

1 **Research article**

2 **Feasibility and promise of circulating tumor DNA analysis in dogs with naturally-occurring sarcoma.**

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15

16 **Abstract**

17 Comparative studies of naturally-occurring canine cancers have provided new insight into many areas of cancer

18 research. The inclusion of pet dogs in the development and validation of circulating tumor DNA (ctDNA)

19 diagnostics may be uniquely informative for human translation for many reasons, including: high incidence of

20 certain spontaneous cancers, repeated access to blood and tumor from the same individuals during the course

21 of disease progression, and molecular heterogeneity of naturally-occurring cancers in dogs. Here, we present

22 a feasibility study of ctDNA analysis performed in 9 healthy dogs and 39 dogs with either benign or malignant

23 splenic tumors (hemangiosarcoma) using shallow whole genome sequencing (sWGS) of cell-free DNA. To

24 enable detection and quantification of ctDNA using sWGS, we adapted two informatic approaches and

25 compared their performance for the canine genome. At presentation, mean ctDNA tumor fraction in dogs with

26 malignant splenic tumors was 11.2%, significantly higher than dogs with benign lesions (3.2%; p 0.001),

27 achieving an AUC of 0.84. ctDNA tumor fraction was 14.3% and 9.0% in dogs with metastatic and localized  
28 disease, respectively although this difference was not statistically significant (p 0.227). In paired analysis,  
29 ctDNA fraction decreased from 11.0% to 7.9% after resection of malignant tumors (p 0.047). Our results  
30 demonstrate that ctDNA analysis is feasible in dogs with hemangiosarcoma using a cost-effective approach  
31 such as sWGS. Future studies are underway to validate these findings, and further evaluate the role of ctDNA  
32 to assess burden of disease and treatment response during drug development.

33

#### 34 **Introduction**

35 Comparative studies of naturally-occurring canine cancers provide a unique opportunity to advance the  
36 care of human cancer patients by answering questions about cancer biology, genomics, diagnosis, and  
37 therapy, that cannot be answered in conventional preclinical cancer models or in human clinical trials  
38 (Paoloni and Khanna 2008; Paoloni et al. 2014; Gustafson et al. 2018). The analysis of somatic mutations in  
39 canine cancers can yield novel insights into pathogenesis of the disease (Lorch et al. 2019). Exome  
40 sequencing of canine splenic hemangiosarcoma (HSA) identified somatic mutations in well-known driver  
41 genes of human cancer (Wang et al. 2017). Unlike most preclinical models, the molecular heterogeneity seen  
42 in spontaneous cancers in dogs mimics human cancer heterogeneity and can serve as a unique model system  
43 for development of novel therapeutic and diagnostic approaches.

44 Analysis of circulating tumor DNA (ctDNA) in dogs with cancer may help further our understanding of  
45 ctDNA biology due to: 1) the high incidence of spontaneous cancer in dogs, including those that are rare in  
46 humans (such as sarcomas); 2) the opportunity to collect serial blood samples of sufficient volume compared

47 to conventional preclinical models and to collect concurrent samples of tumor and normal tissue, using  
48 clinical annotation methods commonly employed in human oncology (such as tumor grade, and patient  
49 stage); 3) comparable relative sizes of tumors between dogs and humans; and 4) the ability to optimize and  
50 compare sample collection and data analysis approaches in the same individual. However, published  
51 experience with ctDNA analysis in dogs is currently sparse. Nonetheless, limited and small-scale published  
52 studies have suggested total cell-free DNA concentrations are higher in dogs with malignant tumors  
53 compared to those with benign disease or healthy controls (Schaefer et al. 2007; Beffagna et al. 2017;  
54 Tagawa et al. 2020). In a recent study of canine mammary carcinoma, ctDNA detection was demonstrated  
55 using digital PCR assays specific to somatic genomic rearrangements in 4 dogs (Beck et al. 2013). In another  
56 study, ctDNA detection was demonstrated in 8 of 11 dogs with urothelial carcinoma using a real-time PCR  
57 assay for a recurrent somatic mutation in BRAF (Tagawa et al. 2020). Earlier, we demonstrated detection of  
58 ctDNA in 2 of 6 dogs with pulmonary adenocarcinoma using a digital PCR assay for a recurrent somatic  
59 mutation in ERBB2 (Lorch et al. 2019). Hence, published demonstrations of ctDNA analysis are limited to a  
60 few dogs and have largely relied on mutation and locus-specific assays. Mutation-specific assays have  
61 limited widespread applicability for ctDNA analysis in dogs or humans because they either rely on highly  
62 recurrent single mutations or require prior analysis of the tumor sample to identify patient-specific mutations.  
63 In this study, we have evaluated an alternative approach through the use of shallow Whole Genome  
64 Sequencing (sWGS) for ctDNA analysis of canine cancer, which relies on direct genome-wide assessment in  
65 plasma DNA for copy number aberrations.

