Master's Thesis

Cell-free DNA as Biomarker in Gastric Carcinoma of Belgian Shepherd dogs

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Canine gastric carcinoma (GC) is rather rare in dogs, accounting for 1% of all cancers, but it has a high mortality rate. Additionally, GC is the fifth most common cancer in humans. This fatal disease is often diagnosed in advanced stages and older age leading to a poor prognosis. Since early detection of GC is crucial for disease outcome, further research is required. Certain breeds, such as the Belgian shepherd dog varieties Tervuren & Groenendael (T&G), are at a higher risk for GC. Canine GC diagnostics commonly requires endoscopy in general anesthesia and is therefore rather invasive and expensive. Using non-invasive liquid biopsies for cell-free DNA (cfDNA) and identifying GC-specific methylation alterations in tissue-derived genomic DNA (gDNA), the early detection of canine GC could be more efficient and cheaper. Plasma cfDNA has been proven to be elevated in dogs with cancer, such as lymphoma. This proof-of-concept study aimed to quantify the plasma cfDNA concentrations of Belgian Shepherd dogs (T&G) diagnosed with GC (n=6), pre-cancerous stages (n=4), gastritis (n=1) and healthy control dogs (n=26). A secondary aim was to identify GC-specific methylation patterns of gDNA in affected and healthy dogs using methylation sequencing. Plasma cfDNA was extracted with Qiagen cfDNA extraction kit and analysed by TapeStation. Total plasma cfDNA concentrations did not show any significant variability between GC, pre-cancer, chronic gastritis and healthy dogs. Unfortunately, sequencing data generated by an external contractor was of insufficient quality to be analysed. In conclusion, cfDNA quantification may not be suitable for early detection of canine GC possibly due to biological characteristics of carcinomas such as low vascularization and tumour shedding rates. However, investigation of GC-specific methylation patterns can provide another, non-invasive solution to current limitations in canine GC diagnostics.

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Mahalaukun karsinooma kattaa noin 1 % koirien syövistä, mutta sen ennuste on yksi huonoimmista. Lisäksi se on globaalisti viidenneksi yleisin syöpä ihmisillä. Mahalaukun karsinooma diagnosoidaan koirilla usein liian myöhään, johtaen huonoon ennusteeseen. Belgianpaimenkoiran muunnokset tervuren ja groenendael (T&G) kuuluvat rotuihin, joilla on kohonnut riski sairastua mahalaukun karsinoomaan. Koirien syöpädiagnostiikka on hidasta ja kallista, minkä vuoksi tarve paremmille diagnostisille menetelmille on suuri. Nestebiopsian ja soluvapaan DNA:n (cfDNA) hyödyntäminen voisi mahdollistaa syövän toteamisen nykyistä nopeammin ja edullisemmin. On todettu, että plasman cfDNA pitoisuus on korkeampi syöpää, kuten lymfoomaa, sairastavilla kuin terveillä koirilla. Tässä tutkimuksessa tarkasteltiin plasma cfDNA konsentraatioita belgianpaimenkoirilla (T&G), joilla on diagnosoitu mahalaukun karsinooma (n=6), sen esiasteita (n=4) tai gastriitti (n=1), sekä verrattiin niitä terveisiin koiriin (n=26). cfDNA:n pitoisuudet määritettiin eristämällä koirien plasmasta cfDNA:ta ja mittaamalla pitoisuus automaattisella sähköforeesitekniikalla. Tämän pilottitutkimuksen perusteella cfDNA ei sovellu biomarkkeriksi mahalaukun karsinoomaan belgianpaimenkoirilla pelkkiä pitoisuustasoja mittaamalla, koska ryhmien välillä ei todettu merkittäviä eroja. Matalat cfDNA arvot voivat liittyä mahalaukun karsinooman biologisiin ominaisuuksiin, kuten matalaan verisuonitiheyteen ja solukuoleman määrään. Tutkimuksen pyrkimyksenä oli lisäksi tutkia belgianpaimenkoirien mahalaukun karsinooman metylaatioprofiileja, mutta valitettavasti ulkoistettu genomisen DNA:n metylaatiosekvensointi epäonnistui tästä tutkielmasta riippumattomista syistä. Mahalaukun karsinooman spesifisten metylaatioprofiilien tutkiminen voisi tarjota vaihtoehtoisen, vähemmän invasiivisen lähestymistavan nykyisten diagnostisten rajoitteiden ratkaisemiseksi.

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TERMS AND ABBREVIATIONS

Terms

Endoscopy	Non-invasive medical procedure that allows direct visualization of internal organs through natural or artificial openings using a camera and light at the tip of a flexible or rigid tube
Aetiology	Refers to the study of the causes or origins of a disease or condition
Gastritis	Inflammation of the lining of the stomach
Neoplastic	Abnormal growth of tissue
Pathogenesis	Biological mechanisms and processes by which a disease develops and progresses

Abbreviations

cfDNA	cell-free DNA
ctDNA	circulating tumour DNA
GC	gastric carcinoma
gDNA	genomic DNA
PCR	polymerase chain reaction
MCED	multi-cancer early detection
NGS	next generation sequencing
QC	quality control
dPCR	digital PCR
qPCR	quantitative real-time PCR

1 INTRODUCTION

1.1 Canine Gastric Carcinoma

Canine gastric carcinoma (GC) accounts for approximately 1 % of all cancers in dogs. However, it is often diagnosed at advanced stages, primarily in older dogs, suggesting that the true prevalence may be higher (Araújo et al. 2022). Furthermore, certain breeds show a notably high incidence of this disease. In both humans and dogs, GC has one of the highest mortality rates among cancers. For example, human GC is the fifth most common cancer globally and ranks third in cancer-related deaths for men and fifth for women (Hugen et al. 2017). The Finnish National Esophago-Gastric Cancer Cohort (FINEGO) has collected data with a population-based, cohort study about declining cases of GC in Finland. While the cases are declining, patients affected are mostly diagnosed at late stages when the prognosis is poor (Kauppila et al. 2020).

In both humans and dogs, gastric carcinoma is heterogeneous; genetic and environmental factors play an important role in the pathogenesis of this disease (Hardas et al. 2021). The exact cause of GC in dogs remains unclear, but research has identified potential links to certain genetic factors, including breed predisposition. Breeds such as Belgian shepherds (Tervuren and Groenendael), rough collies, Staffordshire terriers, Chow Chows and standard poodles have shown a higher tendency for this condition (Candido et al. 2021). Over a ten-year period, the cumulative incidence was 4.7% in the Tervuren and 2.1% in the Groenendael, suggesting a higher risk in the Tervuren variant (Cook et al. 2025). Additionally, GC is commonly diagnosed in older dogs aged between 7-11 years (Araújo et al. 2022). In Tervuren and Groenendael variants of the Belgian Shepherd dog breed, median age at diagnosis is 8.9 years (Kijan et al. 2023, Cook et al. 2025).

