

Next-generation sequencing-based liquid biopsy may be used for detection of residual disease and cancer recurrence monitoring in dogs

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OBJECTIVE

The purpose of this study was to evaluate the performance of a next-generation sequencing-based liquid biopsy test for cancer monitoring in dogs.

SAMPLES

Pre- and postoperative blood samples were collected from dogs with confirmed cancer diagnoses originally enrolled in the CANcer Detection in Dogs (CANDiD) study. A subset of dogs also had longitudinal blood samples collected for recurrence monitoring.

METHODS

All cancer-diagnosed patients had a preoperative blood sample in which a cancer signal was detected and had at least 1 postoperative sample collected. Clinical data were used to assign a clinical disease status for each follow-up visit.

RESULTS

Following excisional surgery, in the absence of clinical residual disease at the postoperative visit, patients with Cancer Signal Detected results at that visit were 1.94 times as likely (95% CI, 1.21 to 3.12; $P = .013$) to have clinical recurrence within 6 months compared to patients with Cancer Signal Not Detected results. In the subset of patients with longitudinal liquid biopsy samples that had clinical recurrence documented during the study period, 82% (9/11; 95% CI, 48% to 97%) had Cancer Signal Detected in blood prior to or concomitant with clinical recurrence; in the 6 patients where molecular recurrence was detected prior to clinical recurrence, the median lead time was 168 days (range, 47 to 238).

CLINICAL RELEVANCE

Next-generation sequencing-based liquid biopsy is a noninvasive tool that may offer utility as an adjunct to current standard-of-care clinical assessment for cancer monitoring; further studies are needed to confirm diagnostic accuracy in a larger population.

Keywords: liquid biopsy, residual disease, cancer recurrence, cancer monitoring, dog

After a patient is diagnosed with cancer and receives therapy, the current standard of care relies heavily on physical examination and imaging to assess disease status and monitor for

cancer recurrence (local recurrence of cancer and/or regional/distant metastasis; hereafter referred to as cancer recurrence). However, these monitoring tools may not be sensitive enough to detect the presence of residual disease following excisional surgery or early evidence of cancer recurrence following therapeutic intervention. Additionally, access to certain imaging modalities may be limited in some

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care settings, and evaluation by imaging may require sedation or anesthesia, posing risks to the patient.

In human oncology, next-generation sequencing-based (NGS-based) liquid biopsy tests for cancer monitoring have been assessed in the postdiagnosis setting. Investigational prognostic accuracy studies where cancer-associated genomic alterations (cancer signal) are detected in a patient's blood after treatment have been evaluated using small sample sizes in a variety of human cancer types (solid as well as hematologic), including colorectal,^{1,2} lung,³ melanoma,^{4,5} bladder,⁶ lymphoma,⁷ and others.⁸⁻¹¹ These studies used NGS-based liquid biopsy in an untargeted approach. Some^{1,11} have detected cancer signals prior to clinical recurrence in human patients; however, such findings have not yet been replicated in veterinary medicine.

The following analyses evaluated the performance of a blood-based liquid biopsy test that has been clinically validated for cancer detection in dogs,¹² as an adjunct tool for cancer monitoring. The study hypothesis was that, in dogs who underwent excisional surgery, patients with a Cancer Signal Detected (CSD) result at a postoperative visit (in the

presence of no clinical residual disease) would have a higher risk of clinical recurrence within 6 months compared to those with a Cancer Signal Not Detected (CSND) result. A secondary objective was to retrospectively determine whether liquid biopsy could detect molecular recurrence of disease prior to or concomitant with clinical recurrence in a subset of patients that were longitudinally monitored.

