Investigation of cell-free DNA in canine plasma and its relation to disease

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ABSTRACT
Background: DNA is released from dying cells during apoptosis and necrosis. This cell-free DNA (cfDNA) diffuses into the plasma where it can be measured. In humans, an increase in cfDNA correlates with disease severity and prognosis.
Objective: It was hypothesized that when DNA in canine plasma was measured by emission fluorometry without prior DNA extraction, the concentration of cfDNA would increase with disease severity.
Animals: The diseased population consisted of 97 client-owned dogs. The clinically normal population consisted of nine client-owned dogs presenting for ‘wellness screens’, and 15 colony-owned Harrier Hounds.
Methods: Plasma cfDNA was measured by fluorometry without prior DNA extraction. The effects of ex vivo storage conditions were evaluated in plasma from two clinically normal dogs. In all other dogs, plasma was separated within two hours of collection. The association between the cfDNA concentration in hospitalized dogs and a variety of clinical, clinicopathological and outcome variables was tested.
Results: The concentration of cfDNA was reliably measured when plasma was separated within two hours of blood collection. The diseased dogs had significantly higher cfDNA than clinically normal dogs (P < 0.001), and the more severe the disease, the higher the cfDNA when severity was categorized according to the American Society of Anesthesiologists (ASA) status (P < 0.001). Dogs that did not survive to discharge had significantly higher cfDNA concentrations than survivors (P = 0.02).
Conclusions/Clinical Importance: The concentration of cfDNA in the plasma of diseased dogs is associated with disease severity and prognosis. Measurement of canine cfDNA could be a useful non-specific disease indicator and prognostic tool.

KEYWORDS
Dog; canine; cell-free DNA; cfDNA; prognosis; biomarker; fluorometry

1. Introduction
In 1948, Mandel and Metais reported the presence of free nucleic acids within human serum for the first time (Mandel & Metais 1948). Due to a lack of understanding of the nature of DNA and its role at the time, this discovery initially received little scientific attention. The presence of intact DNA within the serum was reported in 1973, in patients suffering from systemic lupus erythematosus (Koffler et al. 1973). In 1977, Leon et al. reported high concentrations of cell-free DNA (cfDNA) in the serum of patients suffering from lymphoma, lung, ovary, uterine and cervical neoplasms, and further observed that cfDNA concentrations in serum decreased after successful chemotherapy (Leon et al. 1977). It was hypothesized that disease states causing increased cell necrosis and apoptosis raised the concentration of circulating cfDNA.
Since its discovery, cfDNA has been found to be increased in a variety of neoplastic and non-neoplastic diseases of humans, including myocardial infarction (Chang et al. 2002), rheumatoid arthritis (Zhong et al. 2007), severe viral infections (Ha et al. 2011), stroke (Rainer et al. 2003), sepsis (Martins et al. 2000; Rhodes et al. 2006) and severe trauma (Lo et al. 2000; Lam et al. 2003; Macher et al. 2012). In all these diseases, the cfDNA concentrations have correlated with disease severity and prognosis. cfDNA was also found to correlate with clinical severity within human intensive care units (Saukkonen et al. 2007).
The concentration of cfDNA has been measured in human plasma by various techniques like SYBR Gold fluorescence (Goldstein et al. 2009), PicoGreen direct fluorescence and SYBR Green quantitative polymerase chain reaction (qPCR) (Xue et al. 2009), and using the Qubit nucleic acid quantification system (Invitrogen 2010). Multiple ex vivo factors have been found to affect cfDNA stability, including storage temperature, time till plasma separation from cells and repeated freeze-thawing of the plasma (Jung et al. 2003; Chan et al. 2005). Previously, DNA was isolated from the plasma samples and then amplified by polymerase chain reaction (PCR). However, this DNA extraction
results in a loss of DNA from the sample (Goldstein et al. 2009; Xue et al. 2009; Jung et al. 2010). The amount of DNA lost during extraction depends on the extraction technique but it can be as great as 80% (Xue et al. 2009). In addition, PCR is limited to the amplification of a specific sequence or fragment of the total cfDNA. More recently, studies have focused on non-specific cfDNA quantification using emission fluorometry after the addition of a fluorochrome with specific affinity for DNA (Goldstein et al. 2009; Xue et al. 2009; Invitrogen 2010).

