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frontiers

Non-Invasive Cancer Detection in Canine Urine through *C. elegans* Chemotaxis

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14 **Detection$_4$, Chemotaxis$_5$.**

15 **Abstract**

16 Cancer is the leading cause of death of companion animals, and successful early treatment has been a
17 challenge in the veterinary field. We have developed the Non-Invasive Cancer Screening (N.C.S.)
18 Study to perform cancer detection through the analysis of canine urine samples. The test makes use
19 of the strong olfactory system of the nematode *C. elegans*, which was previously shown to positively
20 respond to urine samples from human cancer patients. We performed a proof-of-concept study to
21 optimize the detection capability in urine samples obtained from dogs with naturally occurring
22 cancers. In this study, we established a scale for identifying the cancer risk based on the magnitude of
23 the chemotaxis index of *C. elegans* towards a canine urine sample. Through validation, the N.C.S.
24 Study achieved a sensitivity of 85%, showing that it is highly sensitive to indicating the presence of
25 cancer across multiple types of common canine cancers. The test also showed a 93% specificity to
26 cancer samples, indicating a low rate of over-identifying cancer risk. From these results, we have
27 demonstrated the ability to perform low-cost, non-invasive cancer detection in companion animals, a
28 method which can increase the ability to perform cancer diagnosis and treatment.

29 1 Introduction

30 There are over 200 million companion animals (dogs, cats, and horses) in the United States, and
31 cancer is the leading cause of death among them (1). Approximately 1 in 4 dogs and 1 in 5 cats will
32 develop cancer in their lifetimes, according to the Veterinary Cancer Society (2). Cancer in
33 companion animals is difficult to treat successfully because few symptoms are evident in its early

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34 stages, and by the time symptoms become apparent, the cancer is usually advanced with a high
35 mortality. It has been shown that approximately half of all canine cancers are treatable if diagnosed
36 early enough, and new therapeutic approaches are continuously being established (3–6). Existing
37 tests are available for cancer screening (7) but can be too expensive or invasive to be conducted
38 regularly for some dog owners. Thus, there is an urgent need for the development of a novel
39 economical and non-invasive method for cancer screening to increase the probability of successful
40 treatment.

41 *C. elegans* is a simple multicellular organism that is often used as a model to study biological
42 phenomena such as cellular signaling, neural development, and aging in higher multicellular animals
43 (8). Breeding isogenic populations of *C. elegans* is straightforward, as nearly all animals in a wild-
44 type population are hermaphrodites that reproduce through self-fertilization and are fed a diet of *E.*
45 *coli*. *C. elegans* possesses a highly sensitive olfactory system to navigate its environment and detect
46 food through the identification of chemical cues (9,10). *C. elegans* encodes at least 1,500 predicted
47 G-protein-coupled receptors (GPCRs) (11). Some of these GPCRs are olfactory receptors that
48 underlie the worm's odor detection capabilities. *C. elegans* has an excellent sense of smell, and
49 possesses approximately 1.5 times as many different types of olfactory receptors as a dog (12).
50 Nematodes such as *C. elegans* rely on their strong sense of smell to search for food and navigate their
51 environments (10). Once *C. elegans* detects an attractive odorant, it aligns with the chemical odorant
52 and travels toward it (a process known as chemotaxis). This acute sense of smell allows for *C.*
53 *elegans* to detect distinct volatile organic compound (VOC) profiles within animal urine.

54 The volatilome is the collection of VOCs which are present in the outputs of a biological organism
55 (13). Cancer cells are known to emit VOCs that produce an odor that is distinguishable from that of
56 non-cancer patients (14,15). Changes in the volatilome of specimen in animals affected with cancer
57 have been measured using both gas chromatography and mass spectrometry (16,17). These odorant
58 signatures are detectable in samples acquired from animals such as dogs (18) and mice (19), and thus
59 could serve as a marker for identifying cancer. It has been shown that *C. elegans* can quantitatively
60 detect the presence of signature VOCs in both in culture media of cancer cells *in vitro* and in urine
61 samples of human cancer patients through chemotaxis assays and through calcium imaging of the
62 AWC neuron (20–23). However, *C. elegans* has not yet been shown to identify cancerous VOC
63 signatures in canine urine samples.

64 Here, we conducted the Non-Invasive Cancer Screening (N.C.S.) Study to measure the differences in
65 *C. elegans* chemotaxis between urine samples from canine cancer patients and urine samples from
66 healthy dogs with no diagnosed cancer. In the first part of the N.C.S. Study, we acquired initial data
67 used to develop a screening method that identifies increased cancer risk through assays of canine
68 urine samples. The study assesses multiple replicates of plate-based chemotaxis assays to measure
69 the olfactory response through a mean chemotaxis index (CI). Based on these results, a risk
70 assessment is made based on how the index relates to that of previously measured cancer and non-
71 cancer samples. To validate the performance of our method, we assessed its ability to identify
72 increased cancer risk using urine samples from dogs with four common types of canine cancer. In
73 doing this, we demonstrate the potential for accurate, rapid, and non-invasive screening for cancer
74 risk using urine samples from canine veterinary patients.

75 2 Materials and Methods

76 2.1 Canine Urine Samples

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77 An initial set of canine urine samples was obtained from Triangle Veterinary Hospital (Durham, NC),
78 Lake Pine Animal Hospital (Apex, NC), New Light Animal Hospital (Wake Forest, NC), Bull City
79 Veterinary Hospital (Durham, NC), Knightdale Animal Hospital (Knightdale, NC), and from the
80 Ohio State University Center for Clinical and Translational Science. Upon acquisition, urine samples
81 were immediately stored at -20 □C until assays are conducted. Each specimen was aliquoted into 100
82 µL portions to minimize repeat freezing and thawing each time an assay is performed.

83 2.2 Maintenance of *C. elegans*

84 *Caenorhabditis elegans* strain N2 and *Escherichia coli* strain HB101 were obtained from the
85 *Caenorhabditis* Genetics Center (University of Minnesota). *C. elegans* was age-synchronized by
86 standard bleaching protocols and was cultured at 20 □C on nematode growth media (NGM) plates
87 seeded with HB101 bacterial lawns. NGM plates were purchased pre-poured from LabExpress (Ann
88 Arbor, MI).

