Recent Advances In Tobacco Science

Volume 37

Challenges in the Development of Biomarkers of Smoking Exposure and Effect

Symposium Proceedings
65th Meeting
TOBACCO SCIENCE RESEARCH CONFERENCE

September 18-21, 2011
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Symposium of the 65th Tobacco Science Research Conference

Challenges in the Development of Biomarkers of Smoking Exposure and Effect

– Symposium Chair –
Edward Robinson

– Editors –
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Edward Robinson

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PREFACE

The Program Editorial Committee of the 65th Tobacco Science Research Conference is pleased to present the 37th volume of Recent Advances in Tobacco Science publication. Each year the Program Editorial Committee of the Conference selects a theme that highlights a scientific or regulatory issue that is relevant, engaging, and thought-provoking for the tobacco community. To review the recent advances made in discovery and measurement of biomarkers related to human exposure to tobacco smoke and the progression of disease caused by that exposure, the main topic chosen for this symposium is "Challenges in the Development of Biomarkers of Smoking Exposure and Effect". Four recognized authorities were invited to share their knowledge and discuss the latest developments in biomarker research. This publication contains the synopses of the symposium presentations and introductory remarks that include a brief biographical sketch of the symposium speakers. Members of the Program Editorial Committee, Tony Gerardi and Ray Robertson, and I, wish to express our sincere appreciation to the speakers, Drs. Mike Milburn, G. L. Prasad, Mohamadi Sarkar, Chris Proctor, and their colleagues for the significant time and effort spent preparing the publications and presentations. Information on ordering additional copies of this publication, as well as previous volumes, is included at the back of this book.

Edward Robinson, Chair
Program Editorial Committee
65th Tobacco Science Research Conference
Lexington, KY, September 18-21, 2011

INTRODUCTION TO THE SYMPOSIUM

Edward Robinson

Lorillard Tobacco Company
Greensboro, North Carolina USA

One of the greatest challenges in tobacco science today is the development of biomarkers that can accurately and specifically measure exposure to cigarette smoke and predict risk of disease development in smokers. The most useful biomarkers would allow early prediction of disease in healthy smokers so that appropriate intervention could be undertaken. The complexity of cigarette smoke, variability in smoking behavior over time, variability in lifestyles, differing susceptibility to disease among smokers, and long delay between exposure and disease onset present particular challenges in determining the potential relationships between exposure and risk from smoking. While there has been extensive research on identifying biomarkers of potential harm associated with the onset of some diseases, most of the identified biomarkers are not specific to cigarette smoke exposure.

It is anticipated that biomarkers will become important in the successful development of modified risk cigarette products by confirming a smoker's reduced exposure to smoke components and the consequent reduction in disease-related biological endpoints. Biomarker studies could be useful as surrogates for epidemiological studies, allowing risk prediction after short periods of smoke exposure versus the decades usually necessary for epidemiological studies.

Our symposium on "Challenges in the Development of Biomarkers of Smoking Exposure and Effect" will provide insight into progress toward the goal of developing biomarkers that are predictive of smoking risk. The program includes four experts in the field of biomarkers related to cigarette smoking who will provide data on technologies for biomarker development, promising biomarkers that have been identified, and strategies for the assessment of risk from cigarette smoking.

Dr. Mike Milburn from Metabolon will be presenting our opening paper entitled "Biomarker Discovery and Validation Using Global Metabolomics." He will describe metabolomics, the study of the small molecule metabolites that are the end product of cellular processes, and its use in overcoming some of the challenges of biomarker development.

Dr. Milburn received his Ph.D. in Biophysical Chemistry at the University of

California, Berkeley and was a research fellow at Harvard Medical School for his post-doctoral work. He has over 20 years of experience in biotech and pharmaceutical companies including over 10 years in senior executive biotechnology positions. He has published more than 100 scientific articles on technology, research and development in the life sciences. Before joining Metabolon, Dr. Milburn was Senior Vice President of Research and Corporate Development at Sirtris Pharmaceuticals where he led the preclinical/clinical development of drugs in the areas of metabolic disease and neurodegeneration. Sirtris Pharmaceuticals, which was acquired by GSK in 2007, pioneered the discovery of drug agents targeting the Sirtuin enzyme family involved in aging-related diseases. Prior to working at Sirtris, Dr. Milburn was Senior Vice President of Research at Plexxikon and was responsible for the development of Plexxikon's proprietary high-throughput scaffold-based chemical technology platform. While at Plexxikon, Dr. Milburn developed lead preclinical and clinical programs in the areas of metabolic disease and cancer. At Metabolon, Dr. Milburn is currently Chief Scientific Officer and is responsible for the global metabolomics technology development and research operations for its service business. Dr. Milburn's group works with over 280 clients at Metabolon and has completed over 1000 commercial fee-for-service studies since 2005.

Dr. G.L. Prasad from R. J. Reynolds Tobacco Company will then present "Smoking Related Biomarkers of Potential Harm/ Effect: Challenges and Opportunities." He will describe a strategy for qualifying and validating methods for discovery and application of biomarkers of effect.

Dr. G. L. Prasad is a Principal Scientist in the R & D department at RJ Reynolds Tobacco Co. (RJRT), Winston-Salem, NC. Some of his current responsibilities include method development, evaluation of the relative effects of combustible and non-combustible tobacco preparations, and identification of tobacco-related biomarkers of effect. He also holds adjunct faculty appointments at the Wake Forest University School of Medicine and Duke University and manages RJRT's Leon Golberg postdoctoral program with both schools. Dr. Prasad obtained his Ph.D. in biochemistry on the enzymology of plant polyamines from the Indian Institute of Science in Bangalore, India. Following a brief tenure as a postdoctoral fellow at the University of Texas Medical Branch, Galveston, TX, he moved to the National Cancer Institute in Bethesda, MD as a Visiting Fellow and later became a Visiting Scientist. At the NCI, he worked on the role of cytoskeleton in neoplastic transformation, which continued as a major focus through his academic research career. His research interests also include tumor immunology, cell signaling, and biomedical applications of nanotechnology, among others. He left NIH in 1996 and established an extramurally funded, independent research lab at the Fels Institute for Cancer Research and Molecular Biology, Temple University, Philadelphia. He was an Associate Professor at both Temple University

and Wake Forest University School of Medicine, until he joined RJRT in 2008. He has published a number of original peer-reviewed papers in PNAS, JBC, Oncogene and other journals and has also written reviews and book chapters. His former laboratory received funding from the Susan Komen Breast Cancer Research Foundation, American Cancer Society, and Department of Defense. He has served on the peer review panels of the American Cancer Society and the Department of Defense. Dr. Prasad is a Project Management Institute certified Project Management Professional and a member of the American Association for Cancer Research, the American Thoracic Society, and other professional Societies.

Following the break, Dr. Mohamadi Sarkar from Altria Client Services will present on the "Utility of Biomarkers in Assessing Exposure to Cigarette Smoke Constituents in Adult Smokers." Dr. Sarkar will discuss results from both a large cross-sectional study of smokers and non-smokers and a clinical study that demonstrate the relative suitability of a number of biomarkers for assessing smoke exposure.

Mohamadi Sarkar, M.Pharm., Ph.D., FCP, serves as Senior Principal Research Scientist for Altria Client Services in Richmond, Va. He has held this position since August 2002. During this tenure, his role has been to enable responsible product development and tobacco-harm reduction by conducting clinical studies related to products made by Altria Group's tobacco companies. He has authored more than 100 scientific peer-reviewed publications and presentations at scientific meetings. He also has participated in several invited seminar presentations and authored a variety of scientific book chapters related to his areas of expertise. Before joining the Altria family of companies, Dr. Sarkar served as Associate Professor, Clinical Pharmacology for the Medical College of Virginia (MCV) at Virginia Commonwealth University (VCU) in Richmond from 1998-2002. He previously served as Director of the Graduate Program in the Department of Pharmaceutics at MCV. Currently, he maintains an affiliation with VCU where he continues to teach Clinical Pharmacology. Between 1990 and 1998, he worked at West Virginia University in Morgantown, W.Va., where he held a variety of positions including: Associate Professor, Clinical Pharmacology; Associate Professor, Genetics, and Developmental Biology; and Associate Professor, Tobacco Research Center, WVU Cancer Center. During the early 1980s, He worked at the University of Bombay in Bombay, India, as a Lecturer in Pharmaceutical Technology as well as a Pharmaceutical Supervisor for SANDOZ (India) Ltd. Dr. Sarkar received his bachelor's degree in Pharmacy from the College of Pharmacy, University of Bombay in 1981, a master's degree in Pharmaceutical Technology from the University of Bombay in 1985 and a Ph.D. in Clinical Pharmacology from the Medical College of Virginia at VCU in 1990.

To close our session, Dr. Christopher Proctor from British American Tobacco will present "A Scientific Framework for Assessing Modified Risk Tobacco Products" which he co-authored with Martin Ward. Dr. Proctor will describe a framework of pre-clinical, clinical, and pre- and post marketing studies that might be used for assessing modified risk tobacco products. He will also discuss the current state of science and future research needs in undertaking such an assessment.

Dr. Proctor is British American Tobacco's Chief Scientific Officer, working at Group Research and Development in Southampton, UK. He is a Ph.D. chemist with postdoctoral research experience at Cornell University, under a Fulbright scholarship, and the University of Kent. In 1983, he joined BAT where he published on the development and application of analytical techniques to the chemical characterization of, and real-life exposure to, environmental tobacco smoke. In 1990, he left BAT to work as a senior scientific advisor to a law firm in Washington DC, before returning to BAT in 1993. Dr. Proctor has represented BAT in public hearings on the World Health Organization's Framework Convention on Tobacco Control and in front of the WHO's Study group of Tobacco product regulation, and has chaired two steering groups of the European Policy Centre on the use of science in regulatory decision making and better regulation. In 2003, he published a book, "Sometimes a Cigarette Is Just a Cigarette" which explores the historic efforts of tobacco companies, research scientists and the public health community to encourage less risky tobacco products. More recently, in 2011, he was part of a panel presenting to an Institute of Medicine committee that is looking into scientific standards for studies of modified risk tobacco products. His recent peer-reviewed publications focus on scientific studies seeking to characterize the risk of novel tobacco products.

The Editorial Committee looks forward to an informative and stimulating symposium. We hope that the presentations provide you with new perspectives on the development and uses of biomarkers in tobacco research and provoke additional questions that may lead to further development in this essential and exciting area of research.

Edward Robinson
65th TSRC Symposium Chair

BIOMARKER DISCOVERY AND VALIDATION USING GLOBAL METABOLOMICS

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Abstract
The search for biomarkers is a hotly studied and active pursuit in a variety of industries. In fact, the future of personalized health interventions or products rests squarely on the ability to discover and validate new biomarkers. The biomarkers themselves can be nearly anything that distinguishes one individual from another. They can be based on a diagnostic test (*e.g.*, glucose or cholesterol measurements), physical characteristics (*e.g.*, BMI), genetics (*e.g.*, SNPs) or any other distinguishing characteristic (*e.g.*, age, diet). Unfortunately, general screening methods for the discovery of new biomarkers have been very challenging with few success stories. The difficulties are mathematical, technological, and/or limited availability of proper sample types and number. One recent promising technology with potential to overcome a number of these issues is metabolomics. The focus of this paper will be to better educate the reader about the importance of understanding biochemical biomarkers and how global metabolomics is an ideal technology for gaining these new insights.

Global Metabolomics
There is a renaissance of renewed interest in understanding metabolism and how biochemical biomarkers can be used in healthcare. Much of this interest is driven from discoveries that numerious oncogenes and tumor suppressors exert their function through alterations of metabolism. This renewed interest is also coinciding with our ability to instantaneously profile 1000's of biochemicals in cells, an approach called metabolomics. The word "metabolomics" (or "metabonomics") first appeared in journal articles in 2000. Only a few metabolomic scientific papers were published that year but by 2009 that number rose to over 1300 published scientific papers reporting metabolomic results. In fact, Nature published in December 2009 that metabolomics would be one of the most influential fields in the next decade from interviews with scientists and policy makers (2010). The major challenge for metabolomics has been to develop a technology that can extract, identify, and quantitate the entire spectrum of small molecules (MW<1500Da) in any biological sample (Blow, 2008). There are between 2500-3000 biochemicals synthesized in humans when one disregards complex lipids or peptides. Importantly, in any one sample matrix (*i.e.*, blood, urine, tissue, etc.) there will always be fewer metabolites than the total number synthesized in the entire organism. Unfortunately, many uses of the word metabolomics cover rather

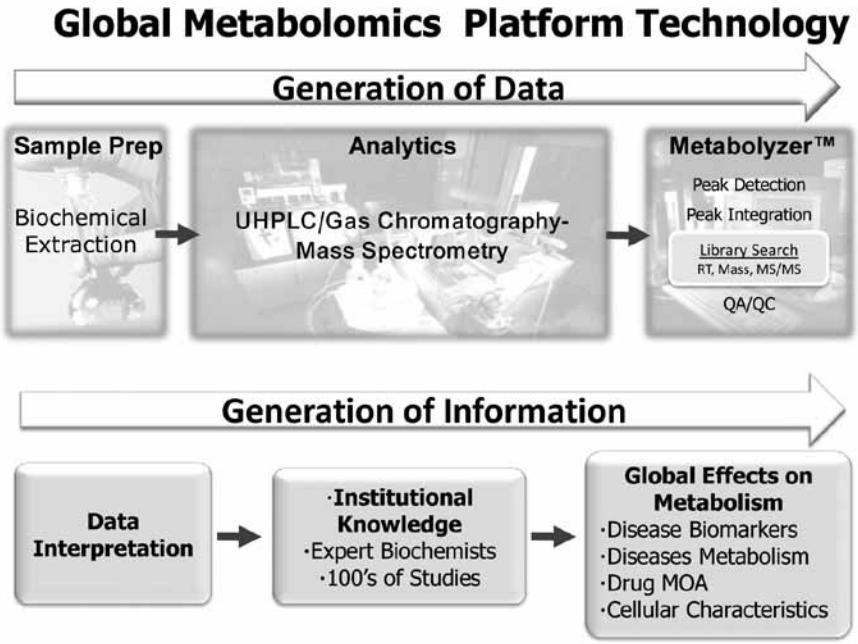
limited attempts to study certain classes of molecules such as amino acids or fatty acids rather than the entire repertoire of available biochemicals. In this chapter we will refer to metabolomics as a technology to obtain as large a snapshot of biochemicals as possible.

"Global" or "unbiased" metabolomics has been plagued by difficulties stemming from the diverse physical properties of small molecules. These properties can vary greatly, with significant differences in solubilities and molecular weights affecting a small molecules ability to be measured and solublilized. A single chromatography method to separate all of the compounds is very difficult and even more difficult to analyze individual compounds without chromatographic separation. Further complications arise if studies are expected to be completed with a reasonable turn-around time. Methods that can only analyze a few samples per day will simply be impractical from a discovery technology and statistical standpoint. These issues are currently being addressed through advanced multi-system approaches where the best separation and detection instrument technologies are being developed to run in tandem. This approach allows for a comprehensive solution achieved by combining principles offered by various best-in-breed technologies. As this new technology develops and its use in biomarker detection studies increases, it is rapidly becoming clear that metabolomics will represent a high impact technology in various healthcare-related fields such as the diagnosis of disease, identification of drug targets, evaluation of the effects of drugs, food products, or external chemical agents, and selection of patients most likely to respond to drug therapy (*i.e.*, personalized medicine) (Zhang *et al.*, 2011, Takei *et al.*, 2010, Barnes *et al.*, 2010).

One method developed for global metabolomics operates in essentially four steps, as shown in Figure 1 (Evans *et al.*, 2009). Step one is extraction of the small molecules from the biological sample. Step two is the chromatography coupled with mass spectrometry and data collection. Step three is the automated and manual QC analysis of the data using visual interfaced software (Dehaven *et al.*, 2010). Step four, the final step, is the statistical and biological interpretation of the data itself. In this method of extracting a wide range of very polar to non-polar compounds from as little as 50ul of blood plasma (Evans *et al.*, 2009). Extracted samples are split into four aliquots for different chromatography and mass spectrometry platforms, two UHPLC methods and one GC method, with one aliquot held in reserve. These three chromatography and MS systems complement each other in the range of biochemicals measured and provide an enhanced biochemical coverage of each sample. Approximately 70-80% of the biochemicals are measured on more than one platform with 30-40% measured on all three platforms. For compounds observed on multiple platforms, the platform with the best analytical characteristics (*e.g.*, fewest interfering peaks or highest signal to noise) is generally used for the analysis of that compound. In general, the

GC method provides better separation of molecules that tend to be more difficult to separate using a typical reverse phase LC method (*e.g.*, carbohydrates).

Figure 1. The top half of the figure depicts the four steps of a global metabolomics method being applied to a biological sample. These three steps, biochemical extraction, multiple chromatography and mass spectrometry analysis, and then a unbiased global informatics methods to reduce the raw machine data to the biochemicals in the sample and the relative concentration of each biochemical in each sample. The bottom half of the analysis involves the data interpretation and statistical analysis that leads to the metabolic understanding available with this method.



After the raw data has been acquired from the instruments, this method utilizes a suite of software packages that automatically integrates each ion across retention time and then uses that ionic information, which may include additional MS/MS fragmentation information and retention time, to identify the compound (Dehaven *et al.*, 2010). After a compound is identified in a sample, one of the characteristic and stronger ions is used to determine a relative concentration of that compound in each sample. This approach assures that the compound will be represented only once in the subsequent statistical analysis. When the software has finished analyzing the samples, all of the data is loaded into a visual user interface that allows a scientist to curate the data for QC purposes and visually inspect how well each compound was identified and verify only those compounds with

the highest degree of confidence for inclusion in the final data set. A variety of statistical approaches can be applied to the final data set at that point, including ANOVA, t-tests, random forest, PCA, etc. The goal of these types of statistical treatments is to identify the biochemicals that best represent the most significant changes in concentration between the groups in the study. One advantage of biochemistry is that multiple compounds in a particular biochemical pathway may often be significantly altered, giving an even higher degree of confidence to the importance of that biochemical change. In this respect, it is important to point out that most statistical treatments assume independent variables when, in fact, we know that certain biochemicals are related to the same or similar pathways. Metabolon is developing a large database of these types of biochemical changes as well as those that result from toxicity, drug mechanism, disease, etc. This knowledge enhances Metabolon's ability to provide a biological interpretation for each study it performs.

Discovering the Mechanism of a Novel Drug Action: GMX1778

One of the more fascinating and important contributions of global metabolomics has been the elucidation of a cancer drug target and mechanism of action. GMX1777 is a soluble pro-drug that is rapidly converted *in vivo* to GMX1778 (CHS828), the active cyanoguanidinopyridine. Although there are many published studies of GMX1778 since its discovery, the actual molecular target of the drug has been elusive. Potent broad spectrum anti-tumor activity has been demonstrated in several tumor types evaluated *in vitro* in a large cell panel and *in vivo* in multiple human xenograft models. The mechanism of action of this small molecule was believed to involve NF-KB inhibition (Olsen *et al.*, 2004). However, substantial NF-KB inhibition did not occur until 24 hrs after treatment with GMX1778 suggesting that NF-KB inhibition might be secondary to some other primary drug action. In an attempt to identify the primary mechanism of action and protein target of the drug, a variety of unbiased proteomic techniques and molecular biology were employed to no avail. Therefore, to attempt to discover the anticancer mechanism a global metabolomics analysis was performed to identify intracellular physiological changes over time.

