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Effect of sample type on plasma concentrations of cell-free DNA and nucleosomes in dogs

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ABSTRACT

Cell-free DNA (cfDNA) and nucleosomes are two biomarkers of cell death and neutrophil extracellular trap formation that are increased in dogs with sepsis, immune-mediated haemolytic anaemia, cancer and following trauma and have diagnostic and prognostic values. cfDNA and nucleosomes are typically measured in plasma samples using DNA-specific fluorophores and ELISA assays, respectively, but their concentrations may be affected by pre-analytical variables such as sample type. The present study aimed to investigate the influence of sample type on the plasma cfDNA and nucleosome concentrations of a heterogeneous group of dogs presenting to an emergency room. Triplicate samples were collected into K_2-ethylenediamine tetraacetic acid, 3.2% citrate and a specialised DNA stabilisation tube (Streck BCT), processed rapidly and frozen for batch analysis. Biomarker concentrations were compared between sample types by calculation of Spearman's correlation coefficients, and with Deming regression, Bland-Altman plots and the Friedman test. Overall, biomarker concentrations were highly correlated between the three sample types. The most concordant results were obtained using citrate samples and the DNA stabilisation tube. Matched cfDNA concentrations between the different sample types were significantly different but there was no significant difference between the nucleosome concentrations in any of the sample types. The present study suggests that cfDNA and nucleosomes can be successfully measured in various sample types, but distinct sample types do not produce interchangeable results. This argues for use of a consistent sample type within studies and suggests standardisation may be useful for the field.

INTRODUCTION

Cell-free DNA (cfDNA) is detectable in the circulation of healthy people,[1–3] and normal dogs.[4] In health, this cfDNA originates from various cells, but particularly from death of haematopoietic cells including those of lymphoid and myeloid origins.[5 6] Some cfDNA may be also released through apoptosis, necrosis or neutrophil extracellular trap (NET) formation,[7–9] through a process called NETosis.[10] Plasma cfDNA has been extensively investigated as a diagnostic and prognostic biomarker in people with various disease processes.[11] Increased plasma cfDNA concentrations have been identified in people with bacteraemia,[12 13] sepsis,[14] neoplasia[15] and burns.[16] In people, concentrations of cfDNA are also increased after myocardial infarction and trauma.[17 18] Increased cfDNA concentrations have been identified in dogs with sepsis,[19] trauma,[20] neoplasia,[21] gastric dilation volvulus (GDV) syndrome[22] and immune-mediated haemolytic anaemia (IMHA).[23 24]

Concentrations of cfDNA are not specific for any one source,[25] but this marker can still provide valuable insights into disease severity, prognosis and pathogenesis.[26] Measurement of cfDNA provides a quantitative measure of DNA release that is applicable to multiple species and can be adapted to high-throughput screening assays.[27 28] Control of pre-analytical factors is important for measurements of cfDNA concentrations.[29] Plasma samples are preferable to serum because release of DNA from leukocytes following collection can falsely increase measured concentrations.[29–31] Other factors that may affect cfDNA concentrations are time between collection and processing,[4 32] centrifugation methods,[29] and storage conditions.[29 33]

Nuclear DNA is coiled inside chromatin by packaging it as DNA–histone complexes consisting of DNA wound around a core of eight histone proteins. These complexes are called nucleosomes and can be released into the circulation during programmed cell death, NETosis and following cell necrosis.[34] As such, cfDNA and nucleosomes share potential origins, but are distinct entities,[35] with differential potential for immune cell activation through pattern recognition receptors.[34] Measuring both biomarkers may provide better insights into disease processes than either alone.[35]

People with septic shock have significantly higher nucleosome concentrations than those with sepsis or fever,[34] and plasma nucleosome concentrations correlate with organ dysfunction severity in septic critically ill people.[36] Nucleosome concentrations are

increased in horses with colic,[37] in dogs with sepsis,[19 21] and in dogs following trauma, where they correlate with injury severity and prognosis.[20] Nucleosome concentrations are also increased in dogs with IMHA,[24 38] a condition in which there may be a link between NETs and thrombosis.[39] This is noteworthy, because nucleosomes may contribute to disease progression by promoting and propagating thrombosis.[40] Histone proteins are highly conserved and hence nucleosomes are typically measured by ELISA methods that enable measurement of these complexes in multiple species. As with cfDNA, however, they are subject to pre-analytical sources of variation because they can also be released by leukocytes post-collection. It should also be recognised that assays employing the picogreen dye (such as that used in the present study) have the potential to recognise double-stranded DNA (dsDNA) that exists as free DNA fragments and also the dsDNA that is bound to histones. The picogreen assay is much more sensitive to free dsDNA fragments, compared with that bound to histones,[41] but some overlap between these two biomarkers exists and may affect the interpretation of results.