Around 70-90 % of canine gastric malignancies cases are adenocarcinomas and in human GC about 90-95% (Abrams et al. 2019, Araújo et al. 2022). Leiomyosarcomas and lymphomas are the second most common types of gastric malignancies in dogs after adenocarcinoma. Gastric adenocarcinoma is commonly

forming tubular structures on the stomach wall with different levels of invasion (Seim-Wikse et al. 2013). In human medicine, carcinomas can be divided into four different subtypes: papillary, mucinous, tubular and signet ring cell, based on their key histological and cytological characteristics. The classification has been adapted for canine GC to categorize these tumours into two primary histological types: diffuse (tubulopapillary) and intestinal (signet ring cell) type (Araújo et al. 2022, Kijan et al. 2023). Canine GC is usually found in the lesser curvature of the stomach. Metastases can be found in liver, omentum, gastric lymph nodes, duodenum, spleen, pancreas, oesophagus, lungs and adrenal glands (Araújo et al. 2022).

Clinical signs of canine GC can include vomiting that may evolve into hematemesis, anaemia, lethargy, anorexia, weight loss, abdominal swelling and discomfort. However, GC has also been found in asymptomatic dogs and can therefore occur without obvious clinical signs (Candido et al. 2021). The prognosis is typically poor with median survival time less than 3 months after diagnosis in untreated patients and metastasis is confirmed in about 70-90 % of cases at the time of diagnosis or death (Hardas et al. 2021). Additionally, in surgically treated patients the median survival time of 178 days (range, 1-1902) has been reported (Abrams et al 2019). Due to the poor prognosis of canine GC, further research is needed to explore methods for better early detection of the disease to improve treatment options, monitoring and outcome.

It has been suggested that canine GC can also serve as a spontaneous disease model for translational research with benefits for human medicine. (Koterbay et al. 2020, Candido et al. 2021, Hardas et al 2021). Recently it was revealed that canine GC seems to be associated with gastric mucosal metaplasia and dysplasia, both changes considered to be preneoplastic in humans (Candido et al. 2021). The aetiology and molecular pathways of canine GC have been largely unknown, but a recent Bayesian genome-wide association study and selection analysis studies identified 15 GC associated loci. Variant filtering revealed germline putative regulatory variants for the *EPAS1*(*HIF2A*) and *PTEN* genes and a coding variant in *CD101* (Cook et al. 2025). Human GC is understood better, but not completely because of its complexity. For example, known risk factors, hereditary cancer syndromes, *Helicobacter pylori* (*H. Pylori*) infection and other gastric diseases play a significant role in human GC (Araújo et al. 2022).

The development of human GC is a complex cascade of multiple steps before cancerous state. Typically, a cascade that can lead to GC starts with chronic superficial gastritis followed by chronic atrophic gastritis, intestinal metaplasia and dysplasia leading to GC. In human GC, *H. pylori* is a frequent initiator of chronic gastritis, the inflammation of the stomach lining. Inflammation changes the microenvironment of stomach epithelia and can activate oncogenic signalling pathways. Activated oncogenic signalling pathways are increasing the development of cellular abnormalities (Ren et al. 2025). However, in dogs *H. pylori* infection is almost non-existing. It has been reported almost absent in dogs, meaning it cannot be linked to canine GC, although many other *Helicobacter* species can be found in dogs (Husnik et al. 2022). Chronic atrophic gastritis has been found alongside gastric neuroendocrine carcinoma in several cases in Norwegian Lundehund (puffin dog), suggesting that in some dogs with unusual disease features, chronic inflammation may accompany malignancy (Qvigstad et al. 2008), but a link between chronic gastritis and canine GC has not been established (Hardas et al. 2025). However, breed disposition linked to canine GC and genome-wide association studies suggest that genetic factors may be involved in addition to possible inflammation-based initiation of cancer development. Our understanding of the mechanism driving the development of the disease and of its underlying causes remains incomplete. Research about canine GC is therefore essential for improving our understanding of disease pathogenesis and for facilitating the development of more effective diagnostic tools.

Treatment options for human GC are more diverse than for canine GC. The suggested treatment for localised canine GC is surgical resection; however, most cases are diagnosed at an advanced stage. For cases that undergo surgery, the overall median survival time after surgery is reported to be approximately 6 months because in most cases, GC has already metastasized or the surgical resection is incomplete at which point surgery alone cannot be curative. Longer survival times have been reported up to 5.2 years (range: 1-1902 days). In untreated dogs, survival times are typically 5-6 months following the onset of clinical signs (Abrams et al. 2019), although this likely reflects the late clinical presentation of the disease. Chemotherapy, either alone or as an adjuvant after surgery, has been reported only in small case studies and may offer rather limited benefit in advanced cases in dogs. Protocols typically include carboplatin, doxorubicin, 5-

fluorouracil, and combinations of cisplatin with 5-fluorouracil and cyclophosphamide (Hugen et al. 2016). To improve diagnostics and treatment methods, more information is needed about the aetiology and development of canine GC. Gathering such information will be made possible when early diagnosis is improved, e.g., by liquid biopsy of cell-free DNA in plasma since this will allow for regular non-invasive follow up examinations of dogs being treated for GC.

1.2 Quantitative cfDNA concentrations as diagnostic tool

Cell-free DNA (cfDNA) is a short, partially degraded double-stranded DNA fragment released into plasma, independent of cells (Chen et al. 2022 & Kim et al. 2021). cfDNA forms when a cell goes through cell death and releases nuclear DNA fragments and nucleosomes into the bloodstream. During cell death, DNA is being cut by enzymes between nucleosomes. cfDNA fragment size range is usually 120 bp to 220 bp, with higher peak at 167 bp. The highest peak mirrors the way DNA is wrapped around proteins called histones. Approximately 150 bp length of DNA can be wrapped around one histone protein and additionally about 20 bp length of linker DNA associated with Histone 1. These basic structures called nucleosomes can be found freely in the blood. In addition, cfDNA can still be bound to multiple histones and therefore, around 300 bp (di-nucleosomal fragments) and 500 bp (tri-nucleosomal fragments) long cfDNA fragments can be detected in blood (Alcaide et al. 2020). cfDNA originates predominantly from hematopoietic cells, with only a minor contribution from non-hematopoietic tissues. The concentration of cfDNA in the blood can vary due to factors associated with cancer, tissue damage, sepsis and inflammation (Chen et al. 2022). Elevated cfDNA concentrations have been linked to several cancers, such as lymphoma and hemangiosarcoma (Flory et al. 2022, Flory et al. 2024, Kim et al. 2021, Prouteau et al. 2021, Rafalko et al. 2023) and proven to correlate with disease development and prognosis (Tagawa et al. 2019).

