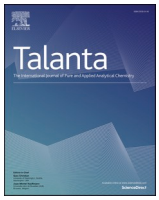
Talanta 250 (2022) 123729

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta



Canine discrimination of ovarian cancer through volatile organic compounds

S.A. Kane [a,1], Y.E. Lee [b,1], J.L. Essler [a], A. Mallikarjun [a], G. Preti [b], V.L. Plymouth [a], A. Verta [a], A. DeAngelo [a], C.M. Otto [a,*]

[a] University of Pennsylvania School of Veterinary Medicine, Penn Vet Working Dog Center, USA
[b] Monell Chemical Senses Center, Philadelphia, USA

ARTICLE INFO

Keywords:
Ovarian cancer
VOCs
Detection dogs
Biomarkers
MP-GC

ABSTRACT

Ovarian cancer has a high mortality rate due to its unclear symptomology and the lack of precise early detection tools. If detected in the first stage, over 90% of patients reach remission. As such, developing a reliable method of early detection is crucial in reducing the mortality rate of the disease. One potential method would be to identify specific biomarkers that are unique to ovarian cancer, which could be detected using a blood test. While this can be done using gas chromatography – mass spectrometry (GC–MS), identifying these biomarkers is an enormous task. One way to expedite the process is to utilize trained scent detection canines. In this study, dogs who were previously trained to respond to positive blood samples from ovarian cancer patients were then tested on their ability to recognize samples prepared by micro-preparative gas chromatography (MP–GC) techniques. MP–GC employed a gradient-cooled glass tube connected to the GC outlet to collect GC eluents containing the plasma-derived volatiles in positive blood samples. These post-column fractions were collected at the exit of the GC according to their eluent times (i.e., 0–15 min, 15–25 min and 25–35 min or 0–35 min) and these full or fractional collections were presented to the trained dogs to judge their responses.

Dogs' time spent investigating the odor was used as an indication of odor recognition and was significantly longer on the early (0–15 min) and middle (15–25 min) fractions of the ovarian cancer than the late (25–35 min) fraction of plasma odorants or either the negative fractions or distractors odorants. These findings suggest that characteristic odor biomarkers of ovarian cancer for dogs may exist in the relatively small and more volatile compounds. Additionally, variation between dogs suggests that there may be a number of different biomarkers that can be used to identify ovarian cancer.

1. Introduction

Epithelial ovarian cancer is the 7th most common form of cancer and the 8th leading cause of death for women worldwide [1]. Despite its high rate of occurrence, there continues to be a lack of effective early screening methods [2,3]. Often, early stages of ovarian cancer have negligible symptoms [4] and advanced stages are characterized by symptoms shared with other common gastrointestinal and gynecological health conditions [3]. The dearth of symptoms, or of unique symptoms, makes an early clinical diagnosis difficult, which is critical to increase a patient's survival [4]. If detected during stage I, when the cancer is still contained in the ovaries, conventional surgical procedures and chemotherapy allow 90% of patients in this stage to achieve remission [5]. The most common and aggressive subtype of ovarian cancer is high-grade serous carcinoma [3], which when diagnosed in an early stage improves a patient's prognosis of a 10-year survival to 55% in comparison to 15% for those diagnosed with an advanced form of this disease [1]. Unfortunately, only 20% of ovarian cancer cases are detected in the initial stage underscoring the lack of sensitive early screening methods [2,3,5]. Detecting ovarian cancer, especially during the early stages is critical for patient survival, thus finding an accurate early screening test is crucial to ensure long term survival.

Current screening methods, including the transvaginal ultrasound (TVUS) and the Serum Cancer Antigen 125 (CA125) blood test [3,6], are

Abbreviations: VOC, volatile organic compound; MP-GC, micro-preparative gas chromatography.

* Corresponding author.
E-mail address: cmotto@upenn.edu (C.M. Otto).

[1] These authors share co-first authorship.

https://doi.org/10.1016/j.talanta.2022.123729
Received 19 April 2022; Received in revised form 1 July 2022; Accepted 5 July 2022
Available online 11 July 2022

effective at detecting ovarian masses but cannot distinguish between malignant and benign masses [2]. As a blood test, the CA125 test is far less invasive than the TVUS test [6] but unfortunately, CA125 is only expressed in 80% of ovarian cancers [6]. Neither of the current screening methods for ovarian cancer are sensitive or specific enough to predictably and correctly identify all cases of ovarian cancer [2,6]. It is possible that other biomarkers unique to ovarian cancer patients could be used for screening, but identifying such biomarkers is a massive undertaking. Gas chromatography and mass spectrometry (GC/MS) can be employed to help identify volatile biomarkers; however, without narrowing the scope of where to look for biomarkers, the breadth of this task is overwhelming [7]. Volatile biomarkers, or volatile organic compounds (VOCs) are byproducts of metabolic processes, inflammatory processes, and oxidative reactions, and can provide a unique olfactory fingerprint of a disease [8]. VOCs are released from the body via breath, urine, feces, skin and blood [8,9] and provide a unique profile for diseases which dogs can detect [10–12].

