An adverse outcome pathway for lung surfactant function inhibition leading to decreased lung function

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ABSTRACT

Inhaled substances, such as consumer products, chemicals at the workplace, and nanoparticles, can affect the lung function in several ways. In this paper, we explore the adverse outcome pathway (AOP) that starts when inhaled substances that reach the alveoli inhibit the function of the lung surfactant, and leads to decreased lung function. Lung surfactant covers the inner surface of the alveoli, and regulates the surface tension at the air–liquid interface during breathing. The inhibition of the lung surfactant function leads to alveolar collapse because of the resulting high surface tension at the end of expiration. The collapsed alveoli can be re-opened by inspiration, but this re-opening causes shear stress on cells covering the alveoli. This can damage the alveolar-capillary membrane integrity, allowing blood components to enter the alveolar airspace. Blood components, such as albumin, can interact with the lung surfactant and further inhibit its function. The collapse of the alveoli is responsible for a decrease in the surface area available for blood oxygenation, and it reduces the volume of air that can be inhaled and exhaled. These different key events lead to decreased lung function, characterized by clinical signs of respiratory toxicity and reduced blood oxygenation. Here we present the weight of evidence that supports the AOP, and we give an overview of the methods available *in vitro* and *in vivo* to measure each key event of the pathway, and how this AOP can potentially be used in screening for inhalation toxicity.

Introduction

Toxicity testing is going through a paradigm shift, from the "black box" thinking, towards a mechanistic understanding of the pathways leading from molecular perturbations, caused by exposure to chemicals and particles, to adverse outcomes. In 2007, the National Research Council outlined a vision and strategy to move towards mechanism-based toxicity testing (National Research Council, Toxicity Testing in the 21st Century: A Vision and a Strategy., 2007). At the same time, there are scientific, regulatory and ethical incentives to move away from animal testing. The concept of the adverse outcome pathway

(AOP) is a useful tool to facilitate the move towards mechanism-based hazard identification relying on non-animal methods.

The AOP concept was first introduced in the context of ecotoxicology (Ankley et al., 2010), but has recently been used extensively in human toxicology, and is endorsed by the Organisation for Economic Cooperation and Development (OECD). Specifically, the OECD AOP programme aims at developing AOPs and it provides guidance for their uptake for hazard identification of compounds (OECD, 2018, 2017). In short, AOPs describe the key events (KEs) leading from a molecular initiating event (MIE) to an adverse outcome (AO), thereby addressing different levels of biological organization from molecules and cells to organs and organisms. The relationship between the change of an

upstream KE to a downstream KE is described as a key event relationship (KER). The mechanistic evidence identified in the context of AOPs can be used to guide the identification and development of methods that target defined key events of the pathway.

Humans inhale airborne chemicals and particles with every breath. Most of these do not affect our lungs, however there are instances where inhaled substances harm the lungs in the short term and/or in the long term. Respiratory symptoms following exposure to airborne substances (chemicals or particles) are reported regularly. In private homes, impregnation products are among the most commonly reported triggers of such symptoms (Lazor-Blanchet et al., 2004; Vernez et al., 2006; Khalid et al., 2009; Scheepers et al., 2017; Tashiro et al., 1998; Duch et al., 2014; Laliberté et al., 1995; Burkhart et al., 1996; Malik and Chappell, 2003). In occupational settings, respiratory clinical signs of toxicity have been observed after workers' exposure to cleaning products (Clausen et al., 2020; Medina-Ramon et al., 2006; Vizcaya et al., 2015) and pesticides (Slavica et al., 2018), among others. The nature of the respiratory symptoms and their severity vary but often include cough, chest tightness and breathing difficulties.

Substances that have the potential for being inhaled are to be tested for acute inhalation toxicity under a number of regulatory schemes, including the biocidal products regulation (European Commision, Regulation no 528/2012, 2012), the plant protection products regulation (European Commision, Regulation no 1107/2009, 2009), and the regulation for the Registration, Evaluation, Authorisation, and Restriction of Chemicals (European Commision, Regulation 1907/2006, 2006). OECD defines the term acute inhalation toxicity as "the adverse effects caused by an airborne test chemical following a single uninterrupted inhalation exposure of less than 24 h" (OECD, 2018). Three OECD TGs for acute inhalation toxicity testing are currently accepted, each relying on inhalation exposure of a group of rodents to the test substance for up to 4 h, followed by an observation period of at least 14 days. These guidelines are designed to assess systemic toxicity after inhalation. The major endpoint in OECD TGs 403 and 436 is the death of the animal. In the most recent OECD TG 433, evident clinical signs of toxicity (such as hypoactivity, tremors, bodyweight loss and irregular respiration) are used as endpoints instead of mortality (Sewell et al., 2018). The major objective of these three OECD TGs is to estimate a value of the concentration that kills 50% of the exposed animals (lethal concentration 50, LC_{50}) in order to classify the test substance in the pre-defined hazard classes according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

Currently, there are no validated alternative methods for hazard identification of airborne substances. The OECD TGs 403, 436, and 433 remain the basis for regulatory decision-making. The development of AOPs that describe the mode of action of airborne substances leading to adverse outcomes is essential for identifying mechanistically and physiologically relevant non-animal methods that can aid the transition from animal to non-animal testing.

In this paper, we describe an adverse outcome pathway (AOP 302, www.aopwiki.org/aops/302) leading to decreased lung function. The pathway defines a portal-of-entry effect that is initiated by inhibition of lung surfactant function in the alveoli. It thereby differs from systemic effects, which are the main endpoints of the OECD test guidelines for acute inhalation toxicity, as detailed in a paper in this journal (Da Silva, et al., 2021). This putative AOP results from several years of investigating clinical signs of respiratory toxicity following inhalation of consumer products and chemicals found in the work environment and in private households. It is accessible on the OECD AOP portal (https://aopkb.oecd.org/) and has been included in the OECD work plan (OECD project 1.87) by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST) AOP committee (http://www.oecd.org/chemicalsafety/testing/projects-adverse-outcome-pathways.htm). The AOP is a living document, and contributions and suggestions to the AOP can be made through the portal and any comments are welcome.

This paper describes each of the individual KEs and their interrelationships in the pathway initiated by lung surfactant function inhibition leading to decreased lung function. Importantly, a weight-of-evidence assessment of this biological pathway is conducted. The biological plausibility of the KERs, and the empirical evidence available are evaluated using data from external exposure to airborne substances and from the pathophysiology of respiratory diseases. An overview of the main triggers (airborne substances that reach the alveolar region upon inhalation and biological components of extra pulmonary origin that enter the alveolar airspace) is provided. Finally, the available methods to measure or estimate each of the key events and key event relationships are briefly described. This is followed by a perspective on how this AOP can be used in screening for inhalation toxicity.