In human medicine, repeated cancer screening is one of the methods used to detect GC in earlier stages. However, physical examination and routine laboratory testing alone are unlikely to detect GC and diagnosis generally requires invasive procedures, such as endoscopy. This highlights the need for non-invasive approaches with improved sensitivity, such as a liquid biopsy (Rafalko et al.

2023). A liquid biopsy detects cancer-related genetic material, such as cfDNA, in bodily fluids like blood. It allows for early cancer detection, monitoring of disease progression, and assessment of treatment response without the need for traditional tissue biopsies. Rafalko and colleagues (2023) stated that non-invasive methods to detect biomarkers offer a way to a faster and cheaper cancer diagnosis (Rafalko et al. 2023). While liquid biopsies are widely used in human cancer diagnostics, their use in canine cancer diagnostics has been limited (Kim et al. 2021). However, liquid biopsy has been recognised as a promising option for canine diagnostics and in recent years the number of studies has increased considerably (Flory et al. 2022, Flory et al. 2024, Kim et al. 2021, Prouteau et al. 2021, Rafalko et al. 2023, Tagawa et al. 2019).

In cancer diagnostics, plasma samples can serve as liquid biopsies because cfDNA and other biomolecules that are released into the bloodstream by tumours, can be extracted from the plasma (De 2021). Tagawa and colleagues (2019) discovered that plasma cfDNA concentrations were significantly elevated in samples of dogs with cancers such as lymphoma and hemangiosarcoma when compared to healthy control samples in dogs (Tagawa et al. 2019). Therefore, elevated cfDNA concentrations can potentially be used as a simple cancer detection method and further studies are warranted to investigate this diagnostic approach.

1.3 Molecular features of cfDNA in Canine Oncology

Studies about cfDNA as a biomarker in canine oncology have become more common in recent years. cfDNA provides multiple applications for investigating cancer genomics. Part of cfDNA, called circulating tumour DNA (ctDNA), is derived from tumour cells. The ctDNA proportion of the total cfDNA is usually very small, commonly around 1%. However, it has been proven that it can correlate with tumour burden and cell turnover in up to 90% of later cancer stages (Bartolomucci et al. 2025, Prouteau et al. 2021). ctDNA contains tumour-associated genetic and epigenetic alterations that reflect cancer development, progression and therapeutic resistance. Cancer treatment is increasingly shifting towards personalized therapeutic approaches tailored to individual patients. This shift requires cancer research to focus even more on genomics and specific characteristics of cancer types.

As mentioned earlier, cfDNA has a specific size profile mirroring its structure around histones. Shi and colleagues (2020) mentioned that nucleosome arrangements or cfDNA size distribution profiles can differ between different tissues. These profiles can be analysed by several approaches, including gel electrophoresis, quantitative real-time PCR (qPCR), atomic force microscopy (AFM) and massively parallel sequencing (MPS) (Shi et al. 2020). cfDNA size distribution profiles have been studied increasingly in cancer research as they could offer a robust method for better cancer diagnostics (Alcaide et al. 2020, Cristiano et al. 2019). In humans, studies have revealed that ctDNA and foetal-derived cfDNA size patterns show higher fragmentation than cfDNA originated from non-cancerous or maternal tissues. Higher fragmentation refers to increased DNA breakdown and results in a higher proportion of small cfDNA molecules in blood (Alcaide et al. 2020).

In human cancer research, ctDNA has been used in many applications. DNA can be studied by determining the exact order of the four nucleotides that are the bases of a DNA molecule. The bases of DNA are Adenine (A), Thymine (T), Guanine (G) and Cytosine (C). Together, these bases constitute a four-letter genetic code that can be decoded using DNA sequencing technologies. Next generation sequencing (NGS) offers high-throughput sequencing of millions of DNA or RNA fragments simultaneously It is faster, more cost-efficient and more versatile than traditional Sanger sequencing.

Blood-based biomarkers in many platforms have been proposed for cancer diagnostics. Commonly, polymerase chain reactions (PCR) and enzyme-linked immunosorbent assays (ELISA) have been used to detect cancer biomarkers. However, cancers are complex systems with many biomarkers which are not usually shared between different cancer types (Wilson-Robles et al. 2022). Flory and colleagues (2022) mentioned that liquid biopsy diagnostic methods using NGS are increasing in human medicine. The multi-cancer early detection (MCED) tests are proven to increase earlier detection of cancers and therefore increase the survival rates for most cancers (Flory et al. 2022). Prouteau and colleagues (2021) mentioned that NGS of ctDNA can reveal genetic alterations that have been linked to cancer. For example, mutations in oncogenes such as EGFR or KRAS in non-small-cell lung cancer, tumour suppressor genes such as TP53 in neck cancer

and chromosomal rearrangements as found in the variable-diversity-joining receptor gene sequences in lymphoma cases (Prouteau et al. 2021).

In recent years, liquid biopsy tests have been developed to be used as a multi-cancer detection tool for canine cancers. Flory and colleagues (2022) performed clinical validation of next generation sequencing (NGS) based multi-cancer early detection liquid biopsy of cfDNA in more than 1000 dogs during the so-called CANDiD (The cancer detection in dogs) study. Detection rate for most aggressive types of cancers (lymphoma, hemangiosarcoma and osteosarcoma) was 85.4%. The detection rate for eight most common canine cancers (mast cell tumour, soft tissue sarcoma, osteosarcoma, hemangiosarcoma, lymphoma, malignant melanoma, mammary gland carcinoma, anal sac adenocarcinoma) was 61.9%. Additionally, cancer signals were detected from one presumably healthy dog, which later was diagnosed with hemangiosarcoma, months before any clinical signs occurred. This suggested that cancer-associated genomic alterations can be shown and are detectable in blood before clinical signs are present (Flory et al. 2022). Following the CANDiD study, McCleary-Wheeler and colleagues (2023) suggested that NGS-based liquid biopsy could be used as a cancer monitoring tool after surgery. After surgery, dogs were divided into two groups; Cancer Signal Detected (CSD) or Cancer Signal Not Detected (CSND). Results indicated that dogs in group CSD had significantly higher risk for cancer recurrence within 6 months than dogs in group CSND (McCleary-Wheeler et al. 2023).

Sequencing of cfDNA and genome mapping can provide information about oncogenic mutation status, somatic mutation allele frequency distribution, mutational signatures of endo- and exogenous mutagens and metagenomic signatures. This information could indicate the following: tissue of origin, malignant disease status and how widespread is the clonal growth (De 2021). In human GC, somatic mutations in specific genes such as TP53 and CDH1 have been documented (Tahara et al. 2016, Cai et al. 2024). However, research on the genetic basis of canine GC is very limited and studying the genetic profile of GC through studying genetics of cfDNA and tissue-derived gDNA could help to identify possible gene alterations related to canine GC.