89 2.3 Chemotaxis Assays

90 Assays are performed using well-fed age-synchronized populations of N2 worms grown at 20 □C for
91 three days, and are conducted on CTX plates (2% Agar, 5 mM KPO4 buffer at pH 6, 1 mM $CaCl_2$, 1
92 mM $MgSO_4$) which were purchased pre-poured from LabExpress (Ann Arbor, MI). Urine samples
93 were thawed and diluted at a ratio of 1:2 of urine to CTX buffer (5 mM KPO4 buffer at pH 6, 1 mM
94 $CaCl_2$, 1 mM $MgSO_4$). Urine samples were centrifuged and were then mixed at a ratio of 2:1 diluted
95 urine to 1 M sodium azide. The urine mixture was then spotted at the “plus” marks on each
96 chemotaxis plate. Control buffer mixture was prepared using a 2:1 ratio of CTX buffer to 1 M
97 sodium azide and was spotted at the “minus” marks on each chemotaxis plate (Supplementary Figure
98 1). Young adult worms were washed from NGM plates using M9 buffer into conical tubes and were
99 allowed to settle. Worms were then washed three times with CTX buffer to remove traces of the
100 bacterial food source. Approximately 75-100 worms were placed at the center of each plate, which
101 were placed in a 23 □C incubator. After one hour, each plate was removed and placed on a backlight,
102 and an image of each plate was acquired using an iPhone X digital camera.

103 2.4 Data Acquisition

104 Data was collected by manually counting the animals in each quadrant using Fiji ImageJ software.
105 Replicates are discarded if one of the three conditions are met: (1) if the total for all four quadrants is
106 less than 55 (2); if the highest total quadrant exceeds the sum of the remaining three quadrants; (3) if
107 the quadrant across from the highest total quadrant has fewer than half the animals of any other
108 quadrant (Supplementary Figure 2). Then, the CI is calculated using the following formula, where Qn
109 is the number of worms in the nth quadrant:

$$CI = \frac{Q1 - Q2 + Q3 - Q4}{Q1 + Q2 + Q3 + Q4}$$

110 A mean CI is calculated from the replicates for each assay that are not discarded.

111 2.5 Statistical Analysis

112 Differences between cancer risk groups were assessed using the Welch t-test for data sets with
113 unequal variance. Thresholds for cancer risk assessment were drawn to optimize the accuracy of the
114 data analysis. 95% confidence intervals were specified based on the following formula

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$$C = t_{95} * \frac{s}{\sqrt{n}}$$

115 Where C is the confidence interval, t_{95} is the t-score, s is the sample standard deviation, and n is the
116 sample size.

117 3 Results

118 3.1 Developing The A.C.D. Test to Distinguish Cancer and Non-cancer Urine Samples

119 We developed the N.C.S. Study based on chemotaxis data that was previously generated showing a
120 slight preference of *C. elegans* for urine samples acquired from cancer patients, as opposed to a slight
121 aversive response to urine from non-cancer patients (20–22). Our first approach was to determine if
122 the procedure previously used for human cancer detection could be applied to canine urine. While
123 performing chemotaxis, we found that individual replicate outliers could cause drastic swings in the
124 calculated mean CI. For this reason, replicates that deviated strongly from the other replicates in the
125 assay were discarded. We chose to discard CI with a difference greater than 0.25 from the closest
126 other replicate within the assay to reduce the distortion from extreme data points on the mean
127 chemotaxis value. Plates with fewer than 55 worms in the four quadrant boundaries were discarded,
128 as plates with fewer worms tended to yield a wider range of CI values, leading to greater distortions
129 in the mean. We also discarded replicates with unusual distributions (Supplementary Figure 2). We
130 defined plates as yielding outlier results when the total number of worms in one quadrant exceeded
131 the total number in all three other quadrants combined, or when the number of worms across from the
132 highest total quadrant is less than 50% the total of any other quadrant. These arrangements indicated
133 migration either towards or away from a particular quadrant rather than a particular chemical
134 stimulus and did not provide reliable data for calculating a mean CI.

135 Through these optimizations, we developed the N.C.S. Study to accurately identify urine samples
136 from canine cancer patients (Figure 1). Previous chemotaxis assays have used anywhere from three to
137 six replicates to determine the mean CI (20–22). For canine urine assays, we often found variance in
138 the response and magnitude in individual replicates within an assay (Supplementary Table 1). We
139 found that it was necessary to acquire at least five non-discarded replicates for one urine test, four
140 replicates if all are positive or negative. From the CI replicates which were not discarded, we
141 calculated the mean CI, which was then used to assess the level of risk.

142 3.2 Determining the Level of Cancer Risk for the A.C.D. Test

143 We performed tests on a series of cancer and non-cancer urine samples to determine if cancer can be
144 detected through positive chemotaxis towards canine urine samples. We initially performed assays on
145 a total of eight cancer samples and fourteen non-cancer samples. We found that *C. elegans* was much
146 more strongly attracted to cancerous urine samples than non-cancer samples (Figure 2A). From these
147 results, we set a threshold for elevated cancer risk determined from a one-way Student t-distribution
148 of the tested non-cancer samples (α=0.005). Mean CI values less than or equal to 0.038 are classified
149 as “low risk”, as that is the range of about 85% of non-cancer samples, while we designated results
150 above the threshold as “moderate to high cancer risk.” Through this approach, we achieved an 88%
151 sensitivity for cancer detection, and a 93% specificity for correctly classifying non-cancer samples
152 (Figure 2B, Supplementary Table 2). We also ran replicates of a cancer and noncancer sample which
153 indicated replicable outcomes of the assay risk classification (Supplementary Table 3). Based on
154 these results, we have shown a preliminary ability to classify the cancer risk of canines through *C.*
155 *elegans* chemotaxis.

