

Heart Allograft Rejection: Detection With Breath Alkanes in Low Levels (the HARDBALL Study)

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Background: We evaluated a new marker of heart transplant rejection, the breath methylated alkane contour (BMAC). Rejection is accompanied by oxidative stress that degrades membrane polyunsaturated fatty acids, evolving alkanes and methylalkanes, which are excreted in the breath as volatile organic compounds (VOCs).

Methods: Breath VOC samples ($n = 1,061$) were collected from 539 heart transplant recipients before scheduled endomyocardial biopsy. Breath VOCs were analyzed by gas chromatography and mass spectroscopy, and BMAC was derived from the abundance of C₄–C₂₀ alkanes and monomethylalkanes. The “gold standard” of rejection was the concordant set of International Society for Heart and Lung Transplantation (ISHLT) grades in biopsies read by 2 reviewers.

Results: Concordant biopsies were: Grade 0, 645 of 1,061 (60.8%); 1A, 197 (18.6%); 1B, 84 (7.9%); 2, 93 (8.8%); and 3A, 42 (4.0%). A combination of 9 VOCs in the BMAC identified Grade 3 rejection (sensitivity 78.6%, specificity 62.4%, cross-validated sensitivity 59.5%, cross-validated specificity 58.8%, positive predictive value 5.6%, negative predictive value 97.2%). Site pathologists identified the same cases with sensitivity of 42.4%, specificity 97.0%, positive predictive value 45.2% and negative predictive value 96.7%.

Conclusions: A breath test for markers of oxidative stress was more sensitive and less specific for Grade 3 heart transplant rejection than a biopsy reading by a site pathologist, but the negative predictive values of the 2 tests were similar. A screening breath test could potentially identify transplant recipients at low risk of Grade 3 rejection and reduce the number of endomyocardial biopsies. *J Heart Lung Transplant* 2004;23:701–8.

More than 61,000 heart transplant operations have been performed since 1967; at least 23,000 of the recipients are presently known to be alive, although the actual number of survivors may exceed 30,000.¹ All these recipients require periodic screening for rejection, a condition that is difficult to detect clinically. Symptoms such as malaise, fatigue, dyspnea, edema and anorexia are uncommon because ventricular function is usually not affected. Right ventricular endomyocardial biopsy is the current “gold

standard” for diagnosis of heart transplant rejection, and post-operative biopsies are generally performed weekly for the first 6 weeks, biweekly until the third month, monthly until the sixth month, then every 1 to 3 months depending on clinical indications. However, most biopsies yield normal or near-normal results that elicit no changes in treatment. Although considered safe, the procedure is invasive and may cause complications such as hematoma, infection, arrhythmia, ventricular perforation and fistulas.

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patents issued and pending on the breath test employed in this study. None of the other authors has any conflict of interest or financial interest with regard to this article.

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Also, a randomized study of biopsy readings by different pathologists found discrepancies between their grading of rejection sufficient to demonstrate adverse treatment implications.² Many attempts have been made to develop non-invasive tests for heart transplant rejection. Several testing procedures have been proposed, including magnetic resonance imaging; antibody imaging; echocardiography; and use of serum markers such as troponin I, troponin T, creatine kinase-MB fraction and C-reactive protein.^{3,4} However the accuracy of these approaches is generally insufficient to guide clinical decision-making for individual patients.

Breath microanalysis has been proposed as a non-invasive test for heart transplant rejection.⁵ The rationale of a breath test is based on 2 observations: first, allograft rejection is accompanied by oxidative stress resulting from increased production of reactive oxygen species (ROS) in the myocardium;⁶ and, second, ROS degrade cellular membranes by lipid peroxidation of polyunsaturated fatty acids (PUFAs), generating alkanes that are excreted in the breath as volatile organic compounds (VOCs).^{7,8} These VOCs may provide markers of the intensity of rejection.