Despite the expanding recent interest in cfDNA in the human literature, there are few investigations of cfDNA in canine plasma. In 2009, Uzuelli et al. reported a significant increase in the concentration of cfDNA in the plasma of dogs with experimentally induced pulmonary thromboembolism (Uzuelli et al. 2009). The increase in plasma cfDNA was linearly correlated with the degree of venous occlusion. The source of the cfDNA appeared to be the thrombus rather than the ischemic tissue, since pulmonary embolism caused by plastic microspheres did not cause a significant rise in cfDNA. Thus, there was a direct relationship between the cfDNA and the total mass of thrombus. In another study, cfDNA was extracted from the plasma of five dogs with mammary carcinoma (Beck et al. 2013). The DNA was then subjected to PCR using primers developed to be specific for known tumor mutations identified in tumor biopsies. In four out of the five dogs, tumor-specific DNA was found in the plasma. The tumor-specific DNA was undetectable following surgical excision of the tumor in four of the five dogs, but persisted in one, which was later attributed to a pulmonary metastasis. Finally, cfDNA concentrations were measured in 40 clinically normal dogs, in 20 dogs with non-neoplastic diseases and in 80 dogs with cancer (Schaefer et al. 2007). Dogs with lymphoma had a significantly higher cfDNA than clinically normal dogs. In addition, the dogs with the highest cfDNA concentrations had shorter remission times than those with lower concentrations. Despite these findings, the authors concluded that the diagnostic sensitivity and prognostic value of the assay were low. Importantly, the authors of these three canine papers used a commercial filtration column based system to purify the plasma DNA prior to measurement, which is likely to have reduced the overall concentration.

The aims of this study were to (1) develop a protocol for the measurement of cfDNA in canine plasma using emission fluorometry without prior extraction, (2) to determine if the concentration of cfDNA was increased in diseased dogs, (3) to determine if any increase was related to disease severity and (4) to determine if the concentration of cfDNA was associated with clinical outcome.

2. Materials and methods

2.1. Determining the effect of storage conditions ex vivo on cfDNA concentration

To determine how ex vivo storage conditions affect cfDNA analysis, blood was collected from two Harrier Hounds from the Massey University Canine Nutrition Unit. The dogs were defined as clinically normal on the basis of having a stable bodyweight, with no significant abnormalities detected on a physical examination, or history of illness in the three months prior to or after collection. Venous blood was collected by needle and syringe, and added to ethylenediaminetetraacetic acid (EDTA) containing collection tubes. A 1 mL aliquot was immediately centrifuged for 15 minutes at 5000 × g, and the plasma was aspirated and frozen at −20 °C (time zero reference sample). The remaining EDTA blood was divided into two series of aliquots. One series of aliquots was held at ambient temperature and the other series was held at 4 °C. At 2 hours (h), 4 h, 6 h, 8 h and 24 h after collection, 1 mL aliquots of the whole blood from both holding conditions were centrifuged and the plasma was separated and divided into two further aliquots of plasma. One aliquot of this resulting plasma was immediately frozen at −20 °C, and the other was held at 4 °C for a further 24 h before being transferred to −20 °C for storage (Figure 1).

2.2. Measurement of cfDNA in clinically normal and diseased dogs

2.2.1. Clinically normal dogs

Plasma samples were obtained from EDTA blood samples collected from nine clinically normal client-owned dogs presented to the Massey University Veterinary Teaching Hospital for health screening. Exclusion criteria were a history of current disease, abnormalities detected on routine clinical examination, or significant abnormalities on a complete blood count (CBC), serum biochemistry or urine analysis. In addition, blood was collected from 15 clinically normal Harrier Hounds at the Massey University Canine Nutrition Unit. In the group of Harrier Hounds, clinically normal was again defined as having a stable bodyweight, with no significant abnormalities detected on a physical examination, or history of illness in the three months prior to or after collection.

2.2.2. Diseased dogs

Plasma samples were collected from canine patients presenting to the Massey University Veterinary Teaching Hospital between July 2013 and February 2014. Inclusion criteria were presence of sufficient EDTA blood after the blood was used for routine hematology, harvesting of plasma within 2 h of collection and the availability of a completed medical record. To
To define disease severity, the physical status classification system of the American Society of Anesthesiologists (ASA status, Table 1) was applied to each diseased dog (Hardie et al. 1995). For assigning the ASA status and for categorizing into neoplastic disease, patients were excluded if the CBC was not performed on the sample at the time of sample collection. Patient data collected for all samples were the patient’s age, sex, breed, CBC and serum biochemistry results, underlying disease process and immediate clinical outcome, which was defined as discharged alive vs. dead or euthanased in hospital. Any patient that had been ill for longer than two weeks was categorized as being chronically diseased, whereas, patients with a shorter disease course were defined as acutely diseased. One of the investigators (D.L. Burnett), blinded to the patients’ cfDNA concentration, categorized all patients into an ASA status retrospectively based on their patient data file. The study protocols were approved by the Massey University Animal Ethics Committee.

