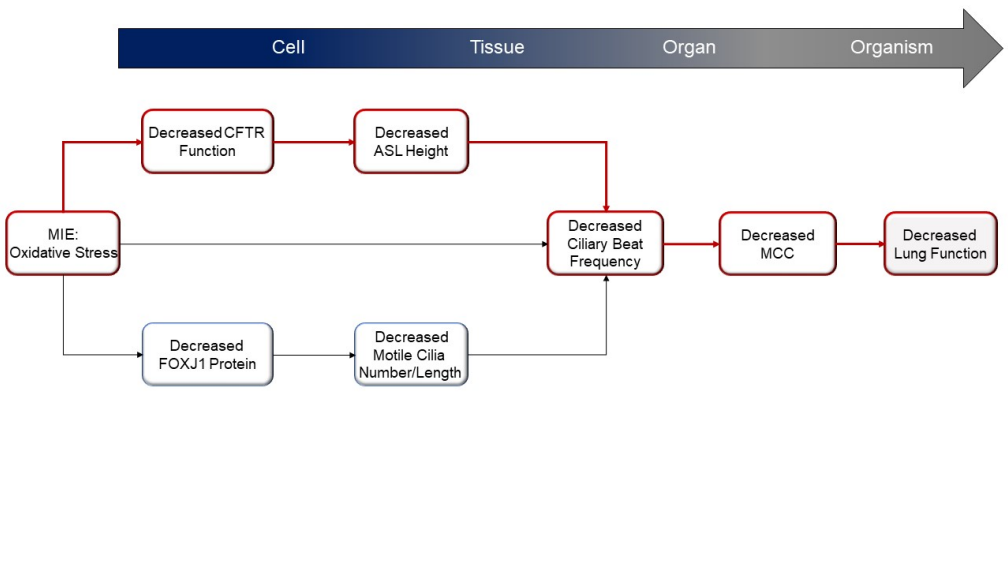
AOP ID and Title:

AOP 424: Oxidative stress Leading to Decreased Lung Function via CFTR dysfunction
Short Title: Ox stress-mediated CFTR/ASL/CBF/MCC impairment

Graphical Representation



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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). For MCC to be efficient, the depth of the ASL has to be constantly adjusted to allow for efficient cilia beating and mucus transport (Antunes and Cohen, 2007). The cystic fibrosis transmembrane regulator (CFTR) is an integral membrane glycoprotein that functions as cAMP-activated and phosphorylation-regulated Cl– channel at the apical membrane of epithelial cells (Farinha et al., 2013) and the major Cl– channel that mediates fluid and electrolyte transport. CFTR function is critical to normal ASL homeostasis. Exposure to inhaled oxidants, such as ozone and cigarette smoke, leads to decreased CFTR gene and protein expression as well as CFTR internalization, thereby reducing or abolishing short-circuit currents (Qu et al., 2009; Cantin et al., 2006a; Cantin et al., 2006b; Clunes et al., 2012; Sloane et al., 2012; Rasmussen et al., 2014). Consequently, ASL height (or volume) decreases. 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 are characterized by decreased lung function and bear a significant risk of increased morbidity and mortality.

Background

With a surface area of ~100 m2 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 425 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	1906	Cystic Fibrosis Transmembrane Regulator Function, Decreased	CFTR Function, Decreased
3	KE	1907	Airway Surface Liquid Height, Decreased	ASL Height, 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	Cystic Fibrosis Transmembrane Regulator Function, Decreased	High	High
Cystic Fibrosis Transmembrane Regulator Function, Decreased	adjacent	Airway Surface Liquid Height, Decreased	High	Moderate
Airway Surface Liquid Height, Decreased	adjacent	Cilia Beat Frequency, Decreased	Moderate	Low
Cilia Beat Frequency, Decreased	adjacent	Mucociliary Clearance, Decreased	High	Moderate
Mucociliary Clearance, Decreased	adjacent	Decrease, Lung function	Moderate	Moderate

Stressors

Name	Evidence
Acrolein	Moderate
Ozone	Moderate
Cigarette smoke	High

Acrolein

Acrolein, a ubiquitous environmental pollutant, is a highly reactive unsaturated aldehyde that exerts toxicity through several mechanism, including oxidative stress (Moghe et al., 2015). Acrolein exposure decreased CFTR-mediated Cl^- transport in primary murine nasal septal epithelia, in human bronchial epithelial cells grown in monolayers and in human Calu-3 lung cancer cells (Alexander et al., 2012; Raju et al., 2013), transiently reduced CBF at low concentrations (0.5–1 mM) and induced ciliostasis at high concentrations (> 1 mM) in rabbit tracheal epithelial cells (Romet et al., 1990). In addition, exposure of Fischer rats to acrolein

caused a left shift in the quasi-static compliance curves and increased lung volumes, indicative of airway obstruction (Costa et al., 1986).

Ozone

Tracheas of Wistar rats exposed to 1.5 ppm ozone for 1 h/day for 3 days exhibited reduced CFTR protein expression. Similarly, at 4 hours following a 30-min exposure to ozone, CFTR mRNA and protein were down-regulated in 16HBE14o- cells. At 24 hours postexposure, a reduction in forskolin-stimulated CFTR Cl– conductance was observed (Qu et al., 2009).

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).

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).

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 ppm; interquartile range 29–45 ppm; range 19–64 ppm) and FEF75 and FEF25–75 in adolescents (aged 18–20 years) (Tager et al., 2005).

Cigarette smoke

CFTR transcript and protein levels were reduced in human Calu-3 lung cancer cells exposed to the gas phase of cigarette smoke (Cantin et al., 2006b), human immortalized bronchial epithelial 16HBE14o- cells treated with 10% cigarette smoke extract (Hassan et al., 2014; Rasmussen et al., 2014; Xu et al., 2015), differentiated primary human bronchial epithelial cells exposed to whole cigarette smoke (Sloane et al., 2012; Hassan et al., 2014), and in airways of smokers compared to non-smokers (Dransfield et al., 2013). Following exposure to cigarette smoke, Cl^- conductance (i.e., CFTR-mediated Cl^- transport) decreased in primary human bronchial epithelial cells grown in monolayers (Lambert et al., 2014), differentiated primary human bronchial epithelial cells (Schmid et al., 2015; Chinnapaiyan et al., 2018), and nasal respiratory and intestinal epithelia of A/J mice (Raju et al., 2013; Raju et al., 2017).

In the lower airways, healthy smokers and smokers with chronic obstructive pulmonary disease (COPD) showed reduced CFTR-dependent Cl^- transport, whereas COPD former smokers showed an intermediate response to chloride-free isoproterenol solution compared to non-smokers. Similarly, amiloride-sensitive lower airway potential difference was also lower in healthy smokers and COPD smokers than in healthy non-smokers. This was linked to reduced CFTR protein levels in the airways of smokers compared to non-smokers, although there were no significant differences between healthy and COPD subjects (Dransfield et al., 2013). CFTR-dependent Cl^- conductance as measured by nasal potential difference was also significantly reduced in healthy and COPD smokers compared to healthy non-smokers or to former smokers with COPD (Sloane et al., 2012). In addition, healthy never-smokers had higher mean sweat chloride concentrations than COPD smokers and COPD former smokers (Raju et al., 2013; Courville et al., 2014).

Multiple studies showed that exposure of primary human bronchial epithelial cells, either undifferentiated or differentiated at the air-liquid interface, to cigarette smoke decreased ASL height (Hassan et al., 2014; Lambert et al., 2014; Raju et al., 2016; Rasmussen et al., 2014; Schmid et al., 2015). Treatment of immortalized bronchial epithelial 16HBE14o- cells with 10% cigarette smoke extract for 48 hours also resulted in a significant reduction in ASL height (Xu et al., 2015).

Treatment of human sinonasal epithelial cells with cigarette smoke condensate 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 with 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).

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. The weakest evidence is for the KER of decreased CFTR function leading to decreased ASL height, due to both insufficient experimental evidence of causality and a scarcity of quantitative data on dose-related responses and temporal concordances. In terms of essentiality, we have rated all KEs 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 flexibleframeworks 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	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	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—with the exception of CFTR function, which is known to vary from species to species (Higgins, 1992)—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), KE1 (Cystic Fibrosis Transmembrane Regulator Function, Decreased), KE4 (Cilia Beat Frequency, Decreased), KE5 (Mucociliary Clearance, Decreased), and AO (Decreased Lung Function), and indicate that they apply to all life stages. There are no data related to ASL regulation and homeostasis relative to organismal health, but it is reasonable to assume that KE3 (Airway Surface Liquid Height, Decreased), through its impact on MCC, can affect 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. Informative evidence on gender differences stems from patients with chronic pulmonary diseases, such as cystic fibrosis, asthma, COPD, and bronchiectasis, that are characterized by decreased lung function. For example, epidemiological data indicate more rapid lung function decline and shorter life expectancy in females with cystic fibrosis (genetic CFTR dysfunction; Corey and Farewell, 1996; Harness-Brumley et al., 2014; Olesen et al., 2010; Rosenfeld et al., 1997), and earlier disease onset, more severe disease and more rapid lung function decline in females with COPD (acquired CFTR dysfunction; Prescott et al., 1997; Sørheim et al., 2010) but higher prevalence of COPD in males although this gender gap is closing (Ntritsos et al., 2018). Considering the expression pattern of CFTR and its function as well as the importance of efficient MCC—brought about by the interactions of ciliary function, ASL homeostasis and mucus properties—for normal physiological function, 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 (also see AOP411), each of which is sufficient to cause the downstream KE to
occur. Oxidant-induced decreases in ASL height via CFTR function decline lead to decreased CBF and decreased MCC. Based on the evidence we judge the
key events MIE (Oxidative Stress), KE1 (Cystic Fibrosis Transmembrane Regulator Function, Decreased), KE4 (Cilia Beat

Frequency, Decreased), and KE5 (Mucociliary Clearance, Decreased) as highly essential and suggest moderate essentiality for KE3 (Airway Surface Liquid Height, Decreased).

