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November 18, 2006

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Baltimore, Maryland 21244
Via e-mail to: Steve.Phurrough@cms.hhs.gov

FORMAL REQUEST FOR A MEDICARE
NATIONAL COVERAGE DETERMINATION (NCD)

Heartsbreath test for heart transplant rejection

Dear Dr. Phurrough:
I append a detailed application for an NCD. I initially met with your colleagues at CMS in February 2004 and presented much of this information to Dr. Jesse Polansky, Ms. Jackie Sheridan-Moore and Dr. Carlos Cano. At that time, I was told that CMS was not considering NCDs for tests with HDE approval from FDA, and that I should apply instead to individual state Medicare contractors. I therefore pursued this application energetically with Medicare contractors in a number of states, particularly New York and New Jersey. These efforts were ultimately fruitless. After more than two years of delays, discussions and meetings, I was ultimately told that I should now return to CMS in order to pursue an NCD. I have therefore now come full circle back to CMS. In view of these protracted delays and their disappointing outcomes, I would be very grateful if you could review this application expeditiously.

Yours sincerely,

Michael Phillips MD, FACP

cc: Sandra-d.Jones@cms.hhs.gov

FORMAL REQUEST FOR A MEDICARE NATIONAL COVERAGE DETERMINATION

HEARTSBREATH TEST FOR HEART TRANSPLANT REJECTION

TABLE OF CONTENTS

1. Requesting organization

Menssana Research, Inc
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Point of contact: Michael Phillips MD, FACP
Telephone and fax: 201 886 7004
e-mail: mphillips@menssanaresearch.com
website: www.menssanaresearch.com

2. Development track

Track #1 is requested

3. Benefit categories

Physician services
Diagnostic tests

4. Description of the Heartsbreath test for heart transplant rejection

The Heartsbreath test is a non-invasive breath test for markers of oxidative stress. It has three components:

a. A breath collection apparatus that collects volatile organic compounds (VOCs) in alveolar breath onto a sorbent trap, and a sample of room air VOCs onto a separate sorbent trap
b. Analysis of the VOCs in alveolar breath and room air by gas chromatography and mass spectroscopy (GC/MS).
c. Interpretation of the results with a proprietary algorithm that predicts the probability of grade 3 heart transplant rejection.

The Heartsbreath test is indicated for use as an aid in the diagnosis of grade 3 heart transplant rejection in patients who have received heart transplants within the preceding year. The Heartsbreath test is intended to be used an adjunct to, and not as a substitute for, endomyocardial biopsy. The use of the device is limited to patients who have had endomyocardial biopsy within the previous month.

5. The target Medicare population

Heart transplant recipients who have

a. received heart transplants within the preceding year, and
b. who have had endomyocardial biopsy within the previous month.

6. Medical condition for which the Heartsbreath test can be used

Detection of Grade 3 heart transplant rejection in heart transplant recipients who have

a. received heart transplants within the preceding year, and
b. had endomyocardial biopsy within the previous month.

7. Supporting medical and scientific information

a. Detection of heart transplant rejection: overview of the problem

Since heart transplantation was first introduced in 1967, more than 61,000 have been performed throughout the world. More than 23,000 people now live with a transplanted heart, and most of them (at least 18,000) live in the USA [1] Between 2,000 and 3,000 heart transplantation procedures are performed in the United States every year. First-year heart transplant recipients are a high-risk subgroup because organ rejection is most common during this period. For this reason, first-year heart transplant recipients must undergo several routine surveillance right ventricular endomyocardial biopsies, the current "gold standard" for diagnosis of heart transplant rejection.

All heart transplant recipients require periodic screening for rejection, a condition which is difficult to detect clinically, but may manifest as sudden death. 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 routine surveillance endomyocardial biopsy is generally performed weekly for the first six weeks after operation, biweekly until the third month, monthly until the sixth month, then every one to three months dependent upon clinical indications.

However, the value of routine surveillance endomyocardial biopsy is limited by a number of problems:

1. The procedure is invasive and expensive.
2. The majority of biopsies yield normal or near normal results which elicit no changes in treatment [2-4].
3. The procedure may cause complications such as hematoma, infection, arrhythmia, ventricular perforation, fistulas and death [5, 6].
4. There is poor interobserver agreement in the reading of biopsies. Randomized studies of different pathologists reading the same biopsies have shown discrepancies between their grading of rejection sufficient to have adverse treatment implications [7, 8 9].

These limitations of endomyocardial biopsy have stimulated research into non-invasive alternative tests for heart transplant rejection. Several have been proposed, including antibody imaging [10], echocardiography, and serum markers such as troponin I, troponin T, creatine kinase-MB fraction, and C-reactive protein [11].; however, their accuracy is generally too poor to guide clinical decision making in individual patients. As Bourge

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Modern immunosuppressive therapy is now so effective that some have argued that routine surveillance endomyocardial biopsies is not justified beyond a year after operation because the incidence of Grade 3 rejection falls so low [3] [2]. White et al stated this view in a report entitled "Routine surveillance myocardial biopsies are unnecessary beyond one year after heart transplantation" [3]. They studied a consecutive series of 235 transplant recipients who survived 1 year or more, and reviewed the results of 1123 routine endomyocardial biopsies performed 1 to 12 years after transplantation. 1115 (99.3%) showed no evidence of significant rejection (i.e. the biopsies were grade 0 or 1). Only seven (0.6%) had evidence of rejection grade 2 or worse. Of the seven abnormal biopsy specimens in seven patients, two occurred at 1 year, two at 2 years, and one each at 4, 7, and 8 years. They concluded that myocardial rejection is rare beyond 1 year after transplantation.

Hausen et al reported a comparable experience; they studied 346 patients who received 382 heart grafts between 1985 and 1992[17]. In the first year the average number of endomyocardial biopsies per patient was 20, with 19% positive for rejection in the first quarter, dropping to 7% by the end of the first year. The percentage of endomyocardial biopsies which were positive for rejection declined annually, year by year: 4.7% in year 2, 4.5% in year 3, 2.2% in year 4, and less than 1% after the fifth year.

The incidence of rejection varies from site to site, but even at sites where grade 3 rejection is more common, a similar fall in incidence has been reported after the first year. Wagner et al performed a retrospective review of 1,169 endomyocardial biopsies performed during a postoperative follow-up period of 2-149 months (median 41 months) [18]. During the first year after heart transplantation, surveillance endomyocardial biopsy detected significant rejection (grade >= 3A) in 18% of biopsies. The diagnostic yield fell during 1-5 yr post-transplantation to 4% in infants,13% in children, 9% in patients with favorable first-year rejection history and 17% in 'frequent rejectors'.

In aggregate, these reports demonstrate that the incidence of grade 3 rejection varies from site to site, but it is uniformly highest at all sites during the first year following heart transplantation.

c. Estimated number of patients who qualify for the test

The following information (Table 1) was obtained from the Organ Procurement and Transplantation Network (OPTN) (http://www.optn.org/data/about/OPTNDatabase.asp)**.** This web site is designed, developed, and maintained by the United Network for Organ Sharing (UNOS) under contract with the U.S. Department of Health and Human Services (HHS) and the Health Resources and Services Administration (HRSA).

Table 1: Number of heart transplants performed in USA

year	n
1988	1676
1989	1706
1990	2107
1991	2126
1992	2171
1993	2297
1994	2340
1995	2363
1996	2343
1997	2294
1998	2348
1999	2188
2000	2199
2001	2202
2002	2154

It is apparent that the number of first year heart transplant recipients has remained fairly steady during the past decade, and has not exceeded the peak of 2363 in 1995 in any one year.

d. General background to breath testing

Breath tests are intrinsically safe, painless, and non-invasive; also, they were used as the earliest chemical probes of metabolism. In the late 18th century, Lavoisier discovered that carbon dioxide is excreted in their breath, the first evidence that food is oxidized in the body. During the 19th century, colorimetric tests demonstrated ethanol in the breath of drinkers, and acetone in the breath of diabetics [19]. A major advance during the 20th century was the development of microanalysis of breath by Linus Pauling, revealing that normal human breath contains a large number of different VOCs in picomolar (10^{-12} M) concentrations [20]. It is now known that a sample of human breath contains around 200 different VOCs, most of them in picomolar concentrations [21]. Microanalysis of breath is a technically difficult procedure which requires concentration of the sample prior to assay by GC/MS. In recent years, breath microassays have opened a new window on to the detection of oxidative stress which results when a cellular injury triggers a cascade of reactive oxygen species (ROS) from the mitochondria. ROS oxidize polyunsaturated fatty acids in membranes to alkanes such as ethane and pentane which are excreted in the breath [14, 15]. Breath tests have demonstrated increased oxidative stress in several conditions including acute myocardial infarction [22], rheumatoid arthritis [23], bronchial asthma [24] and vitamin E deficiency [25].

