# Background information:

Pet dogs spontaneously develop many of the same cancer types as humans making them a valuable comparative cancer model for understanding carcinogenesis and risk factors as well as for running clinical trials on new therapeutic drugs. The detailed sub-classification within specific cancer types is however limited in canine cancers hence the direct translational of specific canine cancer types needs to be improved. Further tools for easily monitoring disease progression or relapse are lacking in the dog. This project focusses mainly on three different types of canine cancer canine mammary tumors, canine osteosarcoma and multicentric lymphoma.

In canines, mammary tumors are the most common type of neoplasia affecting non-neutered female dogs (Egenvall, A., et al. 2005). The tumors can be divided into subgroups mainly based on their histopathological features. Attempts to classify canine mammary tumors using similar classification system as in humans have not been successful as they have been based on immunohistochemistry using poorly validated antibodies or on expression data for a small panel of genes and individuals (Park, Y.Y., et al 2012, Sleeckx, N., et al. 2011, Campos, L.C., et al. 2015[[1]](#footnote-1), , Luder Ripoli, F., et al., 2016, Damasceno, K.A., et al., 2016). The utility of canine mammary tumors as a model for human breast cancer in clinical trials has therefore been limited. Still, the route to metastasis for canine mammary tumors is similar to human breast cancer. Infiltration of the local lymph node or distant metastasis to lung tissue, bone or brain makes the dog a good model for metastatic disease (Sleeckx, N., et al. 2011, Kim, J.H., et al. 2011). Hence, dogs would provide a good model for human metastatic breast cancer if subtyping challenges could be overcome using molecular tools.

## Lymphoma, a heterogeneous cancer in both species.

Among both humans and dogs, diffuse large B-cell lymphomas (DLBCL) make up the majority of non-Hodgkin’s lymphoma cases. Human DLBCL is classified into ABC and GCB subtypes based on gene expression. While similar subtypes have been suggested for canine DLBCL (Richards, K.L., et al., 2013), additional studies are needed to confirm which canine lymphomas are good models for specific human subtypes. In addition, peripheral T-cell lymphoma not otherwise specified (PTCL-NOS) is relatively rare in humans and has a poor prognosis. PTCL-NOS is considerably more common among dogs (Seelig, D.M, 2016), for example in the boxer breed. Since murine transgenic or knock-out PTCL-NOS models vary in how well they recapitulate the human phenotype (Cutucache, C.E. and T.A. Herek, 2016), a canine model with spontaneously occurring disease could greatly improve our understanding. Detailed matching of subtypes between the species will greatly inform the translation of canine clinical data to human research. The social and economic impact of lymphoma is high, both for dogs and humans.

## Osteosarcoma, common in large dog breeds.

Human osteosarcoma is most common in teenagers and young adults. It is a relatively rare, but often deadly disease. In large dog breeds osteosarcoma is more prevalent than in other breeds, with close to 13% of Rottweilers getting osteosarcoma. Like in humans (Ottaviani, G. and N. Jaffe 2009), male dogs are slightly more likely to acquire the disease compared to females (Selmic, L.E., et al. 2014), allowing evaluation of hormone-related factors. Human studies have pointed to a high rate of TP53 mutations and copy number aberrations in human tumors. Still, human studies are limited by the rarity of the disease, and a good model system is needed. Exome sequencing of osteosarcoma in dogs has shown that many somatic genetic aberrations are shared between human and canine osteosarcoma however there are also some unique cancer driving genes in detected in canine osteosarcoma. Further investigating this field could allow sub-classification to match canine tumor subtypes with human osteosarcoma.

# Design

## WP1: Evaluation of ERBB2 (human HER2) copy number variation in canine mammary tumors

**Objective:** To design a copy number variation assay targeting the canine ERRB2 gene (human HER2). To use this assay to estimate the copy number assay in paired canine mammary tumor and normal DNA from the same individual and correlate this with tumor histology.

**Methods:** Primers and probes compatible with droplet digital PCR (Bio-Rad Laboratories, Inc) will be designed using commercially available PCR design tools such as Primer 3 plus (Untergasser et al. 2012). Primer and probes will be designed for targeting unique sequences in the ERRB2 in based on the sequence available in UCSC browser CanFam3.1 (Rosenbloom et al. 2015). Probes and primers will be tested in silico to assure the specificity. Already designed and validated reference probes and primers will be ordered targeting the c7orf28B as previously published (Arendt, M. et al. 2014)

Sample material for the assay will be extracted from the Dog DNA biobank shared between Uppsala University and the Swedish University of Agriculture. All samples included in the study have already been collected with full owner consent.

DNA will be extracted from EDTA blood and RNA fixated tumor tissues from 60 dogs with mammary tumors using Quiagen DNA and RNA extraction kits (Qiagen, Hilden, Germany).

