B. Table of Contents

C. Executive Summary/Abstract

Breath condensates collected from healthy smoking volunteers will be used to explore patterns of phosphatidylcholine (PC) derived lipids and eicosenoids. The critical components will be identified and used to compare pattern differences to matched non-smoking control volunteers. Breath condensate will be collected within an hour following cigarette smoking by breathing ambient air through a disposable mask attached to a piece of tubing cooled to 0°C for 15 –20 minutes or using ECoScreen®, a non-invasive breath collecting system. The lipids extracted from breath condensate will be separated into polar lipids (mostly PC, lyso-PCs), platelet activation factors and eicosanoids. The polar lipids will be analyzed via HPLC/electrospray/tandem mass spectrometry. The eicosanoids will be derivatized to electron-withdrawing pentafluorobenzyl trimethyl silyl derivatives for separation by gas chromatography and analyzed by negative ion mass spectometry.

We expect, like asthma, that there are individuals whose lungs are hypersensitive to the stress of smoking, which should be reflected in the lipid/eicosanoid profile. Target eicosenoids include prostaglandins, (PGE_2, PGD_2, $PCF_{2\alpha}$), thromboxane (TxB_2), and prostacyclins (6-Keto $PGF_{1\alpha}$), as they show differential responses to inflammation. Additionally, the leukotrienes (LTB_4, LTC_4, LTD_4, LTE_4) show responses related to allergy, isoprostanes (8-iso $PGF_{2\alpha}$) are responsive to free radical oxidative stress and HETEs show differential lipo-oxygenase activities. Each of these compounds could play a significant role in establishing a lipid/eicosanoid profile that could be used to monitor the effects of smoking.

In this feasibility study we will correlate medical history of these healthy, smoking subjects to smoking subjects with a history of wheezing, pneumonias, or other manifestations of pulmonary hypersensitivity and peridontitis. We hope to find collaborators for a more in-depth correlation in future studies with Institute for Science and Health particularly directed to testing Potential Reduced Exposure Products if feasibility of breath condensate lipid & eicosanoids prove successful.

D. Introductions and Significance to Science

Introduction

Tobacco use causes 400,000 deaths/year in the USA (1) many of which might be prevented if education focused on abstinence-oriented prevention and cessation strategies were more effective. Nicotine is a remarkably active, addictive drug because small amounts roughly equivalent to a single cigarette can produce long-term stimulation in the dopaminergic reward centers of the mid brain (2). Amongst other alternatives, Institute for Science and Health (IfSH) is supporting developing Potential Reduced Exposure Products (PREPS) for changing cigarette and smoke constituent exposures.

Our proposal is to utilize regulatory lipids and eicosanoids to monitor pulmonary responses to smoke. Of particular interest are lipids and eicosanoids that mediate pulmonary inflammatory responses, immune/allergic responsiveness, activation and chemotaxis of specific white-cells, broncho-constriction, surfactant distribution and composition and blood distribution and coagulability. Additionally, enzymatic oxidative reactivity of the 5-, 12-, and 15-lipooxygenases, the P_{450} detoxification systems and the free radical activities reflected in the isoprostanes and Phosphatidylcholine derivatives (4, 5) could also be significant in monitoring pulmonary responses to smoke.

Our research program proposes to determine the feasibility of utilizing regulatory lipids recovered from breath condensate to monitor specific signals induced by exposure to smoke.

These regulatory lipids would then be used collaboratively with IfSH colleagues to monitor development of PREPS. One of these regulatory lipids, 8-isoprostane, has been found in breath condensate and is considered a "gold-standard" biomarker of oxidative stress (6,). 8-isoprostane is elevated in the breath condensate of smokers (7). Other regulatory lipids have been detected in breath condensate. Our research, supported by the Defense Advanced Research Project Agency (DARPA), has established that at least 6 eicosanoids and 10 phosphatidylcholine surfactant derivatives are present in piglet breath condensate.

We propose to establish the feasibility of using non-invasive breath condensate monitoring of patterns of these critical mediators of tissue responses as predictor biomarkers for toxicity responses. These toxicity response patterns would become quantitative risk assessors that in subsequent collaborations could become cost-effective in predicting risks from modifications in smoke constituent exposures as PREPS are developed.

Significance

Nearly one in 5 deaths in the United States is a result of smoking (8). This ranks smoking as the leading preventable cause of death and brings with it a smoking-related cost to the health care system estimated at $75.5 billion/year (8). Smoking is a significant and complex problem as cession and abstinence are particularly difficult. Some of us are particularly susceptible. Recently one genetic difference in the propensity to nicotine addiction was demonstrated. Mutations in the human CYP2B6 gene product enzyme, that is involved in nicotine metabolism induces increased risk of vulnerability to abstinence symptoms and relapse (9). Is it possible to non-invasively discern especially susceptible subjects or especially toxic smoke constituents? We propose to show the feasibility for non-invasive detection of the patterns of regulatory lipids in breath condensate to help understand smoke constituent toxicity, provide a future capability in defining responses to manipulating PREPS, and detect differences that can possibly be correlated to potential hypersensitivity.

E. Objectives**:** We propose three objectives: 1. Develop quantitative, rapid assays of lipid and eicosanoid regulatory lipid biomarkers from breath condensate as a non-invasive mediators of inflammation, immune reactivity, P450 activity and oxidative stress in healthy tobacco smoker subjects compared to age/sex/medical history matched non-smokers for prediction of consequences of long and short term exposure and harm; 2. Test feasibility of breath condensate lipid and eicosanoid patterns for risk assessment and prediction of success in decreasing exposure and defining hypersensitive subjects. and 3. establish collaborations with IfSH investigators who are testing Potential Reduced Exposure Products (PREPS) or other therapies for changing cigarette and smoke constituent exposures and understanding constituent toxicity by correlating breath condensate lipid and eicosanoid regulatory lipid biomarker patterns to smoke exposures.

F. Overall approach

We propose to demonstrate the feasibility of measuring changes in patterns of breath condensate lipids and eicosaniods between healthy smokers and matched non-smokers as a non-invasive quantitative means to monitor development of Potential Reduced Exposure Products (PREPS) or other therapies for changing cigarette and smoke constituent exposures.

Subjects will breathe ambient air into the cooled non-invasive system for collecting breath condensate in disposable device for 15 –20 minutes at least 1 hour since last cigarette. The lipids from the condensate will be extracted and polar lipids (mostly phosphatidylcholines and platelet activating factors), and eicosanoids analyzed by mass spectrometry. The lipids will be examined utilizing HPLC/electrospray/tandem mass spectrometry. The eicosanoids will be

methoximated, electron-withdrawing pentafluorobenzyl esters generated, and the hydroxyls trimethylsilated prior to separation by gas chromatography and analyzed by capillary gas chromatography/electron capture negative chemical ionization/mass spectrometry (GC/ECNCI/MS) as negative ions (see below). Prostaglandins, (PGE_2, PGD_2, $PCF_{2\alpha}$), thromboxane (TxB_2), and prostacyclin (6-Keto $PGF_{1\alpha}$), show differential responses to inflammation and immune status, the leukotrienes (LTB_4, LTC_4, LTD_4, LTE_4) show responses related to allergy, isoprostanes such as 8-iso$PGF_{2\alpha}$ are responsive to free radical oxidative stress, HETEs show differential lipo-oxygenase activities. One eicosanoid, 8-iso$PGF_{2\alpha}$ is elevated in healthy smokers (6, 7). We expect to show differences in patterns of the regulatory lipids detected in breath condensate between matched non-smokers and smokers with exposure to tobacco.