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} M) concentrations. Existing laboratory instruments cannot detect such low concentrations, so that breath VOCs must be collected and assayed with specialized instruments [19]. 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 [26, 27]. Third, the breath VOC assay must compensate appropriately for VOCs present in ambient air. Since pentane is also present in room air in concentrations comparable to breath, a breath assay for pentane may be skewed by environmental contamination [28, 29]. Fourth, although oxidative stress is known to elicit a variety of alkanes in the breath, most studies have focused on pentane and ethane [15]; no previous studies have investigated whether alkanes with different carbon chain lengths or their methylated derivatives might provide clinically useful markers of oxidative stress.

These difficulties have been surmounted by recent advances in analytical technology. We have reported a portable breath collection apparatus (BCA) and assay which detects VOCs in breath and room air in picomolar concentrations [30]. 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 [21]. We have also reported two new apparent markers of oxidative stress, the breath alkane contour [31] and the breath methylated alkane contour (BMAC), the three-dimensional display of the alveolar gradients of C4-C20 breath alkanes and their monomethylated derivatives [32] .

e. Basic research in breath VOC analysis at Menssana Research, Inc

Phillips has studied microanalysis of breath VOCs for more than 20 years. In 1981, he reported a method for cryogenic capture of breath VOCs in a U-tube chilled in liquid nitrogen; the concentrated sample was then heated, and assay by GC revealed ~100 different VOCs in normal human breath[33]. He subsequently employed sorbent trapping and GC to assay endogenous ethanol[34], acetaldehyde[34] and carbon disulfide[35] in human breath. In the 1990s, he developed a new instrument, a breath collection apparatus (BCA) to collect breath samples outside the laboratory[19, 30, 36] (Figure 1). Studies with the BCA led to the *development of the alveolar gradient concept*. Since ambient room air also contains most of the VOCs observed in breath, analysis of breath VOCs was formerly clouded by uncertainty because it was unclear how much was signal (i.e. VOCs manufactured or degraded in the body) and how much was artifact (i.e. the contribution of background air). Phillips proposed the concept of the alveolar gradient: the concentration of a VOC in breath minus its concentration in room air[29, 35]. Kinetic analysis has shown that the alveolar gradient varies with the rate of synthesis of a VOC and the rate at which it is cleared from the body by metabolism and excretion [21]. In subsequent studies, Phillips evaluated the *qualitative and quantitative range of variation in breath VOCs in normal individuals* [21]. In a study of 50 normals, he detected 204.2 VOCs in an average breath sample and a total of 3481 different VOCs in all subjects, half with positive and negative alveolar gradients respectively. This study

confirmed previous reports of wide inter-individual variations in breath VOCs. It also yielded the new finding that all subjects shared a common core of 27 breath VOCs, predominantly alkanes and methylated alkanes. Further studies of these common core breath VOCs led to *the development of new sets of breath markers oxidative stress*: the breath alkane profile, comprising the alveolar gradients of C4 to C20 alkanes[31], and the breath methylated alkane contour (BMAC) comprising the alveolar gradients of C4 to C20 alkanes and their monomethylated derivatives[32]. All of these VOCs appear to be biomarkers of oxidative stress because spillage of reactive oxygen species from the mitochondria into the cytoplasm causes lipid peroxidation of polyunsaturated fatty acids in cellular membranes. This process liberates volatile n-alkanes that are excreted in the breath.

f. The breath collection apparatus

Background: Breath microanalysis is simple in theory but difficult in practice. While it is child's play to blow up a balloon or a plastic bag, these containers are usually so contaminated that a breath VOC assay yields little useful information. Phillips has identified the major sources of error in breath collection: chemical contamination, resistance to expiration, water condensation, dead-space air dilution, and container adsorption artifact [37]. Pauling employed bench-top apparatus for breath VOC microanalysis, and the first studies of breath biomarkers of lung cancer during the1980s required patients to donate breath samples in a laboratory[38, 39].

Prior to the development of the portable breath collection apparatus (BCA), there was no method available for the routine collection and assay of breath VOCs in picomolar concentrations. Researchers generally employed ad hoc breath collection devices interfaced to a gas chromatograph. Since the patient had to come to the instrument, rather than vice versa, it was not possible to perform large multi-center studies of breath VOCs in various diseases. The BCA has changed this situation by making it possible to collect breath VOC samples in the field. Phillips developed a breath collection apparatus (BCA) that is portable, safe, and simple to use outside the laboratory (Figure 1)[30]. Breath VOC samples are captured onto sorbent traps and analyzed by automated thermal desorption with GC and mass spectroscopy (ATD/GC/MS). This enabled the first large multi-center clinical studies of breath testing[21, 30].

Methods: The method has been described in detail [21, 30] . A picture of the BCA in use is shown in Figure 1.

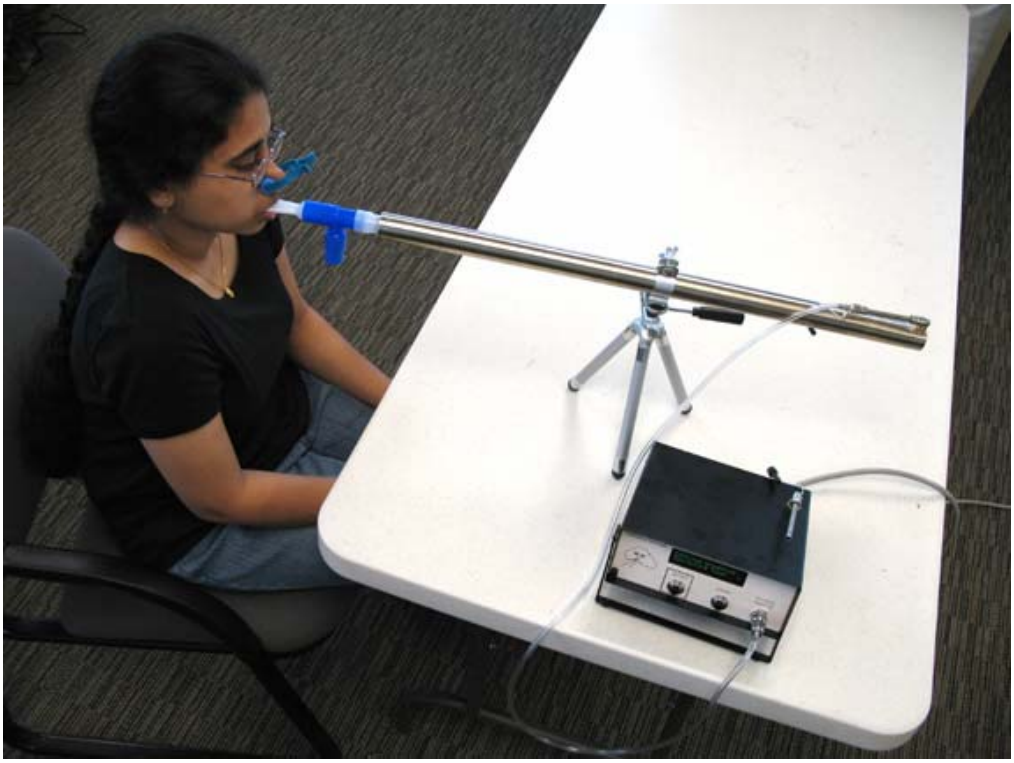
1. 

Figure 1: The breath collection apparatus (BCA 5.0) in use. This latest-generation BCA is controlled by microprocessors. A digital display on the front panel guides the technician through every step of the collection. The donor wears a nose clip and inspires room air through a valved disposable mouthpiece for 2.0 min. Expired breath enters the reservoir tube which is open at its distal end so that the donor does not breathe out against resistance. The breath reservoir separates alveolar from dead space breath, and alveolar breath is pumped from the reservoir through a sorbent trap, a stainless steel tube packed with two grades of activated carbon that capture the VOCs in 1.0 l breath. A 1.0 l sample of room air VOCs is collected onto a second trap. A Mycobacterial filter in the mouthpiece (not shown) prevents bacterial contamination of the system.

