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Cancer detection in clinical practice and using blood-based liquid biopsy: A retrospective audit of over 350 dogs

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Abstract

Background: Guidelines-driven screening protocols for early cancer detection in dogs are lacking, and cancer often is detected at advanced stages.

Hypothesis/Objectives: To examine how cancer typically is detected in dogs and whether the addition of a next-generation sequencing-based "liquid biopsy" test to a wellness visit has the potential to enhance cancer detection.

Animals: Client-owned dogs with definitive cancer diagnoses enrolled in a clinical validation study for a novel blood-based multicancer early detection test.

Methods: Retrospective medical record review was performed to establish the history and presenting complaint that ultimately led to a definitive cancer diagnosis. Blood samples were subjected to DNA extraction, library preparation, and next-generation sequencing. Sequencing data were analyzed using an internally developed

Abbreviations: CANDiD, CANcer Detection in Dogs; CLASSiC, Cancer Lifetime Assessment Screening Study in Canines; CSO, Cancel Signal Origin; IACUC, Institutional Animal Care and Use Committee; MCED, multicancer early detection; NGS, next-generation sequencing.

bioinformatics pipeline to detect genomic alterations associated with the presence of cancer.

Results: In an unselected cohort of 359 cancer-diagnosed dogs, 4% of cases were detected during a wellness visit, 8% were detected incidentally, and 88% were detected after the owner reported clinical signs suggestive of cancer. Liquid biopsy detected disease in 54.7% (95% confidence interval [CI], 49.5%-59.8%) of patients, including 32% of dogs with early-stage cancer, 48% of preclinical dogs, and 84% of dogs with advanced-stage disease.

Conclusions/Clinical Importance: Most cases of cancer were diagnosed after the onset of clinical signs; only 4% of dogs had cancer detected using the current standard of care (i.e., wellness visit). Liquid biopsy has the potential to increase detection of cancer when added to a dog's wellness visit.

KEYWORDS
cancer screening, clinical signs, early stage, incidental, liquid biopsy, multicancer early detection, preclinical

1 | INTRODUCTION

The value of early cancer detection using routine screening protocols has been clearly established in people, with formal guidelines in place over the past few decades for multiple cancer types.[1,2] As such, human medicine employs multiple screening modalities including traditional, site-specific approaches (e.g., mammography, colonoscopy), as well as newer site-agnostic approaches (e.g., blood-based multicancer early detection [MCED] testing[3]) to increase the opportunity for early detection. In veterinary medicine, professional organizations recognize the importance of early cancer detection for optimal patient outcomes,[4,5] but no formal screening guidelines currently exist, and cancer in dogs often is detected at advanced stages. The term "early cancer detection" can be conceptualized as detection of cancer at an early disease stage (i.e., "early-stage detection"), or detection of cancer before the onset of clinical signs (i.e., "preclinical detection").[6] Early-stage detection can occur regardless of the presence of clinical signs, and preclinical detection can occur regardless of formal disease stage.

The current standard of care for cancer detection in dogs is the annual or semiannual wellness visit, which typically consists of a history and thorough physical examination, and may include a minimum database (CBC, serum biochemistry panel, and urinalysis). Recently, a novel, blood-based cancer screening tool called *liquid biopsy* became clinically available for use in dogs. In short, this testing method employs next-generation sequencing (NGS) technology to analyze fragments of cell-free DNA in a dog's blood to identify the presence of cancer-associated genomic alterations.[7-9] Although this technology is hypothesized to increase the detection of cancer when added to a dog's wellness visit, the extent of this benefit has not yet been quantified.

The purpose of our study was 2-fold. The first was to examine how cancer currently is detected in dogs. The second was to determine whether the addition of liquid biopsy to a dog's wellness visit could enhance the number and type of cancer cases detected, with particular focus on preclinical and early-stage cancer detection. To achieve these goals, a retrospective chart review was performed for a cohort of dogs with definitive diagnoses of cancer to determine the presenting complaint that ultimately led to the diagnosis of cancer in these patients, and then the percentage of cases detected by the current standard of care (wellness visit) was examined in relation to the detection rate of liquid biopsy.

2 | MATERIALS AND METHODS

Subjects analyzed for our study were client-owned dogs with definitive cancer diagnoses enrolled in a prospective sample collection program for the CANcer Detection in Dogs (CANDiD) study,[9] which validated a novel blood-based liquid biopsy MCED test. The CANDiD study enrolled dogs with and without cancer at 41 clinical sites across the United States, Canada, Brazil, Netherlands, France, and Hong Kong between November 2019 and August 2021. Collection sites included veterinary specialty practices, university veterinary hospitals, and general practices. All subjects were enrolled under protocols that received Institutional Animal Care and Use Committee (IACUC) or site-specific ethics approval, according to each site's requirements, and written informed consent was obtained from all owners.

Dogs were eligible for the current study if they were enrolled in the CANDiD prospective sample collection program by investigators at any of 5 clinical sites (Veterinary Specialty Hospital of San Diego, Ontario Veterinary College at the University of Guelph, Veterinary Specialty Hospital of North County, Bridge Animal Referral Center, and the College of Veterinary Medicine at the University of Minnesota), if they had complete primary care and specialty records available, if they had macroscopic tumor present at the time of

diagnosis, and if cancer was definitively diagnosed (by cytology or histopathology) in the patient, as described in the CANDiD study.[9] Dogs without evidence of macroscopic disease at the time of enrollment, with a final diagnosis of benign disease, or without a definitive diagnosis of cancer were excluded.