Recently, sample collection tubes containing stabilising agents with the potential to reduce pre-analytical variation in cfDNA and nucleosome measurements have become available.[42–45] Specifically, these tubes aim to limit post-collection release of cfDNA and nucleosomes from leukocytes through the use of preservatives that prevent cell lysis. To the author's knowledge, none of these tubes have been evaluated in dogs to date. Given the considerable interest in measurement of cfDNA and nucleosomes in veterinary medicine and in dogs in particular, it would be valuable to know if these more expensive, specialist tubes perform better than other sample types. The present study aimed to compare the cfDNA and nucleosome concentrations measured in three plasma sample types. It was hypothesised that cfDNA and nucleosome concentrations measured in distinct sample types are different from one another.

MATERIALS AND METHODS

Study population

The present study employed a convenience sampling method to replicate typical conditions in an emergency room where samples for cfDNA and nucleosome measurements might be collected. It was expected that this would produce a range of analyte concentrations and maximise generalisability. Dogs weighing >4 kg with evidence of systemic inflammatory response syndrome (SIRS), or with a disorder expected to cause systemic inflammation (eg, metastatic hemangiosarcoma) were considered potential study subjects. The SIRS criteria used were temperature <38.1°C or >39.2°C; heart rate ≥120 beats per minute; respiratory rate ≥40 breaths per minute; white blood cell count >16×10^9 cells/mL or <6×10^9 cells/mL, or >3% band neutrophils.[46]

Sample collection and processing

Blood samples were collected with informed client consent from intravenous catheters or by venipuncture directly into three separate evacuated tubes containing K2-ethylenediamine tetraacetic acid (EDTA; BD Vacutainer, BD Biosciences, San Jose, CA, USA), 3.2% sodium citrate (Vacuette, Greiner Bio-One, Monroe, NC, USA) and a specific additive tube (Streck Cell-Free DNA BCT, Streck, La Vista, NE, USA) designed for cfDNA measurement. Within 10–15 min of blood collection, plasma samples were generated by centrifugation of whole blood for 10 min at 1370 g (Ultra-8V Centrifuge, LW Scientific, Lawrenceville, GA, USA). Shortly (within 5 min) after centrifugation, plasma was decanted into polypropylene freezer tubes (Polypropylene Screw-Cap Microcentrifuge Tubes, VWR, Radnor, PA, USA) by pipetting. Some plasma was deliberately left in each tube to minimise the risk of disturbing the buffy coats. The plasma samples were then frozen at −80°C pending batch analysis. At the time of batch analysis, samples were thawed at room temperature and then kept at 4°C while in use. Samples were collected within a 24-day period (7 March 2018 to 30 March 2018) with batch analysis on 4 April 2018.

cfDNA and nucleosome measurements

Concentrations of both biomarkers were measured contemporaneously. Concentrations of cfDNA were measured in triplicate using a benchtop fluorimeter (Qubit 3.0 Fluorometer, Life Technologies, Waltham, MA, USA) and relevant reagents (Quant-It HS dsDNA Kit, Life Technologies, Waltham, MA, USA) according to the manufacturers' instructions and as previously described.[19 22] Mean values of the three measurements were used for subsequent analyses. Concentrations of plasma nucleosomes were analysed using a commercial ELISA (Cell Death Detection ELISA Plus, Roche, Indianapolis, IN, USA) scaled against pooled normal canine plasma obtained from healthy canine blood donors, as previously described.[20 24 38] None of the aliquots of pooled normal canine plasma had been previously thawed prior to analysis. Nucleosome concentrations were measured in duplicate and the mean values used for subsequent analyses.

