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TITLE: Developing a Framework for Assessing Respiratory Sensitization: A Workshop Report

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ABBREVIATIONS:

AOP	adverse outcome pathway
APC	Antigen Presenting Cell
BAL	bronchoalveolar lavage
CLP	Classified, Labeling and Packaging Regulation
DC	dentritic cell
DNCB	2,4-dinitrochlorobenzene
DPRA	direct peptide reactivity assay
EC	endothelial cells
ELoC	equivalent level of concern
GARD	Genomic allergen rapid detection
GHS	Globally Harmonized System
ITC	Immunotoxicology Technical Committee
KC	Kupffer cells
LC	Langerhans cells
LLNA	Local Lymph Node Assay
LMW	low molecular weight
LRI	Long Range Research Initiative
MDI	diphenylmethane-4,4'-diisocyanate
MIE	molecular initiating event
MOA	mechanism of action
OA	occupational asthma
OECD	Organisation for Economic Co-operation and Development
OVA	ovalbumin
JCIA	Japanese Chemical Industry Association
PMN	polymorphonucelar leukocytes
PRR	pathogen recognition receptors
REACH	Regulation concerning the Registration, Evaluation, Authorization and Restriction of Chemicals
SAR	structure activity relationship
SVHC	substance of very high concern
TDI	toluene diisocyanate
TMA	trimellitic anhydride

ABSTRACT

Respiratory tract sensitization can have significant acute and chronic health implications. While induction of respiratory sensitization is widely recognized for some chemicals, validated standard methods or frameworks for identifying and characterizing the hazard are not available. A workshop on assessment of respiratory sensitization was held to discuss the current state of science for identification and characterization of respiratory sensitizer hazard, identify information facilitating development of validated standard methods and frameworks, and consider the regulatory and practical risk management needs. Participants agreed on a predominant Th2 immunological mechanism and several steps in respiratory sensitization. Some overlapping cellular events in respiratory and skin sensitization are well understood, but full mechanism(s) remain unavailable. Progress on non-animal approaches to skin sensitization testing, ranging from in vitro systems, –omics, in silico profiling, and structural profiling were acknowledged. Addressing both induction and elicitation phases remains challenging. Participants identified lack of a unifying dose metric as increasing the difficulty of interpreting dosimetry across exposures. A number of research needs were identified, including an agreed list of respiratory sensitizers and other asthmagens, distinguishing between adverse effects from immune-mediated versus non-immunological mechanisms. A number of themes emerged from the discussion regarding future testing strategies, particularly the need for a tiered framework respiratory sensitizer assessment. These workshop present a basis for moving towards a weight-of-evidence assessment.

1. INTRODUCTION

Respiratory sensitization is a health hazard that can occur following exposure to chemical or biological materials. The adverse outcome is an allergic-type response of the airways, mostly asthma or rhinitis. The disease develops in two phases: the sensitization or induction phase in which the immune system is primed and the elicitation phase in which the allergic symptoms occur. Respiratory sensitization/allergy is characterized by a progressive increase in immune system responsiveness, such that sensitized individuals respond to exposures that elicit no effect in non-sensitized populations. Accurate identification of respiratory sensitizers is important because the health effects can be severe and long-lasting. At the same time, incorrect identification of a material as a respiratory sensitizer can result in unnecessarily stringent restrictions on use.

From a toxicological perspective this human health hazard presents a number of challenges, including the uncertainty regarding the mechanisms through which sensitization of the respiratory tract to chemicals is acquired. This has hindered development of methods for the identification and characterization of chemical respiratory allergens. The Globally Harmonized System (GHS) for hazard classification considers evidence from human responses, or “appropriate animal models” which are not standardized. Unlike other hazard endpoints used for classification, there is not an internationally accepted animal test guideline. Different published protocols exist for assessing respiratory sensitization, but no systematic undertaking has validated any of the methods for a broad range of materials. Historically, the guinea pig has been the species of choice for research on respiratory sensitization due to physiological similarities of respiratory reactions compared to humans. Time and cost considerations, as well as a lack of suitable immunochemical or molecular probes for mechanistic evaluations, have led many to look for other animal, and non-animal alternative, test systems. Experimental models using rats and mice have been successful in inducing chemical respiratory sensitization, but the parameters providing best predictive performance remain unknown. Current alternatives face challenges in the form of a relatively limited chemical respiratory sensitizer database and knowledge limitations related to which exposure-response parameters are the best predictors of respiratory sensitization. The ability to accurately detect potential respiratory sensitizers is ultimately hindered by the absence of standard, validated and regulatory

accepted methods to identify potential respiratory sensitizers and distinguish them from irritants and skin sensitizers for hazard identification. The difficulty in distinction is further compounded by absence of generally accepted methods to define dose thresholds for irritation, which may make distinguishing between immune-mediated and non-immunological responses unclear.

The lack of defined approaches for evaluation of respiratory sensitization potential has necessarily represented a major constraint on effective risk assessment and risk management, and on addressing satisfactorily the requirements of regulations such as the Regulation concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). There is increasing regulatory pressure to list respiratory sensitizers as substances of very high concern (SVHC) based on an “equivalent level of concern” as set out in REACH Article 57(f). This approach assumes that in certain cases, the impacts caused by sensitizers (respiratory or dermal) on the health and quality of life of the affected individual and the negative impacts on society as a whole are comparable to those elicited by carcinogens, mutagens, and reproductive toxicants (CMRs). Potential factors for comparison include severity of the effect, delayed onset and/or irreversibility of effects, potency, mode of action, degree of impairment of life quality or uncertainty about the dose-response relationship. As there are currently no applicable guidelines or generally accepted assays that can accurately identify respiratory sensitizers nor distinguish between respiratory and dermal sensitizers, all materials with sensitizing potential, despite their potency, may be inaccurately considered for inclusion as SVHC. If an evidence-based, adverse outcome pathway (AOP)-informed approach to assessment is desired there is an increasingly important need, therefore, to seek integrated approaches to toxicity testing and assessment to bridge this gap.

The Immunotoxicology Technical Committee (ITC) of the International Life Sciences Institute-Health and Environmental Sciences Institute previously organized two activities centered on the state-of-the-science of testing methods to identify proteins and chemicals that pose a risk of immune-mediated respiratory hypersensitivity. An expert roundtable discussion, held in 2003 at the Annual Meeting of the Society of Toxicology in Salt Lake City, Utah, was followed by a two-day international workshop in June 2004 that addressed the appropriate methods for identifying and characterizing respiratory hypersensitivity hazards and risks, and the key gaps and related research needs with respect to respiratory hypersensitivity/allergy

for proteins, low molecular weight drugs, and chemicals (Holsapple et al., 2006). Key research gaps identified for chemical-specific respiratory hypersensitivity included (1) understanding structure activity relationships for chemical allergies, including understanding the mechanism(s) for respiratory hypersensitivity and identifying distinctive characteristics of the respiratory hypersensitivity allergic response, and continuing to build databases of sensitization until chemicals can be clearly identified as respiratory allergens; (2) better understanding of mechanisms for sensitization; and (3) fully characterizing cytokine profiling as a possible approach for hazard identification.

Given a decade's passage and expectation of continuous progress of science, in 2014 the ITC organized a two-day international workshop in Alexandria, Virginia, towards identifying a framework for developing a standard approach for identifying chemical respiratory sensitizers. (The workshop agenda and materials can be found here.) The workshop opened with a presentation on the clinical manifestations of respiratory sensitization. The subsequent series of lectures provided a foundation for the current state-of-the-science for identification and characterization of respiratory sensitizer hazards, using both conventional and non-conventional approaches, and the regulatory and practical needs regarding risk management, with the ultimate aim of identifying near-term and long-term information to facilitate development of validated standard methods and frameworks. The ~75 participants were asked to consider a series of questions that provided a framework for discussions during the break-out sessions. The lectures and break-out discussions provided the foundation for this report, and have been summarized in Sections 2 and 3, respectively.

2. STATE OF THE SCIENCE

2.1 Clinical Aspects of Chemical Respiratory Allergy and Occupational Asthma

In the context of occupational asthma, chemical sensitizers refer to those chemicals that can cause asthma through an immunologic or presumed immunologic mechanism (Bernstein et al., 2013). Besides the potential to cause occupational asthma, some occupational respiratory sensitizers can cause other respiratory allergic responses such as hypersensitivity pneumonitis, eosinophilic bronchitis, and rhinitis.

Chemical sensitization can, in rare instances, trigger life-threatening acute conditions such as anaphylaxis as recently reviewed (Siracusa et al., 2015). The median population attributable risk for asthma from occupation has been estimated to be approximately 15% (Balmes et al., 2003; Toren and Blanc, 2009). Work-related asthma includes occupational asthma (usually new-onset asthma), caused by work, and work-exacerbated asthma, that is asthma caused by other factors but aggravated/exacerbated by work (Tarlo et al., 2008; Tarlo and Lemiere, 2014), but does not necessarily distinguish between immune-mediated and non-immunological agents. The population attributable risk for new-onset asthma that likely reflects occupational asthma was similarly estimated in one large multicenter study as being 10-25% (Kogevinas et al., 2007). Causes include irritant exposures at work (that are usually accidental) (Vandenplas et al., 2014b), and specific responses to a workplace sensitizer (an agent causing a specific immunologic response). Workplace sensitizers can be further classified as high-molecular weight agents (usually proteins) and low-molecular weight chemicals. Specific IgE may not be detected in all symptomatic patients. The lack of universally detected specific immunologic markers of response has made it difficult to determine whether agents such as sprayed cleaning products and air fresheners are acting as specific chemical sensitizers or as airway irritants in studies that have shown increased asthma prevalence among exposed workers (Dumas et al., 2012).

