# Biochemical Markers and Genetic Risk Factors in Canine Tumors

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Cover: Signe and Kajsa two healthy English Springer Spaniel female dogs, four
and nine years old, respectively.
(photo: Ing-Britt Henriksen)

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Abstract

One out of four dogs will develop cancer before the age of 10 years. Many of them will succumb to the disease. Risk factor analysis, tumor prevention, and ways to prognosticate already existing tumors in the individual patient, are important. Canine mammary tumors (CMT) are the most common type of tumor in intact female dogs and constitute about half of all tumors in female dogs. About half of the CMT are malignant. The origin of CMT is considered to be complex, with tumor development likely influenced by both genetic and environmental factors. In Sweden 36% of English Springer spaniels (ESS) are diagnosed with CMT, suggesting a genetic influence in this breed. Several risk factors predisposing to CMT have been identified, but the majority of inherited risk factors remain unknown.

The aim of the research described in this thesis was to study biomarkers, epidemiology and genetic risk factors in canine tumors with the main focus in canine mammary tumors, using a population of a breed at high-risk for this disease in Sweden. In the first study, a study of a serum biomarker in canine tumors, serum thymidine kinase 1 (TK1) –activity in canine malignant lymphoma, leukemia and solid tumors using the standard TK REA and non-radiometric assays was performed. Serum TK1 was proven to be highly effective in diagnosing, prognosticating, and monitoring dogs with hematological neoplasias (lymphoma, leukemia), but the assay was ineffective in detecting TK1 activity in solid tumors such as CMT. Clinical and histological characteristics of CMT were described in a population of ESS dogs in Sweden. The reproductive status was shown as an important risk factor for MT development and age of onset and histological subtype affected the survival time after diagnosis of MT-affected dogs. Genetic risk factors for CMT were further investigated in the following studies. A candidate gene association study in CMT showed two human breast cancer genes, *BRCA1* and *BRCA2* that were associated with CMT in ESS dogs. The potential involvement of the major histocompatibility complex (MHC) class II in CMT development was studied and the results indicated diversity of MHC class II haplotypes and identified a haplotype that protected against MT development in ESS dogs.

In summary, this thesis provides new information about risk factors for CMT development. The identification of genetic risk factors is critical to improvements in prevention, diagnosis and treatment of these tumors.

*Keywords:* Thymidine kinase 1, tumor marker, breast cancer, mammary neoplasia, dog, MHC class II, DLA, candidate gene, comparative oncology, *BRCA1*, *BRCA2*

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*To Annika and my whole beloved family*

"*I know nothing except the fact of my ignorance*"
Socrates

# Contents

# List of Publications

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

I   Von Euler H, Rivera P, Aronsson A, Bengtsson C, Hansson L and Eriksson S. Monitoring therapy in canine malignant lymphoma and leukaemia with serum thymidine kinase 1 activity – evaluation of a new, fully automated non–radiometric assay. *International Journal of Oncology* 34: 505–510, 2008.

II  Rivera P, Fall T, Melin M, Lindblad–Toh K, Häggström J and von Euler H. Canine mammary tumors in a population of English Springer spaniels in Sweden. (Manuscript submitted for publication).

III  Rivera P, Melin M, Biagi T, Fall T, Häggström J, Lindblad–Toh K and von Euler H. Mammary tumor development in dogs is associated with *BRCA1* and *BRCA2*. *Cancer Research* 69: 8770-8774, 2009.

IV  Rivera P, Melin M, Wilbe M, Häggström J, Lindblad–Toh K, Andersson G and von Euler H. Identification of a rare dog leukocyte antigen class II haplotype as a protective factor against canine mammary tumors. (Manuscript submitted for publication).

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# Abbreviations

| | |
|---|---|
| BRCA1 | Tumor suppressor gene (breast cancer 1 susceptibility protein) |
| BRCA2 | Tumor suppressor gene (breast cancer 2 susceptibility protein) |
| CMT | Canine mammary tumor(s) |
| COX-2 | Enzyme cyclooxygenase-2 |
| DLA | Dog leukocyte antigen |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| ESS | English springer spaniel |
| HLA | Human leukocyte antigen |
| LEUK | Leukemia |
| LUPA | A research project of the European commission |
| MHC | Major histocompatibility complex |
| ML | Malignant lymphoma |
| MT | Mammary tumor(s) |
| OR | Odds ratio |
| PCR | Polymerase chain reaction |
| REA | Radio enzyme assay |
| SKC | Swedish kennel club |
| SNP | Single nucleotide polymorphism |
| TK1 | Thymidine kinase 1 |
| TNM | TNM system (tumor size, lymph nodes status and metastasis) |
| WHO | World Health Organization |

# 1  Introduction

The problem of cancer in dogs (*Canis familiaris*) is a serious challenge that veterinarians frequently face. It is estimated that one of four dogs greater than 2 years of age dies of cancer, and certain popular breeds are overrepresented in terms of cancer incidence and mortality (Olson, 2007; Khanna *et al.*, 2006). The prevalence of cancer in dogs has increased in recent years.

Advances in the care of animals have allowed dogs to live longer because of better nutrition, vaccination for common infectious diseases, leash laws that limit automobile deaths, and the availability of more sophisticated diagnostics and treatments for many ailments previously considered to be life-threatening. This improved general health of pets has resulted in an increase in age-related diseases including cancer (Paoloni & Khanna, 2007).

Companion dogs develop tumors spontaneously, are relatively inbred and immunologically competent, and share their living environment with humans. Spontaneous tumors in companion animals represent a valuable and underutilized resource in cancer research. The complex interaction between tumor and host microenvironment associated with tumor development and progression may therefore be effectively captured in these natural models, and as such, provide unique insights into tumor biology. Mammary tumors are one of the most common types of neoplasia affecting intact female dogs (Egenvall *et al.*, 2005; Moe, 2001; Moulton *et al.*, 1986; Priester & Mantel, 1971; Dorn *et al.*, 1968b; Fidler & Brodey, 1967). Mammary tumors (MT) constitute about half of all tumors in intact female dogs, and the proportion of malignant tumors is around 50% (Gilbertson *et al.*, 1983; Moulton *et al.*, 1970). Previous studies have identified many similarities between canine mammary tumors (CMT) and breast cancer in women, in terms of epidemiology, biology, dietary risk factors, and clinical behavior, as well as hormonal dependence (Sorenmo, 2003; Frese, 1985; Chrisp & Spangler,

1980). It has been suggested that the origin of CMT is multifactorial and depends on an interaction between multiple major and minor genetic risk factors and environmental factors. Several risk factors predisposing to CMT have been identified, but the majority of inherited risk factors remain unknown. The overall aim and main focus of the research described in this thesis was to study biomarkers, epidemiology and genetic risk factors in canine tumors with the main focus in canine mammary tumors (CMT) using a population of a breed at high–risk for this disease in Sweden. Common concepts in the field of CMT are summarized in chapter 2. The subsequent chapters (3–7) describe and discuss the four studies included in the thesis.

# 2 Canine mammary tumors

## 2.1 Incidence of canine mammary tumors in Sweden

Mammary tumors are the most common form of neoplasia affecting female dogs (Bonnett *et al.*, 2005). Breast cancer is often familiar in humans, but a similar hereditary pattern has not been described for MT in dogs, although breed predilections have been reported (Egenvall *et al.*, 2005; Cotran, 1994; Dorn *et al.*, 1968b). High-risk breeds vary, depending on study and geographic location. Several spaniel breeds, the Doberman, the German shepherd and the Boxer are predisposed to MT in the Swedish dog population (Egenvall *et al.*, 2005). The ESS breed has the second highest incidence of MT among all dog breeds in Sweden, with 319 dogs per 10,000 years at risk, which represents a hazard ratio of 2.7 compared to the general dog population (Egenvall *et al.*, 2005).

The onset of MT is believed to be influenced by age, hormones, and genetic predisposition. MT are rarely seen in young dogs (<3 years of age) (Egenvall *et al.*, 2005); however, benign cystic hyperplasia can be seen in dogs, involving all mammary glands. Most MT arise in middle-aged intact females, with a peak incidence between 6 and 10 years of age (Egenvall *et al.*, 2005). The median age of occurrence of CMT is reported to be 10-11 years; however, some breeds develop MT at a younger age (Moe, 2001; Boldizsar *et al.*, 1992; Priester, 1979). Studies of age-specific incidence rates have found a higher incidence in old, than in young, bitches (Dorn *et al.*, 1968a). The ESS has been shown to have a relatively low median age of onset at 6.9 years of age in the Swedish dog population aged 3-10 years (Egenvall *et al.*, 2005). MT in males is rare (<1% of all MT occur in males), and female dogs are 62 times more likely to develop MT than male dogs (Saba *et al.*, 2007).

The life-time incidence of MT is reported to be 0.5% in female dogs spayed before their first estrus cycle, but increases to 8% or 26% if spayed after the first or second estrus, respectively (Schneider *et al.*, 1969). If the dog is spayed later than after the second estrus cycle, the risk for malignant MT is earlier reported to be the same as in intact bitches, although the risk for benign MT seems to be reduced by ovariohysterectomy (OHE) even at a later age (Misdorp, 1991).

## 2.2 Pathogenesis of canine mammary tumors

### 2.2.1 Endocrine factors

The development of both canine and human mammary tumors is clearly hormone dependent (Cotran, 1994; Rutteman, 1990; Schneider *et al.*, 1969). The duration of exposure to sex hormones early in life influences the overall mammary tumor risk. Although earlier disputed, there is recent evidence for the importance of *timing* of spaying on survival time on dogs with mammary tumors after surgery. In a study by Sorenmo *et al.*, dogs that were spayed <2 years before MT surgery had a significantly longer overall survival time compared to the group of intact dogs and dogs that were spayed >2 years before MT treatment. This result is, however, contradicted by other studies showing no clinical benefit with OHE at (or in proximity to) mastectomy (Sorenmo *et al.*, 2000; Morris *et al.*, 1998). The correlation between OHE and protection against developing MT indicates priming of the mammary tissue under hormonal influence.

Prolonged, dose-related exposure to synthetic progestins has been shown to induce proliferation of mammary epithelial cells, potentially leading to genetic errors that may subsequently result in the development of MT (benign and malignant) (Stovring *et al.*, 1997; Misdorp, 1991). Administration of diethylstilbestrol is associated with ovarian tumor development, but not mammary tumors (Jabara, 1962). Prevention of heat by hormone administration increases the risk for mammary tumors (Rutteman, 1992). The risk for malignant tumors is especially increased if estrogen-gestagen-combinations in high doses are used (Stovring *et al.*, 1997; Rutteman, 1992; Misdorp, 1991). A protective effect of early pregnancy, well-known in humans (Costarelli & Yiannakouris, 2010; Milne *et al.*, 2010), has not been demonstrated in dogs (Schneider *et al.*, 1969).

The exact mechanisms and interactions at the cellular level that lead to the development of malignant mammary tumors are unknown. Hormonal therapy (anti-estrogen drugs such as tamoxifen) has been shown to delay the onset of metastatic disease in humans with estrogen receptor-positive

tumors. The presence of estrogen, progesterone, and prolactin receptors on canine mammary tissue and tumors has been evaluated (Rutteman & Misdorp, 1993). About 50% of malignant primary tumors were positive for these receptors, and they were infrequently detected on metastatic lesions.

### 2.2.2 Genetic factors

Although hormonal influences are clearly important in MT genesis, the particularly high disease incidence (Egenvall *et al.*, 2005; Rutteman & Misdorp, 1989) reported in certain breeds suggests a significant genetic component to the disease that is inherited. It is widely accepted that in addition to germ-line mutations, the malignant transformation requires the accumulation of somatic genetic alterations, or lesions. The role of acquired alterations in oncogenes and tumor suppressor genes is widely recognized in tumor formation.

Breast cancer is often familial in humans, but a similar hereditary pattern has not been described for CMT, although breed predilections have been reported (Egenvall *et al.*, 2005; Cotran, 1994; Dorn *et al.*, 1968b). Women who have inherited mutations in the *BRCA1* or *BRCA2 (BRCA1/2)* genes have substantially increased risk of breast cancer, with a lifetime risk of 56-84% (Antoniou *et al.*, 2006; King *et al.*, 2003; Ford *et al.*, 1998; Struewing *et al.*, 1997). A majority of the *BRCA1/2* mutations reported cause protein truncation through indels, nonsense mutations, splice variants, or rearrangements (Casilli *et al.*, 2006; Walsh *et al.*, 2006; Couch & Weber, 1996; Tavtigian *et al.*, 1996). A large number of sequence variants with unknown impact on the phenotype have also been detected in *BRCA1/2,* and several studies have tried to determine their clinical significance (Hofstra *et al.*, 2008; Easton *et al.*, 2007).

