Characterizing Circulating Nucleosomes in the Plasma of Dogs with Lymphoma

Christopher Dolan (crdolan@cvm.tamu.edu)
Texas A&M University
Tasha Miller
Texas A&M University
Jarvis Jill
Texas A&M University
Jason Terrell
Volition America LLC
Theresa Kelly
Volition America LLC
Thomas Bygott
Volition Diagnostics UK Ltd
Heather Wilson-Robles
Texas A&M University

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Abstract

Background:

Nucleosomes consist of DNA wrapped around a histone octamer core like beads on a string so that DNA can be condensed as chromatin into chromosomes. Diseases such as cancer or inflammation lead to cell death where chromatin is fragmentated and released as mononucleosomes into the blood. The Nu.Q™ H3.1 assay measures total nucleosome concentration in plasma of humans and has been used to detect and identify cancer even at early stages. The objectives of this study were to determine if nucleosome levels could be used to distinguish between healthy dogs and dogs with various stages of lymphoma (LSA) using the Nu.Q™ H3.1 assay.

A total of 126 dogs diagnosed with LSA and 134 healthy controls were recruited for this study. Plasma was collected from each dog and stored in K2-EDTA tubes. The LSA patient samples were recruited from TAMU or purchased from various biobanks. All control cases were recruited from TAMU. Samples were also collected longitudinally from 3 dogs undergoing treatment for multicentric lymphoma at TAMU as a pilot study to investigate the pattern of nucleosome concentrations in plasma during treatment.

Results:

Dogs with LSA had an approximately 7-fold increase in their plasma nucleosome concentrations compared to controls (AUC 87.8%). Nucleosome concentrations increased with cancer stage and dogs with B cell lymphomas had significantly higher nucleosome concentrations than dogs with T cell lymphomas.

Nucleosome concentrations from serially monitored patients were elevated at diagnosis and progression with subsequent decreases in nucleosome concentration that corresponded to clinically detectable responses to therapy.

Conclusions:

The Nu.Q™ H3.1 assay was able to reliably detect elevated nucleosome concentrations in the plasma of dogs with LSA. Furthermore, it appears that nucleosomes are useful for differentiating cancer from healthy individuals in canines. Results from serially monitored patients indicate that nucleosomes could be an objective monitoring tool for remission status in canine lymphoma patients.

Background

Liquid biopsy is a growing field in human medicine and has significant potential in veterinary medicine as it enables the use of non-invasive techniques and analysis of tumor-derived material including circulating tumor cells, extracellular vesicles, and cell free DNA, among others. Information provided through these tools in cancer patients can provide early detection of neoplastic disease, provide prognostic information, monitor response to treatment, and help identify druggable targets [1, 2]. Furthermore, liquid biopsy assays are much more amenable to serial testing when compared to traditional tissue biopsies or expensive imaging tests.

Nucleosomes are the basic repeating subunit of chromatin consisting of DNA wrapped around a histone core [3]. Nucleosomes regulate several important functions within the cell in part due to a complex network of modifications and regulatory enzymes that control their positioning and stability. Due to the variety and flexibility of modifications, nucleosomes provide the framework for chromatin assembly, epigenetic regulatory mechanisms, while also protecting DNA from damaging agents [4].

Cell free DNA is released into the bloodstream, as nucleosomes, from a variety of cell types that are undergoing apoptosis or necrosis, but are most commonly released from hematopoietic cells as part of normal cellular turnover [6–8]. Low levels of cfDNA have been identified in healthy individuals and increased concentrations are present during various disease processes [9]. Nucleosomes have been shown to have different immunostimulatory potential as compared to circulating free histones and cfDNA [5]. Therefore, while these circulating components are related and share similar origins, they should be considered distinct entities with potentially different functions. Nucleosomes are elevated in humans and dogs with significant inflammation and increased nucleosome concentrations have been shown to be prognostic for survival in dogs experiencing trauma [10–12]. In neoplastic disease processes, nucleosomes are elevated in human patients with colorectal cancer and could help with early detection of this disease [13]. Nucleosomes have also been able to predict response to therapy in patients with advanced non-small-cell lung cancer [14]. Similarly, cfDNA levels are elevated in dogs with various tumor types and cfDNA levels correlated well with clinical stage [15, 16]. While nucleosomes themselves have not been extensively evaluated in canine neoplastic disease, a recent study did show significantly elevated nucleosome concentrations in a small cohort of dogs diagnosed with lymphoma (LSA) [17].

Lymphoma is one of the most frequently diagnosed cancers in the dog and multicentric LSA, characterized by peripheral lymph node enlargement, is the most common clinical presentation of this disease [18]. Often, patients are diagnosed with higher stage disease due to the fact that pet owners often have to recognize the lymph node enlargement before these pets are presented to a veterinarian for diagnosis [19]. The response to therapy for this disease is typically determined by serial measurements of peripheral lymph nodes. Inter- and intra-rater reliability of these measurements are reported to be good to excellent in the clinical setting [20]. Lymph node measurements are helpful in establishing the initial response to therapy evidenced by lymph nodes decreasing in size as well as determining disease progression when the lymph nodes increase in size following therapy. Monitoring peripheral lymph nodes

as the primary indicator of treatment response is lacking as it does not detect minimal residual disease (MRD) after the lymph nodes have returned to a normal size. Previous studies in both humans and dogs have shown a variable amount of MRD following a positive response to therapy and the level of MRD at the end of a chemotherapy protocol has been shown to be prognostic [21, 22]. Lymph node measurements also fail to detect early indicators of disease progression as the disease burden must advance enough to cause lymph node enlargement before the patient is determined to be out of remission. Establishing liquid biopsy techniques, such as measuring nucleosome levels, in canine lymphoma could be helpful by providing objective measures of disease progression or treatment response even if the lymph nodes are normal in size. Such techniques could potentially reveal the MRD as well as provide an early indication of progressive disease prior to detectable lymph node enlargement.

The Nu.Q™ H3.1 Assay detects circulating nucleosomes in the blood of humans that occur with various disease states and has been used to detect and identify cancer even at early stages [13, 23]. This platform is an enzyme-linked immunosorbent assay (ELISA) directed at the histone H3.1 (H3.1) core histone protein. Previous investigations with histone H3.1 have identified cancer-associated mutations which induced nucleosome instability and enhanced cancer cell colony formation [24].

The objectives of this study were to determine whether H3.1 nucleosome concentrations could be used to distinguish healthy dogs and dogs with of LSA using the Nu.Q™ H3.1 assay as well as how nucleosome levels varied across disease stage and with treatment.