There are multiple chemical sensitizers known to cause asthma, both in workers as recently reviewed (Baur, 2013; Baur and Bakehe, 2013), and (less often) in consumers. New formulations and new uses of known agents continue to be reported as well as newly developed agents. The clinical presentation of occupational asthma can mimic other (non-occupational) asthma, and the diagnosis may not be suspected unless the physician takes a careful history of the workplace exposures and timing of symptoms in relation to work. Other diseases can also mimic asthma and therefore objective tests are important for a correct diagnosis of chemical-induced respiratory sensitization. Algorithms have been developed for diagnosis, including immunologic tests where feasible, objective tests for asthma, and objective demonstration of changes in asthma during work periods compared with periods off work (Tarlo et al., 2008; Tarlo and Lemiere, 2014). The most definitive tests are specific inhalation challenges with the suspected agent, but these carry a small safety risk to the subject and are not widely available (Vandenplas et al., 2014a). After diagnosis, workers with occupational asthma are typically removed from

127 further exposure to the sensitizing agent, but often will continue to have asthma to some extent. Outcome
128 of asthma is best with early correct diagnosis and early removal from further exposure (de Groene et al.,
129 2012). Preventive measures include primary prevention by avoidance of worker-exposure to agents that
130 are sensitizers, secondary prevention by early detection of sensitized workers and removal from exposure
131 (by education and medical surveillance), and tertiary prevention by appropriate management of those with
132 occupational asthma.

133 **2.2 Mechanisms of Respiratory Sensitization and Routes of Exposure**

134 A key hurdle, and arguably the most important hurdle, in developing a clearer view of the critical events
135 and immunological pathways required for respiratory tract sensitization is the lack of clarity regarding the
136 role played by IgE antibody. It is legitimate to regard IgE antibody as a potential effector mechanism as it
137 is well established that these antibodies play a pivotal role in allergic responses to proteins, and in allergic
138 asthma. However, it has not been possible to show a clear correlation between symptoms of chemical
139 respiratory allergy and serum IgE antibody in patients with occupational asthma. Nevertheless, even with
140 the diisocyanates, where the detection of IgE antibody among symptomatic patients has proven
141 particularly difficult, there are reports of specific IgE antbody being found in some patients(Kimber et al.,
142 1998).

143 It is this uncertainty about the role of IgE antibody in chemical respiratory allergy specifically, and about
144 the important pathogenic mechanisms generally, that have made it difficult to reach agreement on
145 relevant readouts for predictive test methods (Kimber and Dearman, 2002; Kimber et al., 2014). It is
146 therefore the case that resolution of the role of IgE antibody and/or other immunological mechanisms in
147 the acquisition of sensitization of the respiratory tract to chemical allergens is a major research objective.

148 A second important issue is the route or routes of exposure through which sensitization to chemical
149 respiratory allergens can be acquired. There is growing evidence from experimental animal studies, and
150 from anecdotal information from humans, that skin exposure can result in sensitization of the respiratory
151 tract. That is, the development of sensitization following skin exposure to chemical respiratory allergens is
152 systemic – inducing sensitization of the respiratory tract (Kimber and Dearman, 2002; Kimber et al.,

153 2014).

154 **2.3 What Differentiates Respiratory from Skin Sensitizers? Implications for Predictive Toxicity**
155 **Testing**

156 From a regulatory perspective, it is essential to distinguish respiratory from skin sensitizers. According to
157 GHS, skin and respiratory sensitizers are classified in two different hazard classes that result in different
158 adverse outcomes. Skin and respiratory chemical sensitizers are both low-molecular-weight chemicals
159 that share certain properties needed to provoke an immune response (Kimber and Dearman, 2005). In
160 order to develop predictive test methods that are able to specifically identify respiratory sensitizers, it is
161 essential to identify the unique mechanisms involved in respiratory sensitization. Decades of intensive
162 research have resulted in a good understanding of the key events in induction of sensitization and
163 elicitation of symptoms for similar to skin (Basketter and Kimber, 2010), culminating in a suggested MOA
164 pathway Adler et al. (2011) and AOP for skin sensitization induction (OECD, 2012).

165 Increased airway reactivity, epithelial remodeling and inflammation are adverse physiologic endpoints
166 associated with repeated exposure to some low molecular weight chemicals that may have the intrinsic
167 ability to cause respiratory allergy. As with skin sensitization, this adverse effect can result from an
168 induction of sensitization after exposure to a chemical followed by an elicitation of allergic symptoms upon
169 further exposure with the sensitizing chemical. The most obvious differences between respiratory and
170 skin sensitization to chemicals is that in the classical view exposure of the former involves mucosal
171 surfaces and alveolar macrophages as APCs and triggers (demonstrated or presumed) Th2 cell
172 responses, while the latter involves skin and is Th1 cell oriented (Roggen, 2014). Furthermore, there is
173 some evidence suggesting that under certain circumstances chemical respiratory sensitizers prefer lysine
174 for haptenation, while skin sensitizers favor cysteine (Lalko et al., 2013).

175 In the dermal Local Lymph Node Assay (LLNA) and respiratory LLNA both respiratory and skin
176 sensitizers are able to induce lymphocyte proliferation, but go on to induce the development of distinct
177 effector immune responses. Respiratory sensitizers induce a predominant Th2 response, while skin
178 sensitizers induced a predominant Th1 response (Arts et al., 2008; De Jong et al., 2009; Dearman et al.,

1995; Vandebriel et al., 2000). Glutaraldehyde, a recognized skin and respiratory sensitizer, was negative upon inhalation in the respiratory LLNA, but positive after dermal exposure, inducing a Th2-dominant immune response (van Triel et al., 2011). It was hypothesized that after respiratory exposure glutaraldehyde reacts with the proteins in the mucus layer and is unable to reach the immune system in a sufficiently high dose. This may suggest that there are measurable thresholds of exposure for induction of the sensitized state. Glutaraldehyde is a well-known cause of asthma in humans, but the skin might be an important route of exposure for the induction in environmental or occupational settings. Once sensitized, lower concentrations are sufficient to elicit an allergic response in the respiratory tract. This may support the value of preventing both skin and inhalation exposure to respiratory sensitizers.

In elicitation studies in rats responses to trimellitic anhydride (TMA) and oxazolone, model chemicals inducing respiratory sensitization in rodents, were compared to the skin sensitizer 2,4-dinitrochlorobenzene (DNCB) (Arts et al., 2008; Kuper et al., 2008a; Kuper et al., 2011). Gene expression profiling of the lungs, paralleled with breathing patterns, lung pathology and serum IgE levels revealed interesting mechanistic differences between these chemicals. As expected, the respiratory sensitizers affected breathing patterns and induced lung inflammation and IgE responses in Brown Norway rats. In contrast, DNCB did not affect breathing patterns or serum IgE, but induced an influx of neutrophils in the respiratory tract. Gene expression revealed a difference in regulated pathways, showing that TMA induced the most pronounced regulation of immune-related pathways, followed by oxazolone. DNCB hardly induced any significant pathway regulation. Remarkably, TMA was the only chemical that affected gene expression pathways related to airway remodeling. Oxazolone is a well-known human skin sensitizer, but there are no human reports on respiratory allergy. This could implicate that oxazolone is a false-positive in the animal model or that there is no or low inhalation exposure in man. Since oxazolone's physical state can present as large flakes, the latter explanation is plausible. Interestingly, the gene expression revealed that oxazolone induced more pronounced Th1 genes than TMA (Kuper et al., 2011).

DNCB is a strong human skin sensitizer that was immunogenic in different short-term respiratory animal models (Arts et al., 2008; Kuper et al., 2008b). The significance of these findings in terms of adverse human health effects is unclear. Prolonged and repeated inhalation exposures in Th1-prone Wistar rats

showed that DNCB was able to prime the immune system, as evidenced by DNCB-specific IgG levels. Additionally, DNCB provoked allergic inflammation in the upper respiratory tract, but did not affect functional breathing parameters. Hence, DNCB evoked a different inflammatory response upon inhalation compared to TMA. Whether or not these effects are indicative for adverse effects in humans is unknown, but they do demonstrate that DNCB is immunogenic after inhalation exposures as well (van Triel et al., 2010).

TMA, oxazolone, and DNCB demonstrate that respiratory and skin sensitizers are able to provoke different immune responses in experimental animals. Elicitation models seem especially suitable to demonstrate distinct immune responses, and toxicogenomics proved to be an important tool to increase mechanistic understanding of respiratory sensitization. Application of this knowledge for the development of predictive test methods is yet unclear, since only a few respiratory and skin sensitizers were tested in these animal studies. To become more confident in the type of read-outs that are indicative for respiratory sensitizers, a broader range of skin and respiratory sensitizers should be tested. Besides animal models other information sources, including structure activity relationships (SARs) or *in vitro* models that are currently in development should be included in the development of a predictive testing strategy for respiratory sensitization, a method already demonstrated for skin sensitization. To build such a testing strategy, it is important to map the mechanistic understanding in an AOP as has been done previously for skin sensitization (OECD, 2012).

2.4 Conventional and Non-Conventional Approaches to Assess Respiratory Sensitization

2.4.1 Developing in vivo and in vitro models for Respiratory Sensitization

An overview of two unpublished pilot studies, which were supported by a grant from Japanese Chemical Industry Association – Long-range Research Initiative (JCIA-LRI), of *in vitro* and *in vivo* models for assessment of respiratory sensitizing potential was presented. The JCIA-LRI supported research will establish sensitive cell lines with reduced serine protease inhibitor expression in order to assess chemical-induced hypersensitivity. Basophils and eosinophils secrete abundant serine proteases as well as chemical mediators and cytokines. Serine protease inhibitors have been reported to suppress both

serine protease activity and cytokine production *in vitro*. Both human basophilic cell line KU812-F and human eosinophilic cell line EoL-1 highly produced IL-6 in response to several sensitizers. Based on the allergy protective action of some serine protease inhibitors (Smith and Harper, 2006), these cell lines depleted of any serine protease inhibitor may be ideal candidates for the screening of respiratory sensitizers. Generation of stable cell lines lacking serine protease inhibitors using the inducible short hairpin RNA system may be complimentary to the *in vivo* approach described below.