66        Canine HSA is a relatively common canine cancer with strong clinical similarities to human  
67        angiosarcoma. Both cancers harbor structurally complex genomes that are not associated with recurrent point  
68        mutations, limiting the utility of single mutation ctDNA assays. Hemangiosarcoma commonly develops in  
69        the spleen of dogs and metastasizes to distant organs prior to or early after initial diagnosis. These splenic  
70        lesions are not often discovered until they rupture and the dog presents to the veterinary hospital with acute  
71        abdominal hemorrhage, requiring emergent surgery. This clinical presentation is very similar to dogs with  
72        benign tumors of the spleen or other causes of splenic rupture. Histopathological confirmation of the  
73        diagnosis for these lesions cannot usually be achieved until several days after emergency surgeries are  
74        performed, highlighting the challenges that pet owners face when choosing to pursue aggressive emergent  
75        surgical care and treatment for their dog with an unclear long-term prognosis. An opportunity exists to study  
76        and translate methods for ctDNA analysis in this naturally-occurring cancer model, to ask if ctDNA levels  
77        can distinguish benign from malignant tumors, to understand if ctDNA levels are related to burden of disease  
78        at presentation (such as localized vs. metastatic cancer), and if serial changes in ctDNA levels are effective  
79        surrogates for assessment of treatment response or disease progression. These investigations will be relevant  
80        to the application of ctDNA analysis within human oncology and allow for optimization of diagnostic  
81        methods and technologies. Here, we present a feasibility study evaluating the potential for ctDNA analysis in  
82        dogs with cancer using sWGS to detect ctDNA in dogs presenting with benign or malignant splenic tumors.  
83        We hope our findings and adapted informatics tools will help expand the inclusion of dogs with naturally-  
84        occurring cancers as models for optimal ctDNA assay development and translation, and aid drug  
85        development efforts.

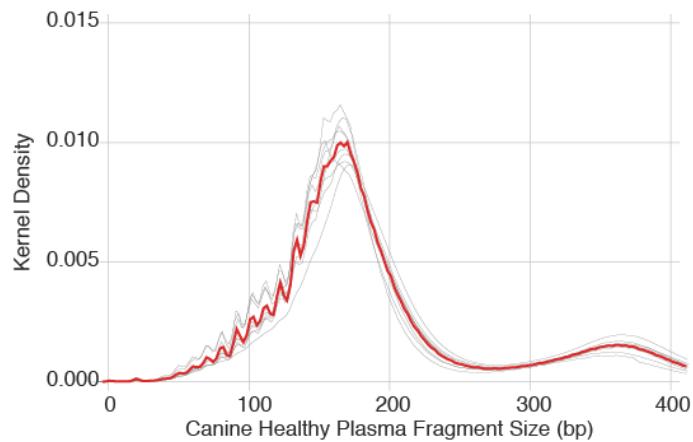
86

87 **Results**

88 Cell-free DNA analysis in healthy dogs

89 In order to establish the feasibility of cell free DNA (cfDNA) analysis in canines, we obtained plasma  
90 samples from 9 healthy dogs including five females and four males. The mean cfDNA concentration was  
91 0.39 ng/ml of plasma (sd 0.38 ng/ml). We prepared sequencing libraries with 1-30 ng of DNA input and  
92 generated a mean of 20.0 million sequencing read pairs per sample. We achieved a mean unique depth of  
93 0.45x per sample (sd 0.15). Following alignment to the dog genome CanFam3.1, we determined the modal  
94 fragment size of 165.6 base pairs (bp), comparable to the known modal fragment size of 166 bp in human  
95 cfDNA (Jiang et al. 2015; Murtaza and Caldas 2016)(Figure 1, Table S1).

96



97

98 **Figure 1.** Plasma cell-free DNA fragment size (base pairs) in nine healthy dogs. Individual samples are show  
99 in grey, all samples combined are shown in red.

