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Developing a framework for assessing respiratory sensitization: A workshop report

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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

1 **ABSTRACT**

2 Respiratory tract sensitization can have significant acute and chronic health implications. While induction

3 of respiratory sensitization is widely recognized for some chemicals, validated standard methods or

4 frameworks for identifying and characterizing the hazard are not available. A workshop on assessment of

5 respiratory sensitization was held to discuss the current state of science for identification and

6 characterization of respiratory sensitizer hazard, identify information facilitating development of validated

7 standard methods and frameworks, and consider the regulatory and practical risk management needs.

8 Participants agreed on a predominant Th2 immunological mechanism and several steps in respiratory

9 sensitization. Some overlapping cellular events in respiratory and skin sensitization are well understood,

10 but full mechanism(s) remain unavailable. Progress on non-animal approaches to skin sensitization

11 testing, ranging from in vitro systems, –omics, in silico profiling, and structural profiling were

12 acknowledged. Addressing both induction and elicitation phases remains challenging. Participants

13 identified lack of a unifying dose metric as increasing the difficulty of interpreting dosimetry across

14 exposures. A number of research needs were identified, including an agreed list of respiratory sensitizers

15 and other asthmagens, distinguishing between adverse effects from immune-mediated versus non-

16 immunological mechanisms. A number of themes emerged from the discussion regarding future testing

17 strategies, particularly the need for a tiered framework respiratory sensitizer assessment. These

18 workshop present a basis for moving towards a weight-of-evidence assessment.

19 **1. INTRODUCTION**

20 Respiratory sensitization is a health hazard that can occur following exposure to chemical or biological

21 materials. The adverse outcome is an allergic-type response of the airways, mostly asthma or rhinitis. The

22 disease develops in two phases: the sensitization or induction phase in which the immune system is

23 primed and the elicitation phase in which the allergic symptoms occur. Respiratory sensitization/allergy is

24 characterized by a progressive increase in immune system responsiveness, such that sensitized

25 individuals respond to exposures that elicit no effect in non-sensitized populations. Accurate identification

26 of respiratory sensitizers is important because the health effects can be severe and long-lasting. At the

27 same time, incorrect identification of a material as a respiratory sensitizer can result in unnecessarily

28 stringent restrictions on use.

29 From a toxicological perspective this human health hazard presents a number of challenges, including the

30 uncertainty regarding the mechanisms through which sensitization of the respiratory tract to chemicals is

31 acquired. This has hindered development of methods for the identification and characterization of

32 chemical respiratory allergens. The Globally Harmonized System (GHS) for hazard classification

33 considers evidence from human responses, or "appropriate animal models" which are not standardized.

34 Unlike other hazard endpoints used for classification, there is not an internationally accepted animal test

35 guideline. Different published protocols exist for assessing respiratory sensitization, but no systematic

36 undertaking has validated any of the methods for a broad range of materials. Historically, the guinea pig

37 has been the species of choice for research on respiratory sensitization due to physiological similarities of

38 respiratory reactions compared to humans. Time and cost considerations, as well as a lack of suitable

39 immunochemical or molecular probes for mechanistic evaluations, have led many to look for other animal,

40 and non-animal alternative, test systems. Experimental models using rats and mice have been successful

41 in inducing chemical respiratory sensitization, but the parameters providing best predictive performance

42 remain unknown. Current alternatives face challenges in the form of a relatively limited chemical

43 respiratory sensitizer database and knowledge limitations related to which exposure-response

44 parameters are the best predictors of respiratory sensitization. The ability to accurately detect potential

45 respiratory sensitizers is ultimately hindered by the absence of standard, validated and regulatory

4

46 accepted methods to identify potential respiratory sensitizers and distinguish them from irritants and skin

47 sensitizers for hazard identification. The difficulty in distinction is further compounded by absence of

48 generally accepted methods to define dose thresholds for irritation, which may make distinguishing

49 between immune-mediated and non-immunological responses unclear.

50 The lack of defined approaches for evaluation of respiratory sensitization potential has necessarily

51 represented a major constraint on effective risk assessment and risk management, and on addressing

52 satisfactorily the requirements of regulations such as the Regulation concerning the Registration,

53 Evaluation, Authorization and Restriction of Chemicals (REACH). There is increasing regulatory pressure

54 to list respiratory sensitizers as substances of very high concern (SVHC) based on an "equivalent level of

55 concern" as set out in REACH Article 57(f). This approach assumes that in certain cases, the impacts

56 caused by sensitizers (respiratory or dermal) on the health and quality of life of the affected individual and

57 the negative impacts on society as a whole are comparable to those elicited by carcinogens, mutagens,

58 and reproductive toxicants (CMRs). Potential factors for comparison include severity of the effect, delayed

59 onset and/or irreversibility of effects, potency, mode of action, degree of impairment of life quality or

60 uncertainty about the dose-response relationship. As there are currently no applicable guidelines or

61 generally accepted assays that can accurately identify respiratory sensitizers nor distinguish between

62 respiratory and dermal sensitizers, all materials with sensitizing potential, despite their potency, may be

63 inaccurately considered for inclusion as SVHC. If an evidence-based, adverse outcome pathway (AOP)-

64 informed approach to assessment is desired there is an increasingly important need, therefore, to seek

65 integrated approaches to toxicity testing and assessment to bridge this gap.

66 The Immunotoxicology Technical Committee (ITC) of the International Life Sciences Institute-Health and

67 Environmental Sciences Institute previously organized two activities centered on the state-of-the-science

68 of testing methods to identify proteins and chemicals that pose a risk of immune-mediated respiratory

69 hypersensitivity. An expert roundtable discussion, held in 2003 at the Annual Meeting of the Society of

70 Toxicology in Salt Lake City, Utah, was followed by a two-day international workshop in June 2004 that

71 addressed the appropriate methods for identifying and characterizing respiratory hypersensitivity hazards

72 and risks, and the key gaps and related research needs with respect to respiratory hypersensitivity/allergy

73 for proteins, low molecular weight drugs, and chemicals (Holsapple et al., 2006). Key research gaps

74 identified for chemical-specific respiratory hypersensitivity included (1) understanding structure activity

75 relationships for chemical allergies, including understanding the mechanism(s) for respiratory

76 hypersensitivity and identifying distinctive characteristics of the respiratory hypersensitivity allergic

77 response, and continuing to build databases of sensitization until chemicals can be clearly identified as

78 respiratory allergens; (2) better understanding of mechanisms for sensitization; and (3) fully characterizing

79 cytokine profiling as a possible approach for hazard identification.

80 Given a decade's passage and expectation of continuous progress of science, in 2014 the ITC organized

81 a two-day international workshop in Alexandria, Virginia, towards identifying a framework for developing a

82 standard approach for identifying chemical respiratory sensitizers. (The workshop agenda and materials

83 can be found here.) The workshop opened with a presentation on the clinical manifestations of respiratory

84 sensitization. The subsequent series of lectures provided a foundation for the current state-of-the-science

85 for identification and characterization of respiratory sensitizer hazards, using both conventional and non-

86 conventional approaches, and the regulatory and practical needs regarding risk management, with the

87 ultimate aim of identifying near-term and long-term information to facilitate development of validated

88 standard methods and frameworks. The ~75 participants were asked to consider a series of questions

89 that provided a framework for discussions during the break-out sessions. The lectures and break-out

90 discussions provided the foundation for this report, and have been summarized in Sections 2 and 3,

91 respectively.