Weight of Evidence Summary

We judge the overall biological plausibility of this AOP as strong. Several KER (i.e., *Oxidative stress leading to decreased CFTR function*, *Decreased CFTR function leading to decreased ASL height*) are supported by multiple studies across different species with ample empirical evidence reflecting both dose-response and time concordance. Other KER, such as *Decreased ASL height leading to reduced CBF*, lack this expanse of empirical evidence, or the evidence does not fully support the causality between the KE (*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 (*Oxidative stress leading to decreased CFTR function*, *Decreased CFTR function leading to decreased ASL height*; *Decreased CBF leading to decreased MCC*).
In some instances, we are less confident in our quantitative understanding. For example, for the KER *Decreased ASL height leading to decreased CBF*, empirical evidence supporting causality between the two KE is lacking as is quantitative evidence. 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., In Press). 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

Key Event Component

Process	Object	Action
oxidative stress		increased

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
Aop:452 - Adverse outcome pathway of PM-induced respiratory toxicity	KeyEvent
Aop:464 - Calcium overload in dopaminergic neurons of the substantia nigra leading to parkinsonian motor deficits	KeyEvent
Aop:470 - Deposition of energy leads to vascular remodeling	KeyEvent
Aop:478 - Deposition of energy leading to occurrence of cataracts	KeyEvent
Aop:479 - Mitochondrial complexes inhibition leading to heart failure via increased myocardial oxidative stress	KeyEvent
Aop:481 - AOPs of amorphous silica nanoparticles: ROS-mediated oxidative stress increased respiratory dysfunction and diseases.	KeyEvent
Aop:482 - Deposition of energy leading to occurrence of bone loss	KeyEvent
Aop:483 - Deposition of Energy Leading to Learning and Memory Impairment	KeyEvent

Stressors

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

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

Taxonomic applicability: Occurrence of oxidative stress is not species specific.

Life stage applicability: Occurrence of oxidative stress is not life stage specific.

Sex applicability: Occurrence of oxidative stress is not sex specific.

Evidence for perturbation by prototypic stressor: There is evidence of the increase of oxidative stress following perturbation from a variety of stressors including exposure to ionizing radiation and altered gravity (Bai et al., 2020; Ungvari et al., 2013; Zhang et al., 2009).

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).

ROS also undermine the mitochondrial defense system from oxidative damage. The antioxidant systems consist of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, as well as antioxidants such as α-tocopherol and ubiquinol, or antioxidant vitamins and minerals including vitamin E, C, carotene, lutein, zeaxanthin, selenium, and zinc (Fletcher, 2010). The enzymes, vitamins and minerals catalyze the conversion of ROS to non-toxic molecules such as water and O_2. However, these antioxidant systems are not perfect and endogenous metabolic processes and/or exogenous oxidative influences can trigger cumulative oxidative injuries to the mitochondria, causing a decline in their functionality and efficiency, which further promotes cellular oxidative stress (Balasubramanian, 2000; Ganea & Harding, 2006; Guo et al., 2013; Karimi et al., 2017).

However, an emerging viewpoint suggests that ROS-induced modifications may not be as detrimental as previously thought, but rather contribute to signaling processes (Foyer et al., 2017).

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).

Sources of ROS Production

Direct Sources: Direct sources involve the deposition of energy onto water molecules, breaking them into active radical species. When ionizing radiation hits water, it breaks it into hydrogen (H*) and hydroxyl (OH*) radicals by destroying its bonds. The hydrogen will create hydroxyperoxyl free radicals (HO_2*) if oxygen is available, which can then react with another of itself to form hydrogen peroxide (H_2O_2) and more O_2 (Elgazzar and Kazem, 2015). Antioxidant mechanisms are also affected by radiation, with catalase (CAT) and peroxidase (POD) levels rising as a result of exposure (Seen et al. 2018; Ahmad et al. 2021).

Indirect Sources: An indirect source of ROS is the mitochondria, which is one of the primary producers in eukaryotic cells (Powers et al., 2008). As much as 2% of the electrons that should be going through the electron transport chain in the mitochondria escape, allowing them an opportunity to interact with surrounding structures. Electron-oxygen reactions result in free radical production, including the formation of hydrogen peroxide (H_2O_2) (Zhao et al., 2019). The electron transport chain, which also creates ROS, is activated by free adenosine diphosphate (ADP), O_2, and inorganic phosphate (P_i) (Hargreaves et al. 2020; Raimondi et al. 2020; Vargas-Mendoza et al. 2021). The first and third complexes of the transport chain are the most relevant to mammalian ROS production (Raimondi et al., 2020). The mitochondria have its own set of DNA and it is a prime target of oxidative damage (Guo et al., 2013). ROS are also produced through nicotinamide adenine dinucleotide phosphate oxidase (NOX) stimulation, an event commenced by angiotensin II, a product/effector of the renin-angiotensin system (Nguyen Dinh Cat et al. 2013; Forrester et al. 2018). Other ROS producers include xanthine oxidase, immune cells (macrophage, neutrophils, monocytes, and eosinophils), phospholipase A_2 (PLA_2), monoamine oxidase (MAO), and carbon-based nanomaterials (Powers et al. 2008; Jacobsen et al. 2008; Vargas-Mendoza et al. 2021).

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. Listed below are common methods for detecting the KE, however there may be other comparable methods that are not listed

- 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 Formation in the Mitochondria assay (Shaki et al., 2012)	"The mitochondrial ROS measurement was performed flow cytometry using DCFH-DA. Briefly, isolated kidney mitochondria were incubated with UA (0, 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."	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."	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." (	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	Intracellular ROS production was measured using DCFH-DA as a probe.		Long/ Easy

hydrogen peroxide production (Yuan et al., 2016)	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)**		

Direct Methods of Measurement

Method of Measurement	References	Description	OECD-Approved Assay
Chemiluminescence	(Lu, C. et al., 2006; Griendling, K. K., et al., 2016)	ROS can induce electron transitions in molecules, leading to electronically excited products. When the electrons transition back to ground state, chemiluminescence is emitted and can be measured. Reagents such as uminol and lucigenin are commonly used to amplify the signal.	No
Spectrophotometry	(Griendling, K. K., et al., 2016)	NO has a short half-life. However, if it has been reduced to nitrite (NO2-), stable azocompounds can be formed via the Griess Reaction, and further measured by spectrophotometry.	No
Direct or Spin Trapping-Based Electron Paramagnetic Resonance (EPR) Spectroscopy	(Griendling, K. K., et al., 2016)	The unpaired electrons (free radicals) found in ROS can be detected with EPR, and is known as electron paramagnetic resonance. A variety of spin traps can be used.	No
Nitroblue Tetrazolium Assay	(Griendling, K. K., et al., 2016)	The Nitroblue Tetrazolium assay is used to measure $O_2^{\bullet -}$ levels. $O_2^{\bullet -}$ reduces nitroblue tetrazolium (a yellow dye) to formazan (a blue dye), and can be measured at 620 nm.	No
Fluorescence analysis of dihydroethidium (DHE) or Hydrocyans	(Griendling, K. K., et al., 2016)	Fluorescence analysis of DHE is used to measure $O_2^{\bullet -}$ levels. $O_2^{\bullet -}$ is reduced to O2 as DHE is oxidized to 2-hydroxyethidium, and this reaction can be measured by fluorescence. Similarly, hydrocyans can be oxidized by any ROS, and measured via fluorescence.	No
Amplex Red Assay	(Griendling, K. K., et al., 2016)	Fluorescence analysis to measure extramitochondrial or extracellular H_2O_2 levels. In the presence of horseradish peroxidase and H_2O_2, Amplex Red is oxidized to resorufin, a fluorescent molecule measurable by plate reader.	No
Dichlorodihydrofluorescein Diacetate (DCFH-DA)	(Griendling, K. K., et al., 2016)	An indirect fluorescence analysis to measure intracellular H_2O_2 levels. H_2O_2 interacts with peroxidase or	No

		heme proteins, which further react with DCFH, oxidizing it to dichlorofluorescein (DCF), a fluorescent product.	
HyPer Probe	(Griendling, K. K., et al., 2016)	Fluorescent measurement of intracellular H_2O_2 levels. HyPer is a genetically encoded fluorescent sensor that can be used for *in vivo* and *in situ* imaging.	No
Cytochrome c Reduction Assay	(Griendling, K. K., et al., 2016)	The cytochrome c reduction assay is used to measure $O_2^{\bullet -}$ levels. $O_2^{\bullet -}$ is reduced to O2 as ferricytochrome c is oxidized to ferrocytochrome c, and this reaction can be measured by an absorbance increase at 550 nm.	No
Proton-electron double-resonance imagine (PEDRI)	(Griendling, K. K., et al., 2016)	The redox state of tissue is detected through nuclear magnetic resonance/magnetic resonance imaging, with the use of a nitroxide spin probe or biradical molecule.	No
Glutathione (GSH) depletion	(Biesemann, N. et al., 2018)	A downstream target of the Nrf2 pathway is involved in GSH synthesis. As an indication of oxidation status, 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).	No
Thiobarbituric acid reactive substances (TBARS)	(Griendling, K. K., et al., 2016)	Oxidative damage to lipids can be measured by assaying for lipid peroxidation with TBARS using a commercially available kit.	No
Protein oxidation (carbonylation)	(Azimzadeh et al., 2017; Azimzadeh etal., 2015; Ping et al., 2020)	Can be determined with enzyme-linked immunosorbent assay (ELISA) or a commercial assay kit. Protein oxidation can indicate the level of oxidative stress.	No
Seahorse XFp Analyzer	Leung et al. 2018	The Seahorse XFp Analyzer provides information on mitochondrial function, oxidative stress, and metabolic dysfunction of viable cells by measuring respiration (oxygen consumption rate; OCR) and extracellular pH (extracellular acidification rate; ECAR).	No

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

Method of Measurement	References	Description	OECD-Approved Assay
Immunohistochemistry	(Amsen, D., de Visser, K. E., and Town, T., 2009)	Immunohistochemistry for increases in Nrf2 protein levels and translocation into the nucleus	No
Quantitative polymerase chain reaction (qPCR)	(Forlenza et al., 2012)	qPCR of Nrf2 target genes (e.g., Nqo1, Hmox-1, Gcl, Gst, Prx, TrxR, Srxn), or	No

		by commercially available pathway-based qPCR array (e.g., oxidative stress array from SABiosciences)	
Whole transcriptome profiling via microarray or via RNA-seq followed by a pathway analysis	(Jackson, A. F. et al., 2014)	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	No

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List of Key Events in the AOP

Event: 1906: Cystic Fibrosis Transmembrane Regulator Function, Decreased

Short Name: CFTR Function, Decreased

Key Event Component

Process	Object	Action
chloride channel activity		decreased

AOPs Including This Key Event

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

Stressors

Name
Acrolein
Ozone
Cigarette smoke
Cadmium

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
epithelial cell

Organ term

Organ term
lung

Evidence for Perturbation by Stressor

Acrolein

Acrolein exhibited a complex dose-dependent response with respect to CFTR-mediated Cl^- transport in primary murine nasal septal epithelia: At 100 µM acrolein, Cl^- currents increased, whereas 300 µM acrolein reduced forskolin-induced total apical Cl^- secretion and 300 µM acrolein abolished all Cl^- transport. These effects were independent of cAMP, suggesting that channel activation was not PKA/cAMP phosphorylation-dependent (Alexander et al., 2012).
Acrolein decreased cAMP-mediated CFTR ion transport in human bronchial epithelial cells grown in monolayers and in human Calu-3 lung cancer cells, where the response was dose-dependent. Repeated, low-level exposure of human bronchial epithelial cells to acrolein (2.5 – 10 ng/mL for 7 days) had a similar effect on CFTR function and was shown to be unrelated to modulation of CFTR expression. Pretreatment with the antioxidant N-acetylcysteine could prevent acrolein-induced CFTR inhibition (Raju et al., 2013). Similar effects on CFTR function (as measured by nasal and intestinal transepithelial potential difference) were elicited by subcutaneous administration of 1 mg/kg acrolein for 4 weeks, and these could also be counteracted by co-treatment with NAC (Raju et al., 2013).