Methods

Assessment of liquid biopsy for the detection of residual disease

The study population comprised 52 dogs originally enrolled in protocol 301 of the CANcer Detection in Dogs (CANDiD) study (**Figure 1**).^{12,13} The CANDiD study was a clinical validation study of NGS-based liquid biopsy in dogs and enrolled presumably cancer-free and cancer-diagnosed dogs into 3 protocols (101, 201, and 301).¹² Protocol 301 included dogs scheduled for biopsy or surgery due to suspected or known cancer. For the current study, each patient had a confirmed diagnosis of at least 1 primary solid

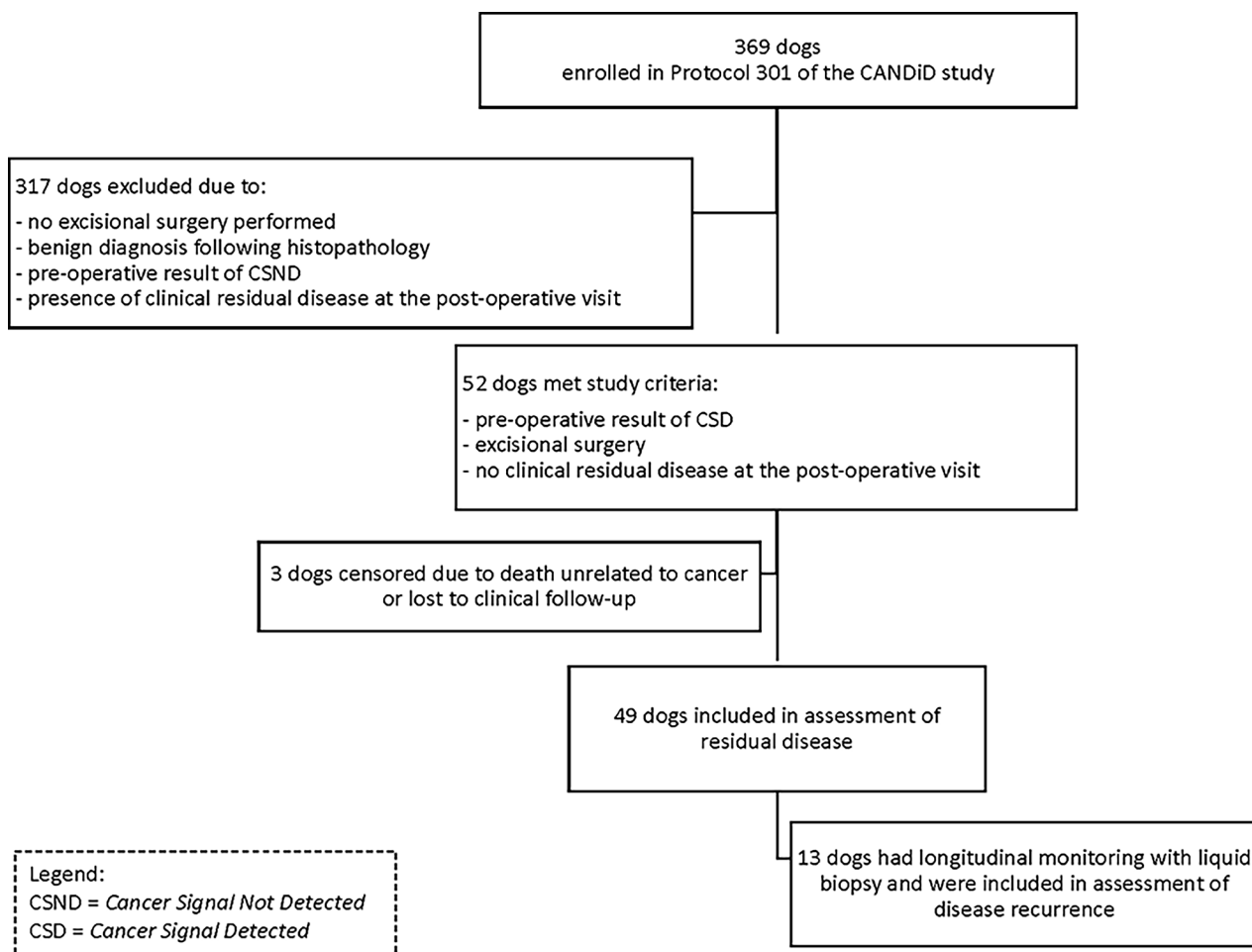


Figure 1—Disposition of patients from enrollment in protocol 301 of the CANcer Detection in Dogs (CANDiD) study through the current study, which evaluated the performance of a next-generation sequencing-based liquid biopsy test as an adjunct tool for cancer monitoring in dogs, specifically for the detection of residual disease following surgery and detection of cancer recurrence.