Statistical methods

A priori sample size calculations were not conducted for this study. Data were assessed for normality using the D'Agostino-Pearson omnibus test. Parametric data are summarised as mean±SD while non-parametric data are summarised as median with IQR. The degree of correlation between cfDNA and nucleosome concentrations measured in different sample types was assessed by calculating Spearman's correlation coefficient (r_s). Strength of correlation was assessed as follows: <0.5 weak, 0.5–0.6 mild, 0.6–0.7 moderate, 0.7–0.8 strong, 0.8–0.9 very strong and 0.9–1.0 excellent. Since no sample type is established as the reference standard, Deming regression was used to analyse the relationship between pairs of sample types.

Bland-Altman plots were constructed to identify constant and proportional biases between sample types. Median cfDNA and nucleosome concentrations were compared between sample types using the Friedman test, with Dunn's adjustment for multiple comparisons. Alpha was set at 0.05. All analyses were conducted using commercial software (Prism 7.0e for Mac OS X, GraphPad, La Jolla, CA, USA).

RESULTS

Patient population

In all 24 dogs were recruited, however, due to two Streck BCT tube failures (one due to breakage and one due to loss of tube vacuum), only 22 complete sets of triplicate samples were collected. The 22 dogs consisted of mixed-breed dogs (n=6), Labrador retrievers (n=4), Golden retrievers (n=3) and one each of the following English bulldog, German shepherd dog, Great Pyrenees, Hungarian vizsla, Mastiff, miniature dachshund, rottweiler, Shetland sheepdog and Staffordshire bull terrier. The mean age and bodyweight were 7.5±3.1 years (IQR 5–10.3) and 31.5±12.3 kg (IQR 23.6–39.4), respectively. On presentation to the hospital, the average temperature, heart rate and respiratory rate were 38.4°C±0.7°C (IQR 38–39), 129±22 bpm (IQR 114–140) and 28 (IQR 24–40) rpm, respectively. The median leucocyte count (n=14) was 10.6×10^9/mL (IQR 5.9–17.2), median neutrophil count was 8.8×10^9/mL (IQR 4.4–13.9), with 0% band neutrophils (IQR 0–4.5). Of the 22 dogs, 13 met at least 2 of 4 SIRS criteria (59.1%) as previously described.[46] Final diagnoses in these dogs were neoplasia (n=9), gastroenteropathy (n=2), intervertebral disc disease (n=2), sepsis (n=2), trauma (n=2), acute kidney injury, fibrocartilaginous embolism, hepatopathy, peripheral neuropathy and urethral obstruction (all n=1).

cfDNA concentrations

The median (IQR) cfDNA concentrations in the three sample types were as follows: citrate plasma 434 ng/mL (298–540); EDTA plasma 519 ng/mL (384–591) and additive tube plasma 532 ng/mL (388–589) (figure 1). The concentrations of cfDNA were significantly lower in the citrate plasma compared with both EDTA plasma and the additive tube plasma (both p<0.0001). There was no significant difference between cfDNA concentrations in EDTA plasma compared with additive tube plasma (p=1.0000). Calculation of Spearman's correlation coefficient and Deming regression showed that there was excellent correlation between the cfDNA concentrations in all three of the sample types (figure 2). Bland-Altman analysis showed evidence of small constant negative biases for the citrate plasma versus other sample types but no proportional biases in any of the sample types. Consistent with the correlation analyses, the best level of agreement was found between the citrate and the additive tube plasma samples (figure 2).