Separation of alveolar from dead space breath: The breath reservoir is open to room air at the downstream end. When a patient expires a single tidal breath of approximately 500cc, the first 150 cc is dead space (from nasopharynx and bronchi), and the following 350 cc is alveolar breath from deep in the lungs. These two phases are separate from one another in the breath reservoir: dead space breath is distal from the mouth, while alveolar breath is proximal. The sampling port is at the end of a tube (not seen in the photograph) which runs down the inside of the breath reservoir, with an opening at the proximal (left) end of the reservoir, close to the mouth. The sample is pumped to the sorbent trap, the small tube appended to the distal end of the reservoir. Breath is collected at 500 cc/min i.e. 8.3 cc/sec, from the alveolar breath sample at the proximal end; the next breath is delivered long before the alveolar breath sample is depleted. The sampling port is exposed to dead space breath only for a fraction of a second during

expiration. Thus, the collected sample comprises more than 99% alveolar breath, and is virtually free of dead space breath.

g. Assay of breath samples

Breath VOCs are assayed by automated thermal desorption/gas chromatography/mass spectroscopy (ATD/GC/MS) using unmodified "off the shelf" instrumentation. The method has been described in detail [21, 30] .

Results: Samples of breath and air usually yield approximately 200 different VOCs, most of them in picomolar concentrations. The composition of normal human breath has now been characterized in detail. More than 3000 different VOCs were observed in a study of 50 healthy volunteers, but only 27 VOCs were present in all subjects [21]

Conclusions: Breath testing has been "democratized" and brought out of the research laboratory and into the clinical arena with the development of a portable breath collection apparatus (BCA). The BCA is portable and user friendly both for the patient and the technician, and has made it possible, for the first time, to perform multi-center clinical studies of breath VOC analysis.

h. Relevant patents

1. Breath Collection Apparatus --U.S. Patent No. 5,465,728--November 14, 1995
2. Breath Test for Helicobacter Pylori--U.S. Patent No. 5,848,975--December 15, 1998
3. Breath Test for Detection of Lung Cancer--U.S. Patent No. 5,996,586--December 7, 1999
4. Breath Test for the Detection of Various Diseases--U.S. Patent No. 6,221,026--April 24, 2001
5. Breath Methylated Alkane Contour: a New Marker of Oxidative Stress and Disease--U.S. Patent No. 6,254,547-- July 3, 2001
6. Breath Test for Detection of Lung Cancer--U.S. Patent No. 6,312,390--November 6, 2001
7. Breath Test for the Detection of Various Diseases--U.S. Patent No. 6,540,691--April 1, 2003
8. Breath collection apparatus - U.S. Patent No. 6,726,637 – April 27, 2004

i. The alveolar gradient concept

Background: Ambient room air contains most of the VOCs observed in breath, in approximately similar concentrations. Thus, any analysis of breath VOCs was formerly clouded by uncertainty because it was not known how much was signal (i.e. VOCs manufactured or degraded in the body) and how much was noise (i.e. the contribution of background air). Phillips had previously proposed the concept of the alveolar gradient: the concentration of a VOC in breath minus its concentration in room air [29].

Methods: VOC flow in the body was analyzed kinetically.

Results: Kinetic analysis demonstrated:

alveolar gradient = $(R_{synthesis} - R_{clearance})/RMV$

where R = rate of movement of VOC (mol/min)
RMV = respiratory minute volume (l/min)

Conclusions: This was an important advance in breath testing because it demonstrated, for the first time, the physiologic basis of the alveolar gradient: it varies with the difference between the rate of synthesis and the rate of clearance of a VOC in the body [21].

j. Breath test results in healthy normal humans

Background: It was known that the composition of VOCs in breath varies widely between individuals, but no previous study had systematically evaluated the qualitative and quantitative range of variation in breath VOCs in a group of normal individuals.

Methods: Phillips performed breath tests in 50 normal humans in order to determine the range of variation in breath VOCs [21].

Results: An average breath sample contained 204.2 VOCs (SD = 19.8, range 157-241). A total of 3481 different VOCs were observed: 1753 with positive alveolar gradients and 1728 with negative alveolar gradients. 27 VOCs were observed in all 50 subjects.

Conclusions: This study confirmed previous reports of wide inter-individual variations in breath VOCs. Two new findings were the comparatively small variation in total number of breath VOCs and the presence of a common core of breath VOCs in all subjects. These common core VOCs were mainly alkanes and methylated alkanes, and appeared to be new markers of oxidative stress.

k. Volatile markers of oxidative stress in breath

k.i. The breath alkane profile

Background: Ethane and pentane in breath are markers of oxidative stress produced by ROS-mediated lipid peroxidation of n-3 and n-6 polyunsaturated fatty acids (PUFAs) but little was known about other n-alkanes in normal human breath. Phillips investigated the spectrum of alkanes in normal human alveolar breath, and their variation with age [31].

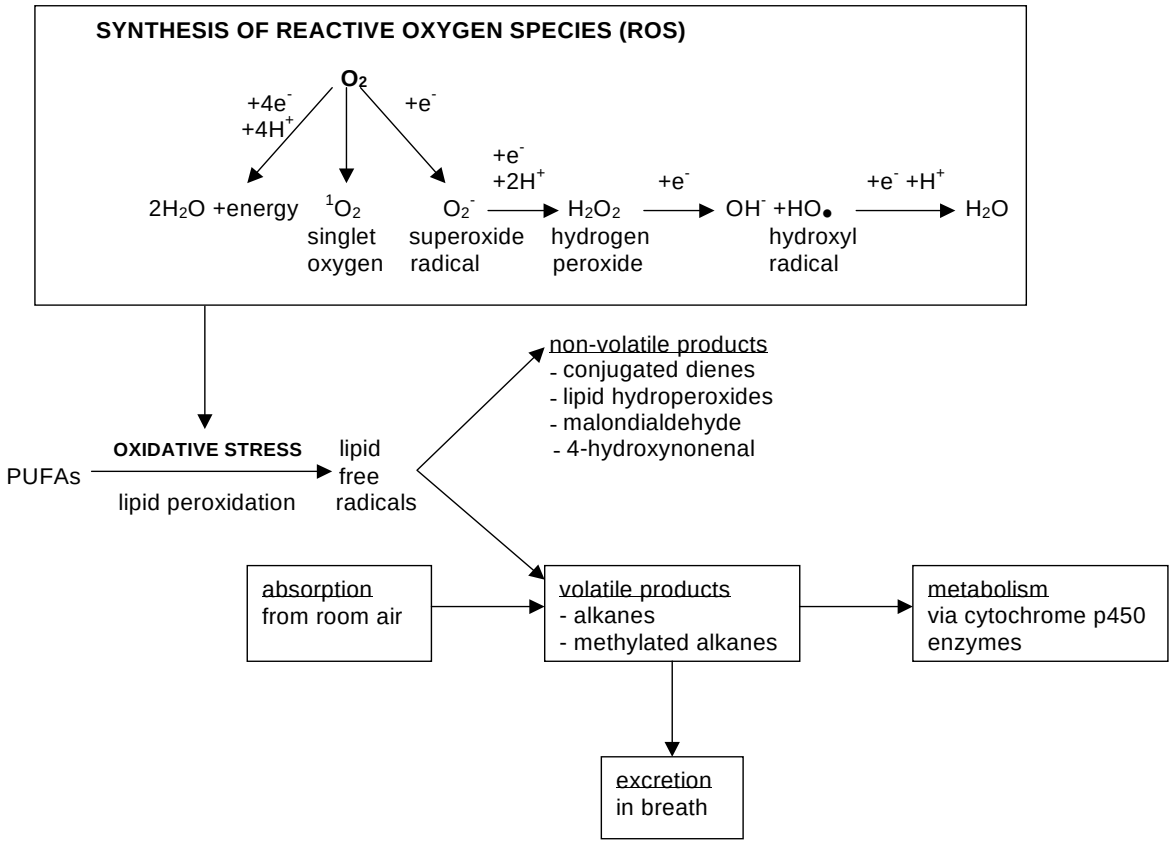
Methods: Breath tests were performed in 50 normal humans (age range 23 to 75, median 35). Volatile organic compounds (VOCs) in alveolar breath were captured on sorbent traps and assayed by gas chromatography and mass spectroscopy. Alveolar gradients (concentration in breath minus concentration in ambient room air) of alkanes were determined .

Results: C4 to C20 alkanes were observed in breath and room air. Their mean alveolar gradients were negative from C4 to C12 and positive from C13 to C20. The mean age of the older half of the group was significantly greater than the younger half (47.56 yrs vs 29.88 yrs, $p<0.0001$), and the mean alveolar gradients of four alkanes (C5, C6, C7 and C8) were significantly more positive in the older subjects ($p<0.05$). There were no significant differences between males and females.