100

101

102 Clinical cohort of dogs with splenic lesions

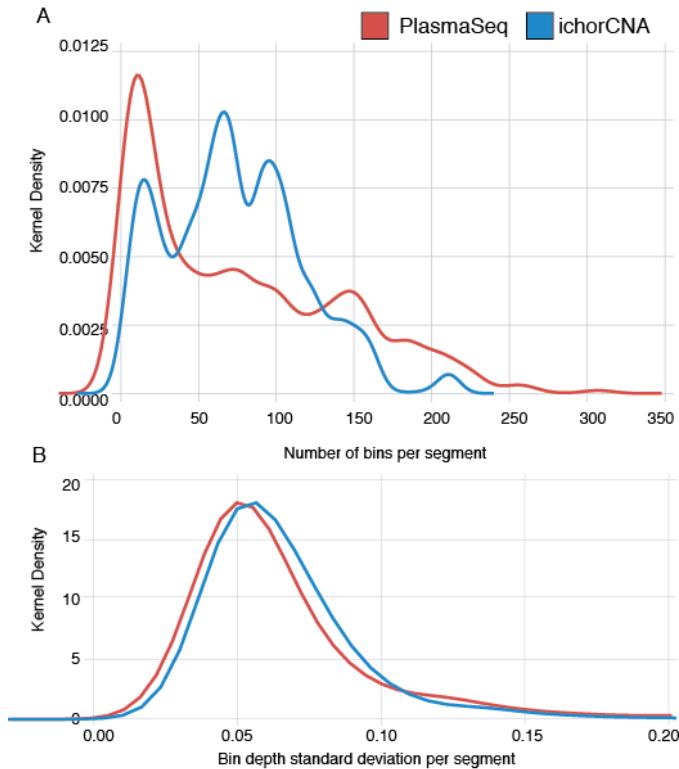
103 To determine the feasibility of cfDNA analysis in dogs, we conducted a prospective clinical trial of dogs  
104 presenting with hemo-abdomen secondary to presumed splenic rupture. The description of this clinical  
105 cohort of dogs, including diagnoses and perioperative outcomes, has been recently reported (Stewart et al.  
106 2020). Samples from 39 dogs from this cohort were evaluable for cfDNA analysis. Twenty five dogs (64.1%)  
107 were classified as having malignant splenic neoplasms and 14 were diagnosed with benign splenic lesions  
108 (35.9%). Among the 25 dogs that were diagnosed with malignant tumors, 23 (92%) were diagnosed with  
109 HSA and 2 were diagnosed with stromal sarcoma. Benign complex nodular hyperplasia represented 78.6%  
110 (11/14) of the benign lesions, while complex hyperplasia with hematoma, hematoma alone and myelolipoma  
111 represented 7.14% each (1/14 each). Two dogs (5.13%) had concomitant benign lesions and malignant tumor  
112 nodules in different portions of the spleen. These were grouped and analyzed as dogs with malignant disease  
113 in the current analysis.

114

115 Comparison of informatic approaches for sWGS

116 In order to investigate the feasibility of cfDNA analysis in dogs using sWGS, we adapted two published  
117 informatic approaches, ichorCNA (Adalsteinsson et al. 2017) and PlasmaSeq (Farris and Trimarchi 2013), to  
118 enable their application to non-human genomes. Both tools infer the presence of tumor-derived copy number  
119 alterations (CNAs) using read depth in large, non-overlapping genomic bins (windows). Bins are then  
120 grouped into segments with the same copy number status. ichorCNA uses fixed-size bins while PlasmaSeq

121 uses a fixed total number of bins and the boundaries are adjusted so that each bin contains approximately the  
122 same number of mappable bases. ichorCNA uses the magnitude represented by the detected CNAs to directly  
123 infer the fraction of tumor DNA found in blood. The canine patient samples were analyzed using 500 kb bins  
124 for ichorCNA. PlasmaSeq analysis was run using 5,500 total bins, with median size of 392 kb (sd 42 kb).  
125 Across all plasma samples from dogs with splenic lesions, the total number of segments was significantly  
126 higher for PlasmaSeq, with an average of 72.6 segments per sample versus 50.0 segments per sample for  
127 ichorCNA. PlasmaSeq had a larger proportion of segments with less than 25 bins and more than 150 bins,  
128 while ichorCNA had a higher proportion of segments with 50-100 bins (Figure 2a). We also quantified the  
129 amount of variation in read depth (log2 values) within bins assigned to a given copy number segment. The  
130 median standard deviation within each segment for ichorCNA and PlasmaSeq were 0.0606 and 0.0569  
131 respectively, (Figure 2b, p 0.004 Mann-Whitney U). Although distinct, we found the two adapted approaches  
132 to be equally useful for cfDNA analysis in dogs.



