

Molecular Properties of Serum Thymidine Kinase 1 and Use of the Canine Enzyme in Disease Monitoring

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Abstract

Thymidine kinase 1 (ATP: thymidine 5'-phosphotransferase, EC 2.7.1.21, TK1) catalyzes the transfer of the gamma phosphate group from a phosphate donor to the 5'-hydroxyl group of thymidine to form thymidine monophosphate, which is converted to thymidine triphosphate and then utilized in DNA synthesis. TK1 activity markedly increases in G1 and S phase of the cell cycle and is found mainly in dividing cells, but also in an extracellular form in the blood. Increased serum TK1 levels are considered as a sensitive and useful marker for cell proliferation and detection of malignancy in clinical medicine.

The aim of this study was to investigate the molecular characteristics of canine thymidine kinase 1 and utilize several anti-TK1 antibodies to a better understanding its role in disease prognostics, risk assessment, and monitoring of cancer development.

Study I was undertaken to clarify the structure of recombinant, cellular and serum TK1 in order to improve the usefulness of serum TK1 (STK1) as a proliferation biomarker in neoplastic diseases. Several forms of STK1 were observed with different enzymatic activities. The active form of STK1 was found mainly as an oligomer. Dimer and tetramer forms of STK1 were also seen in serum by immunoaffinity method, but they were enzymatically less active. Study II described a sensitive method using a [³H]-deoxythymidine phosphorylation assay to measure STK1 activity in dogs and humans with hematologic malignancies and dogs with solid tumors. The results revealed that the dThd phosphorylation assay was suitable for measurement of TK1 in hematologic malignancy (lymphoma and leukemia) and provided valuable information regarding the disease recurrence. Significant correlation was observed between the [³H]-dThd assay and the TK1 activity assays, TK-REA and TK Liaison. However, STK1 activity levels in dogs with solid tumors were very low. Therefore, another method to determine the levels of TK1 in serum based on immunoaffinity techniques was performed in study III. The concentration of STK1 protein in dogs with solid tumors was much higher than expected from the activity. Thus, methods to determine STK1 protein are more sensitive than activity tests in solid tumors. Study IV revealed that serum TK1 activity was elevated in 41% of bitches with pyometra, and thus TK1 determinations may be clinically valuable also in some non-neoplastic diseases.

Keywords: Thymidine kinase 1, blood biomarker, hematologic malignancy, canine pyometra, radio- enzymatic assay, immunoaffinity technique, cell proliferation marker.

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“Allah ‘God’ will raise those who have believed among you and those who were given knowledge, by degrees. And Allah is acquainted with what you do.”

(Holy Quran, 11-Surat Al-Mujadila)

To my beloved family

To my husband

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List of Publications

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

- I Sharif H., Kiran KJ., Wang L., He E., Eriksson S. (2012). Quaternary structure of recombinant, cellular, and serum forms of thymidine kinase 1 from dogs and humans. *BMC Biochemistry Journal* 13 (12), 1-10.
- II Sharif H., Von Euler H., Westberg S., He E., Wang L., Eriksson S. (2012). A sensitive and kinetically defined radiochemical assay for canine and human serum thymidine kinase 1 (TK1) to monitor canine malignant lymphoma. *The Veterinary Journal* (In press).
- III Sharif H*, Kumar KJ*, Westberg S., Von Euler H., Eriksson S. (2012). Determination of serum thymidine kinase 1 polypeptide and activity levels in dogs with hematologic malignancy and solid tumour. (Manuscript).
- IV Sharif H., Hagman R., Wang L., Eriksson S. (2012). Elevation of serum thymidine kinase 1 activity in a bacterial infection: Canine pyometra. (Submitted for publication).

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* First authorship shared

Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APC	Anaphase promoting complex
ATP	Adenosine triphosphate
AZT	3'-azidothymidine
AZTMP	3'-azidothymidine monophosphate
Cdk1	Cyclin dependent kinase 1
cDNA	Complementary DNA
CO ₂	Carbon dioxide
D4T	2'-3'-dideohydro-2'-3'-dideoxythymidine
dAdo	Deoxyadenosine
dCDP	Deoxycytidine diphosphate
dCK	Deoxycytidine kinase
dCMP	Deoxycytidine monophosphate
dCTP	Deoxycytidine triphosphate
dCyd	Deoxycytidine
dGK	Deoxyguanosine kinase
dGuo	Deoxyguanosine
dN	Deoxyribonucleoside
dNDP	Deoxyribonucleoside diphosphates
dNK	Deoxyribonucleoside kinases
dNMDK	Deoxyribonucleoside diphosphate kinases
dNMPK	Deoxyribonucleoside monophosphate kinases
dNTP	Deoxyribonucleoside triphosphates
dTDP	Deoxythymidine diphosphate
dTTP	Deoxythymidine triphosphate
dUDP	Deoxyuridine diphosphate
dUMP	Deoxyuridine monophosphate

dUTP	Deoxyuridine triphosphate
FdUrd	5-fluoro-2'-deoxyuridine
FLT	3'-deoxy-3'-fluorothymidine
GMP	Guanosine monophosphate
IMP	Inosine monophosphates
mtDNA	Mitochondrial DNA
NDP	Ribonucleoside diphosphates
NMP	Ribonucleoside monophosphates
NTP	Ribonucleoside triphosphates
TK1	Thymidine kinase 1, cytosolic
TK2	Thymidine kinase 2, mitochondrial
TMP	Thymidine monophosphate
TS	Thymidylate synthase
UMP	Uridine monophosphate
UTP	Uridine triphosphate

1 Introduction

1.1 Blood biomarkers in veterinary medicine

Disease diagnosis and efficacy of therapeutic interventions are major clinical challenges in veterinary medicine. The ideal biomarker for a certain disease should be sensitive and specific enough to distinguish between healthy and disease states. Thus, provide reliable information about the progression of the disease and the response to therapy as well as predict the recurrence of disease, particularly in tumor.

1.1.1 Infectious disease biomarkers

An infection is a disease caused by the invasion of body tissues by bacteria, viruses, parasites and fungi or results from the toxins that they produce. Hosts mainly fight against these agents via the immune system. Therefore, inflammatory mediators are released into the blood at the site of infection.

The majority of new blood biomarkers are part of the systemic inflammatory response. Many inflammatory actors often use as disease markers in the clinical setting, e.g. white blood cells, neutrophils, monocytes, lymphocytes, eosinophils and basophiles. Any changes in the number, size and morphology of these cells represent inflammation, infection or immune-mediated disease. Low numbers of some of these inflammatory cells indicate bone marrow suppression or septic shock.

The acute phase proteins (APPs) are blood proteins released in response to infection, inflammation or trauma (Young *et al.*, 1991). The acute phase proteins such as C-reactive protein, serum amyloid A, haptoglobin and α_1 – acid glycoprotein are widely used as diagnostic, prognostic and monitoring markers (Ceron *et al.*, 2005; Murata *et al.*, 2004; Petersen *et al.*, 2004). The acute phase proteins are induced by cytokines such as interleukins IL - 1, IL - 6 and tumor necrosis factor alpha (TNF- α), and they stimulate the hepatocytes to produce APPs (Young *et al.*, 1991). The cytokines are themselves considered

as diagnostic markers for systemic inflammation (Song *et al.*, 2012; Rau *et al.*, 2007).

Some biochemical markers are elevated in certain diseases, i.e. elevation of alkaline phosphatase and aspartate transferase, which indicating diseases involving the liver. Elevation of creatinine and blood urea nitrogen indicates kidney disease.

1.1.2 Cell proliferation biomarkers

A tumor is an abnormal mass of tissue that has no function in the body. Tumors are usually classified as benign or malignant. A benign tumor is not cancer. It is slow growing, does not spread or invade surrounding tissue, and once it is completely removed, it usually does not recur. A malignant tumor, on the other hand, is cancer that has the capacity to produce metastatic disease. However, certain "benign" tumors may later give rise to malignant cancers, which result from additional genetic changes in a subpopulation of the tumor's neoplastic cells. Thus, early diagnosis of cancer offers the best opportunity for treatments and increased survival rate.

Tumor markers are any substances that can be found in the body when malignant cells are present. Good tumor markers can significantly reduce the diagnostic lead time and be sensitive enough in screening of the healthy population and populations at risk for the presence of malignancy. This will lead to early detection of tumors and thus rescue or at least prolonged life expectancy. These markers may also help to determine the effectiveness of therapy and predict recurrence of the disease.

Only limited numbers of tumor markers have been used for monitoring proliferation, i.e. Ki-67, Proliferating Cell Nuclear Antigen (PCNA), and argyrophilic nuclear organizing region (AgNOR); these are measured by immunohistochemistry. Other tumor markers that can be measured in serum or plasma, i.e. lactate dehydrogenase (LDH), α -fetoprotein (AFP) and acute phase proteins such as α -1- acid glycoprotein (α_1 AG) and C-reactive protein (CRP). There is a large group of tumor specific markers, e.g. PSA, CA 15-3, CA125 and CYFRA-21-1. They have been used in the clinical setting, but most of them are neither sensitive nor specific enough for screening or diagnosis of cancer without the support of other clinical findings. The levels of these tumor markers often do not increase until the symptoms appear, or they are elevated in diseases other than tumor diseases (Perkins *et al.*, 2003; Bates, 1991).

Ki-67 is a nuclear antigen found in proliferating cells, which can be detected by monoclonal antibodies (Gerdes *et al.*, 1983). For many years, Ki-67 has been used to measure the number of proliferating cancer cells in any particular tumor by immunohistochemistry. Ki-67 is not present in the blood

and is expressed at all stages of the cell cycle except G₀; however, the peak is found in G₂ and M phase (Gerdes *et al.*, 1983). Ki-67 has been used as a cell proliferation marker in human with non Hodgkin's lymphoma (Grogan *et al.*, 1988; Gerdes *et al.*, 1987) and other solid tumors (Sahin *et al.*, 1991). Some studies have been carried out to determine the diagnostic and prognostic value of Ki-67 in veterinary medicine (Ishikawa *et al.*, 2006; Sakai *et al.*, 2002; Roels *et al.*, 1999; Lohr *et al.*, 1997).

PCNA is a DNA clamp holder protein for DNA polymerase delta that plays an important role in DNA replication and repair (Naryzhny, 2008). It is involved in leading strand synthesis and appears in late G₁ and S phase cells (Ishikawa *et al.*, 2006; Bravo & Macdonald-Bravo, 1985).

Drawbacks of PCNA and Ki-67 are the presence of Ki-67 throughout all phases of the cell cycle and the long half life of PCNA (20 h) can create false positive results (Bravo & Macdonald-Bravo, 1985). Furthermore, they cannot be measured in the blood.

AgNORs are proteins associated with a loop of DNA in the nuclei where ribosomal RNA transcription occurs. The amount of AgNORs represents the percentage of cycling cells. It is widely used as a cell proliferation marker and provides information in grading canine malignant lymphoma (Kiupel *et al.*, 1998a; Vail *et al.*, 1996; Preziosi *et al.*, 1995).