Ozone

Tracheas of Wistar rats exposed to 1.5 ppm ozone for 1 h/day for 3 days exhibited reduced CFTR protein expression. Similarly, at 4 hours following a 30-min exposure to ozone, CFTR mRNA and protein were down-regulated in 16HBE14o- cells. At 24 hours post-exposure, a reduction in forskolin-stimulated CFTR Cl– conductance was observed (Qu et al., 2009).

Cigarette smoke

CFTR transcript and protein levels were reduced in human Calu-3 lung cancer cells exposed to the gas phase of cigarette smoke (Cantin et al., 2006b), human immortalized bronchial epithelial 16HBE14o- cells treated with 10% cigarette smoke extract (Hassan et al., 2014; Rasmussen et al., 2014; Xu et al., 2015), differentiated primary human bronchial epithelial cells exposed to whole cigarette smoke (Sloane et al., 2012; Hassan et al., 2014), and in airways of smokers compared to non-smokers (Dransfield et al., 2013).

Following exposure to cigarette smoke, Cl^- conductance (i.e., CFTR-mediated Cl^- transport) decreased in primary human bronchial epithelial cells grown in monolayers (Lambert et al., 2014), differentiated primary human bronchial epithelial cells (Schmid et al., 2015; Chinnapaiyan et al., 2018), and nasal respiratory and intestinal epithelia of A/J mice (Raju et al., 2013; Raju et al., 2017).

In the lower airways, healthy smokers and smokers with chronic obstructive pulmonary disease (COPD) showed reduced CFTR-dependent Cl^- transport, whereas COPD former smokers showed an intermediate response to chloride-free isoproterenol solution compared to non-smokers. Similarly, amiloride-sensitive lower airway potential difference was also lower in healthy smokers and COPD smokers than in healthy non-smokers. This was linked to reduced CFTR protein levels in the airways of smokers compared to non-smokers, although there were no significant differences between healthy and COPD subjects (Dransfield et al., 2013). CFTR-dependent Cl^- conductance as measured by nasal potential difference was also significantly reduced in healthy and COPD smokers compared to healthy non-smokers or to former smokers with COPD (Sloane et al., 2012). In addition, healthy never-smokers had higher mean sweat chloride concentrations than COPD smokers and COPD former smokers (Raju et al., 2013; Courville et al., 2014).

Cadmium

Cadmium (Cd) decreased CFTR protein expression in Calu-3 cells in a dose- and time-dependent manner. CFTR transcript levels, however, appeared to only be transiently affected. Reduced CFTR expression at the plasma membrane was associated with a reduction in CFTR Cl^- conductance. Treatment of cells with NAC did not rescue CFTR expression in Cd-treated cells. In contrast, co-treatment with α-tocopherol prevented CFTR inhibition, and this effect was linked to α-tocopherol suppressing the accumulation of ubiquitinated CFTR (Rennolds et al., 2010).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Phylogenetic analysis of CFTR DNA sequences across multiple species suggests a close evolutionary relationship between human and primate CFTR, followed by rabbit, guinea pig, equine, ovine, and bovine CFTR, whereas rodent CFTR DNA largely diverges from the human DNA (Chen et al., 2001). Of note, CFTR ion permeability differs from species to species (Higgins, 1992). For example, murine CFTR displays reduced channel activity compared with its human counterpart, while ovine CFTR exhibits higher ATP sensitivity, greater single-channel conductance and larger open probability than human CFTR. Moreover, sensitivity to pharmacological agents able to potentiate or block CFTR gating varies greatly from species to species (Bose et al., 2015). Therefore, results from animal studies are not easily and directly transferable to human.

CFTR dysfunction as a consequence of inherited CFTR gene defects is studied in pediatric as well as adult cystic fibrosis patients. Acquired CFTR dysfunction following inhalation exposures (e.g. to cigarette smoke) may also apply to both pediatric and adult populations, depending on the setting and type of exposure.

To our knowledge, the role of gender has not been systematically evaluated in acquired CFTR dysfunction. It is thought that the observed suppression of CFTR expression and impairment of CFTR function in cigarette smokers is a contributing factor to the

pathogenesis of chronic obstructive pulmonary disease (COPD) (Dransfield et al., 2013; Raju et al., 2016b). The main risk factor for COPD is cigarette smoking, and COPD is more common in men than in women, which may be directly related to the higher prevalence of smoking in men, although this gender gap is closing (Hitchman and Fong, 2011; Ntritsos et al., 2018; Syamlal et al., 2014). Nevertheless, the available clinical evidence in support of this AOP suggests that there is no remarkable gender difference.

Key Event Description

The cystic fibrosis transmembrane regulator (CFTR) is a multi-domain membrane protein that belongs to the large family of adenine nucleotide binding cassette transporters consisting of two transmembrane domains, two nucleotide binding domains (NBDs) and a unique regulatory domain (Riordan, 2008). It is an integral membrane glycoprotein that functions as cAMP-activated and phosphorylation-regulated Cl^- channel at the apical membrane of epithelial cells (Farinha et al., 2013).
In respiratory epithelia, CFTR is the major Cl^- channel that mediates fluid and electrolyte transport, and CFTR function is critical to normal ASL homeostasis. Exposure to inhaled oxidants, such as ozone and cigarette smoke, leads to decreased CFTR gene and protein expression as well as CFTR internalization, thereby reducing or abolishing short-circuit currents (Qu et al., 2009; Cantin et al., 2006a; Cantin et al., 2006b; Clunes et al., 2012; Sloane et al., 2012; Rasmussen et al., 2014). Reduced CFTR gene transcription rates were mechanistically linked to mobilization of intracellular Ca^{2+}, resulting in decreased mRNA and protein expression, presumably in a protein kinase-dependent manner (Bargon et al., 1992a; Bargon et al., 1992b). Cigarette smoke exposure of primary human bronchial epithelial cells at the air-liquid interface was shown to rapidly increase intracellular Ca^{2+}, followed by a decrease in cell surface CFTR expression (Rasmussen et al., 2014). Of note, this decrease by CFTR internalization was subsequently linked to decreased active Cl^- transport and a reduction in ASL height/volume (Clunes et al., 2012). Similarly, treatment with pyocyanin, a redox-active virulence factor secreted by Pseudomonas aeruginosa which commonly infects the airways of cystic fibrosis patients, increased hydrogen peroxide levels in CFBE41o- bronchial epithelial cells in a dose- and time-dependent manner, leading to oxidation of the cytosol and inhibited forskolin-stimulated ion transport (Schwarzer et al., 2008). Other possible mechanisms of acquired CFTR dysfunction include direct covalent modification of the protein by cigarette smoke and acrolein (Raju et al., 2013; Raju et al., 2016a) or modulation of channel open probability (Zhang et al., 2013; Woodworth, 2015).

How it is Measured or Detected

In cystic fibrosis patients, who carry a defect in the *CFTR* gene, the determination of the residual levels of normal, full-length CFTR transcripts may have some clinical utility in estimating CFTR function (Amaral et al., 2004). Moreover, decreased *CFTR* mRNA and protein expression were previously shown to result in reduced CFTR-mediated Cl^- transport (Cantin et al., 2006a; Cantin et al., 2006b; Clunes et al., 2012; Sloane et al., 2012; Rasmussen et al., 2014). Therefore, measuring decreased CFTR function could be achieved by a combination of multiple techniques. For example, decreased expression of *CFTR* mRNA and protein in cells and tissues can be directly assessed using RT-PCR, Northern blot and Western blot or immunocyto-/histochemical methods, respectively. Of note, *CFTR* gene expression is generally low as is protein abundance, and protein detection methods in general perform more robustly in cultured cells than in native tissues (Farinha et al., 2004). Other, less frequently used methods include cell surface biotinylation, enabling a distinction between intracellular and cell surface forms of the protein if one wishes to study plasma membrane-expressed CFTR.
In vitro or ex vivo, CFTR channel function can be assessed in real-time using patch-clamping of whole (single) cells or cell patches. In the whole-cell patch-clamp approach, current flow through CFTR can be assessed by voltage-clamp, whereas current-clamping provides insights into the effects of CFTR currents on membrane voltage (Sheppard et al., 2004). Measuring the efflux of radiolabeled tracers is another means of studying CFTR channel function, permitting a higher throughput than patch-clamping (Norez et al., 2004). The most commonly used method to study CFTR ion transport, however, utilizes the Ussing chamber to measure transepithelial voltage or “active transport potential” and short-circuit current (Li et al., 2004).
In vivo, CFTR dysfunction is demonstrated by the chloride sweat test, the gold standard diagnostic tool for cystic fibrosis. The sweat test should be performed according to clinical guidelines using the Gibson and Cooke technique (also known as quantitative pilocarpine iontophoresis sweat test) (Farrell et al., 2017; Smyth et al., 2014). As a complementary diagnostic measure, nasal potential difference (NPD) can be assessed to gauge net transepithelial active ion transport and epithelial ion conductance (Schüler et al., 2004).
An entire issue of the Journal of Cystic Fibrosis dedicated to the Virtual Repository of the CFTR Working Group, including the description of consensus research methods, selected principles, techniques and reagents for the assessment of CFTR expression and function is available here:
https://www.sciencedirect.com/journal/journal-of-cystic-fibrosis/vol/3/suppl/S2

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Event: 1907: Airway Surface Liquid Height, Decreased

Short Name: ASL Height, Decreased

Key Event Component

Process	Object	Action
	epithelial lining fluid	decreased

AOPs Including This Key Event

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

Stressors

Name
Cigarette smoke

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
lung

Evidence for Perturbation by Stressor

Cigarette smoke

Multiple studies showed that exposure of primary human bronchial epithelial cells, either undifferentiated or differentiated at the air-liquid interface, to cigarette smoke decreased ASL height (Hassan et al., 2014; Lambert et al., 2014; Raju et al., 2016; Rasmussen et al., 2014; Schmid et al., 2015). Treatment of immortalized bronchial epithelial 16HBE14o- cells with 10% cigarette smoke extract for 48 hours also resulted in a significant reduction in ASL height (Xu et al., 2015).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	Low	NCBI
Sus scrofa	Sus scrofa	Low	NCBI
Ovis aries	Ovis aries	Low	NCBI
Cavia porcellus	Cavia porcellus	Low	NCBI
Bos taurus	Bos taurus	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Low

Sex Applicability

Sex	Evidence
Mixed	Low

To date, ASL has been investigated in several species including mice, rats, guinea pigs, ferrets, cats, dogs, cows, monkeys, and humans. Although most studies provide data on its composition rather than its height, it is reasonable to assume that regulation of ASL height is equally critical to MCC across these species.