An *in vivo* testing method was developed for identifying respiratory sensitizers and determining their relative sensitizing potency. Known sensitizers, toluene diisocyanate (TDI) and TMA, were used to sensitize female BALB/c mice by intratracheal instillation on five days per week for three weeks. Following subsequent challenge the severity of the lung inflammation increased in dose-related manner for both OVA, TDI, and TMA, but not DNCB. Histological scores dose response evaluation indicated the relative sensitizing potency of each of these known sensitizers in the BALB/c model was similar to the sensitizing potency reported in previous epidemiologic studies. These data suggest that this type of testing method can predict respiratory sensitization and a chemical's relative sensitizing potency, and by extension may provide useful information for the hazard assessment of respiratory sensitizers. Future efforts will expand the evaluation for more sensitizers in order to demonstrate the reliable efficacy of a testing method.

2.4.2 *Mechanistic In Vitro Models for the Assessment of the Respiratory Sensitization Potential of Compounds*

From the mechanistic point of view, our understanding of the toxicity pathways driving both induction of chemical respiratory sensitization and elicitation of symptoms is not as well established as for skin sensitization (Roggen, 2014). In contrast, more is known about the mechanisms underlying protein sensitization and allergenicity (Wills-Karp et al., 2010). The putative key events in an MOA for respiratory sensitization likely include: 1) bioavailability, 2) haptenation, 3) inflammation, 4) dendritic cell activation and maturation, 5) dendritic cell migration, and 6) T-cell proliferation. An overview of novel non-animal tests to assess the key events above is discussed below and in Table 1.

Bioavailability. For a compound to be able to trigger sensitization, it must be present in a bioavailable

form to the relevant effector. Thus, a compound must gain access to the viable epidermis, dermis and vascular network across the bio-barrier (e.g. skin, lung mucosa) (Basketter et al., 2007; Wills-Karp et al., 2010). Several studies correlate pulmonary bioavailability to the lipophilicity, the molecular polar surface area and hydrogen bond donor counts of a chemical. Studies using peptides suggest that the same parameters affect the bioavailability of protein allergens (Cooper et al., 2010).

Haptenation. In contrast to protein allergens which are sufficiently large to be identified as "foreign" by the host innate and adaptive immune systems, low molecular weight chemical sensitizers are generally believed to react covalently with native host protein(s) to form stable neoantigens. The majority of sensitizing chemicals are either inherently reactive, electrophilic chemicals that form covalent bonds with nucleophilic groups on amino acids, or occasionally acquire such reactivity following metabolism. Non-electrophilic mechanisms for protein binding may also occur through disulfide exchange or coordination bonds (e.g. metals) (Chipinda et al., 2011). Compared with skin sensitizers, low molecular weight respiratory sensitizers reacted more readily with lysine rather than cysteine moieties of host proteins (Lalko et al., 2013). Despite the limitation that such methods do not identify the target protein defining the specificity of the immune response (Aleksic et al., 2007), they may, however, provide a useful piece of qualitative (and potentially quantitative) information for hazard identification. Future work to critically evaluate the readiness of haptenation assays may extend their value in hazard identification.

Inflammation. Three potentially useful / *in vitro* test models were discussed for respiratory sensitization testing (Roggen, 2013). These include precision cut human lung slices(Lauenstein et al., 2014); an *in vitro* alveolar-capillary barrier based co-culture system comprised of two human cell lines, H441 and ISO-HAS-1 (Hermanns et al., 2010); and an air liquid interface (ALI) organotypic 3D airway epithelial model employing primary human bronchial epithelial cells (MucilAir™; www.epithelix.com). Although not validated, each of these model systems have been used to discriminate sensitizers from irritants, as well as respiratory from skin sensitizers (dos Santos et al., 2009).

Dendritic cell (DC) activation. It is generally accepted that activation of DCs results in mature antigen-presenting cells having an established Th1-, Th2-, Th17-biased phenotype (Tan and O'Neill, 2005).

Studying the molecular mechanisms behind DC activation and maturation is impeded by the fact that primary DCs constitute a small and heterogeneous population of cells among many functionally specialized DC subpopulations. To circumvent this issue, various human myeloid cell lines (e.g. THP-1, U937, KG-1 and MUTZ-3) were used both for acquiring mechanistic understanding and for development of predictive tests (Larsson et al., 2006; Roggen, 2013). Functional and transcriptional analysis of various myeloid cell lines has clearly demonstrated the significance of the MUTZ-3 cell line as a model for functional studies of inflammatory responses (Larsson et al., 2006; Lundberg et al., 2013). The genomic allergen rapid detection (GARD) test can generate prediction calls of unknown chemicals as skin sensitizers, respiratory sensitizers or non-sensitizers, including irritants (Johansson et al., 2011). In addition to providing an accurate prediction about the sensitizing potential of a chemical, there is growing evidence that the GARD test also provides useful information about the sensitizing potency of the chemical (Albrekt et al., 2014).

The most advanced DC maturation test is the human cell line activation test (h-CLAT). When applied to hazard identification for skin sensitization the test revealed a good concordance (84%) with the LLNA data (sensitivity: 88%; specificity: 75%) (Ashikaga et al., 2010). There are indications that the h-CLAT correlates with the LLNA and may have the potential to provide information about the potency of the test chemical (Ashikaga et al., 2010). The usefulness of this test for assessing respiratory chemicals was not established, but given the potential of the dermal LLNA as a screen for respiratory sensitization potential (*i.e.,* LLNA negatives being unlikely to be respiratory sensitizers), the h-CLAT may provide similar screening potential in the future.

Dendritic cell migration. In an *in vitro* full-thickness tissue-engineered skin model containing fully functional MUTZ-3 derived LCs (MUTZ-LC) (Ouwehand et al., 2008; Ouwehand et al., 2011) can be utilized to assess the impact of irritants and sensitizers on the migration activity of the fluorescently labelled MUTZ-LC. While not evaluated using protein allergens, this *in vitro* DC migration test was found to correctly identify both respiratory and skin sensitizing chemicals (dos Santos et al., 2009).

Summary of Mechanistic *In vitro* Approaches. Novel, but not yet validated, testing methods for

assessment of pulmonary sensitization have been developed. While these assays are functionally plausible, their predictive accuracy remains to be evaluated. The potential application areas for the assays discussed above have been, where possible, integrated into Table 1 below.

2.4.3 *Application of 'Omics' Technologies to Assess Chemical Respiratory Allergy*

Current guidance recommends a weight-of-evidence approach based on human and animal data to identify a potential respiratory sensitizer. The use of 'omics' technologies such as transcriptomics and proteomics can provide an unbiased global assessment of gene-expression and protein network alterations associated with the development of allergic rhinitis and asthma (Park and Rhim, 2011; Sircar et al., 2014). These methods have been used to examine (1) the induction of the sensitized state which includes hapten-protein formation, interaction with epithelial cells impacting dendritic cell activation, Th2-biased maturation, and subsequent lymphoid cell activation, proliferation and differentiation and (2) the elicitation phase where subsequent inhalation exposure enhances localization and amplification of allergic responses. This enhanced response can extend into epithelial remodeling with effector/inflammatory cell influx, mucous cell hyperplasia/metaplasia, development of functional pulmonary responses including airway hyperreactivity, and reversible airflow obstruction.

Toxicogenomics have been applied to the characterization of the elicitation phase. Kuper et al., (2008a; 2008b) reported on the molecular characterization of the respiratory sensitizer TMA and the skin sensitizer DNCB in Brown Norway rats. They performed a whole genome analysis and related the results to physiological and cellular parameters with the aim to improve hazard identification and cross-species comparisons of respiratory allergens through molecular characterization. The presence or absence of notable changes in gene expression were consistent with the physiological/cellular responses to TMA and DNCB. The skin sensitizer DNCB resulted in slight changes in chemokine transcripts but no effects on lung remodeling. Rats dermally sensitized and exposed by inhalation to TMA showed a number of changes associated with lung remodeling similar to that observed in early development of asthma in humans. The authors stated that early lung remodeling genes may be useful in further characterization of molecules capable of causing allergic asthma. The expression profile was generally consistent with genes

regulated in mouse models of asthma and those reported in humans with asthma. These data suggest that changes in gene expression may represent valuable complementary endpoints for the characterization of potential respiratory allergens in sensitization-challenge models.

Proteomic approaches may be used to enhance the identification of respiratory sensitizers (Haenen et al., 2010; Park and Rhim, 2011). Using an OVA sensitized mice, a repeated aerosol challenge was used to induce an elicitation response. Sensitized, OVA-challenged mice had a significant increase in pulmonary eosinophils, and increased airway reactivity to methacholine challenge compared to controls. These changes included upregulation of structural proteins associated with airway remodeling and mammalian chitinases (YM1/YM2) that are induced by IL-13 expression (Jeong et al., 2005). One major strength of proteomics is the ability to evaluate multiple functional tissue compartments in both humans and experimental animals (sputum, BAL, blood) for translational investigations. Additional studies are needed to assess the utility to differentiate respiratory and dermal sensitizers and identify markers that may be used to identify thresholds of sensitization/elicitation or perhaps recovery following removal from exposure (Louten et al., 2012; O'Neil et al., 2011; Park and Rhim, 2011; Zhang et al., 2009).

Toxicogenomic approaches have also been used to examine the induction phase of sensitization to identify respiratory sensitizers. Comparison of a panel of dermal sensitizers (DNCB and alpha-hexylcinnamaldehyde), respiratory sensitizers (TMA and ortho-phthalaldehyde) and non-sensitizing irritants (methyl salicylate and nonanoic acid) identified 4,467 significant gene expression responses, which were in turn categorized (Adenuga et al., 2012; Boverhof et al., 2009). Respiratory sensitizer-specific transcripts were identified, including AKR1c18 (aldo-keto reductase; promotes Th2 cell survival; (Matsuzaki et al., 2005)), Galectin-7 (cell-cell and cell-cell matrix interactions), Mcpt1 and 8 (mast cell protease 1 and 8) and IL-4 (promotes Th2 bias). These data suggest that gene expression changes during sensitization may enhance WoE approaches to distinguish sensitizers from irritants and respiratory sensitizers from dermal sensitizers. There is a need to expand the low molecular weight chemical data set to confirm and extend these data and to expand analyses to upper/lower airway tissues to explore mucosal gene expression signatures.