tumor malignancy and underwent excisional surgery of their cancer along with standard-of-care adjuvant therapy (which may have included chemotherapy, radiation therapy, immunotherapy, etc) at their managing veterinarian's office. Each patient had a baseline (preoperative) blood sample drawn for NGS-based liquid biopsy testing immediately prior to surgical excision of their cancer; all dogs had CSD results at baseline. Patients with CSD results at baseline were chosen because cancer detection by liquid biopsy is known to vary depending on the cancer type present in the patient¹²; therefore, the detection of a cancer signal at baseline (prior to any therapeutic intervention) in a cancer-diagnosed patient suggests that the test can be subsequently used in that patient as a tool for cancer monitoring. Baseline samples were submitted between November 2019 and March 2022, with additional samples collected at follow-up visits as described below. Samples were stored for liquid biopsy testing as previously described.¹²

In addition to the baseline blood sample, each patient had a follow-up blood sample collected after surgery at the postoperative visit (3 to 34 days following surgery) and had no clinical residual disease documented at that visit (**Supplemental Figure S1**). Histopathology reports were reviewed for margin measurement and completeness of excision (when available), and cases were classified as complete or incomplete based on the pathologist report; margins were further categorized as complete and wide if histologically measured ≥ 5 mm and complete but narrow if histologically measured < 5 mm. Excision was considered complete for patients having amputation for a tumor of the long bone or complete organ removal for a tumor confined to an organ (ie, splenectomy for a splenic tumor or nephrectomy for renal tumor) unless specifically denoted as incomplete on the pathology report. Patients that had more than one cancer type were categorized as complete if both cancers were noted to be completely excised and incomplete if either tumor was noted to be incompletely excised. Beyond the postoperative visit, additional clinical outcome data were retrospectively collected for each patient in this cohort through a final patient outcome report completed by the treating veterinarian. Information collected included whether a patient had developed clinical evidence of recurrence, the documented date of recurrence, if the patient had died, and the date and cause of death.

The 52 patients were stratified by their liquid biopsy test results at the postoperative visit into 2 groups, CSD and CSND, and were compared for their (1) rate of recurrence and (2) recurrence-free survival (RFS), in both cases within 6 months of excisional surgery. RFS results were expressed using Kaplan-Meier curves. Patients who were lost to follow-up or died due to non-cancer-related causes within 6 months ($n = 3$) were censored in all calculations.

Assessment of liquid biopsy for the detection of cancer recurrence

Of the aforementioned 52 patients that had surgical resection of their cancer, a subset of 13 patients

had additional blood samples collected longitudinally during follow-up visits on a predefined schedule at 1, 3, 6, 9, and 12 months following the baseline visit (Supplemental Figure S1). Not all patients completed all predefined visits, and, in some cases, additional samples were obtained at nonpredefined visits, eg, when the treating veterinarian noted a change in a patient's clinical disease status. At each follow-up visit, the patient's clinical disease status was evaluated by a specialist (in surgery or oncology) using available standard-of-care methods such as physical exam, bloodwork, and routine imaging (radiography and/or ultrasound). The treating veterinarian assigned a standardized clinical disease status using cRECIST criteria for solid tumors¹⁴ as follows: progressive disease (PD), stable disease (SD), partial response (PR), and complete response (CR); a no evidence of disease (NED) status designation was considered synonymous with CR. These designations, and supporting clinical records, were subsequently reviewed by an ACVIM board-certified veterinary medical oncologist at the laboratory (PetDx) to confirm that the treating veterinarian's clinical disease status designation was aligned with the standardized criteria at each visit. The clinical disease status assigned at each visit was further used to categorize each visit as either the presence of clinical disease (corresponding to SD, PR, or PD) or the absence of clinical disease (corresponding to CR). The clinical team at PetDx that reviewed the clinical disease status designations was blinded to the liquid biopsy test results.

