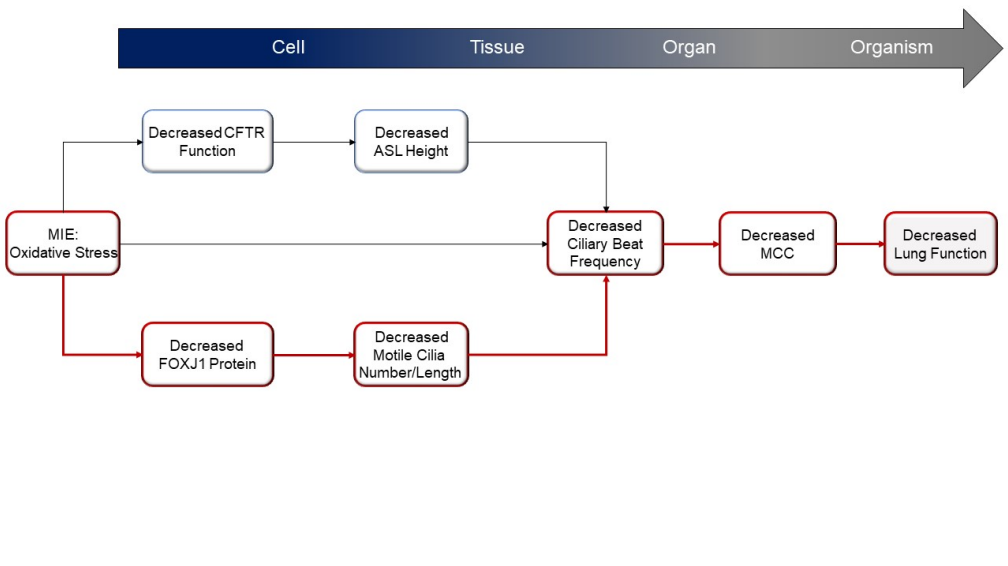
AOP ID and Title:

AOP 425: Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1
Short Title: ox stress-mediated FOXJ1/cilia/CBF/MCC impairment

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
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Abstract

This AOP evaluates one of the major processes known to be involved in regulating efficient mucociliary clearance (MCC). MCC is a key aspect of the innate immune defense against airborne pathogens and inhaled chemicals and is governed by the concerted action of its functional components, the cilia and the airway surface liquid (ASL), which is composed of mucus and periciliary layers (Bustamante-Marin and Ostrowski, 2017). In response to various irritants and pathogens mucus is secreted by goblet cells, and cilia sweep mucus upward by coordinated beating motions thus clearing the airways from these substances. The ciliated airway epithelial cells are typically covered by hundreds of motile cilia. Cilia formation is initiated and coordinated by a distinct gene expression program, led by the transcription factor forkhead box J1 (FOXJ1) (Brody et al., 2000; Zhou and Roy, 2015). FOXJ1 appears to be the major factor in multiciliogenesis, whereby its activity is necessary and also sufficient for programming cells to assemble functional motile cilia (Vij et al., 2012). A decrease in the levels or absence of FOXJ1 protein in cells of the respiratory tract therefore inhibits ciliogenesis, preventing physiological mucus clearance and decreasing MCC. MCC dysfunction is linked to airway diseases such as chronic obstructive pulmonary disease (COPD) or asthma, both of which are characterized by decreased lung function and bear a significant risk of increased morbidity and mortality.

Background

With a surface area of ~100 m^2 and ventilated by 10,000 to 20,000 liters of air per day (National Research Council, 1988; Frohlich et al., 2016), the lungs are a major barrier that protect the body from a host of external factors that enter the respiratory system and may cause lung pathologies. Mucociliary clearance (MCC) is a key aspect of the innate immune defense against airborne pathogens and inhaled particles and is governed by the concerted action of its functional components, the cilia and the airway

surface liquid (ASL), which comprises mucus and the periciliary layer (Bustamante-Marin and Ostrowski, 2017). In healthy subjects, ≥10 mL airway secretions are continuously produced and transported daily by the mucociliary escalator. Disturbances in any of the processes regulating ASL volume, mucus production, mucus viscoelastic properties, or ciliary function can cause MCC dysfunction and are linked to airway diseases such as chronic obstructive pulmonary disease (COPD) or asthma, both of which bear a significant risk of increased morbidity and mortality. The mechanism by which exposure to inhaled toxicants might lead to mucus hypersecretion and thereby impact pulmonary function has already been mapped in AOP148 on decreased lung function. However, whether an exposure-related decline in lung function is solely related to excessive production of mucus is debatable, particularly in light of the close relationship between mucus, ciliary function, and efficient MCC. To date, no single event has been attributed to MCC impairment, and it is likely that events described in this AOP as well as in AOPs 148, 411 and 424 have to culminate to lead to decreased lung function.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	1392	Oxidative Stress	Oxidative Stress
2	KE	1911	FOXJ1 Protein, Decreased	FOXJ1 Protein, Decreased
3	KE	1912	Motile Cilia Number/Length, Decreased	Motile Cilia Number/Length, Decreased
4	KE	1908	Cilia Beat Frequency, Decreased	CBF, Decreased
5	KE	1909	Mucociliary Clearance, Decreased	MCC, Decreased
6	AO	1250	Decrease, Lung function	Decreased lung function

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Oxidative Stress	adjacent	FOXJ1 Protein, Decreased		
FOXJ1 Protein, Decreased	adjacent	Motile Cilia Number/Length, Decreased		
Motile Cilia Number/Length, Decreased	adjacent	Cilia Beat Frequency, Decreased		
Cilia Beat Frequency, Decreased	adjacent	Mucociliary Clearance, Decreased		
Mucociliary Clearance, Decreased	adjacent	Decrease, Lung function		

Stressors

Name	Evidence
Cigarette smoke	High

Cigarette smoke

Whole cigarette smoke exposure or treatment with cigarette smoke extract of normal human bronchial epithelial cells significantly lowered FoxJ1 mRNA and protein levels (Milara et al., 2012; Brekman et al., 2014; Valencia-Gattas et al., 2016; Ishikawa and Ito, 2017). Cigarette smoke extract treatment of normal human bronchial epithelial cells also reduced the expression of cilia-related transcription factor genes, including FOXJ1, RFX2, and RFX3, as well as that of cilia motility and structural integrity genes regulated by FOXJ1, including DNAI1, DNAH5, DNAH9, DNAH10, DNAH11, and SPAG6 (Brekman et al., 2014).

Exposure of human bronchial epithelial cells cultured at the air-liquid interface to cigarette smoke extract during differentiation significantly shortened the average cilia length compared to untreated cultures, and treatment of differentiated cultures prevented elongation of cilia seen in untreated cultures (Brekman et al., 2014). Whole smoke exposure of mouse tracheal epithelial cells differentiated at the air-liquid interface resulted in cilia shortening and also complete loss of cilia at 24 h post-exposure (Lam et al., 2013). Cilia length was also reduced in mouse nasal septal epithelial cells treated with cigarette smoke condensate (Tamashiro et

al., 2009). Cilia length was reduced in endobronchial biopsies and airway brushings of smokers compared to nonsmokers (Leopold et al., 2009) and in COPD smokers compared to healthy smokers and nonsmokers (Hessel et al., 2014). In adults with adults with chronic sputum production, current and former smokers had a higher frequency of axonemal ultrastructural abnormalities than non-smokers and controls (Verra et al., 1994).

Nasomuciliary clearance time (determined by saccharin transit test) was significantly higher in smokers than in non-smokers and correlated positively with cigarettes per day and packs/year index (Proença et al., 2011; Baby et al., 2014; Yadav et al., 2014; Habesoglu et al., 2012; Pagliuca et al., 2015; Xavier et al., 2013; Dülger et al., 2018; Solak et al., 2018; Polosa et al., 2021).

Smoking decreased pulmonary function including forced vital capacity (FVC), forced expiratory volume in one second (FEV1) and FEF25–75 (Kuperman and Riker, 1973; Ashley et al., 1975, Tantisuwat and Thaveeratitham, 2014, Gold et al., 1996; Broekema et al., 2009).

Overall Assessment of the AOP

The experimental evidence to support the biological plausibility of the KERs from MIE to AO is moderate to strong overall for the AOP presented here, while there is a moderate concordance of dose-response relationships. In terms of essentiality, we have rated all of the KEs as either moderate or high.

AOPs such as this one can play a central role in risk assessment strategies for a wide variety of regulatory purposes by providing mechanistic support to an integrated approach to testing and assessment (IATA; (Clippinger et al., 2018)). IATAs are flexible frameworks that can be adapted to best address the regulatory question or purpose at hand. More specifically, this AOP can be applied to the risk assessment of inhaled toxicants, by enabling the development of testing strategies through the assembly of existing information and the generation of new data where they are currently lacking. Targeted approaches to fill data gaps can be developed using new approach methodologies (NAMs) informed by this AOP.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI

Sex Applicability

Sex	Evidence
Mixed	

All KE proposed in this AOP occur and are measurable in several species, including frogs, mice, rats, guinea pigs, ferrets, sheep, and humans. The majority of the supporting empirical evidence derives from studies in rodent and human systems, and experimental findings in animals appear to be highly translatable to humans.

Data regarding the applicability of KE to all life-stages from birth to adulthood are available for the MIE (Oxidative Stress), KE2 (FOXJ1 Protein, Decreased), KE3 (Motile Cilia Number/Length, Decreased), KE4 (Cilia Beat Frequency, Decreased), KE5 (Mucociliary Clearance, Decreased), and AO (Decreased Lung Function), and indicate that they apply to all life stages. It is also worth noting here that age-dependent decreases in CBF, MCC, and lung function have been demonstrated in several species (e.g., guinea pigs, mice, and humans) and reflect normal physiological aging processes (Bailey et al., 2014; Grubb et al., 2016; Ho et al., 2001; Joki and Saano, 1997; Paul et al., 2013; Sharma and Goodwin, 2006).

Gender-specific data relevant to the AOP are not as widely available as species-specific data, and to our knowledge, the role of gender has not been systematically evaluated for all KE described here. Considering the essentiality of FOXJ1 for the ciliogenesis program and the impact of ciliary beating on MCC, we consider this AOP applicable to both genders.

Essentiality of the Key Events

The definition of essentiality implies that the modulation of upstream KEs impacts the downstream KEs in an expected fashion. If blocked or failing to occur, the KEs in the current AOP will not necessarily stop the progression to subsequent KEs. Due to the complex biology of motile cilia formation and function, ASL homeostasis, mucus properties and MCC, the KEs and AO may be triggered because of alternative pathways or biological redundancies. However, when exacerbated, the KEs promote the occurrence of downstream events eventually leading to the AO. The causal pathway starting from the exposure to oxidants and leading to decreased lung function involves parallel routes with KEs, each of which is sufficient to cause the downstream KE to occur. Different mechanisms, such as oxidant-induced decreases in ASL height via CFTR function decline (AOP424) or oxidant-induced decreases in cilia number and length as a result of decreased FOXJ1 levels, lead to decreased CBF and decreased MCC. Each of these pathways contributes to the AO, but their relative contributions are difficult to evaluate. Based on the evidence we

judge the MIE (Oxidative Stress), KE2 (FOXJ1 Protein, Decreased), KE3 (Motile Cilia Number/Length, Decreased), KE4 (Cilia Beat Frequency, Decreased), and KE5 (Mucociliary Clearance, Decreased) highly essential.

Weight of Evidence Summary

We judge the overall biological plausibility of this AOP as strong. The KER *Decreased FOXJ1 protein leading to decreased motile cilia length/number* is supported by multiple studies across different species with ample empirical evidence reflecting both dose-response and time concordance. Other KER, such as *Oxidative stress leading to decreased FOXJ1* lack this expanse of empirical evidence, or the evidence does not fully support the causality between the KE (R*educed cilia number/length leading to decreased CBF, Decreased CBF leading to decreased MCC)* even though the relationship is logical and plausible.

Quantitative Consideration

Overall, our quantitative understanding of the AOP network is moderate.

There is robust evidence that provides an insight into several KER presented here, and the dose response and temporal relationship between the two KE in question are well described and quantified for different stressors across different test systems (Decreased FOXJ1 protein leading to decreased motile cilia length/number; Decreased motile cilia length/number leading to decreased cilia beating frequency; Decreased cilia beat frequency leading to decreased MCC). In some instances, we are less confident in our quantitative understanding. For example, dose response data as well as data supportive of the KE causality are limited for the KER *Decreased MCC leading to decreased lung function*.

Considerations for Potential Applications of the AOP (optional)

Given the individual and public health burden of the consequences of lung function impairment, gaining a greater understanding of the underlying mechanisms is extremely important in the risk assessment of respiratory toxicants. An integrated assessment of substances with the potential to be inhaled, either intentionally or unintentionally, could incorporate inhalation exposure and dosimetry modelling to inform an in vitro approach with appropriate exposure techniques and cell systems to assess KEs in this AOP (EPA's Office of Chemical Safety and Pollution Prevention, 2019). Standardization and robustness testing of assays against explicit performance criteria using suitable reference materials can greatly increase the level of confidence in their use for KE assessment (Petersen et al., 2021). Much of the empirical evidence that supports the KERs in the qualitative AOP described here was obtained from in vitro studies using well-established methodologies for biological endpoint assessment. Being chemical agnostic, this AOP can be applied to a variety of substances that share the AO. For example, impaired MCC and decreased lung function have a long-known relationship with smoking, but little is known about the consequences of long-term use of alternative inhaled nicotine delivery products such as electronic cigarettes and heated tobacco products. This AOP can form the basis of an assessment strategy to evaluate the effects of exposure to aerosol from these products based on the KEs identified here.

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Appendix 1

List of MIEs in this AOP

Event: 1392: Oxidative Stress

Short Name: Oxidative Stress

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:220 - Cyp2E1 Activation Leading to Liver Cancer	KeyEvent
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent
Aop:284 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress leads to chronic kidney disease	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:411 - Oxidative stress Leading to Decreased Lung Function	MolecularInitiatingEvent
Aop:424 - Oxidative stress Leading to Decreased Lung Function via CFTR dysfunction	MolecularInitiatingEvent
Aop:425 - Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	MolecularInitiatingEvent
Aop:429 - A cholesterol/glucose dysmetabolism initiated Tau-driven AOP toward memory loss (AO) in sporadic Alzheimer's Disease with plausible MIE's plug-ins for environmental neurotoxicants	KeyEvent
Aop:437 - Inhibition of mitochondrial electron transport chain (ETC) complexes leading to kidney toxicity	KeyEvent

Stressors

Name
Acetaminophen
Chloroform
furan
Platinum
Aluminum
Cadmium
Mercury
Uranium
Arsenic
Silver
Manganese
Nickel
Zinc

nanoparticles

Name

Biological Context

Level of Biological Organization

Molecular

Evidence for Perturbation by Stressor

Platinum

Kruidering et al. (1997) examined the effect of platinum on pig kidneys and found that it was able to induce significant dose-dependant ROS formation within 20 minutes of treatment administration.

Aluminum

In a study of the effects of aluminum treatment on rat kidneys, Al Dera (2016) found that renal GSH, SOD, and GPx levels were significantly lower in the treated groups, while lipid peroxidation levels were significantly increased.

Cadmium

Belyaeva et al. (2012) investigated the effect of cadmium treatment on human kidney cells. They found that cadmium was the most toxic when the sample was treated with 500 µM for 3 hours (Belyaeva et al., 2012). As this study also looked at mercury, it is worth noting that mercury was more toxic than cadmium in both 30-minute and 3-hour exposures at low concentrations (10-100 µM) (Belyaeva et al., 2012).

Wang et al. (2009) conducted a study evaluating the effects of cadmium treatment on rats and found that the treated group showed a significant increase in lipid peroxidation. They also assessed the effects of lead in this study, and found that cadmium can achieve a very similar level of lipid peroxidation at a much lower concentration than lead can, implying that cadmium is a much more toxic metal to the kidney mitochondria than lead is (Wang et al., 2009). They also found that when lead and cadmium were applied together they had an additive effect in increasing lipid peroxidation content in the renal cortex of rats (Wang et al., 2009).

Jozefczak et al. (2015) treated *Arabidopsis thaliana* wildtype, *cad2-1* mutant, and *vtc1-1* mutant plants with cadmium to determine the effects of heavy metal exposure to plant mitochondria in the roots and leaves. They found that total GSH/GSG ratios were significantly increased after cadmium exposure in the leaves of all sample varieties and that GSH content was most significantly decreased for the wildtype plant roots (Jozefczak et al., 2015).

Andjelkovic et al. (2019) also found that renal lipid peroxidation was significantly increased in rats treated with 30 mg/kg of cadmium.

Mercury

Belyaeva et al. (2012) conducted a study which looked at the effects of mercury on human kidney cells, they found that mercury was the most toxic when the sample was treated with 100 µM for 30 minutes.

Buelna-Chontal et al. (2017) investigated the effects of mercury on rat kidneys and found that treated rats had higher lipid peroxidation content and reduced cytochrome c content in their kidneys.

Uranium

In Shaki et al.'s article (2012), they found rat kidney mitochondria treated with uranyl acetate caused increased formation of ROS, increased lipid peroxidation, and decreased GSH content when exposed to 100 µM or more for an hour.

Hao et al. (2014), found that human kidney proximal tubular cells (HK-2 cells) treated with uranyl nitrate for 24 hours with 500 µM showed a 3.5 times increase in ROS production compared to the control. They also found that GSH content was decreased by 50% of the control when the cells were treated with uranyl nitrate (Hao et al., 2014).

Arsenic

Bhadauria and Flora (2007) studied the effects of arsenic treatment on rat kidneys. They found that lipid peroxidation levels were increased by 1.5 times and the GSH/GSSG ratio was decreased significantly (Bhadauria and Flora, 2007).

