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ABSTRACT

All cells release low molecular weight organic compounds that possess finite vapor pressures at body and/or ambient temperatures. These volatile organic compounds (VOCs) may possess an odor and can be found emanating from all body fluids. As cells turn malignant, analysis of changes in these VOCs can provide insight into cancer onset and diagnosis. Previous studies have demonstrated that dogs can be trained to distinguish ovarian cancer tissues of various stages and grades from normal ovarian tissue and other gynecological malignancies with sensitivity and specificity over 95%. When trained on biopsied tissue, dogs were able to detect the VOC disturbances in peripheral blood samples with the same accuracy. Building on these earlier studies, we examined the VOCs emanating from plasma samples from primary ovarian cancer patients, patients with benign reproductive tract growths, and healthy controls. We used a three-pronged sensor approach to analyze the VOCs from plasma: canines trained on tissue and plasma samples, analysis using solid phase microextraction gas chromatography–mass spectrometry, and novel single stranded DNA-coated carbon nanotube sensor field effect transistors. Each of the three experimental approaches used in this study provided preliminary evidence that plasma from ovarian cancer patients emits a volatile odor signature that can be distinguished from the VOCs of patients with benign ovarian tumors and controls. Our results provide optimism that a diagnostic approach based on the analysis of the VOC odor signature of ovarian cancer is achievable.

I. INTRODUCTION

In 2019, it is estimated that there will be 22,530 new cases of ovarian carcinoma in the US, resulting in an estimated 13,980 deaths. 1,2 Diagnosis of ovarian cancer is severely hindered by the lack of reliable diagnostic tools, despite the importance of early diagnosis to treatment success. Unfortunately, cancers of the ovaries frequently cause no symptoms, and initial symptoms that do occur are often similar to gastrointestinal illness and other common ailments. Moreover, there is no sufficient screening test for the accurate and early detection of ovarian cancer in women of average risk. 3 The lack of reliable screening strategies and lack of obvious symptoms associated with early stage ovarian cancer spurred a large scale investigation into screening, sponsored by the NCI: The Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial, which concluded that annual screening for ovarian cancer with transvaginal ultrasound and CA-125 does not reduce disease-specific mortality in women at average risk for ovarian cancer. 4 Techniques such as transvaginal ultrasound can help find tumors in the ovary, but when used for screening, most of the masses found are non-cancerous.
Studies suggesting that early diagnosis is possible \(^7\) using serum proteinaceous biomarkers appear to be controversial and their findings disputed. \(^2\) In addition, although the level of blood protein CA-125 is elevated in many women with ovarian cancer, it has not proved to be useful as a screening test because there are other common conditions that cause high levels of CA-125. \(^4,8,9\) Studies have also examined a metabolomics approach for characteristic ovarian cancer biomarkers. \(^39\) These have employed both liquid chromatography–mass spectrometry and derivatization of metabolites followed by gas chromatography–mass spectrometry (GC–MS). While several compounds are found in these studies, including possible aberrations in tryptophan \(^1\) and lipid \(^11,12\) metabolism, no screening methods have yet emerged from these efforts.

Consequently, since there are currently no reliable, early detection methods for ovarian cancer, only 15% of all ovarian cancers are diagnosed at a local stage (i.e., before metastasis). When diagnosed early, ovarian cancer has an excellent prognosis with 94% of 5-year survival rate. \(^5\) However, the majority of ovarian cancers are diagnosed at a late stage when the efficacy of therapeutic strategies is limited with resulting high morbidity and mortality. \(^5\) Any reliable technique that can accurately detect ovarian cancer in its early stage can yield better prognoses and have a great impact on overall survival.

Current diagnostics used by modern physicians rely heavily on our sense of sight (direct visualization and multiple imaging modalities), sound (auscultation and ultrasound), and touch (palpation). Olfaction once played a more significant role in diagnosis but has been less prominent as imaging devices and genetic screening, on our sense of sight (direct visualization and multiple imaging modalities). Olfaction once played a more significant role in diagnosis but has been less prominent as imaging devices and genetic screening, and clinical laboratory tests have become more sensitive, specific, and sophisticated. In the past few centuries, physicians used body odors to aid in diagnosis and recognized that many body odors have evolved to convey messages about the individual to others. \(^13,14\) However, man’s sense of smell is relatively inferior to most mammals, and most physicians are not familiar with or trained to work with odors. Therefore, human body odors remain a relatively untapped source for information regarding cancer development and diagnosis.

