Confidential: Circulating cell-free DNA test for monitoring cancer treatments in dogs

Milja Tikkanen[1], Emmi Hurskainen[1], Zoja Verlic[2], Ursa Lamprecht[2], Alenka Nemec Svete[2], Emy Funk[3], Petra Jaakonsaari[1], Bence Berki[1,6], Saila Holopainen[4], Jenni Sukura[4], Hannes Lohi[4], Juha Kere[4], William von der Pahlen[1], Gugan Eswaran[1], Arno Roos[3], Natasa Tozon[3*], and Katja Kivinen[1*§]*

[1] DeepScan Diagnostics, Helsinki, Finland
[2] University of Ljubljana, Ljubljana, Slovenia
[3] EDZ Nieuwegein, Nieuwegein, the Netherlands
[4] University of Helsinki, Helsinki, Finland
[5] Karolinska Institute, Stockholm, Sweden
[6] Present address: Labsystems Diagnostics, Helsinki, Finland

** Authors contributed equally*
§ Send correspondence to katja@deepscandx.com

Abstract

Circulating cell-free DNA (cfDNA) is a promising biomarker for monitoring the patient's response to therapeutic treatments. This study evaluated the utility of cfDNA to assess cancer treatment response. Plasma samples were collected from 55 dogs with cancer and 69 healthy controls. Plasma cfDNA was quantified before the start of the treatment and prior to each treatment cycle for patients, and every three months for healthy controls. cfDNA levels changed rapidly in response to treatment success and cancer recurrence, and stayed stable in healthy dogs. The results suggest that measuring plasma cfDNA throughout a cancer treatment is a powerful biomarker for monitoring treatment success in dogs.

Introduction

Circulating cell-free DNA (cfDNA) is a marker of cell death. It is present at low levels throughout life and becomes more abundant during pathological processes including cancer. Due to its properties, cfDNA has emerged as a promising biomarker for cancer monitoring in both human and veterinary oncology. In dogs, cfDNA offers a non-invasive method for assessing treatment response, detecting minimal residual disease, and predicting cancer recurrence. Monitoring cfDNA dynamics over time can provide valuable insights into the efficacy of therapeutics, complementing imaging and histopathology as monitoring tools. The application of cfDNA-based monitoring is a fast-evolving field with open questions around standardisation, tumour-specific validation, and pre-analytical variability.

Previous studies have explored cfDNA analysis in canine cancers with focus on quantitative measurements and molecular features. Several papers have demonstrated that cfDNA concentrations change in response to therapy, with decreases observed in patients responding to therapy and increases associated with disease progression and relapse. In lymphomas and multicentric cancers, cfDNA levels have been shown to outperform clinical evaluations in early relapse detection by up to six weeks. DNA integrity index, a measure of cfDNA fragmentation, has also been investigated as a potential biomarker for treatment

response, particularly in transmissible venereal tumours (TVT) and oral malignant melanoma (OMM). In OMM, cfDNA concentration and DNA integrity index reflected disease progression and treatment efficacy. Similarly, weekly cfDNA monitoring showed reduced concentration and changes in DNA integrity index during successful treatment.

Beyond cfDNA quantification, studies have examined its broader molecular properties. Fragmentation patterns, such as the prevalence of short (<150 bp) DNA fragments, have been linked to tumour burden and treatment response dynamics. Genomic aberrations including copy number and structural variations have also shown promise for monitoring therapeutic response and cancer recurrence. While epigenetic markers have been proposed as potential high-specificity biomarkers, their application in canine oncology remains largely unexplored.

Several challenges complicate the implementation of cfDNA monitoring in veterinary medicine. Pre-analytical factors, including sample collection and processing protocols, significantly influence cfDNA stability and measurement reproducibility. Additionally, assay sensitivity is affected by tumour type -specific variability with lymphoma, OMM, and TVT being the most well-studied cancers in canine oncology. This body of research suggests that cfDNA has clear potential as a dynamic biomarker for treatment response in canine cancers. However, further studies are needed to optimise standardisation, investigate more tumour types, and refine methodology to improve clinical utility.