With the analysis of multiple regulatory lipids and eicosanoids as proposed herein we expect to be able demonstrate the feasibility to be able to define patterns which can then be correlated to exposures to specific smoke components in proposed continuation collaborations. We also expect that like asthma, that there are individuals whose lungs are hypersensitive to the stress of smoking. In this feasibility study we will correlate medical history of these healthy subjects particularly to history of wheezing, pneumonias, or other manifestations of pulmonary hypersensitivity and peridontitis. We hope to find collaborators for a more in-depth predictive risk assessment correlations in future studies if feasibility of breath condensate lipid & eicosanoid pattern analysis proves successful.

Demonstrations of differential responsiveness to stress in breath condensate lipids and eicosanoids from our research: Detection of multiple PCs and lysoPCs in piglet breath condensate has been repeatedly demonstrated. In an experiment where a piglet was exposed to 30 µg/Kg staphylococcs enterotoxin (SEB), the lipid extract of the breath condensate was subjected to HPLC, and a total ion current measured where the parent ions loosing a fragment of m/z 184^+ (phosphocholine) were recorded. Masses of the major components of each parent ion cluster were then determined. Phosphocholine containing components were detected in the total parent ion current (Q1 scan) at 2-4 min containing major components at m/z 496.4, 520.4, & 522.4; 18-23 min contained m/z 782.7; 26-28 min contained components with m/z 758.7, & 784.7 and at 36-38 min components at 760.7 & 734.8. Piglet breath condensate clearly contains multiple major PC surfactant components. Preliminary evidence shows that the amounts and patterns of these components change in response to different aerosolized stresses. Collection of larger breath condensate samples from humans as proposed in this study will allow determination of the fatty acid components in each of these lipids and additional lyso-PC and other lipid regulatory factors.

Breath condensate lipid components can be detected when recovered with the invasive bronchoalveolar lavage (BAL) technique (10, 11, 12). Eicosanoids recovered by BAL from rats exposed to aerosolized lipopolysaccharide (LPS) showed a differential response to exposure measured at 0, 6, and 24 hours. M. D. Karlstad at The University of Tennessee Medical Center, Knoxville, performed these exposures. We analyzed eicosanoids from 15 ml BAL/rat exposed in a head isolated chambered apartment for breath extraction. Rats showed Neutrophilic white blood cell proliferation in the BAL in response to LPS not seen in controls. Data shown below indicates LPS stimulates 8-ISO, PGE_2, 6-Keto & TxB_2 especially at 24 hrs, PGD_2 only at 24 hrs, whereas LPS has no effect on PGF_{2a}.

Component	Rat	control	LPS	Control	LPS
		25 hrs (# 5)	6 hrs (#7)	5 hrs (# 6)	6 hrs (#8)

	ng/ml	ng/ml	ng/ml	ng/ml
8-ISO	0.030	0.078	0.164	0.220
6 Keto	0.037	0.063	0.080	0.392
TxB_2	0.058	0.115	0.143	0.406
PGF_{2a}	0.005	0.007	0.021	0.018
PGE_2	0.093	0.125	0.108	0.245
PGD_2	0.177	0.175	0.184	0.716

In experiments with breath condensate recovered from piglets, we showed that breath condensate contained 6-keto $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2 , & 8-iso $PGF_{2\alpha}$. TxB_2 and PGD_2 were below detection limits. PGE_2 levels in 3 piglet controls were 1.2, 0.7 and 0.5 ng/mlof breath condensate. Levels of $PGF_{2\alpha}$ and PGE_2 in the breath condensate of one piglet over a 9 hour time period for$PGF_{2\alpha}$ were 0.12, 0.14, 0.12, 0.14, and 0.10 ng/mland for PGE_2 were 0.21, 0.20, 0.21, 0.23, and 0.24 ng/ml indicating reproducibility. There were differential effects of 100 µg/Kg lipopolysaccharide (LPS) and 150 µg/Kg staphylococcal enterotoxin (SEB) on piglet were detected. SEB exposure induced 0.4, 0.2 and 0.7 ng/ml PGE_2 whereas the response to LPS was not detectable (< 0.01 ng/ml). 8-iso $PGF_{2\alpha}$ remained at a constant low level 0.02-0.09 ng/ml after SEB exposure [control 0.05 ng/ml], whereas exposure to LPS induced levels below detection limits except after 8 hours where 8-iso $PGF_{2\alpha}$ increased to 1.6 ng/ml.

G. Experimental Design

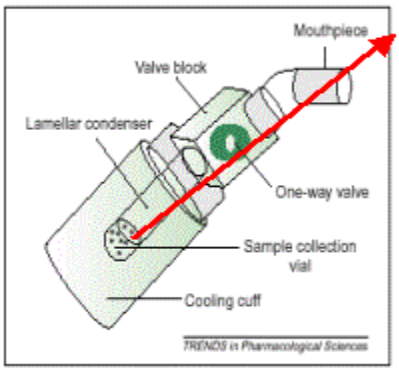
Methodology (sample collection):

About twenty-five healthy adult smoking volunteers with age, sex, and race matched to 25 non-smokers will be recruited. The ages will range between 26-80, both male and female, of African American, Asian-American and Caucasian decent. The volunteers will provide informed consent as approved by The University of Tennessee Institutional Review Board. Subjects will be asked to mark a medical checkoff sheet of past medical history and family history to help in the matching between smokers and non-smokers. Encrypted (www.pgp.com) data on experiments will be maintained by the Principal Investigator and electronically transmitted to A. Peacock at CBA for subsequent analysis. Patients will be assigned a case number to protect confidentiality and case report forms will be used to record clinical data. All research data will be stored in lockable cabinets accessible only to study personnel. External access to the computers hosting neural computing and database applications will be restricted by a *firewall* to secure shell (*ssh*) and *http* ports.

The collaborating participants will be recruited by word-of-mouth for volunteers, or as advertised in the university paper. Volunteers will inhale room air whist sitting quietly and exhale through a valve into a breath condensate collection apparatus for 15-20 minutes. In the initial analytical development phase the collection apparatus for the exhalent gases will be a Hans Rudolph mouth breathing two-way non-rebreathing mask [model 112849] attached to a autoclavable Hytrel smooth bore tube 21.7 mm ID x 4' long, that fits over the 35 mm OD mask outlet that passes through a bucket cooled with ice/water next to the seated subject (10). The mask & valves will be cleaned and sterilized with the clinically approved cleaning procedures developed by Hans Rudolph, Inc., Kansas City, MO. The tubing will be cleaned and autoclaved. With the application phase of the programs initiated, the ECoScreen® apparatus used clinically in Europe to collect breath condensate (11) which has a disposable collection apparatus for each participant will be utilized. The volunteer will then utilize the disposable mouthpiece with a valve separating input air from output air and breathe normally whilst sitting in a chair and wearing a nose clip. Approximately 1-3 ml of breath condensate will be recovered from the tube and frozen for analysis.

The balance between sex, age and ethnic and medical histories origin will be used to balance the approximately 25 smoking and 25 nonsmoking volunteers. The matching, willingness and cooperation of the volunteers will be criteria for selection. We are prepared to pay each participant $20.00 for the inconvenience of the non-invasive test if they desire payment. Some may be asked for multiple tests to help define reproducibility.

