

# Beyond key characteristics of carcinogens: an archetypal MOA-based evidence system for hypothesis testing to advance carcinogen risk assessment

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## ■ ABSTRACT

The key characteristics of carcinogens (KCCs) embraced by the International Agency for Research on Cancer (IARC) have generated considerable discussion. KCCs provide a basis for organizing information, which is inherently attractive to those seeking to simplify the complex process of cancer hazard identification and risk assessment. However, guidance on integrating evidence for decision-making from KCCs is limited and variable. Used in isolation, KCCs do not predict cancer classifications, and additional evidence is presented demonstrating KCCs can result in mislabeling when compared with IARC and U.S. Environmental Protection Agency (EPA) cancer classifications. Scientific understanding of the multifaceted molecular and cellular processes and dose-dependent transitions involved in chemical carcinogenesis is today vastly superior to what it was a half-century ago – or even 20 years ago. It is imperative that these advances in knowledge be applied to risk assessments so that public health can be protected, and at the same time societal benefits of natural and synthetic chemicals can be realized. The forward evolution of the scientific understanding of cancer and risk assessment has consistently been in the direction of greater complexity, and rigorous application of hypothesis testing and causal analysis. Without an integration into this richer tapestry of scientific information and analyses, reliance on simplistic approaches to cancer assessment and regulation, such as the Delaney Clause or use of KCCs in isolation, is decidedly in the wrong direction. Herein, we propose a return to the fundamental scientific principle of hypothesis generation and testing using four previously described and archetypal chemical carcinogenesis modes of action (MOA). We provide an example of an evolved causal evidence scoring system that allows comparison of the extent to which the evidence supports each hypothesized MOA to identify the likely operative MOA so that this likely operative MOA and associated toxicity values can then be taken forward in risk assessments.

**KEY WORDS:** Carcinogenesis; Key characteristics of carcinogens (KCCs); Mode of action (MOA); Risk assessment

## HIGHLIGHTS

- Key characteristics of carcinogens (KCCs) alone can lead to misclassification.
- KCCs cannot predict cancer, can be nonspecific, and may require disaggregation.
- Hypothesis testing of archetypal carcinogenesis mode of actions (MOAs) is an improvement to KCCs.
- Quantitative scoring for alternative MOAs (QMOA) can identify the operative MOA.
- QMOA can discriminate between threshold and linear approaches for risk assessment.

**ABBREVIATIONS:** AOPs, adverse outcome pathways; FCRI, Foundation for Chemistry Research and Initiatives; FDA, US Food and Drug Administration; IARC, International Agency for Research on Cancer; KCCs, key characteristics of carcinogens; MOAs, mode of actions; NAS, National Academy of Sciences.

In response to concerns about the ability of chemicals to cause cancer, in 1958 the Delaney Clause of the Federal Food, Drug and Cosmetic Act banned the addition of any additives to food

that had been shown to be carcinogenic. And several years later (mid-1960s), the International Agency for Research on Cancer (IARC) and the IARC Monographs program were created.<sup>1</sup> Both were successful and influential in identifying chemicals of potential concern, thus allowing authorities to restrict the use of these chemicals or otherwise reduce exposures to levels that were later described by the U.S. Food and Drug Administration (FDA) as *de minimis*, so low as to be inconsequential.<sup>2</sup> The science underpinning these activities in the mid-1960s to 1970s at that time was cutting edge, but largely relied on observational epidemiological studies, and observational high-dose laboratory animal studies. During this time, science continued to advance in cancer hazard identification, and the interplay of physiological systems and cancer dose responses and exposure assessments. These advancements lead to a plethora of ideas and often some confusion in the assessment and management of chemicals. Arguably the first breakthrough in this confusion came in 1983 with the National Academy of Sciences (NAS) of the United States (U.S.) publication of a book<sup>3</sup> on managing risk assessment in U.S. federal agencies (“The NAS Red Book”). Significantly, this breakthrough went beyond the hazard identification process of the Delaney Clause and the IARC approach to include risk assessment – the integration of exposure with hazard under specific scenarios.