Statistical analysis

Normality of data was assessed for age and weight of study patients and for molecular recurrence lead times using Shapiro-Wilk tests and visual inspection using QQ-plots. Comparison of 6-month cancer recurrence in patients with CSD versus CSND results was performed using Fisher exact test. Confidence intervals of proportions were calculated using the Wilson procedure. Differences in progression-free survival as a function of liquid biopsy results were assessed using Kaplan-Meier survival curves. Differences between the survival curves were tested for statistical significance using a log-rank test. In addition, a Cox proportional hazard regression model was used to estimate hazard ratios as a function of liquid biopsy results. Calculations and plotting were performed in R version 4.3.0 (The R Foundation) and Python v3.9 (Python Software Foundation). A P value of < 0.05 was considered statistically significant.

Results

The 52 patients in the study ranged in age from 1.9 to 14.5 years (median 10 years); weights ranged from 9.5 to 60 kg (mean 31.8 kg); 56% of patients were male and 44% were female; 96% of males were neutered and 100% of females were spayed; 42% were purebred and 58% were mixed-breed; and 12 cancer types were represented among the patients

(10 as solitary diagnoses and 2 as part of multiple cancer diagnoses).

NGS-based liquid biopsy for detection of residual disease following excisional surgery

Of the 52 unique patients who underwent excisional surgery and had no clinical residual disease at the postoperative visit, 3 patients were censored due to death unrelated to cancer or because they were lost to clinical follow-up. In the remaining 49 patients (**Table 1**), those with a CSD result at the postoperative visit were found to have an approximately 2-fold higher (1.94 times; 95% CI, 1.21 to 3.12; $P = .013$) likelihood of recurrence within 6 months (12/15, 80%) compared to patients with a CSND result at that visit (14/34, 41%; **Figure 2**). Patients with a CSD result at the postoperative visit also had shorter recurrence-free survival within the 6-month observation period compared to those with a CSND result (log-rank test; $\chi^2 = 5.2$; $df = 1$; $P = .02$). The CSD or CSND result was a significant predictor as estimated by a Cox proportional hazard model ($P = .027$; hazard ratio = 2.4; 95% CI, 1.1 to 5.2).

All 49 patients had assessable margins on histopathology reports (**Supplemental Table S1**): 40 had complete margins (including 3 cases with complete and narrow margins < 5 cm, 6 with complete and wide margins ≥ 5 cm, 28 involving amputation or complete organ removal, and 3 with notation of complete margins but no measurements provided) and 9 had incomplete margins (including 1 case of amputation due to intramuscular hemangiosarcoma, but the presence of neoplastic cells continuing to the tissue margins). Based on margin assessment alone, there was no significant difference in cancer recurrence within 6 months, with 50% (20/40) of patients with complete margins and 67% (6/9) of patients

with incomplete margins showing recurrence within 6 months ($P = .472$). Focusing on the 40 cases with complete margins (which included 25 patients treated with amputation, 20 of which were patients with osteosarcoma), a CSD result at the postoperative visit was associated with a significantly higher rate of cancer recurrence within 6 months (83%; 10/12) compared to cases with complete margins and a CSND result postoperatively (36%; 10/28; $P = .006$). When osteosarcoma cases involving amputation were excluded from analysis, 15 cases with complete margins remained, and a postoperative CSD result was still associated with a significantly higher rate of cancer recurrence within 6 months (80%; 4/5) compared to cases with complete margins and CSND results (10%; 1/10; $P = .017$).

NGS-based liquid biopsy for detection of cancer recurrence following therapy

Thirteen of the 52 surgical excision patients had longitudinal monitoring with liquid biopsy. Within the 12-month observation period, 11 had clinical recurrence; 82% (9/11; 95% CI, 48% to 97%) of patients had a CSD in blood concomitant with ($n = 3$) or prior to (6) clinical recurrence. In the cases where molecular recurrence was detected ahead of clinical recurrence, the median lead time was 168 days (range: 47 to 238 days; **Table 2**).

Specific to osteosarcoma, 7 dogs had amputation (including 1 dog that had a concurrent mast cell tumor involving the same limb). Two patients were reported to be in complete remission throughout the monitoring period (12 months and 9 months, respectively); these dogs had CSND (negative) liquid biopsy results at all follow-up visits. An additional 2 patients assessed to be in CR (with negative liquid biopsy results) following surgery were noted to have clinical recurrence (progressive disease) at

Table 1—Number of patients assessed for residual disease and disease recurrence by cancer type using NGS-based liquid biopsy.