A diagram of EcoScreen® collector and analytical protocol used for samples recovered from is illustrated:



Extraction of Condensate

⬇ Lipids HPLC/ES/MS/MS

Fractionation ➡

FA GC/ECNCI/MS

⬇

Pattern correlation with response

Analysis of the breath condensate:

a. Intact lipids

A portion of the breath condensate will be extracted with the one-phase solvent extraction techniques developed in this laboratory at room temperature (14), and subjected to analysis utilizing high performance liquid chromatography/elecrospray ionization/tandem mass spectrometry (HPLC/ES/MS/MS) for analysis of the phosphatidylcholine containing polar lipids (PCs) as m/z [M+1] will be detected at an LC-Tandem-MS API 365 (Applied Biosystems/MDS SCIEX, Concord, Ontario) by a precursor ion scan of m/z 184 in the positive mode. The samples (10 or 20 µl) will be introduced into the electrospray source by an Agilent 1100 HPLC system at a flow rate of total 50µl min^{-1}. For separation of PCs with different fatty acid composition at the glycerol backbone a reverse phase column will be used (HAISIL RP18, 50x1, 100µm, 15% carbon load) with a constant flow of 40ul min^{-1} MeOH:Water (95:5 + 0.002% piperidine, v/v) as running solvent for 45min. Piperidine in MeOH (0.02%) will be added at 10µl min^{-1} as post column modifier to increase the ionization efficiency in the electrospray (15). Breath condensates will be prepared after thawing by extraction of 250µl with a modified Bligh & Dyer procedure (14). The final methanol extracts will be dried with a gentle stream of nitrogen and reconstituted in 50µl of the running solvent. For most sensitive detection of PC composition in breath condensate samples 20µl extract will be measured without column separation in 3min, as the samples do not contain other compounds yielding a precursor of m/z 184. With extractions the lipid in 1-5 ml of condensate can be concentrated into a few µL of solvent for analysis.

A second portion of the breath condensate will be acidified to pH 3.0 and extracted with a solid phase extraction cartridge, eluted, and analyzed by gas chromatography/electron capture negative ion/mass spectrometry (GC/ECNCI/MS) after derivatization to form the methoximated, trimethylsilated pentafluorobenzyl esters (16-18) for the eicosanoid fatty acids. Between 0.1-0.4 ng/µL authentic tetradeuterated eicosanoids prostacyclin 6-keto-$PGF_{1\alpha}$, thromboxane TXB_2, and the prostaglandins $PGF_{2\alpha}$, PGE_2 and PGD_2 (Caymen Chemical Co., Ann Arbor MI) will be accurately added to a few milliliters of deionized water. The internal standard mix will be added to breath condensate and subsequently acidified to pH 3 with 1N

HCl. This will be loaded onto a pre-conditioned C18 solid phase extractor resin sep-pak and washed with pH 3 water and heptane. The eicosanoids will be eluted with 1:1 ethyl acetate:heptane and dried over sodium sulfate. The concentrated sample will be dissolved in acetonitrile and ketones methoximated with a solution of methoxyamine hydrochloride in water. Following the reaction, the sample will be extracted with ethyl acetate and the concentrated product derivatized with a 10% solution of pentafluorobenzyl bromide (PFBB) and 10% diisopropyl amine to generate the pentafluorobenzyl (PFB) ester. The PFB ester will be purified via thin layer chromatography. The purified products subsequently undergo conversion to trimethylsilyl (TMS) derivatives by reaction with *bis*(trimethylsilyl)trifluoroacetamide (BSTFA) and dimethyl formamide (DMF). The methoximated, PFB esterified and silated products will be brought up in 23 *u*l of undecane and 2 *u*l injected onto a J & W 15m X 0.25 ID X 0.25*u*m DB 1701 capillary gas chromatograph column utilizing negative ion/ chemical ionization gas chromatograph/mass spectrometer (GC/ECNCI/MS) at a constant flow of 1ml/ min, 6.8 psi at 190°C. The initial GC oven temperature will be set at 190°C for 0.5min and then increased at 20°C/min to 300°C and held for 0.75min. 6-keto-$PGF_{1\alpha}$(detected at m/z 614 & 618), 8-iso $PGF_{2\alpha}$(an isoprostane), and $PGF_{2\alpha}$, detected at m/z 569 & 573 from the first level of the thin layer plate; TXB_2 detected at m/z 614 & 618 and PGE_2 detected at m/z 524 & 528 from the second level of the thin layer plate; and PGD_2 detected at m/z 524 & 528. The LOQ for these isoprostanes has been about 0.02 pg/µL.

Statistical methods:

CBA has experience in utilizing biomarkers to correlate with microbial community structure and physiological activities in environmental samples the methods involve ANOVA, MANOVA, linear discriminant analysis, principal components analysis, and artificial neural net analysis (19, 20).

Research plan:

We will collect breath condensate from normal subjects for a supply of constituents to test for expanded panoply of components in the first 4 month of the project. The expanded panoply of components will then be used in the pattern recognition feasibility demonstration scheduled for the last 9 months of the project.

a. *Intact lipids* We will focus on the PCs utilizing our capillary HPLC with the tandem MS/MS concentrating on the phosphocholine containing lipids (defined by parental ions yielding m/z 184^+ (phosphocholine) product ion. The lipid content of pulmonary surfactant (primarily dipalmotyl-PC) is changed by exposure to tobacco smoke (21). Structural features of the PC surfactant lipids in breath condensate can be determined as lithiated products by HPLC/ES/MS/MS (22). Cells are remarkably responsive to lysophospholipids (23). Recently the lyso-PC moieties have been shown to have biological activity as well as being precursors of the lysophosphatidic acid mediators of cell growth (24). Lyso-PCs are readily measured by HPLC/ES/MS/MS (24). Like the PCs and sphingomyelin they can be readily defined as choline containing phospholipids with the product ion at m/z 184^+. The important regulatory lipids derived from sphingomyelin such as sphingosine-1-phosphate are readily detected by HPLC/ES/MS/MS (25). Other regulatory components characterized with a free hydroxyl group such as diglycerides (26) or platelet activating factors (PAFs) (27) can be directly esterified with pentafluorobenzoic anhydride to form the pentafluorobenzyl (PFB) esters. These are readily detected as negative ions with GC/ECNCI/MS at ng/ml sensitivity (utilizing the same system we have for the 6 eicosanoids).

b. *Eicosanoid analysis:* In the initial 4 month period we will focus on facilitating the more complete and consistent recovery of the 8-iso PGF_2, 6-keto-$PGF_{1\alpha}$, TXB_2, $PGF_{2\alpha}$, PGE_2, and PGD_2 The current system was developed in 1987 (18) involves acidification of the condensate to < pH 3, recovery through an activated C-18 solid phase extractor, elution, concentration,

methoximation, esterification to form pentafluorobenzoyl esters, thin layer chromatography to remove the PFB, recovery, trimethylsilization of hydroxyls, followed by GC/ECNCI/MS analysis of three fractions. From analysis of the tetra-deuterated standards the recoveries can vary over 3 orders of magnitude. We have acquired di and 7-^{3}H PGE_2 to quantitatively follow the recovery with scintillation spectrometry. We will replace the SPE cartridges with extraction of the condensate made 4 mM with citric acid and 0.02% antioxidant BHT with an equal volume of hexane/ethyl acetate v/v 1:1, vortex 2 min & centrifuge at 1800g x 10 min at 4^{o}C. Recover the organic phase and repeat twice (28). The eicosanoids can then be derivatized for analysis. Based on our experience the hexane/ethyl acetate will not extract the polar lipids from the aqueous breath condensate. These would be recovered using the one-phase chloroform methanol sovent (14). We will test this with authentic PC. The second problem in recovery is the thin layer chromatography (TLC). The primary function is to remove the unreacted PFBB. A small portion of silicic acid will be added to a capillary tip, pre-washed with hexane:ethyl acetate 1:1 followed by hexane. The PFB reaction mixture will be then added to the capillary tip and the eicosanoids eluted after washing with hexane, hexane/ethyl acetate 1:1, and eluted with hexane/ethylacetate 9:1 (29). These modifications for eicosanoid pattern analysis will precede subsequent extraction of the breath condensate with chloroform:methanol for the lipid PC derivatives.