There are no data related to ASL regulation and homeostasis relative to organismal health, but it is reasonable to assume that decreased ASL, through its impact on MCC, can affect all life stages.

There are no gender-specific data on the regulation of ASL height to our knowledge, but it is reasonable to assume that there is no gender difference.

Key Event Description

The airway surface liquid (ASL) is a liquid layer on the apical side of the respiratory epithelium, reportedly between 5 to 100 µm in depth (Widdicombe and Widdicombe, 1995), and consists of an inner aqueous periciliary liquid layer (PCL) that spans the length of cilia and the outer gel-like mucus layer. The PCL has a low viscosity and enables cilia beating, thereby facilitating the forward movement of the outer mucus layer toward the glottis and, ultimately, its removal by cough or ingestion (Antunes and Cohen, 2007). Both ASL composition and height are considered critical for its function (Fischer and Widdicombe, 2006).
Under physiological conditions, ASL composition and height are regulated via vectorial transport of electrolytes, driven by transepithelial transport and apical secretion of Cl^- by (predominantly) CFTR, resulting in passive H_2O secretion and, consequently, increased ASL height. Absorption of Na^+ at the apical side by the epithelial sodium channel ENaC and ENaC's interaction with the basolateral Na^+/K^+-ATPase exchanging Na^+ for K^+ leads to net absorption of Na^+, which in turn drives fluid absorption and therefore decreases ASL height (Althaus, 2013; Hollenhorst et al., 2011). Impairment of CFTR or ENaC function can lead to the dysfunction of the other ion channel (increased CFTR activity leads to decreased ENaC activity and vice versa) (Boucher R., 2003; Boucher, 2004; Schmid et al., 2011), resulting in permanently perturbed ASL height.

How it is Measured or Detected

There is no standardized protocol for the determination of ASL height. In several experimental in vitro studies, confocal fluorescence microscopy scanning in the vertical plane (i.e., in XZ mode) was used to measure ASL height in human and mouse 3D organotypic airway epithelial models, and changes in ASL height could be calibrated using a fluorophore-dextran conjugate to estimate changes in ASL volume (Garcia-Caballero et al., 2009; Lazarowski et al., 2004; Matsui et al., 1998; Roomans et al., 2004; Saint-Criq et al., 2013; Tarran and Boucher, 2002; Tarran et al., 2005; Tarran et al., 2001; Tarran et al., 2006; Zhang et al., 2013). A similar approach was taken for the measurement of ASL height in freshly excised human trachea and bronchi, excised pig tracheas and mouse tracheas in vivo (Jayaraman et al., 2001; Song et al., 2009). A detailed protocol is provided by (Tarran and Boucher, 2002).
In addition, ASL height was measured using micro-optical coherence tomography in differentiated human bronchial epithelial cells (Raju et al., 2016), synchrotron phase contrast x-ray imaging in excised mouse tracheas (Morgan et al., 2013; Siu et al., 2008) and live mice (Donnelley et al., 2014), and low-temperature scanning electron microscopy in excised, rapidly frozen specimens of bovine tracheal epithelium (Wu et al., 1996; Wu et al., 1998) and guinea pig lungs (Yager et al., 1994). Furthermore, a specifically designed chamber allowed for evaluation of ASL height in excised guinea pig and sheep tracheas using videomicroscopy under a cold light source or strobe lights (Seybold et al., 1990; Shephard and Rahmoune, 1994), whereas a microelectrode technique was employed to determine ASL height in live guinea pigs (Rahmoune and Shephard, 1995).

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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
Level of Biological Organization

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

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).

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).

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: 2449: Oxidative Stress leads to CFTR Function, Decreased

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	Moderate	NCBI
Sus scrofa	Sus scrofa	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Available evidence indicates that this KER is applicable to human, mouse and pig, independent of life stage and gender.

Key Event Relationship Description

Exposure to inhaled oxidants (such as cigarette smoke) leads to decreased CFTR gene and protein expression as well as CFTR internalization, thereby reducing or abolishing open probabilities, short-circuit currents and subsequently ASL height/volume (Cantin et al., 2006a; Cantin et al., 2006b; Clunes et al., 2012; Rasmussen et al., 2014; Sloane et al., 2012). Decreased *CFTR* mRNA expression was previously linked to reduced mRNA stability following treatment with tert-butylhydroquinone (BHQ) (Cantin et al., 2006a) as well as increased intracellular [Ca^{2+}], which is thought to activate protein kinases, thereby decreasing transcription rates (Bargon et al., 1992a; Bargon et al., 1992b; Rasmussen et al., 2014). Other evidence suggests that the STAT1 pathway is involved in CFTR down-regulation following ozone exposure or in the presence of interferon-γ (Kulka et al., 2005; Qu et al., 2009). In addition, transcriptional activation of an antioxidant response element in the CFTR promoter by Nrf2 was shown to regulate *CFTR* gene expression in airway epithelial cells under oxidative stress conditions, leading to an upregulation of transcript levels in the short-term, but a decline in the long-term (Zhang et al., 2015). On the post-transcriptional level, CFTR function was shown to be affected in multiple ways due to oxidative stress: For example, cell surface CFTR expression was drastically diminished in airway epithelial cells following cigarette smoke exposure, involving a change in protein solubility and trafficking to a perinuclear aggresome-like structure rather than to lysosomes or the proteasome (Bodas et al., 2017; Clunes et al., 2012). ATP depletion as a surrogate for lung ischemia also resulted in decreased CFTR expression at the plasma membrane. This was shown to be a consequence of irreversible actin filament depolymerization, which resulted in loss of cell polarity and relocalization of CFTR to the cytoplasm (Brézillon et al., 1997).

Evidence Supporting this KER

Inducers of oxidative stress such as cigarette smoke reduced CFTR expression at both the RNA (Cantin et al., 2006a; Cantin et al., 2006b; Qu et al., 2009; Rennolds et al., 2010) and protein (Cantin et al., 2006b; Qu et al., 2009; Rennolds et al., 2010; Sloane et al., 2012; Hassan et al., 2014; Rasmussen et al., 2014; Xu et al., 2015) level in vitro. CFTR protein expression was lower in the airways of smokers compared to non-smokers (Dransfield et al., 2013). In some of these studies, an accompanying decrease in Cl^- conductance was also observed (Qu et al., 2009; Rennolds et al., 2010; Sloane et al., 2012). There are many studies that support a direct link between oxidative stress and decreased CFTR function in vitro, ex vivo, in vivo and in human subjects. Human primary epithelial cells and cell lines of respiratory epithelial origin have consistently decreased conductance of Cl^- and other ions following exposure to cigarette smoke and other oxidants (Cantin et al., 2006b; Schwarzer et al., 2008; Raju et al., 2013; Lambert et al., 2014; Schmid et al., 2015; Raju et al., 2016; Chinnapaiyan et al., 2018), which could be reversed upon antioxidant treatment (Raju et al., 2013; Lambert et al., 2014; Schmid et al., 2015). Similar observations were made under hypoxic conditions (Brézillon et al., 1997; Zhang et al., 2013; Woodworth, 2015). Antioxidants could also increase Cl^- conductance and anion transport in the absence of oxidant treatment or hypoxia induction in human and murine respiratory cells in vitro and in ex vivo tissues (Azbell et al., 2010; Alexander et al., 2011; Conger et al., 2013). Healthy smokers and smokers with COPD have reduced Cl^- conductance (Sloane et al., 2012; Dransfield et al., 2013) and increased sweat chloride concentrations (Raju et al., 2013; Courville et al., 2014).

Biological Plausibility

A link between CFTR and chronic bronchitis, which shares some of the features of cystic fibrosis lung disease, was proposed more than 20 years ago. Gene association studies, however, found no clear link between CFTR genotype and COPD except for hereditary disseminated bronchiectasis (Artlich et al., 1995; Joos et al., 2002), leading to the ion channel being of more interest in cystic fibrosis than chronic bronchitis research for some time. More recent evidence points toward a mechanism of acquired CFTR dysfunction in the context of cigarette smoking, which remains the leading risk factor for COPD. Multiple studies clearly demonstrated that CFTR Cl^- conductance is significantly inhibited following cigarette smoke exposure in vitro and in vivo, resulting in reductions of ASL height/volume which ultimately impair MCC (Cantin. et al., 2006b; Clunes et al., 2012; Courville et al., 2014; Dransfield et al., 2013; Hassan et al., 2014; Raju et al., 2013; Rasmussen et al., 2014; Sailland et al., 2017; Schmid et al., 2015). Although there appear to be different schools of thought as to how cigarette smoke modulates CFTR (directly or indirectly), the available evidence on the inhibitory effects of cigarette smoke on CFTR anion transport is conclusive. Considering the similarities between cigarette smoke exposure-related oxidative stress in the airways and oxidative stress arising from e.g. the exposure to other inhalation toxicants and pathogens, the described mechanisms are likely to apply to other stressors eliciting an imbalance in the lung's redox state. Moreover, studies with antioxidants such as NAC, resveratrol, genistein and hesperidin confirm the role of oxidative stress in modulating CFTR function (Alexander et al., 2011; Conger et al., 2013; Raju et al., 2013; Woodworth, 2015; Zhang et al., 2013). Taken together, the described cause-effect relationship is biologically plausible, and our confidence in biological plausibility is strong.

Empirical Evidence

CFTR transcript and protein levels were reduced in Calu-3 lung cancer cells exposed to the gas phase of cigarette smoke (Cantin et al., 2006b; Rasmussen et al., 2014), immortalized human bronchial epithelial 16HBE14o- cells treated with 10% cigarette smoke extract (Hassan et al., 2014; Xu et al., 2015), and differentiated primary human bronchial epithelial cells exposed to cigarette smoke extract (Sloane et al., 2012) and whole cigarette smoke (Hassan et al., 2014). Consequently, CFTR membrane localization was decreased as was channel function (i.e., cAMP-dependent ^{125}I efflux or CFTR Cl^- conductance).