Adverse outcome pathway 302

The pulmonary epithelium is covered by a layer of lung surfactant at the interface between the outer environment and the blood circulation. Lung surfactant consists of a complex mixture of 90% lipids (phospholipids and neutral lipids) and 10% surfactant proteins at the air–water surface of the alveoli (Zuo et al., 2008; Perez-Gil and Weaver, 2010). The finely tuned composition of the lung surfactant ensures the formation of a surfactant film at the air–liquid interface that drastically reduces the surface tension and facilitates the mechanics of breathing.

During expiration, the alveoli become smaller as the air leaves, and the surface area is reduced. To prevent collapse of the alveoli, lung surfactant reduces the surface tension to near-zero values at the end of expiration. This reduction in surface tension comes from the enrichment of the surfactant film at the air–liquid interface with surface active components, while the less surface active components of the surfactant are excluded from the interface to reservoirs underneath (Fig. 1-A) (Keating et al., 2012; Possmayer et al., 2012). During inhalation, the alveoli are filled with air and their surface area increases. The surfactant film at the air–liquid interface is then quickly replenished with lung surfactant components from the reservoirs and the surface tension increases again to equilibrium values (Fig. 1-B). These continuous regulation mechanisms ensure effortless breathing. Surfactant proteins SP-B and SP-C stabilize the reservoirs and their connection with the surface to facilitate the replenishment of the interface during inhalation (Cañadas et al., 2020).

The following paragraphs describe each of the individual key events in AOP 302 (Fig. 2).

MIE: Inhibition of lung surfactant function (https://aopwiki.org/events/1672)

Airborne substances that penetrate deep into the lungs and reach the alveoli will come into contact with the thin layer of lung surfactant prior to encountering the alveolar epithelial cells. In addition, blood components (such as albumin) that cross the alveolar-capillary membrane and reach the alveolar airspace can interact with the lung surfactant. The nature of this interaction between substances and lung surfactant depends on the origin (intrinsic versus extrinsic) of the substance, its molecular structure, size, and other physicochemical properties such as hydrophobicity, charge, etc. The interaction can be direct, with certain components of the lung surfactant film at the air–liquid interface i.e. by oxidation or cleaving of the phospholipids (Stachowicz-Kuśnierz et al., 2018; Long et al., 2012), or indirect, via competition with the adsorption of lung surfactant. In many cases, the interaction of substances with lung surfactant at the molecular level is responsible for lung surfactant function inhibition. We refer to the section "Interaction with lung surfactant and inhibition of lung
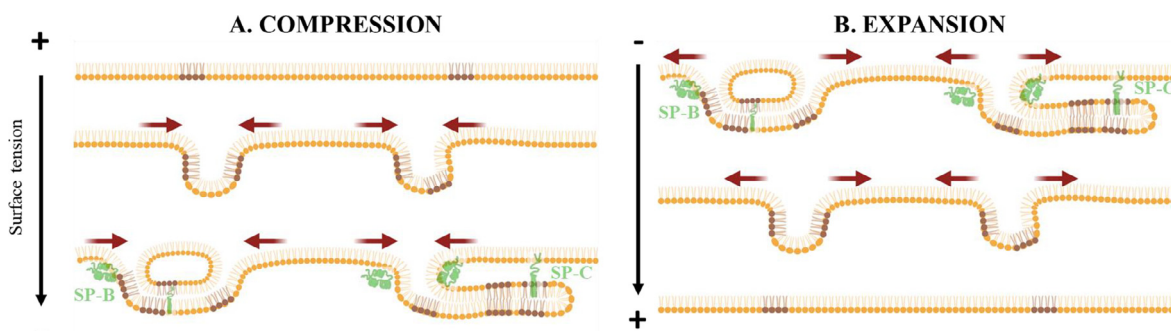
Fig. 1. Compression (A) and expansion (B) of the lung surfactant film at the air–liquid interface. During compression (expiration), there is a re-arrangement of the components in the lung surfactant films: molecules with less surface activity (brown) are excluded from the interface, so that the surface is enriched in molecules with higher surface activitiy (orange). The reservoirs underneath the surface are stabilized by proteins SP-B and SP-C (green). During expansion (inhalation), the less surface active components located underneath the interface spread again at the surface. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
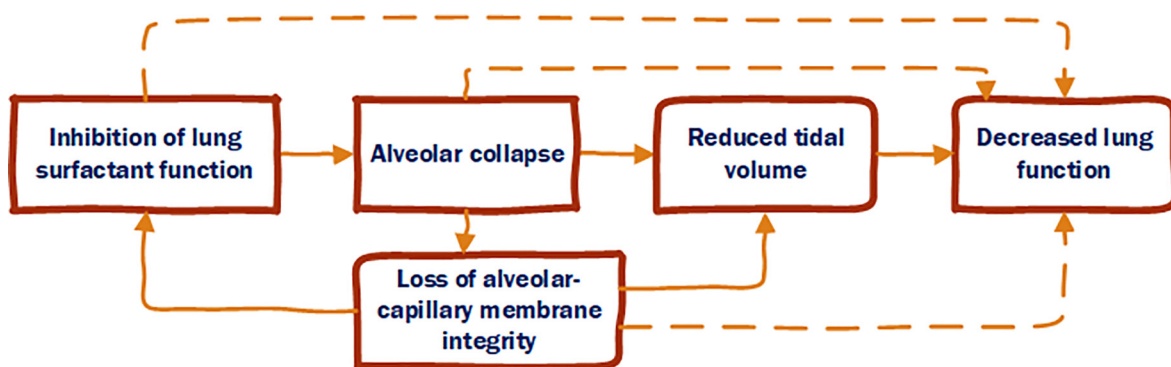

Fig. 2. the AOP 302 starts with the inhibition of lung surfactant function and leads to decreased lung function.

surfactant function", where examples of how substances interact with the lung surfactant are given.

KE: Alveolar collapse (https://aopwiki.org/events/1673)

At the end of expiration, the alveoli are at their minimum volume, and the surface tension is at its lowest. If the surface tension is not sufficiently low at this point, the forces pulling the walls of the alveoli together during expiration cannot be overcome, and the alveoli might collapse (Notter and Wang, 1997). Collapsed alveoli may however be re-opened by the force of air drawn into the lungs during inhalation. As breathing is continuous, the same alveoli can collapse and re-open repeatedly. The consequence of alveolar collapse can be observed as atelectasis upon histological examination, or can be indirectly inferred by reduced tidal volume or perfusion/ventilation mismatch (further details in the "Measurements of alveolar collapse" section).