Conclusions: The spectrum of alkanes in normal human breath contained apparent new markers of oxidative stress. The mean rate of clearance (via cytochrome

P450) exceeded the mean rate of synthesis (by ROS-mediated oxidative stress) for C4-C12 alkanes, while synthesis was greater than clearance for C13-C20 alkanes. The elevated alkane profile in older subjects was consistent with an age-related increase in oxidative stress, though an age-related decline in alkane clearance rate may have contributed.

Figure 2 (below): Oxidative stress and volatile organic compounds (VOCs) in the breath. This diagram shows a typical human cell; the rectangle at the top is a mitochondrion. In mitochondrial oxidative phosphorylation, oxygen accepts electrons, generating water and energy. Oxygen also contributes to the formation of oxygen free radicals and hydrogen peroxide. These compounds are collectively termed reactive oxygen species (ROS). They leak into the cytoplasm where they oxidize most biologically important molecules including DNA, proteins and lipids, a process termed oxidative stress. Oxidation of polyunsaturated fatty acids (PUFAs) in membranes generates alkanes and methylated alkanes which are excreted in the breath.



k. ii. The breath methylated alkane profile (BMAC)

Background: The breath alkane profile was extended by incorporating another molecular dimension - the alkane methylation site - to produce a three-dimensional display, the breath methylated alkane contour (BMAC) [32] (Fig 3). Breath tests for volatile organic compounds (VOCs) offer a rational approach to detection of oxidative stress which occurs when the rate of production of reactive oxygen species (ROS) exceeds their rate of clearance, resulting in electron leak from the mitochondria into the cytoplasm. ROS comprising oxygen free radicals and hydrogen peroxide are highly reactive and toxic, causing peroxidative damage to DNA, proteins, and polyunsaturated fatty acids (PUFAs). Lipid peroxidation of PUFAs in cell membranes liberates volatile alkanes such as ethane and pentane which are excreted in the breath where they provide markers of oxidative stress[14, 15].

Methods: Analytical: The relative abundance (R.A.) of each alkane (C4 to C20) and its monomethylated derivatives were determined from the chromatographic area under the curve (AUC) and the AUC of an internal standard (IS) (1-bromo-4-fluoro-benzene) ($R.A._{VOC} = AUC_{VOC}/AUC_{IS}$). The alveolar gradient of each VOC was determined as $R.A._{breath} - R.A._{room\ air}$.

Methods: Human study: Phillips et al performed breath tests in 102 normal volunteers aged from 9 to 89 years.

In each subject, a breath methylated alkane contour (BMAC) was constructed by plotting alkane carbon skeleton length (x-axis) versus methylation site (z-axis) versus alveolar gradient (y-axis). Subjects were separated into four quartiles by age. Breath data were pooled from subjects in each quartile in order to determine their mean

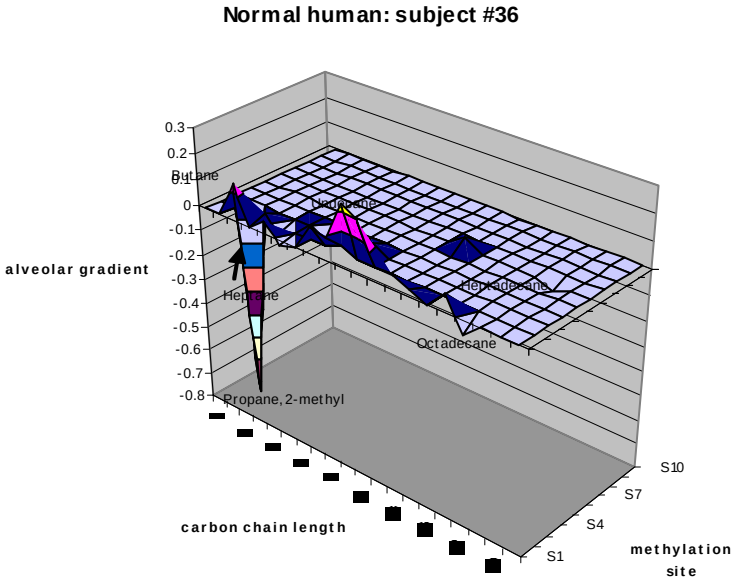


Figure 3 (above): Breath methylated alkane contour (BMAC) in a healthy 30 year old female volunteer. The x-axis is the length of the carbon chain in the alkane or methylated alkane, the z-axis is the site of monomethylation, and the y-axis is the alveolar gradient (abundance in breath minus abundance in room air).

alveolar gradients of alkanes and monomethylated alkanes. Significant differences between quartiles were determined with one-way ANOVA and a Newman-Keuls post hoc test.

Results: The BMAC varied with age: peaks were predominantly negative in the youngest quartile and predominantly positive in the oldest quartile. Changes were statistically significant in the alveolar gradients of several alkanes and methylated alkanes. When subjects were matched for age, there were no significant differences between tobacco smokers and non-smokers; the only difference with sex was 4-methyloctane (greater in females than in males, $p<0.05$, 2-tailed t-test)

Conclusions: These findings were consistent with a previous report that breath pentane increases with age in healthy normal humans [40]. Sagai and Ichinose demonstrated age-related increases in lipid peroxidation in rats, as shown by increased excretion of ethane, butane and pentane in breath [41] and Sohal et al observed [42]

, but not all hydroxylation enzy ilarly affected; there is no age-related decline in the hepatic microsomal hydroxylation of alprazolam [45] or of cutaneous aryl hydrocarbon hydroxylase [46]. It is therefore most likely that the observed changes in the BMAC resulted predominantly from an age-related increase in oxidative stress.

I. Evaluation of breath testing in other disorders

Clinical studies of this breath test for markers of oxidative stress have demonstrated its apparent value as a disease marker in lung cancer[47, 48], breast cancer[49] and unstable angina[50]. In addition, it has demonstrated increased oxidative stress in aging[51] and in oxygen breathing[52]. Phillips has reported clinical studies of breath testing (Table 1). These have yielded an important new insight: increased oxidative stress is *not* a simple and nonspecific increase in a single variable. Previously, oxidative stress had been regarded as a univariate marker, like an erythrocyte sedimentation rate, that could be increased by many different causes e.g. infection, cancer, rheumatoid arthritis etc. However, clinical studies have shown that oxidative stress is a complex multivariate response that affects >100 different biomarkers, and results in different “breath fingerprints” for different diseases

Table 2: Clinical studies of oxidative stress (OS) biomarkers in breath (Phillips)

Heart transplant rejection[53, 54]	Large multicenter study (>1,000 breath tests) Breath test was sensitive and specific for grade 3 rejection Heartsbreath test approved by Food & Drug Administration
Lung cancer[47, 48, 55, 56]	Three multicenter studies Breath test sensitive and specific for primary lung cancer
Breast cancer[49, 57]	Pilot study; breath test predicted breast cancer
Ischemic heart disease[50]	Pilot study; breath test identified unstable angina
Preeclampsia of pregnancy[58]	Pilot study; increased OS in pregnancy and preeclampsia
Diabetes mellitus[59]	Pilot study; increased OS in Type I and Type II diabetes
Pulmonary tuberculosis[60]	Pilot study; increased OS in "sick" patients hospitalized to rule out pulmonary tuberculosis
Aging[31, 32, 51]	Increased OS in normals aged <20 and >40 years
Schizophrenia[61, 62]	Early pilot studies; breath VOCs identified schizophrenia

m. Clinical experience: Breath test for heart transplant rejection

These clinical studies have been published and reported at scientific meetings (see Appendices 10-13) [52,53].

Heart Allograft Rejection: Detection with Breath Alkanes in Low Levels (the HARDBALL study) [52]

Background: More than 61,000 heart transplant operations have been performed since 1967; at least 23,000 recipients are known to be still alive, though the actual number of survivors may exceed 30,000[1]. All of them require periodic screening for rejection, a condition which 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 six weeks, biweekly until the third month, monthly until the sixth month, then every one to three months dependent upon clinical indications. However, the majority of biopsies yield normal or near normal results which elicit no changes in treatment[9]. Although considered safe, the procedure is invasive and may cause complications such as hematoma, infection, arrhythmia,

ventricular perforation, and fistulas. Also, a randomized study of biopsy readings by different pathologists found discrepancies between their grading of rejection sufficient to have adverse treatment implications. Many attempts have been made to develop non-invasive tests for heart transplant rejection. Several have been proposed, including magnetic resonance imaging, antibody imaging, echocardiography, and serum markers such as troponin I, troponin T, creatine kinase-MB fraction, and C-reactive protein[10, 11]; however, their accuracy is generally too poor to guide clinical decision making in individual patients.

