Assessment of In Vitro COPD Models for Tobacco Regulatory Science

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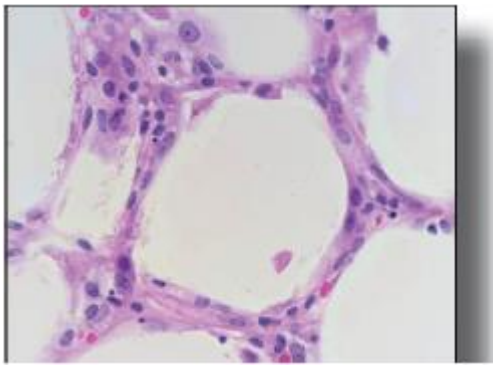

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CONFERENCE TEAM

Conference Director:	**Erin Hill (President, IIVS)**
Principal Investigator:	**Holger Behrsing, Ph.D. (Principal Scientist, IIVS)**
Workshop Chairperson:	**Martha Moore Ph.D. (Senior Manager, Environ)**

Steering Committee Members

Holger Behrsing (IIVS)

Marianna Gaça (British American Tobacco)

Robert Heflich (USFDA - National Center for Toxicological Research)

Martha Moore (Environ)

Richard Phipps (University of Rochester)

Hans Raabe (IIVS)

Kristie Sullivan (Physicians Committee for Responsible Medicine)

Rob Tarran (University of North Carolina)

Acknowledgments

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SUMMARY OF KEY THEMES

- There are several plausible *in vitro* test systems, models, and assay endpoints currently available with the potential to be used for assessing adverse impacts of tobacco product exposure in respiratory tissues. These models and assay endpoints should be further evaluated for their use as hazard assessment tools in a regulatory arena

- Of particular interest are three-dimensional (3D) reconstructed, human, organotypic tissue models of the respiratory airways; these should reduce the need to extrapolate results across species, thus strengthening the relevancy of results

- Standardization of promising test methods should be a high priority

- Relevant reference standards should be developed and made easily available to the research community

- Access to information from non-invasive or minimally invasive clinical studies is needed to bridge the gap between *in vitro* and *in vivo* results.

- Development of test methods for regulatory purposes is unlikely to be funded through the traditional grant process; more direct funding programs should be developed

- Close communication should be maintained between regulators and the research community to be most efficient in the development of useful *in vitro* methods to assess relative risk

INTRODUCTORY PRESENTATIONS

Introduction and Overview of Meeting Plan

Erin Hill, IIVS

This workshop, which is the first in a series, exemplifies what is at the core of IIVS' not-for-profit mission. That is, to bring together stakeholders from a variety of backgrounds in order to identify promising *in vitro* and *in silico* methods and then to standardize and help validate those methods to make them ready for regulatory application. Drawing on the list of research priorities set forth by the Food and Drug Administration's Center for Tobacco Products, we have designed a program where invited experts from academia, government, and industry will present talks and posters covering key areas in utilizing models and assays to investigate the effects of tobacco on human health, such as chronic obstructive pulmonary disease (COPD). The exchange of information is intended to facilitate a better understanding of and forge a route towards the standardization of *in vitro* methods. On the basis of the workshop discussions, conclusions concerning the readiness of current models for regulatory application and approaches to improving models will be drawn and published, thus advancing science-based assessment of tobacco products.

Tobacco Product Regulation and Nonclinical Science

Hans Rosenfeldt, FDA-Center for Tobacco Products

The US Food and Drug Administration (FDA) Center for Tobacco Products (CTP) was set up in 2009 when the FDA was authorized to regulate the manufacture, marketing, and distribution of cigarettes and smokeless tobacco products, in the Family Smoking Prevention and Tobacco Control Act. FDA has the authority to regulate "tobacco products," which are defined, in part, as any products "made or derived from tobacco" that are not "drug", "device", or combination products under the Family Smoking Prevention and Tobacco Control Act. The FDA currently regulates cigarettes, cigarette tobacco, roll-your-own tobacco, and smokeless tobacco. On April 25, 2014, FDA published a proposed rule, *Tobacco Products Deemed to Be Subject to the Food, Drug & Cosmetic Act (Deeming)*. This would extend the FDA's tobacco authority to cover additional tobacco products, including electronic cigarettes (e-cigarettes), cigars, pipe tobacco, nicotine gels, waterpipe (hookah) tobacco, and dissolvables, not already under the Agency's authority and that meet the definition of a tobacco product. The aim of the Act is to protect public health and make tobacco-related disease part of the US's past. Specific aims are to prevent young people starting to use tobacco products, encouraging adults to quit use, and reducing the harm and addictiveness of products for those who continue to use them.

The FDA is using its regulatory authority to gain understanding of tobacco products, restrict changes that might adversely affect public health, prohibit claims of modified risk without sufficient supporting data, ensure compliance with regulation, and educate the public. An important aim is to expand the science base for regulation.

CTP is the newest center at the FDA and has undergone substantial and rapid growth since its inception. Within the CTP, the Division of Nonclinical Science employs scientists in areas such as toxicology, pharmacology, and environmental science, to review product applications, provide scientific input for guidance and regulation

documents, conduct research, and expand knowledge. Unlike other FDA centers, which have regulations that outline the non-clinical studies required of the applicant for clinical studies and product authorization, the CTP has no such regulations. Data from *in vivo*, *in vitro*, *ex vivo*, and *in silico* studies are considered and reviewed to inform the agency on scientific decisions.

The CTP has issued draft guidance that reflects current thinking on non-clinical studies. However, both draft and final guidance documents contain non-binding recommendations which represent the Agency's current thinking. The main difference between a draft and final guidance is that the draft guidance carries an additional disclaimer indicating that Agency thinking on the subject of the guidance is not final. Draft guidance includes suggestions of items to address when preparing applications, similar to previous 'points to consider' documents. Issuing of draft guidance is seen as a good way to share current thinking with stakeholders and to bridge the gap to finalization, which can take a substantial period of time. The following example is found in the guidance for testing of modified risk tobacco products (MRTPs).

"FDA recommends that applicants conduct nonclinical studies to address the known clinical toxicities of tobacco products and evaluate a range of potential toxicities of the product as compared to other tobacco products on the market. Applicants should choose appropriate models for nonclinical studies that are sufficiently sensitive for the evaluation of the selected endpoint and be able to provide support for the model used, including an explanation of the sensitivity and probative value of the model chosen. For in vivo animal studies, researchers should administer the test product to animals by a route representative of human exposure, where feasible. Nonclinical toxicology studies should use methods that are sufficiently sensitive to assess the actual differences between use of the product and use of other tobacco products, or between use of the product and non-use of tobacco products."

Hans Rosenfeldt, PhD, Toxicology Branch Chief, noted the FDA's commitment to the three Rs (replace, reduce, refine) and the inclusion of "modernizing toxicology" as one of the eight priority areas to address.[1,2] The CTP, therefore, is very interested in hearing of current assays that could fit into meeting this goal. These should be relevant to humans and address known and potential clinical toxicities of tobacco products.

Disclaimer: The information in these materials is not a formal dissemination of information by FDA and does not represent agency position or policy.

Key points:

- **CTP is the newest center within the FDA and is interested in information and knowledge on non-clinical methods that could be relevant to tobacco products**
- **Draft and final guidance are issued to share FDA's current thinking with stakeholders**
- **Modernization of toxicology methods, as well as a commitment to the three Rs, is a priority for the FDA**

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[1] http://www.fda.gov/forconsumers/consumerupdates/ucm268201.htm

[2] http://www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/scienceboardtothefoodanddrugadministration/ucm286069.pdf

- **CTP, as part of its commitment to modernization of toxicology and the three Rs, is interested in the investigation into non-animal testing that would be relevant to tobacco products and their toxicity**

Animals Don't Smoke: Ending Tobacco Experiments on Animals

Joseph Manuppello, PETA International Science Consortium, Ltd

Joseph Manuppello discussed PETA's interest in relation to the FDA's Family Smoking Prevention and Tobacco Control Act and what the related strategy and regulation is likely to mean for animal testing. PETA would like to see a tiered approach to regulation introduced that would minimize animal testing use. The FDA guidance to industry lends itself to such an approach, by requesting the submission of clinical data, including endpoints based on validated biomarkers and the use of intermediate clinical endpoints. Crucially, the Act makes no mention of animal testing. Rather, it stipulates that manufacturers undertake well-regulated investigations. Even in the case of MRTPs, which if approved are marketed specifically to reduce harm, animal testing is not mentioned. Nevertheless, the Institute of Medicine (IoM) notes that despite the difficulty in making laboratory animals use tobacco products as humans do and notable interspecies differences that can prevent meaningful extrapolation of data, for MRTPs animal testing might be informative.[3] To temper the use of *in vivo* animal studies, the IoM does recommend that they be preceded by *in vitro* studies and composition and product standards or limits should be set.

As part of the tiered regulatory approach, PETA suggests that for truly new products, not only should pre-clinical *in vitro* studies be done first, but also that the data should be submitted and assessed before any clinical assessments are started. Pre-clinical *in vitro* laboratory tests should also be done of constituents rather than just products. Assays of particular interest are those for cytotoxicity, genotoxicity, cell apoptosis and proliferation, oxidative stress, inflammation, mucus production, and endothelial activation. Only if these assays yield significant findings should animal or human studies be considered. Joseph Manuppello noted that for existing products wishing to make claims of modified risks, epidemiological assessments will play a much more prominent role than laboratory studies, and may be supported by previously reported research on similar products. Likewise, for investigation of addictive potential, human research (after adequate pre-clinical screening) seems the most externally valid approach, owing to difficulties in modelling some kinds of delivery systems, such as snus.

In 2009, when the Smoking Prevention and Tobacco Control Act was introduced, *in vitro* methods were not deemed reliable and were limited to a small number of cytotoxicity and genotoxicity tests. Since then, however, many advances have been made and this is now clearly not the case. Reliable assays are available to also assess air–liquid interface exposure, apoptosis, inflammation, cell transformation, and gene expression. Thus, to support the use of preclinical *in vitro* testing, PETA suggests that the FDA should exercise its authority under the Act to set standards for non-animal pre-clinical testing of tobacco products, including a standard battery of assays.

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[3]Institute of Medicine of the National Academies. Scientific standards for studies on modified risk tobacco products. Washington, DC: National Academies Press, 2012.

It has been suggested that there is a risk that e-cigarettes will be deemed to need relevant animal testing because no products were marketed in 2007, but PETA suggests that there are circumstances that deem this unnecessary. For example, if products have toxicants below certain levels or use food-grade flavors (or flavors are prohibited in given products), etc animal studies should be undertaken only if need is justified by significant *in vitro* findings and agreed to when opened to public comment.

Some countries, such as Belgium, Estonia, Germany, Slovakia, and the UK, have already banned animal testing for tobacco products. Global prohibition, therefore, seems a reasonable goal for manufacturers. Certainly, some manufacturers already avoid animal testing wherever possible.

Key points:

- **Since 2009, the availability, breadth, and reliability of *in vitro* assays has grown substantially**
- **The onus should be on *in vitro* testing of products and constituents where claims of reduced harm are intended**
- **For existing products wishing to make new claims, new epidemiological data and previously published research are more appropriate than new laboratory testing**
- **The FDA should provide a list of standards, including a battery of standard tests, required for regulation**

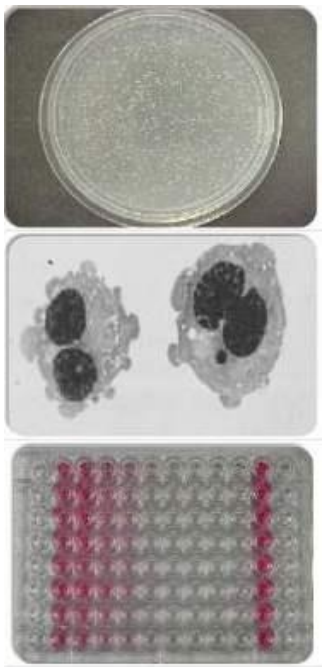
In Vitro Toxicity Testing of Tobacco Products: a Manufacturer's Perspective

Betsy Bombick, R. J. Reynolds Tobacco Co.

Betsy Bombick presented guidance and recommendations on *in vitro* testing, the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) experience, and ways to move forward.

A wide range of samples related to tobacco products can be tested *in vitro*, including cigarette smoke particulate matter, whole smoke, smokeless tobacco, and tobacco product constituents. *In vitro* tests are quicker and less expensive than animal studies and give mechanistic understanding, although they have strengths and limitations and single assays cannot answer all questions. Results, therefore, must be taken in the context of existing data and information. From a regulatory perspective, the use of non-clinical studies (*in vitro*, *in vivo*, and *ex vivo*) is encouraged: Health Canada requires annual toxicity testing of cigarettes sold or manufactured in Canada with three required toxicology tests: Ames bacterial mutagenicity, cytotoxicity in the Neutral Red Uptake assay, and genotoxicity in the micronucleus assay. The FDA is less clear on types of studies that should be used for MRTPs and pre-market products, recommending, respectively, non-clinical studies and "some combination of *in vitro*, *in vivo*, and *ex vivo* studies" in its *Draft Guidance for Industry (Modified Risk Tobacco Product Applications, Applications for Premarket Review of New Tobacco Products)*.

CORESTA, founded in 1956, aims to promote international cooperation in scientific research relative to tobacco in the areas of agronomy and leaf integrity, phytopathology and genetics, smoke science, and product technology. In 2002, the CORESTA *In Vitro* Toxicity Task Force was formed to establish a rationale and strategy for *in vitro* testing of tobacco smoke and to identify key procedures in line with



Images from Ames, micronucleus, and neutral red assays

internationally recognized guidelines but adapted to take into account the unique properties of tobacco smoke. Recommendations related to these objectives were published in 2004.[4] The suggested battery of tests comprised a bacterial mutation assay (Ames), a mammalian cell assay for cytogenetics or mutation (micronucleus, chromosome aberration, or mouse lymphoma), and a cytotoxicity assay with appropriate mammalian cells (Neutral Red Uptake). The defined test item was total particulate matter from mainstream smoke collected on Cambridge filter pads and extracted in DMSO. The report also provided useful background information and references.

Proficiency trial assays were performed to improve study conduct and methods with the approved battery of tests in samples extracted by standardized preparation. Protocols and worksheets were also standardized. The final data were coded and quality was assessed by a quality assurance expert and a statistician. These studies showed that Ames is sufficiently sensitive (excellent concordance between laboratories), that Neutral Red Uptake results replicated well within a particular cell line, and that micronucleus assay results on tobacco samples differ by S9 treatment conditions. Of note, in the neutral red uptake studies differences were noted between results from the four cell lines used, possibly at least partially driven by differences in metabolic capabilities.

On the basis of these findings, the Task Force concluded the following regarding proficiency trials: objectives and rationale must be clear before starting; careful selection of samples is important; and statistical analysis might be challenging. These challenges are compounded by variation in laboratory experience and understanding of strengths and limitation of methods, which might make interpretation and comparison of findings challenging.