Kharroubi et al. (2014) also investigated the effect of arsenic treatment on rat kidneys and found that lipid peroxidation was significantly increased, while GSH content was significantly decreased.

In their study of the effects of arsenic treatment on rat kidneys, Turk et al. (2019) found that lipid peroxidation was significantly increased while GSH and GPx renal content were decreased.

Silver

Miyayama et al. (2013) investigated the effects of silver treatment on human bronchial epithelial cells and found that intracellular ROS generation was increased significantly in a dose-dependant manner when treated with 0.01 to 1.0 µM of silver nitrate.

Manganese

Chtourou et al. (2012) investigated the effects of manganese treatment on rat kidneys. They found that manganese treatment caused significant increases in ROS production, lipid peroxidation, urinary H_2O_2 levels, and PCO production. They also found that intracellular GSH content was depleted in the treated group (Chtourou et al., 2012).

Nickel

Tyagi et al. (2011) conducted a study of the effects of nickel treatment on rat kidneys. They found that the treated rats showed a significant increase in kidney lipid peroxidation and a significant decrease in GSH content in the kidney tissue (Tyagi et al., 2011).

Zinc

Yeh et al. (2011) investigated the effects of zinc treatment on rat kidneys and found that treatment with 150 µM or more for 2 weeks or more caused a time- and dose-dependant increase in lipid peroxidation. They also found that renal GSH content was decreased in the rats treated with 150 µM or more for 8 weeks (Yeh et al., 2011).

It should be noted that Hao et al. (2014) found that rat kidneys exposed to lower concentrations of zinc (such as 100 µM) for short time periods (such as 1 day), showed a protective effect against toxicity induced by other heavy metals, including uranium. Soussi, Gargouri, and El Feki (2018) also found that pre-treatment with a low concentration of zinc (10 mg/kg treatment for 15 days) protected the renal cells of rats were from changes in varying oxidative stress markers, such as lipid peroxidation, protein carbonyl, and GPx levels.

nanoparticles

Huerta-García et al. (2014) conducted a study of the effects of titanium nanoparticles on human and rat brain cells. They found that both the human and rat cells showed time-dependant increases in ROS when treated with titanium nanoparticles for 2 to 6 hours (Huerta-García et al., 2014). They also found elevated lipid peroxidation that was induced by the titanium nanoparticle treatment of human and rat cell lines in a time-dependant manner (Huerta-García et al., 2014).

Liu et al. (2010) also investigated the effects of titanium nanoparticles, however they conducted their trials on rat kidney cells. They found that ROS production was significantly increased in a dose dependant manner when treated with 10 to 100 µg/mL of titanium nanoparticles (Liu et al., 2010).

Pan et al. (2009) treated human cervix carcinoma cells with gold nanoparticles (Au1.4MS) and found that intracellular ROS content in the treated cells increased in a time-dependant manner when treated with 100 µM for 6 to 48 hours. They also compared the treatment with Au1.4MS gold nanoparticles to treatment with Au15MS treatment, which are another size of gold nanoparticle (Pan et al., 2009). The Au15MS nanoparticles were much less toxic than the Au1.4MS gold nanoparticles, even when the Au15MS nanoparticles were applied at a concentration of 1000 µM (Pan et al., 2009). When investigating further markers of oxidative stress, Pan et al. (2009) found that GSH content was greatly decreased in cells treated with gold nanoparticles.

Ferreira et al. (2015) also studied the effects of gold nanoparticles. They exposed rat kidneys to GNPs-10 (10 nm particles) and GNPs-30 (30 nm particles), and found that lipid peroxidation and protein carbonyl content in the rat kidneys treated with GNPs-30 and GNPs-10, respectively, were significantly elevated.

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rodents	rodents	High	NCBI
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Oxidative stress is produced in, and can occur in, any species from bacteria through to humans.

Key Event Description

Oxidative stress is defined as an imbalance in the production of reactive oxygen species (ROS) and antioxidant defenses. High levels of oxidizing free radicals can be very damaging to cells and molecules within the cell. As a result, the cell has important defense mechanisms to protect itself from ROS. For example, Nrf2 is a transcription factor and master regulator of the oxidative stress response. During periods of oxidative stress, Nrf2-dependent changes in gene expression are important in regaining cellular homeostasis (Nguyen, et al. 2009) and can be used as indicators of the presence of oxidative stress in the cell.

In addition to the directly damaging actions of ROS, cellular oxidative stress also changes cellular activities on a molecular level. Redox sensitive proteins have altered physiology in the presence and absence of ROS, which is caused by the oxidation of sulfhydryls to disulfides (2SH àSS) on neighboring amino acids (Antelmann and Helmann 2011). Importantly Keap1, the negative regulator of Nrf2, is regulated in this manner (Itoh, et al. 2010).

Protection against oxidative stress is relevant for all tissues and organs, although some tissues may be more susceptible. For example, the brain possesses several key physiological features, such as high O2 utilization, high polyunsaturated fatty acids content, presence of autooxidable neurotransmitters, and low antioxidant defenses as compared to other organs, that make it highly susceptible to oxidative stress (Halliwell, 2006; Emerit and al., 2004; Frauenberger et al., 2016).

How it is Measured or Detected

Oxidative Stress. Direct measurement of ROS is difficult because ROS are unstable. The presence of ROS can be assayed indirectly by measurement of cellular antioxidants, or by ROS-dependent cellular damage:

- Detection of ROS by chemiluminescence (https://www.sciencedirect.com/science/article/abs/pii/S0165993606001683)

- Detection of ROS by chemiluminescence is also described in OECD TG 495 to assess phototoxic potential.

- Glutathione (GSH) depletion. GSH can be measured by assaying the ratio of reduced to oxidized glutathione (GSH:GSSG) using a commercially available kit (e.g., http://www.abcam.com/gshgssg-ratio-detection-assay-kit-fluorometric-green-ab138881.html).

- TBARS. Oxidative damage to lipids can be measured by assaying for lipid peroxidation using TBARS (thiobarbituric acid reactive substances) using a commercially available kit.

- 8-oxo-dG. Oxidative damage to nucleic acids can be assayed by measuring 8-oxo-dG adducts (for which there are a number of ELISA based commercially available kits),or HPLC, described in Chepelev et al. (Chepelev, et al. 2015).

Molecular Biology: Nrf2. Nrf2's transcriptional activity is controlled post-translationally by oxidation of Keap1. Assay for Nrf2 activity include:

- Immunohistochemistry for increases in Nrf2 protein levels and translocation into the nucleus;

- Western blot for increased Nrf2 protein levels;

- Western blot of cytoplasmic and nuclear fractions to observe translocation of Nrf2 protein from the cytoplasm to the nucleus;

- qPCR of Nrf2 target genes (e.g., Nqo1, Hmox-1, Gcl, Gst, Prx, TrxR, Srxn), or by commercially available pathway-based qPCR array (e.g., oxidative stress array from SABiosciences);

- Whole transcriptome profiling by microarray or RNA-seq followed by pathway analysis (in IPA, DAVID, metacore, etc.) for enrichment of the Nrf2 oxidative stress response pathway (e.g., Jackson et al. 2014);

- OECD TG422D describes an ARE-Nrf2 Luciferase test method;

- In general, there are a variety of commercially available colorimetric or fluorescent kits for detecting Nrf2 activation.

Assay Type & Measured Content	Description	Dose Range Studied	Assay Characteristics (Length / Ease of use/Accuracy)
ROS	"The mitochondrial ROS measurement was performed flow cytometry using DCFH-DA. Briefly, isolated kidney mitochondria were incubated with UA (0,		

ROS Formation in the Mitochondria assay Measuring (Shaki et al., 2012)	50, 100 and 200 µM) in respiration buffer containing (0.32 mM sucrose, 10 mM Tris, 20 mM Mops, 50 µM EGTA, 0.5 mM MgCl2, 0.1 mM KH2PO4 and 5 mM sodium succinate) [32]. In the interval times of 5, 30 and 60 min following the UA addition, a sample was taken and DCFH-DA was added (final concentration, 10 µM) to mitochondria and was then incubated for 10 min. Uranyl acetate-induced ROS generation in isolated kidney mitochondria were determined through the flow cytometry (Partec, Deutschland) equipped with a 488-nm argon ion laser and supplied with the Flomax software and the signals were obtained using a 530-nm bandpass filter (FL-1 channel). Each determination is based on the mean fluorescence intensity of 15,000 counts." (Shaki et al., 2012)	0, 50, 100 and 200 µM of Uranyl Acetate	Long/ Easy High accuracy
Mitochondrial Antioxidant Content Assay Measuring GSH content (Shaki et al., 2012)	"GSH content was determined using DTNB as the indicator and spectrophotometer method for the isolated mitochondria. The mitochondrial fractions (0.5 mg protein/ml) were incubated with various concentrations of uranyl acetate for 1 h at 30 °C and then 0.1 ml of mitochondrial fractions was added into 0.1 mol/l of phosphate buffers and 0.04% DTNB in a total volume of 3.0 ml (pH 7.4). The developed yellow color was read at 412 nm on a spectrophotometer (UV-1601 PC, Shimadzu, Japan). GSH content was expressed as µg/mg protein." (Shaki et al., 2012)	0, 50, 100, or 200 μM Uranyl Acetate	
H_2O_2 Production Assay Measuring H_2O_2 Production in isolated mitochondria (Heyno et al., 2008)	"Effect of $CdCl_2$ and antimycin A (AA) on H_2O_2 production in isolated mitochondria from potato. H_2O_2 production was measured as scopoletin oxidation. Mitochondria were incubated for 30 min in the measuring buffer (see the Materials and Methods) containing 0.5 mM succinate as an electron donor and 0.2 µM mesoxalonitrile 3-chlorophenylhydrazone (CCCP) as an uncoupler, 10 U horseradish peroxidase and 5 µM scopoletin." (Heyno et al., 2008)	0, 10, 30 μM Cd^{2+} 2 μM antimycin A	
Flow Cytometry ROS & Cell Viability (Kruiderig et al., 1997)	"For determination of ROS, samples taken at the indicated time points were directly transferred to FACScan tubes. Dih123 (10 mM, final concentration) was added and cells were incubated at 37°C in a humidified atmosphere (95% air/5% CO2) for 10 min. At *t* 5 9, propidium iodide (10 mM, final concentration) was added, and cells were analyzed by flow cytometry at 60 ml/min. Nonfluorescent Dih123 is cleaved by ROS to fluorescent R123 and detected by the FL1 detector as described above for Dc (Van de Water 1995)"		Strong/easy medium
DCFH-DA Assay Detection of hydrogen peroxide production (Yuan et al., 2016)	Intracellular ROS production was measured using DCFH-DA as a probe. Hydrogen peroxide oxidizes DCFH to DCF. The probe is hydrolyzed intracellularly to DCFH carboxylate anion. No direct reaction with H_2O_2 to form fluorescent production.	0-400 µM	Long/ Easy High accuracy
H2-DCF-DA Assay Detection of superoxide production (Thiebault et al., 2007)	This dye is a stable nonpolar compound which diffuses readily into the cells and yields H2-DCF. Intracellular OH or ONOO- react with H2-DCF when cells contain peroxides, to form the highly fluorescent compound DCF, which effluxes the cell. Fluorescence intensity of DCF is measured using a fluorescence spectrophotometer.	0–600 µM	Long/ Easy High accuracy
CM-H2DCFDA Assay	**Come back and explain the flow cytometry determination of oxidative stress from Pan et al. (2009)**		

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List of Key Events in the AOP

Event: 1911: FOXJ1 Protein, Decreased

Short Name: FOXJ1 Protein, Decreased

Key Event Component

Process	Object	Action
	forkhead box protein J1	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:425 - Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	KeyEvent

Stressors

Name
Cigarette smoke
Irradiation

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term
multi-ciliated epithelial cell

Organ term

Organ term
lung epithelium

Evidence for Perturbation by Stressor

Cigarette smoke

Whole cigarette smoke exposure or treatment with cigarette smoke extract of normal human bronchial epithelial cells significantly lowered FoxJ1 mRNA and protein levels (Milara et al., 2012; Brekman et al., 2014; Valencia-Gattas et al., 2016; Ishikawa and Ito, 2017). Cigarette smoke extract treatment of normal human bronchial epithelial cells also reduced the expression of cilia-related transcription factor genes, including FOXJ1, RFX2, and RFX3, as well as that of cilia motility and structural integrity genes regulated by FOXJ1, including DNAI1, DNAH5, DNAH9, DNAH10, DNAH11, and SPAG6 (Brekman et al., 2014).

Irradiation

Irradiation causes excessive levels of free radicals and associated lipid peroxidation, damage to DNA, proteins, leading to wide-spread cellular damage (Azzam et al., 2012; Koc et al., 2003; Rodrigues-Moreira et al., 2017; Shirazi et al., 2013). Thoracic irradiation reduces FOXJ1 mRNA levels in mouse lungs (Bernard et al., 2012).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Danio rerio	Danio rerio	High	NCBI
Xenopus laevis	Xenopus laevis	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

FOXJ1 is functionally conserved throughout diverse groups of metazoans including flatworm Schmidtea mediterranea, zebrafish Danio rerio, African clawed frog Xenopus laevis (Stubbs et al., 2008; Vij et al., 2012; Yu et al., 2008). Ectopic expression of FOXJ1 triggers ciliogenesis in zebrafish and frog (Stubbs et al., 2008; Yu et al., 2008). Overexpression of FOXJ1 transcription factor in the neural tube of a chick induces cilia formation (Cruz C. et al., 2010). There are multiple studies of FOXJ1 in mice and in human cells (Boon et al., 2014; Brekman et al., 2014; Brody et al., 2000; Chen et al., 1998; Choksi et al., 2014). Furthermore, the target genes of FOXJ1, for example RFX3, are regulated by FOXJ1 across different species (Alten et al., 2012; Didon et al., 2013a).

FOXJ1 function is important for all life stages from embryo through adulthood (Choksi et al., 2014; Stauber et al., 2017).

FOXJ1 is expressed in the airways of both males and females. In addition to respiratory tract and brain, FOXJ1 is functionally important also in male and female reproductive tissues (Hackett et al., 1995).

Key Event Description

The epithelium of the respiratory tract has a powerful defense mechanism against air-borne pollutants due to the combined performance of mucus-producing goblet cells and ciliated cells that are covered with microtubule-based projections, the cilia. In response to various irritants and pathogens mucus is secreted by goblet cells, and cilia sweep mucus upward by coordinated beating motions thus clearing the airways from these substances. The ciliated airway epithelial cells are typically covered by hundreds of motile cilia. Cilia formation is initiated and coordinated by a distinct gene expression program, led by the transcription factor forkhead box J1 (FOXJ1) (Brody et al., 2000; Zhou and Roy, 2015). In addition to the respiratory tract, FOXJ1 is expressed also in the ciliated cells of the reproductive and central nervous systems (Blatt et al., 1999; Hackett et al., 1995; Lim et al., 1997).

The multiple motile cilia assembly factors MCIDAS and GMNC converge in positive regulation of FOXJ1 (Arbi et al., 2016; Berta et al., 2016; Stubbs et al., 2012), whereas NOTCH signaling, IL-13-or EGF (epidermal growth factor)-triggered signaling antagonize FOXJ1-driven multiciliogenesis (Gerovac and Fregien, 2016; Gerovac et al., 2014; Gomperts et al., 2007; Shaykhiev et al., 2013). Various other factors are involved in multiple motile cilia assembly, including MYB (acts early in multiciliogenesis downstream of MCIDAS), RFX3 (can act as a co-factor for FOXJ1), ULK4 (modulates the expression of FOXJ1), Wnt signaling, etc. (Choksi et al., 2014; Liu et al., 2016; Schmid et al., 2017; Tan et al., 2013). Most of these factors act upstream or parallel to FOXJ1. FOXJ1 appears to be the major factor in multiciliogenesis, whereby its activity is necessary and also sufficient for programming cells to assemble functional motile cilia (Vij et al., 2012).

FOXJ1 is a master regulator of motile ciliogenesis and is essential to program cells to grow motile cilia (Zhou and Roy, 2015). This key event represents the decrease in the levels or absence of FOXJ1 protein in cells of the respiratory tract. The decrease in FOXJ1 levels inhibits ciliogenesis in multiciliated cells of zebrafish and Xenopus (Stubbs et al., 2008). The knockdown of FOXJ1 results in almost complete absence of cilia in mouse epithelial cells (Brody et al., 2000; Chen J. et al., 1998). On the other hand, the overexpression of FOXJ1 rescues cigarette smoke-mediated suppression of cilia growth in human airway epithelium (Brekman et al., 2014).

How it is Measured or Detected

FOXJ1 protein levels can be measured by Western blot analysis (Brekman et al., 2014; Didon et al., 2013a; Gomperts et al., 2007; Jacquet et al., 2009; Milara et al., 2012), immunofluorescence (Arbi et al., 2016; Gomperts et al., 2007; Valencia-Gattas et al., 2016) or immunohistochemistry (Abedalthagafi et al., 2016; Danielian et al., 2007; Gao et al., 2015). FOXJ1 protein amounts can be inferred from FOXJ1 mRNA levels that can be measured by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Arbi et al., 2016; Brekman et al., 2014; Didon et al., 2013a; Jacquet et al., 2009; Milara et al., 2012; Stubbs et al., 2012), in situ hybridization (Hackett et al., 1995; Stubbs et al., 2012), and Northern blot analysis (Hackett et al., 1995). In addition, FOXJ1 protein activity can be inferred from FOXJ1 target gene expression levels or from reporter gene expression levels (e.g. luciferase assay) of genes harboring FOXJ1 transcription factor binding sites (Brekman et al., 2014; Lim et al., 1997).