1.4 Genetics of canine GC

Cook and colleagues (2025) investigated the genetic profile of Belgian Tervuren and Groenendael with GC to identify possible genetic risk factors. They identified multiple risk loci using Bayesian analysis and selection scans for canine GC. In this study, high coverage whole genome sequence (WGS) was used with a multi-breed reference panel of 1,143 dogs. Data was presented with Bayesian model GWAS and selection scan analysis of 200 dogs with GC (Tervuren and Groenendael) and 270 healthy control dogs (Tervuren and Groenendael) which showed multiple loci associated with GC. Fifteen loci predict the phenotype of canine GC with 92% discriminatory accuracy, including the tumour suppressor genes *PTEN* and *HDAC2,* which have important roles in transcriptional regulation and cell cycle progression. These genes are known to have a major role in human GC development (Cook et al. 2025).

The *PDZRN3* gene, which is overexpressed in human diffuse type GC, was among the discovered risk loci in canine GC. Two loci (CFA17:54Mb & CFA28:24.8Mb) were associated with intestinal type of GC, however nothing could be linked to diffuse type of GC. Also, a rare variant in *CD101* was linked with the intestinal type of GC. Within high-risk subtypes of Belgian Shepherd dogs, Tervuren and Groenendael, two shared haplotypes were found on CFA4 (CFA4:24.2-24.4Mb) and CFA12 (CFA12:70Mb) loci. The CFA12 locus is known to be a gene-dense region that includes major histocompatibility complex class (MHC) II and some of class I genes. In human GC and autoimmune gastritis, alleles of MHC class II are associated with risk or protection and therefore CFA12 could be important for GC phenotype prediction. Autoimmune gastritis can develop into GC in humans; however, this has not been proven in dogs (Cook et al. 2025). Liquid biopsy offers a tool for different usage of NGS-based methods for studying the genetics of canine GC.

1.5 Methylation sequencing for identifying GC related markers

Methylation sequencing is a method used to identify and quantify epigenetic modifications across the genome. It reveals the methylation or demethylation of cytosines, and it is used to investigate gene activation and silencing mechanisms and their impact on normal cellular function. Methylation sequencing is an

epigenetic method to find the methyl groups in the genome. These alterations with methyl groups can lead to abnormal cellular functions which can lead to the development of cancer. According to Lee and colleagues (2019), methylation changes in human cancers have been found in long interspersed nuclear element-1 (LINE-1). LINE-1s are retrotransposons which means that they can use a "copy and paste" mechanism to move around within the genome, and they take approximately 17% of the human genome. Through this mechanism, LINE-1 elements generate additional copies that insert at new locations in the genome. These elements are mostly silenced, but for example with addition of a methyl group, they can be activated. These activated LINE-1 elements are linked to several cancers including GC (Lee et al. 2019).

Lee and colleagues (2019) stated that changes in DNA methylation play a key role in cancer development and progression. While such alterations can be observed in blood samples of patients, their reliable detection in early-stage cancer remains challenging. The authors studied the methylation patterns of human and canine mammary tumours, emphasizing the similarities between canine and human tumours that highlights the value of investigating canine cancer biomarkers as an alternative research strategy (Lee et al. 2019). Potential human GC methylation markers have been found by using plasma-based digital PCR (dPCR) assays. Methylation of *GHR, GLBR, GATM* were identified only in the tumour tissues and GC cell lines with 90% (95% CI 76.3%–97.2%) specificity and 86,7% (95% CI 75.4%–94.1%) sensitivity for combined data analysis. Additionally, the sensitivity was 81,6% (95% CI 65.7%–92.3%) for stage I human GC, which suggests strong potential for the early diagnosis of human GC (Lee et al. 2025).

1.6 Research aims and hypotheses

The primary objective of this study was to investigate the relationship between plasma cfDNA concentration and the GC status in Belgian shepherd dogs of the Tervuren and Groenendael variants. These were classified into healthy control dogs by clinical history (no clinical signs of gastric disease or any other diseases), and diseased dogs classified by histology of endoscopically taken gastric mucosal biopsies into chronic eosinophilic gastritis, pre-cancerous (metaplastic and dysplastic) changes, or adenocarcinoma. Kim and colleagues (2021) have discovered that cfDNA levels have been higher in dogs with cancer than healthy dogs. This

has been shown in various cancer types, including some carcinomas (e.g. mammary carcinoma, thyroid carcinoma, pulmonary carcinoma, intestinal adenocarcinoma and hepatocellular carcinoma) (Kim et al. 2021).

As a secondary objective, this research aimed to identify potential genetic markers, from cfDNA, specific to GC in Belgian Shepherd dogs by genome-wide sequencing of cfDNA in healthy, pre-cancerous and GC dogs and by methylation sequencing of tissue samples with known GC or precancerous status.

To achieve these aims, a combination of methods was employed, including a comprehensive literature review to identify existing knowledge gaps and a clinical study focusing on the cfDNA plasma concentrations from affected and healthy, privately owned pet dogs. Findings from this study will help determine whether cfDNA could be effectively used for early detection of GC in canine cancer diagnostics.

First, this study tested the hypothesis that dogs with GC have higher plasma cfDNA concentrations than dogs without GC. Secondly, it was tested whether dogs with preneoplastic changes such as mucous metaplasia and glandular dysplasia have higher cfDNA concentrations than healthy dogs but lower than dogs with GC. Thirdly, investigations were planned on whether there are possible genetic markers that can be identified from the plasma cfDNA or genomic DNA from gastric mucosal tissue samples by comparing Belgian Shepherd dogs with and without GC or preneoplastic changes in the gastric mucosa.

2 MATERIALS AND METHODS

2.1 Sample collection

To address the primary objective of the project, this proof-of-concept study about plasma cfDNA was designed as a case-controlled study comprising of EDTA plasma samples from apparently healthy dogs (age 4-15y, no history of signs of gastric disease or any other diseases) serving as the control group and from canine patients being endoscopically screened for GC. Inclusion criteria for patient dogs were written consent of pet owners for endoscopic screening for GC in dogs

older than 5 years independent of whether they had clinical signs of gastric disease or not. Exclusion criteria included diseases associated with an increased risk of adverse events during general anaesthesia required for the endoscopy, as well as a diagnosis of tumours other than GC. The study was ethically approved by the Finnish National Animal Experiment Board (ELLA, Ethical permit ESAVI/40314/2024).

