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Detection of volatile biomarkers of therapeutic radiation in breath

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Abstract

Breath testing could provide a rational tool for radiation biodosimetry because radiation causes distinct stress-producing molecular damage, notably an increased production of reactive oxygen species. The resulting oxidative stress accelerates lipid peroxidation of polyunsaturated fatty acids, liberating alkanes and alkane metabolites that are excreted in the breath as volatile organic compounds (VOCs). Breath tests were performed before and after radiation therapy over five days in 31 subjects receiving daily fractionated doses: 180–400 cGy d^{-1} standard radiotherapy ($n = 26$), or 700–1200 cGy d^{-1} high-dose stereotactic body radiotherapy ($n = 5$). Breath VOCs were assayed using comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry. Multiple Monte Carlo simulations identified approximately 50 VOCs as greater-than-chance biomarkers of radiation on all five days of the study. A consistent subset of 15 VOCs was observed at all time points. A radiation response function was built by combining these biomarkers and the resulting dose-effect curve was significantly elevated at all exposures $\geqslant$1.8 Gy. Cross-validated binary algorithms identified radiation exposures $\geqslant$1.8 Gy with 99% accuracy, and $\geqslant$5 Gy with 78% accuracy. In this proof of principal study of breath VOCs, we built a preliminary radiation response function based on 15 VOCs that appears to identify exposure to localized doses of 1.8 Gy and higher. VOC breath testing could provide a new tool for rapid and non-invasive radiation biodosimetry.

(Some figures may appear in colour only in the online journal)

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Introduction

A biodosimeter that measures tissue responses to ionizing radiation has several potential applications. It could determine the severity of exposure in victims of unplanned nuclear events arising from accidents or terrorism, as well as in passengers and crew of aircraft and spacecraft. Also, since the effects of radiation on normal tissue vary widely between individuals [1, 2], it might also permit more accurate titration of dosage in patients undergoing radiotherapy (RT).

Breath testing offers a rational tool for radiation biodosimetry because gamma irradiation of tissues causes increased leakage of reactive oxygen species (ROS) from mitochondria that specifically accelerate lipid peroxidation of polyunsaturated fatty acids in cell membranes, liberating alkanes and alkane metabolites that are excreted in the breath as volatile organic compounds (VOCs) because of their high vapor pressure at body temperature [3]. Radiation-induced oxidative stress is mediated by changes in intracellular metabolic oxidation/reduction (redox) reactions involving ROS [4], because ionizing radiation generates hydroxyl radicals, either directly by oxidation of water, or indirectly by the formation of secondary ROS [5] which then cause increased oxidative degradation of biological molecules [6]. Studies of irradiated food provide additional evidence for this mechanism: radiation evokes production of a variety of different VOCs consistent with oxidative stress products, including alkanes and alkenes [7, 8], benzene derivatives, and aldehydes [9, 10].

Ionizing radiation has been linked to changes in biomarkers of oxidative stress in humans [11, 12], though previous reports of the effects of radiation on alkane excretion in breath are sparse. Arterbery *et al* reported an increased level of ethane in breath in a patient receiving total body irradiation [13], and Crohns *et al* observed a decreased level of pentane in breath following the first day of RT for lung cancer [14]. Others have proposed nitric oxide in breath as a predictor of radiation pneumonitis [15].

The main advantage of breath VOC biomarkers over biomarkers in blood or other fluids is that breath testing is non-invasive, painless, and safe [16, 17]. For example, gas chromatography–mass spectrometry analysis of breath alkane and furan compounds can detect tobacco smokers [18].

We report here a study of subjects undergoing RT for the treatment of various cancers. We collected breath VOCs onto sorbent traps with a breath collection apparatus (BCA) and analyzed the samples using comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GC × GC-TOF MS) in order to identify biomarkers of radiation exposure and to determine their potential value in radiation biodosimetry.

Materials and methods

Human subjects

Thirty-one subjects were studied; their characteristics are shown in table 1. Subjects received either standard RT in daily fractionated doses of 180–400 cGy over four consecutive days ($n = 26$), or high-dose stereotactic body radiotherapy (SBRT) of 700–1200 cGy over four or five consecutive days ($n = 5$). The Institutional Review Boards at all clinical sites of the study approved the research.