In order to effectively test a wide range of different products, the following considerations are necessary before starting testing:

- Products (differences and similarities)
- Sample production and preparation (smoking regimen, sample type, and phase)
- Sample storage
- Exposure systems (equipment and methods)
- Biological test systems (sensitivity, relevance, strengths, and limitations)
- Interpretation and context
- Utility and application

[4]CORESTA. The Rationale and Strategy for Conducting In Vitro Toxicology Testing of Tobacco. http://www.coresta.org/Reports/IVT_TF_Rationale-IVT-Testing-Tob.-Smoke_Report_Jun04.pdf

Some exciting nnew tools are available to improve the quality of research and further inform the science, such as adverse outcome pathways (AOPs) and systems biology in combination with toxicity methods. *In vitro* models of disease also hold promise.

Key points:

- **A wide range of samples from tobacco products can be tested *in vitro***
- **CORESTA aims to promote international cooperation in scientific research relative to tobacco and supports *in vitro* toxicity discussions and proficiency trials**
- **Proficiency studies with standardized sample preparation and protocols have highlighted the need for planning and understanding of methodological strengths and limitations**

Considerations for Test Method Validation

Rodger Curren, IIVS

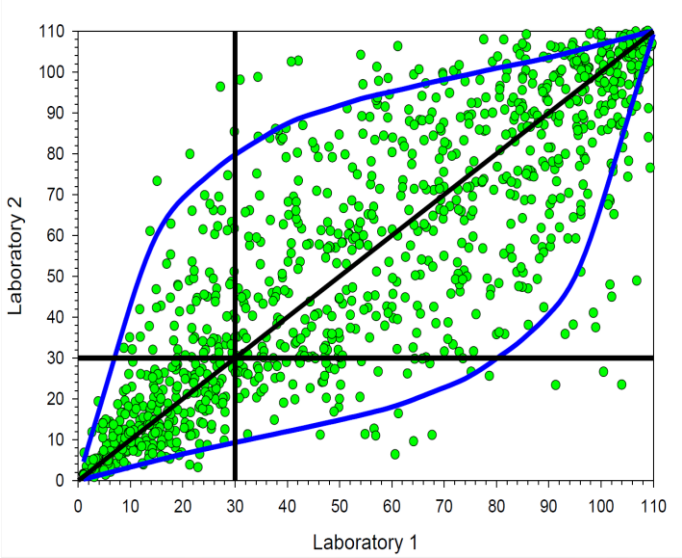
Validation in some form is generally required by regulatory agencies before data from new test methods are accepted. The classic process of validation is demonstration of the reliability (reproducibility) and relevance (extent to which results can correctly predict outcomes) of a new method for a specific purpose. This definition and the classic sequence of events from conception to validation (presented below) are accepted by many international bodies, such as the Organization for Economic Co-operation and Development (OECD).

- Ideas for new test methods come from basic research, but are usually undirected and the suggested methods are are often impractical for routine use
- Optimization of methods increases robustness and ensures performance across multiple laboratories
- Pre-validation, which is a controlled, small-scale study of reproducibility and performance, is conducted
- A final formal validation stage involves multi-laboratory blinded assessment of reliability and relevance

Validation does not need to be done by the agency accepting the test results, but when it is, individual agencies can apply their own standards of rigor to the process. As a result, agencies may vary widely in the number of laboratories required for reproducibility studies, numbers of materials tested, the range of chemistries assessed, and the types of qualitative and quantitative endpoints that must be reported. This highly formal testing is time consuming and very expensive, and is extended by slow review of methods by validation authorities. Even if a method is validated, it might take several years to achieve regulatory acceptance. Thus the process of validation is often severely criticized for the length of time it takes. In this presentation, Rodger Curren explored ways in which the process could be improved.

In 1995, ECVAM proposed a pre-validation stage to assess and refine the validation process, and thereby increase efficiency.[5] Three phases of prevalidation were

[5]Curren RD, Southee JA, Spielmann H, Liebsch M, Fentem JH, Balls M. The role of prevalidation in the development validation and acceptance of alternative methods. ATLA 1995;23:211-217.



Inter-laboratory variation of the Draize rabbit eye test is substantial[9]

suggested. Phase I is protocol refinement through interaction between two designated laboratories to identify potential adjustments needed to optimize the test method. In addition, the protocol can be developed and put into a format compliant with the regulatory agency's requirements. Accompanying standard operating procedures may be developed, reproducibility can be confirmed, prediction models can be proposed, and suitability for the next phase of pre-validation can be confirmed. Phase II involves protocol transfer, in which a third designated laboratory uses the refined protocol and standard operating procedures to test inter-laboratory transferability; further refinement of the protocol is possible at this stage. If phase II results are acceptable, pre-validation moves into phase III, where a limited number of materials are coded for blind testing in a small number of laboratories (at least two) to confirm validity of the prediction model or to suggest its further refinement. If necessary, the method could move on to more rigorous validation involving additional coded materials, or the data obtained during pre-validation could be considered sufficient to establish reliability and relevance.

An efficient validation process can:

- Direct basic research towards gaps in existing testing methodologies and/or knowledge, identified by stakeholders through workshops or directed interactions with regulatory bodies, such as CTP
- Help move useful methods quickly into development and optimization
- Accomodate the use of human biomarkers of effect or disease, rather than—or in addition to—data from animal studies
- Use a pre-validation stage to refine the protocols in competent laboratories, including those associated with Government agencies, such as the US National Center for Toxicological Research or National Institute for Environmental Health Studies
- Include retrospective evaluation of data,[6] including data from robust prior studies (perhaps defined by a regulatory branch, such as the CTP), so that new studies do not have to be conducted unless truly necessary

In determining the value of new test methods, more than just the individual predictive capacity (as assessed during the traditional validation process) should be evaluated. Tests should also be considered as part of integrated testing strategies. For example, combining results from several methods of analysis, such as QSAR, read-across, and

[6]Hartung T, Bremer S, Casati S, Coecke S, Corvi R, Fortaner S, Gribaldo L, Halder M, Hoffmann S, Roi AJ, Prieto P, Sabbioni E, Scott L, Worth A, Zuang V. A modular approach to the ECVAM principles on test validity. ATLA 2004;32;467-472.

one or two other complementary *in vitro* assays might prove to be highly predictive of subsequent human health effects.

To make any validation process truly useful, a meaningful standard against which to evaluate the new test method needs to be established. For many years the gold standard used in toxicology has been the results obtained from animal tests, yet these often suffer from reproducibility problems, and their ability to predict how humans will respond to the same exposure has frequently been called into question. For example, although the Draize rabbit eye test has been viewed as the gold standard for eye irritation for many years, results vary substantially between laboratories[7,8] and are often not predictive of human responses. The figure, from Bruner et al.,[9] illustrates the best correlation relationship that could be expected between two separate laboratories running the Draize test, knowing that the coefficient of variation of this test is around 40%. If this is the highest reproducibility for this animal test, clearly an *in vitro* method could not be expected to perform any better.

In the specific case of validating *in vitro* methods for use in estimating the hazard associated with new tobacco poducts, either combustible or non-combustible, it would clearly be best to validate results against the human not the animal response. A potential way forward to this approach would be to validate against human biomarkers that could be obtained in relatively non-invasive ways during clinical studies. Biomarkers of exposure, effect, or both, should be developed and then used as the gold standards during validation trials with new *in vitro* methods. This approach should be especially useful when used with 3D human tissue constructs that closely resemble the actual human tissues exposed *in situ*. If biomarkers from the *in vitro* systems correlate well with the same biomarkers obtained from individuals in clinical studies after exposure to a given tobacco product, then there would be good evidence that the *in vitro* method would be predictive of either exposure or disease state for a new tobacco product.

In summary, all test methods to assess tobacco products for regulation need to be validated. The exact steps to be taken for each test cannot always be predicted, but however the validation process is conducted it must take into account all research agendas to help the likelihood of regulatory acceptance. Pre-validation performed in competent laboratories and based on guidance documents written by teams with good technical knowledge of the assays, and which address various outcomes, can streamline the process and may eliminate the need for the formal validation studies currently in place.

Key points:

- **Stakeholders should collaborate to direct basic research towards gaps in methodologies**
- **Involvement of the CTP in designing research and selecting facilities for pre-validation testing would be useful**

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[7]Weil CS, Scala RA. Study of intra- and interlaboratory variability in the results of rabbit eye and skin irritation tests. Toxicol Appl Pharmacol 1971;19:276-360.

[8]Cormier EM, Parker RD, Henson C, Cruse LW, Merritt AK, Bruce RD, Osborne R. Determination of the intra- and interlaboratory reproducibility of the low volume eye test and its statistical relationship to the Draize eye test. Regul Toxicol Pharmacol 1996;23:156–161.

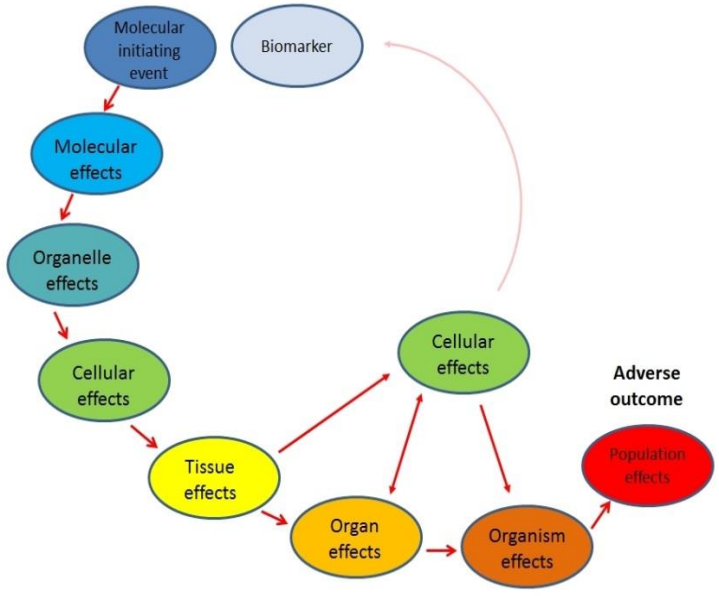
[9]Bruner, LH, Carr, GJ, Harbell, JW, Curren, RD. An investigation of new toxicity test method performance in validation studies: 2. Comparison of three measures of toxicity test performance. Hum Exp Toxicol 2002;21:313-323.

- **The pre-validation stage should be compulsory, with adequate financial support provided for these activities**
- **All research agendas should be taken into account during the development of assays for regulation**

Adverse Outcome Pathways: A Framework for Organizing Mechanistic Information to Improve Chemical Assessment

Kristie Sullivan, Physicians Committee for Responsible Medicine

Kristie Sullivan noted that Tox 21 recommended a strategy that included a wide range of methods of testing. Mapping of toxicity pathways (thought to be a finite number) was intended to be a conceptual framework that would help to predict effects of chemicals without animal testing. Ultimately, it led to AOPs, which are flexible organizational frameworks that outline linear pathways to adverse outcomes and that can link chemical structures to biological response data. There are a variety of uses of AOPs dependent on the amount of information available. For instance, they can highlight research needs (e.g. species or genetic differences and effects on toxicity) and be helpful in the design of testing strategies and frameworks. At the testing level, they can help to identify promising assays, provide mechanistic support for chemical grouping and categorization and hazard or risk assessment, and put assays into biological context, which can remove barriers to regulatory acceptance. Thus, full mechanistic understanding is not necessary for an AOP to be useful.



Example of AOP concept development

The OECD is heavily involved in the development of AOPs. The Extended Advisory Group for Molecular Screening (EAGMST) aims to collect information and enable collaboration, for example with tools such as the AOP wiki,[10] and support regulatory activities.

Concept development can be simple or complex and can be started without knowing exactly what the key events are. Rather, AOPs can be used to try to establish linkages between key events (i.e. what happens to link one to the next). A work plan for the

[10]https://aopkb.org/aopwiki/inex.php/Main_Page

AOP can be proposed and submitted for review by expert groups via the AOP wiki. This can lead to the setting up of workshops and creation of a main team or laboratory to research the AOP, but with contributions from multiple sources (i.e. "crowd-sourcing" of information).

Key points:

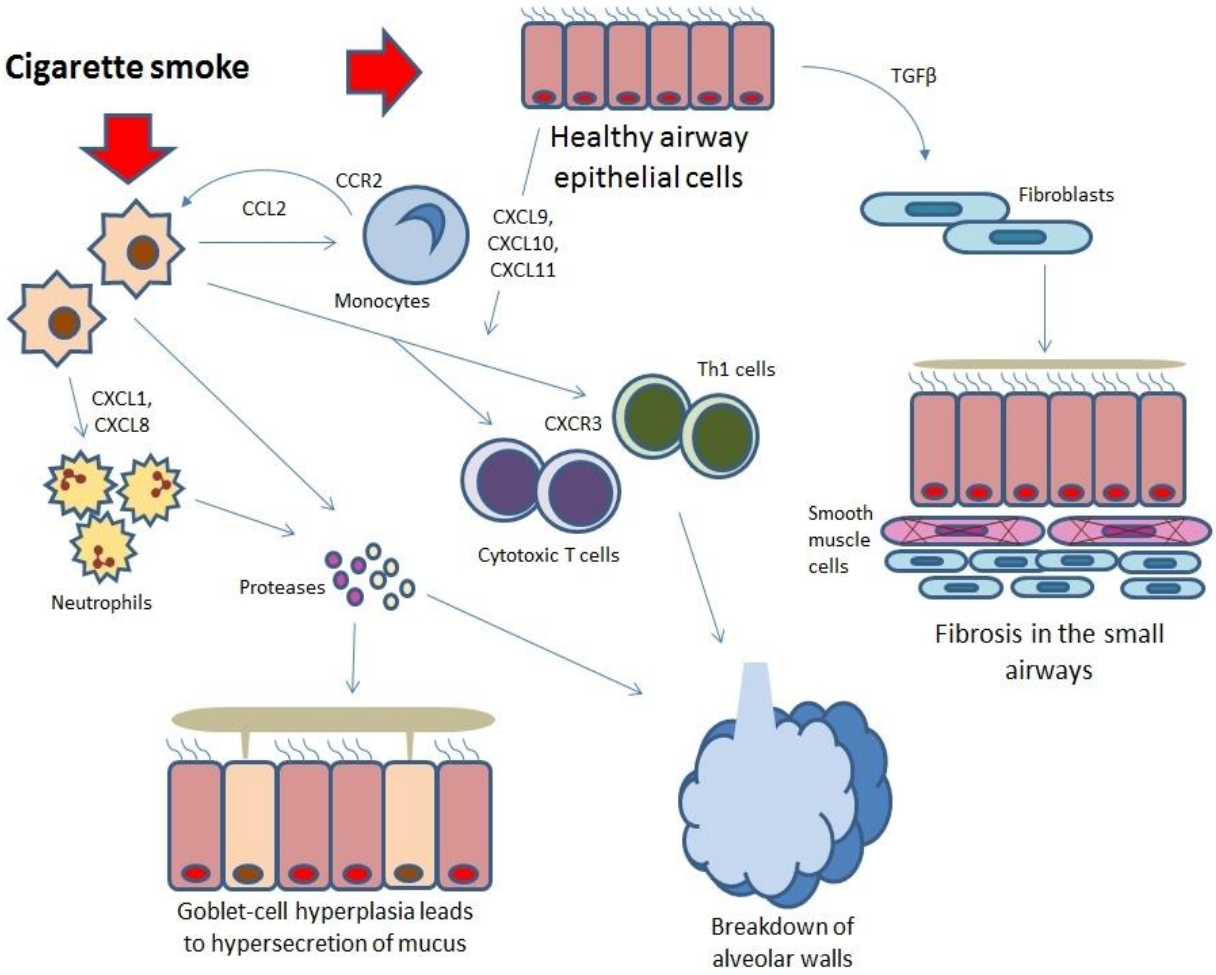
- **AOPs are flexible organizational linear frameworks illustrating pathways to adverse events**
- **They are useful for highlighting gaps in research and knowledge, for chemical grouping, providing context for data or methods, and for test-method development**
- **The OECD AOP wiki allows information to be shared and aids collaboration**

CHRONIC OBSTRUCTIVE PULMONARY DISEASE: OVERVIEW

Etiology of COPD and *In Vitro* Models

Holger Behrsing, IIVS

To start this section of the workshop, Holger Behrsing presented an overview of COPD. Patients with COPD can have one or more symptoms of chronic bronchilitis (excessive mucus production, airway wall thickening, epithelial squamous metaplasia, and leukocyte recruitment), emphysema (airspace enlargement, parenchymal destruction), or both. The distribution varies from individual to individual, but the disease is characterized by (usually) progressive airflow limitation and chronic inflammatory response to noxious gases and particles. The main risk factor is smoking, but sex, genetics, pre-existing airway disease, etc. and environmental factors also contribute to risk. Tobacco smoke exposure changes the lining of the bronchus and leads to oxidative stress: in early stages basal cells begin to crowd out columnar cells, which are ciliated, and, therefore, cilia become reduced in number and efficiency and toxic particles are not cleared effectively; later changes are total displacement of ciliated columnar cells, which raises the risk of infections and exacerbations of airway limitation, and an increase in abnormal sqamous cells, which eventually invade the underlying lung tissue and can become cancerous. Small airways disease, which includes a wide variety of disorders, often includes some form of biochiolitis, bronchiolar inflammation, and metaplasia of the bronchiolar tissues.