133

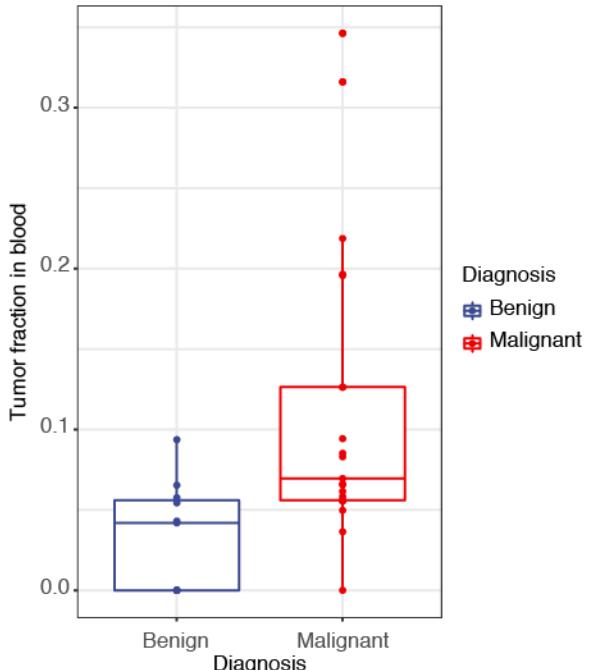
134 **Figure 2.** PlasmaSeq infers a larger proportion of segments with less than 25 bins,  
135 although the depth variation within segments is approximately the same between PlasmaSeq and ichorCNA.  
136 A: The distribution bin counts in each segment across all samples using each tool. B: The distribution of log2  
137 standard deviations across all bins assigned to the same segment.

138

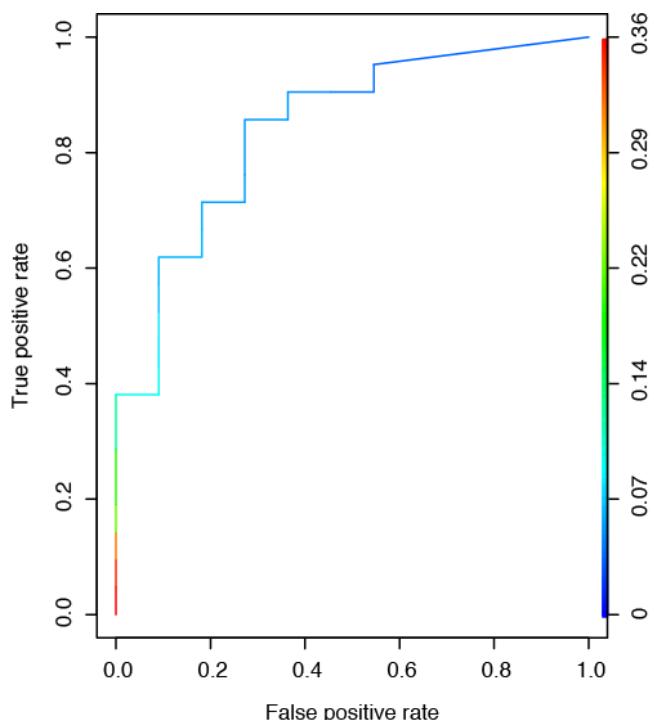
139 Analysis of total cell-free and circulating tumor-specific DNA

140 At presentation prior to any intervention, mean concentration of total cfDNA in plasma samples was  
141 10.6 ng/ml and 20.3 ng/ml in dogs with benign and malignant lesions, respectively (p 0.400). Total cfDNA  
142 concentration after surgery was significantly lower than baseline in both groups (benign p 0.008 and  
143 malignant p 0.017). Within dogs with malignant lesions, mean total cfDNA concentration was 20.9 ng/ml  
144 and 19.5 ng/ml in dogs with localized and metastatic disease, respectively (p 0.830).

145 ctDNA tumor fractions reported here were measured using an adapted version of ichorCNA. Mean  
146 tumor fractions prior to surgery were 3.2% (sd 3.4) and 11.2% (sd 9.1) in dogs with benign and malignant  
147 tumors, respectively (p 0.001; Figure 3, Table S2). Within dogs with malignant tumors, mean ctDNA  
148 fractions in dogs with localized and metastatic disease were 9.0% and 14.3% respectively, although this  
149 difference was not statistically significant (p 0.227). Following splenectomy, patients with malignant tumors  
150 showed a significant decrease in ctDNA levels (paired p 0.047). ctDNA was detected above the previously  
151 reported limit of detection for ichorCNA of 3% tumor fraction in 20/21 dogs with malignant tumors with a  
152 pre-treatment sample available (95.2%). Using tumor fractions in ctDNA, we were able to distinguish blood  
153 samples from dogs with benign and malignant tumors with an area under the ROC curve of 0.84 (Figure 4).  
154 Interestingly, when corresponding tumor biopsies were analyzed using sWGS, we observed mean tumor  
155 fractions of 4.4% (sd 4.9) and 10.1% (sd 12.9) for benign and malignant tumors, respectively, highlighting  
156 the challenges of obtaining high-cellularity splenic tumor biopsies and establishing tissue diagnoses in dogs,  
157 and the additional value potentially offered by ctDNA analysis.



