



2006 Research Symposium
on
Tobacco Science and Health

*Tobacco Harm Reduction
and
Perception of Risk*

9-10 March 2006

Hotel Sacher
Vienna, Austria

Conference Agenda

- 8:00 Check-in (Salon Metternich)
- 8:30 Welcome (Marble Hall)
Forum Chairperson
- 8:45 Keynote Presentation I (Marble Hall)
Dr. Robert Nilsson
Nofer Institute of Occupational Medicine & WHO Collaborating Centre (Professor of Toxicology)
Stockholm University (Professor of Molecular Toxicology & Risk Assessment)
"De Minimus non Curat Lex – Virtual Thresholds for Cancer Initiation by Tobacco-Specific Nitrosamines: Prospects for Harm Reduction by Smokeless Tobacco"
- 9:40 Platform Presentations (IFSH-sponsored Investigators, Marble Hall)
- Dr. Judy Zelikoff
"Prenatal Exposure to Cigarette Smoke and Chronic Airway Disease in the Juvenile and Adult Offspring"
 - Dr. Heidi Foth
"PARP-1 Activity Under Metal Stress in Primary Human Lung Cells"
 - Dr. Albrecht Seidel
"Biomonitoring of Tobacco Smoke-Related Aromatic Amines in Smokers versus Non-Smokers"
 - Dr. Derek Dunn-Rankin
"Comparison between Carbon Monoxide, Nitrogen Oxides and Nicotine in the Mainstream Smoke of Research, Commercial, and PREP Cigarettes"
Each presentation will be allotted 15 minutes (approximately 10 min. to summarize research results and 5 min. for questions).
- 10:45 Break (Salons Metternich & Mayerling)
- 11:15 Poster Session I (11:15 - 12:45, Salons Metternich & Mayerling)
*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 (Salon Prawy / Karajan & Strauss)
- 1:45 Keynote Presentation II (Marble Hall)
Dr. Brad Rodu
University of Louisville (Chair, Tobacco Harm Reduction Research)
"Tobacco Harm Reduction: Smokeless Tobacco Use Can Substitute for Smoking"
- 2:40 Introduction to the *Dietrich Hoffmann Career Development Award Competition*

- 2:45 Platform Session I (DH Career Development Award Competition, Marble Hall)
 Dominique Balharry *(also presenting at Poster #5)*
 Cardiff University, School of Biosciences, Cardiff, United Kingdom
"The Use of Conventional Toxicology to Characterize the Dose Response of a Human Airway Epithelium Model to Tobacco Smoke Components"
[Presentation time = 20 minutes (15 min. for research summary and 5 min. for questions)]
- 3:10 Break (Salons Metternich & Mayerling)
- 3:30 Platform Session II (DH Career Development Award Competition, Marble Hall)
 Abdelrahman Torky *(also presenting at Poster #8)*
 Martin-Luther-University, Institute of Environmental Toxicology, Halle, Germany
"Mediators of Inflammation Modulate Multi-drug Resistance Related Proteins in Cultured Human Lung Cells"
[Presentation time = 20 minutes (15 min. for research summary and 5 min. for questions)]
- 4:30 Cocktail Reception (Salon Prawy / Karajan & Strauss)

Day 2 (10 March 2006)

- 8:30 Keynote Presentation III (Marble Hall)
 Dr. Tong Chen
 The Ohio State University
"Investigation of Inducible Nitric Oxide Synthase as a Chemopreventive Target in Carcinogen-Induced Rat Esophageal Tumorigenesis"
- 9:30 Keynote Presentation IV (Marble Hall)
 Dr. Edmund Maser
 Univ. Medical School Schleswig-Holstein (Director, Institute of Toxicology & Pharmacology for Natural Scientists)
"Significance of Reductases in the Detoxification of the Tobacco-Specific Carcinogen NNK"
- 10:30 Break (Salons Metternich & Mayerling)
- 11:00 Poster Session II (11:00 – 12:30, Salons Metternich & Mayerling)
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 (Salon Prawy / Karajan & Strauss)
- 2:00 *Dietrich Hoffmann Career Development Award* Presentation (Marble Hall)
- 2:30 Concluding Remarks (Forum Chairperson, Marble Hall)
- 2:45 Adjourn

Abstracts

Keynote Speakers

DE MINIMUS NON CURAT LEX – VIRTUAL THRESHOLDS FOR CANCER INITIATION BY TOBACCO-SPECIFIC NITROSAMINES: PROSPECTS FOR HARM REDUCTION BY SMOKELESS TOBACCO

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Whereas the impact of tobacco specific nitrosamines (TSNA) in smokers is obscured by the presence of numerous other carcinogens and promoters, in the case of smokeless tobacco virtually all of the carcinogenic potential is associated with 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) and NNN - N'-nitrosonornicotine (NNN). Exposure to impure products with extremely high TSNA concentrations, typified by Sudanese toombak, are associated with a markedly increased risk for cancers in the oral cavity and pharynx, whereas three large epidemiological studies conducted in Sweden failed to detect any significant increase in neoplastic disease in spite of the fact that more than 20% of the Swedish grown up male population consumes snuff. IARC has classified snuff, including low-nitrosamine Swedish “snus”, as carcinogenic to humans in the same category as wood dust and aflatoxins, and the failure of regulators to realize that these classifications do not consider *levels* of risk has had some unfortunate consequences for EU legislation. In line with U.S. regulatory tradition, this author has analyzed the potential risks from TSNA based on estimation of the level of risk where the mechanism of action is also taken into consideration.