One potential way to narrow down the volatile biomarkers unique to ovarian cancer is to employ the superior olfactory abilities of canines paired with micro-preparative gas chromatography (MP–GC) methodology. The canine olfactory system can detect odors at concentrations of parts per trillion [9]. Dogs have been documented to detect diseases, including ovarian cancer [12,13,16], colorectal cancer [14], lung cancer [10] and prostate cancer [11]. Dogs, as shown by their ability to specifically respond to samples from patients with these diseases, must be using an olfactory cue emanating from the disease, or the bodies' response to the disease, that can be found in the samples. The use of MP-GC can separate sets of VOCs such that dogs can identify particular sets that contain a potentially meaningful biomarker for a disease.

Dogs can effectively detect ovarian cancer and discriminate between malignant and benign ovarian tissues and blood samples [12,13,16]; this suggests that dogs can be utilized along with MP-GC to narrow down potential biomarkers for ovarian cancer. While the sample size in Horvath et al. was limited, this study does provide a 'proof of concept' that some dogs can be trained to detect ovarian cancer. The theory behind Horvath et al.'s findings suggested that the dogs could identify a unique odor 'fingerprint' of the disease; this olfactory profile is likely made up of VOCs. In a more recent study, dogs were found to have a 97% sensitivity and 99% specificity in detecting ovarian cancer positive blood samples [15]. Additionally, dogs can discriminate between ovarian positive blood and tissue samples and healthy controls with a mean proportion of successes (number of correct trials/number of trials) of over 95% [16]. Significantly, this later study found that dogs could discriminate blood and tissue cancer positive samples equally well, indicating that sample type did not hinder detection.

In addition to previous studies showing that dogs can detect ovarian cancer, gas chromatography have been utilized to identify profile VOCs found in cancer samples. VOC biomarkers of gastric cancer [17], lung cancer [10], and colorectal cancer [18]. have been successfully identified. In a GC–MS study of VOCs in ovarian cancer, five potential biomarkers were identified, and together they had a sensitivity of 79% [19]. However, in Hovarth et al., canines have a sensitivity greater than 90%, indicating that dogs are most likely using other biomarkers to identify malignancies [7,12].

The goal of the current study was to pair MP–GC methodology with canine olfaction to identify possible biomarkers of ovarian cancer. MP–GC was used to separate and collect the VOCs from ovarian cancer, and control (non-malignant) plasma samples into different fractions, which were presented to canines trained to discriminate between ovarian cancer samples and benign and normal samples in plasma. The dogs' response to these different malignant cancer fractions was recorded. Canine interest was assumed to represent how much that particular fraction identified with the target odor of ovarian cancer, and thus may contain a biomarker of interest.

2. Methods

This study was approved by the University of Pennsylvania Animal Care and Use Committee, Protocol # 804900.

Five (5) detection canines (3 German Shepherds, 1 Dutch Shepherd and 1 Labrador Retriever) (average age: 39.6 months) from working dog lines were employed in this study. All 5 dogs had previously been trained to discriminate between plasma samples from patients with malignant ovarian cancer, and those with benign growths or no known growths (identified as control patients). These canines participated in over 270 ovarian cancer detection training sessions in 2019, achieving a sensitivity rate of 85% and a specificity rate of 77%. The dogs had been in training to detect ovarian cancer for an average of 17.2 months.

2.1. Blood plasma sample acquisition

The blood plasma from malignant ovarian cancer patients as well as the benign ovarian tumor patients were prepared accordingly [16]. Samples were collected in standard EDTA anticoagulant tubes, spun at 3000 rpm for 10 min at 4 °C and then aliquoted into glass test tubes and frozen at −80 °C before distributed for MP-GC collection and canine detection. For the dogs, samples were further split into 50 μL aliquots dispensed into glass jars, labeled, sealed, and frozen at −80 °C. The plasma samples of non-smoking female control individuals, or those with no known case of ovarian cancer, were also collected and processed to serve as negative controls.

Blood plasma samples (50 μL) were used for the training of the five (5) detection canines. The samples were contained in SKS 1 oz, glass jars (item #4021-01). These samples each contained just one individuals' plasma, or as a pool of up to 6 patients' plasma. All pools contained equal parts blood plasma from each individual. All pooled samples only contained individuals of the same disease status; control, benign or malignant group. Samples were pooled to create novel sample mixes for the dogs to smell, as the study was limited by sample availability. The dogs were never presented with the same sample, or pooled sample twice, however they may have been exposed to one individual's plasma in different pools multiple times. By pooling samples, the intent was that the dogs would not learn an individual's odor, but rather the odor of the disease that was consistent throughout the samples, similar to what was published previously [16]. The samples were presented at very low volumes so the dogs would be able to discriminate odors at very low concentrations, as would be needed for the testing when only VOCs were presented rather than liquid plasma.

During training sessions dogs were presented with up to 2 novel samples, or novel pools of samples of ovarian cancer plasma, and between 2 and 3 novel samples or novel pools of benign and normal plasma samples. Samples were randomly selected based on availability rather than demographic history of the patient. Once samples were pooled, they were stored in a SKS 1 oz, glass jars (item #4021-01) in the −80 °C freezer until thawed for training use. In addition to the blood plasma samples the dogs were presented with one (1) distractor a session. These 'distractors' were lab objects that interacted with the plasma samples such as nitrile gloves, paper towels, vinyl gloves, and 80% isopropyl alcohol.