Ozone exposure decreased CFTR mRNA and protein expression in immortalized human bronchial epithelial 16HBE14o- cells and in tracheas of Wistar rats, leading to a reduction in forskolin-stimulated CFTR Cl^- conductance (Qu et al., 2009).

Cadmium (Cd) treatment decreased CFTR protein expression in Calu-3 lung cancer cells in a dose- and time-dependent manner, but only transiently affected *CFTR* gene expression. However, reduced CFTR expression at the plasma membrane was associated with a reduction in CFTR Cl^- conductance (Rennolds et al., 2010).

Acrolein exhibited a complex dose-dependent response with respect to CFTR-mediated Cl^- transport in primary murine nasal septal epithelia: At 100 µM acrolein, Cl^- currents increased, whereas 300 µM acrolein reduced forskolin-induced total apical Cl^- secretion and 300 µM acrolein abolished all Cl^- transport. These effects were independent of cAMP, suggesting that channel activation was not PKA/cAMP phosphorylation-dependent (Alexander et al., 2012). Acute acrolein exposure also decreased cAMP-mediated CFTR ion transport in human bronchial epithelial cells grown in monolayers and in Calu-3 lung cancer cells, where the response was dose-dependent. Repeated, low-level exposure to acrolein (2.5 – 10 ng/mL for 7 days) had a similar effect on CFTR function and was shown to be unrelated to modulation of CFTR expression. However, pretreatment with the antioxidant N-acetylcysteine could prevent acrolein-induced CFTR inhibition (Raju et al., 2013).

Uncertainties and Inconsistencies

There is currently only limited knowledge about the mechanism by which oxidative stress may affect CFTR expression and function. At least one study indicates that CFTR channel gating is decreased following exposure to acrolein, possibly by protein carbonylation (Raju et al., 2013). Other studies report that cell surface CFTR expression was drastically diminished in airway epithelial cells following cigarette smoke exposure, involving a change in protein solubility and trafficking to a perinuclear aggresome-like structure rather than to lysosomes or the proteasome (Bodas et al., 2017; Clunes et al., 2012b).

Quantitative Understanding of the Linkage

There is a ample quantitative evidence that provides an insight into this KER, with respect to both a response-response relationship and the temporal relationship between the two KEs across different test systems. Based on the evidence presented here, we judge our quantitative understanding to be high.

Response-response relationship

CFTR Gene & Protein Expression

In Calu-3 cells exposed to cigarette smoke (drawn into a syringe and injected into the 5-L exposure chamber at a rate of 35 mL/min) for 10 min every 2 h for a total of four exposures CFTR mRNA expression decreased by approx. half, from 0.87 ± 0.03 to 0.47 ± 0.03 units (CFTR/GAPDH ratio; northern blot). CFTR protein expression decreased from 2.07 ± 0.24 to 1.24 ± 0.14 units (CFTR/actin ratio; western blot) (Cantin et al., 2006b).

Exposure of fully differentiated primary human bronchial epithelial cells (HEBCs) to 30 puffs of whole smoke from 2 cigarettes (generated according to ISO standard) every day for 5 days (total exposure 120 hr) resulted in a ca. 40% reduction in CFTR expression. Exposure of 16HBE14o- airway epithelial cells to cigarette smoke extract (CSE; smoke from one non-filtered Camel

cigarette was bubbled using a peristaltic pump apparatus into 10 mL of complete culture media, defined as 100%) led to a dose-dependent decrease in CFTR protein expression which, with approx. –70% from baseline, was significant for concentrations ≥ 10% (Hassan et al., 2014).

Apical treatment of fully differentiated primary HBECs with 2% CSE (not further described) for 24 h resulted in approx. 30% reduction in CFTR mRNA expression, 50% reduction in total CFTR protein and 30% reduction in CFTR protein cell surface expression (Sloane et al., 2012).

Treatment of 16HBE14o- immortalized HBECs with 10% CSE (smoke from one non-filtered Camel cigarette was bubbled using a peristaltic pump apparatus into 10 mL of complete culture media, defined as 100%) resulted in more than 50% reduction of CFTR total and cell surface protein expression. Co-treatment with 10 mM NAC prevented this decrease in CFTR expression (0.5 mM NAC was not effective) (Xu et al., 2015).

Treatment of fully differentiated primary HBECs with 2% CSE (bubbling 10 puffs of smoke from one 3R4F reference into 1 mL DMSO, at 2 s/10 mL puff, 10 puffs over 3 min; defined as 100%) for 24 h decreased total CFTR expression and cell surface CFTR expression by approx. 20 and 25%, respectively (Raju et al., 2016a).

CFTR protein expression was significantly lower in endobronchial biopsies of healthy smokers (33 [12-54] pack-years; CFTR/tubulin ratio: 0.70), smokers with COPD (52.5 [35-147] pack-years; CFTR/tubulin ratio: 0.50-0.88), and former smokers (45 [39-80] pack-years; CFTR/tubulin ratio: 1.75) with COPD than in healthy non-smokers (0 pack-years; CFTR/tubulin ratio: 4.09 – 4.49) (Dransfield et al., 2013).

Exposure of Calu-3 lung cancer cells to 50 µM cadmium sulfate for 1 or 3 days significantly reduced CFTR protein expression by approx. 20 and 50%, respectively, whereas exposure to lower doses of cadmium (2 µM) for 3 days did not affect CFTR protein levels (Rennolds et al., 2010).

At 4 hours post-exposure to 1.5 ppm ozone for 30 min, CFTR mRNA and protein expression were more than 2-fold decreased (CFTR/GAPDH ratio: 1.35±0.3 (control) vs 0.46±0.07; CFTR/actin ratio: 0.81±0.02 vs 0.33±0.02) in 16HBE14o- airway epithelial cells (Qu et al., 2009).

CFTR Channel Function

Treatment of T84 cells with 100 µM tert-butylhydroquinone (BHQ) for 6 h caused oxidative stress, evidenced by a significant increase in intracellular glutathione concentrations (44.8 ± 0.6 vs. 32.3 ± 0.3 nmol/10^6 cells at 0 hr) and reduced peak cAMP-dependent ^{125}I effluxes in a dose-dependent manner; however, only at BHQ concentrations ≥ 300 µM was the reduction significant (Cantin et al., 2006a).

In murine nasal septal epithelia grown at the air-liquid interface, resting Cl^- secretion was maximally stimulated by 100 µM acrolein (15.9±2.2 vs. 2.4±0.8 µA/cm^2 (control)), whereas forskolin-sensitive ion current was inhibited by 300 µM (13.3±1.2 vs. 19.9±1.0 µA/cm^2 (control); max. and significant) and completely eliminated by 500 µM acrolein (Alexander et al., 2012).

Following exposure of primary HBECs to 3.2 µg/mL acrolein for 24 h, forskolin-stimulated ion currents were inhibited by approx. 50%. Noticeable inhibition occurred at concentrations above 2 µg/ml, and maximum inhibition was seen with ca. 5 µg/mL acrolein; higher concentrations did not further decrease ion currents. Repeated exposure to 10 ng/mL acrolein for 7 days also resulted in an approx. 50% decrease in forskolin-stimulated ion currents (Raju et al., 2013).

Exposure of fully differentiated primary HBECs to whole smoke from four 3R4F reference cigarettes (generated according to ISO standard 3308; Vitrocell VC10 exposure system) resulted in a significant decrease in Cl^- currents (2.1±0.26 vs 4.8±0.71 µA/cm^2 vs 4.8 ± 0.71 µA/cm^2 (control)) (Schmid et al., 2015).

Resveratrol dose-dependently increased CFTR-mediated anion transport in murine sinonasal epithelial cells, with no effect seen for 50 µM, maximal increase (while not affecting total stimulation) seen for 100 µM, and slight inhibitory effects seen for concentrations ≥200 µM (Alexander et al., 2011; Woodworth, 2015). Forskolin-stimulated Cl^- currents increased in murine (14.2±1.5 vs. 0.8±0.2 mA/cm^2 (control)), human (17.4±.7 vs. 1.0±0.2 mA/cm^2 (control)(Woodworth, 2015), 15.69±2.66 vs. 2.49±0.98 mA/cm^2 (control) (Alexander et al., 2011)) and porcine (6.8±0.3 vs. 1.1±0.3 mA/cm^2 (control)) sinonasal epithelial cells following treatment with 100 µM resveratrol. Resveratrol treatment (100 µM) also restored CFTR Cl^- transport in hypoxic murine sinonasal epithelial cells (11.51±0.23 vs. 0.2±0.05 mA/cm^2 (control)) and human sinonasal epithelial cells (10.8±0.7 vs. 0.3±0.05 mA/cm^2 (control)) (Woodworth, 2015; Zhang et al., 2013).
In C57B/L6 mice perfused with 100 µM resveratrol, mean nasal potential difference polarization increased to –4.0±1.87 mV (vs. –0.93±1.69 mV, control), which was slightly higher than results with forskolin (–1.65±1.78 mV), but not significant (Alexander et al., 2011).

Genistein (50 µM) enhanced basal CFTR-mediated anion transport in human sinonasal epithelial cells (23.1±1.8 vs. 0.7±0.2 µA/cm^2 (control)), but had no effect on forskolin-sensitive current (Conger et al., 2013).

Hesperidine dose-dependently increased CFTR-mediated anion transport in murine sinonasal epithelial cells, with the maximum responses seen for 2 mM (16.67±0.43 µA/cm^2) which was not further investigated due to the precipitation of hesperidine. Instead, 1 mM hesperidine was used as it also significantly increased CFTR Cl^- currents (mouse cells: 13.51±0.77 vs. 4.4±0.66 µA/cm^2 (control); human cells: 12.28±1.08 vs. 0.69±0.32 µA/cm^2 (control)). In C57B/L6 mice perfused with 1 mM hesperidine, mean nasal

potential difference polarization was increased (–2.3±1.0 mV vs. –0.8±0.8 mV (control)), similar to results with forskolin (–1.9±1.4 mV) (Azbell et al., 2010).

Basal lower airway potential difference (LAPD) was lower in healthy smokers (-7.71±0.88 mV; 33 [12-54] pack-years) and COPD smokers (-7.33±1.30 mV; 52.5 [32-147] pack-years) than in former smokers with COPD (no data; 45 [39-80] pack-years) or healthy non-smokers (-12.61±1.94 mV)(Dransfield et al., 2013).