Cellular DNA damage that is mis-repaired, or not repaired, constitutes a necessary, although not sufficient prerequisite for the development of malignant growth. Available evidence indicates that the strongly pro-mutagenic DNA adduct O⁶-methylguanine (O6-mGua) plays a major role for cancer induction by NNK, whereas methylation of N⁷ in guanine (N7-mGua) is assumed to be less important. For tumors in the nasal epithelium of rats and mice, 4-hydroxy-1-(3-pyridyl)-1-butyl (HPB) DNA adducts generated by NNK as well as by NNN appears to be involved, although cell proliferation secondary to toxicity seems to be required in addition. Although the data base is incomplete, there seems to be a linear dose-response relationships for O6-mGua in rodent lung Clara cells as well as for associated lung tumors down to the lowest tested dose, whereas non-linear relationships characterize rodent liver and nasal mucosa. However, when the dose response curves for adduct formation are extrapolated to values that correspond to actual uptakes of TSNA from smoking as well as snuff, the expected levels of pro-mutagenic DNA adducts are found to be insignificant.

A background of various types of DNA adducts caused by unknown dietary or endogenous sources are ubiquitously found in healthy humans without known exposures to either tobacco or alkylating agents. When exposure from NNK and NNN is so low that the number of DNA methylations or pyridyloxobutylations will not appreciably affect the background level of such DNA lesions that are normally present, this can be considered as a “virtually safe” dose threshold, irrespective of the actual shape of the dose response relationship.

Based on analyses of various tissues using sufficiently sensitive techniques, the levels of O6-mGua DNA adducts in smokers are not significantly elevated above the levels found in individuals with no known exposure to TSNA. The use of today’s Swedish snuff, where the total intake of NNK

and NNN is about 10 times lower than from smoking 20 cigarettes per day can be expected to result in an even lower impact on the adduct background. The exact nature of DNA HPB adducts is unknown, and pyridyloxobutylations evidently induce several types of DNA lesions including phosphate alkylations. This implies that only a certain fraction of all DNA adducts released as HPB are important in causing mutations. The low impact from TSNA in tobacco smoke on HPB DNA adducts as predicted by rodent studies has been verified by analysis of human biopsies of lung, esophagus and cardia, where the adduct levels in smokers were not significantly elevated above the background found in non-smokers. With a much lower exposure to TSNA in comparison to smoking, intake of currently marketed Swedish brands of snuff will be expected to impact the background levels of HPB DNA adducts to an even lesser extent. Available data demonstrate the presence of appreciable levels of HPB releasing adducts in hemoglobin as well as in DNA from non-exposed subjects, indicating that sources for HPB adducts other than tobacco are important, and where myosmine present in various food products represents a possible candidate

The best conducted of the Swedish studies where no increased risk associated with snuff was found (OR=1.0; CI, 0.7-1.6) included approximately 2 million person-years at risk. Based on rodent bioassays and historical data for the TSNA content of Swedish snuff, the linearized multi-stage model predicts an additional cancer incidence for this population in the interval 48 to 112 cases. Obviously, the rodent data overestimates human risk by an order of magnitude, and possible reasons for this discrepancy will be discussed.

The outcome of the present analysis is that smokers' exposure to NNK and NNN seems to play a minor role compared with other carcinogens and promoters present in tobacco smoke. Taking into consideration that the levels of TSNA in the currently marketed brands of Swedish "snus" have decreased by a factor of 20 during the last decades, one may further safely assume, that use of these products entail a negligible cancer risk, a conclusion that is well supported by experimental as well as epidemiological data.

It can be demonstrated that for 100 million European smokers turned "snus" users, the number of deaths in cancer, chronic obstructive pulmonary and cardiovascular diseases avoided will reach about 120,000 annually after a period of 5 to 10 years. Nicotine products such as nicotine spray or patch are not affordable for large population groups in Central and Eastern Europe. Snuff which has been successfully used for the purpose of smoking cessation offers an affordable and inexpensive alternative. The ban on low-nitrosamine oral snuff in all countries of the European Union except Sweden can be defended neither on medical nor ethical grounds.

TOBACCO HARM REDUCTION: SMOKELESS TOBACCO USE CAN SUBSTITUTE FOR SMOKING

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This presentation will discuss tobacco harm reduction, in which inveterate smokers are informed of alternative delivery systems for nicotine, a powerfully addictive drug that does not cause any of the diseases associated with smoking and may be used as safely as caffeine. A specific focus will be alternative nicotine delivery by smokeless tobacco (SLT) products, for which there is an abundant research literature regarding usage patterns and health consequences.

Oral cancer is the most frequently cited health risk from long-term SLT use, but there are many misconceptions regarding both the putative determinants and magnitude of this risk. Historical levels of tobacco-specific nitrosamines and other contaminants (such as cadmium, formaldehyde, benzo-*a*-pyrene and lead) in moist snuff, dry snuff and chewing tobacco will be compared with those in

contemporary SLT products. SLT use in Eastern societies, associated with substantially elevated risk for head and neck cancers, will be compared with use in the US and Sweden. In addition, the evidence for risks for other smoking-related cancers, cardiovascular diseases and diabetes among SLT users will be reviewed.

There is strong and unambiguous evidence that tobacco harm reduction has been successful in Sweden, where for decades men have smoked at far lower rates than those in other European countries. Data on patterns of tobacco use among adults in northern Sweden from 1986 to 2004 will be presented, and a study on tobacco use among 15-16 year old Swedish schoolchildren will provide perspectives on the impact of prevalent SLT use as a gateway to, or from, smoking.

The presentation will conclude by briefly reviewing the growing discussion of tobacco harm reduction among public health and policy experts in the US and the EU.

