

# Breath Markers of Oxidative Stress in Patients with Unstable Angina

Michael Phillips, MD, FACP\*†, Renee N. Cataneo, MA,\* Joel Greenberg, BS,\* Richard Grodman, MD‡, and Manuel Salazar, MD‡§

**Abstract:** Cardiac chest pain is accompanied by oxidative stress, which generates alkanes and other volatile organic compounds (VOCs). These VOCs are excreted in the breath and could potentially provide a rational diagnostic marker of disease. The breath methylated alkane contour (BMAC), a 3-dimensional surface plot of C4–C20 alkanes and monomethylated alkanes, provides a comprehensive set of markers of oxidative stress. In this pilot study, we compared BMACs in patients with unstable angina pectoris and in healthy volunteers. Breath VOCs were analyzed in 30 patients with unstable angina confirmed by coronary angiography and in 38 age-matched healthy volunteers with no known history of heart disease (mean age  $\pm$  SD,  $62.7 \pm 12.3$  years and  $62.5 \pm 10.0$ , not significant). BMACs in both groups were compared to identify the combination of VOCs that provided the best discrimination between the 2 groups. Forward stepwise entry discriminant analysis selected 8 VOCs to construct a predictive model that correctly classified unstable angina patients with sensitivity of 90% (27 of 30) and specificity of 73.7% (28 of 38). On cross-validation, sensitivity was 83.3% (25 of 30) and specificity was 71.1% (27 of 38). We conclude that the breath test distinguished between patients with unstable angina and healthy control subjects.

**Key Words:** ischemic heart disease, oxidative stress, oxygen free radicals, volatile organic compounds

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There is a clinical need for new diagnostic markers that can accurately differentiate between high-risk patients with cardiac chest pain who require hospitalization and low-risk patients with noncardiac chest pain who can be safely eval-

uated as outpatients. Breath testing has been proposed as a candidate marker of cardiac chest pain because ischemic heart disease is accompanied by increased oxidative stress,<sup>1,2</sup> a condition that elicits production of volatile metabolites, such as ethane and pentane, that are excreted in the breath.<sup>3,4</sup> Increased breath pentane has been reported in patients with acute myocardial infarction<sup>5</sup> and congestive heart failure,<sup>6</sup> but these studies have been challenged because the assay technique may have measured a mixture of isoprene and pentane in the breath.<sup>7</sup> Also, breath pentane is now recognized as a nonspecific marker of oxidative stress that may be increased in other conditions, including rheumatoid arthritis,<sup>8</sup> bronchial asthma,<sup>9</sup> schizophrenia,<sup>10</sup> and vitamin E deficiency.<sup>11</sup>

However, improved microanalytic methods have demonstrated that a typical sample of normal human breath contains more than 200 different volatile organic compounds (VOCs), most of them in picomoles per liter (10–12 mol/L) concentrations.<sup>12</sup> More than 3000 different VOCs have been observed in human breath, including several apparent new markers of oxidative stress.<sup>13</sup> We have identified a comprehensive set of markers of oxidative stress, the breath methylated alkane contour (BMAC), which comprises a 3-dimensional surface plot of the abundance of C4–C20 alkanes and their monomethylated derivatives.<sup>14</sup> The BMAC displayed significant changes with increasing age, heart transplant rejection<sup>15</sup>, and oxygen breathing.<sup>16</sup> We report here a pilot study that compared the BMAC in patients with unstable angina pectoris with that of healthy volunteers.

## MATERIALS AND METHODS

### Human subjects

30 patients with unstable angina (according to Braunwald criteria<sup>17</sup>) were studied at St. Vincent's Medical Center, New York City. All had presented with acute chest pain, and the diagnosis of unstable angina was confirmed by coronary angiography ( $\geq 80\%$  stenosis, with or without associated thrombus, with or without corresponding wall motion abnormality). Breath tests were performed after an overnight fast. Breath tests were also performed in a control group of healthy volunteers in Staten Island, NY<sup>13</sup>; 38 age-matched control

\*From Menssana Research Inc, Fort Lee, NJ; the †Department of Medicine, New York Medical College, Valhalla, NY; and the ‡Department of Medicine, Saint Vincent's Catholic Hospitals of New York, Staten Island, NY. §Present address: 12033 97th Avenue North, Seminole, Florida.

Address reprint requests to Michael Phillips MD, FACP, Menssana Research, Inc, 1 Horizon Road, Suite 1415, Fort Lee, NJ 07024. menssana@bellatlantic.net

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subjects with no known history of heart disease were selected from this data base. The institutional review board approved the research, and all subjects gave their signed informed consent to participate.