Here, we demonstrate the general utility of cfDNA as a biomarker for monitoring canine cancer treatments including haematological and solid tumours, and show that with careful control of pre-analytical variability, it is possible to generate highly reproducible and reliable results.

Materials and Methods

Blood samples were collected from 55 dogs with cancer and 69 healthy controls between February 2023 and December 2024. All samples were collected by veterinary professionals and with the dog owner's consent. Each dog's age, breed, sex, neutering status, diagnosed clinical conditions, and planned treatment were collected using standard questionnaires and entered into an internal database.

Cancer patients were recruited through academic and private animal hospitals in Finland, Netherlands, and Slovenia. Cancer diagnosis was based on clinical examination, blood tests, imaging, and cytology or histopathology, as deemed necessary by the treating veterinarian. A baseline sample was collected from treatment-naive dogs during their first treatment visit. Follow up samples were taken prior to each treatment cycle and for up to 12 months after the treatment had ended. Sample collection frequency was cancer type- and treatment-specific.

Healthy dogs were recruited in Finland in collaboration with breed associations. To be eligible to join the study, dogs had to be clinically healthy, at least five years old, and not pregnant at the start of the study. Samples were taken every three months as part of an ongoing study in which middle aged dogs are followed for two years with serial sampling to assess the effect of preanalytical variability to cfDNA measurements.

For the cfDNA analysis, venous whole blood was drawn as part of the usual blood tests. No separate instructions were given regarding feeding or drinking, as a separate study on pre-analytical variability showed that lipemia did not have a statistically significant effect on the test results *(data not shown - refer to preanalytical factors paper)*.

Internal controls were added to each plasma sample in known quantities before cfDNA extraction as a quality control measure. cfDNA was extracted from thawed plasma using an appropriate kit according to the manufacturer's instructions. cfDNA was eluted into an elution buffer provided by the kit manufacturer and stored frozen until further analysis. cfDNA was analysed using capillary electrophoresis according to the manufacturer's instructions.

For the dogs monitored for cancer treatment, the first sample was collected from a treatment-naive patient and treated as the baseline. Subsequent samples were compared to the first sample to build a trend with fold increase or decrease observed between each pair of samples. Test results were communicated back to the treating veterinarian at each participating clinic who used them as part of their decision-making processes about treatment continuation, adjustment, or termination.

A group of healthy dogs was used to determine the extent of natural variability in cfDNA concentration. These have been described elsewhere *(refer to cfDNA main paper and/or a white paper on the Finnish follow up group)*.

Results

In total, 124 dogs were enrolled in this study. These included 55 dogs with cancer and 69 healthy controls.

The 55 cancer patients consisted of 33% males and 67% females with a median age of 9 years (range 2-16 years). The most common breed group in the cancer patient group was Mixed breed (16 dogs), followed by Belgian shepherd (8), Golden retriever (7), and Bernese mountain dog (5).

The 69 healthy controls consisted of 46% males and 54% females. Their median age was 8 years (range 5-14) and the largest breed groups were Belgian shepherd (13 dogs), Golden retriever (12), Bernese mountain dog (8), Staffordshire bull terrier (8), and Great Dane (8) . Because healthy controls were recruited in collaboration with Finnish breed associations, mixed breed dogs were absent in this group.

The full list of breeds for cancer patients and healthy controls can be found in Table 1.

Table 1. Breed distribution in the cancer patient and healthy control groups.

Patients (n = 55)	**%**	**Controls (n = 69)**	**%**
Mixed breed (9)	16	Belgian shepherd (13)	19

Belgian shepherd (8)	15	Golden retriever (12)	17
Golden retriever (7)	13	Bernese mountain dog (8)	12
Bernese mountain dog (5)	9	Great dane (8)	12
Labrador retriever (4)	7	Staffordshire bull terrier (8)	12
German shepherd (3)	5	Flat-coated retriever (6)	9
Boston terrier (2)	4	Irish wolfhound (6)	9
Boxer (2)	4	German shepherd (5)	7
Cocker spaniel (2)	4	Dobermann (3)	4
Dobermann (2)	4		
Flat-coated retriever (2)	4		
French bulldog (2)	4		
Other (7)	13		

The most common cancer types in the group of cancer patients were mast cell tumours (15 dogs), followed by adenocarcinomas (14) and lymphomas (11). Cancer types studied are listed in Table 2.