KE: Loss of alveolar-capillary membrane integrity (https://aopwiki.org/events/1498)

The thickness, 1 μm (Weibel, 2009), of the barrier between the alveolar airspace and the blood compartment allows gases to diffuse rapidly, but this also makes the barrier vulnerable to injury. During re-opening of alveoli, the alveolar-capillary membrane integrity may be damaged from the shear forces applied to the membrane. The effects of the loss of alveolar-capillary membrane integrity (increased membrane permeability) are observed at different levels: *(i)* extravasation of blood components from the capillaries into the alveolar airspaces, *(ii)* filling of the alveoli with fluid (edema), and *(iii)* impaired gas exchange.

First, loss of alveolar-capillary membrane integrity allows blood components to enter the alveoli. It has been shown that some proteins, such as albumin and fibrinogen, that reach the air–liquid interface, can inhibit lung surfactant function (Rachana and Banerjee, 2004; Gunasekara et al., 2008; Calkovska et al., 2012; Seeger et al., 1985; Gunther et al., 2001; Zuo et al., 2006). As a consequence, additional collapse and re-opening of the alveoli will occur, further damaging the barrier integrity. Second, passive transportation of water across the membrane depends on a functioning sodium gradient. The driving force of alveolar fluid reabsorption (from the airspace to the circulating blood) is the active transport of sodium across the alveolar epithelium by epithelial Na^+ channels (on the apical side) and the Na,K-adenosine triphosphatase (Na,K-ATPase, on the baso-lateral side) (Vadász et al., 2007). As a consequence, loss of alveolar-capillary membrane integrity prevents the efficient reabsorption of fluid from the alveolar space and results in edema. Further aggravation occurs as the oncotic pressure is not compensated for by a sufficiently low surface tension. Alveolar flooding with edema fluid contributes to the impaired gas exchange (Gonzales et al., 2015).

KE: Reduced tidal volume (https://aopwiki.org/events/1677)

At the organ level, when inhalation cannot open the collapsed alveoli, the tidal volume (volume of air inhaled or exhaled) and the alveolar surface area available for gas exchange decrease.

AO: Decreased lung function (https://aopwiki.org/events/1250)

Decreased lung function is characterized by the occurrence of clinical signs of respiratory toxicity immediately after inhalation exposure

or by the decrease in oxygenation of the blood (hypoxemia). Clinical signs of respiratory toxicity include coughing, difficulty breathing, and shortness of breath in humans (Lazor-Blanchet et al., 2004; Duch et al., 2014; Alexeeff et al., 2002; Sørli et al., 2018), and shallow, noisy, or rapid respiration in experimental animals (Da Silva, et al., 2021; Sewell et al., 2015). Besides, the collapse of the alveoli, the loss of alveolar-capillary membrane integrity leading to alveolar flooding, and the reduction in tidal volume reduce the alveolar surface area available for gas exchange and decrease the efficiency of oxygenation of the blood.

Weight-of-evidence assessment of the AOP 302

This section examines the weight-of-evidence assessment of the AOP 302 using the three evolved Bradford Hill criteria by evaluating the biological plausibility of the key event relationships, the essentiality of the key events and the empirical evidence supporting the key event relationships (OECD, 2018; Becker et al., 2015). The biological plausibility of the key event relationships is evaluated by assessing the mechanistic relationships between key events (upstream to downstream). The essentiality of the key events addresses the effects of a modification of an upstream key event on a downstream key event. The empirical evidence for key event relationships can be established by evaluating dose–response relationships, and temporal concordance between key events.

Biological applicability domain of AOP 302

The biological applicability domain of an AOP is informed by the taxonomy, life-stage, and sex of the organisms for which the key events and their relationship are relevant (OECD, 2018). Here, the biological applicability domain of AOP 302 is restricted to the groups of organisms where the structure and the functioning of the pulmonary system, including the lung surfactant, are conserved and relevant. Lung surfactant is a vital component of the lungs found in all major vertebrate groups, but particularly, to sustain the delicate structure of the mammalian lung. The lung surfactant system has a single point of origin and was a prerequisite for the evolution of air breathing (Sullivan et al., 1998). While the composition and function of lung surfactant are conserved in vertebrates, changes in composition among nonvertebrates are noted and likely reflect differences in the structure of the respiratory units (Veldhuizen et al., 1998). Decreased lung function has been observed after exposure to airborne toxicants in humans of all sexes and ages, and in common experimental animal species, such as mice, rats, and rabbits.

Size: A pre-requisite for interaction of inhaled substances with lung surfactant

The molecular target of this AOP is the lung surfactant layer that covers the inside of the alveoli in the deepest parts of the lungs. To reach the alveoli, inhaled substances need to pass through many bifurcations and constantly narrower airways, from bronchi to (terminal and respiratory) bronchioles and alveolar ducts. The lungs are efficient filters, and only very small particles will reach the alveoli (average diameter of about 200 μm). It is a pre-requisite that the aerodynamic diameter of the inhaled substance is less than 4 μm in order to enter the alveoli, where the lung surfactant is located (ISO, ISO 7708:, 1995). Another way to interact with the lung surfactant is to travel from the upper airways to the alveoli via diffusion, which can be facilitated upon association with the air–liquid interface (Hidalgo et al., 2021).

Impregnation spray products are among the most commonly reported triggers of respiratory symptoms in humans. These products are chemically diverse, but have the common function of making sur-

faces of furniture, shoes, clothes, outdoor gear, and building materials water and dirt repellent, and easier to keep clean and waterproof. Impregnation products for household items such as shoes and clothes are mostly used by private consumers, whereas products for building materials are usually used professionally. Impregnation products are often applied by spraying to allow for even distribution on the surface that is to be treated. Spraying creates many small droplets that can be inhaled into the alveolar region, and come in contact with the lung surfactant.

Interaction with lung surfactant and inhibition of lung surfactant function

The biological plausibility and empirical evidence of the inhibition of lung surfactant function by inhaled substances or by blood components reaching the alveolar airspaces, are high. Studies have been conducted for a number of substances including airborne nanoparticles (Chen et al., 2017; Yang et al., 2018; Larsen et al., 2020), airborne chemicals (Da Silva et al., 2021), and biological components such as albumin, cholesterol meconium, and serum (Zuo et al., 2006; Lopez-Rodriguez et al., 2011, 2012, 2013; Zhang et al., 2012; Autilio et al., 2021; Gómez-Gil et al., 2009; Lugones et al., 2018; Gunasekara et al., 2005). For impregnation spray products, the inhibition of lung surfactant function has been reported across multiple methods *in vitro*, such as the lung surfactant bioassay (Sørli et al., 2018), the capillary surfactometer (Sørli et al., 2016), the pulsating bubble surfactometer (Tashiro et al., 1998), and the Langmuir trough (Duch et al., 2014; Larsen, et al., 2014). Further, the causal relationship between interaction with lung surfactant and lung surfactant function inhibition has been demonstrated at the molecular level for some substances, mainly biological components such as cholesterol (Gómez-Gil et al., 2009), or meconium (Lopez-Rodriguez et al., 2011). In one study, the intercalation of airborne chemicals between the phospholipids of the lung surfactant at the air–liquid interface, accompanied by a loss of cohesivity of the multi-layered surfactant structures and an overall loss of the stability of the interfacial film has been shown to inhibit lung surfactant function (Da Silva et al., 2021).

