



**2005 US Research Forum  
on  
Tobacco Science and Health**

*“Biomarkers of Harm, Tobacco Toxicity,  
and  
Emerging Cancer Patterns and Etiology”*

**February 17-18, 2005**

**Renaissance Hotel, Airport  
St. Louis, MO**

# Conference Agenda

## Day 1 (February 17, 2005)

- 8:00 Check-in (Grand Concourse Ballroom D)
- 8:30 Welcome (Grand Concourse Ballroom D)  
Forum Chairperson
- 8:45 Keynote Presentation I  
Stephen Rennard (University of Nebraska Medical Center, Omaha)  
*"Biomarkers of Harm"*
- 9:40 Platform Presentations (Completed IFSH-funded Research Projects from RFA 2002-A)
- David White  
*"Feasibility of Breath Condensate Lipids and Eicosanoids as Non-invasively Collected Biomarker Predictors of Pulmonary Pathology"*
  - Wolfgang Zwicklenpflug  
*"Metabolism of N'-Nitrosonornicotine (NNN) in Rats: Interaction with Nicotinoids and Other Tobacco Constituents"*
  - Oliver Schmitz  
*"CE-LIF Analysis of Endogenous Damage in Mitochondrial and Genomic DNA"*
  - Gerhard Scherer  
*"Influence of Charcoal Filters of Cigarettes on the SH-Reactivity of Smoke and the Urinary Excretion of Thioethers and Mercapturic Acids in Smokers"*
- Each presentation will be allotted 15 minutes (approximately 10 min. to summarize research results and 5 min. for questions).*
- 10:45 Break
- 11:15 Poster Session I (11:15 - 12:45, Lambert Rooms C & D)  
*Open time to view posters. Participants competing for the Dietrich Hoffmann Career Development Award **must** be available to present their poster to the Forum attendees.*
- 12:45 Lunch (Grand Concourse Ballroom C)
- 1:45 Keynote Presentation II (Grand Concourse Ballroom D)  
Karam El-Bayoumy (Penn State Univ. School of Medicine, Cancer Center)  
*"Nutrition and Tobacco-Related Cancer"*
- 2:40 Introduction to the *Dietrich Hoffmann Career Development Award Competition*  
*Sponsored by the Institute For Science And Health*
- 2:45 Platform Session I (DH Career Development Award Competition)  
*Each presentation will be allotted 20 minutes (appx. 15 min. to summarize research results and 5 min. for questions).*
- 3:30 Break

4:00 Platform Session II (DH Career Development Award Competition)

*Each presentation will be allotted 20 minutes (appx. 15 min. to summarize research results and 5 min. for questions).*

5:00 Cocktail Reception (Penthouse Ballrooms A & B, Penthouse Level, 12<sup>th</sup> floor)

## Day 2 (February 18, 2005)

8:30 Keynote Presentation III (Grand Concourse Ballroom D)

Leslie Butler (Univ. of California - Davis)

*"Tobacco and Dietary Sources of Carcinogen Exposure and Risk of Colon Cancer"*

9:30 Keynote Presentation IV

Xifeng Wu (MD Anderson Cancer Center, Univ. of Texas)

*"Tobacco Carcinogenesis: A Pathway-based Approach"*

10:30 Break

11:00 Poster Session II (11:00 – 12:30, Lambert Rooms C & D)

*Open time to view posters. Participants other than those competing for the Dietrich Hoffmann Career Development Award should be available to present their poster to the Forum attendees.*

12:30 Lunch (Grand Concourse Ballroom C)

1:30 *Dietrich Hoffmann Career Development Award* Presentation (Grand Concourse Ballroom D)

2:00 Concluding Remarks (Forum Chairperson)

2:30 Adjourn

# ***Abstracts***

## **Keynote Speakers**

### **BIOMARKERS OF HARM**

Stephen I. Rennard

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Cigarette smoke contributes to the pathogenesis of many diseases. It contributes to morbidity and mortality through adverse effects on several organ systems. Epidemiologic studies have demonstrated the benefits of smoking cessation. Since cigarette smoke shows a “dose response” effect, harm reduction strategies that reduce smoke-derived toxin exposure are also plausible means to reduce the disease burden among continuing smokers. Demonstration of benefits, however, may be difficult as very large numbers of individuals followed for long periods of time are likely to be required to monitor effects on disease. Biomarkers offer an alternate strategy.

Biomarkers may include biochemical, cellular or physiological measures. They can serve as measures of diverse functions including: 1) tobacco exposure; 2) biological response to tobacco smoke; 3) disease risk; and 4) surrogates for disease. Different levels of validation are required for biomarkers to serve these various functions and include not only establishing the accuracy and reproducibility of the measures, but also understanding the relationship between the measures and disease states and the relationship between changes in measures and changes in disease or disease risk. Biomarkers of exposure, including exhaled CO and cotinine are well validated and widely used. Other markers of exposure, including tobacco-specific nitrosamines, have been suggested to reflect cancer risk, as the markers are themselves carcinogens. Biomarkers reflecting either biological response or disease risk have also been assessed to varying degrees of validation in relation to cardiovascular disease, pulmonary disease, fetal disease and malignancy. The wide variety of biomarkers currently under investigation reflects both the myriad effects by which tobacco smoke can cause disease and the interests of investigators pursuing conducting research in this area. As these biomarkers become progressively better validated, their application holds great potential for the assessment of harm reduction strategies to lessen the burden of tobacco-smoke caused disease.

