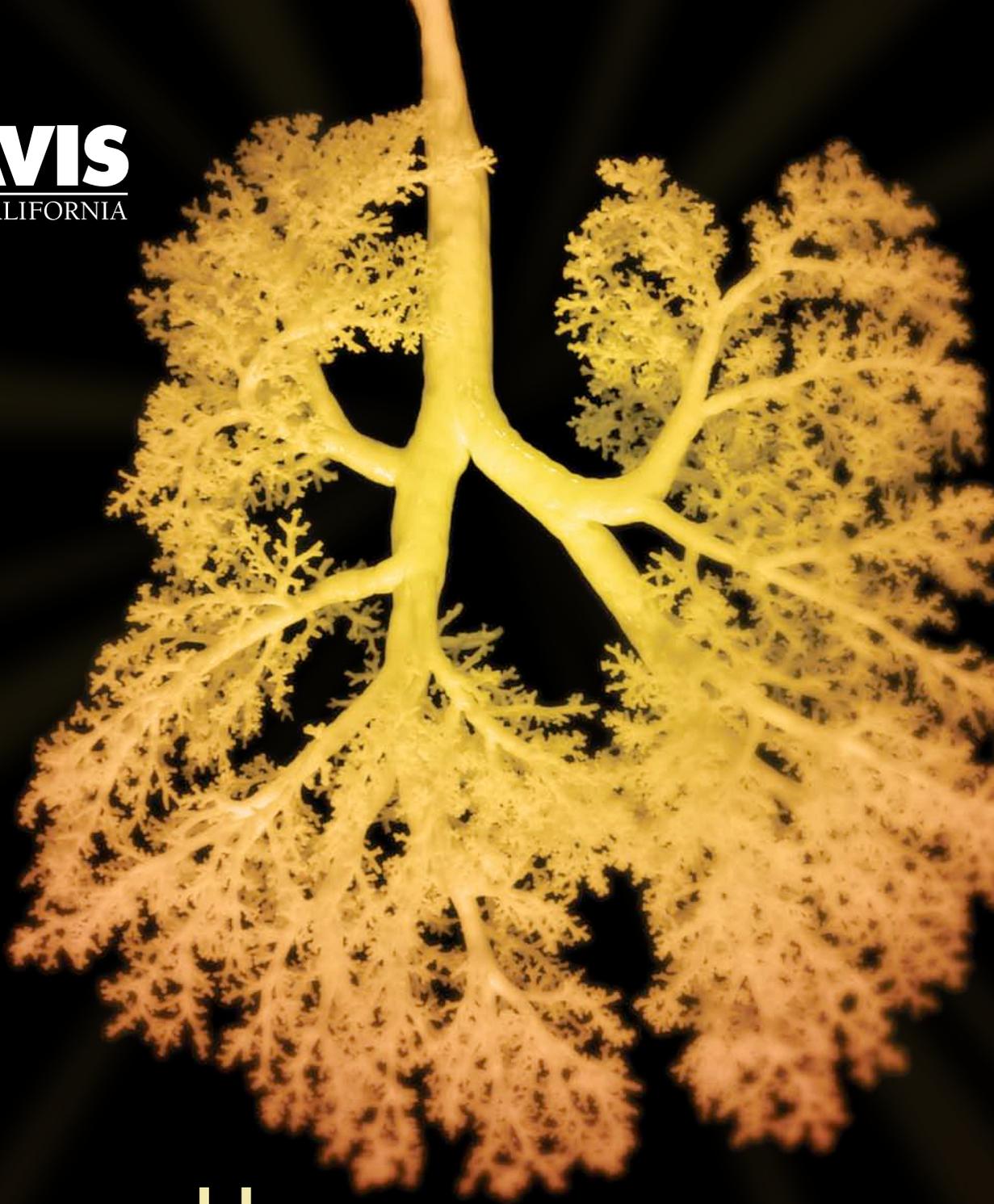


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excellence  
in Pulmonary Research

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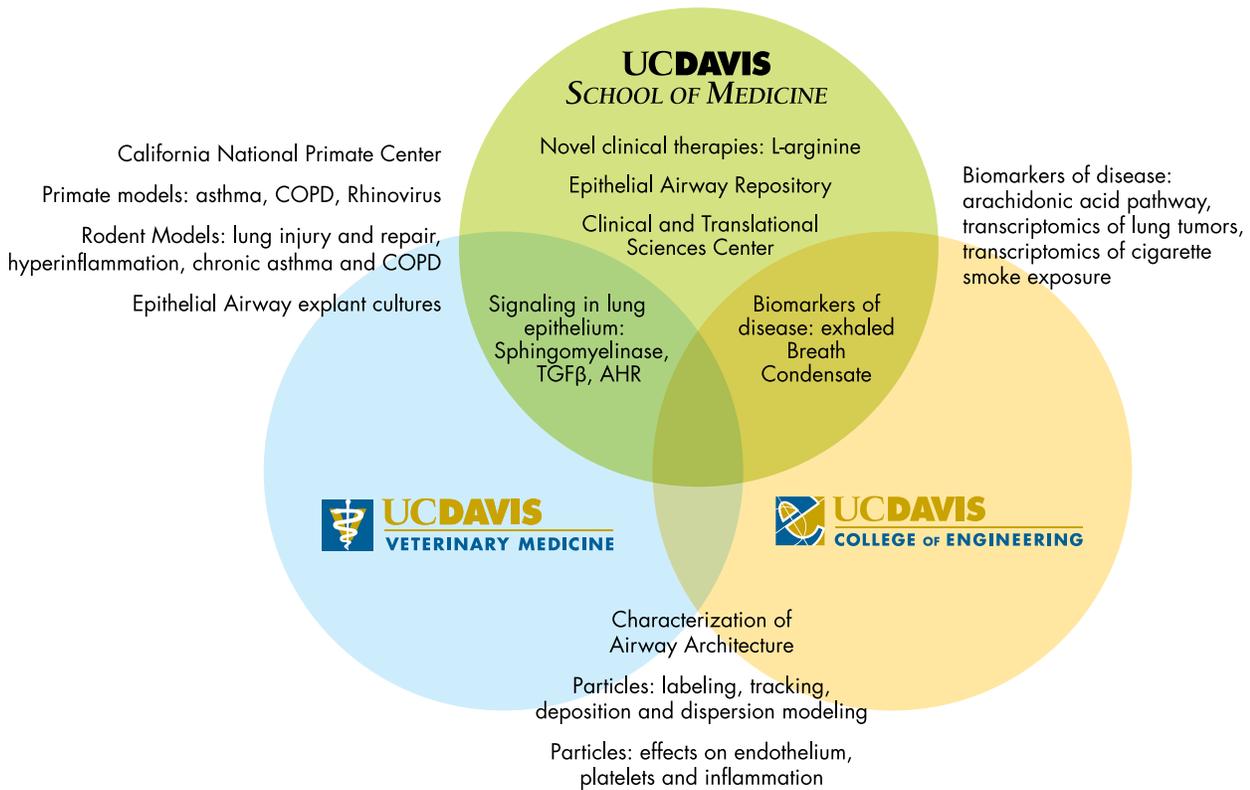
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# SUMMARY

For the past 40 years, UC Davis has been recognized for excellence in research in basic pulmonary toxicology and comparative lung biology. UC Davis investigators have a strong record of developing collaborative research initiatives through the **College of Veterinary Medicine, the California National Primate Center, the School of Medicine, and the College of Engineering.**

Over the past 5 years, the pulmonary principal investigators were granted over **\$70M** in federal and private support, including the Program Project “Pulmonary Effects of Environmental Oxidant Pollutants”- currently in its 34th year—and the T32 “Training program in comparative lung biology”—currently in 31st year.

Our biggest asset is our **MULTIDISCIPLINARY APPROACH** to study respiratory diseases from basic biology and clinical perspective. By utilizing the faculty and resources at several UC Davis schools and NIH funded centers, researchers are able to coordinate efforts to address human lung diseases in unique ways.



# SUMMARY

**California National Primate Resource Center (CNPRC)** is one of eight National Primate Research Centers (NPRCs) funded by the National Institutes of Health, National Center for Research Resources (NIH/NCRR) and is now in the 48th year of operation. CNPRC is located on a 300-acre dedicated tract of land. About 5200 nonhuman primates are housed at the facility, with a breeding colony of about 2500 animals. The CNPRC directly employs 573 people, including 48 academic staff scientists, and 229 research support and animal care personnel. Research and core support facilities are organized in four major units - Brain, Mind and Behavior; Reproductive Sciences; Respiratory Diseases; and Virology and Immunology. The extramural grants of the Staff Scientists are about \$23 million per year and the center supports about \$48 million in extramural support from Affiliate Scientists.

**The UC Davis exposure facilities** include 42 full body chambers at the Primate Center (for primates), and also face mask capability and nose only exposure capabilities, resulting in a unique ability to conduct studies of inhaled toxicants. The Center for Health and the Environment houses chambers for rodents, and UC Davis Human Performance Laboratory has an ozone exposure chamber for human studies.

**The Clinical and Translational Sciences Center (CTSC)** was established in October 2006 with a \$24.8 million award from the NIH. The UC Davis CTSC, one of 38 NIH-supported Centers nationally, is focused on enhancing the study of human health and disease, and bringing new treatments more rapidly to patients and communities ([www.ucdmc.ucdavis.edu/ctsc/](http://www.ucdmc.ucdavis.edu/ctsc/)). The CTSC is a part of a national consortium bridging basic, clinical, and translational research using a transformative approach. The UC Davis CTSC aims to transform research into new collaborative scientific discoveries that will bring new diagnostic techniques and therapies into medical practice, and help to solve complex medical problems.

**Pulmonary Clinical Trials** are performed at the dedicated Clinical Research Center (CCRC). Fifteen UC Davis faculty are involved in pulmonary clinical trials, supported by six Clinical Research Coordinators, two registered Respiratory Therapists and a Research Registered Nurse who assists with inpatient trial procedures (i.e., ECGs, blood draws, medication administration, etc.). Enrollment opportunities in Sacramento area are characterized by high ethnic diversity, and a variety of airway disease phenotypes. In a typical year, the Division of Pulmonary medicine at UC Davis would encounter over 1,000 patient-visits of asthma, over 1,000 cases of chronic bronchitis, and nearly 500 cases of chronic bronchitis with emphysema.

**CTSC Clinical Research Center (CCRC)** consists of a combined inpatient and outpatient unit with 6 rooms and 9 beds. The unit currently employs ten Registered Nurses, a Nurse Practitioner, a Registered Dietitian, an Exercise Physiologist, and a Laboratory technician. CCRC staff nurses provide direct nursing care for all subjects enrolled in various research studies at the CCRC and carries out protocol-specific procedures. All CCRC nurses meet strict hospital and unit-based competencies. They are all ACLS, conscious sedation, and chemotherapy certified; two nurses have a specialty certification from the Infusion Nurses Certification Corporation (INCC); and four others have the Critical Care and Medical-Surgical nursing certifications. CCRC runs over 60 studies/year, with over 2,000 outpatient visits.

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**1.1. Pulmonary toxicology of air pollutants and ingested lung toxicants****Funded grants:**

1. Mechanisms of Particulate Toxicity: Exposure Effects on the Respiratory System (Pinkerton)
2. Health Effects of Concentrated Ambient Particles from the Central Valley of California (Pinkerton)
3. Health Effects of Airborne Particulate Matter and Gases (Pinkerton)
4. Histomorphometric assessment of rat pulmonary tissues after exposure to concentrated ambient particles (CAPs) and sulfur dioxide (Pinkerton)
5. Health Effects of Inhaled Nanomaterials (Pinkerton)
6. Mechanisms of particle toxicity in the respiratory system (Pinkerton)
7. Role of Bioavailable Iron in biological Effects of Inhaled Particles (Pinkerton)
8. Pulmonary Deposition of Ultrafine Particles (Wexler)
9. CRAEMS: Fundamental Studies of Nanoparticle Formation in Air Pollution (Wexler)
10. San Joaquin Valley Aerosol Health Effects Research Center (Wexler)
11. Ion Mobility Analysis of Particulate Matter and Gas Phase Precursors (Wexler)
12. Urban and Regional Particle Number Distribution Modeling (Wexler)
13. Atmospheric Aerosol Chemistry (Wexler)
14. Development of a Broad Spectrum Differential Mobility Aerosol Analyzer for Ambient Aerosol Size Distribution Measurements. (Wexler)

**1.2. Redox biology/xenobiotic metabolism****Funded grants:**

1. Pulmonary Effects of Environmental Oxidant Pollutants (Hyde)
2. Mechanisms of Interaction of Air Pollutants and Airway Remodeling in Asthma (Last)
3. Vitamin E metabolism and lung toxicology (Dr. C.Cross)
4. Nitric Oxide-Mediated Protein Modification in the Lung (Cross)
5. Lung Surface Antioxidant Defenses Against Air Pollutants (Cross)
6. Lung Injury By Naphthalenes (Buckpitt)
7. P450 Mediated Lung Toxicity in Humans and Monkeys (Buckpitt)
8. Metabolomic study of ozone and nitronaphthalene toxicity (Arachidonic Acid cascade) (Hammock)
9. Thioredoxin Localization and its Function in the Airway (Harper)

10. Gonadal vs. Genomic Influences on Xenobiotic Metabolism (Van Winkle)
11. Oxidant induced gene expression in vitamin E deficient transgenic mice (Gohil)
12. Gene Regulation and Injury in Airway by Thioredoxin (Wu)
13. Lung Defense Mechanisms from Environmental Ozone (Schelegle)
14. A Protective Role for Nitric Oxide in Airway Inflammation (Kenyon)

**1.3. Cell biology and vascular biology of lung/signaling/airway remodeling/immunology****Funded grants:**

1. Mechanisms and Treatment of Tobacco Smoke-Induced Pulmonary Inflammation and Epithelial Damage in Rats (Pinkerton)
2. Activation of NF- $\kappa$ B by Cigarette Smoke (Harper)
3. Induction Of Squamous Cell Marker in Airway Epithelium (Wu)
4. Mucous Cell Differentiation in Airway Epithelium (Wu)
5. Programming Airway Epithelial Differentiation by RAD (Wu)
6. IL-17 Mediated MUC Gene Expression in Airway Epithelium (Wu)
7. Mechanisms of Mediators-Regulated Mucin Gene Expression in Airway Epithelium (Wu)
8. Rhinovirus-Induced Gene Expression in Airway Epithelium (Wu)
9. Mechanism of Smoke-Induced MUC5B Gene Expression (Wu)
10. Genomic Responses Of the Lungs to Tobacco Smoke: Roles of Inducible Nitric Oxide Synthase and  $\alpha$ -tocopherol Transfer Protein Genes (Gohil)
11. Dietary Fat and ETS Effects on Lung Epithelial Biology (Van Winkle)
12. Molecular Characterization of Lung Sphingomyelinase (Goldkorn)
13. Proteasome ErbB1 interaction in lung hyperplasia (Goldkorn)
14. Tobacco Oxidants: Ceramide Path and Apoptosis in Bronchial Epithelium (Goldkorn)
15. Tobacco Oxidants Prolong EGF Receptor Signaling in the Lung (Goldkorn)
16. Role of Ion Channels in Regulating Vascular Endothelial Differential Responses to Different Types of Fluid Mechanical Shear Stress (Barakat)
17. Endothelial Cell Adaptive Responses to Sustained Fluid Flow (Barakat)

18. Mechanisms of endothelial cell mechanotransduction (Barakat)
19. Particle-cellular interactions under flow (Barakat)

**1.4. Inflammation****Funded grants:**

1. Effects of Environmental Tobacco Smoke on Pulmonary Allergy (L.Gershwin)
2. Asthma, Gender and ETS: Pathogenic Synergy? (L.Gershwin)
3. Influence of RSV Infection on Immune Responses to Inhaled Antigens in Bovine Lung (L. Gershwin)
4. Mechanisms of Pathogenic Synergy Between BRSV and Haemophilus Somnus (L. Gershwin)
5. Biology of Monocrotaline Induced Pulmonary Hypertension (Dennis Wilson)
6. Effects of Tobacco on Inflammatory Cell Responses (Miller)
7. Effect of Secondhand Smoke on Pulmonary T Cell Recruitment (Miller)
8. Macrophage specific magnetic-fluorescent silicon nanoprobess (Ang.Louie)
9. Immunostimulatory DNA Priming of Lung Innate Immunity (Hyde)
10. Capsaicin and Anaphylactic Reactions in the Guinea Pig Airways (Hyde)
11. Pathobiology of HIV(SIV)-induced severe angioproliferative pulmonary hypertension (Hyde)

**1.5. Lung development****Funded grants:**

1. Prenatal tobacco smoke and gene expression in airways (Van Winkle)
2. Effect of Sidestream Smoke on Bronchiolar Injury and Repair (Van Winkle)
3. Environmental Influences on Perinatal Lung Development (Pinkerton)
4. Passive Smoking and Clara Cell Development in Lungs (Pinkerton)
5. ETS and Newborn Lung Development (Pinkerton)
6. ETS Effects on Airway Development in the Primate Lung (Pinkerton)
7. Regenerative Airway Epithelium by Embryonic Stem Cells (Wu)

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## 2.1. Airway architecture characterization and particle deposition modeling and measurement

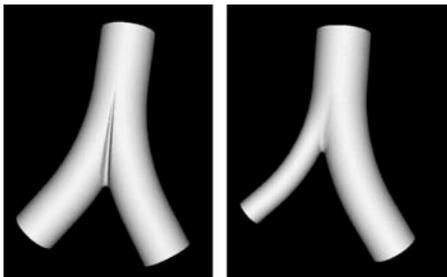
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### (A) Airway Architecture Characterization<sup>1</sup>.

Dr. Wexler has developed techniques for characterizing the details of airway architecture in laboratory animals. The lungs are removed, fixed and casted with silicone. The tissue is eroded to expose the cast. The cast is scanned using a microCT scanner with ~50 um resolution producing a high resolution 3D image of the airways. The image is then analyzed using proprietary software developed in Dr. Wexler's laboratory to extract the lengths, diameters, branching angles and other geometric parameters from the conducting airways. In a typical rat lung, we extract this information for about 5000 airways using 24 hours of computational time on a PC. The figures below show (a) a symmetric and (b) an asymmetric airway bifurcation generated by the software.



The figures above show (a) a symmetric and (b) an asymmetric airway bifurcation generated by the software.

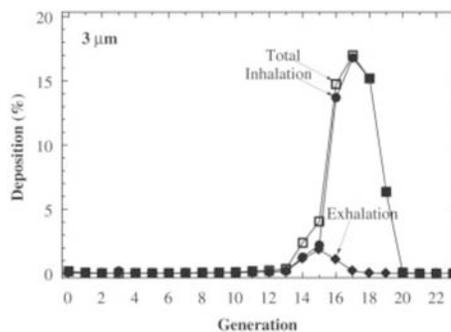
### (B) Particle Deposition Measurements:

Drs. Wexler and Schelegle are developing methods to measure the detailed pattern of size-resolved particle deposition in laboratory animal airways. The animal inhales particles of a given size and composition suitable for the experiment, but fluorescently labeled. The lung is removed from the animal and frozen in liquid nitrogen, freezing the deposited

particles in place. The airway is then warmed to below freezing and casted with a proprietary casting material. The lungs are then refrozen in liquid nitrogen. The lung is then sliced on a cryostat and the block face is imaged with multiple wavelengths of illumination to record the airways and the deposited particles in each airway. The proprietary software described in (A) above is used to identify the airways and the number of particles deposited in each.

### (C) Particle Deposition and Dispersion Modeling:

Dr. Wexler has developed mathematical models of particle dispersion and deposition in human airways<sup>2</sup>. Particles smaller than about 1 um deposit primarily by impaction whereas particles less than about 0.1 um deposition primarily by diffusion in human airways. The deposition of the particles is sufficiently high that it usually occurs on the first breath. Impaction and diffusion do not effectively deposit particles in the intermediate size range so their deposition, if it occurs at all, occurs after multiple breathing cycles and is intimately tied to dispersion of the particles throughout the airways, especially in to the pulmonary regions. The figure below shows the deposition pattern for 0.3 um particles as a function of generation in the human lung.

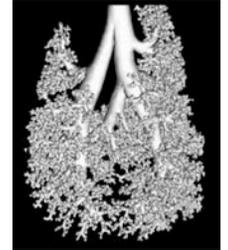


### (D) Particle Bolus Dispersion Measurement:

Bolus dispersion is a sensitive, non-invasive measure of a number of airway functional parameters and is therefore an early indicator of lung disease in humans. Particle bolus dispersion has not been measured in rats due to the small bolus volume and high time resolution required for the measurements. Drs. Wexler and Schelegle are developing a method for measuring particle bolus dispersion in rat airways using high speed data acquisition tools developed in the laboratory for this purpose.

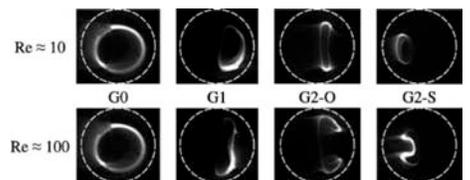
### (E) Airway Architecture Development:

Drs. Wexler, Plopper and van Winkle are exploring how the architecture of juvenile animal airways changes in response to inhaled particles<sup>3</sup>. Newborn rats are exposed to ozone, particles or combinations of the two early in life. The animals grow to adulthood and their lungs are analyzed using the procedures described in section (A). Comparison is made between the detailed architecture of the airways of exposed animals to those breathing filtered air. The figure below is an image reconstructed from a scan of a lung cast.



### (F) Measurements of Flow Patterns in Airways<sup>4</sup>:

Transport and deposition of particles and gases in animal and human airways depends on the flow patterns of air. The airways are very complex leading to complex flow patterns. We have developed methods for measuring details in of the flow patterns in scaled replica of human and animal airways. The figure below is a visualizations of the flow on expiration at low and high Re at various locations in the bifurcation tree.



### Selected Publications

1. Lee, D.Y., S.S. Park, G.A. Ban-Weiss, M.V. Fanucchi, C.G. Plopper, and A.S. Wexler. A bifurcation model for characterization of pulmonary architecture. *Anat. Record* 291:379-389, 2008.
2. Park, S.S. and A.S. Wexler. Size dependent deposition of particles in the human lung at steady-state breathing. *J. Aerosol Sci.* 39:266-276, 2008.
3. Lee, D.Y., M.V. Fanucchi, C.G. Plopper, J. Fung, and A.S. Wexler. Pulmonary architecture in the conducting regions of six rats. *Anat. Record* 291:916-926, 2008.
4. Kelly, J.T., A.K. Prasad, and A.S. Wexler. Detailed flow patterns in the nasal cavity. *J. Appl. Physiol.* 89:323-337, 2000; Fresconi, F., A.S. Wexler, and A. Prasad, Transport Profiles in the Conducting Airways of the Human Lung. *Int'l J. Heat Mass Transfer* 51:5552-5561, 2008.