COPD events after exposure to cigarette smoke

Various models and assays have been proved fit for purpose for COPD. Various features, such as oxidative stress, inflammatory response, ciliary dysfunction, goblet cell hyperplasia (GCH), and small airway and vascular remodeling, are potential targets for *in vitro* testing. Model choices are cell lines of immortalized cells, primary cells, 3D airway tissue cultures, and tissue samples for testing *ex vivo*. These can be employed in drug development, hazard of exposure assessment, etc, and, therefore, the suitability of models must be considered.

Immortalized cells are economic, straightforward to use, and results are reproducible. These cell lines can be expanded and are simple to store, although high passage might be associated with genetic drift. However, cell lines are frequently derived from cancerous tissue and thus do not represent the physiological state as other models do.

Primary cells are often more expensive because they are not immortalized, but are more representative of the population of interest (although subject to donor variability), and may not have much cacpacity to be expanded and stored for future use (i.e. supply is limited).

3D tissue cultures, such as reconstructed airway epithelium, are physiologically relevant, representive of the population of interest, and cell types and functions not available in two-dimensional cell lines might be possible to be assessed. Each culture, however, can take weeks to complete, which can be expensive and reproducibility might vary across donors.

Ex vivo tissue studies can by done using precision-cut lung slices (PCLS). These are more physiologically relevant but are representative only of the part of the organ from which they are cut. They might be representative of a population but frequently are obtained from donors that do not match wider population criteria (e.g. slices are often obtained from lungs discarded from transplant owing to certain disease states). A strength is that they do enable study of multiple cells types, but quality is not always high and might be variable across donors. Reproducibility is also variable across donors and is labor intensive to achieve.

Important considerations in choice of *in vitro* model, therefore, are:

- Cost
- Reproducibility
- Ease of use and accessibility
- Interlaboratory transferability
- Endpoints modeled
- Tissue origin
    - If human, how well does it translate to whole body?
    - If non-human, how well does it extrapolate to humans?
- Amenable to high throughput

Upcoming technologies that might improve *in vitro* testing include lung on chip. The Wyss Institute has created a 'breathing' human lung on chip that recreates expansion and contraction of the airways during breathing to mimic changes in air and blood flow.[11] RTI International, in collaboration with the University of North Carolina, have

[11]http://wyss.harvard.edu/viewpage/404/

created a lung-on-chip model in which distinct cellular layers are stacked to mimic the structure of airway tissue.[12]

Key points:

- **COPD symptoms can vary from individual to individual and are characterized by (usually) progressive airflow limitation and chronic inflammatory response to noxious gases and particles**
- **The major etiological factor is smoking**
- **Various *in vitro* and *ex vivo* models are available that allow relevant testing of exposure to cigarette smoke**
- **Multiple factors must be taken into account when selecting a model; cheaper options might be less physiologically relevant, whereas more expensive options might be time consuming to create or have limited supply**
- **Variability across donors is an issue that needs to be addressed but individuality of tissues may more accurately reflect a population**
- **Emerging technologies such as "lung on a chip" could advance the relevance of models**

Overview of the Clinical Aspects of Chronic Obstructive Pulmonary Disease (COPD)

Sanjay Sethi, University at Buffalo

Sanjay Sethi provided further clinical information about the features and epidemiology of COPD. This disease is the third most prevalent in Canada[13] and USA. It is currently the sixth most prevalent globally and is predicted to reach third by 2020. Despite improvements in treatment and air quality etc., COPD will remain a major problem because of a high rate of undiagnosed disease. Despite being preventable and treatable, the disease is frequently not diagnosed until progression occurs—early stage symptoms are generally not serious and frequently go unrecognized by physicians—and some patients are reluctant to seek treatment, as it is a "self-inflicted" disease. High concentrations of autoantibodies are detected, but whether these are causative is unclear, and autoimmune disease elements need to be explored further. COPD incurs high health costs, particularly in relation to exacerbations. Therapies are mainly symptom driven rather disease modifying, but most patients do not receive the recommended treatment (~60% undertreated, and/or ~65% inappropriately treated). Poorly treated disease is associated with severe extrapulmonary effects and important co-morbid conditions.

Initially, there was a lot of confusion about what constituted COPD. Small-airways disease was overlooked and emphasis was on emphysema, but later it was shown that what happens in the small airways has a notable contribution. In a healthy state the airway is held open by alveolar attachments. In patients with COPD, these attachments are disrupted, which, with mucosal and peribronchial inflammation, fibrosis, and hypersecretion of mucus within the lumen, adds to the impairment of airflow. Currently, most *in vitro* models concentrate on airway epithelium, but given this

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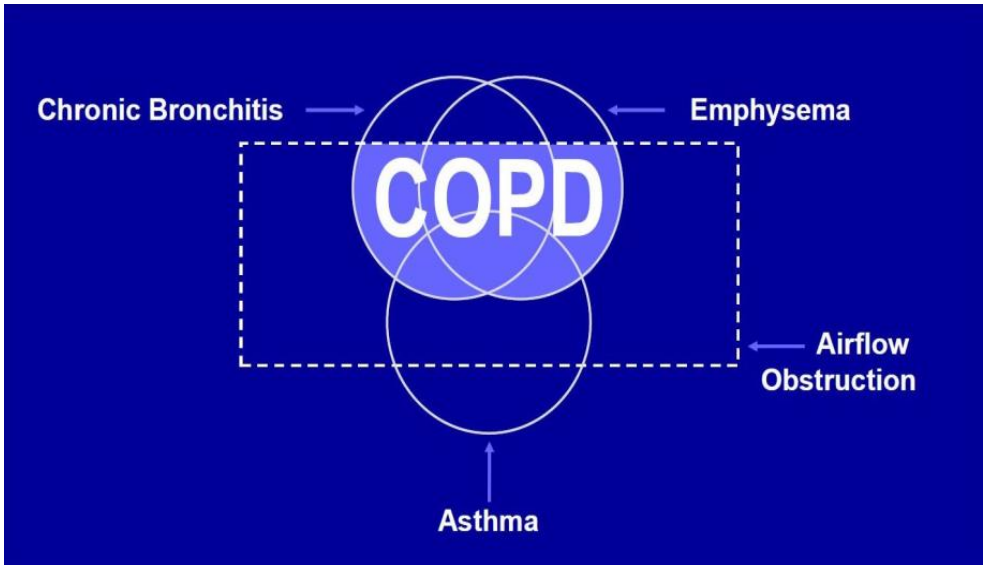
[12]http://www.rti.org/newsroom/news.cfm?obj=503D8210-E344-7120-553A0AD769F494B4

[13]Gershon AS, Warner L, Cascagnette P, Victor JC, To T. Lifetime risk of developing chronic obstructive pulmonary disease: a longitudinal population study. Lancet 2011;378:991-996.

feature of COPD it would be very useful to include alveolar macrophages, especially as attempts to block precursor pathways have so far been unsuccessful.



Spectrum of COPD

Although tobacco smoke is the main risk factor, individual responses to this and other particle exposures vary substantially (e.g. occupational dusts and indoor and outdoor air pollution). Lung growth, oxidative stress, female sex, age, respiratory infection, low socioeconomic status, poor nutrition, and comorbidities are all well-recognized contributing factors and must be taken into account. Genes, however, seem to contribute little to susceptibility.

Diagnosis seems as though it should be easy: symptoms + risk factors + positive spirometry = COPD, but is not that simple owing to variability. Physical examination is rarely diagnostic and spirometry is essential to confirm suspicions and might help to differentiate COPD and asthma. COPD should be considered in patients with any symptoms and a history of exposure to risk factors. Screening might be useful, but as the recommended intervention would remain smoking cessation, is not recommended.

Smoking cessation reduces the risk of COPD and is the only modality known to change the disease course; in some cases, very low FEV_1 might lead to self-perpetuating disease. Lung damage starts early and might be more rapid in these stages than later in the disease course. Even mild disease is associated with exacerbations, airway limitation, and physical impairment.

COPD is staged as mild, moderate, severe, and very severe, initially based on degree of airflow limitation (FEV_1), but this presentation has a high crossover with asthma. Differentiation used to rely on response to bronchodilators (post-bronchodilator $FEV_1/FVC < 0.70$ confirms presence of persistent airflow limitation, and thus COPD), but symptomology should also be taken into account. Asthma and COPD should be viewed and treated as separate diseases.

GOLD Stage	Severity	Degree of Airflow Limitation
1	Mild	FEV_1 ≥80% predicted
2	Moderate	50% ≤FEV_1 <80% predicted
3	Severe	30% ≤FEV_1 <50% predicted
4	Very severe	FEV_1 <30% predicted

Staging of COPD by degree of airflow limitation

The current management guidelines are to reduce risk, to relieve symptoms and improve exercise tolerance and health status and prevent disease progression and exacerbations. Where these latter occur, they should be treated adequately to reduce mortality. The use of non-pharmacological management strategies has grown, but some goals have had more success than others. Smoking cessation is the first priority, and other options are education of patients, rehabilitation (although this is expensive and often not funded), surgery, vaccination, and oxygen therapy. Pharmacological strategy options have also grown, from just short-acting bronchodilators and steroids, to long-acting and anti-inflammatory drugs, and numbers of individual and combinations of drugs continue to grow. Treatment was originally based on FEV_1 staging, but correlation between FEV_1 and quality of life is poor. Therefore, the importance of treating exacerbations has become recognized and now included in treatment planning, although improved guidelines are needed. Discovery of disease-modifying treatments would also be helpful (e.g. anti-inflammatory drugs), as bronchodilators are reaching their capacity of usefulness owing to little difference in outcomes between devices. Treatments to reduce symptoms (such as excess mucus) and risk of infections and those to treat musculoskeletal and gastrointestinal features would also be useful.

Key points:

- **COPD is a preventable and treatable disease**
- **Improvements are required in treatments to relieve symptoms and prevent exacerbations**
- **Increased clinical and basic research offers hope; exacerbations might be a particularly useful target for model development**

INFLAMMATION AND OXIDATIVE STRESS

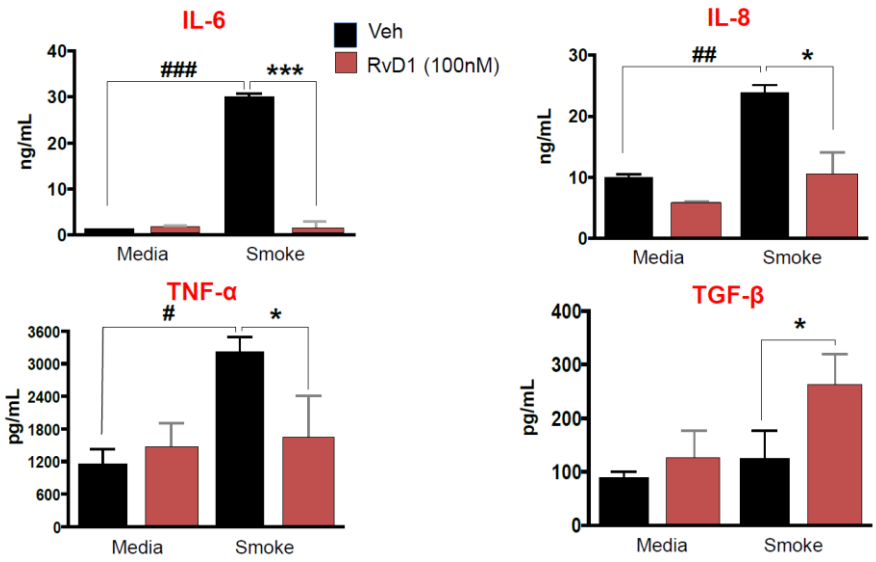
Impact of Tobacco Smoke on Lung Inflammation and Pro-Resolving Pathways in Humans, Mouse Models, and *In Vitro* Models

Richard P. Phipps, University of Rochester

Cigarette smoking is a cause of many inflammation-related pulmonary diseases. Smoke contains more than 4,500 chemicals with various toxic effects, and smoking is associated with around 438,000 deaths per year in the USA. The ideal outcome of inflammation is complete resolution as, unresolved, inflammation becomes chronic and leads to loss of tissue function. Resolution of inflammation was initially thought to be a passive process occurring after removal of the stimulus. However, the lipid-mediator switch concept describes an active process that involves specialized pro-resolution lipid mediators (SPMs), which provide balance against the pro-inflammatory pathway and help to maintain or regain homeostasis. For example, omega-6 fatty acids (e.g. in peanuts) can lead to inflammatory products, whereas omega-3 fatty acids (e.g. in fish) resolve into SPMs and lead to resolution of inflammation. In smoking-related diseases two hypotheses are of interest:

- The normal resolution pathways are dysregulated but may be targeted therapeutically
- Tobacco toxic effects can be gauged on the basis of dysregulation of resolution pathways

Studies of exhaled breath condensate (*c*. 1 mL collected from 10 min breathing) assessed by mass spectrometry have shown the presence and pattern of SPMs, and reveal dysregulation. This non-invasive approach might, therefore, be a useful way to assess inflammation in COPD patients, where endogenous resolution circuitry has been altered.



Resolvin D1 blunts cytokine production by monocyte-derived macrophages in cigarette-smoke-induced inflammation

Mouse inhalation models of acute lung injury have been used to investigate whether pre-treatment with the SMP resolvin D1 (RvD1) alters the inflammatory process. After twice daily exposure to cigarette smoke for 3 days, neutrophil infiltration was inhibited and expression of anti-inflammatory cytokines (e.g. IL-10) and

concentration of macrophage cytokine mRNA were notably increased. Post-treatment 3 days after smoke exposure also encouraged resolution of lung inflammation by a similar mechanism.

