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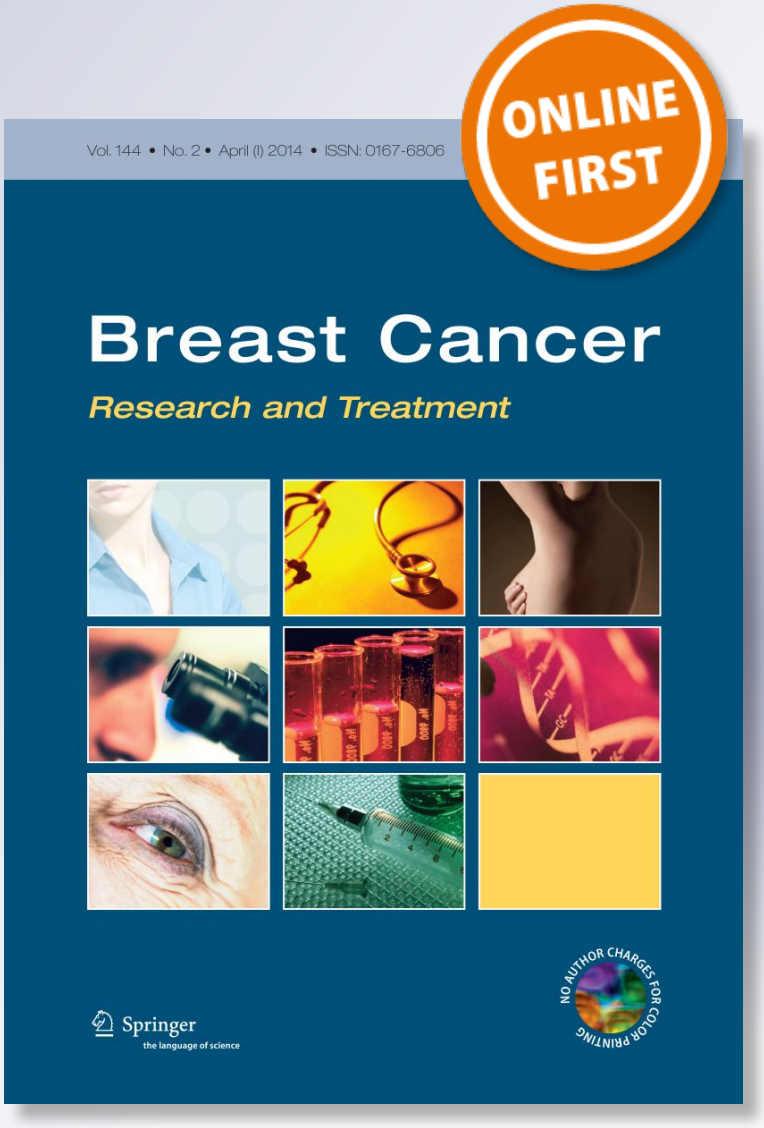

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CLINICAL TRIAL

Prediction of breast cancer risk with volatile biomarkers in breath

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Abstract

Background Human breath contains volatile organic compounds (VOCs) that are biomarkers of breast cancer. We investigated the positive and negative predictive values (PPV and NPV) of breath VOC biomarkers as indicators of breast cancer risk.

Methods We employed ultra-clean breath collection balloons to collect breath samples from 54 women with biopsy-proven breast cancer and 124 cancer-free controls. Breath VOCs were analyzed with gas chromatography (GC) combined with either mass spectrometry (GC MS) or surface acoustic wave detection (GC SAW). Chromatograms were randomly assigned to a training set or a validation set. Monte Carlo analysis identified significant breath VOC biomarkers of breast cancer in the training set, and these biomarkers were incorporated into a multivariate algorithm to predict disease in the validation set. In the unsplit dataset, the predictive algorithms generated discriminant function (DF) values that varied with sensitivity, specificity, PPV and NPV.

Results Using GC MS, test accuracy = 90% (area under curve of receiver operating characteristic in unsplit dataset) and cross-validated accuracy = 77%. Using GC SAW, test accuracy = 86% and cross-validated accuracy = 74%. With both assays, a low DF value was associated with a low risk of breast cancer (NPV > 99.9%). A high DF value was associated with a high risk of breast cancer and PPV rising to 100%.