92

93 **2. STATE OF THE SCIENCE**

94 **2.1 Clinical Aspects of Chemical Respiratory Allergy and Occupational Asthma**

95 In the context of occupational asthma, chemical sensitizers refer to those chemicals that can cause

96 asthma through an immunologic or presumed immunologic mechanism (Bernstein et al., 2013). Besides

97 the potential to cause occupational asthma, some occupational respiratory sensitizers can cause other

98 respiratory allergic responses such as hypersensitivity pneumonitis, eosinophilic bronchitis, and rhinitis.

99   Chemical sensitization can, in rare instances, trigger life-threatening acute conditions such as

100 anaphylaxis as recently reviewed (Siracusa et al., 2015). The median population attributable risk for

101 asthma from occupation has been estimated to be approximately 15% (Balmes et al., 2003; Toren and

102 Blanc, 2009). Work-related asthma includes occupational asthma (usually new-onset asthma), caused by

103 work, and work-exacerbated asthma, that is asthma caused by other factors but aggravated/exacerbated

104 by work (Tarlo et al., 2008; Tarlo and Lemiere, 2014), but does not necessarily distinguish between

105 immune-mediated and non-immunological agents. The population attributable risk for new-onset asthma

106 that likely reflects occupational asthma was similarly estimated in one large multicenter study as being 10-

107 25% (Kogevinas et al., 2007). Causes include irritant exposures at work (that are usually accidental)

108 (Vandenplas et al., 2014b), and specific responses to a workplace sensitizer (an agent causing a specific

109 immunologic response). Workplace sensitizers can be further classified as high-molecular weight agents

110 (usually proteins) and low-molecular weight chemicals. Specific IgE may not be detected in all

111 symptomatic patients. The lack of universally detected specific immunologic markers of response has

112 made it difficult to determine whether agents such as sprayed cleaning products and air fresheners are

113 acting as specific chemical sensitizers or as airway irritants in studies that have shown increased asthma

114 prevalence among exposed workers (Dumas et al., 2012).

115 There are multiple chemical sensitizers known to cause asthma, both in workers as recently reviewed

116 (Baur, 2013; Baur and Bakehe, 2013), and (less often) in consumers. New formulations and new uses of

117 known agents continue to be reported as well as newly developed agents. The clinical presentation of

118 occupational asthma can mimic other (non-occupational) asthma, and the diagnosis may not be

119 suspected unless the physician takes a careful history of the workplace exposures and timing of

120 symptoms in relation to work. Other diseases can also mimic asthma and therefore objective tests are

121 important for a correct diagnosis of chemical-induced respiratory sensitization. Algorithms have been

122 developed for diagnosis, including immunologic tests where feasible, objective tests for asthma, and

123 objective demonstration of changes in asthma during work periods compared with periods off work (Tarlo

124 et al., 2008; Tarlo and Lemiere, 2014). The most definitive tests are specific inhalation challenges with the

125 suspected agent, but these carry a small safety risk to the subject and are not widely available

126 (Vandenplas et al., 2014a). After diagnosis, workers with occupational asthma are typically removed from

7

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.,

9

179 1995; Vandebriel et al., 2000). Glutaraldehyde, a recognized skin and respiratory sensitizer, was negative

180 upon inhalation in the respiratory LLNA, but positive after dermal exposure, inducing a Th2-dominant

181 immune response (van Triel et al., 2011). It was hypothesized that after respiratory exposure

182 glutaraldehyde reacts with the proteins in the mucus layer and is unable to reach the immune system in a

183 sufficiently high dose. This may suggest that there are measurable thresholds of exposure for induction

184 of the sensitized state. Glutaraldehyde is a well-known cause of asthma in humans, but the skin might

185 be an important route of exposure for the induction in environmental or occupational settings. Once

186 sensitized, lower concentrations are sufficient to elicit an allergic response in the respiratory tract. This

187 may support the value of preventing both skin and inhalation exposure to respiratory sensitizers.

188 In elicitation studies in rats responses to trimellitic anhydride (TMA) and oxazolone, model chemicals

189 inducing respiratory sensitization in rodents, were compared to the skin sensitizer 2,4-

190 dinitrochlorobenzene (DNCB) (Arts et al., 2008; Kuper et al., 2008a; Kuper et al., 2011). Gene expression

191 profiling of the lungs, paralleled with breathing patterns, lung pathology and serum IgE levels revealed

192 interesting mechanistic differences between these chemicals. As expected, the respiratory sensitizers

193 affected breathing patterns and induced lung inflammation and IgE responses in Brown Norway rats. In

194 contrast, DNCB did not affect breathing patterns or serum IgE, but induced an influx of neutrophils in the

195 respiratory tract. Gene expression revealed a difference in regulated pathways, showing that TMA

196 induced the most pronounced regulation of immune-related pathways, followed by oxazolone. DNCB

197 hardly induced any significant pathway regulation. Remarkably, TMA was the only chemical that affected

198 gene expression pathways related to airway remodeling. Oxazolone is a well-known human skin

199 sensitizer, but there are no human reports on respiratory allergy. This could implicate that oxazolone is a

200 false-positive in the animal model or that there is no or low inhalation exposure in man. Since oxazolone's

201 physical state can present as large flakes, the latter explanation is plausible. Interestingly, the gene

202 expression revealed that oxazolone induced more pronounced Th1 genes than TMA (Kuper et al., 2011).

203 DNCB is a strong human skin sensitizer that was immunogenic in different short-term respiratory animal

204 models (Arts et al., 2008; Kuper et al., 2008b). The significance of these findings in terms of adverse

205 human health effects is unclear. Prolonged and repeated inhalation exposures in Th1-prone Wistar rats

206 showed that DNCB was able to prime the immune system, as evidenced by DNCB-specific IgG levels.

207 Additionally, DNCB provoked allergic inflammation in the upper respiratory tract, but did not affect

208 functional breathing parameters. Hence, DNCB evoked a different inflammatory response upon inhalation

209 compared to TMA. Whether or not these effects are indicative for adverse effects in humans is unknown,

210 but they do demonstrate that DNCB is immunogenic after inhalation exposures as well (van Triel et al.,

211 2010).

