized by adding the feedback inhibitor dTTP (Welin et al., 2004). Human TK1 crystalizes as a tetramer and each subunit contains two domains (α/β), and a zinc-binding lasso like domain. The active site of the enzyme lies in between these domains (Fig 4A & 4B).

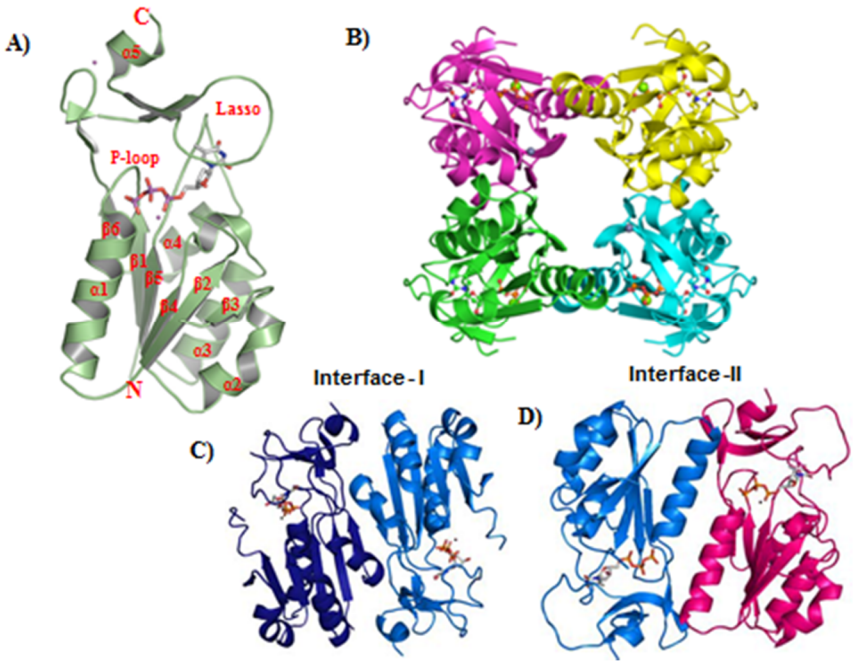

Figure 4. Structures of human TK1 (hTK1), PDB code: 1XBT. (A) Monomer and (B) tetramer structures of hTK1 (C) Dimer one connected by Interface-I, is the most stable dimer (D) Dimer two connected by interface-II, is the weak dimer, and interface-II separates upon dimerization of the tetramer (Welin *et al.*, 2004).

The α/β domain contains six parallel β sheets, which are surrounded by five α helices, along the α-helix and the flexible loop on one side, the remaining three short α-helices lie on the other side (Fig 4A). The α/β domains of TK1 are different from other dNKs and similar to the ATP binding domains of the Rec A-F1 ATPase family proteins (Welin et al., 2004). The zinc-binding domain consists of two perpendicular β ribbons and a lasso loop that covers the substrate binding site of TK1, and they are kept together by the zinc ion, which is coordinated by the two cysteine pairs (153, 155; 186, 188). This region is involved in forming the substrate binding site, thus this site may be sensitive to changes in the redox environment of cells (Birringer et al., 2005; Welin et al., 2004). Zinc has significant role in TK1 expression and it was demonstrated by *Ishikawa et al* that both TK1 expression and the percentage of S phase cells are significantly lower when rats are fed with a diet of 50% reduced zinc compared to controls (Ishikawa et al., 2008).

In the TK1 dimers, there are two different monomer-monomer interfaces labelled as strong and weak (Fig 4C and 4D). The weak interface is stabilized indirectly, whereas the strong interface is stabilized directly by ATP through many polar interactions (Segura-Pena et al., 2007). When the substrate binds

to the TK1 enzyme, most of the changes occur in the conformation of the lasso loop and the P loop, and these conformation changes depend on the substrate (Segura-Pena et al., 2007b; Welin et al., 2004). The small P loop consists of amino acid residues Gly26-Ser33 in hTK1, where the phosphate from ATP binds: once ATP binds to the P loop it becomes rigid and forms a β-hairpin structure (Segura-Pena et al., 2007 b; Birringer et al., 2005; Welin et al., 2004). The interaction between the phosphate-binding site in the loop and ATP is stabilized by a magnesium ion, which is surrounded by the phosphate groups of ATP and a threonine residue (Segura-Pena et al., 2007a; Welin et al., 2004). Phosphorylation of dT to dTMP by TK1 requires coordination of several amino acid residues where the lasso loop is stabilized by dT. Glu98 acts as base which accepts a proton from dT and Lys 32 transfers the phosphate group to dT and the conversion is stabilized by Arg60 (Birringer et al., 2005; Segura-Pena et al., 2007a; Welin et al., 2004). As we mentioned earlier, cysteines in TK1 are important for zinc ion co-ordination. Differences in cysteines between different species can change the enzyme behaviour.

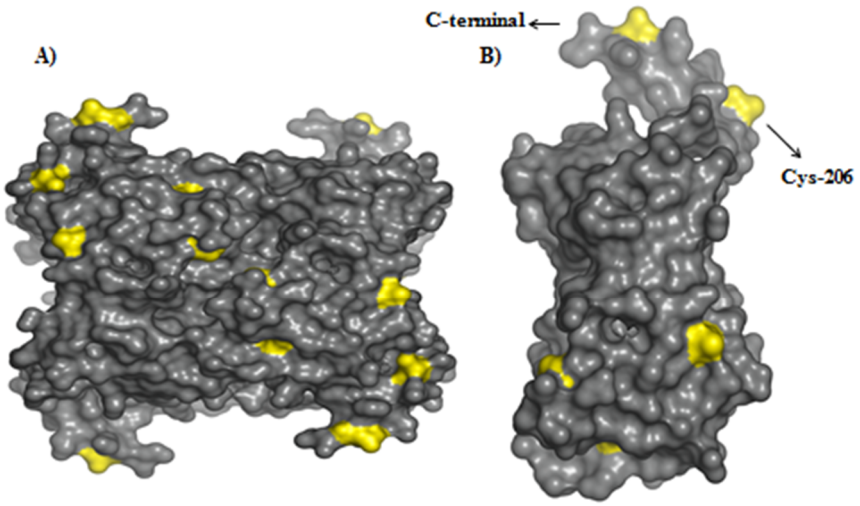

Figure 5. A model of Human TK1 (A) tetramer (B) monomer. Cysteines that are exposed on surface are indicated in yellow colour (provided by M.Welin based on sequence alignments and the structure data from Welin et al., 2004).

For example, Human TK1 contains 11 cysteines whereas canine TK1 contains 8 cysteines. Four of these cysteines in both human and dog plays an important role in coordinating the Zn^{2+} ion and some of them may be involved in

disulfide bridges between the TK1 polypeptides. A 3D model of the structure of full-length human TK1, constructed by M Welin based on sequence alignments and the structure of Ureaplasma TK, illustrates the likely position of the surface exposed cysteines in the tetrameric and monomeric forms of human TK1 (Fig 5). Cysteine 206 in the very C-terminal part of human TK1 (Fig 5B) is a likely candidate for forming disulfide bridges with other exposed cysteine in TK1 or with other interacting protein partners. The extra cysteine 206 in the C-terminal of human TK1 is most likely valine 211 in canine TK1 (Fig 3).

However, ATP binding induces over-all conformational changes in the TK1 structure, and TK1 switches from a dimer of 50 kDa to a tetramer of 100 kDa. This switch is reversible and the transformation from dimer to tetramer can occur in the presence of UTP, GTP, and CTP instead of ATP, indicating that only the phosphate group is involved in the reversible tetramerization (Munch-Petersen et al., 1993; Kuriowa et al., 2000; Munch-Petersen et al., 2009). The tetramerization has significant effects on the TK1 enzyme kinetic behaviour and the catalytic efficiency of the active tetramer increased 30 fold compared to the dimer (Munch-Petersen et al., 1995; Jensen & Munch-Petersen, 1994). Binding ATP also induces a transformation of a closed inactive dimer to an active more open tetramer form (Segura-Pena et al., 2007). The deletion of the C-terminal from TK1 enzyme did not significantly effect the reversible tetramerization induced by ATP (Li et al., 2004). On the other hand, Serine 13 has differential effect on TK1 enzyme activity; a mutation of Serine 13 to Alanine did not alter the enzyme activity, whereas substitution of Serine 13 with Asparagine decreased the TK1 activity and disrupted the ATP dependent tetramerization (Li et al., 2004).

The transformation of dimer to tetramer was not only dependent on ATP but also on TK1 enzyme concentration. Recombinant TK1 concentration higher than 0.2 mg/mL induces the dimer to tetramer switch, and oligomerization was seen at high enzyme concentration, irrespective of ATP (0.4-20 mg/mL) (Fig 6A) (Munch-Petersen et al., 2009; Birringer et al., 2006). This process also depends on enzyme modifications in vitro because immediate analysis after preparation showed TK1 as tetramer even at a low concentration of the protein. However, if the same sample was diluted and stored for 2 weeks before analysis, TK1 was a dimer (Munch-Petersen et al., 2009). The transformation of TK1 from dimer to tetramer does not occur in all organisms. In the case of bacteria, plants and dictyostelium TK1 exists only as dimer whereas in C. elegans and in all vertebrates TK1 is predominantly a tetramer. The regulatory switch of TK1 from dimer to tetramer is functional only in birds and mammals, which indicates a structural evolution of TK1 from a dimer form to a tetramer complex in certain organisms (Mutahir et al., 2013).

Initial studies have been done to determine the molecular forms of cellular TK1 isolated from the human lymphocytes. The molecular weight of native TK ranged from 70-75 kDa in the absence of ATP, whereas in presence of 2 mM ATP it increased to 170-200 kDa (Munch-Petersen, 1984). Later, *Karlstrom et*

al. reported that cellular TK1 from proliferating HeLa cells was only found as a 58 kDa enzyme. The differences in the cellular forms of TK1 in these studies are most likely due to difference in the conditions during preparation and source of enzyme.