Recent studies have sought to exploit the information contained in volatile organic compounds (VOCs) (odorants) emanating from human secretions and excretions to determine if VOCs may provide a target for cancer detection. \(^15\)–\(^36\) In addition, there is evidence from both anecdotal \(^37,38\) and controlled studies, \(^39\)–\(^60\) which have demonstrated that dogs can be trained to use their highly developed sense of olfaction to detect a variety of cancers, including melanoma, \(^39,40\) ovarian, \(^41\)–\(^43\) prostate, \(^47\)–\(^49\) lung, \(^10\)–\(^56\) breast, \(^37,38\) bladder, \(^57,58\) colorectal \(^59\) and cervical \(^60\) cancers. On average, dogs can detect scents many orders of magnitude \((\sim 10^4 \text{ to } 10^5)\) better than humans, roughly 1 part per trillion or the equivalent of 1 drop of chlorine in an Olympic swimming pool. \(^61,62\) Dogs have been used effectively in work environments, relied upon by law enforcement for explosive and drug detection, as well a search and rescue; their skill as biosensors should help expand their role in biomedical applications.

Trained sniffer dogs used in published clinical studies \(^17\)–\(^18,39\)–\(^40\) were able to distinguish the unique odor signature contained within the VOCs emitted from cancer specimens using various source materials including biopsy tissue, \(^41\)–\(^43\) exhaled breath, \(^44\)–\(^50,35,59\) blood, \(^41\)–\(^43\) urine, \(^47\)–\(^49,57,58\) feces, and vaginal secretions’ with remarkable accuracy. Specific to ovarian cancer, dogs were trained to distinguish ovarian cancer tissues of various stages and grades from normal ovarian tissue with sensitivity and specificity over 95%. When trained on tissue samples and/or plasma samples, the dogs were able to detect the VOC disturbances in peripheral blood samples with sensitivity and specificity of greater than 95%. \(^11,42\) Dogs are, thus, able to analyze the signal and ignore confounding outside stimuli. These data suggest that dogs are ideal “biosensors” for the validation of candidate volatile biomarkers identified by organic-analytical techniques and sensor systems.

A traditional electronic nose system employing metal oxide sensors has also been shown to distinguish tissue samples from ovarian cancer from gynecological control tissue (normal fallopian tube and myometrial tissue). \(^1\) These data strongly suggest that ovarian cancer has a unique odor profile, which distinguishes it from the odor signature from tissue and plasma samples from healthy women and suggests that a diagnostic approach based on the analysis of the odor signature is achievable.

Here, we report the initial findings of our multidisciplinary combined sensor approach to detect and characterize the odor signature associated with ovarian cancer. We analyzed pooled plasma samples from patients with ovarian cancer and benign ovarian tumors, as well as pooled plasma samples from age matched controls. We employed trained dogs, organic-analytical chemistry with solid-phase microextraction gas chromatography–mass spectrometry (SPME/GC/MS), and single stranded DNA-coated carbon nanotube (ssDNA-CNT) vapor sensors for detection and identification purposes. Our results suggest significant differences in the VOC profile in the pooled blood plasma samples of the patients with ovarian cancer compared to controls and patients with benign ovarian tumors; these differences were confirmed by all three detection methods: trained detection dogs, organic chemical analysis with SPME/GC/MS, and ssDNA-CNT sensors.