159  
160 **Figure 3.** cfDNA tumor fraction (TF) for benign vs malignant tumors in the baseline sample cohort ( $p =$   
161 0.001).



162  
163 **Figure 4.** Receiver operating characteristic (ROC) curve for tumor fraction detection in cell free DNA from  
164 plasma from 32 dogs with benign and malignant tumors before surgery, with AUC = 0.84.

165 We found a statistically significant bias in the distribution of somatic copy number changes across  
166 chromosomes, although this bias was not consistent across tools. Chromosomes 8, 11, 14, and 26 were more  
167 likely to contain copy number gains called by PlasmaSeq, while chromosomes 5 and 34 were more likely to  
168 have gains called using ichorCNA (Table 1).

169

Chrom	Pvalue	OddsRatio	Method
8	1.19e-12	6.7	PlasmaSeq
14	1.52e-04	2.9	PlasmaSeq
3	4.22e-04	0.1	PlasmaSeq
11	5.90e-04	3.5	PlasmaSeq
26	7.73e-04	3.3	PlasmaSeq
5	7.50e-04	2.0	ichorCNA
34	1.16e-03	2.1	ichorCNA

170 **Table 1.** Copy number variation odds ratio of the chromosomes in the 39 canine patients with splenic  
171 lesions. P values and odds ratios were calculated using Fisher's Exact Test.

