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Volatile biomarkers in the breath of women with breast cancer

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Abstract

We sought biomarkers of breast cancer in the breath because the disease is accompanied by increased oxidative stress and induction of cytochrome P450 enzymes, both of which generate volatile organic compounds (VOCs) that are excreted in breath. We analyzed breath VOCs in 54 women with biopsy-proven breast cancer and 204 cancer-free controls, using gas chromatography/mass spectroscopy. Chromatograms were converted into a series of data points by segmenting them into 900 time slices (8 s duration, 4 s overlap) and determining their alveolar gradients (abundance in breath minus abundance in ambient room air). Monte Carlo simulations identified time slices with better than random accuracy as biomarkers of breast cancer by excluding random identifiers. Patients were randomly allocated to training sets or test sets in 2:1 data splits. In the training sets, time slices were ranked according to their C-statistic values (area under curve of receiver operating characteristic), and the top ten time slices were combined in multivariate algorithms that were cross-validated in the test sets. Monte Carlo simulations identified an excess of correct over random time slices, consistent with non-random biomarkers of breast cancer in the breath. The outcomes of ten random data splits (mean (standard deviation)) in the training sets were sensitivity = 78.5% (6.14), specificity = 88.3% (5.47), C-statistic = 0.89 (0.03) and in the test sets, sensitivity = 75.3% (7.22), specificity = 84.8 (9.97), C-statistic = 0.83 (0.06). A breath test identified women with breast cancer, employing a combination of volatile biomarkers in a multivariate algorithm.

(Some figures in this article are in colour only in the electronic version)

Introduction

Breast cancer is the most common cause of cancer in women, after skin cancer [1]. Throughout the world, more than 1 million women are diagnosed with breast cancer every year [2], and in the USA, the National Cancer Institute estimated that more than 182 000 women would be diagnosed with breast cancer in 2008, and more than 40 000 would die of the disease [3]. Fortunately, deaths due to breast cancer have decreased in recent years, due in part to improved early detection and

to better treatment [4, 5]. However, new tools are needed for early detection of breast cancer because of the limited sensitivity and specificity of current screening methods, as well as the perceived discomfort of mammography and potentially hazardous exposure to radiation [6, 7].

Breath testing has been proposed as a rational screening tool because breast cancer is accompanied by increased oxidative stress [8, 9], which in turn can be detected by increased excretion of volatile alkanes and alkane derivatives in the breath [9, 10]. Hietanen *et al* analyzed the breath of women with breast cancer and found increased concentrations of pentane [10], a volatile biomarker of oxidative stress

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generated by lipid peroxidation of polyunsaturated fatty acids in cell membranes [11]. Members of our group have previously reported a study of breath testing in which multivariate models containing as few as five volatile organic compounds (VOCs) accurately predicted the presence or absence of breast cancer [12, 13]. Breath VOC biomarkers have also been detected in a number of other diseases, including lung cancer [14], pulmonary tuberculosis [15], and an FDA-approved breath test for heart transplant rejection [16].

We performed this study in order to identify volatile biomarkers of breast cancer and to determine the sensitivity and specificity of a breath test for the disease. We compared breath VOCs in women with biopsy-proven breast cancer and in cancer-negative controls.

Materials and methods

Human subjects

Breath VOC samples were collected from female patients attending the Royal Perth Hospital Breast Clinic. They comprised women with biopsy-proven breast cancer and a cancer-free group found either to have no significant abnormalities on routine mammographic screening or who were recalled after screening but subsequently showed not to have breast cancer on further testing [17]. Breath samples were collected from the breast cancer group before they were treated for the disease. All gave their written informed consent to participate in the study, and the research was approved by the Ethics Committees of the University of Western Australia and the Royal Perth Hospital. All breath VOC samples were collected at the Royal Perth Hospital Breast Clinic and sent by express mail for analysis to the Breath Research Laboratory of Menssana Research Inc., Newark, NJ. Studies performed in normal volunteers prior to the clinical study demonstrated that breath VOC samples remained stable with no detectable loss or contamination for up to 4 weeks at room temperature and were not affected by international express shipping. No dietary controls were imposed. Data were not analyzed for comorbidities or medications since both the control group and the cancer group comprised apparently healthy women prior to undergoing routine screening.