INVESTIGATION OF INDUCIBLE NITRIC OXIDE SYNTHASE AS A CHEMOPREVENTIVE TARGET IN CARCINOGEN-INDUCED RAT ESOPHAGEAL TUMORIGENESIS

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Esophageal cancer, a tobacco-related disease, is the third most common gastrointestinal malignancy and the sixth most frequent cause of cancer death in the world. Seventy-five percent of untreated patients with esophagus cancer die within 1 year of diagnosis and 5-year survival rates are only 5-10%. The *N*-nitrosomethylbenzylamine (NMBA)-induced rat model of esophageal cancer has been used extensively in our laboratory to investigate the mechanisms of tumor development in the esophagus and to evaluate the efficacy of potential chemopreventive agents. Nitric oxide (NO) is a single molecular and a high level of NO is synthesized from L-arginine by inducible nitric oxide synthase (iNOS). Increased NO production appears to be associated with many disorders including cancer. Therefore, as shown in many studies, iNOS plays a very important role in carcinogenesis. To investigate the association between iNOS and tumor development in rat esophagus, we conducted a bioassay in which rats were sacrificed at three-week intervals during and following expose to NMBA. Real-time PCR and immunohistochemistry assays have been used to determine mRNA and protein expression of iNOS. The results of this study suggest that overexpression of iNOS is associated with tumor development in the rat esophagus. Based on our findings, we conducted a second bioassay to evaluate a selective iNOS inhibitor, *S,S'*-1,4-phenylene-*bis*(1,2-ethanediyI)*bis*-isothiourea (PBIT), as chemopreventive agent targeting the function of iNOS. We observed a reduction in tumor incidence and multiplicity in rats fed with PBIT when compared to rats fed with regular diet only. PBIT reduced the production of NO in NMBA-induced preneoplastic and papillomatous esophageal lesions when compared with comparable lesions in rats treated with NMBA only. These observations suggest that iNOS plays a role in tumor development, and that its selective inhibitor, PBIT, significantly inhibits esophageal tumor progression presumably through reducing the production of NO.

SIGNIFICANCE OF REDUCTASES IN THE DETOXIFICATION OF THE TOBACCO-SPECIFIC CARCINOGEN NNK

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Smoking is undoubtedly linked to lung cancer, yet only a small fraction of smokers develop this disease. Identification of genetic, environmental and nutritional factors that affect lung-cancer risk might help to explain why some smokers are more likely to develop lung cancer than others. Recent data suggest that the balance between metabolic activation and detoxification is critical in determining the susceptibility to lung cancer upon exposure to the tobacco-specific nitrosamine 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Activation of NNK occurs by cytochrome P-450 (CYP)-mediated oxidation, whereas detoxification in man is initiated by carbonyl reduction of NNK to its corresponding alcohol 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) which is glucuronosylated and excreted into urine or bile. Because carbonyl reduction of NNK is essential for glucuronosylation, producing the detoxified metabolite NNAL-Gluc, the equilibrium between NNK and NNAL is suspected to play a key role in the organospecificity and carcinogenic potency of NNK. While the relationship between lung cancer risk, tobacco smoke and CYP activities is controversial, we focussed on the identification, expression and activity of NNK carbonyl reducing enzymes. Five different enzymes mediating NNK carbonyl reduction in man have been identified: microsomal 11 β -hydroxysteroid dehydrogenase type 1 (11 β HSD1), cytosolic carbonyl reductase (both belonging to the short-chain dehydrogenases/reductases), and three members of the aldo-keto reductases (AKR), namely AKR1C1, AKR1C2 and AKR1C4, previously designated as dihydrodiol dehydrogenases DD1, DD2 and DD4, respectively. All enzymes have been purified to homogeneity from human liver and characterized regarding NNK carbonyl reduction. Since (*S*)-NNAL was discussed to be higher tumorigenic than (*R*)-NNAL in mice, the enantioselectivity of the purified human enzymes has been determined. We propose that the extent of expression and activity of NNK reductases strongly influences the tissue selectivity and interindividual susceptibility to NNK-mediated cancer.

A variety of specific inhibitors of the NNK reductases is known. However, for several reasons, the inhibitor glycyrrhetic acid, a constituent of licorice, deserves some comment. Licorice is derived from the rhizomes and roots of *Glycyrrhiza glabra* L. The extract contains up to 10 % glycyrrhizic acid, a saponin-like glycoside 50 times sweeter than sugar. Importantly, glycyrrhetic acid is not only a potent (in nM concentrations) inhibitor of 11 β -HSD1, but has recently been shown to inhibit (in μ M concentrations) AKR 1C1, 1C2, 1C3 and 1C4. It, therefore, seems obvious that licorice ingestion may affect NNK detoxification by the NNK reductases. Moreover, licorice is used as a tobacco additive to alleviate mucosa irritation upon smoking. Finally, glycyrrhetic acid is known as an inducer of CYPs. The resulting increase in NNK activation would be synergistic to the inhibition of NNK carbonyl reductases, thereby further aggravating the toxicological consequences of smoking.

IFSH-Supported Investigators (Oral platform presentations)

PRENATAL EXPOSURE TO CIGARETTE SMOKE AND CHRONIC AIRWAY DISEASE IN THE JUVENILE AND ADULT OFFSPRING

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(also presented at Poster Board # 1)

Accumulating epidemiologic and toxicologic data indicate that smoking during pregnancy increases the risk of respiratory ailments in the offspring. While these findings carry extensive clinical consequences, no data are available to provide insight into the possible mechanisms by which these effects may occur. Thus, a study is being carried out to test the hypothesis that inhalation exposure to unfractionated (or a particular smoke phase) of mainstream cigarette smoke (MCS) by pregnant mice influences airway reactivity in the offspring. Pregnant CD1 mice were exposed (using an automated cigarette smoking machine) daily for 4 hr/d (5 d/wk) from gestational day 4 to parturition to intact MCS at a concentration equivalent to smoking <1 pack of cigarettes per day (particulate matter = 16 mg/m³; CO = 25 ppm). Under these conditions, inhalation of MCS had no effect on maternal weight gain, pregnancy incidence, gestational duration, litter size or offspring sex ratio. Offspring were evaluated by bronchoprovocation challenge with acetylcholine (ACH) at 5 and 15 wk of age for changes in airway responsiveness (hyperresponsiveness is a diagnostic marker of asthma); biologic parameters known to mediate airway reactivity were also evaluated. Results to date demonstrate that even 5 wk after birth, prenatally exposed offspring have heightened airway reactivity to ACH challenge compared to their air exposed counterparts; effects were observed in the absence of any pulmonary inflammation, lung cell damage or pathology. Studies suggest that MCS-induced effects on lung cytokines may play some role in the observed airway hyperreactivity; IgE levels (an allergy antibody) tended to be elevated in smoke exposed offspring of both age groups and genders. Studies demonstrate that prenatal exposure to MCS may be inducing airway dysfunction in the offspring, and that even modest smoking during pregnancy may increase the offspring's risk of respiratory dysfunction later in life.