Results

Patient Population

A total of 260 dogs were included in this study with 134 in the healthy control cohort and 126 in the LSA cohort. The healthy control cohort ranged in age from 0.83 to 14 years (median 6 years) and the LSA cohort ranged from 2 to 15 years (median 9 years). The healthy control cohort ranged in weight from 2.5 to 55.8 kilograms (median 23 kilograms) and the LSA cohort ranged from 5.0 to 74.5 kilograms (median 28.8 kilograms). The breeds most prevalent in the healthy control cohort were mixed breed (n = 29), Labrador retriever (n = 15), Australian cattle dog (n = 10), pit bull terrier (n = 7), border collie (n = 6), golden retriever (n = 5), dachshund (n = 4), and German shepherd (n = 3). The breeds most prevalent in the LSA population were mixed breed (n = 39), Labrador retriever (n = 10), cocker spaniel (n = 4), golden retriever (n = 8), Shetland sheepdog (n = 4), giant schnauzer (n = 3), and 2 or fewer of a variety of other pure bred dogs such as German Shepherd dogs, boxers, Basset Hounds and terriers. The healthy control cohort had a male to female ratio of 1.05 and a sex distribution including 4 intact females, spayed females (n = 61), intact males (n = 3), and castrated males (n = 65). The LSA cohort had a male to female ratio of 1.86 (82 males and 44 females) and a sex distribution including intact females (n = 3), spayed females (n = 41), intact males (n = 9), and castrated males (n = 73).

Nucleosome Concentration

The nucleosome concentrations in the LSA cohort were significantly higher than those in the healthy control cohort ((median 211.1 ng/ml, mean 570.9 ng/mL, SEM 90.85) and median 31.1 ng/ml, mean 32.07 ng/mL, SEM 1.118, respectively) with a p-value of < 0.0001 (Fig. 1)). According to the receiver operator characteristic (ROC) curve the area under the curve was 87.8% with a sensitivity of 74.6% and a specificity of 100% with a cut off for the healthy range set at 67.5 ng/mL (nucleosome range for all healthy dogs was 6.33–67.42 ng/mL).

To determine whether nucleosome concentrations were elevated across all stages of LSA, we compared nucleosome concentrations between healthy controls and the different stages of lymphoma. Stage of disease was available for all patients included in this study. All stages of LSA, except stage II, had significantly elevated nucleosome concentrations compared to healthy controls (Fig. 2). The median nucleosome concentration for stage I LSA was 104.9 ng/ml (n = 11, mean 691.9 ng/ml, SEM 358, p-value < 0.0001, AUC 87.99%), for stage II LSA was 36.2 ng/ml (n = 7, mean 135.6 ng/ml, SEM 96.45, p-value > 0.088, AUC 69.2%), for stage III LSA was 177.5 ng/ml (n = 37, mean 452.9 ng/ml, SEM 130.4, p-value < 0.0001, AUC 85.1%), for stage IV LSA was 200.2 ng/ml (n = 38, mean 564.2 ng/ml, SEM 167.9, p-value < 0.0001, AUC 91.7%), and for stage V LSA was 421.4 ng/ml (n = 33, mean 763.0 ng/mL, SEM 217.7, p-value < 0.0001, AUC 90.3%).

We next investigated whether elevated nucleosome concentrations were common to both B-cell and T-cell LSA. Immunophenotyping information was available for 61 LSA cases, and nucleosome concentration was compared amongst two immunophenotype groups and healthy controls. Nucleosome concentrations were significantly elevated in both B-cell and T-cell LSA compared to healthy controls (Fig. 3). The median nucleosome concentration for B-cell LSA was 421.42 ng/ml (n = 43, mean 1031.7 ng/ml, SEM 234.2, p-value < 0.0001, AUC 98%) and 153.7 ng/ml for T-cell LSA (n = 18, mean 277.6 ng/ml, SEM 99.4 p-value 0.0006, AUC 74.9%). T-cell LSA patients were found to have a significantly lower nucleosome concentration than B-cell LSA patients (p-value 0.018). In the B cell lymphoma cohort there was one dog with WHO stage I disease (2.3%), no dogs with stage II disease, 13 dogs with stage III disease (30.2%) and 15 dogs each with stage IV (34.9%) and 14 with stage V disease (32.6%). For the T cell lymphoma cohort there were no dogs with stage I disease, one dog with stage II disease (5.6%), 10 dogs with stage III disease (55.6%), 2 dogs with stage IV disease (11.1%) and 5 dogs with stage V disease (27.8%). When using the compressed WHO staging system previously published by Valli et al in 2013, the two have a similar distribution of stage with compressed stage 1 (stages I/II) including one dog each (B cell 2.3% and T cell 5.5%), stage 2 (compressed stages III/IV) the B cell cohort had 28 cases (65.1%) and the T cell cohort had 12 cases (66.6%), finally for the compressed stage 3 (stage V) the B cell cohort had 14 (32.5%) cases and the T cell cohort had 5 cases (27.8%) [19].

A receiver operating characteristic analysis was performed with an established threshold of 67.4 ng/ml which generated an area under the curve of 0.878 (Fig. 4). This threshold produced a sensitivity of 74% at a specificity of 100%. The performance of this threshold for each specific stage was investigated by applying it retroactively to the population of LSA patients. This analysis showed that the threshold could accurately distinguish LSA patients from healthy patients in 63% (7/11) of stage I patients, 14.3% (1/7)

of stage II patients, 75.7% (28/37) of stage III patients, 81.6% (31/38) of stage IV patients, and 81.8% (27/33) of stage V patients. Performance was also evaluated by immunophenotype and the threshold could distinguish LSA patients from healthy patients in 95.3% (41/43) of B-cell LSA and 55.6% (10/18) of T-cell LSA.

Longitudinal Analysis

Since elevated nucleosome concentrations were consistently elevated in patients with LSA we next investigated how nucleosome concentrations changed during the course of disease and treatment in three LSA patients for which longitudinal samples were available. The nucleosome concentration trend of these patients was compared to their clinical response to treatment, obtained from the medical record retrospectively, during therapy along with c-reactive protein (CRP) and thymidine kinase (TK) levels.

Patient 1:

Patient 1, an eleven-year-old male castrated mixed breed dog, was initially diagnosed with multicentric LSA in 2018 and was determined to be out of remission in the spring of 2020 at which time he was restarted on a CHOP chemotherapy protocol. A new heart murmur was detected at the time of progression and an echocardiogram demonstrated dilated cardiomyopathy secondary to a grain-free diet. For this reason, his treatment was changed to a CCNU/L-spar protocol before completing one cycle of CHOP. Clinically, a partial response was noted after starting the CCNU/L-spar protocol. Follow-up with TAMU Veterinary Medical Teaching Hospital (VMTH) was delayed due to COVID-19 and this patient continued to receive treatment with his primary care veterinarian for several visits. Upon returning to TAMU VMTH, he was found to have entered a complete remission.

This patient’s nucleosome concentration was decreased after his second dose of CCNU when he had a partial response clinically (Fig. 5A). His nucleosome concentration was decreased substantially after moving into a complete clinical response. Since achieving clinical remission, his nucleosome concentration has remained in the range of healthy dogs. CRP and TK analysis were performed and compared to Patient 1’s clinical response (Fig. 5A) and were within the normal range during all periods of clinical response. (Fig. 5A).

Patient 2:

Patient 2, a nine-year-old male castrated mixed breed dog, was diagnosed with stage Vb, intra-abdominal, hypercalcemic LSA in February 2020. He was treated with CHOP chemotherapy and was transitioned to a CCNU/L-spar protocol due to lack of response to doxorubicin. Patient 2 also did not respond well to CCNU/L-spar and was transitioned to COP chemotherapy after evaluating his previous responses to vincristine and cyclophosphamide. Due to his primary intra-abdominal disease, the majority of his response was determined by monitoring his calcium concentration and with abdominal ultrasound.