Cancer type	Number of patients assessed for residual disease	Number of patients longitudinally assessed for disease recurrence
Single primary cancer		
Anal sac adenocarcinoma	3	1
Apocrine sweat gland adenocarcinoma	1	0
Fibrosarcoma (bone)	1	0
Hemangiosarcoma	5	0
Hepatocellular carcinoma	2	1
Histiocytic sarcoma	2	0
Malignant melanoma	2	1
Oral sarcoma	1	0
Osteosarcoma (appendicular)	24	6
Osteosarcoma (nonappendicular)	2	0
Soft tissue sarcoma	2	1
Multiple primary cancers		
Hepatocellular carcinoma + insulinoma	1	0
Hemangiosarcoma, hepatocellular carcinoma, soft tissue sarcoma, malignant melanoma	1	1
Hemangiosarcoma, soft tissue sarcoma	1	1
Osteosarcoma, mast cell tumor	1	1
Total	49	13

NGS = Next-generation sequencing.

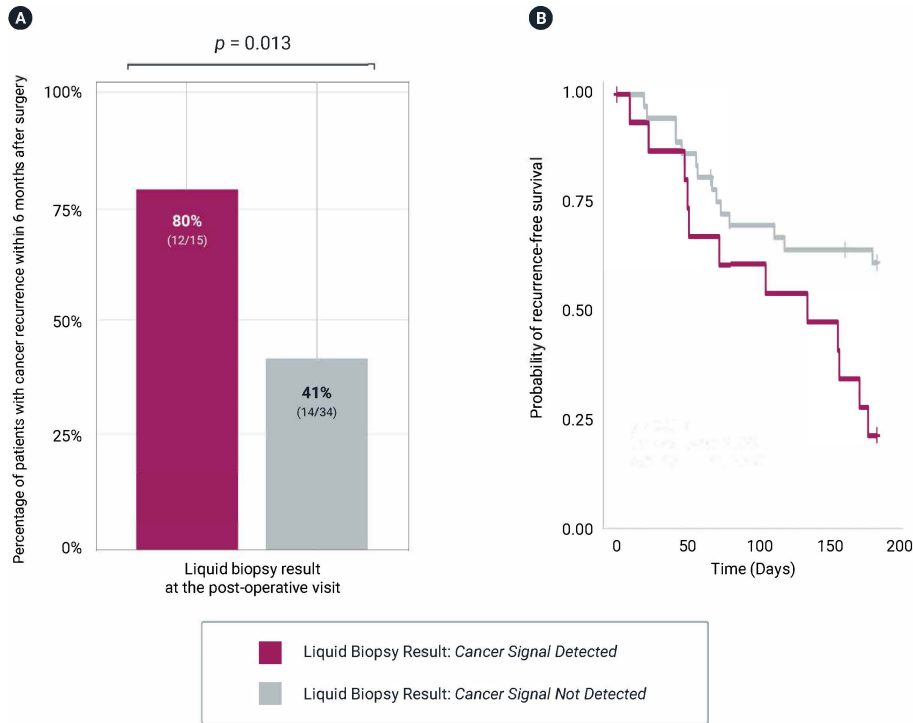


Figure 2—Analysis of outcomes for the 52 unique patients (described in Figure 1) from which detection of residual disease was assessed. Three patients were excluded from final analysis due to death unrelated to cancer or because they were lost to follow-up. A—Evaluation of the 6-month recurrence rate of patients with postoperative Cancer Signal Detected versus Cancer Signal Not Detected liquid biopsy results. B—Evaluation of recurrence-free survival over a 6-month period for patients with postoperative *Cancer Signal Detected* versus *Cancer Signal Not Detected* liquid biopsy results. Tick marks indicate censored cases.

Table 2—Overview of the 13 patients that had longitudinal monitoring with NGS-based liquid biopsy.