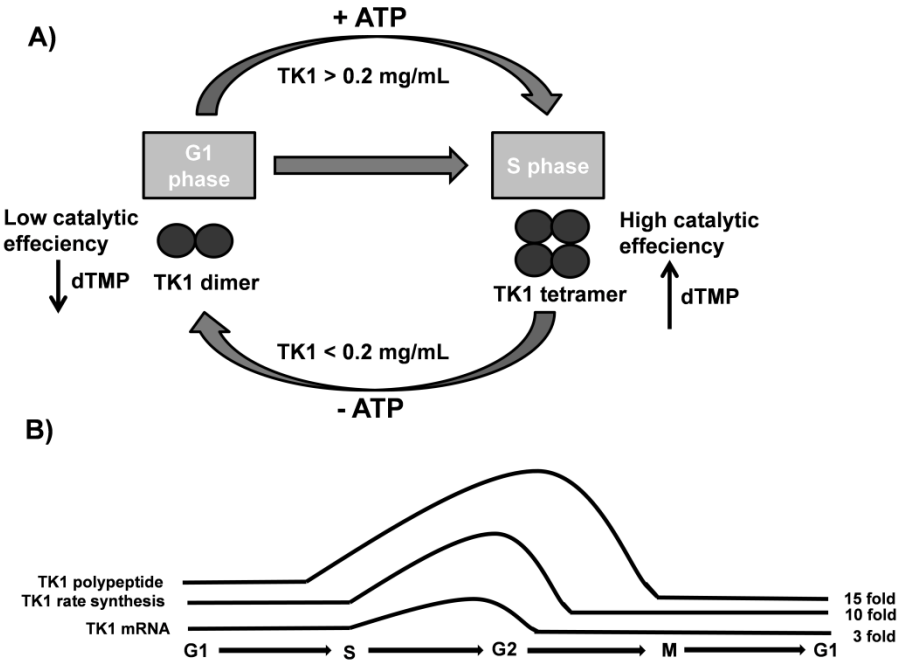

Figure 6. (A) Effects of ATP on concentration depend tetramerization of TK1 (B) Fluctuations in TK1 protein and mRNA levels during different phases of the cell cycle (adopted from Sherley & Kelly, 1988; Munch-Petersen et al., 2009; Birringer et al., 2006).

4.3 TK1 control mechanisms

The activity of TK1 is cell cycle dependent and it fluctuates during the different phases as follows: TK1 activity is very low in G1 phase, starts increasing at the end of G1 and reaches a peak during S phase. In S phase, TK1 increases up to 10-15 fold compared to that in G1 phase cells. In comparison the amount of TK1 mRNA expression shows less cell cycle fluctuation (about 3 fold). Finally, TK1 protein is degraded during late G2/M phase (Fig 6B) (Sherely & Kelly, 1988; Kauffman & Kelly, 1991). Phosphorylation is one common mechanism that targets cell cycle regulated proteins and it was reported that TK1 is heavily phosphorylated in dividing cells and it is hypo-phosphorylated in M phase arrested cells. Thus, phosphorylation may play a role in TK1 regulation (Chang et al., 1994; He et al., 1996; Lin et al., 2003).

The half-life of TK1 protein changes drastically from 40 h to 1-2 h during M phase due to degradation of TK1 protein so that newly divided cells have very low or no TK1 (Sherely & Kelly, 1988). The ubiquitin-proteasome pathway is the main mechanism that regulates cell cycle protein levels. SCF (Skp, Cullin/CDC53 and F box) and APC/C (anaphase promoting complex/ cyclosome) are the two ubiquitin ligases that play a role in targeting proteins for cell cycle dependent proteolysis.

Under normal conditions, TK1 is polyubiquitinylated at the G2/M interface and becomes target for the APC/C degradation pathway. Cdh1 is one of the two activators for the APC/C mediated ubiquitinylation which forms a complex and the APC/C-Cdh1 complex recognizes either a D box or a KEN box in the target proteins (Ke et al., 2004). In the TK1 sequence, a KEN box (residues 203-205 in the human sequence) is conserved in mammals and plays a key role in the mitotic degradation of TK1. The Cdh1 interaction with the KEN box is the rate limiting step for the initiation of this degradation (Ke et al., 2004). Furthermore, Cdh1 mutant has blocked TK1 degradation but this was not observed with the Cdc20 mutant, the other activator of APC/C. Thus, TK1 is degraded via the APC/C-Cdh1 complex (Ke et al., 2004). This degradation process does not occur when dT binds to TK1 (Ke et al., 2007). Although APC/C-Cdh1 mediated degradation explains the mechanism for the low levels of TK1 in G0/G1 cells, transcriptional control via the E2F pathway is apparently responsible for the rapid increase of TK1 levels in S phase (Ke et al., 2004). However, it is likely that the TK1 regulatory switch from inactive dimer to active tetramer is fine-tuned not only by the levels of TK1 protein but also the ATP concentration as discussed previously and this mechanism helps to provide a balanced supply of dTTP for DNA replication (Munch-Petersen et al., 1995; Munch-Petersen et al., 1993).

The phosphorylation of Serine-13 apparently prevents the ATP induced tetramerization of TK1 (Li et al., 2004). Since cdc2 kinase phosphorylates Serine-13 of TK1 in G2/M phase and this phosphorylation regulates TK1 activity levels, leading to inactive TK1 dimers in S phase (Chang et al., 1998; Chang et al., 1994). The truncation of 44 C-terminal amino acids increased the TK1 half-life to 500 min compared to 80 min for wild type TK1 (Zhu et al., 2006). This increased half-life of truncated TK1 demonstrates the role of the C-terminal region (i.e. the KEN box) in TK1 degradation. Mutations in the TK1 binding sites of ATP or dT result in an unstable protein with decreased half-life (Posch et al., 2001).

TK1 is also subject to transcriptional control, not only post-translational modifications as described previously. G quadraplex motifs have been reported to suppress the promoter region of TK1 located between -13 to +8 relative to the transcription start site (TSS) (Basundra et al., 2010). The cell cycle regulatory unit (CCRU) was a 70 bp region between -133 and -64 relative to TSS in the promoter region of TK1. Especially, a 20 bp region from -84 to-64 in CCRU is important because it contains an inverted CCAAT sequence and one GC rich motif, and without this 20 bp region transcription drops to barely

detectable levels (Kim & Lee, 1991; Kim & Lee et al., 1992). In addition, increased binding of cyclin A/p 107 during S phase correlates with increased TK1 mRNA expression (Li et al., 1993). Another protein that is involved in the cell cycle dependent TK1 expression is the CCAAT binding protein which binds to CCAAT sequence in the promoter region of TK1, contributing to the promoter strength (Lipson et al., 1995; Good et al., 1995). Thus, both the transcriptional and post-translational regulation plays an important role in regulation of TK1.

4.4 Physiological role of TK1

TK1 is closely associated with dTTP biosynthesis during the cell cycle as well as in DNA repair. Several studies have shown that TK1 levels increases when DNA is damaged by radiation or by chemotherapeutic agents (Chen et al., 2010; Haveman et al., 2006). Tumour suppressor p53 activation in response to DNA damage is most likely a factor important for TK1 regulation because normal p53 function is required for cell cycle dependent TK1 expression, and loss of the p53 function results in a compensatory increase in TK1 (Radivoyevitch et al., 2012; Schwartz et al., 2004). In relation to this, cellular TK1 levels increase upon DNA damage in $p53^{-/-}$ tumour cells but not in p53 wild type cells (Chen et al., 2010). Furthermore, growth of tumour cells was not effected by TK1 knockdown even though the levels of dTTP decreased significantly. This confirms that the TK1 plays a substantial role is DNA repair rather than production of sufficient levels of dTTP for replication and cell growth (Chen et al., 2010). During DNA damage, TK1 not only interacts with p53 but also with p21, where overexpression of TK1 prevents the reduced cell growth because of p21 dependent growth suppressors (Huang et al., 2001). In this way, TK1 constitutes a resistance mechanism for the survival of tumour cells and depletion of TK1 during DNA recovery results in cell death (Chen et al., 2010).

TK1 has a significant role in the immune system because the TK knockout ($TK^{-/-}$) mice exhibited kidney abnormalities, significantly decrease number of splenic lymphocytes, abnormal lymphoid structure of the spleen and occasional inflammation of arteries (Dobrovolsky et al., 2003). The kidney abnormalities have been reported in an earlier study, which demonstrates that mouse kidney cells mostly depend on nucleotide salvage pathway rather than the *de novo* synthesis (Zaharevitz et al., 1993). Most of tumour cells had higher TK1 activity except in kidney cells where TK1 activity was 3 fold higher in normal kidney cells compared to renal cell carcinoma cells due to increased *de novo* synthesis (Luo et al., 2009; Luo et al., 2010). A recent study demonstrated that TK1 had a potential role in haematopoiesis especially in the maturation of lymphocytes (Alegre et al., 2012). TK1 apparently also plays a role in auto immune diseases especially leukocytes from patients with Hashimoto and Graves' disease had two fold higher TK1 expression compared to healthy

individuals (Karbownik et al., 2003). These results indicate the role of TK1 in normal immune system and kidney physiology.

4.5 Methods for detection of TK1

The tight regulation of TK1 during cell cycle and high levels in S phase explains why TK1 activity is directly associated with cell proliferation. High TK1 activity in rapidly proliferating malignant cells have been demonstrated by determining the TK1 levels with different assays. TK1 detection methods rely on activity assays and protein based assays (Fig 7) as described below.

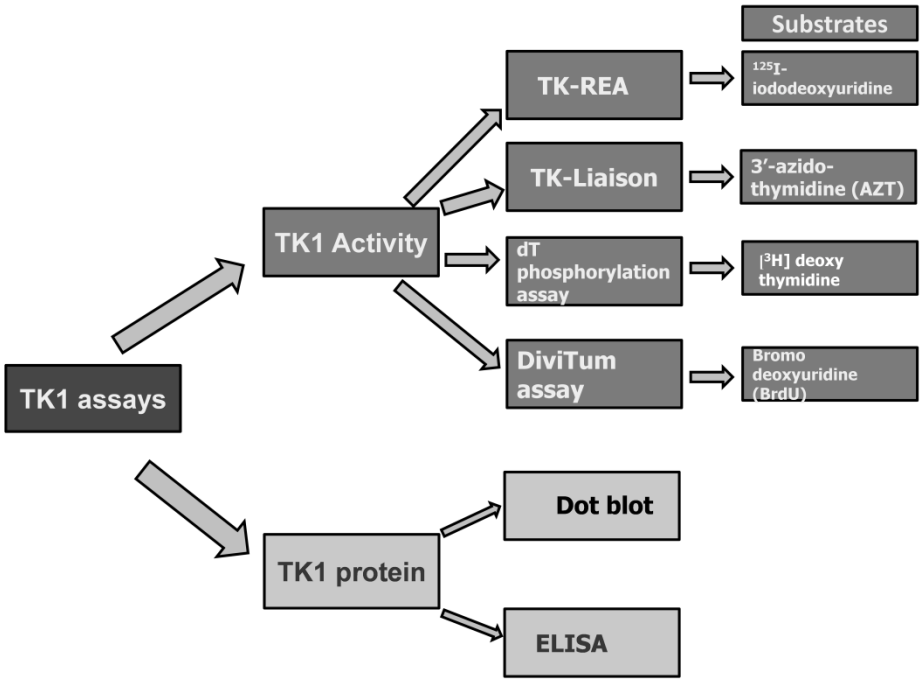

Figure 7. Different TK1 activity and TK1 protein based assays.