Plasma samples from the dogs of the control group have been collected previously by DeepScan Diagnostics. From the endoscopically screened patient group, EDTA plasma samples have been collected prospectively at the University Small Animal Hospital, University of Helsinki at the time of endoscopy. EDTA plasma samples from control and patient dogs were stored at –80°C until cfDNA analysis. To differentiate canine patients into dogs with GC, preneoplastic changes or gastric inflammation, gastric mucosal biopsies underwent histologic examinations at the Veterinary Pathology Unit of the Department of Veterinary Biosciences, University of Helsinki. To address the secondary objective of the project, endoscopically taken gastric mucosa samples from macroscopically changed and unchanged gastric mucosa (two biopsies from each area) were snap frozen in liquid nitrogen and stored at -80°C until shipped on dry ice to an external service provider for identifying GC-specific methylation alterations in tissue-derived gDNA.

In addition to collecting EDTA plasma samples of apparently healthy dogs in the control group, DeepScan Diagnostics provided previously collected data from two Belgian Shepherd dogs diagnosed with GC for statistical analysis of cfDNA. Only cfDNA samples that were extracted with Qiagen QiaAmp MinElute ccfDNA Mini kit were accepted to this study. Additionally, only plasma samples which were collected to EDTA tubes were included in this study.

2.2 Methods

Control dogs were individuals that were apparently healthy based on the owner's information. Canine patients scheduled for GC screening endoscopy underwent physical examination and basic blood work (haematology, blood chemistry organ profiles) to establish fitness for general anesthesia. The endoscopic

examination was performed with the EVIS EXERA III 185 endoscopy system using the Olympus Gastroscope Gif H185 for endoscopic narrow band imaging (Olympus Europa SE & Co. KG, Hamburg, Germany). The patients were classified based on the most severe histologic findings into dogs with GC (GC-Dogs), glandular dysplasia (GD-Dogs), mucous metaplasia (MM-Dogs), and with chronic eosinophilic gastritis (G-Dogs).

EDTA plasma samples of control dogs not undergoing endoscopy and of canine patients undergoing endoscopic GC screening have been obtained by collecting whole blood from the cephalic vein into an EDTA tube. Plasma has been separated by centrifugations within 2 hours of blood collection for EDTA tubes (or blood has been kept refrigerated /on ice until separation has been possible). A double centrifugation protocol has been used in which samples are first centrifuged for 10 minutes at 1800 x g to remove the majority of red and white blood cells. 2 ml of plasma has then been transferred to a clean tube by pipetting while carefully avoiding the buffy coat layer containing white blood cells. The clean tube containing plasma has then been centrifuged again for 10 minutes at 1800 x g to remove as many cell fragments and platelets as possible. 2 ml of clear plasma has been transferred to a fresh 2 ml DNA LoBind tube and stored in -80C until ready to be processed.

cfDNA was extracted from 1 ml of plasma using QiaAmp MinElute ccfDNA Mini kit protocol either manually or partly automated in QiaCube Connect. The manual protocol consisted of dividing 1ml of plasma into 2ml DNA low bind Eppendorf tubes. Then Qiagen magnetic beads, Bead binding buffer and Proteinase K were mixed into the plasma. Tubes were incubated for at least 10 minutes on a rocking platform. After, the solution was shortly centrifuged and put into a magnetic rack for 1 minute or until the solution was clear. Supernatant was distracted and tubes were collected from the magnetic rack. The bead elution buffer was mixed with beads, and the solution was incubated for 5 minutes at RT in the QiaCube heat shaker. Tubes were put to the magnetic rack and after the solution was clear it was transferred into a new 2 ml bead elution tube, and the bead pellet was discarded. 300 µl of Buffer ACB was added to the solution and briefly centrifuged at 1800 x g in a benchtop centrifuge. The mixture was then applied to the QIAamp MinElute column and centrifuged for 1 minute at 6000 x g. The column was placed in a new 2 ml collection tube and flow-through was discarded. 500 µl

of ACW2 was added to the column and centrifuged for 1 minute at 6000 x g. The column was placed in a new 2 ml collection tube and flow-through was discarded. Then the column was centrifuged at full speed (18,000 x g) for 3 minutes. The column was placed into a new 1.5 ml Eppendorf and air dried 3 minutes at 56 °C. Next, 60 µl of Ultraclean water was added to the membrane center and incubated for 1 minute at room temperature. Then it was centrifuged for 1 minute at full speed (18,000 x g). Finally, the column was discarded and the eluted cfDNA was stored at -80 until fragment analysis. Total cfDNA concentration, cfDNA fragment size distribution and the level of gDNA contamination were measured using Agilent TapeStation.

Tissue samples were stored in -80C until ready to be processed. Genomic DNA was extracted using the DNeasy Blood & Tissue kit. Sequencing libraries of gDNA were created using the NEBNext enzymatic Methyl-Seq kit. gDNA samples were sent after internal quality control (QC) to an external sequencing services provider. Sequencing was performed using Illumina technology. Initial processing of sequencing data was performed by Illumina Dragen. Further analysis of methylation sequencing data was planned to be performed by using in-house developed pipelines and R for statistical analysis.

2.3 AI disclosure

In this thesis, the Undermind AI tool was used to support literature search. All writing, analysis and interpretation were conducted independently without using AI tools.

3 RESULTS

3.1 Canine patients

Due to time constraints leading to a short case collection phase, this was conducted as a proof-of-concept study. EDTA plasma samples from 26 T&G dogs served as a control group. All dogs have been considered apparently healthy based on the owner's statements. The healthy group included 15 female and 11 male Belgian Shepherd dogs (14 Tervuren, 12 Groenendael) with a median age

of 7.5 years (range 4-15, Appendix 1.). The patient group included 7 female and 2 male Belgian Shepherd dogs (5 Tervuren, 4 Groenendael) with a median age of 10 years (range 3-13, Appendix 1.) that underwent endoscopy. The median age for patients with GC was 11 years (range 8-12), 8 years with pre-neoplastic changes (range 3-10). The dog with gastritis was 13 years (Appendix 1.). Additional EDTA samples for laboratory analysis from 2 Belgian Shepherd dogs with metaplasia (1 Tervuren) and GC (1 Groenendael) were provided by DeepScan Diagnostics biobank.

As displayed in table 1, the included 11 patient dogs were histologically diagnosed with gastric adenocarcinoma (n = 6), glandular dysplasia (n = 2), mucous metaplasia (n = 2), and chronic eosinophilic gastritis (n = 1).

Table 1. Background information (healthy dogs included in Appendix 1.)

Dog	Breed	Age	Gender	Diagnosis
1.	Groenendael	9y	Male	Gastric adenocarcinoma
2.	Groenendael	8y	Female	Gastric adenocarcinoma
3.	Groenendael	12y	Female	Gastric adenocarcinoma
4.	Tervuren	11y	Female	Gastric adenocarcinoma
5.	Tervuren	12y	Female	Gastric adenocarcinoma
6.	Tervuren	11y	Female	Gastric adenocarcinoma
7.	Groenendael	8y	Female	Gastric dysplasia
8.	Tervuren	8y	Male	Gastric dysplasia
9.	Tervuren	10y	Female	Gastric mucous metaplasia
10.	Tervuren	3y	Female	Gastric mucous metaplasia
11.	Groenendael	13y	Male	Chronic eosinophilic gastritis

For laboratory analysis, cfDNA (6x carcinoma, 2x glandular dysplasia, 2x mucous metaplasia, 1x chronic eosinophilic gastritis) was extracted from EDTA plasma and gDNA from affected and healthy gastric mucosa cells (5x carcinoma, 2x glandular dysplasia, 1x mucous metaplasia, 1x chronic eosinophilic gastritis, 9x healthy).