Subjects were assigned a cancer type, based primarily on anatomic location, as previously described.[9] This classification system was adapted from a veterinary textbook[10] and from the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (Eighth Edition).[11] All cancer-diagnosed dogs underwent complete staging performed by the managing veterinarians according to staging guidelines at the enrolling site for individual cancer types.

Simplified definitions were developed to allow for classification of extent of disease in cancer-diagnosed dogs, given that the process of cancer staging is not standardized, and many cancer types have distinct staging methodologies in veterinary medicine.[10,11] *Localized/regional* was defined as cancer that was limited to the organ of origin or to nearby lymph nodes, tissues, or organs or lymphomas limited to a single lymph node (stage I) or multiple lymph nodes on 1 side of the diaphragm (stage II). *Disseminated/metastatic* was defined as cancer that had spread to areas of the body distant from the primary tumor or lymphomas that involved ≥2 lymph nodes on both sides of the diaphragm or ≥ 1 extra-nodal sites (stages III, IV, and V) or both; or any non-lymphoma hematological malignancy. *Undetermined* was used in a small number of cases where it was not possible to accurately determine the extent of disease, despite a complete cancer staging diagnostic evaluation. These definitions allowed for all cancer-diagnosed cases (whether solid or hematological) to be classified by extent of disease.

A retrospective review of the medical records for all patients was performed by the investigators at the respective clinical sites, as well as by the sponsor's principal investigator (AF, an ACVIM board-certified veterinary medical oncologist), including a review of all records and notes available from the general practitioner or primary care practice, as well as the specialty practice. The presenting complaint at the initial visit that led to a definitive cancer diagnosis was recorded, and each subject was classified into 1 of 3 categories: (1) cancer detected after a sick visit prompted by owner-recognized clinical signs for which cancer was a differential diagnosis, (2) cancer detected after incidental findings while being evaluated for another condition (such as heart disease or osteoarthritis) or during other routine care (such as grooming or a dental cleaning), or (3) cancer detected after findings from a routine wellness visit (conducted with no prior suspicion of cancer). Dogs with cancer detected after a sick visit comprised the clinical group, and dogs with cancer detected incidentally or during a wellness visit comprised the preclinical group. In the context of our study, a wellness visit included a history and physical examination with or without minimum database (CBC, serum biochemistry, and urinalysis). If cancer was detected incidentally while the patient was being evaluated for another condition, that condition was recorded. Cases in which the subject classification by the clinical site did not match the classification assigned by the sponsor's principal investigator were adjudicated between the 2 parties to reach a mutual agreement on classification for the case.

Blood samples were collected from subjects for liquid biopsy testing as part of the CANDiD study. Samples were subjected to DNA extraction, proprietary library preparation, and NGS as previously described.[9] Sequencing data were analyzed using an internally developed bioinformatics pipeline to detect genomic alterations associated with the presence of cancer.

For statistical analyses, calculation of p values was performed using a Mann-Whitney test in the case of continuous variables and Chi-square test for categorical variables; $P < .05$ was considered significant; 95% confidence intervals (CI) were calculated using the Wilson score interval method.

3 | RESULTS

Three-hundred eighty-three dogs enrolled in the CANDiD study across 5 institutions were eligible for inclusion in the study. Twenty-four dogs were excluded due to a benign diagnosis (n = 17), no definitive diagnosis (n = 4), and lack of macroscopic disease at the time of enrollment (n = 3). Three-hundred fifty-nine dogs had confirmed cancer diagnoses and were included in the analysis; 353 of these patients had liquid biopsy results available for review for the study.

3.1 | Subject demographics and how cancer was detected

The 359 dogs consisted of 196 males and 163 females; 176 (90%) of males were neutered and 156 (96%) of females were spayed; 184 (51%) were reported to be purebred and 175 (49%) were reported to be mixed breed. The median age at the time of enrollment was 9.8 years (range, 2.1-16.4 years) and the median weight was 28.7 kg (range, 5.1-119.0 kg). Fifty-four breeds were represented in the study; the most common breeds were Golden Retriever (n = 26), Labrador Retriever (n = 22), German Shepherd (n = 11), Boxer (n = 8), English Bulldog (n = 8), Pembroke Welsh Corgi (n = 7), and Siberian Husky (n = 7). All other breeds were represented by ≤5 dogs. Approximately 40 different cancer types and a range of stages were represented.

In 13 dogs (4%), cancer was detected as a result of findings during a wellness visit with no prior suspicion of cancer (Figure 1). Eight dogs had *localized/regional* disease and 5 dogs had *disseminated/metastatic* disease. Cancer diagnoses included lymphoma in 5 dogs, anal sac adenocarcinoma in 2 dogs, and 1 dog each with mast cell tumor, soft tissue sarcoma, pulmonary carcinoma, a sarcoma that was identified as an osteosarcoma or chondrosarcoma, a squamous cell carcinoma of the oral cavity, and 1 patient that had both urothelial carcinoma of the urinary bladder and cutaneous hemangiosarcoma. Of the 5 dogs that were diagnosed with lymphoma, 1 was stage II, 2 were stage IV, and 2 were stage V; all 5 were substage a; 3 were B-cell and 2 were T-cell.