Cancer type(s)	Number of days from surgery to clinical recurrence	Was molecular recurrence detected? If yes, when?	Lead time (number of days from molecular recurrence to clinical recurrence)
Osteosarcoma patients			
Osteosarcoma	351	Yes, prior to clinical recurrence	168
Osteosarcoma	277	Yes, prior to clinical recurrence	69
Osteosarcoma, mast cell tumor	79	Yes, prior to clinical recurrence	47
Osteosarcoma	83	Yes, concurrent with clinical recurrence	0
Osteosarcoma	84	Yes, concurrent with clinical recurrence	0
Osteosarcoma	N/A (complete remission throughout monitoring period)	No (Cancer Signal Not Detected throughout monitoring period)	N/A
Osteosarcoma	N/A (complete remission throughout monitoring period)	No (Cancer Signal Not Detected throughout monitoring period)	N/A
Nonosteosarcoma patients			
Soft tissue sarcoma	275	Yes, prior to clinical recurrence	238
Hepatocellular carcinoma	198	Yes, prior to clinical recurrence	174
Hemangiosarcoma, hepatocellular carcinoma, soft tissue sarcoma, malignant melanoma	184	Yes, prior to clinical recurrence	168
Malignant melanoma	84	Yes, concurrent with clinical recurrence	0
Anal gland adenocarcinoma	290	No	N/A
Hemangiosarcoma, soft tissue sarcoma	30	No	N/A

Eleven patients had clinical recurrence during the monitoring period; liquid biopsy detected molecular recurrence concurrent with (n = 3) or prior to (6) clinical recurrence in a total of 9 patients. For the 6 patients in which molecular recurrence was identified prior to clinical recurrence, the median lead time was 168 days.

their day 83 and day 84 follow-up visits, respectively. In these 2 patients, liquid biopsy identified a reemergence of cancer signal concomitant with clinical recurrence. The final 3 patients experienced clinical recurrence at postoperative days 79, 277, and 351, but liquid biopsy identified molecular recurrence (ie, a cancer signal in blood prior to clinical recurrence) in all 3 cases, with lead times of 47 days, 69 days, and 168 days, respectively.

Six additional dogs with cancer types other than osteosarcoma had longitudinal monitoring with liquid biopsy. All 6 of these dogs had documented clinical recurrence (30 to 290 days following surgery). In 3 of these patients, molecular recurrence was identified via liquid biopsy prior to clinical recurrence (with lead times of 168, 174, and 238 days, respectively).

Discussion

The results presented herein demonstrate for the first time in veterinary medicine in a cohort of cancer-diagnosed patients that NGS-based liquid biopsy has the potential to be used as a noninvasive adjunct tool for cancer monitoring in dogs. Surgically managed patients with no clinical residual disease but with a CSD result at the postoperative visit had a significantly higher probability of clinical recurrence within 6 months. By comparison, there was no significant difference in cancer recurrence within 6 months based on histologic margin assessment (complete vs incomplete) alone. Future studies that pair NGS-based liquid biopsy with tumor grading and surgical margin assessment may provide further insights into disease recurrence monitoring for specific tumor types and allow clinicians to develop a more personalized monitoring plan.

In surgically managed patients that were followed longitudinally with liquid biopsy and had clinical recurrence of disease while undergoing monitoring, liquid biopsy detected molecular recurrence prior to clinical recurrence in 55% of patients (6/11; 95% CI, 25% to 82%), with a median lead time of over 5 months. This suggests that adding liquid biopsy to standard monitoring methods may aid in the early detection of cancer recurrence in some patients. However, further study is needed to obtain sufficient numbers to assess liquid biopsy's monitoring detection metrics in various tumor types. Of note, the predefined time interval (ie, 1 month after surgery or starting treatment and then every 3 months) for the follow-up visits (where clinical assessments and blood sample collection for liquid biopsy testing were performed) may have affected lead-time estimates. The date of clinical recurrence for each patient was established based on the clinical disease status designation at these predefined visits and the medical records provided by the study sites. Some patients may have had a change in disease status at visits outside of the predefined visit schedule, so the actual date of clinical recurrence may have been different from the recorded date used in this analysis. More frequent liquid biopsy

testing and clinical assessments may provide more accurate estimates for the lead time (from molecular recurrence to clinical recurrence) across various cancer types and therapies.