212 TMA, oxazolone, and DNCB demonstrate that respiratory and skin sensitizers are able to provoke

213 different immune responses in experimental animals. Elicitation models seem especially suitable to

214 demonstrate distinct immune responses, and toxicogenomics proved to be an important tool to increase

215 mechanistic understanding of respiratory sensitization. Application of this knowledge for the development

216 of predictive test methods is yet unclear, since only a few respiratory and skin sensitizers were tested in

217 these animal studies. To become more confident in the type of read-outs that are indicative for respiratory

218 sensitizers, a broader range of skin and respiratory sensitizers should be tested. Besides animal models

219 other information sources, including structure activity relationships (SARs) or *in vitro* models that are

220 currently in development should be included in the development of a predictive testing strategy for

221 respiratory sensitization, a method already demonstrated for skin sensitization. To build such a testing

222 strategy, it is important to map the mechanistic understanding in an AOP as has been done previously for

223 skin sensitization (OECD, 2012).

224 **2.4 Conventional and Non-Conventional Approaches to Assess Respiratory Sensitization**

225 *2.4.1   Developing in vivo and in vitro models for Respiratory Sensitization*

226 An overview of two unpublished pilot studies, which were supported by a grant from Japanese Chemical

227 Industry Association – Long-range Research Initiative (JCIA-LRI), of *in vitro* and *in vivo* models for

228 assessment of respiratory sensitizing potential was presented. The JCIA-LRI supported research will

229 establish sensitive cell lines with reduced serine protease inhibitor expression in order to assess

230 chemical-induced hypersensitivity. Basophils and eosinophils secrete abundant serine proteases as well

231 as chemical mediators and cytokines. Serine protease inhibitors have been reported to suppress both

232 serine protease activity and cytokine production *in vitro*. Both human basophilic cell line KU812-F and

233 human eosinophilic cell line EoL-1 highly produced IL-6 in response to several sensitizers. Based on the

234 allergy protective action of some serine protease inhibitors (Smith and Harper, 2006), these cell lines

235 depleted of any serine protease inhibitor may be ideal candidates for the screening of respiratory

236 sensitizers. Generation of stable cell lines lacking serine protease inhibitors using the inducible short

237 hairpin RNA system may be complimentary to the *in vivo* approach described below.

238 An *in vivo* testing method was developed for identifying respiratory sensitizers and determining their

239 relative sensitizing potency. Known sensitizers, toluene diisocyanate (TDI) and TMA, were used to

240 sensitize female BALB/c mice by intratracheal instillation on five days per week for three weeks. Following

241 subsequent challenge the severity of the lung inflammation increased in dose-related manner for both

242 OVA, TDI, and TMA, but not DNCB. Histological scores dose response evaluation indicated the relative

243 sensitizing potency of each of these known sensitizers in the BALB/c model was similar to the sensitizing

244 potency reported in previous epidemiologic studies. These data suggest that this type of testing method

245 can predict respiratory sensitization and a chemical's relative sensitizing potency, and by extension may

246 provide useful information for the hazard assessment of respiratory sensitizers. Future efforts will expand

247 the evaluation for more sensitizers in order to demonstrate the reliable efficacy of a testing method.

248 **2.4.2** ***Mechanistic In Vitro Models for the Assessment of the Respiratory Sensitization Potential***

249   ***of Compounds***

250 From the mechanistic point of view, our understanding of the toxicity pathways driving both induction of

251 chemical respiratory sensitization and elicitation of symptoms is not as well established as for skin

252 sensitization (Roggen, 2014). In contrast, more is known about the mechanisms underlying protein

253 sensitization and allergenicity (Wills-Karp et al., 2010). The putative key events in an MOA for respiratory

254 sensitization likely include: 1) bioavailability, 2) haptenation, 3) inflammation, 4) dendritic cell activation

255 and maturation, 5) dendritic cell migration, and 6) T-cell proliferation. An overview of novel non-animal

256 tests to assess the key events above is discussed below and in Table 1.

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

258   form to the relevant effector. Thus, a compound must gain access to the viable epidermis, dermis and

259   vascular network across the bio-barrier (e.g. skin, lung mucosa) (Basketter et al., 2007; Wills-Karp et al.,

260   2010). Several studies correlate pulmonary bioavailability to the lipophilicity, the molecular polar surface

261   area and hydrogen bond donor counts of a chemical. Studies using peptides suggest that the same

262   parameters affect the bioavailability of protein allergens (Cooper et al., 2010).

263   **Haptenation.** In contrast to protein allergens which are sufficiently large to be identified as "foreign" by

264   the host innate and adaptive immune systems, low molecular weight chemical sensitizers are generally

265   believed to react covalently with native host protein(s) to form stable neoantigens. The majority of

266   sensitizing chemicals are either inherently reactive, electrophilic chemicals that form covalent bonds with

267   nucleophilic groups on amino acids, or occasionally acquire such reactivity following metabolism. Non-

268   electrophilic mechanisms for protein binding may also occur through disulfide exchange or coordination

269   bonds (e.g. metals) (Chipinda et al., 2011). Compared with skin sensitizers, low molecular weight

270   respiratory sensitizers reacted more readily with lysine rather than cysteine moieties of host proteins

271   (Lalko et al., 2013). Despite the limitation that such methods do not identify the target protein defining the

272   specificity of the immune response (Aleksic et al., 2007), they may, however, provide a useful piece of

273   qualitative (and potentially quantitative) information for hazard identification. Future work to critically

274   evaluate the readiness of haptenation assays may extend their value in hazard identification.

275   **Inflammation.** Three potentially useful / *in vitro* test models were discussed for respiratory sensitization

276   testing (Roggen, 2013). These include precision cut human lung slices(Lauenstein et al., 2014); an *in*

277   *vitro* alveolar-capillary barrier based co-culture system comprised of two human cell lines, H441 and ISO-

278   HAS-1 (Hermanns et al., 2010); and an air liquid interface (ALI) organotypic 3D airway epithelial model

279   employing primary human bronchial epithelial cells (MucilAir™; www.epithelix.com). Although not

280   validated, each of these model systems have been used to discriminate sensitizers from irritants, as well

281   as respiratory from skin sensitizers (dos Santos et al., 2009).

282   **Dendritic cell (DC) activation.** It is generally accepted that activation of DCs results in mature antigen-

283   presenting cells having an established Th1-, Th2-, Th17-biased phenotype (Tan and O'Neill, 2005).

284 Studying the molecular mechanisms behind DC activation and maturation is impeded by the fact that

285 primary DCs constitute a small and heterogeneous population of cells among many functionally

286 specialized DC subpopulations. To circumvent this issue, various human myeloid cell lines (e.g. THP-1,

287 U937, KG-1 and MUTZ-3) were used both for acquiring mechanistic understanding and for development

288 of predictive tests (Larsson et al., 2006; Roggen, 2013). Functional and transcriptional analysis of various

289 myeloid cell lines has clearly demonstrated the significance of the MUTZ-3 cell line as a model for

290 functional studies of inflammatory responses (Larsson et al., 2006; Lundberg et al., 2013). The genomic

291 allergen rapid detection (GARD) test can generate prediction calls of unknown chemicals as skin

292 sensitizers, respiratory sensitizers or non-sensitizers, including irritants (Johansson et al., 2011). In

293 addition to providing an accurate prediction about the sensitizing potential of a chemical, there is growing

294 evidence that the GARD test also provides useful information about the sensitizing potency of the

295 chemical (Albrekt et al., 2014).