The leukotrienes are a major group of eicosanoids found in breath condensate with major effects primarily in allergy and immune system modulation. They will be measured with other eicosanoids as described above. Leukotriene B_4 (LTB_4) is readily analyzed as a trimethylsilated PFB ester by GC/EC/NI/CI/MS (30). The fascinating cysteinyl leukotrienes are readily analyzed by GC/EC/NI/CI/MS after reduction and desulferization of the of the cystinyl derivative (31). For the comprehensive assessment of which eicosanoids will be added to the pallet for pattern testing will be done most efficiently by purchase of authentic eicosanoids from Caymen Chemical Corp., Ann Arbor, MI. They will be derivatized and analyzed by GC/ECNCI/MS. In addition to the leukotrienes, the HETEs, diHETEs, Lipoxins, di- and tetra-nor metabolic products of the TBX_2, PGE_2 and $PGF_{2\alpha}$ will be explored with the generation of their mass spectra. The breath condensate components generated from the GC/ECNCI/MS analysis will be matched to the authentic compounds to create patterns. This task would be made enormously more effective if the increased sensitivity, comprehensiveness, and considerably greater specificity of tandem mass spectrometry were utilized (16). The acquisition of a GC/ECNCI/tandem MS is a major priority for the CBA.

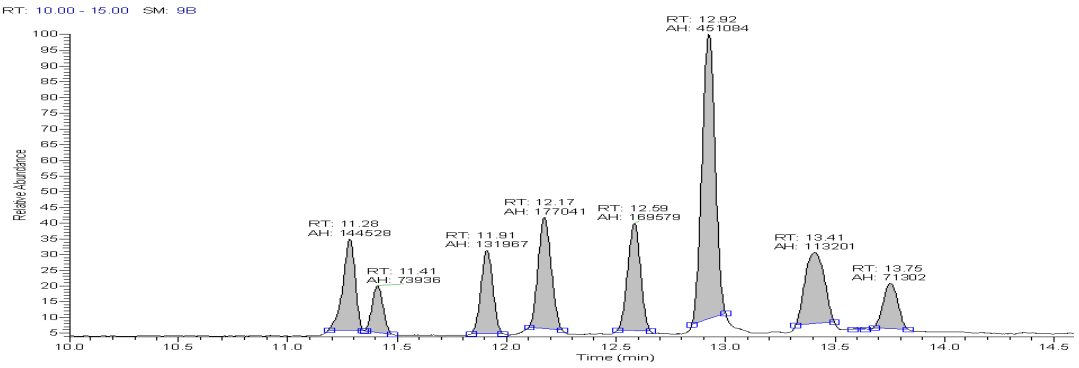
In the subsequent 7 months, the primary focus of the work will be to utilize the expanded pallet of lipid and eicosanoid breath condensate components between the matched healthy smoker and non-smoker volunteers. After detailed correlation analysis the effects of smoking and factors from the medical histories on the relationship to lipid/eicosanoid components in breath condensate will hopefully demonstrate feasibility of this assessment for collaborative arrangements for greatly expanded utilization.

Project Management:

Overall management responsibility will rest with D. C. White. R. Geyer will train J. Cantu in the tricks of mass spectrometry. J. Cantu has worked at CBA for 2.5 years and demonstrated great skill in the GC/EI/MS of lipid biomarkers. He plans to make breath condensate lipid analysis the subject his masters research in the Comparative Experimental Medicine graduate program prior to his Medical School. He plans a career in academic experimental medicine with an MD/PhD.

Leading edge methodology:

The classical methodology for the major eicosanoids requires thin layer chromatographic separation and three separate CG/ECNCI/MS analyses (17, 18), as component of the procedure that allows 12 analyses per day and has a wide variability in the recovery efficiency. R. Geyer developed a 15 minute GC/ECNCI/MS method for baseline separation of the 6 known major eicosanoids utilizing a DB-5 30m, 0.25um film column, with a 2ul, splitless injection, into a constant 1.1ml/min flow of carrier gas, with the injection port at 250ºC , the source at 250ºC, the interface at 280ºC, the column temperature at 190ºC a min, followed by an increase at 25C/min to 300ºC, and a final 10min at 300ºC. Data acquisition begins after 4 minutes. Components (in order from 11.3 min) at 20 pg/µL are authentic 8-iso-PGF_{2a}, an unknown component 2 mass units larger than the 8-iso-$PGF_{2\alpha}$, $PGF_{2\alpha}$, an unknown component, PGD_2, PGE_2 [at 40 pg/µL], TxB_2, and 6-keto- $PGF_{2\alpha}$. Combining this with a streamlined extraction and derivatization methods proposed herein will make assessment of eicosanoids a much more practical technique.



Problems and alternatives: Primary longer-term problems will be in finding the specific PC derived lipids and eicosanoids that form the patterns best correlating with the specific toxicology of the smoke components. The feasibility demonstration requested herein will require a facile method for determining a large suite of PC derived lipids and eicosanoid from breath condensate to find the specific components that will be the major focus of the proposed future effort. The development of facile methods of extraction fractionation of lipids from eicosanoids, and derivatization of the eicosanoids for electron-capture, negative ion chemical ionization mass spectrometry with capillary gas chromatography required for the separation of many isobaric, isomers, enantiomorphs, and conformers will be the major problem. We have proposed a strategy to systematically develop solutions as previously described.

Procedures potentially hazardous:

The CBA meets all University of Tennessee monitored safety standards for protection from hazardous, radioactive, and toxic reagents as well as training requirements. Our hoods are monitored monthly. J. Cantu is the CBA laboratory safety officer.

H. Description of Expected results with measurable outcomes

Timetable:

Detection of new eicosanoid and PC lipids in human breath condensate for healthy volunteers will take 100% of effort during the first 4 months. During ~4-12 mos 70% effort will be designated to the analyses of breath condensate lipids and eicosanoids. Development of detection methods of new eicosanoid components will require 30% effort. In the last month peer-reviewed publications will be prepared.

c. *Expected Results:* We expect to publish our new methodology for recovering and characterizing the regulatory lipids from the breath condensates in peer reviewed journals. If appropriate meetings are found we intend to present our work. The relationships between the patterns of PC derived surfactant lipids and eicosanoids between healthy smokers and age/sex/medical history matched non- smokers will be published in peer reviewed journals and feasibility of utilizing breath condensate assessment will be discussed with potential collaborators. We expect that the system will be useful for other clinical systems and the preliminary data and publications will be a great help in acquiring resources and collaborators for additional studies.