Several other genes are also known to confer increased risk for breast cancer in humans (Walsh & King, 2007). The liability for breast cancer is currently believed to be a polygenic trait, where liability is conferred by a large number of loci, each contributing with a small effect on breast cancer risk (Pharoah *et al.*, 2004). Four genes, *FGFR2, LSP1, MAP3K1,* and *TOX3* were recently found to be associated with a mild increase in risk of breast cancer in humans in a genome-wide association study (Easton *et al.*, 2007). *RCAS1* and *TP53* have been reported to be associated with many types of cancer, including breast cancer (Rousseau *et al.*, 2002). Recent studies in CMT found that 3 of 10 (Chu *et al.*, 1998) and 6 of 40 (Veldhoen *et al.*, 1999) primary malignant tumors contained *p53* mutations, with one dog carrying a germ-line mutation (Veldhoen *et al.*, 1999; Chu *et al.*, 1998). In another study, 17% of 69 canine mammary carcinomas assayed showed a

*p53* gene mutation, and multivariate analysis indicated that this mutation conferred an increased risk of recurrence and death from mammary tumor (Wakui *et al.*, 2001).

*ERBB2* has been shown to have altered expression in human breast cancer and a deletion in the *CHEK2* gene has been reported as associated with a 2-3 fold increased risk of breast cancer (Meijers-Heijboer *et al.*, 2003; Meijers-Heijboer *et al.*, 2002).

Evidence suggests that the enzyme cyclooxygenase 2 (COX-2) also known as the prostaglandin H synthase plays an important role in mammary tumor initiation and progression in human patients (Soslow *et al.*, 2000). This enzyme COX-2 catalyses the rate-limiting step in prostanoid biosynthesis (Hwang *et al.*, 1998). In the dog, there is evidence of a strong relationship between COX-2 expression ("up-regulation") and the malignancy of certain types of cancer (bladder, skin, intestinal, mammary and nasal)(Kleiter *et al.*, 2004; Dore *et al.*, 2003; McEntee *et al.*, 2002; Pestili de Almeida *et al.*, 2001; Khan *et al.*, 2000). Cyclooxygenase 2 is usually absent from normal cells, but can be inducible by growth factors, inflammatory reactions, tumor promoters, and several oncogenes. Induction of COX-2 enzymes, thought to be involved in human breast cancer carcinogenesis, also has been evaluated in normal, benign, and malignant canine mammary tissue. Dore et al., found that COX-2 was not expressed in normal tissues, but showed increased expression in benign (24%) and malignant (56%) tumors, indicating a possible role in tumorigenesis (Dore *et al.*, 2003). An association also has been shown between COX-2 expression levels and CMT histological subtype (Heller *et al.*, 2005). Recent studies in CMT suggest a similar role for COX-2 in the malignant phenotype (Queiroga *et al.*, 2005) and demonstrate a strong link between the level of COX-2 expression and the prognosis of CMT (Queiroga *et al.*, 2009): the higher the expression, the poorer the prognosis.

### 2.2.3 Tumor immunosurveillance

There is evidence suggesting that the immune system has a major task in controlling major histocompatibility complex (MHC) class II-dependent immune responses directed towards tumor cells. Thus, MHC class II molecules have a critical role in tumor surveillance and can play a protective role in tumorigenesis (Ghaderi *et al.*, 2001; Boon *et al.*, 1994). The immunogenicity of a tumor antigen depends on the efficiency of the individual´s MHC class II molecules to present tumor antigens to T-cells. Consequently, tumor development will be influenced by the individual´s MHC class II type and which tumor antigens that are expressed. Considering the importance of immune surveillance during tumorigenesis

(Ferguson *et al.*, 2002; Igney & Krammer, 2002), some individuals who inherit specific alleles or haplotypes of the highly polymorphic human leukocyte antigen (HLA) genes may be predisposed to or may resist specific types of cancers (Wu *et al.*, 2002; Sastre-Garau *et al.*, 1996; Apple *et al.*, 1994; Bidwell *et al.*, 1992; Pellegris *et al.*, 1980).

In dogs, the major histocompatibility complex is referred to as the DLA system and is known to contain class I, II and III genes as well as a large number of other genes, many which are involved in immune regulation.

A number of studies have been published on the role of MHC class II in surveillance against human breast cancer (Baccar Harrath *et al.*, 2006; Biswal *et al.*, 1998; Abrahamova & Majsky, 1988; Bouillenne & Deneufbourg, 1979; Hammond *et al.*, 1979; Subira *et al.*, 1979) and for HLA class II, there is evidence for different alleles conferring both increased and reduced risk and the specific alleles vary with ethnicity (Ghaderi *et al.*, 2001; Chaudhuri *et al.*, 2000; Casoli *et al.*, 1994). Less is known of the possible involvement of DLA in tumor development in dogs.

### 2.2.4 Nutritional factors

Experiments in rodents and epidemiological studies in humans have shown that a high-fat diet, as well as obesity, increased the risk of mammary cancer. A study in pet dogs in the United States found that among spayed dogs, the risk of developing mammary cancer was reduced if the dogs were thin at 9 to 12 months of age OR (0.04) (Sonnenschein *et al.*, 1991). A study in Spain made a similar observation, that obesity at 1 year of age was a risk factor for the development of benign and malignant tumors, without consideration of ovariectomy (Perez Alenza *et al.*, 1998). Somewhat surprisingly, the intake of homemade meals (i.e., red meat) compared with commercial foods also was related to an increased risk. Nutritional factors, therefore, may play a role in CMT development (Perez Alenza *et al.*, 2000).

## 2.3  Presentation, clinical signs, and staging

### 2.3.1 Clinical manifestations

The dog generally has five pairs of mammary glands spread along the ventral aspect of the thorax and abdomen. The two cranial pairs are termed thoracic, the next two abdominal, and the most caudal are inguinal glands. The blood supply originates from the external pudendal arteries and the lateral and internal thoracic arteries. It is generally understood that lymphatic drainage from the thoracic and cranial abdominal glands is to the axillary and sternal lymph nodes, while drainage from the inguinal and caudal abdominal

is to the superficial inguinal nodes. However, the pathways of lymphatic drainage are erratic and some lymph may cross the midline. The fifth or inguinal neoplastic mammary gland usually drains into the ipsilateral superficial inguinal lymph nodes, but rarely does it also drain into the ipsilateral popliteal lymph node and into a lymphatic plexus at the medial aspect of the ipsilateral thigh (Patsikas *et al.*, 2006).

In the non-pregnant, non-lactating bitch the most commonly encountered abnormality of the mammary gland is neoplasia. Dogs with MT may present clinically with solitary or multiple nodules or masses within the mammary gland, developing simultaneously or subsequently. In the case of multiple nodules, each one must be treated individually. Any of the five pairs of glands can develop one or more benign or malignant tumors. The caudal mammary glands are said to be more frequently affected than the cranial ones, probably due to the greater volume of mammary tissue in these glands. Many dogs present with larger nodules. Usually, these nodules are firm on palpation, well circumscribed, and non-painful; they may appear and remain static or grow rapidly. Clinical signs of malignancy include rapid growth, non-circumscribed invasive growth, fixation to skin and/or underlying tissues, and the presence of ulceration. The presence of one or more of these signs is associated with an increased risk for malignancy, but malignancy is not excluded by their absence. It is not possible to differentiate benign from malignant CMT by physical examination alone.

Carcinoma that metastasizes to the inguinal lymph nodes may enter the pudendal lymphatic vessels and spread to the internal iliac nodes. Metastasis from the internal iliac nodes may be palpable and may cause pressure on and compression of the colon. The other common metastatic sites include the lungs, kidneys, and less frequently, bones (Misdorp & Hart, 1979a; Misdorp & Hart, 1979b).

*Figure 1.* Clinical presentation of a simple canine mammary carcinoma before surgical treatment (Courtesy of Dr. von Euler).

Diagnosing of CMT in dogs is relatively straightforward, since it can be made presumptively using a good clinical examination, visual inspection, and palpation by a veterinarian in most cases, but care should be taken to differentiate mammary hypertrophy and mastitis. However, histopathology is needed to determine grade of malignancy, which in turn influences the prognosis. In a study by Hellmén et al., it was shown that the disease entity "MT" is fairly accurately used as a clinical diagnosis in Sweden. Out of 202 histologically and DNA-content-investigated tumors, only nine (4%) were considered as non-MT (four other neoplasias, and five mammary dysplasias) (Hellmen *et al.*, 1993).

### 2.3.2 TNM classification and clinical staging

To stage solitary tumors, we use the *TNM* (tumor, node, and metastasis) staging system. In this system, the body is considered as three anatomical compartments that all have the potential to be invaded by neoplastic cells (T: primary tumor, N: regional lymph node, and M: distant metastasis).
The World Health Organization`s TNM Classification of Tumors in Domestic animals, one of the most widely used and adapted in veterinary medicine, was developed to assist in determining the prognosis of mammary cancer (Owen, 1980). The prognosis for any individual with cancer depends on a number of factors: the histological type of tumor, the grade of the

tumor, the clinical stage or extent of the tumor, and the presence of any tumor-related complications or concurrent disease.

The clinical stage of a tumor describes the anatomical extent of a tumor at a set point in time, and to a large degree, this determines the feasibility of therapy. This TNM system takes account of the size of the primary tumor, the local and regional lymph nodes and distant organs where tumor metastases may develop. The TNM information leads to a division into four clinical stages, with local or regional tumor extension progressing from stages I to II, and distant metastasis being categorized as stage IV.

*Table 1.* Clinical staging of canine malignant mammary tumors. TNM classification modified from LN Owen (Owen, 1980)

| T: Primary tumor size | | |
|---|---|---|
| $T_1$ | <3 cm maximum diameter | |
| $T_2$ | 3-5 cm maximum diameter | |
| $T_3$ | >5 cm maximum diameter | |
| $T_4$ | Inflammatory carcinoma | |
| N: Regional lymph nodes status | | |
| $N_0$ | Histologic or cytologic – No metastasis present | |
| $N_1$ | Histologic or cytologic – Metastasis present to ipsilateral node | |
| $N_2$ | Histologic or cytologic – Metastasis to contralateral lymph node | |
| M: Distant metastasis | | |
| $M_0$ | No distant metastasis detected | |
| $M_1$ | Distant metastasis detected | |
| Stages grouping | | |
| I | $T_1$ | $N_0$ | $M_0$ |
| II | $T_{0-1}$ | $N_1$ | $M_0$ |
| | $T_2$ | $N_{0-1}$ | $M_0$ |
| III | $T_3$ | Any N | $M_0$ |
| | Any T | N2 | $M_0$ |
| IV | Any T | Any N | $M_1$ |
| V | $T_4$ | Any N | Any M |

Determination of the clinical staging of malignant mammary tumors is critical to good decision-making before treatment is undertaken. Before considering any form of definitive therapy, it is important to clinically evaluate the patient; a thorough physical examination, a complete blood cell count, serum chemistry profile, and urinalysis are advised prior to performing surgery. Thoracic radiographs, including ventro-dorsal and right and left lateral views, are recommended for all but the smallest lesions, to

evaluate for potential metastasis. In one retrospective study in Sweden, it was shown that the likelihood of detecting pulmonary metastasis of dogs with MT is low in dogs <8 years of age (Djupsjobacka, 2003). The results of this study give reason to question the benefit of routine thoracic radiography of young dogs with MT. An abdominal ultrasound should be performed for evaluation, if abnormalities are noted on physical examination, or if blood work abnormalities exist.
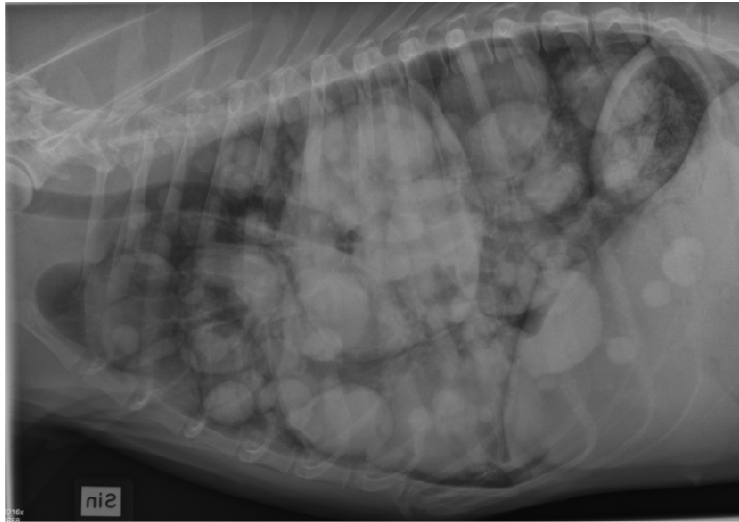


*Figure 2.* Lateral thorax radiograph of a female dog affected by MT that have metastasized to the lungs (Courtesy of Dr. von Euler).