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156 **3.3 Assessing Detection Rate of Four Common Canine Cancers**

157 To further determine the accuracy of the NC.S. Study at detecting the presence of cancer, we
158 performed assays on ten samples of each of four different types of cancer that are commonly
159 diagnosed in domestic dogs: lymphoma, mast cell tumor, melanoma, and hemangiosarcoma (24)
160 (Figure 3, Supplementary Tables 4 and 5). We found that all samples yielded a higher mean CI than
161 for non-cancer samples. By combining the data acquired from these forty samples with that in the
162 preliminary data set, we found that the N.C.S. Study yielded a sensitivity of 85% of identifying at
163 least a moderate risk of cancer in each confirmed cancer patient, as compared to the 7% of non-
164 cancer samples identified as at least a moderate cancer risk (Table 1). Overall, by combining all
165 measured CI values for cancer and non-cancer samples, we achieved an accuracy of 87%. Our results
166 also showed a statistically significant difference between the mean CI for each type of cancer and the
167 non-cancer samples.

168 **4 Discussion**

169 In this study, we assessed how the presence of cancer in canines affects the odor of urine samples as
170 perceived by the nematode *C. elegans* and to determine how it compares to the detected odor in
171 human cancer and non-cancer patients. Additionally, while we were able to replicate the significantly
172 more attractive chemotaxis response in cancer-positive canine samples, we observed a mean CI
173 across all non-cancer samples that was approximately zero. Urine is composed of a mix of salts,
174 minerals, hormones, and other chemicals, all of which contribute to the chemotaxis response for a
175 sample (25,26). Thus, the difference in composition between human and canine urine could yield
176 differing mean chemotaxis responses for non-cancer samples.

177 Each of the four types of cancer for which we screened ten samples was detected at a high rate in the
178 N.C.S. Study, with no cancer type having a sensitivity below 70%. We thus observe that risk of
179 multiple types of cancer can be identified through urine chemotaxis assays. We also performed two
180 assays on samples from patients with soft-tissue sarcoma, a type of cancer which is less prevalent but
181 still occurs in some canines. We found that both samples were detected by chemotaxis assays,
182 showing that it is another type of cancer that potentially can be detected at a high rate using urine
183 chemotaxis assays. While the test can identify the presence of cancer at a high rate, the test does not
184 give any information on which type of cancer a patient has developed. Moreover, a positive result in
185 our test does not constitute a cancer diagnosis, but a warning of a risk that cancer may be present at a
186 certain stage in a canine patient. Further diagnosis and monitoring of symptoms is necessary to
187 confirm the presence of cancer and its type. In addition, it has previously been shown that attractive
188 VOCs are present in the urine during both early and late stages of cancer(20,22), so it is likely that in
189 future assays, we can demonstrate the ability to screen for cancer at the earliest stages.

190 The N.C.S. Study yielded performance metrics that fell into the range of accuracies that was
191 previously determined for human cancer samples. The performance metrics were slightly weaker
192 than what was previously measured by Hirotsu et al, who detected several types of cancer from
193 human urine using *C. elegans* chemotaxis with a sensitivity of 96% and a specificity of 95%, yielding
194 an overall accuracy of 95% (20). However, the performance was comparable to that achieved by
195 Lanza et al. through chemotaxis (accuracy of 86%) (21) and exceeded that achieved by Thompson et
196 al (accuracy of 70%) (22). Thus, our results lend evidence to the hypothesis that patterns in cancerous
197 VOC signatures that have been well-studied in humans are comparable with that in canines. Since
198 VOCs have also been detected in urine samples from cancerous mice (23), there is strong evidence
199 that similar methods can be utilized to detect cancer in feline, equine, and other mammalian

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200 veterinary patients. However, further studies are necessary to determine for which mammalian
201 species this test can be applied, and how effective this method is at distinguishing cancer samples
202 from non-cancer samples.

203 While we were able to measure a significant difference in chemotaxis towards cancer and non-cancer
204 urine samples, in some instances we found a high level of variance between individual replicates
205 within an assay. While we have not identified the source of this variation, these results indicate that
206 the locomotion of *C. elegans* is highly random. However, we also observed that cancer samples
207 producing a strongly positive chemotaxis index will have few if any negative replicates. Nonetheless,
208 assays with a wide range of replicate CI values are more likely to produce different results when
209 repeated. For this reason, by achieving a stronger chemosensory attraction towards cancer urine
210 samples, or by reducing the variance of individual replicates, the accuracy and replicability of cancer
211 detection through odor can be improved. It has previously been shown that a response to positive
212 volatile odorants present in cancerous urine samples can be identified through calcium gradients in
213 the *C. elegans* AWC sensory neuron (27). This characteristic calcium gradient is indicative of the
214 presence of an attractive odorant and is strongly distinguishable from the low gradient that has been
215 measured for noncancer samples. Moreover, a much lower noise level is achieved using this method
216 as compared to odor detection through chemotaxis. We hypothesize that by measuring calcium
217 gradients, we could achieve an even higher accuracy of cancer detection from canine urine samples.

218 In recent years, the number of pet owners in the United States and around the world has undergone a
219 steady increase. This, combined with the strong emotional bonds that owners have with their pets,
220 creates a higher demand than ever for treatments to protect pets from life-threatening illnesses.
221 Collection of urine samples is routine and non-invasive, and the test can be conducted accurately at a
222 high rate in a basic laboratory setting. By detecting more attractive urine chemotaxis in multiple
223 common cancer types, we present evidence towards urine odor as an effective method for cancer
224 screening in canines.

225 **5 Conflict of Interest**

226 Authors Chan Namgong and Daniel Midkiff were employed by the company Animal Cancer Dx.
227
228 The authors declare that this study received funding from Animal Cancer Dx. Employees of Animal
229 Cancer Dx had the following involvement with the study: developed the research idea, performed and
230 conducted experiments, and wrote and edited the article.