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Event: 1912: Motile Cilia Number/Length, Decreased

Short Name: Motile Cilia Number/Length, Decreased

Key Event Component

Process	Object	Action
	motile cilium	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:425 - Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	KeyEvent

Stressors

Name
Cigarette smoke

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
multi-ciliated epithelial cell

Organ term

Organ term
lung epithelium

Evidence for Perturbation by Stressor

Cigarette smoke

Cilia length was reduced in endobronchial biopsies and airway brushings of smokers (average 30 pack-years) compared to nonsmokers (Leopold et al., 2009).

Exposure of human bronchial epithelial cells cultured at the air-liquid interface to 1, 3, and 6% cigarette smoke extract (from the basolateral side) between days 5 and 28 of differentiation significantly shortened the average cilia length of day 28 ALI cultures to 5.7, 5.5, and 4.9 µm, respectively, compared an average cilia
length of 6.7 µm in untreated cultures. Continuous treatment of differentiated cultures with 3 and 6% cigarette smoke extract between days 28 and 42 showed
that ciliated cells in the untreated day 42 cultures had longer cilia than day 28 cultures (ca. +1.5 µm), whereas in the presence of 3 and 6% of CSE, this elongation of cilia was suppressed (+0.5 µm and -0.5 µm, respectively) (Brekman et al., 2014).

Apical exposure of mouse tracheal epithelial cells differentiated at the air-liquid interface to cigarette smoke from 3R4F research cigarettes at a total particular matter concentration of 50 and 100 mg/m^3 for 10 min resulted in cilia shortening (approx. -20% and -50%, respectively) and complete loss of cilia (approx. -25% and -60% of ciliated cells, respectively) at 24 h post-exposure (Lam et al., 2013).

Mean cilia length in the large airway epithelium was 7% shorter in healthy smokers (32.5±10 pack-years) compared to nonsmokers (7.09 vs 7.63 µm), 12% shorter in COPD smokers (39±21 pack-years) compared to healthy smokers (6.16 vs 7.09 µm), and 19% shorter in COPD smokers as compared to nonsmokers. In the small airway epithelium, mean cilia length was 9% shorter in healthy smokers relative to nonsmokers (6.49 vs 7.15 µm), 6% shorter in COPD smokers relative to healthy smokers (6.05 vs 6.49 µm), and 15% shorter in COPD smokers compared to nonsmokers (Hessel et al., 2014).

Exposure of mouse nasal septal epithelial cells to cigarette smoke condensate at concentrations >30 µg/mL for the first 15 days growing at the air-liquid interface inhibited ciliogenesis (ciliated area: 89.9+8.0% in untreated vs 48.8+10.0% [30 µg/mL] and 37.5+12.0% [100 µg/mL]) and resulted in cilia shortening (not quantified) (Tamashiro et al., 2009).

Whole-body exposure of female C57BL/6 mice to mainstream and sidestream cigarette smoke from 1R1 reference cigarettes at 150 mg/m^3 total particular matter for 2 h per day, 5 days per week, for up to 1 year resulted in some areas of sparse or detached ciliated cells by month 6 and an almost complete loss
of ciliated cells by 12 months (Simet et al., 2010).

In a small cohort study in adults with adults with chronic sputum production, current and former smokers had a higher frequency of axonemal ultrastructural abnormalities (16.53 ± 2.66% and 17.66 ± 6.99%, respectively) than non-smokers and controls (5.18 ± 0.9% and 0.7% ± 0.2%, respectively) (Verra et al., 1994).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Danio rerio	Danio rerio		NCBI
Homo sapiens	Homo sapiens	High	NCBI
Xenopus laevis	Xenopus laevis		NCBI
Mus musculus	Mus musculus		NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

The ultrastructural features of human and other mammalian respiratory epithelial cilia and those from lower animals (e.g. flatworms and mollusks) are remarkably similar (Meunier and Azimzadeh, 2016; Wanner et al., 1996). The master regulators of multiciliated cell differentiation, such as NOTCH, GEMC1, MCIDAS, FOXJ1, RFX2/3 are conserved throughout vertebrates (e.g. mammals, Xenopus, zebrafish) and multiple motile cilia across these organisms are functionally similar in generating fluid flow through coordinated beating (Choksi et al., 2014; Meunier and Azimzadeh, 2016; Wessely and Obara, 2008).

The motile cilia numbers reach adult levels in the mouse airway epithelium at day 21 after birth (Rawlins et al., 2007; Toskala et al., 2005). At birth, there is no discernable cilia-generated airway fluid flow in mice (Francis et al., 2009). Between postnatal days 3 and 7 the flow is established in trachea correlating with the increase in the density of ciliated cells in the tracheal epithelia (Francis et al., 2009). After airway fluid flow establishment, the KE is applicable to all life stages.

Key Event Description

Motile cilia are microtubule-based organelles that protrude from the cell surface and generate directional flow of fluid with coordinated beating. 50% to 80% of human respiratory epithelium is comprised of ciliated cells covered with multiple motile cilia that move mucus (together with mucus-trapped substances) upward for clearing the airways (Yaghi and Dolovich, 2016). The ciliated airway epithelial cells are typically covered by more than hundred motile cilia (Bustamante-Marin and Ostrowski, 2017). On average, 150 motile cilia were counted per ciliated human epithelial cell in the study by Mao et al. (Mao et al., 2018). In an earlier report, 200 motile cilia per ciliated cell in human trachea is mentioned (Wanner et al., 1996), and, in a more recent study, a range of 100 to 600 ciliary precursors were counted in fully differentiated mouse tracheal epithelial cells correlated with increasing surface area (Nanjundappa et al., 2019). Cilia are 6–7 µm long and 0.2–0.3 µm in diameter (Brooks and Wallingford, 2014; Yaghi and Dolovich, 2016). Ciliated cell density and the motile cilia length and number per cell correlate with ciliary beating frequency which is routinely used as a predictor of the mucociliary clearance efficiency (King, 2006). Morphological changes of airway cilia are

expected to impact multiple motile cilia functional integrity. This key event represents the decrease in the numbers or absence of motile cilia or reduction in length of motile cilia.

How it is Measured or Detected

Acetylated tubulin is a common ciliary marker (Kim et al., 2013; Piperno and Fuller, 1985), and apical acetylated tubulin staining with subsequent microscope image scoring is a frequently used method of cilia detection and enumeration (Johnson et al., 2018; Mao et al., 2018; Stubbs et al., 2008). Staining of beta-tubulin IV, a protein enriched in motile cilia, is another common method of cilia detection (Brekman et al., 2014; Milara et al., 2012).
Ciliated cells can also be identified by the presence of axonemal structures on the cell surface using scanning electron microscopy (Gomperts et al., 2007).
Mature cilia numbers could be deduced from ciliary precursors in immunofluorescence assays: ciliary precursors can be calculated from three-dimensional superresolution structured illumination microscopy (3D-SIM) images using e.g. a spot detection tool (Nikon Elements AR 4 Software) (Nanjundappa et al., 2019).

For cilia length measurement, the ciliated cells/tissue needs to be stained (Diff-Quik: Dade Behring stain, hematoxylin and eosin staining, labelling with antibodies for ciliary markers such as alpha-tubulin), visualized by microscopy and cilia length quantified (using e.g. ImageJ software or MetaMorph Microscopy Automation & Image Analysis Software) (Brekman et al., 2014; Leopold et al., 2009b; Li et al., 2014). Generally, multiple measurements of one sample and multiple sample preparations of cells/tissues are imaged for reliable quantitation.

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Event: 1908: Cilia Beat Frequency, Decreased

Short Name: CBF, Decreased

Key Event Component

Process	Object	Action
Abnormal ciliary motility	motile cilium	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:411 - Oxidative stress Leading to Decreased Lung Function	KeyEvent
Aop:424 - Oxidative stress Leading to Decreased Lung Function via CFTR dysfunction	KeyEvent
Aop:425 - Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	KeyEvent

Stressors

Name
Cigarette smoke
Acetaldehyde
Acrolein
Nicotine
Ozone
Sex hormone

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

multi-ciliated epithelial cell

Organ term

Organ term

lung epithelium

Evidence for Perturbation by Stressor

Cigarette smoke

Treatment of human sinonasal epithelial cells with cigarette smoke condensate for 3 minutes significantly reduced forskolin-stimulated CBF (Cohen et al., 2009). CBF was also decreased in differentiated normal human bronchial epithelial cells exposed to whole cigarette smoke (Schmid et al., 2015), in cilia-bearing explant adenoid tissues treated with 5 and 10% cigarette smoke extract (Wang et al., 2012), in hamster oviducts treated various mainstream cigarette smoke fractions (Knoll et al., 1995), and in nasal epithelial cells fom smokers with moderate and severe chronic obstructive pulmonary disease (COPD) (Yaghi et al., 2012).

Acetaldehyde

A concentration-dependent decrease in CBF has been observed after treatment with aldehydes. For example inhibition of cilia ATPase activity was observed after treatment with acetaldehyde, in ciliated bovine bronchial epithelial cells (Sisson et al., 1991).

Acrolein

Acrolein, an aldehyde in the gas phase of cigarette smoke, induced ciliostasis at high concentrations (> 1 mM), after 5 min of treatment, and cellular necrosis after 3 hr. However, at lower concentrations (from 0.5−1 mM), acrolein transiently reduced the CBF to 4 Hz (Romet et al., 1990).

Nicotine

Normal human bronchial epithelial cells exposed to aerosolized nicotine showed decreased CFTR and BK conductance, CBF, ASL volume, and decreased expression of FOXJ1 and KCNMA1 (Garcia-Arcos et al., 2016).

Ozone

Continuous, exposure of human nasal epithelial cells to different concentrations of ozone at 37°C for up to 4 weeks slightly (but not significantly) reduced CBF in healthy mucosa (7.1% at 500 µg/m3 and 10.3% at 1000 µg/m3), and significantly in chronically inflamed mucosa (20.5/16.4%) at 2 weeks. During the third and fourth week of exposure at these higher concentrations CBF was significantly reduced in both healthy (after 3 weeks: 18.7/37.5%; after 4 weeks: 11.1/33.3%) and chronically inflamed mucosa (after 3 weeks: 33.8/26.8%; after 4 weeks: 21.4/38.6%). Low ozone concentrations (100 µg/m3) appeared to not have an effect on CBF (Gosepath et al., 2000).

Sex hormone

Female hormones, i.e. progesterone and estrogen, have been shown to have direct effect on CBF, i.e., progesterone reduces CBF, 17β-estradiol and progesterone receptor antagonists counteract progesterone effects, but estradiol alone has also been shown to have no effect on CBF. However, the mechanism by which these hormones modulate CBF is yet to be elucidated (Jain et al., 2012; Jia et al., 2011).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI
Oryctolagus cuniculus	Oryctolagus cuniculus	High	NCBI
Bos taurus	Bos taurus	High	NCBI
Cavia porcellus	Cavia porcellus	Moderate	NCBI
Lithobates catesbeianus	Rana catesbeiana	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	Moderate

Age-dependent decreases in CBF have been demonstrated in several species (e.g. guinea pigs, mice, and human) (Bailey et al., 2014; Grubb et al., 2016; Ho et al., 2001; Joki and Saano, 1997; Paul et al., 2013). In a study with 46 healthy subjects with a wide age distribution (mean 42, range 19–81 years), age was found to be negatively associated with airway clearance of inhaled 6-µm Teflon particles (Svartengren et al., 2005).

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Key Event Description

Cohesive beating of cilia lining the upper and lower respiratory tract is critical for efficient MCC. CBF is influenced by several factors including changes in the physical and chemical properties of the ASL (especially the periciliary fluid), structural modulation in the cilia, concentration of cyclic nucleotides cAMP and cGMP, and intracellular calcium (Ca^{2+}). Formation of cyclic nucleotides such as cGMP is mediated by nitric oxide (NO), which is released by an enzyme family of nitric oxide synthases (NOSs) when the substrate L-arginine (L-Arg) is transformed to L-citrulline. NO activates its receptor protein, soluble guanylate cyclase (sGC), which catalyzes formation of cGMP from guanosine triphosphate (GTP). cGMP then activates protein kinase G (PKG) which has been implicated in the regulation of CBF (Jiao et al., 2011; Li et al., 2000). NO-dependent stimulation of CBF has also been associated with an increase in cAMP-dependent protein kinase A (PKA) (Di Benedetto et al., 1991; Lansley et al., 1992; Salathe et al., 1993; Sanderson and Dirksen, 1989; Schmid et al., 2007; Sisson et al., 1999; Uzlaner and Priel, 1999). An increase in intracellular endogenous cAMP was observed after treatment with isobutyl-1-methylxanthine that also increased CBF (Tamaoki et al., 1989). cAMP accumulation in the airway cilia has been shown to be dependent on Ca2+–calmodulin-dependent PDE1A and indirectly regulates CBF (Kogiso et al., 2018). Increase in CBF after treatment with NO substrate, L-arginine and inhibition of CBF by a NOS inhibitor, N-omega-nitro-L-arginine methyl ester (L-NAME) further provides evidence for the role of NO in increasing CBF (Jiao J. et al., 2011; Sisson J. H., 1995; Uzlaner and Priel, 1999; Yang et al., 1997).
Modulation of CBF is not always accompanied by changes in cAMP levels. PKC activators, phorbol 12-myristate 13-acetate and L-o~-dioctanoylglycerol have been shown to decrease CBF in a concentration- and time-dependent manner in rabbit tracheal epithelial cells (Kobayashi et al., 1989). CBF has been shown to decrease after exposure to inhaled oxidants such as cigarette smoke across different species. A study with 120 subjects showed a significant decrease in nasal CBF following exposure to tobacco smoke (Agius et al., 1998). Exposure to cigarette smoke extract lead to reduction in forskolin-induced CBF in human sinonasal epithelium (Cohen et al., 2009) and isoproterenol- and methacholine-induced CBF in human adenoid tissues (Wang et al., 2012). This decrease in CBF and unresponsiveness to beta-agonist stimulation occurs in parallel to PKC activation and has been shown to be dependent on the duration of exposure to cigarette smoke in mice (Simet et al., 2010). Normal human bronchial epithelial cells exposed to aerosolized nicotine showed decreased CFTR and BK conductance, impaired CBF, ASL volume, and decreased expression of FOXJ1 and KCNMA1 (Garcia-Arcos et al., 2016).
A concentration-dependent decrease in CBF has been observed after treatment with aldehydes. For example inhibition of cilia ATPase activity was observed after treatment with acetaldehyde, in ciliated bovine bronchial epithelial cells (Sisson et al., 1991). Acrolein, an aldehyde in the gas phase of cigarette smoke, induced ciliostasis at high concentrations (> 1 mM), after 5 min of treatment, and cellular necrosis after 3 hr. However, at lower concentrations (from 0.5–1 mM), acrolein transiently reduced the CBF to 4 Hz (Romet et al., 1990).

How it is Measured or Detected

There is no standardized method for measuring CBF. Digital high-speed video imaging with a manual count of CBF in slow motion video play is the most commonly used method for CBF measurement (Kim et al., 2011; Peabody et al., 2018). Photometry and video-microscopy have been used to measure CBF in vitro and ex vivo, including in ciliated bovine bronchial epithelial cells (Allen-Gipson et al., 2011; Sisson et al., 2003; Uzlaner and Priel, 1999), normal human bronchial epithelial cells (Feriani et al., 2017), human nasal epithelial cells (Dimova et al., 2005; Min et al., 1999b), human nasal ciliated epithelium (nasal brushings) (Agius et al., 1998), and mouse tracheal rings (Simet et al., 2010).
CBF measurement in vitro generally involves mounting the tissue at the air-liquid interface on a stage followed by microscopic analysis and acquisition of images and/or video recordings of beating cilia. For in vivo and ex vivo measurements, Doppler optical coherence tomography (D-OCT) can also be applied, a mesoscopic non-contact imaging modality that provides high-resolution tomographic images and detects micromotion simultaneously (Jing et al., 2017). D-OCT has been used to quantitatively measure CBF in ex vivo rabbit tracheal cultures (Lemieux et al., 2015).

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Event: 1909: Mucociliary Clearance, Decreased

Short Name: MCC, Decreased

Key Event Component

Process	Object	Action
mucociliary clearance trait		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:411 - Oxidative stress Leading to Decreased Lung Function	KeyEvent
Aop:424 - Oxidative stress Leading to Decreased Lung Function via CFTR dysfunction	KeyEvent
Aop:425 - Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	KeyEvent

Stressors

Name
Sulfur dioxide
Formaldehyde
PM10
Nitric oxide
Ozone
Cigarette smoke

Biological Context

Level of Biological Organization
Individual

Evidence for Perturbation by Stressor

Sulfur dioxide

SO_2 exposure of dogs dose-dependently decreased CBF and also caused a marked decrease in mean bronchial mucociliary clearance (from 53.7 ± 5.7% to 32.8 ± 7.7%) after 90 min (Yeates et al., 1997). In guinea pig tracheas, SO_2 exposure affected CBF, albeit non-significantly, and mucociliary activity (Knorst et al., 1994).

Formaldehyde

Treatment of frog palate epithelium with different concentrations of formaldehyde induced significant decreases in CBF and MCC (Fló-Neyret et al., 2001; Morgan et al., 1984). Exposure of F344 rats to formaldehyde caused epithelial adaptation of the nasal epithelium, effectively reducing the number of ciliated cells (and hence cilia beating activity) through squamous metaplasia. At the same time, formaldehyde exposure resulted in “ciliastasis” or loss of ciliary activity in a concentration- and exposure duration-dependent manner as well as in a slowing of mucus flow rates (Morgan et al., 1986).

PM10

Incubation of frog palates with PM10 from Sao Paolo, Brazil, for up to 120 min decreased mucociliary transport at concentrations ≥1000 pg/m^3 (Macchione et al., 1999).