In 29 dogs (8%), cancer was detected on the basis of incidental findings identified while being evaluated for another condition, or during other routine care, unrelated to the eventual cancer diagnosis (Figure 1).

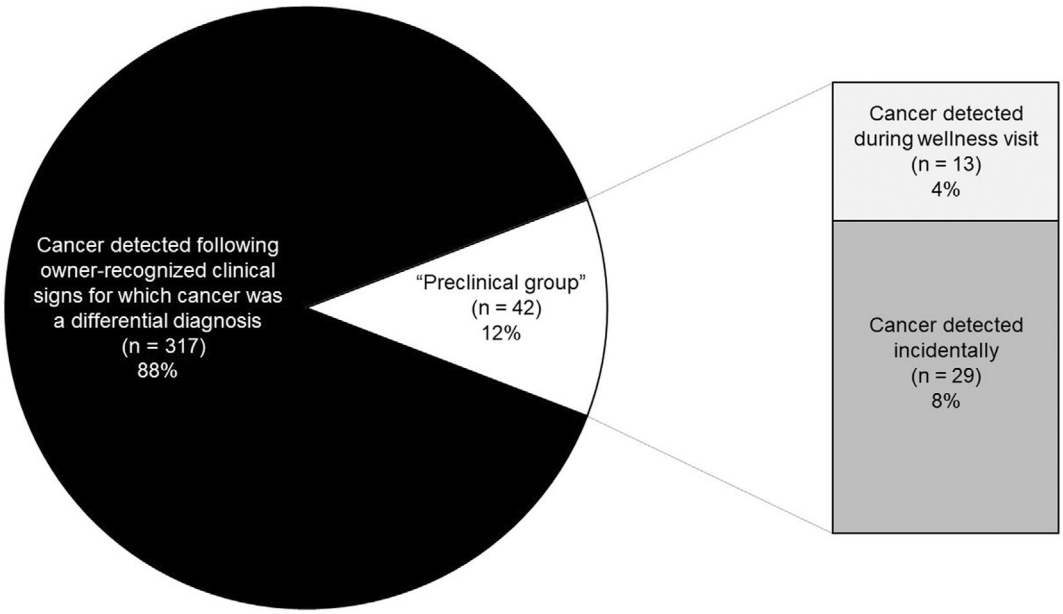

FIGURE 1 Route of detection in a cohort of 359 dogs with a variety of cancer types and stages

Cases with other conditions included 6 dogs with chronic dermatological disease, 5 having pre-dental evaluation, 2 having routine grooming, and 1 each with anemia, cystitis, stertor, chronic pancreatitis, heart disease (myxomatous valve degeneration), osteoarthritis, chronic dental disease, medication monitoring, chronic anal sac impaction, ongoing lameness, inflammatory bowel disease and increased liver enzyme activities, ventral neck abscess, oral mass, coughing and sneezing, lipoma, and re-staging of previous cancer (unrelated to the new primary cancer identified). Eighteen of the 29 cases involved non-hematological malignancies: 14 had *localized/regional* disease and 4 had *disseminated/metastatic* disease. These 18 cases consisted of 5 cases of anal sac adenocarcinoma, 2 cases of liver cancer (1 cholangiocellular carcinoma and 1 hepatocellular carcinoma), 2 cases of mast cell tumors, 2 cases of soft tissue sarcomas, and 1 case each of osteosarcoma, pulmonary adenocarcinoma, prostate carcinoma, and pituitary macroadenoma, respectively; the remaining 3 cases involved multiple primary cancers. Eleven of the 29 cases had hematological malignancies: 9 cases of intermediate to large cell lymphoma (7 B-cell, 2 T-cell; 4 stage III, 5 stage IV). Additionally, there was 1 case of stage V chronic lymphoid leukemia and 1 case of T-zone lymphoma.

In 317 dogs (88%), cancer was detected after a sick visit prompted by owner-recognized clinical signs for which cancer was a differential diagnosis (Figure 1). A total of 177 (56%) of these dogs had *localized/regional* disease, 129 (41%) had *disseminated/metastatic* disease, and extent of disease was *undetermined* in 11 (3%) dogs. There were 207 dogs with non-hematological malignancies (detailed in Supplemental Figure 1), 103 with hematological malignancies, and 7 with multiple primary cancers. Of the group with hematological malignancies, 90 dogs were diagnosed with intermediate to large cell lymphoma, 9 with indolent lymphoma, 2 with acute lymphoid leukemia, and 2 with chronic lymphoid leukemia; 4 stage II, 19 stage III, 38 stage IV, and 42 stage V.

In summary, of the 42 dogs with cancer diagnosed preclinically (either during a wellness visit or incidentally while being evaluated for another condition or during other routine care), 48% had *disseminated/metastatic* disease and, in dogs with cancer diagnosed after the development of clinical signs, 41% had *disseminated/metastatic* disease. No significant difference was found in the proportion of *disseminated/metastatic* cases based on whether detection occurred before or after the development of clinical signs ($P = .4$; Figure 2).

For the cohort of 317 dogs diagnosed with cancer after the development of clinical signs, 11% were presented with a complaint of enlarged lymph nodes, 31% with masses or swellings (other than lymph nodes), and 34% had multiple clinical signs, including ≥1 of the above. The full distribution of clinical signs in these dogs is presented in Figure 3.