A major concern with this marker is the influence of several technical parameters, e.g. fixation, temperature and staining of tissue on the results (Kiupel *et al.*, 1998b).

Serum levels of LDH are used as a prognostic and diagnostic marker in humans with hematologic malignancy (Kornberg & Polliack, 1980; Schneider *et al.*, 1980; Ferraris *et al.*, 1979).

In veterinary medicine, some investigations have been carried out to determine the value of LDH as a screening maker for different malignancies. However, up-regulation of LDH in diseases other than malignancies and fluctuating levels among patients have demonstrated that LDH is of limited value as a marker in canine tumor monitoring (Marconato *et al.*, 2010; Marconato *et al.*, 2009; Von Euler *et al.*, 2006; Zanatta *et al.*, 2003; Nakamura *et al.*, 1997).

Other tumor markers, e.g. α -fetoprotein, which is a fetal antigen, and α_1 AG and CRP, are elevated in some cases such as hepatocellular tumor and lymphoma (Merlo *et al.*, 2007; Lechowski *et al.*, 2002; Ogilvie *et al.*, 1993). However, these markers are also elevated in other than tumor diseases, e.g. inflammation, infection, and trauma (Young *et al.*, 1991).

Over the past several years, many studies in human medicine have been performed and lead to improve diagnosis at an early stage of tumor; one

example is a prostate specific antigen (PSA). It is a protein produced by the prostate glands at low levels in healthy and high levels in men with malignant prostatic disease and shows a correlation with tumor stage and prostate volume. However, it is often also increased in benign prostatic hypertrophy, which limits its value as a screening test (Bates, 1991).

CA 15-3 is widely used as a tumor marker for breast cancer (Duffy, 2006). However, this tumor marker has low sensitivity and relatively low specificity in diagnostics of early stage disease. Its concentration is elevated about 10% in stage I and about 75% in stage IV (Brooks, 2009; Duffy, 2006). Besides breast cancer, CA 15-3 is also up-regulated in other diseases such as liver diseases, pulmonary cancer, gastrointestinal/colonic cancer and ovarian cancer (Brooks, 2009).

CA 125 is a glycoprotein produced by ovarian cancer cells and found in 80% of women with ovarian cancer (Bates, 1991). It provides valuable information regarding prognosis and recurrence of the disease (Diaz-Padilla *et al.*, 2012). However, it has low sensitivity at the early stage of the disease where only 50% of patients expressed CA 125 (Sasaroli *et al.*, 2009). Furthermore, CA 125 is also lacking specificity, since it is also elevated in other tumors, such as endometrial, breast and lung cancer (Bast *et al.*, 1998).

CYFRA-21-1 is a cytokeratin 19 fragment, produced by all types of lung cancers. It is considered as the most sensitive non-small cell lung cancer (NSCLC) marker. CYFRA-21 levels correlate with tumor burden, and it appears to be a reliable biomarker for monitoring disease relapse and assesses the response to chemotherapy (Tufman & Huber, 2010). It is, however, highly expressed in some other diseases such as renal failure (Stieber *et al.*).

2 DNA precursor synthesis

Deoxyribonucleoside triphosphates (dNTPs) are essential for all living cells. They are needed for the synthesis, maintenance and repair of DNA. The DNA precursors can be synthesized either by the *de novo* pathway or the *salvage* pathway, where dNs are reused from degraded DNA and the diet. An overview of the nucleotides metabolism in mammalian cells is shown in figure 1.

2.1 The *de novo* pathway

In *de novo* pathway ribonucleoside monophosphates are formed from ribos-5-phosphate and other molecules such as amino acids and CO₂, in the presence of energy in the form of ATP. IMP is the first purine nucleotide synthesized. Subsequently, it is converted either to GMP or AMP. The ribonucleoside monophosphates (NMPs) will be further phosphorylated to ribonucleoside diphosphates (NDPs); which are then phosphorylated to ribonucleoside triphosphates (NTPs) for RNA synthesis, or reduced by ribonucleotide reductase to form the deoxyribonucleosides diphosphates (dNDPs) (Nordlund & Reichard, 2006; Reichard, 1988; Thelander & Reichard, 1979). The final phosphorylation results in the formation of dNTPs. Deoxythymidine monophosphate is formed through methylation of deoxyuridine monophosphate by thymidylate synthase (TS). After further phosphorylation steps deoxythymidine triphosphate (dTTP) is formed, which is required for DNA synthesis (Montfort & Weichsel, 1997).

2.2 The *salvage* pathway

This is a recycling pathway, where DNA precursors are produced by reusing deoxyribonucleosides (dNs), which come from DNA degradation either inside or outside the cell or from the diet. Specific transporter proteins transport dNs into the cell (Kong *et al.*, 2004; Cass *et al.*, 1999; Plagemann *et al.*, 1988).

2.3 Deoxyribonucleoside kinases (dNKs)

dNKs are enzymes catalyzing the first step of phosphorylation in the salvage pathway by transferring a phosphoryl group from nucleoside triphosphates to deoxyribonucleosides.

Mammalian cells have four different dNKs with overlapping substrate specificities. Two cytosolic dNKs, Thymidine kinase 1 (TK1, E.C. 2.7.1.21) and deoxycytidine kinase (dCK, E.C. 2.7.1.74) and two mitochondrial dNKs, thymidine kinase 2 (TK2, E.C. 2.7.1.21) and deoxyguanosine kinase (dGK, E.C. 2.7.1.113) (Eriksson *et al.*, 2002). The comparison of the amino acid sequences of dCK, dGK, TK2 and TK1 reveals that all dNKs, except TK1, share some conserved motifs. Thus, the dNKs can be divided into two families; the TK1 group and the dCK, dGK, TK2 group (Eriksson *et al.*, 2002).

dNKs are of medical interests as they are involved in several human diseases. For example, TK2 deficiency is associated with mtDNA depletion syndrome and leads to severe myopathy (Eriksson *et al.*, 2002). Lack of dGK is also associated with mtDNA depletion and patients with no dGK activity die in infancy with liver failure and neurological abnormalities (Eriksson *et al.*, 2002). No human cases with TK1 deficiency have been reported; however, TK1 knockout mice have shortened life span and die with kidney failure (Dobrovolsky *et al.*, 2003).

dNKs have important roles in antiviral and anticancer therapy through activation of nucleoside analog drugs. The first step in this activation is phosphorylation of nucleoside analogs by dNKs to nucleoside analog monophosphates (NAMP). Subsequently, the NAMP is phosphorylated to di- and triphosphate by other cellular kinases (Van Rompay *et al.*, 2000). Then the final product can be incorporated into viral or cellular DNA, which interferes with viral DNA synthesis or cellular DNA synthesis and repair, resulting in cell death (Leoni *et al.*, 1998).

dNKs are also important in suicide gene chemotherapy. In this therapy, a kinase gene is delivered and expressed in target cells, followed by treatment with nucleoside analog that are phosphorylated by the introduced kinase. This may result in termination of DNA replication and induces apoptosis in the cells that harbor the specific kinase gene. Many experimental studies have been done to model human cancers using TK from Herpes simplex virus type 1 for the selective activation of nucleoside analogs such as ganciclovir (Sterman *et al.*, 2005; Rainov & Grp, 2000; Ural *et al.*, 2000; Klatzmann *et al.*, 1998).

Deoxycytidine kinase (dCK)

Deoxycytidine kinase (NTP: deoxycytidine 5'-phosphotransferase, E.C. 2.7.1.74) is a cytoplasmic enzyme expressed preferentially in lymphocytic cells (Eriksson *et al.*, 2002; Hatzis *et al.*, 1998; Johansson *et al.*, 1997).

The dCK gene extends over more than 34 kb on chromosome 4q13.3-q21 with 7 exons that vary in size from 90 to 1544 bp (Song *et al.*, 1993; Chottiner *et al.*, 1991). The enzyme has two identical subunits with a molecular weight of 30 kDa. The active form of the enzyme is a homodimer (60 kDa) (Bohman & Eriksson, 1988).

dCK has a broad substrate specificity and can phosphorylate both pyrimidine and purine deoxyribonucleosides and their analogs. These are of importance in antiviral and cancer therapy such as β -L-2'-3'- dideoxy-3'-thiacytidine (lamivudine), arabinosyl cytosine (cytosar), 2- chlorordeoxyadenosine (cladribine, CdA) and diflourodeoxycytidine (gemcitabine), 2-fluoro-arabinofuranosyl adenine (fludarabine, FAraA), 2'-3'-dideoxycytidine (zalcitabine, ddc) and arabinosyl adenine (vidarabine, AraA) (Eriksson *et al.*, 2002; Eriksson *et al.*, 1991a; Eriksson *et al.*, 1991b). The enzyme does not follow Michaelis–Menten kinetics, but shows negative cooperativity with a Hill constant (n) <1. The catalytic efficiency of the enzyme for natural substrates and their analogs are extensively studied (Eriksson *et al.*, 2002). ATP and UTP, as well as other nucleoside triphosphates can serve as phosphate donors (Krawiec *et al.*, 2003; Krawiec *et al.*, 1995).

Deoxyguanosine kinase (dGK)

Deoxyguanosine kinase (nucleoside triphosphate: deoxyguanosine 5'-phospho transferase, EC 2.7.1.113) is a mitochondrial enzyme and can be found in all tissues (Wang *et al.*, 1993). However, dGK is found in the cytosol together with cytochrome *c* during apoptosis (Jullig & Eriksson, 2001).

The gene is located on chromosome 2q13 and encodes a 30 kDa protein. dCK and dGK show similarity in the amino acids sequence, except in the N terminal region, which could be due to that they have different subcellular localization. The active enzyme is a homodimer with a molecular weight of 60 kDa (Wang *et al.*, 1996; Wang *et al.*, 1993). The catalytic efficiency of the enzyme for dGuo, dAdo and dCyd are $6 \times 10^{-4} \text{ M}^{-1}\text{s}^{-1}$, $4 \times 10^{-3} \text{ M}^{-1}\text{s}^{-1}$, and $1 \times 10^{-3} \text{ M}^{-1}\text{s}^{-1}$, respectively (Eriksson *et al.*, 2002). dGK can phosphorylate purines and a number of purines and pyrimidine nucleoside analogs but with the highest efficiency for the purines (Sjoberg *et al.*, 1998). Several nucleoside triphosphates can serve as phosphate donors, and preferentially ATP and UTP (Eriksson *et al.*, 2002).

Thymidine kinase 2 (TK2)

Thymidine kinase 2 (ATP: thymidine 5'-phosphotransferase, E.C. 2.7.1.21) is localized in the mitochondria. TK2 is present in most tissue, and it is expressed independent of the cell cycle (Wang *et al.*, 1993). TK2 has a broad substrate specificity, which differs from TK1, since it can phosphorylate deoxythymidine (dThd), deoxycytidine (dCyd) and deoxyuridine (dUrd) and their analogs (Munch-Petersen *et al.*, 1991). The level of TK2 is low in dividing cells as compared to TK1; however, it is the only dThd phosphorylating enzyme found in non-dividing cells. TK2 can be found in relatively high levels in liver, spleen, brain and pancreas (Eriksson *et al.*, 2002).

