Department of Veterinary Clinical Sciences

Faculty of Health and Mecical Sciences

University of Copenhagen

# Applied canine cancer and comparative genetics - Research Protocol

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# Investigators and responsibilities

|  |  |
| --- | --- |
| **Responsibility** | **Name and Address of Investigator** |
| **Principal Investigator**  **(Sample collection & data stuff. Enrollment and evaluation of canine cancer patients and communication with clients)** | Sophie Agger, DVM, PhD student  Companion Animal Oncology Service and Research Group  Department of Veterinary Clinical Sciences  Faculty of Health and Medical Sciences  University of Copenhagen  Dyrlægevej 16  DK 1870 Frederiksberg C  Email: sesa@sund.ku.dk  Phone: +45 26 35 06 74 |
| **Co-investigator**  **(Sample collection & data stuff. Enrollment and evaluation of canine cancer patients and communication with clients)** | Maja Arendt, DVM, DECVIM-CA oncology, Associate professor  Companion Animal Oncology Service and Research Group  Department of Veterinary Clinical Sciences  Faculty of Health and Medical Sciences  University of Copenhagen  Dyrlægevej 16  DK 1870 Frederiksberg C  Email: maja.arendt@sund.ku.dk  Phone: +45 93 50 95 46 |
| **Co-investigator**  **Overall responsible in Sweden** | Kerstin Lindblad-Toh  Comparative Genetics and Genomics Department of Medical Biochemistry and Microbiology Uppsala Universitet  Husargatan 3  SE 751 23 Uppsala  Email: [imbim@imbim.uu.se](mailto:imbim@imbim.uu.se?subject=e-postadress)  Phone: +46 018 - 471 44 44 |
| **Co-investigator**  Overall responsible in Denmark | Annemarie T Kristensen, DVM, PhD, DECVIM-CA and Oncology, Professor  Companion Animal Oncology Service and Research Group  Department of Veterinary Clinical Sciences  Faculty of Health and Medical Sciences  University of Copenhagen  Dyrlægevej 16  DK 1870 Frederiksberg C  Email: atk@sund.ku.dk  Phone: +45 23 81 24 22 |
| **Veterinary technician** | Mette Hedelund Rasmussen, Veterinary nurse,  Companion Animal Oncology Service and Research Group  Department of Veterinary Clinical Sciences  Faculty of Health and Medical Sciences  University of Copenhagen  Dyrlægevej 16  DK 1870 Frederiksberg C  Email: mera@sund.ku.dk  Phone: +45 21 28 86 59 |
| **Dog biobank** | Susanne Gustafsson, Dog biobank coordinator Swedish University of Agricultural Sciences Email: susanne.gustafsson@slu.se  Phone: +46 018-67 20 12 |

# Introduction

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.

## Mammary tumors

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, 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 (Sleeck, 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

## Part 1:

### Identification of somatic variants by comparing sequencing data from tumor and normal tissue and the relationship with tumor stage, grade and clinical outcome.

**Aim:** 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.

**Objective:** To collect, sequence and analyze tumor/normal samples 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.

## Part 2:

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

**Aim:** 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 high-grade mammary tumors 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).

**Objective:** 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.

# Methods and Materials

## Study population

Dogs will be enrolled from the general patient population at the University Hospital for Companion Animals.

### Inclusion

Dogs can be included without regards for other diagnoses including other tumors.

#### *Part 1:*

Canine cancer patients with any malignant mammary tumor, multicentric lymphoma, or any osteosarcoma where it is possible to take samples without compromising diagnosis or animal welfare.

#### *Part 2:*

Canine cancer patients with malignant mammary tumors where it is possible to take samples without compromising diagnosis or animal welfare.

### Exclusion

Not possible to collect the appropriate amount of blood

BW < 2 kg

Anemia

#### *Part 2:*

The dogs must be able to tolerate follow-up visits including clinical examination and blood draw. If the dog is assessed to be too affected/stressed by these procedures, the dog will be excluded. The dog must not suffer from other cancers

### 

### Number of patients

#### *Part 1:*

A minimum of 20 patients in total are expected and a maximum of 120 patients will be enrolled.

#### *Part 2:*

A minimum of 10 and a maximum of 30 dogs with malignant mammary tumors will be enrolled. The patients from Part 1 can be included in part 2 if they meet the additional inclusion criteria.

## Samples collection:

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.

All samples will be collected with gloves and contact with samples should be minimized.

### Blood samples

Peripheral blood samples will be collected in conjunction with surgical therapy or other procedures.

No extra precautions to avoid DNA contamination during collection is needed. Blood samples will be aliquoted and frozen immediately at -80˚C.

For isolation of circulating cell-free DNA (ccfDNA), at least 4 ml blood will be collected and processed as described in Appendix 1.

### Needle aspirates

For individuals were surgery and biopsies are declined, needle aspirates can be taken in conjunction with diagnostic needle aspirates. The area will be cleaned with ethanol before sampling and the samples will be performed with syringe attached and suction applied. This is both to increase cell yield but also to avoid contamination of the syringe with human DNA. As the suction increases the risk of bleeding in the tumor, aspirates for cytology should be taken first.