A WoE approach is currently required to differentiate respiratory from dermal sensitizers. It is essential to develop and validate robust assay systems to distinguish respiratory sensitizers from both dermal sensitizers and non-sensitizing irritants. A science-based approach to assess respiratory sensitizer potency and thresholds of sensitization/elicitation is critical to address possible hazard classification of respiratory sensitizers, which may be considered as SVHC under the "equivalent level of concern" route set out in Article 57(f) of REACH. Data provided using 'omics' technologies can help identify key cellular and molecular events relevant to development of an adverse outcome pathway for respiratory sensitizers.

2.4.4 Grouping, Read-Across, and Mechanistic Chemistry for Respiratory Sensitization

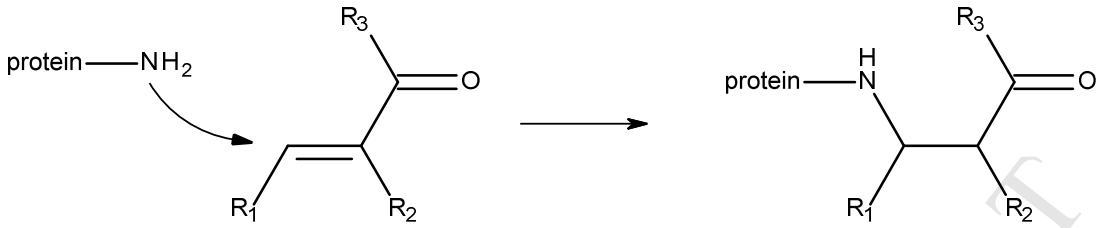
One view on the AOP concept relates a series of key events linking a Molecular Initiating Event (MIE) between a chemical and a biological system to an adverse effect at the organ level. In turn, organ level effects may be linked to predictions of biological system or even the population events. The aim of an AOP is to outline the key processes, some of which can be tested by using either *in silico, in chemico* or *in vitro* methods. The chemistry associated with the MIE can be compiled into '*in silico* profilers', enabling chemicals to be grouped into mechanism-based categories, allowing for predictions of toxicity to be made by using read-across. Such an approach offers an improvement on structural similarity based approaches, which inherently aim to address the possibility of similar *in vivo* chemistries, but without the benefit of applying mechanistic chemistry knowledge to the grouping. This additional mechanistic knowledge is important as simple structural similarity frequently identifies chemicals that are structurally, but not mechanistically similar (in terms of their ability to react with proteins). In addition, such profilers enable chemical inventories to be prioritized for further *in vitro* and/or *in chemico* investigations (rather than testing every chemical in an unstructured manner).

Recent research has led to the development of an *in silico* profiler for respiratory sensitization (Enoch et al., 2012). The profiler was developed from a mechanistic chemistry analysis of a data set of 104 reported in the literature as causing occupational asthma. Initial interest in this area of research stemmed from a study showing that, for some respiratory sensitizers, the most likely MIE was the formation of a covalent bond in the lung (Enoch et al., 2009). An outline was developed for how such mechanistic information

388 could be used to predict respiratory sensitization by read-across for a second, slightly larger, data set of
389 chemicals (Enoch et al., 2010). Both studies highlighted the importance of the underlying mechanistic
390 chemistry as the guiding principle in the process of grouping chemicals, and that there are several key
391 factors that drive the MIE for respiratory sensitization: chemical reactivity (electrophilicity), the ability to
392 cross-link proteins due to the presence of multiple reactive sites within a chemical and chemical volatility
393 (a chemical must be sufficiently volatile to elicit an immune response in the lung following induction which
394 can occur either in the skin or the lung). This analysis also showed that highly electrophilic chemicals
395 cause sensitization without the need for protein cross-linking. By the same token, weakly electrophilic
396 may not cause sensitization.

397 For some mechanistic chemistries there appears to be a “reactivity threshold” for electrophilicity that in
398 part governs whether a chemical is likely to be a respiratory sensitizer (Agius et al., 1991). Consider the
399 two chemicals ethyl cyanoacrylate and methyl tiglate which can both react via Michael addition to form a
400 covalent bond with a protein (Figure 1). Ethyl cyanoacrylate is a potent respiratory sensitiser, whilst there
401 have been no reports of methyl tiglate causing respiratory sensitization in humans (Enoch et al., 2012).
402 This has been rationalized in terms of the differing calculated electrophilicity values of these two
403 chemicals with ethyl cyanoacrylate being the more electrophilic (1.71 versus 1.24 eV – data taken from
404 Enoch et al 2010). This mechanistic rationale is a significant improvement on the previous hypothesis that
405 all chemicals that cause respiratory sensitization must have multiple reactive centers (Agius et al., 1991),
406 and by the same token may explain how relatively weak electrophiles may cause sensitisation if they are
407 also capable of protein cross-linking (for example di-carbonyl conatining chemicals acting via a Schiff
408 base mechanism).).

409 **Figure 1**: Michael addition reaction for ethyl cyanoacrylate (R_1 = H, R_2 = CN, R_3 = OEt) and methyl tiglate
410 (R_1 = R_2 = Me, R_3 = OMe)



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412

413 The availability of the larger data set of respiratory sensitization data enabled further analysis into the
414 detailed mechanistic chemistry associated with the MIE for LMW chemicals (Enoch et al., 2012), resulting
415 in the identification and publication of a set of structural alerts that defined the chemistry associated with
416 covalent protein binding in the lung. An important aspect is the analysis of the associated metadata for
417 each structural alert, which documents the reaction mechanism and supporting peer-reviewed literature.
418 This information is of central importance for profilers, when they are used to group chemicals together in
419 regulatory toxicology.

420 This work in developing *in silico* profilers, and specifically a profiler for respiratory sensitization, offers
421 tools that can be used as part of a chemical assessment, prioritization, hazard assessment and
422 hypothesis generation. As outlined, *in silico* profilers encode the mechanistic information associated with
423 the MIE for organ toxicity. This information can then be used to group chemicals together, and to make
424 predictions via read-across, a process that has been supported at the OECD within the development of
425 the OECD QSAR Toolbox. Chemicals that are not sensitizing in the LLNA are anticipated not to be
426 respiratory sensitizers, a correlation that is empirically supported (Kimber et al., 2007). Yet this approach,
427 at worst, is using a different organ (skin *versus* lung) in a different species (mouse versus human) to
428 predict a chemical's ability to sensitize the human lung. Taking a broader view, the application of more-
429 detailed mechanistic chemistry knowledge can facilitate the development of better, more-relevant, non-
430 animal assays and hazard predictions. Chemistry-driven *in silico* profilers offer one of the key solutions to
431 the problem of making predictions of organ toxicity.

432 ***2.4.5 Dose-Response Models for the OEL-Derivation of Asthmagenic Chemicals***

433 The protocols applied to date for the hazard identification of respiratory sensitizers most commonly
434 employ modelling systems that evaluate the acute etiopathology rather than the chronic allergic airway
435 inflammation typical of asthma. The complex etiopathology has been modelled in the Brown-Norway rat.
436 In this model, initial sensitization is achieved by dermal application of a test compound rather than
437 inhalation. This simplifies the initial induction response, bypassing the inherent tolerogenic response of
438 the lung towards inhaled allergens. Once sensitization is established, subsequent inhalation exposure to
439 the sensitizing antigen serves to localize and amplify the immune response to the lung.

440 Concentration x time (C x t)-response relationships were evaluated on elicitation-based endpoints by
441 employing dose-escalation-like protocols (Pauluhn, 2014). Variables affecting the dosimetry of inhaled
442 irritant and chemically reactive vapors and aerosols (i.e., irritant-related changes in breathing patterns,
443 scrubbing in the upper airways in rodent models) must be thoughtfully observed, otherwise findings
444 cannot readily be translated to humans. Comparing dose-escalation protocols, different designs are
445 suitable for aerosols and reactive vapors. Both concentration and time (C x T) can be used to achieve the
446 desired pulmonary dose. For aerosols a C_{var} x t_{const} challenge protocol is best suited to quantify the lower
447 respiratory tract irritant dose (Pauluhn, 2002; Pauluhn and Poole, 2011) (Pauluhn 2004a,b; Pauluhn et al.,
448 2005). A “minimal irritant’ concentration primes the respiratory tract in predisposed, dermally sensitized
449 rats. For reactive vapors to achieve the desired pulmonary dose, the concentration selected must be high
450 enough to penetrate into the lung regions while retaining stable breathing patterns. This can best be
451 accomplished by varying time used for exposure because increasing the concentration will alter breathing
452 patterns (C_{const} x t_{var}) (Pauluhn, 2014; Pauluhn, 2015). Neutrophilic granulocytes (PMNs) in BAL were
453 considered as the endpoint of choice to integrate the allergic pulmonary inflammation, supplemented by
454 physiological measurements characterizing late-phase asthma-like responses and increased nitric oxide
455 in exhaled breath. The C_{const}x t_{var} regimen yielded the most conclusive dose-response relationship as long
456 as concentration was high enough to overcome the scrubbing capacity of the upper airways. For the
457 known human asthmagens TDI or HDI vapor) and diphenylmethane-4,4'-diisocyanate (MDI) (aerosol), the
458 elicitation threshold-dose was lower than the respective acute irritation threshold dose. Interestingly, a
459 consistent relationship of the elicitation and irritation-threshold dose seemed to exist (Pauluhn, 2008;
460 Pauluhn, 2014; Pauluhn, 2015). The respective 8-hour time-adjusted asthma-related human-equivalent

461 threshold C x t-product (dose), based on 'asthmatic' rats, was estimated to be 3-5 ppb, which is in
462 remarkable agreement of the current ACGIH TLV® of the examined diisocyanates.