I. Detailed Cost Estimate

a. Personnel

D. C. White will supervise and coordinate this grant as part of his supported Distinguished Scientist responsibilities at no charge to the grant, R. Geyer, manager of analytical chemistry at CBA will train and advise J. Cantu in HPLC/ES/MS/MS, J. Cantu will perform the extractions and analyses.

b. Fringe

The University charges an estimated 28% for all fringe benefits

c. Equipment, Supplies and other Expenses

Lease of the ECoScreen® device for collecting breath condensate ($3,827), ultrapure solvents for extraction and HPLC and GC, derivatizations ($1,000), authentic internal standards ($2,000), columns and maintenance of mass spectrometers ($2,000). Volunteer subjects will be paid $20 for samples of breath condensate (~ $1,000).

d. Travel

To Institute for Science and Health meeting by most economical method available.

e. F & A

A rate of 45% of the Modified Total Direct Costs, which is approved by the Department of Health and Human Services.

Budget: [12 Month Seed Grant]

a Personnel	Effort	
D. C. White MD., PhD	10%	contributed
R. Geyer Ph.D.	5%	$1,400
J. Cantu	75%	$24,060
Total Personnel		$25,460
b. Fringe Benefits	@28%	$7,129
c. Equipment, Supplies and other Expenses:		$ 9,827
d. Travel To IfSH meeting		$1,500
Total Direct Cost		$43,916
e. Facilities & Administration costs	@ 45%	$19,762
Total cost		$63,678

J. Addenda

1. Acronym definition

- 6-Keto $PGF_{1\alpha}$ is a prostacyclin
- 8-iso-$PGF_{2\alpha}$ is an isoprostane
- BAL is bronchoalveolar lavage

- GC/ECNCI/MS is capillary gas chromatography/electron capture negative ion chemical ionization/mass spectrometry
- HETE is a mono-hydroxylated eicosanoid
- HPLC/ES/MS/MS is HPLC/electrospray/tandem mass spectrometry
- IfSH is the Institute for Science and Health
- JHU/APL is the Johns Hopkins University Applied Physics Laboratory
- LPS is lipopolysaccaride
- LTB_4, LTC_4, LTD_4, LTE_4 are Leukotrienes
- PC is phosphatidylcholine (PC's have different fatty acids at the *Sn1* and *Sn2* positions)
- PAF are platelet activation factors
- PFB is pentafluorobenzyl (for esterification with strong electron capturing properties)
- PFBB is pentafluorobenzyl bromide the reactant in the PFB-esterification
- PGE_2, PGD_2, PCF_2 are prostaglandins
- PREPS are Potential Reduced Exposure Products
- SEB is staphyloccal enterotoxin
- SPE is solid phase extraction
- TLC is thin layer chromatography
- TxB_2 is a thromboxane

2. Biographical sketches

Name*: David C. White, MD, Ph.D.*

Position/Title: UTK/ORNL Distinguished Scientist, Professor of Microbiology

Address: Center for Biomarker Analysis, 10515 Research Drive Suite 300, Knoxville, TN 37932-2575

Phone: 865-974-8001,**FAX:** 865-974-8027, dwhite1@utk.edu, http://cba.bio.utk.edu

Education:

Dartmouth College, Hanover, NH, A.B., 1951, Chemistry/Geology, Phi-Beta-Kappa,
Tufts University School of Medicine, Boston, MA, M.D; Rockefeller University, New York, NY, Ph.D., 1962;Biochemistry.

Research and Professional Experience:

Assistant Professor to Professor of Biochemistry, University of Kentucky Medical Center, 1962-1972; Professor, Department of Biological Sciences, Florida State University, 1972-1986; Associate Director Program in Medical Sciences, FSU, 1973-1984; [Involved in teaching infectious disease, and in introduction to clinical applications]; University of Tennessee, Knoxville/Oak Ridge National Laboratory Distinguished Scientist 1986-; Professor of Microbiology/Ecology, University of Tennessee, Knoxville, 1986-, Director Center for Biomarker Analysis 2001- , Licensed to practice medicine in Tennessee, formerly in New York State, Kentucky and Florida

Honors: Procter and Gamble award in Applied and Environmental Microbiology sponsored by the American Society for Microbiology for 1993; Athalie Richardson Irvine Clarke Prize for Outstanding Achievement in Water Science and Technology by the National Water Research Foundation, Naval Research Advisory Board 1995-1997, Director, Microbial Task Force, National Water Research Institute 1996-, 1996-1999; Technical Advisory Committee, Strategic Environmental Research and Development Program (SERDP), 1996-1999, Natural Attenuation Program (SERDP) 1996-1998; Distinguished Visiting Scientist at the Jet Propulsion Laboratory, Pasadena CA 1998-1999 [Involved in Planetary Protection for Mars missions].

Selected Peer-Reviewed Publications (of 527 as of December 2002):

White, D. C., C. A. Lytle, Y-D. M. Gan, Y. M. Piceno, M. H. Wimpee, A. Peacock and C. A. Smith 2002. Flash Detection/identification of Pathogens, Bacterial Spores and Bioterrorism

Agents Biomarkers from Clinical and Environmental Matrices. J. Microbial Methods **48:** 139-147.

Lytle, C.A., Y-D. M. Gan, K. Salone, and D. C. White 2001. Sensitive Characterization of Microbial Ubiquinones from Biofilms by Electrospray/Mass Spectrometry Environ. Microbiol. 3 (4): 265-272.

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Chang, Y-J. A. Peacock, P. E. Long, J. R. Stephen, J. P. McKinley, S. J. Macnaughton, A. K. M. Anwar Hussain, A. M. Saxton, and D. C. White. 2001. Diversity and Characterization of Sulfate-Reducing Bacteria in Groundwater at a Uranium Mill Tailings Site. Appl. Environ. Microbiol. **67:** 3149-3160

Hedrick, D. B., A. Peacock, J. R. Stephen, S. J. Macnaughton, J. Bruggemann and D. C. White. 2000. Measuring soil microbial community diversity using polar lipid fatty acid and denaturing gradient gel electrophoresis data. J. Microbial. Methods **41:** 235-248.

White, D. C., M. Fayek, J.. S. Gouffon, A.M. Peacock, S. M. Pfiffner, R.Geyer, and K.L. Sublette. 2002. Facile quantitative detection of microbial biofilms capable of potential *in situ* bioimmobilization of Uranium in aquifers, 2002 International Society for Subsurface Microbiology, Copenhagen Denmark.

Pinkart, H. C., D. B. Ringelberg, Y. M. Piceno, S. J. Macnaughton, and D. C.White. 2002. Biochemical approaches to biomass measurements and community structure analysis. *In* Manual of Environmental Microbiology, 2nd Edition (D. E. Stahl, C. H. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach, and M. V. Walter, eds.) American Society for Microbiology Press, Washington, DC. pp. 101-113.

Almeida, J. S., K. Leung, S. J. Macnaughton, C. Flemming, M. Wimpee, G. Davis, and D. C. White. 1998. Mapping changes in soil microbial community composition signaling for bioremediation. J. Bioremediation. ***1: 255-264.***

White D. C. and D. B. Ringelberg. 1998. Signature Lipid Biomarker Analysis. In Techniques in Microbial Ecology (R. S. Burlage, R. Atlas, D. Stahl, G. Geesey, and G. Sayler edts.), Oxford University Press, New York, NY, pp. 255-272.

Hansen, M. C., Palmer, R. J. Jr., and White, D. C. 2000. Flowcell culture of Porphyromonas gingivalis biofilms under anaerobic conditions. J. Microbial Methods 40:233-239.