II. METHODS

A. Human subjects

All procedures were approved by the Office of Regulatory Affairs at the University of Pennsylvania (IRB No. 818255, 702679), and written informed consent was obtained from each participant (patients and controls). Blood and tissue samples were obtained from the University of Pennsylvania Ovarian Cancer Biorepository. Cancer and benign tumor specimens were obtained from patients presenting for surgical removal of suspected reproductive tract masses. Only primary cases of ovarian carcinoma were studied to eliminate the possibility of confounders from previous treatment modalities: e.g., chemo-therapy or radiation, which may produce anomalous volatile metabolites. From these patients, we analyzed plasma from ten primary ovarian carcinoma cases and ten patients with benign lesions. In addition, plasma samples were obtained from ten healthy, age-matched ovarian carcinoma cases and ten patients with benign lesions. 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TABLE I. Patient characteristics.

| Cohorts | Age | Stage | Origin | Ethnicity | Histology | Comorbidities |
|---------|-----|-------|--------|-----------|-----------|---------------|
| Malignant |     |       |        |           |           |               |
| 1       | 52  | IIIC  | Ov\(^a\) | C\(^c\)   | Serous EOC\(^c\) | None          |
| 2       | 64  | IV    | Ov     | C         | Undifferentiated EOC | GERD\(^d\), HTN\(^e\) |
| 3       | 61  | IIIC  | Ov     | A–A\(^f\) | Well differentiated EOC | Ventral hernia |
| 4       | 64  | IIIC  | Ov     | C         | Serous EOC | Hypothyroidism |
| 5       | 65  | IIIC  | Ov     | C         | Serous EOC | None          |
| 6       | 52  | IA    | Ov     | A–A\(^f\) | Serous EOC | HTN           |
| 7       | 61  | IIIB  | Ov     | C         | Serous EOC | Hyperlipidemia |
| 8       | 69  | IIIIC | PP\(^g\) | C         | Serous PPC\(^h\) | Obesity       |
| 9       | 62  | IIIC  | Ov     | C         | Serous EOC | Hypothyroidism |
| 10      | 59  | IV    | Ov     | C         | Serous EOC | Asthma        |

| Benign |     |       |        |           |           |               |
|--------|-----|-------|--------|-----------|-----------|---------------|
| 1      | 60  | n/a   | Ov     | A–A\(^f\) | Serous cystadenofibroma | None          |
| 2      | 71  | n/a   | Ov     | C         | Fibrothecoma hyperlipidemia | HTN          |
| 3      | 47  | n/a   | Ov     | C         | Hemorrhagic luteal cyst | HTN          |
| 4      | 55  | n/a   | Ov     | C         | Fibrothecoma | None         |
| 5      | 55  | n/a   | Ov     | A–A\(^f\) | Benign Brenner tumor | Zollinger–Ellison sy |
| 6      | 59  | n/a   | Pelvic | C         | Benign tailgut cyst | Urethral diverticulum |
| 7      | 61  | n/a   | Ov     | A–A\(^f\) | Benign Brenner tumor | Diabetes, HTN |
| 8      | 60  | n/a   | Ov     | C         | Serous cystadenoma | GERD         |
| 9      | 48  | n/a   | Ov     | C         | Mucinous adenoma | None         |
| 10     | 67  | n/a   | Ov     | C         | Serous cystadenofibroma | Lung nodule |

| Controls |     |       |        |           |           |               |
|----------|-----|-------|--------|-----------|-----------|---------------|
| 1        | 62  |       |        | C         |           |               |
| 2        | 67  |       |        | C         |           |               |
| 3        | 59  |       |        | C         |           |               |
| 4        | 58  |       |        | C         |           |               |
| 5        | 66  |       |        | C         |           |               |
| 6        | 64  |       |        | C         |           |               |
| 7        | 63  |       |        | C         |           |               |
| 8        | 65  |       |        | C         |           |               |
| 9        | 52  |       |        | C         |           |               |
| 10       | 53  |       |        | C         |           |               |

\(^a\)Ov: Ovarian.
\(^b\)C: Caucasian.
\(^c\)EOC: Epithelial Ovarian Carcinoma.
\(^d\)GERD: Gastro-esophageal reflux disease.
\(^e\)HTN: Hypertension.
\(^f\)A–A: African–American.
\(^g\)PP: Primary peritoneal.
\(^h\)PPC: Primary peritoneal carcinoma.

samples from the same group (ovarian cancer was only used in combination with other ovarian cancer samples).