2.2. Solid-phase micro-extraction collection and validation

Samples of healthy control and malignant plasma were subjected to MP–GC collection at Monell Chemical Senses Center on the same day of canine testing. VOCs from the pooled plasma were first extracted by solid-phase microextraction (SPME) using a modification of a previously described method [26]. 250 μL of a pooled plasma, a stirring bar and 80 mg of NaCl were placed in a 4 mL glass vial. The vial was capped with a silicon/PTFE septa cap and placed in a water bath for equilibration at 37 °C while stirring for 1 h after which time a 1-h SPME collection was made using a 2 cm divinylbenzene (50

μm)/Carboxen/polydimethylsiloxane (30 μm) "Stableflex" solid-phase microextraction (SPME) fiber (Supelco Inc., Bellefonte, PA). These optimal conditions involved adsorption by the SPME fiber and following desorption at the GC were used for all subsequent experiments for the sample preparations. Chromatographic analyses were conducted on a Thermo Scientific Trace gas chromatograph (GC) and single-quadrapole (ISQ) mass spectrometer (MS) (Waltham, MA) equipped with a 30 m × 0.32 mm Stabilwax (1.0 μm film) column (Restek Corp., Bellfonte, PA). The volatile compounds were determined in five duplicates under repeatable conditions with a pooled plasma sample. Each sample was represented by a GC–MS total ion current chromatogram (TIC), and peaks at more than 3 times the signal-to-noise ratio were detected. Each calculate peak area value was recorded for the method validation. Mass spectral peak identifications were assigned based on the library search of the NIST Standard Reference Database and retention time comparisons of commercially available standards. The relative standard deviation (RSD) value was calculated.

2.3. MP–GC fraction preparation

For sample collections, the SPME fiber was immediately transferred to the GC for desorption, separation and fraction collection. The adsorbed compounds on the fiber were desorbed for 2 min at the 230 °C of the injection port. The splitless mode was used. The oven temperature was held for 2 min at 60 °C and then programmed at 5.0 °C/min to 230 °C with a 2-min hold at this final temperature. Helium carrier gas was used at a constant flow of 1.5 mL/min. The total run time was 35 min. The outlet of GC was maintained at 260 °C. A capillary glass tube (3 mm × 2 mm x 12″) was connected to this outlet and 2.5″ × 2.5″ of a pre-cut glass wool was placed to the end of this glass tube for a minimal loss of VOCs. MP–GC fractions were collected in the dry-ice-cooled capillary tube at the exit of the GC. Three separate capillary tubes were used for each collection of early (0–15 min), middle (15–25 min), and late (25–35 min) fractions. A whole fraction (0–35 min) of sample was also prepared in the same manner. Following collection, the glass capillary tube was cut into several pieces, placed in a glass jar and covered with the glass wool for canine testing.

2.3.1. Canine training

To prepare dogs to smell the VOCs of ovarian cancer, each dog was trained to discriminate between blood plasma from patients with malignant ovarian tumors, from individuals with benign tumors, and those with no known ovarian tumors (controls). Using an odor detection wheel (Fig. 1) with only one wheel spoke (a single port) dogs were trained to smell the port at the end of the spoke and indicate if the plasma in that port was malignant (perform a trained final response by standing and staring at the port for greater than 2 s) or a benign or normal sample (leaving the sample and sitting on a platform). This odor detection wheel was set up behind a visual barrier, described later, so that the dog could not get visual cues from handler or researcher turning testing or training settings.

If the dog correctly identified a malignant plasma sample and correctly performed their final response (a "true positive") they were marked by a handler and rewarded. Likewise, if the dog left a sample that was a control or benign sample and sat on the platform (a "true negative"), they were marked and rewarded. If a dog showed a trained final response at a non-malignant sample (a "false positive") they were ignored until they left the sample and got onto the platform at which point they were marked and rewarded. If the dog left a malignant plasma sample (a "false negative") the dog was sent back in to smell the odor again. On the third attempt, if the dog still had not indicated on the sample the handler marked the dog as soon as they put their nose on the sample. One dog on this study was rewarded with a toy (ball), while all other dogs were rewarded with food.

One dog was trained to use a no reward marker, a simple buzzer, which was pressed if the dog was performing the incorrect behavior for one odor, either leaving a positive odor or indicating on a negative odor. The no reward marker was employed to help this dog identify what part of the behavior chain, walking in smelling the sample, and making a decision to alert or leave, was incorrect. This no reward marker was not used in the testing scenario.

Each dog had been imprinting on the ovarian cancer malignant plasma samples at least 6 months prior to the start of this study. Once testing sessions started, dogs continued doing training sessions. Dogs did at least 2 training sessions on plasma between each testing session to reinforce their odor discrimination abilities.