A mouse model of emphysema was also assessed. Exposure required is long term and symptoms begin after c. 6 months, but is faster in the female A/J mice sensitive strain. Some human features of emphysema are mimicked in mice (e.g. alveolar damage, but not airflow restriction), and these outcomes were at least partially ameliorated by pre-treatment with RvD1.

In vitro studies have been done to begin addressing the influence of SPMs on human cells. Primary human cells (macrophages, fibroblasts, and epithelial cells) are preferable. Macrophages, particularly, are of interest because COPD patients are highly susceptible to infection and macrophages play an important role in removing microbes and macrophages are also important in resolution of inflammation. In

human monocyte-derived macrophages, cigarette smoke exposure leads to impaired phagocytosis and increased production of cytokines, chemokines, COX-2 and PGE_2 production. In cells pre-treated with RvD1, production of the inflammatory cytokine IL-6 is blunted, whereas that of TGFβ, a pro-wound-healing cytokine, is increased. Pre-treatment with RvD1 also reduced cigarette smoke extract-induced inflammatory responses in fibroblasts, and in airway epithelial cells of the small airways was associated with reduced production of pro-inflammatory mediators. Human studies show variability in responses, which will be important to take into account for directing responder and non-responder studies. Prevention with SPMs might be useful not only for smokers, but also for inviduals regularly exposed to toxic smoke (eg military, firefighters) or in circumstances of viral-induced cytokine storm.

Dietary uptake of omega fatty acids feeds into these resolution pathways.

Key points:

- **Local tissue inflammation leads to neutrophil infiltration and pro-inflammatory mediators, which in turn lead to apoptosis**
- **SPMs act via several mechanisms to promote resolution of acute and chronic inflammation**
- **Development of SPM therapeutic agents might be a useful route for management of inflammatory lung disease**
- **Measurement of changes in SPMs caused by exposure to tobacco products might serve as an indicator of relative toxicity**

Genetic Variants, Inflammation, and the Mucous Secretory Phenotypes

Yohannes Tesfaigzi, Lovelace Respiratory Research Institute

After epithelial injury, inflammation causes proliferation of non-goblet cells that differentiate into mucus-producing cells (mucous-cell hyperplasia [MCH]) and differentiation of non-goblet cells into mucin-producing goblet cells (mucous-cell metaplasia [MCM]). These changes lead to thickening of the epithelium. The MCH and MCM decrease after about 10 days if inflammation does not continue. The Bcl-2 family of proteins is central in coordinating this resolution process. Bcl-2, which is protective against apoptosis, and Bik, a promotor of apoptosis, are expressed in a timely fashion

to reduce the proliferation of hyperplastic cells. The proportion of Bcl-2-positive mucous cells is increased in chronic airways diseases, such as cystic fibrosis and chronic bronchitis. Bik mRNA levels are reduced but those of other Bcl-2 family genes are not. Thus, the hyperplastic goblet cells do not die and MCM and MCH are not resolved.

Expression of Bcl-2 is suppressed by p53, which destabilizes Bcl-2 mRNA. An inverse relation between Bcl-2 and p53 has been shown in p53-deficient mice, where Bcl-2 levels remain high and resolution of MCM and MCH is obstructed.

The single-nucleotide polymorphism in *TP53* modifies Arg72Pro. This change in amino acid is within the second of five PXXP motifs of the proline-rich domain and encodes two p53 variants, 72Arg and 72Pro. Airway epithelial cells with the 72Pro variant have reduced expression of Bcl-2 and mucus production, thereby attenuating loss in lung function. In individuals with *TP53* Arg72Pro, airway epithelial cells are also more susceptible to death caused by DNA-damaging agents because of reduced Bcl-2 levels. Overexpression of p53Arg increases and of p53Pro decreases mucus production through differential regulation of SPDEF, a transcription factor that drives the mucous differentiation pathway. Humans with COPD and the p53Arg variant, therefore, are at increased risk of chronic disease, and pollutants other than cigarette smoke are likely to have an additive effect.

Therefore, it is important to consider DNA polymorphisms in human disease. This will be important for the development of relevant *in vitro* models.

Key points:

- **The Bcl-2 family of proteins are important in inflammatory lung diseases**
- **They have a variety of actions: guardians, effectors, and sensors**
- **p53 destabilizes Bcl-2 mRNA and is crucial for resolution of MCM**
- **In individuals with the *TP53* Arg72Pro single-nucleotide polymorphism, expression of Bcl-2 and mucus production are reduced**
- **DNA polymorphisms should be considered in the development of *in vitro* models**

Overview of Non-Animal Approaches to Address COPD Pathogenesis Associated with Inhaled Nicotine-Delivering Products

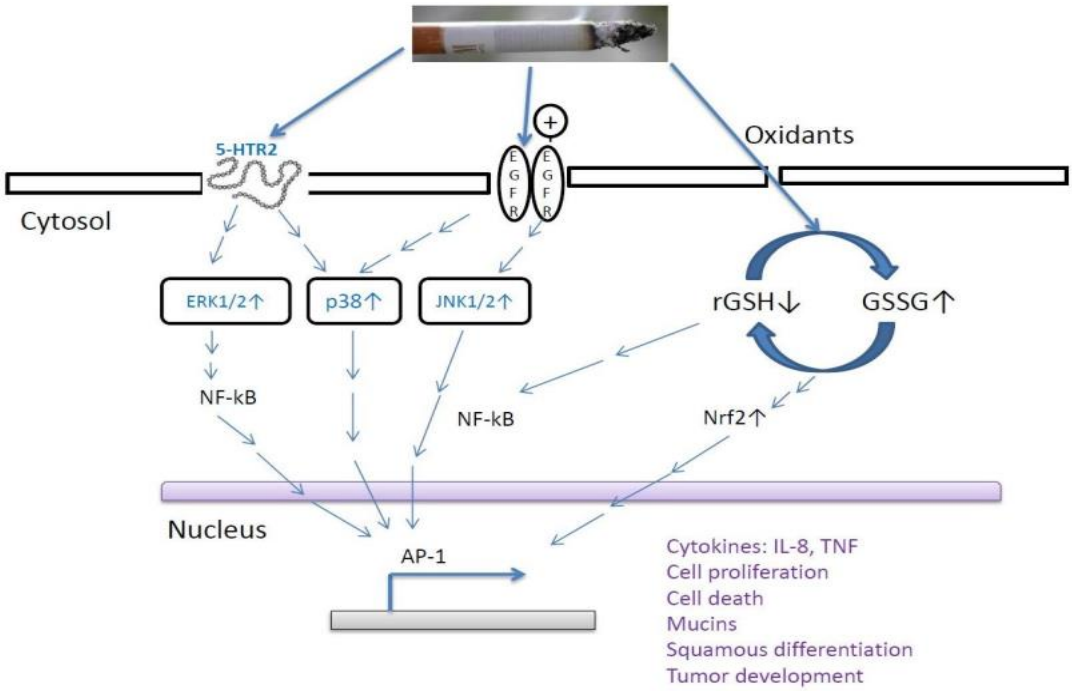
Sherwin Yan, Lorillard

Smoking, the main etiological factor in COPD, is an important exogenous source of reactive oxygen species (ROS) and upregulates release of endogenous ROS. Additionally, activated epithelial cells produce inflammatory mediators, such as TNF, GMCSF, IL-1, and IL-8. Exposure to smoke, however, impairs the innate and adaptive immune responses of the airway epithelium and the likelihood of infections is increased.

COPD is multifaceted and, therefore, no single model can produce all the relevant results. The most frequently used cells types are human sinonasal, lung fibroblast, and small airway and bronchial epithelial cells. Some ready-to-use models are also available, such as fully and pseudo-differentiated epithelial models (basal, goblet, and ciliated cells), human primary epithelial cells co-cultured with fibroblasts (shows interactions of different cells types).

T lymphocytes, especially CD8+ cells and macrophages, are prevalent inflammatory cells in healthy smokers and in smokers with mild COPD. In smokers with severe COPD, CD4 and NF-κB are also overexpressed. Macrophages secrete proteases MMP-2, MMP-9, MMP-12 etc., which are potent activators of inflammation. NF-κB is a pro-inflammatory transcription factor involved in the control of genes for many inflammatory mediators expressed in COPD, and is upregulated in macrophages and airway epithelial cells of people with this disease. Up-regulation of NF-κB in epithelial and endothelial cells may contribute to the differential prevalence of cell infiltration

The MAPK serine-threonine kinase pathway, which regulates inflammation, is heavily discussed in relation to COPD. Within this pathway are three important kinase pathways that can be targeted separately: ERK, JNK, and p38. *In vitro* models have been used to investigate MAPK pathways and cascades. Cigarette smoke activates 5-HTR2, which induces disrupted MAPK cascades through many different types of cellular events in all three pathways. The different components of MAPK, however, have different roles. For example, targeted inhibition of p38 or the ERK kinases MEK 1/2 suppresses IL-8 release after exposure to cigarette smoke, but inhbition of JNK does not. Knockdown of MEK 1 specifically blocks cigarette-smoke-induced release of IL-8.



Oxidative stress and inflammation induced by cigarette smoke

The lung is one of the most vulnerable targets to oxidative damage in the body due to location, anatomy, and function. Oxidant–anti-oxidant imbalance in favor of oxidants increases oxidative stress in COPD. Increased numbers of neutrophils and macrophages in the alveolar space in smokers increases oxidant burden. The imbalance is further increased by increased free Fe^{2+} concentrations (Haber–Weiss reaction) and lipid peroxidation. Antioxidant defence is normally mounted by antioxidants in the respiratory tract lining fluid, such as glutathione, mucins, uric acid, ascobic acid, and albumin. Cigarette smoke reduces antioxidant capacity and actions.

In the acute phase of COPD, reduced intracellular glutathione or increased concentrations of ROS can result in translocation to the nucleus of transcription factor Nrf2. Activation of this transcription factor induces up-regulation of detoxifying genes and pro-inflammatory cytokines.

Overall, the evidence from human studies supports the hypotheses generated by *in vitro* model studies for COPD.

In e-cigarettes the aerosol is much less complex than cigarette smoke, including greatly reduced CO levels because of non-combustible heating. Studies stuggest that there is substantially less inflammation response (IL-8 release) to aerosol than to cigarette smoke. Indeed, there are substantially fewer harmful and potentially harmful consituents in aerosol, and either minimal or no nitrosamines. Reliable, validated methods, however, are needed to assess exposure to nicotine.

Key points:

- **NF-κB is an important factor in the ongoing inflammatory process in COPD**
- **The MAPK pathways are disrupted by exposure to cigarette smoke, which leads to release of pro-inflammatory cytokines, such as IL-8**
- **Human studies support the findings of *in vitro* model studies**

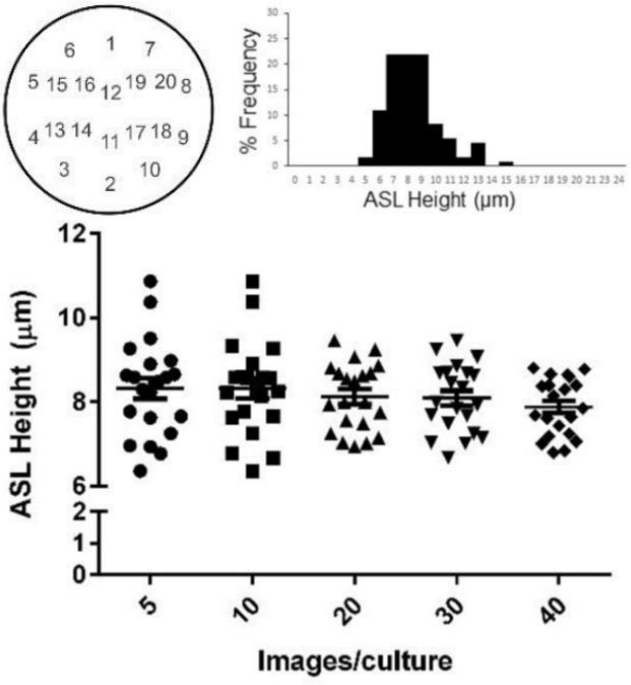
CILIARY DYSFUNCTION AND ION TRANSPORT

Measuring Airway Surface Liquid Volume and Mucus Transport by Fluorescence Microscopy

Robert Tarran, University of North Carolina

Epithelial cells are the first point of contact for cigarette smoke. Research in the past 10 years has shown they regulate immune responses, mainly by mucus and mucin production. Airway surface liquid (ASL) comprises mucus to trap inhaled particles and a peri-ciliary liquid layer that that keeps mucus at an optimum distance from the underlying epithelia for clearance. Ion channels in endothelial cells are damaged by exposure to cigarette smoke, which over the long term can lead to dehydration of ASL in patients with COPD, as is seen in cystic fibrosis soon after birth, and possibly asthma. Increased Na^+ absorption increases mucus dehydration, which has been shown to cause lung disease in mice.

Measurement of ASL height is straightforward to study, and ASL hydration assays have proved extremely predictive of the human *in vivo* effects of cystic fibrosis pharmacology. Additionally, they can be coupled to other assays, such as viability and inflammation assays, to investigate effects of chronic smoke exposure. Cultures differentiate into basal, goblet, and ciliary cells and form the peri-ciliary liquid and mucus layers, but keep growing in height. The cultures can sense changes and alter hydration, as in *in vivo* airways. Thus ASL height can be tracked over time in cultures with use of an inverted confocal microscope. 3D rendering software enables comparison of differences between cultures. As well as ASL volume depletion, cigarette smoke exposure correlates with changes in ciliary beating, which affects the speed of clearance.



Representation of ASL height with automated analysis

Cell cultures of primary airway or alveolar lung cells are best; cell lines can be used if they polarize. Choice of substrate is key, as this has a notable effect on how the cells grow. Fluorescent dextran is mainly used for labeling because there is a wide choice of products for different budgets. Mucus can be labeled with fluorescent latex beads. The size of beads is important, as they will yield different results. Use 100 nm beads to aid visualization of the ultrastructure and 1 µm beads to track speed of movement (e.g. slowing as mucus becomes dehydrated). Effects can be assessed with inexpensive microscope. Fluorescent bacteria can also be used or viruses expressing GFP. These, however, are living organisms, so whether adding them to the culture alters outcomes and/or whether the culture has potential effects on the bacteria must be considered.

Use of an inverted confocal microscope is best for assessment of cultures, but upright scopes with dry or dipping lenses can be used with spinning disc and XZ stacks. XZ line scanning is ideal if the galvo stage is used to enable high-speed scanning. The type of lens greatly influences the quality of images. The optimum is a bright (high numerical aperture) lens with a long working distance, particularly for an inverted microscope, but these can be expensive. Cheaper dry lenses with a good working distance but a lower numerical aperture are good alternatives. More dye in the ASL might be helpful if brightness is an issue.

Cultures in media are sterile. If they are taken out of media, sterility might be lost, so they should only be opened under a tissue culture hood. ASL time courses can run for 2 days and smoke exposure protocols for 2 weeks. Under the hood, the culture may be transferred to a second plate with Ringer glucose solution in media. Experiments should be performed in the second plate, and the culture trasnferred to the original plate after time points are reached (this works >95% of the time).