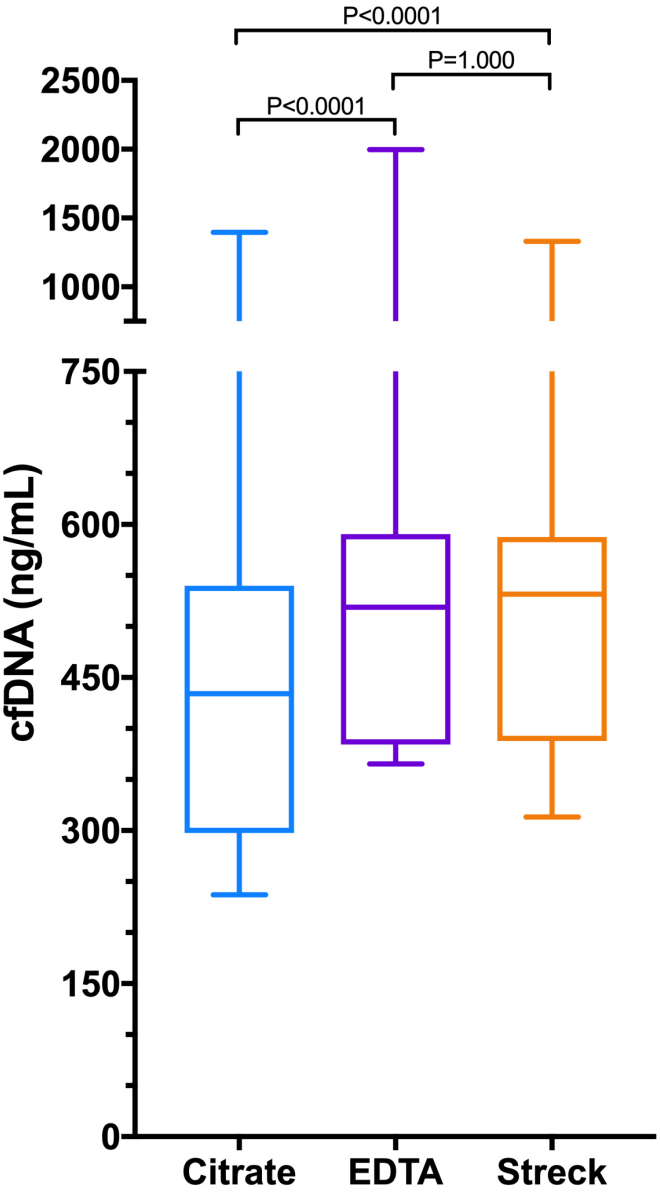

Figure 1 Box and whisker plots comparing cfDNA concentrations in a heterogeneous group of dogs measured in three different sample types (citrate plasma, EDTA plasma, plasma derived from Streck BCT tubes that include a preservative to limit cell lysis). The centre line represents the median, the boxes represent the IQR and the whiskers represent the minimum and maximum values. Concentrations of cfDNA were significantly different in citrate samples compared with both EDTA the Streck tube samples. There was no difference between the cfDNA concentrations in EDTA samples compared with Streck tube samples. cfDNA, cell-free DNA; EDTA, ethylenediamine tetraacetic acid.

Nucleosomes

The median (IQR) nucleosome concentrations in the three sample types were as follows: citrate plasma 4.3 AU (2.1–7.8); EDTA plasma 6.5 AU (2.6–13.9) and additive tube plasma 3.9 AU (1.7–13.0) (figure 3). There were no significant differences in the concentrations of nucleosomes between any of the sample types (p≥0.150).