No significant differences were found in demographic characteristics (i.e., age, weight, proportion of purebred to mixed-breed dogs, proportion of male and female dogs, or proportion of spayed/neutered vs intact dogs) of dogs in which cancer was detected before or after the development of clinical signs (Supplemental Table 1).

3.2 | Liquid biopsy results

Liquid biopsy results were available for 353 of the 359 dogs in the study: 193 dogs (54.7%; 95% CI, 49.5%-59.8%) received a *Cancer Signal Detected* (positive) result and 157 received a *Cancer Signal Not Detected* (negative) result (44.5%; 95% CI, 39.4%-49.7%). Three dogs received an *Indeterminate* result, where genomic alterations were detected but their clinical relevance was unclear.

In the group of dogs in which cancer was detected during a wellness visit, 8 of 13 dogs (61.5%; 95% CI, 35.5-82.3) received a *Cancer*

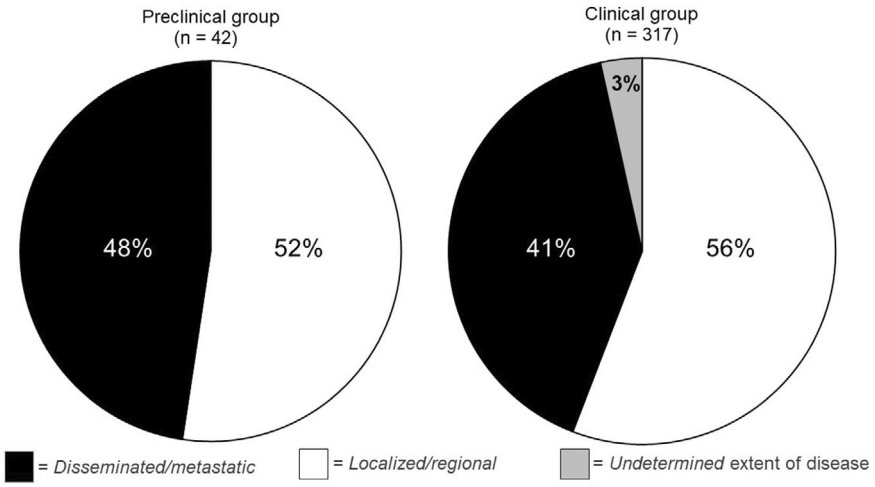

FIGURE 2 Extent of disease in preclinical vs clinical subjects. No significant difference in cases of disseminated/metastatic disease in the preclinical group vs clinical group ($P = .4$)

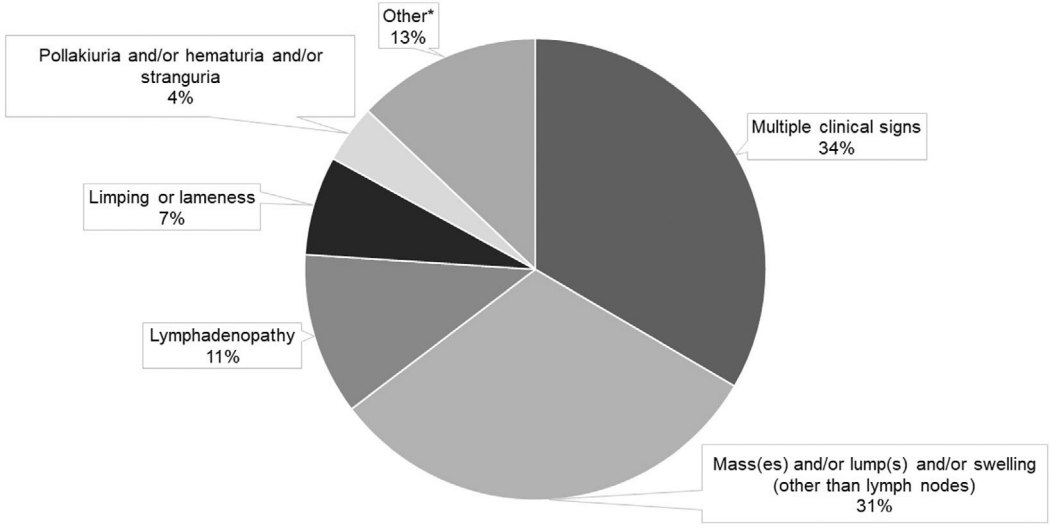

FIGURE 3 Distribution of owner-recognized clinical signs that led to the eventual diagnosis of cancer following presentation to a veterinarian. *Other: Sneezing and/or epistaxis and/or nasal discharge (n = 7), dyspnea or tachypnea and/or coughing (n = 6), nausea and/or vomiting (n = 4), seizures and/or neurologic signs (n = 4), hyporexia or anorexia (n = 3), changes in stool (n = 2), polyuria and/or urinary incontinence (n = 1), lethargy or exercise intolerance (n = 1), unusual odors and/or discharge (n = 1), weakness and/or collapse (n = 1), other signs not captured in prior list (n = 9)

Signal Detected result and 5 received a *Cancer Signal Not Detected* result (38.5%; 95% CI, 17.7%-64.5%). In the group of dogs whose cancer was detected incidentally while being evaluated for another condition or during other routine care, 12 of 29 dogs (41.4%; 95% CI, 25.5%-59.3%) received a *Cancer Signal Detected* result and 17 received a *Cancer Signal Not Detected* result (58.6%; 95% CI, 40.7%-74.5%). No significant difference was found in the detection rate of liquid biopsy for cancers detected during a wellness visit and those detected incidentally ($P = .2$). Considering all dogs in the preclinical group (wellness and incidental), 20 of 42 (47.6%; 95% CI, 33.4%-62.3%) received a *Cancer Signal Detected* result.