296 The most advanced DC maturation test is the human cell line activation test (h-CLAT). When applied to

297 hazard identification for skin sensitization the test revealed a good concordance (84%) with the LLNA

298 data (sensitivity: 88%; specificity: 75%) (Ashikaga et al., 2010). There are indications that the h-CLAT

299 correlates with the LLNA and may have the potential to provide information about the potency of the test

300 chemical (Ashikaga et al., 2010). The usefulness of this test for assessing respiratory chemicals was not

301 established, but given the potential of the dermal LLNA as a screen for respiratory sensitization potential

302 (*i.e.,* LLNA negatives being unlikely to be respiratory sensitizers), the h-CLAT may provide similar

303 screening potential in the future.

304 **Dendritic cell migration.** In an *in vitro* full-thickness tissue-engineered skin model containing fully

305 functional MUTZ-3 derived LCs (MUTZ-LC) (Ouwehand et al., 2008; Ouwehand et al., 2011) can be

306 utilized to assess the impact of irritants and sensitizers on the migration activity of the fluorescently

307 labelled MUTZ-LC. While not evaluated using protein allergens, this *in vitro* DC migration test was found

308 to correctly identify both respiratory and skin sensitizing chemicals (dos Santos et al., 2009).

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

14

310 assessment of pulmonary sensitization have been developed. While these assays are functionally

311 plausible, their predictive accuracy remains to be evaluated. The potential application areas for the

312 assays discussed above have been, where possible, integrated into Table 1 below.

313 **2.4.3 *Application of 'Omics' Technologies to Assess Chemical Respiratory Allergy***

314 Current guidance recommends a weight-of-evidence approach based on human and animal data to

315 identify a potential respiratory sensitizer. The use of 'omics' technologies such as transcriptomics and

316 proteomics can provide an unbiased global assessment of gene-expression and protein network

317 alterations associated with the development of allergic rhinitis and asthma (Park and Rhim, 2011; Sircar

318 et al., 2014). These methods have been used to examine (1) the induction of the sensitized state which

319 includes hapten-protein formation, interaction with epithelial cells impacting dendritic cell activation, Th2-

320 biased maturation, and subsequent lymphoid cell activation, proliferation and differentiation and (2) the

321 elicitation phase where subsequent inhalation exposure enhances localization and amplification of allergic

322 responses. This enhanced response can extend into epithelial remodeling with effector/inflammatory cell

323 influx, mucous cell hyperplasia/metaplasia, development of functional pulmonary responses including

324 airway hyperreactivity, and reversible airflow obstruction.

325 Toxicogenomics have been applied to the characterization of the elicitation phase. Kuper et al., (2008a;

326 2008b) reported on the molecular characterization of the respiratory sensitizer TMA and the skin

327 sensitizer DNCB in Brown Norway rats. They performed a whole genome analysis and related the results

328 to physiological and cellular parameters with the aim to improve hazard identification and cross-species

329 comparisons of respiratory allergens through molecular characterization. The presence or absence of

330 notable changes in gene expression were consistent with the physiological/cellular responses to TMA and

331 DNCB. The skin sensitizer DNCB resulted in slight changes in chemokine transcripts but no effects on

332 lung remodeling. Rats dermally sensitized and exposed by inhalation to TMA showed a number of

333 changes associated with lung remodeling similar to that observed in early development of asthma in

334 humans. The authors stated that early lung remodeling genes may be useful in further characterization of

335 molecules capable of causing allergic asthma. The expression profile was generally consistent with genes

336 regulated in mouse models of asthma and those reported in humans with asthma. These data suggest

337 that changes in gene expression may represent valuable complementary endpoints for the

338 characterization of potential respiratory allergens in sensitization-challenge models.

339 Proteomic approaches may be used to enhance the identification of respiratory sensitizers (Haenen et al.,

340 2010; Park and Rhim, 2011). Using an OVA sensitized mice, a repeated aerosol challenge was used to

341 induce an elicitation response. Sensitized, OVA-challenged mice had a significant increase in pulmonary

342 eosinophils, and increased airway reactivity to methacholine challenge compared to controls. These

343 changes included upregulation of structural proteins associated with airway remodeling and mammalian

344 chitinases (YM1/YM2) that are induced by IL-13 expression (Jeong et al., 2005). One major strength of

345 proteomics is the ability to evaluate multiple functional tissue compartments in both humans and

346 experimental animals (sputum, BAL, blood) for translational investigations. Additional studies are needed

347 to assess the utility to differentiate respiratory and dermal sensitizers and identify markers that may be

348 used to identify thresholds of sensitization/elicitation or perhaps recovery following removal from

349 exposure (Louten et al., 2012; O'Neil et al., 2011; Park and Rhim, 2011; Zhang et al., 2009).

350 Toxicogenomic approaches have also been used to examine the induction phase of sensitization to

351 identify respiratory sensitizers. Comparison of a panel of dermal sensitizers (DNCB and alpha-

352 hexylcinnamaldehyde), respiratory sensitizers (TMA and ortho-phthalaldehyde) and non-sensitizing

353 irritants (methyl salicylate and nonanoic acid) identified 4,467 significant gene expression responses,

354 which were in turn categorized (Adenuga et al., 2012; Boverhof et al., 2009). Respiratory sensitizer-

355 specific transcripts were identified, including AKR1c18 (aldo-keto reductase; promotes Th2 cell survival;

356 (Matsuzaki et al., 2005)), Galectin-7 (cell-cell and cell-cell matrix interactions), Mcpt1 and 8 (mast cell

357 protease 1 and 8) and IL-4 (promotes Th2 bias). These data suggest that gene expression changes

358 during sensitization may enhance WoE approaches to distinguish sensitizers from irritants and respiratory

359 sensitizers from dermal sensitizers. There is a need to expand the low molecular weight chemical data set

360 to confirm and extend these data and to expand analyses to upper/lower airway tissues to explore

361 mucosal gene expression signatures.

362   A WoE approach is currently required to differentiate respiratory from dermal sensitizers. It is essential to

363   develop and validate robust assay systems to distinguish respiratory sensitizers from both dermal

364   sensitizers and non-sensitizing irritants. A science-based approach to assess respiratory sensitizer

365   potency and thresholds of sensitization/elicitation is critical to address possible hazard classification of

366   respiratory sensitizers, which may be considered as SVHC under the "equivalent level of concern" route

367   set out in Article 57(f) of REACH. Data provided using 'omics' technologies can help identify key cellular

368   and molecular events relevant to development of an adverse outcome pathway for respiratory sensitizers.

