Volatile Markers of Breast Cancer in the Breath

Michael Phillips, MD, FACP,*† Renee N. Cataneo, MA,*, Beth Ann Ditkoff, MD,‡ Peter Fisher, MD,§ Joel Greenberg, BS,*, Ratnasiri Gunawardena, MD,¶ C. Stephan Kwon, MD,#, Farid Rahbari-Oskoui, MD,*, and Cynthia Wong, MD†¶

* Menssana Research Inc., Fort Lee, New Jersey, † Department of Medicine, New York Medical College, Valhalla, New York, Departments of ‡ Surgery and § Pathology, Columbia Presbyterian Medical Center, New York, New York, Departments of ¶ Medicine and # Laboratory Medicine, Saint Vincents Catholic Medical Centers of New York, Staten Island Region, Staten Island, New York

Abstract: Breast cancer is accompanied by increased oxidative stress and induction of polymorphic cytochrome P-450 mixed oxidase enzymes (CYP). Both processes affect the abundance of volatile organic compounds (VOCs) in the breath because oxidative stress causes lipid peroxidation of polyunsaturated fatty acids in membranes, producing alkanes and methylalkanes which are catabolized by CYP. We performed a pilot study of breath VOCs, a potential new marker of disease in women with breast cancer. This was a combined case-control and cross-sectional study of women with abnormal mammograms scheduled for a breast biopsy. Breath samples were analyzed by gas chromatography and mass spectroscopy in order to determine the breath methylated alkane contour (BMAC), a three-dimensional display of the alveolar gradients (abundance in breath minus abundance in room air) of C4–C20 alkanes and monomethylated alkanes. BMACs in women with and without breast cancer were compared using forward stepwise discriminant analysis. Two hundred one breath samples were obtained from women with abnormal mammograms and no cancer on biopsy, the breath test identified breast cancer with a sensitivity of 62.7% (32/51) and a specificity of 84.0% (42/50) (cross-validated sensitivity of 60.8% (31/51), specificity of 82.0% (41/50)). The negative predictive value (NPV) of a screening breath test for breast cancer was superior to a screening mammogram (99.93% versus 99.89%); the positive predictive value (PPV) of a screening mammogram was superior to a screening breath test (4.63% versus 1.29%). A breath test for markers of oxidative stress accurately identified women with breast cancer, with an NPV superior to a screening mammogram. This breath test could potentially be employed as a primary screen for breast cancer. Confirmatory studies in larger groups are required.

Key Words: breast cancer, breath, oxidative stress, screening, volatile organic compounds

Cancer of the breast is one of the most common malignancies in women; 10.2% of all white American females develop breast cancer and 3.6% die of the disease. Yet when detected early, breast cancer is also one of the most treatable of all malignancies, and screening mammography in asymptomatic women can reduce mortality by 20–30% (1). However, there is a clinical need for improved methods which can detect the disease in its early stages.

A breath test for markers of oxidative stress might also provide a rational screening tool because increased oxidative stress has been implicated as a risk factor for breast cancer.
cancer (2,3). Oxidative stress occurs when increased quantities of reactive oxygen species (ROS) are produced in the mitochondria and leak into the cytoplasm, where they oxidize biologically important molecules including DNA and proteins (4). ROS also cause lipid peroxidation of polyunsaturated fatty acids in cell membranes, generating alkanes (such as ethane and pentane) and methylated alkanes which are excreted in the breath, where their abundance varies with the intensity of oxidative stress (5,6). Hietanen et al. (7) demonstrated the potential value of breath testing with their finding that breath pentane levels were significantly increased in women with breast cancer. However, pentane is a nonspecific marker of oxidative stress; it is also increased in a number of other conditions including rheumatoid arthritis (8), acute myocardial infarction (9), schizophrenia (10), and bronchial asthma (11).

We have recently reported a breath test for a more extensive set of markers of oxidative stress than pentane alone, the breath methylated alkane contour (BMAC), a three-dimensional display of the alveolar gradients (abundance in breath minus abundance in room air) of C4–C20 alkanes and monomethylated alkanes (6). These breath volatile organic compounds (VOCs) were significantly more abundant in older than in younger healthy humans, a finding consistent with previous reports that aging is accompanied by increased oxidative stress (4,12). These markers were detected with an advanced breath test capable of detecting VOCs present in picomolar concentrations ($10^{-12} \text{ mol/L}$) in the breath (13). We report here a pilot study of the sensitivity and specificity of this breath test as a marker of disease in women with breast cancer.