## 2.2. Multimodal imaging approach for tracking inhaled particles



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Airborne particulate matter less than 10 microns ( $PM_{10}$ ) is now recognized to result in increase morbidity and mortality and high acute exposures have even been linked to cardiac arrest. The mechanisms by which inhaled PM induce the systemic affects, such as heart attack and stroke, are unknown. Two theories are proposed to explain the health affects of these inhaled particles. First, that inhaled particles remain in the lung, triggering the release of inflammatory chemokines that enter the blood stream and affect target organs and this cascade of events results in the morbidity and mortality. Second, that particles translocate out of the lungs into the circulatory system and then accumulate in the heart and vasculature resulting in cardiac death.

We use a multimodal imaging approach to track particles that leave the blood stream

and accumulate systemically. By using complementary imaging techniques we are able to noninvasively track inhaled PM over time. We combine positron emission tomography (PET), which has excellent sensitivity, but lacks anatomical information, with magnetic resonance imaging (MRI) or x-ray computed tomography (CT), both yielding anatomical information.

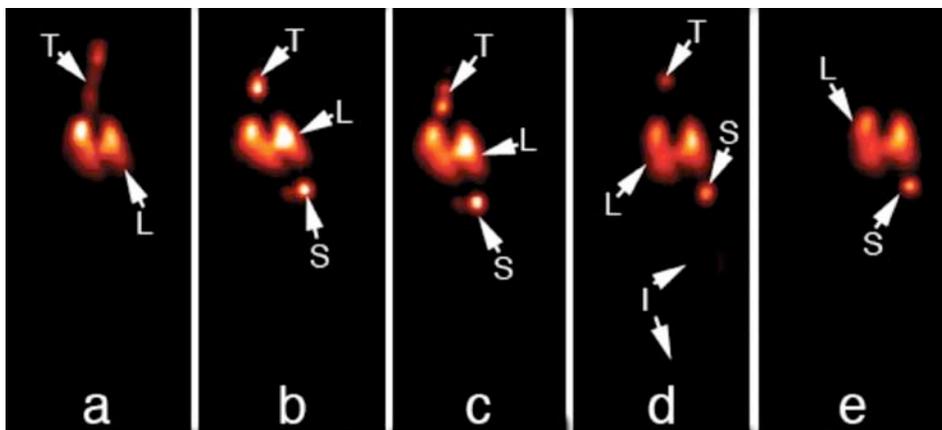
Ultrafine (less than 2.5 microns) model PM are labeled with tracers for PET imaging, such as copper-64, a positron emitting isotope. After exposure to the PET agent, the animals are imaged immediately to determine the initial deposition of PM. We also follow the animals over time to enable visualization of trace amounts of our PM with PET and then co-register the PET data with the anatomical information of MRI or CT to determine where in the body the PM has accumulated.

Our initial results are suggesting that the PM does indeed leave the lungs, enters the blood stream, and accumulates systemically in the liver and heart. We are also testing animals with pre-existing vascular disease to determine if PM will accumulate at sites of vascular inflammation. Epidemiologic studies have demonstrated that people with pre-existing

cardiovascular diseases, such as atherosclerosis (an inflammatory disease of the vessel walls), are more prone to myocardial infarction or stroke after PM exposure than the general population. Therefore, it would be important to image animals with vascular inflammation to see if our PM accumulates at sites of vascular inflammation after inhalation, and therefore lead to destabilization of the vascular injury site, resulting in blockage of a vessel and ultimately myocardial infarction or stroke.

### Selected Publications

Palko, HA, Louie AY. Bio-distribution of Nanoparticle Pollutants using PET and Radiotracer Techniques. Presented as a poster at the World Molecular Imaging Congress in Nice, France (2008).



**Figure 1.** Translocation of 78nm polystyrene nanoparticles over time in the mouse after instillation into the lungs. Bio-distribution is shown at a) 0 hour b) 2 hours c) 4 hours d) 24 hours and e) 48 hours after instillation. Mucociliary clearance is shown from the nasopharynx and tracheo-bronchial regions into the GI tract. T= trachea, L= lung, S= stomach and I= intestines. Imaging Parameters: PET images were obtained on the Focus 120. For the images shown, static scans were taken at 0 hour, 2 hour, 4 hour, 24 hour and 48 hour time points. Acquisition time varied from 15 minutes to 1 hour depending on activity. Images were reconstructed using a maximum a posteriori algorithm (MAP).

### 2.3. The effect of inhaled nanoparticles on human endothelial cells

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Epidemiological studies demonstrate that inhalation of ultrafine particles significantly increases the risk of cardiovascular morbidity and mortality; however, the fundamental mechanisms governing increased predilection to cardiovascular pathology following ultrafine particle exposure remain largely unknown. Acute and chronic inflammation of the vascular endothelium, the monolayer of cells lining the inner surfaces of blood vessels, plays a central role in the development of cardiovascular disease; therefore, we are investigating the effect of particulate matter on endothelial cell function. Furthermore, because endothelial cells *in vivo* are constantly exposed to flow and since flow-derived mechanical forces (pressure and shear stress) regulate various aspects of endothelial cell structure and function, we are interested in establishing how flow modulates particle-cell interactions.

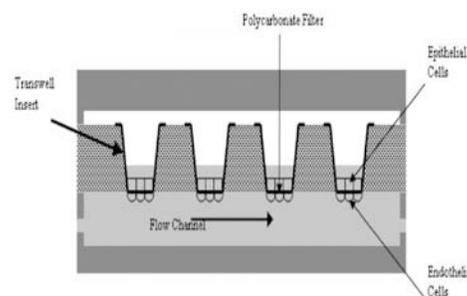
Our studies to date have focused on the effect of metal oxide nanoparticles on human aortic endothelial cells. More specifically, we have studied the impact of 50-100 nm iron oxide ( $\text{Fe}_2\text{O}_3$ ), yttrium oxide ( $\text{Y}_2\text{O}_3$ ), and zinc oxide (ZnO) nanoparticles on the mRNA and protein expression of specific inflammatory

markers in human aortic endothelial cells (HAECs). Our results have demonstrated that under static (no flow) conditions,  $\text{Y}_2\text{O}_3$  and ZnO nanoparticles at concentrations of 10  $\mu\text{g}/\text{ml}$  or greater induce significant inflammation in HAECs, whereas  $\text{Fe}_2\text{O}_3$  particles do not induce inflammation at any concentration (**Figure 1**) [Gojova et al., *Environmental Health Perspectives* 115: 403-409, 2007]. These results suggest that particle composition may be an important element in the inflammatory potential of nanoparticles.

More recently, we have established that HAECs exposed to steady flow for 24 hrs and subsequently exposed to ZnO nanoparticles under static conditions exhibit a similar inflammatory response to cells that had not previously been subjected to flow. On the other hand, HAECs exposed to oscillatory flow for 24 hrs and subsequently exposed to ZnO nanoparticles under static conditions exhibit greatly increased inflammation. *In vivo*, oscillatory flow occurs in regions of disturbed flow near arterial branches and bifurcations, and these areas are particularly prone to the development of cardiovascular pathology. These results suggest that arterial regions prone to cardiovascular disease are particularly sensitive to the inflammatory effects of metal oxide nanoparticles. We are currently investigating the mechanisms underlying these observations.

Interactions between inhaled nanoparticles and the endothelium would only occur if the particles are transported from the pulmonary system to the vasculature. The extent and kinetics of this transport are a matter of controversy. We have devised a Transwell-based system lung epithelial cells are co-cultured with

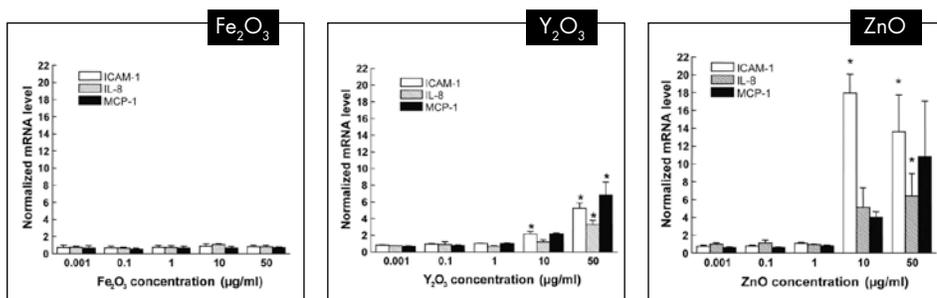
pulmonary microvascular endothelial cells. The co-culture system is subsequently incorporated into a flow chamber as schematically depicted in **Figure 2** below. This system provides the ability to investigate particle transport across epithelial-endothelial co-cultures while the endothelial cells are being subjected to flow as they would be *in vivo*. Preliminary results indicate that nanoparticles are indeed transported across the co-culture system. The effects of flow on this transport are under investigation.



**Figure 2.** Transwell-based system for co-culturing lung epithelial cells with pulmonary microvascular endothelial cells. The co-culture system can be incorporated into a parallel plate flow chamber to probe particle transport while endothelial cells are under flow as they would be *in vivo*.

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- Gautam M, Shen Y, Thirkill TL, Douglas GC, **Barakat AI**. Flow-activated chloride channels in vascular endothelium: shear stress sensitivity, desensitization dynamics, and physiological implications. *Journal of Biological Chemistry* 281: 36492-36500, 2006.
- Barakat AI**, Lieu DK, Gojova A. Secrets of the code: do vascular endothelial cells use ion channels to decipher complex flow signals? *Biomaterials* 27: 671-678, 2006.
- Choi HW, Ferrara KW, **Barakat AI**. Modulation of ATP/ADP concentration at the endothelial surface by shear stress: effect of flow recirculation. *Annals of Biomedical Engineering* 35: 505-516, 2007.
- Gojova A, **Barakat AI**. Regulation of vascular endothelial cell wound healing by shear stress. *Journal of Applied Physiology* 98: 2355-2362, 2005



**Figure 1.** mRNA levels of the three inflammatory markers ICAM-1, IL-8, and MCP-1 in HAECs incubated for 4 hr with  $\text{Fe}_2\text{O}_3$  (A),  $\text{Y}_2\text{O}_3$  (B), or ZnO (C) nanoparticles. Each mRNA value was normalized to corresponding GAPDH value. Ratios relative to control cells (no nanoparticles) are shown; data are mean  $\pm$  SE. \*Statistically significant mRNA level increase relative to control cells ( $p < 0.05$ ) [Ref: Gojova et al., *Environmental Health Perspectives* 115: 403-409, 2007].

## 2.4. Pro-inflammatory effects of particulate matter

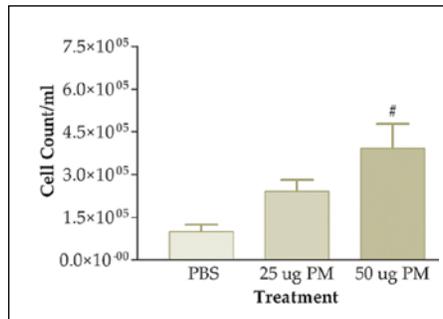


### Contact Information

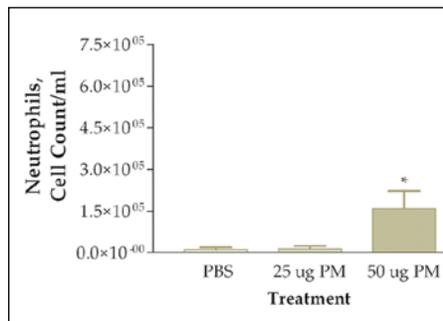
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**Particulate matter (PM)** in ambient air elicits inflammatory and toxic responses in the lung specific to its constituents, which can vary by region, time, and particle size. To identify the mechanism of toxicity in PM collected in a rural area in the San Joaquin Valley of Central California, we studied coarse particles of 2.5–10  $\mu\text{m}$  diameter (PM<sub>2.5</sub>–PM<sub>10</sub>). Potential pro-inflammatory and toxic effects of PM<sub>2.5</sub>–PM<sub>10</sub> in the lung were investigated using intratracheally instilled mice. We determined total and differential cell profiles and inflammatory chemokines in lung lavage fluid, and biomarkers of toxicity resulting from coarse PM exposure. Responses of the mice were readily observed with total doses of 25–50  $\mu\text{g}$  of PM per mouse. Changes in pro-inflammatory cellular profiles and chemokines showed both dose and time responses; peak responses were observed 24 h after PM instillation, with recovery as early as 48 h. Furthermore, macrophage inflammatory protein (MIP-2) profiles following PM exposures were correlated to levels of measured macrophages and neutrophils recovered from lung lavage fluid of PM-treated animals. Our data suggest that pro-inflammatory effects observed from coarse PM collected during the summer months from California's hot and dry Central Valley are driven largely by the insoluble components of the PM mixture, and are not caused by endotoxin.

These methods can be generalized to test any formulation of particulate matter that can be aerosolized into the lung (most conveniently, insoluble PM) for its inflammatory and/or toxic potential by bioassay in mice. Selective use of different mouse strains and/or knockout mice can allow us to test murine analogues of sensitive and resistant populations, to develop highly sensitive bioassays, and to develop mechanistic insights into the mode of action of the materials.



Average cell count of recovered cells in lavage fluid 6 h after instillation of PM (# $p < 0.05$  compared to PBS control, ANOVA). The bars represent  $\pm$  SE; N, the number of mice per experimental group, was 5–6.



Number of neutrophils found in lung lavage fluid 6 h after instillation of PM (# $p < 0.05$  compared to PBS control, ANOVA).

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1. Wegesser TC, Last JA. Lung response to coarse PM: bioassay in mice. *Toxicol Appl Pharmacol.* 2008 Jul 15;230(2):159-66. Epub 2008 Feb 26.
2. Wegesser TC, Pinkerton KE, Last JA. California Wildfires of 2008: Coarse and Fine Particulate Matter Toxicity. *Environmental Health Perspectives* 2009 117:893-897. [Online 2 February 2009]
3. Wegesser TC, Last JA. Mouse Lung Inflammation After Instillation of Particulate Matter Collected From a Working Dairy Barn. *Toxicol. Appl. Pharmacol.* 2009, 236:348-357. PMID: 19272399

## 2.5. Systemic platelet activation in mice exposed to fine particulate matter

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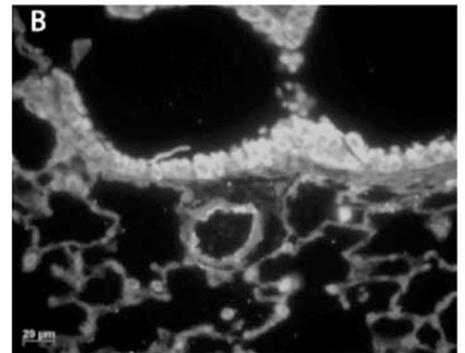
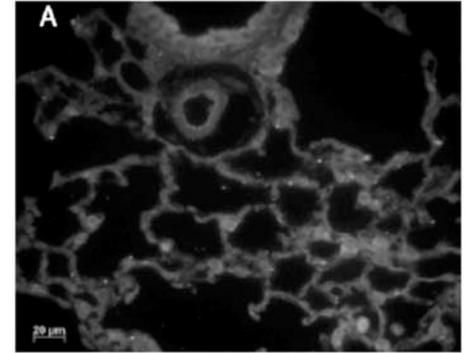


Exposure to air pollution has been shown to be associated with adverse cardiovascular events; however, the mechanisms for this association remain unclear. Many studies point to vascular and thrombotic mechanisms but specific studies of inflammatory mediators of the vascular endothelium and their relationship to endothelial cell and platelet activation are lacking. The potential for platelet activation resulting from inflammatory activation of pulmonary endothelial cells by inhalation of ultrafine particulates is a novel concept that does not appear to have been previously addressed in research investigating their toxicology. Dr. Wilson lab at UC Davis demonstrated *in vitro* that ultrafine particulate matter enters endothelial cells likely through vesiculocaveolar transport. That, and the potential for ROS generation from environmental samples as well as induction of AHR responses suggests that upregulation of endothelial surface molecules may be important intermediaries in stimulating cardiovascular responses to inhaled PM. Work from several laboratories suggests both that ultrafine PM can be absorbed to the systemic circulation after inhalation and that the response to inhalation creates a hypercoagulable state that could predispose to cardiovascular injury.

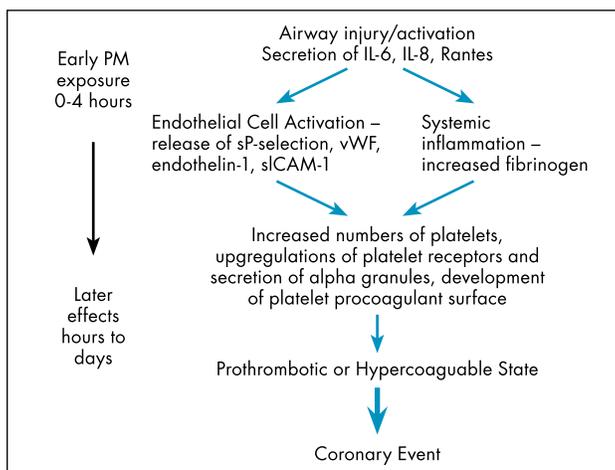
We conducted three separate experiments designed to define the effects of ambient particles on endothelial cell and platelet activation. In the first two experiments, we evaluated the effects of concentrated ambient particles (CAPs) on the vascular and hemostatic systems of a model animal (mouse), and compared them to control animals exposed to filtered air. Our third experiment is based on evidence in humans, that the peak incidence of cardiovascular complications occur 2-3 days after the onset of high particulate matter (PM), in which we examine the role of short term exposures (3 days) in our intra-tracheal instillation studies. Additionally, in this third experiment, we evaluate a recovery period of 3 days determine the reversibility of observed changes and the temporal relationships between pulmonary endothelial cell activation and systemic platelet upregulation leading to pro-thrombotic responses.

Further, in all studies, we aimed to determine the production of inflammatory cytokines, both in the systemic circulation as well as the bronchial alveolar lavage fluid (BALF) and their contribution to the interaction of these cellular systems. Immunocytochemical studies were used to evaluate pulmonary and coronary vascular endothelial activation. Localization and expression of endothelial cell activation markers, VCAM-1, E-selectin and ICAM-1 were assessed both in the pulmonary and coronary vasculature.

In collaboration with Dr. Wilson, we demonstrated upregulation of a set of serum cytokines that have both a pro-coagulant pattern and some predilection for TH-2 type responses. We also find cytokines associated with myelopoiesis. Perhaps more importantly, we find increased numbers and activation of platelets in mice exposed for two weeks to environmentally derived PM. We are now examining the role of pro-inflammatory molecules derived from airway epithelium relative to blood monocytes and the potential upregulation of platelet activation factors in pulmonary endothelium to determine whether platelet activation in PM exposure is a consequence of pulmonary inflammation or systemic responses.



VCAM-1 immunostains of airway epithelium, pulmonary arteriole and parenchyma from mice treated for 2 weeks with filtered air (A) or environmental tobacco smoke (B). Note increased expression of VCAM-1 in terminal airway epithelium as well as the endothelial cell surface of adjacent arterioles. Parenchymal staining of endothelium is only equivocally increased.



### Selected Publications

Items 1 - 20 of 69 Page of 4 Next

- Jandrey KE, Norris JW, MacDonald KA, Kittleson MD, Tablin F. Platelet function in clinically healthy cats and cats with hypertrophic cardiomyopathy: analysis using the Platelet Function Analyzer-100. *Vet Clin Pathol.* 2008 Dec;37(4):385-8.
- Guillaumin J, Jandrey KE, Norris JW, Tablin F. Assessment of a dimethyl sulfoxide-stabilized frozen canine platelet concentrate. *Am J Vet Res.* 2008 Dec;69(12):1580-6.
- Tablin F, Walker NJ, Hogle SE, Pratt SM, Norris JW. Assessment of platelet growth factors in supernatants from rehydrated freeze-dried equine platelets and their effects on fibroblasts *in vitro*. *Am J Vet Res.* 2008 Nov;69(11):1512-9.

## 2.6. TGF $\beta$ family signal transduction and ATF3 in endothelial injury

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The major focus of our laboratory is pulmonary vascular disease. By using the combination of pulmonary and vascular biology we try to understand the relationship between inhalation of environmentally derived fine particulate matter and cardiovascular disease. Our most current hypothesis suggests that airway inflammation drives pulmonary endothelial signaling leading to platelet activation, systemic cytokine release and subsequent alterations in unstable atherosclerotic plaque.

### TGF $\beta$ family signal transduction as a regulator of vascular injury and remodeling.

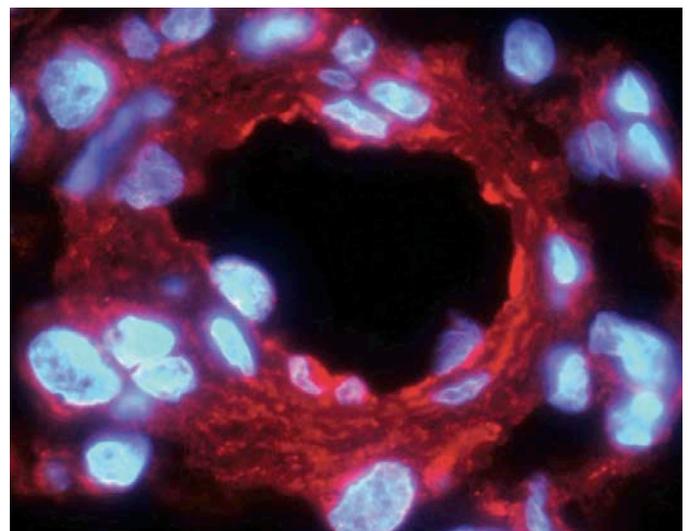
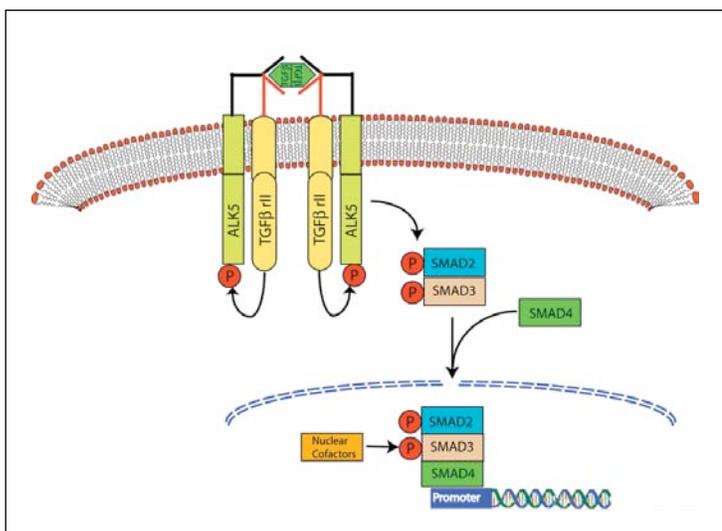
Much of our work in pulmonary hypertension investigates a chemically induced model resulting from treatment of rats with the pyrrolizidine alkaloid monocrotaline (MCT). A key genetic factor in human pulmonary hypertension has been determined to be

mutations in the Bone Morphogenetic Protein type II receptor, a component of the TGF $\beta$  family. Our recent work demonstrates that this receptor is localized to endothelial caveolae and that second messenger signaling resulting from MCT treatment acts through the common TGF $\beta$  SMAD pathway and leads to activation of BMP specific pSMAD1. As part of my ongoing collaboration with Dr. Jack Rutledge in the School of Medicine, we have been investigating the effects of post-prandial lipids on endothelial cell signaling. We have demonstrated that breakdown products of human post-prandial lipids activate the TGF $\beta$  specific pSMAD2 pathway and that this results in loss of endothelial cell barrier function as well as induces endothelial cell apoptosis. Our findings with these two models and complementary signaling pathways fit a paradigm wherein TGF $\beta$  signaling promotes smooth muscle proliferation but induces apoptosis in endothelial cells while BMP signaling does the opposite.