2.3.2. Session layout

Each training session consisted of 10–15 trials, in which between 40 and 60% of the trials did not contain the malignant plasma odor (blank trials). Over the course of four training sessions, 50% of the trials were blank trials. By including these blank trials, the researchers ensured that this training did not present a "forced choice", whereby the dog always believed the sample was present and might guess. The placement of these blank trials was random throughout the session. Fifty percent of the sessions would start with a blank trial. This measure was to prevent dogs from guessing the identity of the odor in the first trial without smelling it, based on their experience in previous sessions. The sample presentation order was randomly selected via a random list generator (random.org).

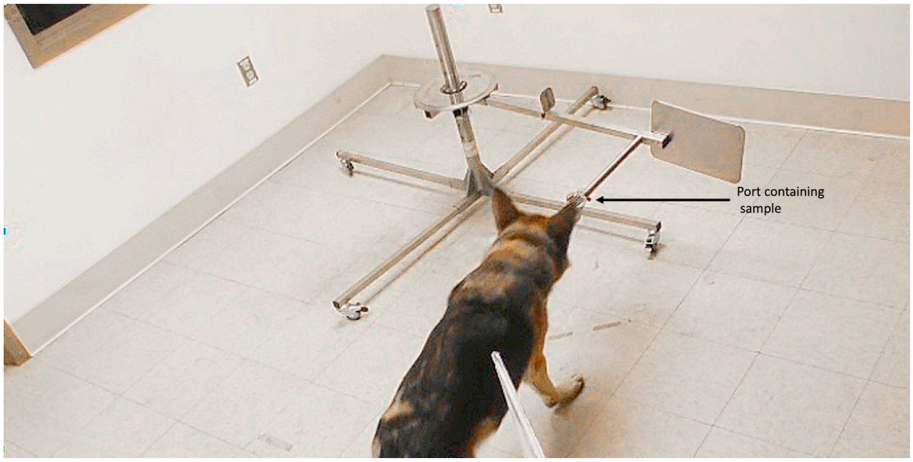


Fig. 1. A photo of the single port wheel set-up. One of the dogs, Osa, is checking the port during a trial.

2.3.3. Room layout

The scent wheel used for training was in a separate room, blocked by a wall and a desk, so the dog could not see the researcher or their handler as they worked. A video feed via Google Hangouts (https://hangouts.google.com) showed a live stream from this room to the researcher's computer so the handler and researcher could watch the dog's performance. In some sessions the handler was blind to the identity of the sample their dog was going to smell, in which case the researcher would indicate if the dog was correct by a thumbs up/thumbs down gesture. In other sessions the handler was told the identity of the sample before the dog was sent into work. All sessions were recorded on a Panasonic camera (HC-V380).

2.3.4. Canine testing with MP–GC samples

Dogs were presented with the MP–GC samples every 2 to 3 training sessions. Every other testing session contained a full VOCs from MP–GC, meaning a full malignant odor run all the way throughout the GC. In these full malignant odor sessions, the dogs were marked and rewarded for performing their trained final response on the malignant sample. For partial MP–GC samples dogs were not rewarded for their response to the odor, other than through praise, to not bias their preference for any partial sample. These partial sample test scenarios were only performed once every two weeks. During both testing scenarios the dog was rewarded for correctly performing a blank behavior, leaving the sample, if the sample was a control, or distractor sample, similar to training protocols.

Partial MP–GC malignant test scenarios only contained 4–5 trials. The dogs were presented with 2 distractors, a blank glass jar from the Monell lab, and a blank capillary tube, and one control sample (plasma from a healthy patient), run through the MP–GC to contain the same fractions as the malignant odor on that day. One of the first three trials contained the malignant odor, if the dog held a trained final response on this odor for more than 2 s they were recalled (directed to return to the handler), however if they passed the sample, they were called out before they could perform their blank response of sitting on the platform. These premature recalls were used in hopes of not giving the dog any information regarding the samples' identity. If the dog were allowed to naturally leave the partial malignant sample after performing a trained final response, it is possible the dog could think that the lack of mark and reward would mean they were incorrect, and so would be less likely to alert on that sample, or a similar partial sample, in the future. If the dog were allowed to do a full blank response and sit on the platform and then called out, they could interpret that response as indicating that the sample was malignant. In short, to avoid cueing the dog recalls were employed. If a dog initially passed the malignant partial fraction, they were sent back to smell it again in a final 5th trial, at which time again they were called out before they could do a trained final response for over 2 s, or perform a full blank behavior. Handlers were not blinded to the identity of the sample the dog was going to smell.

2.3.5. Video coding

All videos of the testing sessions and the 10 previous training sessions for each dog, were analyzed through behavioral coding. While some of these dogs had been working to detect ovarian cancer for several years prior to the start of this project, each dog only had the 10 previous training sessions analyzed to show their level of accuracy prior to the study. Independent coders recorded the length of time each dog spent at port using the free video coding program BORIS [20]. Coders also recorded other port directed behavior, such as licking and pawing at the port.

An inter-rater analysis (ICC) was run in R to determine the reliability of the coders for 20% of all videos recorded, based on a single-rating, consistency, one-way mixed effects model [21]. This model shows that the ICC = 0.844 (95% CI = 0.76 to 0.9), which is considered good reliability [22].