Conclusion Analysis of breath VOC samples collected with ultra-clean balloons detected biomarkers that accurately predicted risk of breast cancer.

Keywords Breath · Breast cancer · Volatile organic compound · Biomarker

Introduction

The incidence of breast cancer in the United States was 426.1 new cases per 100,000 population in 2012, and its prevalence was 0.32% in the screened population [1–3].

Deceased: Jan Huston.

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An ideal screening test for a disease with such low prevalence should be accurate, painless, non-invasive and safe, but women undergoing screening mammography may experience discomfort, anxiety, radiation exposure, false-positive outcomes, and overtreatment [4, 5]. These limitations of mammography have stimulated interest in breath tests for breast cancer because they are intrinsically painless, safe and non-invasive, and their feasibility has been demonstrated with a variety of different analytical tools including gas chromatography mass spectrometry (GC MS) [6, 7], nanosensor arrays [8, 9], and sniffing dogs [10].

We have previously reported clinical studies in which analysis of volatile organic compounds (VOCs) in breath identified women with breast cancer [11–15]. The source of the breath biomarker VOCs may be carcinoma-associated fibroblasts in breast cancer stromal tissue (Fig. 1). These cells produce hydrogen peroxide, a powerful oxidant that induces oxidative stress and tumorigenic alterations in epithelial cells [16, 17]. The resulting peroxidation of arachidonic acid and other polyunsaturated fatty acids in cell membranes liberates volatile n-alkanes (e.g. pentane, hexane, and longer-chain alkanes) with a high vapor pressure that are exhaled in breath [18–20]. Breath VOCs biomarkers may also arise from induced polymorphic cytochrome p450 mixed oxidase enzymes in breast tissues [21, 22].

We report here a study to evaluate the positive and negative predictive values (PPV and NPV) of breath VOC biomarkers as indicators of breast cancer risk. Breath samples were collected with ultra-clean balloons that enabled collection of uncontaminated breath VOC samples in doctors' offices and outpatient clinics. Samples were analyzed by two different methods: GC MS to identify breath mass ion biomarkers, and GC with surface acoustic wave detection (GC SAW) to detect breath biomarkers by their mass.

Methods and materials

Human subjects (Table 1)

We performed breath tests in women with biopsy-proven breast cancer and in asymptomatic controls. Clinical studies were performed at three sites: Saint Michael's Medical Center, Newark, NJ, USA, Hackensack UMC Mountainside, Montclair, NJ, USA, and Universidad de Guadalajara & Instituto Jalisciense de Cancerologia, Guadalajara, Mexico. At each site, an Institutional Review Board approved the research. A physician explained the study to women aged 18 years and older if they fulfilled the inclusion and exclusion criteria, and asked them to gave written informed consent to participate in the study.