### Pathways of stress response in endothelial injury.

The impetus to investigate this comes from our work on the cardiovascular effects of environmentally derived fine and ultrafine particulate matter (PM $<$ 2.5). PM induces reactive oxygen species (ROS) leading to cellular injury. We have been evaluating

the general ROS responses as well as stress response signaling in order to understand the possible implications of systemic circulation of inhaled ultrafine PM. The principal signal responding to ROS is the Nrf2/Keap1 complex a cytoplasmic protein heterodimer that is activated by ROS releasing Nrf2 for nuclear transport as a transcription factor. Using gene array studies to probe the gene responses to post-prandial lipid breakdown products, we identified a transcription factor (ATF3) as the most prominent response element. ATF3 is both a gene product induced by this pathway and a transcription factor stimulating other response elements. Our recent work suggests that siRNA inhibition of ATF3 prevents the upregulation of pro-inflammatory adhesion molecules induced through the JNK pathway by post-prandial lipids thus providing a mechanistic association between the metabolic syndrome and the upregulation of factors that would promote both the formation of atheromas and the rupture of unstable plaque. A final chemical stress response pathway implicated in our work relates to polycyclic aromatic hydrocarbon (PAH) signaling. This suggests that, in addition to ROS responses, the PAH component of environmentally derived particulate matter may be an important factor in biologic responses.



**FIGURE 1** – Smad signaling and Smad 4 localization in endothelial cells of rat lung with MCT PH by IFC. Smad 4 is present within the nuclei of several endothelial cells of a remodeled but patent arteriole MCT = monocrotaline; PH = pulmonary hypertension; IFC, immunofluorescent chemistry (Ref. 1).

## 2.6. TGF $\beta$ family signal transduction and ATF3 in endothelial injury *(continued)*

### **Signaling related to cell cycle regulation and proliferative repair in endothelial cells.**

Our earlier work with MCT demonstrated that this crosslinking compound bound to DNA and induced cell cycle arrest at the G2M phase of the cell cycle. Associated with these investigations, we asked whether proteins were bound by activated metabolites of MCT and whether this was a general phenomenon or alternatively specific protein targeting. We identified several proteins that were specifically targeted based on the availability of soft electrophilic SH groups in their structure. Among these were ER chaperone proteins involved with protein folding and stress responses and an extracellular matrix protein, Galectin-1. Our findings suggest the opposite; prebinding of the reactive intermediates naphthoquinone and monocrotaline pyrrole not only enhance toxicity when transfected into cells but also maintain injury mechanisms, the former leading to oxidative injury while the later recapitulating the apoptotic injury we previously described. Emerging evidence that galectin-1 serves as an

intracellular signal provides a new hypothesis to help explain these results. We now hypothesize that binding of MCT to galectin enhances nuclear transport of the reactive intermediate and the bifunctional covalent capacity of this compound then makes linkages to DNA driving the apoptotic response. Further experiments with this protein now suggest that it has an intracellular role in signaling that relates to growth arrest and contact inhibition.

### **Selected Publications**

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2. Ramos M, Lamé MW, Segall HJ, Wilson DW. Monocrotaline pyrrole induces Smad nuclear accumulation and altered signaling expression in human pulmonary arterial endothelial cells. *Vascul Pharmacol.* 2007 Jun;46(6):439-48. Epub 2007 Feb 2.
3. Ramos M, Lamé MW, Segall HJ, Wilson DW. The BMP type II receptor is located in lipid rafts, including caveolae, of pulmonary endothelium in vivo and in vitro. *Vascul Pharmacol.* 2006 Jan;44(1):50-9. Epub 2005 Nov 3.

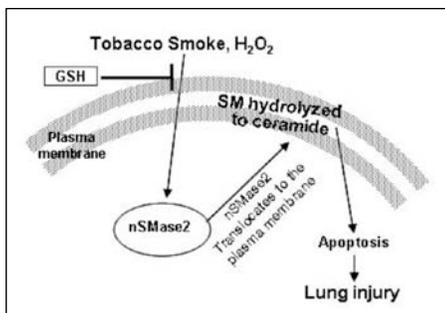
## 2.7. Sphingomyelinases generate ceramide response in lung injury

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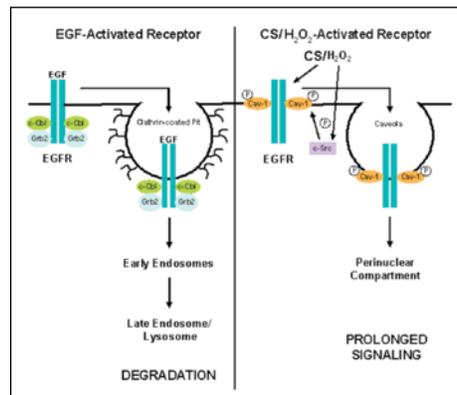
For the past 10 years, we have been studying the mechanisms of apoptosis and hyperplasia in the lung, as these two processes play a critical role in lung cancer and essentially constitute two sides of the same coin of lung injury and repair (14, 21). The research program of our laboratory thus centers both on the mechanisms of cell growth and proliferation in airway epithelial cells (2, 3, 9, 13, 15, 19) and on the ceramide-generating machinery that drives aberrant apoptosis control (1, 4-8, 10-12, 16-18, 20). In simple terms, loss of cells by apoptosis would be expected to be involved in, or perhaps even to initiate the overall tissue destruction normally believed responsible for lung injury.

We found that cigarette smoke (CS) exposure of lung epithelial cells induces aberrant phosphorylation of the EGF receptor, resulting in its lack of ubiquitination by c-Cbl and its impaired degradation. The stabilized CS-activated EGF receptor remains plasma membrane-bound, while a portion of the activated receptor is trafficked via caveolae to the nucleus. Aberrant EGF receptor activation without the feedback regulation of normal degradation leads to uncontrolled cell growth and tumor promotion.



**Fig. 1: Schematic model of nSMase2 modulation by cigarette smoke (CS)-oxidative stress.** CS modulates nSMase2 trafficking, up-regulates its activity, and leads to elevation in ceramide generation (hydrolysis of sphingomyelin (SM)) and thus exacerbated apoptosis in airway epithelial cells which causes lung injury. GSH blocks this effect of CS.

Another breakthrough study demonstrated that nSMase2 is the single target out of a large family of neutral sphingomyelinases (nSMases) responsible for the generation of ceramide in response to reactive oxidant species (ROS) and later on to cigarette smoke (CS) exposures. Even more importantly when nSMase2 was siRNA-silenced, lung epithelial cells COULD NOT undergo apoptosis in response to ROS or CS exposure both with lung epithelial cells and in vivo, with mice. This suggested that nSMase2 is an important target in the ROS/CS lung injury model.



**Figure 2. Schematic model for the role of caveolin-1 in EGFR trafficking under CS-oxidative stress leading to hyperplasia.** When exposed to EGF, EGFR recruits both c-Cbl and Grb2, which are required for the receptor to enter clathrin-coated pits, where it is sorted for lysosomal degradation. Under oxidative stress, EGFR is precluded from entering clathrin-coated pits by its failure to recruit both c-Cbl and Grb2. However, the activated EGFR does accumulate in a perinuclear compartment via caveolae-mediated endocytosis that is dependent on Src phosphorylation of caveolin-1.

Future studies will focus on elucidating new molecular targets that play a critical role in either lung cancer development or in lung injury diseases. Their molecular mechanisms will be characterized in order to better understand their critical role in lung pathogenesis. This will lead to better tools to prevent and cure lung diseases.

### Selected Publications

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- Ravid T, Tsaba A, Gee P, Rasooly R, Medina EA, **Goldkorn T** (2003). Ceramide accumulation precedes caspase-3 activation during apoptosis of A549 human lung adenocarcinoma cells. *Am J Physiol Lung Cell Mol Physiol.* 284(6):11082-1092.
- Ravid T, Heidinger J, Khan EM, Gee P, **Goldkorn T** (2004). C-Cbl-mediated ubiquitinylation is required for epidermal growth factor receptor exit from the early endosomes. *J Biol Chem.* 279: 37153-37162.
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- Castillo, S.S., Levy, M., Wang, C., Thaikoothathil, J., Khan, E.M., and **Goldkorn T** (2007) Nitric oxide-enhanced caspase-3 and acidic sphingomyelinase interaction: a novel mechanism by which airway epithelial cells escape ceramide-induced apoptosis. *Experimental Cell Research* 313(4):816-23.
- Castillo, S.S., Levy, M., Thaikoothathil, J., and **Goldkorn T** (2007) Reactive nitrogen and oxygen species activate different components of the ceramide-generation machinery to induce apoptosis in airway epithelial cells. *Experimental Cell Research.* 313 (12): 2680-6.
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- Raychaudhuri S, Willgohs E, Nguyen TN, Khan EM and **Goldkorn T** (2008) Monte Carlo simulation of cell death signaling predicts cell-to-cell stochastic fluctuation through type 2 pathway of apoptosis. *Biophys J.* 95(8):3559-62.

## 2.8. Modulation of nitric oxide as a therapeutic approach to the treatment of asthma

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Asthma is one important disease that has brought Nitric Oxide (NO) chemistry to the forefront of basic and clinical research. There is clinical wisdom that increased production of exhaled NO by asthmatic patients is a biomarker of disease severity (ATS 1999, 2005). Several levels of evidence by us and other investigators suggest that NO may be protective in inflammatory airways diseases. NO is a weak bronchodilator and may ameliorate airways hyperresponsiveness (AHR). Viral-mediated AHR has been blocked by the administration of the NO precursor, L-arginine (Folkerts et al., 1995). NO has been shown to block methacholine-induced bronchoconstriction in guinea pigs and rabbits (Dupuy et al., 1992, Hogman et al., 1993) and non-specific nitric oxide synthase (NOS) inhibitors have been shown to ameliorate AHR caused by allergen challenge (Ricciardiolo et al., 2000). In one short clinical trial, L-NMMA, an inhibitor of inducible NOS (iNOS, NOS2), reduced bradykinin-induced bronchoconstriction (Ricciardiolo et al., 1996).

L-arginine is a readily available amino acid supplement with a well-documented safety profile in humans. To date, the most promising therapeutic use for arginine is in sickle cell disease patients with acute chest syndrome. Arginine, particularly in combination with hydroxyurea, augments NO production and improves outcomes in this disease, perhaps by correcting an apparent deficiency in eNOS-derived NO that can contribute to red cell sickling and pulmonary vasoconstriction (Morris et al., 2000). Arginine may have therapeutic uses in other diseases, such as high altitude pulmonary edema and cystic fibrosis, and these areas are under investigation. The treatment of refractory asthmatics with L-arginine represents an exciting new avenue of research.

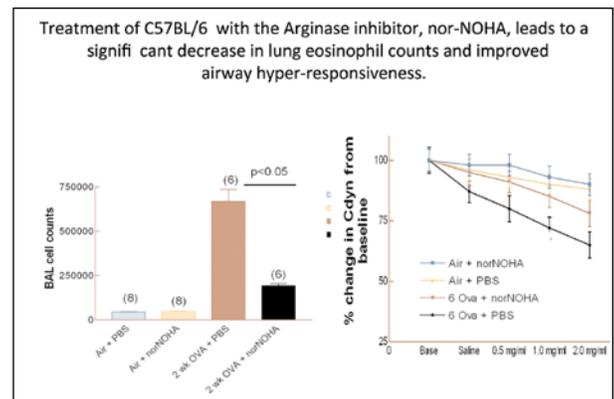
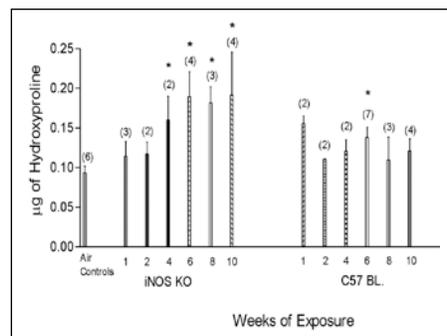
Lastly, the role of the arginase enzymes, the enzymes that convert L-arginine to ornithine, are of renewed clinical interest. Arginase I expression in macrophages is known to be regulated by Th2 cytokines such as IL-4 and

IL-13 (Pauleau et al., 2004). Serum arginase activity is elevated, and plasma arginine levels are decreased, in patients with asthma (Morris et al., 2004). It has been suggested that arginase can contribute to the regulation of NO levels in the lung by its ability to catalyze the diversion of arginine to ornithine (King et al., 2004, Ricciardiolo, 2003, Zimmerman et al., 2003). This theory requires further investigation in human asthma.

To address the protective role of L-arginine and NO in airway inflammation and human asthma further, we currently conduct a randomized, placebo controlled clinical trial of L-arginine supplementation. Severe asthmatic patients, who remain symptomatic despite maximal control medications and are at high risk of adverse outcomes from asthma, can benefit from this type of intervention. NO is a recommended surrogate marker for airways inflammation in asthmatic patients, yet the role of NO remains unclear. NO may protect against the structural and physiological changes that occur in asthmatics. A better understanding of this key molecule is needed. Inhibitors of NO production may not benefit, and indeed might harm, a subset of asthmatics. If we demonstrate that supplementation with L-arginine, a nitric oxide precursor, can improve clinical exacerbations in moderate to severe persistent asthmatics, it will have great implications for future research as well as for the daily lives of asthmatics. It would change our current paradigm of asthma treatment, and put NO in the mainstream of asthma treatment.

### Preliminary Studies

Our central hypothesis derives from our findings in studies with our mouse model of allergic airway inflammation. We have found that



mice lacking the NOS2 (iNOS) gene develop more airway inflammation and airway fibrosis (Figures 1) compared to C57BL/6 mice after exposure to ovalbumin for 4 weeks and longer (Kenyon et al. 2002, 2003a). Furthermore, we determined by PCR that the NOS2 (-/-) mice had more airway collagen I and III mRNA expression than C57BL/6 mice (Kenyon et al. 2003b). Airway arginase I protein levels, as measured by Western blot, are increased in NOS2-/- mice and BALB/c mice compared to C57 mice and correlate with increased lung lavage cell counts and increased AHR.

We have enrolled 15 patients in this study as of January 2006. While we can not fully analyze the data in this blinded cohort, the results suggest that about 25% of the subjects experienced a significant improvement in morning lung function and/or symptoms. Based primarily on the lung function data, I anticipate that L-arginine is responsible for this effect. We anticipate that L-arginine will have its effect either through its anti-inflammatory effects, paradoxical inhibition of the arginase I enzyme, or possibly through anti-oxidant effects.

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Kenyon NJ, Gohil K, Last JA. (2003a) Susceptibility to ovalbumin-induced airway inflammation and fibrosis in inducible nitric oxide synthase-deficient mice: mechanisms and consequences. *Toxicol Appl Pharmacol*. 191:2-11.

Kenyon NJ, Ward RW, Last JA. (2003b) Airway fibrosis in a mouse model of airway inflammation. *Toxicol Appl Pharmacol*; 186:90-100.

Kenyon NJ, Last JA. (2005) Reversible and Irreversible Airway Inflammation and Fibrosis in Mice Exposed to Inhaled Ovalbumin. *Inflammation Res*. 54:57-65.

## 2.9. Identification of lung specific tumour causing gene-networks by comparative transcriptomic analysis

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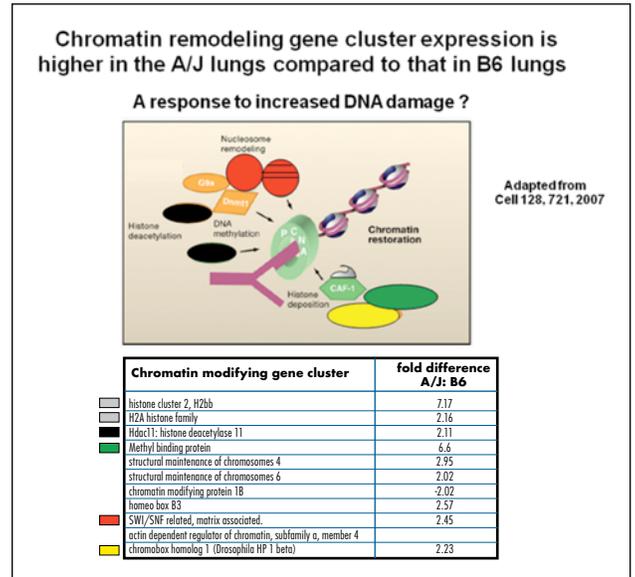
A primary aim of our research is to identify lung specific gene-networks that contribute to tumorigenesis. Previous data indicate that the activities of multiple genes are deregulated simultaneously in tumorigenic processes, and that documentation of single nucleotide polymorphisms and mutations in single genes are poor predictors and therapeutic targets for tumors. Therefore, we chose to use the whole genome analysis, in contrast to a gene-specific focus.

Affymetrix Mouse genome arrays were used to screen the expression of ~8,000 lung genes. To identify tumor causing gene-networks, we compared the lung transcriptomes of young B6 mice with those of AJ-mice; the former are resistant to age and cigarette smoke induced lung tumors whereas the AJ-mice develop tumors with aging, and the carcinogenic process can be augmented by chronic exposure to cigarette smoke. We propose that B6 mice are surrogates for humans that do not develop cancer whereas AJ-mice are surrogates for smokers and non-smokers that develop tumours. Other researchers have shown that AJ-lung tumors have many orthologues of human lung tumors.

Comparative analysis of the lung transcriptomes from young B6 and age-matched, tumor-susceptible but tumor free, AJ-mice identified 567-differentially-expressed genes which are likely to be associated with early stages of tumorigenesis in AJ-lungs. Functional analysis of these genes identified several clusters.

Particularly noteworthy were clusters that included members of protein kinase and phosphatase families; some members of the protein kinase family are displayed below. The data show that some members of functionally related gene-families, such as MAP-kinase are repressed AND induced in the same tissue. These data underscore the challenge of targeting protein kinases for amelioration of carcinogenic processes.

Another remarkable cluster of genes identified by the transcriptomic screen included genes that encode chromatin-remodelling and DNA-repair proteins. Some of the genes and their postulated functions are illustrated in the figure below.



The data in the figure above suggest that, even in the absence of macroscopic tumors, the lungs of AJ-mice display transcriptomic signatures of overactive chromatin remodeling and repair. The significance of these findings is that this cluster of genes may be “diagnostic” of the susceptibility to spontaneous and cigarette smoke induced lung tumors in AJ-mice. Hence this gene-network is likely to be relevant to tumorigenic processes in non-smokers and smokers.



## 2.11. Biomarker analysis of exhaled breath condensate

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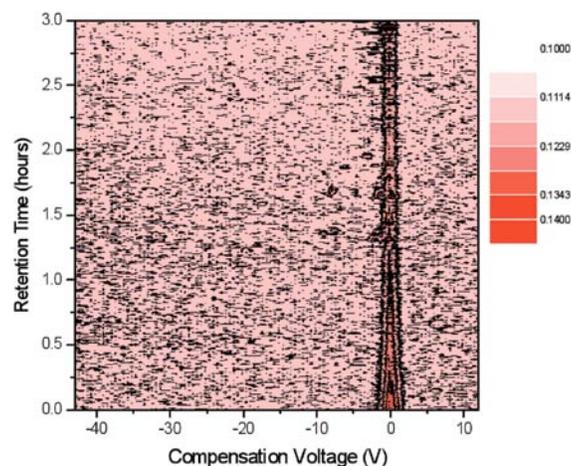


Non-invasive clinical diagnostic tools that are small and portable have the potential to revolutionize modern health care. The long-term, overall goals of our research are two-fold: (1) understand metabolic changes in exhaled breath that predict disease and response to drug therapy, (2) to develop novel, clinic-based sensor systems that will measure a host of compounds in exhaled breath condensate (EBC) and will allow clinicians to accurately diagnose disease. Our methodologies and instrument development approaches represent an exciting advance in the area of non-invasive breath diagnostics that could ultimately be applied to many different diseases, and potentially incorporated into telemedicine networks for at-home monitoring.

Dozens of volatile organic compounds in human breath condensate show promise for diagnosis and management of diseases, but little technical or clinical research has been performed to date (Zarling 1987, Phillips 1992). Some volatile breath biomarkers can be smelled by physicians on the patient's breath, such as ketones in starvation and ketoacidosis, feculent amines in bowel obstruction, and bacterial byproducts in anaerobic infections. Several diagnostic tests measure exhaled hydrogen after a specific sugar/starch load to demonstrate lactose deficiency, malabsorption, bacterial overgrowth of the small bowel, or pancreatic function in cystic fibrosis (Phillips 1992). The office test for *Helicobacter pylori* requires the patient first ingest <sup>14</sup>C labeled urea after which <sup>14</sup>C labeled CO<sub>2</sub> is detected in exhaled breath. Exhaled nitric oxide has also been measured as a marker of inflammation in the lungs (Cheu 1989, Djupesland 2001). Testing of exhaled breath has been proposed as a rapid toxicology test for carbon monoxide and methanol.