Ivanova, I A., J. R. Stephen, Y-J. Chang, , J. Bruggemann, P. E. Long, J. P. McKinley, G. A. Kowalchuk, D. C. White, and S. J. Macnaughton. 2000. A survey of 16S rRNA and amoA genes related to autotrophic ammonia-oxidizing bacteria of the β-subdivision of the class proteobacteria in contaminated groundwater. Canad J. Microbiol. **46:** 1012-1020

Palmer, R. J. Jr. and D. C White. 1999. [11] Spatially resolved, quantitative determination of luciferase activity by photon-counting microscopy. . Biofilms, Methods in Enzymology, Vol 310 (R. J. Doyle, edit). Academic Press, Inc. New York, NY. pp. 152-160.

Navarrete, A., A. Peacock, S. J. Macnaughton, J. Urmeneta, J. Mas-Castella, D. C. White, and R. Guerrero. 2000.. Physiological status and community composition of microbial mats of the Ebro Delta, Spain by signature lipid biomarkers. Microbial Ecology 39: 92-99.

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White, D. C. , J. S. Gouffon, A. D. Peacock, R. Geyer, A. Biernacki, G. A. Davis, M. Pryor, M. B.Tabacco, and K. L. Sublette. 2003. Forensic Analysis by Comprehensive Rapid Detection of Pathogens and Contamination Concentrated in Biofilms in Drinking

Water Systems for Water Resource Protection and Management, Environmental Forensics. In press (March).

Geyer, R., A. Bittkau, M. Gan, D. C. White, and D. Schlosser. 2002 Advantages of lipid biomarkers in the assessment of environmental microbial communities in contaminated aquifers and surface waters. Proceedings of the Third International Conference on Water Resources and Environmental Research (ICWRER) (edited by G. H. Schmitz). Dresden Germany, July 22-25, Eigenvertag des Forum fur Abfallwirtschaft und Altlasten eV, Pirna D-01796 Germany, Volume II, Pp. 163-167.

Gu, B., D.B. Watson, L. Wu, D.H. Phillips, D.C. White, and J.Z. Zhou. 2002. Microbiological characteristics in a zero-valent iron reactive barrier. Environ. Monit. Assess. **77**(3):293-309.

Phillips. R. L., D. R. Zak, W.E. Holms, and D. C. White. 2002 Microbial community composition and function beneath temperate trees exposed to elevated atmospheric carbon dioxide and ozone, Oecologia 131: 236-244.

Myers R. T., D. R. Zak, A. Peacock, and D.C. White. 2000. Landscape-Level Patterns of Microbial Community Composition and Substrate Use in Forest Ecosystems. Soil Science Society of America Journal 65: 359-367.

Lytle, C.A., Y-D. M. Gan, and D. C. White 2000. Electrospray Ionization/Mass Spectrometry Compatible Reversed-Phase Separation of Phospholipids: Piperidine as a Post Column Modifier for Negative Ion Detection. J. Microbial. Methods **41:** 227-234.

Name: Roland Geyer, Ph.D.
Position/Title: Manager, Analytical Chemistry Center for Biomarker Analysis
Address: Center for Biomarker Analysis, 10515 Research Dr. Suite 300, Knoxville, TN 37932-2575
Phone: 865-974-8032, **FAX:** 865-974-8027, **email:** Rgeyer@utk.edu

Education:

Skills Worker for Tannery Weida, Germany 1986-1987
Master of Handcraft for Tannery, Gera, Germany 1987-1991
Diplomate in Biology, Frederich Schiller University, Jena Germany 1990-1996
Major in Microbiology, Minor Biophysics, Ecology and Informatics
PhD in Microbiology, Frederich Schiller University, Jena, Germany 1996-2000
Thesis: Degradation of Chlorophenols by the Basidiomycetes *Trametes versicolor* and *Stropharia rogosoannulata*

Research and Professional Experience:

Developed technology for softer sheepskins utilizing less tanning agent 1987-1990.

Ecology project University of Vienna, Department of Marine Biology, Austria 1993, 6 months.

Project in Landbouw University, Wageningen Netherlands, Horizontal transfer of virus RNA in *Aspergillis nidulans,* 1994 3 months.

Research Assistant, Department of Microbiology, University of Jena, “Application of innovative microbial enzymes for soil and water decontamination” 1996-1999.

Supervisor and Research Assistant, University of Jena, “Analysis of bound residues of TNT after biological treatment and remobilization experiments” 1999-2001.

Post-doctoral Fellow, UFZ Centre for Environmental Research Leipzig-Halle in the Microbiology of Subterrestrial Aquatic Systems Group Sept 2001 –present.

Research Associate and Manager,Analytical Chemistry Center for Biomarker Analysis, University of Tennessee, Knoxville, Jan 2002—.

Peer-Reviewed Publications: as of December 2002:

Schlosser, D, R. Grey(Geyer), and W. Frische. 1997 Patterns pf ligninolytic enzymes in *Trametes versicolor*. Distirbution of extra- andintracellular enzyme activities during cultivation on glucose, wheat straw, and beach wood. Appl. Microbiol. Biotechnol. 47: 412-418.

Grey (Geyer), R., Ch. Hofer and D. Schlosser. 1998 Degradation of 2-chlorophenol and formation of 2-chloro-1,4-benzouinone by mycelia and cell-free crude culture liquids of *Trametes versicolor* in relation to extracellular laccase activity. J. Basic Microbiol. 38: 37-382.

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Gouffon, J. S., R. Geyer, A. D. Peacock, Y-D. Gan, Y-J Chang, K. Salone, C. Lytle, K. L. Sublette, and D. C. White. 2002. Rapid Quantitative Detection of Pathogens & Contamination by Analysis of Biofilms Generated on Coupons in Water Resource Management. Proceedings of the Third International Conference on Water Resources and Environmental Research (ICWRER) (edit. by G. H. Schmitz). Dresden Germany, July 22-25, Eigenvertag des Forum fur Abfallwirtschaft und Altlasten eV, Pirna D-01796 Germany, Volume II, Pp. 305-310.

White, D. C., J. S. Gouffon, A. D. Peacock, R. Geyer, A. Biernacki, G. A. Davis, M. Pryor, M. B.Tabacco, and K. L. Sublette. 2003. Forensic Analysis by Comprehensive Rapid Detection of Pathogens and Contamination Concentrated in Biofilms in Drinking Water Systems for Water Resource Protection and Management, Environmental Forensics in press March 2003.

Name: James M. Cantu, B.S.
Position/Title: Research Assistant
Address: Center for Biomarker Analysis, 10515 Research Dr. Suite 300, Knoxville, TN 37932-2575
Phone: 865-974-8021, **FAX:** 865-974-8027, **email:** jcantu@utk.edu

Education:
University of Tennessee, Knoxville, TN, B.S., 2000, Biology (Biochemistry, Cellular and Molecular Biology)

Research and Professional Experience:
Research Assistant, Center for Biomarker Analysis, University of Tennessee, Knoxville 2000-
Graduate student Comparative and Experimental Medicine, University of Tennessee Knoxville 2003-

Honors:
The National Dean's List 1996-1997; The Chancellor's Award, University of Tennessee, Martin 1996-1997

Peer-Reviewed Publications: as of December 2002:

Peacock, A.D., S.J. MacNaughton, J.M. Cantu, V.H. Dale, and D.C. White. 2002. Soil microbial biomass and community composition along an anthropogenic disturbance gradient within a long-leaf pine habitat *Ecological Indicators*. **1**:113-121.