1. Preparing and pooling of plasma samples

All plasma samples were prepared according to the work of Horvath et al.\(^{42}\) Samples were collected in standard EDTA anticoagulant tubes, spun at 3000 rpm for 10 min at 4\(^{\circ}\)C and then aliquoted into glass test tubes and frozen at −80\(^{\circ}\)C. To establish optimum analysis conditions, we created pooled samples of plasma from each group of patients and healthy controls. 0.2 ml of serum from each of ten individuals within each group were pooled: primary ovarian cancer patients (designated “cancer”), ten benign ovarian disease patients (designated “benign”), and ten healthy age-matched women (designated “controls”) producing 2.0 ml of pooled plasma from each group. From the pooled samples, 0.5 ml aliquots were distributed for GC–MS, nanosensors, and biological/canine detection. For the dogs, pooled samples were further split into 50 µl aliquots dispensed into glass jars, labeled, sealed, and frozen at −80\(^{\circ}\)C. The average ages of patients and controls used for the pools were as
follows: benign group 58.3 ± 7.50 years, ovarian cancer group 60.9 ± 5.43 years, and control group 60.9 ± 5.26 years.

B. Canine training and VOC detection in pooled samples

The canine cancer detection protocol was approved by the Institutional Animal Care and Use Committee (Protocol 804900) and involved the training of four dogs: an English Springer Spaniel (Dog 1), a German Shepherd (Dog 2), and a yellow Labrador (Dog 3). All dogs were between 12 and 20 months of age. One additional dog (a chocolate Labrador) was trained for the study but was eventually removed because of inconsistent performance and lack of focus.

The dogs went through three stages of training: first, the dogs learned to recognize the odor of ovarian cancer (“imprinting”); second, the dogs learned to discriminate between ovarian cancer and controls [empty vials, benign ovarian samples (tissue and plasma) and healthy controls (plasma only)]; and third, the dogs were tested in a double-blind manner, where the dogs performed scent discrimination trials out of sight of the handler (to eliminate any potential cues). For all training and testing, positive reinforcement methods were used; specifically, a verbal marker (“yes”) and a reward (food or toy) were given after correct identification of the sample. In the case of an incorrect identification, the trainer either gave no reward or a no-reward marker (i.e., “wrong” and asked to start again). A trial was considered to begin with the request to “seek” and ended either when the dog made a correct identification or was called off by the trainer due to distractions or failure to alert. All stages were video recorded.

Two of the three successful dogs (dogs 1–2) were imprinted on the odor of the ovarian cancer specimens by encouraging the dogs to play with a plain cotton towel that had been briefly stored with an open vial containing the cancer tissue sample. The towel was then placed into a clean quart sized paint can along with the open plastic freezer vial containing the cancer tissue sample. The dog was directed to find the towel and sample that were placed into one of a series of four clean paint cans, while empty freezer vials were placed in the remaining three cans. The cans were mounted on the ends of two wooden 2 in. x 4 in. boards that were fixed in the middle at 90° angles to create a “scent wheel” with four spokes. Each day the size of the towel placed along with the cancer sample was reduced by 50% until it was 1/32nd of the original size. The dogs were considered to be imprinted on the odor of the cancer, when they were able to identify the paint can with the cancer tissue sample but no toweling. The third dog (dog 3) was imprinted strictly on plasma by exposing the dog to an open vial of pooled plasma from ovarian cancer patients and marking the act of sniffing with a word or a clicker, followed by presentation of a food reward. The dog was then marked and rewarded for sniffing the ovarian cancer sample and ignoring the benign and control samples. This approach was also briefly used to introduce the dogs trained on tissue to the plasma samples.

After imprinting, the first two dogs were trained to discriminate cancer tissue (in the freezer vial) from empty vials (controls). Initially, a single randomly chosen tumor sample was used at a time. After the initial 2 weeks, three randomly selected cancer tissue vials were placed in the same glass holder for training. The goal was to provide increased odor and pooled cancer odor. The order of the dogs and the order of the samples used were randomized. Samples were refrozen after use and used repeatedly. Three different configurations (Fig. 1) were evaluated for testing discrimination with the tissue samples, (1) a rotating stainless-steel scent wheel capable of holding 12 samples [Fig. 1(a)], (2) wall mounted sample rack capable of holding eight samples [Fig. 1(b)], and (3) four wooden discs with PVC piping each holding one sample [Fig. 1(c)]. Only the stainless-steel scent wheel was utilized for training with plasma samples. All devices allowed the dog to put its nose into the odor without disturbing the samples.