## 2.4   Histological classification of canine mammary tumors

### 2.4.1  Histopathology

Histopathological analysis after submission of the whole surgical specimen for evaluation by a pathologist may provide the clinician with information on tumor type(s), the type of growth, and the completeness of surgical removal. Whilst approximately half of the canine MT have been reported to be benign adenomas that are curable with adequate surgery (Gilbertson et al., 1983), about 50% are considered to be malignant and can arise from different structures within the mammary tissue, and half of these malignant tumors express the metastatic phenotype and have metastasized at the time of clinical presentation (Gilbertson *et al.*, 1983; Priester & Mantel, 1971; Moulton *et al.*, 1970).

Epithelial tumors are the most commonly diagnosed histological type of malignant MT, constituting more than 90 % of all MT, with the remainder consisting of sarcomas and mixed tumors (Gilbertson *et al.*, 1983).

Mammary carcinoma (adenocarcinoma) is the most common malignant tumor and is variously described as solid tubular or papillary. They can be simple (epithelium alone) or complex (epithelium and myoepithelium). The infiltrative characteristics of the tumor in conjunction with the degree of invasion into local lymphatics and capillaries are important in determining the long-term prognosis regarding local recurrence and metastatic potential.

The most aggressive carcinomas have lost their identifying characteristics and are described as anaplastic or poorly differentiated tumors; the latter warrant a guarded prognosis.
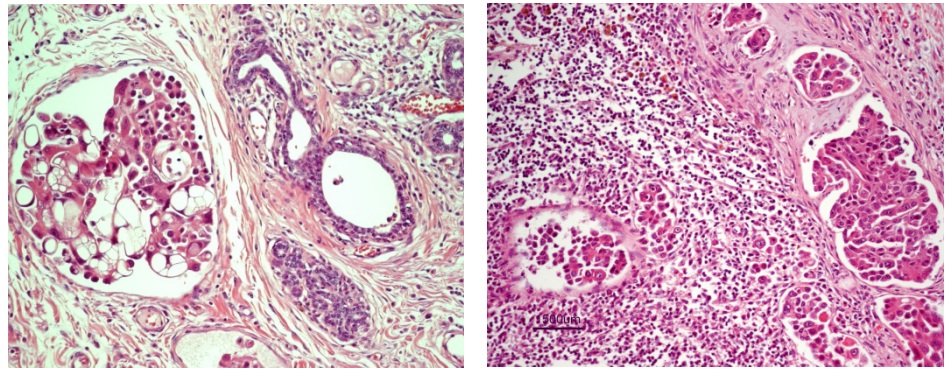


*Figures 3 and 4.* Anaplastic carcinoma in a canine mammary gland composed of large high pleomorphic cells with bizarre nuclei and showing metastasis to the regional lymph node ipsilateral with reactive collagenous stroma (Courtesy of Dr. Grandón).

Inflammatory carcinomas are rare neoplasms with an extremely poor prognosis. These tumors can often be identified at the time of presentation.

Malignant mixed tumors are rare tumors of the mammary gland and the origin within the mammary tissue is unknown. Sarcomas are considered to have poor prognosis; most dogs die within 9-12 months (Hellmen *et al.*, 1993; Misdorp *et al.*, 1971).

### 2.4.2 Histopathological classification

Canine MT has a very detailed histopathological classification, with a confusing variety of different histological types of CMT described. The WHO histological classification aims to internationally standardize terminology and nomenclature of CMT with a descriptive rather than

histogenetic classification, to provide a system that is useful for prognosis (Misdorp *et al,*. 1999).

*Table 2.* From W. Misdorp et al,: Histological classification MT of the dog and cat.

| Malignant tumors |
| --- |
| Noninfiltrating (in situ) carcinoma |
| Complex carcinoma |
| Simple carcinoma |
| Tubulopapillary carcinoma |
| Solid carcinoma |
| Anaplastic carcinoma |
| Special types of carcinomas |
| Spindle cell carcinoma |
| Squamous cell carcinoma |
| Mucinous carcinoma |
| Lipid-rich carcinoma |
| Sarcoma |
| Fibrosarcoma |
| Osteosarcoma |
| Other sarcomas |
| Carcinosarcoma |
| Carcinoma or sarcoma in benign tumor |
| **Benign tumors** |
| Adenoma |
| Simple adenoma (epithelial or myoepithelial type) |
| Complex adenoma |
| Basaloid adenoma |
| Fibroadenoma |
| Benign mixed tumor |
| Duct papilloma |
| **Unclassified** |
| Duct hyperplasia (papillomatosis/epitheliosis) |
| Lobular hyperplasia |
| Adenosis |
| Papillomatosis/epitheliosis |
| Cyst |
| Ductectasia |
| Local fibrosis |
| Gynecomastia |

However, with 14 different malignant types, there is a risk that the general practitioner can be confused. Karayannopoulou et al., in a recent study, evaluated the human "Elston and Ellis grading method" in dogs with mammary carcinomas. In this study, the authors found that histological grading by the Elston and Ellis method was significantly related to prognosis, especially in cases of simple carcinoma. (Karayannopoulou *et al.*, 2005). Histological grade 0 has a 19% recurrence compared to 97% for grade II (Gilbertson *et al.*, 1983). Similarly, with nuclear differentiation, poorly differentiated tumors had 90% recurrence, moderately differentiated had 68%, and well differentiated, 24% (Gilbertson *et al.*, 1983).

In another study performed by Yamagami *et al.* on the prognosis for malignant MT in dogs based on TNM and histology classification, the authors found that the larger the tumor size became (T category), the poorer was the clinical prognosis. The same was described for the dogs with regional lymph node metastasis (N1, N2 category) and/or distant metastasis (M1 category); the survival was lower than in dogs without such involvement. As the grade of TNM staging increased, the prognosis was poorer; however, there were no significant differences in survival rates among subtypes of adenocarcinomas determined by WHO histological classification. It was also noticed in that study that dogs having carcinomas without tubular formation or myoepithelial cell proliferation had a lower survival rate than dogs having carcinomas with those characteristics, and invasive carcinomas into adjacent skin or lymphatic/vascular vessels implied a poorer prognosis than non-invasive ones. The results suggested that a combined practice of TNM system and the evaluation on the above mentioned four histologic features could be useful for prognostic determination of CMT (Yamagami *et al.*, 1996).

Moreover, Sorenmo (2009) recently described in a study the clinical and histological findings in dogs with MT, and compared the histological and clinical evidence consistent with progression from benign to malignant with human breast cancer epidemiology. Dogs with malignant tumors in that study were significantly older than dogs with benign tumors. Dogs with malignant tumors were significantly more likely to develop new primary tumors. These findings suggest that CMT progress from benign to malignant and that malignant tumor may be the end stage of a histological continuum with clinical and histopathological similarities to human breast cancer carcinogenesis (Sorenmo *et al.*, 2009).

## 2.5 Treatment and prognosis

The treatment of choice is surgical excision with appropriate margins, where ideally the extent of surgery (i.e., nodulectomy, mastectomy, regional mastectomy, unilateral or bilateral radical mastectomy) should be guided by clinical appearance and histological grade. The idea is to remove the entire tumor by the simplest surgical procedure (no clinical trial has shown improvement in survival with radical versus local removal). Decisions regarding treatment should be based on a correct TNM staging according to the WHO classification.

Adjuvant treatment is common in women with breast cancer. In CMT there are reports of post-operative chemotherapy, radiotherapy and hormone level-modifying substances. In all, these studies do not show convincing support for combining surgery with any of these to improve treatment outcome. This is probably mainly due to lack of large-scale well-controlled trials.

Current knowledge indicates a major role for COX-2-specific inhibitors as part of the treatment in female dogs affected with malignant CMT. As many CMT do metastasize and the patients eventually die of metastatic disease, there is still a need for improved therapy regimens in dogs. Early detection and rapid therapy are essential to prevent early local or distant dissemination.


## 2.6 Serum biochemical markers in canine tumors

Laboratory diagnosis currently represents an integral part of patient care. The possibility of having strong prognostic and/or predictive markers is of the utmost importance for clinicians, to identify patients at higher risk of relapse and to select the most appropriate systemic treatment for an individual patient. Serum tumor markers are used to obtain prognostic information and to choose and monitor tumor disease treatment both in human and in veterinary medicine. Cellular enzyme thymidine kinase, determining cellular proliferation in connection with the inception, development, and treatment of the disease, belongs to such parameters. Thymidine kinase 1 (TK1) is a deoxyribonucleic acid (DNA) precursor enzyme that is only found in proliferating cells and is one of the key markers for S-phase and G2 cells in cell biology.
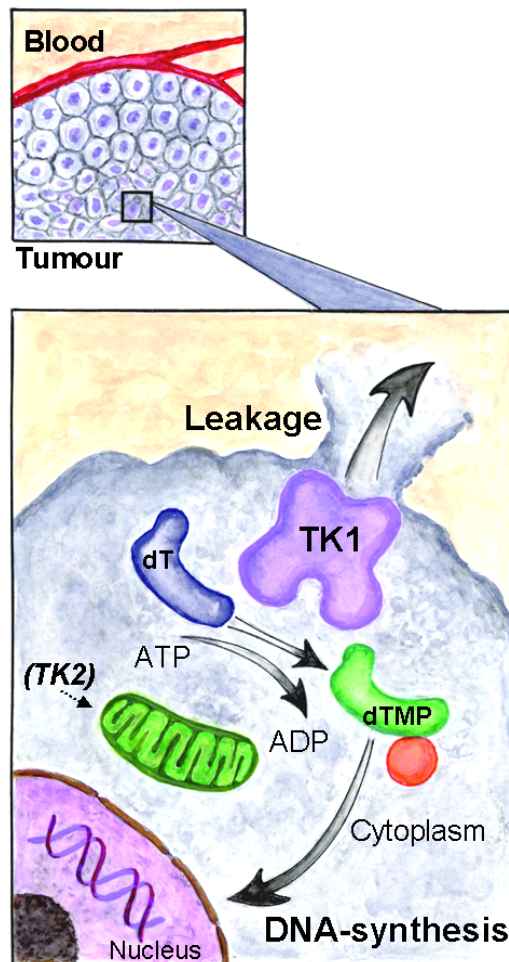
*Figure 5.* The role of thymidine kinase (TK) in the salvage pathway of the pyrimidine synthesis. TK2 is expressed in the mitochondrion and is present in the entire cell cycle. Leakage of TK (i.e., TK1) through the cell membrane reflects either the overall degree of DNA synthesis or the number of cells dying in the replicative stage. In the presence of ATP, TK1 catalyzes the conversion of deoxythymidine (dT) to deoxythymidine monophosphate (dTMP). Deoxythymidine monophosphate is subsequently phosphorylated to its triphosphate analogue (dTTP) before being a substrate for DNA synthesis. Illustration courtesy of Eva Hall (von Euler et al., 2004).

A large amount of biochemical information is now available concerning the structure-function relationships of TK1, which have been of considerable interest for the antiviral and anti-cancer chemotherapy field (Al-Madhoun *et al.*, 2004; Welin *et al.*, 2004; Eriksson *et al.*, 2002). The cell cycle and tissue regulation of TK1 messenger ribonucleic acid (mRNA) and protein, which

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depends on transcriptional and posttranslational processes, has also been studied in great depth (Ke & Chang, 2004; Hengstschlager *et al.*, 1994). A stable serum form of TK1 was identified already in 1980 and serum TK (s-TK) levels serve as biomarker in many different malignant diseases, for example, Hodgkin`s and non-Hodgkin`s lymphoma (NHL), acute myeloid leukemia (AML), lymphocytic leukemia (LL), and chronic LL (Poley *et al.*, 1997; Hallek *et al.*, 1992; Gronowitz *et al.*, 1983). One report describes s-TK activity and lactate dehydrogenase (LDH) activity in dogs with malignant lymphoma (ML) (Nakamura *et al.*, 1997).

More recently, s-TK radio enzyme assay (REA) determinations, including the use of a non-radiometric assay, were shown to provide prognostic and monitoring information regarding disease in canine malignant lymphoma (ML) (von Euler *et al.*, 2006; von Euler *et al.*, 2004). Malignant lymphoma in dogs, in particular, has been shown to share morphological gene expression patterns and clinical similarities with NHL in human patients (Sueiro *et al.*, 2004; Teske, 1994).