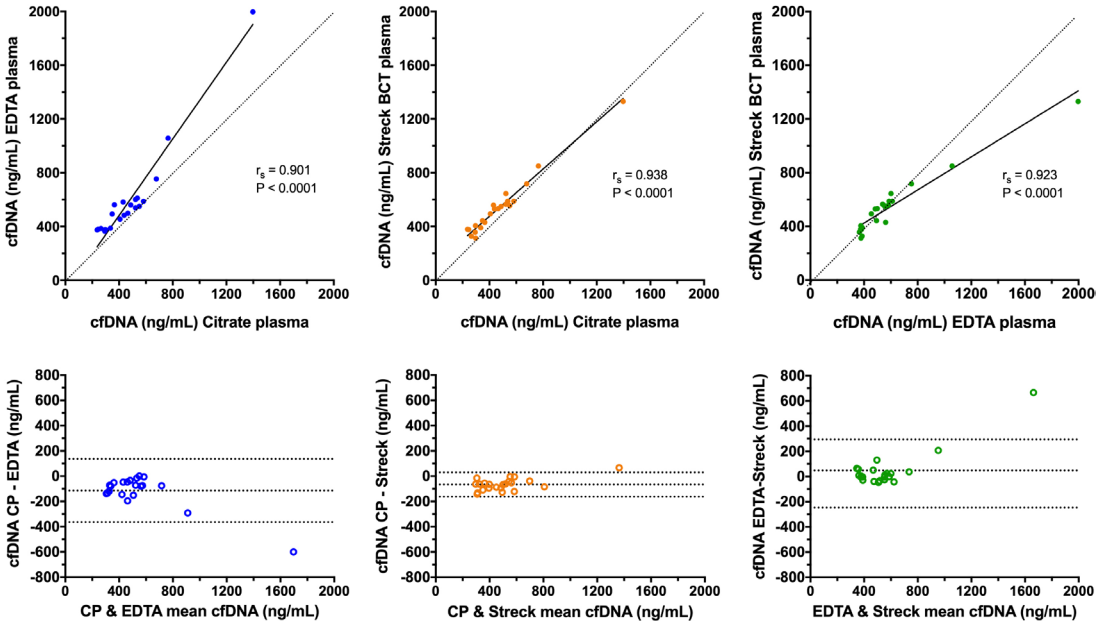

Figure 2 Scatterplots (upper panels) and Bland-Altman plots (lower panels) comparing the cfDNA concentrations in each of the three sample types. In the scatterplots, results from pairs of sample types have been plotted against each other combined with associated values for Spearman's correlation coefficient (r_s) and the relevant significance value. The solid black line represents the line of best fit by Deming regression. Note the r_s value does not correspond to the Deming regression line. The dotted lines are lines of identity, included for reference. In the Bland-Altman plots, the mean value of the two measurements from the two sample types is plotted on the abscissa (x-axis), while the difference between the two sample types is plotted on the ordinate (y-axis). The middle of the three horizontal dotted lines represents the mean difference between the two sets of values, whereas the upper and lower dotted lines represent ±1.96 SD of the mean difference. cfDNA, cell-free DNA.

Calculation of Spearman's correlation coefficient and Deming regression showed that there was very strong correlation between the nucleosome concentrations in all of the sample types (figure 4). Bland-Altman analysis showed a small constant negative bias between citrate samples and the other two sample types, but no evidence of proportional biases in any of the sample types. The best level of agreement was found between the citrate and the EDTA plasma samples (figure 4). There was weak correlation between the cfDNA and nucleosome concentrations in all of the sample types, and none of the correlation coefficients were statistically significant (figure 5). The best correlation between these two biomarkers was present in the additive tube plasma samples (r_s 0.385, p=0.077).

DISCUSSION

The present study aimed to compare three sample types for the measurement of cfDNA and nucleosomes in a population of dogs with disease processes predisposing to inflammation. In addition, the present study aimed to assess the utility of a specific preservative tube designed for cfDNA measurement. The data suggest that all three of the sample types can be used for measurement of cfDNA and nucleosomes. There were statistically significant differences between the concentrations measured in the three sample types for cfDNA, but not for nucleosomes. The differences in cfDNA concentrations might not be clinically relevant, however. For cfDNA measurement, the best correlation and the highest levels of agreement were found between the citrate plasma and the Streck BCT plasma samples, although all of the correlation coefficients were >0.9. For nucleosomes, the best correlation and the highest levels of agreement were found between the citrate plasma and the EDTA plasma samples. This may suggest that either EDTA or citrate plasma samples are reasonable choices for measurement of these biomarkers.

No reference standard (gold standard) method has been established for measurement of these biomarkers and hence the optimal sample type has yet to be determined. This precludes recommending a specific sample type, but data from the present study suggest that either EDTA or citrate plasma would be acceptable choices. The smallest levels of constant bias existed between the Streck and the EDTA plasma samples for both biomarkers. In contrast, the concentrations of cfDNA and nucleosomes consistently measured lower in citrate samples particularly with regard to EDTA plasma. This could be interpreted in two ways. The citrate samples may cause falsely low readings while the other sample types reflect the true concentrations in the patient, or the Streck and the EDTA samples cause falsely increased concentrations relative to the patient. Increased concentrations could be due to cell lysis that may be a source of pre-analytical error. Additional studies in dogs evaluating the quality as well as the quantity of cfDNA are warranted to determine if citrate plasma maintains cfDNA quality better than