In the group of dogs with liquid biopsy results where cancer was detected after the development of clinical signs (n = 311), 173 of 311 (55.6%; 95% CI, 50.1%-61.0%) received a *Cancer Signal Detected* result, 135 received a *Cancer Signal Not Detected* result (43.4%; 95% CI, 38.0%-49.0%), and 3 received an *Indeterminate* result (1.0%; 95% CI, 0.3%-2.8%).

No significant difference was found in the detection rate of liquid biopsy between the preclinical group and the clinical group ($P = .3$; Figure 4).

The liquid biopsy detection rate also was stratified by extent of disease. In the overall cohort, 195 dogs had *localized/regional* disease, of which 63 (32.3%; 95% CI, 26.1%-39.2%) received a *Cancer Signal Detected* result, 131 received a *Cancer Signal Not Detected* result (67.2%; 95% CI, 60.3%-73.4%), and 1 received an *Indeterminate* result (0.5%; 95% CI, .09%-2.9%). There were 148 dogs with *disseminated/*

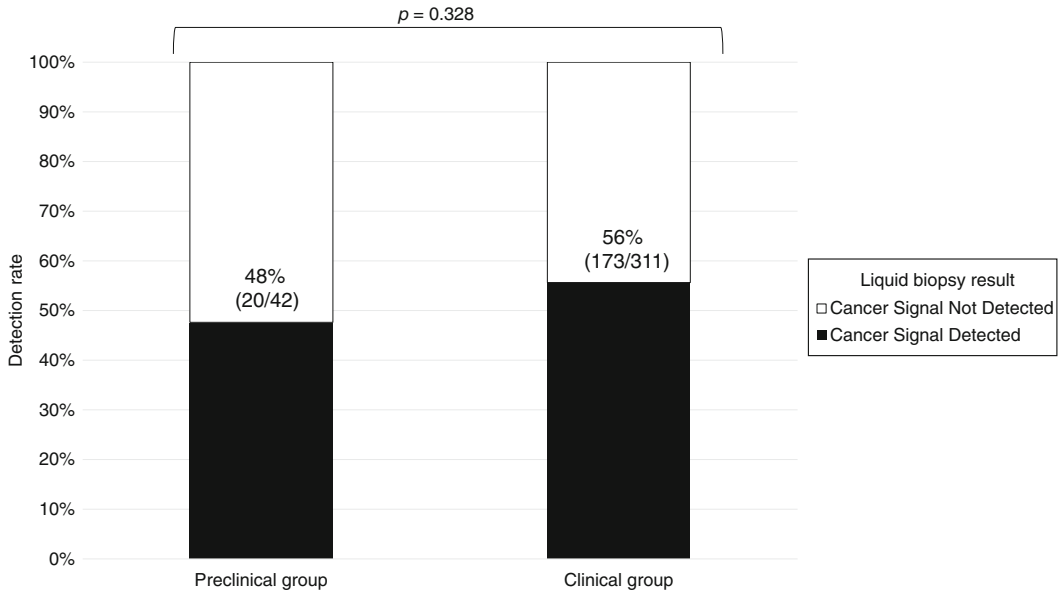

FIGURE 4 Liquid biopsy detection rates in the preclinical and clinical groups

metastatic disease, of which 124 (83.8%; 95% CI, 77.0%-88.9%) received a *Cancer Signal Detected* result, 22 a *Cancer Signal Not Detected* result (14.9%; 95% CI, 10.0%-21.5%), and 2 an *Indeterminate* result (1.4%; 95% CI, 0.3%-4.8%). Ten dogs had an *undetermined* extent of disease; 6 (60.0%; 95% CI, 31.3%-83.2%) received a *Cancer Signal Detected* and 4 received a *Cancer Signal Not Detected* result (40.0%; 95% CI, 16.8%-68.7%). The difference in detection rate of liquid biopsy between the *localized/regional* group and the *disseminated/metastatic* group was significant ($P < .001$; Figure 5A).

In the preclinical group (wellness and incidental), 22 dogs had *localized/regional* disease, of which 4 (18.2%; 95% CI, 7.3%-38.5%) received a *Cancer Signal Detected* result and 18 received a *Cancer Signal Not Detected* result (81.8%; 95% CI, 61.5%-92.7%). Twenty dogs had *disseminated/metastatic* disease, of which 16 (80.0%; 95% CI, 58.4%-91.9%) received a *Cancer Signal Detected* result and 4 a *Cancer Signal Not Detected* result (20.0%; 95% CI, 8.1%-41.6%). The difference in detection rate of liquid biopsy between the *localized/regional* group and the *disseminated/metastatic* group was significant ($P < .001$; Figure 5B).

A full review of liquid biopsy results by extent of disease and route of detection is presented in Supplemental Table 2.