The TK2 gene is located on chromosome 16q22. The TK2 gene spans 45 kb with 10 exons that vary in size from 32 bp up to 1304 bp. The subunit size of mitochondrial TK2 is 28 kDa (Eriksson *et al.*, 2002). The molecular profile of TK2 varies from monomer to hexamer; the first report revealed a dimer with MW of 70 kDa (Lee & Cheng, 1976). In a later report, TK2 had a molecular weight of 98 kDa – 340 kDa as judged by G-200 Sephadex chromatography (Ellims & Van der Weyden, 1980). The monomer with a molecular weight of about 30 kDa and the active dimer has been described more recently (Wang *et al.*, 2003; Munch-Petersen *et al.*, 1991).

The kinetic behavior of TK2 with deoxythymidine does not follow Michaelis-Menten kinetics, but shows negative cooperativity with a Hill constant of $n < 1$ (Wang *et al.*, 2003). However, with other substrates dCyd and dUrd the activity profile follows Michaelis-Menten kinetics (Wang *et al.*, 2003; Munch-Petersen *et al.*, 1991). The K_m values for dCyd and dUrd are 36 μ M and 6 μ M, respectively (Munch-Petersen *et al.*, 1991). ATP and CTP serve as phosphate donors and the final products dTTP and dCTP act as feedback inhibitors (Munch-Petersen *et al.*, 1991).

Thymidine kinase 1 (TK1)

Thymidine kinase 1 (ATP: thymidine 5'-phosphotransferase, EC 2.1.7.21) also catalyses the transfer of γ phosphate group from ATP to Thd (and dUrd) forming thymidine or deoxyuridine monophosphates.

The TK1 gene is located on the long arm of chromosome 17 at position 17q25.2-q25.3 (Petty *et al.*, 1996; Elsevier *et al.*, 1974; Miller *et al.*, 1971). The first human TK1 cDNA was cloned and characterized almost 30 years ago (Flemington *et al.*, 1987; Bradshaw & Deininger, 1984). The entire gene spans 12.9 Kb with 7 exons. The cDNA is 1241 pb, with an open reading frame of 702 pb coding for a protein of 234 amino acids with a molecular weight of 25 kDa (Eriksson *et al.*, 2002).

The recent revolution in gene sequencing has revealed that mammalian and bird TK1;s from canine, mouse, rat, cattle and chicken (Zimin *et al.*, 2009; Boardman *et al.*, 2002; Strausberg *et al.*, 2002) share high sequence identity to human TK1, with 89.3, 86.2, 87.0, 86.7 and 73.2% sequence identity, respectively. Most changes are seen in C-terminal regions (Fig. 2)

Because of the importance of TK1 in different aspects of tumor diagnosis, it has been studied for many years, and studies on the cell cycle regulation, localization in the cell, enzyme structure, reaction kinetics, substrates specificity and medical interest will be described in the following sections.

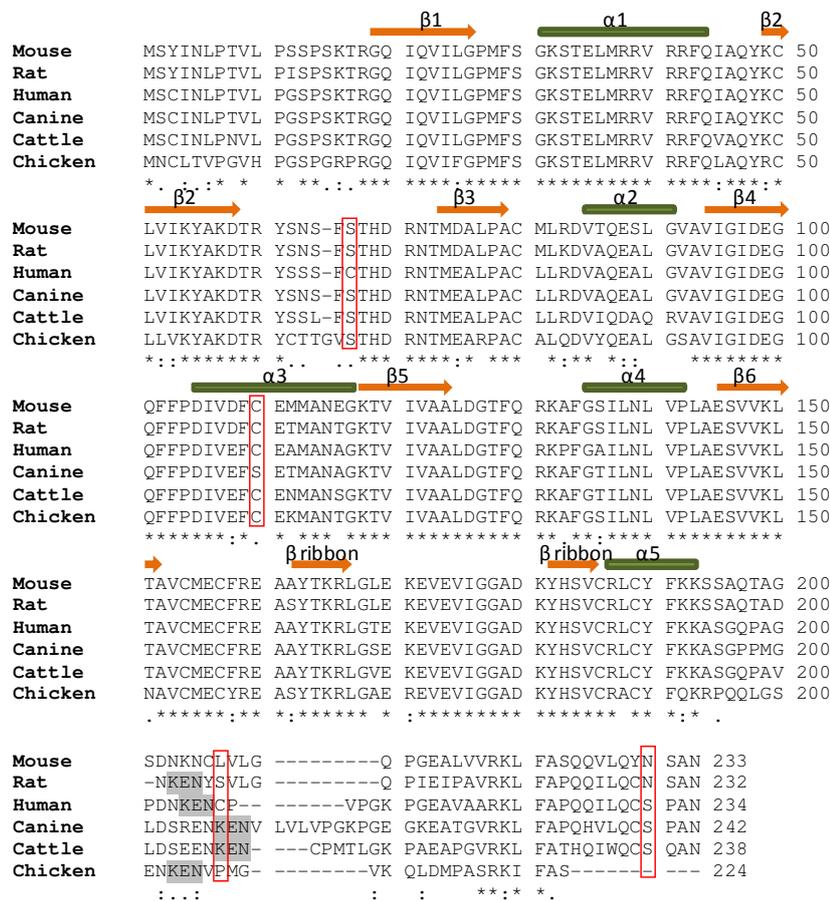


Figure 1. Alignments of TK1 sequences from human, rat, mouse, dog, cow (Zimin *et al.*, 2009; Boardman *et al.*, 2002; Strausberg *et al.*, 2002). Secondary structures of human are marked above the alignment. Red boxes indicate the cysteines difference among the species. (*) identical residues, (.) semi conserved residues, and (:) conserved residues. Gray boxes indicate KEN box motif. The alignment was done by ClustalW 2.1 program.

3 Thymidine kinase 1

Thymidine kinase 1 differs from other deoxyribonucleoside kinases regarding the amino acid sequence, cell cycle regulation, structure, substrate specificity and kinetics. This section will focus on some of these properties.

3.1 Molecular weights and enzyme kinetics

Results on the kinetics behavior and molecular weights of human TK1 vary greatly between different investigations, most likely due to a different source and purity of the enzyme. TK1 purified from acute myelocytic leukemia, and placenta reveals native molecular weights of 90 and 92 kDa, respectively, with apparent K_m values for dThd of 2.6 and 3 μM , respectively (Gan *et al.*, 1983; Lee & Cheng, 1976). In the absence of ATP the molecular weight of TK1, partially purified from lymphocytes, ranged from 70-75 kDa, while in the presence of 2 mM ATP the molecular weight increased 2-3 fold (170-200 kDa) (Munch-Petersen, 1984). It was shown that TK1 phosphorylated FdUrd better than dUrd with K_m values of 2.2 and 9 μM , respectively. AZT and FLT were also phosphorylated by TK1 but not D4T; the K_m values were 0.6 and 2.2 μM , respectively (Munch-Petersen *et al.*, 1991).

In 1993, further studies on the effect of ATP were carried out, and a transition from a dimer with 50 kDa to a tetramer with 100 kDa was observed (Munch-Petersen *et al.*, 1993). This tetramerization was also accompanied by a change in the kinetic behavior of the enzyme. The dimer showed hyperbolic kinetics with negative cooperativity and a $K_{0.5}$ value of 12-16 μM . However, the tetramer form had hyperbolic Michaelis-Menten kinetics with low K_m value (0.4-0.7 μM). Both dimer and tetramer forms had the same maximum velocity and hence, the catalytic efficiency of the tetramer was about 30-fold higher than the dimer (Munch-Petersen, 2009; Munch-Petersen *et al.*, 1995a; Jensen & Munch-Petersen, 1994; Munch-Petersen *et al.*, 1993).

The dimer-tetramer transition was not only dependent on the presence of ATP but also on the enzyme concentration (Munch-Petersen *et al.*, 1995a), since, oligomerization was seen with a high concentration of enzyme (0.2-20 mg/ml) in the absence of ATP (Munch-Petersen, 2009; Birringer *et al.*, 2006).

The K_m values for ATP were 30-50 μM and positive cooperativity was observed with Hill coefficients of 2.0-2.4 (Munch-Petersen, 1984). The K_m for the dimer form was 100-170 μM , and tetramer 10-20 μM (Munch-Petersen *et al.*, 1995a).

The end product dTTP is a feedback inhibitor for TK1. The IC_{50} values were 9-21 μM dTTP at varying concentrations of dThd. The IC_{50} values were 3-36 μM dTTP when different concentrations of ATP were used (Munch-Petersen, 1984). The inhibition kinetics of dTTP were similar for the tetramer and dimer forms with Hill coefficients of 2.5 for the tetramer and 2.7 for the dimers of TK1 (Munch-Petersen *et al.*, 1995b).

3.2 Cell cycle regulation

Unlike other deoxynucleoside kinases, TK1 activity fluctuates greatly during the cell cycle. The enzyme reaches its maximum level during S phase (about 15-fold compared to the minimum level in G1) in actively cycling cells. This increase does not correlate with TK mRNA levels, which increase only 3-fold as compared with G1 phase (Sherley & Kelly, 1988).

In serum stimulated quiescent cells and simian virus 40-infected cells, TK1 activity increased in parallel with increased TK1 mRNA levels (Coppock & Pardee, 1987; Stewart *et al.*, 1987). These findings suggest that the regulatory mechanisms for TK1 expression are controlled at the transcriptional and post-transcriptional level during the cell cycle.

The half life of the TK1 polypeptide during M phase is reduced from 40 h to less than 1 h, resulting in removal of the TK1 protein from newly divided cells (Sherley & Kelly, 1988). This finding indicates a specific degradation mechanism for TK1. Deletion of 40 amino acids at C-terminal region of human TK1 led to stabilization of the enzyme throughout all cell cycle phases. However, no alteration in cell cycle regulation was observed when only 10 C-terminal residues were removed (Zhu *et al.*, 2006; Kauffman & Kelly, 1991).

In 2004, a KEN box motif, which is conserved in chicken and mammalian TK1 sequences (Fig 2), located in The C-terminal region of the TK1 protein was discovered to have a key role in the mitotic degradation of TK1. Through interaction between the KEN box motif and Cdh1, an ubiquitin ligase a complex is formed leading to TK1 proteolysis. Cdh1 is one of the rates limiting factors, which means that the degradation process of TK1 is tightly controlled

in order to balance the dTTP pool and thus, regulate DNA synthesis (Ke & Chang, 2004).

