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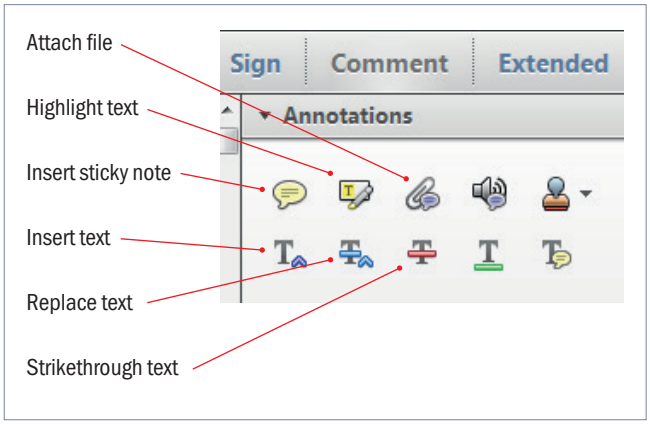

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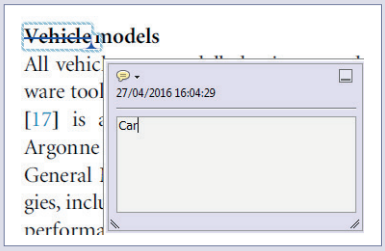

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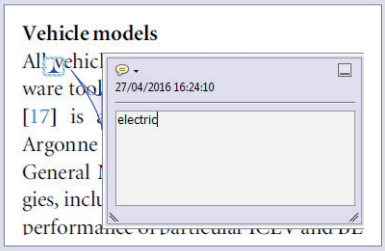

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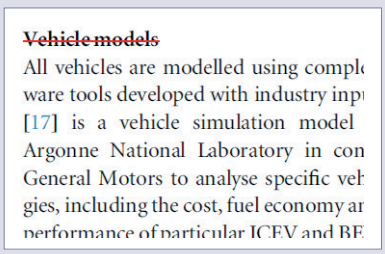

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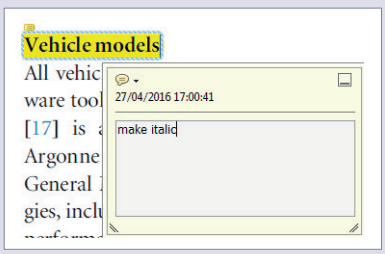

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PAPER

Breath mass ion biomarkers of breast cancer

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Abstract

Breath volatile organic compounds (VOCs) contain biomarkers of breast cancer that are detectable with gas chromatography mass spectrometry (GC MS). However, chemical identification of breath VOC biomarkers may be erroneous because spectral matching can misidentify their structure. Breath mass ions detected with GC MS have been proposed as intrinsically robust biomarkers because they can be identified without spectral matching. We investigated whether breath mass ion biomarkers could identify breast cancer. We re-analyzed data from a previous study of breath VOCs in 54 women with biopsy-proven breast cancer and in 204 healthy controls. Subjects were randomly assigned to a training set (2/3) and a test set (1/3). Chromatograms were processed with metabolomic analysis software (XCMS in R) in order to generate a table listing retention times with their associated ion masses and intensities, and binned into a series of 5 s retention time segments. In the training set, mass ions in each time segment were ranked according to their diagnostic accuracy i.e. the area under curve (AUC) of the receiver operating characteristic (ROC) curve. We employed multiple Monte Carlo simulations to select the biomarker mass ions in each time segment that identified breast cancer with greater than random accuracy and combined those with the highest diagnostic accuracy in a predictive algorithm using multivariate weighted digital analysis (WDA). We then employed this algorithm to predict the diagnosis in the test set. The training set WDA algorithm employing 21 mass ion biomarkers identified breast cancer with ROC curve AUC $=$ 0.79. In the test set, this algorithm predicted breast cancer with ROC curve AUC $=$ 0.77. Breath mass ions biomarkers accurately identified women with breast cancer and could potentially be used in early diagnosis and treatment monitoring.