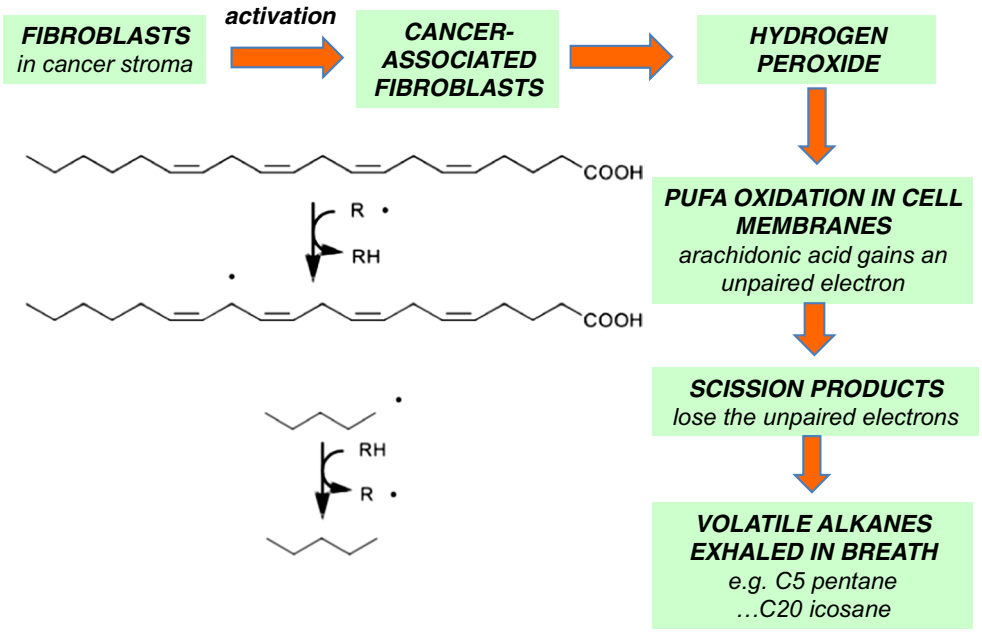

Fig. 1 Source of breath biomarkers in breast cancer: This hypothetical schema postulates that fibroblasts in the cellular stroma surrounding cancer cells are activated to cancer-associated fibroblasts that manufacture hydrogen peroxide. As a result, polyunsaturated fatty acids (PUFA) in cell membranes (e.g. arachidonic acid) are oxidized to free radicals with unpaired electrons. The downstream cascade of oxidation products (not shown) culminates in scission products that lose their unpaired electrons, with the liberation of volatile alkanes. These alkanes undergo subsequent biological transformations including methylation to downstream volatile products with a high vapor pressure that are exhaled in the breath (references in text)

Table 1 Human subjects: women in the breast cancer group had untreated biopsy-proven disease

	GC MS		GC SAW	
	No. subjects	Mean age (years)	No. subjects	Mean age (years)
Breast cancer	54	60.4*	50	61.5**
Normal controls	124	57.0	70	54.6
Total	178		120	

Recruitment site GC SAW	Breast cancer	Controls	Total
Mexico	8	0	8
Montclair, NJ	28	23	51
Newark, NJ	14	47	61
Total	50	70	120

The controls had a normal screening mammogram during the preceding 6 months and no breast-related symptoms. Mean age was higher in the breast cancer GC SAW group than in the normal controls, but this did not significantly affect the sensitivity or specificity of the breath test (see discussion in text)

*NS; **$p < 0.01$

Inclusion criteria

Women in the breast cancer group had untreated disease confirmed on breast biopsy. Women in the control group had a normal screening mammogram during the preceding 6 months and no breast-related symptoms.

Exclusion criteria

Women were excluded from the study if they had any other known serious or potentially life-threatening disease, concurrent acute pulmonary disease (e.g. influenza, or pneumonitis), previous history of cancer of any site (excepting basal cell carcinoma of skin), or if they had received general anesthesia during the 10 days prior to the breath collection.

Ultra-clean balloons (BreathBag™, Menssana Research Inc)

The device is shown in Fig. 2 left panel, and the detailed preparation method has been described [23]. In summary, an inflatable collection bag (e.g. a metallized plastic balloon) was gently inflated with ultra-clean helium in a quantity sufficient to separate its walls, and a reservoir of activated charcoal was introduced through its neck. This reservoir may take different forms, e.g. a perforated tube filled with granules of activated charcoal, or fabric or paper impregnated with activated charcoal. Contaminant VOCs in the bag diffuse into the helium where the activated charcoal captures them by sorbent trapping. Serial analysis of bag VOC contents with GC MS has shown that activated charcoal scavenging prior to collection of a breath sample

Fig. 2 Ultra-clean breath collection balloon. Balloon with activated charcoal reservoir (left hand panel): This figure shows the neck of the balloon with the indwelling reservoir, a paper strip impregnated with activated charcoal that captures > 99% contaminant VOCs by sorbent trapping. The activated charcoal reservoir is removed from the balloon before collection of a breath sample. Collection of a breath sample (right panel). The breath donor inflates the balloon through a drinking straw with a single forced expiration to ensure that the sample comprises alveolar breath from deep in the lungs. The neck of the balloon is sealed with a knot prior to shipping for analysis

removes approximately 99.9% of all contaminant VOCs that were initially detectable.

Breath sample collections

The activated carbon reservoir was removed from the device immediately prior to collecting a breath sample. The user inserted a drinking straw and inflated the bag with a completed forced expiratory exhalation, ensuring that the bag was flushed out with alveolar (deep lung) breath (Fig. 2, right panel). The bag is sealed with a knot tied in its neck, and sent to the laboratory for analysis.