These and other results describing phosphorylation of Ser residue in TK1 during the cell cycle (Li *et al.*, 2004; Chang *et al.*, 1998; Chang *et al.*, 1994) have led to the following hypothesis: in M phase cells, TK1 is hyperphosphorylated, which led to low affinity towards its natural substrate dThd. In S phase cells, TK1 is hypophosphorylated and has 10-fold higher affinity for dThd (Chang *et al.*, 1994). The residue that is phosphorylated during mitosis is Ser 13; however, a mutation of Ser 13 to alanine gave no alteration in the activity of TK1. The wild type TK1 may serve as a good substrate for Cdc2 kinase but not the mutant enzyme (Chang *et al.*, 1998).

Substitution of Ser 13 with Asp to mimic its phosphorylation gave a decrease in the catalytic activity and affinity toward dThd (Li *et al.*, 2004). Furthermore, it prevents the induction of tetramerization of TK1 by ATP. During mitosis, TK1 activity is down-regulated by phosphorylation of Ser 13 giving a reduction of tetramer and dimer ratio, resulting in low dTMP formation (Li *et al.*, 2004).

An overview illustration of this hypothesis concerning the protein expression and catalytic efficiency of TK1 during the cell cycle is presented in figure 3.

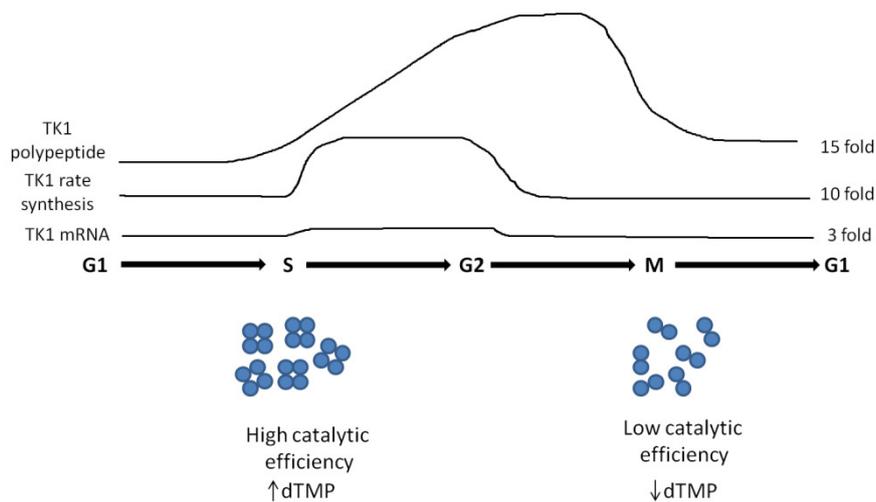


Figure 3. A schematic illustration of the fluctuation of thymidine kinase 1 expression during the cell cycle adapted from Skovgaard, 2009; Li *et al.*, 2004; Sherley & Kelly, 1988.

In G0/G1 cells, the level of TK1 protein is approximately 30-90 ng/ml and it increases up to 4000 ng/ml in S phase cells (Munch-Petersen *et al.*, 1993). This suggests that the dimer with low catalytic efficiency is the dominant form in G0/G1 regardless of the ATP concentration. However, the tetramer is the dominant form in S phase cell, which gives high catalytic capacity of TK1 (Munch-Petersen *et al.*, 1995a; Munch-Petersen *et al.*, 1995b; Munch-Petersen *et al.*, 1993).

In summary, TK1 protein concentrations, intracellular thymidine and ATP concentrations play important roles in the regulation of TK1 activity throughout the cell cycle. Disturbance in this regulation of TK1 may lead to dTTP pool imbalances.

dTTP can be synthesized by *salvage* and *de novo* pathways as described in the previous sections, and the dTTP pool size is 20-fold higher in S phase compared to G0 cells (Spyrou & Reichard, 1989). Down regulation of dTTP in mitosis, is carried out through APC/C ubiquitin ligase mediates degradation of both ribonucleotide reductase R2 subunit and TK1 (Ke & Chang, 2004; Chabes *et al.*, 2003). Loss of APC/C mediated proteolysis of TK1 and RNR R2, results in a significance elevation of the dTTP pool and hence, increased rate of mutations and altered cell growth as well as cell death (Ke *et al.*, 2005).

3.3 The structure of human TK1

In 2004, the first three-dimensional structures were solved for the TK1 type enzymes i.e. human and *Ureaplasma urealyticum* TK1 (Welin *et al.*, 2004). Shortly thereafter, similar structure of human TK1 was presented by Birringer *et al.* in 2005. The initial attempts to crystallize full length TK1 were unsuccessful, but truncated enzymes missing 40 residues in the C-terminal region were used in these studies (Birringer *et al.*, 2005; Welin *et al.*, 2004).

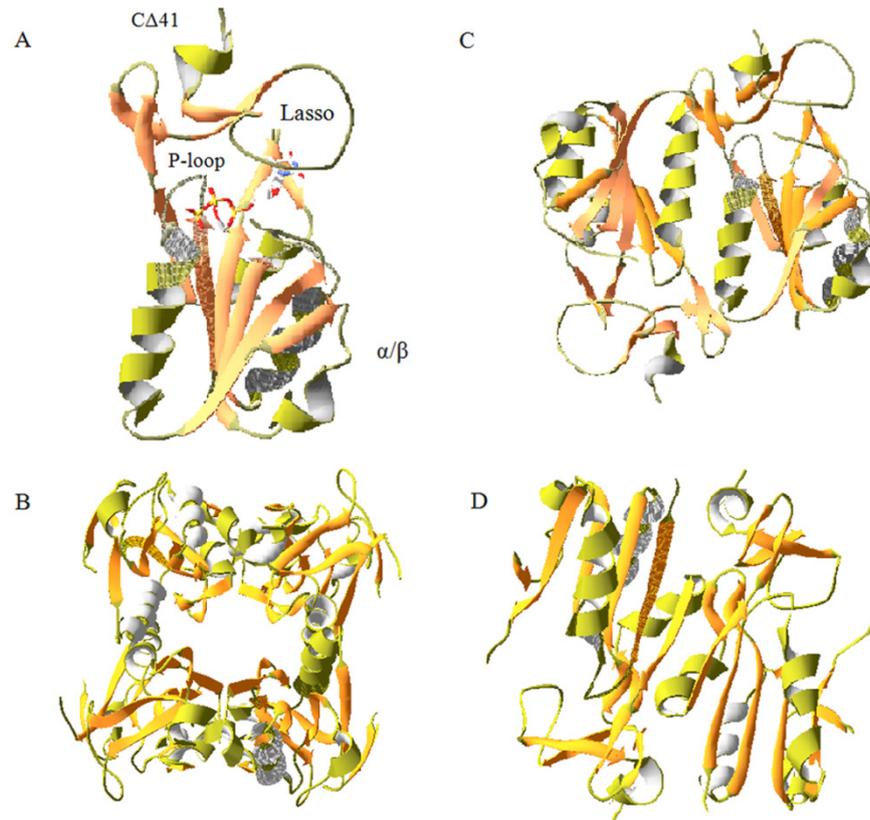
The truncated enzyme is a dimer in the absence of ATP and a tetramer in the presence of ATP similar to the wild type (Zhu *et al.*, 2006; Munch-Petersen *et al.*, 1995a). The enzyme was crystallized in complex with dTTP by Welin and coworkers (Welin *et al.*, 2004). The Swiss group did not include dTTP during crystallization, still dTTP was found in the crystals, most likely originating from the bacterial metabolism (Birringer *et al.*, 2005).

The structure of human TK1 is a tetramer; each subunit is composed of two domains, α/β domain and a lasso-like domain containing structural zinc. The active site is situated between the two domains (Fig 4).

The α/β domain composed of six parallel β - sheets surrounded by five α - helices, which are arranged as follow: on one side, there is a long α - helix and flexible loop, and on the other side, there are three short α - helices, which are

similar to the RecA family proteins (Birringer *et al.*, 2005; Welin *et al.*, 2004) (Fig. 4 C and D).

The tetramer has a central channel covered mainly by polar or charged amino acids. The dimer is formed by two different monomer-monomer interactions: one of these is formed from interaction between the long anti-parallel α -helix from two adjacent subunits, and the second dimer interaction is formed from connecting two anti-parallel β -sheets from two subunits via water molecules (Welin *et al.*, 2004) (Fig. 4 B).



*Figure 2. (A) Monomeric structure of truncated human TK1 with dTTP in the active site. (B) The tetramer structure of human TK1. (C) The dimer interaction between subunits A and B. (D) The dimer interaction between subunits A and C (Welin *et al.*, 2004).*

The zinc domain, from residues 150 to 191, forms part of the substrate binding site; it consists of two β -ribbons and a long lasso-like loop, and they are kept together by the zinc ion. This zinc ion is bound by four cysteines 153, 155, 186 and 188 (Fig. 5). Binding of the zinc to these cysteines is of great importance for the formation of the correct substrate binding site by stabilizing the flexible loop (Birringer *et al.*, 2005; Welin *et al.*, 2004). This region may be particularly sensitive to alterations in the redox environment, i.e. in cells and the blood.

The lasso loop from Leu166 to Lys180 forms a long flexible region that covers the substrate binding site in TK1 (Fig. 5). The domain is kept in its position by hydrogen bonds between the conserved Arg165-Tyr181 and several main chain atoms (Birringer *et al.*, 2005; Welin *et al.*, 2004).

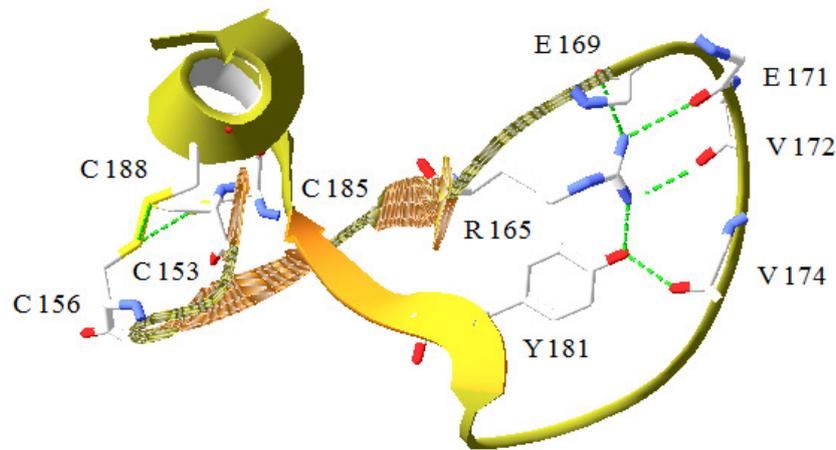


Figure 5. The lasso domain of thymidine kinase 1. Four cysteines involved in binding the zinc containing domain and the lasso loop is stabilized by hydrogen bonds (Welin *et al.*, 2004).

The substrate binding site, where the thymine base and the feedback inhibitor dTTP bind, is buried between the α/β domain and the lasso domain, while the phosphate binding site, which is exposed, is located in the P-loop of the α/β domain.