Table 2. Cancer type distribution in the patient group.

Cancer type (n = 55)	**%**
Mast cell tumour (15)	27
Adenocarcinoma (14)	25
Lymphoma (11)	20
Histiocytic sarcoma (3)	5
Bone tumours (2)	4
Melanoma (2)	4
Plasmacytoma (2)	4
Soft tissue sarcoma (2)	4
Other (4)	7

Median cfDNA concentrations by collection time point are shown in Table 3. Samples from healthy controls showed very low and stable cfDNA concentration values with high reproducibility (median concentration "1" across all time points). Samples from cancer patients showed much wider case-by-case variability, largely driven by lymphoma samples (range 2-10,000x).

Table 3. Median cfDNA concentrations grouped by health status and collection time point. LSA, lymphoma; Adeno, adenocarcinoma; MCT, mast cell tumour; T1-4, time point 1-4. NB. These are not actual concentrations but show typical fold-change differences during for healthy dogs, dogs with cancer, and selected cancer types.

Health status	**T1**	**T2**	**T3**	**T4**
Healthy	1	1	1	1
Cancer (All)	3	1	1	3
Cancer (LSA)	10	8	2	10
Cancer (Adeno)	3	2	2	4
Cancer (MCT)	3	1	1	1

Two main trends were visible in samples from cancer patients. Most adenocarcinomas and mast cell tumours showed modest elevation of cfDNA concentration at diagnosis followed by a continuous downward trend that correlated with treatment success.

In contrast, most lymphoma samples followed a U-shaped curve with high baseline values at diagnosis, an initial downward trend correlating with treatment response, and a rapid increase of cfDNA concentration once cancer became resistant to the treatment. Four cancer cases will be discussed in more detail below and visualised in Figure 1.

Case 1 (Figure 1a) was a 6-year-old female Labrador retriever with high-grade lymphoma. Cancer appeared to respond initially with a rapid decrease in plasma cfDNA concentration but this was followed by a relapse two months later.

Case 2 (Figure 1b) was a 7-year-old female Bernese Mountain Dog with multicentric B-cell lymphoma. In contrast to the previous case, this dog's cancer seemed to have responded well to the treatment for eight months so far.

Case 3 (Figure 1c) was a 9-year-old female Boston terrier with a single mast cell tumour in the vulva area. Typically for a mast cell tumour, there was modest elevation of the cfDNA concentration at diagnosis. Following treatment, cfDNA concentration dropped down to a level seen in clinically healthy dogs of the same age and breed group, and has stayed low for a year. This dog appears to be in full remission.

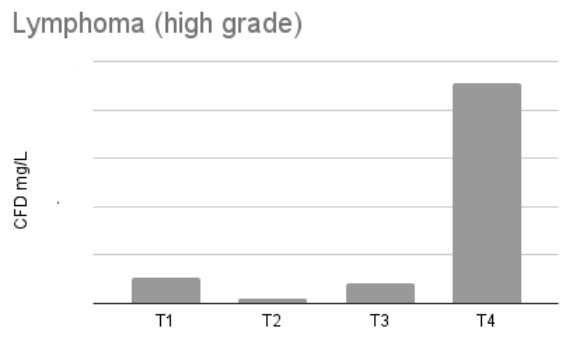
Case 4 (Figure 1d) was a 12-year-old female Belgian shepherd dog with recurring mast cell tumour. The first sample was taken one month after the initial surgery, so the dog was

missing a baseline sample. However, the treatment seemed to have gone well and cfDNA concentration was within the reference range for clinically healthy dogs of the same age and breed group. However, a follow up sample taken eight months after the treatment showed an increase in the cfDNA concentration. Recurrence of mast cell tumour was diagnosed nine months later, the dog underwent surgery, and a follow up sample two months later confirmed that the treatment had again been successful in removing the tumour.