Despite the rational basis of a breath test for heart transplant rejection there are formidable technical obstacles in practice. First, the breath test must be sufficiently sensitive to detect VOCs excreted in picomolar (10^{-12} mol/liter) concentrations. Existing laboratory instruments cannot detect such low concentrations, so that breath VOCs must be collected and assayed with specialized instruments.⁹ Second, the breath VOC assay must be sufficiently specific to distinguish different VOCs from one another. Previous reports have been criticized because breath pentane assays may have been contaminated by isoprene, the most abundant VOC in human breath.^{10,11} Third, the breath VOC assay must compensate appropriately for VOCs present in ambient air. Because pentane is also present in room air in concentrations comparable to breath, a breath assay for pentane may be skewed by environmental contamination.^{12,13} Fourth, most previous studies of oxidative stress markers in breath have focused almost exclusively on only 2 alkanes, ethane and pentane. These VOCs have attracted the most attention because they are the easiest to measure with gas chromatography, but breath contains several other alkanes that are also rational markers of lipid peroxidation and oxidative stress.^{7,8} Despite their potential value in research and clinical diagnosis, alkanes other than ethane and pentane have been largely neglected by researchers because they require more advanced techniques of breath collection and assay.

Most of these problems with breath testing have been surmounted by recent technological advances. We have reported a portable breath collection appa-

ratus (BCA) and assay that detects VOCs in breath and room air in picomolar concentrations.¹⁴ This permits determination of the alveolar gradient, the difference between the abundance of a VOC in breath and air, which varies with the difference between the rates of synthesis and clearance of a VOC.¹⁵ This method also facilitates the collection and assay of C₄-C₂₀ alkanes, thereby extending the spectrum of oxidative stress markers that can be detected in the breath.¹⁶ We have further extended this spectrum with the finding that monomethylated derivatives of C₄-C₂₀ alkanes are also apparent markers of oxidative stress, which increase significantly with age in humans.¹⁷ We have combined all of these VOCs into a comprehensive display of markers of oxidative stress, the breath methylated alkane contour (BMAC), a 3-dimensional surface plot of the alveolar gradients of C₄-C₂₀ breath alkanes and their monomethylated derivatives.¹⁷ In this study, we tested the hypothesis that the BMAC could provide a new marker of rejection in heart transplant recipients.

MATERIALS AND METHODS

Human Subjects

Five hundred thirty-nine heart transplant recipients (mean age 54.3 years, SD 11.8; 411 men, 128 women) were studied over a 3-year period. Technically satisfactory breath VOC samples ($n = 1,061$) were collected on the day of regularly scheduled endomyocardial biopsy, before the procedure. Patients were studied at 7 sites: Columbia Presbyterian Medical Center, New York, NY ($n = 159$); M.S. Hershey Medical Center of the Pennsylvania State University School of Medicine, Hershey, PA ($n = 29$); Mount Sinai Medical Center, New York, NY ($n = 95$); Newark Beth Israel Medical Center, Newark, NJ ($n = 56$); Temple University Hospital, Philadelphia, PA ($n = 47$); University of Alabama at Birmingham, Birmingham, AL ($n = 55$); and University of California Los Angeles Medical Center, Los Angeles, CA ($n = 98$). Thirty-two age-matched healthy volunteers were selected from a group studied in Staten Island, NY¹⁵ (16 men, 16 women; mean age 53.2 years, SD 11.8, with no significant difference compared to heart transplant recipients). The institutional review boards of all participating institutions approved the research.

Breath Collection and Assay

The method has been described previously.^{14,15} In summary, a portable BCA (Breath Meter Technology, Inc., Cleveland, OH) was employed to capture the VOCs in 1.0 liter of breath onto a sorbent trap; VOCs in 1.0 liter of room air were captured on a separate sorbent trap. Subjects wore a nose-clip while breathing in and out of the disposable mouthpiece of the BCA for

2.0 minutes. Light flap valves in the mouthpiece presented low resistance to respiration, and it was possible to collect breath samples without discomfort to patients who were elderly or suffering from pulmonary disease. All sorbent traps were sent to the laboratory for analysis of VOCs by automated thermal desorption, gas chromatography and mass spectroscopy. Analyses were performed by 2 investigators (R.N.C. and J.G.) who were blinded to the pathologic findings. All samples were sent to the central laboratory by express mail and analyzed immediately. Breath test results were generally available within 24 to 48 hours of sample collection.