Normal human breath contains more than 1000 different volatile organic compounds (VOCs), and some have been identified as candidate biomarkers of breast cancer [1–3]. However, breath biomarkers of disease are not yet widely employed in clinical practice, mainly because there is a lack of general agreement about their chemical structures. Probably the best-known example is lung cancer: several clinical studies have found statistically significant evidence of breath VOC biomarkers in patients with lung cancer, but the reported chemical structures of these biomarkers has varied widely between different studies [4–9]. This wide divergence in reported chemical structures may have arisen from several potential causes, including differences in experimental design and methods of sample collection and analysis.

The technical limitations of current analytical tools comprise another potential source of experimental error. The tool most widely employed for breath VOC biomarker discovery is gas chromatography mass spectrometry (GC MS): a sample of concentrated breath VOCs is injected onto a chromatographic column that separates the complex mixture into a series of individual VOCs according to their physicochemical properties such as boiling point and polarity. The separated VOCs then flow into a

detector where they are broken into fragments by a beam of high-energy electrons in a vacuum, and the resulting mass spectrum of fragments comprises a 'fingerprint' that can be used to identify the VOC from a computer-based spectral library.

GC MS is a widely accepted tool, but it can potentially yield erroneous identification of analytes if a mixture as complex and diverse as human breath VOCs overburdens the separation column. If the separation of VOCs is incomplete, then two or more VOCs may enter the MS detector simultaneously, and their combined mass spectra may lead to misidentification of their chemical structures in the spectral library. This may account, in part, for the diversity of breath VOC biomarkers of lung cancer that different investigators have reported.

In response to this problem, we investigated breath mass ions as candidate biomarkers of disease. An individual mass ion may provide an intrinsically more robust biomarker than the mass spectrum of its parent biomarker VOC for two main reasons: first, it is less likely to be affected by coelutions and second, it does not require pattern matching in a spectral library. We recently evaluated this approach in a blinded study of subjects with lung cancer, and found that breath mass ion biomarkers accurately predicted disease [10]. In order to further evaluate the diagnostic potential of breath mass ion biomarkers, we report here a re-analysis of data from a previous study of breath VOC biomarkers of breast cancer [2].

Methods and materials

Human subjects

The clinical study has been previously reported [2]. In summary, we analyzed breath VOCs in 54 women with biopsy-proven breast cancer and 204 cancer-free controls, using gas chromatography/mass spectrometry. Subjects 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. 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 Ethics Committees of the University of Western Australia and the Royal Perth Hospital approved the research.

Breath VOC collection and assay

The method has been described [11]. In summary, we employed a portable breath collection apparatus to capture breath VOCs on to sorbent traps that were analyzed by automated thermal desorption, gas chromatography and mass spectrometry. 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) (BFB).

Re-analysis of data

All subjects and their respective chromatograms of breath VOCs were randomly assigned to either a training set or a test set in a 2/3: 1/3 split.

Selection of breath mass ion biomarkers of breast cancer

This was performed in the training set; the methods have been described [10]. In summary, we reprocessed chromatograms to generate a table of ion masses with their intensities and retention times normalized to BFB. Aligned data was 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 area under curve (AUC) of its receiver operating characteristic (ROC) curve. 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.

XCMS was only used to convert the original data from the chromatograms into a format that can be stored in a database. The data stored had the fields retention time (in 1/100 of a second), ionic component (M/Z) and measured area.

In a first step all retention times were corrected by the retention time of the BFB marker, reducing the variability from around 12 –3 s.

Further, the area of every entry was divided by the area of the BFP peak yielding a value called abundance. This abundance was used for further analysis.

In order to reduce the data for analysis every 5 s were binned together, i.e. 5 s interval, ionic component and abundance. The value 5 s is a compromise between the typical width of a VOC (7 s) and the reduction factor. From then on every interval, M/Z and abundance was investigated as a potential biomarker.