3. Results

This study aimed to identify potential biomarkers of ovarian cancer by presenting different odor fractions prepared by MP–GC to canines trained to detect ovarian cancer. If one specific sample, representing a specific fraction of odor, is more readily identified by the detection canines compared with other sample fractions, it would suggest that there is a unique ovarian cancer biomarker.

4. Method validation

Once the SPME parameters were optimized, the method was evaluated to ensure a level of agreement according to the US FDA guidelines for bioanalytical method validation. A pooled blood sample from 5 ovarian cancer patients was analyzed in order to validate our methodology. The representative VOCs were identified and the RSD values were calculated, as listed in supplementary data Table 1. The RSD values in most of the compounds presented were $< 15\%$ indicating a good level of precision for these SPME–GC methods.

4.1. Control VOC profile compared to malignant VOC profile

The first analysis examined whether there was a difference between the duration of time the dogs spent at the odor containing port for control (healthy) VOC samples as compared to distractors, a full VOC ovarian cancer odor profile, a 0–15 min MP–GC fraction, a 15–25 min MP–GC fraction, and a 25–35 min MP–GC fraction. A linear mixed-effect model generated using the lme() function in R was used for this analysis [23]. Predictor variables included Sample Type (negative plasma as baseline; distractor, Full Cancer, 0–15, 15–25, 25–35). A random intercept of Dog was included.

The duration of time dogs spent at healthy control VOC and distractor items was not significantly different, $\beta_1 = -0.053$, $t(223) = -0.209$, $p = 0.835$.

Dogs spent significantly more time at the complete malignant VOCs of MP–GC fraction (the sample containing all of the VOCs in the malignant sample) than the healthy control VOCs sample, $\beta_1 = 1.056$, $t(223) = 2.735$, $p = 0.007$. This is in line with prior results demonstrating that dogs can differentiate between control samples and malignant samples.

The healthy control VOCs sample was also compared to all three MP–GC fractions. Dogs spent significantly more time at the 0–15 min fraction than the control sample ($\beta_1 = 1.264$, $t(223) = 2.788$, $p = 0.006$), and significantly more time at the 15–25 min fraction than the control sample ($\beta_1 = 1.552$, $t(223) = 2.9$, $p = 0.004$). Interestingly, dogs did not spend significantly more time at the 25–35 min fraction than the control sample, $\beta_1 = 0.718$, $t(223) = 1.58$, $p = 0.115$. This suggests that important volatile markers may be found within the 0–15 min range and 15–25 min range, but not the 25–35 min range (see Fig. 2).

4.1.1. Comparing responses to cancerous VOC profile

A post-hoc Tukey test for comparing a family of four estimates was done using the emmeans() package in R to examine whether dogs spent significantly more time at any one MP-GC fraction than other samples (see Supplementary Table 2). The test shows that there are no significant differences in the amount of time dogs overall spent at any given cancer sample as compared to any other cancer sample.

4.1.2. Variation in canine performance

One more analysis was performed to see if there was variation in canine performance; dogs may have performed similarly and alerted for the same duration on the different cancer samples, or dogs could have shown variation in their performance and shown preferences for different samples. A linear regression was performed in R using the lm() function to determine whether there was a difference between the duration of time the dogs spent at the odor as a function of the type of

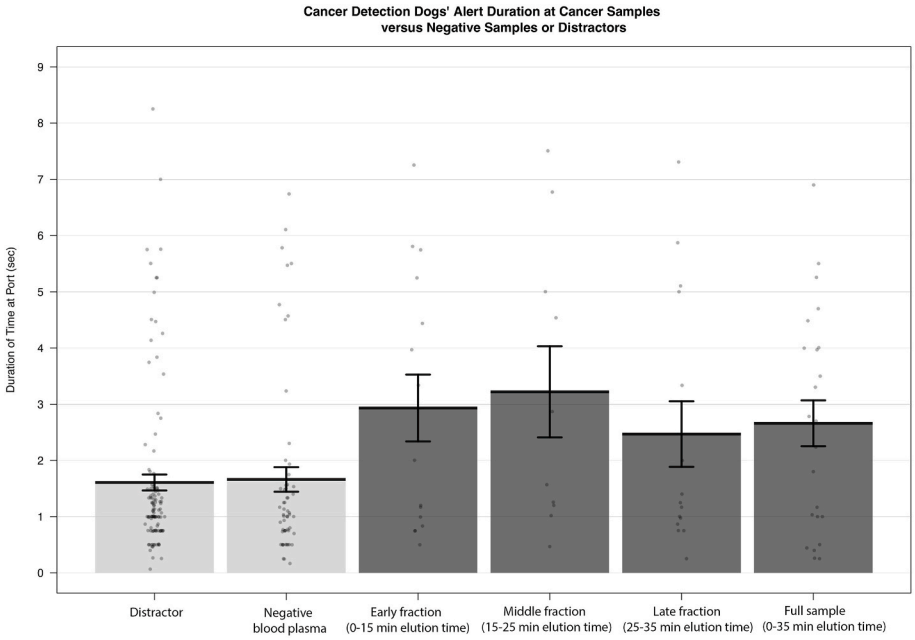


Fig. 2. A graph displaying the alert duration at different sample types. Dogs spent more time at the cancer samples than the distractors or the negative (healthy control) plasma sample. When examining the MP–GC samples, dogs overall spent more time at the early 0–15 and mid 15–25 cancer samples as compared to the late 25–35 cancer sample. This suggests that the biomarkers that identify this cancer may lie in the 0–25 range rather than the 25–35 range.

cancer and the specific cancer detection dog.