3.3 | Lymphoma cohort

Dogs diagnosed with lymphoma at substage a have a better prognosis than those diagnosed at substage b.[12] In this study population, 106 dogs were diagnosed with intermediate to large cell lymphoma (n = 104) or acute lymphoid leukemia (n = 2); 14 of these dogs were from the cohort detected before clinical signs (10 B-cell; 4 T-cell) and 92 were from the cohort diagnosed after owner-recognized clinical signs (51 B-cell, 18 T-cell, 23 Unknown). The percentage of substage a cases was significantly higher in the cohort of dogs with cancer detected before the development of clinical signs ($P = .02$). In the preclinical group, 93% of cases were substage a (13/14) and 7% were substage b (1/14) whereas only 60% (55/92) of cases in the clinical group were substage a and 40% were substage b (37/92). The single lymphoma patient in the preclinical group that was categorized as substage b originally was presented for a pre-dental evaluation at which time increased liver enzyme activities led to a diagnosis of lymphoma; during the diagnostic evaluation the dog developed hyporexia and weight loss.

For the 13 dogs with substage a lymphoma detected before clinical signs, liquid biopsy returned a *Cancer Signal Detected* result in 11 (84.6%; 95% CI, 57.8%-95.7%), and for the 55 dogs with substage a lymphoma detected after the development of clinical signs, liquid biopsy results were available for 54 dogs and returned a *Cancer Signal Detected* result in 47 (87.0%; 95% CI, 75.6%-93.6%). No significant difference was found between the liquid biopsy detection rates for substage a patients across the preclinical and clinical groups ($P = .8$).

In the overall cohort (preclinical and clinical combined) of 105 dogs with lymphoma, no significant difference was found in the detection rate of liquid biopsy for substage a disease (86.6%; 95% CI, 75.6%-93.6%; 58/67) compared to substage b (97.4%; 95% CI, 86.5%-99.5%; 37/38; $P = .07$).

4 | DISCUSSION

Most cancers in dogs in this study were diagnosed when the dogs were presented for evaluation after the onset of clinical signs, and in approximately one-third of these cases, the dogs already had several clinical signs for which cancer was a differential diagnosis. Only a small percentage of dogs (<5%) in this study had cancer detected using the current standard of care (i.e., annual or semiannual wellness visit). Cancer screening using NGS-based liquid biopsy may offer an