The NAS<sup>3</sup> approach clarified the risk assessment landscape, and perhaps more importantly, laid the foundation for improvements in risk assessment methods. Since then, federal agencies, the NAS, and risk assessment scientists throughout the world have followed up with numerous risk assessment guidelines, refined approaches, and frameworks (e.g., see<sup>4–29</sup>). As scientific understanding of biological pathways increased, these evolving risk assessment guidelines, approaches, and frameworks further elucidated approaches for hazard identification and dose response assessment, as well as improved our understanding of the mechanisms of carcinogenic action by various chemicals. This understanding lead directly to the development of several frameworks describing chemical-specific cancer modes of action (MOAs). EPA defines MOA as “... a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation. A ‘key event’ is an empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically-based marker for such an element.”<sup>26</sup> A closely related, and chemical agnostic, concept also developed, called adverse outcome pathways (AOPs). This latter concept can be envisioned as the casual chain of biological responses from molecular-to-cellular-to-organismal that result in an adverse effect, whereas the MOA concept can be seen as how the chemical reacts along the body’s various AOPs. These interrelationships indicate these terms should be interchangeable.<sup>30</sup>

Now, a quarter of the way through the 21<sup>st</sup> century, extensive mechanistic knowledge of biological pathways, dosimetry, and dose-dependent transitions has clearly demonstrated that certain chemicals known to cause cancer under specific conditions of use (exposure) have key events in the MOA that exhibit thresholds (e.g., water disinfectant byproducts such as chloroform and

perchlorate). EPA has determined that for a number of substances that have been shown to cause cancer in animal studies, these are not likely to be carcinogenic in humans unless exposures exceed a threshold that cause certain responses, such as cytochrome P450-2B (CYP2B) induction, irritation responses in mucosal epithelium, formation of urinary bladder calculi, or alteration of rat thyroid hormone homeostasis.<sup>31</sup> A key step in such analyses, as shown by Borgert et al.,<sup>4</sup> is developing alternative potential hypotheses regarding toxicological effects and their underlying MOAs as part of the problem formulation step of the risk analysis. For example, Corton et al.<sup>32</sup> evaluated several MOAs (genotoxicity, receptor-mediated, and cytotoxicity) and showed the utility of determining quantitative thresholds (“molecular tipping points”) for evaluating potential carcinogenic risks. In cases where the likely operative MOA indicates cancer will not arise if exposures are below levels that cause sustained receptor mediated proliferation or below levels that cause repeated cellular toxicity leading to regenerative proliferation, a safe dose level can be derived using the reference dose procedure – recognizing that the underlying methods used for deriving this type of health guidance value have also undergone remarkable improvement.<sup>33</sup> Importantly, the 2016 Frank R. Lautenberg Chemical Safety for the 21st Century Act (15 U.S.C. §§ 2601 et seq.) now requires EPA to use mechanistic information, such as computational toxicology, bioinformatics, high-throughput screening methods, prediction models, weight of evidence, and best available science for evaluating the safety of chemicals and determining whether additional testing or risk management actions are warranted.

Unfortunately, despite subsequent amendments for pesticides, the Delaney Clause remains frozen in time, is unable to keep pace with scientific advances in risk assessment,<sup>34</sup> and leads to risk management actions based on phantom risks. This is clearly illustrated in the January 2025 decision by FDA to ban FD&C Red No. 3 in foods, dietary supplements, and ingested drugs, even though FDA stated “Based on the available data and information, FDA and other regulatory agencies including JECFA, the European Food Safety Authority (EFSA), and the Food Standards Australia New Zealand (FSANZ) have concluded that FD&C Red No. 3-induced thyroid tumors in [male] rats are of limited relevance to humans ... the carcinogenicity of FD&C Red No. 3 was not observed when tested in other animals including female rats and either sex of mice, gerbils, or dogs ...”<sup>35</sup> Ossification is a general feature of inflexible regulatory systems. These inflexible systems also serve as a barrier to progress in the regulatory sciences and contribute to the precipitation of crisis prior to revolution.<sup>36</sup> Risk assessment advances also have implications for more recent regulations (e.g., REACH) that seek to eliminate carcinogens and mutagens in commerce, as well as for authoritative bodies with a remit that still focuses efforts on cancer hazard identification (e.g., IARC) without concomitant efforts devoted to determining scenario-level exposures and developing assessments of potential risks at these relevant levels of human exposures.

In order to update its monograph evaluation procedures, IARC has adapted its hazard-focused approach by developing key characteristics for carcinogens (KCCs).<sup>37,38</sup> Other risk assessment groups are now also considering the use of KCCs. The purpose

of this paper is to briefly review the KCCs, examine their uses and concerns, and discuss scientific advancements and the future of cancer risk assessment. We conclude by proposing a return to the basic scientific principle of hypothesis generation and testing based on a broad consideration of mechanistic evidence with four previously identified archetypal chemical carcinogenesis MOAs,<sup>39</sup> and provide an example of an evolved causal evidence scoring system based on best available science and weight of the scientific evidence to identify the likely operative MOA for subsequent determination of potential risks and setting health guidance values, such as tolerable daily intakes.