One of the most important steps in a mechanism of action study is to appropriately design the sample collection for the study. For most mechanism of action studies it is critically important to evaluate a time course in order to separate the primary effects of the drug from downstream secondary or tertiary effects. Since GMX1778 has maximum activity on a multiple myeloma cell line in 24 hours samples were collected at several timepoints before the maximum activity. IM-9, a sensitive multiple myeloma cell line, was treated with 30 nM GMX1778 or with DMSO (control) for 6-, 13-, 20-, or 27-h in RPMI-1640 media (10% FBS and 0.3 mg/mL L-glutamine), n=6. Frozen cell pellets (2 x 106 cells) were analyzed using a previously published extraction schema followed by GC-MS and LC-

MS metabolomics analysis. The relative standard deviation (RSD) value for a technical replicate of pooled aliquots from the cell samples was 10%. This 10% relative standard deviation represents the total process variation of extraction, chromatography, and quantitation for the biochemicals measured. Using a p-value cutoff of 0.1 and q-value cutoff of 0.2, there were 27, 46, 65, and 65 biochemicals altered relative to the DMSO controls at 6, 13, 20, and 27 h, respectively(Watson et al., 2009). Although more biochemicals changed at the later time points a number of significant biochemical pathway alterations occurred as early as the 6 hr timepoint. All biochemical changes were interpreted physiologically and in a biochemical pathway context using in-house pathways and a biochemical knowledge data base to expedite this process.

The most significant alteration at the earliest timepoint was in the level of NAD. A 60% decrease in intracellular nicotinamide adenine dinucleotide (oxidized) (NAD+) levels was observed after just 6 h treatment with GMX1778(Watson et al., 2009). At 13 h the levels declined by 91% relative to the control and the later time points were below detection limits. Since this was the most signicant early effect of the drug we asked whether other biochemical changes that occurred later could be a result of this primary effect on the levels of NAD.

NAD+ is a cofactor in oxidation-reduction reactions, including ATP generation from glycolysis and oxidative phosphorylation. NAD+ is a substrate for reactions catalyzed by poly (ADP-ribose) polymerase, sirtuins and ADP-ribosyl cyclase. Since NAD is required by three enzymes in the Krebs Cycle we asked whether any of the Krebs Cycle intermediates increased as a result of this NAD inhibition. Indeed, fumarate increased 36%, 79%, 335%, and 244% and malate increased 48%, 87%, 413%, and 268% at the four timepoints measured supporting the idea that the drug inhibited NAD. Additionally pathways involved in glycolysis and alternative glucose metabolism were also supportive of the idea of NAD inhibition.

The next question proposed was how one could validate in an independent experiment that GMX1778 is indeed an inhibitor of NAD biosynthesis. As shown in Figure 2a, in mammals there are two independent pathways for synthesizing NAD using either Niacin or Nicotinamide as substrates.. At least three enzymes in these NAD+ biosynthetic pathways are potential targets of GMX1778: NAD+ synthetase, nicotinamide mononucleotide adenylyl transferase (NMNAT), or nicotinamide phosphoribosyl transferase (NAMPRT). Inhibition of any of these enzymes could account for the observed decreased NAD+ levels. Specific experiments were performed to identify whether GMX1778 inhibited the Niacin or Nicotinamide pathway and to determine which of these three targets was most important for drug action. GMX1778-treated cells were rescued by nicotinic acid as seen in Figure 2b (Roulston *et al.*, 2007). Additional experiments demonstrated that GMX1778 inhibits NAMPRT, the rate-limiting enzyme that

converts nicotinamide (NAM) to nicotinamide mononucleotide (NMN) in vitro and in vivo (Roulston et al., 2007). The apparent Ki of GMX1778 for NAMPRT was 1-3 nM. This correlates well with the low nanomolar IC50 of this compound in multiple human cell lines. Since the crystal structure of NAMPRT is known, it was also discovered that cell lines resistant to the drug were the result of a single amino acid mutation in the active site of the enzyme and presumably interfered with drug binding.

Figure 2a. Two independent pathways for synthesizing NAD used in mammals. Either Niacin or Nicotinamide is used as starting biochemicals to syntheisize NAD. In the validation experiment the study took advantage of these two independent pathways to validate that the drug inhibited only the Nicotinamide pathway and not the Niacin pathway.

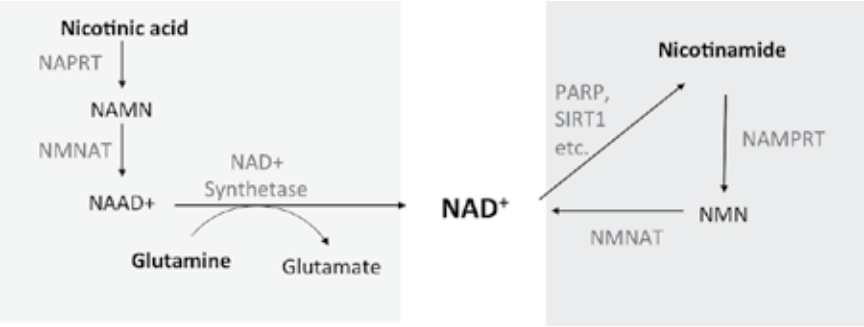
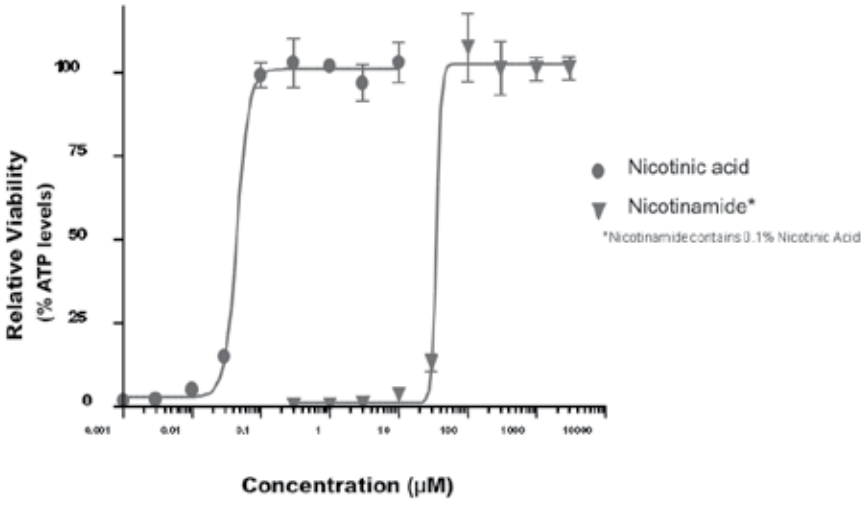


Figure 2b. GMX1778-treated cells were rescued only by nicotinic acid.

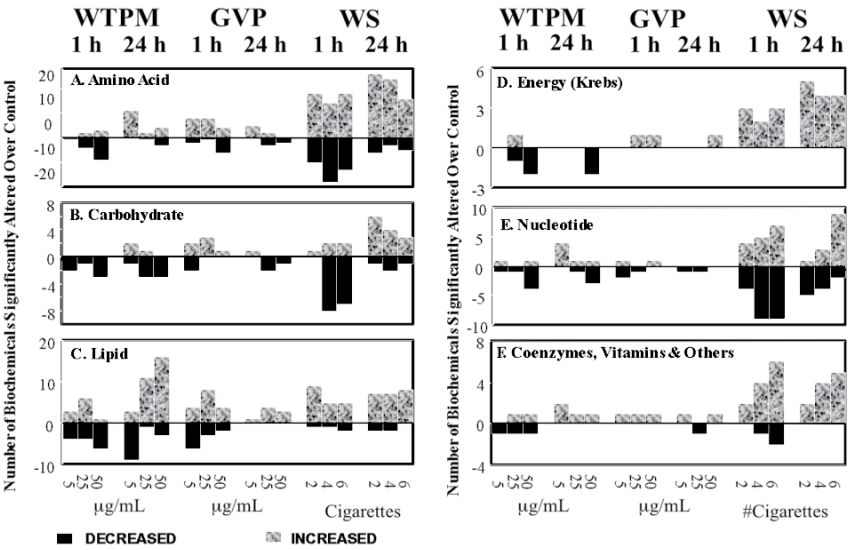


This study illustrates how a global metabolomic approach found the biochemical needle (NAD+) in the hay stack (metabolome containing hundreds of endogenous biochemicals). The study also demonstrated not only how this technology can uncover the mechanism of a drug but also the importance of potential metabolic targets for new cancer therapies. Tumor cells have elevated NAMPRT and a high rate of NAD+ turnover due to high ADP-ribosylation activity required for DNA repair, genome stability, and telomere maintenance, making them more susceptible to NAMPRT inhibition than normal cells (Beauparlant *et al.*, 2009). This novel mechanism supports the clinical use of GMX1777 as an anti-cancer agent and further supports these types of anticancer targets.

Smoke effects on human lung alveolar epithelial carcinoma cells (A549)

Using Metabolon's global metabolomics technology, the A. W. Spears Research Center, explored changes in biochemical profiles of human alveolar epithelial carcinoma (A549) cells following in vitro exposure to mainstream whole smoke (WS) aerosol as well as to wet total particulate matter (WTPM) or gas/vapor phase (GVP), the two constituent phases of WS from 2R4F Kentucky reference cigarettes (Vulimiri *et al.*, 2009). A549 cells were exposed to WTPM or GVP (expressed as WTPM mass equivalent GVP volumes) at 0, 5, 25, or 50 μg/mL or to WS from zero, two, four, and six cigarettes for 1 or 24 h. Cell pellets were analyzed for perturbations in biochemical profiles, with named biochemicals measured, analyzed, and reported in a heat map format, along with biochemical and physiological interpretations. A summary of the significant biochemical changes is shown in Figure 3. Both WTPM and GVP exposures likely decreased glycolysis (based on decreased glycolytic intermediaries) and increased oxidative stress and cell damage. Alterations in the Krebs cycle and the urea cycle were unique to WTPM exposure, while induction of hexosamines and alterations in lipid metabolism were unique to GVP exposure. WS altered glutathione (GSH) levels, enhanced polyamine and pantothenate levels, likely increased beta oxidation of fatty acids, and increased phospholipid degradation marked by an increase in phosphoethanolamine. GSH, glutamine, and pantothenate showed the most significant changes with cigarette smoke exposure in A549 cells based on principal component analysis. Many of the changed biochemicals were previously reported to be altered by cigarette exposure, but the global metabolomic approach offers the advantage of observing changes to hundreds of biochemicals in a single experiment and the possibility for new discoveries. The metabolomic approach may thus be used as a screening tool to evaluate conventional and novel tobacco products offering the potential to reduce risks of smoking.

Figure 3. Effect of Cigarette Smoke Phases and Whole Smoke on Metabolic Pathways of A549 Cells.



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SMOKING RELATED BIOMARKERS OF POTENTIAL EFFECT/ HARM: CHALLENGES AND OPPORTUNITIES

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Abstract

Chronic cigarette smoking causes several diseases such as lung cancer, COPD, cardiovascular disease and oral cancer in some smokers. While smoking has been known to adversely affect multiple cellular and physiological processes, further research is necessary to understand the aberrant physiology that leads to disease in susceptible individuals following decades of smoking. Appropriate biomarkers indicating smoking effects may enhance this understanding. Ideally, the biomarkers of effect would be able to predict harm from smoking in healthy smokers, and thus identify the at-risk individuals. Further, putative biomarkers might be useful in determining whether consumption of potential reduced exposure products (PREPs) or modified cigarettes could reduce harm, or allow comparison of tobacco product categories. Therefore, these assessments should be carried out in short-term cell culture assays, animal models and clinical studies.

Notwithstanding their promise, very few potential biomarkers of effect are currently available, and several challenges remain. For example, the biomarkers need further qualification, and the methods for their quantification need development and validation. In addition, testing the biomarkers in appropriate experimental models and disease-specific clinical studies will be necessary to rigorously validate them prior to their integration into health assessments.

Current cutting-edge discovery technologies such as transcriptomics, epigenomics and metabolomics, together with a more complete understanding of chemistry and biological consequences of smoking hold promise in the discovery and characterization of biomarkers of effect. Given the complexity of smoking-induced biological changes, a diverse array of biomarkers of effect may emerge. A "fit-for-purpose" strategy may be appropriate for qualifying biomarkers and validating methods to expedite the discovery and characterization of smoking-related biomarkers of effect/ harm, and their application for assessing risk to individual consumer and evaluating PREPs.

Background

This brief article aims to provide a critical review of the current status of smoking-related biomarkers of effect/ harm. Cigarette smoking has been attributed as a major cause for developing many diseases such as lung cancer, chronic obstructive pulmonary disease (COPD), cardiovascular diseases (CVD), oral cancer and other diseases in a population of susceptible smokers (1-4). Approximately 15-20% of chronic smokers reportedly succumb to smoking-related illnesses, with about 443,000 deaths every year being attributed to chronic smoking in United States alone. For example, a vast majority of lung cancers and COPD cases are directly linked to cigarette smoking (5). Although smoking rates in the US have fallen from their reported peak of about 42% in 1964 to at or below 20%, smoking still remains a preventable cause of morbidity and mortality (4). Smoking related diseases typically manifest after several decades of smoking, and it is a challenge to predict when and which smokers could fall ill.

RJRT's view of a proper harm reduction strategy calls for: (1) For current never-users of tobacco, remain abstinent; (2) For current tobacco users, quit; (3) For current tobacco users who can't or won't quit, migrate to lower-harm alternatives. The most effective ways of reducing harm from smoking are to avoid or quit smoking. However, a large percentage of smokers are unwilling or unable to quit smoking. Therefore, consideration of alternate approaches to reduce harm from smoking is important (6). Several lines of evidence indicate that different tobacco products present varying levels of risk to users (6, 7). As proposed in the Life Sciences Research Organization (LSRO) report (7), smokers (who cannot or will not stop using tobacco) can reduce their risk of cigarette smoking-related disease by replacing cigarettes with a tobacco product with a lower risk of adverse

health effects. Smokeless tobacco (ST) products are one class of reduced exposure products which have the greatest potential for reduced harm among all the tobacco products because they eliminate the combustion products (6). Further, ST use also eliminates exposure to mainstream and environmental smoke (7). The risk for COPD and lung cancer in consumers of ST is not different from that of non-tobacco consumers, whereas the relative risk for CVD and oral cancer is reduced relative to smokers (7). Although additional research is necessary, the LSRO report concludes with high confidence that ST is a reduced-risk product compared to conventional cigarettes (7).

Earlier efforts to reduce harm by cigarette modification, such as selective filtration or introduction of "low yield" cigarettes have produced mixed results. Although the chemical analyses under the standard machine smoking conditions confirmed reduction in the yields of potential toxicants, compensatory smoking and other smoker behaviors may not have reduced exposure to smoke constituents under normal usage (8). Therefore, appropriate and reliable smoker-derived measures are critical in assessing the potential for reduced harm.

The Institute of Medicine (IOM) report, titled "Clearing the Smoke (8, 9)," and others (10) suggest that epidemiological evidence is perhaps the best measure for evaluating harm and harm reduction strategies. Along the lines of the IOM report, harm reduction may be defined as lowering total tobacco-related mortality and morbidity (8, 9). Given the long-latency for development of smoking related diseases in smokers with a sustained (often several decades) use, epidemiological evaluation of diseases as endpoints of harm reduction will take correspondingly long times and may be complicated by several factors. Hence epidemiological evaluation is unlikely to be beneficial to current smokers in terms of harm reduction. Sole reliance on epidemiology may also hamper development of Potential Reduced Exposure Products (PREPS) which are important components of any harm reduction strategy. Therefore, intermediate measures of exposure and the effects of smoking have been suggested as valuable tools in a harm reduction strategy. One class of these interim measures, in fact, is biomarkers. The IOM report (8), generated at the request of the Food and Drug Administration (FDA), advocates the development of and the use of rigorous biomarkers in the assessment of PREPs.

Cigarette smoke consists of two phases containing approximately 8000 chemicals (11), many of which are highly reactive and transient species with short-half lives. The effects of cigarette smoking extend beyond the sites of direct exposure, i.e., oral cavity and lungs (12). In view of the multitude of cellular and physiological responses to exposure to cigarette smoke and the health effects linked to smoking, chronic smoking appears to elicit systemic effects. For example, cigarette smoke is known to induce oxidative stress, inflammatory responses, DNA damage,

and cell death. Smoking is reported to adversely affect the functions of multiple organ systems of the body over a long period of time (8, 12). Thus, assessment of smoking-related health effects and identification of the events leading to a disease state is challenging.

One approach to evaluate harm reduction strategies and PREPs is to utilize smoking-related biomarkers that would indicate exposure and effect. Smoking-related biomarkers may be broadly grouped as biomarkers of exposure and biomarkers of effect. In the context of this review, the biomarkers of effect are also synonymously referred to as biomarkers of potential harm. Several researchers have reviewed the biomarkers of smoking exposure and effect (13-15) and including in-depth presentations given at recent TSRC conferences (16, 17). Identification of smoking-related biomarkers of exposure and effect is an active and emerging area of research, which holds important implications to public policy, health and the tobacco industry.

Objective

The purpose of this brief review is to discuss the challenges and the opportunities in the development of smoking-related biomarkers of effect. Hence, an exhaustive review of the current literature on smoking-related biomarkers of exposure and all potential biomarkers of effect is beyond the scope of this perspective. Further, the biomarkers that indicate exposure and effect of consumption of smokeless tobacco or nicotine replacement are not discussed, although these products are identified with lower relative risk compared with cigarettes (6-8).

Biomarkers

Although biomarkers have been utilized in routine clinical assessments and research, a brief introduction to biomarkers is provided. In general, biomarkers play a very important role in health care and claims relating to the health benefits of nutritional/consumer products. For example, biomarkers are widely used to monitor a variety of pathophysiological processes. Further, biomarkers are used in drug discovery, routine health care, prediction, prognosis and diagnosis of chronic (example, diabetes, cancer, CVD, COPD) and acute illnesses (example, infectious diseases) and monitoring responses to treatment. Some of the biomarkers that are routinely used in health care include plasma/serum measurement of cholesterol, blood sugar and blood pressure. Therefore, discovery and validation of biomarkers should consist of rigorous, established processes, and the biomarkers should be robust.