4.5.1 TK1 activity assays

In the early 1980s, Ellims et al described a method to measure thymidine kinase levels in plasma from patients with megaloblastic anaemia. The high TK activity levels in megaloblastic anaemia patients compared to normoblastic anaemia encouraged measurements of TK levels in other proliferative diseases

(Ellims et al., 1980). These initial studies rely on dT phosphorylation methods to determine TK activities, similar to those that have been used for many years to measure TK activities in cell and tissue extracts. An improvement in sensitivity of TK activity determinations was described in 1982 where the viral TK activities in herpes simplex virus type 1 and 2 infected cells were measured, using [^{125}I]-deoxyuridine as substrate. The reaction products were applied on DE-81 filter papers and bound dTMP was measured in a gamma counter (Gronowitz & Kallander, 1980). This assay was further developed in order to determine the TK activity in sera from patients with Hodgkin's and non-Hodgkin's lymphomas (Gronowitz et al., 1983). The assay was finally validated and developed as the commercially available TK-REA assay. The other traditional method that was used to measure TK1 activity in serum samples from different malignancies is [^{3}H]-dT phosphorylation assay, which is based on phosphorylation of [^{3}H]-thymidine to thymidine monophosphate (TMP) by using ATP. Similar to previous methods, the [^{3}H]-dTMP was absorbed to ion exchange DE-81 filter paper and radioactivity is measured (O'Neill et al., 1986; Sharif et al., 2012). TK1 only utilizes ATP as phosphate donor whereas TK2 utilizes both ATP and CTP, so the relative contribution of TK1 can be distinguished by using ATP and CTP separately (O'Neill et al., 1986). However, addition of other components to the reaction mixture was essential such as reducing agents to ensure consistency in TK1 activity levels in different samples (O'Neill, et al., 1986; Sharif et al., 2012).

A non-radiometric assay has been developed during the late 90s, based on the substrate analogue AZT instead of labelled thymidine. AZT is phosphorylated to AZTMP by TK1 and the amount of AZTMP formed can be determined by using polyclonal antibody against AZTMP (Öhrvik et al., 2004; von Euler et al., 2009). This assay is commercially available from DiaSorin Inc and is a fully automated competitive form of TK activity assay (TK Liaison assay). The assay correlates very well with the TK-REA and has similar specificity and sensitivity (von Euler et al., 2006; von Euler et al., 2009; Sharif et al., 2012). One advantage is that it is more specific for TK1 than the TK-REA because TK2 present in the samples phosphorylates AZT much less efficiently than ^{125}I-dUrd (Munch –Petersen et al., 1991). However, the performance of the TK Liaison is to some extent dependent on the properties of the TK1 enzyme from different species. For example, human TK1 phosphorylates dT three times more efficiently than AZT (Eriksson et al., 2002; Sharif et al., 2012), whereas canine TK1 phosphorylates AZT and dT with similar efficiency (Hanan et al., 2012). This indicates that TK-Liaison assay may be more suitable for in vitro diagnostics of canine than those of human malignancies. Several studies have also shown that the TK-Liaison assay works well as a diagnostic tool for canine malignancies (von Euler et al., 2009; Thamm et al., 2012). There is one potential problem in the TK Liaison e.g. interfering endogenous antibodies in human serum which may cross react with AZTMP antibodies (Öhrvik et al., 2004). The TK-Liaison assay has been

used for tumour management and healthy screening in dogs with apparently promising results (Veterinary Diagnostics Institute, VDI, USA).

Another different approach to measure serum TK activity is the DiviTum assay, where 5-bromo-deoxyuridine (BrdU) is used as a substrate. TK phosphorylates the BrdU to BrdUMP and subsequently to the BrdUTP, which is further incorporated into a poly-A oligonucleotide immobilized on the bottom of the micro titre plate. Then recombinant reverse transcriptase is used with an oligo (dT) primer. The anti-bromodeoxyuridine antibody conjugated to alkaline phosphatase is used to measure the poly A/bromodeoxyuridine incorporated oligomer which represents the TK1 activity (Nishman et al., 2013). Both the TK-Liaison and DiviTum assays apparently gave similar sensitivity but the DiviTum is a manual assay whereas the TK-Liaison is automatic. Other alternative approaches have been described for determining TK1 activity in clinical samples, e.g. liquid chromatography-MS/MS through phosphorylation of 3-deoxy-3-[^{18}F] flurothymidine (FLT) in hepatocellular carcinoma patients which is another thymidine analogue (Faria et al., 2012). Alternatively, capillary electrophoresis was used to quantify the amount of dTMP formed, following the traditional TK assay procedure instead of DE-81 filters (Tzeng & Hung, 2005). Recent study has described a new PCR based real time assay that measures simultaneously both TK1 and dCK levels in clinical samples (Stalhandske et al., 2013). These alternative methods are still at an early stage development and only a few clinical studies have been done so far.

4.5.2 TK1 protein Assays

Development of antibodies against different regions of TK1 not only suggests alternative assays for TK1 activity but also widens the clinical applications of TK1 as a biomarker. In the TK1 sequence, the N-terminal is conserved among most of the mammals and but not the C-terminal. Therefore, most of the clinically useful TK1 antibodies are raised against the C-terminal region (Wu et al., 2003) or against the active site lasso loop region (Gasppari et al., 2009; von Euler & Eriksson et al., 2011). In 2001, the first study was done with a monoclonal human TK1 antibody, using purified cellular TK1 extract as antigen and an ELISA developed with this antibody showed correlation with the activity assay. However, this antibody ELISA was not developed further until 2014 (Zhang et al., 2001; Alegre et al., 2014). Later high affinity polyclonal chicken IgY antibodies were developed, which are raised against the C-terminal region of human TK1. This antibody is used in dot blot assay to quantify the serum TK1 protein levels. The assay is highly sensitive and it can detect the TK1 protein levels down to 33 pg/mL (Wu et al., 2003). A commercial dot blot kit, produced by SSTK Biotech Inc. was approved by the Supervision Authority for Food and Medicine in China for clinical utility in 2010 (Pan et al., 2010). In the past

decade, a large number of clinical studies were performed with this assay i.e. almost 3,500 different malignant sera and more than 70,000 healthy individuals have been tested and reported (He et al., 2005; Li et al., 2005; He et al., 2006; Zhang et al., 2006; Chen et al., 2008; Xu et al., 2008; Chen et al., 2010; He et al., 2010; Li et al., 2010; Chen et al., 2011; Liu et al., 2011). Recently, a study using the dot blot assay for health screening of 35,365 different working individuals found about 1% of individuals had elevated serum TK1 protein levels, typically corresponding to individuals with potential pre-malignancies (Chen et al., 2011). The main limitation of the dot blot assay is that it cannot be directly applied to the existing clinical routine procedures and requires specially trained staff and a scanning instrument. Therefore, the dot blot method is presently not available outside China.

Some attempts have been made recently to develop more clinically useful antibody assay like a direct ELISA (Alegre et al., 2014) or a sandwich ELISA (Carlsson et al., 2009), but in both cases they have not so far been transformed to a certified in vitro diagnostic tool. Thus, there is still a need for a robust TK1-ELISA assay, adopted to routine clinical practice which can aid disease management of different malignancies. To determine the performance of any diagnostic test that differentiates a group with disease from a group without disease, receiver operating characteristic (ROC) curves are used. Basically the ROC curve is a plot of true positive rates against a function of false positive rates at a series of different cut-off points. The main statistics associated with ROC curve are i.e. the area under the curve (AUC), the sensitivity and the specificity. The AUC in ROC curve quantifies the overall ability of the test to discriminate the disease group from the non-disease group. Sensitivity represents the fraction of positives in test population that can be identified as true positives by the diagnostic test and the specificity indicates the fractions of negatives in test population that are true negatives. In later sections of this thesis, ROC curve analysis for different TK1 assay results was used to determine the sensitivity and specificity values as well as the AUC, giving a quantitative estimation on quality of the various tests.

5. TK1 in human and veterinary medicine

5.1 TK1 determinations in human medicine

In many haematological tumours, serum TK1 (STK1) activity levels are significantly elevated especially in acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), Hodgkin's lymphoma (HL), non-Hodgkin's lymphoma (NHL), myelodysplastic syndrome (MDS), and multiple myeloma (MM).
Significantly elevated levels of STK1 were found in ALL patients compared to healthy controls (Hagberg et al., 1984; O Neil et al., 2007) and the levels of STK1 depends on stage of disease, and in the case of pre-treatment and relapsed patients, high STK1 activities are a sign of tumour progression. In patients with complete remission, no significant difference was observed in STK1 activity compared to healthy individuals (O Neil et al., 2007). Moreover, TK1 activity levels fluctuated in response to treatments, indicating that STK1 is suitable in disease monitoring. In both ALL and AML patients, serum TK1 activity levels are elevated early after treatment, but decrease after 2-4 weeks and stay at low levels during remission. However, increase in STK1 activity was observed in patients with relapse of tumour disease (O Neil et al., 2007). A similar study in children with acute leukemia (34 lymphocytic, 4 myeloblastic) showed high TK1 activity levels at the time of diagnosis, which decreases during therapy and reaches to levels similar as healthy individuals. In relapsed patients, STK1 levels started to increase 1 month before clinical symptoms were detected (Votava et al., 2007).
In CLL patients, STK1 levels correlated with prognosis i.e. overall survival and the STK1 levels could also aid in distinguishing smoldering and non-smoldering disease as well as predicting progression-free survival (PFS). Patients with high TK1 (> 7.1 U/L) had an average PFS of 8 months and for patients with low TK1 (< 7.1 U/L) PFS was 48 months. These results demonstrated the prognostic capacity of STK1 (Hallek et al., 1999). In another study with 188 advanced CLL patients, the STK1 levels before treatment could identify the patients that were most likely to respond to treatment. This study showed that almost all CLL patients had higher levels of STK1 before treatment than after treatment with Fludarabine. Overall, 83% of the CLL

patients with low STK1 levels (< 10 U/L) responded to Fludarabine with a median survival rate of 65%. On the other hand, 45% of the patients with high STK1 levels (> 10 U/L) showed low response to the treatment and a median survival rate of 22% (Di Raimondo et al., 2001). A recent study in CLL patients also demonstrated the prognostic value of STK1 (Konoplev et al., 2010). In the CLL patients, immunoglobulin heavy chain (IgvH) mutations are common, and tests to distinguish mutated IgvH from unmutated IgvH, markers like Zap 70 and CD 38, are often used. A recent study demonstrated the relation between STK1 levels and these markers. Elevated STK1 levels were found in patients with high expression of Zap 70 and CD 38 as well as the stage of disease. Furthermore, STK1 showed significant correlation with white blood cell counts and absolute B-cell counts (Rivikina et al., 2011; Szantho et al., 2014).