### Breath collection and assay

The method has been described previously.<sup>12,13</sup> A portable breath collection apparatus was employed to capture the VOCs in 1.0-L breath on a sorbent trap; the VOCs in 1.0 L of room air were then captured on a separate sorbent trap. Subjects wore a nose clip while breathing in and out of the disposable mouthpiece of the apparatus for 2.0 minutes. Light flap valves in the mouthpiece presented low resistance to respiration, so that breath samples could be collected without discomfort. All sorbent traps were sent to the laboratory for analysis of VOCs by automated thermal desorption, gas chromatography, and mass spectroscopy.

### Derivation of BMACs

The method for generating 3-dimensional surface plots of C4–C20 n-alkanes and their monomethylated derivatives has been described previously.<sup>14</sup> For each breath VOC,  $V_b$  denotes the area under the curve associated with the chromatogram peak, and  $I_b$  denotes the analogous area associated with the internal standard used to calibrate the instrument (0.25 mL of 2 ppm 1-bromo-4-fluoro-benzene (Supelco, Bellefonte, PA).  $V_a$  and  $I_a$  denote corresponding areas de-

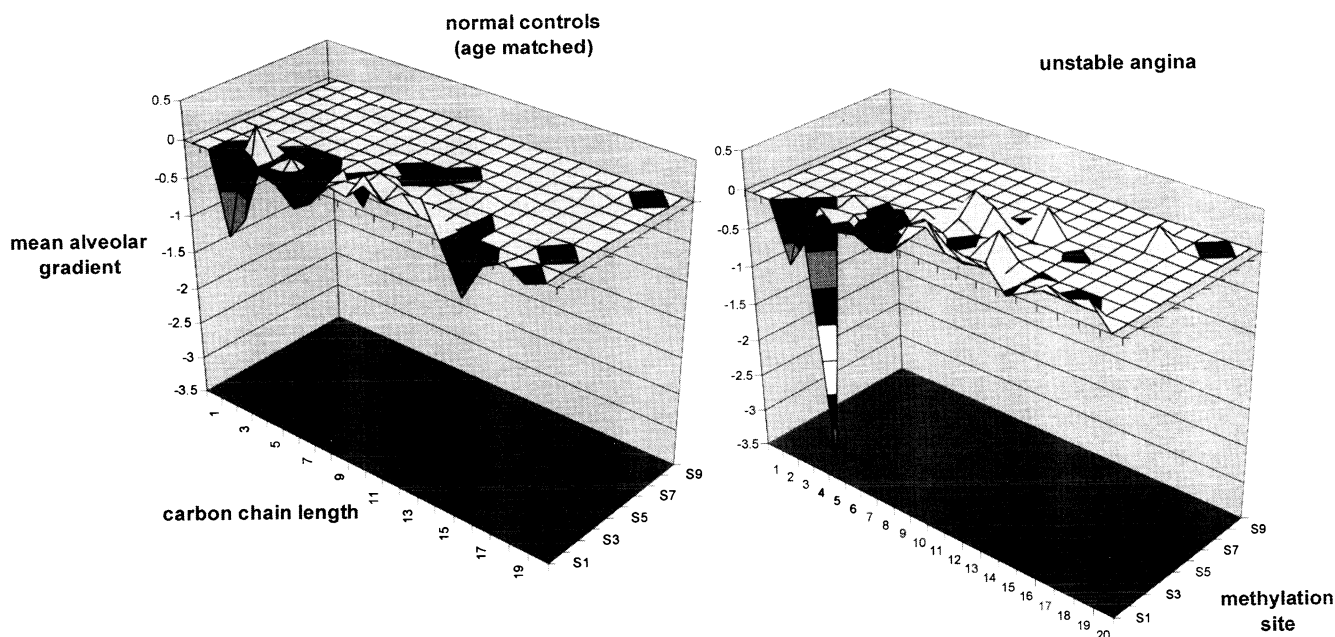
rived from the associated sample of room air. The alveolar gradient<sup>13</sup> of each VOC was then determined using the equation  $\text{alveolar gradient} = V_b/I_b - V_a/I_a$ . The mean alveolar gradients of these VOCs were computed for the patients with unstable angina and for the healthy control subjects, then displayed in 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.

### Statistical analysis of data

Forward stepwise discriminant analysis<sup>18</sup> was used to identify the combination of VOCs that provided the best discrimination between patients with unstable angina and healthy volunteers and to construct a predictive model. The accuracy of this predictive model was first tested by cross-validation using a leave-one-out technique, in which each subject was classified using an equation derived from all other subjects.<sup>19</sup> A receiver operating characteristic (ROC) curve<sup>20</sup> was constructed to display the sensitivity and specificity of the breath test.