## **TOBACCO- AND NUTRITION-RELATED CANCER**

Karam El-Bayoumy

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In the US, nearly two thirds of all cancer deaths can be linked to tobacco and diet. In 1950, the dose response relationship between cigarettes smoked and risk for lung cancer was already established. Laboratory studies with tobacco tar applied on mouse skin and cigarette smoke inhalation experiments with hamsters provided further evidence for this relationship. Cigarette smoking is also causally associated with cancer of the larynx, oral cavity, esophagus, pancreas, renal pelvis and urinary bladder, and it is linked to cancer of the cervix. More recent studies suggest that cigarette smoking could also be linked to colon, breast and prostate cancers. Cigarette smoking among adults in North America and Western Europe has been significantly reduced. Unfortunately, significant increases of cigarette consumption have been observed among adolescents in these countries. An attempt to reduce mortality rates from cancer among cigarette smokers by changing the tobacco composition and make-up of cigarettes was unsuccessful, primarily because of a major increase in smoking intensity and depth of inhalation by the habitual smokers. Clearly, from a public health standpoint, the only harmless cigarette is the one that is not smoked. Thus, smoking control efforts, including the less toxic and carcinogenic cigarettes, need to be intensified. While quitting the smoking habit is considered the surest way to reduce tobacco-related cancers, and help to block the lung cancer epidemic, dietary and/or chemoprevention may offer a plausible alternative for those unable to quit. Moreover, in ex-smokers, chemopreventive agents with dietary modulation may have the potential to reduce the lingering lung cancer risk that remains high for 5-10 years after cessation of smoking and which never reaches the very low level of risk of a never-smoker. However, chemoprevention should never be considered a substitute for primary prevention efforts, but rather a complementary approach. Therefore, we have set up model studies on lung cancer induction and prevention in laboratory animals. The carcinogen in this case is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a nicotine-derived nitrosamine present in tobacco and tobacco smoke; NNK is strongly implicated in the pathogenesis of tobacco-related lung cancer in humans. Epidemiological studies, though these are ambiguous at times, suggest that increased risk for certain human diseases, including cancer is related to insufficient intake of the micronutrient, selenium. In preclinical investigations, we have consistently shown that selenium, in the form of 1,4-phenylenebis(methylene)-selenocyanate (p-XSC), but not selenium-enriched yeast or selenomethionine, inhibits NNK-induced lung tumors in the A/J mouse model. Literature data have shown that chemopreventive agents, although effective against individual tobacco carcinogens, were not effective against lung tumors induced by whole cigarette smoke. Although supplementation of selenium-enriched yeast to humans appears to provide some protection against lung cancer, clinical chemoprevention trials of lung cancer, in general, have been disappointing. Therefore, there is an urgent need to define the true molecular targets, better understand lung cancer biology, and to develop realistic animal models in which candidate chemopreventive agents, as well as therapeutics, could be evaluated prior to the initiation of long-term and costly clinical chemoprevention trials. Studies in our laboratories are aimed toward the above-mentioned goals. In a preliminary investigation we showed that p-XSC inhibits cell growth in vitro using non-small cell lung cancer (NSCLC), which is by far the most common type, occurring in about 80% of all lung cancer cases. To understand the mechanisms responsible for the inhibitory effect of p-XSC, we examined key molecular markers that are known to be critical in the development of NSCLC using Western blot and cDNA microarray approaches, followed by RT-PCR analysis. The results demonstrate that p-XSC is capable of inhibiting several molecular targets that can account for cell growth inhibition and suggest that p-XSC could be a promising candidate for chemoprevention of NSCLC.

# **TOBACCO AND DIETARY SOURCES OF CARCINOGEN EXPOSURE AND RISK OF COLON CANCER**

Lesley Butler

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This presentation will begin with a broad examination of the patterns of cancer incidence in relation to tobacco use trends in California, using data from the Behavioral Risk Factor Surveillance System and the California Cancer Registry. California was the first state to implement a comprehensive tobacco control program, and to date, the program has remained the largest of its kind in the world. The program, which began in 1988 with the passing of the California Tobacco Tax and Health Promotion Act has had striking results. In general, tobacco-related diseases have been declining faster in California than any other U.S. state, and more specifically, lung cancer rates in California declined at three times the rate of decline in non-California Surveillance, Epidemiology, and End Results (SEER) regions from 1988-2001.

The presentation will follow with a focus on diet and non-dietary sources of carcinogen exposure and differences in genetic susceptibility to these exposures in relation to risk of colon cancer. Cigarette smoke is a source of mutagens and potential human carcinogens, such as nitrosamines, polycyclic aromatic hydrocarbons (PAHs), and heterocyclic amines (HCAs) that are introduced from the lungs into the bloodstream. Exposure to these compounds may contribute to carcinogenesis in organs that are not in direct contact with cigarette smoke, such as the kidney, liver, bladder, and possibly the breast and colon. In general, epidemiologic studies of adenomatous polyps, or colorectal cancer precursors have consistently reported modest to strong associations with cigarette smoking by dose, duration, and age at starting to smoke. Such convincing evidence for a known precursor suggests that smoking might also play a role in the etiology of colorectal carcinoma, however, epidemiologic studies of cigarette smoking and colorectal cancer have not been consistent. This paradox may be due to the misclassification of exposure and susceptibility by neglecting the heterogeneity of individual metabolic genotype/phenotype.