Development and evaluation of predictive algorithm

We employed multivariate weighted digital analysis (WDA) [12] to construct a predictive algorithm using the mass ions that identified breast cancer with greater than random accuracy in the training set. This algorithm was then employed to predict the presence or absence of breast cancer in the test set.

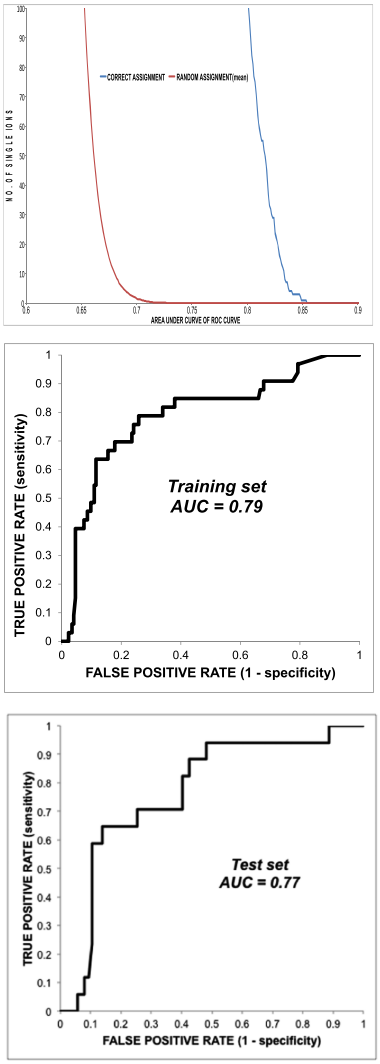

Figure 1. *(Top panel) Monte Carlo statistical analysis of mass ions*. This analysis was performed in a training set that comprised a random selection of two thirds of the study subjects. 21 mass ions exhibited greater than random diagnostic accuracy (C-statistic > 0.6) with correct assignment compared to multiple random assignments. The C-statistic of a mass ion is the area under curve (AUC) of its receiver operating characteristic (ROC). *(Middle panel) receiver operating characteristic (ROC) curve of breath mass ions in training set*. This figure displays the sensitivity and specificity for breast cancer in the training set, employing the multivariate algorithm that was derived from the combination of mass ions exhibiting greater than random diagnostic accuracy. The AUC of a ROC curve (or its C-statistic) indicates the overall accuracy of a test, and may vary from 0.5 (a straight line from bottom left to top right of the graph) to 1.0 (a right angle with its apex at the top left of the graph). A C-statistic of 0.5 indicates that the test performance was no better than random e.g. flipping a coin, while a C-statistic of 1.0 indicates a perfect test with 100% sensitivity and specificity. *(Bottom panel) receiver operating characteristic (ROC) curve in test set*. This figure displays the sensitivity and specificity for breast cancer of the algorithm in the test set that comprised the remaining one third of the study subjects. No data from these subjects was employed in the derivation and construction of the predictive algorithm.

Results

Monte Carlo statistical analysis of mass ions in the training set (figure 1, top panel)

The 'correct assignment' curve displays the number of mass ions as a function of their diagnostic accuracy, defined as the AUC of its associated ROC curve. The 'random assignment' curve similarly displays the number of mass ions as a function of their diagnostic accuracy employing the mean of 50 random assignments of diagnosis ('cancer' or 'cancer-free'). Where the random assignment curve fell to zero at approximately AUC $= 0.6$, 21 mass ions in the correct assignment curve exhibited diagnostic accuracy that was superior to random behavior.

ROC curve in training set (figure 1, middle panel)

The mass ions that exhibited diagnostic accuracy superior to random behavior (identified in top panel) were combined in a multivariate algorithm employing WDA. This algorithm identified breast cancer in the training set with 79% accuracy.

ROC curve in test set: (figure 1, bottom panel)

The multivariate predictive algorithm derived in the test set (top two panels) predicted breast cancer in the test set with 77% accuracy.