High levels of TK have been reported in human breast cancer patients (Romain *et al.*, 1997; O'Neill *et al.*, 1992a; O'Neill *et al.*, 1992b; Spyratos *et al.*, 1992). The serum TK levels in breast cancer patients demonstrated a statistically significant positive correlation with the cancer stage (O'Neill *et al.*, 1992a). They also demonstrated that the total tumor TK levels were significantly higher in breast cancer patients who subsequently had a recurrence than in those who did not. It appears, therefore, that TK is a potentially useful marker in the management of breast cancer in humans (O'Neill *et al.*, 1992a). Carlsson et al. recently demonstrated that the serum concentration of TK1 is increased in human patients with breast cancer (Carlsson *et al.*, 2009).

## 2.7   The dog as genetic model for complex human diseases

The release of an annotated human genome sequence assembly and the emergence of genomics technologies have led to significant advances in our understanding of many human diseases, including cancers. As DNA sequencing technology has become less costly, the field of comparative genomics has progressed rapidly, and attention has turned now to generating whole genome assemblies and dedicated genomic resources for veterinary species. Such progress brings a whole new series of opportunities to advance veterinary medicine. Many human and animal diseases share a pathogenetic basis, and although veterinary species need advances in biomedical research in their own right, the consideration of companion animals as good

comparative models for human disease saw the emergence of the "one medicine" concept. The concept of "one medicine" rests on the translation between human and veterinary medicine in both directions (Breen, 2009). The domestic dog has emerged as an ideal model for gene mapping of human complex diseases, as it has a spectrum of diseases similar to that in man, and also an advantageous population structure (Karlsson & Lindblad-Toh, 2008; Sutter *et al.*, 2007; Sutter & Ostrander, 2004). Dogs have a history of inbreeding, which has resulted in low levels of genetic variation within breeds. The recent breed formation and limited population size has also resulted in a high degree of linkage disequilibrium (LD) within breeds (Lindblad-Toh *et al.*, 2005a; Sutter *et al.*, 2004), particularly in comparison to what is seen in humans (Kruglyak, 1999).

Certain breeds are known to be predisposed to specific disorders and CMT in ESS dogs in Sweden is one such clear example, with 36% of ESS dogs in Sweden being affected by CMT (Egenvall *et al.*, 2005). Due to the small genetic variation, CMT should have a more homogenous origin within a single breed compared to breast cancer in the larger human population. This should allow for an easier identification of risk factors within a breed. As part of the dog genome sequencing project, a power calculation for case-control association within dog breeds was performed, suggesting that with 15,000 single nucleotide polymorphisms (SNPs), the power to detect a locus with a sample of 100 affected and 100 unaffected dogs is 97% for $\lambda = 5$ and 50% for $\lambda = 2$ (Lindblad-Toh *et al.*, 2005a), if the frequency of the associated allele is <20%. This supports the notion that in a genetically isolated population, it is relatively easy to identify a specific associated haplotype, which is significantly more frequent in cases than in controls.

There are now numerous reports of mapping canine diseases genes, both simple and complex, via genome-wide genotyping studies (Wilbe *et al.*, 2010a), illustrating the broader importance of the dog as a comparative model (Breen, 2009).
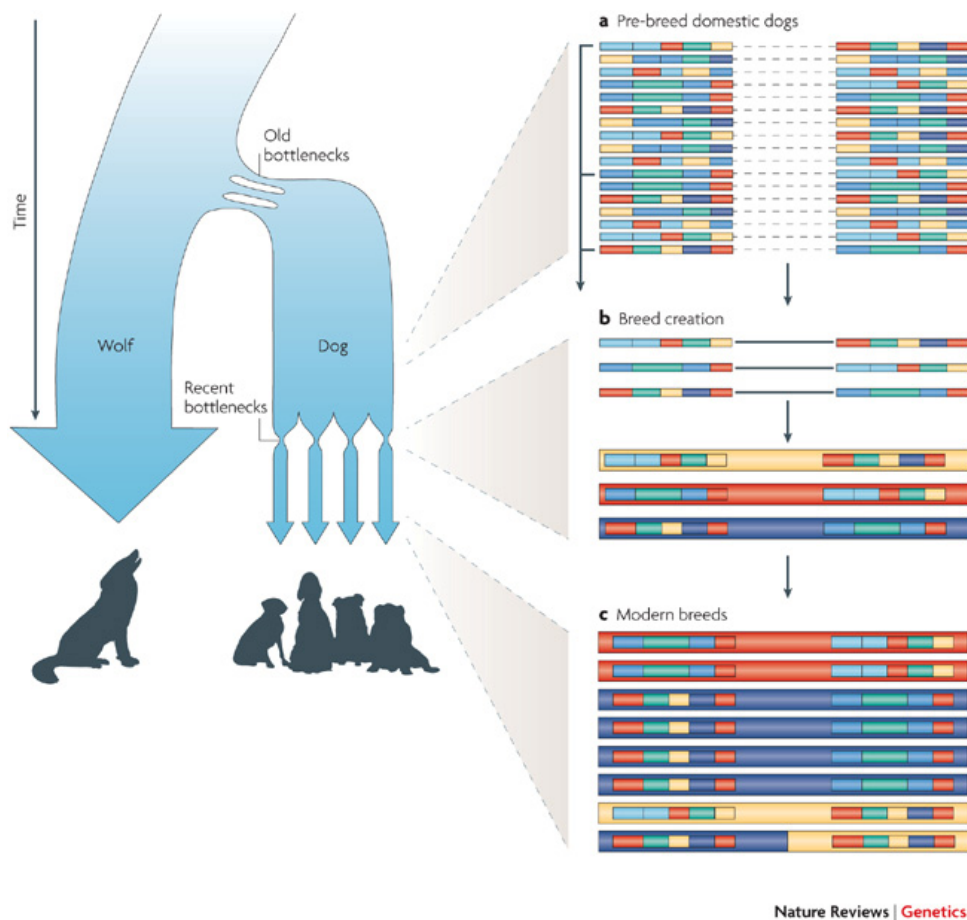
*Figure 6.* Two population bottlenecks in dog population history, one old and one recent, shaped haplotype structure in modern dog breeds. First, the domestic dog diverged from wolves 15,000 years ago, probably through multiple domestication events. Within the past few hundred years, modern dog breeds were created. Both bottlenecks influenced the haplotype pattern and linkage disequilibrium (LD) of current breeds. **a** | Before the creation of modern breeds, the dog population had the short-range LD that would be expected, given its large size and the long time period since the domestication bottleneck. **b** | In the creation of modern breeds, a small subset of chromosomes was selected from the pool of domestic dogs. The long-range patterns that were carried on these chromosomes became common within the breed, thereby creating long-range LD. **c** | In the short time since breed creation, these long-range patterns have not yet been substantially broken down by recombination. Long breed haplotypes, however, still retain the underlying short ancestral haplotype blocks from the domestic dog population, and these are revealed when one examines chromosomes across many breeds. This figure is modified, with permission, from (Lindblad-Toh et al., 2005b) Nature and (Karlsson & Lindblad-Toh, 2008) Nature Genetics © (2005) Macmillan Publishers Ltd. All rights reserved.

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The question is whether research on CMT can contribute to the advancement of knowledge and ultimately the treatment of breast tumors affecting women. Research on spontaneous CMT has been performed for many decades, but still, canine tumors are not very well established as a model for human malignancies. In addition to the high incidence and potential of being fatal, similarities with human breast cancer have prompted several studies on the incidence and factors affecting the incidence of CMT.

Canine mammary carcinomas have epidemiological, clinical, morphological and prognostic features similar to those of human breast cancer, and are therefore suitable models with naturally occurring tumors (Frese, 1985; Chrisp & Spangler, 1980). In both women and dogs, mammary tumors develop with age, rarely occurring before 25 and 5 years of age, respectively (Cohen *et al.*, 1974). Breast cancer is often familial in humans, but a similar hereditary pattern has not been described for mammary tumors in dogs, although breed predilections have been reported (Egenvall *et al.*, 2005; Cotran, 1994; Dorn *et al.*, 1968b). The development of both canine and human mammary tumors is hormone dependent (Cotran, 1994; Schneider *et al.*, 1969). Importantly, the biology of CMT and human breast cancer is very similar in many aspects. Genes involved in tumor development (*MET, IGFIR, KIT*, *mTOR,* and *p53*) have been compared between species, and the canine genome showed greater similarities to the human DNA sequence compared to the mouse (Paoloni & Khanna, 2008; Hoffman & Birney, 2007; Chu *et al.*, 1998), an indication that the canine model can be a resource in tumor research, in general. There are several examples of similar biology and histology of mammary tumors between humans and dogs. For example, mammary sarcomas show the same metastasizing patterns in both species (Gullett *et al.*, 2007; Silver & Tavassoli, 1998; Fong *et al.*, 1993). Another example is a study that evaluated new classifications of human mammary tumors based on gene expression profiling data on canine specimens. The classification was applicable to canine tumors and could predict outcome (Gama *et al.*, 2008).

## 2.8   Comparative oncology

The value of comparative oncology has been increasingly recognized in the field of cancer research, including the identification of cancer-associated genes; the study of environmental risk factors, tumor biology, and progression; and perhaps most importantly, the evaluation of novel cancer therapeutics (Paoloni & Khanna, 2007). Cancer in pets is a spontaneous disease. Pet owners, motivated to prolong the quality of life of their animals,

frequently seek specialized treatment from veterinary oncologists at private veterinary and teaching hospitals. The therapeutic modalities of veterinary cancer patients are similar to those of humans, including surgery, chemotherapy, radiation therapy, and biotherapy.

Many factors contribute to the value of these spontaneous tumors as relevant models for human cancer. The animals share many environmental risk factors, such as housing, food, water, and passive smoking, with their owners, suggesting their value as sentinels of disease. Cancers found in companion animals share tumor biology and behavior with human cancers, and in some cases have identical histology and response rates to conventional chemotherapy. In most cases, the prevalence is sufficient for clinical trials and biological studies (e.g., mammary tumors, osteosarcoma and malignant lymphoma). The size of dogs (and cats) makes multi-modality protocols feasible: the lack of "gold standard" treatments allows for early and humane testing of novel therapies. Finally, early progression, metastatic failure, and shorter life span result in the rapid completion of clinical trials.

The future of many areas of human and veterinary biomedical research is very much interdependent, with one of the closest associations being in oncology.

# 3   Aims of the thesis

The overall aim of this thesis was to further characterize the risk factors for the development of canine mammary tumors, using a population of a breed at high risk for this disease in Sweden.

The following specific aims were set:

> ➢ To study a serum biomarker, thymidine kinase 1 –activity in diagnosing, prognosticating and monitoring dogs with canine malignant lymphoma, leukemia and solid tumors.

> ➢ To describe the clinical and histopathological characteristics of MT in a large, clinically well-defined population of ESS female dogs in Sweden and to assess the influence of potential risk factors for the development of MT and survival.

> ➢ To perform a candidate gene association study in CMT, evaluating 10 human breast cancer genes for association with CMT.

> ➢ To study the potential role of MHC class II genes as genetic risk factors for the development of CMT.

# 4　Comments on materials and methods

Overall issues regarding the dogs and the research methods used in studies (papers) II–IV are outlined in this section. All the dogs used in these four studies were privately owned. For further detailed descriptions, the reader is referred to each individual paper.

## 4.1　Material and methodological considerations (study I)

The study population used in Paper I was 95 dogs in total. Thirty healthy dogs were included as controls, 7 dogs with mast cell tumors, five with osteosarcomas, six with soft tissue sarcomas, 2 with mammary carcinomas, 2 with thyroid carcinomas, 2 with squamous cell carcinomas, 1 dog with transitional cell carcinoma of the bladder, 1 with insulinoma, 29 with malignant lymphoma (ML), and 10 with leukemia (LEUK). In total, the number of blood samples analyzed was 213 (stored at $-20^{0}$C). Originally, 230 samples were blinded and randomly collected from a serum tumor bio bank. All treatments and sampling of dogs were approved by the Swedish Animal Ethics Committee (no. C122/0). The samples originated from a serum sample bank containing specimens from dogs mainly with malignant lymphoma, sampled between June 2006 and December 2007.

Histological/cytological confirmation of diagnosis was procured in all tumors. Each dog tumor was staged according to the classification scheme of the World Health Organization (WHO). Diagnostic methods employed varied, depending on the histologic type of tumor, the anatomic localization of the tumor, and the clinical status of the dog. Since new serum samples were added during the course of chemotherapy, several of the samples eventually proved to come from the same individual, but to have been taken on different occasions. Seventeen samples were excluded due to

insufficient volume, or because they were from an incorrect specie (n = 10). The analyst had no knowledge of the origin of each sample.