This patient’s nucleosome concentration increased after each dose of doxorubicin and decreased after most doses of vincristine or cyclophosphamide (Fig. 5B). There was an increase in nucleosome

concentration after starting the CCNU/L-spar protocol which then decreased again after changing to COP chemotherapy. Consistent with what was observed for Patient 1, CRP and TK analysis did not show any deviation out of the normal range during all periods of clinical response (Fig. 5B)

Patient 3:

Patient 3, an eleven-year-old male castrated Australian cattle dog, was diagnosed with stage IVa multicentric B-cell LSA in February 2020. He was treated with CHOP chemotherapy and in a clinical remission during his cycle 1 week 3 vincristine visit. He experienced some dose delays throughout his protocol due to neutropenias secondary to chemotherapy administration and finished his CHOP chemotherapy protocol in July 2020, remaining in a complete response at his last recheck visit before manuscript preparation in August 2020.

An initial nucleosome concentration was collected at Patient 3's first dose of vincristine for CHOP chemotherapy. Nucleosome concentrations were not available until the start of cycle 2 following a 19-week CHOP protocol due to hospital procedural changes secondary to COVID-19. This patient's nucleosome concentration decreased overall after starting chemotherapy (Fig. 5C). Consistent with what was observed for Patients 1 and 2, CRP and TK analysis did not show any deviation out of the normal range during all periods of clinical response (Fig. 5C).

CRP and TK Correlations:

Spearman's correlation coefficient analysis was performed between CRP, TK, and nucleosome concentrations for each patient. CRP and nucleosome concentration and TK and nucleosome concentration were found to be uncorrelated (Table 1).

Discussion

The current study follows from previous findings in which nucleosome levels helped with early detection of cancer in humans and were significantly elevated in a small cohort of dogs with LSA [13, 17]. As with most studies, there were limitations present in this study. Samples received from the DCTD Canine Tumor Repository had variable amounts of patient demographic, staging, treatment and outcome data available which would have been useful in generating more power for the analysis and better characterization of those cases with low stage disease and T cell phenotypes. The serially followed patients included in this study had variable chemotherapy protocols and, while this variability is more representative of what occurs clinically, more consistent treatment protocols would have allowed for better comparisons amongst patients. Despite these limitations, the authors feel that the data presented here is strong evidence for the utility of nucleosomes as a tool for screening and monitoring dogs with lymphoma when compared to healthy dogs.

As previously described by this group in a small cohort of dogs with lymphoma, nucleosome concentrations were significantly elevated in the cohort of LSA patients when compared to the healthy

control cohort [17]. The median plasma nucleosome concentration in LSA patients was 6.8 times higher than in the healthy controls. Broadly we found that elevated nucleosome concentrations were present at all cancer stages, except stage II, and present in both B and T cell lymphoma. Finally, in three patients examined longitudinally nucleosome concentrations correlated well with clinical response, though some variations in concentration were seen over time. Additionally, these samples were all batched and evaluated retrospectively, which does not recapitulate how active real time monitoring would occur in active patients.

When evaluated by stage, only the dogs with stage II LSA were found to not have significant elevations compared to healthy controls. This subpopulation contained only 7 dogs and the lack of significance is suspected to be due to a population of insufficient size. Another potential consideration is that the tumor burden associated with this stage of disease does not produce more nucleosomes than healthy dogs. However, this is unlikely since the nucleosome concentrations of dogs diagnosed with stage I LSA were significantly elevated compared to healthy controls in this study. Another consideration for the low nucleosome concentration in the stage II LSA cases is a T cell phenotype. The dogs in this study with T cell LSA had significantly lower nucleosome concentrations than those with B cell LSA, however, upon further review, only one of the dogs with stage II LSA had immunophenotype data available and this dog did, indeed, have T cell LSA. Additional collection and analysis of lower stage LSA patients with full characterization of their disease is needed to help further characterize the nucleosome concentrations in these patient populations.

As mentioned above, while both B-cell and T-cell LSA had significant increases in nucleosome concentration compared to healthy controls, B-cell LSA patients had a 2.7 fold higher median nucleosome concentration as compared to T-cell LSA patients. The underlying mechanism of this difference is unknown. One potential explanation is that while T-cell LSA patients often have peripheral lymphadenopathy, it is the authors' experience that their disease burden is subjectively lower than their B-cell counterparts in the clinical setting. The lower nucleosome concentration detected in this study may be the result of an overall lower disease burden that occurs between B-cell and T-cell LSA. In humans, it has been shown that the amount of cfDNA shed by a LSA patient depends on the particular LSA subtype [25]. It is possible this is also true in dogs and the difference between B-cell and T-cell LSA nucleosome concentrations are indicative of underlying pathophysiologic differences between these LSA subtypes. Finally, owing to the fact that many samples for the lymphoma cohort were purchased from a biobank, most of the cases in this population were not characterized by flow cytometry. It is possible that some of the samples in this group were from dogs with indolent T-cell LSA. Standard immunophenotyping (CD3 positivity) would not be able to differentiate the less aggressive T-cell lymphomas from the more aggressive T cell lymphomas. Studies in humans have also shown that the levels of cfDNA are higher in more aggressive subtypes of LSA [25, 26]. If indolent LSA cases were included in the population of T-cell LSA cases, they may have artificially lowered the overall nucleosome concentration in this population.

A sensitivity of 74.6% at a specificity of 100% in distinguishing LSA patients from healthy controls was achieved using nucleosome concentrations with a threshold of 67.5 ng/ml. This indicates that

nucleosomes could be a useful screening tool in the differentiation of dogs with LSA from healthy dogs. The ROC curve demonstrated that some cases of LSA fell below the discrimination line. These cases were of lower stage or had a T cell phenotype and had plasma nucleosome concentrations similar to the healthy control cohort. This is to be expected as nucleosome concentrations are correlated with stage and, therefore, tumor burden in humans [13, 27, 28]. Similar results were found in the dogs evaluated in this study where the nucleosome concentration increased with stage and tumor burden. Despite this finding, the established threshold was successful in discriminating 63.6% of stage 1 LSA patients from healthy controls. This is an encouraging finding as it shows that circulating nucleosomes could be used as a tool for early disease detection and could be helpful when a diagnosis is difficult to establish.