463 In summary, the findings from the Brown Norway model suggests that chemical-induced respiratory
464 sensitization is likely to be contingent on two interlinked, sequentially occurring mechanisms: first, dermal
465 sensitizing encounters high enough to cause systemic sensitization. Second, when followed by recurrent
466 supra-threshold irritant inhalation exposure(s) high enough to initiate and amplify an allergic airway
467 inflammation, then a progression into asthma may occur. The Brown Norway model requires an in-depth
468 knowledge on respiratory tract dosimetry, including the concentration- and/or concentration x time-
469 dependence of respiratory tract irritation and eventually asthma. Animal models suggest occupational
470 asthma may result from exposures of both the skin and respiratory tract. For instance, to acquire
471 diisocyanate asthma, skin-sensitization followed by successive alveolar irritant inhalation encounters
472 seems to be key for the initiation and propagation of this occupational disease (Pauluhn, 2008; Pauluhn,
473 2014; Pauluhn, 2015). The Brown Norway model can deliver a NOAEL on the elicitation-response and
474 can be taken to derive a safe OEL to prevent chemical respiratory sensitization (Pauluhn, 2005; Pauluhn,
475 2008; Pauluhn, 2014; Pauluhn and Mohr, 2005; Pauluhn and Poole, 2011).

476 **2.5 Perspectives and Needs for Identification and Management of Respiratory Sensitizers**

477 ***2.5.1 Current Practices for Risk Management***

478 The risk of respiratory sensitization from exposure to chemicals is managed as with other risks, by
479 performing an assessment beginning with a hazard and exposure evaluation. An example is the
480 diisocyanate industry, where through product stewardship efforts, suppliers visit customer sites where
481 training is given on safe handling and use as well as addressing specific customer related environmental
482 health sciences issues detected during inspection (e.g., engineering controls, administrative controls,
483 work practices). Exposure monitoring for specific applications may be performed and compared to
484 occupational exposure limits. Training is as important for management as for the worker since they play
485 an important role in ensuring the implementation of new procedures/behaviors/technical controls.
486 Education on hazards and exposure reduction is considered so important that industry groups have

created free online training programs as well as readily available brochures in Spanish and English.

Risk management practices have improved over time through improved analytical methods utilizing more efficient collection of vapor & aerosol and the use of more sensitive and specific LCMS for inhalation exposure assessment. Newly developed techniques and methods for improving dermal assessments have increased the understanding of protection degree provided by personal protective equipment. There has been an increased emphasis on dermal protection since dermal exposure may contribute to risk of developing respiratory sensitization. There have also been improvements in biomonitoring techniques where albumin or hemoglobin adducts in blood samples have been shown to be more specific and more sensitive biomarkers of exposure than diamine hydrolysis products in urine.

Current practices have demonstrated that an effective product stewardship program can reduce risk. Significant reductions of diisocyanate-related occupational asthma (OA) cases have been observed globally. Ontario, Canada reported a decrease from 30.5 OA claims/year (1980-1993) to 7.4 claims/year (1998-2002) (Buyantseva et al., 2011). Michigan's Project SENSOR report that the rates of asthma attributable to diisocyanates have fallen from 22.9 cases/yr (1988-1997) to 4 cases/yr (2009-2010) (NIOSH, 2014). Many authors relate the reduction to industry recommendation for medical surveillance, the use of periodic spirometry and examinations targeted to skin and respiratory tract. In addition, using an accepted paradigm to accurately diagnose respiratory sensitization (occupational asthma) by objective measures and the use of specific inhalation challenges when necessary to confirm the relationship of asthma to diisocyanate exposure may have contributed to the lower numbers of new OA cases.

2.5.2 WHO's Guidance on Assessment for Respiratory Sensitization

The WHO Guidance for Immunotoxicity Risk Assessment for Chemicals (chapter 6) provides a framework for conducting of risk assessments for both induction and elicitation of skin allergy, respiratory allergy and oral (systemic) allergy (WHO, 2012). This includes a decision-tree towards developing a WoE based on the available human, laboratory animal and mechanistic data associated with exposures to a potential respiratory sensitizer (Figure 3). The case study (#3) of halogenated platinum salts illustrates how a quantitative risk assessment can be conducted for a chemical respiratory sensitization.

513 When a chemical is characterized as a sensitizer based on the WoE, the data can be applied to a dose-
514 response assessment beginning with selecting the most appropriate end-points and developing point of
515 departures. With regard to halogenated platinum salts, numerous occupational studies report allergic
516 reactions following exposure (WHO, 2012). Respiratory symptoms include airway constriction and
517 inflammation, shortness of breath, wheezing, and rhinitis. Several occupational studies show increased
518 prevalence of workers with respiratory allergy. Merget et al. (2000) provide sufficient exposure data with
519 evidence of health effects for a quantitative risk assessment with a NOEL for respiratory sensitization (3.4
520 ng sol Pt/m^3), which after adjustment for uncertainty resulted in a reference value of 0.012 ng sol Pt/m^3.

521 A similar process can be used to determine elicitation potency. For halogenated platinum salts, however,
522 there is insufficient quantitative elicitation information to proceed with a quantitative risk assessment.
523 Unfortunately, there are few human provocation studies for any potential respiratory sensitizer. In the
524 case of platinum there were studies using positive skin prick tests in sensitized workers as an endpoint,
525 however the range of doses spanned several orders of magnitude. Thus for elicitation only a qualitative
526 assessment is possible.

527 Although a reference value for sensitization from halogenated platinum salts was derived, the case-study
528 also illustrates the several challenges and limitations in the approach. The reference value is applicable to
529 the workplace exposures. However environmental exposures tend to be from a different form of platinum
530 (insoluble complexes). Thus, extrapolation from the workplace to environmental exposures is difficult
531 because of potential differences in chemical form. Overall, the halogenated platinum case study illustrates
532 that a quantitative risk assessment of sensitization is possible (WHO, 2012). However, for many
533 chemicals the data base is insufficient to derive a quantitative assessment and a qualitative or descriptive
534 assessment of sensitization and elicitation is all that is possible.

535 ***2.5.3 EU Regulatory Needs – REACH and CLP***

536 In Annexes VII to X of the REACH Regulation there is no standard information requirement concerning
537 respiratory sensitization, but chemical safety assessment according to Annex I covers sensitization
538 overall. ECHA Guidance chapter R.7.a Endpoint specific guidance describes how to use human and non-

human data in the context of REACH. For sensitizers the chemical safety assessment can be based either on qualitative approach or quantitative risk characterization. Under substance evaluation “Further information” can be requested even beyond the information mentioned in Annexes VII to X of REACH, if there is a concern that a given substance may constitute a risk to human health or the environment, and further information is needed to clarify the concern. For 13 out of 51 substances to be evaluated in 2014, the initial concern (or one of) is respiratory sensitization.

The main risk management options for respiratory sensitizers classified based on Classification, Labeling, and Packaging European Commission Regulation No. 1272/2008 (CLP) under REACH are authorization, restriction or no action (which does not prevent action under other legislation). The prerequisite for subjecting a substance to the authorization requirement is its identification as a SVHC. However, sensitization is not a SVHC criterion itself under REACH Article 57. Therefore, a sensitizing substance must be identified in accordance with Article 57 (f) as giving rise to an equivalent level of concern to the carcinogenic, mutagenic or reprotoxic substances. This requires a case-by-case assessment. The general approach on identification of a sensitizer as having equivalent level of concern under article 57(f) has been agreed with the EU Member States (ECHA, 2013b).

Under CLP Regulation respiratory sensitizers are considered to be among “substances of the highest concern”. In the absence of validated animal models or alternative approaches, the evidence that a substance can lead to specific respiratory sensitization is normally based on human experience, but some animal data can be used in a WoE approach as explained in the CLP guidance (ECHA, 2013a).

In the absence of a validated standard method, case-by-case judgment and weight-of-evidence approaches are necessary for regulatory purposes (hazard information and classification). This is not an optimal situation for a health effect as important as respiratory sensitization. For both REACH processes and CLP, there is a need to differentiate between respiratory sensitizers according to their sensitizing potential and potency and validated methods or approaches would be desirable. In a regulatory framework, an information request (test data or tiered approaches) would, however, need to be such that a reasonably definitive answer to the concern is delivered.

3. RESULTS FROM WORKSHOP BREAKOUT GROUP DISCUSSIONS

3.5 Understanding the Condition

Relevant immunological mechanisms. All breakout groups agreed that Th2 responses predominate in respiratory sensitization. In one of the groups there were divergent views that Th2 responses may not be the sole explanation. The role of IgE is highly likely as well, although IgE is not always detected in humans with occupational asthma and in animal models. Other mechanisms may be involved in the elicitation phase of respiratory allergy, i.e., neurogenic inflammatory responses were mentioned (see below).

There was general agreement on certain essential steps that are required to induce respiratory sensitization, including chemical hapten-protein binding, induction of danger signals by the epithelium, DC activation, maturation and migration, T cell activation and clonal expansion towards a Th2 response and B cell maturation and antibody formation, in some cases IgE. Protein binding was considered to be the molecular initiating event (MIE), similar to that of skin sensitization. Participants acknowledged that the theory on hard versus soft acid base for electrophiles may be useful in discriminating respiratory from skin sensitizers (Enoch et al. 2009, 2010), but that there is a need to define and explore this theory further (i.e., nucleophile chemistry, nucleophile mechanisms that react with cysteine are related to Th1 response).

There was also agreement that many cellular events involved in respiratory sensitization are relatively well understood, i.e., DC activation, T and B cell activation. The cellular sources that deliver the danger signals, however, are not well-defined and different cell types in the airways or skin may be involved in this. Furthermore, the exact mechanisms of action have not been studied in detail and are thus largely unknown. Interestingly, there is an overlap in cellular events with skin sensitization for example protein binding, DC activation, T cell activation. Given that the effector immune response is different, it is important to identify the ‘master switch’ that determines Th2 skewing which will require understanding the key signaling and molecular pathways involved in the adverse outcome. Also, it was proposed that homing of DCs may differentiate between skin and respiratory sensitizers. Despite the general

591 understanding of the cellular events involved, additional gaps and research needs were identified
592 including the role of epigenetics (i.e., DNA methylation, histone modification, microRNA, etc.).