## 4.2   The Swedish Kennel Club database (studies II-IV)

The Swedish Kennel Club (SKC) has kept fully computerized records of pedigrees and ownership of all pure-breed dogs in Sweden since the year 1976. In studies II-IV, their register was accessed in order to find and contact owners of Swedish ESS dogs of a susceptible age for MT, both dogs affected by MT, of any age and any reproductive status (cases), and healthy ESS female dogs older than seven years of age and intact or late-spayed (spayed >2.5 years of age) (healthy controls). The Swedish Kennel Club register was also accessed for pedigree information used for studies III-IV.

## 4.3   The Agria Pet Insurance database (studies II-IV)

The Swedish dog population is unique in that a large proportion of the animals are covered by an insurance plan, and that most dogs are not neutered. The Swedish animal insurance company Agria (Agria Pet Insurance, PO 70306, SE-10723, Stockholm, Sweden) has been shown to cover approximately 30-40% of the entire Swedish dog population (Egenvall et al., 1999). The database was validated and it was concluded that it was acceptable for research purposes (Egenvall et al., 1998). The insured dog population in Sweden reflects the general Swedish dog population with regard to gender distribution and breed structure. However, the mean age of the insured population (5.2 years) is somewhat lower than the general Swedish population (5.7 years), although the median ages are equal (5 years). This is explained by the different age limits that have been applied for the different insurance forms. In general, during the study period for Papers II-IV, life insurance ended at 10 years of age, and veterinary care insurance at 12 years of age. This database was also accessed to obtain additional ESS owners and to find additional clinical information on the dogs.

## 4.4   The Swedish canine mammary tumor project (studies II-IV)

A large number of participating dogs with MT (cases) and respective healthy dogs (controls) were required for the work of this doctoral research. The Swedish canine mammary tumor project was started as a basis for this work in April 2005. Since then, peripheral EDTA blood samples, pathological reports, and medical records have been collected systematically from dogs

diagnosed with MT and healthy dogs of corresponding breeds (ESS, Boxer, Doberman, and German shepherd).

This project is part of the seventh Framework Programme for Research and Technological Development of the European Commission, LUPA (GA-201370). A broad network of veterinarians, researchers, molecular geneticists, bioinformatics specialists, veterinary nurses, breed clubs, and dog owners was established. This network was recruited through articles, television interviews, lectures, and newsletters. The Swedish Kennel Club, the major Swedish dog insurance company, Agria, and various animal hospitals and clinics across Sweden made possible for the project to contact owners of ESS dogs, both MT-affected dogs and healthy controls. In total, peripheral EDTA blood samples from approximately 600 dogs have been collected by the author within the project to date, from veterinary animal hospitals and veterinary clinics throughout Sweden between 2005 and 2010. These samples are stored in a dog genome bio-bank at the department of clinical sciences at SLU in -80$^0$C until analyzed. The owner of every dog involved in the project completed a postal questionnaire, and the veterinarian in charge supplied the medical records to the author. Medical records and questionnaires for all ESS dogs were reviewed, and owners were contacted on several occasions for follow-up information.

All the blood samples and clinical information collected for Papers II-IV were approved by the owners and conformed to the decision of the Swedish Animal Ethics Committee (no. C 62/10), and the Swedish Animal Welfare Agency (no. 31-1711/10).

## 4.5   Clinical information and pathology (studies II-IV)

The epidemiological data for MT in the Swedish dog population (Egenvall et al., 2005) were used to identify a breed at high risk for MT (ESS). In these studies we had access to the SKC, as well as the Agria Pet Insurance Company. The collaboration in the Swedish canine mammary tumor project offers possibilities to access individual dog data with a specific diagnosis (CMT) in the pet insurance data-base and link them with ancestral background at SKC. Information can be retrieved that enable us to contact owners of both dogs with specific phenotypes and related dogs, and to access extensive genealogies and informative pedigrees. These features have already been used not only to identify birth cohorts but also individual dogs to be recruited as cases, as well as controls, and for identification of family material in CMT. The same frame is also used for sampling of related and unrelated healthy controls in our studies. Using questionnaire information

on cases and controls of various phenotypes, we were able to include environmental factors in the analyses of genotypes at risk. Identifying adequate sample sizes of well-phenotyped dogs from population-based epidemiological data, and strict inclusion criteria for the cases, as well as the controls, were essential for these studies. Information was collected regarding possible risk factors for the development of mammary tumors (age of onset, sex, spaying, lactation, and use of contraceptives, diet, pregnancy, disease status and family cancer history), including pathology reports and/or other clinical diagnostic information. All dogs included in these studies were female ESS dogs. All the control dogs were older than seven years, with a confirmed absence of CMT based on palpation of the mammary gland performed by a veterinarian. The ESS cases were selected based on pathology reports, if available, and otherwise based only on physical examination data (the presence of a single or multiple nodules within the mammary gland). Most of these MT were not surgically excised, or excised and not histopathologically evaluated. All the pathological analyses were performed by a veterinary pathologist at one of three central laboratories in Sweden.

## 4.6  Genetic studies (studies III-IV)

For Papers III and IV genomic DNA was extracted from the blood samples using the Qiagen Miniprep extraction kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Prepared genomic DNA was stored in deionized water (stored at $-20^{0}$C). The dogs used in Papers III and IV were subdivided into two study populations. In the first population (dataset 1, n = 192), 99 ESS cases diagnosed with CMT and 92 control dogs older than eight years of age were selected. There were 29 cases with malignant tumors, 57 cases with benign tumors, all confirmed with histopathology performed by a veterinary pathologist at one of three central laboratories, and 13 cases for which the pathology report was unknown. All cases and controls were selected to be unrelated at the parental level.

In the replication population (dataset 2, n = 182) the diagnostic criteria were less stringent, and fewer dogs with diagnosed malignant disease were available. One hundred twenty-one ESS cases were selected based on pathology reports, if available, and otherwise based only on physical examination data (the presence of a single or multiple nodules within the mammary gland). Most of these mammary tumors were not surgically excised, or excised and not histopathologically evaluated. Of the 121 cases,

4 were confirmed as malignant and 39 as benign, by histopathology. Sixty-one control dogs were available. Siblings were allowed in this population.

Regarding candidate gene selection and genotyping for Paper III: Ten human breast cancer genes (*BRCA1, BRCA2, CHEK2, ERBB2, FGFR2, LSP1, MAP3K1, RCAS1, TOX3,* and *TP53*) were selected in the present study as candidate genes for CMT. Sixty-three SNPs were chosen from the 2.5 million-SNP map described by Lindblad-Toh *et al.*, (Lindblad-Toh *et al.*, 2005b). Four to nine SNPs per candidate gene (63 SNPs in total) were selected from the available Dog Genome sequences in the UCSC Genome Browser, Dog May 2005 (CanFam2) assembly. The samples were genotyped for the 63 SNPs using the iPLEX Gold Mass ARRAY according to the manufacturer's protocol (Sequenom, San Diego, CA, USA). The primary genotype data were analyzed using the Typer 4.0 Analyzer User Interface software (Sequenom, San Diego, CA, USA) for cluster analysis. SNPs with a call rate of >75 % and a minor allele frequency (MAF) of at least 5 % were included in each analysis. Samples with a call rate ≤75% were excluded from further analysis.

The primers for amplification and extension were designed using Mass ARRAY Assay Design v.3.1 software. DNA was amplified using PCR, and the remaining nucleotide triphosphates were deactivated by phosphatase treatment (SAP). A single base primer extension (SBE) step was performed, and the allele-specific extension products of different masses were quantitatively analyzed using MALDI-TOF mass spectrometer.

Regarding dog leukocyte antigen (DLA) class II typing performed in Paper IV: The protocol and locus-specific primers used in this study for sequence-based typing of DLA-DRB1, -DQA1, and -DQB1 alleles were based on previous studies (Wilbe *et al.*, 2010b; Wilbe *et al.*, 2009; Kennedy, 2007). The typing method used a polymerase chain reaction (PCR) amplification of the genomic DNA, followed by DNA sequencing in one direction. All the nucleotide sequences for DLA-DRB1, -DQA1, and -DQB1 were analyzed using MatchTools and MatchTools Navigator (Applied Biosystems, Foster city, CA, USA). These sequences were compared to a consensus sequence containing all known polymorphic sites and each polymorphic site was analyzed manually. The corrected sequence was then matched to a reference sequence library (http://www.ebi.ac.uk/ipd/mhc/index.html) using MatchTools, to provide the exact matching allele (Wilbe *et al.*, 2010b; Wilbe *et al.*, 2009; Kennedy, 2007). Haplotypes were first identified in homozygous dogs, and then assigned in the heterozygotes and finally, additional haplotypes not found in homozygotes were identified.

All the lab work and analyses regarding Paper III was performed at the Broad Institute of MIT and Harvard, Cambridge, MA, USA. For Paper IV all the lab work was performed at the Department of Animal Breeding and Genetics, SLU, Uppsala, and the Department of Medical Biochemistry and Microbiology (BMC), Uppsala University, Sweden.

## 4.7   Statistical methods (studies I-IV)

For further detailed information of the statistical methods, the reader is referred to each individual paper.

For study I the TK activities were analyzed both before and after log transformation (natural log) because of skewed distribution. A plot of the difference against the average of the standard (REA) and new measurements (Liaison) was performed using the Bland-Altman method (bivariate fit of difference) (Bland & Altman, 1995). In addition, Spearman`s rank correlation analysis was performed to assess the relationship between the different assays. Comparisons between healthy controls, inflammatory disease, other tumors, malignant lymphoma (ML), and leukemia (LEUK) was done, using one-way analysis (Tukey-Kramer honestly significant difference, HSD test), due to unequal-sized groups with unequal variances. The $\alpha$ level was set to 0.05. Statistical calculations were performed using the JMP$^{®}$ package 7.0 statistical software (SAS Institute, Inc., Cary, NC, USA). Values of $p < 0.05$ were considered indicative of statistical significance.

For Paper II the data were analyzed statistically using the JMP package 8.0.2 statistical software (SAS Institute, Inc., Cary, NC, USA). All statistical tests were two-tailed. Statistical significance was considered at $p < 0.05$. Descriptive statistics are presented for the distribution of age of onset, tumor classification, presence of multiple nodules, ulceration/inflammation of the tumor, surgical treatment for MT, evidence of metastasis, tumor recurrence or new tumors, pregnancy and mastitis at the time of partum, previous hormonal treatments, and reproductive status. Continuous data are expressed as median and interquartile range (IQR). The distribution of discrete characteristics within cases and controls were tested using Fisher`s exact test. Odds ratios were calculated for the different variables comparing cases and controls. Continuous dog characteristic data were tested using Kruskal–Wallis test. A Kaplan Meier survivor function was used to display the age of onset of MT, by reproductive status.

The effects of dog characteristic data on histological subtype in dogs with MT were tested using the same statistical methods as outlined above. Graphs of the Kaplan-Meier survivor function were used to display time-to-event

curves and estimate the median time to endpoint. Endpoints used were all causes of death and death related to MT. Dogs were censored at last date of contact, and in the analysis of death directly related to MT, at death unrelated to MT. A log-rank test with right censoring was used to assess differences between histological subtype groups for survival after diagnosis of MT.

Multivariable Cox Proportional Hazard Models were constructed for investigating the effect of potential risk factors for time to onset of MT and time of survival after diagnosis (all-causes mortality). The models were built in a backward stepwise manner. The analyses started with all risk factors included in the model, which were previous pregnancy, hormonal treatment, and reproductive status. The variable with the highest p-value was eliminated at each step, with reanalysis between steps, until the final model was obtained, which was reached when all the remaining variables had a p-value <0.05. For the survival analysis of time from diagnosis for all-causes mortality, the risk factors included, in addition to the above-mentioned factors, also malignancy and age of onset. The proportional hazard assumption was checked by plotting the survival time against the different variables. No multivariable model was developed for survival after diagnosis for MT-related mortality, because of the low number of events and the consequently low number of degrees of freedom.

For Paper III the association analyses for all comparisons were performed with the PLINK software (Purcell *et al.*, 2007) for single chi-square SNP association, haplotype association, odds ratios, and MAF. Nominal (raw) chi-square and Bonferroni-corrected chi-square p-values were calculated to adjust for the multiple testing that arises from evaluating several SNPs or haplotypes (47, 48). A Bonferroni-corrected p < 0.05, with correction for total SNP number remaining after filtering in each analysis, was considered statistically significant, although this likely *over-corrects* due to the fact that most SNPs within a gene likely are linked to each other and therefore are not unrelated observations.