Breath microanalysis has been proposed as a non-invasive test for heart transplant rejection[12]. The rationale of a breath test is based on two observations: first, allograft rejection is accompanied by oxidative stress resulting from increased production of reactive oxygen species (ROS) in the myocardium[13] and second, ROS degrade cellular membranes by lipid peroxidation of polyunsaturated fatty acids (PUFAs), generating alkanes which are excreted in the breath as volatile organic compounds (VOCs) [14, 15]. 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} M) concentrations. Existing laboratory instruments cannot detect such low concentrations, so that breath VOCs must be collected and assayed with specialized instruments[19]. 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[26, 27]. Third, the breath VOC assay must compensate appropriately for VOCs present in ambient air. Since pentane is also present in room air in concentrations comparable to breath, a breath assay for pentane may be skewed by environmental contamination[28]. Fourth, most previous studies of oxidative stress markers in breath have focused near-exclusively on only two alkanes, ethane and pentane. These VOCs have attracted most attention because they are the easiest to measure with gas chromatography, but breath contains several other alkanes which are also rational markers of lipid peroxidation and oxidative stress[14, 15]. 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 apparatus (BCA) and assay which detects VOCs in breath and room air in picomolar concentrations[30]. 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 [59]. This method also facilitates the collection and assay of C4 to C20 alkanes, thereby extending the spectrum of oxidative stress markers which can be

detected in the breath. We have further extended this spectrum with the finding that monomethylated derivatives of C4-C20 alkanes are also apparent markers of oxidative stress which increased significantly with age in humans[32]. We have combined all of these VOCs into a comprehensive display of markers of oxidative stress, the breath methylated alkane contour (BMAC), a three-dimensional surface plot of the alveolar gradients of C4-C20 breath alkanes and their monomethylated derivatives. 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: 539 heart transplant recipients (mean age 54.3 yr, SD = 11.8, male/female = 411/128) were studied over a three-year period. 1061 technically satisfactory breath VOC samples were collected on the day of regular scheduled endomyocardial biopsy, prior to the procedure. Patients were studied at seven 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), Mt. 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). 32 age-matched healthy volunteers were selected from a group studied in Staten Island, NY [21] (male/female 16/16, mean age 53.2 yr, SD = 11.8, NS 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 [21, 30]. In summary, a portable BCA (Breath Meter Technology, Inc, Cleveland, OH) was employed to capture the VOCs in 1.0 l breath onto a sorbent trap; VOCs in 1.0 l 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 min. 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 RNC and JG without knowledge of the pathological findings. All samples were sent to the central laboratory by express mail and analyzed immediately. Results of a breath test were generally available within 24-48 hr of collection of the sample.
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 ISHLT ratings [16]: absent (Grade 0), mild (Grades 1A, 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 which best represented the diagnostic pathology because it contained the most severe focus of rejection. Two reviewers (JTF and PEF) 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) in order 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: The BMAC was determined in 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 2 ppm 1-bromo-4-fluoro-benzene, Supelco, Bellefonte, PA). V_a and I_a denote corresponding areas derived from the associated air sample. A three dimensional graph of these compounds, the BMAC, displayed the mean value of the alveolar gradient (y-axis) for a specified group of patients versus the carbon skeleton length (x-axis) and the methylation site (z-axis).

Analysis of data: BMACs were compared in three 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 Grade 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 who were studied more than once, repeat breath collections and biopsies were performed at least two 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 zero to 1.0, indicating the probability of Grade 3 rejection. Cross-validation of the patient's classification was performed with SPSS "leave one out" discriminant analysis procedure which predicted whether the patient belonged to the group with Grade 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. Confidence intervals were determined as standard error of percent (SEP).

Results: An overview of the study is shown in Figure 4.

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 to C20 alkanes and methylalkanes in the BMAC, 81 were observed in the breath of at least one heart transplant recipient. Five breath samples were collected from active smokers: four with Grade 0,1 or 2 rejection, and one with Grade 3

rejection. 150 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 1061 jointly agreed ISHLT grades comprised 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 Grade 0,1 or 2 rejection and Grade 3 rejection (respectively 54.7 yr, SD=11.5 and 54.2 yr, SD=14.0, NS).

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 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} and 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), negative predictive value = 96.7% (SEP=0.66) (Table 2 and Figure 4).

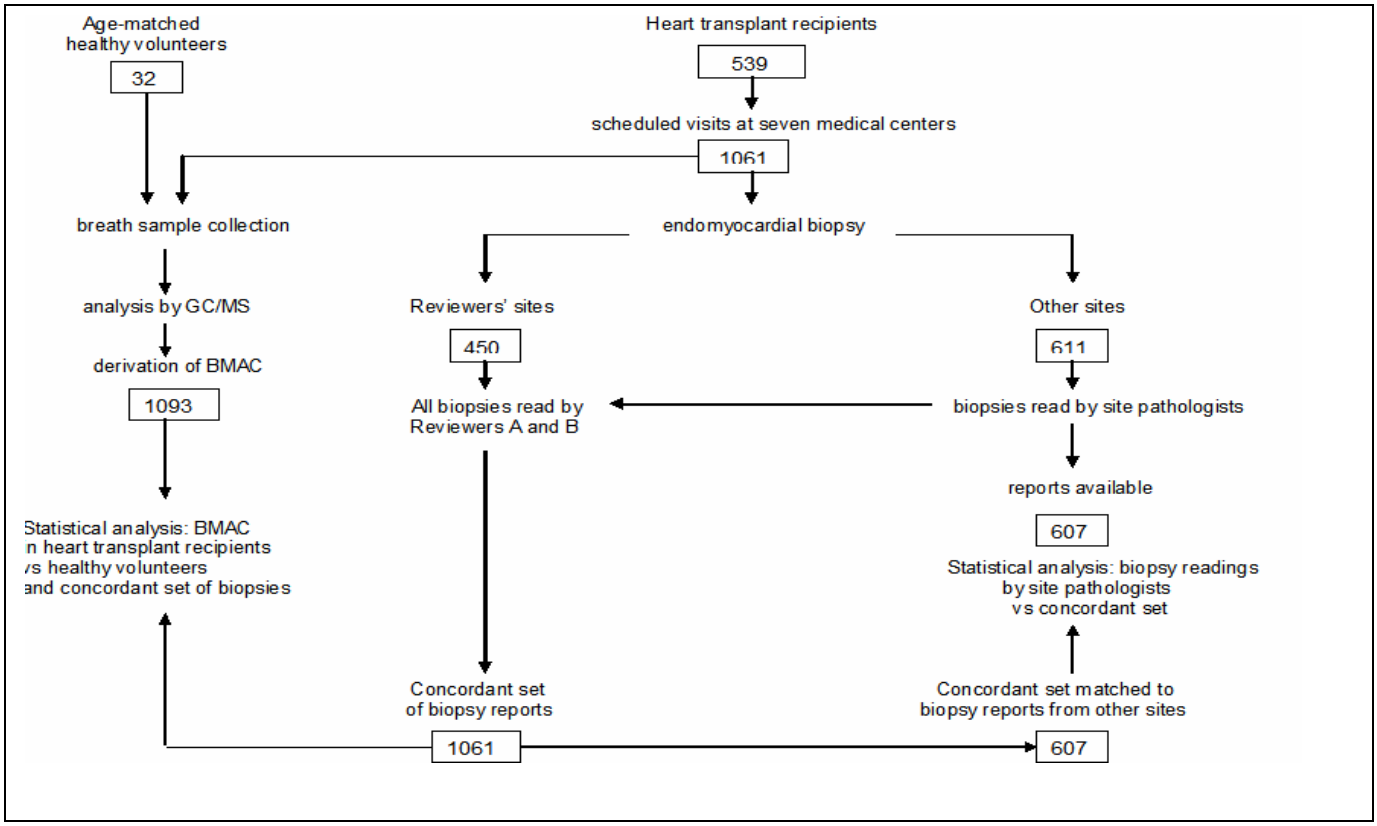


Figure 4: Overview of the research This figure demonstrates how the study groups were derived for statistical analysis. All endomyocardial biopsies were independently graded for degree of rejection by a site pathologist as well as by Reviewer A and Reviewer B. All used standard ISHLT criteria for scoring 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.