When an ATP analog and dThd are bound to TK1, the structures of *Thermotoga maritima*, *Bacillus anthracis*, and *Bacillus cereus* TK;s were very similar to the corresponding TK1-dTTP complexes. Furthermore, the quaternary structure of the bacterial TK;s changed upon binding of only ATP,

where the tetramer showed an open state (Segura-Pena *et al.*, 2007a; 2007b). However, the human TK1 revealed no electron density for an adenosine group when TK1 was crystallized in complex with ATP and thus, no open form of human TK1 has been described so far (Kosinska *et al.*, 2007; Segura-Pena *et al.*, 2007a; 2007b).

TK1 has a relatively small substrate binding site, which explains its narrow substrate specificity compare to other deoxyribonucleoside kinases (Welin *et al.*, 2004). In accordance with the structural studies of different TK;s, described above, small substitution at the 5-position of the base, like bromine, fluorine or ethyl had no effect on enzyme activity. However, large substitutions at this position such as 2- bromovinyl were not accepted as a substrate for human TK1(Eriksson *et al.*, 1991a).

Solving the structures of the human and microbial TK1 is the first step in the design of selective nucleoside analogs or inhibitors, which have low affinity to human TK1, but high affinity for bacterial TK;s (Kosinska *et al.*, 2007; El Omari *et al.*, 2006; Kosinska *et al.*, 2005). These results may lead to development of anti-microbial agents based on nucleoside or non-nucleosides, which is much needed because of the increased antibiotic resistance observed worldwide (Andersson & Hughes, 2012; van Ingen *et al.*, 2012).

3.4 The role of TK1 in DNA repair

DNA damaging agents such as radiation, hypoxia and chemotherapy, which result in loss of normal cell cycle checkpoints control. Subsequently, genomic instability and inappropriate DNA repair that can lead to cell death or transformation to malignancy (Hartwell & Kastan, 1994).

One of the mediators that are induced in response to DNA damage and cell cycle arrest is p53, a tumor suppressor gene (Smith *et al.*, 2003; Hartwell & Kastan, 1994). About 50% of human cancers have alteration or mutation in the p53 gene (Smith *et al.*, 2003). The effect of activation of p53 on cellular growth level and TK1 activity after DNA damage has been defined (Schwartz *et al.*, 2004; Rasey *et al.*, 2002). In p53 positive cultured cells exposed to different doses of radiation, the percentage of S phase as well as TK1 activity, and 3'- deoxy- 3'- flourothymidine (FLT) uptake decreased in parallel. However, in p53 deficient cell the TK1 activity and FLT uptake were high even when S phase percentage decreased (Schwartz *et al.*, 2004). Both p53 positive and negative cell lines show alterations in the cell cycle progression and are arrested in the G2 phase after radiation treatment (Schwartz *et al.*, 2004). Thus, a functional p53 is apparently needed for a normal

synchronization between TK1 expression and S phase percentage (Swartz et al 2004).

Further studies demonstrated that p53 participated in TK1 regulation during DNA damage (Chen *et al.*, 2010). It was reported that during DNA recovery, TK1 was drastically up-regulated in p53 deficient cells, which are arrested at G2 phase, but not in p53 positive cells. Furthermore, a translocation of a fraction TK1 to the nucleus was also observed (Chen *et al.*, 2010).

Accumulation of TK1 in G2 phase is most likely due to lack of mitotic degradation as judged by elevation 4.7-fold in TK1 expression in p21 deficient cells (Chen *et al.*, 2010).

The dTTP pool is expanded in p53 deficient cells, in parallel with an increased TK1 expression after DNA damage, suggesting that TK1 plays a role in DNA repair in p53 deficient cells, providing a better survival of cells. Furthermore, TK1 depletion during DNA recovery leads to cell death (Chen *et al.*, 2010).

3.5 Thymidine kinase 1 as a cell proliferation biomarker

The tight coupling between TK1 activity and the cell cycle described in the previous sections pointed to the usefulness of TK1 as a reporter for cell proliferation and hence, for malignancy. This section will describe the application of TK1 as a cell proliferation biomarker in both human and veterinary medicine.

3.5.1 Quantification of serum thymidine kinase 1

The first method that has been used to quantify TK1 activity in human patients with hematologic malignancy was based on phosphorylation of radiolabel thymidine to thymidine monophosphate with ATP as a phosphate donor (Ellims *et al.*, 1981). The procedure was performed using DE-81 paper discs and processed as previously described (Ellims & Van der Weyden, 1980).

In 1980, an optimized method was used to quantify TK1 activity of herpes simplex virus Type 1 and 2 using [¹²⁵I]-deoxyuridine as a substrate, and the procedure was also based on DE-81 paper discs, and the final product was counted using a gamma counter (Gronowitz & Kallander, 1980). Later, this assay was validated and is commercially available (Prolifigen® TK-REA, DiaSorin) to measure TK1 activity in humans with different malignancies such as Hodgkin's and non-Hodgkin's lymphoma, chronic and acute lymphocytic leukemia (Hallek *et al.*, 1999; Hallek *et al.*, 1992; Gronowitz *et al.*, 1983). The assay is based on conversion the [¹²⁵I]-deoxyuridine to [¹²⁵I]-deoxyuridine monophosphate by TK1 that present in the clinical specimens. The products

are coupled to a granulate matrix, and after several washes, the radioactivity is measured by a gamma counter, and the TK1 activity is expressed as U/L (Gronowitz *et al.*, 1984b).

Recently, an alternative non-radiometric assay was developed based on the thymidine analog AZT. The final product of AZT phosphorylation by TK1, AZTMP, is determined by a polyclonal antibody directed against AZTMP (Ohrvik *et al.*, 2004). The assay was further developed to a fully automated competitive chemiluminescence assay (Liaison TK assay, DiaSorin) (Von Euler *et al.*, 2009). Another ELISA assay, similar to the Liaison technique, is used for monitoring patients with hematologic malignancy. However, 5-Bromo-deoxyuridine is used as a substrate (DiviTum®) and subsequently, the product is incorporated into an oligonucleotide immobilized on the bottom of a microtiter plate; finally recombinant reverse transcriptase present in the DiviTum solution is added. The activity of TK1 is expressed as DiviTum unit per liter (Du/L). This assay is claimed to give 10-fold higher sensitivity compared to the TK-REA assay (http://biovica.com/?page_id=106 26 Jul. 2012).

Recently, different polyclonal and monoclonal antibodies against human TK1 have been produced (Konoplev *et al.*, 2010; O'Neill *et al.*, 2007; Wu *et al.*, 2003; Mao *et al.*, 2002; Zhang *et al.*, 2001; He *et al.*, 2000; He *et al.*, 1996).

An antibody raised against the 31 amino acid C-terminal part of human TK1 is the most efficient and commonly used for serological and immunohistochemical detection of TK1 in human malignancies such as hematologic malignancy, breast, lung and bladder cancers (Aufderklamm *et al.*, 2012; Topolcan & Holubec, 2008; Wu *et al.*, 2003; Mao *et al.*, 2002; Zhang *et al.*, 2001; He *et al.*, 2000; He *et al.*, 1996). An antibody against a 24 amino acid peptide present at the active site of the TK1 has also been used for antibody production and the same applications (Gasparri *et al.*, 2009).

An advanced technique to measure TK1 activity *in vivo* is positron emission tomography (PET), and it can reflect tumor proliferation rates of tumors in the intact animals or persons (Salskov *et al.*, 2007). The most used method is based on administration and monitoring of the thymidine analog 3'-deoxy-3'-[¹⁸F] fluorothymidine (FLT), which is phosphorylated by TK1 into FLT monophosphate, thereby trapped, accumulated and subsequently incorporated into the DNA of proliferating cells. The rate limiting step is in most case the TK1 catalyzed reaction, and this imaging method can directly measure the proliferation rate of tumor cells by following the positron emission after administration of the thymidine analog (Brockenbrough *et al.*, 2011; Salskov *et al.*, 2007; Rasey *et al.*, 2002).

3.5.2 The role of serum TK1 in human medicine

For many decades, elevation of serum thymidine kinase in humans has been observed to be associated with different types of tumors. This section will describe the role of TK1 as a biomarker for the presence of malignancy and its diagnostic and prognostic role in hematologic malignancy and solid tumor diseases.

The levels of serum TK1 in patients with hematologic malignancy

Level of serum TK1 is elevated in a number of hematologic malignancies such as Hodgkin's lymphoma (HL), non-Hodgkin's lymphoma (NHL), acute leukemia, chronic lymphocytic leukemia (CLL), and myelodysplastic syndrome (MDS).

In HL, the levels of STK1 in pre-treated patients showed correlation with clinical parameters such as histopathology and tumor stage (Eriksson *et al.*, 1985). High levels of TK1 were found in advanced stage of the disease and based on its levels; patients in stage IA and IIA could be divided into two groups with regard to disease-free survival (Eriksson *et al.*, 1985).

In patients with NHL and multiple myeloma, the level of TK1 correlated with clinical stage and grade of the disease (Poley *et al.*, 1997; Hallek *et al.*, 1992; Hallek *et al.*, 1988; Gronowitz *et al.*, 1983; Ellims *et al.*, 1981). STK1 levels provided prognostic information regarding response to therapy and predicted patient's overall survival (He *et al.*, 2010; Pan *et al.*, 2010; Hallek *et al.*, 1992; Hagberg *et al.*, 1984a). Transition from indolent to progressive disease could also be predicted in some cases by an increase in the level of TK 1, 6 months before symptoms appear (Hallek M *et al.*, 1990). Similar increases of TK1 levels in NHL patients were reported in a more recent study (TK Liaison) (Ohrvik *et al.*, 2004).

High STK1 has been reported in patients with acute leukemia (Archimbaud *et al.*, 1988; Hagberg *et al.*, 1984c). In a study carried out with children with acute leukemia (34 lymphocytic, 4 myeloblastic), TK1 levels were highly elevated at the time of diagnosis and declined in response to chemotherapy. High levels of STK1 were observed in patients with disease relapse one month before clinical symptoms appear (Votava *et al.*, 2007).

In CLL, TK1 provided valuable information in order to distinguish between smoldering and non-smoldering disease at an early stage. Moreover, patients with non-smoldering CLL can be divided into two groups based on the TK1 levels with different duration of progression-free survival (PFS). STK1 provided prognostic information independently of other known prognostic factors, i.e. patients with high TK1 (> 7.1 U/L) showed shorter PFS than patients with low TK1 level (\leq 7.1 U/L) (Hallek *et al.*, 1999). In 2001, among

188 patients with advanced CLL treated with fludarabine, high level of TK1 was found in 92% of the patients. That study showed that patients with low TK1 level (< 10 U/L) responded to treatment with fludarabine (83%) with a median survival rate of 65%, while only 45% of patients with high TK level (≥ 10 U/L) had a median survival rate of 22% (Di Raimondo *et al.*, 2001). Thus, this study demonstrated the usefulness of TK1 as a prognostic marker for both responses to therapy and duration of survival. Recent studies performed with the chemiluminescence assay (TK Liaison) also showed high prognostic capacity of STK1 in patients with CLL (Konoplev *et al.*, 2010). Similar results were found using the dot blot assay based on anti-TK1 antibodies. These results show that the concentration of TK1 protein in serum can also be used to assess the prognosis of patients with CLL (Konoplev *et al.*, 2010; Xu *et al.*, 2009).