## 2. BRIEF REVIEW OF KCCS, THEIR USE, AND CONCERNS

Table 1 shows the KCCs as identified by IARC.<sup>40</sup> These characteristics are readily recognized as general categories of effects that can be evoked by various chemicals. Not all chemicals, however, can or will evoke all KCCs, and some effects, such as *Alters cell proliferation, cell death, or nutrient supply* (KCC #10), can be elicited by almost any chemical given a high enough dose (e.g., nitrogen asphyxiation due to reduced oxygen supply).

The purpose of the KCCs, first described by Smith et al.,<sup>37</sup> was to “... provide the basis for an objective approach to identifying and organizing results from pertinent mechanistic studies.” Furthermore, these authors proposed “... use of the 10 key characteristics of human carcinogens as a basis for identifying and categorizing scientific findings relevant to cancer mechanisms when assessing whether an agent is a potential human carcinogen.” Smith et al.<sup>37</sup> also stated that “Overall, this categorization facilitated objective consideration of the relevant mechanistic information, thereby advancing analyses of hypothesized mechanisms and toxicity pathways.”

Smith et al.<sup>37</sup> concluded that information collected by using the 10 KCCs could be organized to form hypotheses that could then be evaluated for support of mechanistic events as a function of dose, species, and temporality as more fully described by Guyton et al.<sup>38</sup> Smith et al.<sup>37</sup> further noted, “Mechanistic topics can be included regardless of whether they have been

the subject of prior expert reviews of any particular chemical. This should introduce objectivity that could reduce reliance on expert opinion, as well as facilitate comparisons across agents. Moreover, at its essence, the approach may afford a broad consideration of the mechanistic evidence rather than focusing narrowly on independent mechanistic hypotheses or pathways in isolation.”

However, a broad consideration of mechanistic evidence does not preclude the need for rigorous hypothesis testing. MOAs and AOPs provide detailed frameworks for hypothesis testing,<sup>17,29,41</sup> and their focus is oftentimes mechanistic, or pathway related. We also note that several of the KCCs (e.g., induces oxidative stress, alters cell proliferation) are Key Events common to several MOAs and AOPs, which underscores the importance of not divorcing KCCs from “independent mechanistic hypotheses or pathways.” Cohen et al.<sup>39</sup> present a roadmap and decision tree for modernizing the evaluation of the carcinogenic potential of chemicals that moves away from chronic bioassay based studies to MOA-based screening.

Although the KCCs were proposed as a basis for organizing information, they are attractive to regulatory authorities and stakeholders seeking to simplify the complex process of cancer hazard identification. However, taken to an extreme of oversimplification, KCCs could be used 1) as a checklist that would allow persons with little to no technical expertise to identify what they think are cancer hazards and/or 2) to forgo the conduct of *in vivo* or other assays with a clear causal chain to apical carcinogenicity in favor of bioactivity assays. However, KCCs are neither key nor characteristics of cancer. For example, Doe et al.<sup>42</sup> propose a new approach for cancer classification that segregates substances by both mode of action and potency into three categories. This modernized approach provides useful guidance, based on up to date understanding of the science, for evaluating the carcinogenic potential of chemicals and for regulatory risk management decision-making.

Key characteristics of biological responses, adverse effects, and hallmarks of a disease have proliferated beyond carcinogens<sup>43</sup> and now encompass hepatotoxins, female and male reproductive toxins, cardiovascular toxicants, immunotoxicants, endocrine disruption, and aging.<sup>44</sup> There is considerable redundancy across these various sets of key characteristics for these different adverse outcomes (see Supplemental Material). The overlap of specific key characteristics across the various sets for different toxicants/adverse outcomes is depicted in Table 2. It is evident, for example, that oxidative stress is a key characteristic not only for carcinogens but also for cardiovascular toxicants, female reproductive toxicants, male reproductive toxicants, and hepatotoxicants. Thus, such characteristic are arguably not “key” to a sole specific adverse outcome, but rather, in many cases, would be more aptly described as biological responses associated with general toxicity pathways that are involved in many different types of adverse outcomes. This lack of specificity has been documented and discussed for KCCs.<sup>49</sup> Although it is beyond the scope of this analysis to address the limitations of various key characteristics, an examination published by Borgert<sup>45</sup> noted several “deficiencies in the KC approach to EDCs [endocrine disrupting chemicals].” These include, but are not limited

**Table 1. The key characteristics of carcinogens described by Smith et al. (2016) as cited by International Agency for Research on Cancer (IARC) (2019).**

Ten key characteristics of carcinogens (KCCs)
1. Is electrophilic or can be metabolically activated to an electrophile
2. Is genotoxic
3. Alters DNA repair or causes genomic instability
4. Induces epigenetic alterations
5. Induces oxidative stress
6. Induces chronic inflammation
7. Is immunosuppressive
8. Modulates receptor-mediated effects
9. Causes immortalization
10. Alters cell proliferation, cell death, or nutrient supply