Breath VOC sample collection

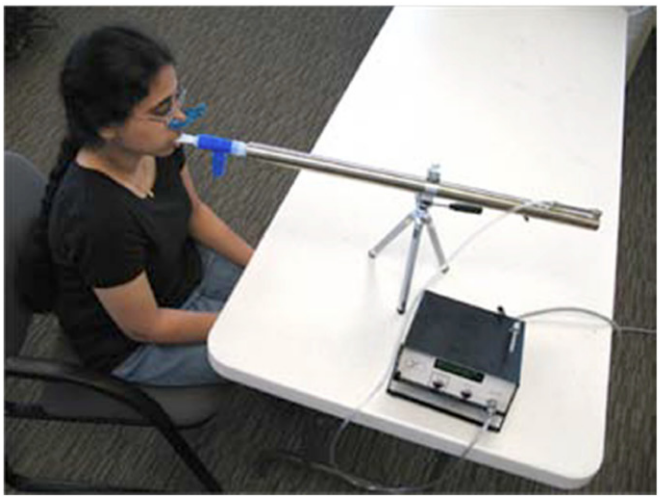
Subjects provided a breath sample before each dose of radiation therapy, and a second sample within 30 min of the conclusion of treatment. The method has been described [19] and the device for sample collection is shown in the left panel of figure 1. In summary, a subject breathed normally for 2.0 min through a disposable valved mouthpiece and a bacterial filter into the breath reservoir of a portable BCA (Menssana Research, Inc., Fort Lee, NJ 07024). VOCs in 1.0 L alveolar breath and 1.0 L room air were captured onto separate sorbent traps containing graphitized carbon black (Supelco, Inc., Bellefonte, PA 16823).

Analysis of samples

VOCs captured on the sorbent traps were analyzed by automated thermal desorption and GC × GC-TOF MS. A Unity 2 thermal desorber (Markes International Inc., DE 19807, USA) was employed to purge water vapor from the sorbent traps with helium, inject 2 ppm of internal standard (1-bromo-4-fluoro-benzene (BFB) from Supelco, Inc., Bellefonte, PA 16823), and then thermally desorb the VOCs onto a cold trap for re-concentration. VOCs were desorbed from the cold trap onto the head of the primary column of a Pegasus 4D GC × GC-TOF MS system equipped with an Agilent 6890 gas chromatograph and a LECO two-stage cryogenic modulator and a secondary oven (LECO Corp., St. Joseph, MI 49085). Two capillary columns were connected in series and separated by the cryogenic modulator so that the relatively non-polar primary column separated VOCs according to their boiling point and the more polar secondary column in the secondary oven separated VOCs according to their polarity. VOCs eluting from the secondary column were detected with TOF MS**.** Helium was used as the carrier gas flowing at a corrected constant rate via automated pressure ramp. We used multiple temperature ramps from 35 to 280 °C to separate the large number of VOCs detected in breath and room air. The sensitivity of the system was monitored using the internal standard BFB injected on each sample tube prior to desorption. Figure 1 (right panel) depicts a typical chromatogram of breath VOCs in a normal human subject.

Data pretreatment

The GC × GC-TOF MS instrument data were first processed using LECO's ChromaTOF software for peak detection and compound identification. VOCs were identified according to their mass spectral signatures matched to a mass spectral library (NIST 2.0, Gaithersburg, MD 20899–1070). The manufacturer's recommended parameters for ChromaTOF were used to reduce the raw instrument data into a metabolite peak list. After retention index filtering [20], all peak lists were