The prognostic relevance of STK1 activity as a proliferation marker in MDS was evaluated in 1995. Patients with high TK1 level (> 38 U/L) had shorter survival time than those with low TK1 level (< 38 U/L). Furthermore, STK1 could predict transformation of MDS to acute myeloid leukemia (AML). Multivariate analysis showed that STK1 was the only efficient parameter that provides prognostic information for both survival and response to therapy (Musto *et al.*, 1995). Similar prognostic efficiency for STK1 measurements was observed in adult T-cell leukemia and AML patients (Sadamori *et al.*, 1995).

Over the past decades, TK 1 has repeatedly been demonstrated to have a diagnostic and prognostic role in hematologic malignancies. By monitoring TK1 levels in patients with these types of malignancies, could provide guidance in the selection of a better treatment regimen.

The levels of serum TK1 in patients with solid tumors

Several studies investigated the clinical applications of TK1 as a proliferation marker in patients with solid tumors such as breast, lung, and bladder, ovarian, gastrointestinal and prostatic cancers.

Breast cancer has been extensively studied, and the activities of cytosolic TK1 in tumor samples from about 1700 breast cancer patients were shown to give very good prognostic information concerning disease specific survival (DSS) and distant-relapse- free interval (DRI) (Broet *et al.*, 2001). In a later study, where the expression of TK1 was assessed by immunohistochemistry and compared with another proliferation marker e.g. PCNA (Mao *et al.*, 2002). The level of TK1 correlated to high stages and grades of the disease while PNCA did not (Mao *et al.*, 2002).

In 1996, He and coworkers developed polyclonal antibodies against the C-terminal region of TK1 that could be used for both serum and immunohistochemistry analyses of TK1 in normal and cancer patients (He *et al.*, 1996). Subsequently, development of similar antibodies using a 31 mer C-terminal peptide for immunization of chickens led to the establishment of a dot blot assay, which could be used as a marker for monitoring the response of therapy in breast cancer patients (He *et al.*, 2000). With the same procedure, increased concentrations of TK1 protein 3 months after operation were observed in patients developing distant and/or loco-regional relapse during the subsequent 5-year period (He *et al.*, 2006). The results were substantiated by immunohistochemistry studies, where a significant increase in TK1 expression was found in patients with high stage and grade of tumor disease. Higher numbers of positive patients were obtained when a combination of the proliferation marker Ki-67 and TK1 was used (He *et al.*, 2004).

High levels of STK1 protein were found in preoperative lung cancer patients with NSCLC, particularly at high stage of the disease, which declined significantly one month after surgery (Li *et al.*, 2005). Poor survival was reported in patients with high TK1 protein levels (Xu *et al.*, 2012).

The role of TK1 protein determinations as a proliferative marker and for monitoring treatment has been reported in bladder carcinoma (Zhang *et al.*, 2006), gastrointestinal cancer (Zou *et al.*, 2002), renal cell carcinoma (Luo *et al.*, 2009), cervical carcinoma (Fujiwaki *et al.*, 2001), ovarian cancer (Fujiwaki *et al.*, 2002), colorectal and prostate cancer (Aufderklamm *et al.*, 2012; O'Neill *et al.*, 2001).

In healthy screening of more than 20 000 apparently healthy persons since 2005, the level of TK1 was determined by the dot blot assay. Only 0.9% had levels of TK1 > 2 pM, which is more than 2 times the SD above the mean TK1 value of known healthy persons, 80% of the positive persons had proliferation related abnormalities in follow up examinations. These studies indicate that STK1 might be able to predict people at risk of developing a neoplastic disease at an early stage (Huang *et al.*, 2011; He *et al.*, 2010; Chen *et al.*, 2008).

PET imaging studies with Thd analogs, have been reported to provide valuable information regarding the survival, tumor aggressiveness and tumor response to therapy in a variety of human tumors, i.e. lung cancer, breast cancer, glioma, head and neck cancer, esophageal cancer, but also in some hematologic malignancies such as in lymphoma (Wu *et al.*, 2012; Brockenbrough *et al.*, 2011; Herrmann *et al.*, 2011a; Herrmann *et al.*, 2011b; Richard *et al.*, 2011; Wardak *et al.*, 2011; Buck *et al.*, 2007; Salskov *et al.*, 2007). However, PET imaging is still not a routine method in most clinical situations and particularly not in veterinary medicine.

3.5.3 The role of serum TK1 in veterinary medicine

The first study was done in 1997 by Nakamura *et al.* and reported high increases in plasma TK1 activities in dogs with lymphoma and leukemia compared with healthy dogs (Nakamura *et al.*, 1997).

Moreover, the level of TK1 declined after chemotherapy correlated with reduction in tumor mass. Two cases had disease relapse, and they showed increased TK1 levels (Nakamura *et al.*, 1997). LDH levels were significantly increased in dogs with lymphoma and leukemia, but they were neither as sensitive nor as specific as TK1 (Nakamura *et al.*, 1997).

Recent studies on dogs with malignant lymphoma (ML) revealed a similar prognostic capability of TK1, where high elevation of TK1 was observed in 55 dogs with malignant lymphoma compared to healthy dogs. Forty-four dogs were followed, and dogs with high TK1 level (> 30 U/L) had shorter overall median survival (1 month), whereas dogs with low TK1 level (< 30 U/L) had longer survival time (9 months). TK1 was also able to predict disease relapse at least 3 weeks before clinical signs appeared (Von Euler *et al.*, 2004).

Further studies by the same group with 213 dogs mainly with ML (2009; Von Euler *et al.*, 2006) demonstrated that the level of TK1 activity in dogs with lymphoma out of remission (7-66 U/L) was significantly higher than dogs in complete remission (0.5-7.0 U/L). Dogs with leukemia had the highest TK1 levels (30.7-263 U/L). Although dogs with leukemia out of remission did not reach the normal reference range, they had lower TK1 level compared to the initial TK1 levels. These studies demonstrate the prognostic capacity of TK1 for monitoring relapse and response to therapy in dogs with ML.

In 2011, a study on 73 dogs with lymphoma showed that only 47% of the cases had TK1 levels above the normal reference with no correlation to the stage of the disease. However, TK1 in dogs that had increased levels, were in the normal range during remission (Elliott *et al.*, 2011).

Some studies have been conducted in dogs with solid tumors with regard to serum TK1. Four dogs with different solid tumors were analyzed by Nakamura *et al.* (mammary tumor, mastocytoma malignant histiocytoma, and anal sac gland tumor) (Nakamura *et al.*, 1997) and fifty solid tumor cases were tested by Von Euler *et al.* (Von Euler *et al.*, 2009). Surprisingly, the levels of TK1 were lower than the normal reference range (6.0 U/L) (Nakamura *et al.*, 1997), and only 3 cases out of 50 had higher TK1 than healthy dogs (Von Euler *et al.*, 2009). The highest value was observed in the dog with advanced stage squamous cell carcinoma (16.2 U/L) and increased levels were found in two dogs with splenic hemangiosarcoma (Von Euler & Eriksson, 2011).

Recent studies on dogs with splenic hemangiosarcoma (HAS) demonstrated that 80% of dogs with HAS had increased TK1 levels, whereas only some dogs with other solid tumors such as osteosarcoma and transitional cell carcinoma had high TK1 level (6.7% and 11%, respectively) (Thamm *et al.*, 2011). The levels of TK1 were highly elevated in dogs with HAS or other malignancies, accompanied by hemoabdomen and a splenic mass, compared to healthy dogs or dogs with benign splenic disease. The conclusion from this study was that serum TK1 might be useful to distinguish between benign disease and HAS in dogs with hemoabdomen and a splenic mass (Thamm *et al.*, 2011).

3.6 Elevation of Thymidine kinase 1 in non-neoplastic diseases

STK 1 activities have also been found to be elevated in some viral infections such as cytomegalovirus (CMV), varicella-zoster virus (VZV), Epstein-Bar virus (EBV) and herpes simplex virus (HSV) (Kallander *et al.*, 1989; Gronowitz *et al.*, 1986; Larson *et al.*, 1986; Gronowitz *et al.*, 1984a; Kallander *et al.*, 1983; Gronowitz *et al.*, 1982; Kallander *et al.*, 1982; Mcguirt *et al.*, 1982).

TK1 was also transiently elevated in patients with morbilli and rubella virus infections (Gronowitz *et al.*, 1984b). Patients with acute hepatitis also had increased STK1, and the highest levels were observed in patients with hepatitis A (Tanaka *et al.*, 1993).

These studies demonstrated that increased STK activities could be due to a virus encoded TK;s (e.g. Herpes virus TK;s) (Gronowitz & Kallander, 1983) but also to host TK1 released as a result of damaged hepatic cells or inflammatory cells (e.g. in viral Hepatitis) (Tanaka *et al.*, 1993).

In some malignancy associated with viral infection such as nasopharyngeal carcinoma, EBV thymidine kinase protein was the most sensitive marker for this type of malignancy (Littler *et al.*, 1991).

In patients with vitamin B 12 deficiency leading to megaloblastic anemia (Hagberg *et al.*, 1984b; Ellims *et al.*, 1980; Hooton & Hoffbrand, 1976), the level of serum TK1 increased often drastically. The rise of STK1 in these patients; is most likely due to that the vitamin deficiency disrupting *de novo* thymidylate synthase and leakage of TK1 from destructed immature proliferating blood cells.

These conditions are relatively easy to identify, and thus false positive results during STK1 analysis in neoplastic disease can be avoided by proper medical examinations.

In veterinary medicine, the activities of STK1 in some dogs with bacterial infection have been described in the literature. i.e. among five dogs with

tumor-unrelated diseases, one case with canine pyometra and one case with tongue necrosis showed increased STK1 levels, while dogs with diabetes mellitus, seborrheic dermatitis, and upper respiratory infection had low TK1 levels (Nakamura *et al.*, 1997). Another study included 14 dogs with unspecified inflammatory diseases, and in this case, the levels of TK1 were all within the normal range (Von Euler *et al.*, 2009).

3.7 Dogs as a model for human diseases

The development of genomic techniques and release of whole genome sequence assemblies have led to a revolution in our understanding of many human and animal diseases, including cancer. The field of comparative oncology has also been advanced greatly because of that complete genome sequence for veterinary species have been generated. Many investigations demonstrate the usefulness of dogs as a comparative model for complex genetic disease, to a large extent as a consequence of mapping of the canine disease genes (Wilbe *et al.*, 2010; Breen, 2009).