**Table 2. Comparison of characteristics across the various sets of key characteristics (KCs) of different toxicants/adverse outcomes.<sup>1,2</sup>**

Characteristic	KCs of carcinogens	KCs of cardiovascular toxicants	KCs of endocrine disrupting chemicals	KCs of female reproductive toxicants	KCs of hepatotoxicants	KCs of immunotoxicants	KCs of male reproductive toxicants	Hallmarks of aging
Electrophilic	x				x	x		
Genotoxic	x			x			x	
Genomic instability	x							x
Epigenetic alterations	x		x	x			x	x
Oxidative stress	x	x		x	x		x	
Inflammation	x	x			x		x	
Immunosuppressive; immune active	x			x		x		
Alters receptor-mediated effects	x	x	x	x			x	x
Immortalization	x							
Alters cell proliferation, cell death	x			x	x	x	x	x
Alters mitochondrial function		x		x	x			x
Cytoskeleton, microtubules				x	x			
Disrupts metabolism			x		x			

<sup>1</sup>See Supplemental Material for complete descriptions of each set of key characteristics. An x indicates the specific characteristic listed in the ordinate is contained in the specific KC set depicted in the abscissa.

<sup>2</sup>Note: Direct verbatim comparisons of key characteristics were not always used. Instead, inference was used in a number of cases to facilitate this elemental side-by-side comparison. For example, the hepatotoxic key characteristics of “causes liver fibrosis” was entered as “inflammation” since inflammation is part of the causal chain of events leading to liver fibrosis. Consequently, others may interpret the similarities of specific characteristics differently.

to, “fails to apply the consensus definition of EDC and is not amenable to empirical testing or validation; Is flexible according to diverse goals, which also ensures inconsistent and unreliable results; lacks a means of distinguishing endocrine-mediated from non-endocrine mediated mechanisms; Lacks a means to reach a negative conclusion about a chemical’s EDC properties and appears to be incapable of distinguishing EDCs from non-EDCs.”

KCCs were not immediately and widely recognized as Key Events and integrated into the ongoing development of MOAs/AOPs. A publication by Becker et al.<sup>46</sup> demonstrated the pitfalls of using KCCs alone to identify cancer hazards. Extensive analyses of prediction modeling showed that, when mapped to ToxCast/Tox21 assays and compared to EPA cancer classifications from traditional data, KCCs were no better than chance in distinguishing carcinogens from noncarcinogens (ibid.). We have now extended this analysis by examining in greater detail the information presented in Guyton et al.<sup>38</sup> As detailed in Table 3, for IARC evaluations conducted between 2017 and 2019, if KCCs are used in isolation (e.g., as a “stand-alone” for cancer hazard identification) “mislabeling” as a carcinogen would occur for certain agents when IARC otherwise considers the animal and human evidence “limited” and/or “inadequate” (e.g., 2,4-dichlorophenoxyacetic acid), as well as “mislabeling” agents as Group 3 that IARC considers to have “sufficient” evidence of carcinogenicity (e.g., consumption of processed meats). Overall, one out of four

IARC Category 1 carcinogens had no associated KCCs, four out of 12 IARC category 2A substances had no associated KCCs, and 10 out of 16 IARC category 2B agents had no associated KCCs. Collectively, 15 out of 32 IARC classified carcinogens had no associated KCCs. This approximately 50% success rate of using KCCs in isolation for classification as a Group 1 or 2 carcinogen is no better than chance and is consistent with Becker et al.<sup>46</sup> Therefore, while KCCs may have utility for searching and organizing the literature, they cannot be used on their own to predict the carcinogenic potential of a chemical to humans. Or, as Bus<sup>47</sup> demonstrated, the KCC approach alone lacks “... robust data integration [that is] fundamental to reasonable mode of action evaluations.”

Other problematic aspects of KCCs have also been pointed out.<sup>30,48,49</sup> The amended January 2019 IARC Preamble<sup>40</sup> explicitly includes KCCs as part of the mechanistic evidence evaluation procedures employed by IARC Working Groups. The IARC Preamble guidance states that mechanistic evidence is classified as “strong” when there is “... Strong evidence that the agent exhibits key characteristics of carcinogens.” Yet, the IARC Preamble fails to specify criteria for how many and which of the KCCs constitute “strong” evidence. The Preamble defines “inadequate” mechanistic evidence as little/no data, questionable studies, and negative results in the available data. This raises the question of how data would be characterized if, for example, there were a large, high-quality, and consistent data set showing a substance is negative

**Table 3. Agents with cancer classifications and number of key characteristics (KCCs).** [Adapted from Guyton et al. 2018 that was published under a Creative Commons License (Attribution Non-Commercial 4.0 International – Creative Commons at <https://creativecommons.org/licenses/by-nc/4.0/>; adaptation consisted of expressing the last column as the number of KCCs.)]