Healthy smokers, current and former smokers with COPD had lower mean sweat chloride than healthy non-smokers (51.3±4.4 [31.08±14 pack-years], 41.9±3.4 [38±19 pack-years], and 39.0±5.4 [44±19 pack-years], respectively, vs. 53.6±3.3 mmol/L). The association between sweat chloride and pack-years was significant (Courville et al., 2014).

Healthy smokers and smokers with COPD exhibited significantly lower nasal potential difference than healthy non-smokers in response to isoproterenol (-6.3±1.4 [33.2; 10-78 pack-years] and -8.0±2.0 [55.1; 35-78 pack-years], respectively, vs. -15.2±2.7 mV (control)) (Sloane et al., 2012).

Treatment of wild-type CFTR expressing HEK293 cells with 1% CSE (bubbling 10 puffs of smoke from one 3R4F reference into 1 mL DMSO, at 2 s/10 mL puff, 10 puffs over 3 min; defined as 100%) decreased CFTR channel-open probability by 59% (Raju et al., 2016a).

Single channel recordings in apical membrane patches of murine sinonasal epithelial cells demonstrated that 100 µM resveratrol significantly enhanced channel-open probability (0.329± 0.116 vs. 0.119±0.059 NPo/N (control)) (Woodworth, 2015).

Time-scale

In T84 cells exposed to 100 µM BHQ, intracellular GSH levels were significantly increased from 6 hours onwards (from 32.3±0.3 to 44.8±0.6 nmol/10^6 cells), up to 24 hours (from 45.5±0.9 to 94.9±2.5 nmol/10^6 cells), whereas CFTR mRNA expression was significantly decreased over time (from 8.2±0.8 to 1.8±0.3 CFTR/GAPDH mRNA ratio at 6 hours). As a consequence, CFTR protein expression was significantly decreased following treatment of T84 cells with 100 µM BHQ for 24 hours (from 101.3±4.6 to 84.7±4.1 CFTR/actin protein, density as % control) (Cantin et al., 2006a).

Exposure of T84 cells to cigarette smoke condensate (CSC; prepared by drawing 35 mL/min cigarette smoke for 5 min into a syringe and injecting this smoke into a tonometer containing 10 mL culture media; defined as 100%) for 10 min resulted in a significant increase in GCLC mRNA expression (from 0.69±0.08 to 2.44± 0.25 GCLC/GAPDH mRNA ratio) and decrease in CFTR mRNA expression (from 0.57±0.04 to 0.21±0.02 CFTR/GAPDH mRNA ratio) at 6 hours post-exposure and a significant increase in GSH at 24 hours post-exposure (from 41.7±1.0 to 89.8±1.5 nmol/mg protein) (Cantin et al., 2006b).

Exposure of immortalized 16HBE14o- airway epithelial cells to 10% cigarette smoke extract (CSE; cigarette smoke from one non-filtered cigarette was bubbled using a peristaltic pump apparatus into 10 mL of complete culture media; defined as 100%) led to an approx. 50% decrease in CFTR mRNA expression and approx. 70% decrease in CFTR protein expression after 24 hours, with little change at later time points (Hassan et al., 2014).

Treatment of Calu-3 lung cancer cells with 50 µM cadmium sulfate significantly reduced CFTR protein expression after 1 day, with maximum decrease observed after 3 days (54 ± 5% of control). Exposure to lower doses of cadmium (2 µM) required 5-day treatment before CFTR protein levels were affected (Rennolds et al., 2010).

Treatment of fully differentiated primary HBECs with 2% CSE (bubbling 10 puffs of smoke from one 3R4F reference into 1 mL DMSO, at 2 s/10 mL puff, 10 puffs over 3 minutes; defined as 100%) for 24 hours decreased total CFTR expression and cell surface CFTR expression by approx. 20 and 25%, respectively, but treatment for 20 minutes did not. A 50% reduction in CFTR channel activity occurred immediately after addition of CSE and lasted for at least 20 minutes (Raju et al., 2016a).

Calu-3 lung cancer cells exposed to cigarette smoke (drawn into a syringe and injected into the 5-L exposure chamber at a rate of 35 mL/min) for 10 min every 2 h for a total of four exposures were loaded with ^{125}I for 1 hour, prior to stimulation with isobutyl methylxanthine, forskolin, and dibutyryl cAMP. cAMP-dependent anion efflux was significantly decreased compared to controls within 3 min of stimulation and remained significantly decreased for a further 3 min (Cantin et al., 2006b).

In untreated monolayers of CFTR-corrected CFBE41o- airway epithelial cells, cAMP-stimulated Cl^- currents remained at stimulated levels for 2 to 3 h, whereas currents were inhibited by 86.0±5.8 and 40.0±2.7% in cells treated with 100 µM pyocyanin or 100 µM H_2O_2, respectively, in the same time period. The effect of pyocyanin occurred at a faster rate than that of H_2O_2; washout of the compounds partly restored cAMP-stimulated Cl^- currents (Schwarzer et al., 2008).

Exposure of fully differentiated primary HBECs to whole smoke (3R4F reference cigarette; inExpose exposure system) resulted in a time-dependent decrease in CFTR short-circuit currents, with significant differences (approx. 15% reduction) from control after 10 min of exposure. Maximal reduction (ca. 50%) was seen after 30 min of exposure (Lambert et al., 2014).

Culture of fully differentiated primary human and murine sinonasal epithelial cells (HSNECs; MSNECs) in 1% O_2 for 12 or 24 h significantly reduced CFTR-mediated (forskolin-sensitive) Cl^- current: HSNECs, 19.55±0.56 mA/cm^2 (12 h); 17.67±1.13 mA/cm^2 (24 h) vs. 25.49±1.48 mA/cm^2 (control); MSNECs, 13.55±0.46 mA/cm^2 (12 h); 12.75±0.07 mA/cm^2 (24 h) vs. 19.23±0.18 mA/cm^2 (control). Transfer of cultures to physiologic O_2 conditions (21%) restored CFTR ion currents (HSNECs, 25.12±1.24 mA/cm^2) after 24 h (Woodworth, 2015).

Known modulating factors

CFTR has also been implicated in transmembrane glutathione transport (Linsdell and Hanrahan, 1998; Roum et al., 1993). Multiple studies suggest that oxidative injury of the lungs, e.g. following inhalation exposures or infections, can be effectively counteracted, if not prevented, by CFTR-mediated elevations of ASL glutathione levels (Day et al., 2004; Gould et al., 2010; Jungas et al., 2002; Kariya et al., 2007; Velsor et al., 2001). The antioxidant properties of glutathione may temporarily delay the acquisition of CFTR dysfunction by neutralizing reactive oxygen species that would otherwise contribute to downregulation of CFTR gene and protein expression.

Known Feedforward/Feedback loops influencing this KER

Not known

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Relationship: 2440: CFTR Function, Decreased leads to ASL Height, Decreased

AOPs Referencing Relationship

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

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

Phylogenetic analysis of CFTR DNA sequences across multiple species suggests a close evolutionary relationship between human and primate CFTR, followed by rabbit, guinea pig, equine, ovine, and bovine CFTR, whereas rodent CFTR DNA largely diverges from the human DNA (Chen et al., 2001). Of note, CFTR ion permeability differs from species to species (Higgins, 1992). For example, murine CFTR displays reduced channel activity compared with its human counterpart, while ovine CFTR exhibits higher ATP sensitivity, greater single-channel conductance and larger open probability than human CFTR. Moreover, sensitivity to pharmacological agents able to potentiate or block CFTR gating varies greatly from species to species (Bose et al., 2015). Therefore, results from animal studies are not directly transferable to human.

To date, ASL has been investigated in several species including mice, rats, guinea pigs, ferrets, cats, dogs, cows, monkeys, and humans. Although most studies provide data on its composition rather than its height, it is reasonable to assume that regulation of ASL height is equally critical to MCC across these species.

CFTR dysfunction as a consequence of inherited CFTR gene defects is studied in pediatric as well as adult cystic fibrosis patients. Acquired CFTR dysfunction following inhalation exposures (e.g. to cigarette smoke) may also apply to both pediatric and adult populations, depending on the setting and type of exposure, and this also applies to decreased ASL height.

To our knowledge, the role of gender has not been systematically evaluated in acquired CFTR dysfunction and its impact on ASL height. It is thought that the observed suppression of CFTR expression and impairment of CFTR function in cigarette smokers is a contributing factor to the pathogenesis of chronic obstructive pulmonary disease (COPD)(Dransfield et al., 2013; Raju et al., 2016). The main risk factor for COPD is cigarette smoking, and COPD is more common in men than in women, which may be directly related to the higher prevalence of smoking in men, although this gender gap is closing (Hitchman and Fong, 2011; Ntritsos et al., 2018; Syamlal et al., 2014). Nevertheless, the available clinical evidence in support of this AOP suggests that there is no remarkable gender difference.

Key Event Relationship Description

Serous and glandular secretions of the airway epithelium contribute to the ASL, and epithelial ion channel (e.g. CFTR, ENaC, CaCC, BK) function is critical to normal ASL homeostasis. Should the PCL decrease in depth, liquid will be absorbed from the mucus layer until the necessary depth is restored. Conversely, the mucus layer will absorb surplus PCL to reduce any increase in its depth. The regulation of these reabsorption processes is complex and not fully elucidated (Boucher, 2004). Experimental evidence suggests that the balance between Na^+ absorption and Cl^- secretion mediated by ENaC and CFTR plays a major role, with the ion channels affecting each other's activity (increased CFTR activity leads to decreased ENaC activity and vice versa) (Boucher, 2003; Boucher, 2004; Schmid et al., 2011). Mechanistic studies with selective CFTR and ENaC inhibitors suggest that the sensors for regulating ASL height lie within the ASL itself (Boucher, 2003; Hobbs et al., 2013). Additionally, ATP, adenosine and other purinergic receptor agonists, adenylate cyclase and cyclic adenosine monophosphate (cAMP)-dependent protein kinases acting on CFTR and/or ENaC ensure that the ASL height is adjusted to the appropriate height, resulting in maintenance of PCL depth at approximately the length of cilia (Antunes and Cohen, 2007). If the CFTR-ENaC interaction is perturbed, the airways become "dehydrated" (i.e., the ASL height decreases), resulting in slowing or inhibition of cilia movement and impaired MCC (Munkholm and Mortensen, 2014).

Evidence Supporting this KER

As a major Cl^- channel in the respiratory epithelium, CFTR levels and function are vital for maintenance of ASL homeostasis. In vitro studies on the effects of cigarette smoke exposure on human lung primary cells and cell lines showed a reduction in ASL height, associated with decreased CFTR levels (Hassan et al., 2014; Rasmussen et al., 2014; Xu et al., 2015; Ghosh et al., 2017) and decreased Cl^- current (Lambert et al., 2014; Raju et al., 2016). Moreover, pharmaceutical stimulation and inhibition of CFTR function and expression directly increased and decreased ASL height, respectively (Song et al., 2009; Van Goor et al., 2009; Van Goor et al., 2011; Tuggle et al., 2014).