PARP-1 ACTIVITY UNDER METAL STRESS IN PRIMARY HUMAN LUNG CELLS

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(also presented at Poster Board # 2)

Activation of poly(ADP-ribose)polymerase-1 (PARP-1) represents one of the immediate responses of eukaryotic cells to DNA damage. (PARP-1) is a well known nuclear protein and DNA binding enzyme and its activity is crucial for genome stability.

We have examined the PARP activity in primary human lung cells heavy metal stress and under prolonged cultivation. Bronchial epithelial cells and peripheral lung cells from lung cancer patients were grown as explant cultures (3-4 weeks) and were seeded on glass coverslides. PARP-1 expression was proved by Western Blotting in normal human bronchial epithelial cells (NHBE). In NHBEs H₂O₂ (100 μM, 5 min) is a stimulating factor for the formation of ADP-ribose-polymers which represent PARP-1 activity (detected by immunofluorescence).

Treatment of NHBEC with copper sulfate (0.05 mM) alone did not trigger PARP-1 activity but decreased PARP-1 activity induced by H₂O₂ (100 µM). Similarly mercury (II) chloride (0.03 mM) exerted no effect on PARP-1 activity and decreased the activity of PARP-1 upon co-incubation with H₂O₂ (0.1 mM).

The inter-individual variation in the first explant (in 4 independent experiments) was up to 30 %. We have analyzed PARP-1 activity in progressive generations and passages of bronchial and peripheral lung cells cultivated for 10 weeks to examine the effect of prolonged cultivation of lung cells. H₂O₂ induced PARP activity was decreased in the higher passages and the levels of PARP activity varied between different cases. This reflects an adaptive response of cells to cultivation.

Further work is to compare the level protein expression and the level of PARP activity under different stress factors in different patients.

This study is supported by IFSH and the DFG Graduate College GRK 416 (MH Ahmad)

BIOMONITORING OF TOBACCO SMOKE-RELATED AROMATIC AMINES IN SMOKERS VERSUS NON-SMOKERS

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(also presented at Poster Board # 3)

Aromatic amines (AA) have been identified as bladder carcinogens and may account for the positive correlation between cigarette smoking and the incidence of bladder cancer in humans. However, only about 50% of attributed risk for bladder cancer is associated with cigarette smoking indicating a remaining exposure to AA not associated with tobacco smoke.

In this cross-sectional study 140 participants will be investigated including 120 non-smokers and 20 smokers. The study is aimed at identifying potential sources of exposure of AA for non-smokers by means of questionnaire, investigating the relationship between excreted AA levels in urine and Hb-adduct levels, and demonstrating a possible association between CYP1A2 phenotype, NAT or GST genotypes and excreted AA levels in urine and Hb adduct levels.

So far the data of 53 non-smoking participants (16 men and 37 women) have been documented and from each individual a 24 hrs-urine as well as a blood sample (to prepare Hb and DNA) have been collected. A protocol of food consumption and beverages covers the time span 24 hrs before urine collection. All participants agreed to take part in a caffeine test for CYP1A2 phenotyping.

At present the profile of AA has been determined in 45 samples. The o-, m- and p-toluidines have been detected in 15, 10, and 8 samples in a range of 0.12-204 µg/L, 1.79-286 µg/L and 0.19-434 µg/L, respectively. 2-amino- and 4-aminobiphenyl have been found in 41 and 16 samples in a range of 0.05-56 µg/L and 0.04-29 µg/L, respectively, whereas 3-aminobiphenyl concentrations were less than LOQ in all samples.

In parallel the determination of AA Hb-adducts and genotyping of each individual for NAT and GST is underway.

COMPARISON BETWEEN CARBON MONOXIDE, NITROGEN OXIDES AND NICOTINE IN THE MAINSTREAM SMOKE OF RESEARCH, COMMERCIAL, AND PREP CIGARETTES

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(also presented at Poster Board # 4)

Potentially Reduced Exposure Products (PREPs) are designed to reduce the health risk associated with cigarette smoking. For this purpose, PREPs utilize modification in tobacco and cigarette design. However, a low nicotine PREP may change the puffing behavior of smokers to achieve nicotine levels comparable to standard cigarettes. Changes in combustion behavior, puffing conditions, composition, and design of cigarettes can alter the characteristics of the smoke and the amount of gases produced. Gases such as carbon monoxide and nitrogen oxides in cigarette smoke have been associated with cardiac and respiratory health problems in smokers. In this work, we have analyzed the gaseous phase for carbon monoxide and nitrogen oxides of a research cigarette (2R4F), a commercial cigarette (Marlboro Medium) and a PREP (Quest1). We have measured these gases using three puffing regimens (1) FTC 35 ml over 2 seconds, (2) short puff 50 ml over 2 seconds, and (3) long puff 100 ml over 10 seconds. Carbon monoxide was measured using an IR gas analyzer and nitrogen oxides (NO and NO₂) were measured using a chemiluminescence gas analyzer. The results showed that more gases were produced using short puffing and long puffing regimens than with the FTC puffing regimen for 2R4F and Marlboro cigarettes. However, Quest1 displayed little effect of puffing variation on gases produced (though more NO₂ was produced in the short puffing than in long puffing and FTC puffing regimens). For estimating the dose of nicotine in the respiratory tract, we measure the nicotine content in size-segregated samples of particulate matter of mainstream smoke from these cigarettes. Since particle size distribution of mainstream smoke aerosol control the deposition behavior of particles in the respiratory tract, a relationship between particle size and chemical composition of nicotine can help in its dose assessment.