Grading of Rejection

A pathologist at each study site evaluated endomyocardial biopsies without knowledge of the results of the breath test, and graded the degree of rejection employing International Society for Heart and Lung Transplantation (ISHLT) ratings¹⁸: absent (Grade 0); mild (Grades 1A and 1B); focal moderate (Grade 2); multifocal moderate to borderline severe (Grade 3), and severe (Grade 4). The site pathologist reviewed all slides obtained from a biopsy, and forwarded for review the slide that best represented the diagnostic pathology because it contained the most severe focus of rejection. Two reviewers (J.T.F. and P.E.F.) also graded rejection by independently reviewing this slide; they had no knowledge of the site pathologist's findings and no clinical information about the patient or the results of the breath test. They reviewed discordant cases jointly (including their own biopsy reports) to establish a concordant set of ISHLT grades for all biopsies.

Masking Procedures

Pathologists reviewing the biopsies had no knowledge of the results of the breath tests.

Derivation of BMACs

BMACs were determined for all subjects. The abundance of each VOC in the BMAC (comprising C₄-C₂₀ *n*-alkanes and their monomethylated derivatives) was determined as:

$$\text{alveolar gradient} = V_b/I_b - V_a/I_a$$

where V_b denotes the area under the curve of the chromatogram peak for that breath VOC, and I_b denotes the area under the curve of the chromatogram peak of the internal standard used to calibrate the instrument (0.25 ml of 2-ppm 1-bromo-4-fluorobenzene, Supelco, Bellefonte, PA). V_a and I_a denote corresponding areas derived from the associated air sample. A 3-dimensional graph of these compounds, the BMAC, displayed the mean value of the alveolar gradient (y axis) for a specified group of patients vs the carbon skeleton

length (x axis) and the methylation site (z axis).

Analysis of Data

BMACs were compared in 3 groups: breath samples from heart transplant recipients with Grade 3 rejection (according to the concordant set of biopsies); the remaining breath samples from heart transplant recipients with Grades 0, 1 or 2 rejection; and age-matched healthy volunteers. BMACs in heart transplant recipients with Grades 0, 1 and 2 rejection were compared to those with Grade 3 rejection using forward stepwise discriminant analysis, employing maximal significance of F to enter = 0.15 and minimum significance of F to remove = 0.20. In patients studied more than once, repeat breath collections and biopsies were performed at least 2 weeks apart, and to maximize the number of data points, repeated tests from the same cases were treated as independent samples. The resulting mathematical model generated a value from each patient's BMAC ranging from 0.0 to 1.0, indicating the probability of Grade 3 rejection. Cross-validation of patient classification was performed with SPSS's "leave-one-out" discriminant analysis procedure, which predicted whether the patient belonged to the group with Grades 0, 1 or 2 rejection or the group with Grade 3 rejection, based on the breath VOC model derived from all the other patients in the study.¹⁹ Confidence intervals were determined as standard error of percent (SEP).

RESULTS

An overview of the study is shown in Figure 1.

Human Subjects and Breath Samples

All subjects recruited for the research were able to donate a breath sample into the BCA, and none reported any discomfort or adverse effects from the procedure. Of the 107 possible C₄-C₂₀ alkanes and methylalkanes in the BMAC, 81 were observed in the breath of at least 1 heart transplant recipient. Five breath samples were collected from active smokers: 4 with Grades 0, 1 or 2 rejection, and 1 with Grade 3 rejection. One hundred fifty breath samples were technically unsatisfactory; these patients and breath samples are not included in Figure 1.

Rejection Grades in Endomyocardial Biopsies

The concordant set of 1,061 jointly agreed ISHLT grades consisted of: Grade 0, 645 (60.8%); Grade 1A, 197 (18.6%); Grade 1B, 84 (7.9%); Grade 2, 93 (8.8%); and Grade 3A, 42 (4.0%). There was no significant difference between the mean ages of patients with Grades 0, 1 or 2 rejection vs Grade 3 rejection (54.7 years, SD 11.5 vs 54.2 years, SD 14.0 [not significant], respectively).