Nucleosomes were monitored serially in three LSA patients undergoing chemotherapy at the TAMU VMTH. It was anticipated that circulating nucleosome levels would trend with the disease response in these patients due to the short half-life reported for most types of cfDNA [29–31]. Though few in number, the results from these patients are encouraging and show elevated nucleosomes at diagnosis and with disease progression (as seen in patient 2) with subsequent decreases in nucleosome concentration in conjunction with clinically detectable responses to therapy. For all three patients examined longitudinally, the nucleosome concentrations correlated well with clinical response. In patient 1, there is an initial response to the first three weeks of the CHOP protocol that correlated with a partial response detected on physical exam. However, there is an increase in the plasma nucleosome concentration between the 1st and 2nd doses of lomustine. These samples were spaced 3 weeks apart, so a rise in plasma nucleosome concentration secondary to chemotherapy induced tumor cell die off, such as that reported in humans with cervical cancer, is unlikely to be the cause [32]. Instead, this may represent tumor cell repopulation due to the three-week break in treatment that is customary with canine lomustine based protocols. A similar phenomenon is seen in patient 3 with the first 14-day rest period after cycle 1 of CHOP. In all three patients the nucleosome concentration was often near (slightly above or slightly below) the established threshold for healthy dogs in this study of 67.5 ng/ml even when the patients were noted to be in a clinical remission based on lymph node measurements or ionized calcium levels. This may be representative of MRD still present in these patients. These results suggest that nucleosomes could be a useful and objective monitoring tool for remission status in canine LSA patients and may be more sensitive for monitoring remission than lymph node measurements. Additional studies evaluating the utility of plasma nucleosome concentrations for longitudinal monitoring of canine LSA patients are warranted.

CRP is an acute phase protein that has been previously reported as a useful tool for evaluating acute inflammation and, when paired with TK, remission status in canines with LSA [33]. Thymidine kinase is an enzyme involved in pyrimidine synthesis, and increases in extracellular TK activity could indicate the overall degree of DNA synthesis and dying cells. TK has also been reported as a useful marker of remission status in canine LSA patients [34]. Both CRP and TK were evaluated along with nucleosome concentration in the longitudinal patients. The CRP and TK values were not found to significantly change during the course of therapy and were not correlated with the clinical response of the patient. These findings show that plasma nucleosome concentrations may be a more sensitive tool for monitoring

clinical response of canine LSA patients than CRP or TK. This also suggests that serial nucleosome concentrations could potentially be utilized to detect a patient moving out of remission prior to changes in peripheral lymph node size.

Conclusion

The results of this study demonstrate that plasma nucleosome concentrations of dogs with LSA are significantly elevated compared to healthy controls. These findings support the use of nucleosomes as a tool for the early detection of LSA in dogs. The longitudinal analyses of three patients also suggests that there may be utility for nucleosomes in monitoring a patient's response to therapy and may be useful in monitoring remission status.

Methods

Healthy Dogs

Dogs were recruited from patients presenting to the Texas A&M University Veterinary Medical Teaching Hospital (TAMU VMTH) for routine wellness exams or from dogs owned by TAMU VMTH personnel. All animal studies were approved by the Texas A&M University Animal Care and Use Committee (AUP #2017-0350). Owners were questioned to determine the health status of each patient. In order to be eligible for inclusion, dogs were required to be over one year of age and healthy. Dogs were excluded if there was any secondary significant inflammatory/infectious disease or history of neoplasia. Information recorded for each patient included signalment, body weight, body condition score, and any relevant comorbidities reported by the owner.

Lymphoma Dogs

The LSA dog cohort was recruited in part from dogs presenting to the TAMU VMTH for treatment of naive multicentric LSA (AUP #2019-0211). The remaining samples were recruited from the National Cancer Institute Division of Cancer Treatment and Diagnosis (NCI-DCTD) Canine Tumor Repository. When available, information including patient signalment, body weight, body condition score, stage of disease, and immunophenotype were recorded.

Samples were collected longitudinally from three dogs undergoing treatment for multicentric LSA at the TAMU VMTH (AUP #2019-0211). These dogs were selected to evaluate the duration and pattern of elevated cancer associated nucleosome concentrations in plasma. Blood was collected at each clinic visit. These samples were collected prior to treatment and labeled according to the visit date. Additional information including treatment protocol and remission status at each visit were recorded.

Sample Collection and Processing

For patients presenting to the TAMU VMTH, blood was collected and immediately placed in K2-EDTA blood collection tubes. Within one hour of collection, samples were centrifuged at room temperature at 3000xg for 10 min. Plasma was then immediately removed without disrupting the buffy coat layer, placed in pre-labeled cryovials and frozen at -80°C to run in batches. Processing samples with this protocol was shown to be appropriate for reliable, consistent nucleosome detection in dog plasma [17]. Samples received from the DCTD Canine Tumor Repository were stored frozen at -80°C to be run in batches.

Nucleosome Assays

Frozen samples were thawed and allowed to come to room temperature for at least 30 minutes prior to analysis. All samples were performed in duplicate. The samples were evaluated using the Nu.Q™ H3.1 ELISA (Belgian Volition, SRL, Isnes, Belgium) and were performed according to the manufacturer's instructions. Briefly, a standard curve was generated using the known standards provided. Before use, the wells were washed 3 times with 200µL of the provided diluted wash solution with excess solution being removed after each wash. Patient and healthy dog plasma samples were vortexed and then centrifuged for 2 min at 11,000xg at 4°C before samples were loaded into the plates. Lymphoma samples were diluted 3-fold in order to ensure that they would register on the plates within the limits of the colorimetric standards. Twenty microliters of patient samples and kit controls were run in duplicate in wells on 96 well plates. Eighty microliters of assay buffer was then added to each well. The plates were sealed with foil and incubated at room temperature for 2.5 hours under agitation at ~700rpm. Plates were emptied and washed as described above. Next, 100µL of HRP labelled detection antibody was added to each well. The plate was sealed with foil and incubated at room temperature for 1.5 hours under agitation at ~700rpm. Plates were then emptied and washed as described above. Next, 100µL of TMB substrate was added to each well. The plate was sealed with foil and incubated at room temperature for 20 minutes in the dark under agitation at ~700rpm. One hundred microliters of stop solution were then added and the plate was shaken gently. Plates were read at an absorbance of 450nm (BioTek Synergy H1 plate reader, BioTek Instruments, Winooski, VT) within 5 minutes of stop solution being added. The standard curve was linearized and fitted to a 5-parameter logistic curve using statistical software (Graphpad Software, version 8, San Diego, CA).

CRP Assays

For the longitudinal LSA patients, samples were submitted to the Texas A&M University Gastrointestinal Laboratory for their commercially available CRP assay if sufficient sample quantity was present. If sample quantity was not sufficient for both nucleosome and CRP analysis, nucleosome assays were given priority.

Thymidine Kinase Assays

The Canine Thymidine Kinase 1 soluble ELISA assay (My Biosource Inc, San Diego, CA) was used to evaluate TK levels in all dogs that were followed longitudinally. The assay was performed according to the manufacturer's protocol. Briefly, 40µl of sample was added to wells followed by 10µl anti-TK1

antibody. Then 50µl streptavidin-HRP was added to each well except the blank control well. The plate was mixed well, covered with sealer and incubated for 60 minutes at 37°C. The plate was then washed 5 times with wash buffer and the wells were soaked with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. Next 50µl of substrate solution A was added to each well followed by 50µl of substrate solution B to each well. The plate was covered with a fresh sealer for 10 minutes at 37°C in the dark. Finally, 50µl of Stop Solution was added to each well. Plates were read at an absorbance of 450nm (BioTek Synergy H1 plate reader, BioTek Instruments, Winooski, VT) within 10 minutes of stop solution being added. The standard curve was linearized and fitted to a 5-parameter logistic curve using statistical software (Graphpad Software, version 8, San Diego, CA).