Several studies have demonstrated the prognostic and diagnostic value of STK1 in NHL (Gronowitz et al., 1983; Hallek et al., 1992). STK1 levels in pre-treatment patients were related to the grade of malignancy (low, medium and high) and provided information regarding response to treatment, and predicted overall survival (Hagberg et al., 1984; Hallek et al., 1992). In some cases, STK1 helped to predict the transition of disease from indolent to progressive before the symptoms appear (Hallek et al., 1990). STK1 had higher capacity to predict the course of disease in NHL patients compared to other serum markers like β_2-macroglobulin, lactic dehydrogenase and haptoglobin (Gronowitz et al., 1983; Hagberg et al., 1984).

In HL, STK1 levels in pre-treated patients correlated with clinical parameters such as cancer stage and histopathology (Eriksson et al., 1985). High STK1 levels corresponded to an advanced stage of disease. Further, STK1 levels could discriminate IA and IIA stages of HL into subgroups based on disease free survival (Eriksson et al., 1985; Hallek et al., 1992).

STK1 levels predicted also the transformation of MDS to AML and multivariate analysis showed that STK1 is the only prognostic marker that provides information about acute transformation potential, response to therapy and overall survival (OS) (Musto et al., 1995). Moreover, MDS patients with STK1 activity higher than 38 U/L had shorter survival times compared to patients with low STK1 level (< 38 U/L) (Musto et al., 1995). STK1 could also distinguish MM from monoclonal gammapathy of undetermined significance (MGUS), where patients with MGUS had significantly lower STK1 levels compared to MM. STK1 levels in MM increased in relation to the disease progression and high pre-treatment STK1 levels strongly associated with short survival (Brown et al., 1989; Back et al., 1993).

The prognostic relevance of STK1 was investigated in various solid tumours such as breast, lung, and bladder, ovarian, gastrointestinal and prostate cancers. Breast cancer has been studied extensively; TK1 activity levels were measured in cytosolic TK1 extraction from tumour tissues of approximately 1700 primary breast cancer patients. TK1 activity correlated with disease-specific survival (DSS), local recurrence-free survival and distant relapse-free survival.

It also could monitor the effectiveness of the chemotherapy (Broet et al., 2001). Another study showed significantly higher STK1 levels in 160 breast cancer (BC) sera compared to sera from healthy women. This study concluded that increases in STK1 levels were associated with staging, grade and vascular invasion. Further, elevated STK1 levels were found in patients with BRCA 1 or BRCA 2 mutations compared to those without mutations and STK1 can be an independent prognostic factor for recurrence free survival (RFS) (Nisman et al., 2010). Recently, another study on breast cancer patients compared the performance of the two commercial assays i.e., the TK-Liaison and DiviTum assays. Despite some differences between the two assays, the study found similar sensitivities and specificities, could predict tumour reoccurrence and showed significant correlation (r =0.85) (Nisman et al., 2013).

Some studies demonstrated the clinical significance of STK1 activity in non-small cell lung cancer (NSCLC). A recent study on the prognostic significance of STK1 in NSCLC found that STK1 activity levels in NSCLC patients are significantly higher than in healthy controls. STK1 levels were also correlated with weight loss and higher STK1 values were associated with decreased PFS and OS. These findings indicate that STK1 can be an independent prognostic factor, but it could not be used for monitoring response to therapy in the case of NSCLC patients (Korkmaz et al., 2013).

In ovarian cancer, STK1 levels were found to differentiate patients with high stage disease from healthy individuals. Moreover, STK1 levels correlated strongly with CA-125, which is a widely used marker for prognosis and disease monitoring in ovarian cancer (Hallek et al., 1997). Similarly, STK1 activity was significantly higher in renal cell carcinoma (RCC) patients and it correlated with clinical stage but not with grade. However, STK1 levels could not differentiate samples from patients with benign kidney tumours from the healthy controls. Overall, high levels of STK1 and TuM2-PK correlated with reduced RFS (Nisman et al., 2010). In prostate cancer, STK1 activity levels (N=92) were also markedly elevated compared to healthy individuals and STK1 activity appeared to be a better marker than PSA in this study (Letocha et al., 1996).

Development of TK1 antibodies provides an alternative approach to the traditional activity assays as described above. Most of these antibodies were developed against the C-terminal region of human TK1 and originally with a 16-mer peptide as antigen (He et al., 1996). A similar type of antibodies using a (31-mer) 31 C-terminal peptide for immunization of chickens lead to development of the commercial dot blot assay (Wu et al., 2003). With the dot blot assay, STK1 protein (STK1p) levels in patients with benign and malignant breast cancer were determined, and significantly higher STK1p were evident in pre-operative patients compared to 3 months after surgery. Patients with metastatic disease had significantly higher STK1p compared to patients with no metastasis. In healthy individuals, STK1p levels were significantly lower as compared to benign and malignant breast cancer patients (He et al., 2000). In another study, CA 15-3 (a breast cancer marker) and STK1p levels were

compared in pre-operative breast cancer patients and 3 months after surgery. STK1p levels increased in patients with recurring disease but this was not clearly seen with CA15-3, indicating that STK1p could be a better marker for early detection of breast cancer recurrence than CA 15-3 (He et al., 2006). In a recent study, STK1p levels were determined in the follow up of 51 patients after surgery and chemotherapy for 44 months. Patients with high STK1p (≥ 2.0 pM) levels 3-6 months after surgery had higher clinical stage, grade and larger tumour size. Furthermore, patients with high STK1p exhibited 11-12 times higher risk for development of metastatic disease compared to those with low STK1p values. Moreover, patients in stage III/IV with low STK1p levels showed significantly improved survival compared to the patients with high STK1p levels (Chen et al., 2013).

Studies have been conducted with the dot blot assay in patients with other different solid tumours such as bladder carcinoma (Zhang et al., 2006), gastrointestinal cancer (Zou et al., 2002), renal cell carcinoma (Luo et al., 2009), cervical cancer (Fujiwaki et al., 2001), ovarian cancer (Fujiwaki et al., 2002) and prostate cancer (Li et al., 2011), and they all suggest that STK1p can play a major role in the management of malignant diseases. The dot blot assay has also been extensively used for health screening in China; the initial test was conducted in 2006 with 72 individuals found that 3 individuals had elevated STK1p levels (1.8, 7 and 7.1 pM) compared to healthy individuals (0.5 pM). The persons with high STK1p had some proliferation related abnormalities as revealed in the clinical examinations (Chen et al., 2008; He et al., 2010). Based on these preliminary results, a larger health screening study has been conducted with 35,000 people from different regions in China. About 0.8% of the individuals had elevated STK1p and of these about 85% had diseases associated with cell proliferation or sometimes with established malignancies compared to individuals with low STK1p values (0.5 pM) (Chen et al., 2011).

Another antibody based sandwich ELISA was developed AroCell AB, and a preliminary study was conducted with breast carcinoma patients subsequent to surgery (N =24) and chemotherapy (N=39). Although STK1p values were significantly reduced 4 weeks after surgery and increased in patients after tumour recurrence, this ELISA had surprisingly low sensitivity and specificity (Carlsson et al., 2009). Recently, another study was conducted with a new form of the TK1- ELISA, based on two monoclonal antibodies by AroCell AB. In this study, breast cancer patients were divided based on TNM classification. Elevated TK1 protein levels were found in breast cancer patients compared to healthy patients, and TK1 protein levels differed significantly between groups based on size (T1<T2<T3). Furthermore, STK1p levels correlated significantly with CA 15-3, this study concludes that STK1p can detect early stages of breast cancer more efficiently but not CA15-3 (www.arocell.se).

In another study with lung carcinoma patients conducted by *Alegre et al*, the STK1p levels were determined in stage I (N=16), stage II (N=17) patients as well as in healthy controls (N=18) by a direct ELISA. The patients with stage I

and stage II showed significantly higher STK1p levels compared to healthy, whereas STK1activity levels only differentiated stage II from healthy but not stage I. These results indicate that STK1p was a more sensitive indicator for lung cancer compared to STK1 activity (Alegre et al., 2014). However, the levels of STK1p determined in this study was about 100 fold higher than those found with the dot blot assay or the AroCell ELISA, so further studies are needed to clarify these discrepancies. Results with another form of TK1-ELISA were conducted and compared with the CA15-3 and CEA (another tumour marker) of breast cancer patients. The percentage of breast cancer patients with high levels of CEA, CA15-3 and STK1p were found to be 62%, 70% and 78%, respectively. It was also demonstrated that combination of these three markers increased the sensitivity to 90% for accurate diagnosis in breast cancer patients (Elfagieh et al., 2012). However, the details of the antibodies used in this assay were not described and no further studies with this assay have been reported.