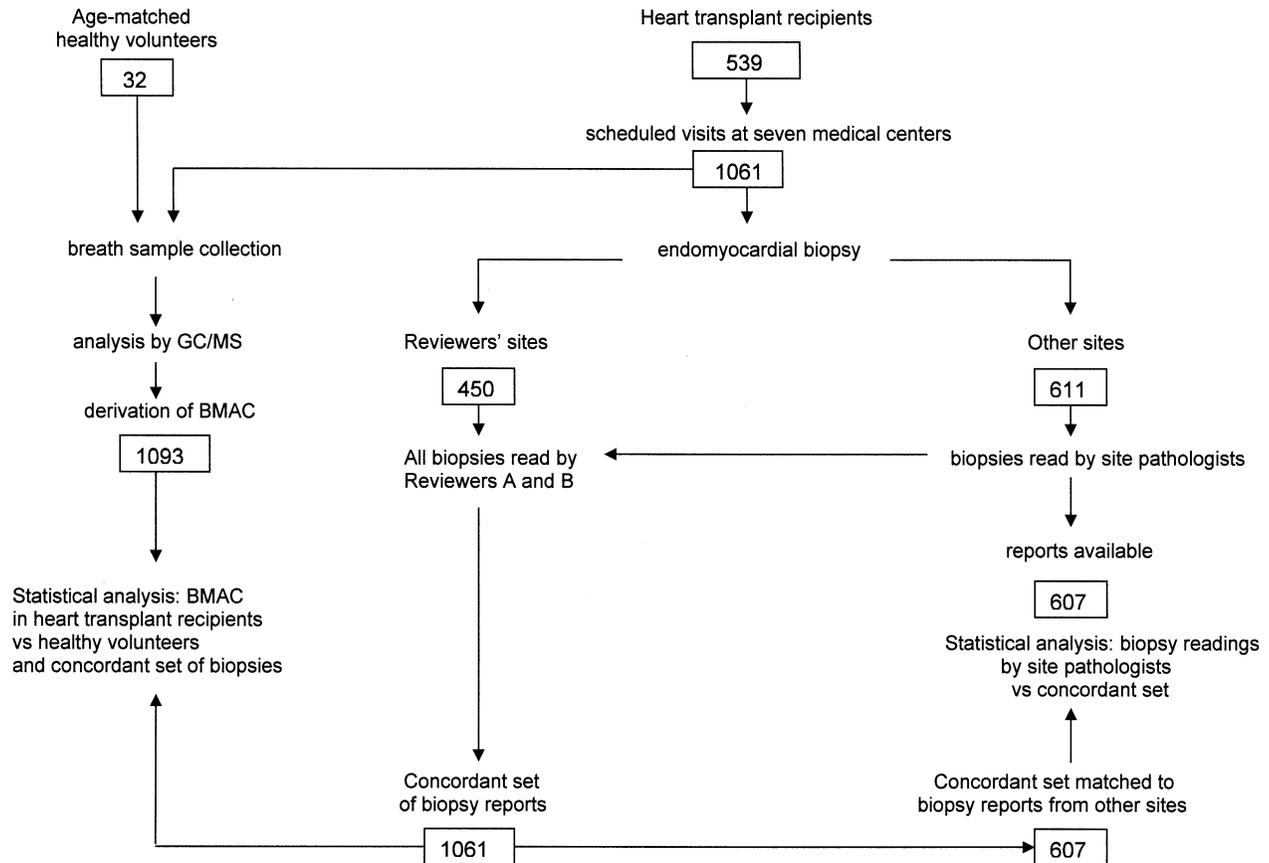


Figure 1. Overview of the research. Study groups were derived for statistical analysis. All endomyocardial biopsies were independently graded for degree of rejection by a site pathologist as well as by Reviewer A and Reviewer B. All used standard ISHLT criteria for scoring rejection. Reviewer A and Reviewer B resolved their disagreements by a joint review of biopsies. The concordant set was then employed as the “gold standard,” against which the breath test and the pathologists at other sites were evaluated. Boxed numbers denote sample sizes.