369   *2.4.4   Grouping, Read-Across, and Mechanistic Chemistry for Respiratory Sensitization*

370   One view on the AOP concept relates a series of key events linking a Molecular Initiating Event (MIE)

371   between a chemical and a biological system to an adverse effect at the organ level. In turn, organ level

372   effects may be linked to predictions of biological system or even the population events. The aim of an

373   AOP is to outline the key processes, some of which can be tested by using either *in silico, in chemico* or

374   *in vitro* methods. The chemistry associated with the MIE can be compiled into '*in silico* profilers', enabling

375   chemicals to be grouped into mechanism-based categories, allowing for predictions of toxicity to be made

376   by using read-across. Such an approach offers an improvement on structural similarity based

377   approaches, which inherently aim to address the possibility of similar *in vivo* chemistries, but without the

378   benefit of applying mechanistic chemistry knowledge to the grouping. This additional mechanistic

379   knowledge is important as simple structural similarity frequently identifies chemicals that are structurally,

380   but not mechanistically similar (in terms of their ability to react with proteins). In addition, such profilers

381   enable chemical inventories to be prioritized for further *in vitro* and/or *in chemico* investigations (rather

382   than testing every chemical in an unstructured manner).

383   Recent research has led to the development of an *in silico* profiler for respiratory sensitization (Enoch et

384   al., 2012). The profiler was developed from a mechanistic chemistry analysis of a data set of 104 reported

385   in the literature as causing occupational asthma. Initial interest in this area of research stemmed from a

386   study showing that, for some respiratory sensitizers, the most likely MIE was the formation of a covalent

387   bond in the lung (Enoch et al., 2009). An outline was developed for how such mechanistic information

17

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)
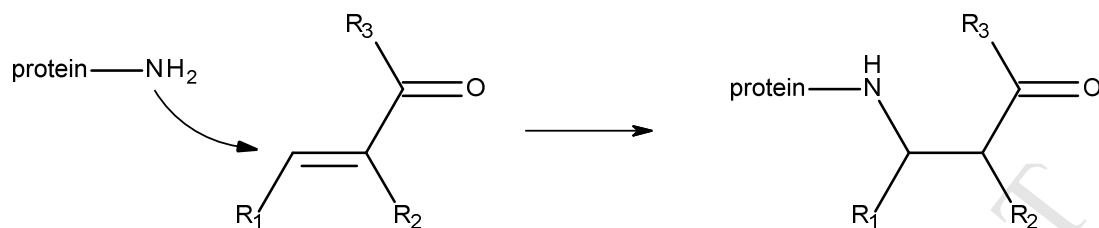
411

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

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

488 Risk management practices have improved over time through improved analytical methods utilizing more

489 efficient collection of vapor & aerosol and the use of more sensitive and specific LCMS for inhalation

490 exposure assessment.   Newly developed techniques and methods for improving dermal assessments

491 have increased the understanding of protection degree provided by personal protective equipment. There

492 has been an increased emphasis on dermal protection since dermal exposure may contribute to risk of

493 developing respiratory sensitization. There have also been improvements in biomonitoring techniques

494 where albumin or hemoglobin adducts in blood samples have been shown to be more specific and more

495 sensitive biomarkers of exposure than diamine hydrolysis products in urine.

496 Current practices have demonstrated that an effective product stewardship program can reduce risk.

497 Significant reductions of diisocyanate-related occupational asthma (OA) cases have been observed

498 globally. Ontario, Canada reported a decrease from 30.5 OA claims/year (1980-1993) to 7.4 claims/year

499 (1998-2002) (Buyantseva et al., 2011). Michigan's Project SENSOR report that the rates of asthma

500 attributable to diisocyanates have fallen from 22.9 cases/yr (1988-1997) to 4 cases/yr (2009-2010)

501 (NIOSH, 2014). Many authors relate the reduction to industry recommendation for medical surveillance,

502 the use of periodic spirometry and examinations targeted to skin and respiratory tract. In addition, using

503 an accepted paradigm to accurately diagnose respiratory sensitization (occupational asthma) by objective

504 measures and the use of specific inhalation challenges when necessary to confirm the relationship of

505 asthma to diisocyanate exposure may have contributed to the lower numbers of new OA cases.

506 ***2.5.2   WHO's Guidance on Assessment for Respiratory Sensitization***

507 The WHO Guidance for Immunotoxicity Risk Assessment for Chemicals (chapter 6) provides a framework

508 for conducting of risk assessments for both induction and elicitation of skin allergy, respiratory allergy and

509 oral (systemic) allergy (WHO, 2012). This includes a decision-tree towards developing a WoE based on

510 the available human, laboratory animal and mechanistic data associated with exposures to a potential

511 respiratory sensitizer (Figure 3). The case study (#3) of halogenated platinum salts illustrates how a

512 quantitative risk assessment can be conducted for a chemical respiratory sensitization.

22

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-

539 human data in the context of REACH. For sensitizers the chemical safety assessment can be based

540 either on qualitative approach or quantitative risk characterization. Under substance evaluation "Further

541 information" can be requested even beyond the information mentioned in Annexes VII to X of REACH, if

542 there is a concern that a given substance may constitute a risk to human health or the environment, and

543 further information is needed to clarify the concern. For 13 out of 51 substances to be evaluated in 2014,

544 the initial concern (or one of) is respiratory sensitization.

545 The main risk management options for respiratory sensitizers classified based on Classification, Labeling,

546 and Packaging European Commission Regulation No. 1272/2008 (CLP) under REACH are authorization,

547 restriction or no action (which does not prevent action under other legislation). The prerequisite for

548 subjecting a substance to the authorization requirement is its identification as a SVHC. However,

549 sensitization is not a SVHC criterion itself under REACH Article 57. Therefore, a sensitizing substance

550 must be identified in accordance with Article 57 (f) as giving rise to an equivalent level of concern to the

551 carcinogenic, mutagenic or reprotoxic substances. This requires a case-by-case assessment. The

552 general approach on identification of a sensitizer as having equivalent level of concern under article 57(f)

553 has been agreed with the EU Member States (ECHA, 2013b).

554 Under CLP Regulation respiratory sensitizers are considered to be among "substances of the highest

555 concern". In the absence of validated animal models or alternative approaches, the evidence that a

556 substance can lead to specific respiratory sensitization is normally based on human experience, but some

557 animal data can be used in a WoE approach as explained in the CLP guidance (ECHA, 2013a).

558 In the absence of a validated standard method, case-by-case judgment and weight-of-evidence

559 approaches are necessary for regulatory purposes (hazard information and classification). This is not an

560 optimal situation for a health effect as important as respiratory sensitization. For both REACH processes

561 and CLP, there is a need to differentiate between respiratory sensitizers according to their sensitizing

562 potential and potency and validated methods or approaches would be desirable. In a regulatory

563 framework, an information request (test data or tiered approaches) would, however, need to be such that

564 a reasonably definitive answer to the concern is delivered.

565 **3.   RESULTS FROM WORKSHOP BREAKOUT GROUP DISCUSSIONS**

566 **3.5 Understanding the Condition**

567 **Relevant immunological mechanisms.** All breakout groups agreed that Th2 responses predominate in

568 respiratory sensitization. In one of the groups there were divergent views that Th2 responses may not be

569 the sole explanation. The role of IgE is highly likely as well, although IgE is not always detected in

570 humans with occupational asthma and in animal models. Other mechanisms may be involved in the

571 elicitation phase of respiratory allergy, i.e., neurogenic inflammatory responses were mentioned (see

572 below).