Cancer detection dog, Helen, had a significantly lower duration at all samples than the other dogs, $t = -3.88$, $p = 0.0008$. She also spent significantly less time at the full cancer sample than the other dogs, $t = 2.597$, $p = 0.01$. Helen had been trained for only six months prior to the start of the study, in comparison with the other dogs that had been training in cancer detection for at least a year.

The analysis shows that dogs did not overall spend similar amounts of time at the same samples; rather, some spent more time at certain samples than others. Cancer detection dogs Ivey and Osa spent a significantly longer time at the late 25–35 min fraction (Ivey: $t = 2.451$, $p = 0.015$; Osa: $t = 2.446$, $p = 0.015$) than the other dogs (see Fig. 3).

5. Discussion

The goal of this current study was to determine if the superior olfactory ability of dogs paired with analytical chemistry technology could help identify a universal biomarker for ovarian cancer. Five (5) working canines trained to discriminate the blood plasma of patients with malignant tumors from those with benign growths or no known cancer (controls), were presented with the VOC profiles of plasma from individuals with ovarian cancer. These VOC profiles contained three different fractions of VOCs as well as one complete VOC profile collected from an ovarian cancer sample. The three partial VOC profiles were prepared by micro-preparative gas chromatography (MP–GC) and collected according to the eluent times of 0–15, 15–25 and 25–35 min respectively.

These VOC fractions were then presented to the dogs, and the time

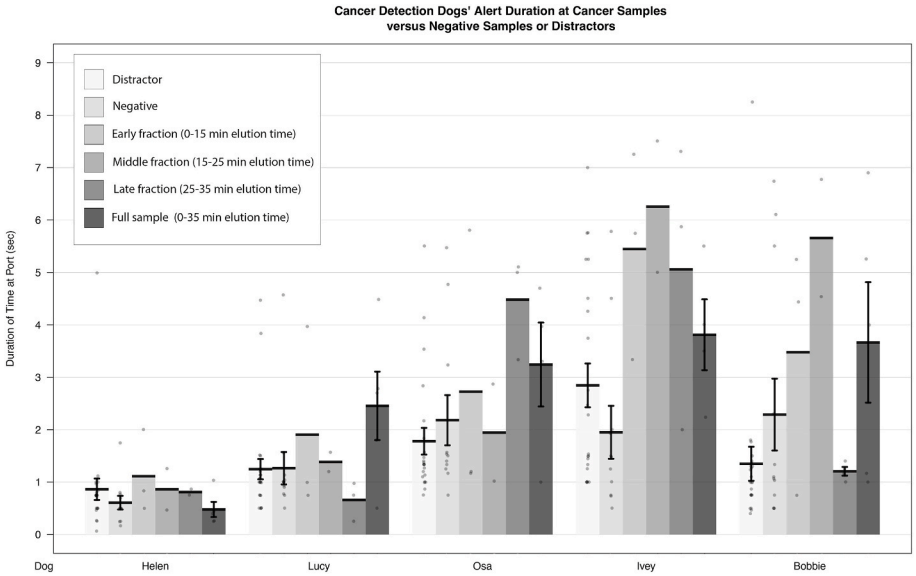


Fig. 3. A graph displaying the cancer detection dogs' average duration of time at the full cancer sample, MP–GC separated cancer samples, negative (healthy control) samples, and distractors. The dogs showed differences in the amount of time they spent at different samples. Ivey and Osa spent significantly longer than other dogs at the 25–35 Late cancer sample. Helen, the dog with the least amount of training, had difficulty discriminating between the negative (healthy control) plasma and the full cancer sample; this suggests she was still learning the odor.

the dogs spent at each fraction was assumed to correlate with how much that profile smelled like ovarian cancer, the target odor these detection canines were trained to discriminate. If all the dogs showed particular interest in one profile, it could be assumed that a universal ovarian cancer VOC biomarker was in that fraction of the VOC profile. Further analysis could then help identify this biomarker. However, if the trained canines did not show any particular interest in one of these profiles, it suggests that dogs are using multiple VOC biomarkers from different fractions to discriminate malignant from control samples, or that individual dogs may be using different VOCs to discriminate. The results of this study suggest the later explanation.

The five (5) cancer detection dogs were able to discriminate between malignant ovarian cancer VOC profiles from distractor and healthy control VOCs. This supports the work done by prior studies [12,15,16,24] demonstrating that dogs can be trained to detect ovarian cancer. However, this is the first study that shows that dogs can be trained to detect the VOC profile of ovarian cancer isolated from biological plasma samples. The dogs showed no difference in interest between distractor and control odors.