231 **6 Author Contributions**

232 Daniel Midkiff grew and maintained stocks of *C. elegans* and conducted all chemotaxis experiments
233 and was the primary author of the article. Chan Namgong developed the research idea, assisted in
234 experimental development, and collected urine samples. Myon-Hee Lee assisted in experimental
235 design and setup and contributed to the writing and editing of the article. Jong Hyuk Kim contributed
236 to data interpretation and the writing and editing of the article.

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247 9 Contribution to the Field Statement

248 Cancer is one of the leading causes of death in companion animals and occurs in approximately one
249 quarter of all domestic dogs at some point in their lives. Despite its prevalence, it has been an
250 immense challenge to treat cancer in canines, as it frequently diagnosed after symptoms are apparent.
251 At this advanced stage, the cancer has often spread too far for successful treatment. In this
252 publication, we have demonstrated that cancer in canines can be detected by identifying the presence
253 of volatile compounds which yield a distinguishable odor. This odorant signature can be identified
254 through the response of organisms such as the nematode *Caenorhabditis elegans*. Here, by measuring
255 the positive response of *C. elegans* to volatile odorants in canine urine samples, we were able to
256 assess cancer risk. These results are replicable in four types of cancer that are common in companion
257 dogs. Through these results, we show that odor detection assays can serve as an accurate, rapid, and
258 noninvasive cancer screening method in dogs. By performing more frequent cancer screening
259 throughout a dog's life, cancer can be detected at an earlier stage and increase the odds of successful
260 treatment.

261 10 References

262 1. Fleming JM, Creevy KE, Promislow DEL. Mortality in North American Dogs from 1984 to
263 2004: An Investigation into Age-, Size-, and Breed-Related Causes of Death. *J Vet Intern Med*
264 (2011) **25**:187–198. doi: 10.1111/J.1939-1676.2011.0695.X

265 2. FAQs | Veterinary Cancer Society. http://vetcancersociety.org/pet-owners/faqs/ [Accessed
266 March 16, 2022]

267 3. Sánchez D, Cesarman-Maus G, Amador-Molina A, Lizano M. Oncolytic Viruses for Canine
268 Cancer Treatment. *Cancers 2018, Vol 10, Page 404* (2018) **10**:404. doi:
269 10.3390/CANCERS10110404

270 4. Park JS, Withers SS, Modiano JF, Kent MS, Chen M, Luna JI, et al. Canine cancer
271 immunotherapy studies: Linking mouse and human. *J Immunother Cancer* (2016) **4**:1–11. doi:
272 10.1186/s40425-016-0200-7

273 5. Nytko KJ, Thumser-Henner P, Weyland MS, Scheidegger S, Bley CR. Cell line-specific
274 efficacy of thermoradiotherapy in human and canine cancer cells in vitro. *PLoS One* (2019)
275 **14**:e0216744. doi: 10.1371/JOURNAL.PONE.0216744

276 6. Horise Y, Maeda M, Konishi Y, Okamoto J, Ikuta S, Okamoto Y, et al. Sonodynamic therapy
277 with anticancer micelles and high-intensity focused ultrasound in treatment of canine cancer.
278 *Front Pharmacol* (2019) **10**:545. doi: 10.3389/FPHAR.2019.00545/BIBTEX

279 7. Flory A, Kruglyak KM, Tynan JA, Mclennan LM, Rafalko Id JM, Christian P, et al. Clinical
280 validation of a next-generation sequencing-based multi-cancer early detection "liquid biopsy"
281 blood test in over 1,000 dogs using an independent testing set: The CANcer Detection in Dogs

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282 (CANDiD) study. *PLoS One* (2022) **17**:e0266623. doi: 10.1371/JOURNAL.PONE.0266623

283 8. Corsi AK. A Transparent window into biology: A primer on Caenorhabditis elegans.
284 *WormBook* (2015)1–31. doi: 10.1895/wormbook.1.177.1

285 9. Melkman T, Sengupta P. The worm's sense of smell: Development of functional diversity in
286 the chemosensory system of Caenorhabditis elegans. *Dev Biol* (2004) **265**:302–319. doi:
287 10.1016/J.YDBIO.2003.07.005

288 10. Bargmann CI. Chemosensation in C. elegans. *WormBook* (2006)1–29. doi:
289 10.1895/WORMBOOK.1.123.1

290 11. Spehr M, Munger SD. Olfactory receptors: G protein-coupled receptors and beyond. *J*
291 *Neurochem* (2009) **109**:1570–1583. doi: 10.1111/J.1471-4159.2009.06085.X

292 12. Hirotsu Bio Science Inc. | HIROTSUバイオサイエンス. https://hbio.jp/en/srv/nnose3
293 [Accessed December 31, 2021]

294 13. Opitz P, Herbarth O. The volatilome - Investigation of volatile organic metabolites (VOM) as
295 potential tumor markers in patients with head and neck squamous cell carcinoma (HNSCC). *J*
296 *Otolaryngol - Head Neck Surg* (2018) **47**:1–13. doi: 10.1186/S40463-018-0288-5/FIGURES/7

297 14. Gordon SM, Szidon JP, Krotoszynski BK, Gibbons RD, O'Neill HJ. Volatile organic
298 compounds in exhaled air from patients with lung cancer. *Clin Chem* (1985) **31**:1278–1282.
299 doi: 10.1093/CLINCHEM/31.8.1278

300 15. Heiden MGV, Cantley LC, Thompson CB. Understanding the warburg effect: The metabolic
301 requirements of cell proliferation. *Science (80-)* (2009) **324**:1029–1033. doi:
302 10.1126/SCIENCE.1160809

303 16. Phillips M, Cataneo RN, Ditkoff BA, Fisher P, Greenberg J, Gunawardena R, Kwon CS,
304 Tietje O, Wong C. Prediction of breast cancer using volatile biomarkers in the breath. *Breast*
305 *Cancer Res Treat* (2006) **99**:19–21. doi: 10.1007/S10549-006-9176-1