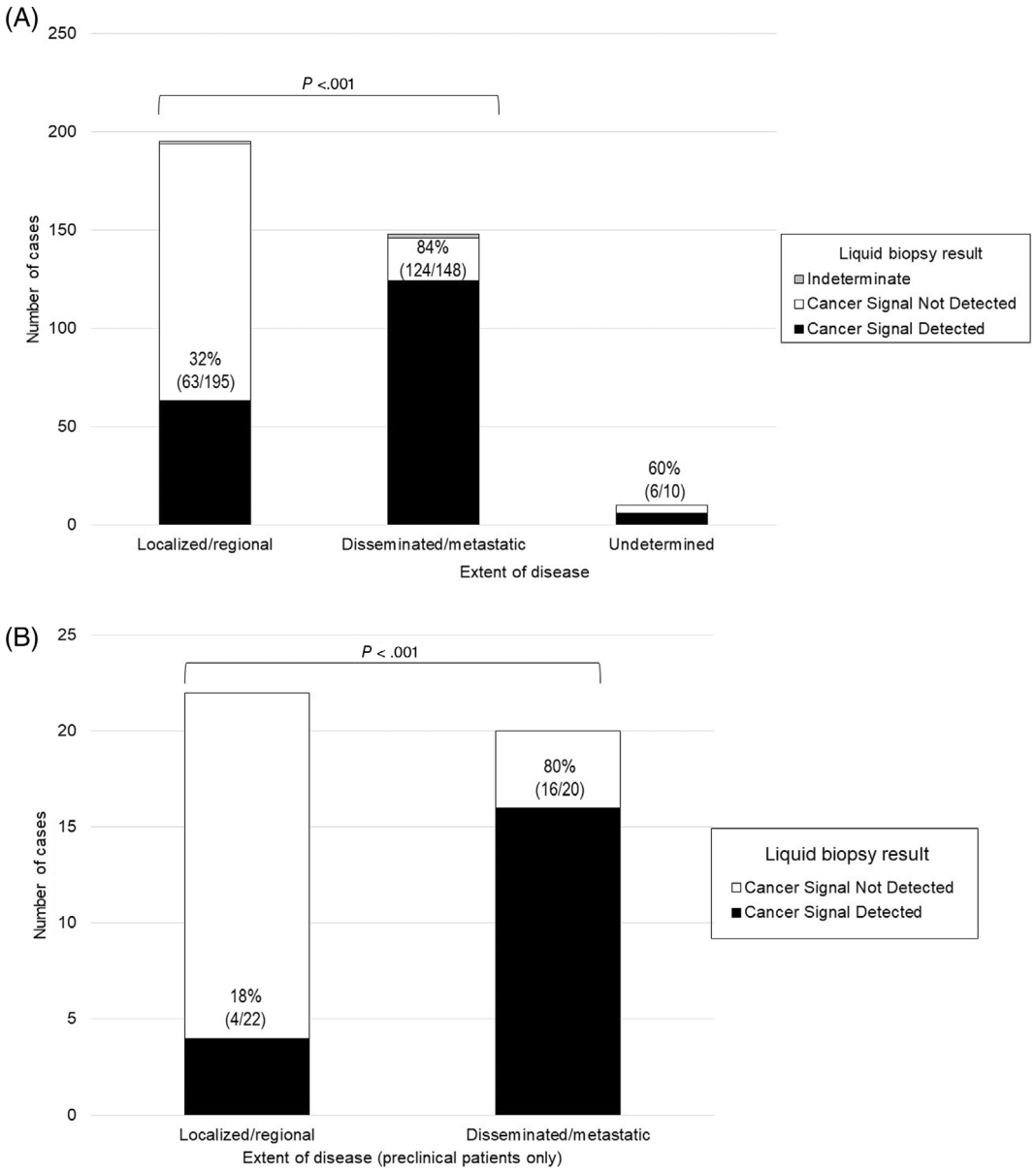

FIGURE 5 (A) Liquid biopsy detection rates by extent of disease in the overall cohort (n = 353). (B) Liquid biopsy detection rates by extent of disease in the preclinical group only (n = 42)

opportunity to enhance detection in patients across a range of cancer types and stages. Even in dogs that were not yet showing clinical signs of cancer, liquid biopsy was able to detect disease in nearly 50% of patients. Therefore, the addition of liquid biopsy to a dog's annual or semi-annual wellness visit (starting at age 7 for all dogs, or earlier for certain breeds[13]) may enhance both preclinical and early-stage cancer detection in dogs and may expand the breadth of cancer types that may be detectable during routine care or at a wellness visit.

The primary benefit of early-stage detection is intuitive. Dogs treated for cancer at early stages have shown improved outcomes for a variety of cancer types, including lymphoma,[14,15] hemangiosarcoma,[16] osteosarcoma,[17] mast cell tumor,[18] soft tissue sarcoma,[19,20] malignant melanoma,[21] mammary gland carcinoma,[22] and anal sac carcinoma.[23] The benefits of preclinical detection may not be as immediately evident but also have been well-documented in the literature. For example, improved outcomes have been associated with lymphoma diagnosed at substage a,[12] non-ruptured hemangiosarcoma,[16] lack of epistaxis in the presence of nasal tumors,[24] lack of seizures or other neurological signs in the presence of brain tumors,[25] lack of recent rapid growth and ulceration in the presence of mast cell tumors,[26] and lack of cough or respiratory signs in the presence of lung tumors.[27] Furthermore, preclinical cancer patients may be easier to manage, both medically and financially, because they do not require stabilization and treatment for their clinical signs in addition to treatment for their cancer. Preclinical detection also affords potential benefits that are not readily quantifiable and not well described in the literature, such as avoiding the pain and suffering associated with unrecognized cancer, the distress and financial burden associated with emergency presentation for care,

unexpected death, and the loss of the opportunity to determine end of life plans on the owner's terms. In short, preclinical detection allows veterinarians and owners to be proactive in the patient's care and provides the opportunity to explore more treatment options, or to start palliative care sooner, in both cases at a potentially lower cost.

In our study, only 12% of dogs had cancer detected preclinically (by wellness visit or incidental findings). In these dogs that were not yet showing clinical signs of cancer, liquid biopsy could detect disease in nearly 50%. Furthermore, no significant difference was found in the performance of liquid biopsy in dogs with cancer detected before or after the development of clinical signs, suggesting that liquid biopsy may be an effective tool for preclinical cancer detection.

Liquid biopsy also may be an effective tool for early-stage cancer detection. In our study, 195 of the 353 dogs with liquid biopsy results had *localized/regional* disease at the time cancer was detected; 32% (63/195) of these early-stage cases were detected by liquid biopsy, with a single test per subject. The cumulative detection rate of a lifetime screening program is typically much higher than the detection rate of a single screening test, because each successive test provides an additional opportunity for detection if cancer indeed is present.[28-31] In general, as a tumor increases in size and becomes more aggressive, more cell-free DNA with cancer-associated genomic alterations will be shed into the circulation, increasing the chances of detection by liquid biopsy.

In addition to early-stage detection, liquid biopsy is highly effective for detecting later-stage disease, which may be of particular importance for patients that are not yet exhibiting clinical signs of cancer despite the advanced stage of their disease. In our study, almost half of the dogs in the preclinical group already had later-stage (*disseminated/metastatic*) disease at diagnosis, and liquid biopsy returned a positive result for >80% of these patients. Detection of advanced-stage cancers in preclinical dogs provides patients with the opportunity for intervention before the development of clinical signs, which may allow a wider array of treatment options, provide families the time necessary to make decisions, improve quality of life through earlier palliation, and potentially lead to better outcomes for these patients. In addition to the opportunity for preclinical intervention in these dogs, liquid biopsy may detect such cases at an earlier stage (resulting in stage migration)[32] when more treatment options are available and outcomes may be further improved, a concept previously demonstrated in human medical oncology.[6]

Based on these observations, adding liquid biopsy to a dog's wellness visit or routine care may increase both overall and early cancer detection, along with expanding the breadth of cancer types detectable during wellness visits. Certain cancer types, such as splenic tumors, hepatic tumors, and lung tumors, are not readily detectable at a preclinical stage even with a thorough physical examination and minimum database, but many of these cancer types may be detectable by liquid biopsy testing.