ASL height can vary substantially from culture to culture and, therefore, for analysis, five areas per culture with different numbers of points should be assessed manually to calculate an average height. ImageJ freeware can be used to process miscroscopy images. For 7 μm ASL, five or six cultures are needed per group to show a 50% decrease in ASL height. Another option to increase output is an automated microscope that can collect 20 images per culture in around 20 s and can be coupled to the automated analysis software.

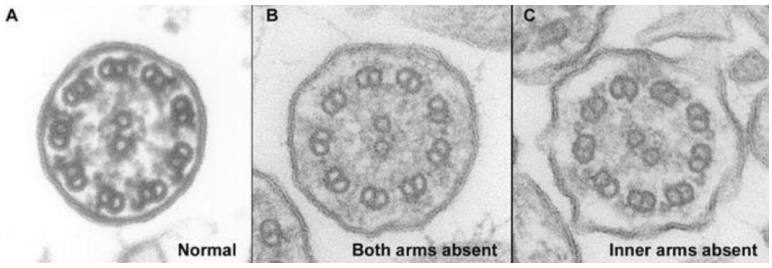
To ensure good results, cultures with good ion transport grown for 3–5 weeks should be used; those grown longer will yield poor images. Primary cultures are best. During growth cultures might need to be washed to ensure too much mucus does not develop, as this will reduce the qualityof ASL height images. When assessing ASL responses, multiple features, such as ion transport and tight junctions, AQPs, GPCRs, etc. should be considered.

Key points:

- **Measurement of ASL height is a straightforward way to measure ciliary function and ion transport, and there is a choice of products to meet different budget needs**
- **Mucus can be labeled with fluorescent latex beads of various sizes to allow visualization of different features, such as ultrastructure and speed of movement**
- **Use of an inverted confocal microscope is best for assessment of cultures, but upright scopes with dry or dipping lenses can be used**
- **To ensure good results, cultures with good ion transport grown for 3–5 weeks should be used; those grown longer will yield poor images**

Assessment of Ciliary Dysfunction in Chronic Obstructive Pulmonary Disease Research

Samuel Constant, Epithelix



Cross-section images of cilia

Sam Constant presented on Epithelix's MucilAir™ airway models and how they can be used in a toxicology setting. Mucus is secreted to trap pollutants, but the cilia comprise a sychronized beating "motor" of the clearance system. Each cilium consists of >600 proteins organized into complexes that work as nanomachines. In healthy individuals the cilia have inner and outer axonemal dynein arms which ensure that cilia move in the same direction. In damaged cilia, the inner arms or both sets might be missing, leading to abnormal function. When arms are missing, movement becomes asynchronous. In patients with airways diseases characterized by primary ciliary dyskinesia, the different frequencies and directions of beating can be visualized with light microscopy. Various mechanisms have been suggested for the sychronization, but the generally accepted theory is that hydrodynamic coupling forces exist between adjacent beating cilia.

Ciliary beating and tracking with micro-beads on a 3D airway epithelium model

Effective mucociliary clearance (MCC) is essential for clear respiratory health. MCC involves the beating cilia but also relies on the properties of the mucus. Mucins are secreted as long strands that interact with globular protein, to create the viscoelastic properties that enable particles to be trapped and transported. The thickness of the layer can be affected by over- and under-hydration because, when the mucus becomes, respectively, not sticky enough or too sticky (and sticks to cells), clearance can be impaired. Thus MCC may be affected at the structural (modificaton of cilia), functional (dys-synchronization of cilia), and physiological (mucus) levels, and might be exacerbated by external pollutants and infections. For instance repeated exposure to pollutants can lead to reduced numbers of ciliary cells and increased numbers of mucus-secreting cells, and influenza viruses can attach to cilia and lead to disorientation or addition and/or decreases in central microtubules. In COPD the cilia become shortened and

are eventually lost due to ROS interacting with ciliary proteins after exposure to cigarette smoke, leading to autophagy of ciliary proteins. Ciliary beating is also depressed in COPD patients.

3D models of the airway epithelium (MucilAir™) have been used to assess cilia dysfunction *in vitro* and have a shelf life of about 1 year due to slow cell turnover in the epithelium. Nasal, tracheal, and bronchial primary human cells can undergo air–liquid differentiation to basal, ciliary, and goblet cells that produce mucus closely resembles host epithelium. Ciliogenesis is seen by 21 days and increases up to 45 days, after which cell numbers remain stable. To ensure correct mucus thickness is maintained, the model should be washed at least twice per month. Cilia beating frequency can be measured with an inverted microscope, high-speed camera, and high-throughput measurement software. Micro-beads seeded on to apical surface can be tracked to calculate the velocity and direction of movement. When mucus is dehydrated and viscous, the cilia still beat, but particles are not moved.

Analysis of cilia dysfunction is relevant for COPD research, as MCC and ciliary beating frequency are informative endpoints of cilia dysfunction. 3D air–liquid interface cultures can also be used to measure chronic effects of airborne xenobiotics on ciliary function and ciliogenesis. Non-destructive methods to measure cilia lengths are still needed. Novel models that better recapitulate the physiology of the human airway for *in vitro* testing (e.g. 3D printing) might also be useful. Another possibility with the 3D model is use of genomic, proteomic, metabolomic, lipidomic, and glycanomic methods to identify relevant biomarkers.

Key points:

- **Effective MCC is essential for clear respiratory health and requires good ciliary beating frequency and correctly hydrated mucus**
- **In COPD the cilia become shortened and are eventually lost**
- **3D models of the airway epithelium may be used to assess particle clearance under normal and altered conditions**
- **Assessment of chronic effects of exposure to pollutants is possible**

Understanding the Impact of Tobacco Smoke Exposure on Ciliary Dysfunction and Ion Transport: The Case for *In Vitro* testing

Gary Phillips, British American Tobacco

Gary Phillips continued the discussion on the use of *in vitro* models to elucidate mechanisms that contribute to the development of COPD. Several publications now advocate the use of *in vitro* methods for toxicity testing based on an AOP approach. In this way, the use of *in vitro* models helps to reduce costs, improve human relevance, and provide a degree of predictability because the test are based on a mechanistic understanding of the toxicity pathway. To date, such models are have been developed and used in various industrial settings, either as a consequence of the ban on animal testing, as is seen in the cosmetic industry, or as a part of the 3Rs approach in the pharmaceutical and chemical industries. In addition, *in vitro* methods that are robust and acceptable for regulatory purposes are being used in the tobacco industry for the assessment of the next generation of nicotine delivery and tobacco-related products.

Studies on MCC have been conducted for more than 40 years and have demonstrated that, as in many disease states, including COPD, the inability of the lung to clear excessive mucus can lead to airflow impairment, inflammation, and a clinically important decline in lung function. Impaired clearance can result as a consequence of many different processes, including overproduction of mucus, increased numbers of mucus-secreting cells, decreased number and function of cilia, and inability of the airway epithelium to maintain adequate hydration of this matrix. Although *in vitro* models of MCC are being utilized more frequently, in many instances the use of cell lines might not be not suitable. They often lack the structural and biochemical features associated with known *in vivo* functions of the MCC. Primary cells and tissue models have helped overcome this problem, as they are able to maintain their *in vivo* metabolic competency *in vitro* and are structurally similar to those cells found in the conducting airways. Tissue samples derived from most areas of the respiratory system (nasal to small airways) can be easily obtained from healthy individuals and those with various disease states, which allows more-relevant studies to be conducted of the effects of exposure to smoke toxicants. More-complex *in vitro* models, such as PCLS and the lung-on-chip system, are also gaining prevalance. These models more closely mimic the complex structural and functional aspects of the lung than cultured models and have a good shelf-life, which allows studies to e conducted that involve chronic and repeated exposures to smoke and aerosol.

When reproducing real-life exposure, how aerosols and particles are collected and presented to *in vitro* models need careful consideration. Testing of cigarette smoke has in the past concentrated on the particulate phase of the aerosol. However, toxicants present in the vapour phase are known drivers of COPD and, therefore, appropriate smoke generation and delivery systems have been developed that allow for more physiologically accurate and approriate aerosol exposures.

In vitro models offer notable benefits in terms of facilitating repeat exposure studies with improved relevance and ethics. Harmonization of approaches is now needed, especially in the way in which aerosols from all tobacco products and nicotine delivery devices are generated, characterized, and delivered to *in vitro* models.

Key points:

- ***In vitro* assays are gaining momentum to address concerns related to ethics and relevance of testing and to meet regulatory requirements**
- **Development of primary cell and tissue models has improved the human relevance of the tests and the ability to address the effects of chronic exposure to tobacco smoke**
- **Many different disease-relevant and toxicologically specific endpoints are being measured, and combination of these endpoints supports understanding of the effects of smoke exposure on the lung**
- **Harmonization is required between all industrial and academic laboratories to agree on the approach to generate smoke aerosols and the *in vitro* models required to address specific disease concerns**

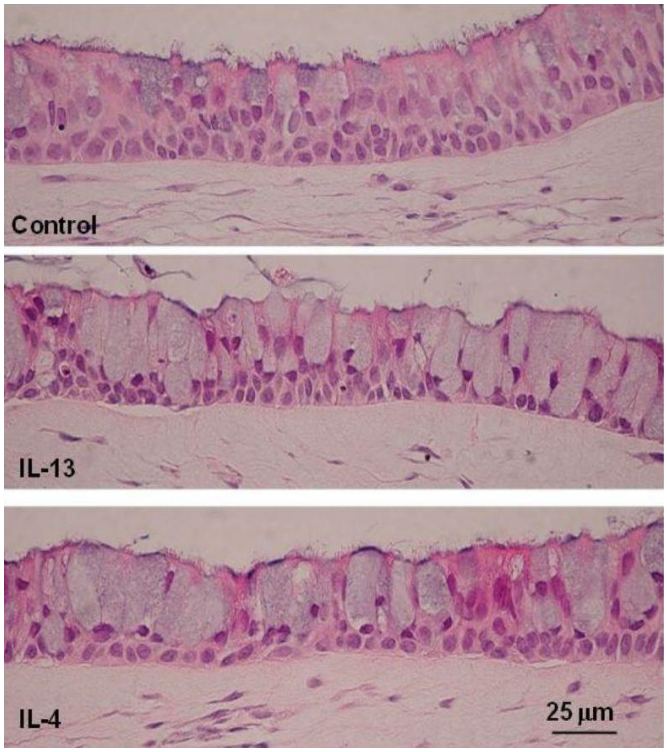
GOBLET CELL HYPERPLASIA AND MUCUS PRODUCTION

In vitro Induction of Airway Goblet Cell Hyperplasia in the EpiAirway Model by Th2 Cytokines, Viral Exposure, or Cigarette Smoke

Patrick Hayden, MatTek Corporation

Patrick Hayden presented on MatTek's 3D human tracheal/bronchial epithelial model (EpiAirway) and a recently developed alveolar model (EpiAlveolar). The EpiAlveolar model is a co-cultured model of alveolar epithelial cells and pulmonary endothelial cells of human origin. Both models are suitable for testing of tobacco products, pharmaceuticals, airborne chemicals, and pathogens. Cultures for both models are grown on microporous membranes so that the apical surface is exposed at the air–liquid interface to mimic *in vivo* exposure conditions. A full-thickness version of EpiAirway incorporates pulmonary fibroblasts in a sub-epithelial stromal matrix. Available formats include individual tissues (6, 12, and 24 wells) and high throughput (24 and 96 wells).

Advantages of the EpiAirway model are a long functional lifespan (>3 months), a pseudostratified morphology (basal, ciliated, goblet, and club cells), functional tight junctions, beating cilia, mucus secretion, and expression of drug-metabolizing enzymes and transporters. Use of primary human cells also avoids potential problems that are often encountered with immortalized cells lines, which might have functional defects or show changes in function over time. Furthermore, the models avoid problematic inter-species differences that are seen with animal studies or animal cells *in vitro*. EpiAirway cultures are typically created from single donors, and a wide selection of donors is available to vary age, sex, ethnicity, smoking history, and disease status. Large cell inventories are available for each donor to support long-term projects. These commercial tissue cultures are cost effective and are supported by a large database of use, supported by numerous peer reviewed papers and abstracts in the literature, and technical support.



Th2-mediated GCH in a 3D epithelial airway model

The EpiAirway and EpiAlveolar models can be used to mimic systemic exposure by additions to the medium, or can mimic inhalation exposure of the apical surface at the air–liquid interface. Thus, the models are relevant for general respiratory toxicology, nanoparticle toxicology, tobacco toxicology, basic airway research, and lung permeation, and to assess the effects of aerosol exposure and inhaled drug delivery.

The availablity of numerous do nors, including those with reported smoking history and disease conditions, such as asthma and COPD, allows for evaluation of individual variablity and research of airway diseases.

A specific application that was presented in detail at the workshop involved reproduction of GCH in the EpiAirway model by exposure to Th2 cytokines (e.g. IL-13 and IL-4), human rhinovirus, and tobacco smoke. The effect on GCH of azithromycin, an antibiotic that also has anti-inflammatory properties, was also investigated. Th2-induced GCH was reproduced by exposure of to IL-4 or IL-13 for 6 days. Pronounced changes could be seen in the number and size of goblet cells, accompanied by notable changes in gene expression. Azithromycin notably reduced the induction of Th2-induced GCH and gene-expression changes. To simulate viral exposure, the cultures were treated with poly(I:C), which induced substantial and unique changes in the full-thickness EpiAirway model, including extensive formation of goblet cells in the middle and at the surface of epithelium. Infection with rhinovirus in non-diseased non-smokers, non-diseased smokers, and asthmatic smokers led to sustained upregulation of *MUC5AC* at 48 h. The response to rhinovirus exposures was stronger in asthmatic smoker donors than in non-asthmatic donors. Exposure of EpiAirway cultures to cigarette smoke showed small but statistically significant differences in GCH induction between exposed tissues in smokers and controls. Some changes in mucin gene expression seen were induced by the smoke exposure (e.g. *MUC5AC*). Azithromycin had less effect on reducing smoke-induced GCH in the full-thickness model than in the partial-thickness model. Taken together, the results demonstrate that the *in vitro* airway models provide translational data that are highly relevant to assessment of human clinical airways disease.

Key points:

- **The use of 3D *in vitro* human airway tissues, including models representing the tracheal/bronchial epithelium and alveolar spaces, can be effectively used for airway toxicological, disease, and basic research and to assess effects of inhaled drug delivery, and respiratory infection**
- **GCH can be induced *in vitro* by Th2 cytokines, viral exposure, or tobacco smoke**
- **GCH can be influenced by co-culture with fibroblasts, individual variability, pre-existing disease (e.g. asthma), and exposure conditions (e.g. smoking history)**

Measurement of Mucin Secretion for Potential Evaluation of the Toxicity in Tobacco Products in Human Air–Liquid Interface Airway Models

Xuefei Cao, FDA National Center for Toxicological Research

Batteries of *in vitro* tests are recommended by the FDA and CORESTA for assessing the cytotoxicity and genotoxicity of cigarette smoke. However, tests for evaluating specific disease outcomes, which could be useful for assessing and predicting the health risks of exposure to cigarette smoke, are not included. In patients with COPD, disturbances of the normal redox state, mucus secretion, extracellular-matrix remodeling, and levels of inflammatory signaling lead to chronic inflammation and airway obstruction. The pathophysiology of COPD potentially could guide the development of *in vitro* tests for assessing disease outcomes of exposure to cigarette smoke.