Analysis of breath VOCs

Bags were heated to > 40 °C to volatilize condensed contents prior to analysis with two different methods.

GC MS analysis

A pump withdrew 700 ml breath from the bag through a dual-bedded sorbent trap (Carbotrap C and Carbopack C, Supelco Inc, Bellefonte, PA) in order to capture the contained VOCs. Using automated instrumentation, VOCs were thermally desorbed from the sorbent trap, cryogenically concentrated, and assayed by GC MS (Perkin Elmer Clarus 500, Waltham, MA). A known quantity of bromofluorobenzene (BFB) internal standard was automatically loaded on to all samples in order to normalize the abundance of VOCs and to facilitate alignment of chromatograms. The method has been described [24].

GC SAW analysis

A portable analyzer (zNose model 4200, Electronic Sensor Technology, Inc, Newbury Park, CA) withdrew 70 ml breath from the bag onto an internal sorbent trap in order to capture the contained VOCs. The concentrated sample was then thermally desorbed onto a GC column coupled to a SAW detector. The method has been described [14]. The analyzer was calibrated daily with an external standard, a mixture of C6 to C22 *n*-alkanes (Restek Corporation, Bellefonte, PA 16823, USA).

Analysis of data

The methods have been described [14, 15]

GC MS chromatograms were processed to generate a table of ion masses with their intensities and retention times normalized to BFB. Aligned data were binned into a series of 5 s retention time segments. We ranked mass ions as candidate biomarkers of breast cancer by comparing their intensity values in subjects with biopsy-proven breast cancer to cancer-free controls. In each 5 s time segment, the diagnostic accuracy of each mass ion was ranked according to the fraction of correct binary patient category classifications for an optimally fixed abundance cutoff. We then employed multiple Monte Carlo simulations to select the mass ion biomarkers in each time segment that identified breast cancer with greater than random accuracy, and these biomarkers were entered into a multivariate predictive algorithm using weighted digital analysis (WDA).

GC SAW chromatograms were aligned and binned into a time series of data segments derived from the SAW detector signal (3013 scans/min), and the diagnostic accuracy of each data segment was ranked according to the fraction of correct binary patient category classifications for an optimally fixed cutoff of detector signal differentials. Biomarkers that identified breast cancer with greater than random accuracy were identified in each time segment In the same fashion as described above for GC MS chromatograms, and entered into a multivariate predictive algorithm.

Cross-validation of predictive models

The same method was employed for GC MS and GC SAW data. The predictive model was trained using multivariate weighted digital analysis (WDA) and Monte Carlo simulation [25]. The WDA model was validated with tenfold cross validation [14] and leave-one-out cross validation. For tenfold cross validation, chromatograms from subjects with breast cancers and cancer-free controls were partitioned randomly into 10 "folds". In 10 trials, predictive models were trained on ninefolds and validated on the remaining fold. The training and validation AUCs were averaged, and their ROCs were interpolated along the specificity dimension and averaged, to produce the reported AUCs and ROC curves. In each training set, multiple Monte Carlo simulations identified the candidate biomarkers that individually performed better than $p < 0.05$ compared to modeling against random reclassification of each subject as one with or without breast cancers. Markers were selected by a cutoff on the fraction of chromatograms that they classified correctly. In each case, the cutoff was selected that produce the best overall WDA training AUC while performing at better than $p < 0.05$ in Monte Carlo simulation.

Determination of positive and negative predictive values (PPV and NPV)

The predictive algorithms derived from the unsplit GC MS and GC SAW data sets were employed to generate discriminant function (DF) values that were correlated with the breath test's sensitivity, specificity, PPV, and NPV,

employing a US prevalence of breast cancer of 0.32% in the screening population [1–3].

Results

Human subjects

Characteristics and recruitment sites are shown in Table 1. None reported any discomfort or adverse effects associated with donation of a breath sample.

Sensitivity and specificity of breath tests (Fig. 3)

Receiver operating characteristic (ROC) curve in complete data sets (Fig. 3 left panel)

The breath VOCs that exhibited diagnostic accuracy superior to random behavior (identified by Monte Carlo statistical analysis) were combined in a multivariate algorithm employing weighted digital analysis (WDA). The algorithms identified breast cancer in the unsplit data sets with 90% accuracy using GC MS analysis, and 86% accuracy with GC SAW.