Several bench-top analytical chemistry approaches are available to investigate biomarkers that are related to metabolite changes in EBC. We are actively working on novel sensor platforms that can be easily adapted to portable breathalyzer sensor platforms. These novel methods enable the rapid detection and identification of compounds that can not be resolved by other analytical techniques. The advantages afforded by this sensor make it attractive as a quantitative detector that is sufficiently low cost to make it practical to use for bedside point-of-care diagnostics in hospital settings.

We have assembled a diverse and talented team bridging the engineering, clinical medicine, chemistry, and bioinformatics fields. Other studies, including our own, have examined biomarkers in exhaled breath for potential diagnostic value. However, all major studies to date have largely ignored potential confounding factors associate with metabolomic changes of the aging process. Our approach can potentially provide ground breaking data to this field.



Exhaled breath condensate (EBC) is a complex human emanation that is comprised of both volatile and non-volatile molecules.

The compounds have the potential to be putative biomarkers of disease, and we have been developing sensor instrumentation to monitor for these non-invasively. In this figure, we used a novel differential mobility spectrometer system to measure chemicals in EBC. Large peaks correspond to chemicals with high abundance, and smaller peaks correlate with medium- and low-abundance compounds. We are using both our novel sensors and other traditional analytical equipment (e.g. mass spectrometers) to identify biomarkers that significantly different between healthy control subjects and those with various pulmonary diseases. We also continue working on shrinking other instrumentation modules that would be needed to assemble into a truly portable hand-held "breathalyzer" for future point-of-care use.

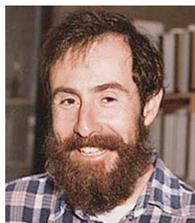
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## 2.12. Arachidonic acid cascade biomarkers in pulmonary diseases

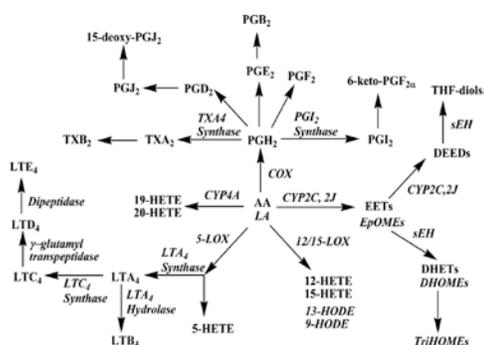
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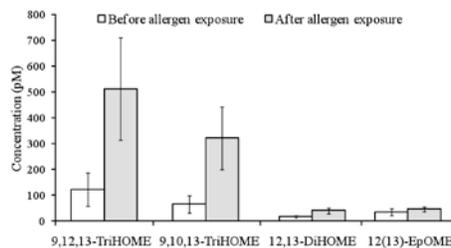
Our lab has developed several LC-MS platforms for monitoring biologically active lipids. The major platform provides quantitative data on 80 eicosanoids and related compounds by negative ion LC-MS. The metabolomic approach allows researching the patterns of multiple bioactive metabolites and thus test hypotheses of interaction. Some of the major targets for this platform are shown below. Other platforms are run on positive ion LC-MS, e.g. leukotrienes, endocannabinoids such as anandamide or 2-AG (the ethanolamide and glycerol derivatives of arachidonic acid), and vitamin D.

Increasingly, vitamin D is being suspected of having a major role in inflammation. The vitamin D metabolic pathway include difficult-to-analyze key intermediates, including biologically active dihydro- D2 and D3. By correlating these oxylipin profiles with biological effects of COPD we were able to evaluate the therapeutic effects of an experimental drug in a rat COPD model. Similarly, the method has also been used to look at the effects of two major components of smog (oxone and nitronaphthalene) in a rodent model. We are expanding our analytical platform to include metabolites of  $\omega$ 3 lipids.



In collaboration with Prof. Cristina Davis, we measured the oxylipins in the exhaled breath condensate of human volunteers (EBC). Exposure of the asthmatic subject to allergen led to increased EBC concentrations of 9, 12, 13-TriHOME, 9, 10, 13-TriHOME, 12, 13-DiHOME, and 12(13)-EpOME when

compared to levels in EBC collected prior to allergen exposure (range = 40 – 510 pM). 12, 13-DiHOME was significantly increased (Student's *t*-test,  $p < 0.05$ ). (figure)



**Figure 1.** Intra-individual variability represented by average levels  $\pm$  standard error for an asthmatic subject following allergen exposure and onset of symptoms ( $n=4$ ) compared to prior exposure occasions ( $n=6$ ). Asterisk indicates significance in comparison to symptom free conditions ( $p < 0.05$ ).

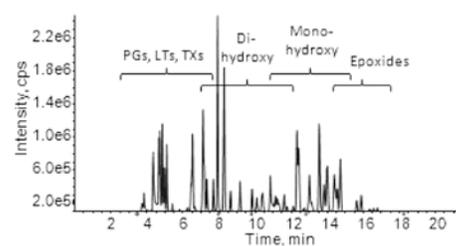
Using LC-MS/MS and an ABI 4000 linear ion trap, we are capable of analyzing 77 components using 200  $\mu$ l of plasma or equivalent tissue. We anticipate continual improvements in the method resulting from adding new analytes, reducing the sample volume and the length of the analytical procedure, improving clean-up, accuracy and precision.

For key biomarkers we develop focused methods with improved sensitivity and reduced costs. Often the 200  $\mu$ l plasma volume needed for a full eicosanoid profile precludes taking multiple samples from rodents. Thus we developed rapid LC-MS methods targeting specifically these metabolites detected in small sample volumes. Similarly, we developed rapid micromethods for the PGE<sub>2</sub> and TXB<sub>2</sub> levels as indicators of pain and cardiovascular side effects, and 5-HETE as indicator of the LOX5 pathway.

A representative HPLC chromatogram of the oxylipin analytes is shown below. The analytes elute according to their polarity with the most polar analytes, prostaglandins and leukotrienes eluting first followed by the hydroxy and epoxy fatty acids. The separated analytes are then quantified by tandem mass spectrometry in multiple-reaction monitoring mode utilizing negative electrospray ionization for the oxylipin profiling and positive electrospray ionization for the endocannabinoids and related fatty acid amides and esters. Surrogate analytes and internal and external standards are used to monitor extraction efficiency and ensure accurate quantitation of analytes. When possible we incorporate heavy atom internal standards.

Random selection of 5% of samples for replicate analysis tracks the method performance in terms of accuracy and precision. In addition, quality control samples are analyzed at a minimum frequency of 10 hours to ensure stability of the analytical calibration throughout a given analysis.

Data are tested for non-normality and outliers by the Shapiro-Wilk and Grubs' tests, respectively and significant differences are tested using ANOVA or Kruskal-Wallis for normal



and non-normal data, respectively. Multivariate statistical techniques such as principle component analysis (PCA) are used to reduce complexity. Additional analyses such as the Tukey-Kramer method or Bonferroni correction are performed to adjust for multiple comparisons.

We also have evidence that soluble epoxide hydrolase inhibitors are potentially useful in the treatment of asthma, cystic fibrosis and other pulmonary inflammatory diseases. We are using classical models of pulmonary inflammation as well as side stream smoke and smog components to create pulmonary inflammation. The sEH inhibitors prevent the resulting inflammation and can also be used therapeutically either alone or synergistically with inhibitors of the Lox and Cox pathways. This work is supported by a fellowship from the Cystic Fibrosis Foundation, the American Asthma Foundation, and NIEHS.

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## 2.13. Variation in the ALOX5 (5-lipoxygenase) gene and response to omega-3 fatty acid supplements

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Both cardiovascular disease and asthma are prevalent in the U.S. and disproportionately affect African Americans. Inflammation is an integral component of these conditions and is mediated, in part, by pro-inflammatory leukotrienes produced from arachadonic acid (AA) via the 5-lipoxygenase (5-LO) pathway. 5-LO is the rate-limiting enzyme in this pathway and is encoded by the gene ALOX5. Naturally occurring variants in the promoter region of the ALOX5 gene appear to cause different levels of expression of 5-LO, and may thus affect an individual's production of leukotrienes. The omega-3 fatty acids eicosapentaenoic acid

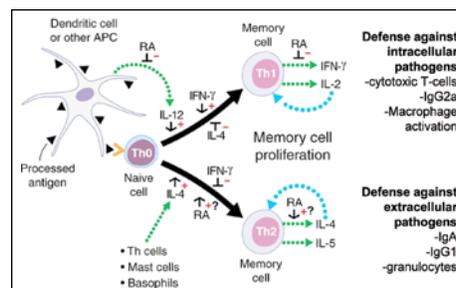
3.0 g/d EPA/DHA from highly refined fish oil for 6 wk. An isocaloric amount of refined olive oil will act as the placebo. The study is being done with collaborators from the University of Southern California (Dr. Hooman Allayee) and the Ethnic Health Institute of the Alta-Bates-Summit Medical Center in Oakland (Dr. Frank Staggers). Recruitment has finished and data analysis is under way.

A similar study is being done among *adults with moderate to severe asthma* with Dr. Nicholas Kenyon in the Department of Medicine at UC Davis using inflammatory, clinical and pulmonary function endpoints. The goal of this 8 month study is to determine if asthmatic subjects with "high-risk" ALOX5 gene variants have less exacerbations of asthma and improved lung function with the addition of omega-3 fatty acid supplements compared to asthmatics with the "low-risk" genotype. Preliminary data from the first 25 participants suggests that the "high risk" gene variants are very prevalent in the severe asthmatics.

## Vitamin A Deficiency, Supplementation and Pulmonary Inflammation

Vitamin A deficiency (VAD) increases infant mortality from some infectious diseases (e.g., diarrhea) (1), presumably by impairing both innate and adaptive immune function (2). For example, VAD impairs adaptive immune responses mediated by T-helper type 2 (Th2) cells (3) while high dietary vitamin A (high VA) enhances such responses (4). However, vitamin A supplementation can increase the severity of some infections (e.g., pneumonia) (5) and supplements containing vitamin A have been linked to an increased risk of asthma (6, 7). These apparent contradictions may result from vitamin A's ability to promote Th2-mediated responses. Such responses may be beneficial when they contribute to protection, as is true for infectious diarrhea, but detrimental when they contribute to disease pathogenesis, as occurs with asthma.

Figure 1. Vitamin A Deficiency "Impairs" Adaptive Immunity by Decreasing Th2 Responses. RA denotes retinoic acid.



The biological activity of vitamin A, apart from vision, is mediated by its active metabolite retinoic acid, which acts via a heterodimer of the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) to modulate gene expression (8). We have developed and characterized mice with T cell-specific disruptions of RXR expression, and found that this RXR deficiency can produce a Th1 bias (9). We have also demonstrated that VAD decreased and high VA increased and the severity allergic pulmonary inflammation and airway resistance in ovalbumin (OVA)-induced mouse model of asthma (Figure 1) (10). We plan to use these dietary and genetic methods to define the mechanisms by which vitamin A regulates this Th2-mediated inflammatory response.

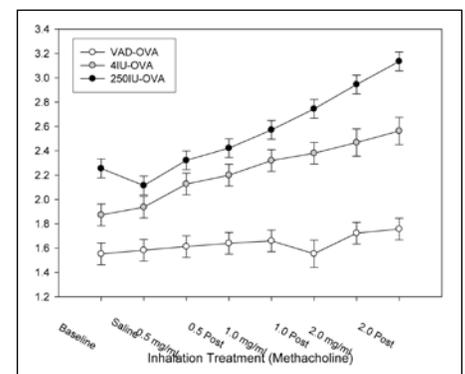
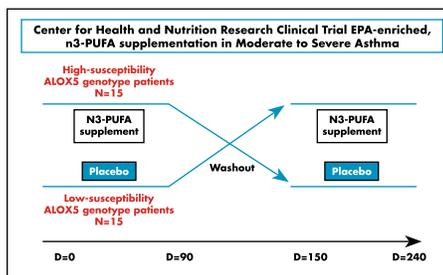


Figure 2. Airway responsiveness in mice fed different levels of vitamin A (VAD: 0 IU/g, 4IU/g and 250IU/g diet) and challenged with OVA aerosol. Baseline resistance was measured before administering saline and serial doses of methacholine (0.5, 1.0, and 2.0 mg/l). The graph shows mean values  $\pm$  SEM for two male and two female mice (except for one male and two females for VAD-OVA) taken over 3 min during and following (Post) methacholine administration.

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(EPA) and docosahexaenoic acid (DHA) decrease production of AA-derived leukotrienes by several mechanisms, including direct competition of EPA with AA as an alternate substrate for 5-LO.

We postulate that subjects with variants in the ALOX5 gene promoter may have different responses to dietary intake of DHA and EPA. Subjects with higher expression may have a greater need for the anti-inflammatory benefits of EPA plus DHA. In two similar studies we are determining if subjects with one or two variant ALOX5 alleles have higher ALOX5 gene expression, higher production of AA-derived leukotrienes, and a "better" response to omega-3 supplements than do subjects homozygous for the common allele. In a study of *healthy adults*, subjects are African Americans 20 – 59 y of age without serious disease. The study is a randomized controlled trial with a 6 x 2 design (6 genotypes x EPA/DHA supplement or placebo). The intervention group will receive

## 2.14. Primary respiratory epithelial cells for biomarker discovery and drug development

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Current research trends indicate an increased need for living primary respiratory tract epithelial cells. Potential uses include:

1. Biomarker discovery
2. Target identification
3. Functional analysis of drug candidates, i.e. activation of signaling pathways in response to drug treatment
4. Preliminary toxicology testing
5. Viral infection

We have an ability to collect and expand primary respiratory cells from both human volunteers and rhesus macaques. UC Davis has one of the largest breeding colonies of rhesus macaques, naturally exposed to various allergens. Some older animals developed respiratory conditions that are analogous to that of humans. In addition, we have an ability to challenge primates with synthetic allergens and environmental tobacco smoke (see page 36).

On the clinical side, our goal is to create a Respiratory Tissue Bank, containing respiratory tract epithelium isolated from various patient groups including those with asthma, COPD, and other airway-specific diseases. We plan to harvest cells from patients undergoing routine bronchoscopy at either the UC Davis Medical Center or the University-affiliated VA Medical Center. Brush biopsies will be taken in subjects already scheduled to undergo elective bronchoscopy. Respiratory tract epithelial cells will be collected with a cytology brush during the procedure. The collection will target the trachea or main bronchi to ensure direct visualization of tissue harvest throughout the procedure. Relevant patient data will be collected from the University or VA Hospital electronic medical record and filed into a separate database specific to this project. Barcodes will be used to link specific patient samples to database information. Based on our current bronchoscopy numbers at both institutions, we anticipate over 1,000 patient collections per year. The Respiratory Tissue Bank

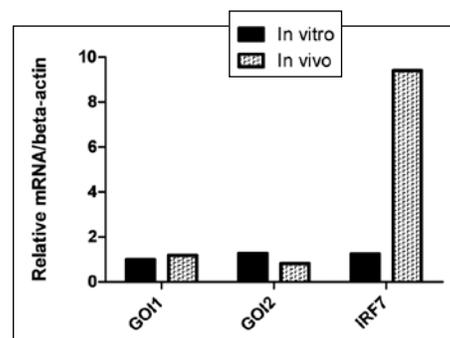
will provide an exceptionally valuable resource to investigate specific translational research questions.

The collected cells will be grown in cell culture conditions for several weeks. Once the cells have filled the dish, we will have sufficiently amplified the cells from an initial harvest of approximately  $1 \times 10^4$  cells to  $2.8 \times 10^6$  cells. At this point, cells can be stored long-term in liquid nitrogen or used directly for experiments. Once amplified, we will have sufficient cell numbers to seed  $12 \times 1 \text{ cm}^2$  or  $24 \times 0.5 \text{ cm}^2$  wells. This will provide adequate parallel samples to, at a minimum, examine protein and RNA expression levels for triplicate samples of four different treatment conditions for each patient. An on-line inventory management system will be designed to support retrieval of barcoded samples on demand.

Originally, this method has been developed at UC Davis by Professor Reen Wu, and has become a routine technique for the isolation and *in vitro* maintenance of conducting airway epithelial cells in a differentiated state. Over time, we have systematically developed a serum-free hormone-supplemented medium for growth of airway epithelial cells derived from the trachea or larger airway branches. The hormonal growth supplements used in our studies include insulin, transferrin, epidermal growth factor, hydrocortisone, cholera toxin, bovine hypothalamus extract, and retinol. Except for retinol, the physiological significance of these supplements *in vivo* is still unknown. Using this defined medium, up to three passages and 25–30 population doublings have been demonstrated in primary airway epithelial cells obtained from an adult human. The other advance in culturing airway epithelial cells *in vitro* is the development of a biphasic culture system in which epithelial cells are maintained in air-liquid medium interface. This new culture system reflects the *in vivo* situation and allows further cell differentiation. Biphasic culture conditions provide the proper milieu for mucociliary differentiation of human and monkey airway epithelial cells; this differentiation is not observed using traditional immersed culture conditions.

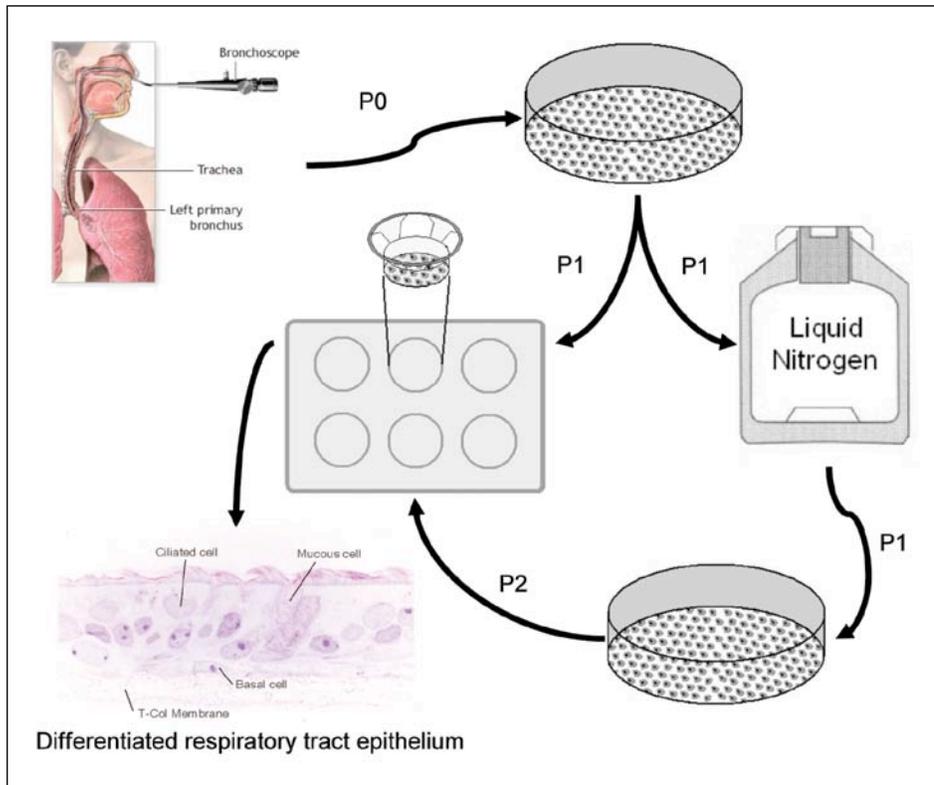
Our long-term experience with this procedure ensures that the cells isolated in this manner maintain phenotypic differences specific to the individual from which the cells were isolated. Using this technique, we have

observed a robust *in vitro* system that closely parallels respiratory tract epithelial cell behavior *in vivo*. As an example, we grew cells harvested from six different individuals in our cell culture system and determined RNA expression levels of several genes that are of interest to our lab. We then isolated RNA directly from a patient that was infected with virus and measured RNA expression levels of the same genes. As shown in figure 1, two genes of interest (GOI1 and GOI2) and beta-actin levels (data not shown) were remarkably similar. However, IRF-7, an important gene for antiviral response, was substantially elevated in the infected patient, but not in the uninfected cell cultures. Subsequent work has demonstrated that, viral infection will induce ten-fold or higher IRF-7 RNA expression levels in our *in vitro* system, on par with IRF-7 RNA levels that were observed after RNA isolation directly from the patient.



**Figure 1** – Relative gene expression levels of three genes normalized to beta-actin. The mean and standard deviation of relative gene expression are plotted for six *in vitro* samples and compared to the values of the same genes isolated directly from a single patient. The mean value for each gene isolated from six *in vitro* samples set to one and compared to the normalized value of each gene from the single patient.

## 2.14. Primary respiratory epithelial cells for biomarker discovery and drug development *(continued)*



**Figure 1** – Overview of cell culture procedure: Respiratory tract epithelial cells of the central airways will be harvested from patients using specialized brushes designed to maximize cell recovery without harm to the patient. These cells will be plated on collagen-coated 100mm dishes for primary culture (passage 0 or P0). These cells can be passaged immediately to porous supports for terminal differentiation or separated into multiple aliquots and stored long-term in liquid nitrogen (passage 1 or P1). Aliquots stored in liquid nitrogen can be re-plated on collagen-coated 100mm dishes to expand cell numbers prior to plating on porous supports for final culture (passage 2 or P2). Using these techniques, a polarized respiratory tract epithelium will form with differentiated cell types that are observed *in vivo*.

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Harper RW, Xu C, McManus M, Heidersbach A, Eiserich JP. Duox2 exhibits potent heme peroxidase activity in human respiratory tract epithelium. *FEBS Lett*. 2006 Oct 2;580(22):5150-4. Epub 2006 Sep 1.

## 2.15. Identifying distinct COPD phenotypes using respiratory epithelial cells

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### Specific Aims

Chronic Obstructive Pulmonary Disease (COPD) is reported to affect 5% of the adult US population. There are a number of existing algorithms to identify and stratify patients with COPD based on symptoms, and spirometry. Eighty percent of those affected by COPD are current or former smokers but conversely 20% are not. The lack of a single diagnostic test points to the conclusion that COPD is not one disease but a spectrum of conditions with overlapping pathophysiologies. To adequately address this challenging diagnostic problem, it is imperative that we better define the factors that cause airflow limitation, disease progression, and the physiologic underpinnings that define the various manifestations of patients with COPD. We will conduct a clinic population-screening to identify individuals who have COPD in its various forms and characterize multiple aspects of these individuals' symptoms and physiology. We will define the airway epithelial cell gene and protein expression profiles that are associated with these clinical characteristics.