The desire to have useful biomarkers often conflicts with the demands of cost and time for developing quality biomarkers. In fact, biomarker discovery and evaluation is an evolving, comprehensive effort that spans basic, translational

and clinical research. At the request of two FDA centers, Center for Food Safety and Applied Nutrition and the Center for Drug Evaluation Research, the IOM recently published recommendations for evaluating biomarkers and surrogate endpoints (18). Notwithstanding the concerted efforts to clarify the ambiguity in biomarker definitions and improve communications among researchers (19), the IOM committee notes there is inconsistent and imprecise definition and use of terms relevant to biomarkers and biomarker evaluation (18). This review will first consider some biomarker definitions and general aspects of biomarker discovery, and later discuss smoking related biomarkers of effect.

Definitions

The nomenclature regarding biomarker discovery, assay and application is fairly well agreed upon within the biomarker research community. For example, the consensus definition of a biomarker is "a factor that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (20). A clinical endpoint is defined as a variable that measures how patients feel, function or survive; an example is death. Considerable effort was invested in defining the surrogate endpoint in regulatory processes (18). One simple definition of a surrogate endpoint is a biomarker that is intended to substitute for a clinical endpoint (20). Another definition, in the context of regulation, states that "surrogate endpoints are risk biomarkers that have been shown to be valid predictors of disease risk and therefore may be used in place of clinical measurements of the onset of the disease in a clinical trial (21)." Other related definitions are presented in Table 1. For additional information, the readers are referred to a recent publication by IOM on the evaluation of biomarkers and surrogate endpoints in chronic disease (18).

Table 1. Selected Biomarker Definitions: The following important definitions have been compiled by the IOM's committee on biomarkers and surrogate endpoints (18). These definitions are generally accepted in biomarker research.

Analytical Validation: "assessing (an) assay and its measurement performance characteristics, determining the range of conditions under which the assay will give reproducible and accurate data."
Biomarker: "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a(n) intervention." Example: cholesterol level.
Chronic Disease: a culmination of a series of pathogenic processes in response to internal or external stimuli over time that results in a clinical diagnosis/ ailment and health outcomes. Example: diabetes.
Clinical Endpoint: "a characteristic or variable that reflects how a patient (or a consumer) feels, functions, or survives". Example: death.
Fit-for-purpose: being guided by the principle that an evaluation process is tailored to the degree of certainty required for the use proposed.
Qualification: "evidentiary process of linking a biomarker with biological processes and clinical endpoints."
Surrogate Endpoint: "a biomarker that is intended to substitute for a clinical endpoint. A surrogate endpoint is expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence." Example: blood pressure for trials of several classes of antihypertensive drugs.

Biomarker development consists of defining a biomarker and its assay methods for a given purpose. The term "validation" refers to analytical method development, whereas "qualification" refers to the evidentiary process linking a biomarker to a biological process and clinical endpoints (19, 20). The FDA has provided guidance in the context of pharmacogenomic data submissions and their validity (22). Three stages have been enunciated: exploratory biomarkers, probable valid biomarkers, and known valid biomarkers. In the context of early detection of cancer, other investigators have proposed to conceptualize biomarker development in five different phases, from preclinical development to cancer control (23).

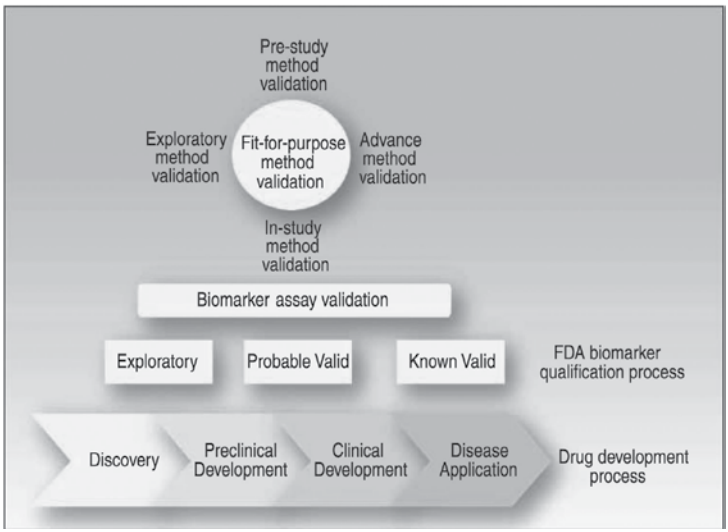
Fit-for-Purpose Strategy

The IOM committee has outlined the scientific basis for PREP assessment (8). Briefly, this paradigm incorporates performing chemical analyses of cigarette smoke, biomarker development, use of preclinical research, conducting clinical studies, and long-term epidemiological studies and surveillance. In terms of smoking (or tobacco)-related biomarkers, clear guidance on biomarker discovery,

validation and qualification is not currently available. Therefore to guide smoking/ tobacco-related biomarker development process, it is useful to consider a simple continuously evolving strategy, known as the "fit-for-purpose" approach which was advanced to support drug development (20, 24). The central idea of this paradigm is that assay validation should be tailored to meet the intended purpose of the biomarker study. This strategy conserves resources at the exploratory stages of biomarker development and characterization, while applying increased rigor of biomarker method validation in the advanced stages. This practical and iterative approach keeps the intended use of the data derived and the appropriate regulatory guidance associated with its use aligned (24).

FDA outlines three degrees in biomarker qualification: exploratory, probable valid and valid (22). As the term implies, exploratory biomarkers provide initial indications, and when suitably qualified, they will evolve to attain the status of probable valid or valid biomarkers (20, 22). Exploratory biomarkers may be generated from any of the Omic approaches (global profiling approaches such as genomics, proteomics and metabolomics) which will need to be qualified further for any potential applications. Thus, a context-based qualification process has been developed (25, 26). The general scheme of biomarker validation and qualification process is outlined in Figure 1 (20). If the biomarker is new, it is possible that appropriate methods may not exist and hence the biomarker qualification may precede the method validation process (26).

Figure 1. Biomarker discovery, qualification and method validation per Fit-for-Purpose approach, wherein assay validation is tailored to meet the intended purpose of the biomarker study (20).

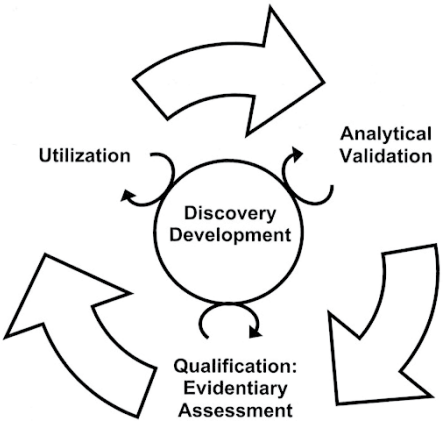


Thus, during the exploratory/discovery phase, research-grade methods may be employed to assay biomarkers. As the candidate biomarkers progressively move through the qualification process, appropriate method development needs to occur. Depending on the phase in which the biomarker is at, and the purpose of the study, consideration must be given to appropriate regulatory standards.

Some of the relevant quality standards frequently referred to in biomarker research are briefly described in the following. Good Clinical Laboratory Practices standards support human sample analysis which can be reported to medical professionals for the purpose of diagnosis and/or treatment of patients or clinical research subjects (reviewed in (27)). Good Laboratory Practices regulations and guidance define laboratory requirements which support non-human laboratory analysis for samples that are primarily derived from animal host systems. Bioanalytical guidance defines laboratory practices, which support the analysis of human research samples, which are not intended to be reported to physicians for the purpose of diagnosis and/or treatment. In any case, implementation of appropriate quality systems is critical for biomarker development.

Further, the IOM biomarker committee (18) has made recommendations on the evaluation framework and harmonization of scientific process. Briefly, two recommendations on the evaluation frame work are: 1) biomarker evaluation process should consist of analytical validation, qualification and utilization as depicted in Figure 2, and; 2) for biomarkers with regulatory impact, the FDA should convene expert panels to evaluate biomarkers and biomarker tests. On the aspect of scientific process harmonization, the FDA was advised to use the same degree of scientific rigor for evaluation of biomarkers across regulatory areas. Additional and ancillary recommendations are also discussed by the IOM panel (18).

Figure 2. Biomarker evaluation framework: Recommendation 1 of biomarker evaluation framework by the IOM committee (18). Biomarker evaluation is an ongoing process, and the steps are interdependent. Analytical validation is the process of assessing how well an assay quantitates a biomarker of interest. The biomarker qualification is further divided into evidentiary assessment and utilization analysis. These describe different processes involved in evaluating evidence (for link between biomarker, the disease pathway and clinical endpoint) and proposed contexts of use.



Smoking Related Biomarkers of Potential Effect/ Harm

Several different categories of measurements to assess PREPs have been proposed by the IOM committee charged with PREP assessment (8). They include: external exposure markers, biomarkers of exposure, biomarkers of biologically effective dose, and biomarkers of potential harm (8, 10). Smoking-related biomarkers of exposure will be discussed in detail by other symposium speakers/reviews. Here we focus on Biomarkers of Potential Harm, which are referred interchangeably with Biomarkers of Effect. The IOM committee defines biomarkers of effect/ harm as "a measurement of an effect due to exposure." These effects could encompass early biological effects, alterations in morphology, structure or function, and clinical symptoms consistent with harm, including preclinical changes. Definitions of smoking related biomarkers are provided in Table 2.

Smoking-related biomarkers are extremely valuable tools and are integral parts of the harm reduction strategy. For example, smoking-related biomarkers: 1) can be useful in predicting the onset of smoking-related illnesses in generally healthy smokers, and thus identify at risk individuals; 2) can be used in distinguishing product categories, evaluation of PREPS and other modified risk products; 3) can be employed in evaluating new products, and 4) are useful in establishing a risk continuum across tobacco products. Since, tobacco biomarker development is an integrated scientific process that spans basic sciences, such as chemistry

and biology, as well as clinical evaluations, judicious utilization of the qualified biomarkers will provide a solid scientific basis for harm reduction strategy and, most likely, tobacco regulation.

Table 2. Smoking-Related Biomarkers (from (8)): Different categories of smoking related biomarkers and their definitions provided by the IOM committee on reducing the harm from tobacco.

Exposure or Biomarker Assessment	Definition
External Exposure marker	A tobacco component or product that may reach or is at the portal of entry to the body
Biomarker of exposure	A tobacco constituent or metabolite that is measured in a biological fluid or tissue that has the potential to interact with a biological macromolecule; sometimes considered a measure of internal dose
Biologically effective dose (BED)	The amount that a tobacco constituent or metabolite binds to or alters a macromolecule; estimates of the BED might be performed in surrogate tissues
Biomarkers of potential harm	A measurement of an effect due to exposure; these include early biological effects; alterations in morphology; structure; or function; and clinical symptoms consistent with harm; also includes "preclinical changes."

Several authors have outlined the attributes and the applications of biomarkers of effect, including in PREP assessment (10, 28, 29). The biomarkers of effect, ideally, should demonstrate adequate sensitivity, specificity and other analytical parameters. Further, the biomarkers should reflect the long-term effects of smoking and disease endpoints to be qualified as surrogate endpoints. In addition, the biomarker should be assayed in a matrix that is obtained through non- (or minimally) invasive procedures. A working definition of biomarkers of harm, in the context of tobacco has been proposed by Gregg *et al* (30): "A significant, objective, measurable, alteration in a biological sample, after smoking a tobacco product, that is known to be on a pathway predictive of pathologic change, or

a surrogate for that pathway, which is altered in a proportion of smokers and is reversible on cessation of smoking." Further, the authors have summarized some of the considerations evaluating smoking-related biomarkers of effect in the following table (Table 3).

Table 3. Selection criteria for biomarkers of effect (30).

Is the proposed biomarker an early- or late-stage marker of carcinogenesis, non-neoplastic respiratory disease or coronary/vascular disease?
Is it a short- or long-term biomarker?
Is there a preferred method for its measurement and has it been validated?
Are there differences between non-, former- and current smokers and with levels of smoking in terms of cigarettes per day or years of smoking?
Which smoke constituent drives the biomarker change?
Is there a scientific consensus about the significance of biomarker alteration?
What tissue or biological fluid is sampled and does this involve an invasive procedure or a clinical stay?

A number of physiological measures (example, forced expiratory volume in one second (FEV1)) and several molecules (example, F2 isoprostanes) have been identified as potential smoking-related biomarkers of effect/ harm. In terms of the molecular nature, the biomarkers of effect/ harm are heterogeneous and not restricted to a select few biological processes. The potential biomarkers of effect/ harm are involved in regulation of inflammation, lipid metabolism, oxidative stress and DNA damage repair (8, 28), which in turn, are aberrantly regulated in smoking-related diseases such as COPD, CVD and cancer. For example, increased levels of F2-isoprostanes are reported by several investigators and are used as markers of oxidative stress. They are also hypothesized to predict the risk of coronary heart disease (31). Some of the proteins such as cytokines (example, interleukin 6, interleukin 8), adhesion molecules (example, soluble intercellular adhesion molecule (sICAM) and C-reactive protein (CRP) are attractive potential biomarkers of effect of COPD and CVD (32-34), and are currently under active investigation in clinical studies (discussed in the later sections of this article). Although Table 4 lists a relatively smaller number of biomarkers of effect (28, 30), a broader list of potential biomarkers of effect exists (8, 28) (Tables 4 and 5). Similarly, a panel of biomarkers which includes biomarkers of exposure and effect/ harm has been proposed (28).

Table 4. Short list of biomarkers of effect (30).

Biomarker	Disease association	Tissue/biofluid
8-epi-PGF2α (F2-isoprostane)	CVD	Urine
Plasma nitrate and nitrite	CVD	Blood
11-Dehydrothromboxane B2	CVD	Urine
Leukotriene B4	CVD	Blood
Plasma interleukin 6	CVD	Blood
Cardiac troponins (cTn)	CVD	Blood
Leukotriene B4	COPD	Exhaled breath
Desmosine/isodesmosine	COPD	BALF/urine
DNA damage (Comet assay)	Cancer	Buccal epithelium
Oxidized DNA repair	Cancer	Urine

Although, in general the effect of chronic smoking on physiology and metabolic pathways has been known (reviewed in (12)), numerous challenges continue to exist in the discovery and characterization of biomarkers of effect/ harm.

Table 5. Panel of useful biomarkers of exposure and potential effect for PREP assessment. The following panel of biomarkers have been hypothesized to show differences between smokers and non-smokers, change with cessation and exhibit a dose-response relationship or that respond to reductions in cigarette consumption (28). This panel also includes biomarkers of exposure, such as NNAL, cotinine and carbon monoxide.

Biomarkers	Measurement of
Cancer	
NNAL and NNAL-Glucs in urine	Carcinogen (NNK) uptake[b]
3-Aminobiphenyl, 4 aminobiphenyl, and other aromatic amine-Hb adducts	Carcinogen (aromatic amines) uptake plus metabolic activation[c]
Urine mutagenicity	Mutagen uptake[d]
Sister chromatid exchange in peripheral lymphocytes	DNA damage[c]
Nonmalignant lung disease	
Macrophages	Inflammation[c]
Cardiovascular disease	
Carbon monoxidea	Chemical uptake[b]
Nicotine/cotininea	Chemical uptake and metabolism[b]
Flow-mediated dilation	Endothelial function[b]

Circulating endothelial precursor cells	Endothelial function[b]
Fibrinogen	Hypercoagulable state[d]
Homocysteine	Hypercoagulable state[d]
White blood cell count	Inflammation[d]
C-reactive protein	Inflammation[d]
slCAM1	Inflammation[d]
Glucose-clamping studies	Insulin resistance[d]
Fetal Toxicity	
Birth weight	Outcome[e]
Neurocognitivie impairment in offspring	Outcome[e]
Maternal exhaled carbon monoxide	Chemical uptake[b]
Maternal cotinine	Chemical uptake and metabolism[b]
Maternal thiocyanate	Chemical uptake and metabolism[b]

Note: [a]Should be included in all studies as general measures of tobacco constituent uptake. [b]Biomarker for exposure. [c]Biomarker for toxicity including biologically effective dose. [d]Biomarker for injury or potential harm. [e]Health outcome.

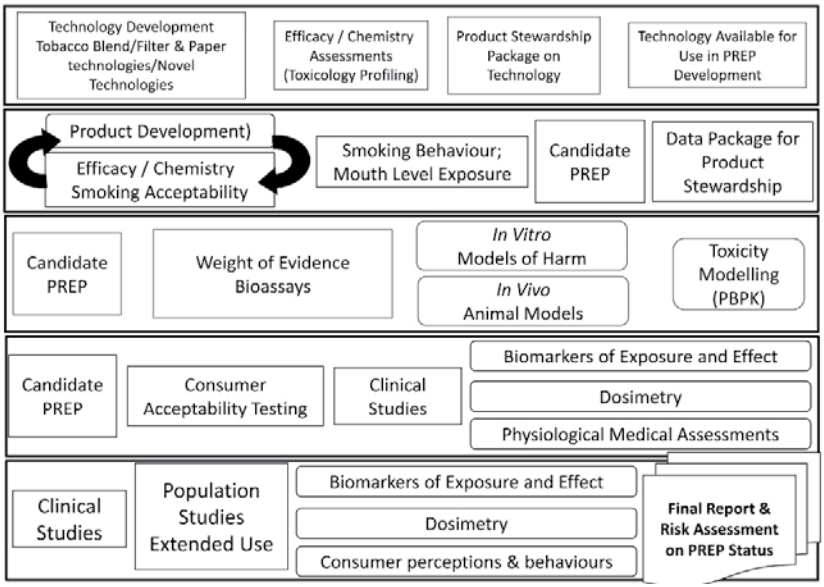
Some of the underlying challenges in identification, qualification and application of biomarkers for harm reduction include (8, 10, 29): 1) the complex chemical nature of cigarette smoke; 2) a wide spectrum of smoking associated health effects and diseases, which compound elucidation of mechanisms; 3) local and systemic effects of smoking; 4) long latency observed in the manifestation of such effects; 5) life style (example, diet, occupation etc.), gender and race of smokers; 6) inter-individual genetic variations (example, genetic polymorphisms) which may influence disease susceptibility of smokers by modifying xenobiotic metabolism through toxin-induced repair of genetic lesions, and; 7) limited availability/ accessibility of tissues for research.

Consequently, the existing potential biomarkers of effect/ harm need further evaluation in the context of smoking-related of harm reduction, under the overall principles of fit-for-purpose strategy. Improvements in methods for assaying novel and putative biomarkers of effect in appropriate biological matrices, generation of useful experimental models, innovative design and harmonization of sample collection and integration of data across clinical studies will expedite the pace of effective integration of the current candidate biomarkers and the discovery of new biomarkers of effect/ harm. A continued and targeted expansion of the scientific knowledgebase into the chemistry and biological effects of smoking is likely to move these potential biomarkers of effect/ harm along the qualification process, and support the development of tools for PREP assessment.

The IOM report, "Clearing the smoke," suggests epidemiological studies as the "gold standard" for definitive qualification measures of harm reduction (8). However, the PREPs need to be widely available to consumers for an extended period so that the epidemiological data can be collected. Moreover, given the required long time for conducting such studies, the use of surrogate biomarkers for evaluation of a reduced harm product is recommended for interim evaluations. Therefore, development of robust biomarkers of effect is a critical and integral component for predicting and reducing harm from cigarette smoking, which is also essential for PREP assessment. A general framework for PREP assessment has been presented recently (35). This suggested algorithm recommends integration of chemical analyses, *in vitro* and *in vivo* evaluation and clinical studies (35).