Agent	Year	IARC Group	Cancer in humans	Cancer in animals	# Studies	# KCCs
Pentachlorophenol (PCP)	2019	1	Sufficient	Sufficient	239	#1, #2, #5, #8, #10
Lindane	2018	1	Sufficient	Sufficient	375	#5, #7
Welding fumes	2018 online	1	Sufficient	Sufficient	189	#6, #7
Consumption of processed meat	2018	1	Sufficient	Inadequate	144 <sup>1</sup>	-
Malathion	2017	2A	Limited	Sufficient	249	#2, #5, #6, #8, #10
Hydrazine	2018	2A	Limited	Sufficient	117	#1, #2, #5, #10
Dichlorodiphenyltrichloroethane (DDT)	2018	2A	Limited	Sufficient	745	#5, #7, #8
N,N-Dimethylformamide (N,N-DMF)	2018	2A	Limited	Sufficient	170	#1, #5, #10
3,3',4,4'-tetrachloroazobenzene (TCAB)	2019	2A	Inadequate	Sufficient	22	#6, #8, #10
Tetrabromobisphenol A	2018	2A	Inadequate	Sufficient	147	#5, #7, #8
Diazinon	2017	2A	Limited	Limited	125	#2, #5
Glyphosate	2017	2A	Limited	Sufficient	146	#2
2-Mercaptobenzothiazole	2018	2A	Limited	Sufficient	19	-
Consumption of red meat	2018	2A	Limited	Inadequate	144 <sup>1</sup>	-
Dieldrin and aldrin metabolized to dieldrin	2019	2A	Limited	Sufficient	237	-
Very hot beverages	2018 online	2A	Limited	Sufficient	30 <sup>2</sup>	-
1-Bromopropane	2018	2B	Inadequate	Sufficient	29	#1, #5, #6, #7, #10
2,4-Dichlorophenoxyacetic acid (2,4-D)	2018	2B	Inadequate	Limited	269	#5
3-Chloro-2-methylpropene (technical grade)	2018	2B	Inadequate	Sufficient	11	#2
Furfuryl alcohol	2019	2B	Inadequate	Sufficient	21	#1
Indium tin oxide	2018 online	2B	Inadequate	Sufficient	22	#6
Melamine	2019	2B	Inadequate	Sufficient	78	#6
1-tert-Butoxypropanol	2019	2B	Inadequate	Sufficient	2	-
2,4,6-Trichlorophenol	2019	2B	Inadequate	Sufficient	35	-
β-Myrcene	2019	2B	Inadequate	Sufficient	34	-
Molybdenum trioxide	2018 online	2B	Inadequate	Sufficient	9	-
N,N-Dimethyl-p-toluidine	2018	2B	Inadequate	Sufficient	19	-
Parathion	2017	2B	Inadequate	Sufficient	209	-
Pyridine	2019	2B	Inadequate	Sufficient	45	-
Tetrachlorvinphos	2017	2B	Inadequate	Sufficient	29	-
Tetrahydrofuran	2019	2B	Inadequate	Sufficient	22	-
Vinylidene chloride	2019	2B	Inadequate	Sufficient	76	-

<sup>1</sup>Number of studies apply to red and processed meat; for red meat, there was strong mechanistic evidence for meat components.

<sup>2</sup>Applies to mate and hot beverages.

for producing DNA reactive species (KCC #1), and negative in inducing mutations (KCC #3) in human cells, and negative in altering cell proliferation (KCC #10). Would an IARC Workgroup characterize such data as “inadequate” for KCCs 1, 3, and 10? Shouldn’t such a data set be regarded as strong counterevidence that would support a downgrade in classification from Group 2B to Group 3, provided evidence in humans was “limited” and evidence in animals was “less than sufficient”? IARC has recognized some limitations of KCCs,<sup>37</sup> noting “Because non-carcinogens can also induce oxidative stress, this key characteristic should be interpreted with caution unless it is found in combination with other key characteristics.<sup>35</sup>” However, IARC goes on to indicate<sup>37</sup> that “Evidence for a group of key characteristics

can strengthen mechanistic conclusions.” We note this is not necessarily the case, as the ability to predict cancer hazard by KCCs whether singly, or in combination, is problematic (Tables 3 and 4) and no better than chance.<sup>46</sup>