*We hypothesize that patients with distinct COPD phenotypes will be identified by genomic and proteomic analyses, and, in turn, that models of such "fingerprints" will identify specific cohorts.* Ultimately, we predict COPD patients in each distinct group will respond similarly to "phenotype-specific" treatments and lead to improved care. Our future goal is to design a prospective, observational and intervention study to test this prediction. Briefly, our aims are:

1. To enroll 100+ ethnically diverse subjects with COPD and stratify patients based on four separate COPD phenotypes (**Table 1**).
2. We will perform a bronchoscopy study on 100 patients - 25 from each of the four clinical phenotypes. We will directly harvest living respiratory epithelial cells for primary culture and for direct measures of RNA and protein.
3. A portion of living respiratory epithelial cells will be cryogenically stored in the UC Davis Clinical Translational Sciences Center (CTSC) core facility to form a biorepository for future analysis

**Table 1 – Clinical COPD Phenotypes**

Phenotype	Primary Defining Criteria
I. Systemic Inflammation	Presence of 2 or more of following co-morbid diseases—cardiovascular disease, diabetes mellitus, skeletal muscle wasting, anemia, osteoporosis, obstructive sleep apnea, and depression
II. Hyperinflation	IC/TLC <25%, or TLC>120% predicted, or CT evidence of bullous emphysema
III. Mucous Hypersecretion	Daily sputum production. More than two exacerbations per year
IV. Airways Hyperreactivity	>12% or > 200 ml improvement in FEV1 with albuterol treatment

At the end of this study, we will have identified patterns to develop new diagnostic indices to test in a prospective, population-based COPD trial, and we will have identified genomic and proteomic markers that may help to explain the progression of the disease. We believe that this approach will allow us to ultimately lead to point-of-care clinical testing and allow us to tailor therapy to the individual patient. In addition, we will have a repository of living respiratory tract epithelial cells that will allow future investigators to ask mechanistic questions on disease-specific patient samples. Using a similar approach, this biorepository will be expanded to include samples from patients with other airway-specific diseases such as asthma, cystic fibrosis, and bronchiectasis.

### Background and Rationale

COPD affects heterogeneous subpopulations of patients that deteriorate functionally over time. Currently, the various molecular mechanisms and pathogenetic processes which contribute to COPD development, progression, and heterogeneity are poorly characterized. COPD subpopulations differ in their composite manifestations because of age, sex, response to tobacco smoke burden, degree of symptoms relative to FEV1, and response to bronchodilators. Phenotypic characterization of COPD subpopulations utilizing clinical parameters in combination with proteomic and metabolomic patterns will identify specific COPD syndromes, the course of these specific syndromes over time, and accelerate clinical trials of specific therapies targeted to these COPD subpopulations [1]. Based on the literature and our clinical experience, we suggest four clinical phenotypes that will capture the majority of patients diagnosed with non-asthmatic airflow limitation (**Table 2**). We suggest that FEV1 measurements alone are insufficient to fully describe the impact of COPD or the effect of treatments on clinical outcomes in COPD clinical trials. We propose

to separate COPD subjects into recognizable clinical phenotypes that are based on eight markers. The markers include outward physical markers (1-4), physiological markers (5-7) and radiological markers (8).

The purpose of this study is twofold. One, we will correlate gene expression and protein profiles isolated from patient respiratory tract samples to specific COPD cohorts. Two, we will establish a patient-derived respiratory tract cell culture tissue core that will provide living cells that can be used long-term to test new hypotheses. We predict that (1) specific "fingerprints" from patient-derived samples will correlate with differential RNA and protein expression and (2) that patient-derived respiratory tract epithelial cells can be sufficiently expanded more than 100-fold for long term storage in liquid nitrogen. We plan to harvest cells from a total of 100 patients over the course of the project. We calculated this number based on the realistic goal of recruiting 25 patients from each of the four clinical phenotype groups over two years.

### Experimental Approach

#### Patient recruitment

We have the patient population at the UC Davis Medical Center Pulmonary clinics (including the COPD Pulmonary Rehabilitation clinic) and the VA Northern California, Mather Medical Center to allow us to easily recruit 100 participants with known COPD. The University of California at Davis is the only major research entity within a hundred mile radius so it is unlikely we will have overlapping recruitment with other unrelated studies. We are planning to recruit a representative sample of participants who cover the spectrum of COPD. We will also use the VANCHCS, Mather center for additional subject recruitment, and that we have a common IRB proposal through a memorandum of understanding with the UC Davis Clinical and Translational Science Center. This enhances the ability to recruit appropriate numbers of

**2.15. Identifying distinct COPD phenotypes using respiratory epithelial cells** *(continued)*

**Table 2 – Differentiation of COPD Phenotype based on Phenotype Markers**

COPD Phenotype Marker	Phenotype I Systemic Inflammation	Phenotype II Hyperinflation	Phenotype III Mucous Hypersecretion	Phenotype IV Airway Hyperreactivity
1. Dyspnea (DOE)	DOE >> decline in FEV1	DOE ≥ FEV1 decline	DOE < FEV1 decline	DOE = FEV1 decline
2. Sex	F > M	M >>F	M = F	F >>M
3. Sputum production	Minimal	Minimal	Copious, daily	Minimal
4. Co-morbidities	Two or more	Less than 2	Less than 2	Less than 2
5. Bronchodilator (albuterol) effect on FEV1	No change or Minimal	No change or Minimal	No change or Minimal	> 12 % or > 200 ml improvement
6. BODE Index	3 to 10	3 to 10	3 to 8	3 to 8
7. IC/TLC & DLco	Mild decrease in IC/TLC Mild decrease in DLco	Moderate to severe decrease in IC/TLC Severe decrease in DLco	Mild to moderate decrease in IC/TLC Mild to moderate decrease in DLco	Mild to moderate decrease in IC/TLC Normal to moderate decrease in DLco
8. Chest CT Scan	Mild thickening of airways on CT scan	Predominance of emphysema/ low attenuation on CT	Prominent thickening with bronchiectasis subpopulation	Mild thickening of airways on CT scan

subjects. Dr. Kenyon is presently using the UC/D/VA Common Consent Form to recruit patients for his studies.

*Our specific plan will be:*

1. Recruit 25 subjects from each of the four cohorts. Our protocol for obtaining airway brushings is based on published protocols. Similar protocols exist at other institutions, and we have obtained copies of these protocols from outside institutions to ensure our methods are consistent with successful, ongoing projects.
2. Utilize the CCRC resources to perform bronchoscopies in a monitored environment with capacity to recover patients after the procedure.
3. Because this is a single-event experience for the patients, we do not anticipate significant resistance in obtaining patient volunteers.

Two brushings in total will be collected from the right main and left main of each patient. One brush will be used for RNA and protein isolation. The other brush will be used for propagation in cell culture conditions. Cultured cells will be processed and passaged to generate approximately 1 x 10<sup>4</sup> cells to 8 x 10<sup>6</sup> cells. Paired samples of normal patients and COPD patients will be passaged to air-liquid interface cell culture conditions for terminal differentiation of cells. The cells then could be manipulated based on the specific hypothesis (i.e. cytokine stimulation) i.e. for harvesting of RNA or protein from each cell population at various time points to determine expression levels for the genes of interest. Air-liquid interface cell culture conditions have been utilized in our laboratory for years, and we do not anticipate any difficulty with this method.

This study will provide a repository of human respiratory epithelial cells which maintain phenotypic differences in cell culture.

**Anticipated Results**

We anticipate that we will successfully recruit 100+ patients for this project. We have demonstrated the ability to harvest living cells from patients under our current IRB protocol (PI: Harper). Cell culture techniques, as outlined for this project have been utilized for several years by Dr. Harper, and we have sufficient expertise to accomplish our cell culture goals. The molecular biology techniques outlined for this study have been performed for years in Dr. Harper’s laboratory. Based on previous experience, we expect that each patient contact will provide, on average, ten aliquots for specific cell culture experiments. Each aliquot will contain approximately 4 X 10<sup>5</sup> cells that can be expanded to 2 X 10<sup>6</sup> terminally-differentiated cells for experiments.

**Timeline**

We anticipate that the initial project will take two years to establish. However, we plan to progress this project to include all patients undergoing bronchoscopy at UC Davis Medical Center. This will exponentially increase the number of patients and represented diseases that will be included in the biorepository over a relatively short period of time.

**Selected Publications**

1. Celli, B.R., Roger s. Mitchell lecture. *Chronic obstructive pulmonary disease phenotypes and their clinical relevance.* Proc Am Thorac Soc, 2006. 3(6): p. 461-5.
2. Forteza, R., et al., *Regulated hydrogen peroxide production by Duox in human airway epithelial cells.* Am J Respir Cell Mol Biol, 2005. 32(5): p. 462-9.
3. Konno, S., et al., *Interferon-gamma enhances rhinovirus-induced RANTES secretion by airway epithelial cells.* Am J Respir Cell Mol Biol, 2002. 26(5): p. 594-601.
4. Kraft, M., et al., *Detection of Mycoplasma pneumoniae in the airways of adults with chronic asthma.* Am J Respir Crit Care Med, 1998. 158(3): p. 998-1001.

	Year 1		Year 2	
Months	1-6	7-12	1-6	7-12
<b>Activity</b>				
Activity #1 (Aim 1-enrollment)	Recruit subjects and collect clinical information	→		
Activity #2 (Aim 2-bronchoscopy)		Perform research bronchoscopy	→	
Activity #3 (Aims 2—Cell culture)		Culture airway epithelial cells and harvest RNA	→	

## 2.16. Ovalbumin rodent model as a model of airway hyperinflammation and hyperresponsiveness

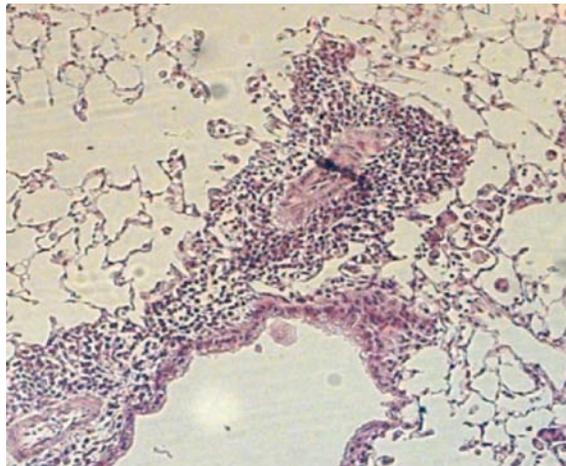
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Several important anatomical differences between human and mice lungs must be considered when making comparisons between the OVA mouse model and human asthma. Such differences include a relatively large airways diameter in mice, an increased airway/ lung parenchyma ratio in mice, and a single lobe in the left lung (Bates *et al.*, 2003). This results in less resistance to airflow in the airways of mice as a percentage of total lung resistance compared to the airways of humans. We can hypothesize that this may explain why mice do not wheeze. Other important differences include the presence of an inflammatory alveolitis and the absence of airway thickening and fibrosis in mice exposed to ovalbumin for 1 week. In addition, mice are obligate nose breathers and measurements of respiratory function in unrestrained mice must factor the contribution of the upper airway. Between 4 to 8 weeks of ovalbumin exposure, airway remodeling is present and thus, may be a better reflection of human asthma (Kenyon *et al.*, 2003b, Temelkovski *et al.*, 1998).

We consider the OVA mouse model to be a model of **allergic airways inflammation and airways hyper-responsiveness** rather than asthma. In general, mice are immunized systemically with an antigen and subsequently challenged with the same antigen through the airways. We immunize mice intraperitoneally with ovalbumin (OVA) of various concentrations plus alum as adjuvant. One immunization is usually sufficient for sensitizing the mice, although two immunizations two weeks apart are commonly employed. The airway antigen challenge is performed typically 14 days after the immunization, either by exposing the mice to the aerosolized antigen once a day for several consecutive days or giving the antigen intranasally a number of times. The aerosol is generated in a plexiglas chamber connected to a nebulizer. In a model resembling a chronic asthma with structural changes characteristics of airway remodeling (airway wall thickening and increased



Section of lung from a Balb/c mouse sensitized and exposed to ovalbumin for two weeks showing subepithelial eosinophilic and lymphocytic inflammation. (H & E stain)

collagen), sensitized mice are exposed to aerosolized antigen daily for three weeks or three times a week for six to eight weeks.

This OVA mouse model is invariably associated with airway eosinophilia developed after the airway antigen challenge. Bronchoalveolar lavage (BAL) fluid from the mice contains a large number of eosinophils as well as elevated levels of different Th2 cytokines. The model also exhibits airways hyper-responsiveness (AHR) and reversible airflow obstruction, two cardinal features of human allergic asthma. Mice demonstrate increased AHR in response to methacholine compared to mice exposed to filtered air (Hamelmann *et al.*, 1997, Temelkovski *et al.*, 1998, Kenyon *et al.*, 2003a). Furthermore, when these methacholine-exposed mice are subsequently allowed to breathe the filtered air or are treated with a bronchodilator, airflow obstruction is ameliorated and airways resistance decreases to baseline levels.

Measurement of AHR in mice exposed to OVA is done by monitoring anaesthetized, unrestrained mice in a whole body plethysmograph. Plethysmograph pressure changes are measured and analyzed with changes in the respiratory pattern. We believe that unrestrained plethysmography is useful for screening larger groups of mice, particularly when testing potential therapeutic inhibitors (Kenyon *et al.*, 2003b). This technique can limit the usage of animals in such trials. Any promising results in these screening trials should be repeated with measurements of lung mechanics by more formal methods (e.g., Zhang *et al.*, 1997, Kenyon *et al.*, 2005, Zuberi *et al.*, 2003). The most well-

studied and accepted measurements of lung mechanics in mice are respiratory system impedance, resistance, and compliance that are measured before and after exposure to methacholine. For these measurements, mice are deeply anesthetized, often paralyzed, and mechanically ventilated through a tracheotomy cannula. A single pressure transducer is needed to determine impedance, while pressure and flow transducers are needed to calculate respiratory system resistance and compliance. Impedance is determined by measuring the change in pressure at the tracheotomy tube, while the mouse is ventilated at a constant flow rate on a volume-cycled mode. The more conventional technique for determining compliance and resistance requires independent measurements of pressure and flow. Generally, the pressure transducer is in-line with the ventilator, while the flow transducer is connected to the sealed mouse plethysmograph. Dynamic compliance, for example, is calculated by dividing the change in volume, derived from the flow-time signal, over the change in pressure.

### Selected Publications

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- Kenyon NJ, Gohil K, Last JA. (2003a) Susceptibility to ovalbumin-induced airway inflammation and fibrosis in inducible nitric oxide synthetase-deficient mice: mechanisms and consequences. *Toxicol Appl Pharmacol*. 191:2-11.
- Kenyon NJ, Ward RW, Last JA. (2003b) Airway fibrosis in a mouse model of airway inflammation. *Toxicol Appl Pharmacol*; 186:90-100.
- Kenyon NJ, Last JA. (2005) Reversible and Irreversible Airway Inflammation and Fibrosis in Mice Exposed to Inhaled Ovalbumin. *Inflammation Res*. 54:57-65.

## 2.17. Lung injury and repair using naphthalene model

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Conducting airway epithelial injury and repair include several stages that are in common with wound healing in other epithelial tissues such as skin and gut. These stages include injury, squamation of surviving uninjured cells, proliferation to repair damaged areas, migration and re-differentiation to restore cellular density and return to normal differentiated function. Thus, epithelial injury and repair can be thought of as studying wound healing in the lung.

In the past, lung wound healing has been studied mostly in the context of the impact of oxidant pollutants on lung cell kinetics, and specifically in targeting ciliated cells.

Although a portion of the lung disease incidence has been linked to tobacco use, exposure to chemicals in the air, water and food also may play a role. A number of chemicals undergo bioactivation to produce highly selective lung injury in rodent models. Our work focuses on naphthalene and 1-nitronaphthalene, which are combustion byproducts present in air from mobile sources. Naphthalene is a

significant component of jet fuel and is an important component of both mainstream and sidestream tobacco smoke. Naphthalene and 1-nitronaphthalene undergo metabolic activation to electrophilic intermediates which become bound covalently to proteins in target cells. Both chemicals produce species, cell and site selective toxicity but there is very little information to indicate whether the human is a susceptible species.

Toxicity of naphthalene arises from a metabolic activation via the cytochrome P-450 monooxygenase system in Clara cells. The extent of the injury and the sites involved can be varied depending on the route of exposure and the dose administered.

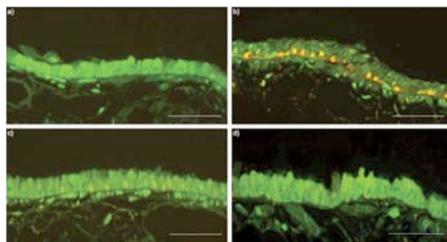
We use the naphthalene injury model to study **factors that can protect from injury and enhance repair**. The model may also be used for **specific phases of the repair process**, including the role of specific stem cell niches, as well as to investigate how other exposures might **compromise normal repair**. The ease of administration of naphthalene by injection and the high reproducibility of the model have led to its wide adoption as a method to study cell biology in the lung in relation to transcriptional control and progenitor/stem cell relationships. Because the phases of acute injury, cell proliferation and cellular redifferentiation are separated by several days, this model can be used to study the role of various growth factors and experimental therapeutics in mediating different components of airway epithelial repair. This model is an example of lung injury and repair that occurs in the absence of a significant

influx of inflammatory cells and in which the epithelium returns to steady state following a single acute injury. In this model, inflammation is clearly secondary to injury.

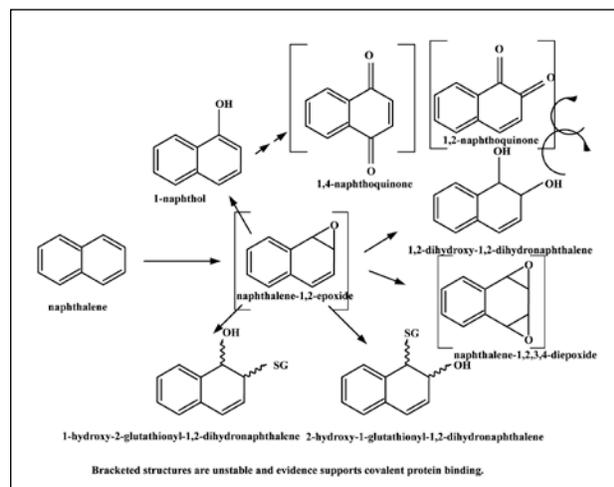
To define damaged conducting airway epithelial cells in situ in the injured lung we utilize a novel imaging method. A fluorochrome is instilled into the lung allowing for detection of the spatial pattern of cellular injury in the entire microdissected conducting airway tree. This can be quantified and the cells that are injured can be characterized using high resolution histology on resin sections. The fluorochrome remains embedded in the damaged cells.

Following the *i.p* administration of naphthalene, a site specific injury occurs specifically in the most distal airways, terminal bronchioles. As doses increase, the sites containing Clara cell damage increase to involve larger and larger airways, eventually spreading to Clara cells in the trachea and lobar bronchus. The pattern of cellular repair has a defined temporal sequence with the basic elements of proliferation and re-differentiation separated by days/weeks. This allows testing of interventions for wound healing in the lung during specific phases of repair. Our recent results demonstrate that pre-treatment with N-terminally truncated recombinant human keratinocyte growth factor protects against naphthalene-induced injury. This suggests that KGF exerts its beneficial effect through a decrease in the expression of cytochrome P<sub>450</sub> isoform 2F2<sup>6</sup>.

Another area of interest is the role of sex hormones on metabolic activation and detoxification in the lung. By analysis of acute injury using differential permeability to fluorescent nuclear dyes and high-resolution histopathology, injury in female mice was found to be more extensive, occur earlier, and include permeable cells in proximal airways, including airway bifurcations. HPLC analysis of the products of cytochrome P-450 (CYP)-mediated metabolism in microdissected airways indicated that although both genders produced a predominance of products from CYP2F2, female mice produced more naphthalene dihydrodiol in distal airways, the primary



**Figure 2.** Ethidium homodimer-1 staining (red-orange) of the distal airways of mice. Mice were treated with a, c) corn oil and b, d) naphthalene (200 mg/kg body weight<sup>-1</sup>) with (c and d) or without (a and b) N-terminally truncated recombinant human keratinocyte growth factor (ΔN23-KGF: 10 mg/kg body weight<sup>-1</sup>) pre-treatment. Pre-treatment with ΔN23-KGF protected Clara cells against naphthalene-induced necrosis. The images are representative of four distal airways per mouse (n=3 mice per group). Scale bar=50 μm.



## 2.17. Lung injury and repair using naphthalene model

(continued)

sites of injury. We conclude that there are clear gender differences in susceptibility to naphthalene induced injury and that differences in metabolism of naphthalene may play a role in elevated susceptibility in female mice<sup>4</sup>.

We also employ both active cycling and gonadectomized animals to investigate the role of steroid hormones in lung metabolism<sup>7</sup>. Recent studies have found shifts in the amount of airway epithelial glutathione and CYP2F2 metabolism by estrous cycle stage in mice. We conclude that the hormonal patterns associated with different stages of the estrous cycle 1) alter metabolism of naphthalene in the lungs of mice and 2) alter naphthalene metabolism differentially in extrapulmonary versus intrapulmonary airways. Furthermore, the pattern of changes suggests that naphthalene conjugate formation may be inhibited by estradiol, but dihydrodiol formation in proximal airways is influenced by other hormones. Our study suggests that hormonal regulation of enzymes (such as P450s or epoxide hydrolase), or their cofactors, in the lung of females influences metabolism of naphthalene. Formation of increased levels of toxic metabolites may, in turn, influence the extent of toxicity or tumor formation, issues that may underlie the differential susceptibility of females to lung cancer.

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1. Van Winkle LS, Isaac JM, Plopper CG. Distribution of epidermal growth factor receptor and ligands during bronchiolar epithelial repair from naphthalene-induced Clara cell injury in the mouse. *Am J Pathol*. 1997 Aug;151(2):443-59.
2. Coppens JT, Van Winkle LS, Pinkerton K, Plopper CG. "Distribution of Clara cell secretory protein expression in the tracheobronchial airways of rhesus monkeys." *Am J Physiol Lung Cell Mol Physiol*. 2007 May; 292(5):L1155-62. Epub 2007 Jan 19.
3. West JA, Van Winkle LS, Morin D, Fleschner CA, Forman HJ, Plopper CG. Repeated inhalation exposures to the bioactivated cytotoxicant naphthalene (NA) produce airway-specific Clara cell tolerance in mice. *Toxicol Sci*. 2003 Sep;75(1):161-8. Epub 2003 Jun 12.
4. Van Winkle LS, Gunderson AD, Shimizu JA, Baker GL, Brown CD. Gender differences in naphthalene metabolism and naphthalene-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol*. 2002 May;282(5):L1122-34.
5. Phimister AJ, Williams KJ, Van Winkle LS, Plopper CG. Consequences of abrupt glutathione depletion in murine Clara cells: ultrastructural and biochemical investigations into the role of glutathione loss in naphthalene cytotoxicity. *J Pharmacol Exp Ther*. 2005 Aug;314(2):506-13. Epub 2005 Apr 21.
6. Yildirim AO, Veith M, Rausch T, Müller B, Kilb P, Van Winkle LS, Fehrenbach H. Keratinocyte growth factor protects against Clara cell injury induced by naphthalene. *Eur Respir J*. 2008 Sep;32(3):694-704. Epub 2008 Apr 2.
7. Stelck RL, Baker GL, Sutherland KM, Van Winkle LS. Estrous cycle alters naphthalene metabolism in female mouse airways. *Drug Metab Dispos*. 2005 Nov;33(11):1597-602. Epub 2005 Aug 5.