Indirect evidence for the biological plausibility of the relationship between interaction with lung surfactant molecules and lung surfactant function inhibition comes from *in vitro* experiments where addition of certain surfactant components post-exposure to an inhibitory agent restored, at least partially, lung surfactant function. For instance, one study investigated changes to the biophysical properties of lung surfactant after exposure to an impregnation spray product, and found that addition of the surfactant protein SP-B (but not SP-C) restored most of the function (Larsen, et al., 2014). SP-B is involved in the stabilization of the lung surfactant layer at the air–liquid interface. Knowing that the proximity of SP-B to DPPC (the main component of lung surfactant) is essential for its function, the authors studied the localization of SP-B in organic-buffer emulsifications using DPPC as the emulsifier in the presence or absence or the impregnation spray product. They observed the transfer of SP-B from the interface (its site of action) into the organic phase in the presence of impregnation product. The results of this study suggest that the inhibition of lung surfactant function is, at least partially, mediated by a direct interaction of the impregnation spray product with SP-B, leading to perturbations of molecular lipid-protein or protein–protein interactions with this surfactant protein (Larsen, et al., 2014).

KER: Lung surfactant function inhibition leading to alveolar collapse

The biological plausibility of a causal relationship between lung surfactant function inhibition and alveolar collapse is high, but the empirical evidence is moderate (Jefferies et al., 1988; Nørgaard, et al., 2010). In one case, histology of lungs performed after inhalation exposure to an impregnation spray product known to inhibit lung surfactant function, revealed areas of atelectasis (Nørgaard, et al., 2010).

Additional evidence comes from the pathophysiology of the acute respiratory distress syndrome, where the inhibition of lung surfactant function and the collapse of the alveoli are key characteristics (Gunther et al., 2001; Enhorning, 2001).

KER: Alveolar collapse leading to loss of alveolar-capillary membrane integrity

The biological plausibility of the relationship between alveolar collapse and loss of alveolar-capillary membrane integrity is high, but the empirical evidence is moderate. In addition to the knowledge from the pathophysiology of the acute respiratory distress syndrome (Gunther et al., 2001; Enhorning, 2001; Ware and Matthay, 2000), a series of *in vitro* experiments showed that the re-opening of collapsed alveoli damaged the epithelial membrane and caused breakdown of the membrane integrity (Jacob and Gaver, 2012; Bilek and Dee, 2003).

KER: Loss of alveolar-capillary membrane integrity leading to inhibition of lung surfactant function

Although there is a high biological plausibility of the correlation between the loss of capillary-membrane integrity and inhibition of lung surfactant function, relevant empirical evidence is moderate. In experiments where rabbits were anaesthetized and ventilated, the collapse and reopening of the alveoli weakened the alveolar-capillary membrane integrity (measured as a loss of radioactive tracer from the lungs). This loss of barrier function was associated with inhibition of lung surfactant function, observed as leakage of blood components into the lung (Taskar et al., 1997). *In vitro*, blood components inhibit the lung surfactant function (Zuo et al., 2006; Lopez-Rodriguez et al., 2012; Autilio et al., 2021; Lugones et al., 2018).

KER: Loss of alveolar-capillary membrane integrity leading to reduced tidal volume

Although the biological plausibility of the correlation between the loss of alveolar-capillary membrane integrity and reduced tidal volume is high, relevant empiric evidence is low. In one study, the loss of capillary membrane integrity, measured as protein level in bronchoalveolar lavage fluid and presence of blood in the alveolar airspace, revealed by histological analysis, was associated with reduced tidal volume (Nørgaard, et al., 2010).

KER: Alveolar collapse leading to decreased tidal volume

The biological plausibility of the relationship between alveolar collapse and decreased tidal volume is high, but the empirical evidence is low.

KER: Key events leading to decreased lung function

As described earlier, decreased lung function is characterized by the occurrence of clinical signs of respiratory toxicity (both in exposed humans and experimental animals) and by the reduced oxygenation of the blood (hypoxemia). The biological plausibility of the key event relationship between alveolar collapse and hypoxemia, and between loss of alveolar-capillary membrane integrity and hypoxemia is high. Hypoxemia has been observed in animals following exposure to inhibitors of lung surfactant function (Jefferies et al., 1988), in animals where the alveolar-capillary membrane integrity was damaged by repeated collapse and reopening of the lungs (Taskar et al., 1997), and in humans who have experienced decreased lung function shortly after being exposed to impregnation products (Lazor-Blanchet et al., 2004; Duch et al., 2014; Burkhart et al., 1996; Ware and Matthay, 2000; Woo et al., 1983; CDC, 1993; Sawamoto et al., 2018). Moreover, indirect evidence comes from the improvement of blood oxygenation

following administration of exogenous lung surfactant therapy (Meng et al., 2012; Markart et al., 2007). Evidence of loss of alveolar-capillary membrane integrity measured by the mean diffusing capacity of the lung for carbon monoxide was also provided in humans after exposure to humidifiers in private homes (Hong et al., 2014).

Besides, the biological plausibility of the key event relationship between the non-adjacent events lung surfactant function inhibition and clinical signs of respiratory toxicity is high. In animals, there is extensive evidence from *in vivo* studies for a broad range of airborne substances. In one study (published in this journal), 26 industrial chemicals with potential to be inhaled that had already been tested according to OECD TGs 403 or 436 for acute inhalation toxicity testing were selected. The 26 registration dossiers were used to identify occurrence of decreased lung function, observed as clinical signs of respiratory toxicity, occurring within 2 h post-exposure. The inhibition, or not, of lung surfactant function was assessed using the lung surfactant bioassay and accurately predicted the occurrence of clinical signs of respiratory toxicity for 21 out 26 chemicals (Da Silva, et al., 2021). Furthermore, a number of nanomaterials were tested for ability to inhibit lung surfactant *in vitro*, upon intratracheal instillation in mice. The inhibition of function correlated with histological findings of lung collapse in the exposed animals (Yang et al., 2018). In addition, the key event relationship between lung surfactant function inhibition *in vitro* leading to rapid decrease in tidal volume *in vivo* has also been demonstrated for zinc oxide nanoparticles (Larsen et al., 2020), bile salt enhancers for drug formulation (Sørli et al., 2018), and impregnation products (Tashiro et al., 1998; Duch et al., 2014; Sørli et al., 2018; Nørgaard, et al., 2010, 2014). In some cases, when the animals were given clean air to breathe, the decrease in tidal volume was not reversed, indicating that the damage was too severe to be restored by the lungs in the absence of exposure (Duch et al., 2014; Larsen, et al., 2014; Nørgaard, et al., 2010).