TK1 antibodies have been used in a number of immunohistochemistry (IHC) studies to determine the TK1 expression in tissues and compared with other proliferation markers such as PCNA and Ki-67. TK1 and PCNA were both overexpressed in malignant tissues compared to normal tissues but only TK1 expression significantly increased with grade and stage in breast, liver, lung, colon, kidney, prostate, thyroid and stomach cancer tissue samples (Wu et al., 2000; Mao et al., 2002; Wu et al., 2003; Mao et al., 2005; Alegre et al., 2012). TK1 expression showed a strong positive correlation with Ki-67 expression in breast atypical ductal hyperplasia (ADH), infiltrating ductal breast carcinoma (IDC) and in NSCLC (He et al., 2004; Brockenbrough et al., 2009; Guan et al., 2009). TK1 expression in breast cancer tissues was also examined in order to identify the pre- malignant diseases. For ADH, IDC, ductal carcinoma *in-situ* (DCIS) and usual ductal hyperplasia (UDH), the TK1 positive rates are significantly increased in the following order: UDH<ADH<DCIS<IDC. TK1 expression also correlated with histological grade and pathological stage. These IHC results strongly suggest that TK1 is a promising proliferation marker for pre-cancer as well as different forms of malignant breast cancer (Guan et al., 2009). TK1 expression was also compared with Ki-67 expression in cell cycle studies with Array Scan analysis. The results showed that TK1 starts to increase earlier than Ki-67 after stimulation of growth arrested normal human fibroblasts (Gasparri et al., 2009). Expression of TK1 and Ki-67 were also examined in ovarian adenocarcinoma and the expressions of both these markers correlated with pathological stage, tumour size and grade. Further analysis showed that patients with advanced disease and low TK1 expression had better survival compared to patients with high TK1 expression (Liu et al., 2011).

In a recent study, TK1 expression was investigated in prostate cancer patient samples (N=103) and compared to the Gleason scores and prostate specific antigen (PSA) levels in the same patients. TK1expression was significantly higher in prostate tissues compared to normal tissues and TK1 expression

correlated with the Gleason score and disease staging. It was concluded that TK1 expression not only differentiated tumour tissues from normal tissue but also provided predictive information concerning biochemical relapse and onset of metastatic disease (Aufderklamm et al., 2012).

In summary, both STK1 activity and STK1p levels could give information related to survival, recurrence and response to treatment. For pT1 lung adenocarcinoma patients, high TK1 expression is significantly associated with worse 5-year survival (Xu et al., 2012). In addition, TK1 expression was able to identify the node-negative breast cancer patients that were at a high risk for metastasis (Romain et al., 2001). Studies reported that TK1 activity from tissue extract is 12-fold higher in ovarian carcinoma, and 2-fold higher in bladder carcinoma compared to normal controls. Finally, it was also shown that carcinomas with low tissue TK1 activity had longer RFS (Demeter et al., 2001; Mizutani et al., 2002).

Serum TK1 determinations and TK1 expressions are often combined or compared with imaging techniques like Positron Emission Tomography (PET), which utilizes labelled thymidine analogues. PET can provides valuable information regarding tumour size and localization, and the aggressiveness, and response to treatment of various human cancers such as breast, lung, head & neck cancer, gliomas as well as lymphomas (Buck et al., 2007; Salskov et al., 2007; Herrmann et al., 2011; Richard et al., 2011). Similarly, some studies were done with PET for monitoring FLT uptake in relation to TK1 and Ki-67 expressions, and it was found that low FLT uptake was surprisingly associated with high TK1/Ki-67 expression (Zhang et al., 2012). Another study reported that FLT uptake is strongly correlated with TK1/Ki-67 IHC expression but not with tissue TK1 activity (Brockenbrough et al., 2011). However, other studies showed that FLT uptake correlates with TK1 expression as well as with tissue TK1 activity (Rasey et al., 2002; Barthel et al., 2005; Kameyama et al., 2011). These controversies has been further expanded since a recent study concluded the FLT uptake and retention could not be used to measure tumour cell proliferation rates because FLT phosphorylation did not correspond to TK1 expression or tissue TK1 mRNA levels (Shinomiya et al., 2013). Thus, the relation between TK1 expression and FLT uptake in tumour cells is still unclear.

5.2 TK1 determinations in veterinary medicine

The first study describing TK1 determinations in dogs was done in 1997 by *Nakamura* and colleagues. They reported that plasma TK1 activities in dogs with lymphoma and leukemia (N=20) were significantly higher compared to clinically healthy dogs (N=13). The plasma TK1 activity values in dogs with lymphoma decreased in parallel with the reduction of tumour mass after treatment, whereas two dogs with tumour relapse showed increased plasma TK1 activity levels. Furthermore, plasma TK1 activities in sera from dogs with lymphoma and leukemia dogs were compared with the lactate dehydrogenase (LDH) levels, and a significant correlation was found (r = 0.64). Moreover, plasma LDH activity was neither as sensitive nor as specific as the TK1 activities (Nakamura et al., 1997). The second study by *von Euler* et al on dogs with malignant lymphoma was designed to determine the prognostic capacity of the STK1. STK1 activity in dogs with lymphoma (n=52) was significantly higher compared to healthy dogs. Following treatment, STK1 activity was determined in 44 dogs at regular intervals and no significant differences were found between the mean STK1 activities of dogs with complete remission (CR) and healthy dogs. Furthermore, dogs with high STK1 levels (> 30 U/L) had shorter OS (1.5 months) compared to dogs with low STK1 levels (< 30U/L) having a median survival of 9 months. Finally, increased STK1 levels could predict the relapse 3 weeks before onset of clinical symptoms (von Euler et al., 2004).

Further studies on STK1 were done using the TK-Liaison assay (von Euler et al., 2006; von Euler et al., 2009) in large groups of dogs with malignant lymphoma (N=213) and leukemia (N=35). It was concluded that STK1 activity levels in dogs (N=56) with complete remission (0.5-7.0 U/L) were similar to healthy dogs, whereas dogs (N=28) with progressive disease had significantly higher STK1 levels (7-66 U/L). Dogs with leukemia had highest STK1 activity (30-263 U/L) and STK1 levels in dogs under remission after chemotherapy were reduced compared to the initial TK1 levels. These studies demonstrate the prognostic potential of STK1 and its value for response monitoring after treatment of dogs with malignant lymphoma. Furthermore, the non-radioactive TK-Liaison assay gave results similar to TK-REA and the correlation between the two assays was excellent (r = 0.97).

In a separate study, STK1 levels were determined in 73 dogs with lymphoma and 46 out of 73 dogs were also immune-phenotyped. Overall, 47% of the dogs with lymphoma showed TK1 levels higher than the normal reference range. Dogs with naive B-cell lymphoma had significantly higher TK1 activity levels than dogs with naive T-cell lymphoma. There was no association between STK1 activity and clinical stage of disease. However, dogs with high STK1 levels were in the normal range during remission (Elliott et al., 2011).

A recent study was done on STK1 activity in dogs with malignant lymphoma (ML) by an optimized [^{3}H]-dT phosphorylation assay. The performance of assay was compared with the commercially established TK1 enzyme assays

such as TK-REA and TK-Liaison. STK1 activity was assayed in 29 dogs with ML and STK1 activity was significantly higher in sera from dogs with ML compared to healthy dogs. ROC curve analysis showed that the assay had a sensitivity of 0.94 and a specificity of 0.68. There was a significant correlation between [^{3}H]-dT phosphorylation assay with other TK activity assays such as TK-REA ($r=0.92$) and TK-Liaison assays ($r=0.96$, $p<0.0001$) (Sharif et al., 2012).

The above mentioned studies also included samples from dogs with solid tumours, i.e. STK1 activity levels were determined in sera from 4 dogs with solid tumours by Nakamura et al (1997), 50 dogs with solid tumours in the study by von Euler et al (2009) and in 35 solid tumour dogs by Sharif et al (2012). Surprisingly, the STK1 levels were below the normal reference range in the first study, and 3 out of the 50 dogs with solid tumours had high STK1 levels in von Euler et al., (2009). Similarly, in the study by Sharif et al (2012) 4 out of 35 had higher STK1 levels compared to the cut-off value from healthy dogs. The conclusion was that the STK1 activities in dogs with solid tumours differ significantly from those in healthy dogs only in very few cases and therefore STK1 activity measurements are not useful clinically with these patients.

Another study on the STK1 levels in dogs with hemangiosarcoma (HSA), demonstrated significantly higher STK1 activities in sera from HSA patients compared to those in healthy dogs, but not in dogs with benign disease. Still, the ROC analysis demonstrated only a sensitivity of 0.52 and specificity of 0.93 for the patient groups with HSA compared to the healthy groups. Patients with other solid tumours were also tested and only 6.7% of osteosarcoma and 11% of transitional cell carcinoma had increased STK1 levels. These results suggest that STK1 can be a marker for differentiating between benign disease and HSA (Thamm et al., 2011). STK1 activity has also been tested along with canine CRP in healthy screening projects for dogs and this combination could be used to predict the onset of tumour disease. A study with 360 dogs was done for 6 months and both STK1 activity and CRP levels were determined at regular intervals. Both the STK1 and CRP concentrations were higher in dogs with cancer compared to healthy dogs. In addition, ROC curve analysis based on STK1 activity 6 months prior to the onset of cancer disease, showed a sensitivity of 0.73 and a specificity of 0.84. A Neoplastic Index (NI) was constructed, which used both STK1 and CRP levels, and this index showed higher sensitivity compared to each marker alone. The conclusion from this study was that serum TK1 can be used to predict early stages of cancer whereas NI can predict onset of cancer more efficiently (Selting et al., 2013).

The first study of STK1 activity levels in feline lymphoma was recently published. The study included cats with lymphoma (N=33), inflammatory diseases (N=55), non-hematopoietic neoplasia (NHPN) (N=34) as well as clinically healthy cats (N=49). Cats with lymphoma had significantly higher mean STK1 activity (17.5 U/L) than healthy cats (2.2 U/L) or cats with inflammatory disease (3.4 U/L) and cats with NHPN (4.2 U/L). These results

indicate that STK1 could be a biomarker for feline lymphoma (Taylor et al., 2012). Another recent study was published on clinical significance of STK1 activity in equine lymphomas. STK1 activity was measured by TK-REA in healthy horses (N=37), lymphoma horses (N=23) along with few samples from other infections also. STK1 activity was significantly higher in horses with lymphomas compared from healthy horses. The results suggested that STK1 can be a potential biomarker in equine lymphomas (Larsdotter et al., 2015). Both these studies on clinical use of STK1 activity in cats and equines are preliminary, but the prognostic role of STK1 in tumour management is still unclear.