FIG. 1. Configurations for scent training using ovarian tissue samples. (a) A rotating stainless-steel scent wheel capable of holding 12 samples. (b) A wall mounted sample rack capable of holding eight samples. (c) Four wooden discs with polyvinyl chloride (PVC) piping each holding one sample.
Both dogs imprinted on tissue reached >90% mean proportion of success (number of correct trials/total number of trials) distinguishing cancer tissue from control vials within 3 months. They were then introduced to tissue samples from women with benign ovarian neoplasms designated as benign tumors. All tissue samples were monitored for the frequency of use, duration of thaw, and any change in weight. After approximately 6 months, when dogs attained ≥90% success on tissue samples, they were introduced to pooled plasma samples in the rotating stainless-steel scent wheel.

1. Canine training sessions with plasma

Training was transitioned to pooled plasma samples due to the limited supply of tissue. Cancer and control plasma samples were introduced first. Dogs were trained on ovarian cancer, and control normal plasma for approximately 45 days before benign plasma samples were introduced.

Each day of training represented a session; within each session, the dogs typically performed between 7 and 10 trials. Dogs were trained in random order 3–4 times per week. Each vial containing pooled plasma (50 μl) was thawed and only used on 1 day. The same set of samples (i.e., one pooled ovarian cancer sample, one pooled normal sample, one pooled benign ovarian disease sample, and various controls including gloves, cotton, paperclips, and empty vials) was used for all dogs tested on a single day. Each vial was handled using nitrile gloves and placed directly into an arm of the scent wheel, then covered with a perforated stainless steel cage. The wheel and covers were cleaned using 70% isopropyl alcohol. Between each trial, a metal tool was used to spin the wheel to change sample placement and prevent visual cues. Double-blind testing commenced when the dogs were performing at >85.

2. Mean proportion of success

During testing, the data were taken as follows: a true positive when the dog correctly identified the experimental sample, a true negative when the dog passed a healthy or benign control without indicating, a false positive when the dog incorrectly indicated at either the healthy or benign control, and a false negative when the dog passed (and sniffed) the cancer sample without indicating. The mean proportion of success was calculated as described by Willis et al. and below for each dog’s session of trials. We then compared each dog’s mean proportion of success to the expected value for the task (PVC: 1 in 4; Wall: 1 in 8; and Scent Wheel: 1 in 12) using the following formula: mean proportion of success = No. of true positives/No. of total trials.

C. GAS chromatography/mass spectrometry (GC/MS)

1. Preparation of plasma samples for GC/MS

Pooled plasma samples were aliquoted into several portions to develop optimal analysis conditions as well as to gain knowledge of what exogenous and endogenous compounds were present in the collected VOCs; 0.25 ml aliquots of plasma from each pool were used before settling upon the following analysis method. The plasma (0.25 ml) was added to a 4 ml clear glass vial (Supelco Corp., Bellefonte, PA). To this vial, we added ~80 mg of NaCl and a micro-stir bar. The vial was capped with a white silicone/TFE septum-containing screw cap and incubated in a 37 °C water bath with stirring using a magnetic stirrer. After 15 min of stirring, a solid-phase microextraction (SPME) device was inserted through the septum cap and the collection fiber, a 2 cm, 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (“Stableflex” fiber; Supelco Corp., Bellefonte, PA) exposed. The fiber remained exposed to the headspace VOCs for an additional 60 min at 37 °C. The SPME fiber was then inserted into the injection port of a GC/MS, and the VOCs were desorbed for 1 min at 230 °C.

2. GC/MS analysis of pooled plasma samples

Analyses were performed using a Thermo Scientific ISQ single quadrupole GC/MS (Waltham, MA) with the Xcalibur software (Thermo Electron Corp.) and equipped with a Stabilwax column (30 m × 0.32 mm with 1.0 μ coating; Restek, Bellefonte, PA), which was used for separation and analysis of the desorbed volatiles. We employed the following chromatographic protocol for separation before MS analyses. The injection port was set at 230 °C. The oven was held at 60 °C for 4 min and then increased at 6 °C/min to 220 °C with a 20-min hold at this final temperature. Column flow was constant at 1.5 ml/min. Operating parameters for the mass spectrometer were as follows: ion source temperature 200 °C, ionizing energy at 70 eV, and scanning frequency was 2 scans/s with a range of 40–400 m/z.