In humans, there is extensive evidence of the key event relationship between inhibition of lung surfactant function by inhaled substances and decreased lung function. The clinical signs of respiratory toxicity that are reported include coughing, shortness of breath and tightness in the chest (Alexeeff et al., 2002). In one specific case, inhalation exposure to an impregnation product sprayed on tiles in a supermarket resulted in decreased lung function in more than 40 people (Duch et al., 2014). Using a Langmuir balance *in vitro*, it was shown that the minimum surface tension reached upon compression was higher when lung surfactant was mixed with the impregnation spray product compared to mixing to a control solvent (Duch et al., 2014). In a follow-up study, a total of 21 products were studied using the lung surfactant bioassay. Each of the 6 products that were responsible for decreased lung function in humans inhibited the function of lung surfactant *in vitro* (Sørli et al., 2018).

Empirical evidence of dose–response relationships between the estimated amount of substance inhaled and impairment of lung function also provides strong evidence for linkage of the two events. In the context of this AOP, such evidence is not yet available. Nevertheless, reported findings describe the dose–response relationship between exposure (amount of substance, duration of exposure) and response (for a given key event or adverse outcome). For example, there is a clear correlation between both the aerosol concentration and the duration of exposure, and the severity of the reduction in tidal volume of exposed mice: the higher the concentration of test substance in air, or the longer the duration of exposure, and the more severe and more rapid the decrease in tidal volume (Sørli et al., 2018; Larsen, et al., 2014; Nørgaard, et al., 2010). Similarly, *in vitro*, the higher the amount of test substance depositing onto the drop of lung surfactant and the more severe and rapid the inhibition of lung surfactant function (increase in minimum surface tension) (Sørli et al., 2018). In human case reports, the dose–response relationship is more difficult to assess as the exposure is always described retrospectively, often through second-hand information, e.g. via doctors that have treated the exposed individuals. Nevertheless, case reports suggest a trend

between higher exposure (both concentration of the test substance in air and time) and adversity of health effects. For example, in an outbreak of decreased lung function after exposure to an impregnation spray product, the three individuals with the most severe clinical signs of toxicity included one person directly operating the spray gun and two individuals that were not in the area during spraying but that stayed over a longer period of time (Duch et al., 2014). Generally, the safe use of substances, i.e. that do not cause adverse outcomes, is not reported. As a consequence, disclosed cases of adverse outcomes following exposure to substances are associated with high levels of exposure, use over extended periods of time, or in an enclosed or poorly ventilated space (Lazor-Blanchet et al., 2004; CDC, 1993; Sawamoto et al., 2018; Walters, et al., 2017; Harada et al., 2017; Hashimoto et al., 2009; Thibaut et al., 1983; Muller-Esch et al., 1982; Schicht et al., 1982; Christensen et al., 1984; Yamashita et al., 1997; Bracco and Favre, 1998; Bonte et al., 2003; Kikuchi et al., 2015; Kobayashi et al., 2006; Epping et al., 2011).

There is indirect evidence of the relationship between lung surfactant function inhibition and decreased lung function from the pathophysiology of neonatal lung diseases, acute respiratory distress syndrome and from the study of lung surfactant *ex vivo* isolated from animals with respiratory diseases. Mutations in the ABCA3 gene or in the gene encoding surfactant protein SP-B are lethal at birth (Nogee et al., 1993; Shulenin et al., 2004; Brasch et al., 2006). Both proteins are crucial for the biogenesis of the lung surfactant system, indicating that complete absence of functional lung surfactant is incompatible with life. In premature babies, insufficient amount of surfactant as a consequence of lung immaturity is the cause of neonatal respiratory distress, characterized by an increased work of breathing and accelerated respiratory rate among other signs of decreased lung function (Reuter et al., 2014). Meconium aspiration syndrome in infants at birth is characterized by severe respiratory distress requiring intensive care (Singh et al., 2009). It is now well-documented that lung surfactant function in patients with meconium aspiration syndrome is inhibited (Lopez-Rodriguez et al., 2011; Kopincova and Calkovska, 2016) and that the phospholipids and proteins profiles are altered (Autilio et al., 2020). Acute respiratory distress syndrome is characterized by a widespread injury of the alveolar–capillary membrane, resulting in alveolar flooding with edema fluid, decreased lung function, and severe hypoxemia, which is refractory to oxygen treatment and requires assisted ventilation (Gunther et al., 2001; Enhorning, 2001; Ware and Matthay, 2000). Lung surfactant isolated from the broncho-alveolar lavage fluid of ARDS patients is not functional, contains high amount of proteins and cholesterol and has altered phospholipid profile and altered distribution of large and small surfactant aggregates (Gunther et al., 2001; Autilio et al., 2020; Gregory et al., 1991; Echaide, et al., 2017) .

Indirect evidence of the link between lung surfactant function inhibition and decreased lung function comes from the observation of positive effects of lung surfactant replacement therapy in patients. In neonates, the administration of exogenous surfactant has been shown to prevent respiratory failure, decrease neonatal mortality and reduce the frequency of serious pulmonary air leak syndrome (Hallman et al., 2001). Since there is evidence that lung surfactant function is inhibited in ARDS patients (Gunther et al., 2001; Gregory et al., 1991), it has been suggested to treat patients by delivering exogenous surfactant. However, this has proven challenging (Meng et al., 2012; Raghavendran et al., 2011). It has been suggested that surfactant therapy would be more successful for treating patients with direct injury (e.g. inhalation of toxic compounds) rather than indirect systemic injury (e.g. sepsis) where the exogenous material would be challenged by strong inhibitory conditions (Spragg, 2007).

Alternative pathways

A number of adaptive processes exist that make temporal concordance of upstream events with downstream events (including the adverse outcome) unclear. Lung surfactant function inhibition can be

aggravated by the alveolar collapse itself: when the alveolar-capillary membrane integrity is lost, inhibitory proteins from the blood compartment, such as albumin and fibrinogen, enter the alveolar airspace and further inhibit lung surfactant function (Rachana and Banerjee, 2004; Gunasekara et al., 2008; Calkovska et al., 2012; Seeger et al., 1985; Gunther et al., 2001; Zuo et al., 2006).