## RESULTS

Mean ages of patients with unstable angina and age-matched healthy volunteers were 62.7 (SD = 12.3) and 62.5 years (SD = 10.0) respectively (not significant). Breath samples were collected from all subjects without adverse



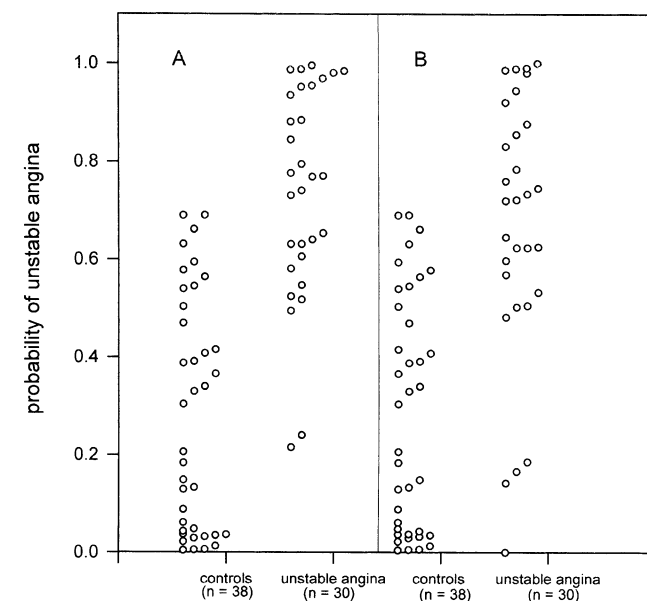
**FIGURE 1.** Surface plots of breath test results. The mean alveolar gradient (concentration in breath minus concentration in room air) is shown on the vertical axis for C4–C20 alkanes and their monomethylated derivatives. The horizontal axes identify the specific VOC (eg, the combination of carbon chain length of 4 and methylation site at S2 corresponds to 2-methylbutane). A number of the mean alveolar gradients seem increased in patients with unstable angina; those selected as optimal discriminators between the 2 groups are listed in Table 1.

effects. The mean BMACs in patients with unstable angina and in healthy control subjects are shown in Figure 1. Forward stepwise entry discriminant analysis selected 8 VOCs to construct a predictive model (Table 1). Retrospective and cross-validated classification of subjects with this model is shown in Figure 2 and the ROC curves are shown in Figure 3. Where the sum of sensitivity and specificity was maximal, this model correctly classified patients with unstable angina with sensitivity of 90.0% (27 of 30) and specificity of 73.7% (28 of 38). Cross-validation of this model using a leave-one-out technique predicted unstable angina with sensitivity of 83.3% (25 of 30) and specificity of 71.1% (27 of 38). A similar analysis employing 6 VOCs correctly classified patients with unstable angina with sensitivity of 90.0% (27 of 30) and specificity of 65.8% (25 of 38) [cross-validated sensitivity of 83.3% (25 of 30) and specificity of 65.8% (25 of 38)].

## DISCUSSION

We found that breath markers of oxidative stress were more abundant in patients with unstable angina than in age-matched healthy control subjects, and a statistical model employing these breath markers accurately distinguished between the 2 groups. These findings are consistent with previous reports of increased oxidative stress in ischemic heart disease as shown by elevations in a variety of different blood and urine markers, including thiobarbituric acid reactive substances,<sup>21,22</sup> biopyrrins (oxidative metabolites of bilirubin),<sup>23</sup> platelet lipid peroxidation,<sup>24</sup> malondialdehyde-modified low-density lipoprotein,<sup>25</sup> thioredoxin,<sup>26</sup> xanthine and hypoxanthine,<sup>27</sup> lipid hydroperoxides,<sup>22,28</sup> conjugated dienes, and total radical-trapping antioxidant capacity.<sup>28</sup>

Oxidative stress is a condition caused by increased leakage of reactive oxygen species (ROS) from the mitochondria



**FIGURE 2.** Predictions of the discriminant model in patients with unstable angina and healthy volunteers. The scatter diagram indicates the probability of unstable angina predicted by the discriminant model employing the 8 breath VOCs listed in Table 1. Left, results obtained with retrospective analysis of data; right, predictions of the cross-validated model.