Breath collection and assay (overview in figure 1)

Collection. The method has been described in [12, 18]. A portable breath collection apparatus (BCA) was employed to capture the VOCs in 1.0 L breath and 1.0 L room air on to separate sorbent traps. The geometry of the breath reservoir of the BCA ensured that the sample comprised >99% alveolar breath. Subjects wore a nose-clip and respired normally for 2.0 min through a disposable and previously unused valved mouthpiece with a bacterial filter to prevent contamination of the instrument. The mouthpiece and filter presented low resistance to respiration, ensuring that samples were collected without discomfort to patients. Prior to the clinical study, breath samples collected with and without a bacterial filter from normal volunteers demonstrated that the filter introduced no detectable changes into the breath VOC profile.

Analysis. The method has been described in [18, 19]. VOCs captured in the sorbent traps were analyzed in the laboratory by automated thermal desorption, gas chromatography and mass spectroscopy. In order to quantify peak areas and to control for drift in instrument performance, an internal standard was run with every chromatographic assay of breath and air (0.25 mL 2 ppm 1-bromo-4-fluorobenzene, Supelco, Bellefonte, PA).

Masking procedures. Laboratory staff members who performed the breath VOC assays had no clinical information about the subjects, and the research assistants who collected breath samples were not informed of the results of the assays. Masking was not broken until data were analyzed.

Analysis of data and statistical methods

Chromatographic data. Chromatograms were converted into a series of data points by segmenting them into a series of 900 time slices, each with 8 s duration and 4 s overlap. The alveolar gradient (abundance in breath minus abundance in ambient room air) of each time slice [AG_{TS}] was determined as $AG_{TS} = T_b/I_b - T_a/I_a$, where T_b is the integrated area under the curve (AUC) of the time slice in the alveolar breath chromatogram, I_b is the AUC of the internal standard peak, and T_a and I_a are corresponding values derived from the associated chromatogram of room air [18, 19].

Monte Carlo simulations. Chromatographic time slices were ranked as candidate biomarkers of breast cancer according to their C-statistic values (i.e. the AUC of the receiver operating characteristic (ROC) curve [20]). In order to minimize the risk of including random identifiers of disease, Monte Carlo simulations were employed to select the chromatographic time slices that identified breast cancer with better than random accuracy. The average random behavior of breath VOCs was determined by randomly assigning subjects to the 'breast cancer positive' or 'breast cancer negative' groups, and performing 200 estimates of each breath VOC's C-statistic value. For any given value of the C-statistic, it was then possible to compare the mean number of VOCs exceeding that value by correct assignment or by random assignment.

Construction and cross-validation of multivariate algorithms. Patients were randomly allocated to a training set or a test set in a 2:1 data split. In the training set, each time slice was evaluated as a candidate biomarker by comparing its alveolar gradient values in subjects with and without breast cancer and determining the value of its C-statistic. Time slices were ranked according their C-statistic values, and the top ten were combined in a multivariate algorithm employing weighted digital analysis (WDA), a multivariate analysis procedure [21]. The algorithm was cross-validated in the test set. The procedure was repeated ten times, with unique selections of patients in the training and test sets.