573 There was general agreement on certain essential steps that are required to induce respiratory

574 sensitization, including chemical hapten-protein binding, induction of danger signals by the epithelium, DC

575 activation, maturation and migration, T cell activation and clonal expansion towards a Th2 response and

576 B cell maturation and antibody formation, in some cases IgE. Protein binding was considered to be the

577 molecular initiating event (MIE), similar to that of skin sensitization. Participants acknowledged that the

578 theory on hard versus soft acid base for electrophiles may be useful in discriminating respiratory from skin

579 sensitizers (Enoch et al. 2009, 2010), but that there is a need to define and explore this theory further

580 (i.e., nucleophile chemistry, nucleophile mechanisms that react with cysteine are related to Th1

581 response).

582 There was also agreement that many cellular events involved in respiratory sensitization are relatively

583 well understood, i.e., DC activation, T and B cell activation. The cellular sources that deliver the danger

584 signals, however, are not well-defined and different cell types in the airways or skin may be involved in

585 this. Furthermore, the exact mechanisms of action have not been studied in detail and are thus largely

586 unknown. Interestingly, there is an overlap in cellular events with skin sensitization for example protein

587 binding, DC activation, T cell activation. Given that the effector immune response is different, it is

588 important to identify the 'master switch' that determines Th2 skewing which will require understanding the

589 key signaling and molecular pathways involved in the adverse outcome. Also, it was proposed that

590 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

617 route, the dose metric used both in human as well as in animal studies is ppm. Where testing in animal

618 models is not permitted (*i.e.,* cosmetics) dose response assessment will likely have to turn to *in vitro*, *in*

619 *chemico*, and *in silico* methods (several of which are described above) to estimate dose responses.

620 **3.6 Overview of available models**

621 **Strengths, weaknesses, opportunities, and challenges.** The need for rapid, inexpensive and validated

622 in silico and in vitro model systems was identified as a key area where considerable progress has been

623 made but where significant challenges remain. QSAR models are available for dermal sensitizers but not

624 respiratory sensitizers. Structural profiling has the potential to provide insight into possible differences in

625 the initial molecular interactions between low molecular weight chemicals and host proteins. These may

626 include preferential binding of chemicals to specific amino acid residues based on well characterized

627 physical properties (acid/base theory, electrophilicity, etc.). These expert systems may eventually be used

628 to identify and differentiate potential skin and respiratory sensitizers from irritants and toxicants, however,

629 the predictive power of these *in silico* models must be validated against known sensitizing chemicals. This

630 may be difficult in the case of respiratory sensitizers since, compared to skin sensitizers, there are

631 relatively few known human respiratory sensitizers and these are represented by only a few chemical

632 classes (e.g., isocyanates, aldehydes, anhydrides and dyes and platinum salts). It is likely that numerous

633 models will be developed and disseminated. One potential challenge may come if the availability and use

634 of new profiling tools proceeds more rapidly than their validation. Confidence in the predictive power of

635 newly developed tools will only come through repeated confirmatory testing in other in vitro and in vivo

636 model systems, building a WoE case for the models. For instance, the direct peptide reactivity assay

637 (DPRA) is a means to assess protein reactivity of electrophilic chemicals. DPRA is an *in chemico* method

638 which determines the reactivity of chemicals to peptides containing nucleophilic cysteine and lysine

639 residues. It has been hypothesized that chemicals that preferentially bind to lysine form molecular

640 interactions with host proteins that result in the development of a Th2-bias, characteristic of respiratory

641 sensitizers. This is a testable hypothesis that can may be used to discriminate between skin (cysteine-

642 binding) and respiratory (lysine-binding) sensitizing chemicals.

643 **Distinguishing events associated to local irritation and systemic sensitization.** A number of *in vitro*

644 cellular systems were described that help probe specific key events in the development of the sensitized

645 state. The KeratinoSens assay can be used to identify skin sensitizers through their ability to activate the

646 Keap1-Nrf2-antioxidant/electrophile response element (ARE). Dendritic cells, either freshly isolated or

647 cell lines, may be used to differentiate chemicals leading to a Th1- or Th2-bias. Organotypic 3D airway

648 epithelial cell cultures, grown at the air/liquid interface (ALI) may be used to investigate the role of the

649 epithelium in "reading" the hapten-protein complex and expressing specific signals that may interact with

650 the innate immune system to influence the maturation and Th1/Th2-bias of mucosal dendritic cells. These

651 *in vitro* cellular systems are valuable tools to examine specific cellular responses, but more complex co-

652 culture systems may need to be developed to explore the cell-cell interactions involved in development of

653 the sensitized state. Precision cut lung slices may provide short term test systems to explore cell-cell

654 interactions, however they cannot be maintained in culture long, and dosimetry is complicated and limited

655 by diffusion due to the need to inflate the lung with agar prior to cutting the slices. Organotypic ALI

656 cultures overcome the dosimetry problem, but do not contain all of the cell-types found in the intact lung.

657 *In vivo* model systems have been developed to assess the induction of the sensitized state. The LLNA

658 measures proliferation in the draining lymph node to identify sensitizing chemicals. It is a widely used and

659 validated method that can identify sensitizing chemicals, but it cannot distinguish respiratory from skin

660 sensitizers, and care must be used to differentiate irritant responses from immune-responses. The LLNA

661 can provide data on potency and when coupled with direct exposure of the respiratory tract and analysis

662 of the appropriate draining lymph nodes may provide insight into local immune responses in the

663 upper/lower respiratory tract. The LLNA has been coupled with "omics" endpoints in an attempt to identify

664 gene expression profiles characteristic of skin and respiratory sensitizers and irritating chemicals.

665 Progress has been made but a definitive profile that can uniquely identify and differentiate sensitizing

666 chemicals has not been identified. Part of the problem may be a lack of standardization of the exposure

667 and sampling protocols between laboratories. Evidence suggests that even the most basic of

668 experimental variables, such as dose, dose number and timing and sample time, may influence the

669 results. Cytokine profiling and IgE expression are likewise valuable complementary assays with which to

670 build a WoE case for respiratory sensitizers on a case by case basis.

671 **Confounders and strategies to reduce their effect.** *In silico*, *in chemico*, *in vitro* and most *in vivo* model

672 systems available at this time examine only the induction or sensitization phase. This is important

673 because of the profound importance of being able to identify which chemicals may be skin or respiratory

674 sensitizers, differentiate between the two, and assess potency as it impacts thresholds of sensitization. A

675 clear and definitive identification of a no-effects threshold, perhaps using a combination of *in vitro* assays

676 targeted at key events in the AOP, would provide immediate impact on the safe use of chemicals with

677 sensitizing potential. Some may argue that from a regulatory perspective, due to the wide range of human

678 sensitivities to chemical exposures, that one must assume that induction of sensitization is a given. The

679 question then becomes whether a threshold of elicitation can be identified so that a sensitized individual

680 can be protected from progression of the disease through preventing repeated bouts of secondary

681 elicitation reactions. Evidence from the widespread use of isocyanates and biocides suggests that these

682 sensitizing agents may be safely used if the potential for exposure is controlled. At this time, only the

683 Brown Norway rat model has been demonstrated to be useful to address thresholds of elicitation. This

684 model is time consuming and expensive, requires expertise in the conduct of inhalation exposures that

685 may not be available in many laboratories and presents challenges in regional dosimetry related to both

686 the physical/chemical nature of the test chemical and the anatomy of rats which are obligate nose

687 breathers. It is, however, justified on a case by case basis in order to derive a safe level of exposure in

688 occupational settings.