However, the dogs universally did not show any affinity toward one particular VOC fraction. Overall, the dogs did show higher interest in the 0–15 and 15–25 min fractions of the odor, as indicated by length of time investigating these samples. The dogs spent less time at the 25–35 min section of the VOC profile, suggesting that the compounds these dogs use to discriminate ovarian cancer samples from control or distractor odors exist in the earlier eluted volatiles. Due to the dogs' interest in multiple sections of odor profile, it suggests that there was not one universal biomarker these dogs were using to discriminate. This finding suggests that ovarian cancer results in changes in multiple VOCs and any biomarker strategy will need to encompass the span of VOC alterations, whether the change is in absolute amount or in the pattern of VOC release.

Another explanation for the dogs' interest in multiple odor fractions is that individual dogs use different VOCs to discriminate ovarian cancer. DeGreeff et al. noted that individual dogs had different generalization and discrimination patterns across a series of structurally similar odorants [25]. Researchers found that these differences were correlated with previous training experience with the odorants, and breed [25]. This could also explain the variable performance of some of these dogs to the full VOC profile of ovarian cancer. If some dogs rely on VOCs that are present in lower concentration in some samples, they would have a lower overall sensitivity to the overall profile of those samples. A future study, with more dogs could help tease apart some of these individual differences between dogs.

The outcome of the current study indicates that dogs can discriminate ovarian cancer VOC profiles from control and distractor VOC profiles. However, trained dogs do not seem to use one universal biomarker to discriminate ovarian cancer VOC profiles from those of control samples. The results do suggest that further study is necessary to understand individual differences in dogs' ability to detect ovarian cancer, and to determine if there is a correlation between dogs' accuracy, and the identity of the VOCs those dogs use to discriminate malignant from non-malignant odors.

Author credits

S. A. Kane: Investigation; Writing – original draft; Writing: review and editing Y. E. Lee: Data curation; Formal analysis; Writing – original draft J. L. Essler: Investigation; Methodology; Data curation; Writing – original draft A. Mallikarjun: Data curation; Formal analysis; Visualization; Writing – review & editing. G. Preti: Conceptualization, Methodology, Supervision, Funding acquisition; Dr. Preti passed away March 3rd, 2020, but his work on this project, and previous studies has paved the way for furthering our understanding of early ovarian cancer detection systems. V. L. Plymouth: Investigation; Writing – review & editing. A. Verta: Investigation, Writing – review & editing. A. DeAngelo: Methodology; Writing – review & editing. Dr. C. M. Otto: Methodology; Conceptualization; Supervision; Funding acquisition; Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was funded by the Robert J Kleberg Jr and Helen C Kleberg Foundation, Ovarian Cancer Symptom Awareness, the Robert J. Kahn Foundation, Kaleidoscope of Hope, Trustees Council of Penn Women and Lucy was sponsored by Kaleidoscope of Hope and Bobbie was sponsored by Merck Animal Health.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.123729.

References

[1] M.A. Lisio, L. Fu, A. Goyeneche, Z.H. Gao, C. Telleria, High-grade serous ovarian cancer: basic sciences, clinical and therapeutic standpoints, Int. J. Mol. Sci. 20 (2019), https://doi.org/10.3390/ijms20040952.
[2] K.B. Mathieu, D.G. Bedi, S.L. Thrower, A. Qayyum, R.C. Bast, Screening for ovarian cancer: imaging challenges and opportunities for improvement, Ultrasound Obstet. Gynecol. 51 (2018) 293–303, https://doi.org/10.1002/uog.17557.
[3] T. Motohara, K. Masuda, M. Morotti, Y. Zheng, S. El-Sahhar, K.Y. Chong, N. Wietek, A. Alsaadi, M. Karaminejadranjbar, Z. Hu, M. Artibani, L.S. Gonzalez, H. Katabuchi, H. Saya, A.A. Ahmed, An evolving story of the metastatic voyage of ovarian cancer cells: cellular and molecular orchestration of the adipose-rich metastatic microenvironment, Oncogene 38 (2019) 2885–2898, https://doi.org/10.1038/s41388-018-0637-x.
[4] M.A. Rossing, K.G. Wicklund, K.L. Cushing-Haugen, N.S. Weiss, Predictive value of symptoms for early detection of ovarian cancer, J. Natl. Cancer Inst. 102 (2010) 222–229, https://doi.org/10.1093/jnci/djp500.
[5] R.C. Bast, B. Hennessy, G.B. Mills, The biology of ovarian cancer: new opportunities for translation, Nat. Rev. Cancer 9 (2009) 415–428, https://doi.org/10.1038/nrc2644.
[6] A.R. Simmons, C.H. Clarke, D.B. Badgwell, Z. Lu, L.J. Sokoll, K.H. Lu, Z. Zhang, R. C. Bast, S.J. Skates, Validation of a biomarker panel and longitudinal biomarker performance for early detection of ovarian cancer, Int. J. Gynecol. Cancer 26 (2016) 1070–1077, https://doi.org/10.1097/IGC.0000000000000737.
[7] K.A. Kouremenos, M. Johansson, P.J. Marriott, Advances in gas chromatographic methods for the identification of biomarkers in cancer, J. Cancer 3 (2012) 404–420, https://doi.org/10.7150/jca.4956.
[8] M. Shirasu, K. Touhara, The scent of disease: volatile organic compounds of the human body related to disease and disorder, J. Biochem. 150 (2011) 257–266, https://doi.org/10.1093/jb/mvr090.
[9] C. Angle, L.P. Waggoner, A. Ferrando, P. Haney, T. Passler, Canine detection of the volatilome: a review of implications for pathogen and disease detection, Front. Vet. Sci. 3 (2016), https://doi.org/10.3389/fvets.2016.00047.
[10] B. Buszewski, T. Ligor, T. Jezierski, A. Wenda-Piesik, M. Walczak, J. Rudnicka, Identification of volatile lung cancer markers by gas chromatography-mass spectrometry: comparison with discrimination by canines, Anal. Bioanal. Chem. 404 (2012) 141–146, https://doi.org/10.1007/s00216-012-6102-8.
[11] J.N. Cornu, G. Cancel-Tassin, V. Ondet, C. Girardet, O. Cussenot, Olfactory detection of prostate cancer by dogs sniffing urine: a step forward in early diagnosis, Eur. Urol. 59 (2011) 197–201, https://doi.org/10.1016/j.eururo.2010.10.006.
[12] G. Horvath, G.A.K. Järverud, S. Järverud, I. Horváth, Human ovarian carcinomas detected by specific odor, Integr. Cancer Ther. 7 (2008) 76–80, https://doi.org/10.1177/1534735408319058.
[13] J.L. Essler, C. Wilson, A.C. Verta, R. Feuer, C.M. Otto, Differences in the search behavior of cancer detection dogs trained to have either a sit or stand-stare final response, Front. Vet. Sci. 7 (2020) 1–8, https://doi.org/10.3389/fvets.2020.00118.
[14] H. Sonoda, S. Kohnoe, T. Yamazato, Y. Satoh, G. Morizono, K. Shikata, M. Morita, A. Watanabe, M. Morita, Y. Kakeji, F. Inoue, Y. Maehara, Colorectal cancer screening with odour material by canine scent detection, Gut 60 (2011) 814–819, https://doi.org/10.1136/gut.2010.218305.
[15] G. Horvath, H. Andersson, S. Nemes, Cancer odor in the blood of ovarian cancer patients: a retrospective study of detection by dogs during treatment, 3 and 6