Poster Presentations

Poster Board # 5

THE USE OF CONVENTIONAL TOXICOLOGY TO CHARACTERIZE THE DOSE RESPONSE OF A HUMAN AIRWAY EPITHELIUM MODEL TO TOBACCO SMOKE COMPONENTS

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A novel toxicological tool consisting of a differentiated, 3-D, in vitro model of human respiratory epithelia (EpiAirway-100 cells; MatTek Corp., USA), is being utilized to examine the early gene response(s) following exposure to tobacco smoke components (TSC). Surrogate solutions of TSC (Nicotine, Formaldehyde, Cadmium, Urethane) will be tested for their capacity to up- and/or down-regulate genes in the respiratory epithelia following acute exposure. It is imperative that the final doses used are sub-toxic, since the objective is to identify important biomarkers involved with toxicant stress rather than studying dead or dying cells.

Conventional toxicological analysis (e.g. transepithelial electrical resistance [TEER], cell viability [MTT] and protein assay) was used to establish the dose of TSC needed to cause changes in epithelial resistance, secreted surface proteins and release of inflammatory markers. Following pre-conditioning of the human respiratory epithelial tissue, the cells were exposed to TSC. Individual TSC were prepared in sterile phosphate buffered saline and exposed to the apical surface of the cells (n = 3 wells per dose). A range of TSC doses were used to elicit a classic dose response (max. TD100). By applying conventional toxicology techniques to this range of doses, the toxic effect of the TSC has been characterized. All four TSC appeared to induce similar stress responses within the model. As expected, at high doses MTT and TEER values decreased but at low doses a peak in TEER measurements (20–60% increase from control) was observed. This peak coincided with an increase in protein secretion, suggesting a role for mucin production as an early protective response following exposure to the TSC. As such, this dose range was considered important for gaining an understanding of potential protective mechanisms, and will be utilized in further toxicogenomics investigations.

Poster Board # 6

HYPERMETHYLATION OF TUMOR SUPPRESSOR GENES IN LUNG CANCER PATIENTS AS BIOMARKER OF HARM

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Tumor suppressor genes (TSG) are important factors in carcinogenesis and TSG inactivation by hypermethylation deserves more and more attention. TSG inactivation might be acquired through yet unknown environmental factors.

We have investigated different tissue samples from lung resection of lung cancer patients. All patients were active or former smokers and therefore the impact of tobacco smoke would have targeted the morphologically normal epithelium as well as the region of tumor development. Genes of interest were RASSF1A (Ras association domain family 1-isoform A), p16 and DAPK (death associated

protein kinase). The DNA is pre-treated with bisulfite in order to distinguish methylated from unmethylated DNA in a subsequent methylation specific PCR (MSP).

We have observed that RASSF1A was methylated in 4 of 11 tumor tissues samples and not methylated in the paired samples of morphologically normal lung tissue. For p16 a methylation of DNA in the tumor- and matching healthy lung tissue was found in 3 of 11 cases. P16 plays an important role in the regulation of the cell cycle. DAPK which is involved in apoptosis was methylated in 3 of 3 bronchial epithelial tissues, in 7 of 11 lung tissues and in 7 of 11 lung tumor tissues.

Usually TSGs are unmethylated in all normal tissues. The frequent hypermethylation of TSGs in lung, bronchial epithelial and tumor tissues from lung cancer patients shows that aberrant methylation patterns are possible markers of harm.

Our further aim is to enlarge the study group and investigate the sensitivity of normal lung epithelium towards hypermethylating factors like cigarette smoke condensate *in vitro*.

Poster Board # 7

GENOTOXICITY AND EFFECTS ON THE EXPRESSION OF MRP-TRANSPORTERS OF CU(II) AND FE(II) IN HUMAN LUNG CELLS IN CULTURE

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Various sources contribute to an inhalational exposure of humans to heavy metals (e.g. motor vehicle traffic and smoking). Recently tobacco smoke ingredients were reported to facilitate the iron-uptake into lung cells.

Human lung has to cope with exposure to low doses of heavy metals and the chronic uptake might lead to adaptation. Primary cultures of normal human bronchial epithelial cells (NHBE) and the lung tumor cell lines H322 and A549 were exposed to Cu(II) and Fe(II). Transition metals like Cu and Fe generate reactive oxygen species via Fenton-reaction and might lead to DNA-damage, therefore we analyzed Fe(II) and Cu(II) treated cells in the COMET-assay. In NHBE incubation with 100 μ M for 24h Fe(II) was not toxic (MTT-assay) but led to significant DNA-damage. In A549 cells DNA-damage occurred above 1000 μ M (24h). Cu(II) induced DNA-damage above 150 μ M in NHBE and at 125 μ M in A549.

As MRP-transporters export a variety of substrates they might protect cells by extrusion of metal complexes. The effect of Cu(II) on expression of MRP-transporters was analyzed using real-time PCR. Incubation with Cu(II) (25 μ M; 24h) increased MRP5 expression 1.3 fold in NHBE, while it had no effect on MRP-expression in H322. Three days of exposure (5 μ M) increased MRP2 (1.4 fold) in H322, but decreased MRP3. In H322 seven days of exposure decreased MRP5 (0.7 fold). Incubation of NHBE with Cu(II) for 5d showed strong variations between donors. These results show, primary cells react different towards metals compared to tumor cells and that long term is different from short term response.