Although cigarette smoke is the major non-dietary source of benzo[a]pyrene (BaP) exposure, both PAHs and HCAs are also formed during the cooking of meat. Consumption of pan-fried, grilled or barbecued well-done meat are surrogates for HCA and PAH exposure, and may be positively associated with colon cancer. In a population-based, case-control study of African-Americans and whites in North Carolina, we examined the association between colon cancer, smoking and meat intake categorized by level of doneness, cooking method, and estimated meat levels of HCAs, BaP, and mutagenicity. We also examined joint effects between these exposures and genetic susceptibility at the following polymorphic loci involved in the metabolic pathway of these compounds: N-acetyltransferase (NAT) 1 and 2, microsomal epoxide hydrolase (mEH), UDP-glucuronosyltransferases (UGTs).

## **TOBACCO CARCINOGENESIS: A PATHWAY-BASED APPROACH**

Xifeng Wu

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Using examples from various ongoing studies, I will explain our pathway-based molecular epidemiologic approach for studying the role of genetic variations in genes involved in three major DNA repair pathways (NER, BER, and DSB repair) and cell cycle control in tobacco carcinogenesis. In addition, I will discuss a series of phenotypic-based assays we are applying to study DNA damage and repair, latent genetic instability, cell cycle checkpoints, and telomere length. Phenotypic assays are crucial because even though we use a pathway-based approach, we still cannot identify all the SNPs and genes that may modify these cellular events; there are still many unknown genes, unidentified polymorphisms, and functionally unclear polymorphisms. Phenotypic assays can bypass these limitations and measure the summary results of genetic variations. Finally, I will discuss the application of these assays in pharmacogenetics, an emerging discipline that attempts to predict treatment response based on inherited genetic variants.

## **IFSH-Funded Researchers (Oral platform presentations)**

### **FEASIBILITY OF BREATH CONDENSATE LIPIDS & EICOSANOIDS AS NON-INVASIVELY COLLECTED BIOMARKER PREDICTORS OF PULMONARY PATHOBIOLOGY**

David C. White<sup>1</sup>, Sung Chan Jo<sup>1</sup>, James M. Cantu<sup>1</sup>, Edilberto Bermudez<sup>2</sup>, and Owen R. Moss<sup>2</sup>

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Breath condensates (BC) collected non-invasively from healthy smoking and age-matched non-smoking volunteers are being used to explore patterns of phosphatidylcholine (PC) derived lipids, eicosanoids and cytokine regulatory biomarkers. BC are collected, within an hour following cigarette smoking, by breathing ambient air through a disposable mask with a one-way valve into a counter current collection device cooled to 0°C for 15–20 minutes or using ECoScreen<sup>®</sup>, a non-invasive breath collecting system. The lipids are recovered from the BC with solid phase extraction and the PC-based lipids separated by high performance liquid chromatography (HPLC). Phosphocholine containing lipids are identified as progenitors of positive product ions at m/z 158 with tandem mass spectrometry. Sensitivity is pg/ml BC. To achieve this sensitivity for eicosanoid fatty acids a new derivatization procedure to increase electrospray ionization efficiency was required. Eicosanoids are derivatized after activation with 1,1'-Carbonyldiimidazole, to form an eicosanoid-iImidazolide that is reacted with 2-hydroxymethylpyridine to form the picolinyl-eicosanoid that is then methylated with methyl iodide to form the positively charged N-methylpicolinium-eicosanoid derivatives. These derivatives are separated by HPLC and detected after electrospray ionization with tandem mass spectrometry as the progenitor ions generating a neutral loss at m/z 107 (methyl-pyridinium). Cytokines are detected from BC concentrated with Amicon filters utilizing dyed microspheres conjugated with monoclonal antibody specific for each target cytokine in a multiplex assay (BioPlex<sup>®</sup>). Antibody-conjugated beads react with the concentrated breath condensate sample and a secondary, detection, antibody in a microtiter well to form a capture immunoassay read in an array reader. Thus far breath condensate has been collected from 9 female (av. age 28), and 11 male smokers (av. age 26) and matched to 9 female (av. age 26) and 12 male (av. age 25) with an approved IRB protocol. Preliminary studies indicate no diacyl PC surfactant lipids are present. Yet to be identified eicosanoids are detectable. Immune detection of cytokines IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17; G-CSF, and GM-CSF, IFN- $\gamma$ , MCP-1(MCAF), MIP-1 $\beta$ , TNF- $\alpha$  will be initiated once the 50 samples are collected. We expect, as with asthma, that there are individuals whose lungs are hypersensitive to the stress of smoking, which should be reflected in the biomarker profile. Initial targets are: platelet activation factors and prostaglandins (PGE<sub>2</sub>, PGD<sub>2</sub>, PCF<sub>2 $\alpha$</sub> ), thromboxane (TxB<sub>2</sub>) and prostacyclins (6-keto PGF<sub>1 $\alpha$</sub> ), that show differential responses to inflammation, leukotriene (LTB<sub>4</sub>) that responds to allergy, and isoprostane (8-iso PGF<sub>2 $\alpha$</sub> ) that responds to free radical oxidative stress. These targets in conjunction with the cytokine panel provide a non-invasive means to monitor inflammation of the lung. In this feasibility study we will correlate the inflammatory responses of these healthy, smoking subjects to non-smoking subjects as a baseline demonstration of feasibility. We hope to find collaborators for a more in-depth correlation in future studies with the Institute for Science and Health, particularly directed to testing Potential Reduced Exposure Products if feasibility of BC biomarkers proves successful.