In opposition, some of the key events can be counteracted by several processes in the lungs. These include replacement of disrupted lung surfactant by newly released material (stimulated by stretching of the type II cells (Andreeva et al., 2007), and rapid repair of the alveolar-capillary membrane by cells lining the alveoli (Hogan et al., 2014).

Further, independently of lung surfactant function inhibition, there are several other events leading to decreased lung function. These include for example direct damage to the epithelial cells of the different regions of the lungs, disruption of the alveolar-capillary membrane integrity (independently of alveolar collapse and re-opening), activation of the immune system and inflammation, and interaction with the nervous system in the lungs. Notably, some of these events can interact with lung surfactant. For example, inflammatory damage of the respiratory epithelium, and activation of immune cells, have been described to liberate inhibitors of the lung surfactant function, such as proteases, lipases, C-reactive protein, and neutrophil extracellular traps (NETs) (Arroyo et al., 2019).

Inconsistencies observed in the pathway to decreased lung function

Although there is a strong causal relationship between inhalation of certain substances (e.g. impregnation spray products) and decreased lung function, some inconsistencies can be observed where unexpected outcomes occur. This paragraph addresses the role of dosimetry and individual susceptibilities in explaining these apparent inconsistencies.

For impregnation products, there are products that have a large market share and thus have frequent use, but only occasionally cause decreased lung function, or where many people are exposed and only a few develop symptoms (Scheepers et al., 2017; Cormican and Rees, 2006). The explanation for this could have to do with dosimetry, i.e. there could be a threshold to be crossed before the effects appear. In most cases of use, the concentration of the substance in the air may not become high enough, or the person is not exposed for long enough. This threshold is almost impossible to determine because the products are used under highly varying conditions. Decreased lung function often occurs following improper use (e.g. application in an area without ventilation or by devices that are not designed for application of the particular product). Improper use could result in accidentally higher dose rates or prolongation of exposure.

Further complicating the matter is a highly variable individual susceptibility. It is well known that lung diseases, such as asthma and chronic obstructive pulmonary disease, might affect lung surfactant function, independently of exposure to airborne substances, and may substantially increase susceptibility to respiratory challenges. In addition, smoking or environmental exposure to air pollution may affect baseline function of the lung surfactant. Age has been recently revealed to increase susceptibility for lung surfactant-dependent respiratory problems (Yazicioglu et al., 2020; Pineiro-Hermida, et al., 2020).

The following section details the techniques that can be used to measure the different key events and it is followed by perspectives on the implications and applications of this AOP in hazard identification of inhaled substances.

Technical details and methodologies

Measurements of lung surfactant function inhibition

The inhibition of lung surfactant function can be measured *in vitro* by evaluating the surface activity in dynamic assays that mimic the

continuous compression and expansion of the surfactant films at the air–liquid interface in the alveoli during breathing. Values of minimum surface tension, i.e. the lowest value of surface tension reached upon compression of the surfactant film, is a good indicator of the proper functioning of the lung surfactant. Maximum surface tension, i.e. the highest value of surface tension reached upon expansion of the lung surfactant film, reflects the effective re-adsorption of the lung surfactant at the interface. This parameter was shown to be less sensitive than the minimum surface tension to identify inhibitors of lung surfactant function (Da Silva et al., 2021; Valle et al., 2015). These tests can be performed in different setups.

Constrained drop surfactometer

In the constrained drop surfactometer (CDS) a droplet of lung surfactant is deposited on a sharp-edged pedestal, so that a surfactant film is formed at its air–water surface. The adsorbed lung surfactant film is cycled continuously, to mimic breathing (Zuo et al., 2008; Yang et al., 2018; Sørli et al., 2016; Valle et al., 2015). A camera continuously takes pictures of the droplet before and during exposure to aerosols of the test substance at the air–liquid interface. Alternatively, the lung surfactant and the test substance can be mixed prior to deposition on the pedestal (Sørli et al., 2020). Surface tension values are obtained by analysis of the drop shape in real-time (Yu et al., 2016). The main advantages of this method include the accessibility of the air–liquid interface for exposure to airborne substances, flexibility in controlling cycling rates, and ease of determination of the surface tension in real-time while cycling the surfactant film. The constrained drop surfactometer has been applied to the exposure to a broad range of substances, including nanoparticles (Larsen et al., 2020; Valle et al., 2015; Fan et al., 2011; Yang et al., 2018; Wang et al., 2020), individual chemicals (Da Silva et al., 2021), mixtures of chemicals (Da Silva, et al., 2021; Sørli et al., 2018, 2016), excipients for drug formulation (Sørli et al., 2018), per- and poly-fluoroalkyl substances (Sørli et al., 2020), or plasma (Autilio et al., 2021).

Captive bubble surfactometer

In the captive bubble surfactometer (CBS), the lung surfactant film is formed at the air–liquid interface of an air bubble suspended in liquid. The function can be studied by injecting the test substance in the proximity of the surfactant layer at the interface between the air bubble and the surrounding liquid, or by mixing the substance and the surfactant *prior to* injecting the lung surfactant into the chamber. The captive bubble surfactometer allows study of the rapid initial adsorption of the lung surfactant at the air–liquid interface, post-expansion adsorption, surface activity during dynamic and quasi-static cycles, and stability of the surfactant film to mechanical perturbations (Autilio and Perez-Gil, 2019). The method has been applied to investigation of industrial chemicals (Da Silva et al., 2021), nanoparticles (Bakshi et al., 2008), cholesterol (Lopez-Rodriguez et al., 2012; Gómez-Gil et al., 2009; Gunasekara et al., 2005), meconium (Lopez-Rodriguez et al., 2011, 2012), plasma (Autilio et al., 2021), serum (Lopez-Rodriguez et al., 2012, 2013; Lugones et al., 2018), corticosteroids (Hidalgo et al., 2017), or cyclodextrines on lung surfactant (Al-Saiedy, et al., 2018).

Pulsating bubble surfactometer

In the pulsating bubble surfactometer (PBS), an air bubble suspended on a capillary tube is formed in a chamber containing lung surfactant and is periodically compressed and expanded by a piston pulsator (Enhorning, 2001; Autilio and Perez-Gil, 2019). The method has been used to study the effects of nanoparticles (Schleh et al., 2009), bacterial lipopolysaccharides (Kolomaznik et al., 2018), glucocorticoids (Cimato et al., 2018), or meconium (Stichtenoth et al., 2006) on lung surfactant. The pulsating bubble surfactometer was also used to investigate the surface activity of lung surfactant from patients

with acute respiratory distress syndrome (Markart et al., 2007; Gregory et al., 1991).