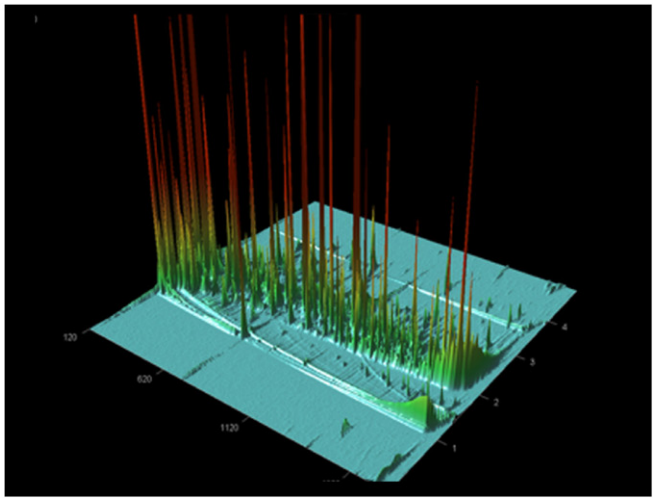


Figure 1. Breath VOC collection and analysis. The BCA (left-hand panel) collects samples of volatile organic compounds (VOCs) in alveolar breath and in air onto separate sorbent traps. The subject breathes normally for 2.0 min through a disposable valved mouthpiece. The BCA then automatically switches over to collect a sample of room air VOCs on to a separate sorbent trap. Breath VOC analysis of sample (right-hand panel) is performed using comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry. Separation with non-polar and polar columns reveals around 2 000 different VOCs in a sample of human breath.

Table 1. Characteristics of subjects.

Cancer group	*n*	Age (yr) Mean (SD)	Mean daily dose (Gy) Standard RT	SBRT	Mean total dose (Gy) Standard RT	SBRT
Breast	20	55.8 (11.6)	1.9		7.6	
Prostate	7	63.0 (11.0)	1.8	7.0	7.1	21.0
Lung	4	67.8 (5.4)		11.0		46.0

RT = radiation therapy, SBRT = stereotactic body RT.

aligned using an improved version of DISCO software [21], where the peaks with low quality were filtered and multiple peak entries were merged using the default values specified in DISCO, and the spectral similarity was calculated using the method described in [22]. For each breath VOC, the value of the alveolar gradient was determined as $V_b/I_b–V_a/I_a$ where V_b = area under curve (AUC) of the chromatographic peak of a VOC in breath, I_b = AUC of the internal standard (BFB) peak in the same chromatogram, and V_a and I_a denote the corresponding values in the associated room air sample [19].

Identification of biomarkers of radiation exposure

Multiple Monte Carlo simulations were employed to identify the breath VOCs that identified radiation exposure with greater than random accuracy. The method has been previously described [23]. In summary, the alveolar gradients of all breath VOCs were compared before and after radiation and ranked as candidate biomarkers according to their C-statistic values, i.e. the AUC of the receiver operating characteristic (ROC) curve [24]. The average random behavior of the alveolar gradients of all breath VOCs was determined with multiple Monte Carlo simulations by randomly assigning subjects to the before or after radiation group, and performing 40 estimates of the C-statistic value. Differences of the C-statistic values obtained between the correct assignment and the random assignment indicate the power of using breath VOCs to differentiate the radiation exposure from the random [25, 26].

Dose-effect response

Breath VOCs identified as biomarkers of radiation with greater than random accuracy were entered into a third order polynomial regression to correlate the response function with total dosage of radiation.

Binary dose response to radiation

All breath VOC data were randomly assigned to either a training set or a test set. In the training set, multiple Monte Carlo simulations were employed to identify the biomarkers of radiation that distinguished between groups of subjects receiving either <1.8 or $\geqslant 1.8$ Gy with greater than random accuracy. These VOCs were analyzed with weighted digital analysis (WDA) and the resulting binary algorithm was employed to predict dosage in the test set. The procedure was repeated in groups of subjects receiving <5 or $\geqslant 5$ Gy.

Results

Heatmaps of breath VOC responses to radiation

Figure 2 displays heatmaps of breath VOC response to radiation and the number of subjects studied at each time point. The 1000 breath VOCs with the greatest abundance were selected for this display. The mean abundance of each breath VOC in all subjects is compared to its baseline