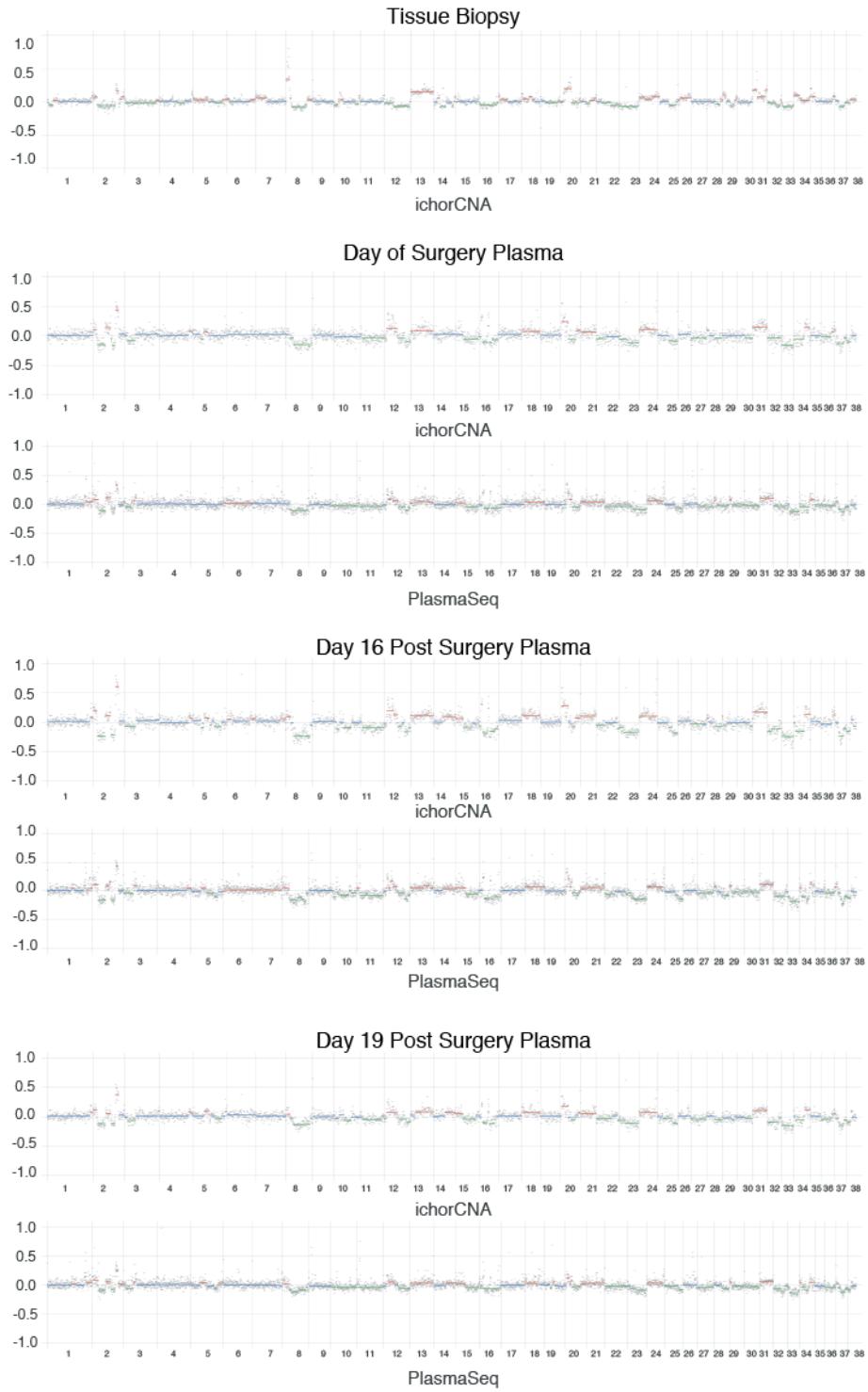
172

173 In cases where corresponding tumor DNA and cfDNA samples both showed adequate tumor fraction, copy  
174 number aberrations observed were largely concordant. For example, in one dog with metastatic malignant  
175 hemangiosarcoma, copy number aberrations observed in tumor biopsy are also observed in blood samples  
176 obtained on days 0, 16 and 19 after splenectomy, using both informatic approaches (Figure 5).

177

178

179



180  
181 **Figure 5.** Genome-wide copy number variation plots from the metastatic HSA canine patient, dog no. 26,  
182 representing the plasma and tumor biopsy sWGS from day of surgery, and plasma sWGS from days 16 and  
183 19, both after the splenectomy procedure.  
184

185 Follow-up blood samples from two dogs classified as having a) benign complex nodular hyperplasia and  
186 b) complex nodular hyperplasia with hematoma, had unexpectedly high tumor fractions in plasma (21.4%  
187 and 46.9%). However, these observations were made at 392 and 345 days after surgery, respectively. In both  
188 dogs, earlier samples had ctDNA levels consistent with those observed in dogs with benign disease (5.4%  
189 and 4.8%). The source of these elevated ctDNA levels observed later in follow-up is unclear. In these older  
190 clinical patients, it is possible that elevated ctDNA levels almost a year after initial diagnosis are a result of  
191 clinically silent unrelated cancers. Indeed, post hoc analysis revealed new neurologic clinical signs that were  
192 localized to the right cerebral cortex 415 days following surgery in one of the two dogs suggestive of distinct  
193 intracranial neoplasia. However, further investigation was not pursued to confirm this diagnosis. There were  
194 no clinical signs suggestive of neoplasia in the second dog. Nonetheless, the presence of indolent neoplasia  
195 (such as indolent lymphoma) in this clinical cohort of older large breed dogs cannot be ruled out.

196

## 197 **Discussion**

198 ctDNA analysis in pet dogs with cancers provides an opportunity to develop and validate methods, and to  
199 optimize pre-analytical conditions for molecular and computational analysis of liquid biopsies. Analysis of  
200 naturally-occurring cancers with similar complexities of cancer heterogeneity as human patients can  
201 potentially generate novel insights and allow rapid translation back to human oncology. However, published  
202 experience with ctDNA analysis in canine oncology has been very limited. In the current study, we  
203 demonstrate that sWGS is useful as a tumor-independent approach for ctDNA detection in dogs with cancer.  
204 We validate the application of informatic methods adapted for the canine genome and make these adapted

205 versions available for future use to accelerate the development and deployment of ctDNA analysis in dogs  
206 (Farris and Trimarchi 2013; Adalsteinsson et al. 2017). We provide proof-of-principle results suggesting  
207 potential utility of ctDNA analysis using sWGS in dogs presenting with structurally complex cancers to  
208 specifically distinguish malignant cancers from benign disease and as a potential surrogate of disease burden.  
209 The analysis of ctDNA levels in the same dog before and after resection of the splenic tumors yielded  
210 significant differences in ctDNA levels, whereas comparison of dogs with localized versus metastatic disease  
211 (noted at the time of surgery) were not statistically distinct. Given the relatively limited size of our cohort,  
212 comparison of ctDNA levels with metastatic disease burden requires further analysis in a larger cohort of  
213 dogs. In future studies, we plan to include measurement of tumor volumes to account for quantitative  
214 differences in burden of metastatic disease and size of primary tumors, and how these may correlate with  
215 observed differences in ctDNA levels. Furthermore, we plan to perform paired comparisons of sample  
216 processing workflows and analytical approaches to optimize measurement of ctDNA levels as a surrogate for  
217 disease burden. With further clinical validation in larger studies, ctDNA analysis can aid diagnostic  
218 dilemmas such as those investigated here and may be included in studies that can explore the feasibility and  
219 value of early diagnostic approaches, better prognostication, and improved treatment monitoring for rapid  
220 translation to human oncology.