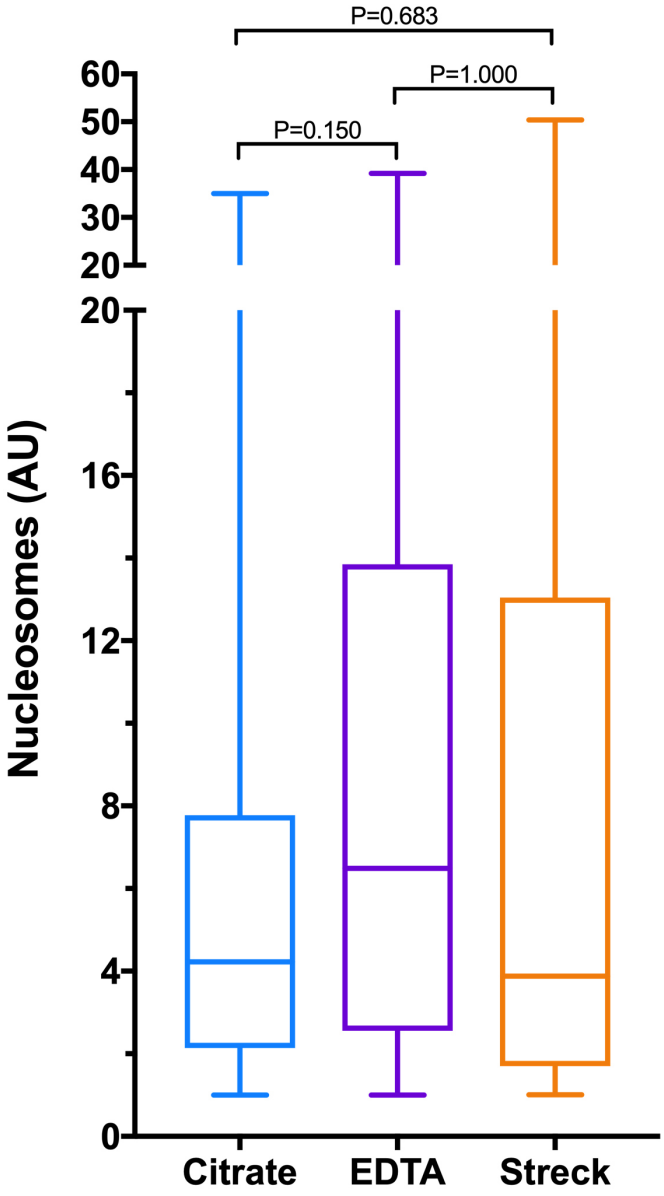

Figure 3 Box and whisker plots comparing nucleosome concentrations in a heterogeneous group of dogs measured in three different sample types (citrate plasma, ethylenediamine tetraacetic acid plasma, plasma derived from Streck BCT tubes that include a preservative to limit cell lysis). The centre line represents the median, the boxes represent the IQR and the whiskers represent the minimum and maximum values. There were no significant differences between the concentrations of nucleosomes in any of the sample types.

does EDTA, as has been described for people.[47] Such additional studies might also evaluate whether there is an impact of diagnosis or disease severity on the sensitivity of biomarker detection in distinct sample types. For instance, future studies might investigate whether one sample type is more sensitive in sepsis, while another has greater sensitivity in trauma.

The Streck tube additive is designed to reduce the likelihood of release of cfDNA after sample collection. If those tubes work correctly in dogs, then this might suggest that the biomarker concentrations in the citrate samples are falsely low. At this time, it is not possible to determine which of these scenarios is correct. Irrespective, data from the present study argue that while different sample types might produce similar results, they are not interchangeable. Thus, any study measuring cfDNA or nucleosome concentrations in dogs should use a single sample type consistently throughout the study period.

Concentrations of cfDNA can be measured at the point of care and because they correlate with illness severity indicators such as bacteremia,[19] and outcome in diseases including sepsis and IMHA,[19 23] this marker may become a useful clinical tool for emergency and critical care practice. In this respect, use of a sample type that is readily available, such as EDTA or citrate, may be preferable to a more expensive, single-purpose tube such as the Streck BCT.

Although the initial descriptions of direct cfDNA measurement were performed on serum,[48] plasma samples are now recommended.[29] This is because serum samples can generate falsely increased concentrations,[31 49] due to lysis of leukocytes during the ex vivo clotting process.[50 51] Some authors have suggested that EDTA tubes may be the best choice where plasma processing must be delayed.[52] In human medicine, the need for sample storage or shipping prior to processing led to the development of cfDNA stabilisation tubes such as the ones evaluated in the present study.[53–55] The medical literature suggests that the Streck BCT tubes enable blood collection in a range of clinical settings,[42] and extend the time available for sample processing. However, they may not offer substantial advantage over conventional sample types such as EDTA when processing occurs within 6 hours.[56] Data from the present study also suggest that these stabilisation tubes perform comparably to other sample types when samples are processed and frozen rapidly after collection. Future studies in dogs might evaluate the effects of sample types in the presence of delayed processing, storage or shipping.