A similar strategy that integrates the use of biomarkers of exposure and effect in PREP evaluation has also been articulated recently by Ward and Gregg (36). This iterative, inter-disciplinary approach outlines various steps; for example, product development through clinical studies and population studies in the development and assessment of PREPs. It is important to note the integrated use of biomarkers of exposure and effect in this scheme (Figure 3). Further, this approach will require appropriate level of understanding of the chemical nature of the PREP and biological pathways which are affected by the consumption of PREP to put the ideal biomarker "in the context" of usage (18, 26), as discussed above.

Figure 3. Summary of PREP Assessment Framework (36): Biomarkers of exposure and effect are integral part of PREP assessment. The image is presented in grayscale.



Similarly, discovery and qualification of biomarkers of exposure and effect typically involve a comprehensive strategy that integrates evidence from *in vitro*, *in vivo* and clinical studies. The biomarkers of effect are expected to measure biological consequences of long-term smoking in generally healthy consumers, and predict the potential harm. The challenges of developing valid predictive markers are deemed to be greater compared to mechanistic biomarkers (26). For example, a qualified biomarker of effect should be able to detect differences in current smokers, non-tobacco consumers, former smokers and the consumers of PREPs. Further, the biomarker should be able to predict who among the smokers is susceptible to smoking-related illnesses. Thus, it is possible that a panel of biomarkers of effect, rather than a single "magic" biomarker, is likely to emerge in a successful harm reduction strategy.

Smoking-related biomarkers of effect: current approaches and challenges

Cellular studies

Cytotoxicity (cell death) assays performed in cell culture are relatively rapid, inexpensive and are useful as one of the first biological tests for evaluating potential biomarkers of effect (35). Since cytotoxicity (or cell proliferation) are cumulative endpoints of the overall growth status, these assays alone may not yield significant mechanistic information which is desired of a robust biomarker. Further, the phenotype and differentiation status of the test cells, the chemistry and dosage of the tobacco preparations employed warrant careful consideration, in addition to other well recognized limitations of cell culture studies (35). High doses of smoke preparations, such as TPM or whole smoke, are employed to determine the potential cytotoxicity in short-term assays (typically 24-72h), and these studies have provided valuable information on the acute effects of tobacco preparations (summarized in (12, 35)).

Since smoking-related pathophysiological changes manifest after sustained exposure, it is necessary to evaluate biomarkers of effect under non-cytotoxic conditions, after long-term treatment with tobacco preparations. At sub-toxic doses, where cells adapt to the effects of smoke extracts, the cell death related pathways are unlikely to overwhelm the cell physiology. Thus, cell cultures may also need to be treated with lower doses and/or for a longer time with the smoke preparations for evaluating candidate biomarkers of effect to gain insights to smoke (preparation)-induced perturbations in cell biology (example, (37)). Recently developed multi-parameter high content screening tools will be useful to evaluate the cellular effects of exposure to cigarette smoke (38).

Clinical Studies

A recent review by Hatsukami *et al* summarized the clinical trial methods for evaluation of PREPs (39). A number of potential biomarkers of exposure and

effect (listed in Tables 4 & 5) have been evaluated in some of these clinical studies. Many potential biomarkers of effect are able to differentiate smokers from non-smokers. However, the existing biomarkers of effect appear to be limited in detecting differences due to the duration of smoking, the number of cigarettes or the type of cigarettes smoked. The following examples illustrate the current status of assessments of biomarkers of effect in clinical studies.

In a pilot exploratory study, Zedler *et al* (40) investigated biomarkers of exposure and potential effect in adult smokers of lower tar delivery cigarettes and non-smokers. The potential biomarkers of effect included surrogate markers of cardiovascular disease (total cholesterol, HDL-cholesterol and LDL-cholesterol, fibrinogen and high sensitivity-C-reactive protein (hs-CRP)). Other biomarkers were urinary 8-epi-prostaglandin F2α (8-epi-PGF2 α) and 11-dehydro-thromboxane B_2 (TxB_2), which are markers of oxidative stress and platelet activation, respectively. Among these, except for LDL-cholesterol, all of the biomarkers clearly distinguished adult smokers from non-smokers. The study also noted inter-subject variability and observed gender differences in the biomarker levels.

In a cross-sectional study involving smokers (one group who smoked 10 or fewer cigarettes and a second group consisted of smokers who smoked 20 or more cigarettes), former smokers and never-smokers, Lowe *et al* (41) addressed the effect of increased smoke exposure (based on the number of cigarettes smoked per day) and the reversibility of biomarker levels in individuals who quit smoking. The biomarkers of exposure were found to be significantly different in smokers and non-smoking cohorts. Further, significant differences in the levels of biomarkers of exposure were reported between the smoker groups who differed in the number of cigarettes smoked per day. However, of the 18 biomarkers of effect tested, only 7 (TXB2, 2,3 dinor-thromboxane B2, 8-epi-PGF2 α, 8-hydoxy-2'deoxyguanosine, cis-thymidine glycol, LDL-cholesterol and total IgG) showed significant differences between smoking and non-smoking groups. While statistical differences in the levels of some of these biomarkers of effect (example, 8-epi-PGF2 α and LDL cholesterol) were detected between the two smoking cohorts, the authors note the need for conducting studies with a larger number of subjects to increase the statistical power for detecting smaller differences.

Two other publications (42, 43) reported results from a cross-sectional study that recruited healthy, moderate and heavy smokers who smoked lower tar and higher tar delivery cigarettes, respectively. The biomarkers of exposure (CO, nicotine metabolites and NNAL) were elevated in smoking groups and showed incremental increases in the group who smoked more cigarettes. However, biomarkers of effect generally revealed only subtle differences, although TXB_2, c-TG, 8-OH-dG levels were significantly elevated in heavy smokers. The markers associated with CVD,

however, generally were not consistent with the levels found in overt clinical disease (43).

To evaluate biomarkers of exposure and effect that are hypothesized to be associated with cardiovascular diseases, a cross sectional study was conducted in generally healthy, age stratified cohorts of chronic smokers, moist snuff consumers and non-tobacco consumers (44-46). A large suite of blood and urine biomarkers of exposure and effect was investigated after consumption of a tobacco product (as appropriate to the cohort), and fasting from food and tobacco for 8-10 h captured acute and chronic changes in biomarkers, respectively.

As expected, biomarkers of exposure significantly differed in tobacco and non-tobacco cohorts, and the biomarkers of combustion by-products distinguished smoker versus moist snuff-consumers. A notable finding of the study was that several biomarkers of effect detected differences in study cohorts. The levels of urinary 8-epi-PGF2 α (iPF2α), isoprostane and TXB_2 were significantly elevated in smokers relative to non-smoking subjects, further confirming their ability to distinguish smokers from non-smokers, oral tobacco consumers included. Among the blood biomarkers of effect, interleukin-12 (p70), interleukin-8, and the soluble intercellular adhesion molecule-1 (sICAM-1) provided distinction among study cohorts. However, some potential biomarkers of effect were unable to differentiate cohorts. The physiological assessments, *viz.*, flow mediated dilation (FMD) and carotid intima-media thickness (CIMT) and ankle-brachial index, were not able to distinguish the study cohorts for chronic usage, although CIMT revealed an age effect.

Since the above studies have investigated biomarkers of effect in generally healthy subjects, it is possible that the biomarkers reflected their general health state. The selected markers of oxidative stress and inflammation (example, 8-epi-PGF2 α and TXB2) are elevated indicating the underlying smoking-related changes, which may indicate early steps in the pathophysiology of smoking-related illnesses. Further, some of the known biomarkers of effect tested may not be sensitive enough to detect changes in healthy smokers, and they are confounded by other factors.

The next example illustrates the complexities and limitations of existing smoking-related biomarkers of effect. Roethig *et al* (47) reported the results of a large cross sectional study, known as the Total Exposure Study (TES), on cigarette smoke exposure among the US population across four tar delivery categories of cigarettes, and a control non-smoking group. This study enrolled 3585 adult smokers and 1077 non-smokers, and the study subjects were stratified by gender, age and BMI (48). Twenty nine biomarkers of effect, presumed to indicate cardiovascular disease, oxidative stress and COPD were assayed in the TES subjects, and the differences between the smokers and non-tobacco consumers were noted. While this large

smoking-focused biomarker study took into consideration of several confounding factors, such as age, gender, smoking duration and BMI, the study enrolled healthy subjects. Statistically significant differences were found in the levels of many potential biomarkers of effect between smokers and non-tobacco consumers in this study. Three previously known biomarkers of effect, 8-epi-PGF2 α , TXB_2 and white blood cell count emerged as the top three significant biomarkers of effect that are dependent on smoking-related variables. Interestingly for many other potential biomarkers of effect, BMI was a more important factor. Thus, while the TES provided valuable insights into smoking-related biomarkers, as discussed above, it has also highlighted the limitations of current biomarkers of effect.

For example, C-reactive protein (CRP) is a widely considered marker of inflammation, which has been reported to be elevated in smokers and is known to be associated with CVD risk (49). CRP is an acute phase plasma protein induced rapidly in response to general inflammation. In the CVD biomarker study the CRP levels were significantly higher in smokers, relative to non-tobacco consumer cohort (44, 46). The levels of CRP in the TES (48) were modestly higher in smokers relative to non-smokers; however, BMI, not smoking, emerged to be a more important contributing factor. Considering that CRP levels are influenced by multiple factors, such as gender, age, genetic factors and diet, there is ongoing debate whether CRP levels are useful as a predictive/prognostic biomarker of CVD and other smoking-related diseases (49, 50). Lipid peroxidation products, such as F2-isoprostanes, are indicators of oxidative stress and lipid peroxidation, and are considered to be potential independent risk factors for coronary heart disease (31). Since lipid peroxidation also generates numerous other products, additional research is suggested for identifying other lipid peroxidation products which could serve as important biomarkers (31), and how their levels may be altered in relation to smoking status. Thus, in view of the limited number of existing candidate biomarkers of effect, there appears to be a consensus for additional research to identify new smoking-related biomarkers of effect/ harm (6, 41, 48).

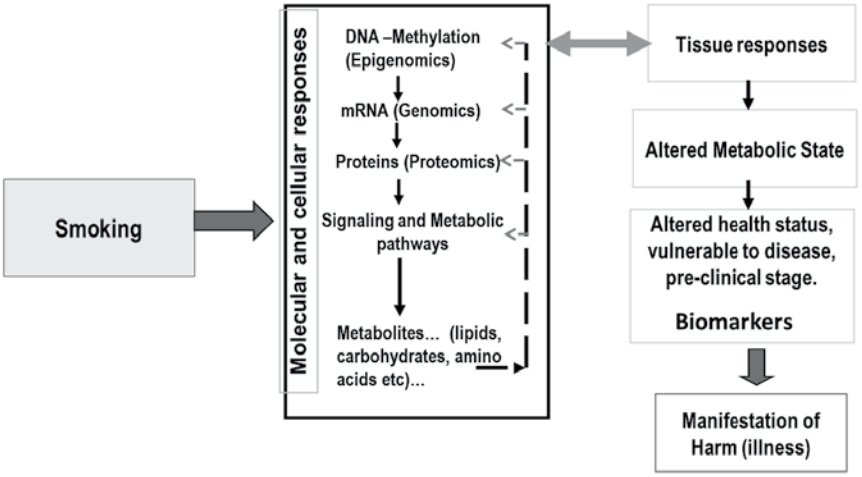
Smoking-related biomarkers of effect: new technologies and opportunities

From the foregoing, it is clear that discovery and evaluation of smoking-related biomarkers of effect is a challenging, but a critical component of a harm reduction strategy. In addition to continuing investigations into the existing panels of potential biomarkers of effect, new avenues of discovery and identification of novel biomarkers will be required to support a harm reduction strategy. The emergence of new technologies, based on global profiling, is likely to provide new opportunities in the discovery of biomarkers of effect, and consequently in reducing harm from smoking.

Considering that pathophysiological effects of smoking extend beyond local areas of exposure (oral cavity and lung) to systemic circulation through inflammatory

responses caused by oxidative stress (12, 51) (52, 53), new lines of research may lead to the discovery of novel biomarkers. For example, smoking is known to epigenetically regulate expression of many genes, and alter gene expression, as evident by mRNA and protein changes, which culminates in the deregulation of signaling and metabolic pathways. As a result, the metabolic state is perturbed, resulting in altered metabolite profiles, which in turn, could potentially contribute to further exacerbation of the effects of smoking (Figure 4). In terms of the available Omic technologies, methylation profiling, proteomics, and metabolomics are reaching the maturity of mRNA profiling and a number of fee-for service options are available. The next generation of biomarkers of effect could potentially reflect qualitative or quantitative changes in epigenetic, gene expression, or metabolite profiles/levels.

Figure 4. A simplified view of Putative Biomarkers of Potential Effect/ Harm. The potential biomarkers could include any changes in the levels of the biological macromolecules, metabolites or some alteration in their regulation. Ideally, the biomarkers of potential effect/ harm would be indicative of preclinical changes, as the changes in a disease state (illness) are likely to harbor many biological events unrelated to smoking, and hence complicate biomarker qualification process.



In addition, more subtle changes, such as differential post-translational modifications or subcellular locilazation of regulatory proteins may prove to be mechanistic potential biomarkers of effect. For example, smoke exposure induces nitrosylation (54-57) and carbamylation (58-61); post-translational modifications, such as phosphorylations, are key drivers of cellular signaling mechanisms (55, 62). Similarly, altered subcellular localization of key regulatory proteins such as kinases and transcription factors are well recognized mechanisms (63, 64), which

may add a new dimension in the efforts of discovering biomarkers of effect. Since the effects of smoke exposure on post-translational modifications and other subtle regulatory mechanisms, at this point, are primarily obtained from cell culture studies, further research is essential to determine whether they can be explored as potential biomarkers of effect/ harm.

Since, the putative biomarkers of effect should indicate the health consequences of smoking and be able to predict harm from smoking, inclusion of long-term smokers who are generally healthy and older, may be appropriate and most likely will reflect the effects of sustained exposure to cigarette smoke. Thus, conducting clinical studies exclusively in generally healthy smokers is an important first step for elucidating the effects of smoking for discovery and evaluation of biomarkers of effect/ harm. It is critical to follow up with case-control studies where the cases should consist of subjects with a smoking-related illness, such as COPD, lung cancer, CVD or other diseases, or employ other study designs (65). Study subjects with advanced diseases, however, may have undergone pathophysiological changes beyond those that are relevant for the intended purpose of discovering and evaluating biomarkers of effect. Further, various treatment regimens given to the subjects with clinically significant and advanced disease may also confound the biomarker discovery process. Therefore, careful consideration should be given to the cases.

Thus, recruitment of older subjects who are sustained long term smokers, higher risk for disease, or pre-disease states (example, COPD stage 0 or 1) may benefit biomarker discovery; inclusion of appropriate control cohorts, such as non-smokers, healthy smokers and those with early, measurable disease would strengthen the effort. Because of the complexity and enormity of these exploratory efforts, a fit-for-purpose approach, as discussed in the preceding sections of this manuscript, will be best suited in the biomarker discovery and harm reduction strategies. In fact, many researchers have embarked on an integrated strategy of interrogating candidate markers and employing cutting-edge global profiling methods in the smoking-related biomarker area. Application of toxicogenomics and other emerging technologies in elucidating mechanisms of smoking-induced health effects and biomarker discovery have been reviewed as a symposium presentation at the 2010 TSRC (66).

The Omic Approaches

It is well recognized that epigenetic modifications, particularly gene methylation and histone acetylation, are important early and reversible changes in carcinogenesis. It is also known that exposure to cigarette smoke induces methylation of tumor suppressor genes and other key regulatory genes, which may contribute to diseases such as lung cancer, COPD, CVD and oral cancer (37, 67-81). Further, inflammation is reported to induce epigenetic changes (82, 83). Identification of

gene methylation signatures that are reversible upon smoking cessation and/or predictive of disease state may lead to a category of exploratory biomarkers of effect (81, 84, 85).

Similarly, changes in gene expression have been investigated by several researchers. Particularly, recent efforts have led to a substantial knowledgebase on smoking-induced gene expression changes (86-88) (89-93). Further, simultaneous transcriptomic and proteomic profiling of bronchial epithelial cells derived from smokers and non-smokers has been performed and a good correlation between transcriptomic and proteomic data sets has been reported (90). Separately, several investigations of proteomic profiling have identified many candidate biomarkers (94-102).

Post-transcriptional gene regulation by microRNAs has also been shown to play a role in smoking-induced pathophysiological changes (103-106). MicroRNAs are small non-coding RNA species that bind to the 3' untranslated regions of mRNAs and down regulate gene expression. A more recent technology (107) that profiles small molecular weight biochemicals in biofluids, known as metabolomics, has also been applied to the discovery of smoking-related biomarkers of metabolic changes (108-110).

Omics and Beyond

The Omics technologies discussed above are powerful and useful in the discovery of smoking-related biomarkers of effect. It is relevant to consider some of the standard methods that follow global profiling experiment. The global profiling technologies (epigenetics, transcriptomics, proteomics, metabolomics and micro RNA profiling) typically identify a fairly large number of candidates as presumptive biomarkers (commonly known as "hits") in their respective classes of molecules. It is important to confirm and qualify those in separate, independent follow up studies; otherwise the "false positives" will overwhelm and complicate the discovery process. Therefore, it is important to distinguish candidate markers which are truly different among study samples/cohorts. The candidate markers (hits) obtained in the first round of global profiling (early discovery stage) may be confirmed in a second independent global profiling study using a different sample set. Alternatively, expression levels of the candidate markers may be tested using techniques that specifically measure the levels of selected candidates.

In a recent study on the evaluation of smoking-related methylation changes in peripheral blood, two different technologies were employed to discover and qualify a single candidate locus of F2RL gene. The discovery phase employed Illumina methylation arrays, whereas MALDI-ToF mass spectrometry was used in the qualification process (84, 85). Other commonly used independent techniques of confirmation of the targets for mRNA and epigenetic "hits" are PCR-based

quantitative RT-PCR and methyl-specific RT-PCR, respectively. Additional confirmation can also be derived from immunoblotting, enzyme-linked immunosorbent assay, flow cytometry or other suitable methods. Alternatively, the profiling study may be repeated in a different set of study subjects as a method of confirming the candidate genes. In any case, the candidate markers will need to be qualified by independent methods prior to investing additional time and effort.

Identification of changes in gene expression, methylation states, and metabolite levels are interesting and important early phases in the biomarker discovery process. However, it is critical to qualify those changes, recognize the underlying biological significance and link them to potential pathophysiological changes and disease mechanisms to be able to predict harm from smoking. Understanding the biology behind a biomarker is an important source of information on biomarker's relevance, specificity, and robustness (18, 111). This understanding is particularly important because qualifying a predictive biomarker is considered to be more difficult than qualifying a mechanistic marker (26).