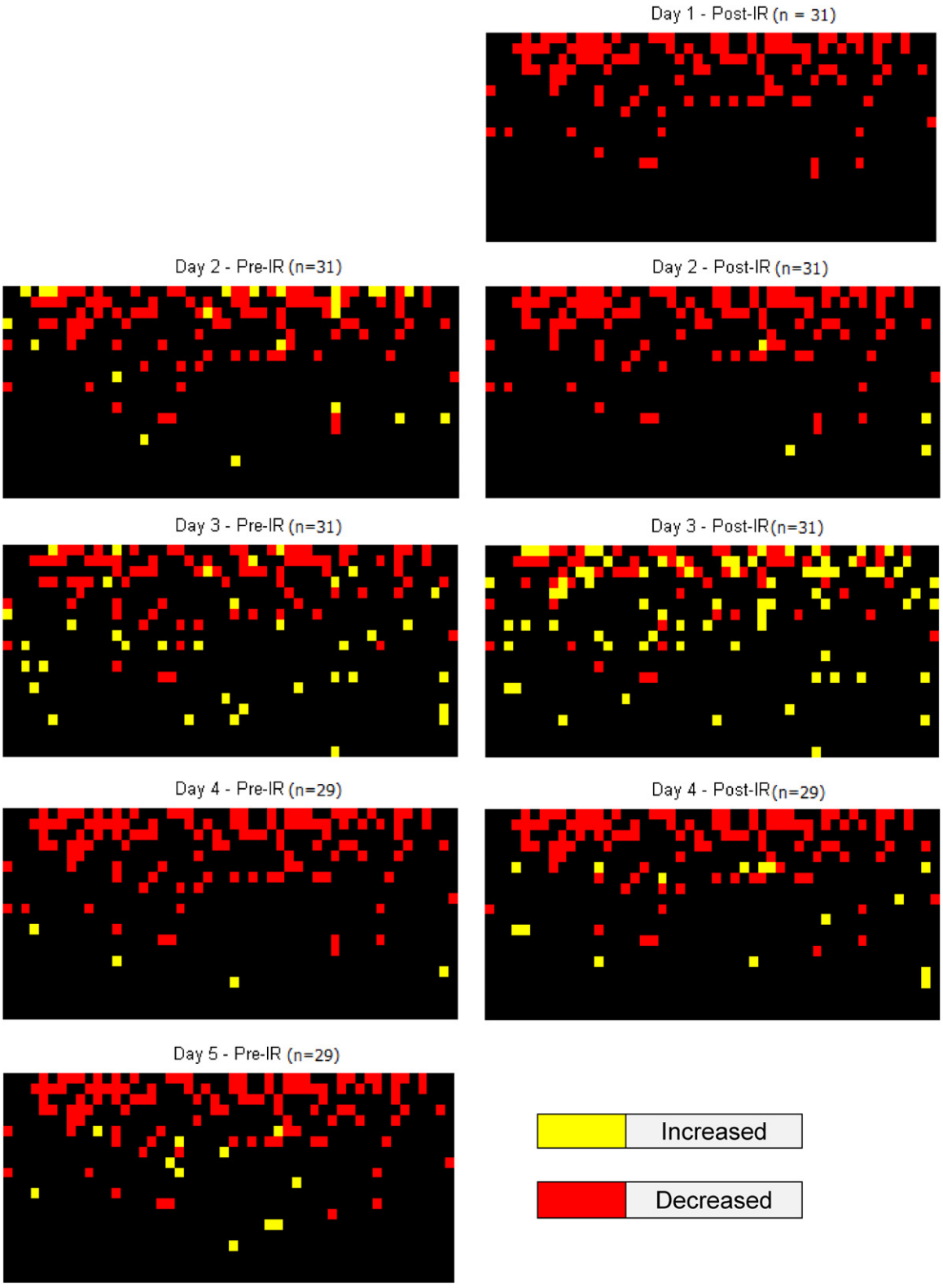


Figure 2. Heatmaps of breath VOC responses to radiation. Subjects received either standard radiotherapy over four consecutive days ($n = 26$), or high-dose stereotactic body radiotherapy over four or five consecutive days ($n = 5$). The 1000 breath VOCs with the highest abundance based on their alveolar gradient are displayed in order of chromatographic retention time like words on a page, i.e. from left to right and from top to bottom in each panel. The change in mean abundance of each breath VOC in all subjects is shown. Binary changes are shown: a yellow or a red cell respectively indicates increased or reduced mean abundance of a VOC compared to its baseline pre-radiation level. These heatmaps demonstrate that radiation elicited major changes in the mean abundance of a wide variety of breath VOCs throughout the course of the study. However, not all of these changes may have been statistically significant, and the Monte Carlo simulations in subsequent figures identified those changes in the heatmap that were greater than could be accounted for by chance alone. IR = irradiation. The number of included subjects is shown on each heatmap. No post-radiation heatmap is shown for day 5 since only two subjects were studied.