The lack of a significant correlation between the nucleosome concentrations and the cfDNA concentrations in these dogs is noteworthy. As discussed above, these two biomarkers are similar but distinct, in terms of their structure, origin and pathophysiological effects. The picogreen assay used in the present study has the potential to recognise both biomarkers. The lack of correlation between the two biomarker concentrations suggests that the cfDNA assay may be relatively insensitive to the nucleosomes in these plasma samples, as has been previously reported.[41] In studies of other canine patient populations, these biomarkers have been determined to be positively correlated,[19 20] but the strength of the association in those studies was weak. These weak associations may indicate related but distinct origins for release of these biomarkers and suggests that measuring both biomarkers may be worthwhile. Fortunately, the results of the present study confirm they can both be measured in the same sample type.

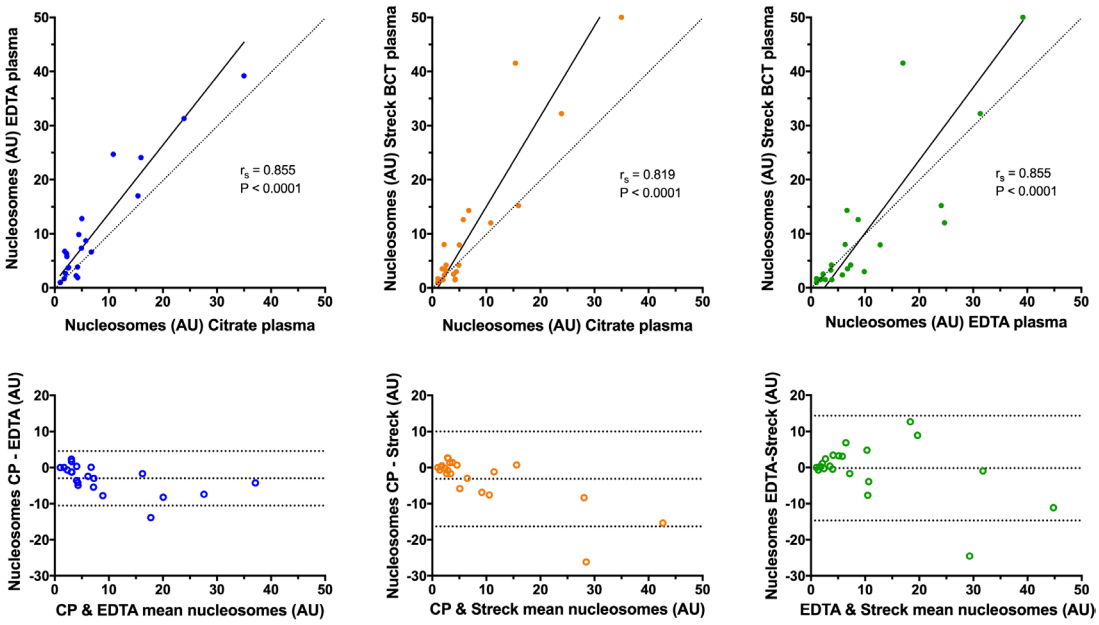

Figure 4 Scatterplots (upper panels) and Bland-Altman plots (lower panels) comparing the nucleosome concentrations in each of the three sample types. In the scatterplots, results from pairs of sample types have been plotted against each other combined with associated values for Spearman's correlation coefficient (r_s) and the relevant significance value. The solid black line represents the line of best fit by Deming regression. Note the r_s value does not correspond to the Deming regression line. The dotted lines are lines of identity, included for reference. In the Bland-Altman plots, the mean value of the two measurements from the two sample types is plotted on the abscissa (x-axis), while the difference between the two sample types is plotted on the ordinate (y-axis). The middle of the three horizontal dotted lines represents the mean difference between the two sets of values, whereas the upper and lower dotted lines represent ±1.96 SD of the mean difference.