3.2 Plasma cfDNA concentration comparison between Belgian shepherd dogs with GC, -precancerous changes and healthy control dogs

Plasma cfDNA concentrations were analysed from 6 dogs with diagnosed GC, 4 dogs with precancerous gastric glandular dysplasia or mucous metaplasia, 1 dog with chronic eosinophilic gastritis and 26 healthy control dogs. Median cfDNA concentration for dogs with GC was 8 pg/µl (2-15 pg/µl), with precancerous changes 4 pg/µl (2-14 pg/µl), with chronic eosinophilic gastritis 6 pg/µl and for presumably healthy dogs 6 pg/µl (2-12 pg/µl), revealing a wide overlap of results between groups (Figure 1., Appendix 1.)

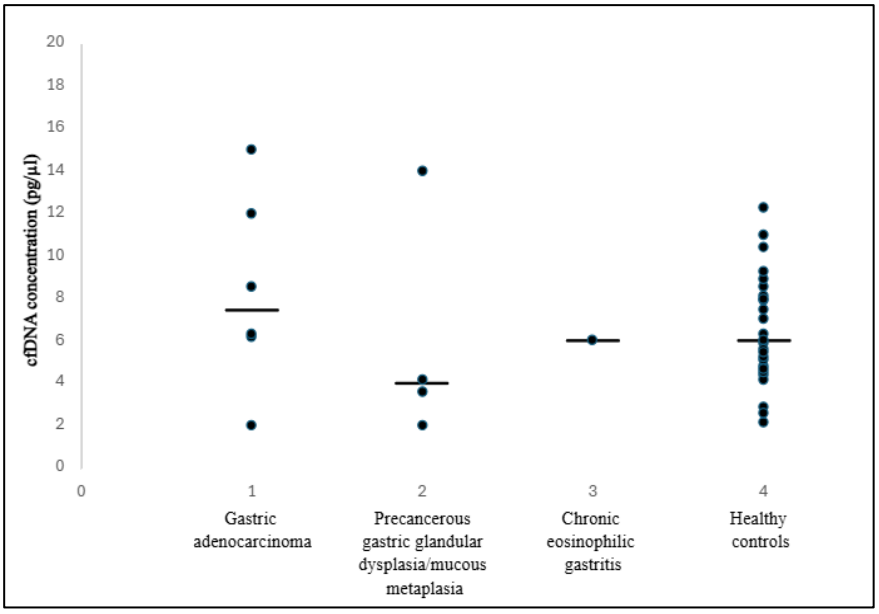

Figure 1. Plasma cfDNA concentrations (pg/µl) of gastric adenocarcinoma (n=6), precancerous gastric glandular dysplasia / mucous metaplasia (n=4), chronic eosinophilic gastritis (n=1) and healthy controls (n=26).

3.3 Methylation sequencing of gDNA and cfDNA

Sequencing library preparation and sequencing were outsourced to an external service provider due to time constraints. Unfortunately, sequencing data generated by the external provider were of insufficient quality to allow full analysis,

and it was financially impossible to send the samples to another service provider for a second sequencing attempt. The main complication was inefficient fragmentation of gDNA before library preparation, and lack of QC measures to flag up the issue to the sample processing team before sequencing.

Methylation sequencing is a relatively new method and therefore not yet frequently used at facilities providing molecular analyses. Therefore, it was financially not possible to send all the gDNA and cfDNA samples to another place leading to the postponement of this analysis and with this to an exclusion of respective date from this study.

4 DISCUSSION

The objective of this study was to investigate the relationship between cfDNA concentration and the GC status in Belgian shepherd dogs of the Tervuren and Groenendael variants, classified into healthy control dogs by clinical history (no clinical signs of gastric disease or any other diseases), and into patient groups by histology of endoscopically taken gastric mucosa tissue biopsies into GC, precancerous changes (metaplastic and dysplastic), and chronic eosinophilic gastritis. The aim of the study was to test the hypothesis that the measurement of cfDNA concentrations in EDTA-plasma might be a way to diagnose GC earlier and in a less invasive matter than gastric endoscopy in general anaesthesia. The hypothesis was based on studies which have highlighted increased plasma cfDNA concentrations in cancer patients, both in humans and in dogs (Flory et al. 2022, Flory et al. 2024, Kim et al. 2021, Prouteau et al. 2021, Rafalko et al. 2023, Tagawa et al. 2019).

4.1 cfDNA concentrations in GC of Belgian Shepherd dogs

Based on this proof-of-concept study, there is no indication that cfDNA concentrations mirror the status of canine GC in dogs that underwent endoscopy with gastric mucosal biopsy for histological classification. Plasma cfDNA concentrations of all groups, including healthy dogs and dogs with GC, preneoplastic gastric mucosal changes or chronic eosinophilic gastritis, were low with a large overlap of concentrations between all groups. The results disproved the study's

hypothesis that cfDNA concentrations would reflect the GC status of Belgian Shepherd dogs as assumed due to previous findings linking elevated plasma cfDNA levels to canine cancers such lymphoma and hemangiosarcoma (Flory et al. 2022, Flory et al. 2024, Kim et al. 2021, Prouteau et al. 2021, Rafalko et al 2023, Tagawa et al. 2019). Based on this study, there is no evidence that cfDNA concentration would increase as the disease develops.

When reflecting these results, several limitations concerning the study design, number of investigated cases, type of investigated cancer and certain preanalytical variables in a canine cancer liquid biopsy need to be taken into consideration. Proof-of-concept studies are performed, when there is no information available that allows for a power analysis to estimate the number of cases needed for a reliable statistical assessment. For a new diagnostic test, such studies allow an early-stage clinical trial to gather preliminary evidence whether the test holds promise for the given diagnosis. The advantage of proof-of-concept studies is their small sample size: it allows researchers to evaluate a diagnostic method's promise before deciding to proceed with or modify future clinical trials. Proof-of-concept studies have been conducted in canine oncology to evaluate the feasibility of novel diagnostic approaches. For example, a proof-of-concept study demonstrated that using NGS-based method to investigate plasma cfDNA could detect tumour-associated genomic alterations in dogs with cancer and therefore supporting the potential of liquid biopsy as a non-invasive diagnostic tool in veterinary medicine (Kruglyak et al. 2021).