DNA will be digested with a restriction enzyme which will cut out the targeted areas for both the reference and the target probe. Droplet digital PCR will be run using the BioRAD Digital PCR (Bio-Rad Laboratories, Inc) instrumentation and reagents according to the manufacturers protocol. Initial validation and optimization steps will be performed on a test samples, to optimize DNA input concentration and reaction temperature.

**Milestone 1:** Evaluate ERRB2 copy number in 60 tumor normal tissues from dogs with mammary tumors and compare this with histopathology. Summarize results in research paper.

Centrifuge cost -15%, or rotor.

**Risk assessment:** Please see risk assessment in the later section.

**WP2: Identification of somatic variants by comparing exome data from tumor and normal tissue and the relationship with tumor stage, grade and clinical outcome.**

**Objective:** To classify tumors based on their mutational landscape and to compare this to human subtypes. Focus will be on three canine tumor types mammary tumors, osteosarcoma and multicentric lymphoma and their comparative human diseases. To understand the relationship between the mutation profiles and the clinical characteristic of the individual cancers.

**Materials and methods:** Veterinarians from Scandinavia which are part of the Scandinavian Veterinary Oncology Consortium (SVOC) and veterinarians in the US as well as the PhD student will participate in collecting sample material for the work in this work package. Peripheral blood samples and tumor tissue biopsies will be collected in conjunction with surgical therapy or other procedures. For individuals were surgery is declined needle aspirates can be taken as an alternative and stored in lysis buffer. Samples will only be collected from owners from which a written informed consent has been signed. Clinical parameters will be collected for each patient, including animal age, stage, breed, histopathological diagnosis, tumor location, tumor size, co-morbidities and current medication. Blood samples will be aliquoted and frozen immediately at -80. Tissue samples will be put in RNAlater and stored 24 hours at 4C and then frozen at -80 until further usage. Needle aspirates will be stored in lysis buffer and frozen immediately at -80C.

When 40 tumor and normal samples have been collected from a particular tumor type then DNA and RNA will be extracted will be extracted from tissue and DNA will be extracted from peripheral blood using the Quiagen AllPrep kit and the Qiagen DNA mini kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). Exome libraries will be prepared using the Nimblegen (Roche Molecular Systems, Inc) canine exome liquid capture kit. Exome libraries will be sequenced using Illumina sequencing. Sequencing data will be aligned using the GATK best practices and the UCSC CanFam 3.1 reference genome (Untergasser, et al. 2012). Somatic mutations will be called from the tumor tissues using the Mutect2 software (do Valle, I.F., et al. 2016). Clinical parameters will be evaluated in view of the tumor driving mutations and mutational signatures.

**Milestone 3:** To collect, sequence and analyze at least 40 tumor / normal pairs from one of the selected tumors. To compare these to tumor normal data from equivalent human tumor types and to summarize the results in a scientific paper.

**Risk assessment**: See section on risk assessment below.

## WP3: Identification of somatic mutations in liquid biopsies and the use of these for diagnosis, monitoring disease relapse and progression.

**Objective:** To identify somatic mutations in liquid biopsies (blood samples) from dogs with mammary tumors and the use of these for monitoring disease stage, relapse and progression.

**Materials and methods:** Dogs being treated with surgery for a high grade mammary simple carcinoma will be enrolled in the study. Only dogs for which the owner has consented to participate in the study will be included. Blood samples will be collected at the time of surgery and immediately after and stored at -80C for further usage. A tissue biopsy will be collected at the time of surgery and frozen in RNAlater. The patient will be followed by a clinical exam every 2nd month for the first year and then every 6 months for a year. Tumor recurrence will be estimated by palpation of the mammary tissue and regional lymph nodes as well as a complete clinical exam. Chest radiographs and abdominal ultrasound will be performed if strong suspicion of metastasis is present.

Cell free DNA and cellular DNA will be extracted from all blood samples and tumor DNA will be extracted from the tumor. The cellular DNA and tumor DNA will be sequenced to identify somatic mutation in the tumor tissue. The cell free DNA fraction will be sequenced at low coverage for screening (1x) to evaluate if there are tumor DNA present in this fraction. If reproducible somatic mutations are found in the cell free DNA fraction, then deeper coverage sequencing will be pursued (10x-30x).

**Milestone 2:** Document the presence of cell free tumor DNA in the peripheral blood samples (liquid biopsies) from 10 dogs with malignant mammary tumors at the time of diagnosis and at the time of relapse. Summarize the results and the materials used in a scientific paper and reflect on further usage of this method for diagnostic purposes.

**Risk assessment**: See section on risk assessment below.

1. [↑](#footnote-ref-1)