221 This feasibility study has several limitations. ctDNA studies in human oncology have largely come to  
222 rely on gold standard pre-analytical processing focused on rapid separation and adequate processing of  
223 plasma samples. In on-going studies, we have ensured implementation of standard processing protocols to  
224 isolate plasma rapidly after venipuncture. In future work, ctDNA analysis in dogs may provide an

225 opportunity to evaluate and optimize pre-analytical factors using paired tumor samples and multiple blood  
226 samples from cancer patients, such as blood collection tubes, DNA extraction methods, and storage  
227 conditions. An additional limitation was our observation of significantly elevated levels of ctDNA detectable  
228 in two dogs with benign cancers in follow-up blood samples. These dogs were shown to have benign splenic  
229 lesions at the time of initial diagnosis and surgery but hematological or other malignancies present  
230 concomitantly or arising later may only become apparent during sufficient clinical follow-up. In one of these  
231 dogs, concomitant neoplasia of the brain was suspected, but not confirmed, based on the subsequent  
232 development of clinical signs suggestive of a space occupying mass. Given the clinical characteristics of this  
233 cohort of larger older dogs, we speculate that a non-splenic cancer, such as indolent lymphoma, may explain  
234 these findings.

235 In summary, our results demonstrate that ctDNA analysis is a promising approach for improving  
236 diagnostics in veterinary oncology. In addition, ctDNA analysis of naturally-occurring cancers in dogs can  
237 enable further optimization for diagnostic applications in human oncology including noninvasive tumor  
238 genotyping, early detection, and monitoring of treatment response.

239  
240

241 **Methods**

242 Ethics statement

243 Blood and tissue samples were collected with the approval and consent of the canine patient owners. The  
244 study was launched following approval from the animal care and use committee convened by Animal  
245 Clinical Investigation (Chevy Chase, MD).

246

247 Sample collection

248 Blood samples were collected from the jugular or cephalic vein from 39 canine patients that were presenting  
249 acute hemoperitoneum secondary to splenic neoplasm rupture. All dogs underwent preoperative staging, the  
250 results of which have been previously reported (Stewart et al. 2020). The whole blood was transferred to Cell  
251 Free DNA BCT Streck tubes (Streck, Inc.), processed as per manufacturer's instructions and the collected  
252 plasma and buffy coat were stored at -80° C. The blood sample collections were performed prior to the  
253 splenectomy procedure and during the subsequent clinical visits. Biopsy sections of 1cm x 1cm x 1cm were  
254 collected from three distinct regions of the spleen: primary tumor site, tumor periphery and visually normal  
255 splenic tissue, and were stored in 10% formalin at 4° C for histopathological analysis. Additional biopsy  
256 sections were collected from the same regions and at two distinct areas of the tumor were placed in  
257 Allprotect Tissue Reagent (Qiagen, Hilden, Germany) and stored at 4° C for DNA extraction and evaluated  
258 histopathologically. Through the clinical and histopathological examinations, the patients were diagnosed  
259 with malignant splenic neoplasms (n = 25) and benign splenic neoplasm (n = 14). All histopathologic  
260 diagnoses were verified through post hoc medical record reviews and the clinical data was locked to confirm

261 the diagnoses. Blood samples were also collected in Cell Free DNA BCT Streck tubes (Streck, Inc.) from  
262 nine healthy dogs and the plasma frozen at -80° C. The plasma samples were used for cfDNA extraction and  
263 the tissue biopsy for total DNA extraction, which were used for shallow whole genome sequencing.

264

265 DNA extraction from tumor, white blood cells and plasma

266 Cell-free DNA extractions from 1-4 ml of plasma were performed with MagMAX Cell-Free DNA Isolation  
267 Kit (Thermo Fisher Scientific, Austin, TX) or the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden,  
268 Germany), as per manufacturer's instructions. The tissue biopsy (30 mg) was rinsed in 1X phosphate  
269 buffered saline, homogenized with Bullet Blender Bead Lysis Kit (NextAdvance, Troy, NY) and the  
270 supernatant passed through QIAshredder columns (Qiagen, Hilden, Germany) before been used for tumor  
271 DNA extraction using the Allprep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany), according  
272 to the manufacturer's instructions. All extracted DNA were stored at -20° C until use. The cfDNA and tumor  
273 DNA concentration, integrity and purity were assessed with the Bioanalyzer (Agilent Technologies, Santa  
274 Clara, CA), Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Austin, TX) and 4200 TapeStation genomic  
275 DNA assay (Agilent Technologies, Santa Clara, CA). DNA from the white blood cells were extracted from  
276 200 µl of buffy coat with the DNeasy Blood Tissue Kit (Qiagen, Hilden, Germany), according to the  
277 manufacturer's instructions, and stored at -20° C until further processing.