5.3 TK1 determinations in non-neoplastic diseases

Serum TK activity levels are elevated in some viral infections such as cytomegalovirus (CMV), varicella-zoster virus (VZV), Epstein-Barr virus and herpes simplex virus (HSV) (Gronowitz et al., 1982; Kallander et al., 1982; Gronowitz et al., 1984; Gronowitz et al., 1986; Kallander et al., 1989). Even though patients with acute hepatitis showed high STK1 levels, only hepatitis-A patient had the highest STK1 activity (Tanaka et al., 1993). The increase in STK1 could be due to virus encoded TK released during the infection (Gronowitz & Kallander 1983) or TK1 originating from damaged host cells (Tanaka et al., 1993). STK1activity also increased significantly in patients with vitamin B_{12} deficiency and this is most likely because of interference in *de novo* pathway due to vitamin B_{12} deficiency which causes the leakage of TK1 from immature proliferating blood cells as described earlier. The STK1 levels normalize rapidly after vitamin B_{12} supplement therapy (Hagberg et al., 1984; Ellims et al., 1980; Hooton & Hoffbrand et al., 1976).

Some early studies on dogs with bacterial infection and unspecified inflammatory diseases have been done and the STK1 activity levels were within the normal reference range (Nakamura et al., 1997; von Euler et al., 2009). However, a study of 54 dogs with pyometra showed that STK1 levels were significantly increased in a subset of these dogs. The other finding was that 6 out of 10 dogs with pyometra had significant reduction in STK1 activity after ovariohysterectomy. Furthermore, toxic neutrophils and monocytes counts showed a trend towards being correlated with the STK1 activities, indicating that the increase in STK1 activity could be associated with the severity of infection (Sharif et al., 2013).

The STK1 activity levels in bovine leukosis (BL) were found to be increased, most likely associated with the leukocytosis. Of 20 cows with bovine leucosis virus infection 19 had significantly higher STK1 activities compared to healthy cows, whereas 7 out of 79 cows with other diseases had elevated STK1 levels. In ROC curve analysis, the sensitivity and specificity of STK1 for diagnosis of bovine leukosis was 95% and 95.9%, respectively (Sakamoto et al., 2009). In another study, STK1 activity was demonstrated to be a useful as diagnostic tool for BL with difficult diagnosis and clinically confirmed BL.

High STK1 activity was found in 84% of cows with difficult diagnosis of BL and 97% in cows with confirmed BL, whereas in cows with other tumours and inflammatory diseases the sensitivities were 15 and 21%, respectively (Tawfeeq et al., 2013). Thus, it seems possible that STK1 measurements could be a valuable tool also in ruminant medicine.

6. Aims of the thesis

The main aim of these studies is to widen the clinical utility of TK1 as a biomarker in veterinary medicine. Specific attention was given to an antibody based assay that can determine the serum TK1 protein levels in different canine malignancies. These antibody based assays provides better understanding about the molecular forms of serum TK1 in canine lymphomas and mammary tumours. This information will help to improve the clinical use of TK1 activity TK1 protein assays for in vitro diagnostic procedures in canine oncology and in comparative medicine.

The following specific aims were set:

- To determine the TK1 26 kDa protein levels in sera from dogs with malignant diseases by using an antibody based immunoaffinity assay. Furthermore, to evaluate the TK1 antibody assay in comparison with [^{3}H]-dT phosphorylation assay for experimental and clinical purposes.
- To determine the effect of reducing agent on TK1 activity and TK1 peptide antibody performance in the immunoaffinity assay.
- To study the molecular forms of human, dog recombinant TK1 and compare them with the cellular and serum forms of TK1 from humans and dogs. Further to determine the enzyme behavior in presence of reducing agents.
- To assess the possibility of using STK1 protein levels as a biomarker in dogs with mammary tumours. To determine the differences in the molecular forms of cellular and serum TK1 in canine acute lymphocytic leukemia and canine mammary tumours.
- To develop a sandwich TK1-ELISA and evaluate its performance in comparison with [3H]-dT phosphorylation assay in different canine malignancies.
- To make a preliminary assessment on clinical performance of the new TK-ELISA in dogs with lymphomas and solid tumours.

7. Main results and discussion

A detailed discussion of each specific study is given in the respective papers. In the general discussion below, the main results and conclusions of the investigations are summarized. For further details, see Papers I-IV.

7.1. Paper I

High levels of inactive thymidine kinase 1 polypeptide detected in sera from dogs with solid tumours by immunoaffinity methods: Implications for in vitro diagnostics.

This study concerns about the relation between serum TK activity and TK1 protein in sera from dogs with different malignancies and healthy dogs. A new TK1 antibody based assay is described and used to determine TK1 polypeptide levels in dogs with haematological neoplasia (lymphomas and leukemia) as well as solid tumours. The effect of a reducing agent (DTE) on serum TK1 binding to an anti TK1 peptide antibody Sepherose was also studied. Determinations of STK1 activity levels had so far relied on TK-REA, TK-Liaison and [^{3}H]-dT phosphorylation assays. These assays demonstrated that dogs with haematological tumours have significantly higher levels of TK1 compared to healthy dogs but this was not seen in sera from dogs with solid tumours (Sharif et al., 2012; von Euler et al., 2009; Nakamura et al., 1997). This clearly limits the clinical use of STK1 as a diagnostic tool in veterinary medicine.
Development of antibody based assays for TK1 protein in human medicine inspired the approach used here to establish an immunoaffinity assay for canine TK1 protein (Wu et al., 2003; He et al., 2005). The TK1 26 kDa protein was measured by reacting serum samples or recombinant dog TK1 with an anti TK1 peptide antibody Sepharose, followed by a washing procedures and elution of the bound proteins. They were then analysed by western blot using a polyclonal anti TK1 antibody for detection of the dog TK1 sub unit of 26 kDa protein (Fig 8). The band intensities were measured by densitometry and the corresponding protein levels were determined with different concentrations of recombinant dog TK1 as standard. The study comprised 47 samples from dogs

with different malignancies. i.e., leukemia (n=3), lymphoma (n=5), solid tumours (n=21) as well as healthy dogs (n=18).

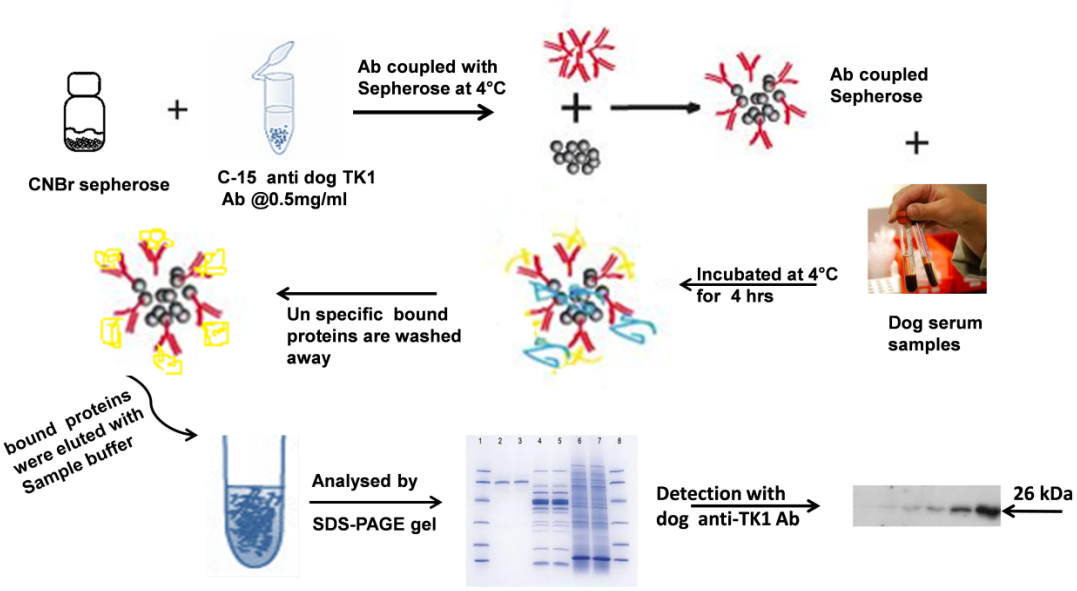

Figure 8. Isolation of TK1 polypeptide from dog serum samples by immunoaffinity assay.

The TK1 26 kDa polypeptide was quantified in sera from dogs with hematologic malignancies, solid tumours and healthy dogs in parallel with STK1 activity measurements. The results are shown in Table 2.

Table 2. Summary of serum TK1 activity and TK1 protein levels in dogs.

Tumour type	N	STK1 protein (ng/mL)			STK1 activity (pmol/min/mL)		
		Range	Mean	Median	Range	Mean	Median
Without DTE							
Leukaemia and lymphoma	8	15 - 47	33	37	16 -255	65	38
Solid tumours	21	13 - 60	30	31	0.6 - 2.9	1.1	1
Healthy	18	5 - 18	10	8	0.3 - 1.6	1	1
DTE pre-incubation							
Leukaemia and lymphoma	8	10 - 30	28	27	6 - 282	60	32
Solid tumours	21	6 - 35	17	14	0.2 - 1.5	0.7	0.7
Healthy	18	3 - 14	7	6	0.2 - 1.1	0.7	0.7

The STK1 activity levels from dogs with haematological tumours were 40 fold higher than TK1 activity in sera from dogs with solid tumours. However, the mean serum TK1 protein levels in these two cases did not differ significantly (33, 30 ng/mL, Table 2).
Furthermore, there was no significant difference in STK1 activity between dogs with solid tumours and healthy dogs in accordance with previous results (von Euler et al., 2009; Sharif et al., 2012). However, the STK1 protein levels were significantly different in healthy dogs compared to those found in patients with solid tumours. There was a significant correlation between STK1 activity and TK1 protein levels in sera from dogs with solid tumour sera (*rs* = 0.51. P = 0.01), but no correlation was observed for the haematological tumours. This may be due to the small number of samples tested. Pre-incubation of serum samples with the reducing agent (DTE) gave significant reduction in both the STK1 activity and protein levels in all sera, except in the leukemia patient H3, where both STK1 activity and protein levels increased after treatment.
The immunoaffinity assay had good accuracy with patient samples from dogs with haematological tumours and solid tumours. ROC curve analysis showed an area under curve of more than 90%. Overall the assay showed high sensitivity of 72% compared to activity assay with only 44%. The specificity of the immunoassay was 94% similar to TK1 activity assay. Thus, the TK1 protein assay has higher sensitive compared to the TK1 activity assay. The specific activity of serum TK1 was subsequently determined based on the median STK1 activity and protein values, i.e. nmol [^{3}H]-deoxythymidine monophosphate dTMP)/min/mg of TK1 protein. The results showed that the TK1 in haematological malignancies had about 30-fold higher specific activity compared to healthy dogs. In contrast, the TK1 in healthy dogs showed 2.5-fold higher specific activity compared to dogs with solid tumours. These results indicate that there is a large fraction of inactive TK1 in sera particularly in dogs with solid tumours. Further studies are needed to determine the diagnostic value of the TK1 protein assay in canine oncology.