593 Participants also proposed that information from protein sensitizers could be used for further identification
594 of key signaling and molecular pathways, but no consensus was reached on this topic. For protein
595 sensitization much more mechanistic studies have been performed, hence data are available that might
596 support information on chemicals and improve mechanistic understanding. However, there were concerns
597 regarding whether the mechanisms and the inflammatory responses are the same for induction and
598 elicitation. Neurogenic and neuro-immuno mechanisms are involved in the elicitation phase of respiratory
599 allergy. These mechanisms are involved in bronchoconstriction, for example. The role of these
600 mechanisms in the induction of allergy is not fully understood.

601 An updated, contemporary view of skin and lung physiology delineating the key similarities and
602 differences between organs to benefit understanding of underlying immunological mechanisms was also
603 suggested. As discussed above, there are clear differences in potential consequence for immune
604 activation in skin versus lung. In skin the immediate effects may be localized with reduced potential for
605 systemic impact, whereas inflammation in the lung may have greater systemic impact owing to its role in
606 gas exchange and the volume of blood flow through the lung.

607 **A unifying dose metric to address induction/respiratory elicitation and/or help threshold**
608 **identification.** No workgroups identified a potential unifying dose metric for dermal and respiratory
609 sensitizers. While such a metric could prove useful in study design and interpretation, current knowledge
610 of the underlying processes resulting in induction and elicitation appear inadequate to arrive at a
611 consensus, or even minority, view. In the absence of such a metric, the workgroups generally considered
612 a WoE approach to be the realistic option for threshold identification. Where available, human dose
613 response data would represent the “gold standard,” as demonstrated with halogenated platinum salts.
614 Such data is relatively rare, so in practice the information most likely to be applied in dose response
615 assessment comes from dermal LLNA or inhalation toxicology studies. In case of dermal induction
616 studies, the dose metric used for skin sensitizers can be used, which is $\mu g/cm^2$ skin. For the respiratory

route, the dose metric used both in human as well as in animal studies is ppm. Where testing in animal models is not permitted (*i.e.,* cosmetics) dose response assessment will likely have to turn to *in vitro*, *in chemico*, and *in silico* methods (several of which are described above) to estimate dose responses.

3.6 Overview of available models

Strengths, weaknesses, opportunities, and challenges. The need for rapid, inexpensive and validated in silico and in vitro model systems was identified as a key area where considerable progress has been made but where significant challenges remain. QSAR models are available for dermal sensitizers but not respiratory sensitizers. Structural profiling has the potential to provide insight into possible differences in the initial molecular interactions between low molecular weight chemicals and host proteins. These may include preferential binding of chemicals to specific amino acid residues based on well characterized physical properties (acid/base theory, electrophilicity, etc.). These expert systems may eventually be used to identify and differentiate potential skin and respiratory sensitizers from irritants and toxicants, however, the predictive power of these *in silico* models must be validated against known sensitizing chemicals. This may be difficult in the case of respiratory sensitizers since, compared to skin sensitizers, there are relatively few known human respiratory sensitizers and these are represented by only a few chemical classes (e.g., isocyanates, aldehydes, anhydrides and dyes and platinum salts). It is likely that numerous models will be developed and disseminated. One potential challenge may come if the availability and use of new profiling tools proceeds more rapidly than their validation. Confidence in the predictive power of newly developed tools will only come through repeated confirmatory testing in other in vitro and in vivo model systems, building a WoE case for the models. For instance, the direct peptide reactivity assay (DPRA) is a means to assess protein reactivity of electrophilic chemicals. DPRA is an *in chemico* method which determines the reactivity of chemicals to peptides containing nucleophilic cysteine and lysine residues. It has been hypothesized that chemicals that preferentially bind to lysine form molecular interactions with host proteins that result in the development of a Th2-bias, characteristic of respiratory sensitizers. This is a testable hypothesis that can may be used to discriminate between skin (cysteine-binding) and respiratory (lysine-binding) sensitizing chemicals.

Distinguishing events associated to local irritation and systemic sensitization. A number of *in vitro* cellular systems were described that help probe specific key events in the development of the sensitized state. The KeratinoSens assay can be used to identify skin sensitizers through their ability to activate the Keap1-Nrf2-antioxidant/electrophile response element (ARE). Dendritic cells, either freshly isolated or cell lines, may be used to differentiate chemicals leading to a Th1- or Th2-bias. Organotypic 3D airway epithelial cell cultures, grown at the air/liquid interface (ALI) may be used to investigate the role of the epithelium in "reading" the hapten-protein complex and expressing specific signals that may interact with the innate immune system to influence the maturation and Th1/Th2-bias of mucosal dendritic cells. These *in vitro* cellular systems are valuable tools to examine specific cellular responses, but more complex co-culture systems may need to be developed to explore the cell-cell interactions involved in development of the sensitized state. Precision cut lung slices may provide short term test systems to explore cell-cell interactions, however they cannot be maintained in culture long, and dosimetry is complicated and limited by diffusion due to the need to inflate the lung with agar prior to cutting the slices. Organotypic ALI cultures overcome the dosimetry problem, but do not contain all of the cell-types found in the intact lung.

In vivo model systems have been developed to assess the induction of the sensitized state. The LLNA measures proliferation in the draining lymph node to identify sensitizing chemicals. It is a widely used and validated method that can identify sensitizing chemicals, but it cannot distinguish respiratory from skin sensitizers, and care must be used to differentiate irritant responses from immune-responses. The LLNA can provide data on potency and when coupled with direct exposure of the respiratory tract and analysis of the appropriate draining lymph nodes may provide insight into local immune responses in the upper/lower respiratory tract. The LLNA has been coupled with "omics" endpoints in an attempt to identify gene expression profiles characteristic of skin and respiratory sensitizers and irritating chemicals. Progress has been made but a definitive profile that can uniquely identify and differentiate sensitizing chemicals has not been identified. Part of the problem may be a lack of standardization of the exposure and sampling protocols between laboratories. Evidence suggests that even the most basic of experimental variables, such as dose, dose number and timing and sample time, may influence the results. Cytokine profiling and IgE expression are likewise valuable complementary assays with which to build a WoE case for respiratory sensitizers on a case by case basis.

Confounders and strategies to reduce their effect. *In silico*, *in chemico*, *in vitro* and most *in vivo* model systems available at this time examine only the induction or sensitization phase. This is important because of the profound importance of being able to identify which chemicals may be skin or respiratory sensitizers, differentiate between the two, and assess potency as it impacts thresholds of sensitization. A clear and definitive identification of a no-effects threshold, perhaps using a combination of *in vitro* assays targeted at key events in the AOP, would provide immediate impact on the safe use of chemicals with sensitizing potential. Some may argue that from a regulatory perspective, due to the wide range of human sensitivities to chemical exposures, that one must assume that induction of sensitization is a given. The question then becomes whether a threshold of elicitation can be identified so that a sensitized individual can be protected from progression of the disease through preventing repeated bouts of secondary elicitation reactions. Evidence from the widespread use of isocyanates and biocides suggests that these sensitizing agents may be safely used if the potential for exposure is controlled. At this time, only the Brown Norway rat model has been demonstrated to be useful to address thresholds of elicitation. This model is time consuming and expensive, requires expertise in the conduct of inhalation exposures that may not be available in many laboratories and presents challenges in regional dosimetry related to both the physical/chemical nature of the test chemical and the anatomy of rats which are obligate nose breathers. It is, however, justified on a case by case basis in order to derive a safe level of exposure in occupational settings.

3.7 Identification of research needs

Multiple research needs were identified regarding improving our understanding of chemical respiratory allergy, including mechanistic and clinical questions. With regard to mechanistic research questions the areas of interest identified were related to potential differences in tissue level responses (such as development or evasion of immune tolerance, bioavailability, homing responses) and cell level responses (mechanistic chemistry, the role of danger signals, and potential for differences in DC activation). The potential for differential responses between skin and respiratory sensitizers related to immune tolerance was considered. From a generic immunological perspective the creation of non-self epitopes via protein adduct formation appears similar for skin and respiratory sensitizers (albeit with different amino acid

698 preferences discussed above), yet many of the skin sensitizers are “tolerated” by the respiratory tract. If
699 some feature of chemical respiratory sensitizers breaks tolerance, that may aid in correct identification of
700 hazards. Differences in bioavailability, in the context of access to a complete immune system, were
701 considered as a possibility. Empirical evidence supports differences in cysteine and lysine reactivity, size,
702 and reactivity may alter the localization of responses to chemical sensitizers, in some cases limiting the
703 availability of non-self epitopes to the adaptive immune system. It is tempting to speculate that differences
704 in these parameters for chemical respiratory sensitizers, when integrated into a cumulative activation
705 signal to the immune system, either generate a qualitatively different signal or exceed some unelucidated
706 threshold that skin sensitizers do not. Along the lines of bioavailability is the potential for chemical-specific
707 differences in homing associated with different phenotypes. Put simply, if dermal induction does not result
708 in responsive immune cells arriving in the lung a reduced likelihood of response might be expected.

709 At the cellular level a clearer understanding of the mechanistic chemistry, and subsequent biological
710 responses, underlying sensitization responses was considered desirable. While *in silico* and *in chemico*
711 approaches to assessment have improved, the further extension and refinement of those approaches
712 could continue to contribute to hazard identification and assessment. Similarly, while several cytokines
713 have been strongly implicated as key factors in differentiating sensitization responses, a wide range of
714 “danger signals” for the immune system exist. Which signals contribute or control the resulting cellular
715 phenotype remains a promising area. One key cell type in guiding immune response is the DC. Whether
716 skin and respiratory sensitizers result in differential activation or phenotype in DC may provide useful
717 mechanistic understanding of the subsequent immune response.

718 Multiple clinical research questions were also identified in the breakout groups. At a basic individual level,
719 clarity on what fraction of chemical respiratory sensitized individuals also develop positive skin patch
720 responses could benefit or clarify the diagnostic value of the test. At a higher population level a global
721 compilation of data on actual human chemical respiratory sensitization cases may be fruitful in
722 understanding the condition. While multiple national databases exist (Canada, United Kingdom,
723 Germany, and United States), they operate independently. A powerful illustration of value in unifying
724 databases can be seen in cancer registries. Regional registries have limited power for use in research,

but national (or international) registries can be more useful (Steliarova-Foucher et al., 2015). Such an effort naturally comes with challenges including definition or criteria for classifying cases, but may be worth the additional effort.