Nitric oxide

In New Zealand white rabbits exposed to 3 ppm NO_2 for 24 h, the average CBF decreased from 764 beats/min to 692 beats/min and the transport velocity decreased from 5.23 mm/min to 3.03 mm/min (Kakinoki, 1998).

Ozone

Acute exposure (2 h) of adult ewes to 1.0 ppm ozone significantly reduced tracheal mucus transport velocity (TMV) at 40 min and 2 h post-exposure. Repeated exposure to 1.0 ppm ozone for 5 hper day, for 4 consecutive days showed a progressively significant decrease in TMV on the first and second days, and stabilized over the third and fourth days, around values ranging from -42% to -55% of the initial baseline. TMV remained depressed even after the end of exposure, persisting up to 5 days post-exposure (Allegra et al., 1991).

Cigarette smoke

Nasomuciliary clearance time (determined by saccharin transit test) was significantly higher in smokers than in non-smokers 8 h after smoking (16 ± 6 min vs 10 ± 4 min) and insignicantly higher immediately after smoking (11 ± 6 min vs 10 ± 4 min). Nasomuciliary clearance time correlated positively with cigarettes per day and packs/year index (Proença et al., 2011).

In a small Indian cross-sectional study, the mean nasomuciliary clearance (determined by saccharin transit test) in smokers was significantly higher than that of nonsmokers (481.2 ± 29.83 s vs 300.32 ± 17.4 s). In addition, mean nasomuciliary clearance increased as the duration of smoking increased (NMC in smoking <1 year = 492.25 ± 79.93 s, NMC in smoking for 1-5 years = 516.7 ± 34.01 s, and NMC in smoking >5 years = 637.5 ± 28.49 s) (Baby et al., 2014).

Nasomuciliary clearance (determined by saccharin transit test) in active and passive smokers was significantly higher than in non-smokers (23.08 ± 4.60 min; 20.31 ± 2.51 min vs 8.57 ± 2.12 min) (Yadav et al., 2014).

Nasomuciliary clearance (determined by saccharin transit test) was significantly higher in active smokers than in passive smokers and non-smokers (23.59 ± 12.41 min vs 12.6 ± 4.67 min; 6.4 ± 1.55 min) (Habesoglu et al., 2012).

Nasomuciliary clearance time (determined by saccharin transit test) in smokers was significantly higher than in former smokers and non-smokers (15.6 min vs 11.77 min and 11.71 min, respectively) (Pagliuca et al., 2015).

Moderate and heavy smokers had higher saccharin transit test times than light smokers and non-smokers, and there was a positive correlation between STT and cigarettes/day (Xavier et al., 2013).

The median nasal mucociliary clearance time (determined by saccharin transit test) was significantly higher in smokers (who smoked a mean of 20.6 cigarettes (median: 20) per day) than in nonsmokers (12 (interquartile range: 5–33) min vs 9 (interquartile range: 4–12) min) (Dülger et al., 2018).

Nasal mucociliary clearance time (determined by saccharin transit test) in smokers was significantly higher than in non-smokers (536.19 ± 254.81 s vs 320.43 ± 184.98 s) and correlated with the numbers of cigarettes per day, pack-years and smoking duration (Solak et al., 2018).

Current smokers had a median (IQR) mucociliary clearance transit time (determined by saccharin transit test) of 13.15 (9.89–16.08) min, which was significantly longer compared with that of never smokers at 7.24 (5.73–8.73) min, former smokers at 7.26 (6.18–9.17) min, exclusive e-cigarette users at 7.00 (6.38–9.00) min, and exclusive heated tobacco product users at 8.00 (6.00–8.00) min (Polosa et al., 2021).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Sus scrofa domesticus	Sus scrofa domesticus	Moderate	NCBI
Ovis aries	Ovis aries	Moderate	NCBI
Cavia porcellus	Cavia porcellus	Moderate	NCBI
Canis lupus	Canis lupus	Moderate	NCBI
Rana catesbeiana	Rana catesbeiana	Moderate	NCBI
Oryctolagus cuniculus	Oryctolagus cuniculus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence

Mixed High
Sex Evidence

Key Event Description

In healthy adults, tracheal mucus movement varies from 4 to >20 mm/min (Stannard and O'Callaghan, 2006), whereas mucociliary clearance (MCC) in the small airways is slower due to the lower number of ciliated cells (fewer cilia) and their shorter length (Foster et al., 1980; Iravani, 1969; Wanner et al., 1996).
Since optimal MCC is dependent in multiple factors, including cilia number and structure as well as ASL and mucus properties, any disturbances of these can lead to impaired MCC. While high humidity or infection can enhance MCC, long-term exposure to noxious substances (e.g. cigarette smoke) lead to decreased mucus clearance from the airways. In most instances this is reflected by decreased mucus transport rates or velocities.

How it is Measured or Detected

In humans, MCC has been assessed traditionally following inhalation of radio-labeled particles such as 99Tcm-labeled polystyrene particles, resin particles or serum albumin and following their clearance at regular intervals by radioimaging using gamma cameras (Agnew et al., 1986; Kärjä et al., 1982). Taking into account inhalation volumes and flow rates, lung airflow, particle deposition and retention, clearance rates can be calculated and effects of e.g. drugs on MCC can be examined. Alternatively, since MCC occurs at a similar rate in the nose to that in trachea and bronchi (Andersen and Proctor, 1983; Rutland and Cole, 1981) and for ease of use, measurements of MCC can be restricted to that of nasal MCC only. Probably one of the simplest methods is the saccharin transit test (STT). For this test, a small particle of saccharin is placed behind the anterior end of the inferior turbinate. The saccharin will be transported by mucociliary action toward the nasopharynx, where its sweet taste is perceived. When MCC is impaired, saccharin transit times will increase, with a 10- to 20-minute delay being considered a clinical sign of decreased MCC. Using the same principle, the test can also be performed or complemented with dyes such as indigo carmine or methylene blue (Deborah and Prathibha, 2014).

In experimental animals, MCC has been evaluated by gamma-scintigraphy (Greiff et al., 1990; Hua et al., 2010; Read et al., 1992), fluorescence videography/fluoroscopy (in explanted tracheas etc.) (Grubb et al., 2016; Rogers et al., 2018), or by 3D-SPECT (Ortiz Belda et al., 2016). Direct observation of particle movement across airway epithelia to determine mucus velocity or transport rates by using a fiberoptic bronchoscope may be helpful when working in larger animals such as dogs (King, 1998).
In vitro, freshly excised frog palate preparations have been used to assess cilia function and mucociliary transport by videomicroscopy (Macchione et al., 1995; Macchione et al., 1999; Trindade et al., 2007). Murine and human nasal, bronchial and small airway epithelial models grown at the air-liquid interface are also suitable in vitro test systems for determining mucus transport by tracing inert particle movement with a set-up similar to that used for assessing CBF (Benam et al., 2018; Fliegauf et al., 2013; Knowles and Boucher, 2002; Sears et al., 2015).

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List of Adverse Outcomes in this AOP

Event: 1250: Decrease, Lung function

Short Name: Decreased lung function

Key Event Component

Process	Object	Action
respiratory function trait		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:148 - EGFR Activation Leading to Decreased Lung Function	AdverseOutcome
Aop:302 - Lung surfactant function inhibition leading to decreased lung function	AdverseOutcome
Aop:411 - Oxidative stress Leading to Decreased Lung Function	AdverseOutcome
Aop:418 - Aryl hydrocarbon receptor activation leading to impaired lung function through AHR-ARNT toxicity pathway	KeyEvent
Aop:419 - Aryl hydrocarbon receptor activation leading to impaired lung function through P53 toxicity pathway	AdverseOutcome
Aop:424 - Oxidative stress Leading to Decreased Lung Function via CFTR dysfunction	AdverseOutcome
Aop:425 - Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	AdverseOutcome

Stressors

Name
Ozone
Nitric oxide
Cigarette smoke
Diesel engine exhaust
PM10

Biological Context

Level of Biological Organization
Individual

Evidence for Perturbation by Stressor

Ozone

Acute exposure of healthy young adult subjects (aged 19 to 35 years, non-smokers) to 0.06 ppm ozone for 6.6 h resulted in a 1.71 ± 0.50% (mean ± SEM) decrease in FEV1 and a 2.32 ± 0.41% decrease in FVC compared with air exposure (Kim et al., 2011).

A US-based study found inverse associations between increasing lifetime exposure to ozone (estimated median: 36; interquartile range 29–45; range 19–64) and FEF75 and FEF25–75 in adolescents (aged 18–20 years) (Tager et al., 2005).

Nitric oxide

In a Dutch cross-sectional study in school children (aged 7–13 years), NOx exposure from industrial emissions per interquartile range of 7.43 µg/m^3 had a significantly lower percent predicted peak expiratory flow (PEF) (-3.67%, 95%CI -6.93% to -0.42%). Children exposed to NOx (per interquartile range of 7.43 µg/m^3) also had a significantly lower percent forced vital capacity (FVC) and percent predicted 1-s forced expiratory volume (FEV1) (– 2.73 95%CI -5.21 to -0.25) (Bergstra et al., 2018).

The European Study of Cohorts for Air Pollution Effects (ESCAPE), a meta-analysis of 5 cohort studies on the association of air pollution with lung function, found that a 10 µg/m^3 increase in NO_2 exposure was associated with lower levels of FEV1 (–14.0 mL, 95% CI –25.8 to –2.1) and FVC (–14.9 mL, 95% CI –28.7 to –1.1), and an increase of 20 µg/m^3 in NOx exposure was associated with a lower level of FEV1, by –12.9 mL (95% CI –23.87 to –2.0) and of FVC, by –13.3 mL (95% CI –25.9 to –0.7) (Adam et al., 2015).

Cigarette smoke

A smoking history of > 20 pack-years decreased pulmonary function including forced vital capacity (FVC), forced expiratory volume in one second (FEV1), FEV1/FVC, and forced expiratory flow at 25–75% (FEF25–75%) (Kuperman and Riker, 1973).

In the Framingham Heart Study, cigarette smoking showed an inverse association with FVC and FEV1% (Ashley et al., 1975).

In the international Seven Countries Study, there was a dose-effect relationship between pack-years and forced expiratory volume in 0.75 s (FEV0.75) in continuous smokers without chronic bronchitis (Pelkonen et al., 2006).

In 34 male subjects aged between 15–18 years who smoked FVC was lower than in an age-matched male group that did not smoke. The most common duration of cigarette smoking was 1–3 years (47%) and the maximal number of cigarettes smoked per day was less than or equal to 10 cigarette(s) per day (88%) (Tantisuwat and Thaveeratitham, 2014).

A dose–response relation was found between smoking and lower levels of FEV1/FVC and FEF25–75 in children between 10-18 years of age (Gold et al., 1996).

In a study of 147 asthmatics, FEV1%predicted was significantly lower in ex-smokers and current smokers compared with never-smokers (Broekema et al., 2009).

In a 6-year longitudinal study in Japanese-American men, FEV1 was lowest in current smokers (2702 mL) and in former smokers (2817 mL) at baseline. These 2 groups experienced a steeper annual decline in FEV1 (-34.4 and -22.8 mL/year, respectively, adjusted by height and age at baseline) compared with never-smokers (-20.3 mL/year) (Burchfiel et al., 1995).

Diesel engine exhaust

In a study of 733 adult females who had lived in the Tokyo metropolitan area for more than 3 years, the higher the level of air pollution, the more significantly the FEV1 was reduced (Sekine et al., 2004).

In a study in 29 healthy subjects, exposure to DE inside diesel-powered trains for 3 days was associated with reduced lung function (Andersen et al., 2019).

In workers who tested diesel engines in an assembly unit of a manufacturing plant, FEV1, FEV1/FVC, FEV25-75 and MEF were significantly reduced compared to non-exposed workers (Zhang et al., 2017).

PM10

A Taiwanese study in 1016 children between 6 and 15 years of age reported that lifetime exposure to to 25–85 µg/m^3 PM10 were associated with lower FEV1, FVC, and FEF25-75 (Tsui et al., 2018).

The Swiss Study on Air Pollution and Lung Diseases in Adults (SAPALDIA) found that an increase of 10 µg/m^3 in annual mean concentration of PM10 was associated with 3.4% lower FVC and 1.6% lower FEV1 (Ackermann-Liebrich et al., 1997).

In the Health Survey for England, a 10 mg/m^3 difference in PM10 across postcode sectors was associated with a lower FEV1 by 111 mL, independent of active and passive smoking, social class, region and month of testing (Forbes et al., 2009).

A 7 µg/m^3 increase in five year means of PM10 (interquartile range) was associated with a 5.1% (95% CI: 2.5%–7.7%) decrease in FEV1, a 3.7% (95% CI: 1.8%–5.5%) decrease in FVC in the German SALIA study (Schikowski et al., 2005).

The ESCAPE project, a meta-analysis of 5 European cohorts/studies from 8 countries, reported that an increase of 10 µg/m^3 in PM10 was associated with a lower level of FEV1 (–44.6 mL, 95% CI:–85.4– –3.8) and FVC (–59.0 mL, 95% CI: –112.3– –5.7)

(Adam et al., 2015).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	High

Sex Applicability

Sex	Evidence
Mixed	High

Pulmonary function tests reflect the physiological working of the lungs. Therefore, the AO is applicable to a variety of species, including (but not limited to) rodents, rabbits, pigs, cats, dogs, horses and humans, independent of life stage and gender.

Key Event Description

Lung function is a clinical term referring to the physiological functioning of the lungs, most often in association with the tests used to assess it. Lung function loss can be caused by acute or chronic exposure to airborne toxicants or by an intrinsic disease of the respiratory system.

Although signs of cellular injury are typically exhibited first in the nose and larynx, alveolar-capillary barrier breakdown may ultimately arise and result in local edema (Miller and Chang, 2003). Clinically, bronchoconstriction and hypoxia are seen in the acute phase, with affected subjects exhibiting shortness of breath (dyspnea) and low blood oxygen saturation, and with reduced lung function indices of airflow, lung volume and gas exchange (Hert and Albert, 1994; and How it is Measured or Detected;). When alveolar damage is extensive, the reduced lung function can develop into acute respiratory distress syndrome (ARDS). This severe compromise of lung function is reflected by decreased gas exchange indices (PaO_2/FIO_2 ≤200 mmHg, due to hypoxemia and impaired excretion of carbon dioxide), increased pulmonary dead space and decreased respiratory compliance (Matthay et al., 2019). Acute inhalation exposures to chemical irritants such as ammonia, hydrogen chloride, nitrogen oxides and ozone typically cause local edema that manifests as dyspnea and hypoxia. In cases where a breakdown of the alveolar capillary function ensues, ARDS develops. ARDS has a particularly high risk of mortality, estimated to be 30-40% (Gorguner and Akgun, 2010; Matthay et al., 2018; Reilly et al., 2019).

Lung function decrease due to reduction in lung volume is seen in pulmonary fibrosis, which can be linked to chronic exposures to e.g. silica, asbestos, metals, agricultural and animal dusts (Meltzer and Noble, 2008; Cheresh et al., 2013; Cosgrove, 2015; Trethewey and Walters, 2018). Additionally. decreased lung function occurs in pleural disease, chest wall and neuromuscular disorders, because of obesity and following pneumectomy (Moore, 2012). Decreased lung function can also be a result of narrowing of the airways by inflammation and mucus plugging resulting in airflow limitation. Decreased lung function is a feature of obstructive pulmonary diseases (e.g. asthma, COPD) and linked to a multitude of causes, including chronic exposure to cigarette smoke, dust, metals, organic solvents, asbestos, pathogens or genetic factors.

How it is Measured or Detected

Pulmonary function tests are a group of tests that evaluate several parameters indicative of lung size, air flow and gas exchange. Decreased lung function can manifest in different ways, and individual circumstances, including potential exposure scenarios, determine which test is used. The section outlines the tests used to evaluate lung function in humans (https://www.nhlbi.nih.gov/health-topics/pulmonary-function-tests, accessed 22 March 2021) and in experimental animals.

Lung function tests used to evaluate human lung function

The most common (“gold standard”) lung function test in human subjects is spirometry. Spirometry results are primarily used for diagnostic purposes, e.g. to discriminate between obstructive and restrictive lung diseases, and for determining the degree of lung function impairment. Specific criteria for spirometry tests have been outlined in the American Thoracic Society (ATS) and the European Respiratory Society (ERS) Task Force guidelines (Graham et al., 2019). These guidelines consist of detailed recommendations for the preparation and conduct of the test, instruction of the person tested, as well as indications and contraindications, and are complemented by additional guidance documents on how to interpret and report the test results (Pellegrino et al., 2005; Culver et al., 2017).

Spirometry measures several different parameters during forceful exhalation, including:

- Forced expiratory volume in 1 s (FEV1), the maximum volume of air that can forcibly be exhaled during the first second following maximal inhalation
- Forced vital capacity (FVC), the maximum volume of air that can forcibly be exhaled following maximal inhalation
- Vital capacity (VC), the maximum volume of air that can be exhaled when exhaling as fast as possible
- FEV1/FVC ratio
- Peak expiratory flow (PEF), the maximal flow that can be exhaled when exhaling at a steady rate
- Forced expiratory flow, also known as mid-expiratory flow; the rates at 25%, 50% and 75% FVC are given
- Inspiratory vital capacity (IVC), the maximum volume of air that can be inhaled after a full expiration

A reduced FEV1, with normal or reduced VC, normal or reduced FVC, and a reduced FEV1/FVC ratio are indices of airflow limitation, i.e., airway obstruction as seen in COPD (Moore, 2012). In contrast, airway restriction is demonstrated by a reduction in FVC, normal or increased FEV1/FVC ratio, a normal spirometry trace and potentially a high PEF (Moore, 2012).