3. GC/MS pooled plasma data analysis

Identification of compounds was performed using both the NIST ’02 library and a manual comparison of mass spectra with those reported in the literature. In addition, comparisons of relative retention times using a homologous series of linear ethyl esters and mass spectra of commercially available standards were performed when available. Standard chemicals for structure and retention time confirmation were obtained from Sigma-Aldrich (St. Louis, MO).

For this qualitative examination of the data from the pooled samples, compounds found in each were separately normalized in the following manner: we examined the mass spectra of all peaks ~1% above baseline in each total ion chromatogram (TIC) to eliminate components arising from siloxanes, room air, fragrances, cosmetics, soaps, solvents (e.g., traces of chloroform), column and septa, and solvents commonly employed in cosmetic room air products, e.g., 2-butoxy ethanol as well as linear and branched alkanes >C5, and aromatic hydrocarbons (see Ref. 64 for further details regarding criteria for excluding exogenous compounds).

D. Single-stranded DNA-decorated single-walled carbon nanotubes (ssDNA-CNT)

Electrical contacts for carbon nanotube (CNT) field-effect transistors (FETs) with channels 10 μm long and 25 μm wide were patterned by photolithography and metallized with Cr/Au via thermal evaporation. After O₂ plasma cleaning to remove residual photoresist, a 3-aminopropyltriethoxysilane (APTES) monolayer was deposited using atomic layer deposition (Savannah 200, Cambridge Nanotech) with surface pretreatment by introduction of H₂O vapor to increase the concentration of hydroxyl groups. Semiconducting NTs were deposited from solution (Nanointegris, Isonanotubes-S 98%) by pipetting onto the surface of the chip and incubation in a humid atmosphere for 20 min. The NT-FET arrays were cleaned...
by immersion in isopropanol followed by immersion in a deionized (DI) water bath and then annealed at 200 °C for 1 h to improve the electrical contacts.

Devices were functionalized by incubation in a solution of ssDNA (Invitrogen) in deionized water for ~30 min to allow self-assembly of a nanoscale layer of ssDNA on the CNT sidewall, driven by the attractive π–π stacking interaction.

1. Response of DNA-CNTs to VOCs from pooled plasma samples

The concentration of VOCs in these small samples (a few 100 μl) of human plasma was much lower compared to our prior experience analyzing tissue culture VOCs. In order to maximize the concentrations of volatiles in the headspace of the samples, we adapted the technique used for SPME-VOC collections to suit our sensor arrays. First, a 500 μl plasma sample was transferred to a 25 ml two-neck round-bottom flask. We then added 125 mg of NaCl to the flask in order to make the plasma less hospitable for VOCs and help drive them into the vapor phase. The sample was then heated to 45 °C and stirred vigorously with a miniature stir bar. A mass flow controller (MFC) was connected to the inlet of the round-bottom, and a check-valve was connected to the outlet, keeping the headspace isolated until any carrier gas was pulsed through. The closed headspace was left to accumulate volatiles for 30 min, after which nitrogen carrier gas was passed through the MFC, pushing a stream of VOCs from the plasma sample out of the round-bottom toward the sensor chamber. The vapor flow was delivered to the sample while monitoring its electrical resistance. Array measurement was conducted at high speed by routing multiple signals to an analog to digital converter (AD card) for transmission to a computer. A two-way valve was used as a switch to flush the sample chamber with pure carrier gas and ready the array for the next sample.

The sensor array consisted of 18–25 carbon nanotube sensors functionalized with one of four sequences of single-stranded DNA. The four different, randomly selected DNA sequences used in this research were as follows: sequence (Seq) 1: 5′ GAG TCT GTG GAG GAG GTA GTG 3′; Seq 2: 5′ CTT CGT TCT TGA TGT TTG TCA A AC 3′; Seq 4: 5′ CCC GGT GTT ATG GGA GTC GAG TGC 3′; and Seq 5: 5′ GTA CGG ACT GTG AAT GCCGT TAG 3′.