For Paper IV the statistical analyses were performed using VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html). A 2 x 2 contingency table was used to calculate odds ratios, and p-values for each allele, haplotype, and genotype in ESS. For the $\chi^2$ test Yates p-value and a 99% confidence interval (CI) were used. We analyzed all cases versus all controls in data set 1 and data set 2 and together (datasets 1 & 2), to investigate whether a single allele, haplotype, or genotype was present at a significantly higher or lower frequency in cases compared to controls, and thus associated with CMT.

# 5 Main results and discussion

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

## 5.1 A serum biochemical tumor marker (TK1) in canine malignant lymphoma, leukemia and solid tumors (Paper I)

The first study in this thesis evaluated the TK1 activity as a potential biochemical tumor marker, not only in dogs with response in hematological neoplasia (malignant lymphoma, leukemia), but also in those with solid tumors. Determinations of TK levels had so far relied on radio enzyme assay (REA) and experimental ELISA methods, which limited the clinical use of this biomarker, although recent studies in dogs with ML have demonstrated its wide potential.

In this study we also evaluated a new fully automated non-radiometric assay, the Liaison TK assay (DiaSorin, Vercelli, Italy). The aim in investigating the new Liaison TK assay was both to validate a new non-radioisotope-based fully automated assay vs. the gold standard TK–REA and also to see if the Liaison assay had a higher sensitivity for detecting solid tumors.

The study population in this first study comprised 95 dogs in total. Sera from healthy dogs (n = 30), and dogs with leukemia (LEUK) (n = 35), malignant lymphoma (ML) (n = 84), non-hematological tumors (n = 50), and inflammatory disease (n = 14) were tested using both methods. Lymphoma and leukemia samples were available before and during chemotherapy. In total, the number of blood samples analyzed was 213.

*Table 3.* Serum thymidine kinase 1 activity is summarized in this table

|  | Mean | Median | Range |
|---|---|---|---|
| Normal (n = 30) | 2.3 | 1.7 | 0.4-6.1 |
| Inflammatory disease (n = 14) | 2.1 | 2.0 | 0.7–3.0 |
| Other tumors including CMT (n = 50) | 3.3 | 1.7 | 0.5–16.2 |
| ML in complete remission (n = 56) | 3.0 | 2.6 | 0.5–7.0 |
| ML out of remission (n = 28) | 22.0 | 18.6 | 7.2–66.3 |
| LEUK in remission (n = 11) | 21.6 | 54.0 | 12.8–26.4 |
| LEUK out of remission (n = 24) | 88.1 | 57.3 | 30.7–263.0 |

Table 3. Inflammatory diseases, non-hematological neoplasias, and malignant lymphoma in complete remission (CR) have TK 1 activity levels not significantly different from those of healthy dogs, while the mean TK activity in malignant lymphomas (ML) out of remission and leukemias (LEUKs) is 10- to 20- fold higher than the means of the other groups. None of the dogs with LEUK reached CR, only partial remission. This shows the clinical benefit of monitoring hematological neoplasias with serum TK (s-TK), as well as indicating a high specificity of the Liaison assay.

The TK1 levels measured during chemotherapy were different between dogs in complete remission and dogs out of remission. A Tukey-Kramer analysis showed that all LEUKs and MLs out of remission differed significantly from the others groups.

The Liaison TK assay showed high precision, high sensitivity, and a good correlation to the TK REA. Although initially developed for human purposes, both the TK REA and the Liaison TK assay are species-independent, since they measure enzyme activity. This warrants further investigations in veterinary medicine. The Liaison TK assay is the first non-radiometric assay that is able to accurately measure s-TK1 activity in dogs, being fully automated for large-scale use, and provides valuable clinical information in the treatment and management of canine LEUK and ML, with a potential to be further validated in human trials; but, the Liaison assay was not effective in detecting solid tumors in the study samples (there were, however, only two MT cases in this study). Due to the low sensitivity and specificity for the serum biomarker TK1 regarding activity in canine solid tumors, another approach was chosen.

Canine MT was picked as model for further studies from the solid tumor group. This choice was based on the high prevalence in Sweden (Egenvall *et al.*, 2005), but also because it is considered a good comparative model for human breast cancer. To expand the utility of biomarkers, a slightly different approach was chosen, as molecular/genetic biomarkers were used instead of serum proteins. With this tool, risk factors/predisposing genes

may be found. At a later stage, sub-populations of dogs having a higher risk of developing, for example, CMT, can then be recommended for more frequent physical examinations for the presence of tumors and likely have a diagnosis as early as possible, enhancing the possibility for a successful treatment. This is in contrast to serum biomarkers, which discover tumors that are already developing. Moreover, knowledge of predisposing genetic risk factors can be beneficial for breeding purposes, to reduce the number of susceptible dogs in the high-risk breed.

This leads us to investigate other biomarker tools, for example to screen for potential genetic risk factors involved in the pathogenesis of CMT.

## 5.2   Risk factors for CMT in Swedish ESS (Paper II)

In this case-control study (II), we investigated potential risk factors for mammary tumors and survival after MT in the largest uniform population of dogs described from one high-risk breed tested so far (488 Swedish ESS female dogs in total for study I). We obtained information regarding 299 MT cases and 189 controls, obtained from questionnaires, pathology reports, and medical records of ESS dogs recruited from the Swedish Kennel Club's database. The distribution of various clinical and pathological variables and survival times were compared between dogs with or without MT and between dogs with malignant or benign tumors.

### 5.2.1  Age of onset

The median age at MT diagnosis was found to be 7 years in study II. This is a relatively low age of onset compared to other breeds, and this finding is in agreement with a previous study (Egenvall *et al.*, 2005).

### 5.2.2  Reproductive status

Of the 488 dogs in this study, 384 (79%) were sexually intact dogs. The majority of dogs spayed in this study were spayed at a relatively late age and mainly due to medical reasons, for example, pyometra, with a median age of spaying of 6.8 years. Only less than 1% of the dogs in this study were spayed early in life as prophylaxis for MT, making it impossible to examine the effect of early spaying. More intact female dogs in this study had MT compared to spayed dogs. Fifty-three dogs in the case group were spayed and 51 dogs in the control group. The OR for MT was 1.7 for intact female dogs compared to spayed dogs (p=0.01). We did not find a protective role of spaying for survival after diagnosis.
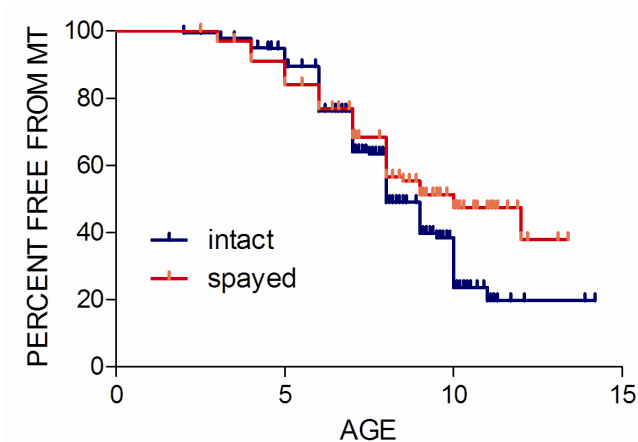
*Figure* 7. Kaplan-Meier function for age of onset of MT by reproductive status (n=299, 20 missing and 207 censored subjects, which are marked with ticks). Spayed dogs had a significantly higher age of onset compare to intact female dogs (log-rank test, p = 0.01)

### 5.2.3 Hormonal risk factors

Only 6% of the population in this study was treated with gestagens to prevent estrus and only 5% received aglépristone for pregnancy termination, and their mammary glands were not exposed to prolonged hormonal stimulation. Failure to show a statistically significant difference in MT development in these groups may be due to the small number of dogs exposed to hormone treatment. Similarly, there was no influence on the development of MT by pregnancy and lactation in the dogs in this study.

### 5.2.4 Histopathology

Benign MT was found in 122 dogs (41%), malignant MT was found in 55 dogs (18%), and 122 dogs had MT with unknown histopathology (41%). The tumor classification in this study revealed that in the group of dogs with a histopathology report (n = 177), 68% had benign tumors, and 32%, malignant. The higher frequency of benign tumors in this study may be due to a relatively low age of onset (median of 7 years); this agrees with findings by Sorenmo *et al.*, (2009), which showed that the majority of the dogs in her study had only benign tumors and that these dogs were significantly younger.

As a comparison to human breast cancer, similar to what we found in the dogs, younger women are more likely to have benign tumors and the risk for malignancy increases with age (Miltenburg & Speights, 2008). However,

it is possible that some of these benign tumors would have progressed and become malignant if opportunity (i.e., time) had been provided.

One might speculate that early benign tumor development is more easily triggered by hormonal effects, but that malignancy is less dependent on hormones, due to a loss of steroid dependency for malignant MT (de Las Mulas *et al.*, 2005; Millanta *et al.*, 2005). In addition, the genetic underpinnings may be slightly different between the early benign and late malignant forms. This might be tentatively supported by the fact that *BRCA1* was more strongly associated in malignant than benign tumors in ESS, whereas *BRCA2* had similar association to both benign and malignant disease (Rivera *et al.*, 2009). Sorenmo *et al.* recently described that CMT progresses from benign to malignant, and that the malignant tumor may be the end stage of a histological continuum with clinical and histopathological similarities to human breast cancer carcinogenesis (Sorenmo *et al.*, 2009).

### 5.2.5 Survival after MT diagnosis

In the Cox Proportional Hazard Model for survival after diagnosis, a higher age of onset was associated with shorter survival times, with a hazard ratio of 1.67 for each additional year of age ($p < 0.001$). Benign tumors were associated with a more favorable outcome compared to malignant or unknown tumors with Hazard ratios of 0.53 and 0.47, respectively ($p = 0.038$).
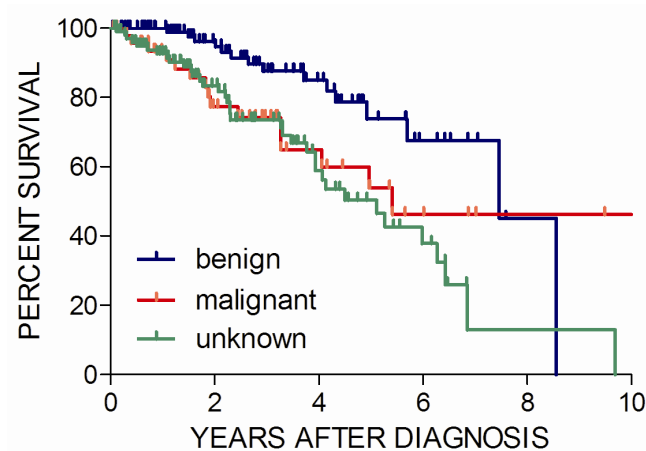


*Figure 8*. Kaplan–Meier survival function for overall survival time sorted by malignancy (all cause of death) (n = 66 deaths, 41 missing and 192 censored subjects, which are marked with ticks). Dogs with malignant MT or dogs with unknown MT malignancy had a shorter survival after diagnosis than dogs with benign MT, where the endpoint was any cause of death (log-rank test, $p = 0.021$).
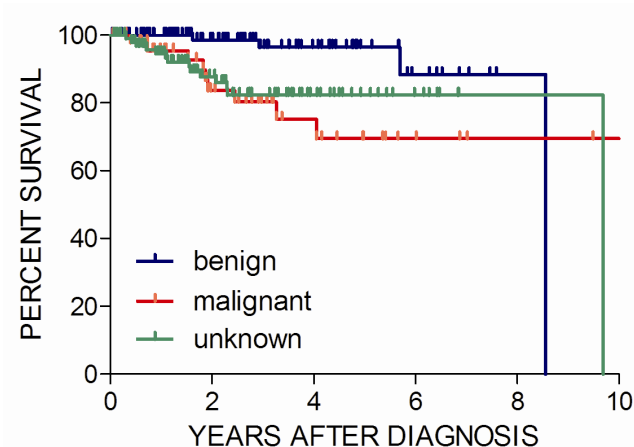
*Figure 9.* Kaplan–Meier survival function for overall survival time sorted by malignancy (death related to MT). (n = 20 deaths, 41 missing and 231 censored subjects, which are marked with ticks). Dogs with malignant MT had a shorter survival after diagnosis than dogs with benign MT where the endpoint was death due to MT (log-rank test, p = 0.018).

The survival data shows that dogs with malignant MT had a significantly shorter survival time after onset, regardless of whether the endpoint was any cause of death (log-rank test and multivariate Cox Proportional Hazard analysis), or death due to MT (log-rank test), than dogs with benign MT. One simple explanation may be that persistent or recurrent cancer is more likely to shorten the dog`s life span, but may not have been recorded as related to MT. Furthermore, the knowledge that the dog has MT could also affect the tendency for the owner to choose euthanasia, rather than attempt treatment in case of development of other disease. A large difference in survival time was found with respect to age of onset, a noted risk factor in women with breast cancer (Host & Lund, 1986).