278

279 cfDNA and tumor DNA next generation sequencing

280 The cfDNA library construction was performed with the SMARTer ThruPLEX Plasma-Seq Kit and DNA  
281 HT Dual Index Kit (Takara Bio USA, Mountain View, CA), as per manufacturer's instructions. Sequencing  
282 libraries were purified with SPRI magnetic beads (Beckman Coulter, Brea, CA). Library sizes and  
283 concentrations were measured using a genomic DNA assay on the TapeStation 4200 (Agilent Technologies,  
284 Santa Clara, CA). Tumor DNA was fragmented to a target size of 200 bp on E220 Focused-ultrasonicator  
285 (Covaris, Woburn, MA). 20 ng of sheared DNA was used for the library construction with ThruPLEX DNA  
286 Seq Kit and DNA HT Dual Index Kit (Takara Bio USA, Mountain View, CA). Plasma and tumor DNA  
287 libraries were sequenced using paired-end 50 bp reads generated on the NovaSeq 6000 Sequencing System  
288 (Illumina, San Diego, CA).

289

290 Somatic copy number analysis

291 Raw sequence data was converted to fastqs using illumina's bcl2fastq v2.20.0.422. Reads were trimmed  
292 using fastp v0.2 (Chen et al. 2018) and then aligned to the canFam 3.1 (Hoeppner et al. 2014) genome  
293 assembly using bwa-mem. WGS bams from nine healthy canine plasma cfDNA samples were used as  
294 controls for ichorCNA and PlasmaSeq analysis of large-scale copy number changes. GenMap (Pockrandt et  
295 al. 2019) was used to calculate mappability on the canFam 3.1 genome assembly. PlasmaSeq analysis was  
296 conducted using our implementation of the algorithm using the julia programming language v1.1. Usable  
297 bases were identified using the mappability data, and boundaries for the fixed 5,500 bins were selected to  
298 reduce the variability of usable bases across all bins. The number of bins was selected to be approximately  
299 the same as the number of bins used by ichorCNA. For relative bin depth normalization, bams from the nine

300 healthy control samples were merged. Read depths for PlasmaSeq bins were calculated using bedtools  
301 v2.28.0 (Quinlan and Hall 2010).  
302 ichorCNA analysis was conducted using a modified version of ichorCNA v0.3.2. Functions that  
303 standardized chromosome names for human genomes were removed, as they caused errors with unexpected  
304 chromosome names. A canFam 3.1 panel of normals was generated using the nine healthy control samples  
305 with 500 kb bins. Mappability and GC content calculations were performed as for PlasmaSeq. HMMcopy  
306 (Lai et al. 2020) was used to calculate read depths per bin. Initial normal proportion range was set to  
307 [0.7,0.8,0.9,0.95,0.99]. Ploidy was fixed at 2, with a max copy number of 3 and subclone state options of  
308 [1,3]. Without these restricted parameter ranges, high tumor fraction inferences with no obvious copy  
309 number changes and inferred ploidy of 3 were very common across samples.

310

### 311 Statistical analysis

312 Patient groups were compared using non-parametric tests including Kruskal-Wallis and Wilcoxon Rank-Sum  
313 tests using the R package *stats*, and plots were prepared using the packages *ggpubr*, *magrittr*, *ggplot2*, *ggsci*  
314 and *scales*. Specificity, sensitivity and test performance were calculated with the packages *ROCR* and  
315 *cvAUC*. All means, standard deviations and confidence intervals were calculated with the R package *stats*.  
316 The Fisher Exact Test was performed with the julia package *HypothesisTests*.

317

318

319 **Data Access**

320 Upon manuscript acceptance, the sequencing data generated in this study will be submitted to the Sequence  
321 Read Archive (SRA) and the accession number will be updated. Adapted versions of ichorCNA and  
322 PlasmaSeq will be deposited to github and accession numbers will be updated here.

323

324 **Acknowledgments**

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326

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331 MM.

332

333 **Author Contributions**

334 CK, MM, JT and WH conceptualized and designed the study. PFF, BRM, TCC and MM developed methods.  
335 SDS, JC and CK designed and conducted prospective clinical studies. PFF, TCC, and SW generated data.  
336 BRM and PFF analyzed sequencing data. PFF, SDS, BRM, CK and MM interpreted data. PFF, SDS, BRM,  
337 CK and MM wrote the paper with assistance from JC, TCC, SW, WH and JT. All authors approved the final  
338 manuscript.

339

340 **Disclosure declaration**

341 The authors declare no conflicts of interest.

342

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