Only one DNA sequence was used in each experiment, and each set of sensors was exposed to the accumulated VOCs from each of the pooled plasma samples (e.g., controls, benign, and cancer) in the following manner. Headspace VOCs from the plasma samples were alternately pulsed into the sensor chamber for 2 min each, with 2 min breaks in between to allow the chamber to be purged. Vapor from each headspace was measured four times. By varyling the flow through the water bubbler, the total flow rate into the chamber was held constant. Water vapor was used instead of dry air in order to minimize the variation in humidity in the chamber when they flow through the plasma sample.

III. RESULTS

A. Canine detection using pooled plasma samples

Dogs could be trained to recognize ovarian cancer plasma samples using either tissue samples or plasma samples. Results from testing of the dogs (dogs 1 and 2 for tissue samples, and dog 1, dog 2, and dog 3 for pooled plasma) are displayed in Figs. 2 and 3. The initial two dogs displayed consistently high mean proportion of success during each session of trials with the tissue samples, regardless of the type of scent detection task used (PVC, Scent Wall, and Scent Wheel). In addition, our results demonstrated that our dogs’ ability to identify the malignant cancer sample was significantly greater than chance, which was 1 in 4 and 1 in 12 for the PVC and Scent Wheel tasks, respectively (PVC: dog 1: z = 2.59, p = 0.0096 and dog 2: z = 2.74, p = 0.006; Scent Wall: dog 1: z = 3.08, p = 0.0021 and dog 2: z = 3.22, p = 0.0013; and Scent Wheel: dog 1: z = 4.21, p = 0.000 03 and dog 2: z = 4.16, p = 0.000 03). We also examined whether there was a difference in the mean proportion of success between the use of tissue vs plasma samples and found no significant difference (p = 0.764). Furthermore, these results are consistent with and reproduce the results of Horvath and co-workers.42,43 The dogs were able to detect an odor signature from the pooled ovarian cancer plasma.
with a high mean proportion of success (dog 1: 95.1 ± 15.8%; dog 2: 98.7 ± 5.1%; and dog 3: 93.3 ± 20.1%) and discriminate it from samples from patients with benign ovarian tumors and from healthy controls.

B. SPME-GC/MS analyses of VOCs from pooled plasma samples

Pooling and analyzing the samples allows us to observe what may be average amounts of VOCs in each category of patients and controls as well as obvious differences attributed to subject status. Any suspected differences will be further tested in the analyses of individual samples. In addition, creating the plasma pools gave two of us (G.P. and K.P.-P.) the opportunity to appreciate any obvious odor differences in samples from the different groups: most samples were odorless or possessed faint/weak odors. No consistency in these odorous samples could be described. Duplicates of each pool were analyzed using the analysis method described above. Samples from each pool look remarkably similar in their major components, with the largest being acetone that elutes at 1.55 min (see Fig. 4), which features three total ion chromatograms (TICs), one from each group. Consequently, we undertook a careful analysis of all components, regardless of size from the onset of data collection at time “zero” through 40 min in each GC/MS analysis. These data suggest the following. There are many compounds present from background/laboratory air (e.g., chloroform, “Texanol®,” and

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**FIG. 5.** The top three plots show differences in the same selected region in the total ion chromatograms obtained from analyses of SPME-collected VOCs from the three pools of samples (top: control; middle: benign tumor; and bottom: ovarian cancer). This higher resolution view suggests specific quantitative differences between the three pooled samples. The bottom two plots show the mass spectrometry data for the two compounds that show the strongest differences. Each has been identified as 3,4-dimethylbenzaldehyde (top mass spectrum with a molecular ion at m/z 134 and large ions found at m/z 133 and 105) and dimethylsulfone (bottom mass spectrum with major ions at m/z 79 and 94 (molecular ion)).
phthalates) and sampling materials (e.g., carbon disulfide from the vacutainer tubes). There do not appear to be qualitative differences between the VOCs in the controls and patient groups. Some obvious quantitative differences may be present, which delineate the three groups. For example, there are differences in the areas of the TICs presented in Fig. 5, which focuses on a smaller, 10 min portion (14–24 min) of the three TICs shown in Fig. 4.