## 2.18. A chronic asthma model in the young guinea pig using environmental tobacco smoke (ETS) combined with allergen challenge.

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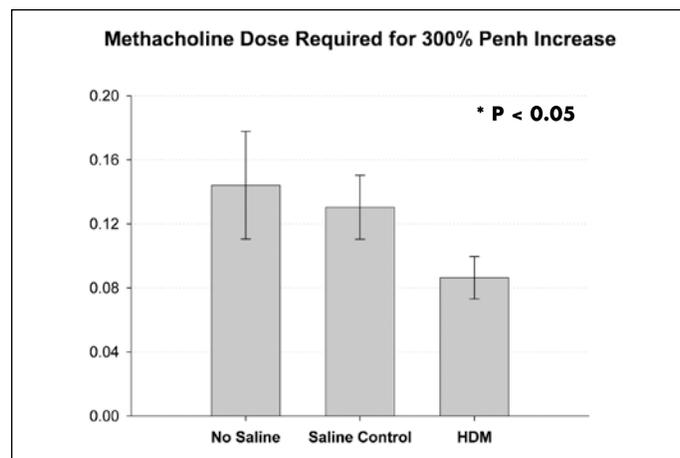


A chronic asthma model in the young guinea pig has been developed using a combined exposure regimen of environmental tobacco smoke (ETS) and allergen challenge to house dust mite. This model is established

within a nine week period to yield hyper-reactive airways (**Figure 1**) and enhanced eosinophilia, particularly within the airway walls and epithelium (**Figure 2**). The allergen used is *Dermatophagoides farinae*, given once/week by intranasal instillation. Exposure to ETS is administered following antigen sensitization over a 5-week period to enhance the classical features of an asthmatic-like condition.

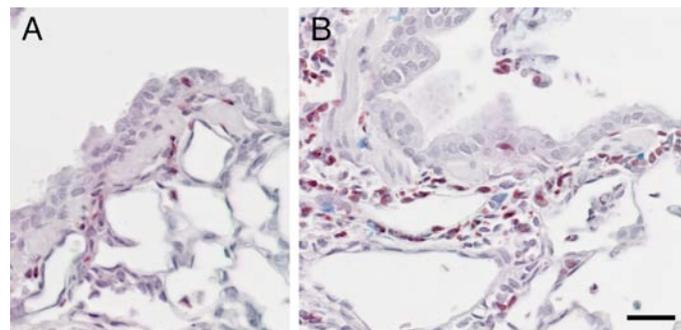
### Selected Publications

Mouadeb DA, Belafsky PC, Birchall M, Hood C, Konia T, Pinkerton KE. 2009. The effects of allergens and tobacco smoke on the laryngeal mucosa of guinea pigs. *Otolaryngol Head Neck Surg*. 140:493-497.



### Figure 1.

Methacholine challenge to demonstrate airway hyper-reactivity, requiring a smaller methacholine dose to elicit an increase in Penh activity.



**Figure 2.** Micrographs of the guinea pig airways and lung parenchyma (A, saline control and B, house dust mite and ETS). Eosinophils found in the epithelium and underlying airway wall of guinea pigs (A) are significantly increased with house dust mite challenge and further enhanced with exposure to ETS (B).

## 2.19. A rodent model of chronic obstructive pulmonary disease (COPD). Tobacco smoke-induced lung changes in spontaneously hypertensive rats.

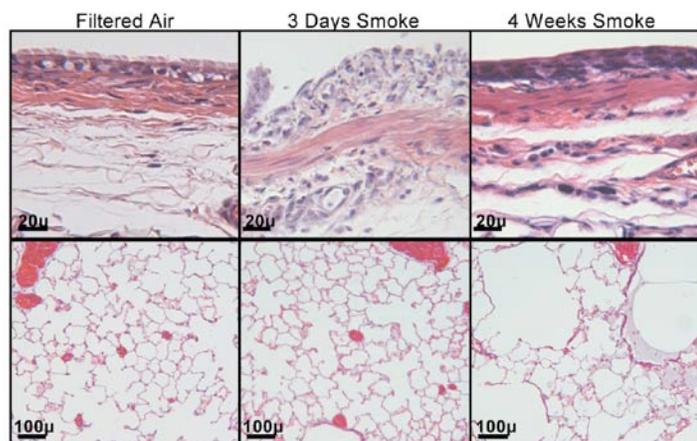
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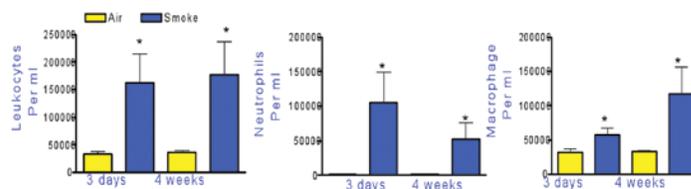


We have developed tobacco smoke exposure regimens via inhalation in the male spontaneously hypertensive rat (SHR) to create a powerful model for lung inflammation, airway remodeling/epithelial metaplasia, mucus hypersecretion and alveolar airspace enlargement. The model demonstrates reproducible acute and persistent inflammation, enhanced apoptosis, and induction of the five classical signaling pathways leading to inflammation, chronic bronchitis and cell differentiation and dysregulation (Zhong et al, 2005; Zhong et al, 2008).

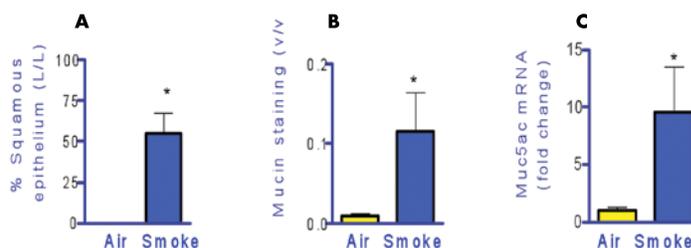
Histological changes in the lungs are illustrated in **figure 1** for airway epithelial injury and remodeling, accompanied by parenchymal alterations consistent with COPD. Patterns of cell inflammation induced by exposure to tobacco smoke in the lungs are graphed in **figure 2**. Quantitative measurements of altered epithelial cell type, intracellular composition and mucin gene expression are provided in **figure 3**.



**Figure 1. Tobacco smoke induced damage to the lung.** Hematoxylin and eosin staining of the bronchial wall (top panels) and lung parenchyma (lower panels) following exposure to filtered air or to tobacco smoke. Following three days of tobacco exposure significant epithelial cell desquamation of the airways is noted. After four weeks of smoke exposure, striking changes in squamous cell metaplasia of the airway epithelium and alveolar airspace enlargement of the lung parenchyma are observed.



**Figure 2. Inflammatory cells recovered from the lungs by bronchoalveolar lavage (BAL) in SH rats following three days or four weeks of tobacco smoke exposure.** Total leukocytes, neutrophils, and monocyte/macrophages recovered from the BALF after three days or four weeks of tobacco smoke exposure. Significant increases in total leukocyte number are due to the influx of neutrophils and additional macrophages to the lungs \* p value <0.05 vs. filtered air.



**Figure 3. Airway epithelial cell changes. Alterations in airway epithelial cell composition (A), mucosubstance content (B) and mRNA levels for Muc 5ac (C) following exposure to tobacco smoke for 4 weeks in the SH rat.** Approximately 50% of the airway epithelium in the second to fourth generation intrapulmonary airways of the lungs is replaced by stratified squamous cell (A), while the more distal airways of the lungs undergo mucus cell hyperplasia (B), accompanied by significant increases in gene expression for mucin (C) \* p value <0.05 vs. filtered air.

### Selected Publications

- Zhong, C.Y., Y.M. Zhou, G.C. Douglas, H. Witschi, and K.E. Pinkerton. 2005. MAPK/AP-1 signal pathway in tobacco smoke-induced cell proliferation and squamous metaplasia in the lungs of rats. *Carcinogenesis* 26(12):2187-2195.
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## 2.20. Asthma model in non-human primates

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### Uniqueness of the Rhesus Monkey Model of Asthma

The rhesus monkey model of asthma is unique because of the genetic and physiologic similarity to humans that allows pulmonary function measurements commonly used in humans. The airway anatomy and especially the similarities during postnatal development of the lung are another major advantage in using rhesus monkeys. The rhesus monkey has more specific reagents available for use compared with other nonhuman primates (NIH Nonhuman Primate Reagent Resource; <<http://NHPReagents.bidmc.harvard.edu>>).

The HDM-rhesus monkey model involves subcutaneous injection of a common human allergen, followed by intranasal instillation and repeated aerosol challenges of the allergen. The rhesus monkey model shares many of the key features of human allergic asthma, including: allergen-specific IgE and skin test positivity, eosinophils and IgE+ cells in airways, a Th2 cytokine profile in airways, mucous cell hyperplasia, subepithelial fibrosis, basement membrane thickening, and persistent baseline hyperreactivity to histamine or methacholine. In our rhesus model of asthma a fully human Ab against hOX40L demonstrated inhibition of antigen-driven Th2 inflammation.

### Airway Physiology

Following aerosol challenge with HDM, sensitized rhesus monkeys show cough, rapid shallow breathing, and increased airway resistance, which can be reversed by albuterol aerosol treatment. Compared with nonsensitized monkeys, there is a fourfold reduction in the dose of histamine aerosol necessary to produce a 150% increase in airway resistance in sensitized monkeys.

### Airway Generation-Specific Immune and Inflammatory Cells and Cytokines

After aerosol challenge, serum levels of histamine are elevated in sensitized monkeys. Sensitized monkeys also exhibit increased levels of HDM-specific IgE in serum, numbers of eosinophils and exfoliated cells within lavage,

and elevated CD25 expression on circulating CD4+ lymphocytes. In HDM-challenged monkeys, the volume of CD1a+ dendritic cells, CD4+ T helper lymphocytes and CD25+ cells, IgE+ cells, eosinophils, and proliferating cells is significantly increased in the airways. All of these cell types accumulate within airways in unique patterns of distribution, suggesting compartmentalized responses with regard to trafficking. Although cytokine messenger ribonucleic acid levels are elevated throughout the conducting airway tree of HDM-challenged animals, the distal airways (terminal and respiratory bronchioles) exhibit the most pronounced upregulation.

### Airway Generation-Specific Remodeling

Intrapulmonary proximal and distal bronchi of sensitized and challenged monkeys have focal mucous goblet cell hyperplasia, thickening of the BMZ, hypercellularity in the epithelium and subepithelial connective tissue, and increases in smooth muscle and bronchial microvessels. These changes are site specific and vary greatly within various portions of the airway wall in the same airway generation, between airway generations in an individual sensitized and challenged monkey, and from animal to animal in the sensitized and challenged group. A mouse model of allergic airways did not show airway generation-specific remodeling using airway microdissection-based sampling but did show changes in the proximal airways that were significantly different from control mice.

### Epithelial Changes

A characteristic of asthma is the sloughing of sheets of columnar epithelium from the airways leaving basal cells attached to the basal lamina. The desmosomal attachments between sloughed epithelial cells are intact, suggesting that failure of the desmosomal attachment between basal and columnar cells is responsible for sloughing of airway epithelium in asthmatic subjects. There is a reduction in the anchoring mechanisms between columnar cells and basal cells in the airways of asthmatic humans. Whether this change, combined with the inflammatory and immune cell trafficking, is responsible for cell sloughing is not known. To study this response, one must use an animal model that includes a multilayered epithelium, resembling the human epithelium, with abundant basal cells throughout the majority of the airway tree. Basal cells are more sparsely distributed in mice than in the rhesus monkey. We are currently investigating the signaling

mechanisms in the epithelium responsible for epithelial sloughing and subsequent increased production of growth factors and chemokines from the basal cells following sloughing of the columnar cells.

### Airway Wall Matrix/Basement Membrane

A characteristic of asthma is a massive increase in BMZ thickness. The BMZ is the central structure of the EMTU. Attenuated fibroblasts beneath the BMZ are thought to synthesize the collagen components of the BMZ. Remodeling of the epithelial BMZ, such as occurs in asthma, involves increased deposition of collagen in the BMZ. Thickening of the BMZ is thought to protect against airway narrowing and air trapping. FGF-2 is the main growth factor stored in the BMZ through binding with perlecan, an intrinsic constituent of the BMZ. FGF-2 forms a ternary signaling complex with the cell surface receptors FGFR-1 and syndecan-4. In the airway, these receptors are found only on basal cells. In the rhesus monkey, (1) FGF-2 is associated with synthesis of the BMZ in development and injury; (2) perlecan regulates FGF-2 and is necessary for normal development of the BMZ; and (3) the molecular signal associated with a thick BMZ persists during recovery; however, the role of FGF-2 released from the BMZ and its interactions with basal cells of the airway epithelium has not been determined.

### Fibroblasts

Attenuated fibroblasts are a distinct morphologic category of cells lying directly under the epithelium of the conducting airways of humans and rhesus monkeys. They exist as a layer of large flat cells covering about 70% of the BMZ. In the bronchi of normal humans, these cells also exist as a discontinuous layer composed of attenuated fibroblasts and myofibroblasts. In asthmatics, the number of myofibroblasts in this fibroblast layer is more than double that of normal individuals and the cell layer is more than twice as far from the epithelial basal lamina owing to subepithelial fibrosis. The functional role of this layer of cells is not known. Attenuated fibroblasts play a pivotal role in the pathogenesis of asthma based on morphologic evidence of subepithelial fibrosis in asthmatic human airways and the well-known role of collagen produced by these cells in tissue compliance and mechanics. The same appears to be the case for the rhesus monkey.

## 2.20. Asthma model in non-human primates (continued)

### Smooth Muscle

Smooth muscle hypertrophy and hyperplasia are characteristics of the distribution of airway smooth muscle in remodeling airways in humans. Type I asthmatics have increased smooth muscle mass only in large bronchi, largely owing to hyperplasia. Type II asthmatics have smooth muscle thickening from large bronchi to small bronchioles owing to both hypertrophy and hyperplasia. Increases in airway smooth muscle may increase airway contractility, as is found in AHR and in airways taken from asthmatic patients. The same is true for infant rhesus monkeys with allergic airways disease that also exhibit altered orientation and increases in muscle bundle mass. Increased airway smooth muscle is a hallmark of asthma, but whether environmental factors such as HDM allergen or O<sub>3</sub> exposure can cause this increase, by altering development in neonates or repair in adults, can best be defined in a species with an extensive period of postnatal airway development such as the rhesus monkey.

### Bronchial Vasculature

The bronchial vasculature of the EMTU contributes to the development of the human asthma phenotype via microvascular leakage and delivery of inflammatory mediators and leukocytes in the airways and is a prerequisite for airway remodeling in bronchial asthma. Studies in patients with asthma have described an increase in bronchovascular density; in conjunction with this finding, patients with asthma have been shown to have elevated vascular endothelial growth factor (VEGF) in their sputum and VEGF-producing cells in the mucosa assessed by endobronchial biopsy. Even patients with impaired left ventricular function have been shown to have increased AHR. Rhesus monkeys with HDM-induced allergic airways also show an increase in bronchovascular density that was airway generation specific.

### Airway Nerves

Substantial reductions in sensory nerve innervation of midlevel intrapulmonary airways of infant rhesus monkeys episodically exposed for 6 months to HDM allergen and/or ozone were observed, followed by a compensatory hyperinnervation after a 6-month recovery

period. One explanation is that the initial reduction and consequent hyperinnervation are the result of the disruption of normal growth factors and cues during the postnatal development of airway epithelial innervation and the consequent overexpression of growth factors within the airway EMTU. Paralleling these observations of altered midlevel airway epithelial nerve density and distribution are the observations of disruption of the conducting airway basement membrane and the associated growth factor FGF-2 after exposure for 6 months to HDM allergen and/or O<sub>3</sub>. Also paralleling changes in mid-level airway epithelial innervation are the observations that the disruption of basement membrane and FGF-2 continue to be overexpressed during a 6-month recovery period. Given that FGF-2 has been shown to be a potent nerve growth factor in the lung and other organ systems, we propose that FGF-2 released from the basement membrane directly affects nerve density and morphology within the airways.

These studies demonstrate that allergic asthma can be produced experimentally in a nonhuman primate, the rhesus macaque monkey, using a recognized human allergen, HDM, and provide a valuable model for human asthma.

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## 2.21. Development of rhinovirus infection model in non-human primates

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Rhinovirus (RV) is an important pathogen present in the respiratory tract epithelium of a significant proportion of patients with asthma and chronic obstructive pulmonary disease during severe inflammatory exacerbations. Controversy exists as to whether rhinovirus infection during infancy drives the asthma phenotype later in life, or if rhinovirus infection elicits the asthma phenotype which already exists. Our long-term objective is to understand the pathogenic role of rhinovirus infection in the etiology of asthma and exacerbation of chronic airways inflammation in humans. Although rodent models have been developed for respiratory syncytial virus infection, human rhinovirus infection has not been successful in this species because of host cell tropism. We propose to develop an experimental animal model of human rhinovirus infection using the rhesus macaque monkey (*Macaca mulatta*). Towards this end, the first step is to establish susceptibility of rhesus monkeys to human rhinovirus infection. The second step is to determine if experimental infection of rhesus monkeys with human rhinovirus can induce the clinical manifestations of human infection.

An *in vivo* primate model will provide the essential experimental foundation needed to explore the clinically relevant relationship between viral infection and the development of asthma during the first year of life. In addition, a primate model for adult rhinovirus infection will provide the means to perform needed mechanistic *in vivo* studies to determine the inflammatory/immune pathways that contribute to acute exacerbations of atopic asthma or chronic obstructive pulmonary disease. Completion of these studies will address fundamental mechanisms of mucosal immunity and airways disease that cannot be approached by epidemiologic studies or current *in vitro* model systems.

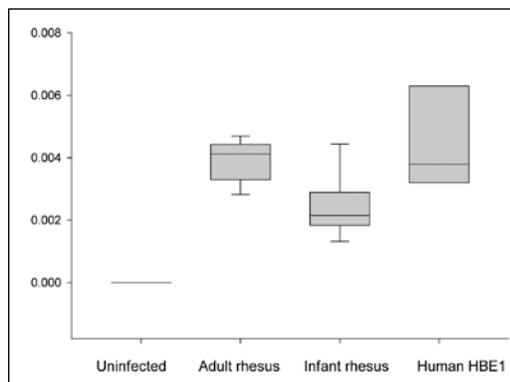
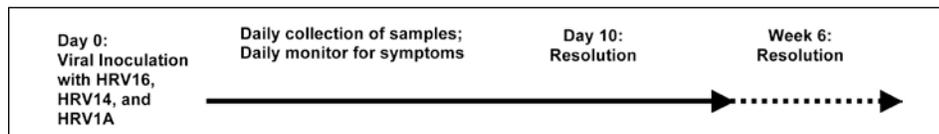
### Preliminary data:

A major obstacle to develop a non-human primate model is viral tropism with both major and minor serotypes of human rhinovirus. We recently demonstrated that two human rhinovirus serotypes will infect and replicate in primary cell cultures of tracheobronchial epithelium isolated from rhesus monkeys (**Figure 1**). Primary cultures of rhesus monkey airway epithelium from post-mortem infant and adult monkeys are readily available using a well-established method that has been pioneered by faculty at UC Davis. Our findings suggest that human rhinovirus isolates can recognize cellular receptors expressed on rhesus monkey epithelium (ICAM-1 and low density lipoprotein receptor, respectively). From our experience in the rhesus monkey model of allergic asthma, we know that non-human primates can generate airway inflammatory/immune profiles and physiological responses that are comparable to the responses observed in humans. Further, the vast majority of antibodies and molecular reagents used to investigate immune phenotype, function, and airways remodeling in humans work effectively in the rhesus monkey. Given that the immune system and lung anatomy of the rhesus macaque monkey are very similar to that of the

human, we anticipate that successful respiratory virus infection will produce similar results: an acute neutrophilic response and mild airways obstruction.

### Experimental outline

We propose to infect and monitor age-matched infant male rhesus monkeys and adult age-matched male rhesus monkeys with a series of three HRV serotypes in a longitudinal fashion (six animals per cohort). Virus will be administered by direct instillation of liquid culture media in anesthetized monkeys. Prior to each inoculation, animals will be evaluated by rectal temperature, chest radiography, nasal lavage, complete blood counts (CBC) and serum collection. Clinical signs will be measured daily for a total of ten days, followed by evaluation at six weeks post inoculation (total of 12 evaluations per infection period, per HRV serotype). Nasal lavages and blood collection will take place at each timepoint for clinical observations. We will also collect blood at 12-14 days post infection to assess immunoglobulin responses. Nasal lavages will be performed by instillation of 0.5 ml endotoxin-free PBS into each nostril, followed by aspiration into a sterile container.



**Figure 1: Similar HRV infectivity between human and rhesus monkey cell culture models.** Two isolates of primary rhesus tracheobronchial epithelial cells and transformed human tracheobronchial epithelial cells, HBE1, were grown in submerged cell culture conditions until confluent and infected with HRV16 at an MOI of 1 for one hour followed by removal of viral particles by three PBS washes. Real-time quantitative PCR of RNA harvested from all three cell types 24 hours after initial infection demonstrated similar levels of HRV16 vRNA transcripts.

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## 2.22. Overview of COPD as phenotypically heterogeneous disease

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### Introduction

Chronic obstructive pulmonary disease (COPD) is the most common respiratory disease of adults and is characterized by the progressive development of airflow limitation that is not fully reversible<sup>1</sup>. COPD is the fourth leading cause of death in the United States and is the only cause of death in the top ten that are on the rise. Chronic Obstructive Pulmonary Disease (COPD) is reported to affect 5% of the adult US population. COPD is a clinical term used to characterize a collection of multiple phenotypes that share the physiologic abnormality of decreased airflow. There are a number of existing algorithms for identifying and stratifying patients with COPD based on symptoms, signs and spirometry. Eighty percent of those affected by COPD are current or former smokers but conversely 20% are not. The lack of a single diagnostic test points to the conclusion that COPD is not one single disease but multiple conditions with different etiologies and overlapping pathophysiology.