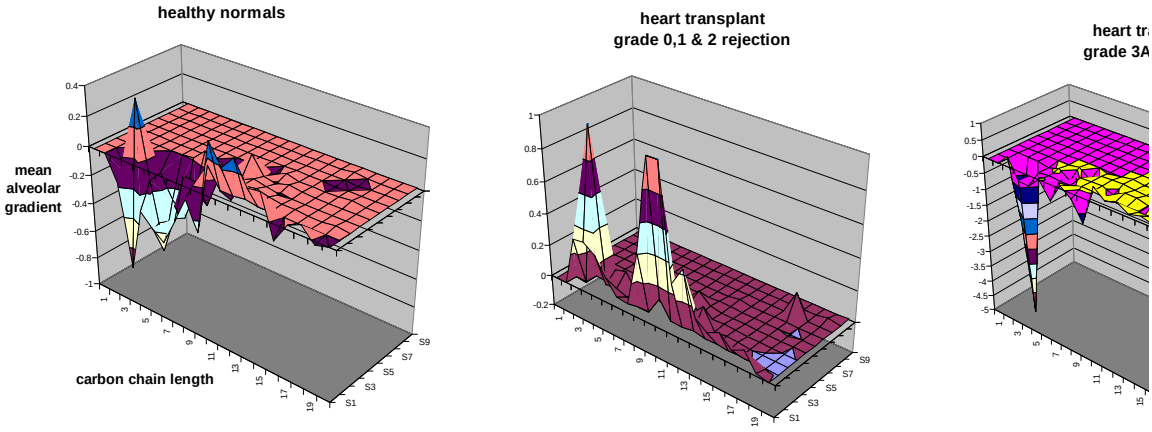


Figure 5. Surface plots of breath test results. The BMAC was constructed from all breath samples and surface plots of the mean BMACs are shown for three groups: healthy normals, heart transplant recipients with grade 0,1&2 rejection, and heart transplant recipients with grade 3 rejection. The alveolar 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 which provided optimal discrimination between grade 0,1&2 rejection and grade 3 rejection are listed in Table 1. The volume under curve (VUC) of each surface plot is shown in Figure 6.

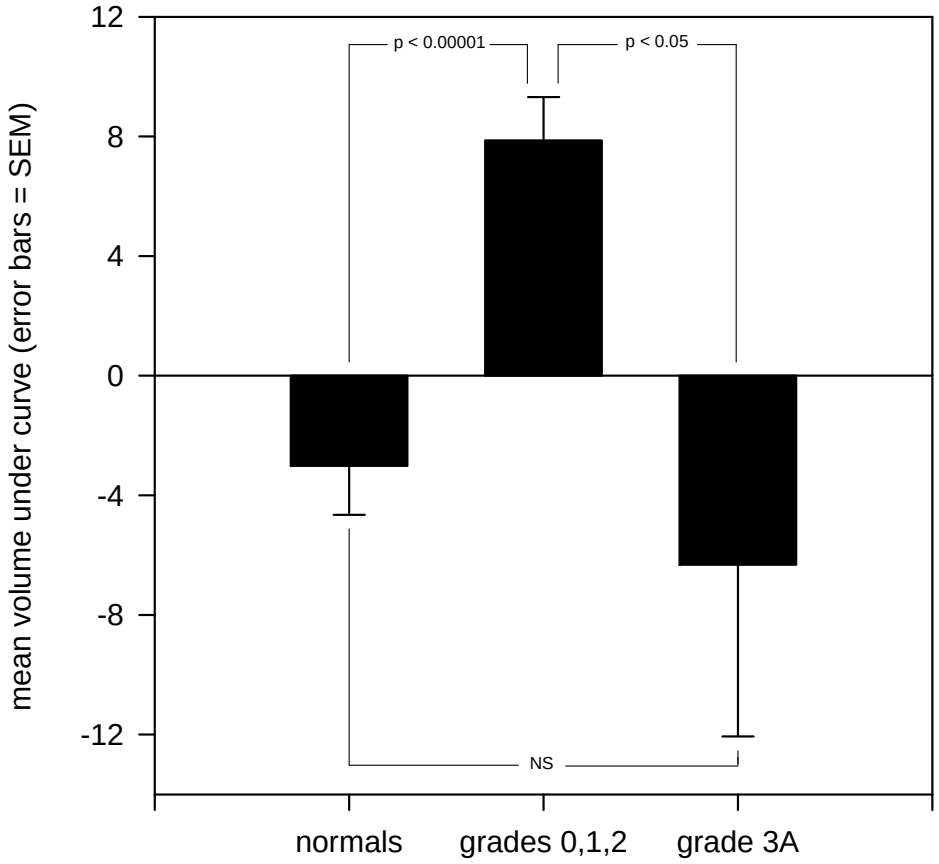


Figure 6: Volume under curve of BMAC surface plots. The mean volume under curve (VUC) of BMAC surface plots shown in Figure 2 is shown for three groups: healthy normals, heart transplant recipients with grade 0,1&2 rejection, and heart transplant recipients with grade 3 rejection (bar = SEM). Compared to healthy normals, the VUC was significantly greater in the group of heart transplant recipients with grade 0,1&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 pseudonormalization and not true normalization, since Figure 2 demonstrates that the distribution pattern of individual VOCs was not identical in the two 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 3. VOCs used to identify patients with Grade 3 heart transplant rejection. Alkanes and methylated alkanes were selected by forward stepwise discriminant analysis to generate a statistical model which predicted 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).

	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

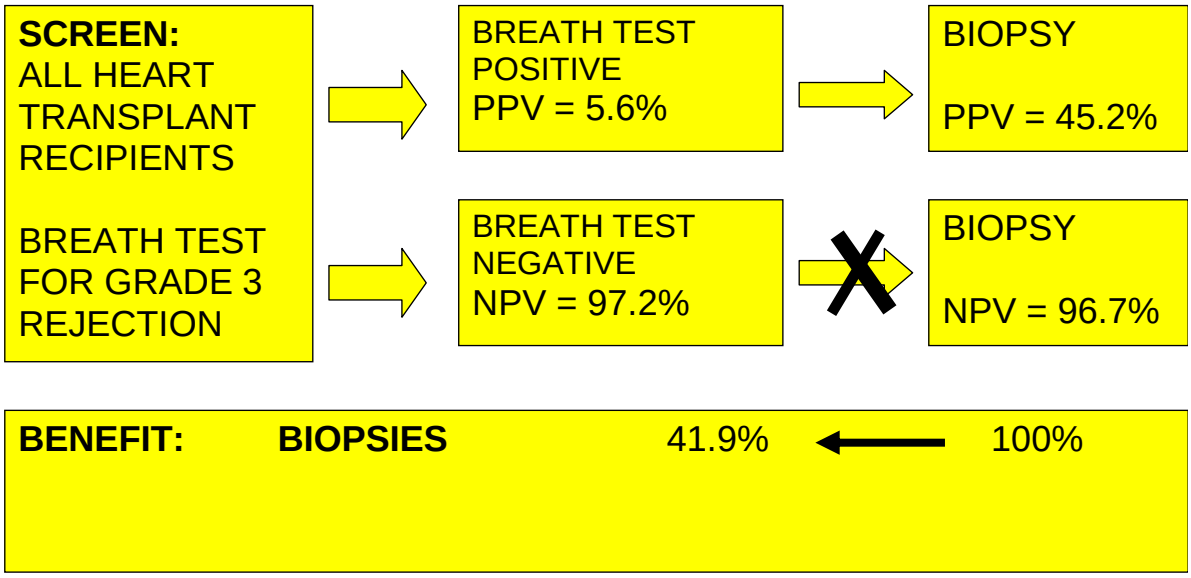


Figure 7: Screening breath test in clinical practice. This figure demonstrates the expected results of screening all heart transplant recipients with a breath test for Grade 3 rejection. If the breath test result is positive, it is appropriate to proceed to endomyocardial biopsy because the positive 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 more than one half.

Discussion: This study demonstrated three main findings: first, breath markers of oxidative stress were significantly more abundant in heart transplant recipients with Grade 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 Grade 0, 1 or 2 rejection was consistent with previous reports; increased myocardial oxidative stress has been detected in stored hearts and within hours following transplantation; these acute changes may be due to increased cytokine production and cytochrome c release[63 64]. Following 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 glutathione peroxidase, some of which persisted for up to 6 years after transplantation[13]. Coenzyme Q10 is depleted in transplanted human hearts, and mitochondrial respiratory chain function and energy production vary with the histological severity of rejection[65]. These findings are consistent with an abnormally high level of chronic oxidative stress in the transplanted heart, possibly resulting from chronic subclinical 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 5 and 6). 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 since 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 e.g. by drugs such as barbiturates [66] as well as by alkanes 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 physiological response to a toxin [67-69]. Several microorganisms also respond to high concentrations of alkanes and hydrocarbons with accelerated catabolism; the responsible genes have been characterized and cloned in the yeast Yarrowia lipolytica[70]. 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 with changes in cytochrome P450 3A activity[71]. El Gamel et al studied a group of heart transplant recipients treated with a standard dosage of cyclosporine and observed a significant decline in trough blood levels in patients who progressed from Grade 0 to Grade 3A rejection[72]. Other studies have also demonstrated significantly lower levels of cyclosporine in Grade 3 rejection than in Grade 0 rejection[73, 74]. It is possible that induced cytochrome P450 activity might explain reductions in the levels of both cyclosporine and breath VOCs in Grade 3A rejection..