Companion animals, primarily dogs and cats with spontaneously occurring cancers share many features with human malignancies, e.g. mammary tumors, osteosarcoma, lung cancer, soft tissue sarcoma and non-Hodgkin's lymphoma (Macewen, 1990). Furthermore, pets share to a large extent the same environmental carcinogenic factors as their owners. Increased incidences of certain types of tumors in veterinary medicine, such as lymphoma in dogs, provide a large population, shorter lifespan and the relatively large body size which are advantages that contribute to the value of dogs as models for human oncology (Vail & MacEwen, 2000; Macewen, 1990).

Although pets with natural occurring cancers are an underused entity presently, further investigations can provide excellent opportunities to study many aspects basic oncology, e.g. regarding etiology, cancer progression, pharmacodynamics, pharmacokinetics, biomarkers and response to treatment.

4 Aims of the thesis

The overall aim of this thesis was to provide a better molecular understanding of the serum form(s) of thymidine kinase 1, primarily in dogs with lymphoma. This information is essential in deciding how this cell proliferation biomarker can be used in cancer treatment and prevention.

Specific aims were as following:

- To define the difference in enzymatic and molecular properties of recombinant dog and human TK1, including characterization of the native molecular forms of cytosolic and serum TK1.
- To assess the possibility of use a [³H]-dThd phosphorylation assay to monitor and evaluate the prognosis of dogs with canine lymphoma.
- To evaluate the protein levels of serum TK1 in dogs with hematologic malignancies and solid tumors by immunoaffinity techniques. Furthermore, to try to assess the correlation between the protein level and serum TK activity.
- To determine the effect of reducing agents on the structure and activity of TK1.
- To determine whether TK1 activity can be used as a clinical biomarker in dogs with bacterial infection diseases such as canine pyometra.

5 Main results and discussion

Materials and methods that used in the present investigation are described in details, in papers I-IV and will not be described here.

5.1.1 Paper I: Quaternary structure of recombinant, cellular, and serum forms of thymidine kinase 1 from dogs and humans

In the first paper, the molecular forms of the cellular and serum thymidine kinase 1 protein is studied and compared with the pure recombinant dog as well as human enzymes. The enzyme preparations were mainly characterized by size exclusion chromatography and immunoaffinity methods.

Dog and human recombinant TK1 were cloned and expressed in an *E.coli* system as described in the methods section of paper I. The substrate specificities of both enzymes were determined, as well as their thermal stability and pH dependence. The velocity was determined at different concentrations of dThd and thymidine analog (AZT) in the presence of a constant concentration of ATP. When the data was fitted to the Michaelis-Menten equation, hyperbolic kinetics was observed with K_m values for dThd and AZT of 0.9 μM and 0.7 μM , respectively for dog TK1. In case of human TK1, the K_m was 0.8 μM for dThd and 0.65 μM for AZT, in agreement with what was previously reported (Munch-Petersen *et al.*, 1995b). Both enzymes showed a tendency of positive cooperativity with dThd and AZT with Hill coefficients of 1.3 and 1.4, respectively.

The specificity constant (k_{cat}/K_m) for AZT with dog TK1 was 3-fold higher than that with human TK1. This result may explain why the TK Liaison assay, which is based on AZT phosphorylation, gives so reliable results measuring TK1 activities in canine leukemia and lymphoma (2009; Von Euler *et al.*, 2006).

Dog recombinant TK1 was more stable, with only 16% of the specific activity reduced at 50 °C, while with human TK1, the reduction was about 40% compared to the specific activity at optimal condition (37 °C).

The subunit composition of recombinant enzyme was determined by size exclusion chromatography using Superose 12 column. Because of the total recovery of dog recombinant TK1 in the absence of the reducing agent was very low, it was difficult to determine the exact molecular weight of the dog recombinant TK1. However, when the enzyme was pre-treated with DTE, both the TK1 activity and protein were detected, and it was revealed that dog recombinant TK1 is in an oligomeric form.

Oligomer forms were found with human recombinant enzymes, in addition to tetramers and dimers, as observed by measuring both TK1 enzyme activities and protein levels. This profile differed from what was previously described by Munch-Petersen *et al.* 2009, where they observed that the recombinant enzyme occurred mainly as dimers in the absence of ATP and tetramers in the presence of ATP or at a high TK1 concentration in the absence of ATP (Munch-Petersen, 2009). When the enzyme was pre-treated with DTE, a shift toward a low molecular weight could be observed, in agreement with the previous study (Karlstrom *et al.*, 1990).

Serum samples from dog and human patients with acute lymphocytic leukemia were also characterized by gel filtration. The enzyme activity profile of both dog and human serum TK1 was similar, eluting as oligomers in agreement with the only previous study describing the molecular weight of human STK1 (Karlstrom *et al.*, 1990). By western blot analysis, TK1 protein bands were found in the same fractions. However, a large fraction of inactive tetramer and dimer forms of human TK1 protein was detected.

Dog sera treated with DTE showed a reduction in both the activity and protein levels of TK1, but with no shift in the apparent size of the peaks. However, by western blot, a minor peak with lower molecular weight was observed, and inactive dimer forms of TK1 protein were detected. Human STK1 showed minor changes in the MW profile after DTE treatment with a reduction in the total activity. However, no bands could be detected in the western blot, probably due to that the total recovery of the protein during this procedure was very low. These results suggested that disulfide bonds are involved in the large complex formation as suggested previously (Karlstrom *et al.*, 1990). Dog TK1 has three cysteines less than human TK1 in the primary sequences (Fig 2). This fact may be responsible for the difference in response to reducing agents. Further studies are needed to determine which of the cysteines are involved in the formation of the large complex forms of STK1.

5.1.2 Paper II: A sensitive and kinetically defined radiochemical assay for canine and human serum thymidine kinase 1 (TK1) to monitor canine malignant lymphoma

In this study, we determined if the TK1 activity in serum could serve as a proliferation biomarker in dogs and humans with different types of tumours using an optimized [³H]-deoxythymidine phosphorylation assay. The assay was compared with the commercially established TK1 enzyme assays, i.e. the TK-REA and TK Liaison.

The kinetic behavior and optimal conditions for determination of TK1 activity in serum from both healthy and diseased dogs and humans were evaluated.

Sera from normal dogs and dogs with lymphoma, having different TK1 activity levels were studied using different substrate concentrations. Data was fitted to the Michaelis-Menten equation, and the mean (\pm SD) V_{max} and K_m values for Thd were 42.1 ± 34 and 1.2 ± 0.4 , respectively, in diseased dogs, whereas in healthy dogs, they were 5.7 ± 2 and 2.5 ± 0.8 , respectively. In addition, the results with human sera from patients with hematologic malignancy or from healthy subjects showed a substrate inhibition pattern, i.e. at Thd concentration $\geq 50 \mu\text{M}$.

Inter-assay and intra-assay variations (CV%) as estimated from the variability of replicated samples were low, i.e. in the range of 2-5%.

The population in this study was composed of 100 dogs (34 healthy, 29 with lymphoma, 2 with leukemia, and 35 with solid tumors) and 37 humans (18 healthy, 9 with chronic lymphocytic leukemia, 10 with myelodysplastic syndrome). Some dogs with malignant lymphoma were monitored during chemotherapy courses.

Table 1. Summary of serum thymidine kinase 1 activities in dogs (pmol/min/ml)

	Mean	Median	Range
Healthy subjects (n=34)	0.99	0.8	0.5- 2.4
Hematologic tumor patients (n=31)	24.2	5.5	0.5-243
Solid tumor patients(n=35)	1.61	0.96	0.4-11.8

The [³H]-dThd phosphorylation assay had a good accuracy with both dogs and humans samples from patients with hematologic malignancy, with the area under the curve (the ability of the assay to discriminate between diseased and

non diseased subjects) of 88% and 94%, respectively. The assay also showed high sensitivity (probability that the test result will be positive when the disease is present, i.e. the true positive rate) of 94% for dogs and 89% for humans. The specificity (probability that the test result will be negative when the disease is not present, i.e. the true negative rate) was 67% for dogs and 74% for humans.

By using the Man-Whitney *U* test, which was applicable since the TK1 activity values were not normally distributed, the STK1 activity was significantly higher in sera from dogs with hematologic tumors compared to sera from healthy dogs; $P \leq 0.0001$.

Lymphoma dogs with low STK activities were in most cases in complete remission, whereas when the disease relapsed, high S-TK activity levels were observed.

It has been shown that TK1 activity values significantly correlated with the clinical stages of dog lymphoma (Von Euler *et al.*, 2004). Although, the sample size was limited in this study, there was a tendency of higher TK1 activity in stage IV and V cases (Fig. 6).

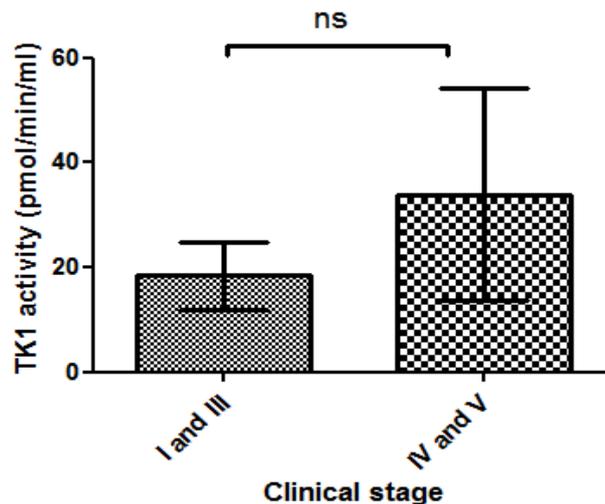


Figure 6. Correlation between serum TK1 activities (pmol/min/ml) and different clinical stages of malignant lymphoma; stage I and III (n= 18) and Stage IV and V (n= 12). Error bars represent SEM.

The [³H]-Thd-assay correlated very well with results obtained with TK-REA and TK Liaison ($r = 0.92$ and 0.96 , $P \leq 0.0001$, respectively). Hence, the

assay provided fast and a sensitive alternative way to measure TK1 activity, avoiding the drawbacks of the other methods, such as complexity and long experimental time in the TK-REA (Gronowitz *et al.*, 1983) or interference by other serum antibodies and dependence on a particular instrument as in the TK Liaison assay (Ohrvik *et al.*, 2004).

The effectiveness of measurements of STK1 activity in sera from patients with solid tumors was low; although four cases in this study showed increased TK1 activities compared to the cut-off value, i.e. synovial cell sarcoma, histiocytic sarcoma, thymoma, and mammary gland tumor with metastasis in the lung. It is not known why these four cases had high STK1 activities while the rest did not. However, this result is similar to what has been reported previously regarding the activity of STK1 in dogs with solid tumors (Von Euler *et al.*, 2009; Nakamura *et al.*, 1997).

Dog STK1 activity was checked at different time points in different assay conditions. The enzyme was stable at 4 °C for two weeks and at -20 °C for up to seven months and 24 hours at room temperature.

Human samples showed significantly increased STK1 activities in the hematologic tumor patients group. The highest STK1 values were observed in sera from patients with myelodysplastic syndrome. The levels of STK1 most likely give an indication of the severity of the disease and the risk to develop acute lymphocytic leukemia (Musto *et al.*, 1995).