Liquid biopsy testing has limitations and should not be viewed as a replacement for a thorough physical examination, minimum database, and other clinical evaluation methods that might be part of the standard of care at individual clinical sites. The liquid biopsy test evaluated in our study leverages multiple classes of genomic data to generate a binary positive or negative result of *Cancer Signal Detected* or *Cancer Signal Not Detected*. In the current form, the test is limited to a Cancer Signal Origin (CSO) prediction for hematological malignancies, with potential for expanding CSO prediction to additional cancer types with further development. A positive result requires a confirmatory cancer evaluation to achieve a definitive diagnosis, including cancer type and stage. Also, performance of liquid biopsy can vary by cancer type and stage because of various underlying biological reasons. For instance, certain tumors do not readily shed cell-free DNA into blood, limiting the opportunity for detection by liquid biopsy.[9] Furthermore, small localized tumors may shed very small amounts of cell-free DNA into circulation, which may be below the limit of detection of liquid biopsy. Some of the cancer types with lower detection rates by liquid biopsy may include small cutaneous tumors and anal sac adenocarcinomas, both of which are often readily detectable on physical examination. In fact, in our study, 5 dogs were diagnosed by wellness examination with such cancers, but received negative results from liquid biopsy. Therefore, the wellness visit and liquid biopsy testing should be considered complementary screening approaches to increase the detection of cancer in dogs, in the context of each patient's unique clinical presentation.

Our study had some limitations. One limitation was referral bias. The dogs analyzed in our study originated from 5 clinical sites, all of which were specialty hospitals or academic centers. An unknown number of dogs in which cancer was detected by various means and at various stages at the general practitioner's office may not have been referred for care to a specialty clinic or academic center. For instance, mast cell tumors, small soft tissue sarcomas, anal sac adenocarcinomas, and lymphomas (among others) may be detected and treated (medically or palliatively) by the general practitioner without referral. Therefore, the population of patients in our study may represent a biased cohort of cancer types and stages and may not reflect the detection rates of cancer by wellness examination across the full spectrum of veterinary care.

Another limitation is that the detection of cancer using liquid biopsy may have been augmented in some cases by the time that elapsed between the dog's presentation to the general practitioner and referral to the specialist where liquid biopsy testing was performed. With continued tumor growth over time, detection by liquid biopsy may have been aided by increased cell-free DNA shedding from the tumor. The number of cases that would have been detectable by liquid biopsy had the blood sample been collected at the time the disease was first detected by the patient's primary care practitioner is unknown.

Similarly, a patient's extent of disease was established at the time the dog was seen by a specialist. This designation may not accurately reflect the extent of disease at the time the dog was presented to the general practitioner and initially received a cancer diagnosis. Therefore, the percentage of dogs with *disseminated/metastatic* disease may be overrepresented in our study population.

Lastly, the extent of a wellness visit can vary from provider to provider. Although clinical history and physical examination are likely

to be part of any wellness visit, a minimum database may not always be performed. On the other hand, some clinics may perform routine imaging as part of geriatric wellness visits. The inclusion of multiple screening elements into the wellness visit is likely to result in increased detection, but the elements of each of the individual wellness examinations performed were not known to the investigators.

Our retrospective study determined that liquid biopsy could detect cancer in a population of dogs diagnosed with the disease before the onset of clinical signs. A separate prospective study, the Cancer Lifetime Assessment Screening Study in Canines (CLASSiC; PetDx, Inc, La Jolla, California) is currently underway to evaluate the ability of liquid biopsy to detect cancer over time in a large cohort of dogs at higher risk of cancer because of age and breed but with no current evidence of cancer. These dogs will be followed longitudinally with physical examination and liquid biopsy testing as frequently as every 6 months to determine the appropriate interval for cancer screening and evaluate the ability of liquid biopsy to prospectively detect preclinical cancer in a typical screening population.[33,34]

Early detection of cancer is crucial to optimizing outcomes for patients in both human and veterinary medicine. Human medicine employs multiple screening modalities (e.g., mammography, colonoscopy, newer blood-based MCED testing) to increase the opportunity for early detection. Cancer screening tests and guidelines do not exist for dogs, and most patients are presented for evaluation after the development of clinical signs. The availability of liquid biopsy testing using NGS presents veterinarians, owners, and patients with a novel noninvasive option for cancer screening that may enhance the preclinical detection of cancer in dogs (often at earlier stages of the disease) and may expand the range of cancer types routinely detectable at a wellness visit or during routine care.

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CONFLICT OF INTEREST DECLARATION

Andi Flory, Lisa McLennan, Betsy Peet, Marissa Kroll, Kristina M. Kruglyak, Angela L. McCleary-Wheeler, Ashley Phelps-Dunn, Lilian K. Wong, Chelsea D. Warren, Gina Brandstetter, Michelle C. Rosentel, Lauren DiMarzio, Allison L. O'Kell, Todd A. Cohen, Daniel S. Grosu, Jason Chibuk, Dana W. Y. Tsui, Ilya Chorny, and Jill M. Rafalko are all employees and shareholders of PetDx, Inc. No other authors have a conflict of interest.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All subjects were enrolled under protocols that received IACUC or site-specific ethics approval, according to each site's requirements. All subjects were client-owned, and written informed consent was obtained from all owners.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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