Lung capacity or lung volumes can be measured using one of three basic techniques: 1) plethysmography, 2) nitrogen washout, or 3) helium dilution. Plethysmography consists of a series of sequential measurements in a body plethysmograph, starting with the measurement of functional residual capacity (FRC), the volume of gas present in the lung at end-expiration during tidal breathing. Once the FRC is known, expiratory reserve volume (ERV; the volume of gas that can be maximally exhaled from the end-expiratory level during tidal breathing, i.e., the FRC), vital capacity (VC; the volume change at the mouth between the positions of full inspiration and complete expiration), and inspiratory capacity (IC; the maximum volume of air that can be inhaled from FRC) are determined, and total lung capacity (TLC; the volume of gas in the lungs after maximal inspiration, or the sum of all volume compartments) and residual volume (RV; the volume of gas remaining in the lung after maximal exhalation) are calculated (Weinstock and McCannon, 2017).

The other two techniques used to measure lung volumes—helium dilution and nitrogen washout—are based on the principle of conservation of mass: [initial gas concentration] x [initial volume of the system] = [final gas concentration] x [final volume of the system]. The nitrogen washout method is based on the fact that nitrogen is present in the air, at a relatively constant amount. The subject is given 100% oxygen to breathe, and the expired gas, which contains nitrogen in the lung at the beginning of the test, is collected. When no more nitrogen is noted in the expirate, the volume of air expired and the entire amount of nitrogen in that volume are measured, and the initial volume of the system (FRC) can be calculated. In the helium dilution method, a known volume and concentration of helium is inhaled by the subject. Helium, an inert gas that is not absorbed significantly from the lungs, is diluted in proportion to the lung volume to which it is added. The final concentration of helium is then measured and FRC calculated (Weinstock and McCannon, 2017).

Measurements of lung volumes in humans are technically more challenging than spirometry. However, they complement spirometry (which cannot determine lung volumes) and may be a preferred means of lung function assessment when subject compliance cannot be reasonably expected (e.g. in pediatric subjects) or where forced expiratory maneuvers are not possible (e.g. in patients with advanced pulmonary fibrosis). There are recommended standards for lung volume measurements and their interpretation in clinical practice, issued by the ATS/ERS Task Force (Wanger et al., 2005; Criée et al., 2011).

Finally, indices of gas exchange across the alveolar-capillary barrier are tested by diffusion capacity of carbon monoxide (DLCO) studies (also referred to as transfer capacity of carbon monoxide, TLCO). The principle of the test is the increased affinity of hemoglobin to preferentially bind carbon monoxide over oxygen (Weinstock and McCannon, 2017). Complementary to spirometry and lung volume measurements, DLCO provides information about the lung surface area available for gas diffusion. Therefore, it is sensitive to any structural changes affecting the alveoli, such as those accompanying emphysema, pulmonary fibrosis, pulmonary edema, and ARDS. Recommendations for the standardization of the test and its evaluation have been outlined by the ATS/ERS Task Force (Graham et al., 2017). An isolated reduction in DLCO with normal spirometry and in absence of anemia suggests an injury to the alveolar-capillary barrier, as for example seen in the presence of pulmonary emboli or in patients with pulmonary hypertension (Weinstock and McCannon, 2017; Lettieri et al., 2006; Seeger et al., 2013). Reduced DLCO together with airflow obstruction (i.e., reduced FEV1) indicates lung parenchymal damage and is commonly observed in smokers and in COPD patients (Matheson et al., 2007; Harvey et al., 2016), whereas reduced DLCO with airflow restriction is seen in patients with interstitial lung diseases (Dias et al., 2014; Kandhare et al., 2016).

Lung function tests used to evaluate experimental animal lung function

Because spirometry requires active participation and compliance of the subject, it is not commonly used in animal studies. However, specialized equipment such as the flexiVent system (SCIREQ®) are available for measuring FEV, FVC and PEF in anesthetized and tracheotomized small laboratory animals. Other techniques such as plethysmography or forced oscillation are increasingly preferred for lung function assessment in small laboratory animals (McGovern et al., 2013; Bates, 2017).

In small laboratory animals, plethysmography can be used to determine respiratory physiology parameters (minute volume, respiratory rate, time of pause and time of break), lung volume and airway resistance of conscious animals. Both whole body and head-out plethysmography can be applied, although there is a preference for the latter in the context of inhalation toxicity studies, because of its higher accuracy and reliability (OECD, 2018a; Hoymann, 2012).

Gas diffusion tests are not frequently performed in animals, because reproducible samplings of alveolar gas are difficult and technically challenging (Reinhard et al., 2002; Fallica et al., 2011). Modifications to the procedure employed in humans have, however, open possibilities to obtain a human-equivalent DLCO measure or the diffusion factor for carbon monoxide (DFCO)—a variable closely related to DLCO, which can inform on potential structural changes in the lungs that have an effect on gas exchange indices (Takezawa et al., 1980; Dalbey et al., 1987; Fallica et al., 2011; Limjunyawong et al., 2015).

Regulatory Significance of the AO

Established regulatory guideline studies for inhalation toxicity focus on evident clinical signs of systemic toxicity, including death, or organ-specific toxicity following acute and (sub)chronic exposure respectively. In toxicological and safety pharmacological studies with airborne test items targeting the airways or the lungs as a whole, lung function is a relevant endpoint for the characterization of potential adverse events (OECD, 2018a; Hoymann, 2012). Hence, the AO “decreased lung function” is relevant for regulatory decision-making in the context of (sub)chronic exposure (OECD, 2018b; OECD, 2018c).

Regulatory relevance of the AO “decreased lung function” is evident when looking at the increased risk of diseases in humans following inhalation exposure, and because of its links to other comorbidities and mortality.

To aid diagnosis and monitoring of fibrosis, current recommendations include both the recording of potential environmental and occupational exposures as well as an assessment of lung function (Baumgartner et al., 2000). The latter typically confirms decreased lung function as demonstrated by a loss of lung volume. As the disease progresses, dyspnea and lung function worsen, and the prognosis is directly linked to the decline in FVC (Meltzer and Noble, 2008).

Chronic exposure to cigarette smoke and other combustion-derived particles results in the development of COPD. COPD is diagnosed on the basis of spirometry results as laid out in the ATS/ERS Task Force documents on the standardization of lung function tests and their interpretation (Pellegrino et al., 2005; Culver et al., 2017, Graham et al., 2019). Rapid rates of decline in the lung function parameter FEV1 are linked to higher risk of exacerbations, increased hospitalization and early death (Wise et al., 2006; Celli, 2010). Reduced FEV1 also poses a risk for serious cardiovascular events and mortality associated with cardiovascular disease (Sin et al., 2005; Lee et al., 2015).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

[Relationship: 2450: Oxidative Stress leads to FOXJ1 Protein, Decreased]

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Mixed	

Key Event Relationship Description

Oxidative stress (such as that caused by cigarette smoke exposure or irradiation) leads to decreased forkhead box J1 (FOXJ1) gene and protein expression, as well as to decreased FOXJ1 target gene expression (Brekman et al., 2014; Garcia-Arcos et al., 2016; Ishikawa and Ito, 2017; Milara et al., 2012; Valencia-Gattas et al., 2016). FOXJ1 is a key factor of multiple motile cilia assembly in the respiratory airways (Zhou and Roy, 2015). Thus oxidative stress blocks the multiple ciliogenesis program in the airway epithelium.

Evidence Supporting this KER

Cigarette smoke-induced oxidative stress downregulates FOXJ1 expression at both the gene and protein levels in human lung cells in vitro (Milara et al., 2012; Brekman et al., 2014; Valencia-Gattas et al., 2016; Ishikawa and Ito, 2017). Oxidative stress induced by human respiratory syncytial virus reduces FOXJ1 mRNA levels, which can be restored by treatment with antioxidants or the phosphodiesterase 4 inhibitor roflumilast N-oxide (Akaike et al., 1990; Geiler et al., 2010; Mata et al., 2012). In mice, thoracic irradiation results in free radical generation and subsequent reduction in FOXJ1 mRNA expression (Bernard et al., 2012). Many genes that are transcriptionally regulated by FOXJ1 are also downregulated following exposure to cigarette smoke, which implies a reduction in FOXJ1 transcriptional activity (Brekman et al., 2014).

Biological Plausibility

The negative association between cigarette smoke exposure and FOXJ1 levels in airways was shown in multiple studies and can be estimated as a strong linkage. Yet, the notion that oxidative stress as a result of cigarette smoke exposure is leading to decreased FOXJ1 levels is not well demonstrated. As a complex mixture of thousands of chemicals, cigarette smoke exposure could lead to reduced FOXJ1 levels via different routes. Indirect evidence, such as antioxidant molecules that restore cigarette smoke exposure-reduced FOXJ1 levels, as well evidences from other oxidative stress generating insults that decrease FOXJ1 levels add confidence to this KER. However, studies showing a link between oxidative stress generating agents and reduced FOXJ1 levels are scarce. Collectively, the empirical evidence and uncertainties of the linkage imply a moderate ranking for the KER.

Empirical Evidence

Whole cigarette smoke exposed normal human bronchial epithelial cells (HBECs) had significantly lower FoxJ1 protein levels than air-exposed controls (by immunofluorescence). In addition, *FOXJ1* mRNA levels were reduced in whole smoke-exposed differentiating and differentiated HBECs (by TaqMan quantitative RT-PCR)(Valencia-Gattas et al., 2016).

Treatment with cigarette smoke extract (CSE) significantly down-regulated FOXJ1 mRNA and protein levels in differentiating human airway basal cells in air–liquid interface (ALI) cultures (by TaqMan qRT-PCR and Western blot analysis) (Brekman et al., 2014).

Treatment with cigarette smoke extract (CSE) reduced FOXJ1 gene and protein expression in differentiated HBECs through an IL13-mediated mechanism (by RT-qPCR and Western blot analysis) (Milara et al., 2012). Treatment with roflumilast N-oxide (which reduced intracellular reactive oxygen species levels) prevented FOXJ1 loss in CSE-treated cells (Milara et al., 2012).

Exposure of 3D co-cultures of HBECs and fibroblasts to whole cigarette smoke decreased FOXJ1 gene expression in a concentration-dependent manner (by TaqMan quantitative RT-PCR) (Ishikawa and Ito, 2017).

Human respiratory syncytial virus (RSV) infections involve reactive oxygen intermediates (ROIs) that cause cellular damage (Akaike et al., 1990; Mata et al., 2012). Treatment with the free radical scavenger N-acetylcysteine (NAC) reduced the RSV inflammatory response (Geiler et al., 2010). RSV infection reduced *FOXJ1* gene expression (by RT-PCR), which was restored in a dose-dependent manner by NAC treatment (Mata et al., 2012). In another study, *FOXJ1* mRNA levels were consistently low after RSV infection and were restored with roflumilast N-oxide (Mata et al., 2013).

Thoracic irradiation reduced *FOXJ1* mRNA levels in mouse lungs (Bernard et al., 2012). Irradiation causes excessive levels of free radicals and associated lipid peroxidation, damage to DNA, proteins, leading to wide-spread cellular damage (Azzam et al., 2012; Koc et al., 2003; Rodrigues-Moreira et al., 2017; Shirazi et al., 2013).

The expression of cilia-related transcription factor genes, including *FOXJ1*, *RFX2*, and *RFX3* was significantly down-regulated by CSE treatment. The expression of cilia motility and structural integrity genes, including *DNAI1*, *DNAH5*, *DNAH9*, *DNAH10*, *DNAH11*, and *SPAG6* was also significantly down-regulated by CSE treatments (Brekman et al., 2014). Many of these genes (*RFX2*, *RFX3*, *DNAI1*, *DNAH9*, *DNAH11*, *SPAG6*) are transcriptionally regulated by FOXJ1 (Causal biological network database, 2019). The downregulation of FOXJ1-controlled genes infer reduced FOXJ1 transcription factor activity. Indeed, overexpression of FOXJ1 led to partial restoration of CSE treatment-induced downregulation of cilia-related genes (Brekman et al., 2014).

Uncertainties and Inconsistencies

Schamberger et al. did not find any alterations in *FOXJ1* mRNA levels or FOXJ1 target gene (*DNAI1*, *DNALI1*, *SPAG6*, *TEKT1*) transcription upon exposure of HBECs to 2.5% or 5% CSE for 28 days. However, in this study, cigarette smoke exposure reduced ciliated cell numbers (Schamberger et al., 2015).

The evidences listed suggest several mechanisms on how oxidative stress could lead to decreased FOXJ1 levels, including EGFR-, MCIDAS- or IL-13-mediated mechanisms. Most of the studies, however, do not corroborate on how oxidative stress mechanistically leads to reduced FOXJ1 levels. Since there are several other factors (GMNC, NOTCH, ULK4 etc.) known to regulate FOXJ1 levels, further pathways might be involved in passing the oxidative stress signal to FOXJ1.

Quantitative Understanding of the Linkage

High oxidative stress causes reduction in FOXJ1 levels measured at 24 h and for up to 15 days after exposure to an oxidative stress-causing agent. The data on cigarette smoke-reduced FOXJ1 levels are convincing. Indirect evidence such as antioxidants restoring CS-reduced FOXJ1 levels suggest that oxidative stress plays a major role in the CS-induced effects (Milara et al., 2012). However, given the complexity of the CS mixtures (Baumung et al., 2016), we cannot exclude that factors other than oxidative stress are involved in FOXJ1 level reduction. Other sources of oxidative stress such as RSV infection or GY radiation reduce FOXJ1 levels to similar degree as CS-caused FOXJ1 reduction. Based on the available evidence, we classify the quantitative understanding of this KER as moderate.

Response-response relationship

Normal HBECs were exposed to whole cigarette smoke from 3R4F research grade cigarettes using the Vitrocell® VC 10® Smoking Robot (35-mL puff volume, 2 s duration and 1 min between puffs or air as a control). Differentiated cells were exposed every 2 d for 5 d (3 exposures), and samples were collected 48 h after treatment. Differentiating cells were exposed 3 times per week to smoke from 1 cigarette, and samples were collected after 14, 21 and 27 days. FOXJ1 protein and mRNA levels decreased 2.5-fold in differentiated and 2-fold in differentiating normal HBECs. There was a significantly lower percentage of FOXJ1-positive cells following whole smoke exposure at day 27 (4.3 +/- 4.2% vs. 13.0 +/- 7.3%, air) (Valencia-Gattas et al., 2016).

CSE was obtained from one Marlboro Red commercial cigarette bubbled in 12.5 mL of differentiation medium that was then filtered (0.2-µm pore filter). The absorbance was measured at 320 nm on a spectrophotometer, and the optical density of 1 was defined as 100% CSE. HBECs were differentiated at the air-liquid interface while being exposed to 0, 3, and 6% CSE between days 5 and 28. Treatment with 3% and 6% CSE reduced *FOXJ1* mRNA levels to approx. 65% and 55% of control levels, respectively. Treatment of differentiating cultures with 3% CSE reduced FOXJ1 protein levels by 2-fold by day 28 (Brekman et al., 2014).

The smoke of one 2R4F research cigarette was bubbled into a flask containing 25 mL of pre-warmed (37°C) differentiation medium using a respiratory pump model (Harvard Apparatus Rodent Respirator 680, Harvard Apparatus, Holliston, MA, USA) that generates three puffs/min; 35 mL per each puff of 2-s duration with a volume of 0.5 cm above the filter. The solution was then filtered (0.22 µm pore size) to remove particles and the tar phase. The resulting sterile solution was defined as 100% CSE and used within 30 min of preparation. Treatment of differentiated human bronchial epithelial cells with 10% CSE decreased FOXJ1 expression by about 40% at 24 h and 70% at 72 h exposure (Milara et al., 2012).

3R4F reference cigarettes were smoked in accordance with the ISO smoking protocol (35-mL puffs of 2 s each minute). Whole cigarette smoke, generated by a VC10 smoking robot, was released into a mixing device in 2.8-s exhaust and diluted with humidified clean air at 1.0 L/min dilution flow. Diluted smoke was introduced into the CULTEX RFS module and guided into the exposure chamber (5 mL/min) using a vacuum pump to expose 3D co-cultures of HBECs and fibroblasts. *FOXJ1* mRNA levels were reduced to 60% and 40% of the control after exposure to CS from 1 or 4 cigarettes, respectively (Ishikawa and Ito, 2017).

RSV infection elicits reactive oxygen intermediate-mediated effects manifested by changes in the expression of *NRF2* and *HMOX-1* genes (approx. 6- and 9-fold increase at day 15 post-RSV infection, respectively), H_2O_2 generation (7-fold increase in intracellular levels at day 15 post-infection) and severe reduction in total antioxidant capacity. These data together indicate the presence of

oxidative stress following infection which leads to decreased *FOXJ1* mRNA levels (ca. 25% of control at 15 days post-RSV infection (Mata et al., 2012) and ca. 45% of control at 10 days after RSV infection (Mata et al., 2013).

FOXJ1 mRNA levels were reduced by 50% in murine lungs 14 days after thoracic irradiation at 15 Gy (Bernard et al., 2012).

Time-scale

Whole cigarette smoke exposure from one 3R4F research grade cigarette using the Vitrocell® VC 10® Smoking Robot (35-mL puff volume, 2-s duration and 1 min between puffs or air as a control) once a day on alternate days for 5 days decreased FOXJ1 mRNA levels by 2.5-fold in differentiated HBECs (Valencia-Gattas et al., 2016).

Whole cigarette smoke exposure from one 3R4F research grade cigarette using the Vitrocell® VC 10® Smoking Robot (35-mL puff volume, 2-s duration and 1 min between puffs or air as a control) for 3 times per week for 4 weeks (27 days) decreased FOXJ1 mRNA levels by 2-fold in differentiating HBECs (Valencia-Gattas et al., 2016).