We assigned all biopsies into one of two groups - Grades 0, 1 and 2 rejection or Grade 3 rejection, in order to identify the group at greatest need of increased immunosuppressive therapy. A subset of 9 VOCs in the BMAC identified patients with Grade 3 rejection, with a high negative predictive value. Table 2 demonstrates the comparative results of screening heart transplant recipients with a breath test or an endomyocardial biopsy 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 prior 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. Since 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 more than one half. 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 expressed 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 two major limitations: First, it is accompanied by a high degree of interobserver variability; studies of experienced pathologists reading an identical series of biopsy specimens revealed major

discrepancies between their ISHLT grading of rejection[7, 8]. 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 two 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 pathological information which guided their assessment of the grade of rejection.

We conclude that a breath test for markers of oxidative stress provides new evidence that oxidative stress is chronically increased in the majority of heart transplant recipients. A subset 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 the majority of 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.

Prediction of heart transplant rejection with a breath test for markers of oxidative stress [54]

We have reported a breath test for oxidative stress[32] which identified grade 3 rejection in heart transplant recipients in the HARDBALL study (Heart allograft rejection: detection with breath alkanes in low levels)[53]. We report here a follow-up analysis of the results of the HARDBALL study in which we determined the sensitivity, specificity and predictive value of the breath test as a marker of grade 3 heart transplant rejection.

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We employed a breath collection apparatus to collect 1061 breath samples from 539 heart transplant recipients prior to endomyocardial biopsy over a 3 year period at 7 medical centers. Volatile organic compounds (VOCs) in breath and room air were analyzed by gas chromatography and mass spectroscopy, and the breath methylated alkane contour (BMAC), a three-dimensional display of the abundance of C4-C20 alkanes and monomethylated alkanes was constructed for every patient[32]. The institutional review boards of all participating institutions approved the research.

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. The resulting mathematical model generated a value from each patient's

BMAC ranging from zero to 1.0, indicating the probability of grade 3 rejection. Cross-validation of the patient's classification was performed with a "leave one out" discriminant analysis procedure which predicted whether the patient belonged to the group with grade 0, 1 or 2 rejection or the group with grade 3 rejection. The predicted probability of grade 3 rejection was evaluated as a marker of disease by determining its sensitivity and specificity (shown in an ROC curve in Figure 8) and its positive predictive value (PPV) and negative predictive value (NPV) (Figure 9).

These findings demonstrate that the breath test divided the heart transplant recipients into three groups: positive for grade 3 rejection, negative for grade 3 rejection, and intermediate. In clinical practice, results in the intermediate group could be reported as the probability of grade 3 rejection, with the positive or negative predictive value of the test, dependent upon whether the probability was greater or less than 0.5

Breath testing provides a non-invasive new test for grade 3 heart transplant rejection which could potentially reduce the number of endomyocardial biopsies performed, and consequently reduce patient morbidity and health-care costs.

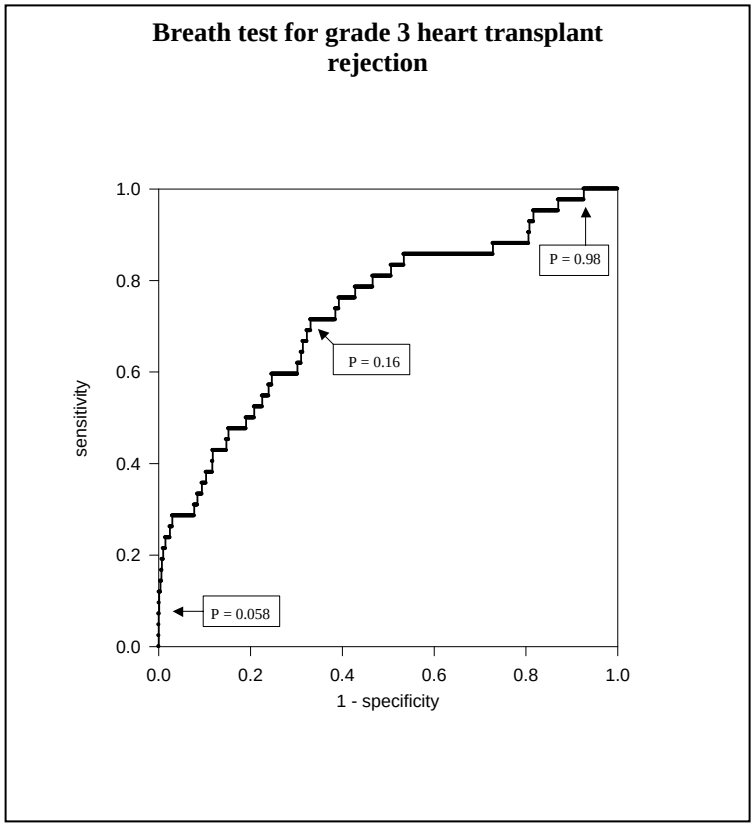
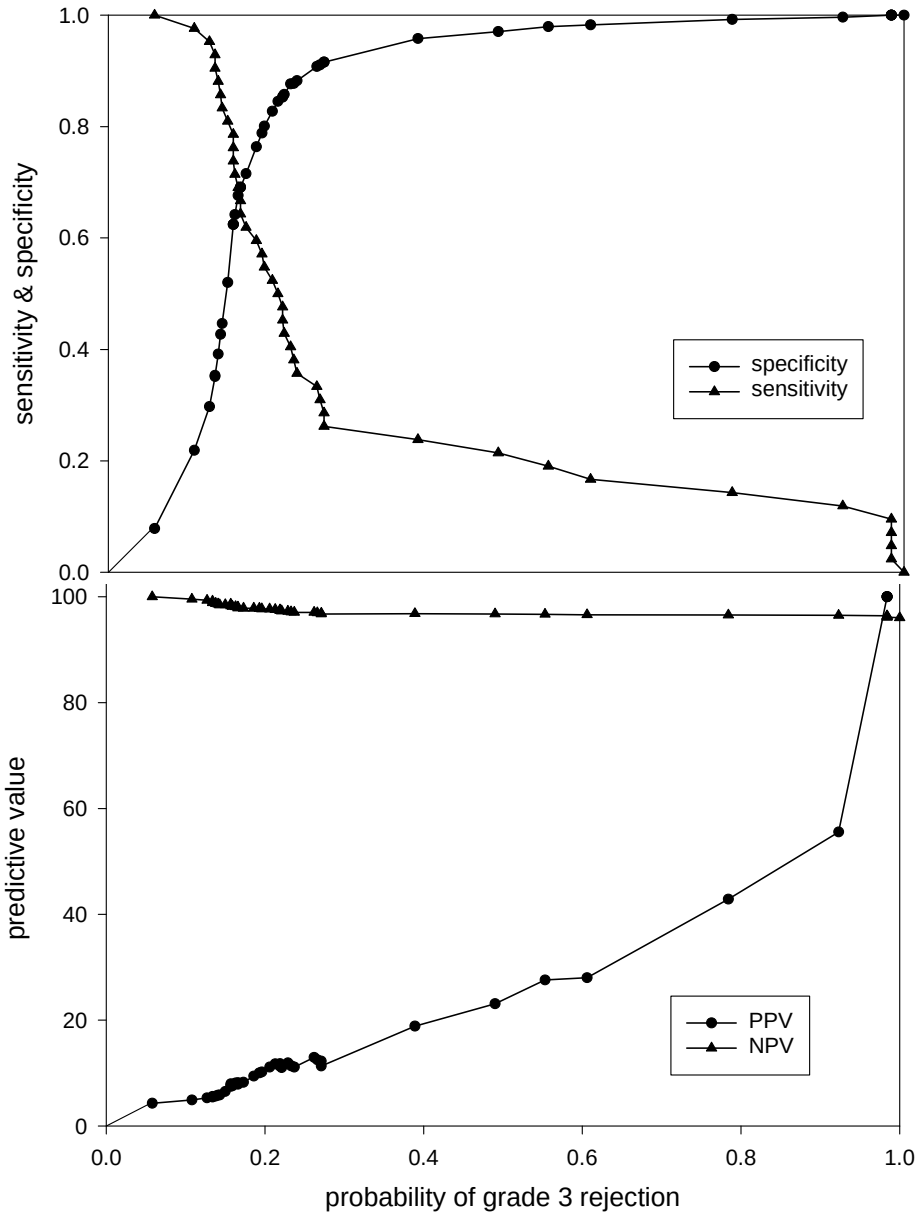


FIGURE 8. Receiver-operating characteristic of breath test for grade 3 heart transplant rejection. The breath test was 100% sensitive for grade 3 heart transplant rejection when the predicted p value was >0.98. Specificity was 100% when the predicted p value was <0.058. At the shoulder of the curve (predicted p <0.16), the sensitivity was 71.4% and the specificity was 62.4%.