## **METABOLISM OF *N'*-NITROSONORNICOTINE (NNN) IN RATS: INTERACTION WITH NICOTINOIDS AND OTHER TOBACCO CONSTITUENTS**

Wolfgang Zwickenpflug

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*(also presented at Poster Board # 6)*

In contrast to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), no data exist on interactions between *N'*-nitrosonornicotine (NNN) and other tobacco constituents. Therefore, in acute experiments groups of male F344 rats have been treated with <sup>3</sup>H-NNN alone (30 nmol/kg bw) or in combination with either nicotine (15 μmol/kg bw), cotinine (150 μmol/kg bw), β-phenethylisothiocyanate (PEITC, 1 μmol/g diet), nornicotine, anatabine, anabasine (15 μmol/kg bw each), myosmine (3 μmol/kg bw), *N'*-nitrosoanatabine or *N'*-nitrosoanabasine (300 nmol/kg bw each). Variations in metabolic pattern have been studied by quantification of the urinary metabolites 4-hydroxy-4-(3-pyridyl)butanoic acid (hydroxy acid), 4-oxo-4-(3-pyridyl)butanoic acid (keto acid), NNN-*N*-oxide, 4-(3-pyridyl)butane-1,4-diol (diol) and nornicotinine besides unmetabolised NNN via radio-HPLC. After acute co-administration of nicotine, cotinine, PEITC and nornicotine levels of keto acid were significantly decreased (32% to 27-29%) in favor of hydroxy acid (44% to 48-50%). In subacute experiments (28 days) rats received 1st NNN alone (30 nmol/kg bw, last day <sup>3</sup>H-labelled) or in combination with myosmine (3 μmol/kg bw) or nornicotine (15 μmol/kg bw) and 2nd myosmine alone (3 μmol/kg bw, last day <sup>3</sup>H-labelled) or in combination with NNN (30 nmol/kg bw). Co-administration of NNN with nornicotine or myosmine resulted in a significant rise of keto acid (32% to 33-34%). In urine of myosmine control rats, 3-pyridylacetic acid (16%), keto acid (58%), 3-pyridylmethanol (7%), 3'-hydroxymyosmine (2%) and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, 4%) have been identified. Levels of keto acid in myosmine metabolism were significantly reduced (58% to 50%) after 28 days co-administration of NNN.

In summary, acute co-treatment of rats with nicotine, cotinine and nornicotine significantly reduced 2'-hydroxylation of NNN, the only metabolic pathway currently known to induce DNA damage in rodent tissues, in favor of 5'-hydroxylation. Only little effects on the NNN metabolism resulted after subacute co-administration of nornicotine or myosmine, whereas co-treatment with NNN caused a remarkable decrease of keto acid formation from myosmine.

## **CE-LIF ANALYSIS OF ENDOGENOUS DAMAGE IN MITOCHONDRIAL AND GENOMIC DNA**

Oliver J. Schmitz

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Reactive oxygen molecules are formed in vivo as by-products of normal aerobic metabolism. All organisms dependent on oxygen are inevitably exposed to these species so that DNA damage can occur, in both genomic and mitochondrial DNA (mtDNA). In order to determine endogenous DNA damage we have developed an analytical method that involves the isolation and hydrolysis of genomic DNA or mtDNA, the labeling of modified and unmodified nucleotides and micellar electrokinetic chromatography with laser-induced-fluorescence detection. With this method we have found etheno-adenine, thymine glycol, uracil, hypoxanthine and 5-methylcytosine. These were identified by addition of internal standards to the genomic or mitochondrial DNA. In the DNA of a patient with chronic

lymphocytic leukemia (CLL) high levels of etheno-adenine and uracil were found, which can perhaps be explained by the influence of chemo-therapy.

## **INFLUENCE OF CHARCOAL FILTERS OF CIGARETTES ON A) THE SH-REACTIVITY OF SMOKE AND B) THE URINARY EXCRETION OF THIOETHERS AND MERCAPTURIC ACIDS IN SMOKERS**

Gerhard Scherer

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In this project, we developed a simple, cell-free assay, which detects the SH-reactivity of the gas phase, particulate phase and whole mainstream smoke of cigarettes. We compared the SH-reactivity of 25 commercial cigarette brands. Charcoal (CC) filter tipped cigarettes showed generally lower SH-reactivities compared to cellulose acetate (CA) filter tipped cigarettes with similar tar yields. The differences between CC and CA filter tipped cigarettes were most pronounced for the gas phase. Four pairs of CA/CC filter tipped cigarettes with similar tar and nicotine yields but significantly different SH-reactivities of the gas phase were selected for application in a brand-switching study with 39 human smokers. Twenty subjects smoked CA filter tipped cigarettes during the first week of the study, the remaining 19 subjects smoked CC filter tipped during the first week. In the second week, the subjects switched to the corresponding brand with the other filter type. Saliva samples were collected on each of the 14 study days, 24-h-urine samples were collected on Day 3, 6, 10 and 13. Smoking behavior, as determined by the daily cigarette consumption and butt length, did not significantly change when switching to the cigarettes with the other filter type. The same was true for the carbon monoxide level in exhaled air, salivary cotinine and trans-3'-hydroxycotinine as well as the molar sum of the six major nicotine metabolites in urine. Urinary excretion rates of 3-hydroxypropyl-mercapturic acid (metabolite of acrolein), 3-hydroxy-1-methylpropyl-mercapturic acid (metabolite of crotonaldehyde), monohydroxybutenyl-mercapturic acid (metabolite of 1,3-butadiene) and S-phenyl-mercapturic acid (metabolite of benzene) were significantly lower when smoking CC compared to CA filter tipped filter cigarettes. Other mercapturic acids and thioethers (a biomarker indicating the exposure to electrophilic compounds) were not or only slightly reduced upon smoking CC filter tipped cigarettes.