Summary (Paper I)

- An antibody based assay is described, which showed that TK1 protein levels are significantly higher in dogs with solid tumours compared to healthy dogs.
- STK1 activity levels in sera from dogs with solid tumours did not differ from those in healthy dogs (P = 0.63).
- ROC curve analysis demonstrated that the TK1 protein assay was as sensitive as the TK1 activity assay for discrimination of healthy dogs from those with haematological tumours.
- ROC curve analysis of serum TK1 protein assay showed 3-fold higher sensitivity compared to the TK1 activity assay for dogs with solid tumours.

- The specific activity determination based on TK1 activity and TK1 protein levels demonstrated that sera from healthy dogs and dogs with haematological tumours had significantly higher specific activity compared to dogs with solid tumours.

7.2. Paper II

Quaternary structure of recombinant, cellular and serum forms of thymidine kinase 1 in dogs and humans

In this study, the molecular forms of cellular, serum TK1 were studied and compared with recombinant TK1. Differences in molecular forms of recombinant, cellular and serum TK1 between humans and dogs were studied by size exclusion chromatography. Human and dog recombinant TK1 were cloned and expressed in E. coli and further tested for their substrate specificities, thermal stabilities and the effects of reducing agents. The substrate specificity of human and dog recombinant TK1 was investigated with different concentrations of the AZT and the dT. AZT phosphorylation efficiency was 3-fold higher for dog TK1 compared to human TK1. These enzymes showed positive co-operativity with dT and AZT with Hill coefficients of 1.3 and 1.4, respectively. Dog TK1 was somewhat thermally more stable than human TK1. Pre-incubation of recombinant enzymes with different concentrations of DTE (0. 2 and 20 mM) on ice for 30 min did not significantly effect enzyme activities. However, at higher concentration of DTE (100 mM) both the enzymes lost around 50 % of their activity.
The subunit composition of recombinant, cellular and serum TK1 from humans and dogs were determined by size exclusion chromatography. In the case of dog recombinant TK1, total recovery was low in the absence of DTE so that we could not determine the subunit composition. However, when the enzyme was pre-incubated with DTE, recombinant dog TK1 was in high MW oligomers and both TK1 activity and TK1 28 kDa protein followed the same elution pattern. Human recombinant TK1 behaved in a different manner. It eluted as two peaks: a major peak between 720-300 kDa and minor peak between 200-50 kDa in absence of DTE. However, pre-incubation of human TK1 with DTE shifted the major peak towards the minor peak and in both cases, TK1 activity and TK1 subunit of 25 kDa followed a similar pattern. These results differ from a previous study, where human TK1 was a dimer in the absence of ATP and a tetramer in presence of ATP (Munch- Petersen., 2009). However, these experiments were performed with lower recombinant protein concentrations and in the presence of reducing agents. Cellular TK1 both from dog and human cells eluted mainly as tetramers, and pre- incubation with DTE inhibited dog TK1 activity and had little effect on human TK1 activity.

Serum samples from human and dog patients with acute lymphoid leukemia were also analysed by size-exclusion chromatography. In case of the dog patient, serum TK1 activity eluted as a single large peak with a MW range of 720-300 kDa. Western blot analysis demonstrated a TK1 polypeptide in the same MW range but there was no direct correlation between TK1 activity and TK1 protein in these fractions. Pre-incubation of dog sera with DTE had reduced both serum TK1 activity and TK1 protein significantly. There was no shift in dog TK1 activity peak whereas western blot demonstrated a minor peak in the low molecular weight region. However for human TK1, the activity eluted as two peaks: a major fraction in the MW range of 720-300 kDa similar to dog serum TK1 and a minor peak in the MW range of 200-50 kDa. These results were similar to what was observed in a previous study, which demonstrated serum TK1 in oligomeric forms (Karlstrom et al., 1990). Western blot analysis showed TK1 polypeptide of 25 kDa in two peaks with more intense bands in the low MW region. Pre-incubation with DTE significantly shifted the major activity peak to the minor peak region and no TK1 polypeptide of 25 kDa bands was detected in western blot analysis.
Human and dog recombinant TK1 showed different quaternary structure as the quaternary structure of serum TK1. Furthermore, human and dog serum TK1 behave differently in presence of reducing agents. This could be due to that human TK1 contains more cysteines (11 cysteines-human & 8 cysteines-dogs TK1) which can form surface S-S bridges resulting in oligomers formation. Dog TK1 most likely has a similar over all structure, but lacks Cys-206 at C-terminal, and therefore may be less likely to form inter and intra disulfide bonds compared to human TK1. However, further studies are needed to understand the role of cysteines in formation of large MW complexes of TK1 in serum.

Summary (Paper II)

- Dog recombinant TK1 was successfully cloned and expressed in E.coli and dog TK1 had 3 fold higher substrate specificity for AZT compared to human TK1.
- Recombinant dog TK1 existed as oligomers after pretreatment with DTE and it is difficult to determine the MW of the active enzyme in the absence of reducing agent.
- Recombinant human TK1 eluted as a major peak in the high MW fractions and DTE pre-incubation shifted the peak towards a lower MW region.
- Cellular TK1 from humans and dogs eluted mainly as tetramers and pre-incubation with DTE inhibited dog cellular TK1 activity and had little effect on human cellular TK1.

- Human and dog serum TK1 from patients with acute lymphocytic leukemia eluted as high MW oligomers and pre-incubation with DTE showed a reduction of the TK1 activity peak.
- There was a proportion of 25 kDa TK1 polypeptide in fractions with no activity particularly for human serum TK1, demonstrating the presence of inactive TK1 protein in serum.
- Extra cysteines in human TK1 could explain the differential behavior of TK1 from human and dogs in presence of reducing agents.

7.3. Paper-III

Properties of cellular and serum forms of thymidine kinase 1 (TK1) in dogs with acute lymphocytic leukemia (ALL) and canine mammary tumours (CMTs): implications for TK1 as a proliferation biomarker.

In this study, the TK1 activity and TK1 protein levels in sera from dogs with mammary tumours were determined as well as the differences in the molecular forms of serum TK1 in canine lymphocytic leukemia and mammary tumours. An immunoaffinity assay described in Paper I was used to measure the TK1 polypeptide (26 kDa protein) levels in CMT sera, and TK1 activity levels were determined by using optimized [^{3}H]-dT phosphorylation assay as described by Sharif et al. (2012). Fresh blood sample from ALL dogs and tissue from mammary tumour dogs as well as sera from the same dogs were collected and analysed for the cellular and serum forms of TK1 by using size exclusion chromatography.
In total 20 healthy dogs and 27 dogs with CMTs classified as benign and malignant based on histological examination were included in the study. Overall, serum TK1 activity levels in CMTs were in the range of 0.5-1.8 pmol/min/mL, which is significantly higher compared to the levels in healthy dogs. Serum TK1 protein levels were also significantly higher in sera from CMT patients compared to healthy dogs (Table 3).

Table 3. Summary of serum thymidine kinase 1 activity and protein levels.

		STK1 activity (pmol/min/mL)		STK1 protein (ng/mL)	
Sample group	N	Median	Range	Median	Range
Healthy	20	0.7	0.4 - 1.4	8	3 - 18
Mammary adenoma	11	0.9	0.5 - 1.5	26	12 - 39
Mammary carcinoma	13	0.9	0.6 - 1.6	31	13 - 54
U.C (Un classified)	3	0.7	0.5 - 1.8	17	9 - 28

A significant correlation was found between STK1 activity and TK1 protein levels in healthy and CMT samples (r = 0.52, P = 0.01; r = 0.41, P = 0.03, respectively). Furthermore, the TK1 protein assay differentiated sera from mammary adenomas efficiently from those of healthy dogs and adenomas from carcinomas. In contrast the TK1 activity assay could not distinguish sera from healthy dogs compared to mammary adenomas, and adenomas from carcinomas. ROC curve analysis showed that TK1 activity had sensitivity of 22% while the TK1 protein assay of 80%.

To understand the reason for high TK1 protein levels in CMTs, we collected CMT tissue along with sera from same patient and analysed TK1 by size exclusion chromatography. In parallel, we collected fresh blood sample from ALL dogs, isolated leukocytes and separated serum. The leucocytes (containing a high fraction of leukemic cells) were lysed and cytosolic proteins isolated and analysed.

The TK1 enzyme activity and protein profiles in the cellular extract from the ALL patients showed a major peak in the MW range of 40-66 kDa, which is similar to what was observed in a previous study (Sharif et al., 2012). However, in the cellular extract from CMT tissue, a major peak corresponding to MW of 40-100 kDa and minor peak corresponds to 720-400 kDa were found. Western blot analysis demonstrated the TK1 polypeptide of 26 kDa in the fractions corresponding to the major peak and faint bands in the high MW region.

The serum TK1 activity from the ALL patient eluted as a single peak in the high MW region (720-300 kDa) and western blot analysis showed the TK1 polypeptide in same factions as the TK1 activity. In sera from CMT patients active TK1eluted as single peak similar to in the ALL case, but western blot analysis showed the TK1 protein bands in almost all fractions, indicating that TK1 exits in multiple forms in CMT (oligomers, dimers, and tetramers), with a large proportion of apparently inactive protein. The difference in the molecular forms of TK1 in ALL and CMT may explain the reason for high TK1 protein levels in the CMT sera but with relatively low TK1 activity. In healthy dogs, both TK1 activity and TK1 protein eluted as single peak in the high MW region. Moreover, the specific activity of serum TK1 (nmol dTMP/min/mg of TK1 of 26 kDa), based on the immunoaffinity assay and [^{3}H]-dT activity measurements in healthy dogs was 2.5-fold higher than dogs with mammary tumours. Thus, there is a large fraction of inactive TK1 in CMTs. These results may improve the clinical utility of TK1 protein assays but further studies are warranted to determine the prognostic and monitoring capacity of TK1 protein assays.