The human airway expresses around 10 mucin genes,with MUC5AC and MUC5B being the major mucins secreted by airway epithelium. Well-differentiated human *in vitro* air–liquid interface models of airway tissue possess many of the structural and physiological characteristics of the human bronchial epithelium, including mucin production and, thus, may be suitable for investigating the pathological roles of the mucin proteins in the progression of respiratory diseases. Quantitative measurement of mucin secretion could conceivably provide valuable information for risk assessment on the toxic effects of inhaled toxicants, such as cigarette smoke. A pilot study was performed to compare the mucin-inducing effects of two sets of whole-smoke solutions prepared with the International Organization for Standardization (ISO) or Health Canada Intense machine smoking regimens. Each set contained two whole-smoke solutions generated by machine smoking of 60 sticks of either Marlboro Red (R60) or Marlboro Silver (S60) cigarettes. The apical surface of the air–liquid-interface model was exposed to whole-smoke solutions for 4 h each day for 5 days. Mucus was collected before the start of each exposure. After the last treatment, the cultures were allowed to recover for 2 weeks, during which time mucus was collected at the end of each week (the apical surface was still washed daily). Secretion of mucin proteins was measured with mucin ELISA assays.

With whole-smoke solutions prepared under the ISO smoking regimen, MUC5AC expression was increased significantly by the R60 and S60 samples, with the amount of mucin generally dependent upon doses of whole-smoke solution and the number of treatments. The kinetics of MUC5B secretion differed from those of MUC5AC in that its induction was delayed until the third treatment, at which point the induction of MUC5B was significantly higher than that in the vehicle-treated control. In general, mucin induction by R60 was stronger than that with S60 on the basis of the lowest effective dose. With whole-smoke solutions prepared with the samples obtained under the Health Canada intense regime, the induction patterns of MUC5AC and MUC5B were similar to those seen with the ISO whole-smoke solutions, but the differences between R60 and S60 were diminished. Furthermore, compared with ISO whole-smoke solutions, the Health Canada intense samples appeared to be stronger inducers of MUC5B secretion, since changes in MUC5B induction were seen after one treatment. During the recovery period, secretion of the two mucin proteins decreased after 1 week and returned to baseline levels at 2 weeks.

Changes in goblet cell density and morphology also were examined after exposure to Health Canada intense whole-smoke solutions. Five daily treatments with the highest doses resulted in a significant increase in GCH compared with the vehicle-treated control. Treatment with the Health Canada intense whole-smoke solutions also led to morphological changes, such as decreased size and misshapened cells (hypotrophy). These observations correlated well with those made on mucin induction. Recovery following the five daily treatments with whole-smoke solutions resulted in gradual loss of GCH, hypothrophy, and size alterations.

Concurrent exposure with N-acetylcysteine significantly decreased MUC5AC hypersecretion induced by ISO whole-smoke solutions, which suggests that mucin induction is a downstream effect of oxidative stress.

In summary, mucin measurement is a sensitive and quantifiable endpoint for human *in vitro* air–liquid interface cultures exposed to whole-smoke solutions. The findings are relevant to the progression of smoking-related diseases, specifically COPD, which makes this endpoint potentially useful for evaluating the health impact of exposure to cigarette smoke. A major challenge for this measurement is to develop smoke exposure regimens, including the appropriate machine smoking protocols, exposure

doses, and exposure schedules, to better mimic *in vivo* cigarette exposure in humans. To use such an assay for regulatory testing, standard operating procedures will need to be established for culture exposure conditions, methods and schedules for mucin collection, and ELISA assay procedures. Furthermore, donor variation in primary airway epithelial cells should be taken into account and a sample size should be established to ensure a desired detection sensitivity with minimum donor effects.

Key points:

- **Methods to assess disease outcomes would be useful for evaluating the health impact of inhaled toxicants, such as cigarette smoke**
- **Exposure of human airway air–liquid interface cultures to whole-smoke solutions resulted in increased levels of mucin secretion**
- **The levels of mucin protein secretion are consistent with morphological and density changes in goblet cells**
- **Oxidative stress is involved in cigarette-smoke-induced mucin protein hypersecretion**
- **Procedures need to be established for optimizing the sensitivity of mucin measurements while mitigating donor effects**

Combining Systems biology, Computational Approach and Human Organotypic *In Vitro* Models Exposed to Whole Cigarette Smoke: an Example of 21st Century Toxicology *Assessment*

Carole Mathis and Julia Hoeng, Philip Morris International R&D

Systems toxicology is an approach intended to decode the toxicological blueprint of active substances that interact with living systems.[14] It is an integrative approach that harnesses multidisciplinary expertise to gain a detailed understanding of toxicants. The systems aspect involves transcriptomics, genomics, proteomics, and lipidomics. An important aim is to develop dynamic AOPs that include detailed mechanisms information, predict adverse outcomes, and provide a new paradigm for risk assessment, and direct research towards safer products and environmental protection. Methods used are computational models, apical and molecular measurements, and application of -omics technologies.[15]

Knowing that smoking causes serious disease, but that a proportion of the population continues to smoke, manufacturers are developing MRTPs. The biological impact of novel products and related disease risks are being compared by mechanism with those of conventional cigarettes to assess the differences in effects from conventional cigarettes and smoking cessation. The approach allows for systematic generation of experimental data, by measurement of responses in tissues that are contextualized by molecular measurements in pathway models (mechanisms), and outcomes and risks

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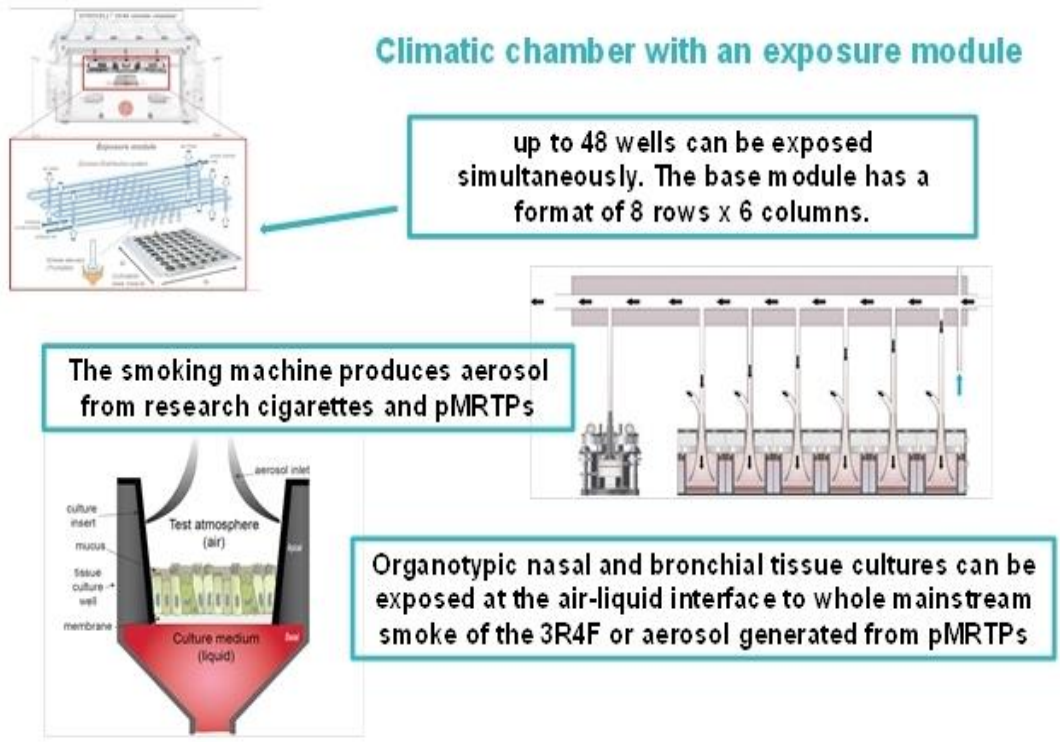
[14]Sturla SJ, Boobis AR, Fitzgerald RE, Hoeng J, Kavlock RJ, Schirmer K, Whelan M, Wilks MF, Peitsch MC. Systems toxicology: from basic research to risk assessment. Chem Res Toxicol 2014;27:314-329

[15]Hoeng J, Kenney RD, Pratt D, Martin F, Sewer A, Thomson TM, Drubin DA, Waters CA, de Graaf D and Peitsch MC. A network-based approach to quantify the impact of biologically active substances. Drug Discov Today 2012;17:413-418.

are quantified to compute biological impact factors. Many of the protocols and data will be made publicly available.[16]

To try to establish reliable *in vitro* systems to support the 3Rs principle, work is being done to translate findings between other species and human experimental systems. Studies so far have included data from rats and human cellular models, and the scientific community has been asked to help identify which systems are translatable.



In vitro whole smoke exposure experiments

Owing to bans in various countries on animal testing for tobacco products, manufacturers have moved towards *in vitro* assays for genotoxicity and cytotoxicity testing. PETA expressed a wish that should the boundaries of non-animal testing should be pushed further to test whole-product and whole-system effects. As part of its *in vitro* program, Philip Morris International is looking for organotypic models. Sampling the bronchial epithelium to identify potential biomarkers of exposure response and disease has yielded significant insights and shown that many of the smoking-related changes in the bronchial epithelia are also present in nasal and buccal epithelium. With a climatic chamber exposure model, whole-smoke and aerosol experiments have been performed in nasal and bronchial tissue cultures. Linear correlations were found for nicotine and eight different carbonyls, which is of note owing to changes in aerosol.[17] A difficulty with this method is understanding how to achieve reliable exposure.

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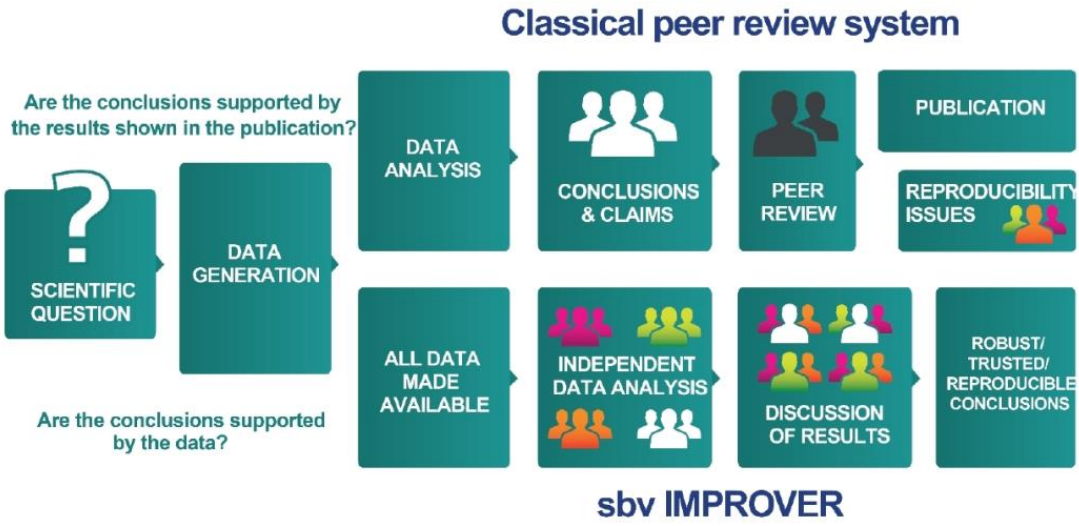
[16]http://www.jove.com/video/52325/impact-assessment-repeated-exposure-organotypic-3d-bronchial-nasal

[17]Majeed S, Frentzel S, Wagner S, Kuehn D, Leroy P, Guy PA, Knorr A, Hoeng J, Peitsch MC. Characterization of the Vitrocell® 24/48 in vitro aerosol exposure system using mainstream cigarette smoke. Chem Cent J 2014;8:62.

Of clinical importance is what actually reaches the tissue from smoke and aerosol. Computational fluid dynamics has been used in an attempt to model transport and evolution of aerosols and compute deposition at the air–liquid interface. Features to take into account are droplet size, number, and intensity, the required residence time for the aerosol to reach a uniform particle number concentration in the exposure system, the stability and physical characteristics of aerosol in the dilution system, and the influence of operating systems and conditions. Many different substances are involved, but the degree of exposure can be measured with the above system and characterized with gas chromatography time of flight analysis. Exposure of organotypic human primary bronchial epithelial cell cultures to cigarette whole smoke clearly shows dose-response with 3R4F in terms of inflammatory cytokine release but also for other endpoints, such as gene expression, microRNA, MMP-1 release, and cell counts and survival.[18]



Translation of *in vivo* smoking exposure based on a specific gene signature was assessed in thousands of inserts, based on four publicly available bronchial datasets for smokers and non-smokers. Similar results were obtained with the *in vitro* organotypic bronchial epithelial model. Results for microRNA were also assessed, but only one dataset was available for comparison. However, differentially expressed microRNAs were found after exposure to cigarette smoke that were common to the *in vivo* and *in vitro* datasets, and were associated with functions, such as inflammation and cell cycle processes.

As interest has been expressed in global biological responses, the similarities of nasal and bronchial tissue gene expression changes have been assessed. There is some overlap, but nasal tissue can't necessarily be used to predict COPD. However, this tissue can be used to identify smokers. Culture studies with bought cultures show increases in changes with increasing exposure time and dose. Likewise changes have been shown in cellular composition.

Transcriptomic studies have also shown striking similarities in responses to cigarette smoke in the bronchial and nasal tissues. The observation suggests the xenobiotic responses in the bronchial and nasal epithelial cells of smokers were similar to those

[18]Mathis C, Poussin C, Weisensee D, Gebel S, Hengstermann A, Sewer A, Belcastro V, Xiang Y, Ansari S, Wagner S, Hoeng J and Peitsch MC. Human bronchial epithelial cells exposed in vitro to cigarette smoke at the air-liquid interface resemble bronchial epithelium from human smokers. Am J Physiol Lung Cell Mol Physiol 2013;304:L489-L503.

observed in their respective organotypic models exposed to cigarette smoke. Furthermore, the results suggest that nasal tissue is a reliable surrogate to measure xenobiotic responses in bronchial tissue.[19] Effects of exposure to 8% whole smoke on nasal organotypic cultures resembles those in bronchial cultures. Thus these are promising experimental systems to reduce the need for animal research.

In an attempt to obtain independent verification of findings, the Sbv IMPROVER program has been set up.[20] The scientific community is able to look at entire datasets to complement the classic peer-review system. As there is little to no standardization in operating procedures, assays, etc so far, this approach helps to provide a measure of R&D quality control and to highlight gaps in knowledge or the research pipeline. It has potential applications in risk assessment to enhance insight into mechanistic understanding and improve product development. Thus, moving forward, data for 3R4F and some prototypic products could be assessed in this way.

Key points:

- **Many biological functions known to be directly affected by exposure to cigarette smoke were identified by transcriptomic analysis *in vivo* and *in vitro***
- **Human bronchial epithelial cells exposed *in vitro* to cigarette smoke at the air–liquid interface resemble bronchial epithelium from human smokers**
- **Gene expression profiling has demonstrated a remarkably similar transcriptomic response to cigarette smoke in nasal and bronchial tissues from current and never smokers**
- **Modeling transport and evolution of aerosol droplets is important for understanding aerosol deposition in the *in vitro* exposure system**

[19]Iskandar AR, Martin F, Talikka M, Schlage WK, Kostadinova R, Mathis C, Hoeng J, Peitsch MC. Systems approaches evaluating the perturbation of xenobiotic metabolism in response to cigarette smoke exposure in nasal and bronchial tissues. BioMed Res Int 2013;2013:512086.
[20]Meyer P, Alexopoulos LG, Bonk T, Califano A, Cho CR, de la Fuente A, de Graaf D, Hartemink AJ, Hoeng J, Ivanov NV, Koeppl H, Linding R, Marbach D, Norel R, Peitsch MC, Rice JJ, Royyuru A, Schacherer F, Sprengel J, Stolle K, Vitkup D, Stolovitzky G. Verification of systems biology research in the age of collaborative competition. Nat Biotechnol 2011;29:811-815.