## Poster Presentations

Poster Board # 1

### **HUMAN LUNG CELLS CULTURED UNDER DRY/WET CONDITIONS**

Ekkehard Stehfest

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The lung represents not only the entrance organ for gases and airborne particles but also partly works as a filter. Therefore, it is the first organ, which is exposed to possible harmful substances on the respiratory way. That makes the lung one of the most prominent organs for investigations of possible damage and also detoxification mechanisms caused by cigarette smoke.

An appropriate system to investigate such mechanisms is human lung cell models. In our institute we work on well-defined human bronchial epithelial and peripheral lung cell cultures, which are developed from resects obtained from lung surgery. As lung cells are an important component of the blood-air barrier with two surfaces, it is necessary to overcome the submerge situation by a different culture system.

We are able to establish a cell culture system which allows the cultivation of lung cells under more physiological conditions. Insert dishes with a semi-permeable membrane provide a culture system with a wet (medium) and a dry (air) surface. These cells are characterized by immunohistochemistry and have a different morphology compared to submerge cultures. Peripheral lung cells in dry/wet cultures seem to be organized in alveolar-like structures which are very similar to lung cut slides from the organ. Investigations by electron microscopy show that bronchial epithelial cells start to redevelop cilia again under dry/wet conditions.

Eventually, differences on the protein level can also be found in subject to the culture conditions. By confocal laser scan technology we can show the distinct localization of multi drug resistance related proteins (MRPs) in both culture systems.

Due to their dry surface, lung cells cultured under dry/wet conditions provide an ideal model for investigations of the effects of airborne substances on the human lung, such as caused by cigarette smoke.

Poster Board # 2

### **TOBACCO SMOKE-INDUCED INFLAMMATION AND CYCLOOXYGENASE-2 INDUCTION IN THE LUNGS OF RATS**

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Cigarette smoking is associated with pulmonary inflammation, epithelial damage, and remodeling of the airways resulting in chronic obstructive pulmonary disease (COPD). Chronic airway inflammation plays a key role in the development and progression of tobacco smoke-induced lung disease. However, the molecular mechanisms responsible for changes leading to COPD are as yet undetermined. We have previously demonstrated exposure of spontaneously hypertensive (SH) rats to tobacco smoke results in a striking degree of early pulmonary inflammation persisting throughout multiple weeks of exposure. Exuberant mucous cell hyperplasia is also present. Continued exposure results in further epithelial airway hyperplasia, and some alveolar distension within centriacinar sites of

the lung parenchyma, suggestive of centrilobular emphysema. These features are typical of changes in the lungs of human smokers with COPD. Reactive oxygen species, present in tobacco smoke, are known to activate transcription factors, resulting in the synthesis of inflammatory mediators which play a critical role in the initiation and progression of inflammation. We have previously demonstrated that repeated exposure of SH rats to tobacco smoke is associated with upregulation of the inflammatory mediator cyclooxygenase-2 (COX-2) in the lungs. Use of the SH rat in a model of smoke-induced pulmonary changes affords us the unique opportunity to elucidate more precisely those molecular, cellular, and pathological processes involved in smoke-induced lung disease. During this one-year pilot study we will test the hypothesis that reactive oxygen species in tobacco smoke induce overexpression of COX-2 leading to elevated inflammation and remodeling of the airway epithelium. We hypothesize that these events can be blocked by antioxidants or inhibitors specific for COX-2. Changes will be measured using histopathologic, morphometric, and biochemical techniques.

*Poster Board # 3*

## **PRENATAL EXPOSURE TO CIGARETTE SMOKE AND CHRONIC AIRWAY DISEASE IN THE OFFSPRING: TOXIC IMPACT OF THE PARTICULATE AND VAPOR PHASE**

Judith T. Zelikoff

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While the effects of gestational exposure to cigarette smoke (CS) on childhood asthma is being debated, accumulating epidemiologic and toxicologic data indicated that smoking during pregnancy increases the risk of airway hyperresponsiveness/respiratory impairment in the offspring. Unfortunately, no data are available to provide insight into the particular smoke constituent(s) or possible mechanism(s) by which these effects may occur. Thus, a toxicological study was proposed to test the hypothesis that inhalation exposure to mainstream CS - more specifically, its particulate phase - by pregnant mice influences airway reactivity in the offspring, and that these effects are gender-related and persistent. Using CD1 mice exposed daily (4 hr/d, 7 d/wk from gestational d 3 to birth) to either CS minus the particulate phase or intact CS, the following objectives were proposed to: assess the degree to which exposure of pregnant mice to each CS atmosphere, at a realistic concentration, modulates airway reactivity in normal, unsensitized juvenile and adult male and female offspring; and, determine the effects of such exposures on the inducibility of allergen responses in cockroach antigen-sensitized offspring in response to specific bronchoprovocation stimuli. For each of the above aims, a dedicated subset of offspring will be used to ascertain whether biological parameters important in mediating airway reactivity (lavage cell profiles, levels of endogenous biological mediators, antigen-specific serum antibodies and, lung tissue-associated eosinophil and mast cell number) might be modified so as to determine more specifically some of the mechanisms by which CS (especially CS-associated particulates) might produce the observed outcomes.

## **TRANSPOSABLE ELEMENTS: A POSSIBLE LINK BETWEEN TOBACCO-PRODUCT-INDUCED TOXINS AND CARCINOGENESIS?**

Wolfgang D. Schmitt

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Today, we all are exposed to environmental poisons, which are produced by industry, traffic and consumers. Especially the usage of tobacco-products is not only often unpleasant for the individual environment, but also an important source for suspect compounds, that may lead to cancer of various organs, like the lung, larynx or esophagus.