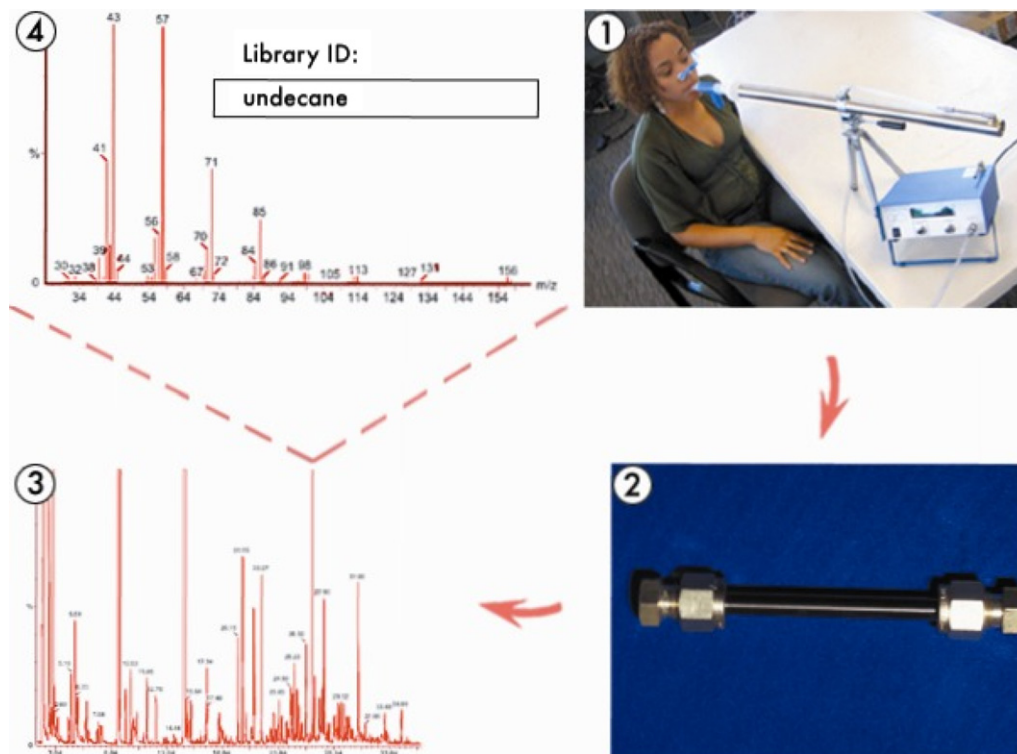


Figure 1. Collection and analysis of breath VOCs. (1) Breath collection apparatus. A subject wears a noseclip and respire for 2.0 min through a disposable-valved mouthpiece unit and a bacterial filter. (2) Sealed sorbent trap. VOCs in 1.0 l alveolar breath and room air are collected on to separate sorbent traps which are hermetically sealed for shipping to the laboratory. (3) Gas chromatogram of breath. Samples are desorbed onto an automated thermal desorber that concentrates the VOCs almost 1 million-fold prior to analysis by gas chromatography and mass spectroscopy. A chromatogram of breath typically contains 150–200 peaks, each eluting with a different retention time. Each peak usually, but not invariably, represents one VOC. (4) Mass spectrum of one chromatogram peak. The probable chemical structure of the VOC is inferred from its resemblance to another mass spectrum in the computer-based NIST library. Every VOC has a unique mass spectrum that serves as its ‘fingerprint’, but VOCs with near-similar chemical structures may have near-similar mass spectra. The library generates a list of tentative identifications that are ranked according to the resemblance between the mass spectra of the sample VOC and the library VOC. In this example, undecane was determined to be the most likely correct identification. In order to identify candidate biomarkers of breast cancer, VOC retention times in chromatogram time slices were employed for primary identification, and mass spectra for secondary identification.

Tentative identification of biomarker VOCs. The chemical identity of the major VOC in each time slice in one training set was tentatively identified in the chromatograms with the Turbo Mass Software (PerkinElmer Life and Analytical Sciences, Waltham, MA 02451).

Results

Human subjects

Patient characteristics are shown in table 1. A total of 258 technically satisfactory breath VOC samples were collected, and no patient reported any adverse consequences of the procedure.

Tentative identifications of VOC biomarkers of breast cancer

Table 2 displays tentative mass spectroscopic identifications of the VOCs in the top ten time slices ranked according their C-statistic values in a training set.

Table 1. Patient characteristics.

	Breast cancer	Normals
Number	54	204
Mean age (SD)	55.1 (7.3)	54.8 (10.8) NS
Smokers/non-smokers/unknown	2/38/10	26/177/1

NS = not significant on the two-tailed *t*-test.

Monte Carlo simulations

Figure 2 displays the C-statistic of each time slice employing either randomized or correct assignment to the breast cancer group, in single and multiple simulations. There was an excess of correct over random time slices, consistent with the presence of non-random biomarkers of breast cancer in the breath.

Multivariate algorithms

Figure 2 displays the ROC curves of a training set and a test set employing the top ten time slices identified as biomarkers of breast cancer.