Cross validation

The outcome of tenfold cross validation is shown in Fig. 3, right panel. The 10 multivariate predictive algorithms derived in the training sets identified breast cancer predicted disease in the GC MS and GC SAW validation sets with accuracy of 77 and 74% respectively.

Predicted outcomes of algorithms

Figure 4 displays association of algorithm predictions (DF values) with sensitivity, specificity, and positive and negative predictive values of the GC MS and GC SAW algorithms. Low DF values were associated with a low risk of breast cancer NPV > 99.9% and high DF values were associated with a high risk of breast cancer (PPV rising to 100%).

Effects of age

Age of subjects is shown in Table 1. The AUC of age as a predictor was approximately 0.63. The respective standard error values of the AUC for the WDA model and age, trained on the full data set, were approximated with a formula reported by Hanley and McNeil [26]. Using these standard error estimates, a *t* test was performed to compare the AUCs. For a single tailed test, the two AUCs were different with $p \leq 0.00035$. The 95% confidence interval of difference between the two AUCs was approximately 0.097–0.36, which provides high confidence that the model predicting breast cancer represents signal beyond the effects of age. Inclusion of age as an independent variable with the breath VOC dataset did not change the ROC curve AUC values.

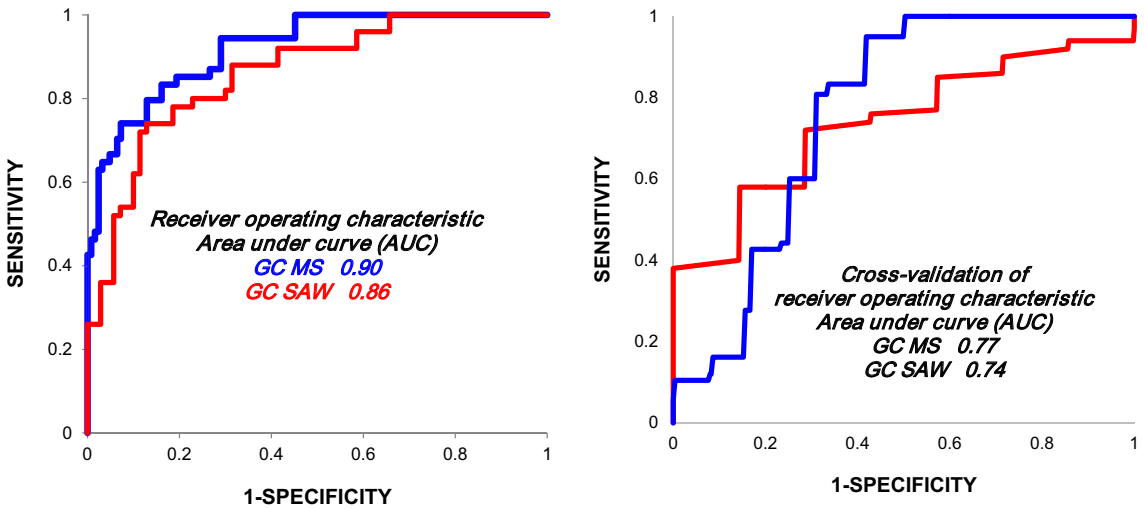

Fig. 3 Sensitivity and specificity of breath tests. Receiver operating characteristic (ROC) curves in complete data sets (left panel). This figure displays the sensitivity and specificity of the breath test for breast cancer. Multivariate algorithms that were derived from breath VOC biomarkers detected with GC MS (blue) and GC SAW (red). The area under curve (AUC) of the ROC curve indicates that the algorithms identified breast cancer with 90 and 86% accuracy, and using GC MS and GC SAW respectively. Cross-validation of ROC curves *(right panel)*. In tenfold cross validation, the cancer and control chromatograms were partitioned randomly into 10 "folds" of 5 cancers and 7 controls. In 10 trials, predictive models were trained on ninefolds and validated on the remaining fold