MEDIATORS OF INFLAMMATION MODULATE MULTIDRUG RESISTANCE RELATED PROTEINS IN CULTURED HUMAN LUNG CELLS

Abdelrahman Torky

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The respiratory tract is affected by stressors in tobacco smoke that induce irritation and inflammation. Mediators of inflammation such as prostaglandins are candidates for modulation of multidrug resistance proteins (MRP-transporter) which might be crucial for the ability to cope with these stressors. We have compared the pattern of MRP-expression in A549 lung tumor cells and primary human lung cells and have studied the effect of prostaglandins on both MRP-mRNA expression and function.

Normal human bronchial epithelial (NHBE) and peripheral lung cells (PLC) were cultivated under conventional and dry-wet circumstances. MRP1 and MRP3 were localized to the cell membrane in tested lung cells. In contrast to that MRP2, MRP4 and MRP5 could be described as intracellular proteins in NHBE and PLC. MRP1-5 isoforms could be characterized in A549 tumor cells as membrane proteins. Furthermore, MRP1 and 2 showed adaptive expression response toward different culture circumstances. The pattern of MRP-mRNA isoforms was different between the tumor and normal lung cells.

The receptors of prostaglandins (EP 1-4) were found in protein extracts of NHBEs, and PLCs using immunoblotting. The pattern of PG receptors was different between A549 and primary lung cells. PG F2a did not alter MRP1-mediated transport in NHBE, PLC and A549 cells (5-10 μ M, 1d and 4d treatment). On the other hand PGE2 modulated MRP1 expression and function in NHBE but not in PLC and A549 cells. COX1 and II inhibitors (Indomethacin – COX I/ II – 5, 10 μ M and Celecoxib- COX II- 5, 10 μ M) could decrease the transport activity of MRP1 in NHBE, PLC and A549 in both 1 and 4 day trials.

Taken together, there is evidence that inflammation increases MRP-expression and function in lung cells. This may lead to increased absorption of MRP compounds through basolateral membrane transport.

THE RELEVANCE AND SIGNIFICANCE OF O6-, N7-ALKYLGUANINES AND N3-ALKYLADENINE DNA ADDUCTS FROM TOBACCO SMOKE

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DNA adducts induced by tobacco smoke may give a clue on the onset of cancer. Tobacco smoke represents a mixture of 3800 organic and inorganic compounds, already recognized in main stream, side stream, and secondhand tobacco smoke, more than 40 are known to be carcinogenic in animals (polycyclic aromatic hydrocarbons, aza-arenes, aromatic and heterocyclic amines, aldehydes, N-nitrosamines, inorganic compounds, benzene, vinylchloride, styrene and 1,3-butadiene) (Vineis et al. 1995). Major concern is devoted tobacco specific nitrosamines (TSNA), arising from the metabolism of the nicotine (Hecht et al. 2003), the potent human carcinogens (head and neck, lung cancer) being N-nitrosornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). Methyl diazohydroxide, product of nitrosation

of nicotine (Tyroller et al. 2005), is strongly methylating O6- and N7- positions of guanine and N3- position of adenine. These exogenous methylations may be mutagenic and carcinogenic, since N7-guanines and N3-adenines result in GC-TA transversion (N7-MeG) and AT-TA transversion (N3-MeA). Less abundant O6-MeG results in GC-AT transition (Vodicka et al. 2002). Furthermore, 7-alkylguanines may be converted into corresponding formamidopyrimidines, resulting in mispairing. The biological consequences of above alkylations are related to the efficiency of DNA repair (N7-MeG and N3-MeA via BER, O6-MeG via MGMT pathway). N7-alkylguanines also represent the most abundant adduct for other tobacco smoke carcinogens like styrene and 1,3-butadiene (BD). Biotransformation of styrene and BD generate reactive electrophilic intermediates (styrene-7,8-oxide, monoepoxybutene, diepoxybutane and epoxybutanediol). Prominent styrene-induced adducts are regioisomeric N7-gua, N6-ade, N3-ade, N3-ura, while BD epoxides give rise N1-(2,3,4-trihydroxybutyl)adenine (N1-THB-ade), prone to rearrangement to N6-(2,3,4-trihydroxybutyl)adenine (N6-THB-ade), and 7-(MEB)-gua. Only N1- (N6-) adenine adducts of both compounds were determined in humans exposed to both styrene (mean 100 mg/m³) and BD (5 mg/m³) so far; BD was almost 100-fold more effective in DNA adducts formation (Zhao et al. 2000). It is a question, where present detection limits enable the determination of any DNA adducts, including N7-G, in individuals exposed to styrene and BD from tobacco smoke (1 cigarette results in 10 µg of styrene). This applies for studies, in which DNA adducts were analyzed in white blood cells. To our knowledge, no attempt was undertaken to detect DNA adducts of styrene and BD in target tissues of heavy smokers (laryngeal or lung autopsies).

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Poster Board # 10

CHARACTERIZATION OF SELECTED HOFFMANN ANALYTES IN TOBACCO SMOKE AND THERAL DESORPTION OFF-GASES FROM TOBACCO APPLYING ON-LINE MASS SPECTROMETRY WITH SOFT PHOTO-IONIZATION TECHNIQUES