BMACs in Different Groups

The mean BMACs in healthy volunteers, heart transplant recipients with Grades 0, 1 and 2 rejection, and heart transplant recipients with Grade 3 rejection are shown in Figure 2. The volume under the curve (VUC) of these BMACs is shown in Figure 3.

Identification of Grade 3A Rejection by Breath Test and by Site Pathologists

A combination of 9 VOCs in the BMAC identified Grade 3 rejection with sensitivity 78.6% (SEP 6.33) and specificity 62.4% (SEP 1.18), where the sum of sensitivity and specificity was maximal (cross-validated sensitivity 59.5% [SEP = 7.57], specificity 58.8% [SEP = 1.54], positive predictive value 5.6% [SEP = 1.09], negative predictive value 97.2% [SEP = 0.66]) (Table 1 and Figure 4). Site pathologists identified the same cases with sensitivity 42.4% (SEP 8.6%), specificity 97.0% (SEP 0.74), positive predictive value 45.2% (SEP 8.94) and negative predictive value 96.7% (SEP 0.66) (Figures 4 and 5).

DISCUSSION

This study demonstrated 3 main findings: first, breath markers of oxidative stress were significantly more abundant in heart transplant recipients with Grades 0, 1 or 2 rejection than in healthy normals; second, this increase was apparently reversed in patients with Grade 3 rejection; and, third, breath markers of oxidative stress identified patients with Grade 3 rejection with a high negative predictive value.

The observed increase in breath markers of oxidative stress in heart transplant patients with Grades 0, 1 or 2 rejection was consistent with previous reports; increased myocardial oxidative stress has been detected in stored hearts²⁰ within hours after transplantation, and these acute changes may be due to increased cytokine production and cytochrome *c* release.²¹ After transplantation, myocardial oxidative stress may be both intense and prolonged: Schimke et al found increased levels of oxidative stress markers in endomyocardial biopsies, including total copper/zinc and manganese superoxide dismutase, lipid peroxides and