Statistical Analysis

Descriptive statistics for the patient populations were performed using Microsoft excel for Mac (v. 16.16.27, 2016). For data sets containing only two cohorts, such as the healthy controls versus all LSA cases, a Wilcoxon rank sum test was used to compare the medians of the data sets. For data sets where multiple conditions were compared such as disease stage, a two-way ANOVA for repeat measures with a Tukey's multiple comparisons test was performed. This part of the analysis was performed using GraphPad Prism version 8.0.0 for Macintosh, GraphPad Software, San Diego, California USA, www.graphpad.com. Spearman's correlation, ROC curves and specificity/sensitivity calculations were performed using R version 3.4.3 and the pROC package [35, 36].

Abbreviations

1. LSA - Lymphoma
2. CRP - C-Reactive Protein
3. TK - Thymidine Kinase
4. TAMU VMTH – Texas A&M University Veterinary Medical Teaching Hospital
5. ROC – Receiver Operating Characteristic
6. MRD – Minimal Residual Disease

Declarations

Ethics approval and consent to participate:

All animal studies were approved by the Texas A&M University Animal Care and Use Committee (AUP #2017-0350). Client-owned animals were evaluated with the informed consent of the owner (AUP #2019-0211).

All methods were carried out in accordance with relevant guidelines and regulations.

No humans were involved in this study.

No agents were administered to dogs in any way in this study.

Consent for publication:

Not applicable

Availability of data and materials

All relevant data are within the paper.

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Authors' contributions

Conceptualization: Heather Wilson-Robles.

Data curation: Christopher Dolan, Heather Wilson-Robles, Tasha Miller, Jill Jarvis.

Formal analysis: Heather Wilson-Robles, Theresa Kelly, Thomas Bygott.

Funding acquisition: Heather Wilson-Robles, Jason Terrell.

Investigation: Christopher Dolan, Heather Wilson-Robles, Jill Jarvis.

Methodology: Heather Wilson-Robles, Tasha Miller.

Project administration: Heather Wilson-Robles, Jill Jarvis.

Resources: Heather Wilson-Robles, Tasha Miller, Jill Jarvis, Jason Terrell, Theresa Kelly, Thomas Bygott.

Software: Heather Wilson-Robles, Thomas Bygott.

Supervision: Heather Wilson-Robles.

Validation: Heather Wilson-Robles, Tasha Miller.

Visualization: Heather Wilson-Robles.

Writing – original draft: Christopher Dolan, Heather Wilson-Robles.

Writing – review & editing: Christopher Dolan, Heather Wilson-Robles, Jason Terrell, Theresa

Kelly, Thomas Bygott.

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Authors' information (optional)

Not applicable.

References

1. Rubis GD, Krishnan SR, Bebawy M. Liquid Biopsies in Cancer Diagnosis, Monitoring, and Prognosis. Trends in Pharmacological Sciences. 2019 Mar;40(Nat. Rev. Genet. 17 2016):172–86.
2. Zhang W, Xia W, Lv Z, Ni C, Xin Y, Yang L. Liquid Biopsy for Cancer: Circulating Tumor Cells, Circulating Free DNA or Exosomes? Cell Physiol Biochem. 2017;41(2):755–68.
3. Luger K, Mäder AW, Richmond RK, Sargent DF, Richmond & TJ. Crystal structure of the nucleosome core particle at 2.8Å resolution. Nature. 1997;(389):251–60.
4. Cutter AR, Hayes JJ. A brief review of nucleosome structure. Febs Lett. 2015;589(20PartA):2914–22.
5. Marsman G, Zeerleder S, Luken BM. Extracellular histones, cell-free DNA, or nucleosomes: differences in immunostimulation. Cell Death Dis. 2016;7(12):e2518–e2518.
6. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch R-D, et al. DNA Fragments in the Blood Plasma of Cancer Patients: Quantitations and Evidence for Their Origin from Apoptotic and Necrotic Cells. Cancer Research. 2001;(61):1659–65.
7. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA Comprises an In Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin. Cell. 2016;164(1–2):57–68.
8. Lui YYN, Chik K-W, Chiu RWK, Ho C-Y, Lam CWK, Lo YMD. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. Clin Chem. 2002;48(3):421–7.
9. Alborelli I, Generali D, Jermann P, Cappelletti MR, Ferrero G, Scaggiante B, et al. Cell-free DNA analysis in healthy individuals by next-generation sequencing: a proof of concept and technical validation study. Cell Death Dis. 2019;10(7):534.
10. Letendre J-A, Goggs R. Concentrations of Plasma Nucleosomes but Not Cell-Free DNA Are Prognostic in Dogs Following Trauma. Frontiers Vet Sci. 2018;5:180.
11. Letendre J, Goggs R. Determining prognosis in canine sepsis by bedside measurement of cell-free DNA and nucleosomes. J Vet Emerg Crit Car. 2018;28(6):503–11.

12. Phan T, Mcmillan R, Skiadopoulos L, Walborn A, Hoppensteadt D, Fareed J, et al. Elevated extracellular nucleosomes and their relevance to inflammation in stage 5 chronic kidney disease. Int Angiol. 2018;37(5).
13. Rasmussen L, Christensen IJ, Herzog M, Micallef J, Nielsen HJ. Circulating cell-free nucleosomes as biomarkers for early detection of colorectal cancer. Oncotarget. 2017;9(12):10247–58.
14. Kumar S, Guleria R, Singh V, Bharti AC, Mohan A, Das BC. Plasma Nucleosome Levels Might Predict Response to Therapy in Patients With Advanced Non–Small-Cell Lung Cancer. Clin Lung Cancer. 2010;11(1):36–44.
15. Tagawa M, Shimbo G, Inokuma H, Miyahara K. Quantification of plasma cell-free DNA levels in dogs with various tumors. J Vet Diagn Invest. 2019;31(6):836–43.
16. Beffagna G, Sammarco A, Bedin C, Romualdi C, Mainenti M, Mollo A, et al. Circulating Cell-Free DNA in Dogs with Mammary Tumors: Short and Long Fragments and Integrity Index. Plos One. 2017;12(1):e0169454.
17. Wilson-Robles H, Miller T, Jarvis J, Terrell J, Dewsbury N, Kelly T, et al. Evaluation of nucleosome concentrations in healthy dogs and dogs with cancer. Biorxiv. 2020;2020.07.08.193466.
18. Zandvliet M. Canine lymphoma: a review. The Veterinary quarterly. 2016 Jun;36(2):76–104.
19. Valli VE, Kass PH, Myint MS, Scott F. Canine Lymphomas. Vet Pathol. 2013;50(5):738–48.
20. Childress MO, Fulkerson CM, Lahrman SA, Weng H -Y. Inter- and intra-rater reliability of calliper-based lymph node measurement in dogs with peripheral nodal lymphomas. Vet Comp Oncol. 2016;14(S1):74–81.
21. Rambaldi A, Lazzari M, Manzoni C, Carlotti E, Arcaini L, Baccarani M, et al. Monitoring of minimal residual disease after CHOP and rituximab in previously untreated patients with follicular lymphoma. Blood. 2002;99(3):856–62.
22. Yamazaki J, Takahashi M, Setoguchi A, Fujino Y, Ohno K, Tsujimoto H. Monitoring of Minimal Residual Disease (MRD) after Multidrug Chemotherapy and Its Correlation to Outcome in Dogs with Lymphoma: A Proof-of-Concept Pilot Study. J Vet Intern Med. 2010;24(4):897–903.
23. Bauden M, Pamart D, Ansari D, Herzog M, Eccleston M, Micallef J, et al. Circulating nucleosomes as epigenetic biomarkers in pancreatic cancer. Clin Epigenetics. 2015;7(1):106.
24. Arimura Y, Ikura M, Fujita R, Noda M, Kobayashi W, Horikoshi N, et al. Cancer-associated mutations of histones H2B, H3.1 and H2A.Z.1 affect the structure and stability of the nucleosome. Nucleic Acids Res. 2018;gky661.
25. Schroers-Martin JG, Kurtz DM, Soo J, Jin M, Scherer F, Craig A, et al. Determinants of Circulating Tumor DNA Levels across Lymphoma Histologic Subtypes. Blood [Internet]. 2017 Dec 7;130(Supplement 1):4018–4018. Available from: https://doi.org/10.1182/blood.V130.Suppl_1.4018.4018
26. Hohaus S, Giachelia M, Massini G, Mansueto G, Vannata B, Bozzoli V, et al. Cell-free circulating DNA in Hodgkin's and non-Hodgkin's lymphomas. Ann Oncol. 2009;20(8):1408–13.