Biological Plausibility

Impaired function of the CFTR and ENaC ion channels results in enhanced Na^+ absorption and reduced Cl^- secretion, and as a consequence, reduced ASL height. This phenomenon is well-known from studies in models of cystic fibrosis and acquired CFTR deficiency, even though the exact mechanism of the interaction between these two channels remains to be elucidated (Boucher, 2003; Hassan et al., 2014; Raju et al., 2016a; Rasmussen et al., 2014; Tarran et al., 2001a; Woodworth, 2015; Zhang et al., 2013). Additionally, evidence from studies with pharmacological agents that enhance CFTR expression and/or function or perturb the interaction between CFTR and ENaC provide further support for strong biological plausibility of this KER (Lambert et al., 2014; Van Goor et al., 2009; Van Goor et al., 2011).

Empirical Evidence

Addition of 2% cigarette smoke extract (CSE) to differentiated primary human bronchial epithelial cells (HBECs) stimulated with

forskolin acutely decreased Cl^- transport, which was associated with reduced ASL height and cilia beat frequency (Raju et al., 2016a).

Exposure of differentiated primary HBECs to cigarette smoke for up to 120 h significantly reduced plasma membrane CFTR expression and ASL height (Hassan et al., 2014) in one study, and reduced intracellular cAMP levels, Cl^- conductance, ASL volume and cilia beat frequency in another study (Schmid et al., 2015).

Exposure of primary HBECs grown in monolayers to cigarette smoke caused a dose- and time-dependent decrease in forskolin-stimulated Cl^- transport, which could be partially restored by treatment with roflumilast via a cAMP-dependent mechanism. Smoke exposure also decreased ASL height, and this effect could also be ameliorated by roflumilast (Lambert et al., 2014).

Treatment of immortalized 16HBE14o- airway epithelial cells with 10% cigarette smoke extract for 48 h resulted in significant reduction in CFTR total and cell surface protein expression as well as in ASL height (Xu et al., 2015).

Repeated exposure of differentiated primary HBECs to smoke from little cigars significantly decreased CFTR protein expression and ASL height (Ghosh et al., 2017).

CFTR protein expression was also reduced following exposure of baby hamster kidney cells expressing human CFTR (BHK^{CFTR}) or Calu-3 lung cancer cells to cigarette smoke, resulting in cigarette smoke-induced increases in intracellular Ca^{2+}, CFTR internalization and subsequent reduction in ASL height (Rasmussen et al., 2014).

In excised pig and human trachea preparations, addition of amiloride and CFTR activators increased ASL height. In pig trachea, CFTR inhibition prevented the increase in ASL height produced by forskolin/3-isobutyl-1-methylxanthine (IBMX). This study did not investigate dose or time responses (Song et al., 2009).

Experiments with pharmacological compounds shown to enhance CFTR cell surface expression or channel open probability in primary human bronchial epithelial cells demonstrated enhanced Cl– currents and increased ASL height (Van Goor et al., 2009; Van Goor et al., 2011).

ASL height in the excised tracheas of rats without functional CFTR expression was approx. half that of wild-type animals (Tuggle et al., 2014).

Exposure of differentiated primary HBECs to supernatant from mucopurulent material (SMM) of cystic fibrosis airways decreased amiloride-sensitive Cl– current in CFTR wild-type but not CFTR mutant HBECs. Forskolin-induced Cl^- conductance was absent in treated CFTR mutant HBECs and enhanced in wild-type HBECs, where this effect was both dose- and time-dependent. Enhanced UTP-induced currents were seen in HBECs, independent of CFTR genotype. SMM treatment furthermore induced an increase in ASL height in CFTR wild-type but not mutant HBECs (Abdullah et al., 2018).

Knockdown of SPLUNC1, an allosteric regulator of ENaC, in human bronchial epithelial cells significantly reduced ENaC currents and rapidly and significantly reduced ASL height (Garcia-Caballero et al., 2009). SPLUNC1 is degraded in airway epithelia of cystic fibrosis patients, resulting in failure of ENaC internalization and, consequently, increased ENaC activity. Addition of recombinant SPLUNC1 or sputum supernatants from healthy subjects significantly elevated ASL height in HBECs (Hobbs et al., 2013; Webster et al., 2018).
Knock-down of β-ENaC mRNA in HBECs resulted in significantly reduced amiloride-sensitive short-circuit (Na+) currents and significantly increased ASL height (Gianotti et al., 2013). Knockdown of ENaC mRNA in BMI-1 transduced cystic fibrosis BECs resulted in nearly 50% reduction in amiloride short-circuit currents, a ca. 1.5 fold increase in ASL height and increased cilia beat frequency (Tagalakis et al., 2018).
Inhibition of channel activating proteases, including prostasin, matriptase, and furin, in ΔF508 cystic fibrosis airway epithelia by QUB-TL1 increased intracellular ENaC, decreased ENaC-mediated Na^+ transport and increased ASL height (Reihill et al., 2016).
In excised tracheas and bronchi from mice overexpressing the β-ENaC subunit in lower airway epithelia, amiloride-sensitive Na^+ transport was increased ca. 2- to 3-fold, and ASL was significantly depleted (Mall et al., 2004).

Uncertainties and Inconsistencies

The process of reabsorption of excess liquid to regulate ASL height is also known as “isosmotic volume hypothesis” or “isotonic volume transport/mucus clearance hypothesis” and implies that CFTR assumes a critical role in regulating ASL height by inhibiting ENaC activity (Ganesan et al., 2013; Matsui et al., 1998). However, an alternative, opposing hypothesis exists, the “hypotonic hypothesis” which states “*that normal airway epithelia are covered by an ASL with a [NaCl] sufficiently low (≤ 50 mM NaCl) to activate defensins and create an antimicrobial “shield” on airway surfaces*”, and there is evidence to both support and refute it (Cowley et al., 1997; Goldman et al., 1997; Jayaraman et al., 2001; Knowles et al., 1997; Landry and Eidelman, 2001; Matsui et al., 1998; Tarran et al., 2001a; Tarran et al., 2001b; Verkman et al., 2003). Other studies suggest the involvement of additional ion channels such as alternative chloride channels (Grasemann et al., 2007) and cyclic nucleotide-gated cation channels, particularly in the alveolar epithelium (Schwiebert et al., 1997; Wilkinson et al., 2011) in the regulation of ASL height.
In addition, one study showing that instillation of Pseudomonas aeruginosa-laden agarose beads into excised swine tracheas significantly increased ASL height, and that this increase could be blocked by pre-incubation with the CFTR inhibitor CFTRinh172 (100 µM, 30 minutes) (Luan et al., 2014) presents an inconsistency with the available evidence presented here.

Quantitative Understanding of the Linkage

While there are convincing quantitative data in support of this KER, it becomes clear from the review of the evidence that the downstream KE can only be modulated to a certain extent (e.g., maximal decrease in ASL height did not exceed 50% in the majority of studies), independent of the extent of change in the upstream KE. In addition, the available temporal data indicates that acute exposures predominantly cause a transient, rather than a lasting change in the downstream KE. Since chronic treatment data are not available, we judge our quantitative understanding as being moderate.

Response-response relationship

Treatment of fully differentiated primary human bronchial epithelial cells (HBECs) with 2% cigarette smoke extract (CSE; bubbling 10 puffs of smoke from one 3R4F reference into 1 mL DMSO, at 2 s/10 mL puff, 10 puffs over 3 min; defined as 100%) for 20 minutes reduced CFTR channel activity by 50% and ASL by approx. 2-fold (Raju et al., 2016).

Apical treatment of primary HBECs grown in monolayers with 2% CSE (not further described) for 24 h decreased forskolin-induced Cl^- currents by ca. 20% and ASL height by approx. 25%, and this could be counteracted by co-treatment with 10 µM ivacaftor, a CFTR potentiator known to significantly augment cAMP-mediated ion transport activity (Sloane et al., 2012).

Exposure of primary HBECs to cigarette smoke (5 min, ca. 12 puffs at 1 puff every 30 s; generated according to ISO standards) resulted in efficient removal of CFTR from the plasma membrane and a ca. 2-fold reduction in ASL height (Xu et al., 2015).

Exposure of primary HBECs, differentiated at the air-liquid interface, to cigarette smoke from 1 cigarette (ten 35-mL puffs, 2R4F reference cigarette) nearly abolished responses of the transepithelial electric potential difference Vt to ADO (i.e., blocking the ADO-A2b-cAMP-CFTR- active ion transport) and significantly decreased ASL volume/height by approx. 2-fold after 30 min (Clunes et al., 2012).

Exposure of fully differentiated primary HBECs to 30 puffs of whole smoke from 2 cigarettes (generated according to ISO standards) every day for 5 days (120 h) resulted in a ca. 40% reduction in CFTR expression and approx. 50% reduction in ASL height (Hassan et al., 2014).

Exposure of primary human airway epithelial cells grown in monolayers to whole smoke (3R4F reference cigarette; inExpose exposure system; 3L/min) resulted in significant reduction of CFTR Cl^- currents (ca. 40% for a 30-min exposure) and significantly decreased ASL depth from 11.4± 4.1 to 5.6± 2.0 µm (Lambert et al., 2014).

Exposure of fully differentiated primary HBECs to smoke from 1 cigarette or little cigar every day for 5 days (1 × 35 ml puff per 30 second, up to a butt length of 36 mm) significantly reduced CFTR protein expression by ca. 2- to 4-fold and ASL height by 10 to 20% (Ghosh et al., 2017).

Cell surface CFTR protein expression was reduced by ca. 70% following exposure of baby hamster kidney cells expressing human CFTR (BHK^{CFTR}) to cigarette smoke for 10 min (3R4F reference cigarette, 1 puff per min according to ISO standards). This was accompanied by a significant reduction in ASL height by approx. 50% (Rasmussen et al., 2014).

Treatment of primary HBECs from a cystic fibrosis patient with the ΔF508 mutation, grown as monolayer at the air-liquid interface, with the CFTR corrector VX-809 for 48 h increased CFTR maturation by ca. 8-fold and enhanced Cl^- transport by approx. 4-fold, from 1.9±0.4 to 7.8±1.3 µA/cm^2. VX-809 treatment for 5 days increased ASL height from 4.5±0.2 to 6.7±0.5 µm. Addition of 3 µM VX-770 further increased the ASL height to 9.2±0.2 µm (Van Goor et al., 2011). Treatment of primary HBECs from a G551D/ΔF508 cystic fibrosis patient, grown as monolayer at the air-liquid interface, with the CFTR potentiator VX-770 (10 µM) for 72 h dose-dependently increased forskolin-mediated Cl^- currents by ca. 10-fold to 27±2 µA/cm^2 and ASL volume to 125% that of controls (Van Goor et al., 2009).