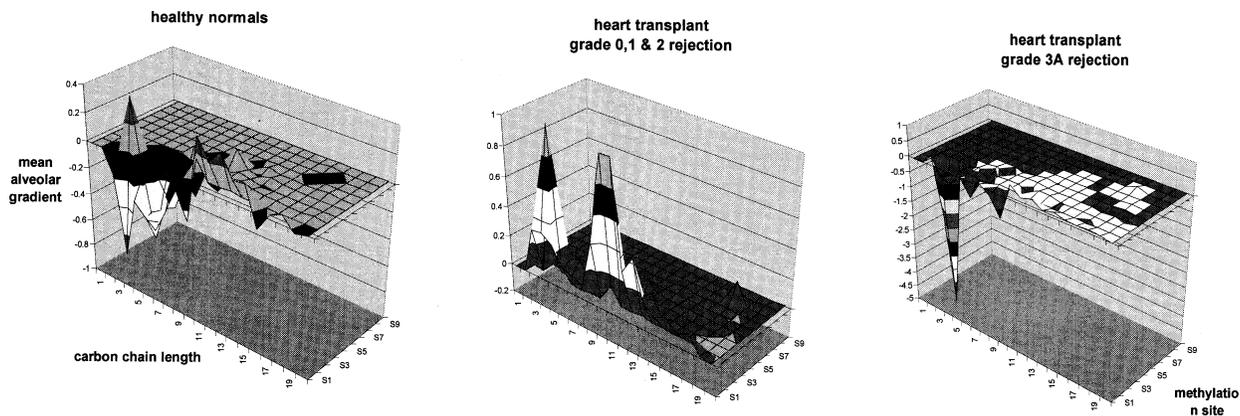


Figure 2. Surface plots of breath test results. The BMAC was constructed from all breath samples, and surface plots of the mean BMACs are shown for 3 groups: healthy normals; heart transplant recipients with Grades 0, 1 and 2 rejection; and heart transplant recipients with Grade 3 rejection. The alveolar gradient (abundance in breath minus abundance in room air) is shown on the vertical axis for C4–C20 alkanes and their monomethylated derivatives. The horizontal axes identify the specific VOC (e.g., the combination of carbon chain length = 4 and methylation site = S2 corresponds to 2-methylbutane). The VOCs that provided optimal discrimination between Grades 0, 1 and 2 rejection and Grade 3 rejection are listed in Table 1. The volume under the curve (VUC) of each surface plot is shown in Figure 3.

glutathione peroxidase, some of which persisted for up to 6 years after transplantation.⁶ Coenzyme Q10 is depleted in transplanted human hearts, and mitochondrial respiratory chain function and energy production vary with the histologic severity of rejection.²² These findings are consistent with an abnormally high level of chronic oxidative stress in the transplanted heart, possibly resulting from chronic sub-clinical inflammation and/or rejection.

There was a paradoxical reversal of the polarity of the BMAC markers of oxidative stress in heart transplant recipients with Grade 3 rejection (Figures 2 and 3). This was an unexpected finding because we had anticipated that patients with the most severe heart transplant rejection would also exhibit the highest levels of oxidative stress markers in their breath. However, the phenomenon was statistically significant, and appears to be clinically important because it is consistent with known pathways of alkane metabolism as well as with previous reports of reduced drug levels in heart transplant rejection.

The most likely mechanism of this paradoxical reversal is that the progression to Grade 3 rejection was accompanied by accelerated catabolism of the alkanes and methylated alkanes comprising the BMAC. Alkanes are catabolized by cytochrome P450 (CYP)-mixed oxidase enzymes, which are highly inducible by drugs such as barbiturates²³ and alkanes, and which induce their own catabolism. In animal studies, exposure to high levels of alkanes and hydrocarbons induces production of CYP2E1, resulting in accelerated catabolism of these compounds as a physiologic response to a toxin.^{24–26} Several microorganisms also respond to high concentrations of alkanes and hydrocarbons with accelerated

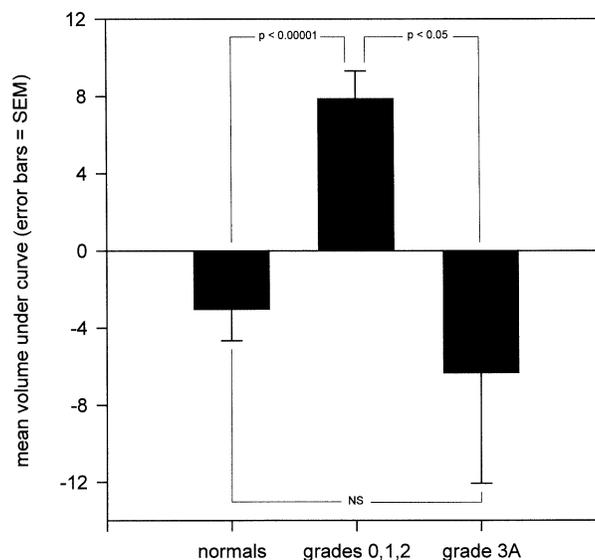


Figure 3. Volume under curve of BMAC surface plots. The mean volume under the curve (VUC) of BMAC surface plots shown in Figure 2 is shown for 3 groups: healthy normals, heart transplant recipients with Grade 0, 1 and 2 rejection, and heart transplant recipients with Grade 3 rejection (bar = SEM). Compared with healthy normals, the VUC was significantly greater in the group of heart transplant recipients with Grades 0, 1 and 2 rejection, demonstrating a global increase in the abundance of volatile markers of oxidative stress in this group. Heart transplant recipients with Grade 3 rejection exhibited an apparent paradoxical reversal of the VUC to levels resembling those in healthy normals. However, this was pseudo-normalization and not true normalization, because Figure 2 demonstrates that the distribution pattern of individual VOCs was not identical in the 2 groups. Oxidative stress was probably most intense in the group with Grade 3 rejection. The resulting higher levels of alkanes may have triggered increased activity of inducible cytochrome P450 enzymes, thereby accelerating the catabolism of alkanes and reversing the VUC.