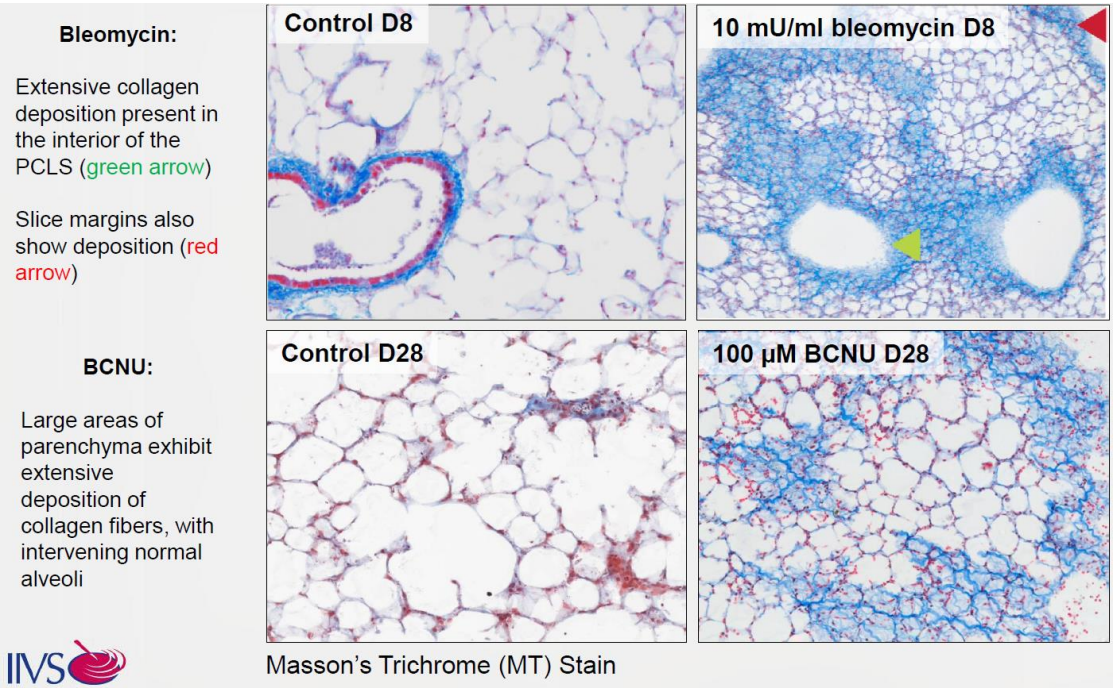
PARENCHYMAL/BRONICHAL TISSUE DESTRUCTION AND REMODELING

Detection of Inflammation and Parenchymal Damage Using Precision-Cut Lung Slices

Holger Behrsing, IIVS

Organ slice culture has been around for nearly 100 years, but precision cutting only became available in the past 30–40 years. The advantages of using PCLS are that they can be cultured for days to weeks and that endogenous cell types are retained, which helps with assessment of complex tissue responses.

PCLS are taken from whole lungs that are inflated with agarose solution that gels. Use of whole lungs allows proper inflation and means that samples can be obtained from multiple regions. Many of the lungs used, however, are transplant discards, meaning that disease could make the region of interest unusable. Thus, acceptance criteria (e.g. age, pretreatment, etc.) should be set and agreed with the vendor. Slices are obtained from tissue cores. Methods of cutting slices are similar across laboratories, but how slices are processed and cultured differ (e.g. roller systems, shaking flasks, rocker platforms, and well inserts).



Collagen deposition on lung slices

At 8 days and also much later at 28 days, using the roller culture method, endogenous cellularity is fairly well preserved and a range of cells types are present. Key is the retention of activated macrophages, which is important in COPD research by mediating inflammation and illustrating inflammatory response through secretion of several pro-inflammatory cytokines (e.g. TNF, IL-1β, and IL-6). Exposure to carmustine is associated with increased numbers of macrophages that infiltrate the alveolar walls by day 7. Exposure to bleomycin results in numerous diffuse activated macrophages plus clusters that fill the alveolar spaces. Collagen desposition is also seen in sloughs and ribbon-like patterns that vary from slice to slice. Thus, these features and biomarker content (e.g. ALP and LDH) can be used to demonstrate toxic

effects to the lungs. Human PCLS may also be used to assess parenchymal damage. For instance, aminoflavone and IL-1β exposure leads to cytokine increase then severe tissue damage by around day 7, although meaningful results could be obtained on earlier days.

Of note, the process of creating PCLS leads to increased cytokine (and chemokine) production owing to mechanical disruption of the tissue, but values resolve to baseline levels after around days 2–3. Thus, exposure should not be started until after day 2 if one wants to avoid confounding results of cytokine expression.

Establishing whether there is a no-effect level with the exposure compound is important. For example, exposure to Phortress, a pro-drug in the class of CYP-activated DNA-interactive anti-cancer agents, was associated with acute cytokine/chemokine increases. After low-dose exposure (25 μM) was stopped, these effects were reversible, whereas with higher doses the levels continued to increase after exposure removal. A dose of 10 μM was shown to have no toxicological effect.

PCLS are an attractive model for COPD because the native architecture is retained and long-term culture is possible. The important features, such as cytokine/chemokine induction, inflammation, collagen deposition, and parenchymal destruction can be measured in PCLS. Other features, such as ciliary function, increased mucus secretion, reduced airflow (diameters of airways), and chronic inflammation, should be measureable in a manner reflecting tobacco-induced changes that may lead to COPD. PCLS might also be suitable for a much wider range of biomarker and other assessments. Disadvantages are that the hardware rate of slice production, experimental capacity, and methods for exposure of PCLS to smoke could all be improved, but few significant changes have been made for many years. Obtaining slices from human lungs would be ideal to avoid the use of animals and avoid the need for cross-species extrapolation, but the supply of good-quality human lungs is limited. How utilization and storage could be improved to extend use of this model need to be explored (e.g. freezing of slices so more can be taken from individual donor lungs for use in a later study).

Key points:

- **PCLS have been utilized in research for many years and retain native lung architecture and endogenous cell types that allow the study of complex cell interactions that may be required for the COPD disease state**
- **Current culture methods allow PCLS culture for weeks and the assessment of biomarkers of acute and chronic exposure, such as early cytokine release, activation of macrophages, and collagen deposition – markers associated with tobacco exposure**
- **The PCLS model could benefit from modernizing equipment used to create and culture slices, a greater pool of human donor tissue available for research, and the ability to cryopreserve samples**

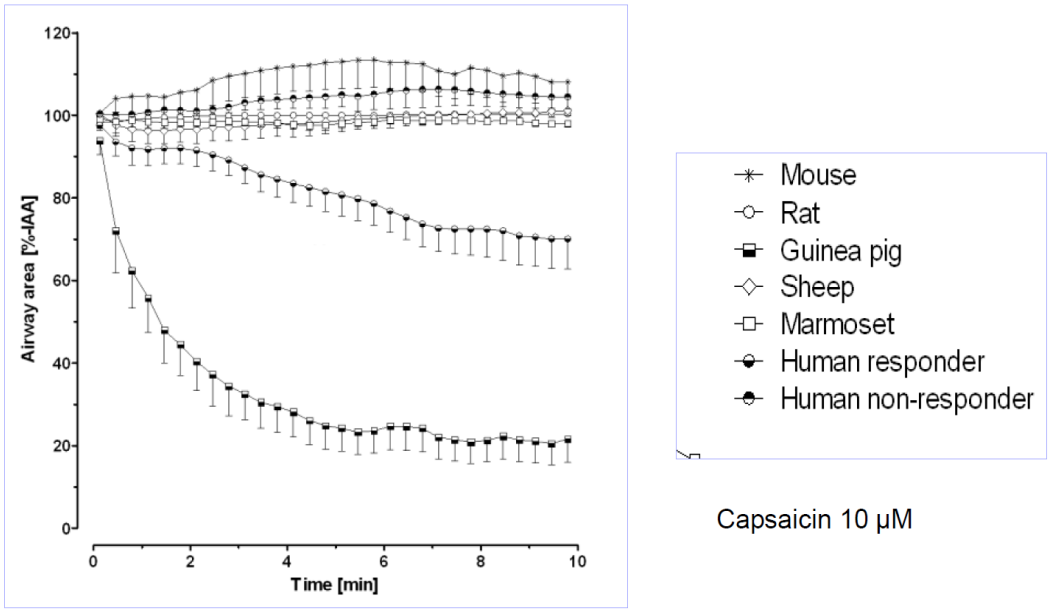
Use of Precision Cut Lung Slices to Test Physiological and Pathophysiological Lung Response

Armin Braun, Fraunhofer Institute for Toxicology and Experimental Medicine

Armin Braun discussed what PCLS can offer compared with simple cell cultures, and whether inflammation is the main driver of disease

In PCLS, the tissue is kept alive, is 3D, and contains many of the types of cells expected to be present in the lungs, including smooth-muscle cells, fibroblasts, and mast cells, as well as mucous glands, microvessels, and other structures. Thus, these *ex vivo* samples bridge the gap between cell lines and *in vivo*.

Airways and lungs are affected by many exposures, including cold, heat, biologicals, endogenous stress, etc., which might be physiological or pathophysiological. Neurogenic inflammation is stimulated by endogenous and exogenous activators. With PCLS, these can be mimicked *ex vivo*.



Results in PCLS vary substantially between species
Figure reproduced from Schlepütz et al. PLoS One 2012; 7(10): 347344, published under Creative Commons Attribution CC BY.

PCLS can be tested as part of an integrated strategy. Ideally, this would include a test system with standard operating procedures, clear endpoints, a pre-determined data interpretation procedure, and comparison of results with those for reference substances. The results should be used to develop prediction models to provide a point of reference for validation studies. For example, exposure of human lung to lipopolysaccharide followed by segmental lavage showed a strong correlation between *in vivo* and *ex vivo* responses, which provided a point of reference. Bronchoconstriction after the addition of agents to PCLS can be seen microscopically. These tissue samples, therefore, provide a useful way to test and rank the prevention of airway constriction with anti-COPD drugs. Of note, human PCLS are not consistently available, but there are substantial variations in PCLS reactions between species, so careful selection of the samples and the questions to be addressed are important.

Standardization of testing methods is important. To assess standardization between laboratories, a joint study was done by Fraunhofer ITEM, BASF and the University of

Aachen. Twenty chemicals were applied to PCLS followed by 24 h of no exposure in three laboratories, yielding 927 fitted curves. EC_{50} values obtained for the WST-1, LDH, and BCA assays were very similar in all participating laboratories. Differences between toxic substances and those not yielding dose-response curves could be clearly seen and showed excellent correlation with *in vivo* data. After intensive training for about 2 years, the technique was fully reproducible and transferable between laboratories.

A limitation of the above method is that it only measured acute responses. Longer-term experiments were done and showed that lung function decreased over time. Both changes were related to dose. Live/dead staining in a Triton repeat-dose study showed clear dose responses that did not differ very much over time.

A new exposure system for gases and aerosols enables customization of exposure within a single air–liquid interface plate and has a compact design, although it has some difficulty with longer-term exposures (high cell death after 3 h). Good results have been seen for cytotoxic and inflammatory responses. Additionally, establishment of viral infection, such as rhinovirus, could be assessed in human PCLS, as performed in the framework of the IMI EU project U-BIOPRED. Human PCLS infected with rhinovirus showed differences between donors that is likely to reflect individual susceptibility, while response within each donor was robust. Transcriptomics of human rhinovirus can be used to produce heat maps to indicate the antiviral response fingerprint.

In summary, PCLS can be used to test different types of toxic effects in various disease models after exposures to various chemical or biological agents, including cigarette smoke and infections.

Key points:

- **PCLS are suitable for acute and repeat-dose testing, but inter-species differences must be considered at the research planning stage**
- **PCLS have been used to assess bronchoconstriction and the effects of anti-COPD drugs that might affect tissues**
- **Exposure systems are available to test multiple exposures simultaneously in PCLS**

BREAKOUT DISCUSSION GROUPS

Overview

Moderated breakout groups were held in three subject areas: inflammation and oxidative stress, ciliary function and ion transport, and GCH and mucus production. All groups had the same goals:

- Confirm that the biological effects addressed by the group are indeed relevant to tobacco-induced COPD
- Characterize any proposed *in vitro* models and test methods for their relevance to predicting the key events
- Identify the limitations of the test method and propose activities to address those limitations or gaps in mechanistic understanding

Each group was provided with the following set of questions to guide the discussions:

A: What is the similarity of the model to any part of the human respiratory tract?

1. Is it of human origin?
2. Is it 3D?
3. Does it have similar morphology?
4. Are all appropriate cell types present?
5. What area of the human respiratory tract is modeled?
6. Is it proprietary?
7. Is it suitable for long-term studies?
8. Does it respond appropriately to known stimulants or inhibitors?
9. Does the model have any known shortcomings?

B: What is the relationship of the assay endpoint(s) to the clinical manifestations of COPD?

1. Is the endpoint measurable in humans? Quantitatively?
2. Is it a precursor to the disease state or a constituent?
3. Does it measure progression to the disease?
4. Is it useful for screening out either active or inactive materials?
5. Does the assay have any known shortcomings?

C: How well characterized is the assay?

1. Ease of use and throughput?
2. Intra-laboratory reproducibility established?
3. Number and relevance of materials tested?
4. Used by (available to) other laboratories?
5. Portability to other laboratories?
6. Inter-laboratory reproducibility established?
7. Used with tobacco-related materials (chemicals)?

D: What research activities can be proposed to address any gaps and limitations?

1. For models?
2. For assays?

Inflammation and Oxidative Stress

Model characteristics

- Cell lines
    - Suitable for screening for exposure effects as part of a larger package of tests (potentially human and non-human cell lines)
    - Everyone can use the same cell lines, provided no genetic drift has occurred between sources of cells or between laboratories
    - They are easy to grow and can be sensitive (possible advantage and disadvantage)
    - Metabolic activity is often low, which limits applicability
    - Characteristics of some cells change when brought to the air–liquid interface (e.g. formation of tight junctions at confluence; possible advantage and disadvantage)
    - Signal transduction pathways are abnormal
    - Cells might be altered or abnormal in different, unknown ways
    - Cell lines can only demonstrate potential for a process rather than the process itself
    - For ROS measurement, it might even be possible to utilize a reporter system, such as Nrf.