2.3. Sample handling

For all samples collected from clinically normal and diseased client-owned dogs, plasma was separated from whole blood within 2 h of sample collection, by centrifugation for 15 minutes at 5000 × g. The plasma was then immediately frozen at −20 °C.

2.4. cfDNA analysis

Plasma samples were thawed in batches of 15–50 samples for cfDNA analysis. The quantification of cfDNA was performed utilizing the Qubit® dsDNA HS Assay Kit and a Qubit® 1.0 fluorimeter (Life Sciences, Carlsbad, CA, USA), according to manufacturer specifications. The Qubit assay utilizes a dye that fluoresces with a higher intensity when bound to dsDNA, where the recorded amount of fluorescence is proportional to the amount of dsDNA within the sample. A total volume of 20 μL of plasma was utilized for each analysis. The concentration of the cfDNA was calculated using the dilution algorithm provided by the manufacturer within the Qubit® 1.0 (Life Sciences, Carlsbad, CA, USA). The Qubit® 1.0 was calibrated with the provided standards before each run. A single sample with a previously measured concentration of cfDNA, which had been previously separated into aliquots and stored at −20 °C, was assayed with every batch of samples as an inter-assay control. Samples collected from the clinically normal Harrier Hounds were assayed in triplicate. Samples collected from client-owned dogs were assayed once. Intra-assay precision was analyzed by determining coefficient of variation (CV) on 22 samples run in triplicate.

2.5. Statistical analysis

The results are expressed as means ± standard error of the mean (SEM). Data were not normally distributed when tested using the Shapiro–Wilk test and,
therefore, data were inverse transformed to normalize. Repeated measures two-way analysis of variance (ANOVA) was used to determine the significance of the effect of storage conditions on cfDNA concentration. Comparisons between categorical variables (e.g. disease status, sex, survival, chronicity and neoplasia) were performed using a Welch’s Two Sample t-test, and comparisons between handling conditions were made using a paired t-test. A simple linear model was used to test the relationship between the concentration of cfDNA and the ASA status. A multiple linear regression model with stepwise exclusions was developed to test the association between the concentration of cfDNA and all hematological variables; serum glucose, creatine kinase (CK), aspartate aminotransferase, alanine aminotransferase, bilirubin, albumin, globulins, urea, creatinine, sodium, potassium, chloride, age and sex. Outliers were recognized visually by inspection of scatter plot and histogram confirmed by a Cook’s distance of greater than 1, and were removed from the analysis. An alpha-value of $P < 0.05$ was used to define statistical significance.

3. Results

The intra-assay CV for the measurement of cfDNA ranged from 0.04% to 6.5% with a mean CV of 2.0%. The inter-assay CV of six aliquots of the same sample held at $-20 \, ^\circ C$ over a four-month period was 4.7%.

3.1. Ex vivo handling conditions on canine cfDNA

When whole blood from two clinically normal Harrier Hounds was stored at $4 \, ^\circ C$ or at ambient temperature prior to plasma separation, the concentration of cfDNA in the plasma decreased during the first 4 h, then appeared to rise again after 4–6 h (Figure 2(A)). The effect of time was significant ($P < 0.001$), as was the storage temperature prior to plasma separation ($P = 0.0378$), and the concentration of cfDNA from the sample held at ambient temperature was significantly higher than the $4 \, ^\circ C$ sample at 24 h. There was no significant difference between the cfDNA concentration in plasma when separated immediately and when it was removed 24 h later, regardless of whether the blood was held at room temperature or refrigerated (Figure 2(B)).

Once separated from the cellular component, the concentration of cfDNA did not significantly alter when the plasma was stored for 24 h at $4 \, ^\circ C$ ($P = 0.0584$) (Figure 2(B)). However, there was a trend towards a very small (1.05%) decrease in cfDNA concentration in samples stored for 24 h (median: 570 $\mu g/L$ at 0 h and 564 $\mu g/L$ at 24 h). Thus, for testing of clinical samples, it was decided that plasma should be removed from EDTA blood within 2 h of collection, and stored at $-20 \, ^\circ C$ if not assayed immediately.