Navarrete, A. J. Urmeneta, J. Cantu, E. Vegas, D.C. White, R. Guerrero Analysis of Signature Lipid Biomarkers of Microbial Mats of the Ebro Delta (Spain), Camargue Delta and Etang de Berre (France): an assessment of biomass and activity. *Microbial Ecology* IN REVIEW

3. Disclosure of related funding:

DARPA/DSO, NBCH1020006, *"Rapid identification of biochemical signatures of infection with semi-volatile expired breath condensate analysis*", CoPI's, D. C. White, R. Geyer, $300,000, 03/01/02-09/30/03. This program operates in collaboration with the Walter Reed Army Institute of Research and CIIT Centers for Health Research which provide animal models (mice, rats and piglets) exposed by inhalation of toxins and infectious agents whose breath and breath condensate is analyzed for volatile components by Stanford Research Institute (SRI) International, for lipid regulatory molecules and bacterial signature lipids in condensate by us at CBA, University of Tennessee Knoxville, for cytokines/proteins/DNA in condensate by Johns Hopkins University Applied Physics Laboratory (JHU/APL), for breath condensate peptides with new instrumentation by Johns Hopkins University School of Medicine. The data is collected at JHU/APL and analysis/algorithm development done at the Johns Hopkins University Whiting School of Engineering.

The project manager is M. Donlon, at DARPA/DSO. The goal of the program is to demonstrate feasibility that breath and breath condensate analysis can provide rapid detection and determination of the etiology of infectious or toxin exposure. We expect that additional funding may become available for extension of this work and that funding for a tandem GC/EC/NI/CI/MS/MS or GC/EC/NI/CI/Ion trap mass spectrometer would be provided to CBA to enable us to explore differential patterns with the 200 or so eicosanoids detected in human and animal fluids. All of the six eicosanoids we initially selected in the feasibility study showed differential responses to lipopolysaccharide exposure.

4. Facilities/equipment

The CBA Pellissippi-laboratory has 15,000 sq. feet of laboratory space. Agilent 1100 HPLC gradient apparatus with autosampler, and detectors for fluorescence, UV-Vis (diode array), the Dionex LC Packing ULTINATE capillary HPLC with FAMOS autosampler and SWITCHOS column switching system that can be coupled to a PE-Sciex API 365 tandem quadrupole mass spectrometer capable of electrospray or atmospheric pressure ionization with intergraded data system. The system has syringe pumps for nanospray injections. For accelerated extraction, CBA has a Supercritical fluid extraction (SFE) device consisting of two ISCO syringe pumps, a controller and SFX 2-10 module, and a Dionex ASE-200 Accelerated Solvent extractor with a 24 station autosampler. The laboratories at CBA contain 4 capillary gas chromatographs, equipped with auto-samplers, and flame ionization detection. One of these is configured for the MIDI bacterial identification system with the software to compare fatty acid patterns to over 8000 known fatty acid patterns for identification with a data collection system. CBA has a Hewlett Packard bench-top 6890/5973 GC/MS with autosampler and a VG Platform II GC/HPLC/electrospray/atmospheric pressure chemical ionization quadrupole mass spectrometer. Walk-in cold rooms, laminar flow hoods, autoclaves, a dark room, refrigerated centrifuges, liquid scintillation counters, lyophilizers, flash evaporators, and freezers are also available. Two state-of-the-art thermal cycler ("Robocyler", Statagene), image analyzer, blotting apparatus and hydridization oven are available for nucleic acid analysis, with easy access to nucleic acid sequencing apparatus. Continuous culture apparatus for flow controlled coupon exposure are in operation. A SPEX fiberoptic spectrofluorimeter with dual gratings for both excitation and emission is available for on-line analysis of biofilms in a laminar flow test system with controlled shear. The Center contains facilities for the culture of organisms with laminar flow hoods, autoclaves and bioreactors. Glove boxes for anaerobic culture and biofilm

generation are available. Continuous culture apparatus for flow controlled coupon exposure are in operation. electroporator, bioimaging analyzer for gels, pulsed field electrophoresis, radioscan analyzer for TLC plates, and various ultracentrifuges are available.

The microscopy facility contains a Leica TCS-4D confocal scanning laser microscope: three fluorescence channels, upright-inverted interconvertable, galvanometer-driven Z stage (10 nm steps; 40 nm reproducibility), fiber-optic delivery of Krypton/Argon mixed-gas laser illumination (75 mW total output at 488, 568, and 647 nm). Leica's 3-D volume-rendering software can be used to produce reconstructions of confocal stacks. The instrument is configured as an FTP site so that users may download their data from their office computers. The Leica microscope is also a research-grade upright/inverted transmitted light and epifluorescence instrument with photomicrograph and video output. A Hammamatsu Argus 50/VIM-3 photon-counting camera (with center-of-gravity board) mounted on a Zeiss Axioplan microscope for quantitative bio- and chemiluminesence measurements and a Dage-MTI 70 video camera plus VCR for real-time video microscopy.

CBA recently acquired quantitative volatile organics analysis system composed of the Entech 7100 3-stage preconcentrator, a 4 position autosampler and a 400 ml canister cleaner coupled to the Hewlett-Packard 6890/5973 GC/MS. We have apparatus to collect human end-expiratory breath analyses (over 3400 components at ppb have been identified), crime scene, soil gas, food spoilage, indoor air organics, toxic inhalant analyses, volatile fatty acids for bioremediation monitoring and a host of other analyses.

5. Human Use.

Letter of IRB approval (see PDF file named White-IRBLtr)

Consent form

Feasibility of breath condensate lipids as response predictors

INTRODUCTION

You are invited to participate in a research study whose purpose is to measure the effects of new technology to measure the components of breath condensate. The objective of this study is to expand quantitative non-invasive detection of breath condensate analysis.

INFORMATION ABOUT PARTICIPANTS INVOLVEMET IN THE STUDY

First time participants will be shown a demonstration. You will be asked to fill out a medical history checklist to help us match smokers with non-smoking subjects. You may leave blank questions if your wish. If you want to be a subject then the collaborating staff will offer potential volunteers the opportunity to exhale through an open tube with a disposable mouthpiece tube and check valve whilst comfortably sitting in a chair. There is no pain, discomfort and the test takes 5 to 30 minutes requiring you to just breath normally whilst the condensate is collected. We anticipate you will have a single sample collected. You may read, listen to tapes or work on your laptop. Volunteers will be paid $20.00 for the inconvenience of this one-time non-invasive test if they desire payment.

RISKS

The mouthpiece is disposable and the apparatus will be sterile or disinfected between each use so there is no risk of contamination from other participants. There are no other risks.

BENIFITS

This proposed non-invasive quantitative assessment of oxidative stress levels and inflammatory response levels could be extremely valuable in modulating smoking exposure s well as demonstrating feasibility of the technique to the participant and to medical science. The

breath condensate analysis has the potential to be very useful in the determination of risk from exposure to tobacco smoke and potentially in the rapid diagnosis of infection generated by bio-terrorists.

CONFIDENTIALITY

Participant's records will be held confidential by the Center for Biomarker Analysis (CBA). They will be identified by number code. No reference will be made in oral or written reports that could link participants to the study.

CONTACT INFORMATION

If you have any questions about the study or its procedures, (or you experience adverse effects as a result of this study) you may contact Dr. David C. White at 10515 Research Drive, Site 300, Knoxville, TN 37932-2575, 865-974-8001. If you have any questions about your rights as a participant, contact the Research Compliance Service

_____Participants initials Section at the Office of Research at (865) 974-3466.

PARTICIPATION

Your participation in this study is voluntary; you may decline to participate without penalty. If you decide to participate, you may withdraw from the study at any time without penalty and without loss of benefits to which you are otherwise entitled. If you withdraw from the study before data collection is completed your data will be returned to you or destroyed.