Summary (Paper III)

- Serum TK1 protein and TK1 activity levels were significantly higher in dogs with mammary tumours compared to healthy dogs.

- The TK1 protein assays were able to differentiate dogs with mammary adenomas from healthy dogs, while the TK1 activity assays were not.
- Cellular TK1 from ALL patients exists mainly as dimers while the serum TK1 from the same dog was mainly as high MW oligomers.
- In CMT patients, TK1 from tumour tissue extracts eluted mainly as tetramer while serum TK1 from same patients exists in multimeric forms (dimer, tetramer and oligomers).
- In healthy dogs, serum TK1 could only be detected in the high MW oligomer.
- Differences in the molecular forms of serum TK1 in healthy, ALL and CMT dogs provides valuable information with clinical implications for the use of TK1 as a biomarker in veterinary medicine.

7.4. Paper IV

Development of a new ELISA for determining serum thymidine kinase 1 protein levels in canine malignancies and its clinical significance

The goal was to develop an ELISA for determining TK1 protein levels in sera from dogs with different malignancies. We also tested whether the TK1 protein levels could serve as a marker for monitoring treatment of canine lymphoma. The performance of TK1-ELISA was compared with that of the [^{3}H]-dT phosphorylation assay.
The TK1-ELISA is based on two antibodies raised against TK1 peptides from two of different regions, i.e. the C-terminal region of dog TK1, a highly conserved part of the active site of human TK1. Recombinant dog TK1 was used as standard. Dog specific polyclonal antibody was used to coat the 96-well plates for catching serum TK1 and it is raised against the C-terminal region of canine TK1. The second biotinylated antibody, used for detection of the bound dog TK1, was a mouse monoclonal antibody raised against a peptide in the active site of human TK1 (Gasparri et al., 2009). Inter-assay and intra-assay variations (CV %) of the prototype ELISA was estimated from the variability of duplicated samples is 5-15% and 5%, respectively.
Serum samples from 30 healthy, 31 dogs with lymphoma, 5 dogs with leukemia and 40 dogs with solid tumours were analysed with the dog TK1 ELISA. Furthermore, serum TK1 protein levels were also monitored in six lymphoma patients during chemotherapy. The results using STK1 activity assay and TK1-ELISA showed significantly higher TK1 levels (activity & protein) in sera from haematological malignancies compared to sera from healthy dogs ($P < 0.0001$) (Table 4). ROC curve analysis of TK1 activity assay had an AUC of 83% with a sensitivity of 75% and specificity of 96%. A

similar analysis of TK1-ELISA results showed an AUC of 94% with a slightly higher sensitivity of 78% and specificity of 96%. For dogs with solid tumours, there was no significant difference in STK1 activity levels compared to the healthy group. However, the STK1 protein levels were significantly higher in sera from dogs with solid tumours compared to healthy dogs ($P < 0.0001$). Sera from solid tumours were sub grouped as mammary tumours, mastocytomas, melanomas and others. The mean STK1 activity and STK1 protein levels in different sub groups were shown in Table 4. Solid tumour sub groups did not have higher median STK1 activity above the cut-off based on healthy. In contrast, STK1 protein levels of sub groups were above the healthy cut-off. These results were clearly demonstrated by ROC curve analysis as TK1 activity assay had a sensitivity of 27% whereas the TK1-ELISA showed a sensitivity of 62%. Overall, the TK1-ELISA results correlated with the TK1 activity values ($rs = 0.64$, $P < 0.0001$).

TK1 protein levels along with TK1 activity were measured in lymphoma patients during chemotherapy and the TK1 protein levels returned to the normal range after treatment but in one dog with progressive disease the TK1 protein and TK1 activity values continued to increase after treatments. These results indicate that TK1 protein determination during chemotherapy may help clinicians to monitor treatment and detect reoccurrence of tumours in early stages.

Table 4. Serum thymidine kinase 1 activity and TK1 protein levels in dogs measured with the prototype ELISA.

		STK1 activity (pmol/min/mL)		STK1-ELISA (ng/mL)	
Group	N	Range	Median	Range	Median
Healthy	30	0.7 - 1.4	1.02	0.07 - 0.47	0.25
Lymphoma	31	0.54 -29.8	2.43	0.25 - 4.38	0.78
Leukemia	5	1.33 - 58.7	38.3	0.33 - 4.19	3.92
Mammary tumours	16	0.62 - 1.83	1.07	0.22 -1.5	0.50
Mastocytoma	8	0.51 - 1.52	0.85	0.25 - 1.1	0.50
Malignant Melanoma	11	0.52 - 1.52	1.15	0.21 - 3.40	0.53
others	5	0.53 - 1.78	1.08	0.34 -1.13	0.67

In this study, the TK1 activity values in sera from dogs with solid tumours were low, similar to previous studies (Sharif et al., 2012; von Euler et al., 2009; Nakamura et al., 1997). The higher TK1 protein levels in dogs with solid tumour strongly suggest that TK1 protein determinations are preferable to TK1

activity assays. The availability of a new ELISA should increase the clinical utility of TK1 as a proliferation biomarker.

Summary (Paper IV)

- A sandwich TK1-ELISA was developed using antibodies against from different regions of TK1 sequence.
- TK1-ELISA is sensitive enough as TK1 activity assay to differentiate dogs with hematological tumors from healthy dogs.
- In lymphoma patients, serum TK1 protein followed a similar pattern as the TK1 activity values during chemotherapy.
- The TK1 activity assay could however not differentiate dogs with solid tumours from healthy dogs.
- TK1-ELISA may be an alternative to TK1 activity assay for diagnosis of dogs with solid tumours.

8. Conclusions

The results of this doctoral thesis contribute basic information about molecular properties of canine and human TK1 as well as provide new detection techniques of particular relevance for different canine malignancies.

A major contribution was to establish immunoaffinity methods to determine the levels and molecular properties of the canine as well as human TK1 polypeptide. These results indicate that STK1 activity assay would be valuable clinically for diagnosis and monitoring of haematological tumours. However, dogs with solid tumours had a low TK1 activity level, which in turn makes the clinical use of TK1 as biomarker less valuable. Here we developed an antibody based assay that measures TK1 subunit of 26 kDa protein levels in different canine malignancies. STK1 protein levels are significantly higher in dogs with haematological tumours and solid tumours compared to healthy dogs. This indicates that TK1 protein assays can be an alternative for TK1 activity assay especially for dogs with solid tumours.

These methods were also used to define the differences between the molecular forms of recombinant, cellular and serum TK1 of humans and dogs by size exclusion chromatography. Recombinant and serum thymidine kinase 1 were shown to exist as high molecular weight oligomers of more than of 700 kDa as determined by both activity and the immunoaffinity assays. However, cellular TK1 from human or dog cells eluted mainly as dimers and tetramers. The presence of reducing agents produced important differences between the chromatographic behaviour of human and dog TK1 complexes, probably dependent on differences in inter and intra disulfide bonds. These differences could at least partly be explained by a lack of a cysteine in the C-terminal region of canine TK1.

The results presented here showed that the TK1 protein assay had significantly higher ability to differentiate healthy dogs from dogs with mammary tumours compared to the activity assays, suggesting a clinical benefit with using the TK1 protein assay. This may thus help to identify tumour progression at an early stage. Furthermore important new basic information was obtained by size- exclusion chromatography, which convincingly demonstrated that there is a large fraction of inactive TK1 protein in case of sera from dogs with

mammary tumours. The reason for high TK1 protein in mammary tumours could be due to the tumour micro environment, characterized by oxidative stress, hypoxia and abnormal blood flow, which can induce changes in the structure of TK1 before it enters into the blood stream. This may results in inactivation of serum TK1. However, for ALL TK1 is directly released into blood from damaged cells, apparently leading to a more active form of serum TK1. This was shown for the first time in this thesis since we were able to determine the specific activities of serum TK1 in samples from dogs with ALL and mammary carcinoma.

Even though TK1 protein assay appears to be valuable, it needs to be in a clinically compatible assay format allowing routine measurements. Attempts were therefore made to convert the immune affinity assay into an ELISA format. A prototype form of a sandwich TK1-ELISA, using TK1 antibodies from different regions, was developed. The TK1-ELISA showed similar sensitivity as the TK1 activity assay and could be used to clearly differentiate dogs with haematological tumours from healthy dogs. More importantly, the TK1 ELISA was also able to detect increased levels of SK1 in sera from dogs with several types of solid tumours, overcoming this previously shown limitation of the TK1 activity assay. Therefore, the TK1-ELISA is most likely clinically valuable for prognosis and monitoring of different malignant diseases in dogs.

9. Future research

- One future goal is to make a mutational analysis of the cysteine residues in dog and human TK1. Human TK1 contains three additional cysteines and it is likely that Cys206 in the C-terminal region can form disulfide bridges with other proteins including other TK1 subunits. There is no cysteine in the homologous position in dog TK1. A systematic mutational analysis can help to define the role of cysteines in the TK1 oligomerzation process.
- Even though the molecular forms of STK1 in sera from dogs with different tumours have been studied, further studies need to be done to clarify the relationship of these TK1 forms and staging.
- Moreover, a detailed analysis of serum TK1 from ALL and CMT patients by 2D gel electrophoresis coupled with mass spectrometry should give important information on the nature of TK1 amino acid modifications. Further studies on the influence of different tumour microenvironment factors in various tumour types and stages should be carried out. This would give information about whether factors such as blood supply and oxygen availability can play vital role in the inactivation of serum TK1 in dogs with mammary tumours and other solid tumours.
- The TK1-ELISA has to be validate further with a large group of clinical samples from different canine malignancies i.e. lymphomas, mammary tumours, malignant melanomas and other tumours, with serum samples taken at the time of diagnosis and during chemotherapy to determine the prognostic and monitoring ability of the TK1 protein assay. Furthermore, TK1-ELISA should most likely also be developed into a commercially available assay.
- There is a need to determine whether the anti -TK1 antibodies can be used for histo-chemical expression studies and some attempts have already been made (von Euler & Eriksson, 2011).

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