The role of IgE in respiratory sensitization remains unresolved. There is a long history of difficulties in assessing the role of IgE as related to chemical respiratory sensitization (Kimber and Dearman, 2002; Kimber et al., 2014; Kimber et al., 1998), yet the potential application in hazard identification for humans remains attractive. Lacking methods to address how (e.g., serum chemical-specific or total IgE by ELISA) and when to measure (e.g., anytime or only after challenge), and how to apply IgE assessment in predictive animal models, continues to be a scientific challenge.

A concept shared across breakout groups was the value of a consensus list of respiratory sensitizers. To that end, developing such a list could begin with agreement on assessment criteria, then build through the identification and consideration of data compared to the criteria (Selgrade et al., 2012). While there are widely accepted examples of respiratory sensitizers from which the list could begin (e.g., TDI, MDI, TMA), a consensus list would ideally not necessarily identify entire classes of chemical. It is unclear how representative TDI and the like are of all members of their categories (e.g., diisocyanates and acid anhydrides). As discussed above, chemicals from similar categories or with similar reaction mechanisms can vary in their electrophilic potential for protein reactivity. In some cases, despite having functional groups associated with hazard, the inherent reactivity for some chemicals may be too low to induce sensitization.

Multiple groups also consider skin likely to be relevant to induction. As discussed above, in the light of potential physiologic consequences from robust immunologic responses in airway versus skin (airway responses can have a systemic adverse effect owing to impaired gas exchange, whereas skin responses generally have local adverse effect) it appears all the more plausible that local skin exposure followed by systemic immune memory could play a contributory, if not major, role in the etiology of chemical respiratory sensitization.

Among the breakout groups there was divergence regarding whether protection from irritation also

provides protection from elicitation, and if a single elicitation threshold exists for each chemical. As summarized above, empirical data derived with TMA support the case that protection from irritation also covers elicitation, but extending those findings to a broader range of chemicals has yet to happen. Regarding single elicitation thresholds for each chemical, workgroups were uncertain about whether current models would be suitable for such efforts.

3.8 Future Strategies for Testing

Concerns, legislation and research needs have precipitated developments such as the MoA concept, the Tox21 strategy, the concept of Pathways of Toxicity and the AOP framework. The common goal of these developments is toxicity assessment based upon in-depth understanding of the *in vivo* physiological and toxicological processes in humans and on their relation to specific key molecular events or toxicological endpoints (Ankley et al., 2010; US National Research Council, 2007). This workshop addressed new technologies and paradigms that are currently transforming these concepts into applicable animal-free toxicity testing systems by implementation of libraries of generic profiles of genes (genomics), proteins (proteomics) and metabolites (metabonomics) describing molecular initiators, pathways and key events of toxicity within tissues, organisms and biological systems (Berg et al., 2011). The key themes for future testing strategies that emerged from the workshop discussions are described below.

Threshold Assessment. While the expectation of thresholds for both induction and elicitation was communicated from multiple participants in the workshop, it was also recognized that the methods to quantify those thresholds are currently limited. The workgroups identified several approaches to identification of thresholds for elicitation. In assessing potential for elicitation thresholds in animals there is data to support use of the Brown Norway rat . Beyond animal models, workgroups identified approaches combining human exposure data with clinical assessment to help delineate thresholds. Examples include workplace exposure monitoring, post-implementation assessment of engineering controls, and post-exposure evaluations correlated to clinical assessments.

It was generally recognized that identifying a threshold for induction is challenging, and the potential for thresholds was recognized during the meeting as potentially divergent among groups. The challenge may

in part be understood in the potential for a reciprocal dose response to chemical sensitizers, wherein low doses may trigger induction but require high doses for elicitation and high doses triggering induction lead to reduced doses causing elicitation. Such a relationship creates challenging questions for experimental assessment of thresholds. One of the justifications for considering respiratory sensitizers as substances of equivalent concern to carcinogen, mutagens, or reproductive hazards is deriving "a safe concentration may not be routinely possible and any figure derived would be associated with large uncertainty." This stems from difficulties in measuring induction and elicitation thresholds, particularly because the induction dose may vary depending on the individual.

Role of the LLNA. Multiple workgroups identified the dermal LLNA as a potentially key piece of information in assessing respiratory sensitization potential. One gap in knowledge identified was that while the LLNA provides a very solid tool for potency ranking, which appears to translate well between species, whether the animal model EC3 value is directly, quantitatively translatable to humans is not fully understood. Several caveats or complications to application of the LLNA were identified:

1. LLNA is capable of identifying proliferation, but simply assessing proliferation may lead to occasional false positives.
2. Some compounds, particularly corrosive materials, may not be tested in the LLNA. In such a scenario it may still be possible to conduct an experiment if non-corrosive formulation (*i.e.,* diluted) test materials can still be used.
3. Due to the Cosmetics Directive the use of the dermal LLNA (i.e. animal testing) is not permitted in some nations.

Toward a Tiered Framework. The workgroups generally considered a tiered framework for assessment of respiratory sensitization potential a possibility, particularly for the purpose of screening and prioritization. Where the data is available, the dermal LLNA and standard inhalation toxicology testing was considered an appropriate first point in an assessment. If the LLNA indicates the material is not a skin sensitizer, it was considered unlikely to be a respiratory sensitizer and an assessment could stop there. By themselves standard inhalation toxicity studies would not typically be expected to provide much

information regarding sensitization potential; however, they may contribute to a WoE if there is no pathological findings or if pathologic findings indicate immunologic engagement (*e.g.,* post-nasal inflammation or lymph node alterations). If the LLNA results indicate sensitizing potential, or there is no data available, one turns to SAR and in vitro data next. All the existing available data can be used to build a WoE assessment, which may also assist in the prioritization of any decisions to develop additional experimental data.

Based on collated feedback from the workgroups, Table 1 was developed to identify potentially useful information in WoE assessment. Data from the dermal LLNA, if available, could be considered a useful starting point in an assessment. If results indicate sensitizing potential, or no data is available, assessors might progress down the table to consider what data is available. Tools for *in silico* analysis are readily and freely available (*e.g.,* OECD QSAR Toolbox), so in cases where data is not already available it can be obtained with relative ease. While the table suggests a progression from *in chemico, in vitro,* and finally to *in vivo* models, the relative contributions are not necessarily rank ordered in Table 1. Consider a hypothetical example where bioavailability may be low on the basis of *in vitro* measures, but other tests suggest effects on DC activation or T cell proliferation. While a simple example, it illustrates why WoE assessment can be challenging. In some cases deriving *in vivo* data may not be possible, a challenge facing the cosmetics industry, and thus would likely need to be built upon *in silico*, *in chemico*, and *in vitro* data.

Table 1. Summary of Non-conventional methods to assess respiratory sensitization

Method(s)/Model(s)	Strengths and Weakness	Key Reference(s)
Non-Conventional Methods		
***In silico* profiling** • Mechanistic chemistry evaluation • Structure activity relationships[1]	+ Rapid + Low-cost + Suitable for diverse range of structures + Suitable for diverse range of chemistries - Model dependent on being in domain - Need for expert interpretation of borderline results - Not always empirically confirmed	(Enoch et al., 2012)
Direct chemical methods • Peptide reactivity (DPRA/PPRA/CPRA)	+ Rapid + Low-cost + Quantitative measure of reactivity + Provides information on amino acid preference - Limitations on metabolic functionality in system	(Aleksic et al., 200; Lalko 2013)

	-	may lead to false negatives Small database of observed results	
Bioavailability • Human cell based assay for pulmonary absorption of chemicals	+ -	Provides indication of chemical access to immune system Not an indicator of immune endpoint	
Epidermal Inflammation (cytokine profile or stress pathway activation) • human precision cut lung slice • *In vitro* alveolar-capillary barrier • Air liquid interface organotypic 3D airways epithelial model	+ + - -	Indicator of danger that may influence immune response May discriminate irritants from sensitizers Metabolic capability may be uncertain Some require high degree of technical expertise	(dos Santos et al., 2009; Hermanns et al., 2010; Roggen, 2013). www.epithelix.com
DC Activation or Maturation • skinGARD • respGARD • h-CLAT	+ -	Direct measure of immune system engagement May overlook compounds requiring metabolism	(Ashikaga et al., 2010)
DC Migration[2] • Langerhans Cell Skin Equivalent model	+ + -	Direct measure of immune system engagement Reflective of tissue organization that may influence response May overlook compounds requiring metabolism	(dos Santos et al., 2009; Ouwehand et al., 2011)
Basophil/eosinophil • IL-6 responses in myeloid cell lines	+ - -	Indicator of responses that may lead to airway reaction May be insensitive to chemical effects early in AOP Unclear how many respiratory sensitizers have direct activity on mast cells	
Genomics • Gene expression array analysis	+ -	Unbiased, systematic assessment of cellular response Requires high degree of technical expertise	(Adenuga et al., 2012; Boverhof et al., 2009; Kuper et al., 2008a; Kuper et al., 2008b)
Proteomics • 2D electrophoresis and MALDI-TOF	+ -	Unbiased, systematic assessment of cellular response Requires high degree of technical expertise	(Haenen et al., 2010; Jeong et al., 2005; Louten et al., 2012; O'Neil et al., 2011; Park and Rhim, 2011; Zhang et al., 2009)
Conventional Methods			
Dermal LLNA	+ + + - -	Strong empirical evidence for absence of false negatives Incorporates entire immune function Can demonstrate hyperresponsiveness Does not discriminate skin and respiratory sensitizers May be confounded by irritants	
Cytokine Profiles	+ -	Provides useful information to assess Th1/Th2 skewing May not show hyperresponsive shift	
Standard Inhalation Toxicology Studies[4]	+ + - - - -	Frequently available for inhaled chemicals Generally rigorous examination of airway health Does not evaluate hyperresponsiveness May be difficult to distinguish irritant and sensitizer response Exposure paradigm not designed for sensitizer assessment Commonly tested strain tend towards Th1 responses	
Subacute repeat exposure with challenge	+ +	May provide comparative potency Anchored to observable, adverse responses	

	-	Limited empirical data to characterize domain - Intratracheal exposure may not model real-world conditions	
Subchronic repeat exposure-rest block with challenge (Brown Norway Model)	+	May provide suitable point of departure for deriving reference values (i.e., thresholds, NOAELs) + Exposure model reflects periodic nature of real world + Design allows for assessing development of hyperresponsive phenotype - High cost and technical expertise requirements - Limited empirical data to characterize domain	(Pauluhn, 2008; Pauluhn, 2014; Pauluhn, 2015)

One of the key challenges to applying *in vitro* tests to an assessment is incorporating a systems biology perspective to the results. Results in DC's may be confounded if keratinocyte metabolism is a key event in a chemical's mode of action. The importance of system-level consideration may be one of the strengths of the PCLS model. Because it contains multiple cell types in system coherent structure it may be a particularly rigorous tool for use in an assessment. Workgroups also identified dosimetry as a key consideration for interpreting results from *in vitro* models. While the *in vitro* models may allow for testing at non-physiologic concentrations, the value of the results may be reduced for developing a solid WoE assessment.