ASL depth in the excised tracheas of rats without functional CFTR expression was approx. half that of wild-type animals (Tuggle et al., 2014).

Knockdown of mRNAs for the α- and β-ENaC subunits resulted in a ca. 70% decrease in amiloride-sensitive currents and a significant increase in ASL height from 6.8±0.5 and 7.4±0.5 µm to 9.8±0.6 and 9.6±0.8 µm in non-CF and CF epithelia, respectively (Gianotti et al., 2013).

Knockdown of α-ENaC mRNA in BMI1-transduced cystic fibrosis bronchial epithelial cells resulted in ca. 50% reduction in protein expression, reduction in amiloride-sensitive short-circuit current from 11.5 (siRNA control) to 6.4 µA/cm^2 and increase in ASL height from 7.9 (siRNA control) to 12.1 µm (Tagalakis et al., 2018).

Overexpression of the β-ENaC subunit in mouse airways increased basal and amiloride-sensitive short-circuit currents approx. 2-fold (excised tracheas; compared to wild-type) and significantly reduced ASL height in bronchi and tracheas (by approx. 2 µm) (Mall et al., 2004).

Time-scale

Treatment of fully differentiated primary HBECs with 2% CSE (bubbling 10 puffs of smoke from one 3R4F reference into 1 mL DMSO, at 2 s/10 mL puff, 10 puffs over 3 minutes; defined as 100%) for 24 h decreased total CFTR expression and cell surface CFTR expression by approx. 20 and 25%, respectively, but treatment for 20 min did not. A 50% reduction in CFTR channel activity

occurred immediately after addition of CSE and lasted for at least 20 minutes. A 2-fold reduction in ASL height was seen after 20 minutes, and ASL height was only partially restored at 1 h after CSE treatment (Raju et al., 2016a).

Following exposure of primary HBECs, differentiated at the air-liquid interface, to cigarette smoke from 1 cigarette (ten 35-mL puffs, 2R4F reference cigarette), ASL volume/height was significantly decreased by approx. 2-fold after 30 min. This decrease lasted for >2.5 h, and ASL height was restored at 4 h post-exposure (Clunes et al., 2012).
Exposure of BHK^{CFTR} cells to cigarette smoke for 10 min (3R4F reference cigarette, 1 puff per min according to ISO standards) resulted in a reduction in ASL height by approx. 50% within 30 min; the decrease lasted for up to 1 h post-exposure (Rasmussen et al., 2014).

Exposure of fully differentiated primary human HBECs to 30 puffs of whole smoke from 2 cigarettes (generated according to ISO standards) was sufficient to decrease ASL height by approx. 50% within 1 h of exposure, and following daily exposure for another 4 days, ASL height remained at around this level (Hassan et al., 2014).

Exposure of fully differentiated primary HBECs to whole smoke from four 3R4F reference cigarettes (generated according to ISO standard 3308; Vitrocell VC10 exposure system) resulted in a small, non-significant increase in ASL volume 4 h post-exposure. ASL volume decreased to baseline levels 7 h post-exposure and continued to drop below baseline levels until 24 h post-exposure (Schmid et al., 2015).

ASL height of primary HBECs dropped within 30 min of exposure to cigarette smoke (5 min, ca. 12 puffs at 1 puff every 30 seconds). ASL height stayed at that reduced level up to until 70 min post-exposure (Xu et al., 2015).

The maximum effect of VX-809 treatment on Cl^- currents of primary human bronchial epithelial cells, grown as monolayer at the air-liquid interface, occurred following 24 h, and Cl– transport returned to uncorrected levels within 48 h of compound washout (concurrent ASL data not available) (Van Goor et al., 2011).

Known modulating factors

Unknown

Known Feedforward/Feedback loops influencing this KER

Unknown

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Relationship: 2441: ASL Height, Decreased leads to CBF, Decreased

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	Moderate	NCBI
Sus scrofa	Sus scrofa	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	Moderate

Key Event Relationship Description

The airway surface liquid (ASL) is a liquid layer on the apical side of the respiratory epithelium, reportedly between 5 to 100 µm in

depth (Widdicombe and Widdicombe, 1995), and consists of an inner aqueous periciliary liquid layer (PCL) that spans the length of cilia and the outer gel-like mucus layer. Under physiological conditions, ASL composition and height are regulated via vectorial transport of electrolytes, driven by transepithelial transport and apical secretion of Cl^- by (predominantly) CFTR, resulting in passive H_2O secretion and, consequently, increased ASL height. Absorption of Na^+ at the apical side by ENaC and ENaC's interaction with the basolateral Na^+/K^+-ATPase exchanging Na^+ for K^+ leads to net absorption of Na^+, which in turn drives fluid absorption and therefore decreases ASL height (Althaus, 2013; Hollenhorst et al., 2011). Decreased ASL height or ASL dehydration, if not rebalanced, results in cilia collapse and thereby effectively hinders coordinated ciliary beating (Knowles and Boucher, 2002; Matsui et al., 1998; Tarran et al., 2001)

Evidence Supporting this KER

Concurrent ASL height and CBF decreases were noted in human 3D airway epithelial cultures following exposure to cigarette smoke (Åstrand et al., 2014; Xu et al., 2015) and following the addition of large dextran molecules, low-melting point agarose or endogenous mucus (Button et al., 2012). Treatment of human airway epithelial with an ENaC inhibitor prevented the cigarette smoke effect on ASL height and CBF (Åstrand et al., 2014). In addition, treatment of cystic fibrosis airway cultures with a CFTR-modifying drug increased both ASL height and CBF (Van Goor et al., 2009).

Biological Plausibility

Boucher states that "*Morphological studies of normal cultures under these steady-state conditions reveal that the 7 µm height is optimal for the extension and beating of cilia and, therefore, is physiologically suited to efficient mucociliary clearance.*" (Boucher R., 2003). The link between decreased ASL height and reduced cilia beating has been established in multiple in vitro and in vivo studies (Van Goor et al., 2009; Xu et al., 2015; Zhang et al., 2014), and even though the evidence does not describe causality between these two events, this KER is biologically plausible (Button et al., 2012; Mall, 2008).

Empirical Evidence

When increasing the osmotic modulus in human bronchial epithelial cells (differentiated at the air-liquid interface) by treatment with large dextran molecules, low-melting point agarose or endogenous mucus, ASL height decreased and cilia collapsed. Although the cilia were still beating, they did not do so at their full height (Button et al., 2012).

Uncertainties and Inconsistencies

Although the empirical evidence suggests a link between decreased ASL height and reduced cilia beating, causality between the two KEs has not been proven nor has this KER been systematically examined or quantified yet.

Quantitative Understanding of the Linkage

The evidence provided here stems from studies reporting on the effects of stressors such as cigarette smoke on both ASL height and CBF. Although the empirical evidence suggests a link between decreased ASL height and reduced ciliary beating, causality between the two KEs has not been proven nor has this KER been systematically examined or quantified yet. Our quantitative understanding of this KER is therefore poor (weak).

Response-response relationship

Osmotic compression of the ASL between 300 and 800 Pa (using large dextran molecules, endogenous mucin or low-melting point agarose) had minimal effects on cilia height or cilia beating. At osmotic pressures exceeding 800 Pa, the ASL became compressed from 7 to less than 2 µm, and cilia height decreased to approx. the same extent (consequently, cilia were not beating at full height) (Button et al., 2012).

Exposure of primary human airway epithelial cells to cigarette smoke (5 min, ca. 12 puffs at 1 puff every 30 seconds) resulted in a ca. 2-fold reduction in ASL height. CBF decreased from 4.19 ± 0.24 Hz (in air control) to 1.28 ± 0.06 Hz. Replenishment of the ASL by addition of 50 µL PBS restored CBF in air- and smoke-exposed cultures (6.04 ± 0.3 Hz vs 6.82 ± 0.37 Hz) (Xu et al., 2015).

Treatment of murine nasal septal epithelia with Sinupret, a phytomedicine, significantly increased ASL depth from 5.25±0.38 to 9.14±0.42 µm and increased the mean CBF from 1.52±0.10 to 2.05±0.15 when applied apically, from 0.99±0.04 to 1.37±0.09 when applied basally, and from 1.53±0.09 to 2.17±0.12 when applied to both compartments (Zhang et al., 2014).

An experimental compound targeting ENaC termed "compound A" dose-dependently increased ASL height in ASL-depleted cultures (absorptive mode analysis). Following exposure to cigarette smoke (1 2R4F cigarette, ISO smoking regimen), ASL decreased by approx. 4 µm within 30 min compared to air controls, and this could be prevented by a 2.5-h pre-treatment with 1 µM compound A. In the same cultures, CBF was significantly decreased by more than 1 Hz following cigarette smoke exposure, whereas pre-treatment with compound A completely prevented this (Åstrand et al., 2014).

Time-scale

ASL height of primary human airway epithelial cells dropped within 30 min of exposure to cigarette smoke (5 min, ca. 12 puffs at 1

puff every 30 s). ASL height stayed at that reduced level up until 70 min post-exposure. Significant decreases in CBF in cigarette smoke-exposed cultures were seen 3 h post-exposure (Xu et al., 2015).

Treatment of primary human bronchial epithelial cells from a cystic fibrosis patient with the G551D/ΔF508 genotype, grown as monolayer at the air-liquid interface, with the CFTR potentiator VX-770 (10 µM) for 72 h increased ASL height by approx. 25%, and treatment for 5 days more than doubled CBF (Van Goor et al., 2009).

Exposure of human bronchial epithelial cells to cigarette smoke decreased ASL height by approx. 4 µm within 30 min, whereas pre-treatment with 1 µM compound A prevented this decrease. When compound A was added 30 min after exposure to cigarette smoke, ASL height returned to normal levels significantly more quickly. In the same exposed cultures CBF was decreased by more than 1 Hz within 1 h (Åstrand et al., 2014).

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

Evidences for this KER are derived from studies carried out in dog, gunea pig, rat, frog, sheep, rabbit model systems as well as in human epithelial cell cultures. MCC and CBF were observed to decrease with age 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; Yager et al., 1978), but evidence by (Agius et al., 1998) suggests that age does not have a major effect on CBF.

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

The evidences for this KER come from and therefore apply to humans.

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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