**MATERIALS AND METHODS**

The study design flow sheet is shown in Figure 1.

**Human Subjects**

Three groups were studied: women with breast cancer found in a breast biopsy, women with no histologic evidence of breast cancer in a breast biopsy, and healthy volunteers. The first two groups comprised 201 women undergoing open surgical biopsy to exclude malignancy. Examination prior to surgery included a physical examination, mammogram, and additional breast imaging where clinically indicated. The women were recruited at two sites: Columbia Presbyterian Medical Center, New York, NY (23 subjects) and Saint Vincents Catholic Medical Centers of New York, Staten Island Region (178 subjects). Patients were eligible to participate if they were ≥18 years of age, had no history of previously diagnosed cancer at any site, and could give written informed consent to participate. The third group comprised healthy volunteers who were recruited from members of the general population with no history of cancer or other chronic disease. One hundred two healthy volunteers were recruited in Staten Island, NY (6), from which an age-matched subgroup of 42 women was selected to serve as a control group for the patients with breast cancer. The institutional review boards of all participating institutions approved the research.

**Detection of Breast Cancer**

All biopsy slides were independently reviewed by two pathologists (P.F. and S.K.) and assessed according to standard criteria for breast cancer (14). There were discordant readings in 3 of 201 biopsies and these were excluded from the data analysis.

**Breath Collection and Assay**

The method has been described in detail elsewhere (13). Samples were collected with a portable breath collection apparatus. The VOCs in 1.0 L of breath and 1.0 L of room air were captured onto separate sorbent traps. The subject wore a nose clip while breathing in and out of the disposable mouthpiece of the apparatus for 2 minutes. Breath samples could be collected without discomfort because light flap valves in the mouthpiece presented low resistance to respiration. Breath samples were collected prior to breast biopsy. Sorbent traps were sent to the laboratory for analysis of VOCs by automated thermal desorption, gas chromatography, and mass spectroscopy.

**Masking Procedures**

Neither pathologist had any knowledge of the breath test results when they examined the biopsies. Research assistants in the laboratory (R.N.C., J.G.) had no knowledge of the clinical or pathologic findings when they assayed the breath samples.

**Derivation of BMAC**

The BMAC in each subject was constructed using alveolar gradients of C4–C20 n-alkanes and monomethylated alkanes. Values were derived from the equation

$$\text{alveolar gradient of a VOC} = \frac{V_b}{I_b} - \frac{V_a}{I_a},$$

where $V_b$ denotes the area under the curve of the VOC peak in the breath chromatogram and $I_b$ denotes the area under the curve of the internal standard used to normalize
the data \(0.25\text{ ml 2 ppm 1-bromo-4-fluoro-benzene (Supelco, Bellefonte, PA)}\). \(V_a\) and \(I_a\) denote corresponding areas derived from the associated air sample (15). The mean alveolar gradients of these VOCs were then computed for the three study groups and the results displayed in a series of surface plots showing the carbon chain length on the \(x\)-axis, the methylation site on the \(z\)-axis, and the mean alveolar gradient on the \(y\)-axis.

### Data Analysis

The plan is shown in Figure 1. Women with breast cancer were compared to age-matched sets of healthy women (model 1) and women with an abnormal mammogram (model 2). Forward stepwise discriminant analysis was performed with SPSS to identify the combination of alkanes and monomethylated alkanes that provided the best discriminators of disease (16). This multivariable technique produced a predictive model (or equation) which estimated the probability of disease for each study subject (17). Cross-validation of the patient’s classification was performed with the SPSS “leave one out” discriminant analysis procedure, which predicted the group to which the patient belonged based on the breath VOC model derived from all the other patients in the model (18).