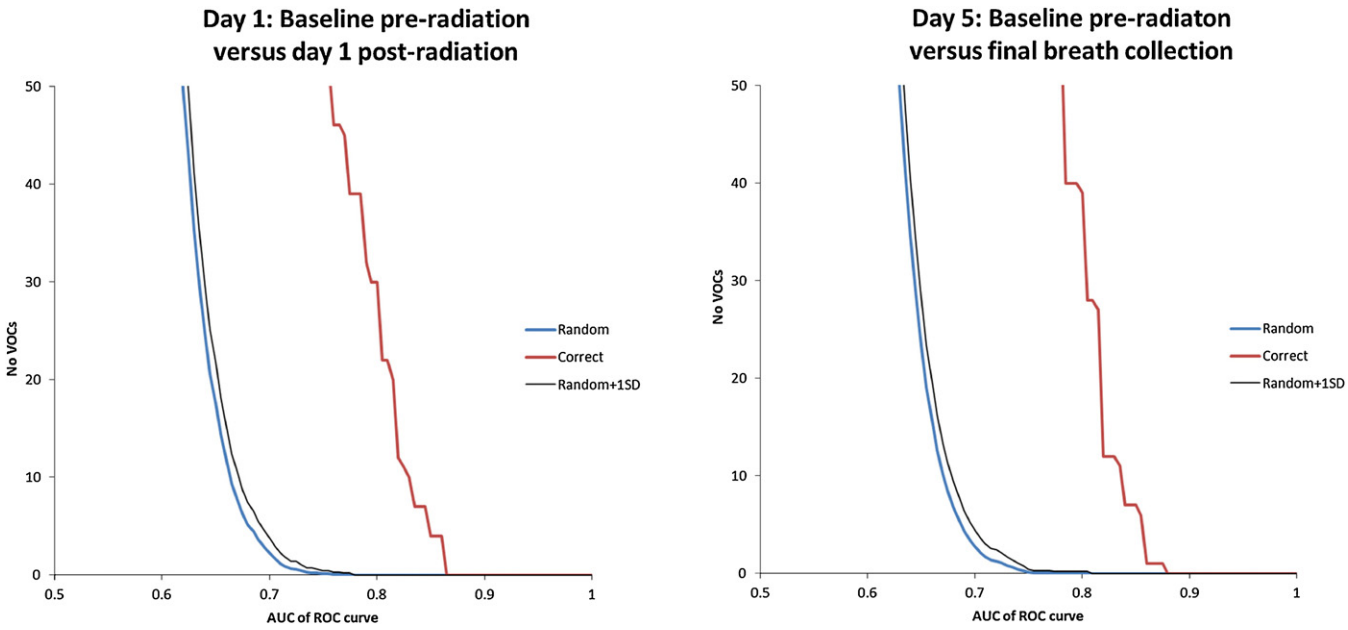


Figure 3. Identification of biomarkers of radiation with multiple Monte Carlo simulations. Day 1: baseline pre-radiation versus day 1 post-radiation (left-hand panel). Day 5: baseline pre-radiation versus final breath collection (right-hand panel). This figure displays the number of breath VOCs as a function of their accuracy as biomarkers of radiation exposure. The criterion of a biomarker's accuracy was the AUC of its ROC curve, a value that ranges between 0.5 (no better than a random coin toss) and 1.0 (a perfect test with no false positive or false negative results). In this figure, the number of VOCs starts at a high value when AUC = 0.5 because it includes all VOCs in the sample, and then progressively declines as the AUC threshold increases. The 'correct assignment' curve shows the outcome when the subject was correctly assigned to the appropriate group (pre-radiation or post-radiation). The 'random assignment' curve shows the mean outcome of 40 Monte Carlo simulations in which subjects were randomly assigned to the pre-radiation or post-radiation group. In both panels, the number of VOCs in the mean random assignment curve fell to <1 when ROC curve AUC = 0.75. However, more than 50 breath VOCs in the correct assignment group had ROC curve AUC values >0.75, demonstrating that these VOCs identified radiation exposure with greater than random accuracy (>5 SD).

pre-radiation level. Changes were seen within 30 min of the first exposure to radiation and persisted through day 5.