27. HOLDENRIEDER S, STIEBER P, BODENMÜLLER H, BUSCH M, PAWEL J, SCHALHORN A, et al. Circulating Nucleosomes in Serum. Ann Ny Acad Sci. 2001;945(1):93–102.
28. Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer. 2011;11(6):426–37.
29. Gauthier VJ, Tyler LN, Mannik M. Blood clearance kinetics and liver uptake of mononucleosomes in mice. J Immunol Baltim Md 1950. 1996;156(3):1151–6.
30. Fatouros IG, Jamurtas AZ, Nikolaidis MG, Destouni A, Michailidis Y, Vrettou C, et al. Time of sampling is crucial for measurement of cell-free plasma DNA following acute aseptic inflammation induced by exercise. Clin Biochem. 2010;43(16–17):1368–70.
31. Lo YMD, Zhang J, Leung TN, Lau TK, Chang AMZ, Hjelm NM. Rapid Clearance of Fetal DNA from Maternal Plasma. Am J Hum Genetics. 1999;64(1):218–24.
32. Trejo-Becerril C, Oñate-Ocaña LF, Taja-Chayeb L, Vanoye-Carlo A, Cetina L, Duenas-Gonzalez A. Serum nucleosomes during neoadjuvant chemotherapy in patients with cervical cancer. Predictive and prognostic significance. Bmc Cancer. 2005;5(1):65.
33. Nielsen L, Toft N, Eckersall PD, Mellor DJ, Morris JS. Serum C-Reactive Protein Concentration as an Indicator of Remission Status in Dogs with Multicentric Lymphoma. J Vet Intern Med. 2007;21(6):1231–6.
34. Euler H, Einarsson R, Olsson U, Lagerstedt A, Eriksson S. Serum Thymidine Kinase Activity in Dogs with Malignant Lymphoma: A Potent Marker for Prognosis and Monitoring the Disease. J Vet Intern Med. 2004;18(5):696–702.
35. R Core Team. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www R-project org. 2010 Jan 1;
36. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, et al. pROC: an open-source package for R and S + to analyze and compare ROC curves. Bmc Bioinformatics. 2011;12(1):77.

Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

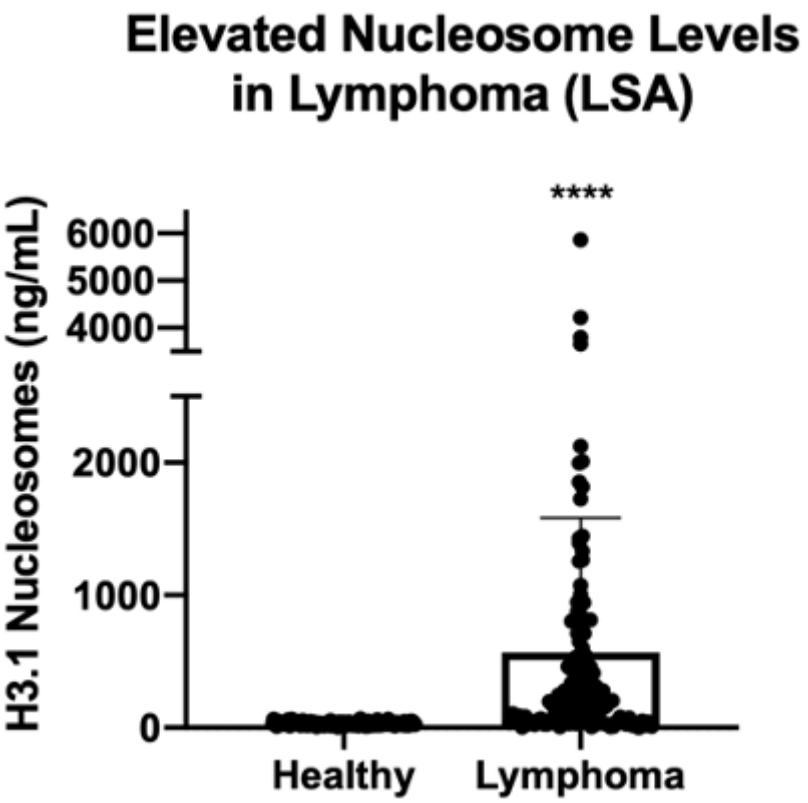

Figure 1

Elevated Nucleosome Levels in LSA. Mean plasma nucleosome concentrations (ng/mL) were significantly higher in LSA dogs compared to healthy controls.