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Laser mass spectrometry based on resonance multiphoton ionization (REMPI) and single photon ionization (SPI) combined with time-of-flight mass spectrometry (TOFMS) has been applied for the direct real time analysis of health relevant smoke components (Hoffmann analytes) such as acetaldehyde, isoprene, benzene, and PAH) in tobacco related smoking chemistry. First, thermal desorption-pyrolysis/mass spectrometry from pure tobacco samples (Burley, Oriental, and Virginia) have been carried out. Samples were placed in a quartz glass liner and sequentially heated to 190°C, 250 °C, and 310 °C. Respective evolved gaseous compounds at each temperature were guided by means of helium as carrier gas via a heated transfer line into the ion source of the time-of-flight mass spectrometer. Ionization was carried out by REMPI and SPI, respectively. The resulting spectra revealed distinct differences in the product pattern of the different tobaccos. The obtained data has been taken as source for statistical analysis for investigation of marker compounds for a fast on-line distinction of the tobaccos. Additionally, mainstream smoke of a 2R4F research cigarette has been analyzed on-line with SPI-TOFMS on a real time puff by puff basis. Mass spectra were recorded with a temporal resolution of 100 ms. Since the duration of a puff is two seconds, twenty mass spectra could be recorded during one single puff, enabling a sub puff time regime investigation of mainstream

smoke. A first analysis exhibited slightly different puff structures especially between the first puff and subsequent puffs. Finally, single photon ionization with a novel electron beam pumped excimer lamp as light source was applied to analysis of mainstream smoke and human breath. This allows the usage of a more compact setup without selectivity and sensitivity losses. Typical marker compounds for smoking such as 2,5-dimethylfuran, butadiene, and benzene could be detected solely in the smoker's breath in real time.

Poster Board # 11

VALIDATION OF BIOMARKERS IN EXHALED BREATH CONDENSATE (EBC) FOR THE SMOKING-RELATED AIRWAY INFLAMMATION: ESTABLISHMENT OF ANALYTICAL METHODS

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EBC is a non-invasively derived biological matrix, which is used for the detection of biochemical changes in the respiratory tract of patients with lung diseases. Biomarkers in EBC could be also used as early indicators of smoking-related biological effects in the airways. There are at least three major challenges in EBC biomarker analysis: (1) EBC is a highly diluted biological fluid (10000 – 20000-fold compared to plasma) requiring sensitive analytical methods for quantitation; (2) these ultra-low concentrations are most sensitive to exogenous (e.g. from the sampling device) and endogenous (saliva) contaminations; (3) only a limited volume of EBC (ca. 2 ml collected in 15 min) is available. To overcome these problems, we developed a series of analytical methods for the following groups of biomarkers: (a) Markers for controlling the dilution and salivary contamination of EBC: Cations (Na⁺, K⁺, Ca²⁺, Mg²⁺; method: ion chromatography), urea (GC-MS after derivatisation), amylase (enzymatic test); (b) biomarkers for oxidative stress and lipid peroxidation: Hydrogen peroxide (fluorimetry), arachidonic acid metabolites (8-isoprostane, LTB₄; GC-MS), aldehydes (acrolein, malondialdehyde, hexanal, heptanal, nonanal, 4-hydroxy-hexenal, 4-hydroxy-nonenal; LC-MS/MS and SPME-GC-MS); (c) biomarkers for inflammation processes: Nitrite/nitrate (photometry), 3-nitrotyrosine (LC-MS/MS after derivatization). In addition, two different sampling devices for EBC collection have been tested: ECoScreen (Jaeger GmbH, Hoechst, Germany) and RTube (Respiratory Research, Inc., Austin, Texas, USA). The latter system appears to have advantages in terms of pre-cleaning of the critical parts of the device in order to avoid contamination, particularly when larger numbers of EBC samples are to be collected.

The validated methods will be applied to EBC samples from non-smokers and smokers. Suitable biomarkers in EBC would be valuable for testing the effectiveness of potentially reduced exposure products (PREPs).

THE DEVELOPMENT OF AN ANALYTICAL METHOD TO DETERMINE CYTOSINE METHYLATION AS AN IMPORTANT PART IN EPIGENOMICS

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In addition to conventional genes there exist at least two further sources of information. First, there is the huge group of “non-coding” DNA sequences between and within the genes, which for a long time were referred to as “junk DNA”. Epigenetic phenomena constitute a further source of information. Amongst others, these include modifications of the DNA and the proteins surrounding them.

In this work we present a capillary electrophoretic method for determination of the global DNA methylation level. To increase the sample throughput, an array system was constructed that allows the parallel run of three analyses. In addition, a new spectrometric detector and a chemical sample preparation were developed, which improve the limit of detection by a factor of 100 and allow us to determine the methylation level in 10 and perhaps even in 1 ng DNA. Thus the analysis of gene-specific methylation could be possible in future.

Furthermore we have shown that in both cell culture and animal experiments changes in the methylation level could already be determined 48 h after treatment with carcinogens. As a result of the improved sensitivity of the analytical method we could also detect other DNA modifications. For example, patients with chronic lymphocytic leukemia (CLL) show a drastic increase in the etheno-adenosine concentration. This confirms that CLL is a neoplastic disease susceptible to antioxidant enzyme alterations and oxidative stress. Perhaps this analysis will be useful for early diagnosis and for assessment of tumor progression.

IN-VIVO EXTRACTION OF LEAD AND CADMIUM FROM FOUR BRANDS OF SWEDISH SNUS COMPARED TO NICOTINE EXTRACTION

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Background. The content of lead (Pb) and cadmium (Cd) in North American moist snuff and Swedish snus are well studied. Their extraction from snus in-vivo, however, has not been reported in the literature. Adverse health effects may occur at lower exposure than previously anticipated, primarily as kidney damage and osteomalacia of Cd and neurotoxicity of Pb (Ref). Cd oxide from burning cigarettes is highly bioavailable, 10% is deposited in lung, and 30-40% into the blood circulation. Pb levels in blood and hair are significantly higher in smokers than non-smokers. The dietary Cd and Pb absorption rates are about 5% to 10% and could be similar for moist snuff.

Aim. To estimate the Pb and Cd extraction from Swedish snus.

Methods. In an open label, randomized, two-way cross-over study, 32 male healthy regular snus users were given repeated doses of four different brands of portion snus: A:1g“General”, B:1g“Catch”, C:0.5g“Catch Mini” and D:0.3g“Catch Dry Mini”. Analysis and calculation of the extracted amount of nicotine, Pb and Cd from each sachet of used and unused snus were performed.