CONSENT

I have read the above information. I have received a copy of this form. I agree to participate in this study.

Patients Signature _____ Date _____

Investigator's Signature _____Date _____

i. Invitation to participate & medical check list.

INVITATION TO PARTICIPATE IN AN IMPORTANT STUDY BY EXHALING THE GASEOUS COMPONENTS OF BREATH AND COLLEVCTING BREATH CONDENSATE TO BE ANALYZED FOR BIOMARKERS

You can volunteer and be compensated $20.00 for the inconvenience to provide a single breath condensate sample through a disposable mouthpiece whilst breathing normally whilst you read, listen to the radio or work on a computer as your exhaled breath is condensed in a plastic tube in an ice bucket or with the EcoScreen breath collector used widely in clinics in Europe. You will be asked to fill out a medical history checklist to help us match smokers with non-smoking subjects. You may leave blank questions if your wish. The breath condensate will be analyzed at Center for Biomarker Analysis (CBA) at the University of Tennessee. The condensate will be extracted and the regulatory fatty acids that you exhale as an aerosol will be analyzed by tandem mass spectrometry. We will explain how the breath and condensate samples are recovered and the Informed Consent form you need to sign explains the procedure, risks, benefits, and the confidentiality of the data about you that remain in your medical record at the clinic. The procedure is totally non-invasive.

The data will be used anonymously to establish what dietary and life style interventions are most effective in decreasing the damage caused by oxidative stress.

Thanks so very much.

David C. White M.D. Ph.D., 865-94-8001, dwhite1@utk.edu

Check list

Please Print **MEDICAL HISTORY** DOB _____

Assigned # _____ Appointment Date_____

1. Are you currently under a doctor's care? Yes No

2. Do you have any allergies? Yes No List:_____

_____

3. Do you have a history of the following:

Allergy	Yes	No	Chest Pain	Yes	No
Post-nasal Discharge	Yes	No	High Blood Pressure	Yes	No
Sinus Infection(s)	Yes	No	Stroke	Yes	No
Nose Bleeds	Yes	No	Diabetes	Yes	No
Headaches	Yes	No	Ulcers	Yes	No
Dizziness	Yes	No	Other Stomach Disease	Yes	No
Tinnitus (ringing in ear)	Yes	No	Anemia	Yes	No
Hearing Loss	Yes	No	Gum Disease	Yes	No
Difficulty Breathing	Yes	No	Glaucoma, Visual Problems	Yes	No
Difficulty Swallowing	Yes	No	Thyroid Disease	Yes	No
Asthma	Yes	No	Hepatitis (Liver)	Yes	No
Hayfever	Yes	No	Kidney Disease	Yes	No
Tuberculosis	Yes	No	Convulsive Disorder	Yes	No
Lung Disease	Yes	No	Positive HIV Testing	Yes	No
Heart Trouble	Yes	No	Exposure to HIV	Yes	No

Comments _____

_____

_____

_____

_____

4. Do you drink alcohol? Yes No Frequency / Amount _____

5. Tobacco use: Current: Yes No #pack(s) per day_____ #years_____

6. Any history of alcohol or drug dependency? Yes No

7. List all of the medications you are taking and the dosage you take (including over-the-counter medications such as aspirin, birth control pills, etc. _____

_____

8. List all previous surgeries or major illnesses along with appropriate dates. _____

_____

_____

_____

9. Do you have a history of increased bleeding tendency? Yes No _____

10. Have you ever had a blood transfusion? Yes No _____

11. Have you been treated for any mental or emotional disorders? Yes No _____

Please Print Assigned #_____ Appointment Date_____

12. Family History

Have any members of your family had the following?

Allergies Yes No _____

Bleeding Problems Yes No _____

Cancer Yes No _____

Diabetes Yes No _____

Heart Disease Yes No _____

17. (For Women) Is there ANY POSSIBILITY you are pregnant? Yes No

Last Menstrual Period _____

18. Please list any other information you think important to this analysis

_____

_____

_____

_____

_____

_____

_____

Signature of Patient (by number) _____ Date_____

Witness Signature_____ _____

7. Animal use No animals are proposed for this study.
8. Certificate of Environmental Compliance.

January 16, 2003

TO: Whom it May Concern

FROM: John E. Shanks, Administrator

SUBJECT: Environmental Regulatory Compliance

Based on my knowledge and assessments, the Center for Biomarker Analysis at The University of Tennessee is in full compliance with the environmental regulations of The University of Tennessee, Knoxville.

John F. Shanks, Administrator
The University of Tennessee
Environmental Health and Safety
916 22nd St.
Knoxville, TN 37996-3503
865-974-5984
jshanks@utk.edu

9. References:

1. Bowers,M. 2001. Tobacco Control Programs: what works and what's possible. Pulmonary Reviews 6(3): April 1-5.
2. Begany, T. 2000. What makes nicotine so addictive? Pulmonary Reviews 5(11) November.
3. Murphy, R. C. Free-radical-induced oxidation of arachidonyl plasmalogen phospholipids: Antioxidant mechanism and precursor pathway for bioactive eicosanoids. Chem Rev. Toxicol. 14: 463-472.
4. Funk, C. D., 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294: 1871-1875.
5. Morrow, J. D., and L. J. Roberts.1997. The isoprostanes: unique bioactive products of lipid peroxidation. Prog. Lipid Res. 36: 1-21.
6. Montuschi P. , J. V.Collins , G. Ciabattoni, N. Lazzeri, M. Corradi, S. A. Kharitono, and P. J. Barnes. 2000. Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with COPD and healthy smokers. Am. J. Respir. Crit. Care Med: Sep162(3 Pt 1):1175-1177
7. Dietrich, M., G. Block, M. Hudes, J. D. Morrow, E. P. Norkus, M. G. Taber, C. E. Gross, and L. Packer. 2002. Antioxidant supplementation decreases lipid peroxidation biomarker F(2)-isoprostanes in plasma of smokers. Cancer Epidemiol Biomarkers Prev. 11(1):7-13
8. Hughes, S. 2003 Cutting through the smoke. Pennsylvania Gazette 101: 24-29.
9. Lerman C. , P. G. Shields, E. P. Wileyto, J. Audrain, A. Pinto, L. Hawk, S. Krishnan, R. Niaura, and L. Epstein. 2002. Pharmacogenetic investigation of smoking cessation treatment. Pharmacogenetics. 12(8): 627-634.
10. Mutlu, G. M., K. W. Garey, R. A Robbins,L. H. Danziger, I. Brubinstein. 2001. Collection and analysis of exhaled breath condensate in humans. Am J. Crit Care Med. 164: 731-737.

11. http://www.spiropharma.com/dm/grafik/4_20011224154023_100x70.pdf
12. Barnes, P. J. 2000. Chronic obstructive pulmonary disease. New England J. Med. 343 (4): 269-280.
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16. Tsikas, D. 1998. Application of gas chromatography-mass spectrometry and gas chromatography-tandem mass spectrometry to assess in vivo synthesis of prostaglandins, thromboxane, leukotrienes, isoprostanes and related compounds in humans. J. Chromatog, B, 717: 201-245.
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20. Almeida, J. S., A. Sonesson, D. B. Ringelberg, and D. C. White. 1995. Application of artificial neural networks (ANN) to the detection of *Mycobacterium tuberculosis*, its antibiotic resistance and prediction of pathogenicity amongst *Mycobacterium spp.* based on signature lipid biomarkers. Binary--Computing in Microbiology **7:** 53-59.
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