While consideration of hazard alone may have been a state-of-the-science practice a half-century ago, it is not today. The understanding of exposure and dose-response relationships is fundamental to assessing the potential for harm which is essential for regulatory decision-making (e.g., setting safety standards) and public health strategies (e.g., determining interventions to reduce exposure and mitigate risks). Further, in understanding dose-response relationships, there have been scientific advances in mechanistic toxicology that indicate dose-dependent transitions

**Table 4. Summary of key characteristics (KCCs) for IARC Group 3 (“the agent is not classifiable as to its carcinogenicity in humans”) and EPA Groups D & E (noncarcinogens: “not classifiable as to human carcinogenicity and evidence of noncarcinogenicity in humans,” respectively).**

Agent	IARC Group 3 or EPA Group D or E	Putative KCCs identified by authors from literature search of abstracts <sup>1</sup>
Aldicarb	IARC Group 3	#2, #5
Atrazine <sup>2</sup>	IARC Group 3	#2, #5
Chloroacetonitrile	IARC Group 3	#2, #5
Coal dust	IARC Group 3	#2, #5
Decabromodiphenyl oxide	IARC Group 3	#2, #4, #5
Dicofol	IARC Group 3	#5
Hepatitis D	IARC Group 3	#2
Hydroquinone	IARC Group 3	#5
Jet fuel	IARC Group 3	#2, #4, #5, #7
Pentavalent antimony	IARC Group 3	#2, #5
Permethrin <sup>3</sup>	IARC Group 3	#2, #5
Simazine <sup>2</sup>	IARC Group 3	#6, #7
Sulfur dioxide	IARC Group 3	#5
Thiram <sup>2</sup>	IARC Group 3	#2, #5
Zineb	IARC Group 3	#2, #5
DEET	EPA Group D	#5, #7
Kathon	EPA Group D	#2, #6
Chlorpyrifos	EPA Group E	#4, #5
Prometryn	EPA Group E	#2

<sup>1</sup>The number in this column corresponds to the specific key characteristics (KCC) listed in Table 1. For example, the entry #2 indicates published literature has reported results of “is genotoxic.”

<sup>2</sup>EPA classification (as of 2018) “Not Likely To Be Carcinogenic to Humans.”

<sup>3</sup>EPA classification (as of 2018) “Suggestive Evidence of Carcinogenic Potential.”

EPA states, “Note: Chemicals that were classified under previous EPA guidelines (e.g., chemicals with 1986, 1996, or 1999 cancer classifications) cannot be directly compared to the 2005 cancer classification descriptors. Each system designation refers to the reviews and criteria it contains...” <https://www.epa.gov/system/files/documents/2023-06/2023%20ARC%20WoE%20Guidance.pdf>

occur in mammals. A study by Zhang et al.<sup>50</sup> identified the molecular signaling motifs that underlie dose-dependent transitions at the cellular level. Importantly, all transitions had thresholds, and observed linearity could occur at concentrations higher than the transition concentrations.

Conceptually, thresholds will occur when exposure is so low as to not cause a response, or the response elicited is considered within the homeostatic range. A dose-dependent transition would occur when there is higher exposure, or a greater dose-time intensity, which is sufficient to overcome such homeostatic resilience. Dose-response modeling is critical in evaluating both apical effects data and mechanistic data streams since it is well established that dose-dependent transitions exist in mechanisms of toxicity.<sup>51</sup>

Another shortcoming of hazard-based approaches, such as the KCCs, is that they do not explicitly incorporate understanding of the causal linkages of the sequence of key events and

biological responses (including dose-response and temporal relationships) involved in carcinogenesis. For incorporating mechanistic data into cancer hazard evaluations, the AOP<sup>52</sup> and MOA frameworks<sup>14</sup> are prescriptive by articulating toxicity pathways comprised of sequences of key events, starting with an initial molecular event, followed by a series of key events linked to one another temporally, ultimately resulting in a specific adverse outcome.<sup>13,14</sup> The causal relationships between Key Events (KEs) and tumor development is a critical aspect of MOA and AOP framework evaluation, and this is what application of the KCC approach often lacks.

Along these lines, Rusyn and Wright<sup>44</sup> also found that genotoxicity and cell proliferation were strongly associated with IARC decisions on whether mechanistic data was impactful on the final cancer hazard classification. Perhaps not surprisingly, these two categories are also often the key events on the pathway to cancer as described in the MOA/AOP approaches. This observation provides fertile ground from which to suggest a new path forward (discussed below in Section 4).