## 5.3 Identification of genetic risk factors for CMT, using a candidate gene approach (Paper III)

The third study in this thesis is a candidate gene approach to CMT, in which we evaluate ten human breast cancer genes (*BRCA1, BRCA2, CHEK2, ERBB2, FGFR2, LSP1, MAP3K1, RCAS1, TOX3,* and *TP53*) for association with CMT. Sixty-three SNPs (4-9 SNPs per gene) were genotyped by Sequenom iPLEX in two sets of ESS female dogs (212 CMT cases and 143 controls), and association analysis was performed.

*Table 4.* Association of the best single SNP in each gene to CMT risk.

| Gene | Best $p_{raw}$ dataset 1 | Best $p_{raw}$ dataset 2 | Best $p_{raw}$ total | Best $p_{Bonf}$ total | Odds ratio total |
|------|------|------|------|------|------|
| *BRCA2* | **0.0032** | **6.7x10$^{-4}$** | **3.9x10$^{-6}$** | **1.4x10$^{-4}$** | 4.24 |
| *BRCA1* | **0.012** | 0.18 | **1.3x10$^{-4}$** | **0.0049** | 3.74 |
| *FGFR2* | **0.018** | 0.11 | **0.0047** | 0.18 | 1.88 |
| *TOX3* | 0.29 | 0.023 | **0.014** | 0.52 | 1.80 |
| *CHEK2* | 0.37 | 0.015 | **0.034** | 1.0 | 1.40 |
| *MAP3K1* | **0.025** | 0.54 | **0.042** | 1.0 | 1.43 |
| *LSP1* | **0.036** | 0.64 | 0.11 | 1.0 | 1.45 |
| *RCAS1* | 0.25 | 0.063 | 0.16 | 1.0 | 1.42 |
| *TP53* | **0.010** | 0.80 | 0.20 | 1.0 | 1.23 |
| *ERBB2* | 0.30 | 0.33 | 0.24 | 1.0 | 1.45 |
| $N_{cases}$ | 89 | 122 | 212 | 212 | 212 |
| $N_{controls}$ | 85 | 59 | 143 | 143 | 143 |

Abbreviations: $p_{raw}$: The best chi-square single SNP p-value obtained for each gene. Nominal association ($p_{raw}$ < 0.05) is indicated in bold. $p_{Bonf}$: Bonferroni-corrected p-values. Significant association is indicated in bold.

Two genes, *BRCA1* and *BRCA2,* were significantly associated with CMT (Bonferroni-corrected p = 0.005 and p = 0.0001, respectively) (indicated in bold in Table 4). Borderline association was seen for *FGFR2*. Benign and malignant cases were also analyzed separately. Germ line mutations in *BRCA1* and *BRCA2* are thought to account for 5% to 10% of all breast cancers in women (Ochiai *et al.*, 2001; Martin *et al.*, 2000), and our results suggest that they also predispose to CMT based on candidate gene association.

In this study we were able to detect association to risk factors conferring ~4-fold increased risk for both *BRCA1* and *BRCA2* to CMT, using data from 212 cases and 143 controls. This is in concordance with previous power calculations stating that canine complex traits can be mapped with a few hundred dogs (Lindblad-Toh *et al.*, 2005a), and demonstrates the advantages of mapping genetic risk factors in dogs compared to humans.

However, expanding the cohort in our study could possibly generate significant associations for additional genes, since the disease frequency is as high as ~36% in the ESS breed. This increased disease frequency could be caused either by many different risk factors accumulated within the breed or by a few risk alleles of very high frequency. The latter notion is supported by the high frequency (~90%) of both the *BRCA1* and *BRCA2* risk alleles in the healthy ESS dogs. Despite this high allele frequency, both risk factors

confer a ~4-fold increased risk, suggesting a complex etiology of multiple strong risk factors for this disease.

## 5.4  The potential role of MHC class II as a genetic risk factor in CMT (Paper IV)

In the last study, we investigated the potential role of MHC class II as a potential genetic factor for the development of CMT. It has long been recognized that genetic susceptibility to cancer may, in part, be due to inherited variations in MHC genes (Wu *et al.*, 2002; Sastre-Garau *et al.*, 1996; Apple *et al.*, 1994; Bidwell *et al.*, 1992; Pellegris *et al.*, 1980). The dog leukocyte antigen system (DLA) is believed to play a key role in the tumor cell`s escape from immune surveillance. In this study, we evaluated the role of MHC class II in controlling the immune response towards CMT tumor antigens, and we investigated the role of DLA class II in CMT development in dogs.

We performed sequence-based genotyping of the polymorphic exon 2 of DLA-DRB1, -DQA1, and -DQB1 class II loci, using DNA samples from 363 dogs. Comparison of allele and haplotype distribution between cases and controls revealed a statistically significant protective effect in dogs carrying the rare haplotype DLA-DRB1*00101/DQA*100201/DQB*01303 (odds ratio of 0.09, 99% CI 0.013 – 0.64) and the incidence of CMT. This haplotype confers an ~11-fold decreased risk for developing the disease and was present in 0.5% of the cases (only one dog with benign disease) and 4.8% of the controls (14 chromosomes). The haplotype includes, not surprisingly, the two rare significant DRB1 and DQA1 alleles (DRB1*00101 and DQA1*00201), combined with DQB1*01303. The DQB1*01303 allele is common and present in 43.1% of the total population (40.1% of the cases and 47.6% of the controls) and also found on haplotypes not associated with CMT. Our results indicate the DRB1 and DQA1 loci as the major contributors to the negative association with only minor influence from DQB1.

*Table 5*. Haplotype frequencies and association with CMT risk in the total population.

| No | DRB1/DQA1/DQB1 | Total % | Cases % | Controls % | Odds Ratio | $p_{\mathrm{raw(Yates)}}$ | 99% CI |
|----|----------------|---------|---------|------------|------------|---------------------------|--------|
| | | Datasets 1 + 2 (total) | | | | | |
| 1 | 01501/00601/00301 | 22.6 | 23.6 | 21.0 | 1.16 | 0.47 | 0.725–1.86 |
| 2 | 00501/00301/00501 | 9.4 | 9.9 | 8.6 | 1.16 | 0.66 | 0.588–2.288 |
| 3 | 00101/00201/01303 | 2.2 | 0.5 | 4.8 | **0.09** | **0.0002** | **0.0123–0.643** |
| 4 | 01201/00401/01303 | 34.7 | 32.8 | 37.6 | 0.81 | 0.21 | 0.539–1.219 |
| 5 | 02001/00601/00301 | 18.2 | 20.0 | 15.5 | 1.36 | 0.16 | 0.817–2.282 |
| 6 | 01501/00601/02002 | 6.2 | 6.9 | 5.2 | 1.35 | 0.46 | 0.585–3.135 |
| 7 | 02001/00401/01303 | 3.3 | 2.8 | 4.1 | 0.66 | 0.42 | 0.225–1.912 |
| 8 | 00601/005011/00701 | 2.2 | 3.0 | 1.0 | 2.94 | 0.14 | 0.558–15.489 |
| 9 | 01501/00601/01101 | 1.0 | 0.7 | 1.4 | 0.50 | ⋆ | 0.069–3.578 |
| 10 | 01201/00401/DQB013+017 | 0.3 | 0.0 | 0.7 | ⋆ | ⋆ | ⋆⋆ |
| | Total number (2n) | 726 | 436 | 290 | | | |

⋆ Sample size too small in this category for $X^2$ analysis.
Significant association 99% CI is indicated in bold.

Mechanistically, it is not known how the DLA association protects against CMT development. However, it is worth noting that the detected CMT association could be due to the DLA genes in themselves, or explained by linkage disequilibrium between the markers analyzed and other protective genes. One might speculate that in CMT the immune system plays an important role in protecting against tumorigenesis, as specific genetic variants may be better suited for facilitating removal of aberrant neoplastic cells. This is in concordance with previous studies showing the significance of immune surveillance against tumor development and progression (Ferguson *et al.*, 2002; Igney & Krammer, 2002). The immune response could potentially be directed against mutant self-proteins (Wolfel *et al.*, 1995) or against proteins expressed in a highly tissue-specific manner in the tissue of origin of the tumor (Coulie *et al.*, 1994; Brichard *et al.*, 1993). A third category of tumor antigen is represented by proteins that are expressed at high levels only in some tumors (Boel *et al.*, 1995; van der Bruggen *et al.*,

1991), including melanoma antigen MAGE1 in breast carcinomas (Brasseur *et al.*, 1992). The associated haplotype could possibly influence the efficiency by which cells present tumor antigens to T-cells.

Recent research has focused on the functional characteristics and the biological significance of MHC class I and II gene product expression and its relationship to processing and presentation of tumor antigens, as well as their role in anticancer treatment strategies against solid tumors. Feinmesser *et al.*, have shown that it is likely that the individual HLA specificities affect both the quality and quantity of antitumor immune response (Feinmesser *et al.*, 2000). Tissue typing in female patients has demonstrated an alteration of MHC antigen expression of breast carcinomas, which is usually related to prognosis (Concha *et al.*, 1991a; Concha *et al.*, 1991b). It will be interesting to see if the expression varies between the protective and other HLA haplotypes detected in this study, suggesting a connection to tumor surveillance relating to the relative levels of DLA antigens.

## 5.5 Some reflections from the author, based on the main findings of the thesis

Activity measurement of thymidine kinase 1 in serum was proven to be valuable for staging and monitoring treatment response in hematological neoplasia in both dogs and humans. Detecting expression of TK1 in serum, and especially tumor tissue, would likely expand usefulness of TK1 in solid tumors, as well. This could be clinically relevant, allowing refined grading of lymphomas and also solid tumors, including MT. This enzyme has an obvious potential as proliferation marker, via both activity measurements and protein expressions in the same tumor type. The comparative aspects are also obvious with the high homology to human TK1.

Cancer is a complex disease of all vertebrate species that can be considered as a breakdown in cellular homeostasis leading to uncontrolled cell division and proliferation, which ultimate leads to a disease state. Cancer formation is the phenotypic end result of a whole series of changes that may have taken a long period of time to develop. Cancer is a genetic disease, involving fundamental changes at the genetic level. Despite major efforts, identifying susceptibility genes for common diseases such cancer in humans remains extremely difficult. This is likely due to the complexity of the underlying causes, including genetic and environmental heterogeneity, and gene-by-gene and gene-by-environment interactions. Very large disease cohorts genotyped for hundreds of thousands of SNP markers are likely to be required to achieve satisfactory power. In order to accelerate the

discovery pace, studies that directly target human populations are therefore advantageously complemented by studies of more tractable animal models of human disease. Until recently, the only widely used model organisms for that purpose have been the mouse and rat. Dogs have a number of unique features that have the potential to make them a superior genetic system in which to study the molecular basis of cancer.

Changes in oncogenes or tumor suppressor genes may contribute to carcinogenesis. Changes or mutations in genes can lead to either a stimulatory or inhibitory effect on cell growth and proliferation. The stimulatory effects are provided by the proto-oncogenes, where mutations of these genes produce positive growth and proliferative signals, leading to uncontrolled cellular growth. In contrast, tumor formation can result from a loss of inhibitory functions associated with mutation of another class of cellular genes called the tumor suppressor genes.

The high frequency of MT in the Swedish ESS population (36%) (Egenvall *et al.*, 2005) might be explained by the inbreeding and accumulation of genetic risk factors within the breed. It has been suggested that the origin of MT is multifactorial and depends on an interaction between multiple major and minor genes and environmental factors. In study II we found that reproductive status of the dog appears to significantly influence MT development and that age of onset and histological subtype affects the survival time after diagnosis. These results were useful in better defining the phenotype, which facilitated the genetic analysis. The association data obtained in study III indicate that the two suppressor genes *BRCA1* and *BRCA2* are involved in the development of CMT. In their wild-type, or non-mutated state, the role of these two tumor suppressor genes is to inhibit cellular proliferation and growth. We also identified in study IV a rare MHC class II haplotype as a protective genetic factor against the development of CMT. One might expect a small number of strong risk factors driving up the frequency of MT in the Swedish ESS population, such as reproductive status, age of onset, and major genetic risk factors, for example, the common genetic variations of *BRCA1* and *BRCA2* associated with MT in this population. Alterations in these genes cause early onset MT in women, and it is possible that the ESS has an earlier onset of MT in this dog population with similar pathogenesis. The results also suggest a potential moderation of the frequency of MT based on the protective MHC haplotype in this population.