To adequately address this problem, it is imperative that we better define the factors which cause airflow limitation, how it progresses, and the genetic and physiologic underpinnings that define the various manifestations of patients with COPD. Having a representative model of COPD would allow for more accurate and relevant assessment of causative pathophysiology. In order to generate a relevant model of COPD it is important to recognize the various phenotypes of COPD that exist clinically.

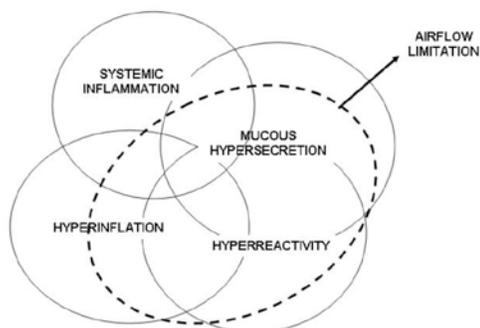


Figure 1: COPD Phenotypes

### COPD Phenotypes

COPD consists of heterogeneous subpopulations of patients that deteriorate functionally over time. Currently, the various molecular mechanisms and pathogenic processes which contribute to COPD development, progression, and heterogeneity are poorly characterized. COPD subpopulations differ in their composite manifestations because of age, sex, response to tobacco smoke burden, degree of symptoms relative to FEV<sub>1</sub>, and response to bronchodilators. Phenotypic characterization of COPD subpopulations utilizing clinical parameters in combination with genomic, proteomic and metabolomic patterns will identify specific COPD syndromes, the course of these specific syndromes over time, and accelerate clinical trials of specific therapies targeted to these COPD subpopulations.<sup>2</sup> Figure 1 is a schematic representation of the distinct phenotypes that are seen clinically.

#### Phenotype I – Systemic Inflammation

COPD subjects with this phenotype have evidence of COPD and concomitant diseases such as coronary artery disease (CAD), diabetes mellitus, or osteoporosis. Spirometry and lung volume changes are moderate with lower incidence of static hyperinflation, and less than 12% improvement in FEV<sub>1</sub> after bronchodilator challenge. The severity of dyspnea is significantly greater than is expected based on the decline in FEV<sub>1</sub> for this phenotype. These patients tend to have high systemic inflammation markers such as C-reactive protein. Currently we do not have a monkey model for this phenotype.

#### Phenotype II – Hyperinflation

COPD subjects with this phenotype have evidence of COPD with significant airflow limitation resulting in high degree of “air trapping” that worsens during exercise. A subpopulation of this phenotype will also have a pulmonary vascular component with resultant pulmonary arterial hypertension (PAH). These patients tend to have more emphysema than chronic bronchitis.

When damage to the lungs involves mostly parenchyma including the alveoli and small airways, there can be a loss of the elastic recoil of the lung. This loss of elastic recoil leads to a limitation of airflow resulting in a chronically hyperinflated lung. This particular phenotype is likely to have a higher degree of emphysema and small airway damage. Symptomatically the limitation of exercise is profound and the degree and perception of symptoms is progressive. A subset of this phenotype may have a degree of pulmonary vascular disease which can

be associated with a shortness of breath that is out of proportion to the degree of airflow limitation. Overproduction of mucus is not a major part of this phenotype.



The hyperinflated phenotype is represented in pulmonary function testing by airflow limitation, increase in RV/TLC and decrease in IC/TLC. The decrease in IC/TLC is the baseline static hyperinflation. Upon exercise, this ratio becomes even smaller reflecting dynamic hyperinflation. This phenotype is also likely to have a substantial decrease in diffusion capacity (DLCO). In a cohort of COPD outpatients in Spain the IC/TLC ratio was found to be an independent predictor of mortality<sup>3</sup>. The current generation of CT scans is able to quantify areas of low attenuation (LAA) as well as areas with airway thickening. Patients with the hyperinflation phenotype are more likely to have LAA than to have airway wall thickening. The degree of LAA has been shown to correlate with the FEV<sub>1</sub>%, FEV<sub>1</sub>/FVC ratio and DLCO<sup>4</sup>. A small subset of these patients can have evidence of pulmonary hypertension. Patients with true pulmonary arterial hypertension will typically have a low DLCO that is out of proportion to the degree of airflow limitation.

Since tobacco smoke inhalation is in large part responsible for the dramatic increase in COPD, what are the potential mechanisms of the oxidant contribution? Emphysema, one of the major causes of the hyperinflation phenotype of COPD, is defined as “a condition of the lung characterized by abnormal, permanent enlargement of airspaces distal to the terminal bronchiole, accompanied by destruction of their walls with or without obvious fibrosis.”<sup>5</sup> Inflammation and excessive proteolysis via an elastase/antielastase imbalance has been proposed for over 35 years to be the downstream consequence of chronic cigarette smoking resulting in interalveolar septal destruction, but animal models have yielded variable results.<sup>5</sup> The downstream consequences of direct injury to interalveolar septal cells by cigarette smoke have not been fully explained by elastase/antielastase imbalance (inflammatory cell hypothesis).<sup>6</sup> The presence of apoptosis and decreased VEGF protein levels in human emphysematous lungs<sup>7</sup> and the induction of emphysema in rats by blocking VEGF receptors that is associated

**2.22. Overview of COPD as phenotypically heterogeneous disease (continued)**

with increased apoptosis have provided additional data for a more attractive hypothesis.<sup>8</sup> Direct induction of interalveolar septal cell apoptosis in mice by instillation of active caspase-3 also resulted in emphysema.<sup>9</sup> Together, the studies of Kasahara and colleagues and Aoshiba and colleagues support the interdependence of interalveolar septal structure on both epithelial and endothelial integrity and that disruption of either leads to the same result of an imbalance in homeostasis with the consequence of emphysema.<sup>8,9</sup> There is also considerable evidence of markers of oxidative stress in smokers' lungs, presumably a direct effect of cigarette smoke or by chronic inflammation.<sup>10</sup> Since oxidative stress alters cellular signaling, particularly those involved in apoptosis and elastase/antielastase imbalance, it is also an important component of the pathogenesis of COPD. Oxidative stress and apoptosis, which cause lung cellular destruction in emphysema, were induced by VEGF receptor blockade with destructive emphysema in focal areas of rat lungs.<sup>11</sup> In this model of emphysema, both a caspase inhibitor and a superoxide dismutase mimetic blocked the development of emphysema and reduced markers of oxidative stress and apoptosis, thereby implying an additive effect between apoptosis and oxidative stress.<sup>11</sup>

**Phenotype III – Mucous Hypersecretion**

COPD subjects with this phenotype have evidence of COPD characterized by daily sputum production and frequent exacerbations (more than two per year). A subset of this phenotype will have chronic airway infections and bronchiectasis on CT scans. Chronic bronchitis (mucous hypersecretion) is an inflammatory condition in which CD8+ T-lymphocytes, neutrophils, and CD68+ monocytes/macrophages predominate. The condition is defined clinically by the presence of chronic cough and recurrent increases in bronchial secretions sufficient to cause expectoration. There is enlargement of mucous-secreting glands and goblet cell hyperplasia.<sup>12</sup> During airflow limitation, caused by bacteria, viruses, or environmental factors, patients may have increased rates of exacerbations. During exacerbation, there are changes observed in sputum, where increases in the level of IL-6, IL-8, and TNF- suggest an intense inflammatory burst.<sup>13</sup> In patients with COPD, 69.6% of exacerbations were associated with a bacterial pathogen, most commonly *Haemophilus influenzae*.<sup>14</sup> The rise in bacterial load at exacerbation showed a significant correlation with increased sputum interleukin (IL)-8 and decreased FEV1.<sup>14</sup>

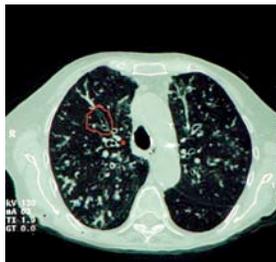
It also appears that stable state airway bacterial colonization modulates the severity and frequency of COPD exacerbations.<sup>15</sup>

**Phenotype IV - Airway Hyperreactivity**

COPD subjects with this phenotype have evidence of COPD and abnormal airway hyperresponsiveness (AHR), but not bronchial asthma or atopy. This particular phenotype shares many features of asthma but tends to be only partially reversible. The rhesus monkey model of airway hyperreactivity is established by episodic exposure to house dust mite allergen and ozone over a 5 month period. The airways of these monkeys show nonspecific airways hyperresponsiveness to histamine, increased baseline airways resistance, mucous cell metaplasia, basement membrane thickness, smooth muscle bundle size, bronchial microvessels and inflammatory cells.<sup>16,17</sup> In infant monkeys exposed to this regimen, the airways also show narrowed bronchioles.<sup>18</sup> Although some of these phenotypes may co-exist in single patient they represent distinct pathophysiology that could be modeled with an appropriate animal model.

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## 2.23. Development of COPD model in rhesus macaques

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### Rationale

Our assumption is that common environmental tobacco smoke in combination with environmental ozone exposure will mimic the typical exposure experienced by the average smoker. In order to accelerate the biology of this exposure the ability of the lung to generate a neovascular response to injury will be blocked. This approach will partially select for the "vascular" phenotype of COPD, but it is our hope that the combined exposure to ozone and ETS will effectively contribute to airway injury leading to parenchymal and airway pathology analogous to the clinical condition of COPD.

**Specific Aim** Combining an inhibition of vascular response to injury with episodic exposure to ozone and ETS will generate a COPD-like disease in rhesus macaque

A. Rhesus macaque treated with ETS and ozone will exhibit physiologic, radiologic, and pathologic features of COPD

B. Airway epithelial cultures from rhesus macaque treated with ETS and ozone will exhibit the characteristic biochemical features seen with airway epithelial cells obtained from chronic smokers.

### Preliminary Data

In a pilot project we exposed rhesus macaques to a combination of ozone and endotoxin over a 36 hr period of time to generate an inflammatory response. Four animals had this exposure while two animals were exposed to ozone only to serve as a background control. Two of the four animals exposed to ozone and endotoxin were treated with a low dose of SUGEN, inhibitor of the VEGF receptor flk-1. All animals were then imaged for neovascular responses in the lobes where endotoxin was instilled bronchoscopically. The two animals that received endotoxin and ozone acutely (endo group) had a robust neovascular response while the two animals that were pretreated with Sugen had a diminished neovascular response.

These results are summarized in **Figure 1** which a plot of the standardized uptake value for each animal. The results demonstrate a high peaked uptake over time for the endo group and a lower, below control, uptake for the endo block group. *The significance of this data is that we have demonstrated the ability to block neovascularization that occurs in response to lung injury.*

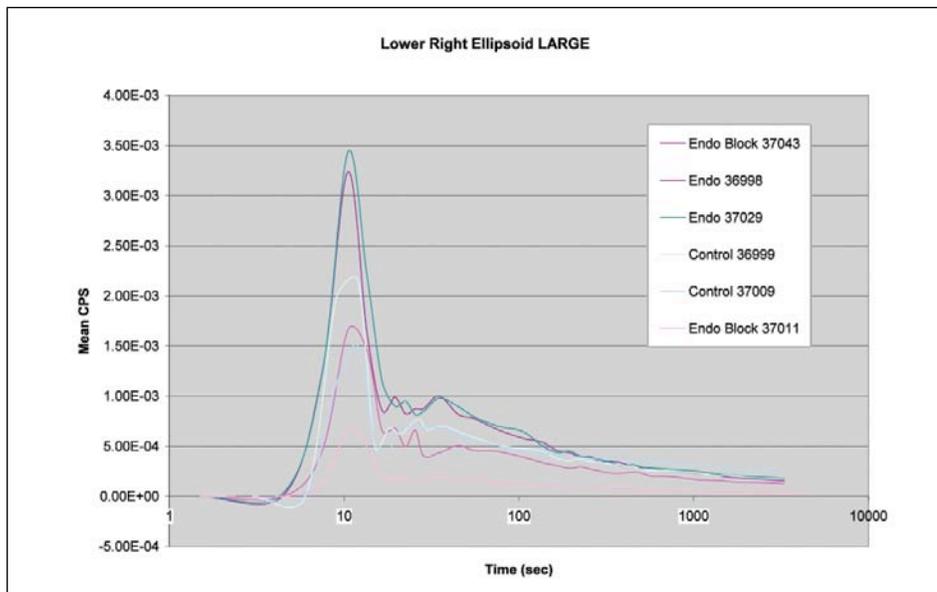
### COPD Experimental Plan

**Animal Protocol.** The animal protocol has been evaluated in terms of cost and feasibility with animal services. We will use two groups of 6 monkeys each. Group 1 will be controls that receive subcutaneous vehicle injections and filtered air in similar chambers to the other exposure groups. Group 2 will receive subcutaneous injections of SUGEN, inhibitor of the VEGF receptor flk-1 at a dose of 20 mg/kg at the beginning of the experiment and each month and 11 cycles of environmental tobacco smoke (ETS) plus ozone. Each cycle will be 2 weeks in duration with ETS and ozone delivered in chambers 8 hours a day for the first 10 days of the cycle and filtered air the remaining 4 days. This type of episodic exposure with ozone inhalation shows greater injury and less repair than continuous exposure and better mimics how humans are exposed to tobacco smoke. Each monkey at baseline and at the end of the 6 month exposure, will receive pulmonary function tests, blood samples and undergo bronchoalveolar lavage. Two monkeys in each group will have their final bronchoalveolar lavage and blood collection at necropsy and we will instill OCT 1:1 in RNAase-free buffer into the left caudal lung lobe, RNA later into the left cranial lung lobe and fix (1% glutaraldehyde/1%paraformaldehyde in cacodylate buffer) the remainder of the right lung for pathology and morphometric measurements relevant to COPD.

**Pulmonary Function** Quasi-static lung mechanics will be preformed using a Buxco Research Systems whole body plethysmograph and system software for forced maneuvers. Measures obtained from this system include total lung capacity, vital capacity, functional residual capacity, inspiratory capacity, residual volume and lung compliance. Airway resistance before and after treatment with the bronchodilator atropine will be obtained with a Pulmonac Instruments transfer impedance system that will provide measures of central and peripheral airway resistance, central airway compliance, and tissue resistance and compliance. Whole lung diffusing capacity and pulmonary capillary volume will be determined using a multiple gas, multiple breath method.

**Bronchoscopy** Bronchoscopy will be performed by Dr Mark Avdalovic, and adult pulmonologist with the assistance of the veterinary staff for conscious sedation. Dr Avdalovic has performed over 500 bronchoscopies in humans and approximately 50 bronchoscopies on rhesus macaques.

**Figure 1**



## 2.23. Development of COPD model in rhesus macaques *(continued)*

**Cell Count** Bronchoalveolar lavage will be spun down with the supernatant saved for future use. Cells will be resuspended, total cells estimated with a hemocytometer and a differential cell count done. Peripheral blood will be evaluated by an automated cell counter and a differential leukocyte count done. Cells can also be saved for subsequent analysis by Flow Cytometry.

### Anticipated Results

We would expect that rhesus macaque in the treatment group will have classic irreversible airflow obstruction and predictable airway hyperreactivity. Tissue analysis will demonstrate airway fibrosis, typical mucous hyperplasia, loss and alteration of alveoli. Morphometric analysis of the airways and parenchyma will confirm a loss of vascular density in the airways and parenchyma. Finally cell culture analysis of airway epithelial cell culture will match the biochemical phenotype that will be in a clinical cohort of patients with moderate to severe COPD. We feel this will be the cornerstone of linking this animal model to the clinical disease of COPD. It will serve as the foundation for translational research in COPD.

### Selected Publications

1. Schelegle, E. S., L. A. Miller, L. J. Gershwin, M. V. Fanucchi, L. S. Van Winkle, J. E. Gerriets, W. F. Walby, V. Mitchell, B. K. Tarkington, V. J. Wong, G. L. Baker, L. M. Pantle, J. P. Joad, K. E. Pinkerton, R. Wu, M. J. Evans, D. M. Hyde, and C. G. Plopper. 2003. Repeated episodes of ozone inhalation amplifies the effects of allergen sensitization and inhalation on airway immune and structural development in Rhesus monkeys. *Toxicol.Appl.Pharmacol.* 191:74-85.
2. Schelegle, E. S., L. J. Gershwin, L. A. Miller, M. V. Fanucchi, L. S. Van Winkle, J. P. Gerriets, W. F. Walby, A. M. Omlor, A. R. Buckpitt, B. K. Tarkington, V. J. Wong, J. P. Joad, K. B. Pinkerton, R. Wu, M. J. Evans, D. M. Hyde, and C. G. Plopper. 2001. Allergic asthma induced in rhesus monkeys by house dust mite (*Dermatophagoides farinae*). *Am J Pathol.* 158:333-341.
3. Fanucchi, M. V., C. G. Plopper, M. J. Evans, D. M. Hyde, L. S. Van Winkle, L. J. Gershwin, and E. S. Schelegle. 2006. Cyclic Exposure to Ozone Alters Distal Airway Development in Infant Rhesus Monkeys. *Am J Physiol Lung Cell Mol.Physiol* 291:L644-L650.
4. Schelegle, E. S., W. F. Walby, M. F. Alfaro, V. J. Wong, L. Putney, M. Y. Stovall, A. Sterner-Kock, D. M. Hyde, and C. G. Plopper. 2003. Repeated episodes of ozone inhalation attenuates airway injury/repair and release of substance P, but not adaptation. *Toxicol.Appl.Pharmacol.* 186:127-142.

### 3. California national primate resource center and exposure facilities

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The California National Primate Resource Center (CNPRC) is one of eight National Primate Research Centers (NPRCs) funded by the National Institutes of Health, National Center for Research Resources (NIH/NCRR) and is now in the 48th year of operation. CNPRC is located on a 300-acre dedicated tract of land. Approximately 85 acres are currently used for research, administration, and indoor and outdoor animal holding facilities. The overall facility is over 500,000 square feet, which includes research laboratories (~16,000 square feet), indoor animal housing and specialty testing space (~51,000 square feet), outdoor animal housing (~443,000 square feet) and administrative/research support space. In addition, planned construction and renovation projects will increase the CNPRC administrative and laboratory space and outdoor housing by 70,000 square feet. About 5200 nonhuman primates are housed at the facility, with a breeding colony of about 2500 animals. The CNPRC directly employs 573 people, including 48 academic staff scientists, and 229 research support and animal care personnel. This does not include employees funded by grants administered in other UC Davis departments. Research and core support facilities are organized in four major units - Brain, Mind and Behavior; Reproductive Sciences; Respiratory Diseases; and Virology and Immunology. The extramural grants of the Staff Scientists are about \$23 million per year and the center supports about \$48 million in extramural support from Affiliate Scientists.



#### 3.1. The inhalation exposure facility

The Facility, located at the CNPRC, is one of the largest in existence on a university campus. It permits unique human health-related pulmonary research opportunities using nonhuman primates. Capabilities exist for exposure of a wide variety of biological entities from cells to populations of organisms to a great range of compounds that can be delivered by inhalation. The expertise is available for exposures to oxidant gases, reactive gases, aerosols, mixed gas and aerosols, allergens, microbes and various drug-containing entities. This permits an integrated, comparative approach to defining mechanisms of respiratory system injury and repair. The pulmonary function laboratory offers a comprehensive array of testing for infant through adult nonhuman primates. Emphasis in the design of the exposure facilities has been on long-term exposure to carefully controlled pollutant atmospheres. The facilities are located in a 454 m<sup>2</sup> building at the (CNPRC).

The predominant inhalation chamber types are large stainless steel and glass units, 3.5 m<sup>3</sup> or 4.2 m<sup>3</sup> in volume. A central air handling system supplies chemical, bacteriological, and radiological (CBR) filtered air to each chamber at a flow rate of 2.1 m<sup>3</sup> per minute for a complete air change every two minutes. The exhaust systems are composed of bag house dust collectors and CBR filters. The high rate of ventilation causes rapid chamber atmosphere equilibration and lowers the level of airborne contaminants from the animals housed within. Close temperature control is maintained, and if needed, humidity can be controlled in most of the chambers as well. The university provides responsive around-the-clock emergency service for the large air handling and electrical equipment necessary to support a core of this size. A fast response emergency electrical generator keeps the core fully operational when power failures occur. Limit controls are employed so that pollutants are turned off and an alarm is activated if preset levels are exceeded.

#### Ozone Exposures

For ozone exposures, ozone is produced by electric discharge ozonizers from vaporized liquid medical grade oxygen. Both the ozone and oxygen are mixed at the inlet of the exposure chamber. The ozone concentration is monitored with an ultraviolet ozone analyzer. Calibration of the analyzers is performed according to the national reference method located at the California Air Resources Board Quality Assurance Laboratory in Sacramento, CA. Proportional ozone control systems are also used to dynamically stabilize exposure concentrations. These feedback loops fully automate chamber equilibration and control the mean level very precisely.

Individual exposures must be replicated with very high precision. We were able to precisely replicate an episodic 154 day regimen with two groups of infant monkeys receiving ozone exposure at a target concentration of 0.5 ppm. The mean ozone concentration of values logged every 4 minutes for each group was  $0.500 \pm 0.005$  ppm (mean  $\pm$  standard deviation,  $n = 6,273$  and  $n = 6,522$ ). The maximum of the range of ozone values was held to 5.8% above the mean concentration for each group.

Ozone exposure of monkeys can also be maintained by using a humidified atmosphere of air with the oxygen content

### 3.1. The inhalation exposure facility (continued)

increased to an adjustable percentage and delivered through a tracheal tube and a nasal cannula to an anesthetized animal. To create this atmosphere, adjustable flows of purified compressed air and medical grade oxygen are mixed. This gas mixture was humidified to about 75% relative humidity at 25° C by bubbling the stream through deionized and distilled water. The humidified gas mixture is then filtered through a Teflon™ membrane to eliminate the possibility of water droplets in the flow. This gas mixture is introduced to the zero air inlet of an ultraviolet ozone analyzer configured to control an ultraviolet ozone generator. The stream, now containing ozone, is then conveyed through tubing to a 0.5 liter volume gas bag and then to a manifold to which a tracheal tube and a nasal cannula were connected. Excess flow from the manifold is exhausted through tubing with the tip under 4 cm of water to maintain a slight positive pressure in the exposure system. The gas bag serves as a compliant reservoir for breathing.