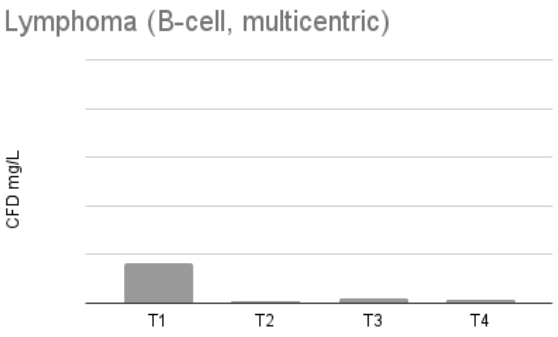
Figure 1 also shows two representative sample series collected from clinically healthy dogs. The first (Figure 1e) was a 8-year-old male Flat-coated retriever and the second (Figure 1f) was a 7-year-old female Great Dane. Both dogs participate in the two-year follow up group to ascertain the effect of preanalytical factors to cfDNA concentration in healthy dogs.

Figure 1. Monitoring cfDNA concentrations in plasma from dogs with a) high-grade lymphoma; b) multicentric B-cell lymphoma c) and d) mast cell tumours. Additionally, two examples of serial sampling in healthy controls are shown in d) and e).

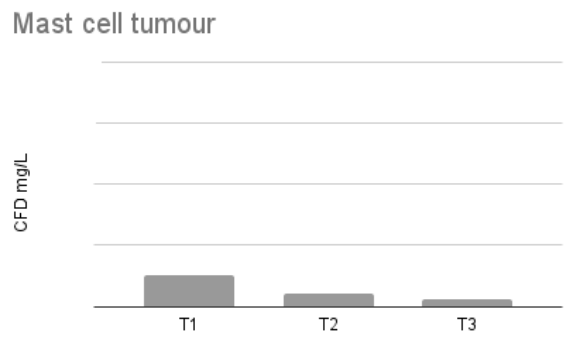
a.



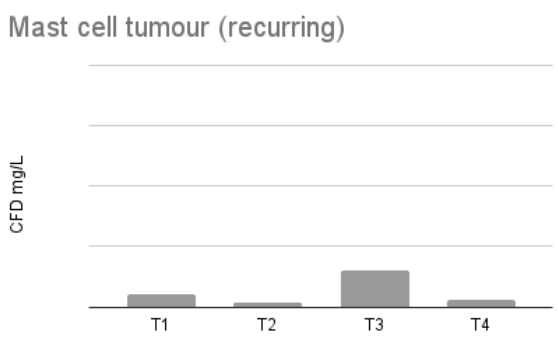
b.



c.

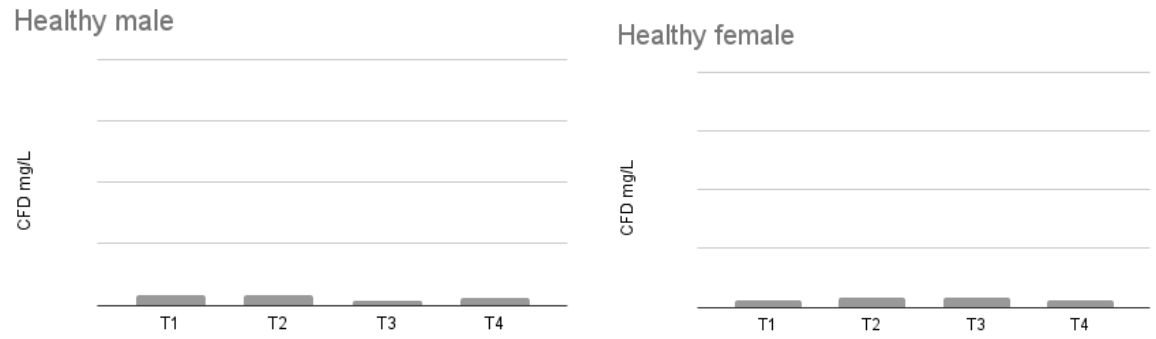


d.



e.

f.