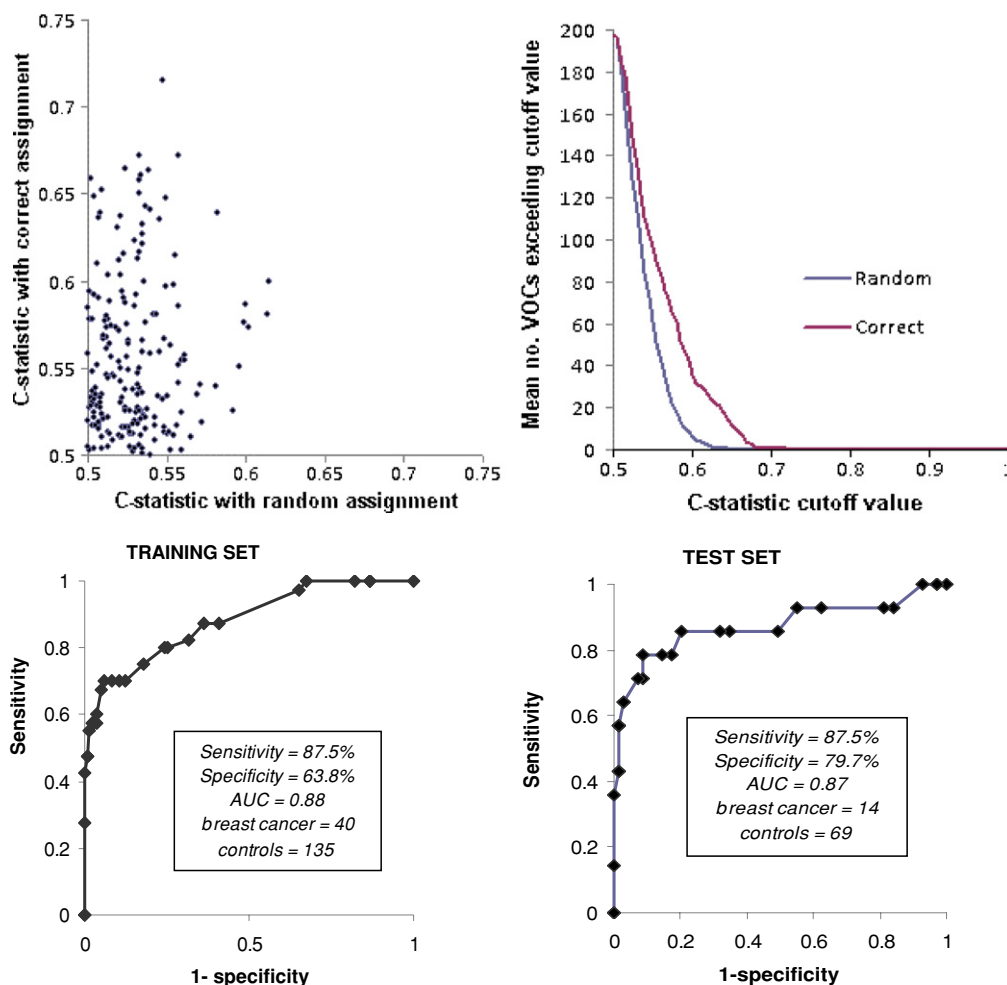


Figure 2. Biomarker identification and evaluation as predictors of breast cancer. Outcome of a single Monte Carlo simulation (top-left panel). Each chromatographic time slice was evaluated as a candidate biomarker of breast cancer by comparing its alveolar gradients in subjects with and without disease. This figure displays the C-statistic of each time slice employing either randomized or correct assignment to the breast cancer group on the *x*- and *y*-axes, respectively. In this particular simulation, there were more than 25 time slices with C-statistic > 0.62 with correct assignment, versus none with randomized assignment. Outcome of multiple Monte Carlo simulations (top-right panel): 200 unique Monte Carlo simulations were performed as shown in the left panel. These curves display the mean number of time slices exceeding a given AUC cutoff with random and correct assignment to the breast cancer group. When AUC cutoff = 0.65, there was a mean of 20 time slices with correct assignment and zero time slices with random assignment. The excess of correct over random time slices identified non-random biomarkers of breast cancer in the breath. Receiver operating characteristic (ROC) curve—training set (bottom-left panel). Patients were randomly assigned to a training set or a test set in a 2:1 data split. This training set ROC curve displays the performance of the multivariate algorithm employing the ten time slices with highest C-statistic values (their contained VOCs are tentatively identified in table 2). Receiver operating characteristic (ROC) curve—test set (bottom right panel). This test set ROC curve displays the performance of the training set algorithm as a predictor of breast cancer in a separate group of patients. The outcome of ten similar random data splits was determined (results in the text). The risk of erroneous results arising from an ‘overfitted’ multivariate algorithm was minimized by the high ratio of experimental subjects to variables (175:10, i.e. 17.5:1) in the training set, as well as by validation of the algorithm in an independent test set.