months afterward, BMC Cancer 13 (2013), https://doi.org/10.1186/1471-2407-13-396.
[16] N. Kybert, K. Prokop-Prigge, C.M. Otto, L. Ramirez, E. Joffe, J. Tanyi, J. Piltz-Seymour, A.T.C. Johnson, G. Preti, Exploring ovarian cancer screening using a combined sensor approach: a pilot study, AIP Adv. 10 (2020), https://doi.org/10.1063/1.5144532.
[17] H. Wu, R. Xue, Z. Tang, C. Deng, T. Liu, H. Zeng, Y. Sun, X. Shen, Metabolomic investigation of gastric cancer tissue using gas chromatography/mass spectrometry, Anal. Bioanal. Chem. 396 (2010) 1385–1395, https://doi.org/10.1007/s00216-009-3317-4.
[18] M. Mal, P.K. Koh, P.Y. Cheah, E.C.Y. Chan, Metabotyping of human colorectal cancer using two-dimensional gas chromatography mass spectrometry, Anal. Bioanal. Chem. 403 (2012) 483–493, https://doi.org/10.1007/s00216-012-5870-5.
[19] H. Amal, D.Y. Shi, R. Ionescu, W. Zhang, Q.L. Hua, Y.Y. Pan, L. Tao, H. Liu, H. Haick, Assessment of ovarian cancer conditions from exhaled breath, Int. J. Cancer 136 (2015) E614, https://doi.org/10.1002/ijc.29166. –E622.
[20] O. Friard, M. Gamba, BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations, Methods Ecol. Evol. 7 (2016) 1325–1330.
[21] M.E. Wolak, D.J. Fairbairn, Y.R. Paulsen, Guidelines for estimating repeatability, Methods Ecol. Evol. 3 (2012) 129–137, https://doi.org/10.1111/j.2041-210X.2011.00125.x.
[22] T.K. Koo, M.Y. Li, A guideline of selecting and reporting intraclass correlation coefficients for reliability research, J. Chiropr. Med. 15 (2016) 155–163, https://doi.org/10.1016/J.JCM.2016.02.012.
[23] D. Bates, M. Maechler, B. Bolker, S. Walker, Fitting linear mixed-effects models using lme4, J. Stat. Software 67 (2015) 1–48.
[24] G. Horvath, H. Andersson, G. Paulsson, Characteristic odour in the blood reveals ovarian carcinoma, BMC Cancer 10 (2010), https://doi.org/10.1186/1471-2407-10-643.
[25] L.E. DeGreeff, A.G. Simon, K. Peranich, H.K. Holness, K. Frank, K.G. Furton, Generalization and discrimination of molecularly similar odorants in detection canines and the influence of training, Behav. Process. 177 (2020), https://doi.org/10.1016/j.beproc.2020.104148.
[26] J. Kwak, M. Gallagher, M.H. Ozdener, C.J. Wysocki, B.R. Goldsmith, A. Isamah, G. Preti, Volatile biomarkers from human melanoma cells, J. Chromatogr. B 931 (2013) 90–96.