689 **3.7 Identification of research needs**

690 Multiple research needs were identified regarding improving our understanding of chemical respiratory

691 allergy, including mechanistic and clinical questions. With regard to mechanistic research questions the

692 areas of interest identified were related to potential differences in tissue level responses (such as

693 development or evasion of immune tolerance, bioavailability, homing responses) and cell level responses

694 (mechanistic chemistry, the role of danger signals, and potential for differences in DC activation). The

695 potential for differential responses between skin and respiratory sensitizers related to immune tolerance

696 was considered. From a generic immunological perspective the creation of non-self epitopes via protein

697 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,

725 but national (or international) registries can be more useful (Steliarova-Foucher et al., 2015). Such an

726 effort naturally comes with challenges including definition or criteria for classifying cases, but may be

727 worth the additional effort.

728 The role of IgE in respiratory sensitization remains unresolved. There is a long history of difficulties in

729 assessing the role of IgE as related to chemical respiratory sensitization (Kimber and Dearman, 2002;

730 Kimber et al., 2014; Kimber et al., 1998), yet the potential application in hazard identification for humans

731 remains attractive. Lacking methods to address how (e.g., serum chemical-specific or total IgE by ELISA)

732 and when to measure (e.g., anytime or only after challenge), and how to apply IgE assessment in

733 predictive animal models, continues to be a scientific challenge.

734 A concept shared across breakout groups was the value of a consensus list of respiratory sensitizers. To

735 that end, developing such a list could begin with agreement on assessment criteria, then build through the

736 identification and consideration of data compared to the criteria (Selgrade et al., 2012). While there are

737 widely accepted examples of respiratory sensitizers from which the list could begin (e.g., TDI, MDI, TMA),

738 a consensus list would ideally not necessarily identify entire classes of chemical. It is unclear how

739 representative TDI and the like are of all members of their categories (e.g., diisocyanates and acid

740 anhydrides). As discussed above, chemicals from similar categories or with similar reaction mechanisms

741 can vary in their electrophilic potential for protein reactivity. In some cases, despite having functional

742 groups associated with hazard, the inherent reactivity for some chemicals may be too low to induce

743 sensitization.

744 Multiple groups also consider skin likely to be relevant to induction. As discussed above, in the light of

745 potential physiologic consequences from robust immunologic responses in airway versus skin (airway

746 responses can have a systemic adverse effect owing to impaired gas exchange, whereas skin responses

747 generally have local adverse effect) it appears all the more plausible that local skin exposure followed by

748 systemic immune memory could play a contributory, if not major, role in the etiology of chemical

749 respiratory sensitization.

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

31

751   provides protection from elicitation, and if a single elicitation threshold exists for each chemical. As

752   summarized above, empirical data derived with TMA support the case that protection from irritation also

753   covers elicitation, but extending those findings to a broader range of chemicals has yet to happen.

754   Regarding single elicitation thresholds for each chemical, workgroups were uncertain about whether

755   current models would be suitable for such efforts.

756   **3.8 Future Strategies for Testing**

757   Concerns, legislation and research needs have precipitated developments such as the MoA concept, the

758   Tox21 strategy, the concept of Pathways of Toxicity and the AOP framework. The common goal of these

759   developments is toxicity assessment based upon in-depth understanding of the *in vivo* physiological and

760   toxicological processes in humans and on their relation to specific key molecular events or toxicological

761   endpoints (Ankley et al., 2010; US National Research Council, 2007). This workshop addressed new

762   technologies and paradigms that are currently transforming these concepts into applicable animal-free

763   toxicity testing systems by implementation of libraries of generic profiles of genes (genomics), proteins

764   (proteomics) and metabolites (metabonomics) describing molecular initiators, pathways and key events of

765   toxicity within tissues, organisms and biological systems (Berg et al., 2011). The key themes for future

766   testing strategies that emerged from the workshop discussions are described below.

767   **Threshold Assessment.** While the expectation of thresholds for both induction and elicitation was

768   communicated from multiple participants in the workshop, it was also recognized that the methods to

769   quantify those thresholds are currently limited. The workgroups identified several approaches to

770   identification of thresholds for elicitation. In assessing potential for elicitation thresholds in animals there is

771   data to support use of the Brown Norway rat . Beyond animal models, workgroups identified approaches

772   combining human exposure data with clinical assessment to help delineate thresholds. Examples include

773   workplace exposure monitoring, post-implementation assessment of engineering controls, and post-

774   exposure evaluations correlated to clinical assessments.

775   It was generally recognized that identifying a threshold for induction is challenging, and the potential for

776   thresholds was recognized during the meeting as potentially divergent among groups. The challenge may

777 in part be understood in the potential for a reciprocal dose response to chemical sensitizers, wherein low

778 doses may trigger induction but require high doses for elicitation and high doses triggering induction lead

779 to reduced doses causing elicitation. Such a relationship creates challenging questions for experimental

780 assessment of thresholds. One of the justifications for considering respiratory sensitizers as substances

781 of equivalent concern to carcinogen, mutagens, or reproductive hazards is deriving "a safe concentration

782 may not be routinely possible and any figure derived would be associated with large uncertainty." This

783 stems from difficulties in measuring induction and elicitation thresholds, particularly because the induction

784 dose may vary depending on the individual.

785 **Role of the LLNA.** Multiple workgroups identified the dermal LLNA as a potentially key piece of

786 information in assessing respiratory sensitization potential. One gap in knowledge identified was that

787 while the LLNA provides a very solid tool for potency ranking, which appears to translate well between

788 species, whether the animal model EC3 value is directly, quantitatively translatable to humans is not fully

789 understood. Several caveats or complications to application of the LLNA were identified:

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

797 **Toward a Tiered Framework.** The workgroups generally considered a tiered framework for assessment

798 of respiratory sensitization potential a possibility, particularly for the purpose of screening and

799 prioritization. Where the data is available, the dermal LLNA and standard inhalation toxicology testing was

800 considered an appropriate first point in an assessment. If the LLNA indicates the material is not a skin

801 sensitizer, it was considered unlikely to be a respiratory sensitizer and an assessment could stop there.

802 By themselves standard inhalation toxicity studies would not typically be expected to provide much

33

803   information regarding sensitization potential; however, they may contribute to a WoE if there is no

804   pathological findings or if pathologic findings indicate immunologic engagement (*e.g.,* post-nasal

805   inflammation or lymph node alterations). If the LLNA results indicate sensitizing potential, or there is no

806   data available, one turns to SAR and in vitro data next. All the existing available data can be used to build

807   a WoE assessment, which may also assist in the prioritization of any decisions to develop additional

808   experimental data.