Results. The mean nicotine extraction from A, B, C and D was 2.4 (25%), 2.2 (26%), 1.4 (30%) and 0.7mg/sachet (13%), respectively. The mean Cd extraction was 0.03 (10%), 0.02 (8%), 0.006 (6%)

and 0.005µg/sachet (3%), respectively. The Pb content did not differ between used and unused snus for any brand.

Conclusion. The percent Cd extraction was below 10% and Pb extraction was negligible for all brands. Swedish snus therefore appears safe with respect to in-vivo Cd and Pb extraction.

Poster Board # 14

U.S. SMOKELESS TOBACCO COMPANY ("USSTC") SURVEY DATA REGARDING ADULT CIGARETTE SMOKERS' PERCEPTIONS OF THE COMPARATIVE HEALTH RISKS OF TOBACCO PRODUCTS

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A number of tobacco harm reduction proponents suggest that cigarette smokers who do not quit and do not use medicinal nicotine products should switch completely to smokeless tobacco, although others disagree. We sought to understand adult cigarette smokers' views of the comparative health risks of cigarette smoking and smokeless tobacco use and assess opinion in the scientific and public health communities, as expressed in the published scientific literature, regarding how those views may affect implementing a tobacco harm reduction strategy that includes smokeless tobacco.

One significant impediment to effectively implementing such a tobacco harm reduction strategy, according to published opinion, is adult cigarette smokers' perceptions of the comparative health risks of tobacco products. To explore this issue, consumer research conducted for USSTC investigated U.S. adult cigarette smokers' opinions on the relative health risks of tobacco products. Adult smokers were asked to rate, on a scale of one to ten, how dangerous they thought certain tobacco products are to a person's health. The results suggest that adult cigarette smokers view cigarettes and smokeless tobacco as posing nearly equal health risks. Data reflecting these results will be made available at the symposium.

There is support in the scientific and public health communities for providing adult cigarette smokers with accurate and relevant information regarding the options that are available to reduce the potential risks to their health, although there are also those who disagree. Further, among proponents of tobacco harm reduction there is opinion that unless cigarette smokers are provided with such information the status quo with respect to their smoking will remain.

Poster Board # 15

POLONIUM-210 AND LEAD-210 AS BIOMARKERS OF ACTUAL DOSE OF INHALED CIGARETTE SMOKE

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Evaluation of risk of disease from cigarette smoking requires valid estimates of inhaled dose, for both current and past smoking. We hypothesize that direct estimates of the inhaled dose are available via Pb-210 and Po-210 concentrations measured in the urine of cigarette smokers. Both of these naturally occurring radioactive components of the smoke are elevated in the urine of cigarette smokers as compared with non-smokers. This work will examine whether the amount excreted correlates with the intensity of smoking. Both Pb-210 and Po-210 are associated with smoke tar, the primary agent in

smoking related carcinogenesis. Pb-210 is potentially a useful biomarker for long-term dose, and Po-210 for short-term dose. To our knowledge, there are no available biomarkers related to long-term integrated dose of cigarette smoking.

We have recruited 125 of a planned total of 250 healthy subjects in China with different levels of smoking intensity and history of long-term smoking. Smoking history is assessed by interview. A single 24-hour urine sample is collected from each subject. Samples are assayed for creatinine, cotinine, Po-210, and Pb-210 concentrations.

To date we have recruited 33, 30, 29, 19 and 14 subjects who report smoking 0, 1-18, 19-29, 30-40, and >41 cigarettes per day. The mean subject age is 46.7 years (range 20-82). Analysis for cotinine and creatinine indicate that the total urinary cotinine excreted in 24 hours is reasonably well correlated with the measured ratio of cotinine to creatinine. Neither fully predicts the reported average number of cigarettes smoked per day, or the number smoked during the 24 hours of sample collection. Dose-response relationships will be established between the surrogate indicators of exposure and the measured concentrations of the Pb and Po.

Poster Board # 17

OXIDATIVE DNA DAMAGE INDUCED BY CIGARETTE SMOKE

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Cigarette smoke is a complex mixture of thousands of chemicals distributed between the particulate or 'tar' phase and the vapour phase which collectively is termed 'whole smoke'.

It is believed that components of cigarette smoke including free radicals can play a part in the oxidative damage of biomolecules such as DNA. Oxidative stress is reported to be a contributing factor to a number of diseases associated with smoking including chronic obstructive pulmonary disease (COPD) and lung cancer.

A novel exposure system has been employed to investigate the effects of whole cigarette smoke on oxidative DNA damage in H292 cells cultured at the air-liquid interface using a modified *in vitro* Comet assay incorporating the lesion specific enzyme formamidopyrimidine glycosylase.

Exposure of cells to whole smoke, followed by a 20 hour cellular recovery period resulted in a dose-dependent increase in oxidative DNA damage. This procedure has now been adopted for the investigation of the effects of cigarette smoke on oxidative DNA damage.

Poster Board # 18

PM AND BAT ANTI-SMOKING ACTIONS IN GEORGIAN SCHOOLS

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Objective: To learn new aims of representatives of international tobacco industry representatives (PM-Altria and BAT) actions with youth population of Georgia regarding fighting against smoking.

Methods: Interviewing representatives of Ministry of Education and schoolchildren, where PM and BAT provided anti-smoking action and learn information from global sources regarding real face of tobacco industry.

Results: In the years of 2002-2004 the PM and BAT offices provided aggressive policy with youth population of Georgia. Ordinary it was done in schools of big cities: Tbilisi and Kutaisi. First time they wanted to sign the agreement with Ministry of Education, but after our strong efforts the Ministry changed positions, but some schools on the basis of financial interest organized actions in school for stopping smoking until 18. General aim was to popularize brands of such companies and they recommended to schoolchildren begin the smoking after 18. It is one of the parts of aggressive policy of transnational companies. Until such action they created coalition of NGOs, which received money from the industry. They create billboards and organized actions in schools.

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