3.2. cfDNA in clinically normal and diseased animals

In the clinically normal dogs, the median concentration of cfDNA was 583 $\mu g/L$ with a 25%–75% inter-quartile range (IQR) of 550–610 $\mu g/L$. A total of 97 diseased dogs met the inclusion criteria. The dogs ranged from six months to 15 years of age. The median concentration of cfDNA in the diseased dogs was 733 $\mu g/L$ (25% and 75% IQR: 622–818 $\mu g/L$), which was significantly higher ($P < 0.001$) than the normal dogs (Figure 3).

When the diseased population was divided into patients with neoplastic and non-neoplastic disease, there was no significant difference between the two disease groups ($P = 0.5174$) (Figure 4). When the diseased population was divided into those with acute and chronic illness, the patients with acute illness had significantly higher cfDNA than those with chronic disease (median: 813 $\mu g/L$, 25% and 75% IQR: 664–981...
mL and median: 696 mL, 25% and 75% IQR: 603–779 mL, respectively, P < 0.0103) (Figure 5).

Of the 97 diseased dogs, 89 patients survived till discharge, 8 animals were euthanased due to their underlying disease process and 1 died spontaneously in hospital. When the diseased population was divided into those that survived to discharge and those that died or were euthanased in the hospital, it was found that the patients that died or were euthanased in the hospital had significantly higher cfDNA than those animals that survived to discharge (median: 934 mL/mL, 25% and 75% IQR: 771–1158 mL/mL vs. median: 727 mL/mL, 25% and 75% IQR: 619–804 mL/mL, P < 0.02) (Figure 6). Categorization into an ASA status was possible for 77 of the diseased hospital patients, as well as for the 24 clinically normal controls. The concentration of cfDNA was positively associated with the ASA status of the patients (P < 0.0001) (Figure 7). The results of the linear regression model are presented in Table 2.

After stepwise exclusion to determine the variables independently associated with the cfDNA concentration, only band neutrophils and activities of aspartate amino transferase (AST) and CK remained in the final model. The regression output for the inverse transformed cfDNA concentration is presented in Table 3. Whilst AST and band neutrophils were positively associated with cfDNA (or negatively associated with the inverse transformed data, Table 3), CK was negatively associated.

The concentration of cfDNA was then assessed for its potential as a non-specific screening test for disease. The 95% upper confidence interval (CI) of the mean of clinically normal animal population (598 mL/mL) was
used as a cut-off, which resulted in a sensitivity of 84% and a specificity of 58% for disease detection. The positive and negative predictive values of this test as a non-specific indicator of disease were 89% and 47%, respectively. The concentration of cfDNA was also assessed for its potential as a prognostic indicator test within the diseased dogs. A cut-off of the 95% upper CI of the mean of the diseased dogs that survived to discharge was used (778 μg/L). Using that cut-off, the sensitivity and specificity for predicting outcome were 75% and 65%, respectively. Although the positive predictive value of this test as a prognostic indicator was only 16%, the negative predictive value of the test was 97%.

### 4. Discussion

The principle finding in this study is that the concentration of cfDNA is elevated in a number of canine disease states. Dogs with a cfDNA of higher than 598 μg/L had an 84% likelihood of being diseased. An increase in cfDNA concentration was also significantly associated with the severity of the underlying disease process, as categorized by the animal’s ASA status. This might be due to an increase in cell death resulting in an increased number of DNA fragments diffusing into circulation. The results from this analysis demonstrate that acute disease processes had significantly higher elevations in cfDNA concentrations than chronic ones. This result is indicative of an increased rate of necrotic and apoptotic cell death in acute compared with chronic diseases. These findings suggest that measuring the concentration of cfDNA could be a useful indicator of disease severity in a clinical setting.

The increase in cfDNA concentration was also significantly associated with short-term survival (defined as discharged alive), suggesting the potential for the measurement of cfDNA concentration to be utilized as a prognostic indicator. Using a cut-off of 778 μg/L, the positive predictive value for short-term survival of a hospitalized patient was 97%. However, the negative predictive value of this test was only 16%, indicating that although cfDNA may be useful in practice to convey a favorable prognosis to owners, a high cfDNA would not in itself be an indicator of a negative prognosis. It should also be noted that evaluation of survival in veterinary patients is complicated by euthanasia of treatable conditions. Certainly, it can be postulated that some of the euthanased patients may have survived considerably longer, had their owners not elected for euthanasia at that period of time.

The multiple linear regression model demonstrated a significant positive correlation between cfDNA and both the band neutrophil count and AST activity. An explanation for this positive correlation is that these clinical pathology parameters would have been increased in animals with increasing disease severity and increasing cell death. It is noteworthy that there was no association with the total white cell count, which suggests that even when there is a leukocytosis, the circulating cells are not significant contributors to the cfDNA component. The weak negative association between CK activity and cfDNA is more puzzling. One possible explanation may be that animals with more severe underlying disease processes and higher cfDNA, may have had reduced activity and, therefore, may have had reduced muscle mass, leading to an overall decrease in CK activity. Alternatively, it is possible that the increased cfDNA concentration is demonstrative of the poorer prognosis of hospitalized patients with low body muscle (Thibault & Pichard 2010).