The present study is not without limitations. The sample size was relatively small, but despite this significant differences between sample types for cfDNA concentrations were detectable. The present study aimed to recruit a heterogeneous population of dogs with disease processes predisposed to inflammation to reflect a typical population of dogs in which cfDNA and nucleosomes might be measured. The final diagnoses for most dogs were not known at the time of recruitment and hence the population enrolled contained more dogs with neoplastic diseases than anticipated. The overall cfDNA and nucleosome concentrations were lower than reported for other patient populations,[19 20 22] perhaps reflecting a lower disease severity in this cohort compared with others. Despite this, the cfDNA and nucleosome concentrations covered a wide range (~twofold for cfDNA and ~sevenfold for nucleosomes) and provided a solid dataset to assess for evidence of proportional biases between sample types. Inspection of the cfDNA scatterplots suggests that one sample with a high concentration of cfDNA in all three sample types may have disproportionately affected the Deming regression line (high leverage point). Reanalysis of these scatterplots without this point (not shown) did not alter the conclusions drawn, but reduced all of the correlation coefficients. Thus, data from all 22 dogs are presented here. The present study did not evaluate all of the commercially available tubes that purport to reduce

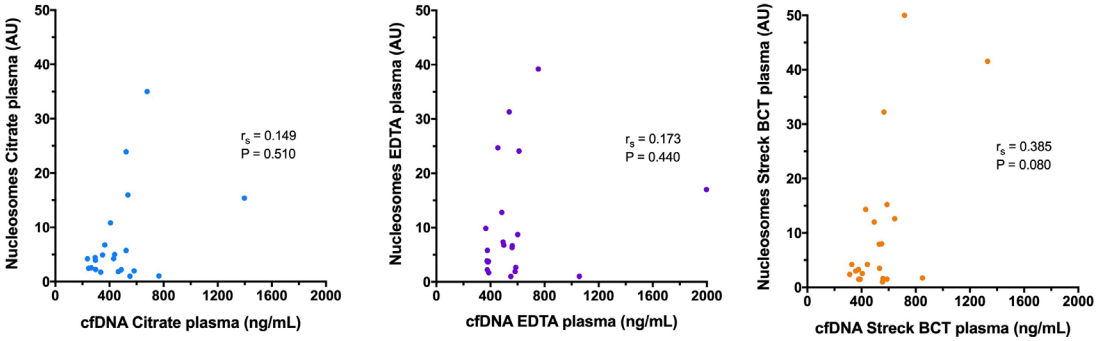

Figure 5 Scatterplots comparing the cfDNA and the nucleosome concentrations in each of the three sample types. In the scatterplots, results from pairs of sample types have been plotted against each other. Also displayed are the associated values for Spearman's correlation coefficient (r_s) and the relevant significance value. Note none of the r_s values were statistically significant at p<0.05. cfDNA, cell-free DNA.

pre-analytical variation. Future studies might compare the Streck BCT tube with other similar products from other manufacturers.

The samples tested were frozen for varying amounts of time before batch analysis, but the maximum duration of storage was less than 1 month. It is possible, but unlikely that sample deterioration occurred within this time period. cfDNA concentrations are reportedly stable in serum samples repeatedly frozen and thawed. In a study by Goldshtein *et al*, there was a decrease in the mean cfDNA concentrations, but the difference was not statistically significant.[48] A study of serum samples from human cancer patients measured nucleosomes after 6–9 months of storage at −70°C and then again ~5.5 years later. That study identified a significant decrease of 32% in measured concentrations between the first and the second analyses.[57]

In summary, in the present study, cfDNA and nucleosome concentrations were similar in all three sample types tested. Distinct sample types do not produce equal cfDNA concentrations, however, and should not be considered interchangeable. Thus, a consistent sample type should be used for any given study. In settings where sample processing and analysis can be conducted rapidly, there may be little advantage to using a more expensive and less widely available additive tube for cfDNA or nucleosome measurement. EDTA or citrate plasma samples appear to be reasonable choices for cfDNA or nucleosome measurement in dogs. It may be useful to consider standardisation of cfDNA and nucleosome assays in the future to maximise the comparability of results across studies and between groups, as has been recommended in human medicine.[29]

Contributors All authors listed fulfilled the following criteria: (1) substantial contributions to conception and design, acquisition of data or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content. Specifically, RG designed and conducted the research, analysed the data and wrote the manuscript. All authors approved the final version of the manuscript.

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Competing interests None declared.

Ethics approval This study was carried out in accordance with the recommendations of the Cornell University College of Veterinary Medicine (CU-CVM), Institutional Animal Care and Use Committee (Ithaca, NY, 14853). Approval for this study was granted by the local Institutional Animal Care and Use Committee (IACUC-2014–0053).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article.

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