Considering the broad effects of smoking, it is likely that the putative biomarkers of effect could be derived from a combination of genetic, epigenetic, proteomic and metabolomics signatures through concerted efforts. Indeed, a set of recent publications demonstrate the progress towards discovery of biomarkers of COPD in a multi-pronged approach using genomic, proteomic and metabolomics technologies (112-115). The authors report the discovery of a number of genes, peptides and metabolites that differentiated the study cohorts.

Future Opportunities

As discussed above, because of the diverse effects of smoking, the putative smoking-related biomarkers of effect potentially can span a wide range of physiological processes, and different types of molecules could be developed into biomarkers. While this unique situation presents multiple opportunities for the biomarker development, it requires the development of assay methods and qualifying a large number of candidate biomarkers through the biomarker discovery process (Figures 1 & 2). Further, the IOM panel on biomarker evaluations recommends rigorous biomarker testing (18). This comprehensive discovery process will eventually be a resource intensive effort. By developing collaborative partnerships through which scientific information can be exchanged freely and verified among researchers, progress in biomarker development can be expedited and valuable resources may be more effectively utilized, which will ultimately enhance the probability of success.

An excellent example for successful collaborative efforts in biomarker research is the Predictive Safety Testing Consortium (PSTC) (116). The PSTC is a collaboration of public and private partnerships. Under the advisement of the FDA and European

Medicines Agency, they share and validate each other's safety testing methods. The members of the consortium share internal experience with pre-clinical and clinical safety biomarkers in six workgroups: carcinogenicity, kidney, liver, muscle, vascular injury, and cardiac hypertrophy. Several new promising biomarkers of kidney injury were qualified by the nephrotoxicity working group (117) and have been highlighted in the May 2010 issue of Nature Biotechnology (116).

In addition to molecular and cellular biomarkers, other types of measures, such as Patient Reported Outcomes (PROs) could also be useful in assessing the effect of smoking. For example, in cooperation with the FDA and Critical Path Institute, Patient-Reported Outcome Consortium was formed for the purpose of developing, evaluating, and qualifying PRO instruments with the FDA for use in clinical trials designed to evaluate the safety and efficacy of medical products. (118). Thus, appropriately designed specific and sensitive PRO instruments could provide valuable assessment of PREPs, and could contribute to harm reduction strategies. In fact, the usage of various PROs (termed scales) in tobacco research has been reviewed recently (119).

Summary and Conclusions

In summary, a better understanding of smoking-induced physiological, cellular and molecular changes, combined with the application of new technologies and qualification of biomarkers through the fit-for-purpose approach, should markedly expedite the discovery, qualification and application of smoking-related biomarkers to reduce the harm from smoking. The data obtained from measuring biomarkers of effect ideally should reflect/relate to that obtained with biomarkers of exposure. Further, the biomarker qualification process should include post-market surveillance studies to measure the effect of sustained exposure in "real world" situations.

This short review article discussed wide ranging aspects of biomarker discovery and application in reducing smoking-related harm and PREP assessment. The main conclusions of this article are:

1. The use of biomarkers will be a key component of harm reduction strategies and PREP development.
2. There are limited numbers of potential biomarkers of effect available at present. Thus, there is a clear need for new biomarkers to assess health effects related to tobacco use, and in evaluation of new products.
3. Biomarker discovery, qualification and utilization require an integrated, iterative and methodical approach.
4. New approaches, such as global profiling techniques, are powerful tools in the discovery of novel biomarkers of effect/ harm. The emerging biomarkers are likely to consist of metabolites, nucleic acids (example, genomic, epigenomic and RNA) and proteins, and PROs.

5. Achieving a better understanding of the biological changes arising from exposure to cigarette smoke and investigating potential biomarkers of effect in the context of smoking-related diseases will realize the overall goal of harm reduction.

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A SCIENTIFIC FRAMEWORK FOR THE ASSESSMENT OF MODIFIED RISK TOBACCO PRODUCTS

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Abstract
Given that tobacco is a leading cause of morbidity and mortality the development and assessment of potential reduced-exposure products (PREPS) or modified risk tobacco products (MRTPs) is a research imperative. To assess the potential impact of a MRTP on both individual and population health risks will require a wide range of pre-clinical, clinical and pre- and post marketing studies, with evaluations staged over time. Pre-clinically this would involve chemical, physiochemical and biological characterisation (for example, through *in vitro* models of disease) and behavioural studies. The clinical phase would assess the impact of product switching to biomarkers of exposure and of biological effect, while a pre-marketing phase would assess likely tobacco user and non-user reaction to the product and any claim made. PMS would evaluate both the longer term effect on individual health status and population dynamics of users and non-users. At each stage a weight-of evidence approach should be adopted. This paper describes the current state of science in being able to undertake such an assessment and identifies research needs.

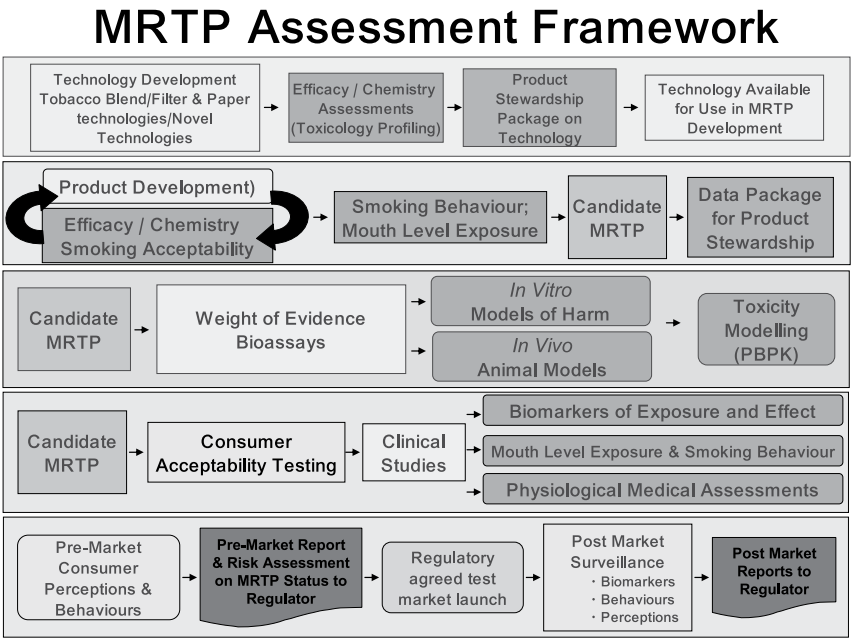
Introduction
Many research groups are working on developing a range of scientific tools that could help assess whether "modified risk tobacco products" (MRTPs) could help reduce individual and population health impacts of tobacco use. We have a scientific research programme which seeks to develop a range of new tobacco products that might substantially reduce health risks related to tobacco use. As part of our efforts we are undertaking a wide range of research that we hope will contribute to the scientific evidence base which should aid the development of a framework for assessing MRTPs or, as a previous committee of the US Institute of Medicine (IOM) termed them, "Potential Reduced-Exposure Products" or PREPs (1).

We have been developing a framework to guide us in the type of research that we believe needs to be completed in order to allow accurate scientific assessment of a candidate MRTP. The concept behind this framework was to set out both the types of scientific test that can be performed today and to look forward in time and assume that a variety of additional tests had become available to validate a

reduced-risk profile for a candidate product. We then sought to determine the scientific and other evidence that should be provided to appropriate regulators. Our framework takes into account criteria discussed in the IOM's Report (1), and we have sought to produce a practical guide for implementing an assessment of a candidate PREP/MRTP. An early version of this framework was presented at the 14th Annual Meeting of the Society for Research on Nicotine and Tobacco in Portland in 2008 (2), and an evolution of this framework was presented at the CORESTA Congress 2010 (3).

Our current framework is shown in Figure 1. This paper sets out our approach, and makes recommendations for additional research.

Figure 1: A scientific framework for the assessment of candidate modified risk tobacco products.



Product Composition

We believe that manufacturers should provide detailed information about the composition of a candidate MRTP. This should include information on the list of product characteristics being considered by the World Health Organisation in the context of the Framework Convention on Tobacco Control (4). However, we also believe that details should be given of any product modifications that have been included in the candidate product with the purpose of having the potential

to modify the risk profile of the product. Examples of this approach include our recent publications on toxicant reduction technologies (5, 6, 7). Information on the stability of the technologies and the product against defined performance criteria should also be give. For example, technologies with adsorption properties included in filters to reduce toxicant levels in smoke may reduce in adsorption efficiency over time.

Chemical characterisation - tobacco products involving inhalation
Studies of MRTPs that produce smoke, or any form of aerosol expected to be inhaled, should include research on the chemical contents of the smoke. Smoke chemistry studies should be conducted for three main reasons:

- to demonstrate lower levels of toxicologically relevant constituents in the smoke from a product intended for human exposure;
- as part of a toxicological profiling assessment for a toxicant reduction technology prior to its incorporation into a finalised product design; and
- establishing whether the modified product generates new constituents, or modifies the smoke emissions in a way that raises toxicological concern.

It is well established that machine smoking data do not accurately reflect the relative quantities of smoke taken by individual smokers (8), and are therefore of limited value in assessing human exposure to toxicants. Clinical studies have proven of great value in establishing exposure to toxicants amongst users of tobacco products. However, the number of toxicants for which validated and useable biomarkers of exposure have been identified is relatively small. In contrast, machine smoking methods have been used historically to rank and compare different products under controlled conditions, and analytical methods are available for a considerably wider range of smoke constituents (9). We therefore believe that machine chemistry data if used in an appropriate way has the potential to provide valuable insights into the characterisation of an MRTP.

(a) Which harmful and potentially harmful smoke constituents should be measured?

There are a considerable number of toxicants in tobacco smoke and scientific knowledge has yet, with any degree of certainty, to determine which toxicants are the most important in relation to the various diseases caused by tobacco use. A number of researchers have compiled lists of toxic constituents (*e.g.* Hoffmann & Hoffmann (10, 11, 12), Fowles & Dybing (13), Rodgman & Green (14), IARC (15), Rodgman & Perfetti (16), the FDA Tobacco Products Scientific Advisory Committee (TPSAC) (17), and Talhout *et al.* (18)). These lists were created for a variety of purposes and so it is not surprising that they vary in both the number of constituents and the identities of the smoke constituents. Furthermore, quantitative predictions of the incidence of smoking related disease from these

lists of prioritised toxicants significantly underestimate the observed incidences in population surveys (13, 19). Consequently, at the current time, it is necessary to measure a wide range of smoke toxicants when seeking to characterise the smoke composition of an MRTP. However, despite these uncertainties, we believe that there are certain fundamental approaches that can help define which smoke constituents should be examined.

First, a number of constituents found in cigarette smoke are included in the lists of chemicals evaluated and categorized by IARC (20) and the USEPA (21). For example, IARC has categorized over 900 chemicals, complex mixtures, physical agents, biological agents, occupational exposures and lifestyle factors, including compounds found in cigarette smoke. On a less extensive level, a number of compounds found in smoke have been identified as respiratory or cardiovascular toxicants. This approach, together with some other considerations, has been used by TPSAC (17) as the basis of identifying an initial draft list of harmful or potentially harmful constituents of smoke. This approach may be of use in defining constituents that should be measured for MRTPs, though we recognise that validated analytical methods are not currently available for some of these constituents.

Second, we believe that there is value when considering MRTPs in taking account of the list of smoke constituents required for annual disclosure by Health Canada (22) and ANVISA (23) in Brazil. The rationale for this is two-fold. First, methods for the analyses of these species in smoke are widely available (9), and some of these methods are internationally standardised. Second, there exists a substantial database of published yields in the literature for these species under ISO 4387 (24), Massachusetts (25) and Health Canada Intense (26) smoking regimes. These data sources provide a useful comparator with which to assess the yields of smoke constituents from MRTPs.

(b) Developing standardised analytical methods

Whichever smoke constituents are considered appropriate in studying MRTPs, the analytical challenges should not be underestimated. The cigarette smoke toxicants described above are present in ranges from milligrams (*e.g.* CO) to femtograms (210-Po) per cigarette. Cigarette smoke contains over 5300 identified but not necessarily quantified constituents (16), and probably a much greater number of unidentified constituents (27, 28). Given the complexity of the smoke matrix and low yields of many smoke toxicants it is essential that validated analytical methods are used to measure toxicant yields. Ideally, internationally standardised validated methods would be employed. However, at the current time the number of methods that have achieved this status is relatively small.

Hence, it may be necessary to consider data from analytical methods that have been developed within a single analytical laboratory; however, we believe that data should only be accepted from methods developed and validated according to minimum standards of analytical excellence (*ie* IUPAC (29)), developed in laboratories operating under an effective quality management system. The methods should demonstrate robustness, selectivity, consistency and be quantitative over the range of yields relevant to cigarette smoke. We believe that details of the analytical methodology and the validation data should be made available as part of the submission documentation, in order that the quality of the method used to determine the yields can be critiqued and that independent verification of smoke yields can take place if necessary. Kentucky Reference product data, analysed at the same time as the MRTP, should also be disclosed to allow inter-laboratory variability parameters to be established.

(c) Choice of smoking regime

Three smoking regimes are currently in use for regulatory reporting purposes. The ISO regime (24, 30) is widely used in the European Union and elsewhere; the Commonwealth of Massachusetts and the State of Texas mandate use of a more intense method with 50% filter ventilation blocking (25) whereas the Canadian Federal government (26) requires smoke yield testing to be conducted using both ISO4387 and a "maximum emission" testing protocol (with 100% ventilation blocking) intended to "provide data that reflects the emissions that are actually available to the consumer" often referred to as Health Canada Intense (HCI).

The ISO regime for machine smoking cigarettes generally underestimates the mean yields of smoke obtained from cigarettes by smokers. We (31) and others (32) have reported that the HCI regime generally overestimates most human behaviour or mouth level exposure (MLE) to smoke constituents.

We have conducted a comparison of mouth level exposure (MLE) to tar and nicotine from smokers of cigarettes covering the ISO yields available in cigarette products on the German market with yields from the same cigarettes when smoked on smoking machines, using the ISO, ISO/TC126 WG9 Option B (33), Massachusetts regime (25), HCI (26), and using the Kozlowski and O'Connor compensating regime (34). The results of this comparison showed that the Massachusetts regime provided data that were most similar to mean MLEs for those products smoked by German smokers. The "Compensating" and ISO TC126 WG9 Option B regimes provided data that were similar to MLEs for some products but the HCI regime over-estimated most mean MLEs.

We have presented data from a clinical study (35) during which we compared 24 hour urinary levels of a number of biomarkers of exposure ("BoE") to machine

measured smoke yields from five types of low ISO tar cigarettes tested under ISO, Health Canada Intense, ISO/TC126 Working Group 9 Option B, and a smoking regime equivalent in parameters to HCI but with ventilation unblocked. Strong correlations were identified between average 24 hour urinary biomarkers of exposure to toxicants and the product of cigarettes smoked per day and machine yields of toxicants - suggesting that machine smoke yields measured under an appropriate regime, together with mean smoking consumption data, can be used successfully to estimate mean smoker exposure to toxicants. Overall, the strongest correlations were obtained using measurements of smoke yields generated using the smoking regime "Option B" proposed by ISO/TC126 Working Group 9. Importantly the correlations with WG9B machine yields were stronger than other regimes for volatile smoke toxicants; constituents whose yields are strongly influenced by the level of ventilation in a cigarette. Stronger correlations were also obtained between BoE levels and machine yields than between BoE levels and ratios of yields to nicotine; however, the limited range of nicotine yields in this study weakens this latter analysis.

It therefore appears that two alternative approaches can be advocated for the choice of smoking regimes used to generate and measure smoke yield data for MRTPs. The first is use of a bracketing approach wherein two smoking regimes are employed, one such as ISO which on average underestimates smokers' exposure to cigarette smoke, and one such as HCI which on average overestimates smokers exposure. This approach, which has been proposed by the TPSAC (17) is reasonable in this context as it covers much of the range and extremes of human exposure. The approach is limited, however, in that one tool for the reduction of smoke yields, filter ventilation, is eliminated in the HCI regime and hence may produce unrealistic data particularly with gas-phase constituents. A second approach would be to use a regime that approximates average human exposure for a population. This approach is simpler to interpret but is currently not viable, as despite indications that regimes featuring 50% ventilation blocking provide good indications of relative human exposure to a range of smoke toxicants from different products, this work has yet to identify an optimum set of parameters relevant to the range of cigarette designs and smoking behaviour likely to be encountered on a global basis.

(d) Other analyses

Sidestream smoke analyses of the MRTP help to more fully characterise emissions from the product. Sidestream emissions measured using the ISO regime generally provide higher yields than intense regimes such Health Canada Intense, and we believe that sidestream yields measured under ISO are sufficient to characterize emissions from a MRTP.

As part of the toxicological evaluation of a toxicant reduction technology, we recommend assessing unintended changes in smoke characteristics arising from the product changes. The nature of the changes to the tobacco product should define the scope of the smoke chemistry assessment. We recommend that smoke profiling methods are used to identify any new smoke species that result from the product technologies. Examples of these profiling methods could include particulate and vapour phase chromatographic sweeps; we have established methods for these analyses and are working to extend their capability as analytical technologies advance. Profiling techniques are rarely quantitative or reliable to the same degree as dedicated analytical methods, and further specific analysis and quantification may be required to establish the yields of any newly introduced compounds resulting from the product changes.

(e) Comparator products and benchmarking against existing products

A challenge in establishing reductions in exposure to toxicants is identification of appropriate comparator products. With extended chemical analysis, *in-vitro* assays, *in-vivo* animal testing, and clinical studies it is necessary to identify a small number of relevant control products.

Comparator products may comprise scientific controls, Kentucky reference cigarettes, one or more commercial products of similar (or different) machine tar or nicotine yields, or ultra-low ISO tar products. Scientific controls are of greatest use in assessing the effectiveness of a toxicant reduction technology, but are of questionable relevance to consumer intake from real-world products; there are also challenges in identifying appropriate scientific controls when multiple design features are used simultaneously. Use of Kentucky reference products also do not reflect consumer intake from commercial products, but have utility in examining analytical method performance. Commercial products with similar smoke yields enable a direct assessment of changes relevant to a particular consumer group, and help identification of an appropriate subject group for clinical trials; however, the conclusions from the comparison may be difficult to extrapolate to a wider population. Finally, comparison to ultra-low tar products may be a useful comparator to extremes of conventional cigarette design, but such comparisons provides only limited information about performance characteristics relative to the majority of conventional cigarette products. We recommend that appropriate commercial products are used as the control or reference products for pre-clinical studies, as they provide data relevant to the consumer groups to which the product will be marketed or from which clinical subjects will be sourced.