The goals of early identification of residual disease or disease recurrence during cancer monitoring are to achieve better disease control and improve patient outcomes by taking an individualized approach to patient care. In dogs, the clinical benefits of proactive intervention based on liquid biopsy results in the monitoring setting are yet to be established; therefore, specific recommendations for changes to patient treatment cannot yet be made. However, closer clinical monitoring of patients who receive a positive liquid biopsy result in the postsurgery or posttreatment setting appears prudent. For these patients, it may be reasonable to restage following a positive result, focusing on the primary anatomic site of the patient's cancer, as well as common metastatic sites associated with the patient's cancer type. If restaging has already been completed, options might include offering additional diagnostics and/or more frequent monitoring.

Despite the novelty of these findings, this study faces limitations. The major confounder is the lack of data from a larger cohort of dogs with the same tumor type(s). The number of dogs in the CANDiD study was one of the most substantial sums of patients reported in a single canine cancer genomics study, with the goal of validating liquid biopsy as a mult-cancer detection tool. Therefore, the enrollees represented a variety of diverse cancer types, each with differing biological behavior. Future monitoring studies would ideally focus on single tumor histologies, as sites and the likelihood of recurrence (whether local or distant metastasis) vary across cancer types. This would be necessary to extrapolate these data to a clinical setting, changing monitoring or treatment recommendations and potentially prognosis in specific tumor types.

Additionally, this study had predetermined follow-up time points that did not necessarily coincide with standard tumor-specific monitoring schedules and/or clinical progression time points. Adjuvant therapy also widely varied, due to differing standard of care recommendations by oncologists for the various tumor types represented. Future studies are needed to describe the ideal timeline of follow-up for patients with specific cancer types. Another limitation of this study was that only a subset of CANDiD patients was eligible for analysis (ie, those amenable to surgery), so the number of dogs with individual cancer types was low in some cases. Finally, the recurrence monitoring in the entire cohort was performed post hoc.

In addition to the limitations of the study, it is important to note that liquid biopsy, as a potential diagnostic tool, also faces certain limitations. In the cancer monitoring setting, liquid biopsy is intended to be used as an adjunct to, rather than a replacement for, standard-of-care monitoring procedures. False positive and false negative indices are currently

unknown for the test, and the detection rate for different cancer types is variable. It is important to note that the test cannot currently distinguish between the recurrence of the patient's original cancer and the development of a new cancer in the patient. The test reports a CSD or CSND result and does not indicate the extent of the disease or whether a particular therapy is effective in reducing tumor burden. In the postdiagnosis setting, liquid biopsy may be most useful for the detection of residual disease and the detection of disease recurrence in patients who had a CSD result at baseline (prior to any therapeutic intervention). In the absence of a baseline sample, the relative reassurance of a negative result during cancer monitoring is directly related to the detection rate for that cancer type, as described in the CANDiD study.¹² Finally, significant trauma, including tissue damage secondary to surgical procedures, can result in the temporary release of high amounts of cell-free DNA (cfDNA) from damaged or necrosed normal cells and/or tumor cells¹⁵; in dogs undergoing surgery for tumor removal, high release of normal (noncancer) cfDNA from damaged healthy tissues immediately after the procedure could dilute the residual tumor cfDNA fraction and impair the test's ability to detect a cancer signal. Previous studies in humans¹⁶ and in dogs¹⁷ have shown that cfDNA fragments are usually cleared from circulation within a few days. Therefore, it is recommended to wait a minimum of 3 days, but conservatively 7 days, after surgery to perform liquid biopsy for the purpose of residual disease detection.

Future studies are needed to evaluate the impact of liquid biopsy testing on clinical decision-making and clinical outcomes across various cancer types and therapeutic interventions. Ideally, the investigation should center on single tumor types using standard monitoring practices, with the installment of liquid biopsy at predetermined clinically relevant time points to assess diagnostic accuracy. If liquid biopsy is shown to be superior to standard routine monitoring (eg, thoracic radiographs for detection of pulmonary metastasis in osteosarcoma), additional studies should evaluate divergent monitoring and treatment decisions in a standardized fashion.

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Disclosures

The authors of this study are all current or former employees of PetDx and hold vested or unvested equity in PetDx. This does not alter the authors' adherence to AJVR policies. The authors declare no additional conflicts of interest.

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Supplementary Materials

Supplementary materials are posted online at the journal website: avmajournals.avma.org