Needle aspirates will be gently suspended in 600 μl RLT plus buffer by gently suctioning the RLT plus buffer in and out of the syringe. The sampling can be repeated and resuspended in the same RLT plus buffer. Samples are stored in 600 μl of lysis buffer (RLT plus) and frozen immediately at -80˚C.

A matching cytology slide will be evaluated by the Cytology service, Central Laboratory, University of Copenhagen to ensure the samples are representative.

### Tumor tissue biopsies

0.3 x 0.3 x 0.3 cm tumor tissue biopsies will be collected in conjunction with surgical therapy or other procedures. Tissue samples will be put in RNAlater and stored 24 hours at 4˚C and then frozen at -80˚C until further usage. If possible, an adjacent tumor sample will be fixed in 10% formalin and sent to a laboratory for histopathological evaluation

### 

### Post processing

When tumor/normal samples have been collected, the DNA and RNA will be extracted from tissue and extracted from peripheral blood using the Quiagen AllPrep kit and the Qiagen DNA mini kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). If exome sequencing is chosen, 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.

## Diagnostics and procedures

In order to maximize the phenotypical information about each patient, any abnormalities found during work-up and staging will be recorded. The project does not pay for any paraclinical tests per default and therefore, the amount of work-up is at the discretion of the owner. If the owner is unwilling to perform any diagnostic tests, the project will pay for cytology/histopathology.

### Blood and Urine analysis

Routine blood analysis consists of hematology and biochemistry, routine urine analysis will consist of specific gravity. Analysis will be performed by the Central Laboratory, University of Copenhagen and to their internal standards.

### Staging and Grading Procedures

Staging and grading procedures are performed using standard procedures according to WHO and current literature for the malignancy under investigation (Appendix 2). Grading will be performed by the pathologists at the laboratory (either SLU or Idexx).

# Appendix 1: Procedure for isolation of ccfDNA

For additional details see QIAamp ® MinElute ® ccfDNA Handbook 08/18.

## Plasma Separation and Storage for isolation of ccfDNA[[1]](#footnote-1)

To isolate circulating, cell-free nucleic acids from blood samples, we recommend following this protocol, which includes a high g-force centrifugation step to remove cellular debris and thereby reduce the amount of cellular or genomic DNA and RNA in the sample. Whole blood must be stabilized to separate circulating nucleic acids from genomic DNA. Appropriate tubes are the **BD Vacutainer (or other primary blood tubes) containing EDTA** as an anti-coagulant, or Streck Cell-Free DNA BCT.

Procedure

1. Collect at between 4 and 10 ml of blood in EDTA tubes with a maximum of 1.6 ml / kg including any samples taken for paraclinical tests.
2. Centrifuge the blood samples for 10 min at 1900 x g (3000 rpm) with temperature set to 4°C (the large centrifuge in vet-lab can perform this).
3. Carefully aspirate plasma supernatant from each tube without disturbing the buffy coat layer.
4. Transfer aspirated plasma into the required number of eppendorph conical-bottom tubes.
5. Centrifuge the plasma samples for 10 min at 13 000 rpm (maximum speed) on the small centrifuge in room next to the large centrifuge with temperature set to 4°C.
6. Using a pipette, carefully transfer the supernatant from all eppendorph tubes into the necessary number of cryo-tubes without disturbing the pellet.
7. Freeze at –80°C. Before using the plasma for circulating nucleic acid extraction, thaw plasma tubes at room temperature.

## Protocol: Purification of Circulating DNA from 1–4 ml Serum or Plasma using the QIAamp MinElute ccfDNA Mini Kit

### Procedure

1. Mix components according to Table 1 in a 15 ml tube (not provided), and incubate for 10 min at room temperature (15–25°C) while shaking (slow speed) end-over-end.
2. Spin briefly (30 s at 200 x g) to remove any solution in cap.
3. Place the tube containing bead solution into a magnetic rack for 15 ml tubes. Let stand for at least 1 min, until the solution is clear. Discard supernatant.
4. Remove the tube from the magnetic rack and add 200 µl Bead Elution Buffer to the bead pellet. Vortex to resuspend beads, and pipet up and down to mix and rinse residual beads from the tube wall. Transfer the mixture (including beads) into a Bead Elution Tube. Incubate for 5 min on a shaker for microcentrifuge tubes at room temperature and 300 rpm.
5. Note: If the same shaker for microcentrifuge tubes is to be used in step 10, remove the tubes after the room temperature incubation and equilibrate the shaker to 56°C.
6. Place the Bead Elution Tube containing the bead solution into a magnetic rack for 2 ml tubes. Let stand for at least 1 min, until the solution is clear.
7. Transfer the supernatant into a new Bead Elution Tube. Add 300 µl Buffer ACB, and vortex to mix. Briefly centrifuge the tube to remove drops from inside the lid. Discard the bead pellet.
8. Pipet the supernatant–Buffer ACB mixture from step 6 into a QIAamp UCP MinElute column, and centrifuge for 1 min at 6000 x g. Place the QIAamp UCP MinElute column into a clean 2 ml collection tube, and discard the flow-through.
9. Add 500 µl Buffer ACW2 to the QIAamp UCP MinElute Column, and centrifuge for 1 min at 6000 x g. Place the QIAamp UCP MinElute column into a clean 2 ml collection tube, and discard the flow-through.
10. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.
11. Place the QIAamp UCP MinElute column into a clean 1.5 ml elution tube (provided), and discard the 2 ml collection tube from step 9. Open the lid, and incubate the assembly in a shaker for microcentrifuge tubes at 56°C for 3 min to dry the membrane completely.
12. Carefully pipet 20–80 μl of Ultra-clean Water into the center of the membrane. Close the lid and incubate at room temperature for 1 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min to elute the nucleic acids.