306 17. Silva CL, Passos M, Cmara JS. Investigation of urinary volatile organic metabolites as
307 potential cancer biomarkers by solid-phase microextraction in combination with gas
308 chromatography-mass spectrometry. *Br J Cancer* (2011) **105**:1894–1904. doi:
309 10.1038/BJC.2011.437

310 18. McCulloch M, Jezierski T, Broffman M, Hubbard A, Turner K, Janecki T. Diagnostic
311 accuracy of canine scent detection in early- and late-stage lung and breast cancers. *Integr*
312 *Cancer Ther* (2006) **5**:30–39. doi: 10.1177/1534735405285096

313 19. Matsumura K, Opiekun M, Oka H, Vachani A, Albelda SM, Yamazaki K, Beauchamp GK.
314 Urinary Volatile Compounds as Biomarkers for Lung Cancer: A Proof of Principle Study
315 Using Odor Signatures in Mouse Models of Lung Cancer. *PLoS One* (2010) **5**:e8819. doi:
316 10.1371/JOURNAL.PONE.0008819

317 20. Hirotsu T, Sonoda H, Uozumi T, Shinden Y, Mimori K, Maehara Y, Ueda N, Hamakawa M.
318 A Highly Accurate Inclusive Cancer Screening Test Using Caenorhabditis elegans Scent
319 Detection. *PLoS One* (2015) **10**:e0118699. doi: 10.1371/JOURNAL.PONE.0118699

320 21. Lanza E, Rocco M Di, Schwartz S, Caprini D, Milanetti E, Ferrarese G, et al. C. elegans-based
321 chemosensation strategy for the early detection of cancer metabolites in urine samples. *Sci*

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322 *Reports 2021 111* (2021) **11**:1–16. doi: 10.1038/s41598-021-96613-z

323 22. Thompson M, Feria NS, Yoshioka A, Tu E, Civitci F, Estes S, Wagner JT. A Caenorhabditis
324 elegans behavioral assay distinguishes early stage prostate cancer patient urine from controls.
325 *Biol Open* (2021) **10**: doi: 10.1242/BIO.057398

326 23. Ueda Y, Kawamoto K, Konno M, Noguchi K, Kaifuchi S, Satoh T, et al. Application of C.
327 elegans cancer screening test for the detection of pancreatic tumor in genetically engineered
328 mice. *Oncotarget* (2019) **10**:5412. doi: 10.18632/ONCOTARGET.27124

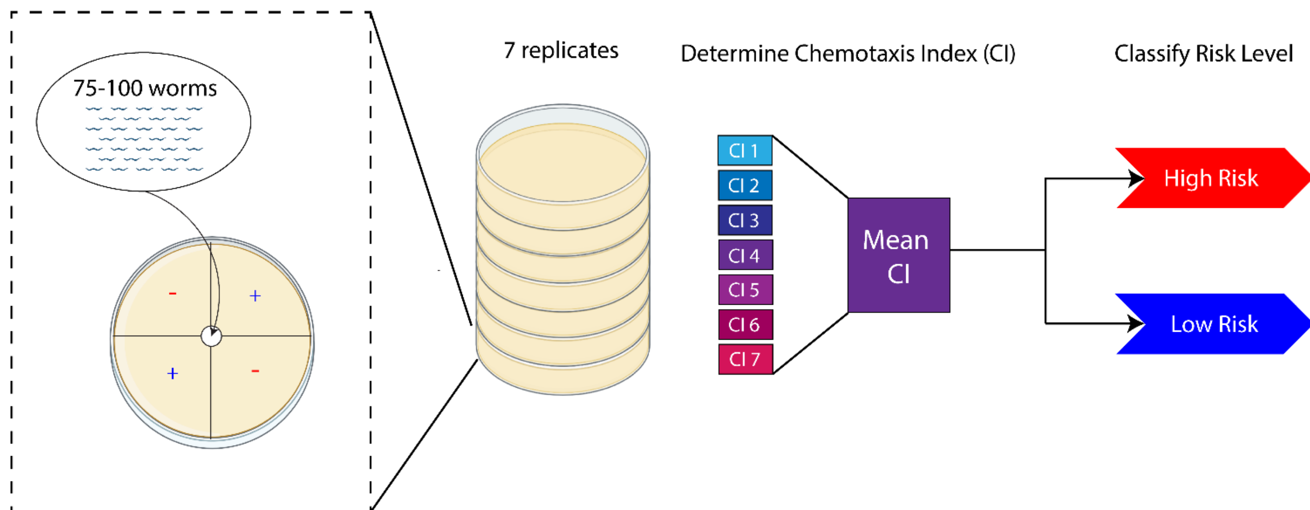
329 24. Common Cancers In Dogs - Flint Animal Cancer Center.
330 https://www.csuanimalcancercenter.org/2019/11/14/common-cancers-in-dogs/ [Accessed
331 March 31, 2022]

332 25. Stevenson AE, Markwell PJ. Comparison of urine composition of healthy Labrador retrievers
333 and miniature schnauzers. *Am J Vet Res* (2001) **62**:1782–1786. doi:
334 10.2460/AJVR.2001.62.1782

335 26. Bouatra S, Aziat F, Mandal R, Guo AC, Wilson MR, Knox C, et al. The Human Urine
336 Metabolome. *PLoS One* (2013) **8**:e73076. doi: 10.1371/JOURNAL.PONE.0073076

337 27. Lanza E, Rocco M Di, Schwartz S, Caprini D, Milanetti E, Ferrarese G, et al. C. elegans-based
338 chemosensation strategy for the early detection of cancer metabolites in urine samples.

339 11 Figures



340

341 **Figure 1**: The A.C.D. Test is conducted by placing 75-100 worms on each assay plate. A total of
342 seven assay replicates are conducted, from which a mean CI is calculated and used to assess the level
343 of cancer risk.