5.1.3 Paper III: Determination of serum thymidine kinase 1 polypeptide and activity levels in dogs with hematologic malignancy and solid tumour

In this study, we wanted to determine the level of TK1 protein in serum from dogs with hematologic malignancies, solid tumors and healthy subjects, and to assess the correlation between the protein level and TK activity. The effect of addition of reducing agents was also studied.

The study comprised 14 samples from dogs with different malignancies, i.e. leukemia (n=2), lymphoma (n=4), solid tumor (n=8) as well as healthy dogs (n=7). The TK1 26 kDa subunit in sera from dogs with hematologic malignancies, solid tumors and healthy subjects were quantified in parallel with STK1 activity measurements. Because of the uncertainty of the recovery of the immunoaffinity methods for recombinant and STK1, the protein levels were determined by measuring the bands' intensities, and the values were expressed as an arbitrary unit (AU).

Based on the activity, there was a significant difference between hematologic malignancy compared to the healthy subjects, but not in the solid

tumor group (Sharif *et al.*, 2012b; Von Euler *et al.*, 2009; Nakamura *et al.*, 1997). Similar results were presented in this study, since there was no difference in STK1 activity between the solid tumour group and healthy subjects. Moreover, the mean value of the activity in hematologic malignancy was approximately 43-fold higher than those found in the solid tumor group. However, when the STK1 protein levels were measured, a clear elevation in STK1 protein in solid tumor and hematologic tumor relative to the healthy controls were observed. Interestingly, the mean value did not differ in the hematologic malignancy group compared to the solid tumor group (59 and 58 AU, respectively).

The relative specific activity of the STK1 protein was determined here for the first time, and the result showed about 22-fold higher specific activity in case of hematologic malignancies compared to those from patients with solid tumors ($P= 0.0004$). The level of protein in the healthy group was very low, so we could not determine the specific activity in this group; however, it must be several folds higher than that determined in the solid tumor group.

Furthermore, there was a significant correlation between STK1 activity and TK1 protein concentrations in solid tumor samples ($r = 0.85$, $P < 0.05$), in agreement with some earlier studies that was carried out in certain human tumours, e.g. leukemia, breast cancer, and gastric cancer (O'Neill *et al.*, 2007; He *et al.*, 2005; Zhang *et al.*, 2001). However, no correlation was observed in the hematologic malignancy group, which is most likely due to the small number of samples in this study. A recent study using immunohistochemistry and [^{18}F]-FLT PET studies demonstrated that there was no correlation between TK1 activity and TK1 protein expression in 25 lung cancer patients (Brockenbrough *et al.*, 2011).

The effect of the reducing agent DTE was in accordance with what was previously observed (Sharif *et al.*, 2012a), i.e. both the activity and protein levels decreased in response to reducing agents in most samples, except in one case with acute myelocytic leukemia, where both the STK1 activity and protein levels were elevated.

The preliminary results are shown here, with a limited number of cases, strongly suggest that there is a large fraction of inactive STK1 in solid tumors, and STK1 protein seems to be a more sensitive marker for the presence of solid tumor compare to STK1 activity.

5.1.4 Paper IV: Elevation of serum thymidine kinase 1 activity in a bacterial infection: Canine pyometra

In this study, we aimed to determine whether the level of TK1 is altered in non-neoplastic diseases such as canine pyometra. The relationship between TK1 levels and other biochemical markers as well as hematological parameters were also investigated.

The optimized assay that has been described in paper II was used in this study to measure STK1 activity in serum and plasma samples. The study included 94 female dogs (54 dogs with pyometra and 40 healthy dogs). Only apparently healthy dogs with hematologic and physical parameters within the normal range were included in the control group.

High correlation between serum and plasma TK1 activity was demonstrated ($R^2 = 0.96$, $P < 0.0001$); hence, both serum and plasma can be used to measure TK1 activity. STK1 activities revealed significant differences between the pyometra group and the control group $P < 0.05$ (Table 2). This result differs, to some extent, from what was previously reported by Von Euler *et al.* 2009, where healthy dogs and dogs with unspecified inflammatory diseases were analyzed by the TK-REA assay (Von Euler *et al.*, 2009). In that study, no significant differences were observed in the TK1 levels. In another study, only one case with pyometra was reported, and that case showed an elevation of TK1 activity (Nakamura *et al.*, 1997).

Table 2. Summary of thymidine kinase 1 activities (pmol/min/ml)

	Mean	Median	Range
Pyometra group (n=54)	4.0	1.4	0.5-38.6
Healthy group (n=40)	1.1	1.0	0.66-1.9

Plasma TK1 activities were analyzed after ovariohysterectomy in some cases and the data revealed a significant reduction in TK1 activity in 6 of 10 diseased dogs one day after surgery. This effect was especially observed in individuals that had high TK1 activities (> 1.0 pmol/min/ml), which strongly suggest that the increased TK1 levels are caused by the presence of the infected uterus. However, the direct mechanism(s) that led to high TK1 activities is still unknown.

Physical examinations, hematologic, and biochemical parameters, e.g. C-reactive protein (CRP) and prostaglandin $F_{2\alpha}$ metabolites (PGM) were analyzed in most of the pyometra cases. These results were used to determine

their correlation with the TK1 levels in the same individuals. No significant correlations were found with any of the mentioned parameters.

It has been shown previously that the activity of TK1 is significantly correlated with leukocytes in humans (Suehiro *et al.*, 1992). However, in this study, no general leukocytosis was observed, since only 54% of the pyometra cases showed high leukocyte counts. The fact that a large group of the dogs did not have increased leukocytes is most likely linked to the bone marrow suppression as a result of the severe infection. Furthermore, toxic neutrophils and monocyte numbers showed a trend towards a correlation with TK1 activity levels ($r_s=0.55$, $P=0.06$ and $r_s=0.3$, $P=0.053$, respectively). This result may indicate a direct link between increased TK1 activity and the severity of the infection as reflected by the elevated numbers of band neutrophils and/or the monocytosis. If this is due to the leakage of TK1 from destruction of infected cells or highly proliferating inflammatory cells remain to be determined.

6 Conclusions and future perspectives

Overall, the results of this thesis contribute to a better understanding of the role of serum thymidine kinase 1 as a cell proliferation biomarker in veterinary medicine. More precise conclusions are as following:

- Serum thymidine kinase 1 was found as active oligomers with a molecular weight of > 700 kDa and inactive dimer and tetramer complexes as judged by gel filtration analysis and immunochemical methods. Intra and inter molecular disulfide bonds most likely participate in the formation of the different complexes of serum thymidine kinase 1.
- An optimized [³H]-Thd phosphorylation assay was sufficiently sensitive for measuring serum thymidine kinase 1 in canine hematologic malignancies to be of clinical value. The assay showed a significant correlation with the commercially available TK1 assays, e.g. TK-REA and TK Liaison. The assay was not sensitive enough for routine use in solid tumor diseases.
- A 26 kDa polypeptide was observed in sera from dogs with different tumors, i.e. both hematologic and solid tumor diseases, using the anti-dog TK1 antibody based method. Furthermore, serum thymidine kinase 1 protein concentrations appear to be effective in distinguishing between healthy dogs and dogs with malignancy, including solid tumors in contrast to serum TK1 activity measurements.
- Serum thymidine kinase 1 was significantly elevated in 41% of dogs with pyometra caused by bacterial infection.

The findings in this thesis increase our knowledge about the role of TK1 in both canine and human medicine, which can be of great help in further comparative studies. The short life span of pet animals gives an advantage in studies of the progression of many diseases, as well as the effectiveness of therapy. Moreover, a health screening test may be designed in dogs where the values of diagnostic tests, e.g. serum TK1 determinations, as early disease markers can be evaluated much more effectively and at lower costs than in humans.

The new results presented here concerning the determination of STK1 activity, and protein levels in cells and serum in healthy and diseased individuals encourage the use of both these parameters in veterinary and human medicine.

As presented in this investigation, low specific STK1 activity in the solid tumor group may or may not mirror the proliferation rate of this type of tumors. Hence, the availability of another parameter, i.e. the STK1 protein concentration may overcome the drawback of the other TK1 assays.

The difference in both activity and protein level of STK1 between hematologic malignancy and solid tumor might be related to the abnormal structure and function of tumor blood vessels, resulting in abnormal tumor blood flow, increased blood pressure and hypoxia. These conditions most likely could change the structure of STK1, e.g. to inactive dimers (Munch-Petersen, 2009; Carmeliet & Jain, 2000). In contrast, in the hematologic malignancy where TK1 is directly released into the blood, oligomer complexes with high TK1 activity are found (Sharif *et al.*, 2012a; Karlstrom *et al.*, 1990). The mechanism behind the release of TK1 from solid tumors is not known, but is most likely related to tumour cell's destruction (Luo *et al.*, 2009). The abnormal blood and lymphatic vessels lead to increased intra-tumor blood pressure, as well as high blood vessel's permeability, which most likely are responsible for the release of inactive TK1 from tumor cells (Jain *et al.*, 2007; Carmeliet & Jain, 2000).

Fluctuating levels of TK1 in response to chemotherapy provide a measure of the effectiveness of therapy and thus, an opportunity to choose the best treatment course for the patients. Furthermore, STK1 activity and protein determinations are good prognostic tools giving information about disease relapse, most likely improving rescue treatment.

Elevation of STK1 activity in certain bacterial diseases must be considered as an alternative during tumor diagnostic procedures. However, larger studies are needed in order to confirm and explain the role of TK1 in bacterial infections.

The results presented in this thesis provide a starting point for many new studies and some of the most urgent and obvious ones are suggested below.

Future perspective and remaining investigations needed:

- A mutational analysis of the cysteine residues in dog and human TK1;s to determine which cysteine residues that participated in disulfide cross linking and TK1 oligomer formation.
- Develop an ELISA to determine the dog TK1 protein levels in blood, based on the anti-dog TK1 antibodies presented in this investigation.
- Determine the levels of serum TK1 in a large group of canine pyometra cases, which sample at an early stages of the disease, and assess the correlation with other disease markers including those for endotoxemia to understand the mechanism responsible for increasing serum TK1 activities. Furthermore, evaluate the level of TK1 in other bacterial infection diseases in different animal species such as cat, cattle, calf and horse.

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إلى والدي و والدي الحبيبين من الصعب أن أجد الكلمات التي تعبر عما في داخلي من حب و تقدير
لجهودكما و تضحيتكما لكي أصل إلى ما أنا عليه الآن. شكراً على كل ما قدمتماه لي و بارك الله فيكما و
رزقني بركما و أطال الله عمركما سنين طويلة. أحبكما ♥
ليلي، نادية، حليلة، بشير، شهيرة، مروة، محمد و غادة شكراً على الدعاء و الدعم و الحب الذي قدمتموه
لي طيلة هذه المدة.
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إلى عائلة نويجي، شكراً على كل ما قدمتموه لي من حب و دعم.

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