Note: To maximize yield eluted in 20–80 µl, reapply the eluate to the column for reelution. Place the QIAamp UCP MinElute Column in a clean 1.5 ml elution tube (not provided). Aspirate the eluate in the 1.5 ml elution tube from step 9 and reload it onto the center of the membrane. Close the lid, and incubate 1 min at RT. Centrifuge 1 min at full speed (20,000 x g; 14,000 rpm).

# Appendix 2: Staging

## Osteosarcoma

Standard clinicial examination

Palpation of regional lymph nodes and cytology if enlarged.

3-view thoracic radiographs or thoracic CT

### Staging system

The Enneking[[2]](#footnote-2) system will be used for stage grouping.

Histologic grade

G1: Low grade

G2: High grade

Tumor

T1: Intracompartmental

T2: Extracompartmental

Regional lymph node involvement

N0: No regional lymph node involvement

N1: Regional lymph node involvement

Distant metastases

M0: No distant metastases

M1: Distant metastases

Table 1: Staging system for canine osteosarcoma

|  |  |  |  |
| --- | --- | --- | --- |
| Stage | Grade | Tumor | Metastasis |
| IA | G1 | T1 | M0 |
| IB | G1 | T2 | M0 |
| IIA | G2 | T1 | M0 |
| IIB | G2 | T2 | M0 |
| III | Any | Any | M1 |

## Lymphoma

Standard clinical examination

Lymph node cytology to diagnose

At least 3 different lymph nodes, preferably not lnn. maxillaris

Flowcytometry to confirm cytologic diagnosis and subtyping

At least 3 different lymph nodes, preferably not lnn. maxillaris

3-view thoracic radiographs or thoracic CT

Abdominal ultrasound

Bone marrow biopsy on indication

### Staging system

The staging system applied is the World Health Organization’s Clinical Staging System for lymphoma in Domestic Animals.

Table 2: Staging system for canine lymphoma

|  |  |
| --- | --- |
| Anatomical site | |
| A | Generalized |
| B | Alimentary |
| C | Thymic |
| D | Skin |
| E | Leukemia (only blood and bone marrow involved) |
| F | Others |
| Stage | |
| I | Involvement limited to a single node or lymphoid tissue in a single organ [[3]](#footnote-3) |
| II | Involvement of many lymph nodes in a regional area (± tonsils) |
| III | Generalized lymph node involvement |
| IV | Liver and/or spleen involvement |
| V | Manifestation in the blood and involvement of bone marrow and/or other organ systems |
| a | Without systemic signs |
| b | With systemic signs |

## Mammary tumors

Standard clinical examination.

Lymph node cytology to diagnose of regional and/or enlarged lymph nodes if feasible.

For M1-2: Bilateral lnn. axillaris  
 For M3: Bilateral lnn. axillaris and inguinales superficialis  
 For M4-M5: Bilateral lnn. Inguinalis superficialis

3-view thoracic radiographs or thoracic CT

Abdominal ultrasound

### Staging system

The staging system applied is a modified version of the system published by Owen in 1980[[4]](#footnote-4).

Tumor

T1: < 3 cm

T2: 3-5cm

T3: > 5 cm

Regional lymph node involvement

N0: No regional lymph node involvement

N1: Regional lymph node involvement

Metastases

M0: No distant metastases

M1: Distant metastases

Table 3: Staging system for canine mammary tumors

|  |  |  |  |
| --- | --- | --- | --- |
| Stage | Tumor size | Lymph node involvement | Metastasis |
| I | T1 | N0 | M0 |
| II | T2 | N0 | M0 |
| III | T3 | N0 | M0 |
| IV | Any | N1 | M0 |
| V | Any | Any | M1 |

1. QIAamp MinElute ccfDNA Handbook 08/2018 [↑](#footnote-ref-1)
2. Enneking WF, Spanier SS, Goodman MA: A system for the surgical staging of musculoskeletal sarcoma, Clin Orthop Relat Res 106–120, 1980. [↑](#footnote-ref-2)
3. Excluding bone marrow [↑](#footnote-ref-3)
4. Rutteman G, Withrow S, MacEwen E: Tumors of the mammary gland. In Withrow S, MacEwen E, editors: Small animal clinical oncology, ed 3, Philadelphia, 2001, WB Saunders. [↑](#footnote-ref-4)