Concerning the number of investigated dogs, the low number of affected dogs must be taken into consideration. In addition to small sample size, this study included single time-point sampling which could be a limitational factor to detect differences in plasma cfDNA concentrations. Healthy dogs did not go through endoscopy and therefore missing histologic confirmation of gastric health should be considered. GC is often difficult to diagnose and can be present in dogs without clinical signs (Candido et al. 2021). Patient-specific variation in plasma cfDNA concentrations could be another factor affecting the results as the patient cohort is limited.

Concerning type of investigated cancer, it needs to be taken into consideration that tumour characteristics can vary significantly between tumour types. Tumour cell turnover, meaning cell death frequency, has proven to be different

between cancer types. Tumour burden and location can influence the cell turnover in tumours and therefore affect how much cfDNA is released into the bloodstream. Tumours can be high shedding or low shedding, which influences how much tumour-derived material it releases into the bloodstream (Rouvinov et al. 2026, Sheriff et al. 2025). Shedding type of an investigated tumour could be one of the reasons for variations in plasma cfDNA levels of different types of cancers.

Haematological malignancies such as multicentric lymphoma, leukaemia, histiocytic sarcoma and vascular solid tumours such as hemangiosarcoma are proven to shed more cfDNA to the blood than more localized solid tumours. Canine GC is a highly localized solid tumour such as soft tissue sarcoma, osteosarcoma and mast cell tumour. These are proven to be more low shedding due to various factors. In addition, patients with mammary carcinomas have been proven to be showing low plasma cfDNA. Although, the grade of mammary carcinomas is linked to increasing plasma cfDNA concentration (Beffagna et al. 2017, Tagawa et al. 2019, Wilson-Robles et al. 2022). Wilson-Robles and colleagues stated that tumours of haematopoietic origin (lymphoma, histiocytic sarcoma, hemangiosarcoma) often caused nucleosome elevations in the blood while localized tumours (e.g. GC) did not. This highlights that differences in systemic versus localized tumour behaviour may influence cfDNA concentration in the blood (Wilson-Robles et al. 2022).

Possible explanation for the lower plasma cfDNA levels of GC could be associated to tumour morphology and biological behaviour of the disease. Solid tumours, such as GC, soft tissue sarcoma or mammary tumours, can have different levels of vascularisation. The density of capillaries and structure of blood vessels can differ within a tumour. Neovascularization, formation of new blood vessels, has been linked to increased tumour metabolism, vasodilation and inflammation (Ribatti & Pezzella, 2021).

Vascular integration can affect the shedding level of tumours. Visceral and pulmonary histiocytic sarcoma (HS), a highly vascular cancer, commonly shows higher plasma ctDNA detection than non-pulmonary HS when screening for mutations. Elevated plasma ctDNA levels may contribute to an overall increase in total plasma cfDNA and total shedding (Prouteau et al. 2021). Additionally, dogs with metastatic hemangiosarcoma have higher ctDNA fractions than localized disease. Suggesting that dogs with metastasis have higher plasma cfDNA and

more degraded fragments versus those without metastasis (Tagawa et al. 2019, Favaro et al. 2022). This indicates that capillary density and direct blood-tumour contact could be one of the factors that affect tumour shedding and plasma cfDNA levels. Additionally, Wilson-Robles et al. (2022) mentioned that localized solid tumours are commonly using regional vasculature and lymphatic drainage for metabolic processes rather than systemic circulation. As these more localized tumours mainly rely on nearby blood vessels instead of systemic circulation, the release of tumour-derived DNA into the bloodstream might be limited, which could explain their low cfDNA shedding (Tagawa et al. 2019, Wilson-Robles et al. 2022).

However, these interpretations are mainly theoretical and there could be many factors contributing to this phenomenon. For example, pre-analytical variables in a canine cancer liquid biopsy have not been systematically studied yet. Although there are some broader studies about analytical factors affecting cfDNA yields in liquid biopsy. Goggs (2019) studied the effects of sample type on plasma cfDNA concentrations in dogs. Plasma cfDNA levels were compared between EDTA, citrate and Streck BCT plasma tubes, EDTA and citrate being the most promising. Pre-analytical factors such as tube type can factor into plasma cfDNA levels (Goggs, 2019). In this pilot study, only EDTA tubes were used exclusively to ensure that all samples were collected and kept under the same conditions.

In addition to sample tubes, pre-analytical factors such as the timing of plasma separation can affect the plasma cfDNA levels. Burnett and others (2016) suggested that plasma should be collected within 2 hours of blood collection to be reliably measured (Burnett et al. 2016). However, there is no gold standard for the pre-analytical methods or measurement of plasma cfDNA for dogs and therefore should be kept in mind when comparing data and results from other studies related to plasma cfDNA measurements.

Alcaide and others (2020) provided more insight on limitations of cfDNA as a diagnostic tool for dogs. Common methods for measuring plasma cfDNA do not provide reliable information about sample purity and therefore can affect downstream analyses negatively. gDNA contamination can shift the ratios of short and long cfDNA fragments (Alcaide et al. 2020). Lee et al. (2019) also mentioned that low plasma cfDNA concentrations makes it harder to extract from

blood and can cause bias and inconsistency for the results, although at the same time stating cfDNA as very promising biomarker for breast cancer detection (Lee et al. 2019). Despite its limitations as a quantitative biomarker for localized solid tumours such as GC, cfDNA shows considerable potential in the detection and monitoring of systemic and non-localized canine cancers.

There are certain limitations of using cfDNA as a biomarker in canine cancer diagnostics. cfDNA might not be a suitable quantitative biomarker for all cancer types. Many factors, including tumour location, high vs. low shedding, vascularization, greatly influences the plasma cfDNA levels. As discussed above, GC is a localized solid tumour with low shedding characteristics. It has been established that the result of this study is not supporting quantification of plasma cfDNA as a reliable method for detecting canine GC.

4.2 Methylation patterns of gDNA as a biomarker for canine GC

The other hypothesis of this study was that there could be detectable GC-specific methylation patterns in gDNA. Methylation patterns have been associated with cancer development, and it is the most studied epigenetic modification in animals. Unusual methylation patterns in DNA, such as hypomethylation, have been linked to early phases of cancers such as leukaemia and lymphoma in dogs. Genome-wide hypomethylation has been observed frequently in canine mast cell tumours, especially in grade III. It has been discovered in dogs with lung cancer and metastatic osteosarcoma (Xavier et al. 2020). Methylation sequencing provides tissue-specific information, and methylation patterns are often altered in the early stages of tumour development. Despite this potential, there are currently no studies on methylation patterns in canine GC.