Despite this overwhelming knowledge about the final effects of smoking, much less is known about the precise disease-inducing effects of the suspected compounds. At this point, it should be mentioned that this is also true for a remarkable number of well established carcinogens. Especially the classical theory of cancer development, the somatic mutation hypothesis, can hardly explain the carcinogenic effect of all these compounds, since a substantial number of them are no mutagens.

Here we present a different theory of carcinogenesis. We propose that cancer initiation might be the result of activated retrotransposons which are induced by chronic cellular stress. Retrotransposons belong to the family of transposable elements, which contribute to almost half of our genome. Among them, only the LINE1 subgroup is able to transpose independently, whereas Alu elements are dependent on the enzyme machinery of LINE1-elements.

But activity of retrotransposons does not only lead to relocation of these elements themselves, it also influences gene expression and induces large-scale rearrangements. The summative effects may result in genetic instability and complex abnormalities in gene expression as observed in cancers. Finally, we present some possibilities to detect LINE1-activity to investigate our theory in the future.

## **TRAPPING GENES ASSOCIATED WITH DEVELOPMENTAL EXPOSURE TO TOBACCO SMOKE**

William H. Hanneman, Marie E. Legare and Wen Li

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The use of tobacco products is known to increase an individual's risk for disease. The Surgeon General of the United States has succinctly summarized that risk: "Today, more than one of every six American deaths is the result of cigarette smoking. Smoking is responsible for an estimated 40 percent of all cancer deaths, including 87 percent of lung cancer, the leading cause of cancer mortality; 21 percent of deaths from coronary heart disease; 18 percent of stroke deaths; and 82 percent of deaths from chronic obstructive pulmonary disease." Smoking not only has an impact on the health of the smoker, but also on those indirectly exposed to tobacco smoke, specifically, the developing fetus of smoking mothers.

While there is sufficient data that reports the negative effects of smoking in mature adults there is little to no data that reports the effects of tobacco smoke on fetal gene expression. One of primary reasons for this lack of knowledge is that defining the effects of tobacco smoke on developmental gene expression is tantamount to finding the proverbial "needle in a haystack". We have effectively overcome this problem through the molecular tagging of the "needle". The strategy we have adopted is one of gene trapping mouse embryonic stem (ES) cells. Briefly, ES cells have been transfected with

a promoterless reporter gene construct containing *LacZ*. Successful transfection of a cell with this construct will “knockout” the endogenous gene, and the reporter gene (*LacZ*) will be active and under the control of the endogenous genes promoter. The reporter gene itself is then used as the starting point for sequencing of the endogenous genes. Genetic sequences from the “trapped” genes are compared to those in the GenBank database to identify novel genes as well as previously known genes that are dysregulated by tobacco smoke. In addition, mutant embryonic stem cells can be further utilized to generate knockout mice.

As our data will show to date, we have trapped a large number of unique ES cell clones that have been characterized for their responsiveness to cigarette smoke condensate (CSC). Moreover, we have generated knockout mice based on trapped ES cell clones which we are presently analyzing.

Poster Board # 6

## **METABOLISM OF N'-NITROSONORNICOTINE (NNN) IN RATS: INTERACTION WITH NICOTINOIDS AND OTHER TOBACCO CONSTITUENTS**

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In contrast to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), no data exist on interactions between *N*-nitrosornicotine (NNN) and other tobacco constituents. Therefore, in acute experiments groups of male F344 rats have been treated with <sup>3</sup>H-NNN alone (30 nmol/kg bw) or in combination with either nicotine (15 μmol/kg bw), cotinine (150 μmol/kg bw), β-phenethylisothiocyanate (PEITC, 1 μmol/g diet), nornicotine, anatabine, anabasine (15 μmol/kg bw each), myosmine (3 μmol/kg bw), *N*'-nitrosoanatabine or *N*'-nitrosoanabasine (300 nmol/kg bw each). Variations in metabolic pattern have been studied by quantification of the urinary metabolites 4-hydroxy-4-(3-pyridyl)butanoic acid (hydroxy acid), 4-oxo-4-(3-pyridyl)butanoic acid (keto acid), NNN-*N*-oxide, 4-(3-pyridyl)butane-1,4-diol (diol) and norcotinine besides unmetabolised NNN via radio-HPLC. After acute co-administration of nicotine, cotinine, PEITC and nornicotine levels of keto acid were significantly decreased (32% to 27-29%) in favor of hydroxy acid (44% to 48-50%). In subacute experiments (28 days) rats received 1st NNN alone (30 nmol/kg bw, last day <sup>3</sup>H-labelled) or in combination with myosmine (3 μmol/kg bw) or nornicotine (15 μmol/kg bw) and 2nd myosmine alone (3 μmol/kg bw, last day <sup>3</sup>H-labelled) or in combination with NNN (30 nmol/kg bw). Co-administration of NNN with nornicotine or myosmine resulted in a significant rise of keto acid (32% to 33-34%). In urine of myosmine control rats, 3-pyridylacetic acid (16%), keto acid (58%), 3-pyridylmethanol (7%), 3'-hydroxymyosmine (2%) and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, 4%) have been identified. Levels of keto acid in myosmine metabolism were significantly reduced (58% to 50%) after 28 days co-administration of NNN.

In summary, acute co-treatment of rats with nicotine, cotinine and nornicotine significantly reduced 2'-hydroxylation of NNN, the only metabolic pathway currently known to induce DNA damage in rodent tissues, in favor of 5'-hydroxylation. Only little effects on the NNN metabolism resulted after subacute co-administration of nornicotine or myosmine, whereas co-treatment with NNN caused a remarkable decrease of keto acid formation from myosmine.