Table 1. Volatile organic compounds used to identify patients with Grade 3 heart transplant rejection

	Function	
	1	2
Propane, 2-methyl	0.418	0.144
Octadecane, 5-methyl	9.301	-19.316
Octadecane, 6-methyl	-4.730	0.318
Heptadecane, 2-methyl	6.221	14.796
Octane	0.714	-0.010
Heptane, 2-methyl	-1.193	-0.166
Undecane, 3-methyl	0.121	0.239
Octadecane, 2-methyl	-7.237	16.031
Hexadecane, 2-methyl	-5.637	14.908
(Constant)	-0.007	0.042

Alkanes and methylated alkanes were selected by forward stepwise discriminant analysis to generate a statistical model that could predict the probability of Grade 3 heart transplant rejection. VOCs are ranked according to their discriminatory power as markers of rejection. Discriminant functions are shown (function 1 is for all grades of rejection other than 3A) and function 2 is for Grade 3A rejection).

catabolism; the responsible genes have been characterized and cloned in the yeast *Yarrowia lipolytica*.²⁷ Grade 3 rejection may have been accompanied by intense oxidative stress, which generated high levels of alkanes sufficient to induce activity of cytochrome P450 enzymes; the resulting acceleration in alkane catabolism may account for the changes observed in the BMAC.

This hypothesis is supported by previous reports of apparently analogous changes in cyclosporine levels in heart transplant recipients suffering from severe rejection. Turgeon et al studied heart transplant recipients with an erythromycin breath test, and found that their daily dosage requirement for cyclosporine correlated

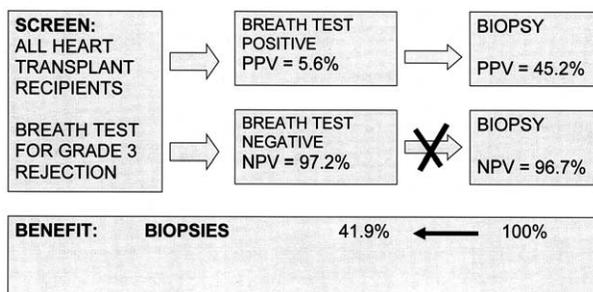


Figure 4. Screening breath test in clinical practice. The expected results of screening all heart transplant recipients with a breath test for Grade 3 rejection. If the breath test result is positive, it is appropriate to proceed to endomyocardial biopsy because the positive predictive value (PPV) increases from 5.6% to 45.2%. However, if the breath test result is negative, a biopsy need not be performed because the negative predictive value (NPV) stays virtually the same. If employed as an alternative to routine surveillance endomyocardial biopsy in all patients, a screening breath test would reduce the number of biopsies performed by >50%.

		Concordant set: ISHLT Grade of rejection	
		0, 1 & 2	3
Site pathologist assignment	0, 1 & 2	TN = 557	FN = 19
	3	FP = 17	TP = 14
Breath test assignment	0, 1 & 2	TN = 600	FN = 17
	3	FP = 419	TP = 25
	Site pathologist	Breath test	
Sensitivity	14/33 = 42.4%	25/42 = 59.5%	
Specificity	557/574 = 97.0%	600/1019 = 58.8%	
Positive predictive value	14/31 = 45.2%	25/444 = 5.6%	
Negative predictive value	557/576 = 96.7%	600/617 = 97.2%	

Figure 5. Detection of Grade 3 rejection by site pathologists and by breath tests. As shown in Figure 1, 2 reviewers read all endomyocardial biopsies and reached joint agreement on ISHLT grading of rejection. This comprised the concordant set, which was employed as the “gold standard” of rejection. The accuracy of Grade 3 rejection assignment by site pathologists (607 biopsy readings) and by breath testing (1,061 samples) was evaluated against the concordant set. Breath testing was more sensitive and less specific than biopsy reading by a site pathologist, and the negative predictive values of both tests were similar.