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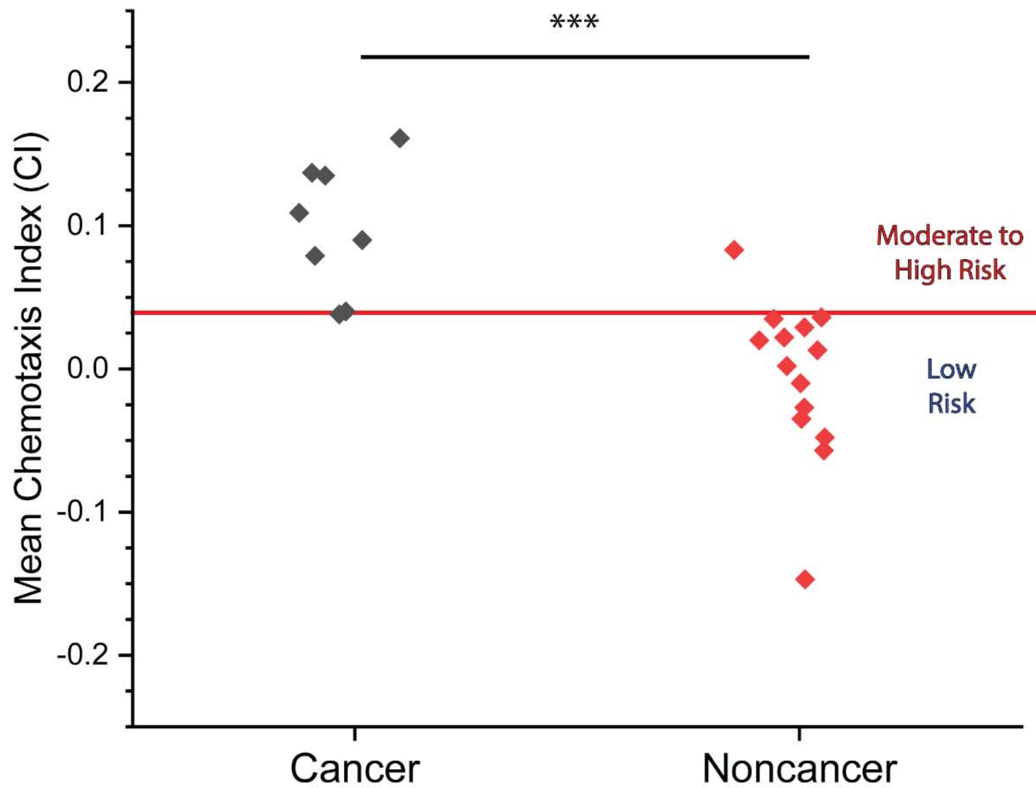

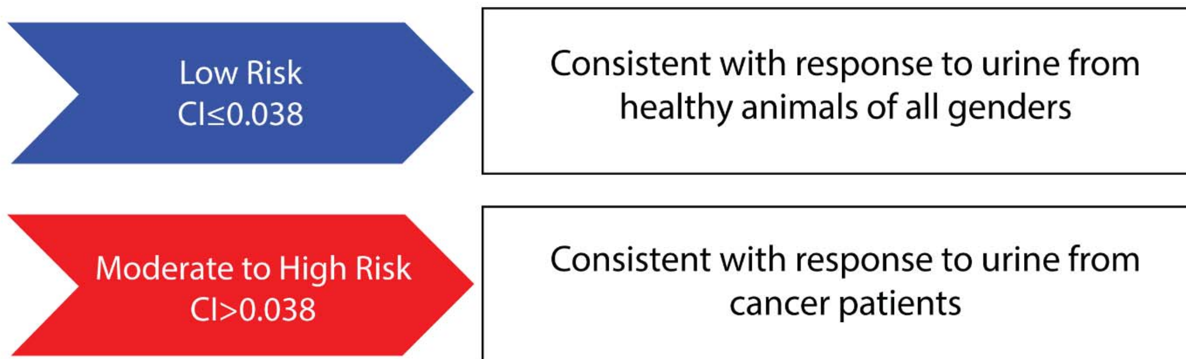

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345 **Figure 2:** (A) Mean CI plotted for eight cancer and 14 non-cancer samples for which the A.C.D. Test
346 was conducted. A mean CI of 0.099±0.038 for cancer samples versus a mean CI of -0.006±0.032 in
347 non-cancer samples (p=0.0002) Red line indicates moderate to high cancer risk classification
348 threshold. ***p<0.001 (B) Levels of cancer risk set at the following range: Low Risk (<0.038) and
349 Moderate to High Risk (>0.038)

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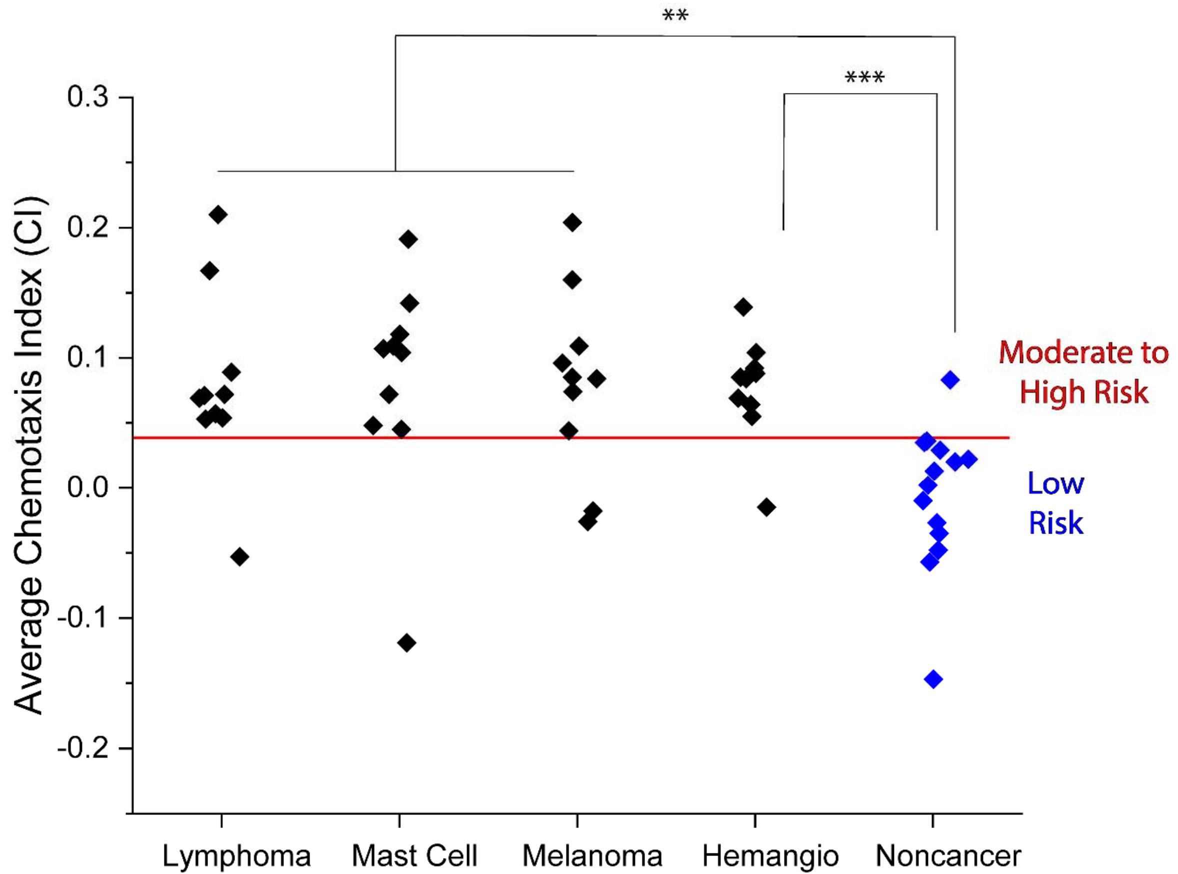