Capillary surfactometer

In the capillary surfactometer (CS), surfactant is deposited in a capillary tube of uneven diameter that simulate the cylindrical surfaces of the terminal conducting airways a constant airflow is led through the capillary. The percent of time with an open passage is used to assess the functionality of lung surfactant (Sørli et al., 2016; Larsen, et al., 2014; Enhorning, 2001).

Surfactant adsorption test

The surfactant adsorption test is a fluorescence-based method that measures the extent and rate of adsorption of lung surfactant at the air–liquid interface. Lung surfactant is labelled with a fluorescent probe, and injected into the wells of a multi-well plate containing a light-absorbing agent (typically brilliant black). The plates are shaken and the fluorescence (of the lung surfactant sample reaching the surface of the wells) is measured. The fluorescence of the lung surfactant sample in the bulk (not adsorbed at the interface) is quenched by the light-adsorbing agent. This method is high-throughput compared to the biophysical assays described above and it allows to measure the effects of physiologically relevant factors, such as temperature, surfactant concentration, or presence of inhibitors in a high number of samples (Ravasio et al., 2008). However, this assay does not measure other biophysical properties like pressure-area isotherms, compressibility etc. The method has been used to study how albumin at the air–liquid interface hinders adsorption of lamellar body like particles (Hobi et al., 2014).

Investigation of the interaction of a substance with lung surfactant

The interaction between a substance (exogenous airborne substances or biological components) and lung surfactant components can be investigated at the molecular level *in vitro* and estimated *in silico*. The methods rely on lung surfactant models, ranging from simple monolayers of dipalmitoylphosphatidylcholine (DPPC, the main surface-active component of lung surfactant), to the more complex native surfactant, obtained from broncho-alveolar lavage fluid or minced lung tissue. In most methods, a film of lung surfactant is formed at air–liquid interfaces and exposed to the substance of interest via aerosolisation or deposition. In some cases, the lung surfactant model is mixed directly with the test substance before spreading of the film.

Atomic force microscopy

The topography of surfactant structures formed at respiratory-like air–liquid interfaces upon exposure to test substances can be studied by atomic force microscopy on fixed samples.

This method has been extensively used with nanoparticles (including gold nanoparticles, graphene oxide and carbon nanotubes) in order to characterize their presence within surfactant films, to identify interactions with surface-associated structures, and to study the retention at the interface upon film compression (Valle et al., 2015; Yang et al., 2018; Sachan et al., 2012; Tatur and Badia, 2012; Hu et al., 2013). Atomic force microscopy has also been used to compare the molecular organization and lateral structure of surfactant models in the presence and absence of corticosteroids (Wang et al., 2012).

Langmuir-Blodgett films

Langmuir-Blodgett films are interfacial films of surfactant transferred from the air–liquid interface onto solid supports. They are used to gain information about the distribution of lipids and proteins within the surfactant film and the effect of the interaction with test substances (Cruz and Perez-Gil, 2007). Surfactant films deposited at the air–liquid interface of a trough filled with liquid can be compressed by reducing

the surface area of the trough. A sensor plate measures the variation in surface pressure over compression to yield surface pressure – area isotherms. It should be noted that in addition to the traditional Langmuir trough, the Langmuir-Blodgett technique has been adapted in the constrained drop surfactometer to study adsorbed surfactant films (Xu et al., 2020). The comparison of such isotherms in the presence or absence of the test substance gives insights in the interaction of a substance with lung surfactant at the molecular level. Shifts in the surface pressure-area isotherms are identified most easily using simple models such as DPPC monolayers, but can also be seen using the more complex lung surfactant. Structural changes can be identified during compression of the film when combined with epifluorescence or atomic force microscopy. These methods have been applied successfully to the study of changes induced by resin acids (Jagalski et al., 2016), nanoparticles (Wang et al., 2020), soot particles (Fang et al., 2020), volatile organic substances (Zhao et al., 2019), industrial chemicals (Da Silva et al., 2021); tobacco smoke constituents (Stenger et al., 2009), e-cigarette components (Przybyla et al., 2017), spray products (Larsen, et al., 2014), corticosteroids (Wang et al., 2012), and biological components like cholesterol (Zhang et al., 2012; Taeusch et al., 2005), or meconium (Lopez-Rodriguez et al., 2011).

Cryogenic transmission electron microscopy

In aqueous dispersions, lung surfactant forms vesicles. Cryogenic transmission electron microscopy allows visualizing morphological and structural changes at the single membrane vesicle level. After incubation with the test substance, the surfactant model is applied onto a carbon grid and vitrified in liquid ethane cooled by liquid nitrogen. Changes in the size, circularity or lamerality of the vesicles indicate disruption of the three-dimensional surfactant structures. Cryogenic transmission electron microscopy was applied to study the effects of industrial chemicals (Da Silva et al., 2021), resin acids (Jagalski et al., 2016), meconium (Autilio et al., 2021; Gross et al., 2006), and nanoparticles (Fan et al., 2011) on native surfactant.

Differential scanning calorimetry

Differential scanning calorimetry allows the study of phase transitions occurring in lipid membranes (such as lung surfactant) over changes in temperature (Demetzos, 2008). It can be used to characterize the thermotropic phase behaviours of phospholipids in the surfactant models in the absence or presence of interacting substances. Associated enthalpy, transition temperature, and cooperativity can be estimated from the thermograms. It is a very sensitive method when working with simple models such as pure DPPC bilayers. The method is much less sensitive when using complex lung surfactant models. This is because several transitions overlap in membranes made of complex mixtures, each occurring at different temperature so it is difficult to identify one specific variation. Differential scanning calorimetry has been used to investigate the effects of meconium (Lopez-Rodriguez et al., 2011), cholesterol (Roldan et al., 2017), industrial chemicals (Da Silva et al., 2021), or resin acids (Jagalski et al., 2016) on various membrane models.

Other methods

A range of other methods allows to investigate the interaction of a substance with lung surfactant. Binding of lung surfactant components on nanoparticles was shown by proteomics and lipidomics analysis of the corona after incubation *in vitro* (Gasser et al., 2010) or after exposure of rodents and broncho-alveolar lavage fluid isolation (Kapralov et al., 2012). Molecular dynamics simulations *in silico* have been successfully used to investigate the interaction of atmosphere components with lung surfactant (Yuan et al., 2020), particularly single-wall carbon nanotubes (Xu et al., 2017), and hydrophilic (hydroxyapatite, silver) and hydrophobic (polystyrene) nanoparticles (Hu et al., 2013, 2017).