Actual use of the KCCs by IARC Working Groups from 2015 to 2022 for 73 agents has been statistically analyzed by Rusyn and Wright.<sup>52</sup> This analysis included the strength of evidence for each KCC for each agent. The authors found no data for the majority (approximately 65%) of KCC/agent pairwise comparisons, with strong and moderate KCC/agent pairs for about 13% each (for a total of about 26%) and weak KCC agent/pairs at less than 10%. This indicates a relatively low availability of strong or moderate mechanistic evidence based on KCCs for these 73 agents. In terms of strength of mechanistic evidence, KCC #2 (is genotoxic) was reported as strong evidence more times than any other KCC. With the exception of KCC #8 (receptor-mediated) and KCC #10 (alters cell proliferation/death/nutrient supply), KCCs tended to decrease in strength from Group 1 to Group 3. However, strong KCCs did not differ between Group 1 and Group 2A, although differences were present between Group 1 and Group 3 and Group 2A and Group 3. It is important to note that even when KCCs were available, they were not always used by IARC Working Groups. For example, 75% of agents classified as Group 3 nevertheless showed moderate to weak evidence of genotoxicity<sup>52</sup> (Figure 3). This is consistent with the authors<sup>44</sup> statement that KCCs are not used by IARC “... to predict cancer ... or to determine carcinogenic potency ...” The authors additionally noted that ToxCast data, when used, tended to be used mostly for ruling out KCCs for an agent. We recognize that the revised IARC preamble does allow for mechanistic evidence (which presumably includes KCCs) to be used when human or animal evidence is limited, inadequate, or less than sufficient (Table 4). As such, we note use of KCCs and *in vitro* data by IARC Work Groups could change in the future as confidence in KCCs and/or confidence in *in vitro* data increase.

### 3. COULD THE USE OF KCCS PRODUCE FALSE POSITIVES?

In order to determine if use of KCCs alone for cancer classification could potentially produce false positives, we conducted a literature search in January of 2024 in PubMed<sup>®</sup> using the following

search string: “electrophile OR electrophilic OR hyperplasia OR DNA adduct OR protein adduct OR genotoxic OR DNA repair OR DNA replication OR genomic instability OR epigenetic alterations OR DNA methylation OR histone modification OR oxidative stress OR oxidative damage OR chronic inflammation OR immunosuppression OR immunosuppressive OR immortalization OR DNA damage OR gene mutation OR DNA strand break OR DNA-protein cross-link OR unscheduled DNA synthesis OR intercalation OR chromosome aberration OR base excision OR elevated white blood cell OR altered cytokine OR chemokine production OR decreased immunosurveillance OR immune system dysfunction OR altered cell proliferation OR leukocytosis OR modulates receptor-mediated”. This “KCC Master List” produced over 2 million hits. We then selected particular Group 3 agents from IARC Monographs 1–135 or pesticides EPA classified as Group D or E. (The EPA descriptors/classifications from the 1986 Guidelines were selected because these appeared to us more similar to IARC’s descriptors than EPA descriptors/classifications according to the more recent 2005 Guidelines). For each particular agent/pesticide, we ran a PubMed search and then identified the studies common to both of these lists by selecting the “Add” query and using the “Add with AND” function. The abstracts of the studies common to both lists were then examined manually to identify KCCs.

The results are summarized in Table 4. There are 15 agents deemed not classifiable as to carcinogenicity (IARC Group 3, EPA Group D) or classified as having evidence of noncarcinogenicity to humans (EPA group E) for which 1 or more putative KCCs were identified. This analysis, while not exhaustive, nonetheless strongly suggests that cancer classification based on KCCs alone could produce false positives. These findings should be viewed as illustrative not exhaustive; further insight could be obtained by expanding the analysis to the more than 200 pesticides classified by EPA using the more current descriptor as “Not Likely To Be Carcinogenic To Humans.”<sup>31</sup> Also, since KCCs are not predictive of cancer,<sup>46</sup> even if a battery of tests that identify all the KCCs are all negative, this does not rule out the potential for a carcinogenic response.

#### 4. BEYOND KCCS – AN MOA-BASED FRAMEWORK AND EVIDENCE SCORING SYSTEM FOR HYPOTHESIS TESTING TO IDENTIFY THE LIKELY OPERATIVE MOA FOR USE IN HUMAN HEALTH RISK ASSESSMENTS

Without a suitable framework for hypothesis generation and testing, use of KCCs as a stand-alone for cancer hazard identification may lead to “tunnel vision” by constraining consideration of the entirety of the evidence, including species-specific AOPs and consideration of dose-response and exposure. This is evident for chemicals causing cancer in experimental animals that is explained by AOPs that are not relevant to humans. Examples include  $\alpha$ 2u-globulin kidney tumors in male rats,<sup>53</sup> phenobarbital,<sup>54</sup> or acrylamide-induced tunica vaginalis mesotheliomas in rats.<sup>55</sup> Species-specific differences in MOAs are also observed between experimental animals and humans, for example, chemical induction of PPAR-alpha in mice with resulting liver tumors<sup>56</sup> or induction of thyroid tumors in rats.<sup>57</sup> As we

have mentioned in previous examples, situations also exist where chemicals not known to cause cancer nevertheless evoke one or more KCCs (i.e., high levels of oxygen inducing oxidative stress; see also Table 1).