Treatment of differentiating HBECs (between days 5 and 28 of the air-liquid interface) with 3% CSE reduced FOXJ1 protein levels by 2-fold by day 28. Treatment of differentiating HBECs with 6% CSE reduced FOXJ1 protein levels by approx. 55% by day 28 (Brekman et al., 2014).

Treatment of differentiated human bronchial epithelial cells with 10% CSE decreased FOXJ1 expression by about 40% at 24 h and ca. 70% at 72 h (Milara et al., 2012).

Repeated exposure of 3D bronchial epithelial cultures to whole smoke of 4 cigarettes (every other day, treatment started on ALI culture day 7) resulted in a 2.5-fold decrease of FOXJ1 mRNA levels by day 21 (Ishikawa and Ito, 2017).

At 15 days post-RSV infection, FOXJ1 mRNA levels were 4-fold reduced compared to untreated samples (Mata et al., 2012). In another study from the same group, FOXJ1 mRNA levels were reduced to 45% of the FOXJ1 levels in uninfected sample at 10 days post-RSV infection (Mata et al., 2013).

At 7 and 14 days after 15 GY thoracic irradiation, FOXJ1 mRNA levels were reduced to approx. 70% and 50% of controls, respectively (Bernard et al., 2012).

Known modulating factors

Unknown

Known Feedforward/Feedback loops influencing this KER

Unknown

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Relationship: 2447: FOXJ1 Protein, Decreased leads to Motile Cilia Number/Length, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI
Xenopus laevis	Xenopus laevis		NCBI
Mus musculus	Mus musculus		NCBI
Schmidtea mediterranea	Schmidtea mediterranea		NCBI
Danio rerio	Danio rerio		NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Mixed	

Key Event Relationship Description

Forkhead box J1 (FOXJ1) is a master regulator of motile ciliogenesis which is necessary and also sufficient to program cells to grow functional motile cilia (Vij et al., 2012; Zhou and Roy, 2015). Studies in different model organisms have shown that the loss of FOXJ1 results in a loss of motile cilia (Brody et al., 2000; Chenet al., 1998; Stubbs et al., 2008; Vij et al., 2012).

Evidence Supporting this KER

Homozygous null mutation of Foxj1 results in complete absence of cilia in mouse respiratory epithelium (Chen et al., 1998; Brody et al., 2000). In a previous study, wild-type mice had approximately 20% heavily ciliated cells in the proximal pulmonary epithelium, while explanted Foxj1$^{-/-}$ mouse trachea had no ciliated cells (Gomperts et al., 2004). Loss of FOXJ1 orthologs FoxJ1–4 in flatworm Schmidtea mediterranea results in loss of ciliation of the ventral epithelium which closely resembles the human airway epithelium (Rompolas et al., 2009; Vij et al., 2012). Loss of Foxj1 activity in *Xenopus* and zebrafish—through antisense morpholino oligonucleotides—reduces cilia formation, while, conversely, ectopic Foxj1 overexpression results in formation of multiple motile cilia (Stubbs et al., 2008; Yu et al., 2008). There is a strong correlation between FOXJ1 and expression of the FOXJ1 ciliogenesis program genes in zebrafish, Xenopus and mouse cells (Abedalthagafi et al., 2016).

Treatment with cigarette smoke extract downregulates FOXJ1 mRNA and protein expression, which is accompanied by a reduction in cilia length and number in human bronchial epithelial cells in vitro (Milara et al., 2012; Brekman et al., 2014). This can be prevented by overexpression of FOXJ1 (Brekman et al., 2014) or treatment with roflumilast N-oxide, which reduces intracellular free radical levels and increases FOXJ1 mRNA and protein expression (Milara et al., 2012).

Biological Plausibility

The requirement of FOXJ1 for cells to grow functional motile cilia was demonstrated in 1998 in a mouse model study where targeted disruption of FOXJ1 resulted in the absence of motile cilia in the respiratory epithelium, oviduct, haploid sperm, and choroid plexus (Chen et al., 1998). Subsequently, many research groups consistently showed FOXJ1 requirement for cilia growth in various model organisms (Brody et al., 2000; Gomperts et al., 2007; Stubbs et al., 2008; Vij et al., 2012; Yu et al., 2008). In addition, overexpression of FOXJ1 in ectopic locations prompted cilia growth (Stubbs et al., 2008; Yu et al., 2008), and FOXJ1 overexpression could rescue cigarette smoke extract-caused cilia growth suppression in human airway epithelium (Brekman et al., 2014). The causal association of FOXJ1 to ciliogenesis gene expression program was computationally reinforced (Abedalthagafi et al., 2016). Taken together, the empirical support for this KER based on the research in the motile ciliogenesis field implies a high (strong) confidence for the biological plausibility of the linkage.

Empirical Evidence

Two research groups independently showed that homozygous null mutation of Foxj1 causes complete absence of cilia in the respiratory airways of mice (Brody et al., 2000; Chen et al., 1998).

Loss of Foxj1a activity in zebrafish (antisense morpholino oligonucleotides designed to block Foxj1a protein translation) compromises formation of motile cilia (Yu et al., 2008). In the same study, Foxj1a ectopic expression in the neural tube (through hyperactivation of the hedgehog pathway using dominant negative PKA) triggered motile cilia development (Yu et al., 2008).

In Xenopus, FoxJ1 morpholino oligo treatments dose-dependently reduced cilia formation, and FoxJ1 overexpression in ectopic locations was sufficient to drive multiciliogenesis (Stubbs et al., 2008). In addition, authors also used a zebrafish model where FOXJ1 morpholino resulted in cilia number and length reduction (Stubbs et al., 2008).

The closest ortholog of mammalian FOXJ1 in the flatworm *Schmidtea mediterranea* is FoxJ1-4 which is expressed in the motile ciliated cells of the ventral epithelium. Flatworm ventral epithelium closely resembles the mammalian airway epithelium (Rompolas et al., 2009; Vij et al., 2012). S. mediterranea deficient for foxJ1-4 (RNAi) lost the ciliation of the ventral epithelium (Vij et al., 2012).

Abedalthagafi et al. defined the genes that comprise the FOXJ1 ciliogenesis program using public mRNA expression profiling data from Xenopus, zebrafish and mouse cells. A strong correlation of FOXJ1 expression and the expression of the FOXJ1 ciliogenesis program was confirmed (Abedalthagafi et al., 2016).

Cigarette smoke extract (CSE) down-regulated FOXJ1 mRNA and protein levels and cilia length in differentiating human airway basal cells in air–liquid interface (ALI) cultures, while lentivirus-mediated FOXJ1 expression prevented CSE-elicited cilia growth suppression (Brekman et al., 2014).

Wild-type mouse tracheas cultured at air-liquid interface demonstrated nearly 20% of the proximal pulmonary epithelial cells to be ciliated with abundant cilia on each cell. In explanted Foxj1$^{-/-}$ tracheas no ciliated epithelial cells were observed (Gomperts et al., 2004).

Exposure of differentiated human bronchial epithelial cells to CSE decreased FOXJ1 expression alongside with reduction of the average number of cells with cilia motility. Roflumilast N-oxide (which reduced intracellular reactive oxygen species levels) prevented the CSE-elicited decrease in the expression levels of Foxj1 mRNA and protein. Concurrently, roflumilast N-oxide partly prevented the loss in cells with cilia motility (Milara et al., 2012).

FOXJ1 overexpression increased the percentage of ciliated cells in mouse trachea organ culture 1.51-fold (Johnson et al., 2018).

Uncertainties and Inconsistencies

Foxj1 overexpression failed to promote ciliogenesis in mouse polarized epithelial cell lines and primary cultured alveolar epithelial cells (You et al., 2004). Also, the overexpression of Foxj1 in wild-type airway epithelial cells did not enhance the total number of ciliated cells. However, delivery of Foxj1 to null cells resulted in cilia formation (You et al., 2004).

Quantitative Understanding of the Linkage

There is ample empirical evidence of FOXJ1 requirement for motile cilia formation, where complete removal of FOXJ1 results in cilia loss, and different levels of FOXJ1 downregulation result in proportional reduction in cilia number and length. Accordingly, FOXJ1 overexpression leads to higher cilia numbers. Based on these data of strong causality between upstream and downstream KEs, we judge our quantitative understanding to be high.

Response-response relationship

Complete removal of FOXJ1 by means of homologous recombination in mouse embryonic stem cells resulted in absence of cilia in mouse airways (as well as in other typically multiciliated tissues such as oviduct, haploid sperm, choroid plexus and epithelial cells

of the brain but not in embryonic node) (Brody et al., 2000; Chen J. et al., 1998).

Newly fertilized zebrafish eggs were injected with antisense morpholino oligonucleotides designed to block Foxj1a protein translation. Motile cilia numbers were severely reduced in Kupffer's vesicle (KV), the floor plate and pronephric ducts 14 and 24 hpf (Yu et al., 2008).

Downregulation of Xenopus FoxJ1 produced a dose-dependent defect in skin cilia formation. When 20 ng or 40 ng morpholino oligonucleotides were injected, cilia formed, but were reduced in number and shortened in length. After injection of 75 ng morpholino oligos, most cilia were lost. Cilia length decreased from ~11 microns to 4 microns (Stubbs et al., 2008). Morpholino oligo knockdown of zebrafish FoxJ1 caused a two-fold decrease in the number of KV cilia and a 3.5-fold decrease in the average length of KV cilia (Stubbs et al., 2008).

RNAi against *Schmidtea mediterranea* foxJ1-4 substantially reduced the expression levels of foxJ1-4 which lead to almost complete loss of motile cilia (Vij et al., 2012).

In the presence of CSE, in FOXJ1 overexpressing human airway epithelial cells the average cilia length was significantly higher (5.2 µm) than in lentivirus-control–infected cells (4.1 µm) (Brekman et al., 2014). CSE was obtained from one Marlboro Red commercial cigarette bubbled in 12.5 ml of differentiation medium that was then 0.2 mm pore filtered. The absorbance was measured at 320 nm on a spectrophotometer and the optical density of 1 was defined as 100%. Homozygous FOXJ1 mutant mice were obtained by mating foxj1$^{+/-}$ male and female animals. The explanted trachea of the foxj1$^{-/-}$ mice harbors no motile cilia in contrast to wild-type trachea (Gomperts et al., 2004).

Exposure of differentiated human bronchial epithelial cells to 10% CSE decreased FOXJ1 expression by about 40% at 24 h and 70% at 72 h exposure. The smoke of one 2R4F research cigarette was bubbled into a flask containing 25 mL of pre-warmed (37°C) differentiation medium using a respiratory pump model (Harvard Apparatus Rodent Respirator 680, Harvard Apparatus, Holliston, MA, USA) that generates three puffs min–1; 35 mL per each puff of 2 s duration with a volume of 0.5 cm above the filter. The CS solution was filtered (0.22 µm pore size) to remove particles and the tar phase. The resulting sterile solution was defined as 100% CSE and used within 30 min of preparation. Exposure to CSE concentration- and time-dependently reduced the average number of cells with cilia motility, which was significant after 3 days of incubation with CSE at 2.5% (about 30% inhibition), and reached a maximum of about 75% inhibition versus control after 7 days of incubation with CSE at 10% (Milara et al., 2012). Roflumilast N-oxide at 2 nM or 1 µM concentration-dependently prevented the decrease in the expression levels of Foxj1 mRNA and protein following 3 days of exposure of differentiated bronchial epithelial cells to CSE at 10%. Concurrently, roflumilast N-oxide partly prevented the loss in cells with cilia motility (Milara et al., 2012).

Electroporation using negative control (GFP-only) plasmid resulted in 45±1.4% (mean±s.e.m.) GFP+ ciliated cells in mouse trachea organ culture. FOXJ1 significantly increased the percentage of GFP+ ciliated cells to 68±3.6% (1.51-fold) (Johnson et al., 2018).

Time-scale

14- or 24-hr treatment with antisense morpholino oligonucleotides designed to block Foxj1a protein translation results in absence of motile cilia in zebrafish (Yu et al., 2008).

Xenopus embryos were injected with FOXJ1 morpholino oligos at two-cell stage (1.5 h of embryo life) and the embryos were analyzed at stage 26 (1 day, 5 h and 30 min of embryo life) for cilia phenotype (Stubbs et al., 2008).

Schmidtea mediterranea worms received three feedings of foxJ1-4 RNAi (2 days in between feeds) and were analyzed for cilia phenotype 14 days after the last feed. RNAi against Schmidtea mediterranea foxJ1-4 substantially reduced the expression levels of foxJ1-4 which lead to almost complete loss of motile cilia (Vij et al., 2012).

Exposure of differentiated human bronchial epithelial cells to CSE concentration- and time-dependently reduced the average number of cells with cilia motility, which was significant after 3 days of incubation with CSE at 2.5% (about 30% inhibition), and reached a maximum of about 75% inhibition versus control after 7 days of incubation with CSE at 10% (Milara et al., 2012).

Known modulating factors

Regulatory factor X3 (RFX3) is a transcriptional co-activator of FOXJ1 (Didon et al., 2013) and is involved in motile cilia biogenesis (El Zein et al., 2009). Fluctuations in RFX3 levels can modulate the outcome that the upstream KE has on the downstream KE.

Known Feedforward/Feedback loops influencing this KER

Unknown

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Relationship: 2448: Motile Cilia Number/Length, Decreased leads to CBF, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Danio rerio	Danio rerio		NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Mixed	

Key Event Relationship Description

The cilia in the respiratory epithelium beat in a coordinated fashion at a frequency of approximately 10 to 16 Hz, propelling mucus upwards (Joki et al., 1998; Smith et al., 2012). Many factors, including cilia length, number, structure, orientation as well as mucus viscosity, temperature, pH, chemicals, airway surface liquid height, exposure to bacterial and viral pathogens have been shown to affect ciliary function (Clary-Meinesz et al., 1998; Ho et al., 2001b; Jing et al., 2017; Joki et al., 1998; Kanthakumar et al., 1996; Mall, 2008; Smith et al., 2012; Snyder et al., 2017). Alteration from normal physiological conditions and from healthy cilia number/length/structure typically reduces the cilia beat frequency (CBF) (Clary-Meinesz et al., 1998; Jayathilake et al., 2012).

Evidence Supporting this KER

In Chlamydomonas, ciliary motion is directly related to the length of the cilia (Bottier et al., 2018). Similar observations have been made in zebrafish, where modulation of cilia length and number by FOR20 (centrosomal protein 20) deletion/knockdown directly impairs ciliary motility (Xie et al., 2019). There is also a positive correlation between cilia number and CBF in sinusitis patients (Joki et al., 1998), while cilia number, length and orientation correlate positively with mucociliary transport rate in patients with recurrent or longstanding respiratory infections (Toskala et al., 1995; Joki et al., 1998). Comparisons of strips of normal and disrupted ciliated epithelium have shown that CBF is decreased in the latter (Thomas et al., 2009).
Mathematical models and simulations have shown that periciliary liquid and mucus velocity are directly affected by cilia number and length (Lee et al., 2011; Jayathilake et al., 2012; Jayathilake et al., 2015).

Biological Plausibility

Many research groups have shown positive correlations between cilia number/length and ciliary function in studies dating as early as 1995. In some cases, the authors measured mucociliary clearance as the endpoint, thus the evidence does not describe causality between cilia number/length and CBF. However, the commonly held assumption is that reduced ciliary function, i.e., reduced CBF, leads to decreased mucociliary clearance. Based on the evidence, we judge the biological plausibility of this KER as moderate.

Empirical Evidence

Cilia beating frequency analysis in sinusitis patient samples showed a positive correlation between cilia number and CBF (Joki et al., 1998).

Cilia study from patients with recurrent or longstanding respiratory infections demonstrated a correlation between mucociliary transport rate and cilia number, length, and orientation (Toskala et al., 1995).

According to a numerical model formulated by Jayathilake et al., the average periciliary liquid layer (PCL) velocity decreases when cilia are shortened (Jayathilake et al., 2015; Jayathilake et al., 2012).

A study of *Chlamydomonas* cilia motion concluded that cilia shorter than 4 µm show variable or no periodicity of beating. For cilia longer than 4 µm, the ratio of frequency estimated from body motion to frequency of cilia motion is very close to 1.0, as expected. In short (2 µm, 4 µm) cilia, the ratio was significantly different from 1; apparently, even if the short cilium beats periodically, the frequency of beating is not reliably transmitted to the body motion (Bottier et al., 2018).

In a simulation study by Lee et al., an increase in cilia number increased mucus velocity (Lee et al., 2011).

In zebrafish, deletion or knockdown of FOR20 caused reduced number and length of cilia. Ciliary motility is impaired in animals with reduced FOR20 (Xie et al., 2019).

Cilia beating frequency measurements on normal ciliated or disrupted epithelial strips of tissue revealed lower CBF for tissues with cilia disruptions (Thomas et al., 2009).

Uncertainties and Inconsistencies

Although the majority of studies discuss the effect of shorter cilia on ciliary function, there are also reports on longer than normal cilia impairing cilia function (Jayathilake et al., 2015; Li et al., 2014b). A range of factors other than cilia length and number can influence cilia beat frequency. Often a combination of two or more factors affect ciliary function making it difficult to discern the impact of an individual factor on CBF (Toskala et al., 1995; Xie et al., 2019).

Quantitative Understanding of the Linkage

Cilia beating frequency correlates with cilia size and numbers such that higher number of motile cilia with a healthy length show efficient CBF and a reduction of cilia number and/or length results in a proportionate reduction of CBF. In some studies, mucociliary clearance was measured as an indicator of CBF. Based on evidence presented here, we judge the quantitative understanding to be moderate.

Response-response relationship

A study in nasal mucosa samples from sinusitis patients demonstrated that reduced cilia numbers account for decreased CBF. In normally ciliated samples the CBF was 11.2±3.7 Hz, in samples with some ciliated cells CBF was 8.9±6.3 Hz, and in samples with no detectable cilia CBF was 2.1±3.8 Hz (Joki S. et al., 1998).