Dimethylsulfone, a commonly occurring mammalian metabolite of methionine appears to differ among groups with the largest amounts being present in the controls (top TIC) and cancer patients (bottom TIC). The level of this compound has been shown to vary on human skin with age\(^67\) and has been suggested as an indicator of skin cancers.\(^68\) The large component eluting just prior to dimethylsulfone is butylated hydroxytoluene (commonly referred to as BHT).

C. ssDNA-CNT analysis of pooled plasma samples

Current passing through the ssDNA-CNT sensors was monitored as a function of time. When the VOCs from the plasma headspace were introduced into the chamber and bound to the ssDNA-CNTs, the current passing through the sensors decreased, and when the VOCs desorbed the current recovered. The baseline response of the sensors to clean air showed a slow drift over time that was removed using a smooth fit. Sensor responses were quantified using the normalized change in device current $\Delta I/I_0$, as we have done previously.\(^65,66,69\) By averaging the output from multiple devices, a clear trend emerged. ssDNA-CNT sensors based on Seq 1 showed differential responses to the headspace vapor from the three pooled samples, all measured in the same data run (Fig. 6). The other sequences tested led to ssDNA-CNT sensors that showed a differential response to the control pool (Seq 2), the benign tumor pool (Seq 5), and no observed discrimination power (Seq 4). Figure 6 shows the examples of the current changes seen for Seq 1 as well as the summed, average current change-responses for each sequence.

IV. DISCUSSION

The pilot data presented provide compelling evidence that ovarian cancer has an odor signature that can be distinguished from that of patients with benign ovarian tumors and from controls. This signature emanating from pooled plasma samples can be detected and analyzed by the complimentary approaches described above: trained detection dogs, analytical-organic chemistry, and ssDNA-CNT sensors.

One potential concern is that the dogs may have learned the pattern of odors of our specific pooled samples and this detection ability may not be generalizable to screening fresh samples. The fact that unique differences were also detected by the ssDNA-CNT sensors makes this less likely, and we are currently performing studies of multiple unique individual samples to evaluate this issue.

The analyses of samples by GC/MS suggest subtle quantitative differences in plasma VOCs, which reflected, in part, our use of plasma as a substrate. Plasma is strongly buffered to remain within a narrow pH window ($\sim 7.4 \pm 0.40$), and so we would not expect to see
a variety of acidic components in its headspace. In addition, plasma has not been seen as an abundant source of VOCs relative to skin or breath; however, plasma is less likely to be contaminated by confounding environmental compounds. The analytical results from the pooled samples suggest that the greatest differences in VOCs may be found between odorants emanating from the plasma samples taken from individuals with benign growths vs cancer or controls. However, relative differences in VOCs that compose the odor signature of ovarian cancer are presented, as suggested by the trained canines and ssDNA-CNT sensors. Certainly, further analysis of a wider range of individual samples is warranted.

Remarkably, despite using randomly chosen DNA oligomers, our ssDNA-CNT nanosensor devices were able to discriminate between cancer and control samples. There were clear differences in the abilities of specific DNA sequences to discriminate, and further research using individual plasma samples will try to determine optimum arrays of oligomers to maximize the differential response between the diagnostic groups.

Despite the preliminary nature of this investigation, the results from both the canines and the ssDNA-CNT nanosensor devices provide preliminary evidence that ovarian cancer may be detected by analyzing VOCs. At this initial stage, however, we cannot be sure if the VOCs detected are unique to ovarian cancer or represent changes that may overlap with other cancers. These pilot data are sufficiently compelling to encourage us to proceed with analysis of individual plasma samples from a larger, more diverse patient population. Discrimination of individual plasma-borne, cancer-specific volatile biomarkers using both GC/MS and trained dogs will enable translation to specific and sensitive ssDNA-CNT sensor devices. Since the trained canines are able to analyze the cancer-specific signals and ignore confounding outside stimuli, we plan to employ them to validate candidate molecules for both analytical biomarker discovery and selection of DNA oligomers used for sensor development. Ultimately, we will work toward an array of ssDNA-CNT nanosensors that may provide a sensitive and specific high throughput, rapid, non-invasive screening for ovarian cancer by harnessing the VOC odor signature of ovarian cancer.

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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