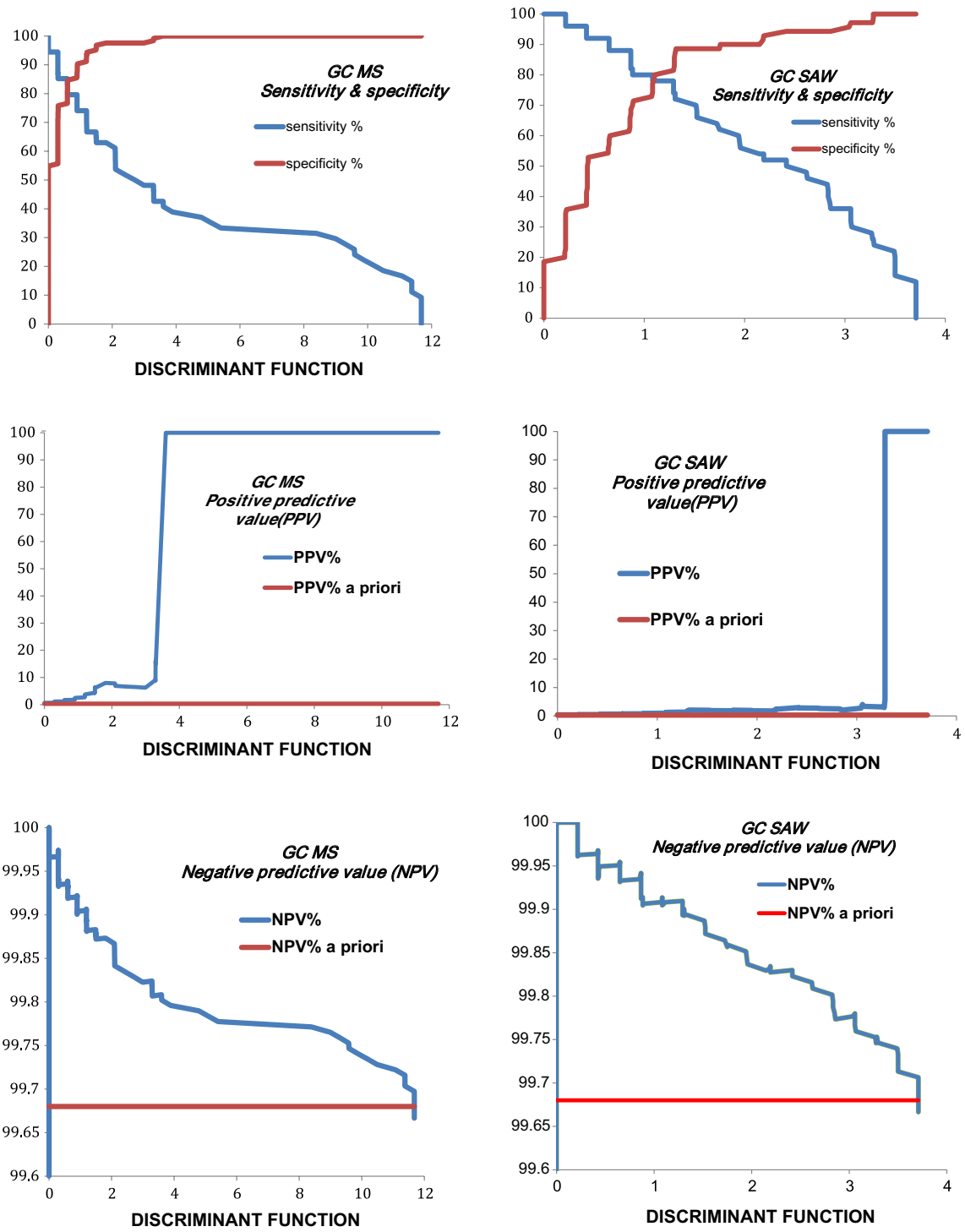

Fig. 4 Variation in sensitivity, specificity and predictive value with discriminant function (DF). The predictive algorithms employed the input of a subject's chromatogram to generate an output termed the discriminant function whose value varied with the risk of disease. Predictive values were derived from the sensitivity and specificity of the breath tests for breast cancer in the unsplit datasets, and the prevalence of breast cancer (0.32%) in a US screening population[1–3]. Sensitivity and specificity. Figure 4, top left and top right panels displays sensitivity and specificity of GC MS and GC SAW tests as they varied with DF. Positive predictive value (PPV) Fig. 4, left middle and right middle panels displays PPV of GC MS and GC SAW tests as they varied with DF. Negative predictive value (NPV) Fig. 4 bottom left and bottom right panels displays NPV of GC MS and GC SAW tests as they varied with DF