Patients with recurrent or longstanding respiratory infections were divided into 3 groups based on mucociliary transport rates (MTR). The group with slowest MTR had the biggest number of non-ciliated columnar cells in the nasal mucosa, and the highest number of short and disoriented cilia. Loss of ciliated cells was seen in 50% of specimens with good MTR (7 to 10.8 mm per minute), in 71% with moderate MTR (3 to 6.9 mm per minute) and in 86% with poor MTR (0 to 2.9 mm per minute). The percentages of short cilia were 1% in the first group, 6% in the second, and 10% in the third. In this study, ciliary disorientation also contributed to the low MTR (Toskala et al., 1995).

Jayathilake et al. developed a two-dimensional numerical model for computing how the length of cilia is affecting fluid flow. In the model, the cilia length was reduced by 5%, 15%, 25%, and 35% from 6 µm while other parameters were kept unchanged. The average periciliary layer (PCL) velocity in stream-wise direction decreases when cilia are shortened. When the cilia are shortened by 10%, the average stream-wise PCL velocity is reduced by about 11%, while the average span-wise PCL velocity is reduced by about 62% (Jayathilake et al., 2012). In a similar study by the same group, the length of cilia was defined as 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130% and 140% of the length of the healthy cilia (i.e., 6 µm). The mucus velocity reaches its maximum value when the ciliary length is around the length of the healthy cilia (or standard length of 6 µm) (Jayathilake et al., 2015).

Cilia shorter than 4 µm have anomalies in the ciliary beating periodicity. Cilia shorter than 2 µm were never found to beat periodically. Cilia between 2 and 4 µm in length exhibited more variable periodicity. Cilia with lengths between 4 and 12 µm beat periodically with conserved frequency (Bottier et al., 2018).

Mucus velocity increased in concordance with cilia number increase. Mean mucus velocity increased from 26.82 µm/s to 49.53 µm/s when cilia number grew from 10 to 24 (Lee et al., 2011).

FOR20 deletion or knockdown in zebrafish reduced the number and length of cilia. Cilia in FOR20 morphants had impaired ciliary motility. When cilia length was reduced from an average 5.5 µm to an average 3 µm due to FOR20 depletion, about 80% cilia displayed consistently paralyzed or arrhythmically beating pattern (Xie et al., 2019).

Nasal brush biopsy samples from pediatric patients with primary ciliary dyskinesia were analyzed. The median CBF was 13.4 Hz for normal ciliated epithelia, 11.4 Hz for the ciliated edge with minor projections and 8.7 Hz for the ciliated edge with major projections (Thomas et al., 2009).

Time-scale

No data

Known modulating factors

Unknown

Known Feedforward/Feedback loops influencing this KER

Unknown

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Relationship: 2442: CBF, Decreased leads to MCC, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative stress Leading to Decreased Lung Function	adjacent	High	Moderate
Oxidative stress Leading to Decreased Lung Function via CFTR dysfunction	adjacent	High	Moderate
Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus		NCBI
Canis lupus	Canis lupus		NCBI
Cavia porcellus	Cavia porcellus		NCBI
Ovis aries	Ovis aries		NCBI
Lithobates catesbeianus	Rana catesbeiana		NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Key Event Relationship Description

Synchronized ciliary action transports mucus from the distal lung to the mouth, where it is swallowed or expectorated (Munkholm and Mortensen, 2014). In addition to ASL and mucus properties, the speed of ciliary movement, and hence the effectiveness of mucociliary clearance (MCC), is dependent on ciliary amplitude and beat frequency (Rubin, 2002). CBF itself is influenced by several factors, including changes in the physical and chemical properties of the ASL (especially the periciliary fluid), structural modulation in the cilia, concentration of cyclic nucleotides cAMP and cGMP, and intracellular calcium (Ca^{2+}). Aside from genetic defects leading to ciliopathies, there is ample evidence for prolonged exposure to noxious agents, such as cigarette smoke, nitrogen oxide and sulfur dioxide, playing a major role in decreasing CBF and hampering efficient MCC.

Evidence Supporting this KER

A decrease in CBF resulting from sulfur dioxide exposure reduced mucociliary clearance in dogs (Yeates et al., 1997) and mucociliary activity in guinea pig tracheas (Knorst et al., 1994). In rats, formaldehyde inhalation exposure resulted in lower numbers of ciliated cells, while ciliary activity and mucus flow rates were decreased in a dose and time-dependent manner (Morgan et al.,

1986). In humans, CBF positively correlates with nasal mucociliary clearance time (Ho et al., 2001), and bronchiectasis patients have lower nasal CBF and slower mucociliary transport (MCT) (Rutland and Cole, 1981). Administration of nebulized CBF inhibitors and enhancers quantifiably decreased or increased mucociliary clearance, respectively (Boek et al., 1999; Boek et al., 2002). Increased CBF and MCT was also noted in human sinonasal epithelial cell cultures treated with Myrtol®, an essential oil distillate (Lai et al., 2014) and in sheep tracheas and human airway epithelial cultures subjected to temperature changes (Kilgour et al., 2004; Sears et al., 2015). Exposures of frog palate epithelia to formaldehyde and PM10 reduced MCC and mucociliary transport, but only formaldehyde-treated epithelia showed decreases in CBF (Morgan et al., 1984; Macchione et al., 1999; Fló-Neyret et al., 2001).
Ex vivo treatment of sheep trachea with acetylcholine and epinephrine increased CBF, but only acetylcholine increased surface liquid velocity, while both parameters were decreased upon incubation with platelet-activating factor (Seybold et al., 1990).

Biological Plausibility

Ciliary function and mucus transport are invariably linked to effective mucus transport along the mucociliary escalator (Bustamante-Marin and Ostrowski, 2017; Mall, 2008). Therefore, this KER is biologically plausible.

Empirical Evidence

Studies in animal models of ciliopathies and in individuals with genetic disorders causing cilia defects demonstrate that absent or asynchronous cilia beating results in defective mucus clearance from the lungs, consequently leading to respiratory infections that may be chronic recurrent in nature and ultimately lead to declining lung function (Knowles et al., 2013; Munkholm and Mortensen, 2014; Tilley et al., 2015). Similarly, indirect effects of airway inflammation, caused for example by respiratory infections or allergies, are known to be responsible for changes in cilia beating and hence mucus clearance (Almeida-Reis et al., 2010; Hisamatsu and Nakajima, 2000; Maurer et al., 1982). Finally, airway epithelial injury following exposure to inhalation toxicants can also damage cilia and inhibit cilia function and thereby impair MCC (Iravani and Van As, 1972; Wanner et al., 1996).

A decrease in CBF resulting from sulfur dioxide exposure reduced mucociliary clearance in dogs (Yeates et al., 1997) and mucociliary activity in guinea pig tracheas (Knorst et al., 1994). In rats, formaldehyde inhalation exposure resulted in lower numbers of ciliated cells, while ciliary activity and mucus flow rates were decreased in a dose and time-dependent manner (Morgan et al., 1986). In humans, CBF positively correlates with nasal mucociliary clearance time (Ho et al., 2001), and bronchiectasis patients have lower nasal CBF and slower mucociliary transport (MCT) (Rutland and Cole, 1981). Administration of nebulized CBF inhibitors and enhancers quantifiably decreased or increased mucociliary clearance, respectively (Boek et al., 1999; Boek et al., 2002). Increased CBF and MCT was also noted in human sinonasal epithelial cell cultures treated with Myrtol®, an essential oil distillate (Lai et al., 2014) and in sheep tracheas and human airway epithelial cultures subjected to temperature changes (Kilgour et al., 2004; Sears et al., 2015). Exposures of frog palate epithelia to formaldehyde and PM10 reduced MCC and mucociliary transport, but only formaldehyde-treated epithelia showed decreases in CBF (Morgan et al., 1984; Macchione et al., 1999; Fló-Neyret et al., 2001).

The available evidence does not interrogate the direct relationship between CBF and MCC, but rather evaluates both outcomes in parallel. However, because of the intrinsic linkage of cilia function and MCC, we find the empirical evidence in support of this KER to be moderate.
Ex vivo treatment of sheep trachea with acetylcholine and epinephrine increased CBF, but only acetylcholine increased surface liquid velocity, while both parameters were decreased upon incubation with platelet-activating factor (Seybold et al., 1990).

Uncertainties and Inconsistencies

Although ciliary function is considered a primary determinant for effective MCC (Duchateau et al., 1985; Gizurarson, 2015), there is evidence that suggests that MCC can be impeded by other factors that do not affect CBF. For example, nasal CBF in cigarette smokers regularly exhaling through the nose was not significantly different from that of nonsmokers, although they exhibited significantly longer nasomuciliary clearance times compared to nonsmokers. Possible explanations offered for this discrepancy were a potential loss of cilia in the nasal epithelium or increased mucus viscoelasticity (Stanley et al., 1986). Similarly, formaldehyde exposure of rats resulted in decreased cilia numbers and slower mucus flow rates (Morgan KT et al., 1986). On the other hand, there are a number of pharmacological compounds that improve mucociliary clearance through reductions in mucus viscosity, but have no effect on CBF (Jiao and Zhang, 2019), or through increases in CBF, but have no effect on mucociliary clearance (Phillips et al., 1990).

Quantitative Understanding of the Linkage

There are several studies providing insights into the negative effect of inhalation exposures on CBF and MCC, that are in line with the current thinking on how these two KEs connect. Additionally, pharmacological studies demonstrated that stimulation of CBF typically results in stimulation of MCC. However, since most studies usually evaluated the KEs in parallel, and even though some results support both dose response and temporal sequence of the KEs, none of the available data affirms causal linkage between CBF and MCC. Our understanding of the evidence is therefore moderate.

Response-response relationship

CBF decreased sequentially with increasing SO_2 doses in dogs. CBF decreased from 6.3 ± 0.2 (SE) Hz at baseline to 5.7 ± 0.2 Hz at 5.5 ppm SO_2. Five ppm SO_2 delivered to both the trachea and tracheobronchial airways for 20 min also caused a marked decrease in mean bronchial mucociliary clearance from 53.7 ± 5.7% to 32.8 ± 7.7% after 90 min (Yeates et al., 1997).

The effects of 30-min exposure to SO_2 on mucociliary activity (MCA) and ciliary beat frequency (CBF) were studied in 31 guinea pig tracheas. A 63% reduction in mean MCA and statistically insignificant changes in CBF were recorded at concentrations of 2.5 ppm SO_2. Higher SO_2 concentrations caused further impairment of MCA as well as a dose-dependent decrease in CBF: At 5 ppm SO2, CBF decreased by 45%, at 12.5 ppm by 72%. The maximum decrease in MCA (81%) was observed with 7.5 ppm SO_2; the highest SO_2 concentration did not decrease MCA further. The decrease in MCA was associated with an impairment of CBF only at SO_2 concentrations ≥5.0 ppm (Knorst et al., 1994b).

Administration of a nebulized CBF inhibitor (0.9% NaCl) to 15 healthy volunteers significantly decreased mucociliary transport (MCT) from 7.9±1.5 mm/min (SEM) to 4.5±1.6 mm/min. Salbutamol, a CBF enhancer, significantly increased MCT from 8.0±1.4 to 12.5±1.1 mm/min (Boek et al., 2002; Boek et al., 1999).

Cooling human airway epithelial cultures grown at the air-liquid interface from 37°C to 25°C over the course of approx. 20 min decreased CBF from 12 to 6 Hz and mucociliary transport (MCT) from 140 to 90 µm/s. Extending the range of temperature tested, CBF was found to increase by 0.49±0.06 Hz for every temperature increase by 1°C, and this was mirrored by an increase in MCT. MCT increased on average between 5 and 11 µm/s for every Hz increase in CBF. This study also showed that CBF decreased with increasing mucin concentration, dropping from 12.4 Hz at 2% bovine submaxillary mucin (BSM) to 10.1 Hz at 8% BSM, concurrent with a ca. 70% reduction in MCT. In addition, treatment with 10 µM basolateral forskolin reproducibly increased CBF by 19.3±2.1% and MCT by 24.4±3.1% over baseline (Sears et al., 2015). In sheep trachea CBF and mucus transport velocity (MTV) were 9.8±2.7 beats/s and 5.7±2.6 mm/min, respectively, at baseline. Temperature reductions from 37°C to 34°C caused a progressive decline in CBF (ca. –20% at 2 h and –90% at 4 h) and MTV (ca. –50% at 2 h and –90% at 4 h), which was further exacerbated by additional temperature decreases (30°C; CBF: ca. –75% at 2 h; MTV: –80% at 2 h) (Kilgour et al., 2004).

Frog palate preparations were incubated with 1.25, 2.5 and 5.0 ppm formaldehyde. At formaldehyde doses of 2.5 and 5 ppm, CBF decreased by ca. 25% compared to baseline within 30 min and by 35-50% within 60 min (Fló-Neyret et al., 2001).
Incubation of frog palates with PM10 from Sao Paolo, Brazil, for up to 120 min did not affect CBF but decreased MCT at concentrations ≥1000 pg/m3 (Macchione et al., 1999)
In freshly excised sheep tracheas, a 60-min incubation with 10 µM platelet-activating factor caused a 6% decrease in CBF and a dose-dependent decrease in surface liquid velocity, reaching a maximum of 63% (Seybold et al., 1990).

In patients with bronchiectasis, nasal CBF was 12.8±1.3 Hz and nasal clearance time was 31.8± 18.4 min. In comparison, in healthy controls, nasal CBF was 14.0±1.3 Hz and nasal clearance time was 17.6± 8.3 min (Rutland and Cole, 1981).

Following basolateral treatment of human sinonasal epithelial cell cultures grown at the air-liquid interface with Myrtol®, a phytopharmaceutical mixture of distillates of rectified essential oils of eucalyptus, sweet orange, myrtle, and lemon as the active ingredients, increased CBF in a dose-dependent manner, with a maximum stimulation with 0.1% of 48±7% after 30 min. The same concentration caused a 46±16% increase in MCT at 40 min (Lai et al., 2014).

In New Zealand white rabbits exposed to 3 ppm NO_2 for 24 h, the average CBF decreased from 764 beats/min to 692 beats/min, and the transport velocity decreased from 5.23 mm/min to 3.03 mm/min (Kakinoki, 1998).

Time-scale

A 20-minute exposure of dogs to SO_2 caused a decrease in mean bronchial MCC after 90 min (Yeates et al., 1997).

Frog palate epithelia were incubated with 1.25, 2.5 and 5.0 ppm formaldehyde. At formaldehyde doses of 2.5 and 5 ppm, CBF decreased by ca. 25% compared to baseline within 30 min and by 35-50% within 60 min (Fló-Neyret et al., 2001).

Incubation of freshly excised sheep tracheas with 10 µM platelet-activating factor caused a maximal decrease in CBF of 6% after 60 min and decrease in surface liquid velocity of ca. 30% at 20 min, ca. 50% at 40 min and 63% after 60 min (Seybold et al., 1990).

Following basolateral treatment of human sinonasal epithelial cell cultures grown at the air-liquid interface with different concentrations of Myrtol®, CBF increased rapidly within the first 30 min and then declined thereafter. The maximum response for MCT was seen after 40 min (Lai et al., 2014).

Known modulating factors

Physiological factors such as age, sex, posture, sleep, and exercise were shown to affect MCC, although study findings are not always concordant (Houtmeyers et al., 1999). MCC and CBF, for example, were observed to decrease with age in several species in numerous studies (e.g. guinea pigs, mice, and human) (Bailey et al., 2014; Grubb et al., 2016; Ho et al., 2001; Joki and Saano, 1997; Paul et al., 2013; Yager et al., 1978), but evidence by (Agius et al., 1998) suggests that age does not have a major effect on CBF.

Known Feedforward/Feedback loops influencing this KER

Unknown

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Relationship: 2443: MCC, Decreased leads to Decreased lung function

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Oxidative stress Leading to Decreased Lung Function	adjacent	Moderate	Moderate
Oxidative stress Leading to Decreased Lung Function via CFTR dysfunction	adjacent	Moderate	Moderate
Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Key Event Relationship Description

It is very well known that patients suffering from motile ciliopathies, such as primary ciliary dyskinesia, have impaired or absent MCC and lower lung function (reduced FEV1 and FVC) compared to their healthy counterparts (Halbeisen et al., 2018; Marthin et al., 2010; Wallmeier et al., 2020). In cystic fibrosis patients, decreased MCC (due to reduced airway hydration and changes in mucus chemical and viscoelastic properties) causes mucus build-up leading to mucus plugging in the airways and consequently to

decreased lung function over time (Kerem et al., 2014; Mossberg et al., 1978; Regnis et al., 1994; Robinson and Bye, 2002; Szczesniak et al., 2017; Wanner et al., 1996). Mucus plugging due to decreased MCC is also considered a major cause of airway obstruction and airflow limitation in COPD patients (Dunican et al., 2021; Okajima et al., 2020) and asthmatics (Kuyper et al., 2003; Maxwell, 1985).