Outcome of multiple random data splits

The mean outcome of ten random 2:1 data splits was similar to that of the single split shown in figure 2: *training set*: mean values (with standard deviation) of C-statistic (area under the curve of the receiver operating characteristic) = 0.89 (0.03), sensitivity = 78.5% (6.14), specificity = 88.3% (5.47). *Test set*: C-statistic = 0.83 (0.06), sensitivity = 75.3% (7.22), specificity = 84.8% (9.97). Sensitivity and specificity were determined from the point on the ROC curve of each data split where their sum was maximal.

Stratification of outcomes by stage of breast cancer

These results are shown in table 3.

Discussion

The main finding of this study was that a breath test accurately identified women with breast cancer, when a combination of volatile biomarkers was employed in a multivariate algorithm. These findings were consistent with the outcome of a previous study [12, 13].

Table 2. Tentative identifications of VOCs in top ten time slices.

Rank	Tentative identification	CAS no
1	Cyclopropane, ethylidene	18631-83-9
	1,4-Pentadiene	591-93-5
	1,3-Butadiene, 2-methyl-	78-79-5
2	Cyclotetrasiloxane, octamethyl-	556-67-2
	3-Ethoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy)trisiloxane	18030-67-6
	Benzoic acid, 4-methyl-2-trimethylsilyloxy-, trimethylsilyl ester	NIST # 153593
3	D-Limonene 5989-27-5	1461-27-4
	Cyclohexene, 1-methyl-5-(1-methylethenyl)-, (R)-	13898-73-2
	Cyclohexene, 1-methyl-5-(1-methylethenyl)-	13898-73-2
4	Benzene, 1,2,4,5-tetramethyl-	95-93-2
	Benzene, 1,2,3,5-tetramethyl-	527-53-7
	Benzene, 1-ethyl-3,5-dimethyl-	934-74-7
5	Tridecane	629-50-5
	Dodecane	112-40-3
	Undecane	1120-21-4
6	Dodecane, 2,7,10-trimethyl-	74645-98-0
	Dodecane, 2,6,11-trimethyl-	31295-56-4
	Tridecane	629-50-5
7	Tetradecane	629-59-4
	Tridecane	629-50-5
	Pentadecane	629-62-9
8	(+)-Longifolene	475-20-7
	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-	72747-25-2
	Longifolene-(V4)	61262-67-7
9	2-Hexyl-1-octanol	19780-79-1
	1-Octanol, 2-butyl-	2/8/3913
	Trifluoroacetic acid, <i>n</i> -octadecyl ester	79392-43-1
10	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	719-22-2
	2,5-di-tert-Butyl-1,4-benzoquinone	2460-77-7
	Acetic acid, 2,6,6-trimethyl-3-methylene-7-(3-oxobutylidene)oxepan-2-yl ester	NIST # 185414

This table displays tentative identifications of the VOCs in the top ten time slices that were ranked as the best biomarkers of breast cancer, according to their C-statistic values in the training set shown in figure 1. These identifications were obtained by comparing the mass spectrum of the predominant VOC in each time slice to a library of mass spectra, and identifying the three compounds with the best concordance. CAS numbers are shown (or NIST number where CAS number unavailable). Closely similar alternative structures of some VOCs (e.g. variants of longifolene) as well as the inclusion of chiral compounds were identifications generated by the NIST library of mass spectra.

Table 3. Breath test outcomes stratified by stage of breast cancer.

Stage of breast cancer	No of patients	C-statistic
IDC	4	0.86
1	21	0.83
2	10	0.93
3	8	0.96
4	1	0.99
All with staging data	44	0.88
All patients	54	0.81
Staging data unavailable	10	

All diagnoses of breast cancer were histologically confirmed by breast biopsy. IDC = intraductal carcinoma. The C-statistic (area under curve of the receiver operating characteristic) indicates the accuracy of a diagnostic test. It varies between 0.5 (no better than random results) and 1.0 (a perfect test with no false-positive or false-negative results). These values were obtained by applying the algorithm derived from the training set to each of the different subsets of patients. Since each subset contained only a small number of patients, it is possible that some values might have been artificially high.

The accuracy of a diagnostic test, or its likelihood of delivering the correct answer in a randomly selected population, is indicated by the value of its C-statistic [20], which may range from 0.5 (no better than a random coin flip) to 1.0 (a perfect test, with no false-positives or false-negatives). The breath test had a mean C-statistic value of 0.88 in all women with breast cancer, ranging from 0.86 in intraductal carcinoma to 0.99 in stage 4 disease. This degree of accuracy compares favorably with the results of breast imaging; a large study of asymptomatic women presenting for screening mammography reported mean C-statistic values of 0.74 for film mammography and 0.78 for digital mammography [22].