Unfortunately, methylation sequencing did not provide any data in this study because of analytical problems at the externalized sequencing provider and resulting funding limitations. The gDNA of all Belgian Shepherd groups were extracted from the affected and healthy tissue samples collected by FINSAGAS. cfDNA was extracted in the laboratory provided by DeepScan Diagnostics. The methylation patterns of gDNA were supposed to be compared based on the methylation sequencing data. The library preparations for methylation sequencing were tested in the DeepScan Laboratories, however because of time

constraints, the library preparation and sequencing were outsourced to an external service provider. Internal QC was done before sending the gDNA samples to out. For reasons outside our control, sequencing data received from the service provider was of inadequate quantity and quality to develop and test in-house pipelines for data analysis. However, methylation sequencing data could provide deeper insights into the molecular mechanisms of GC in Belgian Shepherd dogs. Consequently, the next phase of the Canine Gastric Carcinoma project involves sequencing a larger cohort of affected and healthy dogs by a trusted Finnish partner, supported by a long-term collaboration between DeepScan Diagnostics and the FIN-SAGAS research group to collect cases with proven gastric pathologies.

4.3 Future aspects

Future studies of canine GC should focus on larger cohorts, multiple sampling time-points and the use of qualitative rather than quantitative methods for cfDNA assessment. The investigation of GC-specific methylation patterns in cfDNA could improve the sensitivity and accuracy of cfDNA-based diagnostics for canine GC. More research is needed on localized and low shedding canine tumours, such as GC, to determine whether missing differences in cfDNA between patient groups are because of low shedding due to tumour biology or because of technical detection limitations.

Although the quantification of total plasma cfDNA concentration is not enough to distinguish between cancerous, pre-cancerous and healthy dogs in this proof-of-concept study, plasma cfDNA can still be considered as a potential biomarker for canine GC. Plasma cfDNA has many qualities and applications with the simplest cfDNA-based diagnostic tool being the measurement of total plasma cfDNA to support the diagnosis of canine lymphoma and hemangiosarcoma (Tagawa et al. 2019, Kambayashi et al. 2024). However, in recent years, tumour-specific cfDNA fragment analyses have enabled the investigation of cfDNA characteristics and even the determination of tissue of origin (Alcaide et al. 2020, Cristiano et al. 2019). In recent studies, the focus has shifted from cfDNA to ctDNA with more in-depth information of tumour genetic profile (Alcaide et al. 2020, Prouteau et al. 2021). Currently, there has been limited research on the canine GC genetic profile, but it should be more in focus due to its potential as a

spontaneous disease model for human GC (Hugen et al. 2016, Pinho et al. 2011). Investigating the epigenetic characteristics of canine GC may be the next important step in identifying GC-specific biomarkers for early detection (Ishizaki et al. 2020), as abnormal DNA methylation patterns have been widely explored as diagnostic biomarkers in human GC and breast cancer (Lee et al. 2019, Lee et al. 2025).

Early detection of canine GC is crucial for better prognosis of this mostly fatal disease since it is often diagnosed too late when the cancer has already metastasized. Current detection methods for canine GC are invasive and expensive for owners. There is a need for minimally invasive diagnostic approaches that enable earlier detection of canine GC and allow repeated sampling without significant burden to the patient or owner. As discussed earlier, cfDNA provides a promising alternative to conventional diagnostic methods enabling the analysis of tumour-derived DNA from blood samples. In the case of canine GC, cfDNA could potentially provide information about tumour-specific molecular alterations. While total plasma cfDNA concentration may lack sufficient sensitivity for canine GC, qualitative features of cfDNA, such as methylation, fragmentation and epigenetic modifications, hold promise of a possibly greater diagnostic value. In addition, these methods may also support disease monitoring and provide insight into tumour biology within comparative oncology.

5 CONCLUSIONS

Quantitative plasma cfDNA concentrations did not show any significant variability between GC, pre-GC, chronic gastritis and healthy Belgian Shepherd dogs. Low plasma cfDNA concentrations may be due to biological characteristics of canine GC. Factors such as tumour vascularization, compartmentalization (localized versus systemic), cellular turn-over and tumour shedding rates could influence plasma cfDNA levels.

This study highlights the knowledge gaps in the biological characteristics of canine GC, particularly regarding its genetic and molecular features, which remain poorly understood. Non-invasive and sensitive diagnostic approaches are essential for the detection of this disease, which remains particularly difficult to

detect in early stages. Qualitative cfDNA detection method should be considered over quantitative cfDNA detection for canine GC.

This thesis was done as a part of the larger Canine Gastric Carcinoma project, which will continue to study the methylation patterns of canine GC and the possibility to discover GC-specific alterations from plasma cfDNA. Overall, cfDNA sequencing and methylation analysis represent promising non-invasive methods for addressing current diagnostic limitations in canine GC.

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APPENDIX 1. TOTAL PLASMA CFDNA CONCENTRATION DATA

Table 2. Total plasma cfDNA concentration data

Group/Diagnosis	Variant	Age	Sex	cfDNA concentration (pg/µl)
GC	GROENENDAEL	9	MALE	12
GC	GROENENDAEL	8	FEMALE	6
GC	GROENENDAEL	12	FEMALE	9
GC	TERVUREN	11	FEMALE	6
GC	TERVUREN	12	FEMALE	2
GC	TERVUREN	11	FEMALE	15
Dysplasia	GROENENDAEL	8	FEMALE	4
Dysplasia	TERVUREN	8	MALE	4
Metaplasia	TERVUREN	10	FEMALE	2
Metaplasia	TERVUREN	3	FEMALE	14

Gastritis	GROENENDAEL	14	MALE	6
Healthy	TERVUREN	10	FEMALE	4
Healthy	TERVUREN	9	FEMALE	2
Healthy	TERVUREN	11	MALE	6
Healthy	TERVUREN	5	MALE	7
Healthy	TERVUREN	5	FEMALE	6
Healthy	TERVUREN	9	FEMALE	10
Healthy	TERVUREN	12	MALE	8
Healthy	TERVUREN	7	MALE	8
Healthy	TERVUREN	6	FEMALE	3
Healthy	TERVUREN	9	MALE	6
Healthy	TERVUREN	6	MALE	5
Healthy	TERVUREN	10	FEMALE	5
Healthy	TERVUREN	4	FEMALE	9

Healthy	TERVUREN	12	FEMALE	9
Healthy	GROENENDAEL	4	FEMALE	12
Healthy	GROENENDAEL	9	FEMALE	6
Healthy	GROENENDAEL	6	MALE	3
Healthy	GROENENDAEL	5	FEMALE	7
Healthy	GROENENDAEL	15	MALE	5
Healthy	GROENENDAEL	12	MALE	9
Healthy	GROENENDAEL	8	FEMALE	4
Healthy	GROENENDAEL	5	FEMALE	8
Healthy	GROENENDAEL	6	MALE	5
Healthy	GROENENDAEL	6	FEMALE	5
Healthy	GROENENDAEL	5	MALE	5
Healthy	GROENENDAEL	14	FEMALE	11