Evidence Supporting this KER

Changes in MCC rate are typically paralleled by effects on lung function in several studies where both endpoints have been assessed. In patients with primary ciliary dyskinesia, absence of cilia motion prevents normal MCC and consequently, lung function is reduced (Denizoglu Kulli et al., 2020). In cystic fibrosis patients, the ASL is depleted resulting in impaired MCC (Boucher, 2004). Although the known CFTR genotypes can result in a variety of phenotypes (Derichs, 2013), clinical data indicate that some specific gene defects, such as the p.Phe508del variant, are more frequently associated with decreased lung function indices (e.g. FEV1 % predicted, FVC % predicted, FEF25-75) (Kerem et al., 1990; Johansen et al., 1991; Schaedel et al., 2002). Both cigarette smoking and occupational exposure to biomass fumes led to slower MCC and reduced FEV1 % predicted and FEV1/FVC (Ferreira et al., 2018). Nasomucociliary clearance was slower in COPD smokers compared to former smokers with COPD or to nonsmokers (Ito et al., 2015). Allergen challenge in asthma patients resulted in both reduced MCC and FEV1, which could be reversed by inhalation of hypertonic saline solution (Alexis et al., 2017). In cystic fibrosis patients, treatment with mucolytic agents (Laube et al., 1996; McCoy et al., 1996; Quan et al., 2001; Elkins et al., 2006; Amin et al., 2011; Donaldson et al., 2018) or a CFTR potentiator (Rowe et al., 2014) improved both MCC and lung function (FEV1, FVC and FEF25-75).

Biological Plausibility

Lung function is known to decrease with age, and several studies showed that mucus transport rates also decrease in older compared to younger individuals (Goodman et al., 1978; Uzeloto et al., 2021). Impaired MCC is also seen in chronic smokers, even prior to a clinically significant drop in lung function and the detection of small airway disease (Clunes et al., 2012a; Goodman et al., 1978; Lourenço et al., 1971; Uzeloto et al., 2021; Vastag et al., 1986), and in patients with obstructive lung disease and hence, poor lung function (Cruz et al., 1974; Vastag et al., 1986). Adult asthmatics also displayed decreased mucus transport rates/velocities in addition to decreased lung function (Ahmed et al., 1981; Bateman et al., 1983; Foster et al., 1982; Mezey et al., 1978).
In patients with primary ciliary dyskinesia, absence of cilia motion prevents normal MCC and consequently, lung function is reduced (Denizoglu Kulli et al., 2020). In cystic fibrosis patients, the ASL is depleted resulting in impaired MCC (Boucher, 2004a). Although the known CFTR genotypes can result in a variety of phenotypes (Derichs, 2013), clinical data indicate that some specific gene defects, such as the p.Phe508del variant, are more frequently associated with decreased lung function indices (e.g. FEV1 % predicted, FVC % predicted, FEF25-75) (Johansen et al., 1991; Kerem et al., 1990; Schaedel et al., 2002). Unsurprisingly, results from studies with pharmacological agents aimed at restoring CFTR function do not only indicate enhanced MCC but also support improvements in lung function (Bennett et al., 2018; Donaldson et al., 2018; Rowe S. M. et al., 2014a). While the available data link these two KEs, causal evidence is not always available, and some inference is present. Therefore, we judge the biological plausibility of this KER as moderate.

Empirical Evidence

Occupational exposure to biomass combustion products resulted in slower MCC and reduced FEV1 % predicted and FEV1/FVC (Ferreira et al., 2018).

Compared to healthy controls, current smokers without airway obstruction and current smokers with COPD exhibited longer saccharin transit times, indicative of impaired MCC, and lower FEV1 % predicted and FEV1/FVC (Uzeloto et al., 2021). Similarly, nasomucociliary clearance was slower in COPD smokers compared to former smokers with COPD or to nonsmokers (Ito et al., 2015). Additionally, mucus plug density—assessed by CT imaging—and mucoid (rather than watery) consistency were inversely related to FEF25–75% and associated with increased RV/TLV (Kesimer et al., 2018).

Asthma patients responded to allergen challenge with a reduction in both MCC and FEV1 (Bennett et al., 2011; Mezey et al., 1978), which could be rescued by inhalation with hypertonic saline solution (Alexis et al., 2017).

Multiple studies interrogating the effect of mucolytic agents such as hypertonic saline solution or recombinant DNase on mucus transport rates or mucus clearance in patients with cystic fibrosis report improvements in both, mucus transport velocities or rates and lung function indices, including FEV1, FVC and FEF25-75 (Amin et al., 2011; Donaldson et al., 2018; Elkins et al., 2006; Laube et al., 1996; McCoy et al., 1996; Quan et al., 2001).

Both MCC and lung function (FEV1, FVC and FEF25-75) improved in cystic fibrosis patients treated with ivacaftor, a CFTR potentiator that increases the channel open probability (Rowe et al., 2014b).

Some studies with mucolytics such as N-acetylcysteine, bromhexine, theophylline/ambroxol or sorebrol demonstrated improved MCC was connected with small improvements in lung function (FEV1, FVC and FEV1/FVC) in patients with chronic bronchitis (Aylward et al., 1980; Castigiioni and Gramolini, 1986; Thomson et al., 1974; Würtemberger et al., 1988).

Uncertainties and Inconsistencies

Genetic defects leading to motile ciliopathies or defects in CFTR function are linked to impaired MCC. However, because of the genetic variety, not every defect, for example in the CFTR gene, also expresses an overt pulmonary phenotype. Other factors, such as low-level chronic inflammation may drive lung pathology by pathways independent of MCC. This might also explain the absence

of differences in MCC between healthy smokers and smokers with COPD (Fleming et al., 2019).
Not all studies looking to elucidate the effect of mucolytics on MCC report an improvement of lung function, even though mucus transport rates or tracheobronchial clearance significantly improve. These studies include, for example, some on the effects of hypertonic saline solution, NAC, ambroxol and 2-mercapto-ethane sulphonate (Clarke et al., 1979; Ericsson et al., 1987; Millar et al., 1985; Robinson et al., 1997; Würtemberger et al., 1988). This could be, at least in part, related to the fact that a sudden drop in lung function served as an indicator of patient distress in these studies, and interventions were halted when they occurred to ensure patient safety (Robinson et al., 1996). Another reason could be related to the mechanisms underlying mucus solubilization that may be completely independent of lung function.
MCC is only one means by which mucus can be cleared from the lungs. Another one is cough clearance, and it is highly dependent on the properties of the ASL, in particular the ASL height (Knowles and Boucher, 2002).

Quantitative Understanding of the Linkage

The available data, though not causally linking decreases in MCC with decreased lung function, provide a good insight into the importance of the physiological role of MCC in maintaining normal lung function. In at least some studies, impairment of MCC correlated with the drop in FEV1 or FEF25-75. Although clinically valuable benefits can be seen in studies with pharmacological agents such as mucolytics and CFTR modifying drugs, they do not cover a wide range of dose responses nor are they supportive of the KER causality. Therefore, we judge our quantitative understanding as moderate.

Response-response relationship

Sixteen Brazilian sugarcane workers aged 25±4 years, with a BMI of 24±3 kg/m2, with exhaled CO of 2.1±1.5 ppm, were examined during the non-harvest season and during the sugarcane burning harvest season. There was a non-significant decrease in saccharin transit time (from 8±1 min to 3±1 min) and a significant decrease FEV1/FVC ratio (from 88.62±5.68 to 84.90±6.47) and %FEV1 (from 92.19±13.24 to 90.44±12.76) during harvest compared with the non-harvest season (Ferreira et al., 2018).

12 (6M/6F) mild allergic, non-smoking asthmatics ages 20–39 with skin sensitivity to house dust mites (HDM) and normal baseline lung function (FEV1 %pred > 80, FEV1/FVC ratio >0.70) inhaled sequential doses of inhaled HDM extract (D. farinae, Greer®, Lenoir, NC) delivered as 5 inhalations from a Devilbiss 646 nebulizer (mass median aerodynamic diameter of 5 um, GSD = 2.0). Five of the 12 patients responded to the allergen challenge with >10% reductions in FEV1 % predicted and reduction in whole lung MCC as evidenced by increased retention rates (mean Central TB Ave120Ret increased from 0.69 to 0.79 for baseline vs. allergen challenge respectively). This reduction in MCC significantly correlated with the post challenge 24 hour FEV1 (Bennett et al., 2011).

Treatment of patients with chronic bronchitis with bromhexine (3 x 16 mg/day) for 14 days resulted in mean changes in FEV1, FVC and FEV1/FVC of + 0.047 L + 0.033 L and +0.6%, respectively, with MCC at 6 h being 6.8% greater after treatment compared to baseline (Thomson et al., 1974).

Treatment of patients with chronic bronchitis with ambroxol alone (2 x 30 mg/day) or with theophyllin (2 x 400 mg/day) and ambroxol (2 x 30 mg/day) for 7 days MCC/h improved from 18.3 ± 11.1% to 23.3 ± 13% and 29.6 ± 15.7%, respectively whereas lung function remained nearly unchanged with FEV1 predicted of 86.0 ± 9.78 at baseline vs 83.7 ± 9.27 (ambroxol only) and 83.1 ± 11.07 (combination) (Würtemberger et al., 1988).

Treatment of chronic bronchitics with N-acetylcysteine (4 mg/day by metered dose inhaler) for 16 weeks significantly improved sputum viscosity (-0.53 vs -0.67; differences between medians to placebo: 0-14 (-0.77 0.64)) and minimally improved FVC (3.0±0.21 vs 2.9± 0.18 L/s) and PEF (356.7 ±29.64 L/min vs 354.6±25.07) but not FEV1 (1.9±0.18 vs 2.0± 0.13 L/s) (Dueholm et al., 1992).

Treatment of asthmatics with salmeterol improved tracheobronchial clearance rates (AUC: 333±24%h vs 347±30%h in placebo) as well as FEV1 (76 ± 8), FVC (100 ± 5) and PEF % predicted (100 ± 7) compared to placebo (73 ± 8; 95 ± 5; 94 ± 7) (Hasani et al., 2003).

Treatment of mild-to-moderate bronchitics with 42 µg salmeterol slightly enhanced whole lung clearance in 2 hr (not significant; C10–2= 25±11% vs 22±10% in placebo), significantly increased mean peripheral lung clearance (C10–2= 22±9% vs 17±10% in placebo) and significantly increased FEV1 %pred and FEF25–75 at 2 h compared to baseline (93±18%predicted, 2.45 ± 1.08 L/s vs 88±19%predicted, 2.27 ± 0.98 L/s in placebo) (Bennett et al., 2006).

Sputum induction by inhalation of hypertonic saline solution (5%) in asthmatics at 6 hr following challenge with LPS significantly improved FEV % predicted by approx. 20% and was accompanied by a ca. 6-fold increase in whole lung clearance (from 0.1 %/min to 0.6%/min) (Alexis et al., 2017).

133 cystic fibrosis patients (age (mean [SD]) was 21.1 (11.4) years and 46.4% were female. All participants had one copy of the G551D mutation, and 72.2% were compound heterozygous with F508del on the other allele.) completed a 6-month course of ivacaftor. Lung function improved from baseline FEV1% predicted of 82.6 (25.6) to 90.1 (25.0) (mean change, 6.7; 95% CI, 4.9–8.5). In a subgroup of 22 patients, particle clearance from the whole right lung was markedly increased. Average clearance through 60 minutes at 1 month post-treatment was more than twice the baseline value, reflecting substantially improved MCC (Rowe et al., 2014b).
Inhalation of hypertonic saline solution (7%, 4 mL twice daily for 48 weeks) by cystic fibrosis patients improved FVC (by 82 mL; 95 percent confidence interval, 12 to 153) and FEV1 (by 68 mL; 95 percent confidence interval, 3 to 132) values, but not FEF25–75 (Elkins et al., 2006).

In cystic fibrosis patients that inhaled hypertonic saline solution without amiloride twice a day over a period of 14 days one-hour mucus clearance rates improved from baseline (9.3±1.6%) to 14.0±2.0% and increased FEV1 by 6.2%. FVC and FEF25-75 also improved by 1.8% and 13.1%, respectively (Donaldson et al., 2006).

Dornase alfa (recombinant human DNase) is currently used as a mucolytic to treat pulmonary disease in cystic fibrosis. It reduces mucus viscosity in the lungs, promoting improved clearance of secretions (Yang and Montgomery, 2021). In children with cystic fibrosis (mean: 8.4 yrs of age with FEV1 ≥95% predicted) treated with dornase alfa for 96 weeks, FEV1 % predicted improved by 3.2 ± 1.2, FVC % predicted improved by 0.7 ± 1.0, and FEF25-75 % predicted improved by 7.9 ± 2.3 compared to placebo (Quan et al., 2001). In young patients with cystic fibrosis (6-18 yrs of age with FEV1 ≥80% predicted) treated with dornase alfa for 96 weeks, FEF25-75 % predicted improved by 6.1±10.34 compared to placebo (Amin et al., 2011). In 10 adult cystic fibrosis patients receiving 2.5 mg rhDNase twice a day for 6 days, FEV1 and FVC increased by an average of 9.4 ± 3.5% and 12.7 ± 2.6%, respectively, as compared with a decrease of 1.8 ± 1.7% and an increase of 0.4±1.1% in the placebo group, respectively, although there were no significant changes in MCC (Laube et al., 1996). In 320 cystic fibrosis patients (7 to 57 yrs of age), dornase alfa treatment at 2.5 mg/day for 12 weeks (McCoy et al., 1996).

Saccharin transit times (a marker of nasal MCC) were higher in healthy current smokers and COPD smokers than in healthy controls (10.87 [7.29–17] min and 16.47 [8.25–20.15] min, respectively, vs 8.52 [5.54–13.91] min). These groups also differed in their lung function indices: FEV1 % predicted was 101.4 ± 12.37 in healthy controls, 96.41 ± 12.3 in healthy current smokers, and 67.96 ± 24.02 in COPD smokers. FVC % predicted was 103.1 ± 13.45 in healthy controls, 97.51 ± 12.88 in healthy current smokers, and 90.33 ± 29.27 in COPD smokers. FEV1/FVC % predicted was 82.15 [78.5–85] in healthy controls, 82.20 [79.2–84.1] in healthy current smokers, and 61.1 [55.3–67.2] in COPD smokers (Uzeloto et al., 2021).
Saccharin transit time of smokers with COPD (16.5 [11–28] min, median [interquartile range 25–75%]) was slightly longer than that of current smokers (15.9 [10 –27] min), and both were longer compared with exsmokers with COPD (10.2 [6 –12] min) and nonsmokers (8 [6 –16] min). Lung function parameters for the groups were as follows: nonsmokers, FEV1/FVC 0.84 ± 0.09, FEV1 % predicted 103.2 ± 11.5, FVC % predicted 102.2 ± 13.3; current smokers, FEV1/FVC 0.76 ± 0.05, FEV1 % predicted 90.7 ± 7.4, FVC % predicted 96.3 ± 13.9; former smokers with COPD, FEV1/FVC 0.49 ± 0.08, FEV1 % predicted 46.8 ± 12.6, FVC % predicted 76.8 ± 18.5; current smokers with COPD, FEV1/FVC 0.66 ± 0.16, FEV1 % predicted 48.7 ± 16.8, FVC % predicted 71.7 ± 13.0 (Ito et al., 2015).

Time-scale

Six asymptomatic patients with bronchial asthma and a history of allergic pollenosis and episodic bronchospasm consistent with ragweed hypersensitivity wer challenged by inhalation of an aqueous, short ragweed antigen extract (Greer Laboratories, Lenoir, N.C.), diluted with a phosphate-buffered saline solution. Mean tracheal mucus velocity (TMV) decreased to 72% of baseline immediately after challenge when specific airway conductance (SGaw), and FEV1 showed a maximal decrease, with a further decrease to 47% of baseline after 1 h, when SGaw and FEV1 had returned to baseline values (Mezey et al., 1978).

Treatment of chronic bronchitics with N-acetylcysteine (3 x 200 mg/day) for 4 weeks significantly decreased sputum thickness, increased sputum pourability from 650% glycerol time (at baseline) to 320% glycerol time on day 21 and PEFR on days 28 (+5%), 35 (+6%) and 42 (+7%) and FEV1 on days 21 (+2%), 28(+3%), 35 (+4%) and 42 (+5%) compared to baseline (ca. 33% predicted and 28% predicted, respectively) (Aylward et al., 1980).

Treatment of mild-to-moderate bronchitics with 42 µg salmeterol slightly enhanced whole lung clearance in 2 hr (not significant; C10–2= 25±11% vs 22±10% in placebo), significantly increased mean peripheral lung clearance (C10–2= 22±9% vs 17±10% in placebo) and significantly increased FEV1 %pred and FEF25–75 at 2 h compared to baseline (93±18%predicted, 2.45 ± 1.08 L/s vs 88±19%predicted, 2.27 ± 0.98 L/s in placebo) , and significantly increased FEV1 %pred and FEF25–75 at both 1 (92±19%predicted, 2.44 ± 1.14 L/s) and 2 h (93±18%predicted, 2.45 ± 1.08 L/s) compared to baseline (pre-dose; 90 ± 20%predicted, 2.16 ± 0.92 L/s) (Bennett et al., 2006).

In cystic fibrosis patients on a 6-month ivacaftor regimen, FEV1% improvement was detectable as soon as the 1-month follow-up visit (mean change, 6.7; 95% CI, 5.2–8.3) (Rowe et al., 2014b). MCC remained at elevated level at the month 3 visit (Donaldson et al., 2018).

One-hour mucus-clearance rates in cystic fibrosis patients receiving hypertonic saline with placebo were significantly faster than in the group receiving hypertonic saline with amiloride (14.0±2.0 vs. 7.0±1.5 %), and the durability of response following the inhalation of hypertonic saline with placebo was ≥8 hours (Donaldson et al., 2006).

Known modulating factors

Invariably, if mucus viscosity increases (independent of whether that results from increased mucus production (hypersecretion), depletion of the ASL or another cause) and MCC decreases, another mechanism comes into action to clear excess mucus: cough clearance. Cough constitutes a “backup” host defense by which acutely or chronically accumulated mucus is expelled through forceful, high-velocity airflow (Button et al., 2018; King, 2006). Our current understanding of the mechanical principles and biology of cough suggest that failure of cough clearance may also be a contributor to decreased lung function.

Known Feedforward/Feedback loops influencing this KER

Unknown

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