### Estimation of Predictive Value

The expected positive predictive value (PPV) and negative predictive value (NPV) of a screening breath test and a screening mammogram were compared. A group of 10,000 apparently normal women 60–69 years of age may be assumed to contain 39 women with undiagnosed breast cancer based on a prevalence of 3.3–3.9 cases of breast cancer per thousand patients (19). The expected PPV and NPV were determined for the breath test employing derived values of the sensitivity and specificity, and for screening mammography employing a sensitivity of 75% and a specificity of 94% (20).
RESULTS

Human Subjects and BMACs

Characteristics of the study subjects are shown in Table 1. The mean BMACs of healthy controls, women with breast cancer, and women with negative biopsies are shown in Figure 2.

Women with Breast Cancer versus Healthy Volunteers (Model 1)

We employed statistical analysis to address the question, could a predictive model employing the BMAC distinguish between women with breast cancer and healthy volunteers? Forward stepwise discriminant analysis identified eight VOCs in the BMAC as the best markers of breast cancer (Table 2) and generated a predictive model of disease employing these VOCs. The diagnostic cutoff point or dividing point between a “positive” and “negative” breath test was designated as the point where the sum of sensitivity plus specificity was maximal (Figs. 3 and 4). The model exhibited a sensitivity of 94.1% (48/51) and a specificity of 73.2% (31/42) when the probability of disease was 0.48. When cross-validated using the leave-out jackknife procedure and retaining the same probability of disease as the threshold, the sensitivity was 88.2% (45/51) and the specificity was 73.8% (31/42). Neither smoking status nor tumor histology (in situ carcinoma versus invasive carcinoma) were significant confounders of these results (Table 3).

Women with Breast Cancer versus Women with Abnormal Mammograms (Model 2)

We employed statistical analysis to address the question, could a predictive model employing the BMAC
distinguish between women with breast cancer and women without breast cancer who had an abnormal mammogram? Forward stepwise discriminant analysis identified 10 VOCs as the best set of markers of disease (Figs 1 and 4). The model exhibited a sensitivity of 62.7% (32/51) and a specificity of 84.0% (42/50) when the sum of sensitivity plus specificity was maximal. Cross-validation of the model exhibited a sensitivity of 60.8% (31/51) and a specificity of 82.0% (41/50).

**DISCUSSION**

This study demonstrated three main findings. First, breath markers of oxidative stress distinguished between women with breast cancer and healthy controls. Second, these breath markers also distinguished between women with breast cancer and women without breast cancer who had an abnormal mammogram. Third, the estimated NPV of a screening breath test was superior to a screening mammogram.

A subset of eight VOCs in the BMAC accurately distinguished between women with breast cancer and healthy volunteers. These observations are consistent with previous

<table>
<thead>
<tr>
<th>Smoking Status</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmokers</td>
<td>83.9% (26/31)</td>
<td>80.0% (16/20)</td>
</tr>
<tr>
<td>Smokers</td>
<td>91.2% (11/12)</td>
<td>33.3% (1/3)</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>83.3% (5/6)</td>
<td>81.8% (9/11)</td>
</tr>
<tr>
<td>In situ carcinoma</td>
<td>100.0% (7/7)</td>
<td></td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>86.4% (38/44)</td>
<td></td>
</tr>
</tbody>
</table>

The predictions of the breath test developed in model 1 (women with breast cancer versus healthy age-matched controls) are shown stratified according to smoking status and tumor histology. Tobacco smoking history was available for 49 of 51 breast cancer patients and 24 of 42 controls. All possible pairs were compared by chi-squared test and none achieved statistical significance.

<table>
<thead>
<tr>
<th>Table 3. Effects of Smoking Status and Tumor Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>Nonsmokers</td>
</tr>
<tr>
<td>Smokers</td>
</tr>
<tr>
<td>Ex-smokers</td>
</tr>
<tr>
<td>In situ carcinoma</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
</tr>
</tbody>
</table>