An additional approach can be used to more fully establish the extent to which cigarette-like MRTPs offer reduced machine yields in comparison to a wide range of commercial cigarettes. For example, toxicant yields from the cigarette-

like MRTPs can be directly compared with published smoke yield data from commercial cigarettes obtained under the same smoking regime. Reasonably extensive published datasets exist under ISO (Department of Health Australia and Ageing (36), Gregg *et al.* (37), Health Canada (38), Counts *et al.* (39), Borgerding *et al.* (40)), and Health Canada Intense regimes (Department of Health Australia and Ageing (36), Health Canada (38), and Counts *et al.* (39)). While extensive, the currently available published datasets are unlikely to fully reflect the range of cigarette products on-sale globally; in the future it is likely that further information will be published and increasing datasets will broaden the scope of the comparator datasets. Comparisons may be made either on a toxicant-by-toxicant basis or by totaling the yields from all toxicants on an absolute or normalized basis to give a total toxicant yield. Such comparisons must be treated with caution due to the known difficulties based on limited standardisation between laboratories for the analysis of smoke constituents other than NFDPM, nicotine and CO (9, 37, 39), nevertheless they provide a valuable perspective on the reduced yields from the cigarette-like MRTP. We have recently submitted for publication a paper exemplifying the use of these techniques to contextualise the machine smoke yields from a reduced toxicant prototype product (41).

(f) Variability of products over time

There is considerable variability in the composition of the base material for any cigarette-type MRTP, the tobacco. As an agricultural crop the chemistry of tobacco leaf will depend on many factors including the tobacco variety, soil content, climatic conditions while growing, use of fertilizers and the method of tobacco curing (42). Additional variability may be introduced during both manufacture of products and with the use of additional materials such as charcoal in filters. In our view sufficient testing should be performed to assess the likely variability in key toxicants both pre- and post-marketing.

Chemical characterisation: Smokeless tobacco products

Smokeless tobacco products cover an extremely wide range of tobacco compositions, product configurations, manufacturing techniques, additives and patterns of use (43, 44, 45). Many scientists have attempted to group this broad range of products into a collective entity; such an approach fails to acknowledge the fundamental differences between the different product styles. For example, the use of some smokeless tobacco products types from South Asia and the US is associated with oral cancer (44) while in Sweden substantial epidemiology finds no increased risk for oral cancer amongst snus users (45). These products also have quite different chemistries, for example, Swedish style snus typically contains much lower levels of tobacco-specific nitrosamines (44).

(a) Chemical analysis

In the case of smokeless tobacco products therefore, chemical analysis of their composition may provide an important contribution towards understanding their potential toxicities. Several thousand compounds are present in tobacco (16), and a number are carcinogens or toxicologically active (44).

A number of groups have identified constituents of smokeless tobaccos which may be of toxicological concern. The FDA TPSAC identified a draft proposed list of over 40 constituents of smokeless tobacco products covering a range of chemical groups (17). IARC, in Monograph 89 reported 28 chemical agents or carcinogens in smokeless tobaccos (44). Swedish Match have created a quality standard ("GothiaTek") limiting the levels of 12 compounds in snus (46) and the European Smokeless Tobacco Council (ESTOC) have evolved this quality standard into an industry standard for European smokeless tobacco products (47). The WHO Study Group on Tobacco Product Regulation (TobReg) has proposed limits on the levels of (NNN+NNK) and benzo[a]pyrene in smokeless tobaccos (48).

We have investigated the chemical profiles of different contemporary smokeless product types from the US and Sweden, and have examined these products for the presence and levels of approximately 100 chemical substances (49, 50, 51, 52, 53, 54, 55). Our studies have shown significant differences across these product types amongst these constituents, and a range of constituent levels from milligram to not detectable for many of those constituents.

We recommend that the GothiaTek standard (46) is a starting point for the contents of modified smokeless tobacco products. Given the broad range of product styles encompassed by the smokeless tobacco descriptor, the GothiaTek standard can only be a baseline position for further chemical analysis depending upon the characteristics of the product.

(b) Stability during storage

A further consideration is the stability of the smokeless tobacco product over the period from manufacture to consumption. Studies (44) have shown that the nitrosamine contents of some US smokeless tobacco styles increases during several weeks' storage at ambient temperatures, and in Sweden many products are refrigerated until they are sold (46). We recommend that chemical stability data are included in the product characterisation analysis for smokeless tobacco products.

Regulatory Toxicology

As a complementary approach to chemical characterisation of cigarette-like

MRTPs we would suggest data are also collected using a standard package of *in vitro* tests for genotoxicity and cytotoxicity. A number of standardised *in vitro* tests are now available, and they are widely used in different industrial sectors. They have an important regulatory role in risk assessments, especially in detecting potential carcinogens, though have currently limited use in the assessment of relative risk.

International guidelines (56, 57) recommend *in vitro* genotoxicity tests for chemicals and pharmaceuticals. These include the Ames test (strains TA98, TA100, TA102, TA1535, TA1537 +/- S9), the micronucleus test (V79 cells) and the mouse lymphoma assay (L5178Y cells). The results obtained from these *in vitro* tests are used to characterize the genotoxicity of particulate matter collected from tobacco smoke.

It is important when deploying these assays with cigarette smoke to ensure that the experimental approach is validated for the matrix under examination. We are engaged in a method development programme to support the use of *in vitro* genotoxicity assays with cigarette smoke. For example, the stability of tobacco smoke particulate matter when stored frozen at -80°C has been assessed (58). The discriminatory power of the *in vitro* genotoxicity assays has been defined and the numbers of concentrations/replicates used within the assays has been optimized (59).

We are also developing methods to expose cultured cells to tobacco smoke aerosol and its volatile constituents with a longer term view to assess the genotoxic potential of the whole smoke aerosol rather than just the particulate matter. Preliminary data on the effects of different smoking regimes has been presented (60). We are part of a CORESTA *In Vitro* Task Force which works co-operatively to measure and to reduce the inter-laboratory variability of these methods.

Methodology has also been developed to assess the genotoxic potential of extracts of snus tobacco. In this case, extracts of tobacco have been prepared using the medium of artificial saliva and a suite of genotoxicity/cytotoxicity assays have been employed to test the extracts (61).

Although we do not routinely use *in vivo* toxicology tests, where deemed necessary we would consider a standard 90 day rat inhalation protocol is undertaken, essentially as specified in OECD Technical Guideline 413. All such *in vivo* toxicology tests would be conducted by external GLP-accredited Contract Research Organisations.

Computational Toxicology

As previously mentioned, tobacco smoke is a complex mixture of over 5300

identified chemicals (16, 62) of which at least 150 are known to have specific toxicological properties and can be termed 'tobacco smoke toxicants'.

From the perspective of potential regulatory frameworks and product modifications aimed at tobacco harm reduction, it would be advantageous to identify which of these tobacco smoke toxicants are the most important in relation to each of the diseases caused by cigarette smoking, and what dose response relationships are likely (and hence what size of reduction is necessary to have the potential to reduce risks). Dose metrics of individual toxicants can be generated through the use of predictive computational models, which can make use of biomonitoring and dosimetric data. By integrating all relevant information, it may then be possible to develop a risk assessment paradigm that is appropriate for the assessment of MRTPs.

We believe that there are three research strands that need to be progressed in this area; margin of exposure, mode of action and physiologically based pharmacokinetic modelling.

(i) Margin of Exposure.

The current trend has been to develop a risk assessment strategy which can be used to rank tobacco smoke toxicants on the basis of their impact on human health. One approach is to take the concept of Margin of Exposure (MOE) developed by the European Food Safety Authority (EFSA) for genotoxic and carcinogenic compounds and examined the applicability of this approach to tobacco smoke toxicants.

The MOE approach has been examined by the European Commissions' Scientific Committee on Emerging and Newly Identified Health Risks and the Scientific Committee on Health and Environmental Risks and was selected as the preferable method for prioritisation of measures to reduce risk in certain circumstances (63). Whilst the MOE procedure was initially established for use with genotoxic and carcinogenic compounds in the area of food (64, 65), the procedure is also being applied within other areas of risk management and other industries, such as risks associated with pesticide use (66), as well as to non-cancer risks (63, 67).

The MOE methodology uses a reference point, or point of departure (POD), calculated from available toxicology data corresponding to a daily dose that causes a low but detectable increase in tumour incidence. This dose is then divided by an estimate of human exposure to yield a dimensionless ratio known as the MOE (68).

MOE = POD / Estimated human intake.

According to the guidelines developed by EFSA, MOEs are made up of the lower 95% confidence interval of a 10% benchmark dose (*i.e.* the recommended POD), divided by the estimated human intake. In our application of the MOE methodology, an upper boundary estimate for human intake of a toxicant is estimated from Health Canada Intense machine-smoking yields (36), multiplied by a value for the average number of cigarettes consumed in a single day, divided by the average daily human default breathing rate (3 13, 69).

In the context of food safety assessment, MOE values >10,000 "might be considered a low priority for risk management actions" (64). To determine the consistency of conclusions based on MOEs it is possible to employ the concept of using all available toxicological studies to generate PODs for each of the data sets, thus giving rise to a range of MOE values. The estimation of MOEs for individual tobacco smoke toxicants represents a first step towards assessing the biological relevance of reductions in toxicant yields from MRTPs or identifying the toxicants for which significant reductions in yields may reasonably be expected to reduce the risk of one or more specific diseases or other adverse health effects, although the development of more complex and physiologically relevant risk assessment paradigms is recommended.

(ii) Mode of Action.

Tobacco smoke is a highly complex mixture and as such any risk assessment technique that may be used to investigate groups of toxicants may be particularly relevant. Certain chemical classes of compound, *e.g.* aldehydes, that are found in tobacco smoke may well have common modes of action and therefore the MOE approach described here could be further refined to incorporate groups of such structurally (and toxicologically) related chemicals leading to the concept of a group MOE. To proceed with such a model requires careful consideration of the mode of action for groups of structurally related chemicals together with a more rigorous evaluation of the tissue dose for individual chemicals. Mode of action reviews can be based on the current framework as proposed by the International Programme on Chemical Safety (IPCS)(70).

A mode of action (MOA) is described as being "a biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data" (71). It requires the description of key events, which must be measurable and ultimately result in a carcinogenic or non-carcinogenic endpoint. MOAs differ from mechanism of action in that a detailed description of the molecular basis for an effect is not requirement of a MOA.

In 2007, the IPCS published a framework for constructing MOAs for both carcinogenic and non-carcinogenic compounds (70). We believe, the MOA

framework is a useful tool to systematically evaluate available data (a weight of evidence approach) on a specific response (also known as a key event) to a chemical in a transparent manner. The framework as described by the IPCS is constructed from the key headings (70):

- Postulated MOA – what are the theoretical events leading to the final endpoint of interest?
- Identification of the key events – what are the key events which are critical to the development of the final endpoint. The assessor should ensure that the body of evidence supporting these key events is consistent.
- Dose-response relationships – it should be possible to evaluate the available dose-response relationships according to the Bradford-Hill criteria and subsequently correlate the dose-response with the incidence of the key event.
- Temporal association – key events should occur prior to the final endpoint and the temporal relationship between the two should be evident.
- Strength, consistency, and specificity of association of tumour response with key events – the weight of evidence linking the key events to interim lesions prior to the formation of the final endpoint should be discussed.
- Biological plausibility and coherence – this section may be of particular interest if the weight of evidence supporting the key events and the final endpoint are not in the species of interest.
- Other modes of action – discuss if there are any other plausible MOAs available for the compound of interest.
- Uncertainties, inconsistencies, and data gaps – should be discussed for each of the key events.
- Assessment of postulated mode of action – in this section the confidence in the postulated MOA should be discussed.

MOA reviews can be used as a method for hazard characterisation within any given risk assessment methodology, and therefore may be of use in assessing MRTPs. In particular, they can be used to inform the quantitative assessment of risk to humans and may (where possible) be used to understand how similarly acting chemicals can be grouped together, which may aid the process of developing a mixture based risk assessment (*i.e.* using a combined MOE methodology, as described previously). In particular, MOA reviews may be utilised when considering the development of physiologically relevant models.

(iii) Physiologically Based Pharmacokinetic Modelling.

Physiologically based pharmacokinetic (PBPK) modelling is a mathematical technique for predicting the absorption, distribution, metabolism and excretion (ADME) of a compound in humans and other animal species. A PBPK model is

made up of a series of discrete compartments, relating to different organs or groups of similar tissue types with approximations of volumes. These compartments are connected by blood flows and (where possible) metabolism pathways are included.

The model itself is a series of equations, which account for parameters such as time of exposure, blood flow rates and partition coefficients for the compound of interest in different tissues. Each compartment has its own set of mass-balance differential equations which represent biological processes. Each set of equations are then solved by using tissue time-course concentrations of the compound of interest and its metabolites (72).

PBPK modeling has the potential to add significantly to a risk assessment through a number of advantages. It can help to give access to internal body concentrations of a compound (and/or its metabolites) of interest, in particular generating detailed information regarding tissue interactions, which can aid the estimation of the dose of a compound seen in the tissues of interest. Tissue doses which align closely with tissue responses are called dose metrics (73). It is these dose metric values which can then be used in the dose-response section of a risk assessment, enabling a more realistic view of exposure. PBPK modeling can also be important when there is little to no human data available. Experimental data can be used to optimise an animal based PBPK model, from which it may then possible to extrapolate to a human exposure. The differences between experimental and epidemiological data sets may not just be due to variations between species (weight, metabolic capacity, etc), but also between the range of doses required to result in a particular endpoint or lesion of choice. Even if enough epidemiological data is available, there may still be differences in exposure duration and route of administration between the modeled data sets and the exposure scenario of interest and again PBPK modeling can be used for these extrapolations.

The profile of PBPK modeling has increased over recent years, as alternatives to the use of animals are considered. A review of the technique has been undertaken by the IPCS as part of the harmonisation project (74). In the same manner as with the MOA framework, a set of guidelines relating to "Principles of Characterizing and Applying PBPK Models in Risk Assessment" have been developed. The application of these guidelines should ensure a consistent approach across the risk assessment community.

In vitro models of disease

We believe that there is an important role for *in vitro* models of disease not only for the pre-market evaluation of MRTPs but also to help develop a better understanding of mechanisms of disease related to smoke exposure and to cross-evaluate disease-related biomarkers. We accept that the state of scientific art in this

field is still in a developmental stage, but believe that any assessment framework of MRTPs should include the possibility of acquiring useful data from *in vitro* models of disease. On-going research seeks to develop and validate appropriate *in vitro* models to form part of a framework for assessing MRTPs, to generate rapid, initial data that will give general insight into the biological effects of test products and materials as part of a weight of evidence approach, and into disease mechanisms.

In vitro models of disease can be considered to include a battery of tests that examine various cell types and modes of action associated with smoking-related disease processes including inflammation and oxidative stress linked to lung cancer, chronic obstructive pulmonary disease (COPD), and cardiovascular disease (CVD). In general we investigate changes in disease related mediators in response to cigarette smoke at the protein and gene level using the multiplex ELISA (Meso Scale Discovery®) platform, RT-PCR, Superarray and Affymetrix® Gene Chip technologies.

(a) In vitro model validation

In vitro models should be demonstrated to be fit for purpose through internal, and ultimately external validation. Validation criteria for *in vitro* tests have been developed by three international validation agencies: the Organization for Economic Cooperation and Development (OECD), the European Centre for the Validation of Alternative Methods (ECVAM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). These three agencies have worked together to harmonize validation criteria so that there are no major differences between them. Ultimately acceptance of validated tests should result in the development of an OECD Test Guideline (TG) and appropriate regulatory approval. However, many of the *in vitro* models of disease are not currently validated and a number of standards need to be established.

One approach to model development and internal validation includes:

- Literature reviews to provide a scientific rationale for model selection and inclusion of new or major advancement in model development, cell lines, comparison of published protocols, and study of any available commercial kits.
- Determination of the mechanistic relevance of the *in vitro* test endpoint defined relative to effects observed *in vivo*, including cell type and expected response including changes in morphology, protein or gene expression of disease specific markers, including chemokines, cytokines, adhesion molecules, matrix remodelling factors and transcription factors.
- Experimental methods developed to measure key disease endpoints/

biomarkers and tested with a small number of appropriate positive and negative control agents to establish responsiveness and sensitivity. Smoke from a reference cigarette, 3R4F and/or any other reference cigarette is tested. As an initial proof of concept a dose response for cigarette smoke is generated and characterized alongside a common positive control agonist.
- Assessment of suitability of the *in vitro* model by the generation of robustness and repeatability data with a series of test agents including reference cigarettes and cigarettes with different major design features.
- External peer-review following the publication of the development, 'internal validation' and application of the *in vitro* model.
- Scientific engagement with external scientists in relevant fields to confirm or suggest mechanisms or biomarkers of disease end-points that can be modelled.
- External collaborations.

This takes into consideration evaluation of the cells utilised, for example the use of authenticated commercially available cells and tissues. Human material is preferred, particularly the use of primary cells and differentiated reconstituted tissues (using endogenous cell types), as some *in vivo* functionality may be maintained, including metabolic competency. As a caveat, it is essential that cells supplied for *in vitro* model development are characterised for metabolic activity.

We are working with developers and users of *in vitro* models with supporting organisations (Centre for Alternatives to Animal Testing–EU (CAAT-EU), the European Commission's Partnership on Alternative Approaches (EPAA) ECVAM Stakeholder Forum (ESTAF), and the *In Vitro* Testing Industrial Platform (IVTIP)) to support and consider reproducibility and retrospective testing as part of a pre-validation submission to regulators. Generally we are trying to take the approach advocated by the National Research Council (NRC) in "Toxicity Testing in the 21st Century- A vision and a strategy (75). We are working with and supporting other organizations such as Fund for the Replacement of Animals in Medical Experimentation (FRAME) and IVTIP, to identify the practical steps that are needed to make the NRC vision a reality (76). Validation and acceptance can only be achieved through closer working with the developers, users and producers of these systems along with better and more communication with regulators and opinion leaders in the field of *in vitro* testing and application.

(b) Smoke generation and exposure systems

The response of cells to cigarette smoke in *in vitro* bioassays are sensitive to both the method of smoke generation and the fraction or sample of smoke to which cells are exposed.

Previous work has shown that cell responses to cigarette smoke change when different smoking regimes are used (77). Therefore we recommend that smoke is generated from cigarettes machine-smoked under specific smoking regimes, such as ISO and Health Canada Intense, as discussed for smoke chemistry evaluations.

The way in which cigarette smoke is trapped or sampled also influences its chemical composition, and therefore potentially the responses of *in vitro* assays to cigarette smoke. There are three main sampling approaches that have been used in *in vitro* model test systems:

- Particulate matter - Routine *in vitro* testing of cigarette smoke traditionally assesses the particulate phase activity of cigarette smoke. The particulate phase can be easily trapped by Cambridge filter pads and extracted and re-suspended in a solvent. However this approach does not allow an evaluation of contributions from gases and vapours present in smoke.

- Aqueous extracts - Components of the gas phase may also induce toxicological and biological effects. As the compounds and some components of the particulate phase are water-soluble, aqueous cigarette smoke extracts can be generated by bubbling cigarette smoke through cell culture media (78).

- Whole smoke - We have developed a cell culture device (79) that allows *in vitro* cultures to be exposed to freshly generated whole cigarette smoke using commercially available human cells and tissues. This system allows all of the phases of cigarette smoke - particulates, gases and vapours - to be assessed in combination, or singly with suitable adaptation to the machine. This enables a more physiologically relevant assessment of the effects of cigarette smoke (79, 80).