Measurements of alveolar collapse

There are approximately 480 million alveoli in the lungs (Ochs et al., 2004). Alveolar collapse and re-opening can only happen in intact lungs in a living organism and thus cannot be measured *in vitro*. Further, because of their small diameter of approximately 200 μm in diameter (Ochs et al., 2004), it is virtually impossible to measure the collapse and re-opening at the level of individual alveoli with any certainty. However, areas of atelectasis can be observed in experimental animals after staining of lung tissue from exposed animals (Jefferies et al., 1988; Nørgaard, et al., 2010; Yamashita and Tanaka, 1995).

Measurements of the loss of alveolar-capillary membrane integrity

The loss of alveolar-capillary membrane integrity can be measured *in vivo* by collecting broncho-alveolar lavage fluid from exposed animals, or in human subjects with acute lung injury (Gunther et al., 2001; Nakos, et al., 1998). Broncho-alveolar lavage fluid can be analyzed for total protein (elevated due to extravasation of blood components into the alveolar airspace), specific markers for cell injury such as detection of tight-junction proteins in lung tissue by immunofluorescence (Herrero and Matute-Bello, 2015) or markers of endothelial injury (Johnson and Matthay, 2010). Patients with acute respiratory distress syndrome have significantly higher concentrations of albumin in the lung lining fluid than healthy controls or patients with cardiogenic pulmonary edema (Ishizaka et al., 2004).

On the other hand, the presence of SP-D, a protein normally found in the airspaces of the lungs, in blood samples is taken as an indication that the alveolar-capillary membrane integrity has been compromised (Sorensen, 2018). In cell cultures, the trans epithelial electrical resistance (TEER) can be measured as an indicator of the barrier integrity (Sørli et al., 2018; Mathis et al., 2013; Sauer et al., 2013; Neilson et al., 2015; Bengalli et al., 2017; Balogh Sivars, et al., 2018). The alveolar-capillary barrier can also be visualized and examined under electron microscopy after sample fixation (Knudsen et al., 2012).

The exchange of gasses across the alveolar-capillary membrane can also be used as a measure of integrity. Haemoglobin preferentially binds carbon monoxide and the test can be used to determine the integrity of the barrier in humans (Macintyre et al., 2005) and experimental animals (Limjunyawong et al., 2015).

Measurements of reduced tidal volume

Reduced tidal volume can be measured in humans by spirometry (Miller et al., 2005; Pellegrino et al., 2005) and in animals by plethysmography (Sørli et al., 2018a, 2018b; Larsen et al., 2020). Reduced tidal volume and total lung capacity has been observed in humans after to exposure to an impregnation spray products (Lazor-Blanchet et al., 2004; Khalid et al., 2009), and in exposed experimental animals (Duch et al., 2014; Sørli et al., 2018a, 2018b; Nørgaard, et al., 2014).

Measurements of decreased lung function

Usually, lung function is evaluated in humans by volumetric measures of the forced-expiratory volume, and the forced vital capacity. In the context of this AOP, decreased lung function is evaluated by the occurrence of respiratory clinical signs of toxicity (reported by the patients, or observed in animal studies), and by the reduction in blood oxygenation (hypoxemia).

Several parameters can be measured to evaluate blood oxygenation: arterial oxygen saturation (SaO_2, percentage of hemoglobin saturated with oxygen), arterial oxygen tension (PaO_2, dissolved oxygen in the plasma), oxygenation index (OI, based on the mean airway pressure, fractional concentration of inspired oxygen, and arterial oxygen tension). In humans, blood oxygenation is measured easily by pulse

oximetry (non-invasive method), or by arterial blood test (invasive). A test of the "diffusing capacity of the lung for carbon monoxide" allows evaluation of diffusion impairment. Decreased partial pressure in oxygen and saturation in oxygen in arterial blood were observed after exposure to spray products (Lazor-Blanchet et al., 2004; Khalid et al., 2009). Reduction in the diffusion capacity for carbon monoxide was also shown after exposure to household products (Lazor-Blanchet et al., 2004; Khalid et al., 2009; Hong et al., 2014). Rodent oximeter sensors exist to measure blood oxygenation in experimental animals.

Perspectives: Potential significance and applications of the AOP

The previous section described the evidence supporting the AOP on lung surfactant function inhibition leading to decreased lung function, and how specific key events can be measured. Currently, testing for inhalation toxicity is conducted in experimental animals. This presents many challenges, including high cost and labour intensity of the relevant *in vivo* methods, and a number of ethical considerations. However, developing AOPs that describe the pathways leading to inhalation toxicity, can show where new approach methodologies (NAMs) can be used to predict the toxicity of the test substance.

Regarding inhalation exposure to airborne substances, several AOPs (at different stages of development) describing adverse outcomes have been entered in the AOPwiki. The adverse outcomes include lung fibrosis (https://aopwiki.org/aops/173), lung cancer (https://aopwiki.org/aops/303) and atherosclerotic plaque formation (https://aopwiki.org/aops/237) (all described in (Halappanavar et al., 2020), sensitisation of the respiratory tract (https://aopwiki.org/aops/39), sensory pulmonary irritation by activation of the TRPA1 receptor (https://aopwiki.org/aops/196), and decreased lung function by activation of epidermal growth factor receptor (EGFR) (https://aopwiki.org/aops/148).

Common KEs have already been identified across several of these AOPs, i.e. "hub KEs", and more will be as AOPs are further developed. For example, the assessment of a collection of AOPs relevant for inhalation of nanomaterials (including AOP 302) revealed that the AOPs could be connected in a network through hub KEs (Halappanavar et al., 2020). The next step in AOP development is to understand the quantitative relationships between the different KEs. Quantitative AOPs can be used to identify specific KEs that can be measured and used as indicators of the likelihood of the adverse outcome happening. If NAMs can be developed for hub KEs these can potentially be combined with other relevant NAMs into an alternative strategy for inhalation toxicity testing (Nymark et al., 2020).

AOP 302 clearly identifies the event "lung surfactant function inhibition" as a relevant step that is well documented and can be measured *in vitro* to predict the adverse outcome of "decreased lung function". Ultimately, NAMs that are identified when defining an AOP could fulfil some of the information requirements set under REACH regulation by predicting downstream events. In the context of inhalation toxicity, predicting decreased lung function is relevant for acute inhalation toxicity testing and for repeated dose toxicity via inhalation. Outside of regulatory toxicity, the development of NAMs for inhalation toxicity is useful for early product development.

CRediT authorship contribution statement

Emilie Da Silva: Investigation, Visualization, Writing - original draft. **Ulla Vogel:** Writing - review & editing. **Karin S. Hougaard:** Writing - review & editing. **Jesus Pérez-Gil:** Writing - review & editing. **Yi Y. Zuo:** Writing - review & editing. **Jorid B. Sørli:** Conceptualization, Investigation, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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