350

351 **Figure 3**: Average CI for urine samples obtained from lymphoma, mast cell tumor, melanoma, and
352 hemangiosarcoma patients as compared to non-cancer samples. Lymphoma: 0.079±0.050, mast cell
353 tumor: 0.081±0.059, melanoma: 0.081±0.051, and hemangiosarcoma: 0.077±0.028 versus non-
354 cancer: -0.006±0.032). Red line indicates moderate to high cancer risk classification threshold.
355 **p<0.01, ***p<0.001

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364 **12 Tables**

365 **Table 1:** Data summary for each classification of cancer versus non-cancer urine samples.

Type	Sample Size	Detected	Detection Rate
Mast Cell tumors	13	12	92%
Lymphoma	11	10	91%
Melanoma	11	8	73%
Hemangiosarcoma	11	9	82%
Soft tissue sarcoma	2	2	100%
Total	48	41	85%
Non-cancer	14	13	93%

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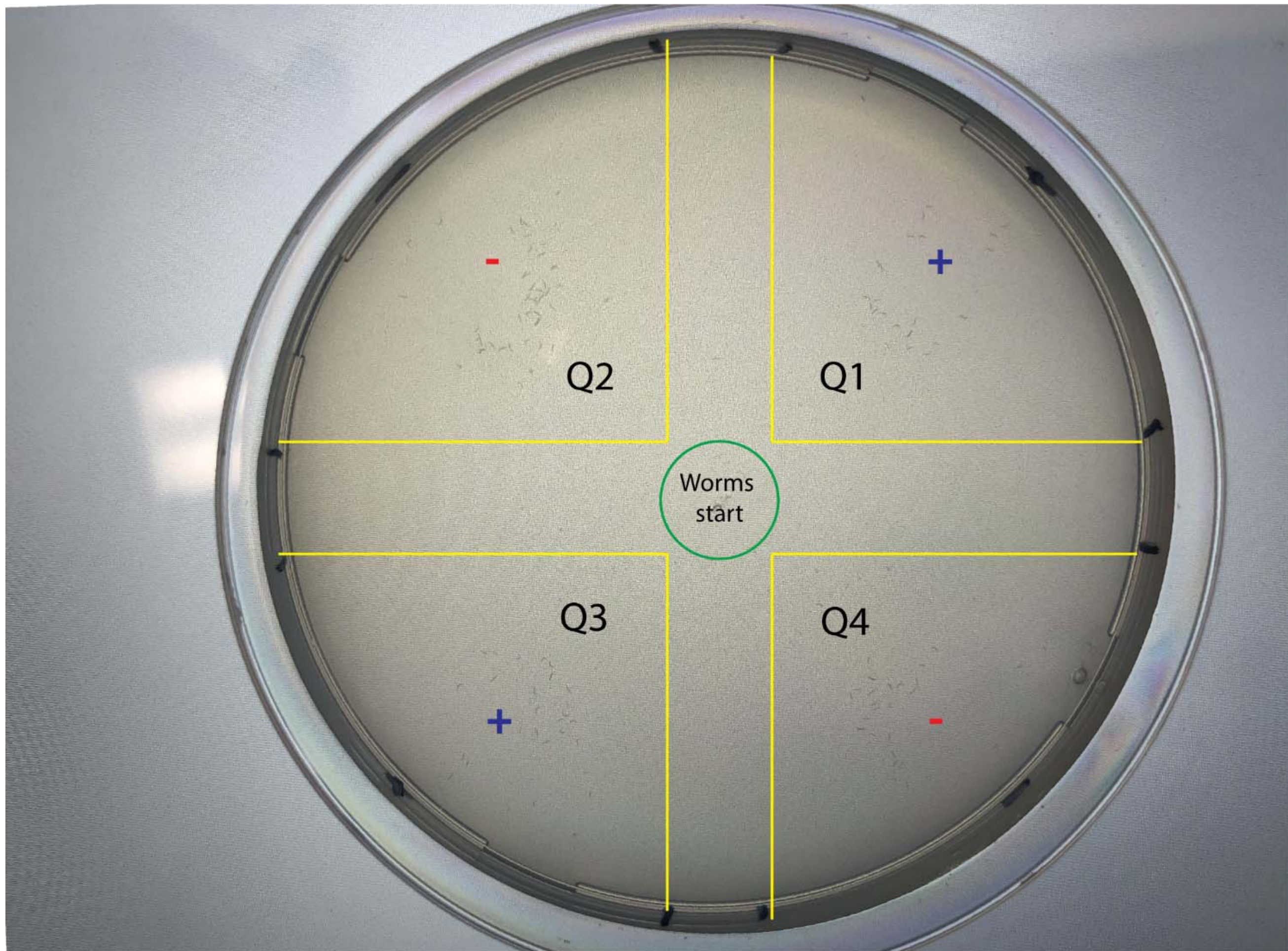
Supplementary Material