Discussion

The main finding of this study was that volatile biomarkers in breath accurately predicted a woman's risk of breast cancer. The risk of disease varied with the numerical value of the discriminant function generated by a predictive algorithm employing a chromatogram of biomarkers in breath. Results were similar when breath samples analyzed either with GC MS or with GC SAW: women with a low algorithm score value had a low risk of breast cancer and a high NPV > 99.9%, while those with a high score value had a high risk of breast cancer and a PPV that rose to 100%.

The values of PPV and NPV were derived from the sensitivity and specificity of the breath test observed in this study and from the US prevalence of breast cancer of 0.32% in the screened population [1–3]. In view of the comparatively small size of the study population, the calculated values of PPV and NPV should be regarded with caution as preliminary estimates. These estimates will be re-evaluated in a larger clinical study that is now in progress [27].

A novel feature of this study was the use of ultra-clean breath balloons to collect samples suitable for assay of breath VOCs. Previous studies employing GC analysis to detect breath biomarkers of breast cancer have required specialized and expensive sorbent trapping devices to collect and store technically usable samples with low background VOC contamination [14, 15, 28]. In contrast, breath VOC collections with ultra-clean bags were simple to perform, safe, and comparatively inexpensive. These ultra-clean breath balloons are simple to use in low-technology settings such as an outpatient clinic, a doctor's office, or a patient's home.

We attempted to minimize potential confounding variables in the experimental design that might have skewed the results of this study. First, we collected breath samples from both the cancer patients and the cancer-free controls in the same room at each site, in order to minimize potential effects of site-dependent variables such as ambient room air contamination. Second, we employed Monte Carlo analysis of data in order to minimize the risk of "voodoo correlations" that may arise by chance alone when large numbers of candidate biomarkers are "overfitted" to small numbers of experimental subjects, yielding results that are statistically true but clinically meaningless [29]. Third, we cross-validated the test results by randomly splitting the data into training sets to develop the predictive algorithms, and then testing the testing the algorithms in independent validation sets.

Despite these precautions, we could not eliminate all potential confounding variables. An intrinsic limitation of a cross-sectional experimental design is that the results are susceptible to demographic and physiological differences between the two groups. The women with breast cancer were older than the cancer-free controls but age did not exert a statistically significant effect on the algorithm predictions.

Women in the control group were deemed to be cancer-free on the basis of a normal screening mammogram during the preceding 6 months and if they had no breast-related symptoms. Women in the disease group were deemed to have breast cancer based on the results of a breast biopsy. Consequently, it is possible that breath VOCs might also have been influenced by factors such as anxiety and breast tissue trauma. These, and other potential confounding variables associated with a cross-sectional experimental design could be minimized in a blinded prospective clinical study in which breath tests are performed before the diagnosis is determined, and such a study is now in progress [27].

The pathophysiologic source of breath VOC biomarkers in breast cancer remains hypothetical. As shown in Fig. 1, activation of breast stromal fibroblasts may result in increased oxidative stress with consequent liberation of volatile n-alkanes including ethane and pentane and other abnormal metabolic products that are expired in the breath [16, 19, 20]. A previous study employing GC MS identified n-alkanes in breath (nonane, tridecane) and methylated derivatives of n-alkanes (5-methyl undecane, 3-methyl pentadecane) as candidate biomarkers of breast cancer [11]. Also, headspace analysis of VOCs derived from breast cancer cells cultured in vitro has demonstrated a variety of unique products, some of which may have arisen from induced cytochrome p450 activity [30].

Breast cancer is the most commonly diagnosed cancer in women, in whom it is second only to lung cancer as a cause of cancer death [31]. The National Cancer Institute estimated that more than 232,000 US women would be diagnosed with breast cancer in 2013 and nearly 40,000 would die of the disease [32]. Breath VOC biomarker analysis offers a new approach to screening women for risk of breast cancer; a breath test could potentially stratify a screening population into groups at low, intermediate, or high risk of breast cancer. Breath tests are painless, cost-effective, and completely safe, and they could potentially reduce the number of needless mammograms that are now performed.

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