## BLACK RASPBERRIES AND CANCER CHEMOPREVENTION: BIOLOGIC MECHANISMS AND PRACTICAL IMPLICATIONS

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Our research group has been determining the molecular events involved in the development of esophageal squamous cell carcinoma, a tobacco-related cancer, and identifying chemopreventive agents for this disease. One of the most prevalent molecular changes induced by the nitrosamine carcinogen, *N*-nitrosomethylbenzylamine (NMBA), is mutational activation of the *Ha-ras* oncogene, where virtually all tumors contain *Ha-ras* transition mutations. Activation of *ras* genes results in the induction of multiple transcription factors including C-Jun, a member of the activated protein-1 (AP-1) family. Inducible nitric oxide synthase (iNOS), which has an AP-1 binding site, is overexpressed after treatment of rat with NMBA. In the present study, we evaluated the chemopreventive potential of black raspberries (BRB) and determined whether berries influence the expression of C-Jun. F-344 rats were given s. c. injections of NMBA (0.25 mg/kg body weight) three times per week for 5 weeks. One week later, they were fed a synthetic diet containing 5% freeze-dried BRB until the end of the bioassay (25 weeks). At weeks 9 and 15, 5 rats from each group and at week 25, all remaining rats were sacrificed. Esophagi were collected and evaluated for expression of C-Jun by Real-Time RT-PCR, immunohistochemistry and Western Blot analysis. BRB reduced the incidence of esophageal cancer and tumor multiplicity and significantly reduced the expression of C-Jun mRNA in esophagi treated with BRB+NMBA versus NMBA only. Immunohistochemistry and Western blot analysis revealed a similar reduction in C-Jun protein. These results indicate that the chemopreventive effect of BRB is associated with a reduced expression of C-Jun. Natural food products such as berries may be a useful approach to the prevention of cancer including cancer of the esophagus.

## NICOTINE DEMONSTRATES GENOTOXICITY IN HUMAN TONSILLAR TISSUE AND LYMPHOCYTES

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The addictive component of tobacco and tobacco smoke nicotine was suggested to contribute to human carcinogenesis by recent studies. The present study focuses on possible genotoxic effects in human probes.

The DNA damaging effect of this alkaloid on human lymphocytes and target cells from lymphatic tissue of the palatine tonsils from 10 healthy patients was tested with the alkaline single cell microgel electrophoresis (Comet) assay. The degree of DNA migration, a measure of possible DNA single strand breaks, alkali labile sites and incomplete excision repair sites, was expressed as the Olive tail moment and the percentage of DNA in the tail.

One hour exposure to nicotine at 0.125, 0.25, 0.5, 1, 2, 4 mM induced a statistically significant dose-dependent increase of DNA migration up to 3.8-fold and 3.2-fold in tonsillar cells and lymphocytes, respectively. The lowest concentration eliciting a significant DNA damage was 0.5 mM for tonsillar cells and 0.25 mM for lymphocytes. The genotoxic effect was confirmed in a second series of experiments using nicotine of high purity from two different suppliers. There were no significant

differences between both series excluding artifacts due to the source of nicotine. Finally, DNA damage by nicotine was compared in cells incubated in media strictly adjusted to neutral pH with non adjusted media becoming alkaline with increasing nicotine concentrations. Again no differences concerning DNA migration were observed.

The data indicate that nicotine expresses significant direct genotoxic effects in human target cells. This result warrants further investigations on the contribution of nicotine to tobacco carcinogenesis.

*Poster Board # 9*

## **IDENTIFICATION OF INTELLIGENT BIOMARKERS OF EXPOSURE AND HARM IN THE RESPIRATORY EPITHELIA TO TOBACCO SMOKE COMPONENTS**

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A novel toxicological tool, which consists of a differentiated, 3-D, in vitro model of human respiratory epithelia, (EpiAirway-100 cells; MatTek Corp., USA), will be utilized to examine the early gene response(s) following exposure to tobacco smoke components (TSC). EpiAirway-100 cells will be exposed at the air/liquid interface to representative particle and vapour phase components of cigarette smoke. The TSC selected represent an overview of the compounds found in cigarette smoke that induce thrombogenic events (nicotine), cell toxicity (cadmium) and produce reactive metabolites during xenobiotic metabolism (formaldehyde and urethane). Surrogate solutions of TSC will be tested for their capacity to up- and/or down-regulate genes in the respiratory epithelia following acute exposure, as a means to identifying intelligent biomarkers of exposure and harm. Conventional toxicological analysis will be used to establish the dose of the various TSC needed to cause changes in epithelial resistance, secreted surface proteins and release of inflammatory markers. Following establishment of the dose required to achieve these different biological endpoints, toxicogenomic investigations will be initiated. Macroarray technology will be employed to compare the patterns of mRNA expression of human genes associated with stress, simultaneously from control and TSC treated lung tissue. Major candidate genes will be classified (growth factors, inflammation, xenobiotic metabolism) and associated with the biological endpoints. It is anticipated that stringent lists of candidate genes associated with these changes will be generated, thereby providing data on the mechanisms of the biological endpoints, i.e. intelligent biomarkers. Proteomic analysis will be used to correlate candidate gene response with a specific protein. The interest will be to study the proteins involved in the early stress phase of toxicant challenge. Once protein function has been identified, this will provide insight into the mechanism of action of the responsive genes and hence, the "intelligent biomarkers" of TSC exposure in lung epithelia.