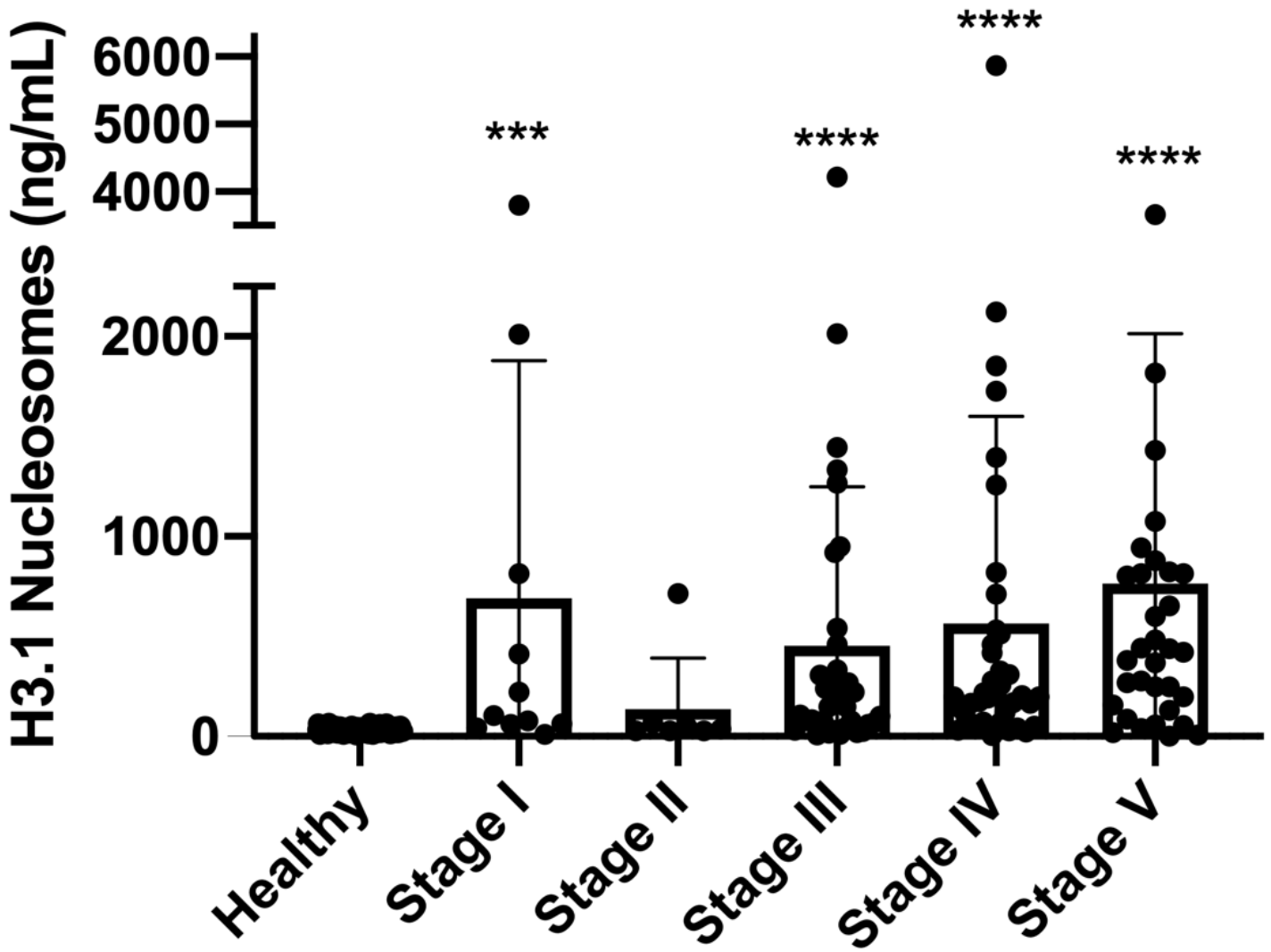

Figure 2

Mean Nucleosome Concentrations: LSA by Stage. Mean plasma nucleosome concentrations (ng/mL) in all LSA stages (except stage II) were significantly higher than healthy controls.

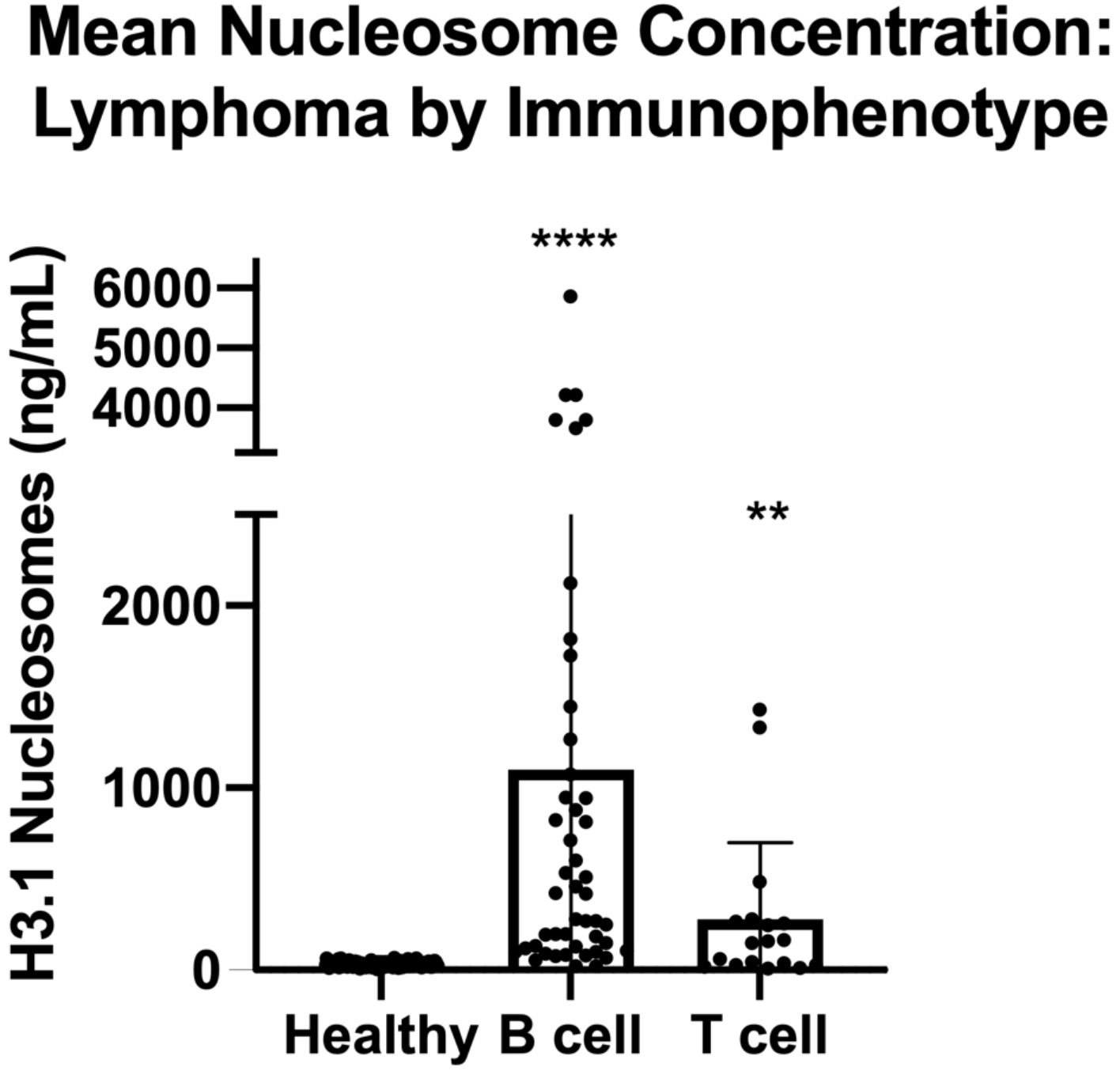

Figure 3

Mean Nucleosome Concentrations: LSA by Immunophenotype. Mean plasma nucleosome concentrations (ng/mL) in B- and T-cell LSA were significantly higher than healthy controls. B-cell LSA mean nucleosome concentrations were significantly higher than T-cell LSA.