One of the more promising approaches to WoE assessment for sensitization potential is in Bayesian approach to the available information. In Bayesian analysis each new piece of information can be used to refine a prediction. The value of a Bayesian analysis for sensitization has been demonstrated for skin (Jaworska et al., 2013), which demonstrated a 95% accuracy for hazard classification and 86% accuracy for potency classification. One of the greatest strengths of Bayesian analysis is that as more data becomes incorporated overall prediction accuracy may be improved. However, a similar effort for respiratory sensitizers has not been undertaken to date.

Methodological Considerations. The workgroups noted several methodological characteristics that warrant consideration when assessing respiratory sensitization potential:

- The maximum tolerated dose for a dermal LLNA may be at the irritation threshold.
- Irritation and induction may be difficult to differentiate using current approaches; applying –omics technologies may aid the differentiation, but the available datasets are still relatively limited in

scope.

- Standardizing inhalation induction models may be exceedingly difficult owing to individual chemical characteristics. Highly reactive chemicals may be scrubbed higher in the airway, irritating chemicals may change the breathing pattern and thus deposition pattern, and classic inhalation toxicology challenges related to aerosol versus vapor behaviors will apply.
- For definitive *in vivo* inhalation studies there are endpoints beyond the typical measures that can benefit an assessment. In particular bronchoalveolar lavage fluid characterization, exhaled nitric oxide measurement, enhanced histology (immunohistochemistry), lung function measures (e.g., methacholine challenge), and even lung weights (wet and dry) were identified as potentially useful.

Case of the Brown Norway Rat Model. Multiple workgroups considered the Brown Norway rat a potential model for assessing elicitation. As promising as it appears, its current iteration would make it a technical challenging model to use, requiring sophisticated inhalation exposure and assessment technologies conducted over a relatively long study period (66 days). Given the resources necessary to apply such a model, it is conceivable that the model is better suited to deriving a point of departure for risk assessment than routine screening for hazard identification.

4. CONCLUSIONS AND FUTURE WORK

While scientific progress has moved forward in the period between the 2004 and 2014 workshops, a satisfactory answer to the question of how to best assess and characterize chemical respiratory sensitization remains elusive. Substantial progress has occurred in the development of non-traditional assessment models (*in silico* and *in vitro*), whereas an understanding of some of the more fundamental pathophysiologic characteristics such as the role of IgE in human chemical sensitization remain largely where it was a decade ago. The uneven advancement may reflect several challenges, particularly the high investment cost and complexity of animal models for chemical respiratory sensitization coupled with the relative rarity (whether real or perceived) of the hazard property. A rational path forward for research would be studies designed to support or refute key events in an AOP for chemical respiratory

869 sensitization (Kimber et al., 2014).

870 Despite uneven advancement, the near term future for assessment of chemical respiratory sensitizer
871 potential appears situated to capitalize on multiple lines of evidence to arrive at a conclusion. While not
872 the simplest method to assess hazards, the WoE approach remains the best available option in the
873 absence of validated methods for assessment.

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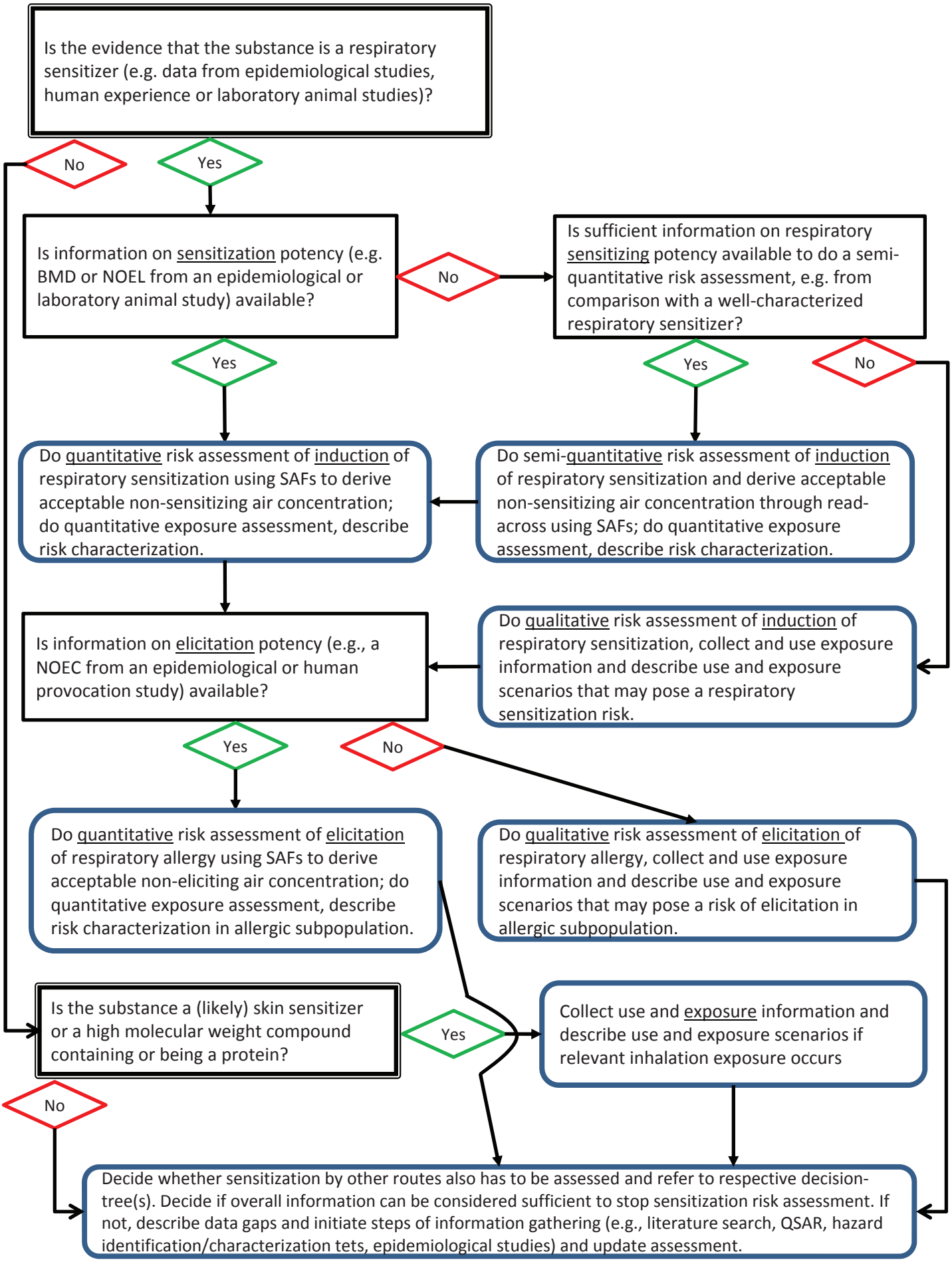
Is the evidence that the substance is a respiratory sensitizer (e.g. data from epidemiological studies, human experience or laboratory animal studies)?
No
Yes
Is information on sensitization potency (e.g. BMD or NOEL from an epidemiological or laboratory animal study) available?
No
Is sufficient information on respiratory sensitizing potency available to do a semi-quantitative risk assessment, e.g. from comparison with a well-characterized respiratory sensitizer?
Yes
Yes
No
Do quantitative risk assessment of induction of respiratory sensitization using SAFs to derive acceptable non-sensitizing air concentration; do quantitative exposure assessment, describe risk characterization.
Do semi-quantitative risk assessment of induction of respiratory sensitization and derive acceptable non-sensitizing air concentration through read-across using SAFs; do quantitative exposure assessment, describe risk characterization.
Is information on elicitation potency (e.g., a NOEC from an epidemiological or human provocation study) available?
Do qualitative risk assessment of induction of respiratory sensitization, collect and use exposure information and describe use and exposure scenarios that may pose a respiratory sensitization risk.
Yes
No
Do quantitative risk assessment of elicitation of respiratory allergy using SAFs to derive acceptable non-eliciting air concentration; do quantitative exposure assessment, describe risk characterization in allergic subpopulation.
Do qualitative risk assessment of elicitation of respiratory allergy, collect and use exposure information and describe use and exposure scenarios that may pose a risk of elicitation in allergic subpopulation.
Is the substance a (likely) skin sensitizer or a high molecular weight compound containing or being a protein?
Yes
Collect use and exposure information and describe use and exposure scenarios if relevant inhalation exposure occurs
No
Decide whether sensitization by other routes also has to be assessed and refer to respective decision-tree(s). Decide if overall information can be considered sufficient to stop sensitization risk assessment. If not, describe data gaps and initiate steps of information gathering (e.g., literature search, QSAR, hazard identification/characterization tets, epidemiological studies) and update assessment.