## CHARACTERIZATION AND MAPPING OF DNA DAMAGE INDUCED BY REACTIVE METABOLITES OF 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK)

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The nitrosamine NNK is an important tobacco-specific carcinogen associated with lung cancer. Its complex activation leading to methyl- and pyridyloxobutyl (POB)-modified DNA, makes DNA damage difficult to characterize and quantify. Therefore, we use the analogue NNKOAc to study and induce (POB)-modified genomic DNA, and to map the sites and frequency of adducts formation at nucleotide resolution using ligation-mediated polymerase chain reaction (LMPCR) and terminal transferase-dependent polymerase chain reactions. NNKOAc induced single-strand breaks in a concentration-dependent manner. Post-alkylation treatments with chemical or DNA repair-enzymes did not increase the level of DNA breaks in NNKOAc-treated DNA. Detection of DNA damage using LMPCR was possible only when POB-DNA was 5'-phosphorylated prior to the LMPCR procedure. NNKOAc generated damage at all four bases with the decreasing order guanines > adenines > cytosines > thymines. Analysis of damage distribution patterns, reveals a high frequency of damage in the p53 gene in codons 241 and 245 and a lower frequency of damage in codon 248. We analyzed the 3'-termini of the NNKOAc induced single-strand breaks using a post-labeling assay or a nucleotide exchange reaction at the 3'-termini. Both methods indicate that the 3'-termini of the single-strand breaks are not hydroxyl groups and are blocked by an unknown chemical structure. These data are consistent with POB-phosphotriester hydrolysis leading to strand breaks in DNA. The POB-damage could be mutagenic because NNKOAc produces single-strand breaks with the products being a 5'-hydroxyl group and a 3'-blocking group and strand breaks that could be slowly repaired. Pyridyloxobutyl adducts and/or SSB generated by NNKOAc at the sites frequently mutated in lung cancer can reflect the action of NNK as an important etiological factor and possible marker of genotoxicity of NNK exposure.

## SIZE SEGREGATED CHEMICAL COMPOSITION OF MAINSTREAM TOBACCO SMOKE AEROSOL FROM REGULAR CIGARETTES AND POTENTIALLY REDUCED EXPOSURE PRODUCTS (PREPS)

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Potentially Reduced Exposure Products (PREPs) are intended to reduce the health risks associated with cigarette smoking. These products utilize modification in tobacco processing and cigarette design for this purpose. Variations in the processing, design and use produce changes in the chemical composition of cigarette smoke and in the size of aerosol particles containing particular chemicals. The particle size distribution of mainstream smoke aerosol controls the deposition behavior of particles in the respiratory tract, and therefore, if there is a relationship between particle size and chemical composition, a simple measure of total concentration will not give an entire picture of the link between smoke properties and biological effects. Our project studies the variation in the chemical composition of mainstream smoke as a function of particle size distribution in order to compare risk

associated with regular cigarettes and PREPs. The work will utilize research cigarettes and commercial cigarettes for comparison with the PREPs. The chemical constituents of major toxicological significance in smoke include nicotine, NNK, NNN, NDEA, NDMA, B(a)P, benzo(b)fluoroanthene, benzo(k)fluoroanthene. These species, along with gaseous species like CO, NO<sub>x</sub> and CO<sub>2</sub>, will be measured in our study. Mainstream smoke generated by the cigarettes will be collected through cascade impactors to obtain size-segregated samples of aerosol. These samples will be quantitatively analyzed for the aforementioned constituents using various chromatographic techniques (GC-TEA, GCMS, HPLC) and gas analyzers. To emulate the effect of various inhalation patterns by smokers, these chemical constituents will also be analyzed from smoke generated using different puffing regimens (FTC, long, short). The results will be used to recognize chemical species whose particle size may play a role in determining aerosol behavior, e.g. deposition efficiency and location in the lung.

Poster Board # 12

## **IN VITRO AND IN VIVO ACTIVATION OF THE TOBACCO ALKALOID MYOSMINE**

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For a long time myosmine was considered to be one of the minor tobacco alkaloids without significant relevance. Identification of myosmine in various foods has disproven its limitation to tobacco products. In addition, myosmine has been detected in human saliva and blood plasma in ng/ml amounts. This suggests that *in vivo* myosmine levels are even higher than extrapolated from dietary myosmine uptake based on currently identified sources. Therefore, research on myosmine metabolism and on *in vitro* as well *in vivo* activation pathways has been intensified. After oral administration to female Wistar rats 3-pyridylacetic acid, 4-oxo-4-(3-pyridyl)butanoic acid (keto acid), 3-pyridylmethanol, 3'-hydroxymyosmine and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) have been identified as urinary metabolites. The identified metabolites give rise to speculations about their metabolic formation. Myosmine is easily nitrosated in human gastric juice after addition of NaNO<sub>2</sub> yielding *N'*-nitrosornicotine (NNN) and HPB. The precursor of HPB, 4-(3-pyridyl)-4-oxobutyl diazohydroxide, releases 4-(3-pyridyl)-4-oxobutylcarbenium species for which hemoglobin and DNA adduct formation has been found. A covalent DNA binding has been shown *in vitro* for myosmine under nitrosation conditions. HPB-releasing DNA adducts were increased in different rat tissues including esophagus after p.o. administration of myosmine. HPB-releasing DNA adduct levels of human esophagus and cardia did not correlate with the individual smoking status but a strong correlation was found with increasing body mass index. Myosmine was genotoxic for human target cells in the Comet assay. Under consideration of its imine structure, peroxidation of myosmine seems to be a promising additional activation pathway. In preliminary studies using H<sub>2</sub>O<sub>2</sub> and acetic acid anhydride, besides other reaction products HPB has been identified. In conclusion, DNA damage is likely to arise from myosmine activation. Therefore, further research should focus on revelation of myosmine activation pathways.

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