with changes in cytochrome P450 3A activity.²⁸ El Gamel et al studied a group of heart transplant recipients treated with a standard dose of cyclosporine and observed a significant decline in trough blood levels in patients who progressed from Grade 0 to Grade 3A rejection.²⁹ Other studies have also demonstrated significantly lower levels of cyclosporine in Grade 3 rejection than in Grade 0 rejection.^{30,31} It is possible that induced cytochrome P450 activity can explain reductions in the levels of both cyclosporine and breath VOCs in Grade 3A rejection.

We assigned all biopsies to one of 2 groups—Grades 0, 1 and 2 rejection or Grade 3 rejection—to identify the group in greatest need of increased immunosuppressive therapy. A sub-set of 9 VOCs in the BMAC identified patients with Grade 3 rejection, with a high negative predictive value. Figure 5 demonstrates the comparative results of screening heart transplant recipients with a breath test or an endomyocardial biopsy as read by a site pathologist. The breath test was more sensitive and less specific than a biopsy reading by a site pathologist, and the negative predictive values were similar in both tests (97.2% and 96.7%, respectively). In practice, a negative breath test would convey essentially the same clinical information about the absence of

Grade 3 rejection as a negative biopsy reading by a site pathologist.

These findings carry implications for clinical care. Routine surveillance endomyocardial biopsy is the current standard of care in heart transplant recipients, but patients could benefit from previous screening with a breath test. As shown in Figure 4, if the breath test result were positive, it would be appropriate to proceed to an endomyocardial biopsy because this would increase the positive predictive value of Grade 3 rejection from 5.6% to 45.2%. However, if the breath test result were negative, there would be no indication to perform an endomyocardial biopsy because it would confer no meaningful increase in negative predictive value for Grade 3 rejection. Because a negative breath test could be expected in 58.2% of screened patients, a decision not to perform a biopsy in these patients would reduce the total number of biopsies performed by >50%.

Consequently, a screening breath test could potentially reduce both the morbidity associated with endomyocardial biopsy and the costs of health care. In practice, it would not be difficult to implement routine screening breath tests for heart transplant recipients. Breath VOC samples would be collected, as in this study, at the clinical care site, then express-mailed to a central laboratory for analysis and interpretation. Results would generally be available to the clinician by the next day.

We encountered a challenging problem during the design phase of this study: to what "gold standard" of transplant rejection should the breath test be compared? Endomyocardial biopsy is the currently accepted gold standard, but it has 2 major limitations: First, it is accompanied by a high degree of inter-observer variability; a study of experienced pathologists reading an identical series of biopsy specimens revealed major discrepancies between their ISHLT grading of rejection.² Second, the severity of allograft dysfunction may not necessarily coincide with the severity of abnormalities seen on an endomyocardial biopsy; other factors such as infection or systemic inflammation also play an important role. Breath markers of oxidative stress provide an entirely different approach to detection of allograft rejection and/or dysfunction; however, we were concerned that even if this marker proved to be clinically useful, it need not necessarily correlate strongly with the results of an endomyocardial biopsy.

Despite these concerns, we were constrained by the absence of any other widely accepted gold standard for allograft rejection. We therefore elected to employ a concordant set of biopsy readings derived by 2 unbiased, trained pathologists who were untainted by any extraneous information. Their readings were highly dependable but probably not infallible, and it is possible

that in some cases the site pathologists may have possessed additional clinical or pathologic information that guided their assessment of grade of rejection.

We conclude that a breath test for markers of oxidative stress provides new evidence that oxidative stress is chronically increased in most heart transplant recipients. A sub-set of these breath markers of oxidative stress identified Grade 3 rejection with a high negative predictive value. The test is non-invasive, safe and acceptable to patients. Breath testing could identify most heart transplant recipients at low risk of Grade 3 rejection and could potentially reduce the number of endomyocardial biopsies performed, with a consequent reduction in patient morbidity and health-care costs.

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