We have performed a genome-wide association (GWA) study using the Illumina 100K SNP arrays in 100 cases and 100 controls in Swedish ESS dogs and identified several candidate regions. Here we plan to complement

this dataset with an additional unrelated 100 cases and 100 controls. In total, 11 regions have been identified for further examination. Several of these contain plausible, but novel, candidate genes. We also plan to replicate the candidate genes identified in additional samples by fine mapping in ESS and other breeds with increased disease frequency (Boxer, German shepherd and Doberman dogs).

The identification of additional disease-associated markers could permit the future development of a genetic test for carriers of MT in these high-risk breeds. The suspected high frequency of these risk factors for CMT development in this breed could be problematic, because removal of breeding animals carrying these genetic alterations could create a serious genetic bottleneck, which would threaten the viability of the breed. Therefore, careful planning and testing is required to increase the frequency of the rare protective haplotype found in study IV, while avoiding the common alterations in *BRCA1* and *BRCA2* in these high-risk dog populations (study III).

In addition, further identification of the specific mutation(s) responsible for MT could permit further studies of the biological cause underlying mammary carcinoma in dogs, eventually allowing exploration of more effective therapies. The author also believes that it could be interesting to find whether tumors of histological classes are inherited together within a family. Our current analysis of a small set of familial cases also suggests that different tumor classes can be seen within a family or even a single individual. If this is generally true, a more basic predisposing genetic risk factor might be present, in addition to genetic risk factors determining tumor class. Thus, it would be reasonable to include different histological tumor types in the mapping effort to aim to identify more fundamental genetic mammary tumor risk factors: If our full analysis shows that different histological tumor types are not broadly inherited together, we would focus on a particular subset of tumors for the mapping effort.

It could be also interesting to perform large studies of gene expression in clinical samples of spontaneously occurring CMT (and others) in the near future. Hopefully, this can also be useful in a comparative fashion to enhance the understanding of tumorigenesis and metastatic pathways in both dogs and humans. By looking both at the inherited risk factors and the effects in expression by whole pathways, one might be able to link certain predisposing risk factors to specific changes in the cell. Knowing the faulty pathway might allow specific treatment options for each dog based on its genetic make-up, a little like personalized medicine.

Knowledge of the predisposing genes in dogs is bound to provide insights into the pathogenesis of the corresponding human breast cancer and to provide novel targets for drug development that would improve both canine and human health.

On the basis of the main findings in this doctoral thesis, the author would like to suggests that CMT is an excellent model for human breast cancer, indicating that both humans and dogs can benefit from further comparative studies and provide a foundation for further studies, which are necessary to understand the functional mechanisms whereby this protective factor influences the development of mammary tumors in dogs, and possibly also in humans. Veterinary oncology will benefit enormously from data derived from genomics, and this era will see a huge shift in the ways in which companion animal cancer patients are evaluated and consequently treated (Breen, 2009). The future seems very promising for veterinary clinical oncology, if we succeed in communicating the value of using tumors in companion animals as comparative models for human cancers, including mammary tumors.

# 6 Conclusions

Overall, the results of this doctoral thesis contribute new knowledge on canine mammary tumors (CMT). More precisely, the main conclusions were as follows:

➢ Serum thymidine kinase 1 was proven to be effective in diagnosing and monitoring dogs with hematological neoplasias (lymphoma, leukemia), but the Liaison assay was not effective in detecting solid tumors.

➢ Reproductive status is an important risk factor for MT development, and age of onset and histological subtype affect the survival time after diagnosis of MT affected dogs.

➢ Two human breast cancer genes, *BRCA1* and *BRCA2,* were associated with CMT in ESS dogs. The study suggests that CMT is an excellent model for human breast cancer, indicating that both humans and dogs can benefit from further comparative studies.

➢ A rare MHC class II haplotype, DLA (DRB1⋆00101/DQA1⋆00201/DQB1⋆01303), was identified as a protective genetic factor against the development of CMT in ESS dogs. This haplotype confers an ~11-fold decreased risk for developing CMT.

# 7 Future research

New insights have been gained regarding the characteristics of canine mammary tumors. More knowledge tends to generate even more questions, and that is the case with this thesis, also. There are several research areas that have been identified in which studies are ongoing or further research is needed:

➢ Activity measurement of TK1 in serum has been proven to be valuable for staging and monitoring treatment response in hematological neoplasia in both dogs and humans. Expression of the enzyme in serum, and especially tumor tissue, would likely expand usefulness of TK1 measurement in solid tumors, as well. Further studies are required to investigate the TK1 activity in solid tumors, including CMT to investigate its potential clinical utility.

➢ Further studies are necessary to investigate the role of DLA class II in the development of CMT, and to understand the functional mechanisms whereby this protective haplotype influences the development and malignancy of this disease in ESS dogs, and possibly also in humans.

➢ We have performed a genome-wide association (GWA) study using the Illumina 100K SNP arrays in 100 unrelated cases and 100 unrelated controls in Swedish ESS female dogs and identified several candidate regions. Here, we plan to complement this dataset with an additional unrelated 100 cases and 100 controls. In total, 11 regions have been identified for further examination. Several of these contain plausible, but novel, candidate genes. We also plan to replicate the candidate genes identified in additional samples by fine

mapping in ESS and other breeds with increased disease frequency (Boxer, German shepherd, and Doberman dogs). We aim also to characterize identified susceptibility genes in CMT, to establish mechanism and biological significance in dogs and humans. We also aim to perform relative risk assessments using the results from the candidate gene study, DLA, and the genes resulting from the GWA study together, when the exact mutations are known.

➢ A future goal is to transfer our results to human breast cancer cohorts and investigate whether the novel genes and pathways identified in the dog are important also in human breast cancer. This could provide a unique opportunity to improve prevention, diagnosis, and treatment of human breast cancer.

# 8 Populärvetenskaplig sammanfattning

## 8.1 Juvertumörer hos hund (kort bakgrund om sjukdomen)

Olika former av cancer är idag en av de vanligaste dödsorsakerna hos hundar. Såväl diagnostik som metoder att behandla cancer hos hund och katt har utvecklas dramatisk de senaste åren.

Tumörer i bröstvävnad, som hos hund kallas för juvertumörer, är den vanligaste typen av tumör både hos kvinnor och tikar, men sjukdomen är ungefär tre gånger vanligare hos hund än människa. Juvertumörer är den vanligaste tumörtypen hos icke-kastrerade tikar och den kan även i sällsynta fall uppträda hos hanhundar. Framförallt drabbas medelålders och äldre tikar.

Drygt 50 % av juvertumörerna är godartade och sprider sig inte till andra delar av kroppen. Dessa juvertumörer växer långsamt utom under löpperioderna då de på grund av påverkan från könshormonerna växer snabbare.

Elakartade tumörer har en potential att sprida sig med uppkomst av dottersvulster i andra organ och vävnader som följd. Tiken får ofta tumörer i flera juverdelar samtidigt och dessa tumörer kan vara av olika typer. Detta gör det svårt att på ett enkelt sätt diagnostisera om tumören är elakartad innan en eventuell operation. Det enda säkra sättet att ställa en definitiv diagnos är via en mikroskopisk vävnadsundersökning av tumören (biopsi). En biopsi ger också viktig information om huruvida man lyckats avlägsna all tumörvävnad eller inte. Detta prov kan ge säkrare upplysning om tumörtyp och vilken risk för återfall/spridning det finns efter operationen samt info om det är lönt att operera tiken igen och ta bort en större bit juvervävnad.

Juvertumörer behandlas ofta genom ett kirurgiskt ingrepp då hela tumören avlägsnas. Prognosen efter operation är individuellt mycket olika och beror på tumörens karaktär. Hos människa behandlas ofta nyopererade

kvinnor med cellgifter eller strålning. Detta är en behandling som idag används mycket sällan hos hund.

Den enda åtgärden som kan ha verkligt stor betydelse för att undvika att hunden får juvertumörer är en tidig kastrering. En tik som kastreras tidigt, helst före sitt första löp, får en dramatiskt minskad risk att drabbas av juvertumörer senare i livet. För att kastrering av tiken ska skydda mot juvertumörer är det viktigt att den utförs tidigt i livet, ju tidigare desto bättre. Detta beror på att tumören är beroende av könshormoner, som försvinner i och med kastrering. Risken för juvertumör ökar om hunden får p-sprutor eller annan hormonell behandling. Varken dräktighet, skendräktighet eller kastrering sent i livet påverkar risken för juvertumörer.

## 8.2 Hundar som modell för bröstcancerforskning

Tumörsjukdomar är genetisk komplexa vilket innebär att flera gener ligger bakom sjukdomsutvecklingen i kombination med oftast okända miljöfaktorer. Genom att hundens hela arvmassa numera är klarlagd har möjligheterna till genetisk forskning ökat markant. Hundar är idealiska för studier av sjukdomars genetiska bakgrund eftersom individerna inom varje hundras är genetiskt mycket lika och därför kan tänkas bära på samma genvarianter som ger ökad risk för sjukdom. Eftersom hundar är genetiskt mer lika människor än möss som vanligtvis används i forskning är hunden ett relevant modelldjur för grundforskning om bröstcancer**.**

Juvertumörer är överrepresenterade hos vissa hundraser och en ärftlig bidragande orsak misstänks därför. Exempel på hundraser som drabbas oftare än andra är engelsk springer spaniel, boxer, schäfer och dobermann. Korthårig collie är emellertid en hundras som sällan drabbas. Med kunskapen om vilka genetiska komponenter som ligger bakom sjukdomen kan bättre diagnostiska och behandlingsmetoder utvecklas som kan komma till nytta både för hunden som människan.

## 8.3 Sammanfattning av avhandlingsarbetet

Syftet med avhandlingsarbetet var att öka kunskapen om juvertumörer samt att identifiera olika riskfaktorer för utvecklingen av dessa tumörer hos hund. För att uppnå dessa mål utfördes fyra studier.

I den första studien studerades aktiviteten av *thymidinkinas* (TK) i serum hos hundar som drabbat av malignt lymfom, leukemi samt andra tumörer bl.a. juvertumörer. Sedan en längre tid har man känt till att framförallt hematopoetiska tumörer ger upphov till förhöjda nivåer av proteinet

*thymidinkinas* (TK) i serum hos människa. TK är ett enzym som är involverat i DNA-syntesen. Det produceras i celler före celldelning och har visat sig vara en god indikator för ökad celldelning. Thymidinkinas frigörs ut till cirkulationen när snabbt delande celler dör och cellmembranen går sönder. TK är distinkt kopplade till tumörförekomst och grad av malignitet. Thymidinkinas är en etablerad celldelningsmarkör som används kliniskt på humansidan.

Vi fann att detta enzym var effektivt för diagnosticering samt monitorering av hundar med blodcancer (lymfom, leukemi). Denna studie visade dock att detta test var otillräcklig för att kunna användas för att upptäcka solida tumörer i det studerade materialet. Detta ledde oss till att studera andra potentiella riskfaktorer för utvecklingen av juvertumörer hos hund för att kunna ta fram bättre diagnostiska verktyg.

I de följande tre studierna, användes hundar av rasen Engelsk springer spaniel för att studera olika risk faktorer för utveckling av juvertumörer hos hund. Vi fann i den andra studien att kastrering minskar risken för juvertumörer samt att hundens ålder vid diagnos och typ av juvertumör påverkar hundens överlevnadstid efter diagnos.

I den tredje och fjärde studien undersöktes olika genetiska riskfaktorer för utveckling av juvertumörer hos svenska ESS. I dessa studier försökte vi hitta gener som predisponerar för utveckling av juvertumörer. Genom att jämföra arvsmassan hos sjuka och friska hundar hittade vi genvarianter som påverkar risken för juvertumörer hos ESS.

I den tredje studien konstaterades för första gången att de sedan tidigare kända riskfaktorerna för utveckling av bröstcancer hos människa, *BRCA*1 och 2) även utgör riskfaktorer för juvertumörer hos hund.

I den sista studien undersökte vi om olika varianter av immungenerna Major Histocompatibility Complex II (MHC) klass II har någon påverkan på förekomst av juvertumörer i ESS hundarna. MHC klass II är en typ av antigenpresenterande molekyler som finns på antigenpresenterande celler i kroppen. Vår studie visade att en variant av MHC (DRB1*00101/DQA1*00201/DQB1*01303) hade en skyddande effekt mot juvertumörer.

Fortsatta studier gällande den genetiska komplexiteten av juvertumörer hos hund pågår för närvarande. Genom att förutsättningslöst jämföra variation i hela genomet hos sjuka och friska hundar, hoppas vi kunna identifiera ej tidigare kända riskfaktorer för uppkomst av juvertumörer. Genom att identifiera riskfaktorer för juvertumörer hoppas vi öka förståelsen för sjukdomen vilket förhoppningsvis även kan bidra till en ökad förståelse av bröstcancer hos människa.

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