We are working with others in this field to agree on standardised equipment and methodologies for smoke generation and cell exposure

(c) Cancer models

There are a number of cancer models available, but currently none have been validated for use with cigarette smoke. Although lung cancer is of particular importance in tobacco research, most *in vitro* cancer models are not organ-specific. We and others are assessing the suitability of these accepted models for use with cigarette smoke. This includes research into the Syrian Hamster Embryo (SHE) two-stage transformation assay, which might give insights into the initiation and promotion stages of carcinogenesis following exposure to cigarette smoke (81). While the SHE cell transformation assay is becoming the method of choice for the

study of the early stages in cell transformation (*i.e.* morphological transformation), we believe that an anchorage-independent assay may provide additional information on the later stages of cell transformation (*e.g.* immortalisation, cell migration). Research is currently seeking to develop an anchorage-independent growth (AIG) assay using whole smoke in BEAS-2B human bronchial cells, and preliminary data show that whole smoke induces AIG in this cell line. Whole smoke is also the exposure agent of choice for investigating oxidative stress-induced DNA damage using the Comet assay with the lesion-specific enzyme formamidopyrimidine N-glycosylase (FPG). Data shows that oxidative DNA damage occurred in H292 human bronchial epithelial cells following exposure to whole smoke. Further, when cells were allowed to recover, the levels of non-oxidative DNA damage were reduced in a time-dependent fashion, and a clear FPG-associated oxidative dose response was revealed after a 16 hour recovery period. The data further suggest that single-strand DNA breaks repair more quickly than oxidative lesions (82). A number of other models and assays are also being developed to provide additional tools to evaluate the potential carcinogenic activity of modified cigarette smoke. These include the epithelial-mesenchymal transition model and the investigation of cell cycle and DNA damage parameters in lung epithelial cells exposed to cigarette smoke.

(d) Models of COPD

There are many models available for COPD, but currently none have been validated for use with cigarette smoke. We are making progress in the development of *in vitro* models of processes occurring in the lung diseases covered by the term COPD. Goblet cell hyperplasia (GCH) is a characteristic feature of the lung epithelium in hypersecretory diseases such as bronchitis, and is thought to contribute to the over-production of mucus. In the *in vitro* model of GCH that we have developed, Human Bronchial Epithelial Cells (HBECs) are cultured at the air-liquid interface over 28 days resulting in pseudostratified mucociliary differentiated epithelium containing goblet, ciliated and basal cell populations. These morphological changes have been confirmed in our cultures using electron microscopy (83). This model can be used to investigate changes in the goblet cell population in response to cigarette smoke. In particular, it is possible to assess the expression of MUC5AC using immunostaining and the different cell types by flow cytometry. Using either the HBECs or human bronchial epithelial H292 cells, changes in disease-related mediators at the protein level, following exposure to cigarette smoke, can be evaluated.

(e) Models of Cardiovascular disease

Cigarette smoking is an important risk factor associated with the development of atherosclerosis. Cigarette smoke has been shown to regulate the activity of

numerous cardiovascular system cells including endothelial cells, smooth muscle cells, monocyte/macrophages and platelets and each of these cell types plays an important role in disease development and progression. However, no models have currently been validated.

An important initiating step in atherosclerosis is endothelial cell damage and activation. We are developing *in vitro* models to investigate the responses of endothelial cells to cigarette smoke (84). Static *in vitro* cultures of human umbilical vein endothelial cells (HUVECs) respond to cigarette smoke exposure by upregulating the expression of a number of CVD-related markers including adhesion molecules and pro-inflammatory markers such as members of the family of interleukins (85). These can be measured using protein profiling technologies such as the Meso Scale Discovery (MSD) platform or by gene expression assays such as the Superarray technology.

Furthermore, the wound-healing capability of endothelial cells is impaired when cells are exposed to cigarette smoke extracts, as demonstrated in our endothelial cell migration assays using the IncuCyte platform. This assay reflects the lack of endothelial repair capacity seen in smokers.

Using a Microfluidics platform, we can simulate haemodynamic stresses and expose endothelial cells to atherogenic laminar or oscillatory shear stress in combination with cigarette smoke. This model allows us to investigate morphological changes in cells, to assess the expression of disease mediators such as pro-inflammatory markers and to monitor adhesion of monocytes to endothelial cells (86). Using this system this is achieved under more physiologically-relevant conditions.

Physiological angiogenesis is a means by which coronary and cerebral blood flow is restored following an infarction. This process is impaired in smokers and this likely contributes to long-term harm in those who smoke. We assess angiogenesis *in vitro* using HUVECs cultured on Matrigel plates and have demonstrated an impairment of angiogenesis when these cells are exposed to cigarette smoke extracts.

Alongside the smoke exposure systems described above we are also currently developing methods with which we can expose cells to sera from smokers and non-smokers. Preliminary data suggest that cells respond differently when exposed to these different sera and we propose that serum is a useful exposure agent for *in vitro* studies of cardiovascular disease.

(f) Models of Inflammation and oxidative stress

Oxidative stress within cells exposed to cigarette smoke can be investigated using

commercially available kits and fluorescent probes (87). These assays include the DCF assay to directly measure free radicals and the GSH-Glo assay to indirectly assess oxidative stress by measuring the levels of the antioxidant glutathione (GSH).

Cigarette smoking induces a number of inflammatory processes within cells. Pro-inflammatory cytokines are produced by cells in response to smoke toxicants and, in turn, these chemical messengers recruit inflammatory cells, including neutrophils (88). We believe assays may be developed to investigate the responses of inflammatory cells and cascades in cigarette smoke treated cells, in particular the effects of cigarette smoke extracts on a human monocyte/macrophage cell line, THP-1.

(g) Genomics

Genomics research - especially the application of transcriptomics technologies such as microarrays or Affymetrix® GeneChips - gives a molecular insight into a cell's response to external stimuli. By linking molecular measurements to standard biological observations, it may be possible to find robust ways of understanding the disease processes which are likely to be triggered or exacerbated by cigarette smoke constituents. In future genomics may give an insight into molecular pathways that could be useful as a screen or as part of a weight of evidence approach for assessment of candidate MRTPs. As part of this development an Affymetrix® microarray facility can be used for gene expression analysis (transcriptomics), resulting in gene expression patterns unique to whole cigarette smoke exposure *in vitro*. Studies on the global genomic response of human bronchial primary epithelial cells (HBECs) in culture to whole cigarette smoke should provide a basis for establishing an expected baseline gene expression signature in clinical studies (89).

***In-vivo* animal models**

We do not have any active programme of *in vivo* research, but recognise that others do and that such testing may be expected by some regulators. Our approach is to support the *In Vitro* Testing Industrial Platform (IVTIP), an informal forum for European chemical, pharmaceutical, cosmetics and contract research companies with an active research around *in vitro* testing to be used in regulatory and safety assessment or in compound discovery and product development. Currently there are over 30 companies who participate in this forum. IVTIP participants are supportive of applying, where possible, the 3Rs principle of Replacement, Reduction and Refinement of animal testing in the field of *in vitro* testing.

Pre-clinical assessment of consumer exposure to toxicants

Candidate MRTPs exhibiting substantial reduction in priority toxicants through

standard machine smoking measurements should be evaluated by a sample of the target population prior to and/or during a clinical study.

The objective of such assessments is to understand how smokers use the candidate MRTP, estimate yields taken from the candidate MRTP during real-world use conditions, and establish deposition properties of smoke from the candidate MRTP during inhalation.

a) Consumer acceptability testing

It is important to assess the acceptability of the candidate MRTP with consumers prior to conducting a clinical study, as products with low levels of acceptability may invalidate any clinical study through subjects rejecting the product, changing their normal smoking patterns, stopping use before the end of the trial, or co-smoking commercially available products. All manufacturers have internal procedures for assessing consumer acceptability of commercial products, and these can form the basis of candidate MRTP assessment approaches. What is currently less clear is the acceptability threshold in these types studies that can act as a decision point for a clinical study.

b) Puffing behaviour measurement

The quantity of smoke that consumers obtain from combustible tobacco products depends on a number of variables, but a significant influence is how the product is used. Consumer smoking behaviour includes measurable parameters such as the puff volume drawn through the cigarette, the puff duration and puff frequency.

One way to gain insights into this is to use a smoke analyser device, such as the SA7, which is able to record a consumer's puffing behaviour under laboratory conditions (90). In order to do this the cigarette is smoked through a special holder. The SA7 also estimates in real time the amount of Total Particulate Matter (TPM) known as 'Optical Tar', by measuring the light obscuration properties of the smoke. The 'Optical Tar' calibration is product specific, and requires correlation with NFDPM values obtained via traditional machine smoking.

The use of laboratory conditions and cigarette holders can introduce changes into the puffing behaviour of smokers, therefore tests under real-world conditions are also desirable to provide estimates of human smoke yields.

c) Mouth Level Exposure

The yields of smoke obtained from cigarettes by human smokers can be estimated using a technique known as Mouth Level Exposure (MLE). Such studies also

provide an indication of the range of exposures in a group of smokers in real-world use conditions.

A filter analysis method has been developed from which the MLE can be estimated in an unobtrusive manner (91, 92). The technique involves analysing a mouth end portion of the cigarette filter from human smoked cigarettes for the 'tar' and nicotine retained therein.

Subjects use a filter cutter to cut a mouth end section from the filter of a smoked cigarette. The cut portion of the filter must be downstream of any ventilation holes or charcoal or carbon sections. The cut section of the filter drops into a collection box attached to the cutter. This box is sealed with anti-tamper labels to prevent smokers interfering with the samples. The filter tips are extracted in solution and then analysed for nicotine and nicotine-free dry particulate matter (NFDPM or "tar") by gas chromatography and ultraviolet absorbance on liquid chromatography, respectively.

Calibration is achieved through machine smoking of cigarettes of the same brand and batch as those smoked by the subjects. The smoking machine can be set to smoke cigarettes at a wide range of puff volumes and frequencies that encompass most human behaviours. Cut filters from calibration cigarettes are analysed alongside those from human-smoked cigarettes to avoid differences in ageing effects. The particulate matter from the cigarettes is captured on a glass fibre pad, also called a Cambridge filter. These pads are weighed before and after smoking to calculate the total particulate matter (TPM) trapped. Further analysis of the nicotine and water content from the trapped smoke allows the NFDPM to be calculated.

The smoke nicotine and NFDPM yields from the calibration smoking are plotted against the nicotine content of filter tips and UV absorbance of tip extracts respectively to produce regression equations. The measured human filter tip values are used with these equations to provide estimates of the MLE to nicotine and NFDPM.

Smoking behaviour studies of the kind described in the previous section can help to understand how the consumer is able to obtain the MLE.

This methodology has been applied successfully in several studies to estimate consumer MLE (Mariner *et al.* (31), Ashley *et al.* (93), Cote *et al.* (94), and Pauly *et al.* (95)) and been noted as having potentially utility as a proxy estimate of toxicant exposure (95).

d) Smoke Deposition

Most measures of exposure to tobacco smoke relate to systemic exposure and not the exposure of individual regions or organs. Region specific exposure would potentially help in the assessment of a biologically relevant dose of tobacco smoke toxicants.

Deposition studies (96) are conducted to estimate smoke particulate deposition in various regions of the smokers' respiratory system. The primary aim of these studies is to identify any significant differences between the deposition patterns of test cigarettes and commercially available cigarettes, to understand whether any reduction in systemic exposure is applicable to all respiratory regions. This is demonstrated by estimating the regional deposition in the volunteers pre- and post switching to the test product from a suitable control product.

Volunteers smoke cigarettes through an SA7 smoke analyser to establish puffing profiles and optical tar values. The respiratory profile and the optical tar of exhaled smoke are measured by the Breathe In - Breathe Out system (BIBO). The fraction of smoke depositing in the respiratory system per volunteer is calculated by comparing the optical tar puffed from the cigarette to the optical tar exhaled by the volunteer, taking into consideration hygroscopic growth of the smoke particles in the high humidity atmosphere of the deep lung and any expected chemical changes due to evaporation of volatile components from the smoke particles. The hygroscopic growth potential of the smoke is determined in laboratory studies on a product by product basis.

A particle deposition model developed in-house is used to estimate deposition in five anatomical regions of the respiratory system. The model uses the puffing profile recorded by the SA7 and the respiratory profile measured by the BIBO to predict the transport, hygroscopic growth and deposition of smoke in the respiratory system on a puff by puff basis. Differences in deposition rate between volunteers relating to anatomical differences are accounted for by applying one volunteer specific factor to the model per volunteer. This factor is calculated to provide the best fit between the modelled prediction of and the measured exhaled optical tar for each volunteer.

(e) Exposure from Smokeless Tobacco products

Smokeless tobacco products are used in a range of ways including chewing, placing between lip and gum, to dissolving in the mouth; use of some smokeless tobacco products involves expectoration; whereas other products are removed from the mouth largely intact after use. Products are also used for different periods of time (97, 98) and are used in different quantities both within and across product

categories. All of these factors will influence the degree of exposure of consumers to product constituents. We therefore believe that understanding of the usage and behaviour patterns of smokeless tobacco products is important for establishing exposure of users to tobacco toxicants, and recommend that product usage data be part of a pre-clinical characterisation package for smokeless tobacco products. We have published our approach for understanding behaviour and useage patterns for snus users in Norway and Sweden, and these may provide the basis of methods to understand consumption patterns with other smokeless products (97, 98).

We have developed techniques to examine snus users' exposure (or "transfer") to constituents from tobacco (99), and monitored their nicotine uptake through a pharmacokinetic study (100). Such studies help to establish the level of tobacco constituent bio-availability during use, and approaches of this kind are recommended as part of a pre-clinical/clinical product assessment.

Clinical Studies

(a) Clinical study approach

Clinical studies should be conducted to the highest standards and in accordance with the latest version of the Declaration of Helsinki and the International Conference on Harmonisation (ICH) Guidelines for Good Clinical Practice (GCP). We intend as a matter of policy to seek independent ethics committee (EC) approval for any of our clinical studies. The studies are conducted in conjunction with clinical departments of Contract Research Organisations and universities and only take place following approval by ECs and receipt of signed informed consent from participating volunteers. Current and future clinical studies will be registered on the WHO-prescribed clinical trials databases (such as the International Standard Randomised Controlled Trial Number Register (ISRCTN) or ClinicalTrials.gov) and the results will be published in the scientific literature.

(b) Biomarkers of Exposure

It is widely recognised that biomarkers of exposure comprise an effective way to measure exposure to environmental chemicals. Biomarkers of exposure have been and are being developed as tools to assess exposure to tobacco and tobacco smoke constituents in humans. The data generated by this form of exposure assessment can be used to support a weight of evidence approach for the evaluation of candidate MRTPs (1, 101).

Ideally, a biomarker of exposure should be (1, 102):

- specific to the source constituent,

- reproducibly and accurately measurable at the levels that tobacco users are exposed to,
- show differences in the levels found between tobacco users and non-tobacco users,
- correlate with exposure dose, and shows a dose–response relationship that can be understood on a mechanistic basis.

In addition, the usefulness of biomarkers must be established for different consumer populations (*i.e.* those varying in behaviour, sex, age, genetics and tobacco use). When the chemical entity is present in its free form in a biofluid, it is considered to be a marker of internal dose. When the chemical entity has been transformed and is chemically linked to macromolecules it is considered to be a marker of effective dose.

Nicotine metabolites are good biomarkers of exposure for tobacco use. However, no single biomarker is likely to be able to satisfy all assessment needs and, therefore a suite of biomarkers are being developed which may reflect exposure to a range of tobacco sourced toxicants. Biomarkers of exposure are currently available (Table 1) for a variety of tobacco smoke toxicant chemicals, such as nitrosamines, aldehydes, aromatic amines, and polyaromatic hydrocarbons. A selection of these biomarkers may also be applicable in the assessment of smokeless products, such as snus. We, and others, are working to extend the range of biomarkers of exposure that could be used in clinical studies. Use of literature reviews of pathways of metabolism for smoke toxicants, and capabilities for investigating the metabolism of smoke toxicants (*e.g.* metabolomics) are helping to identify new biomarkers of exposure. Additional biomarkers are need though, for example to assess exposure to acetaldehyde and formaldehyde.

Table 1. Potential biomarkers of exposure for assessing the toxicant exposure from candidate MRTPs.

Smoke Constituent	Biomatrix	Biomarker of Exposure
Nicotine	Urine	Total Nicotine Equivalents (Sum of nicotine and 5 metabolites)
Nicotine	Saliva	Cotinine
Nicotine	Plasma	Cotinine
NNK	Urine	NNAL
NNN	Urine	NNN
NAB	Urine	NAB
NAT	Urine	NAT
4-aminobiphenyl	Urine	4-aminobiphenyl
3-aminobiphenyl	Urine	3-aminobiphenyl
o-toluidine	Urine	o-toluidine
2-aminonaphthalene	Urine	2-aminonaphthalene
Crotonldehyde	Urine	HMPMA
Acrolein	Urine	3-HPMA
1,3-butadiene	Urine	MHBMA
Pyrene	Urine	1-hydroxypyrene
Fluorene	Urine	2-hydroxyfluorene
Naphthalene	Urine	1-hydroxynaphthalene
Naphthalene	Urine	2-hydroxynaphthalene
Phenanthrene	Urine	2-hydroxyphenanthrene
Phenanthrene	Urine	3-hydroxyphenanthrene
Phenanthrene	Urine	4-hydroxyphenanthrene
Phenanthrene	Urine	1+9-hydroxyphenanthrene

Validation of candidate biomarkers is important prior to use in clinical studies. Guidelines on biomarker validation have been provided by the US FDA (103).

Inter-laboratory ring trials are being used to assess the robustness of biomarker analysis methods. We are also examining the stability of the biomarkers under different storage conditions.

Proof-of-concept studies are being used to test whether biomarkers of exposure can differentiate between smokers and non-smokers and whether a dose-response

can be measured upon exposure to smoke. For example, a variety of biomarkers have been assessed for their ability to discriminate between levels of toxicant exposure in smokers of different ISO tar yield cigarettes, and their applicability in evaluating future candidate MRTPs (104, 105). We have reported that potential maximum MLE results from the analysis of spent cigarette filters for smoke chemicals correlate well with the corresponding biomarkers of exposure using clinical correlation studies. Data from these studies have also demonstrated dose-response relationships between levels of specific urinary, plasma and salivary biomarkers and indicators for daily smoke exposure (106, 107). Some of our recent research has involved evaluating the extent to which these biomarkers of exposure (crotonaldehyde, acrolein, NNK, pyrene and 1,3-butadiene) correlate with nicotine exposure.

These studies demonstrated that we have the tools and appropriate study designs to assess human exposure to smoke toxicants (107, 108). The simple non-invasive nature of the filter analysis method lends itself to large-scale, late phase studies into human smoke toxicant exposure whereas validated biomarkers of exposure are the 'gold standard' measure of smoke toxicant exposure.