One potential limitation of this study is the fact that the majority of normal controls was Harrier Hounds (15 of 24 animals) and, therefore, may not have been a true representation of the normal canine population. Due to the large variety of breeds analyzed in this study, the presence of any significant breed variation in cfDNA concentration was unable to be determined.
The previous study on canine cfDNA by Schaefer et al. found no impact of breed on cfDNA concentration, although that study also had too few of any particular breed for a firm conclusion (Schaefer et al. 2007). Thus, a breed effect cannot be excluded at this stage and requires further investigation.

Analysis of the ex vivo effects on cfDNA concentration in canine plasma suggested that a delay in separation of plasma of more than 2 h may have a detrimental effect on cfDNA concentration. In the samples collected from the two clinically normal dogs, there was an initial drop, followed by an increase in the cfDNA concentration, most notable when the whole blood was held at room temperature prior to separation. This dynamic process of continued cell death and DNA degradation ex vivo in samples prior to plasma isolation has also been found to occur in human samples and, similarly, is least marked when plasma is separated less than 2 h post sample collection (Jung et al. 2003; Xue et al. 2009). To minimize the impact of ex vivo variation in this study, all plasma was separated from blood samples in less than 2 h. However, once plasma has been separated from the cells, storage for 24 h prior to cfDNA analysis at 4 °C did not cause a biologically relevant change in plasma cfDNA concentration. This implies that measurement of cfDNA concentration could be utilized in general veterinary practices without the purchase of extra equipment. To ensure accurate results, blood would need to be promptly centrifuged, the plasma refrigerated and sent to a laboratory for analysis within 24 h. Nonetheless, it is clear that further study is required to more fully describe the changes in cfDNA concentration ex vivo with different handling conditions and times.

The concentration of cfDNA was increased in dogs with both neoplastic and non-neoplastic diseases. This contrasts with the findings of Schaefer et al. (2007), who reported that the cfDNA concentration was only elevated in dogs with lymphoma (Schaefer et al. 2007). It is most likely that the contrast is due to the difference in sample handling between the two studies. In the study by Schaefer et al. (2007), a column filtration based DNA extraction protocol was used, whereas, no filtration step was used in the present study. Although column filtration greatly improves DNA purification, it removes a significant quantity of DNA in the process (Goldstein et al. 2009; Xue et al. 2009; Jung et al. 2010); thus, it is possible that an increase in cfDNA concentration in non-neoplastic patients was not seen by Schaefer et al. (2007) because of the extraction technique. In the present paper, cfDNA was analyzed without DNA extraction, thus, eliminating the loss of small DNA fragments in the extraction process. Using the technique described in this manuscript, the lowest concentration of cfDNA was 439 µg/L, whilst the range of concentrations reported by Schaefer et al. (2007) was 1–91 µg/L, which was similar to the experimental data reported by Uzuelli et al. (2009), and in both studies, DNA extraction was performed with QIAamp DNA blood mini Kits, prior to DNA quantification. Standard DNA extraction protocols are extremely insensitive at conserving small DNA fragments (<100 bp), resulting in up to eight-fold variations in the human literature in cfDNA concentrations when DNA extraction was performed using standard extraction techniques (Jung et al. 2010). Thus, the recommendation that could be made from the results of this research is that quantification of cfDNA in plasma only be performed on unaltered plasma.

5. Conclusions

The assaying of cfDNA in canine plasma is economical, simple and easy, using emission fluorometry without prior DNA extraction. The concentration of cfDNA in separated plasma was stable for 24 h if kept at 4 °C prior to freezing. The concentration of cfDNA correlated with measures of clinical severity and prognosis in canine patients. As such, there is potential for cfDNA measurement to be utilized as a prognostic indicator in general clinical veterinary practice similar to currently used acute prognostic indicators such as lactate or C-reactive protein in human and veterinary medicine. However, it is likely that further analysis of the cfDNA fraction such as sequence-specific analysis of cfDNA or determination of the fragment size distribution may prove more useful for the diagnosis and monitoring of various diseases, as has been shown in human medicine (Benesova et al. 2013; Mattos-Arruda et al. 2013).

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Disclosure statement

The authors declare that they have no competing interests.

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