809   Based on collated feedback from the workgroups, Table 1 was developed to identify potentially useful

810   information in WoE assessment. Data from the dermal LLNA, if available, could be considered a useful

811   starting point in an assessment. If results indicate sensitizing potential, or no data is available, assessors

812   might progress down the table to consider what data is available. Tools for *in silico* analysis are readily

813   and freely available (*e.g.,* OECD QSAR Toolbox), so in cases where data is not already available it can

814   be obtained with relative ease. While the table suggests a progression from *in chemico, in vitro,* and

815   finally to *in vivo* models, the relative contributions are not necessarily rank ordered in Table 1. Consider a

816   hypothetical example where bioavailability may be low on the basis of *in vitro* measures, but other tests

817   suggest effects on DC activation or T cell proliferation. While a simple example, it illustrates why WoE

818   assessment can be challenging. In some cases deriving *in vivo* data may not be possible, a challenge

819   facing the cosmetics industry, and thus would likely need to be built upon *in silico*, *in chemico*, and *in vitro*

820   data.

821   ***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	(dos Santos et al., 2009; Hermanns et al., 2010; Roggen, 2013). www.epithelix.com
	+	May discriminate irritants from sensitizers	
	-	Metabolic capability may be uncertain	
	-	Some require high degree of technical expertise	
DC Activation or Maturation • skinGARD • respGARD • h-CLAT	+	Direct measure of immune system engagement	(Ashikaga et al., 2010)
	-	May overlook compounds requiring metabolism	
DC Migration[2] • Langerhans Cell Skin Equivalent model	+	Direct measure of immune system engagement	(dos Santos et al., 2009; Ouwehand et al., 2011)
	+	Reflective of tissue organization that may influence response	
	-	May overlook compounds requiring metabolism	
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	(Adenuga et al., 2012; Boverhof et al., 2009; Kuper et al., 2008a; Kuper et al., 2008b)
	-	Requires high degree of technical expertise	
Proteomics • 2D electrophoresis and MALDI-TOF	+	Unbiased, systematic assessment of cellular response	(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)
	-	Requires high degree of technical expertise	
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)	(Pauluhn, 2008; Pauluhn, 2014; Pauluhn, 2015)
	+	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	

822

823 One of the key challenges to applying *in vitro* tests to an assessment is incorporating a systems biology

824 perspective to the results. Results in DC's may be confounded if keratinocyte metabolism is a key event

825 in a chemical's mode of action. The importance of system-level consideration may be one of the strengths

826 of the PCLS model. Because it contains multiple cell types in system coherent structure it may be a

827 particularly rigorous tool for use in an assessment. Workgroups also identified dosimetry as a key

828 consideration for interpreting results from *in vitro* models. While the *in vitro* models may allow for testing

829 at non-physiologic concentrations, the value of the results may be reduced for developing a solid WoE

830 assessment.

831 One of the more promising approaches to WoE assessment for sensitization potential is in Bayesian

832 approach to the available information. In Bayesian analysis each new piece of information can be used to

833 refine a prediction. The value of a Bayesian analysis for sensitization has been demonstrated for skin

834 (Jaworska et al., 2013), which demonstrated a 95% accuracy for hazard classification and 86% accuracy

835 for potency classification. One of the greatest strengths of Bayesian analysis is that as more data

836 becomes incorporated overall prediction accuracy may be improved. However, a similar effort for

837 respiratory sensitizers has not been undertaken to date.

838 **Methodological Considerations.** The workgroups noted several methodological characteristics that

839 warrant consideration when assessing respiratory sensitization potential:

840 • The maximum tolerated dose for a dermal LLNA may be at the irritation threshold.

841 • Irritation and induction may be difficult to differentiate using current approaches; applying –omics

842 technologies may aid the differentiation, but the available datasets are still relatively limited in

843   scope.

844   • Standardizing inhalation induction models may be exceedingly difficult owing to individual

845   chemical characteristics. Highly reactive chemicals may be scrubbed higher in the airway,

846   irritating chemicals may change the breathing pattern and thus deposition pattern, and classic

847   inhalation toxicology challenges related to aerosol versus vapor behaviors will apply.

848   • For definitive *in vivo* inhalation studies there are endpoints beyond the typical measures that can

849   benefit an assessment. In particular bronchoalveolar lavage fluid characterization, exhaled nitric

850   oxide measurement, enhanced histology (immunohistochemistry), lung function measures (e.g.,

851   methacholine challenge), and even lung weights (wet and dry) were identified as potentially

852   useful.

853   **Case of the Brown Norway Rat Model.** Multiple workgroups considered the Brown Norway rat a

854   potential model for assessing elicitation. As promising as it appears, its current iteration would make it a

855   technical challenging model to use, requiring sophisticated inhalation exposure and assessment

856   technologies conducted over a relatively long study period (66 days). Given the resources necessary to

857   apply such a model, it is conceivable that the model is better suited to deriving a point of departure for risk

858   assessment than routine screening for hazard identification.

859   **4. CONCLUSIONS AND FUTURE WORK**

860   While scientific progress has moved forward in the period between the 2004 and 2014 workshops, a

861   satisfactory answer to the question of how to best assess and characterize chemical respiratory

862   sensitization remains elusive. Substantial progress has occurred in the development of non-traditional

863   assessment models (*in silico* and *in vitro*), whereas an understanding of some of the more fundamental

864   pathophysiologic characteristics such as the role of IgE in human chemical sensitization remain largely

865   where it was a decade ago. The uneven advancement may reflect several challenges, particularly the

866   high investment cost and complexity of animal models for chemical respiratory sensitization coupled with

867   the relative rarity (whether real or perceived) of the hazard property. A rational path forward for research

868   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 <u>sensitization</u> potency (e.g. BMD or NOEL from an epidemiological or laboratory animal study) available?

No →

Is sufficient information on respiratory <u>sensitizing</u> potency available to do a semi-quantitative risk assessment, e.g. from comparison with a well-characterized respiratory sensitizer?

Yes / **No**

Yes

Do <u>quantitative</u> risk assessment of <u>induction</u> of respiratory sensitization using SAFs to derive acceptable non-sensitizing air concentration; do quantitative exposure assessment, describe risk characterization.

Do semi-<u>quantitative</u> risk assessment of <u>induction</u> 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 <u>elicitation</u> potency (e.g., a NOEC from an epidemiological or human provocation study) available?

Do <u>qualitative</u> risk assessment of <u>induction</u> of respiratory sensitization, collect and use exposure information and describe use and exposure scenarios that may pose a respiratory sensitization risk.

Yes / **No**

Do <u>quantitative</u> risk assessment of <u>elicitation</u> of respiratory allergy using SAFs to derive acceptable non-eliciting air concentration; do quantitative exposure assessment, describe risk characterization in allergic subpopulation.

Do <u>qualitative</u> risk assessment of <u>elicitation </u>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 <u>exposure</u> 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.