Our first candidate MRTP clinical study was a short term (6 week) switching study that focused on exposure. It was conducted in Germany on our first generation of candidate MRTP cigarettes which had, in the laboratory, shown significant reduction in some of the tobacco smoke toxicants. The study demonstrated significant reductions in smoke toxicant 'dose' in smokers, as determined using a range of biomarkers of exposure, following a switch from conventional cigarettes to these candidate MRTP cigarettes (109) (ISRCTN 72157335).

The next logical step is to conduct clinical studies that include both biomarkers of exposure and of biological effect. The latter will necessitate an extended switching period to allow time for changes in these biomarkers to occur. The primary objective of such studies will be to determine whether longer term use of a candidate MRTP results in continued exposure reduction and a reduction in biomarkers of biological effect.

This approach is in line with the recommendation of Hatsukami *et al.* (110) who recommended a series of clinical studies of both short and intermediate length in the assessment of candidate MRTPs.

(c) Biomarkers of biological effect

An important part of any assessment of whether a candidate MRTP has the potential to reduce health risks associated with tobacco use will be clinical studies that include biomarkers of biological effect.

Biomarkers of biological effect are indicators of the body's response to cigarette smoke exposure. These biomarkers indicate early sub-clinical changes which, if sustained, may have pathological consequences. While there are currently no biomarkers of effect which are qualified and validated to the extent that they can predict the risk of disease we believe that such measurements, together with information on smoke chemistry and biomarkers of exposure, will contribute to a weight of evidence approach to the assessment of any modification of the risk potential of candidate MRTPs relative to conventional cigarettes (1, 111, 112, 113).

For the purposes of assessment of a candidate MRTP, a biomarker of effect would need to be robustly altered in response to cigarette smoking and minimally affected by inter-individual variability. For those biomarkers of effect that change following cessation of cigarette smoke exposure, there will also be a reasonable expectation that they will change to a certain extent if a smoker switches from a conventional control product to a candidate MRTP.

Our belief is that consideration must be given to determine the most appropriate comparators for benchmarking a candidate MRTP. We will approach this by taking two "bookends" to the comparison, both of which are reasonably well defined by epidemiology. When considering risk reduction, at the highest risk end would be regular long term cigarette smoking and at the lowest risk end would be the risk profile associated with quitting tobacco use. There may also be risk profiles between these two ends of the spectrum, such as that for smokeless tobacco use, which can act as comparators. Taking this approach allows relative assessments, whether that is of exposure or of biological effect, and may help frame any risk communication.

Ideally, candidate biomarkers would be related to a disease-specific endpoint, but this requirement may not necessarily be achievable in the context of biomarkers of effect for smoking-related diseases in shorter term studies. Instead, the biomarkers that can be employed in such studies will reflect biological responses caused by inflammation (local or systemic), oxidative stress and DNA damage.

An issue surrounding biomarkers of biological effect is that most are not currently validated as having a predictive use with respect to the actual risk of developing disease. Therefore, we believe that further work is needed to validate biomarkers of biological effect in order that they can be utilised to predict individual disease risk and as such act as biomarkers of potential harm.

One approach to identifying suitable biomarkers of effect would be through:

- in-house research

- literature reviews to identify cessation studies in which biomarkers have been monitored by others
- scientific engagement
- external collaborations

In a recent study samples were collected to study any differences in the biomarker profiles of smokers who smoked more cigarettes per day compared to those who smoked less (114). In this study, some biomarkers appeared to be associated with cumulative cigarette smoke exposure across groups of smokers, former smokers and never-smokers. These included total white blood cell count, serum IgG, urinary 11-dehydrothromboxane B2, 2,3-dinor thromboxane B2, 8-epi prostaglandin F2 alpha, 8-hydroxy 2'deoxyguanosine and cis-thymidine glycol.

It is important to determine whether smoking cessation has any effect on these biomarkers, rather than simply reflecting a general perturbation of biological systems. Cessation studies should be informative in assessing the applicability of biomarkers of biological effect and we are currently considering such studies.

In another study, a small group of monozygotic twins in which one twin smoked and the other did not were studied as a way of trying to identify informative biomarkers for candidate MRTP assessment in the absence of genetic variation (115). In brief, in this study 11-dehydrothromboxane B2, 2,3-dinorthromboxane B2, 8-epi-prostaglandin F2α, hydroxyproline, fibrinogen, white blood cell, neutrophil and lymphocyte counts and heart rate were statistically significantly increased in the smoker compared to the non-smoker twins. More work is needed to identify biomarkers that demonstrate either reversibility or a decreased rate of change following cessation, which we believe would be the most useful in clinical studies of candidate MRTPs.

Biomarker research can be supported by a number of tools including databases such as BiomarkerCenter (Thomson Reuters), which enabled us to mine relevant regulatory information in the context of biomarker qualification. In addition, software applications such as MetaCore (GeneGo) allow us to explore our experimental or clinical study data in combination with publicly available literature to identify novel biomarker candidates with potential utility for candidate MRTP assessment.

It is necessary to test the usefulness of selected biomarkers according to the criteria proposed by independent experts in tobacco research and sponsored by the National Cancer Institute of the United States (116). The suggested criteria that biomarkers should meet are:

- Differences in the biomarker levels between tobacco users and non-users

- A change in biomarker levels as a consequence of cessation
- A dose-response relationship between extent of exposure and levels of biomarkers
- A change in levels of biomarkers as a result of tobacco use reduction.

Utilising this approach in combination with the literature reviews and experimental studies described above, we have identified a series of biomarkers of effect associated with inflammation, oxidative stress and DNA damage that are relevant to cardiovascular disease, chronic obstructive pulmonary disease, and cancer. These biomarkers range from biomolecules found in tissues or body fluids, physiological measurements such as lung function or vascular reactivity (see Table 2) and Quality of Life questionnaires.

Table 2. Potential biomarkers of biological effect for assessing the risk potential of candidate MRTPs.

Biomarker	**Type**	**Biomatrix**
F_2-isoprostane (PGF2α)	Oxidative stress, COPD	Urine/sputum
Superoxide dismutase activity	Oxidative stress	Plasma/erythrocytes/ plasma/serum
Glutathione peroxidase activity	Oxidative stress	Plasma/erythrocytes
Ascorbic acid	Oxidative stress	Plasma/serum
Dehydroascorbic acid	Oxidative stress	Plasma
Glutathione reductase activity	Oxidative stress	Plasma/erythrocytes
Total antioxidant capacity	Oxidative stress	Plasma/serum
Catalase activity	Oxidative stress	Plasma/erythrocytes
Malondialdehyde	Oxidative stress	Plasma/serum/urine
8-OHdG	Cancer	Urine
cis-thymidine glycol	Cancer	Urine
Flow-mediated dilatation	CVD	Physical measurement
sICAM-1, sVCAM-1	CVD	Plasma/serum
Endothelial progenitor cells	CVD	Fresh blood
C-reactive protein	CVD	Plasma or serum
White blood cells	CVD	Fresh blood
Lipid profile (oxLDL, HDL)	CVD	Plasma/serum

Fibrinogen	CVD	Plasma/whole blood
11-dehydrothromboxane B2	CVD	Urine
PGF1α	CVD	Urine
Leukotriene E4	CVD	Urine
FEV_1	COPD	Physical measurement
Neutrophils, macrophages	COPD	Sputum/whole blood
IL-6, IL-8, IL-1b, IL-16, GRO-α, MCP-1, MIP-1	COPD, inflammation	Sputum/plasma/ serum
Neutrophil Elastase	COPD	Sputum/plasma/ serum
MMP-1, MMP-9, TIMP-1, TIMP-2	COPD	Sputum/plasma/ serum
LTB4	COPD	Sputum/plasma/urine
GM-CSF	COPD	Sputum/plasma/urine
TNF-α	COPD	Sputum/plasma/ serum
VEGF	COPD	Sputum/plasma/ serum

It is necessary to take such a broad approach because developing reliable and predictive biomarkers of biological effect will be one of the most difficult scientific challenges. Data from physiological measurements and quality of life questionnaires lead to greater confidence in the value of biomarkers. The biomarkers listed are neither exclusive nor exhaustive and the choice of biomarkers for a given study would depend on many factors such as the study length and the availability of specialist techniques for measuring a particular biomarker. Some potential biomarkers, for example endothelial progenitor cells, require developmental work for them to be routinely included in candidate MRTP testing due to technological limitations in assaying their levels in samples obtained from large cohorts.

(d) Dosimetry

Dosimetry measurements, such as smoking behaviour and mouth level exposure measurements, of the kind described for pre-clinical assessment are valuable data to be obtained during a Clinical Study. These data can provide contextual understanding and aid interpretation of results obtained with the clinical study population and individual study subjects.

(e) Physiological and Biochemical Medical Assessments

A suite of physiological and biochemical assessments are conducted on subjects prior to commencement of a clinical study. These assessments are to ensure that study participants meet the study inclusion criteria. Assessments are also carried out throughout the study to ensure that participants remain healthy and compliant with the inclusion criteria. Physiological assessments include pregnancy tests, blood pressure, ECG (12 lead), lung function (FEV_1), height, weight and body mass index. Clinical chemistry assessments include urine analyses for kidney function, a biochemical panel for liver function, cardiac enzymes, and general haematology to ensure subjects are free from inflammatory or infectious events, and blood cell counts for coagulation disorders and anaemia.

Pre-market consumer assessment of consumer communications
We believe that assessment of the understanding of any exposure or health related communications to be delivered with an MRTP should be evaluated with various groups of volunteers, with a focus on the potential adopters of the product (current adult smokers). This pre-market assessment should focus on consumer understanding of the communications and in particular on their understanding of the health risks posed by product use, the advantages of complete cessation, and any uncertainties surrounding any proposed reductions in risk. The studies should consider all aspects of the product that is intended to be sold, including any branding and packaging and the regulated health warnings. Studies should evaluate all proposed forms of communication.

We think that such studies will give an indication of understanding of the communication, and should help in the development of the communication, but are unlikely to be clearly predictive of behaviour post-marketing (which will involve many other factors including price, convenience, consumer preference and relative choice of products).

We believe that it will be very difficult to properly assess the reactions of ex-smokers or non-smokers/non-tobacco users to the product communication, but if this were to be required it should focus on whether the communication was likely to impact on a return to tobacco use in the case of ex-smokers or an initiation of tobacco use in non-tobacco users.

It will be important to correlate results from these studies with data from the post market surveillance studies to determine the validity of this research.

Post Market Surveillance
Balancing the risks and benefits of MRTPs presented as alternatives to conventional cigarettes will be challenging. An MRTP that decreases risk for individuals who

switch to this product instead of smoking conventional cigarettes could also increase risk for individuals who use the MRTP instead of remaining tobacco free, making assessment of an overall public health benefit to a population difficult to assess. Consequently, it is critical to conduct independent research on the design, composition and health effects of new products, and to put in place a comprehensive surveillance system that aims to understand consumers' knowledge, attitudes, usage patterns and behaviours regarding these products, in combination with clinical and epidemiological methods to determine the effects of a MRTP on population risk. While some analyses aimed at understanding population impacts may be conducted prior to marketing an MRTP, the most useful data will be generated after the product has entered the marketplace. Therefore, planned post-marketing population assessment studies will be necessary to detect unintended public health consequences that may result from the introduction of a MRTP as well as any realised reductions in individual and/or population risks. A robust surveillance programme will serve both as an early warning system for identifying problems associated with MRTPs, and also as an epidemiological tool for evaluating long-term health consequences of individuals. The quality of both surveillance and epidemiological studies should be high enough so that the results can permit modification of any exposure or health risk-related claims as understanding of these products perform develops over time.

Although different post-marketing evaluation approaches are well established in the pharmaceutical and food arenas, the framework for monitoring a tobacco product will necessarily be quite different, due to the known risks of using conventional cigarettes. One key difference will be the additional need to capture data about individuals who choose the candidate MRTP, specifically if they were never smokers, or this switch has delayed an attempt to quit tobacco product use. Similar to a pharmaceutical product or functional food, which are claimed to treat a particular disease indication or have a particular function, the claim for an MRTP might be that it reduces risk relative to continued use of cigarettes or other comparison tobacco product, and therefore both safety and efficacy evaluation are needed to monitor any unintended health events, as well as to assess whether its commercial availability is effectively associated with a reduction in harm to the population as a whole. Typical post-marketing surveillance methods for pharmaceutical drugs, medical devices and foods are passive or observational in nature (for example using patient discussions or self-reporting by healthcare workers concerning adverse effects to establish a post-market safety and efficacy profile). Similar methods need to be developed for MRTPs to evaluate their harm reduction potential both to the individual and the population as a whole. Implementing a scientifically sound programme capable of detecting both positive and negative effects (decreases or increases in population risk and/or harm) from the use of a candidate MRTP in a timely fashion will be challenging for any MRTP.

Evaluating MRTPs could more closely resemble functional foods in that the main sources of information regarding the product's harm-reduction profile (analogous to the concept of safety and functionality in a novel food product) would originate through relatively direct contact between the consumer and the manufacturer, with the possible contribution of public health agency positions. This adds a level of complexity that does not exist with pharmaceuticals. With a new drug, a patient visits their doctor, often undergoes a battery of tests that, along with medical history, leads to the doctor prescribing that drug. The doctor then monitors the patient during the course of treatment and in the case of any adverse effects may alert the manufacturer of the drug. Because blood or other samples were collected at the point of diagnosis, there is a great deal of data about the patient already available and can be shared with the manufacturer. With functional foods as well as candidate MRTPs these products would be sold directly to consumers – that is, there would be no intermediary physician or healthcare worker involved, and therefore not nearly as much data would be available. This in turn creates a requirement for an active system to be set up to gather similar information from individual consumers. Active surveillance systems are more resource intensive and in this case would involve data collection on the MRTP consumers, and product marketing.

In our current view, a post market surveillance approach for an MRTP would have both passive and active design elements similar to those applied in pharmaceuticals and functional foods and interventional clinical studies. In the context of an MRTP some elements of a post-marketing surveillance effort must be by definition "interventional", as information about individual consumers will need to be gathered proactively (for instance, biomarkers monitored in blood and urine samples), rather than being able to rely on this information already having been collected. For this information to be meaningful, it needs to be collected from individuals who "switch" from another product to the candidate MRTP as close as possible to the time of switching to provide baseline measures for comparison.

There should also be elements of the post-marketing approach that are more traditionally observational, including a Quality of Life questionnaire, sales data provided by marketing as well as a product usage questionnaire. Collectively these observational tools will help provide information about perceived health benefit, as well as information on rates of initiation, cessation and prolongation (which are crucial to evaluating the overall health effect on the population as a whole).

In order to generate sufficient data for meaningful analysis, it will be crucial to identify and follow a minimum number of adult consumers who switch to this candidate MRTP. In order to collect this data, an active system will need to be set up to monitor and record the information from the observational elements along with contracting organizations to conduct the interventional piece involving the

collection of biomarkers and physical data.

While only specific large-scale epidemiological studies of sufficient duration will ultimately provide definitive evidence of population health effects of an MRTP, clinical biomarker and behavioural studies may help identify unanticipated adverse effects associated with a product and provide key information about potential short term outcomes that implicate a long term effect. Surrogate indicators associated with tobacco-related diseases can be potential short-term measures of the risks associated with a MRTP. Surveys provide behavioural indicators, such as prevalence of smoking, concurrent product use, initiation and quitting rates, and preferences. Such information constitutes short-term data which can then be used to predict the potential public health impact associated with the commercialization of the MRTP. This body of evidence may help predict the harm and/or risk reduction potential of an MRTP while long-term epidemiological evidence is collected.

Discussion

The assumption which we have made in developing this framework is that clinical biomarker data on exposure and biological effect would be obtained for a candidate MRTP after completing a series of pre-clinical tests, including chemistry, regulatory toxicology, *in vitro* assays modeling disease pathways and some initial consumer behaviour. Some of these tests are available today, and others are being developed as part of ours and others research programmes.

The framework also reflects our expectation that post-market surveillance research would follow any regulatory agency approval to market a MRTP for which the submitted data supports a conclusion that the new product substantially reduces exposure to one or more tobacco toxicants and can reasonably be expected to reduce the risk of one or more tobacco-related diseases as compared to a conventional tobacco product. We also believe that studies on consumer understanding of any health-related communication, including uncertainties, are important in both pre- and post-market phases.

We propose a weight of evidence approach, with a clear preference for clinical data on biomarkers of toxicant exposure and, where available, biomarkers of biological effect from controlled studies of subjects using the new products over an extended period. We propose laboratory, toxicological and chemical studies on the novel products that may provide support for the referenced clinical findings, and, where available, biomarkers of biological effect from studies involving subjects who have used the new products over an extended period. We think laboratory toxicological and chemical studies on the products would provide support for the clinical findings.

We do not believe that the full range of scientific assessment tools necessary to determine whether a modified risk tobacco product (MRTP) will reduce the risks of long-term chronic disease from present levels are currently available. In particular, biomarkers of potential harm that could predict the risk of chronic disease have, in the main, not been developed and validated. This is true for both tobacco and other consumer products such as functional foods. In this paper, we set out the breadth of research that we are currently undertaking to develop an MRTP assessment framework. We recognise that many others are also researching this area. Given the magnitude of the risks of tobacco use and its impact on public health, it is important not to wait until science is fully developed in some of these areas, but rather take a weight of evidence approach within the context of the state of scientific art at the time. This means that some uncertainties will exist with respect to the potential for reduced risks associated with any new product. In our view, these can be counter-balanced by pre-market assessment of communication, and robust systems of post-market surveillance to ensure that such uncertainties are communicated properly. In this regard, we believe that a regulatory process can provide an important mechanism of oversight.

A framework for the assessment of MRTPs needs also to account for quite disparate risk profiles of different styles of tobacco products, but should not ignore any one product style. For example, we know from epidemiological studies that cigarettes are associated with a high level of risk for many diseases, including lung cancer and chronic obstructive pulmonary disease. While switching to snus (in the absence of quitting tobacco use altogether) is likely to reduce individual risks dramatically, many smokers choose not to switch. Thus, even though the potential for reducing risks of combustible cigarettes with current technology may not be as great as would result from wide-scale switching to snus, in our view it remains important to research ways to reduce risks to cigarette smokers through evaluating cigarette-like MRTPs.

We believe that an assessment framework for MRTPs should be informed by the experience in other sectors. For example, a European Commission supported project "Process for the assessment of scientific support for claims on foods (PASSCLAIM)" (117) has set out a scheme for the evaluation of claims related to functional foods which has some positive elements of flexibility. It proposes that different types of studies be required for enhanced functional claims as compared to reduction of disease risk claims. It also addresses proper combination of generally accepted data with product specific data. Tobacco product use, with its associated inherent health risks, poses different challenges than do pharmaceuticals, medical devices or functional foods. While experience from these other sectors helps inform and assessment framework, we believe that tobacco products require their own scheme of assessment.

Given the above, we believe that while the scientific standards for studies on MRTPs need to be set high, tobacco product assessments should be flexible enough to account for developments in science, the disparate range of risks associated with different types of tobacco product and uncertainties in assessing various products' risk profiles.

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