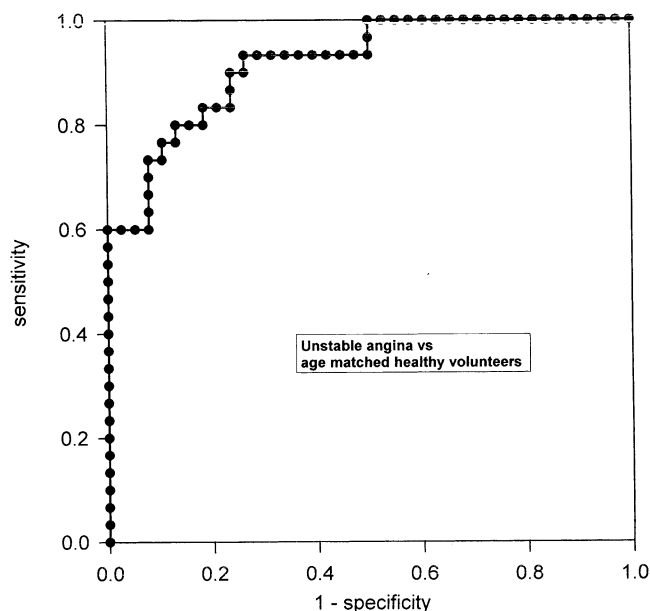
dria into the cytoplasm of a cell.<sup>29</sup> ROS energetically oxidize polyunsaturated fatty acids, DNA, proteins, and other biologically important molecules; this diversity of target molecules accounts for the variety of different breakdown products that have been employed as markers of oxidative stress.<sup>30</sup> Lipid peroxidation of polyunsaturated fatty acids generates volatile alkanes and methylated alkanes that are excreted in the breath, where they provide noninvasive markers of the intensity of oxidative stress.<sup>3,4</sup>

Oxidative stress in the ischemic myocardium may arise from a combination of increased formation of ROS and decreased antioxidant reserve. The increase in ROS generation has been variously ascribed to impaired mitochondrial reduction of molecular oxygen, secretion of ROS by white blood cells, endothelial dysfunction, auto-oxidation of catecholamines, and exposure to radiation or air pollution.<sup>31</sup> The major deleterious effect of ROS seems to be damage to subcellular organelles, resulting in intracellular overload of calcium.<sup>1</sup> These findings provide a rational basis for therapeutic trials of antioxidant drugs and diets in patients with ischemic heart disease<sup>32-34</sup> but the efficacy of this approach is still inconclusive. Clinical studies of the therapeutic and diagnostic role of oxidative stress in ischemic heart disease are complicated by the effects of aging, which independently increases the abundance of components of the BMAC<sup>14</sup> as well as other markers such as isoprostanes<sup>35</sup> and pentane.<sup>36</sup>

**TABLE 1.** VOCs Selected as Discriminators of Unstable Angina

Component*	P
Octane, 4-methyl-	<0.0001
Decane, 4-methyl-	<0.0001
Hexane	<0.001
Pentadecane, 5-methyl-	<0.001
Hexadecane, 7-methyl-	<0.001
Propane, 2-methyl-	<0.001
Pentane	<0.001
Butane, 2-methyl-	<0.02

\*These components of the BMACs shown in Figure 1 were selected by forward stepwise discriminant analysis as the best discriminators between patients with unstable angina pectoris and age-matched healthy controls. VOCs are ranked according to the probability that they were not selected by chance.



**FIGURE 3.** Classification of patients with ischemic heart disease. The ROC curve displays the predictions of the retrospective model employing 8 breath VOCs. Where the sum of sensitivity and specificity was maximal, the retrospective model correctly classified patients with unstable angina with sensitivity of 96.7% (29 of 30) and specificity of 92.1% (35 of 38). The cross-validated model predicted unstable angina with sensitivity of 83.3% (25 of 30) and specificity of 86.6% (33 of 38).

The study was limited by the method of statistical analysis. An important feature of multivariate analysis is that accuracy of the classification model (unstable angina versus control subjects) generally improves as more variables are employed in the model. We observed better specificity with an 8-VOC model than with a 6-VOC model, though sensitivity was not affected. Generally, it is common to employ at least 5 subjects for each variable employed in a classification model. Because this was a comparatively small pilot study, it is possible that future studies of larger patient populations may improve both the sensitivity and specificity of the breath test because it would permit a larger number of VOCs to be employed in the classification model.

Another limitation of this study was the lack of clinical information about heart disease in the healthy control subjects. All denied any history of ischemic heart disease or chest pain, but in view of their mean age of 62.5 years, a number of them may have suffered from occult coronary artery disease. Future studies will require larger numbers of subjects with documented unstable angina, control subjects with confirmed freedom from ischemic heart disease, and an evaluation of potential confounding factors such as tobacco smoking and concomitant disease. Also, because patients

with unstable angina/non-ST-segment elevation myocardial infarction present with a wide spectrum of risk for death and cardiac ischemic events, it would be worthwhile to correlate the results of future studies of breath markers of oxidative stress with risk factors such as the thrombolysis in myocardial infarction (TIMI) score.<sup>37</sup>

We conclude that breath markers of oxidative stress accurately distinguished between patients with unstable angina and healthy control subjects. Prospective clinical trials are required to evaluate this breath test for its ability to differentiate between cardiac and noncardiac chest pain.

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