Identification of biomarkers of radiation with multiple Monte Carlo simulations

Figure 3 displays outcomes of multiple Monte Carlo simulations on day 1 (baseline pre-radiation versus day 1 post-radiation) and on day 5 (baseline pre-radiation versus final breath collection). Similar results for pre-radiation and post-radiation breath tests were also observed on day 2, 3 and 4, respectively. The simulations identified approximately 50 VOCs in all time points as better-than-chance biomarkers of radiation employing a five sigma criterion.

A subset of 15 VOCs was observed at seven out of eight time points. These VOCs mainly comprised methylated and other derivatives of alkanes, alkenes, and benzene. However, they could only be identified tentatively because of the potential confounding factors that may compromise identification of unknown compounds with the NIST library [27]. Figure 4 displays the dose-effect response observed when these 15 biomarker VOCs were entered into a third order polynomial regression and the response function was correlated with total dosage of radiation.

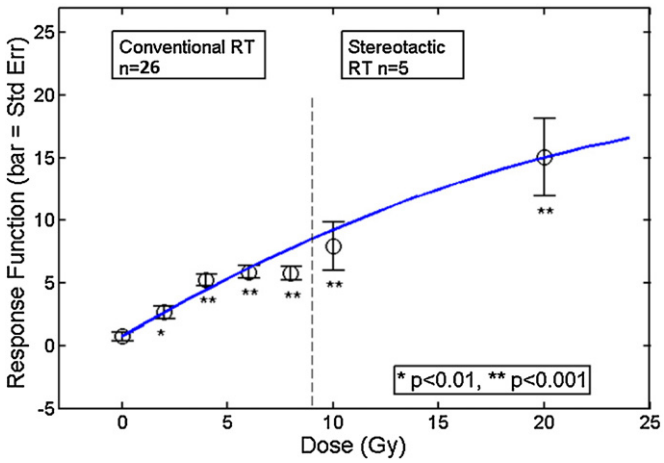


Figure 4. Dose-effect response of breath test to radiation. Radiation response function (+/− standard error) versus dosage. The 15 biomarker VOCs shown in table 1 were converted to a radiation response function by third order polynomial regression and the response function was correlated with total dosage of radiation (Gy). The elevation in response function compared to the pre-radiation level was significant at all exposures of 1.8 Gy and greater.

Binary dose response to radiation

Figure 5 displays the binary dose-effect training sets and test sets observed at cutoff points of 1.8 and 5 Gy