- Primary cells (two-dimensional mono-layer)
    - Cells are closer (more "normal") than cell lines to those in the *in vivo* population of interest and, therefore, have greater predictive power
    - The potential of stem-cell-derived primary cells exists
    - Signal transduction pathways are typically retained more than in cell lines
    - Some heterogeneity exists between donors, which could be an advantage or disadvantage
    - There is some control over the donor population (e.g. diseased vs. healthy or by sex)
    - The lifespan is limited and accessibility to human donors is finite
    - Primary cells are more expensive and harder to grow and maintain than cell lines

- 3D models (typically constructed of primary cells)
    - Availability of healthy or diseased donor tissue sources allows assessment of disease states in COPD research.
    - Relevant structures can be maintained, although not necessarily the full structure and, therefore, models potentially need to increase in complexity to improve physiological relevance. However, complexity can lead to cell–cell cross-talk
    - Cell types (e.g. monocytes, macrophages, neutrophils, and ciliary and mucous cells) should be included to enable investigation of downstream effects; some key cell types (e.g. dendritic cells and macrophages) are missing; gene expression and metabolic pathways are retained
    - The cultures remain reasonably stable, which makes them suitable for chronic or repeated-dose experiments
    - The possibility that dosimetry will not be consistent throughout the model must be taken into account
    - The model can be technically challenging and costly to create/utilize in research

  - Inter-donor variability of model characteristics and response to challenge requires consideration

- *Ex vivo* (i.e. PCLS)
  - Advantages are similar to those for 3D cultures, plus organelles, biomarkers, etc. are easy to label and disease characteristics, such as broncho-obstruction/constriction, collagen deposition, ciliary beating, alveolar scarring, MMP secretion, differentiation between irritation, and sensitization, can be viewed as they were *in vivo*
  - The lung region of interest can be selected from the whole lungs
  - Disadvantages are similar to those for 3D models, plus availability is one human donor per experiment and PCLS have only short- to medium-term stability
  - The question was raised of whether human lung tissue would be made available for tobacco research
  - Few historical data are available compared with for some of the other models
  - Long-term stability needs to be studied further

All models are capable of showing changes related to inflammation and oxidative stress. Whatever model is used, quantitative sensitivity is crucial and inter-species translation might need to be considered if human cells or cultures are not available. For the simple detection of oxidative stress, non-cellular (chemical-reaction-based) detection was suggested but this system would not capture cell-generated ROS that might play into more complex cellular mechanisms.

Assay characteristics
Lists of markers of inflammation and oxidative stress related to COPD were identified:

- Inflammation
  - NfκB
  - Reporter for NfκB
  - Interleukins
  - Pro- and anti-PPARG (blocks NfκB)
  - Chemokines
  - Cytokines
  - γ-interferon (not regulated by NfκB)
  - Toll-like receptors – driven by NfκB and dysregulation of toll-like receptors
  - MMP-2 and MMP-9
  - Aryl hydrocarbon receptor (AHR)
  - Lipid and lipid mediators (prostaglandins, resolvins) – PGE2
  - Reporter assay for MAPK and ERK

- Oxidative stress
  - Glutathione (reduced vs. oxidized) expression level (HIP pathway)
  - γ- GCS (related to glutathione enzyme)
  - Nrf2
  - Hif-1α
  - Mitochondrial ROS (quantitative by flow cytometry or dyes)
  - F2 isoprostanes
  - Lipid peroxidation
  - Hemoxygenase 1

Whether all would need to be tested or whether it is possible to select a specific representative panel (e.g. clinically relevant markers) was highlighted as an issue. All the markers listed are related to early biological events, but, even if they are strongly associated, none is unique to COPD and, therefore, the predictive value needs to be investigated. The choice of markers and changes thereof need to impart a reasonable expectation of disease risk. Certain markers might need to be considered in specific sequences (e.g. if NfκB is not induced, a host of other markers will not be present).

Although different approaches in testing for oxidative stress and inflammation were described (small sets of markers vs. large sets by different participants), specific positive controls were identified:

- Oxidative stress: H_2O_2, potassium bromate, and L-sulforaphane
- Inflammation: LPS, IL-1β, TNF

Research recommendations
As regulatory bodies want tests to show safety, but because product development requires more screening of effects, it was suggested that a consensus should be sought about which markers might be most useful to include in testing panels. As not enough information is available to easily eliminate possible cell models, the following paths to reduce the marker panel size were proposed:

- Focus on those that predict the clinical situation
- Base the panel on the assays that are already available to measure them
- Review the literature to investigate whether biomarkers in the clinic have been reported as suitable for laboratory assessment
- Consider whether they contribute to prediction models to aid interpretation of data

General recommendations
- Creation of collaborative partnership to evaluate endpoints and to provide recommendations on which might be most useful
- Standardization of methods used for models, exposure protocols (including dosimetry), and reference materials
- Positive or negative cutoffs should be determined for assay sensitivity, through development of prediction and data interpretation models
- Correlation of *in vitro* to *in vivo* correlation is extremely important

Conclusions
- Many *in vitro* models and assays can be used to detect adverse events, such as oxidative stress and inflammation. These events, however, can be resolved and when assessed in an acute manner may not reflect downstream events associated with progression of COPD pathology
- More correlation between acute, initiating events and downstream key COPD events is needed, and/or focus should be assigned to long-term *in vitro* models capable of modeling chronic states of oxidative stress and inflammation

Ciliary Dysfunction and Ion Transport

Model characteristics

- 3D bronchial models
    - Bronchial epithelial models cultured from human cells retain morphology similar to that found *in vivo*
    - Any reconstructed model should include basal, goblet, and ciliary cells as standard; co-culture with fibroblasts might be useful as long as these cells do not change the phenotype from what is found *in vivo*; other desirable cells to add might be macrophages and club cells
    - Having a profile of phase I and phase II metabolizing enzymes similar to that found in the human respiratory tract is important for the testing of xenobiotics
    - Some 3D models of the upper respiratory tract can be used for long-term studies (e.g. ≥6 weeks) and respond to known toxicants and stimulators of hyperplasia and increased mucus production
    - Some commercial, proprietary models are available, but they are expensive and information about all medium constituents might not be available. Lack of definitive information about the medium could make the interpretation of certain experiments very difficult
    - Shortcomings of the current models are that they lack glands and are not capable of mimicking bronchial constriction

- Nasal tissue models
    - Nasal epithelial cells can be obtained directly from individuals by swab and tissue inserts can be created from the samples
    - Most of the properties are similar to those for 3D bronchial models
    - There is a need to distinguish nasal respiratory and nasal olfactory epithelia. Presently there do not seem to be any good models for latter
    - A shortcoming is that if excised tissues are required for studies, they are better taken from bronchial epithelium, as samples are better and larger

- Small airways models
    - Although of human origin, the current small airways models consist of cells grown in a mono-layer rather than 3D more complex models
    - Some commercial, proprietary cultures are available
    - Despite probably being the tissue most relevant to COPD, since the small airways of the lung is where the disease starts to develop, few models are available because samples are difficult to obtain, even from lung transplant discards, and because micro-dissection is required

- Alveolar models
    - Human type 2 epithelial cells can be isolated and de-differentiated in to type 1 cells, but this method of obtaining type 1 cells is not ideal
    - Cell types present in most of the models are type 1 epithelial cells, other endothelial cells, and macrophages
    - Some commercial, proprietary models are available
    - Responses to known toxicants are unclear, although some data are available on ion channel effects. Of note, ASL is generally thicker than *in vivo*, which might alter the results obtained from *in vitro* models.

- PCLS that can be used *ex vivo* can be derived from humans and other species; the latter might be helpful to correlate *ex vivo* and existing animal *in vivo* data after similar exposures. Animal tissues will likely be easier
- PCLS are useful in terms of the mixture of cell types (basal, interstitial, goblet, ciliary, fibroblasts, macrophages, etc. [although not neural cells]) they contain and the fact that they retain the endogenous structure. They can also be obtained from different lung regions of interest
- Once prepared, PCLS can be used to assess inflammatory responses over several weeks, but there is a short initial timeframe for collection and culture. Some functions decline quite rapidly if they are being used to assess MCC or bronchoconstriction, etc. Scarcity of human tissue donors hinders the possibility for collection, culture, and experimentation
- The tissues show reasonable response to known toxicants
- Because it is difficult to orient the axis of the cutting, PCLS are often in the wrong plane to be able to study ciliary movement. However, assessment, ciliary beating frequency has been reported in some studies
- Additional shortcomings are:
  - Most tissues are obtained from lungs that are already unhealthy (e.g. the lungs might contain tumorigenic tissue)
  - Achieving a consistent air–liquid interface for the duration of the experiment can be difficult
  - Sensitivity and specificity, for instance in tracking inflammatory responses, might be hindered by the rich variety of cell types

Assay characteristics

- Important endpoints are MCC, ciliary beating frequency, ion transport, and ASL, which cover progressive changes that appear before full disease development. All endpoints can be used for screening
  - MCC is easy to use and has good intra-laboratory reproducibility (inter-laboratory not established), although throughput is low. Has been tested quite extensively with multiple drugs and some individual compounds (e.g. nitrosamines). Suitable for use with cigarette smoke and smoke condensate, but whether can distinguish between similar products is unclear. PCLS have been shown to distinguish between the effects of MRTPs and reference cigarettes
  - Ion transport assays have good intra- and inter-laboratory reproducibility and are suitable for use with many materials, including tobacco-related products (although few data are published for the latter)
  - ASL is simple to use but only with the correct equipment. Thus, although reproducibility is good and assays can be used with tobacco product samples and constituents, use might not be widespread

Research recommendations

- For 3D models, investigate new supports and flexible substrates to enable assessment of mechanical properties of airway constriction

- Models to assess MCC require improved hardware and software for assessment and standardization of test conditions and controls
- Comparisons of *in vitro* and *in vivo* data need to be made to provide background information for mechanisms of disease development and resolution, speed of transfer of particles, etc.
- Expansion of ion channel models to enable assessment of individual smoke constituents and validation of the assays for *in vitro* testing would be useful
- Finding ways to overcome the difficulties with creating small airway models would be an important advance
- More bridging is needed between the *in vitro* and *in vivo* responses for these parameters. This could be accomplished with non-invasive studies (nose, lung?) to compare clinical versus *in vitro* findings. Such data should be compiled so that they are easily accessible by all researchers

General recommendations

- Standardization of *in vitro* assays for regulatory purposes is necessary. This includes protocols, standard operating procedures, acceptance criteria, performance standards, and a discussion of donor variability for PCLS
- A process should be developed to make human pulmonary tissue more available to researchers
- Efforts should be made to understand how induced pluripotent stem cell technology can contribute to human tissue models
- Close interactions between regulators and researchers would dramatically shorten the development of useful *in vitro* models
- Development of pulmonary toxicology AOPs for tobacco products would be beneficial and should include molecular and cellular network information
- Funding for practical, regulatory-focused research should be made available
- Interactions with investigators in the field of air pollution and aerosolized pharmaceuticals should be encouraged

Conclusions

- MCC is a key event in a COPD AOP and can be measured *in vitro*. Hence, the development of standardized and qualified *in vitro* assays to measure proxies of MCC has clear potential to predict and inform on tobacco-product hazards.
- There are practical considerations that govern the use of individual *in vitro* assays. Therefore, depending on the specific purpose of the study, various combinations of assays will likely be used.

Goblet Cell Hyperplasia and Mucus Production

Model characteristics

- 3D models
    - Are commercially available (not proprietary) and can be developed in house from human primary cells
    - Show organized and stratified epithelium
    - Morphology is analogous to that in the human airway epithelium *in vivo*
    - Dependent on the specific model, the requisite functional airway epithelial cells responsible for mucus production and clearance are present (i.e. basal epithelial cells, ciliated epithelial cells, and goblet

cells). Co-culture with fibroblasts may be helpful to enhance responses, which might modulate the magnitude of mucus production. Other cells types, such as club cells, might also be desirable, but their roles in chemically induced GCH and changes in mucus production need to be better understood. In general, the complexity of the model should not exceed the experimental requirements
    - Theoretically, the models address GCH and mucus production throughout the respiratory tract, but the specific modeling may be that of the upper large airway epithelium, as the cells are typically derived from the tracheal or bronchial epithelium. The breakout group members recognized that there are notable differences between the upper and lower respiratory tracts (e.g. the reduction or absence of cilia in the small airways). However, to meet the objectives of establishing a model for evaluating chemically induced GCH and changes in mucus production, these differences were noteworthy but not known to be limiting to the applicability
    - Models have been demonstrated to be sufficiently stable to allow for repeat exposure or dosing over several weeks, and have demonstrated GCH and changes in mucus production to known stimulants and inhibitors, including cigarette smoke, in as little as a few days

- PCLS
    - The breakout group members recognized that PCLS have the potential to be used to evaluate chemically induced GCH and changes in mucous production, similar to assessments done in 3D reconstructed human respiratory epithelial models, but considered this model to be early in the conceptual process
    - Further understanding of GCH and mucus production will help in the evaluation and understanding of the potential strengths and limitations of this model

Assay and endpoint characteristics

- The two main overall endpoints for clinical manifestations and disease progression are GCH/metaplasia (increases in numbers of goblet cells and changes in morphology) and changes in mucus production. These endpoints can be determined *in vivo* by collecting airway biopsy samples or sputum or bronchial lavage samples
- PAS staining of histology sections and specific immunofluorescence staining of mucins were deemed useful for qualitative assessment of changes in mucous production
- Histology sections would enable comparative goblet cell counts and evaluation of changes in goblet cell morphology
- ELISA technology may be used to quantify specific mucins (such as MUC5AC and MUC5B profiles), but availability/quality of commercial ELISA kits may be limited; it might be possible to develop assays in house. Other useful detection methods could include -omics technologies and chromatography
- The production of airway cultures and the subsequent repeat exposures of the test systems is expected to be time and labor intensive and, therefore, adequate time must be budgeted for these activities

Research recommendations

- A better fundamental understanding of the mechanisms of action of normal and chemically induced GCH and mucin production

- Population of a database with high-quality *in vivo* data of GCH and mucin production parameters in humans, optimized to improve *in vitro* model and endpoint development
- Improved understanding of how or whether any of the *in vivo* endpoints are predictive of the progression towards COPD and whether the relationships, if any, will need to be investigated
- Overall performance characteristics of models and quality-control need to be evaluated, and test system (i.e. tissue model) and assay endpoint quality and intra- and inter-laboratory reproducibility need to be characterized
- Optimization and standardization of assay endpoints
- Development of performance standards for the tissues
- Assessment of suitability of models to assess combustible tobacco smoke and aerosol exposures; it is envisioned that the tissue model platforms and the smoke and aerosol or vapor exposure systems will need to be optimized to enhance compatibility
- Extend availability of specific ELISA assays for detecting and quantifying mucin profiles
- Establish sensitivity and dynamic range in GCH and mucus production, cell counts, and other metrics
- Establish reference substances and positive and negative controls (e.g. poly(I:C), air pollutant material [provide to NIST for analysis, storage, and distribution] standard whole smoke extract, total particulate matter, stable extract tobacco from standard smokeless tobacco products, etc) for use in evaluating the performance of the test systems and assay endpoints

Conclusions

- The breakout group accepted the premise that one can model goblet cell hyperplasia and changes in mucous production *in vitro* using relevant 3D reconstructed human airway models, and that currently available technologies can be utilized for qualitative and quantitative endpoint measurements
- To meet the widespread acceptance of these prototypic test methods, it was recommended that considerable development activities should be conducted to define the requisite characteristics of the systems/tissue models, develop and optimize the test system exposures, and optimize the various endpoint methodologies.
- Establish reference materials to aid in the evaluation of the proposed test models