Taken together, these results show that clinically healthy dogs shed very low levels of cfDNA and that these levels remain relatively stable over time. Lymphoma represents systemic cancers that show large variations in cfDNA concentration. Variability is more modest in mast cell tumours but it is still measurable and consistent. Adenocarcinomas appear to be a more heterogeneous group with some (pulmonary, renal, prostate) adenocarcinomas showing more marked changes in cfDNA concentrations than others (gastric, mammary).

Regardless of the cancer type, serial measurements allowed monitoring cancer treatment efficacy and disease recurrence with high accuracy. Initial cfDNA concentration measurement in treatment-naive patients was used as a baseline, and subsequent samples were compared to the baseline to establish a trend. The oncologists treating dogs used these trends to decide whether to continue, discontinue, or change the treatment regime.

Discussion

Our study demonstrates the value of cfDNA analysis as a practical, non-invasive method for monitoring cancer treatment efficacy and disease recurrence in dogs. The results show that cfDNA concentrations remain level in healthy dogs and differ markedly between healthy dogs and those diagnosed with cancer. Furthermore, these levels respond dynamically to treatment outcomes. The clear differentiation in cfDNA behavior across tumour types - particularly the high variability observed in lymphoma compared to the more modest shifts in mast cell tumours and the heterogeneity of adenocarcinomas - highlights the versatility and potential of cfDNA monitoring in diverse clinical contexts.

The findings align with prior studies indicating that cfDNA concentrations rise in response to tumour burden and fall following effective therapy. Our data specifically strengthen the evidence for cfDNA as an early marker of therapeutic response and disease recurrence, particularly in systemic cancers like lymphoma. The U-shaped cfDNA trajectory seen in most lymphoma cases - characterized by high levels at diagnosis, reduction during initial treatment response, and subsequent rise upon treatment resistance - underscores its potential as a real-time disease activity tracker.

One of the key strengths of this study lies in its longitudinal design and inclusion of a robust healthy control group. Regular cfDNA sampling in healthy dogs revealed low and stable cfDNA concentrations, reinforcing the biomarker's specificity for pathological states. Importantly, the consistency of cfDNA measurements in this group, despite the lack of fasting protocols and environmental standardisation, supports the feasibility of implementing

cfDNA testing in real-world clinical settings. Moreover, the use of internal controls and rigorous sample processing protocols ensured reproducibility and minimized pre-analytical variability - a known challenge in cfDNA assays.

Interestingly, adenocarcinomas showed a more heterogeneous cfDNA profile, with some subtypes exhibiting substantial changes during treatment while others remained relatively static. This highlights the need for tumour-type specific validation in future research and suggests that cfDNA dynamics may depend not only on tumour burden, but also on tumour location, vascularisation, and cell turnover. The observed correlation between cfDNA trends and clinical decisions made by oncologists further supports the clinical utility of this biomarker as a complementary tool in treatment planning.

Nonetheless, several limitations should be acknowledged. The sample size for each cancer subtype was relatively small, limiting the ability to perform detailed subtype-specific analyses or robust statistical comparisons. Additionally, although cfDNA concentration trends provided actionable insights, the lack of molecular characterisation means the full diagnostic potential of cfDNA was not explored. We are developing tests specific for each cancer subtype and are testing different source materials (plasma, urine, saliva) to further our understanding about the suitability for each material type for cfDNA analysis.

Conclusions

cfDNA concentration is a valuable biomarker for monitoring treatment response and detecting recurrence in canine cancers. Its non-invasive nature, responsiveness to treatment dynamics, and reproducibility in healthy controls make it a powerful adjunct to traditional monitoring methods such as imaging and clinical evaluation. This study confirms its clinical utility across common cancer types, particularly lymphomas, mast cell tumours, and adenocarcinomas.

As cfDNA testing continues to evolve, integrating quantitative measurements with molecular and fragmentomic profiling may unlock further diagnostic potential. Broader studies with larger sample sizes, additional tumour types, and harmonised protocols will be critical for translating cfDNA monitoring into routine veterinary oncology practice. cfDNA has the potential to personalise treatment strategies, improve patient outcomes, and reduce the burden of invasive monitoring procedures in canine cancer care.