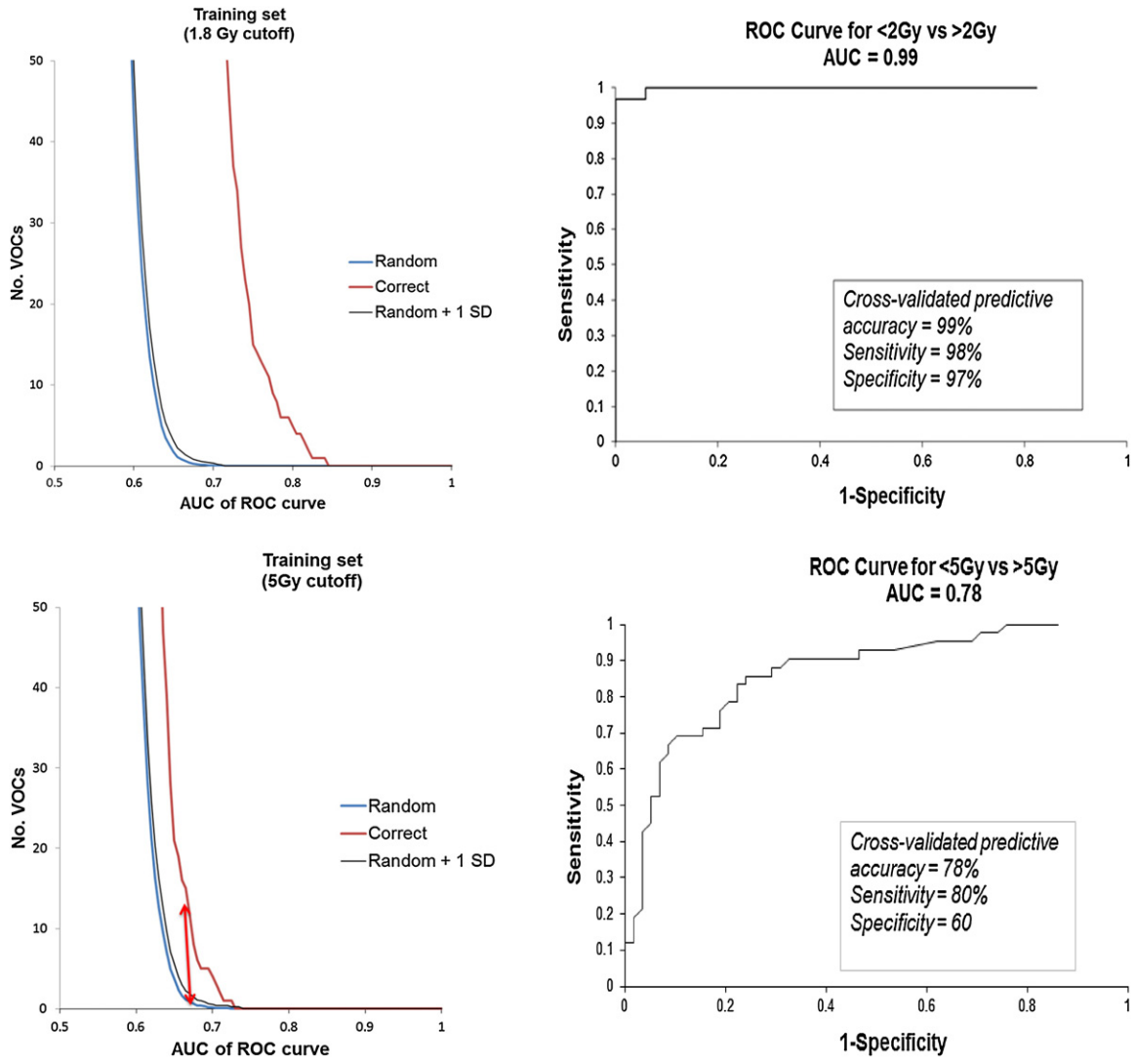


Figure 5. Binary dose-effect response *Upper panels*: training set and test set with cutoff value of 1.8 Gy. *Lower panels*: training set and test set with cutoff value of 5 Gy. Breath VOC data were randomly assigned to either a training set or a test set, and multiple Monte Carlo simulations were employed in the training set to identify the biomarkers that identified radiation exposure in excess of a specific cutoff value. More than 50 VOC biomarkers distinguished between exposures of $<$1.8 Gy compared to $\geqslant$1.8 Gy with greater than random accuracy ($>$5 SD). 30 VOC biomarkers similarly distinguished between exposures of $<$5 Gy compared to $\geqslant$5 Gy. These VOCs were analyzed with weighted digital analysis (WDA) and the resulting binary algorithms were employed to predict dosage in the test sets. The cross-validated prediction accuracy of the binary dose-effect response was 99% at 1.8 Gy and 78% at 5 Gy.

Effect of site of radiation

The radiation response data plotted in figure 4 was stratified by diagnosis and by dosage, and there were no statistically significant differences between subjects with cancer of breast, prostate and lung.

Discussion

The main finding of this study was that therapeutic radiation elicited major changes in the abundance of breath VOCs. These changes were highly significant: compared to their baseline pre-radiation levels, the abundance of 50 breath VOCs increased or decreased by more than five standard deviations in excess of variation arising by chance, corresponding to a p-value of less than 3×10^{-7}.