We employed a Monte Carlo simulation technique to select a set of breath biomarker VOCs based on their individual C-statistic values, and to determine the lower limit of the accuracy of the multivariate WDA model. Monte Carlo methods are computational algorithms that achieve their results by using repeated random sampling. Originally

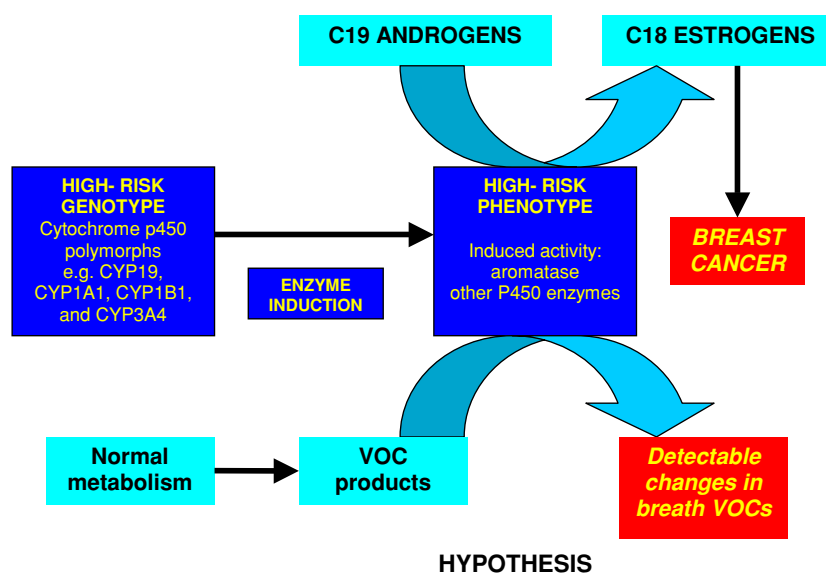


Figure 3. Hypothetical basis of the breath test for breast cancer. A high-risk genotype comprising cytochrome P450 polymorphs may be activated to a high-risk phenotype, with induced activity of several cytochrome P450 enzymes including aromatase. Activation of aromatase results in accelerated biosynthesis of estrogen, with increased risk of breast cancer. Induced cytochrome P450 activity may simultaneously accelerate the clearance of endogenous VOCs, including alkane products of oxidative stress, resulting in detectable changes in composition of the breath.

developed to simulate physical and mathematical systems, the term was coined in the 1940s by physicists working on nuclear weapon projects in the Los Alamos National Laboratory [23]. Monte Carlo simulations have been increasingly employed in recent years for biological applications such as the identification of biomarkers [24, 25].

The biological mechanism of production of volatile biomarkers of breast cancer remains speculative. Time slices and VOCs identified as biomarkers were not unique to patients with breast cancer, but were also observed in the cancer-free controls in greater or lesser abundance. Previous studies of breath biomarkers of disease yielded similar findings: in patients with lung cancer, breath biomarkers were apparently generated by accelerated catabolism of normal metabolic products, consistent with cancer-associated induction of cytochrome P450 enzymes [26]. We propose a similar hypothesis that may account for the volatile biomarkers of breast cancer, associated with altered metabolism of estrogen (figure 3). Estrogens promote the proliferation of both normal and neoplastic breast epithelium cells, and their role as breast carcinogens has been confirmed by epidemiological studies [27, 28]. The carcinogenic role of estrogens is supported by the finding that aromatase is expressed at a higher level in human breast cancer tissue than in normal breast tissue [29, 30]. Aromatase (estrogen synthase) is the cytochrome P450 enzyme complex that catalyzes estrogen production by converting C19 androgens to C18 estrogens [31]. Other cytochrome P450 enzymes are also activated in breast cancer, including CYP1A1, CYP1B1 and CYP3A4 [27, 32]. Cytochromes P450 are hemoproteins encoded by a superfamily of genes nearly ubiquitously distributed in different organisms from all biological kingdoms. The reactions carried out by P450s are extremely diverse and contribute to bioconversion of xenobiotics, alkanes, terpenes

and aromatic compounds [33]. Since normal human metabolism generates a wide variety of VOC products including alkane products of oxidative stress [34], the induced cytochrome P450 activity associated with breast cancer may have modulated the composition of VOCs excreted in the breath.