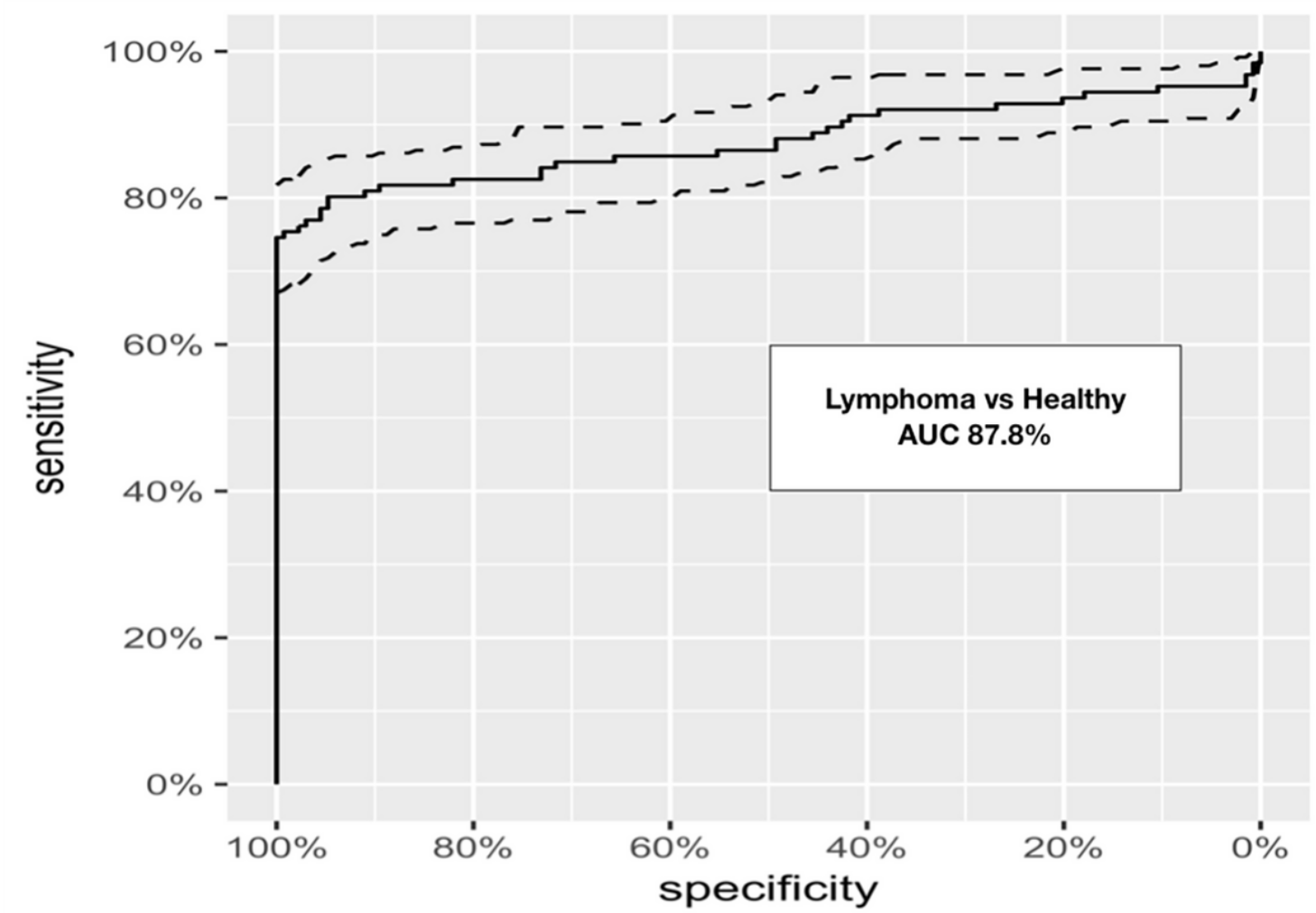

Figure 4

Discriminating LSA from Healthy Controls. ROC analysis with an established threshold of 67.5 ng/ml generated an area under the curve of 0.878. This threshold produced a sensitivity of 74.6% at a specificity of 100%

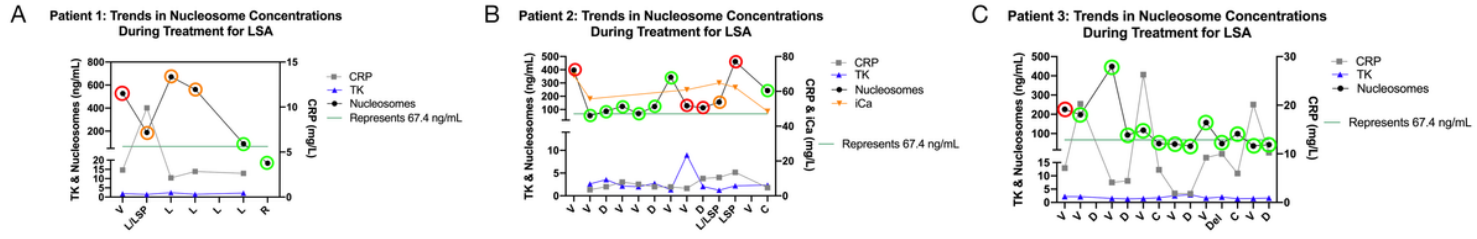

Figure 5

Trends in Nucleosome, CRP, and TK Concentrations During Treatment for LSA. Clinical response is represented for each patient with red time points indicating either the initial diagnosis or progressive disease. Orange time points indicate a partial response or stable disease, and green time points indicate a complete clinical response. The left y-axis represents nucleosome concentration and TK concentration while the right y axis represents CRP concentration and iCa concentration. The nucleosome concentration

and TK concentration are plotted as ng/mL, and the CRP and iCa concentrations are plotted as mg/L for comparison. The x-axis represents visits to the hospital along with administered treatment. V – vincristine, C – cyclophosphamide, D – doxorubicin, L – lomustine (CCNU), LSP – L-asparaginase, R – recheck visit (no treatment), Del – Delay visit (no treatment). A. Trends shown for Patient 1. Nucleosome concentrations decreased after the second dose of lomustine when a partial response was noted clinically. The nucleosome concentration decreased substantially after moving into a complete response and has since remained in the range of healthy dogs. The CRP and TK were within the normal range during all periods of clinical response and were uncorrelated with nucleosome concentration. B. Trends shown for Patient 2. Nucleosome concentrations increased after each dose of doxorubicin and decreased after most doses of vincristine. Another increase is seen after starting CCNU/L-spar chemotherapy and a decrease occurs after changing to COP chemotherapy. The CRP and TK were within the normal range during all periods of clinical response and had no correlation with nucleosome concentration. C. Trends shown for Patient 3. Nucleosome concentration decreased overall after starting chemotherapy. The CRP and TK were within the normal range during all periods of clinical response and had no correlation with nucleosome concentration.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1LSAH3.1NucleosomeConcentration.pdf