The significantly altered breath VOCs included a stable subset of 15 compounds mainly comprising methylated and other derivatives of alkanes, alkenes, and benzene. The chemical structures of these VOCs could only be identified tentatively because of the inherent limitations of identifying unknown VOCs with the NIST library. Stein has identified the major factors affecting confidence in the identification process of mass spectra with the NIST library as prior probability, risk of false negative results, risk of false positive results, and the problem of 'unknown unknowns' [27]. These findings are consistent with previous reports of the effects of oxidative stress in which peroxidation of lipids generates alkanes and methylated alkanes that are excreted in the breath [3, 16], and with the VOCs reported in irradiated food products [7–10]. A dose-effect curve employing these 15 VOCs in a multivariate algorithm demonstrated significant responses to radiation at

doses of 1.8 Gy and higher. The cutoff points of 1.8 and 5 Gy were selected because of their relevance to screening and triage: whole-body acute exposure greater than 2 Gy is associated with increased risk of hematopoietic syndrome, while doses greater than 5 Gy may be lethal [28].

We found no statistically significant differences between different diagnostic groups at any dosage; however, the comparatively small number of subjects with prostate and lung cancer provided insufficient statistical power to differentiate on diagnosis.

In addition, cross-validated binary algorithms identified radiation exposures $\geqslant$1.8 Gy with 99% accuracy, and $\geqslant$5 Gy with 78% accuracy. The decline in accuracy at $\geqslant$5 Gy may have been induced by a statistical effect of the comparatively small number of subjects in this group.

These findings are consistent with previous reports of radiation-induced changes in biomarkers of oxidative stress [11, 12]; also, the early global decline in breath VOCs (figure 2) was consistent with the previously reported fall in breath pentane levels following the first day of RT for lung cancer [14]. The fluctuating pattern of increasing and decreasing breath VOCs observed during the five-day course of radiation is not yet understood and merits future investigation. These changes may have arisen from a complex interaction of acute and chronic inflammatory responses to radiation injury resulting in fluctuating levels of oxidative stress and altered release of biomarkers of inflammation. However, this figure should be interpreted with caution because of an intrinsic limitation of heatmaps: even when they display visually striking changes in the evolution of a data set, not all of the apparent changes may be statistically significant.

The relevance of these findings should be interpreted with caution because of the selection of subjects with acute medical concerns and the fact that the effects of partial-body exposure employed in therapeutic radiation may differ from the effects of whole-body radiation resulting from a nuclear event or irradiation in aircraft and spacecraft. Also, a number of potential confounding factors may have affected responses to radiation in members of the study population, including differences in age, gender, type of cancer, stage of disease, preceding treatment with antioxidants or other drugs, and the effects of dosage schedule (e.g. short-term high exposure versus longer-term lower dosage). All of these factors merit future investigation, but they were beyond the scope of this pilot study which was targeted to establishing proof of principle. It is obviously desirable to follow this study with both animal studies and research of human subjects undergoing total body irradiation.

These findings suggest future areas of research that were not addressed in this report. For example, it is not yet known if different results might be observed in subjects receiving high exposure for a short period compared to those receiving low exposure for a long period. This merits future studies in animals since it is not feasible to perform such studies in humans.

Large populations could be exposed to acute radiation injury in the event of an unplanned nuclear event resulting from an accident or from military or terrorist activity [29]. Early responders would require a rapid triage strategy to identify those in urgent need of acute medical care and determine their exposure using some form of radiation biodosimetry [30, 31]. Different techniques have been proposed as candidate biomarkers of radiation exposure, including detection of induced chromosomal abnormalities in peripheral blood lymphocytes [32], electron paramagnetic resonance in teeth [33], as well as changes in the proteome [34] and genome [35].

In this study, breath VOCs provided a preliminary set of accurate biomarkers of radiation exposure in humans. A breath test for radiation biodosimetry has several advantages over the tests requiring blood or urine: they are rapid, non-invasive, and completely safe. In addition, this technology could be applied in a cost-effective point-of-care platform that does not require operators with laboratory expertise, similar to the point-of-care breath test for active pulmonary tuberculosis that employs a mobile gas chromatograph [36].

However, further studies are required to validate these findings in humans and in animals, and also to determine the time-course of breath VOC responses to radiation. In addition, it will require further investment in development of instruments and methods to migrate this technology to a point-of-care platform that could be employed by the first-responders. We conclude that a breath test detected a set of volatile biomarkers of radiation in human subjects receiving radiation therapy, and that this test could potentially be employed to estimate exposure to radiation in other settings.

Acknowledgment

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