In addition, a number of the candidate VOCs listed in table 2 included alkanes (e.g. tridecane, dodecane) and methylated alkane derivatives, which are products of oxidative stress produced by lipid peroxidation of polyunsaturated fatty acids [11, 19]. Increased oxidative stress has been implicated as a risk factor in women with breast cancer [35, 36], and increased breath pentane, another alkane, has been reported in women with breast cancer [37].

The site of origin of these VOC biomarkers is not yet known. In a previous study of breath biomarkers of lung cancer, the breath test remained positive in a subset of patients after their tumors had been surgically resected, suggesting that the biomarkers were generated in an extrapulmonary site such as the liver [26]. The same may be true of breath biomarkers of breast cancer, but confirmation must await a future study in patients before and after surgical excision of their tumors.

Biomarkers in clinical medicine have traditionally relied upon a single pathognomic variable (e.g. HIV serology in AIDS) that is both sensitive and specific for a particular disease. There are justifiable concerns about 'the risk of determining risk' with multivariate algorithms that are susceptible to errors such as overfitting of data to the model and type II errors arising from inclusion of random identifiers [38, 39]. These risks associated with multivariate modeling have grown in recent years with the advent of new techniques of metabolomic analysis such as breath testing that generate huge data sets where the underlying physiology is complex or unknown [40]. In order to minimize these risks, we first

employed a Monte Carlo technique to control for random identifiers. The outcome of multiple unique Monte Carlo simulations demonstrated an excess of correct over random time slices in women with breast cancer, consistent with the presence of non-random biomarkers of breast cancer in the breath. Second, we employed multiple random splits of data in order to generate a unique multivariate algorithm in the training sets, and then cross-validate these algorithms in separate groups of patients in the test sets. The consistent similarity of the ROC curves demonstrated that the multivariate models delivered similar accuracy in both the training and test sets of data.

Modern breath testing dates from 1971, when Linus Pauling first detected large numbers of VOCs in low concentrations in cryogenically concentrated samples of normal human breath. Assays using gas chromatography/mass spectroscopy have since identified more than 3000 different VOCs in human breath [41]. However, identification with mass spectroscopy is susceptible to errors, and analysis of VOCs by chromatographic retention times alone may provide an intrinsically more robust technique. 30 years ago, Stern *et al* employed time slice chromatography to differentiate between different strains of *Enterobacteriaceae* and *Yersinia enterocolitica* [42, 43]. Chromatograms are converted to a time series of data points by partitioning into a series of time slices, each of equal duration, and by determining the integrated abundance of chromatographic peaks in each time slice. Multivariate analysis of data may then identify the differences between chromatographic time slices derived from two populations. This approach minimizes the risk of experimental error in identifying breath biomarkers, without precluding tentative identification of the major VOC in each biomarker time slice using mass spectroscopy.

The intrinsic robustness of this technique ensured that the diagnostic accuracy of the breath test was not affected by possible inaccuracies in the GC/MS identification of VOCs. The multivariate algorithms employed the integrated abundance of VOCs in each chromatographic time slice, and required no knowledge of their chemical structures. The chemical identities of the compounds shown in table 2 should be regarded as tentative because their structures were inferred from resemblances between their mass spectra and mass spectra in the computer-based NIST library. Although widely employed as an analytical tool, this method is susceptible to error. For example, it is unlikely that the derivatives of siloxane and trifluoroacetic acid were derived from biological sources. The biological significance of limonene in humans is unknown; it may have been ingested from foodstuffs, and it has been reported as a constituent of the breath of fasting monks [44]. Benzene and benzene derivatives have been previously observed in the breath [45] and may have been ingested as environmental pollutants. Future studies will be required to confirm the chemical identities of candidate VOC biomarkers of breast cancer by comparing their retention times and mass spectra to those obtained from pure compounds.

We conclude that breath contains volatile biomarkers of breast cancer and that these biomarkers can identify

women with the disease. A breath test for breast cancer has potential clinical value since its accuracy appears comparable or possibly superior to established methods of imaging with film or digital mammography. In addition, a breath test is intrinsically safe, painless and free from exposure to radiation. Breath testing may prove useful as an adjunct to population screening with mammography by identifying a low risk group which can be offered less intensive screening. However, further studies in larger populations are required in order to confirm these findings.

Acknowledgments

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