366 **1 Supplementary Figures**



367

368 **Supplementary Figure 1.** Each assay plate is divided into four quadrants. The positive quadrants
369 (Q1, Q3) are marked with a "plus" sign, which designates the location of the tested urine sample. The
370 negative quadrants are marked with a "minus" sign, which designates the location of the control
371 buffer. *C. elegans* are placed at the center of the green circle at the beginning of the assay. After the
372 assay is complete, animals are counted in each quadrant within the bounds of the yellow lines to
373 avoid counting those that are close to the quadrant borders or that remain at the center of the plate.

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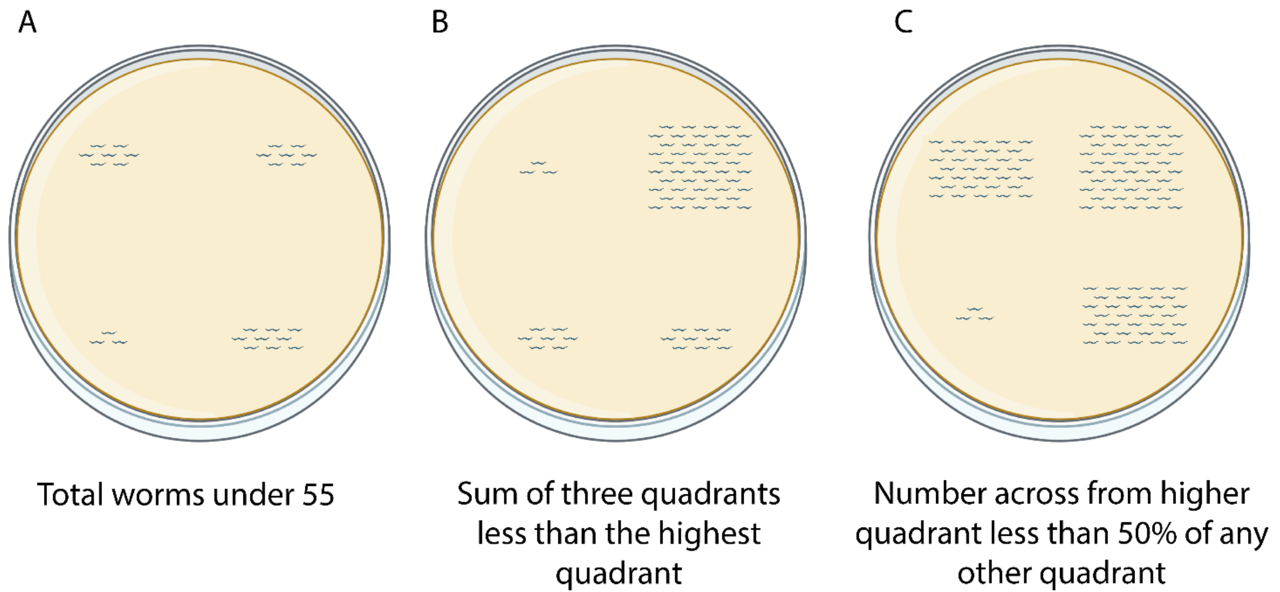

374

375 **Supplementary Figure 2.** Examples of conditions in which plates are discarded. (A) The total
376 number of animals on the plate is less than 55:

$$Q1 + Q2 + Q3 + Q4 \leq 55$$

377 (B) The sum of the total animals in three quadrants is less than the total number of animals in the
378 fourth quadrant, e.g.

$$Q1 \geq Q2 + Q3 + Q4$$

379 © The number of animals across from the quadrant with the highest total has 50% or less than the
380 number of animals on any other quadrant, e.g.

$$Q1 \geq Q2|Q3|Q4, Q3 \leq \frac{Q1}{2}\left|\frac{Q2}{2}\right|\frac{Q4}{2}$$

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390 ## 2 Supplementary Tables

391 **Supplementary Table 1.** CI replicates for initial set of cancer and non-cancer assays. The CI for
392 each non-discarded replicate is listed beneath the name of each cancer and non-cancer patient.

393 **Supplementary Table 2.** Chemotaxis data summary for initial cancer and non-cancer data
394 comparison.

395 **Supplementary Table 3.** Sample assay repeats for dog patients with (P03) and without (P09)
396 diagnosed cancer. Each of the assay repeats shows a mean CI above and below the moderate risk
397 threshold for cancer and non-cancer patients, respectively.

	P03 (Mast Cell Tumor)			P09 (Non-cancer)		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
Replicate 1	0.123	0.223	0.109	-0.159	-0.051	0.294
Replicate 2	0.233	0.278	0.052	-0.153	0.163	-0.294
Replicate 3	0.12	-0.042	0.045	-0.167	-0.136	-0.127
Replicate 4	0.174	0.286	0.071	0.031	0.164	0.147
Replicate 5	0.102	0.147	0.211	0.096	-0.31	-0.143
Replicate 6	0.082		0.027	0.056		
Replicate 7	0.109			0.053		
Mean CI	0.135	0.178	0.086	-0.035	-0.034	-0.025

398

399 **Supplementary Table 4.** CI replicates for ten samples of lymphoma, mast cell tumor, melanoma,
400 and hemangiosarcoma. The CI for each non-discarded replicate is listed beneath the name of each
401 cancer and non-cancer patient.

402 **Supplementary Table 5.** Chemotaxis data summary for ten samples of lymphoma, mast cell tumor,
403 melanoma, and hemangiosarcoma.

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