*In vitro* ozone exposures of cultured cells are also conducted. A relatively large scale *in vitro* exposure system enables the simultaneous exposure of cells to three ozone levels with a filtered air control and is very well suited for dose response studies. Culture plates with wells containing the cells on inserts are exposed to ozone or filtered air in specially designed cylindrical glass vessels 3.66 liters in volume. Atmospheres contain 95% air and 5% carbon dioxide by volume and are saturated with water vapor at 37.5° C. This mixture flows through each vessel at a total rate of 15 liters per minute. In the lid of each vessel, a diffuser plate with 19 symmetrically located holes, each 1.6 mm in diameter, is incorporated to distribute the flow evenly. Exhaust is taken from a central point below a perforated desiccator plate on which the culture plate is placed. Vessel geometry and flow patterns have proven to be critical in ensuring that a homogeneous pollutant concentration is maintained within and that each culture receives the same concentration. The biological response data indicates that homogeneous exposure is achieved (5, 9). Three vessels are used for ozone and one control vessel is used without provisions for pollutant exposure. Pollutant levels in the three exposure vessels are independently adjustable. Concentrations in every vessel are monitored for two minutes every eight minutes.

#### Allergen Exposures

House dust mite (*Dermatophagoides pteronyssinus*) allergen (HDMA) is diluted in phosphate buffered saline. This solution is

nebulized with a high flow rate, compressed air nebulizer, which is operated at 3.5 kg/cm<sup>2</sup>, for a flow rate of 20 liters/min and immersed in an ice-water bath during operation to reduce water evaporation from the solution. From the nebulizer, polydisperse droplets about 2 µm in diameter are diluted with a 48.3 liters/min stream of dry air and conveyed upward through a 33.6 liter volume krypton-85 discharging column to reduce electrostatic charge. The aerosol is finally mixed with the inlet air stream of a 4.2 m<sup>3</sup> volume exposure chamber, producing in the chamber an aerosol of solid particles composed of the allergen with salt residue. Each HDMA aerosol exposure is typically conducted for 130 minutes.

The HDMA aerosol is characterized by samples drawn from the animal breathing zone of the chamber. Total mass concentrations are measured by weighing collected samples. Selected samples are also submitted to the UC Davis Molecular Structure Core to measure the protein concentration. The particles collected on the filters are extracted and the protein content is determined by amino acid analysis. Aerodynamic size distributions are determined from samples collected with a Mercer-type cascade impactor. On each of the seven impactor stages and the after-filter, the content of chloride anion derived from saline residue in the particles is measured by ion chromatography. A lognormal distribution is fitted to each sample set of data. The values reported are the mass median aerodynamic diameter (MMAD) and the geometric standard deviation ( $\sigma$ ) of the fitted distributions. Typically, for these HDMA particles, the MMAD is 1.4 µm with a  $\sigma$  of 2.6. Particles can also be collected for microscopic examination on polycarbonate membrane filters or with an electrostatic precipitator on electron microscope grids. An optical photometer is used to continuously monitor the concentration of particles during exposure and to indicate when the chamber can be safely opened after exposure. Similar generation and characterization is used for ovalbumin aerosols used for reactive airway models in rodents.

#### Bioaerosol Exposures

The expertise of the Inhalation Exposure Core is available for exposures to bioaerosols, including exposures with immunostimulatory sequence DNA, proteins, endotoxin, cystic fibrosis genes in liposomes or adenoviral vectors, and other viruses including respiratory syncytial virus. High concentration aerosols containing these agents are delivered via

mask to sedated monkeys, with a concurrent flow spirometry aerosol inhalation exposure system. This is a closed system in which respirable aerosol is generated with a nebulizer and conveyed directly through 22 mm ID tubing to a mask, forming an air-tight seal with the muzzle. The mask is conical shaped clear glass to permit observation of aerosol respiration. To create effective sealing over the nose and mouth of each animal, the mask is equipped with a flexible rubber diaphragm and a secondary seal of latex dental dam. Exhaust is drawn from an outlet port on the mask through tubing and across a breathing circuit filter. A heated pneumotachograph is connected to a tee immediately upstream from the exhaust filter. As the monkey inhales and exhales the aerosol, pressure changes in the mask are automatically compensated and held to a minimum by the changes in a bias flow across the pneumotachograph. These changes are measured with a pressure transducer and computer based pulmonary physiology platform that provides real-time measurements of respiratory rates and volumes during the dosing period. With aerosol concentration, aerodynamic size, estimated deposition fraction and volume inhaled by the animals, the dose delivered during an inhalation period can be estimated. To ensure that potentially infectious or toxic aerosol does not escape into the room where exposures are conducted, the system is operated at a slight negative pressure, breathing circuit filters are used on the main exhaust and spirometer leg of the system, and a HEPA cartridge filter is used downstream from the breathing circuit filter on the main exhaust line. In addition, a high flow exhaust duct with HEPA filtration is used to ventilate the area around the monkey's head.

#### Aged and diluted cigarette smoke exposure

Conditioned 2R4F research cigarettes are smoked in a smoking machine. The cigarettes are alternately puffed at a 35 ml volume for 2 seconds every minute. The mainstream smoke from each puff is added to the side stream smoke, and the mixture is drawn into an aging chamber. Here, equal streams are drawn into the inlets of two 3.5 m<sup>3</sup> exposure chambers. The smoke in the chamber is further diluted at a flow rate of 875 liters/min. The total aerosol mass concentration of the chamber, or "total suspended particulate (TSP)" is maintained at 1 mg/m<sup>3</sup> by adjusting the number of cigarettes burning and by exhausting a small flow of smoke from the aging chamber.

### 3.1. The inhalation exposure facility *(continued)*

Carbon monoxide is continuously monitored with a non-dispersive infrared absorbance analyzer. Total mass concentrations are measured by weighing samples collected on pre-weighed, Teflon® coated glass fiber filters. A piezobalance aerosol mass monitor is also used during these exposures. Half-hourly mass concentration measurements of the aged and diluted smoke were made with the mass monitor, which completed a measurement after a two-minute sampling period. It was used to adjust chamber concentrations during exposures and allowed problems with smoke generation to be detected rapidly. Air samples from the chamber are drawn through XAD-4 sorbent tubes for nicotine analysis. The collected sample is extracted and analyzed by gas chromatography.

#### Exposure Capacity

Chamber or System Type	Number	Exposure Capability
4.2 m <sup>3</sup> volume stainless steel	18	Aerosols and gases, large or small animals for long-term
3.5 m <sup>3</sup> volume stainless steel	2	Aerosols and gases, large or small animals for long-term
0.55 m <sup>3</sup> volume stainless steel	2	Indirect calorimetry metabolic rate measurement of a monkey for 24 hours
0.44 m <sup>3</sup> volume stainless steel	3	Aerosols and gases, small animals or single monkey for short-term
Modular clean air building with CBR (chemical, bacteriological and radiological) air filtration	1	House non-human primates in clean air
Concurrent flow spirometry aerosol inhalation system	1	High efficiency aerosol delivery by inhalation to a monkey or other large animal with simultaneous measurement of respiratory volumes and rates for estimation of dose
Oxygen and ozone inhalation system	1	Adjustable oxygen percentage with or without ozone delivered to a monkey via nasal cannula and tracheal tube
Inhaled steroid delivery system	6	Deliver steroid or other aerosols by mask to awake infant monkeys for 5 to 10 minute dosing periods
PennCentury™ MicroSprayer®	1	Administer liquid spray with droplet size of 16 to 22 µm directly into the lung through a bronchoscope
<i>In vitro</i> exposure system	1	Expose cell culture or explant preparations simultaneously to as many as three levels of ozone or nitrogen dioxide
Isolated-perfused lung exposure system	1	<i>In vitro</i> exposure of isolated and perfused rat lungs to ozone and other gases (located in Dr. Joad's laboratory)
Nose-only rodent exposure system	1	Nose-only exposure of up to 48 rats to aerosols

#### Major Gaseous Pollutant Monitoring Equipment

Equipment Available	Function
9 Teledyne-API ultraviolet ozone analyzers	Continuous analyzers
2 Dasibi ultraviolet ozone monitors	Continuous analyzers
Dasibi oxides of nitrogen analyzer	Continuous analyzer
Teledyne-API dynamic dilution calibrator	Manual or automatic calibration of ozone, oxides of nitrogen, carbon monoxide and sulfur analyzers; absolute ozone photometer; gas phase titration of ozone and nitric oxide
Dasibi programmable multi-gas calibrator	Manual or automatic calibration of ozone, oxides of nitrogen, carbon monoxide and sulfur analyzers; absolute ozone photometer; gas phase titration of ozone and nitric oxide
6 Meloy sulfur analyzers	Continuous analyzers for sulfur dioxide and other sulfur compounds
Teledyne-API carbon monoxide analyzer	Continuous analyzer
10 Proportional controller systems	Extremely precise (<1%) feedback control of chamber ozone concentration
4 Multiple chamber gas samplers	Sequentially samples up to four chambers with a single monitor
Dell 4500 computer with GE Fanuc data acquisition system	Logs exposure concentrations, temperatures and other parameters for exposure data reports or emergency alarm notification

**3.1. The inhalation exposure facility** *(continued)***Aerosol Characterization Equipment**

Method	Comments
Total filter	Mass determination of all particle sizes in conjunction with chemical or gravimetric analyses; a variety of fiber and membrane filters are used depending on the analysis. A microbalance with filter weighing chamber can be used. Filters can be examined by microscopy.
Inertial impaction	7-stage, Mercer-type single jet cascade impactors with an after filter; effective cut-off aerodynamic diameter (ECAD) of final stage is 0.3 $\mu\text{m}$ . For aerodynamic size determination, used in conjunction with chemical analyses; Sierra ambient cascade impactor for gravimetric or chemical determinations; Berkeley Controls quartz crystal microbalance cascade impactor for automated measurement of aerodynamic size.
Ion chromatograph	Dionex unit for the chemical separation and determination of anionic and cationic constituents of aerosol samples
Photometer	TSI DustTrak™ light scattering aerosol monitors; continuous monitor of particle concentrations from 0.001 to 100 mg/m <sup>3</sup>
Scanning Mobility Particle Sizer (SMPS)	TSI system for continuous measurement of particle size distributions from 0.004 to 0.9 $\mu\text{m}$ and number concentration (from AQRC, UC Davis College of Engineering)
Optical particle counter	Climet single particle light-scattering counter for number concentration and optical equivalent size distribution; continuous monitor for particles 0.4 $\mu\text{m}$ to greater than 3.0 $\mu\text{m}$ . Sizes in five ranges, but also can be used with pulse height analyzer for greater resolution.
Mass monitor	Kanomax (TSI) unit combines a piezobalance and an electrostatic precipitator for rapid determination of mass concentrations.
Electrostatic precipitator	Collects particles for geometric size distribution analysis in conjunction with transmission or scanning electron microscopy
Condensation particle counter (CPC)	TSI units used to continuously monitor number concentration of particles too small to detect with an optical particle counter.
Diffusion battery	Used with CPC to measure size distribution of particles from 0.004 to 0.5 $\mu\text{m}$

### 3.2. Human exposure chamber

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The human inhalation exposure chamber consists of 9 by 8 foot stainless steel walk-in chamber equipped with environmental controls for the regulation of temperature and humidity that is housed in UC Davis Human Performance Laboratory. Currently the chamber is equipped with ozone generation and monitoring instruments that allows for the precise control of chamber ozone concentrations. The chamber is also equipped with a treadmill, cycle ergometer, computerized spirometer and expired breath condensate collection equipment.

In an effort to translate some of the observations in animal models, Dr. Schelegle has undertaken a series of studies examining the mechanism leading to ozone-induced pulmonary function

decrements in humans. We have completed a study examining the effects of inhaled anesthetic aerosols on ozone-induced pulmonary decrements, rapid shallow breathing and subjective symptoms. We recently completed a study examining the differences in time course and profile of inflammatory mediators following ozone inhalation in subjects varying greatly in ozone responsiveness. The most significant finding of our current so far is that the inhalation of 0.070 ppm ozone for 6.6 hours in normal healthy subjects results in significant subjective symptoms of respiratory discomfort and decrements in pulmonary function. This is noteworthy since the current 8 hour national standard is 0.080 ppm.

#### Selected Publications

1. Alfaro MF, Walby WF, Adams WC, Schelegle ES. Breath condensate levels of 8-isoprostane and leukotriene B4 after ozone inhalation are greater in sensitive versus nonsensitive subjects. *Exp Lung Res.* 2007 Apr-May;33(3-4):115-33.
2. Schelegle ES, Eldridge MW, Cross CE, Walby WF, Adams WC. Differential effects of airway anesthesia on ozone-induced pulmonary responses in human subjects. *Am J Respir Crit Care Med.* 2001 Apr;163(5):1121-7.
3. Adams WC, Brookes KA, Schelegle ES. Effects of NO2 alone and in combination with O3 on young men and women. *J Appl Physiol.* 1987 Apr;62(4):1698-704.

#### Exposure Core Test Atmospheres

Gases	Generation	Concentration Measurement
Ozone	Electric discharge or UV	UV absorption, absolute ozone photometer, gas phase titration
Carbon monoxide	Cigarette smoke Endogenous production by monkeys	NDIR absorption, span gas Gas chromatographic with reduction gas detector for very low ppb range concentrations
Carbon dioxide	Endogenous production by monkeys	IR absorption, span gas
Oxygen	Consumption by monkeys	High temperature galvanic cell, span gas
Nitrogen dioxide	Gas cylinders or liquid N <sub>2</sub> O <sub>4</sub>	Chemiluminescence, span gas, gas phase titration
Sulfur dioxide	Gas or liquid cylinders	Flame photometry, span gas, permeation tube
Aerosols	Generation	Concentration Measurement
Dust-mite allergen	High flow rate nebulizer	Gravimetry, ion chromatography, protein analysis
Endotoxin (LPS)	Nebulizer, concurrent flow spirometry aerosol inhalation system	Biological effects; estimated and controlled total inhaled endotoxin units
Ethylene combustion products including ultrafine particles, both low and high PAH modes	Controlled burner system	Gravimetry, thermal oxidation, GC/mass spectrometry
Ovalbumin allergen	High flow rate nebulizer	Gravimetry, ion chromatography, protein analysis
Immunostimulatory sequence DNA	Nebulizer, concurrent flow spirometry system	Biological effects, DNA analysis, ion chromatography
Budesonide (inhaled steroid)	Nebulizer	HPLC for aerosol; plasma levels by LCMS

### 3.3. Pulmonary function lab

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#### Services Offered

- Baseline Airway Resistance Testing
- Airway Responsiveness Testing
- Allergen Responsiveness Testing
- Static Lung Mechanics Testing
- Physiologic Monitoring – Aerosol Therapy
- CT scans with pulmonary mechanics

*Pulmonary testing could be applied for:*

- 1) Evaluating the progressive development of responsiveness to inhaled house dust mite allergen (HDMA), histamine, and methacholine, a cholinergic non-specific stimulus. Responsiveness is evaluated by examining the effect of these inhaled challenges on airway resistance, breathing pattern, and arterial oxygen saturation.
- 2) Evaluating the progressive change in static lung mechanics following experimental interventions.
- 3) Evaluating chronic changes in respiratory function using surgically implanted telemetry devices.
- 4) Coordinating the time course of the experimental protocol and collating the skin test, clinical, pulmonary responsiveness, static lung mechanics and telemetry data.
- 5) Developing new and refining existing measures of pulmonary function, including the development of the lung diffusing capacity in primates.

#### Methods and Procedures

- 1) *Sedation and Anesthesia.* Monkeys are sedated using Telazol (8 mg/kg, IM) maintained if necessary with Ketamine hydrochloride (5-10 mg/kg, IM). The monkeys are anesthetized using Diprivan (0.1 - 0.2 mg/kg/min, IV) with the dose adjusted as deemed necessary by the attending veterinarian. Monkeys are intubated with an appropriately sized cuffed endotracheal tube (2.5-5.0 mm), or their mouths are held open with rubber bite inserts and they have facemasks secured

to their faces using elastic straps. Each monkey is then placed in a head-out, body plethysmograph, and the intubation tube or facemask attached to a pneumatic four-way valve/pneumotachograph assembly. For static lung mechanics measurements, all animals are intubated with a cuffed endotracheal tube.

- 2) *Static Lung Mechanics.* Sedated monkeys are placed into the whole-body plethysmograph, connected to a 3-way valve assembly, and given supplemental O<sub>2</sub> during the procedure. The semi-automated software, Maneuvers XA (Buxco Electronics), controls positive pressure inflation and negative pressure deflation to the lung in order to calculate the following parameters: standard static lung volumes/capacities, forced expiratory volumes and flows, quasi-static lung compliance, functional residual capacity and thoracic gas volume. This procedure takes approximately 10 minutes to complete.
- 3) *Aerosol Challenges.* All challenges are administered as aerosols at a set tidal volume and breathing frequency (size and age appropriate) using a compressed air nebulizer (Vortran, Inc., Miniheart Model) in series with a positive pressure ventilator (Bird Mark 7A respirator). Allergen challenge is done using a set concentration of house dust mite allergen (0.02 mg protein/ml) delivered for 1 minute followed by 4 minutes of data collection and repeated up to 12 times. Allergen challenge is terminated when airway resistance (Raw) doubles or arterial oxygen saturation falls below 75%. Data are expressed as the cumulative dose of allergen (mass concentration of allergen in mg protein/ml x tidal volume in ml x number of breaths) that produces a 150% increase in Raw (CDA150Raw). Histamine or methacholine challenge are done using repeated 30 second challenge periods separated by 4 minute data collection periods. Histamine or methacholine challenges are done with the initial concentration being 0.0625 mg/ml and ending at 64.0 mg/ml. The final concentration of histamine or methacholine to be delivered will be the one that produces a doubling in Raw or a decrease in O<sub>2</sub> saturation to 75%. The data is expressed as the concentration that produces a 150% increase in Raw (EC150Raw).

The CDA150Raw and EC150Raw are determined by linear interpolation on the log-log plot of the dose response curve with the response being expressed as the percent of baseline Raw.

- 4) *Breathing Pattern and Arterial Oxygen Saturation.* Tidal volume (VT) and breathing frequency are recorded on a breath-by-breath basis by integrating the output of the pneumotachograph using a digital data acquisition system. Arterial oxygen saturation (O<sub>2</sub>Sat%) is recorded at the beginning and ending of each data collection period. This monitoring is done routinely during aerosol challenges as well as during aerosol administration of standard or new therapeutic agents.
- 5) *Pulmonary Mechanics via Transfer Impedance.* Pulmonary mechanics are measured using a transfer impedance method (24). The monkey breathes spontaneously through the pneumotachograph (Fleisch no. 2) while the thorax of the monkey is vibrated using a pseudo-random noise waveform produced by two speakers mounted in the walls of the head-out plethysmograph (Pulmetrics Group, Boston, MA), and encompassing frequencies of 2 to 128 Hz. The small changes in flow produced at the mouth, along with the changes in pressure inside the plethysmograph are measured using a Microswitch transducer (model 743PC). This technique allows the monkey to breath spontaneously while pulmonary mechanics measurements are made at 4 second intervals. Transfer impedance (Z<sub>tr</sub>) is calculated as the ratio of the Fourier transform of pressure inside the box versus the Fourier transform of airway flow at each frequency. Impedance data is fit to the six-element model of the respiratory tract proposed by DuBois et al (J. Appl. Physiol. 8:587-594, 1956) using a gradient optimization technique. This six-element model includes central airway resistance (Raw), airway inertance (I<sub>aw</sub>), alveolar gas compressibility (C<sub>g</sub>), tissue resistance (R<sub>ti</sub>), tissue inertance (I<sub>ti</sub>), and tissue conductance (C<sub>ti</sub>).

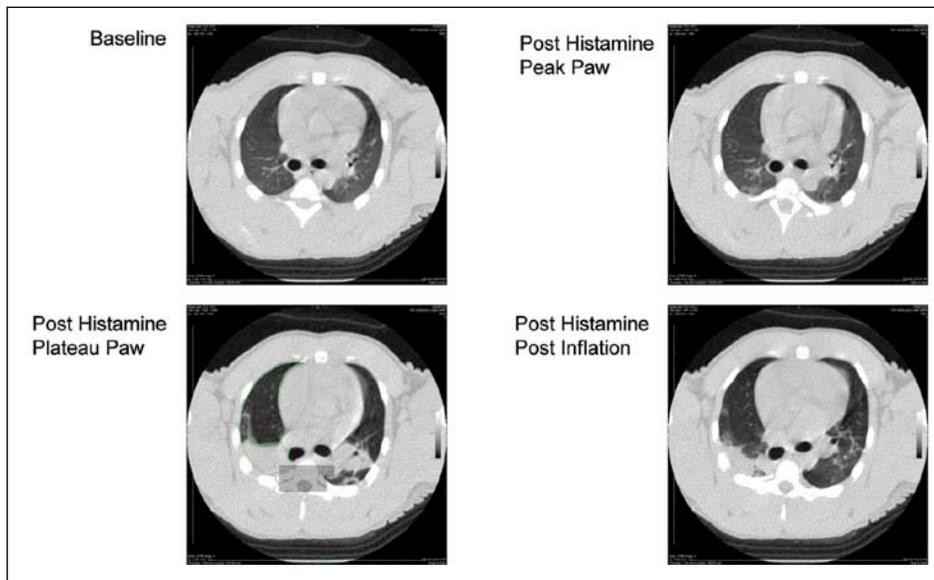
Since only five elements of the six elements of the model can be uniquely estimated using this technique, we restrict C<sub>g</sub> to a constant value. The value of C<sub>g</sub> is determined using a published regression equation relating functional residual capacity (FRC) and body weight for rhesus monkeys.

**3.3. Pulmonary function lab** *(continued)*

6) *Monitoring Respiratory Function via Telemetry.* Using standard surgical techniques, the CNPRC veterinarians implant radio-telemetry transmitters into the abdominal cavity of monkeys identified for study. Indices of breathing effort and indirect changes in airway caliber are determined by measuring 1) intrapleural pressure utilizing a pressure transducer placed in the subserosal layer of the esophagus, 2) changes in thoracic volume assessed by a pressure sensor placed across the rib cage, and 3) diaphragmatic EMG activity to determine changes in breathing effort. The data from these sensors allow us to calculate breathing frequency, tidal volume, and compliance in non-tethered conscious animals.

7) *New techniques.* We plan on developing new techniques for evaluating pulmonary function in the rhesus monkey—pulmonary transfer impedance and multiple gas diffusing capacity. Pulmonary transfer impedance will be a refined measure of the respiratory system transfer impedance we currently measure, and should provide an assessment of changes in pulmonary tissue resistance and compliance that is not available using the present technique. The development of a multiple gas diffusing capacity measurement increase our capability of evaluating the effect that altered lung development and pulmonary pathologies have on gas exchange and at what level these changes occur.

**Figure 1: Utilizing CT for measurement of Atelectasis Volume**







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