Example of mass ion biomarkers in cancer patients and controls

Table 1 displays the aggregated abundance of mass ion biomarkers in a segment of the chromatogram (1350–1375 s) in six randomly selected subjects, three with breast cancer and three cancer-free controls.

Discussion

The main finding of this study was that breath mass ions biomarkers accurately identified women with breast cancer. The training set algorithm employing 21 mass ion biomarkers identified breast cancer with ROC curve AUC $= 0.79$. In the test set, this algorithm predicted breast cancer with ROC curve AUC $= 0.77$. These findings are consistent with previous reports that biomarkers in breath can identify women with breast cancer.

The pathophysiologic mechanisms underlying breath biomarkers of breast cancer are not known. However, increased oxidative stress in breast tissue provides a plausible mechanism that links cancer development with altered biomarkers in breath. In breast cancer, almost 80% of stromal fibroblasts acquire an activated phenotype that produces hydrogen peroxide, which induces tumorigenic alterations in epithelial cells and fuels the growth and survival of cancer cells [13]. Hydrogen peroxide is a powerful oxidant that causes oxidative stress in tissues, which is known to peroxidate polyunsaturated fatty acids in

Table 1. Example of mass ion biomarkers in cancer patients and controls: this table displays the aggregated abundance of mass ion biomarkers (M/Z 56 to 61) in a segment of the breath VOC chromatogram (1350–1375 s) in six randomly selected subjects, three with breast cancer and three cancer-free controls. It is apparent that the abundance of these mass ions was consistently lower in the breast cancer subjects than in the controls.

MZ value	56	57	58	59	60	61
Control #1	2 653 243	3 206 919	3 687 286	487 286	441 990	525 678
Control #2	7 310 221	3 232 056	7 027 963	555 546	238 182	199 806
Control #3	7 607 665	2 467 155	1 887 122	369 912	307 363	135 114
Breast cancer #1	1 600 950	605 261	96 075	63 801	235 209	34 914
Breast cancer #2	1 367 103	365 763	72 178	44 597	605 709	44 103
Breast cancer #3	308 432	284 385	160 276	29 227	99 903	32 764

cell membranes, liberating volatile *n*-alkanes (e.g. ethane and pentane) that are expired in the breath [14]. Future studies of stromal fibroblasts *in vitro* could test the hypothesis that they are a source of the VOCs and mass ions observed in breath as biomarkers of breast cancer.

We previously identified breath mass ion biomarkers of lung cancer employing instruments and methodology similar to those employed in this study [10], and other researchers employing different instrumentation have reported mass ions in breath and in bacteria that also have potential clinical applications for detection of disease [15–17].

We conclude that breath mass ion biomarkers appeared to provide accurate new biomarkers of breast cancer. We employed mass ion biomarkers in order to control for potential coelutions of VOCs that may result from separation with one-dimensional gas chromatography. However, the newer technique of two-dimensional gas chromatography provides improved selectivity of separation of VOCs and could potentially reduce the need for this approach in future studies [1].

The findings of this pilot study are consistent with previous reports of breath biomarkers of breast cancer that employed different detection methods, but this will require validation in future prospective blinded clinical studies.

The ability to detect early breast cancer with a simple breath test provides opportunities to improve patient outcomes. For example, when two tests with different biological mechanisms are employed together to screen for the same disease, the combination may provide greater accuracy than either test when it is used alone [18]. Consequently, it may be possible to increase the sensitivity and the specificity of breast cancer detection by combining a breath test with a screening mammogram. We are currently testing this hypothesis in a prospective clinical study to determine the effects of an ancillary breath test on the sensitivity and specificity of mammography (https://clinicaltrials.gov/ct2/show/NCT02888366).

This includes enhancing the sensitivity and specificity of population mammographic screening, early detection of recurrent cancer to allow change in management, and monitoring response to cancer treatment, enabling early discontinuation of futile, often toxic, therapies, and a switch to potentially more efficacious treatments, thus improving both quality and quantity of life for people with breast cancer.

Acknowledgments

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