FIGURE 9. Variation in sensitivity, specificity, and predictive value of the breath test. This figure demonstrates the variation in sensitivity and specificity *(upper panel)*) and positive (PPV) and negative predictive values (NPV) *(lower panel)* of the breath test, depending upon the predicted probability of grade 3 rejection. The PPV of the breath test for grade 3 rejection (i.e., its ability to rule in grade 3 rejection) increased to 100% when the predicted probability was >0.98. However, the NPV of the breath test (i.e., its ability to rule out grade 3 rejection) showed less variation because of the low prevalence of the disorder.



8. Benefits of the Heartsbreath test

a. ***Medical:*** The anticipated medical benefits of the Heartsbreath test are shown in Figure 7. This figure demonstrates 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 could reduce the number of biopsies performed by more than one half.

b. ***Financial:*** Evans et al reported the costs of endomyocardial biopsy (American Journal of Transplantation 2005; 5 (6):1553):

Average outpatient biopsy cost: $3297 (excluding professional fees)

Average reimbursement: Medicare $3581
Private payers $4140

The average heart transplant recipient has several endomyocardial biopsies performed in the first year:

		cumulative
Week 0 – 6	*Weekly*	*6*
3 months	*biweekly*	*9*
6 months	*monthly*	*13*
12 months	*every 1-3 months*	*19*

Consequently, the average cost of endomyocardial biopsies to Medicare in first year is:

19 biopsies @ $3581 = $68,039

In the majority of patients, the Heartsbreath test will be negative. When employed as an ancillary test, a transplant cardiologist reviewing a clinically stable patient with a negative Heartsbreath test (the majority) might elect to perform biopsies only on alternate months. As a result, the number of biopsies could potentially be dramatically reduced, with a consequent reduction in costs, yet without compromising the quality of patient care (Figure 10). This figure assumes a cost of $500 for the Heartsbreath test.

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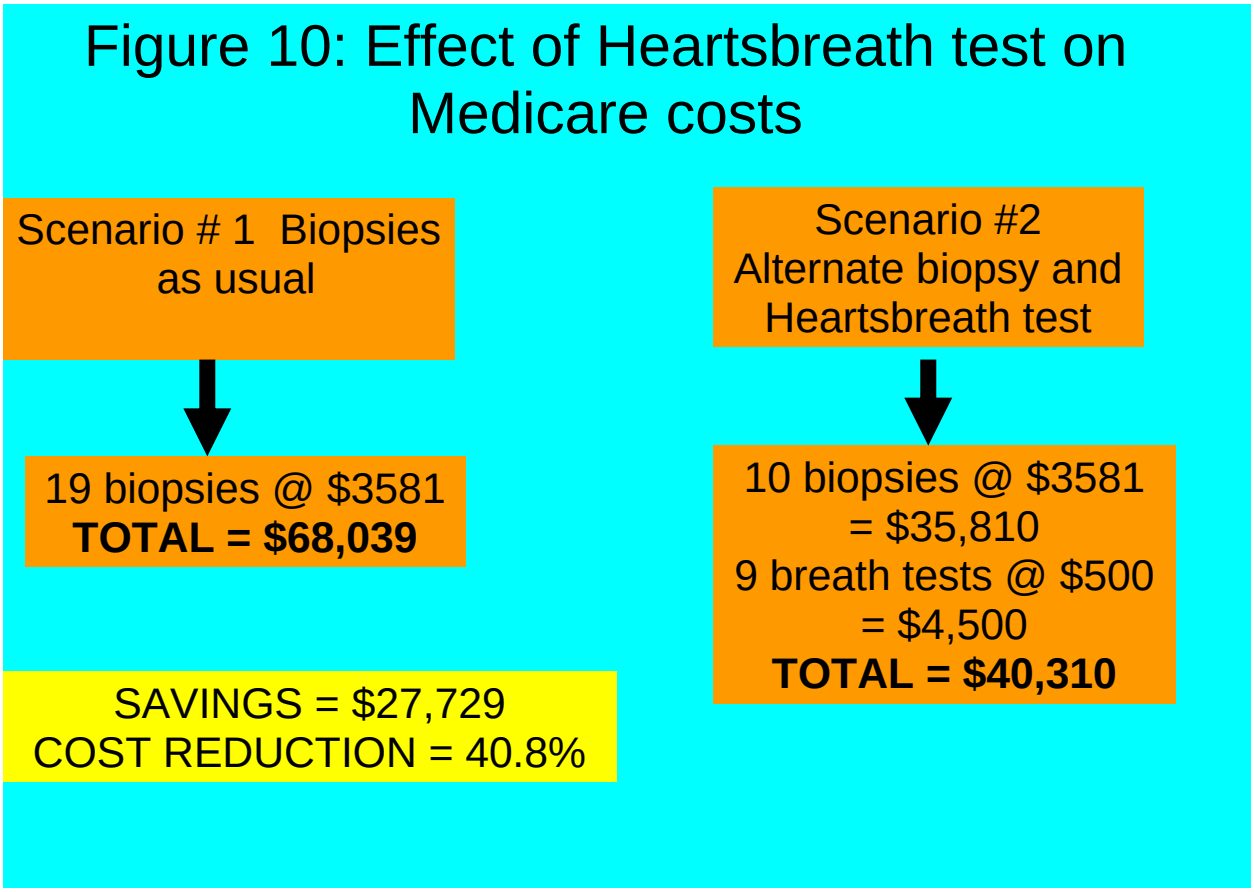

9. FDA approval of the Heartsbreath test

a. Humanitarian Device Approval of the Heartsbreath test: The following information is displayed on the FDA website at
http://www.fda.gov/cdrh/MDA/DOCS/H030004.html

Heartsbreath - H030004 *FDA approved this device under the* Humanitarian Device Exemption (HDE) program*. See the links below to the Summary of Safety and Probable Benefit (SSPB) and other sites for more complete information on this product, its indications for use, and the basis for FDA's approval.*

Product Name: Heartsbreath
Manufacturer: Menssana Research, Inc.
Address: 1 Horizon Road, Suite 1415, Fort Lee, NJ 07024-6510
Approval Date: February 24, 2004
Approval Letter: http://www.fda.gov/cdrh/ode/H030004sum.html

What is it? The Heartsbreath test is a breath test that is used along with a traditional heart (endomyocardial) biopsy on patients who have received a heart transplant within the past year. This test measures possible organ rejection in heart transplant patients.

How does it work? The Heartsbreath test works by measuring the amount of methylated alkanes (natural chemicals found in the breath and air) in a patient's breath.

- The patient breathes into a plastic mouth piece that is attached to a breath collecting device.
- The device subtracts the amount of methylated alkanes in a patient's breath from the amount of methylated alkanes in the room air.
- The value generated by the device is compared to the results of a biopsy performed during the previous month to measure the probability of the implanted heart being rejected.

When is it used? The Heartsbreath test may be used in patients who have had heart transplants within the past year. The results of the Heartsbreath test should be compared to a heart biopsy performed during the previous month.

What will it accomplish? The Heartsbreath test may be used along with the results of a heart biopsy to help guide short term and long term medical care of heart transplant patients.

The test's greatest value may be in helping to separate less severe organ rejection (grades 0, 1, and 2) from more severe rejection (grade 3).

When should it not be used? The Heartsbreath test should not be used for patients who:

- have received a heart transplant more than one year ago, or
- have a grade 4 heart transplant rejection because Heartsbreath has not been evaluated in these patients.

Additional information:

SSPB and Labeling: http://www.fda.gov/cdrh/ode/H030004sum.html

Illustrated anatomy of the heart:
http://www.tmc.edu/thi/anatomy2.html

Fact sheet for heart transplant patients from the American Heart Association:
http://circ.ahajournals.org/cgi/reprint/106/14/1750.pdf

b. Other relevant FDA documentation

Part 1 - Approval Order is shown in Appendix 5

Part 2 - Summary of Safety and Probable Benefit is shown in Appendix 6

Part 3 - Professional Labeling is shown in Appendix 7

Part 4 - Consumer Labeling is shown in Appendix 8

Other Consumer Information is shown in Appendix 9

References

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