In women 60–69 years of age the prevalence of breast cancer is 3.3–3.9/1000, so that a group of 10,000 women will include 39 with previously undetected breast cancer (19). The table shows the predicted outcome of screening this group with a breath test (sensitivity 88.2%, specificity 73.8%) or a mammogram (sensitivity 75%, specificity 94%) (20). The breath test is more sensitive and less specific than a screening mammogram, and a screening breath test would exhibit a higher negative predictive value (NPV) in a lower positive predictive value (PPV) than a screening mammogram.
reports that breast cancer is accompanied by increased oxidative stress (21) and induction of cytochrome P-450 mixed oxidase enzymes (22,23). Alkanes and methylated alkanes are markers of oxidative stress because they are the degradation products of membrane polyunsaturated fatty acids (PUFAs) which have undergone lipid peroxidation by reactive oxygen species (ROS) liberated from mitochondria (5,24). Disruption of membranes by oxidative stress may progress to cell dysfunction and death. The evolved VOCs are either degraded by cytochrome P-450 (CYP) enzymes or excreted in the breath. We have previously reported changes in the abundance of these VOCs in the breath of patients with lung cancer, some of which were consistent with induced CYP activity (25).

Compared to age-matched healthy women, several breath VOCs were either increased or decreased in abundance in women with breast cancer. This finding is consistent with two different mechanisms operating simultaneously: increased oxidative stress may account for the VOCs whose abundance was increased, and increased cytochrome P-450 activity may account for the VOCs whose abundance was decreased.

The cytochrome P-450 (CYP) system comprises a group of inducible mixed-function oxidase enzymes that metabolize drugs and the VOCs produced by oxidative stress. Several P-450 genes are polymorphic and are associated with an increased risk of cancer development in specific tissues, while individual P-450 enzymes are over-expressed in different types of tumors (21). CYP enzymes are also present in human breast tissue, where they appear to be activated in cancer. Murray (22) reported that cytochrome P-450 CYP1B1 was expressed in cancers of the breast as well as other tissues. Huang et al. (23) detected activity of the xenobiotic-metabolizing CYP1, CYP2, and CYP3 subfamilies of cytochrome P-450 in human breast tissue.

The expected NPV of a screening breath test was superior to the NPV of a screening mammogram. If validated, this finding could potentially influence clinical practice. A woman with a negative screening breath test may not need to proceed to a screening mammogram because the additional test may provide no additional clinical evidence that disease has been ruled out. Since the breath test would be expected to be negative for breast cancer in 7356 of 10,000 women more than 60 years old, a screening breath test could potentially reduce the number of screening mammograms performed in this group by more than 70%.

The BMACs demonstrated differences between healthy volunteers and women with an abnormal mammogram whose biopsies were negative for cancer (Fig. 2). This finding suggests that markers of oxidative stress were abnormal in women with abnormal mammograms even though no tumor was detectable. It is possible that non-malignant conditions sufficient to cause detectable lesions on mammography also induced abnormal oxidative stress and/or induction of CYP mixed-oxidase enzymes. It is also possible that a number of women in this group may have had premalignant conditions or tumors that were not detected at biopsy.
There may be a continuum of abnormality in markers of oxidative stress in which abnormal mammograms are associated with changes intermediate between normality and breast cancer. However, this hypothesis requires further investigation and validation.

The main limitation of this pilot study was the comparatively small number of subjects. The sensitivity and specificity of a statistical model constructed with discriminant analysis generally improves with the number of variables employed, but the number of VOCs in the breast cancer model was limited to eight because of the generally accepted convention that at least five subjects are required for each variable in the model. Consequently, future studies employing larger numbers of subjects are likely to generate predictive models with greater sensitivity and specificity.

Concomitant disease, such as infection or inflammation in other tissues, could potentially act as a confounding source of oxidative stress and skew the results of the breath test. We attempted to control for this possibility with an experimental design in which we prospectively studied a group of women with abnormal mammograms who were subsequently found to be either positive or negative for breast cancer. Provided that any concomitant disease (such as a bacterial infection) was not a consequence of breast cancer, the a priori prevalence of concomitant disease should have been equal in both groups, so the outcome of the study should not have been affected. We recommend that future studies of breath markers of breast cancer incorporate a prospective design in order to control for concomitant diseases which might potentially skew the clinical findings.

We conclude that a breath test for volatile markers of oxidative stress appeared to provide a sensitive and specific set of biomarkers for breast cancer. Breath testing could potentially provide a noninvasive, safe, and cost-effective screening test for breast cancer. However, these findings will require validation in larger clinical studies.

Acknowledgments

This research was supported by STTR grant no. 1 R41CA81842-01 from the National Institutes of Health. We thank Eugene Sersen, PhD for statistical consultation and review.

REFERENCES