The pros and cons of approaches using KCCs, MOAs and AOPs have been the recent focus of many publications that debate these approaches.<sup>37,38,46–49,58,59</sup> Unfortunately, this debate remains largely unresolved. However, a path forward has been proposed where the original intent of the KCCs is integrated into the existing prescriptive MOA and AOP approaches. Meek and Wikoff<sup>30</sup> present an example of such an integration by first considering the three traditional evidence streams, toxicology, epidemiology, and mechanistic, independently, and then melding these into a chemical-specific MOA.<sup>a</sup> This approach is similar to the hypothesis-based weight of evidence approach.<sup>60–62</sup> If sufficient information is available in experimental animals and/or humans, then this MOA is placed within a species-specific AOP. KCCs are then seen as Key Events within this integration, leading to a fuller appreciation of the underlying complexity of the causal pathways leading to carcinogenic risk in humans.

Thus, while evidence from any particular stream, such as a human observational study(ies), may show a statistically significant correlation with cancer and a particular compound, such a correlation needs to be substantiated by a supportive MOA, AOP, and/or positive findings from other evidence streams, in order for such a finding to form the basis of a credible hazard identification. Moreover, the designation that a chemical causes cancer, while an important step in the risk assessment process as highlighted early on by the Delaney Clause and IARC, is only the first step. The NAS<sup>3</sup> and numerous additional publications and multiple agency guidelines now dictate considerations of dose response and scenario-level exposure assessment in a biological pathway relevant to humans prior to the development of a risk characterization. And of course, the integration of this evidence into a risk characterization is needed prior to any risk management decision.

And what might this integration of evidence in a biological pathway relevant to humans look like? One approach, gleaned from the last 70 years of research, is based upon the body of scientific evidence as summarized by Cohen et al.,<sup>39</sup> that:

- “Cancer is due to mistakes occurring in the DNA (usually in somatic cells but can be inherited through germ cells).
- More than one mistake in the DNA is necessary.
- All of the mistakes need to accumulate in a single cell (clonal origin of cancer).

<sup>a</sup>EPA<sup>26</sup> defines MOA and Key Events as follows: “The term ‘mode of action’ is defined as a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation. A ‘key event’ is an empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element.” OECD<sup>68</sup> defines Key Event Relationships (KERs) as “A scientifically based relationship that connects one key event to another, defines a causal and predictive relationship between the upstream and downstream event, and thereby facilitates inference or extrapolation of the state of the downstream key event from the known, measured, or predicted state of the upstream key event.”

**Table 5. Archetypal modes of action (MOA) of chemical carcinogenesis.**

Mode of action (MOA)	Characteristic of this MOA
Mutagenic	A chemical (or metabolite) directly interacts with DNA and causes mutations which can be carried forward in dividing cells.
Cytotoxicity	A chemical (or metabolite) interacts with cellular processes to induce toxicity which induces regenerative proliferation from repeated cellular injury. Chemicals acting via this MOA will have no effect on carcinogenicity at dose levels below which cytotoxicity occurs.
Receptor-mediated induction of proliferation (mitogenesis)	A chemical (or metabolite) interacts with receptor processes to induce cellular proliferation (at an intensity that exceeds homeostasis). Chemicals acting via this MOA will not be carcinogenic at dose levels at which the increased cellular proliferation does not occur.
Immunosuppression	A chemical (or metabolite) decreases the ability of immune system to fight infections that cause cancer (or impaired immune surveillance of neoplastic cells).

- The cell population at risk are generally the tissue pluripotent (stem) cells.
- Every time DNA replicates, permanent mistakes could occur.
- Carcinogenesis is a stochastic process.”

Based on this accumulated wealth of scientific knowledge and data, two fundamental ways exist to increase the risk of cancer by any chemical, either by 1) damaging DNA directly<sup>63,64</sup> or by 2) increasing the number of stem cell replications<sup>65–67</sup> because with each replication spontaneous mutations occur. These two fundamental pathways to cancer can be organized into four archetypal MOAs<sup>69</sup> to enable broad consideration of mechanistic evidence, as summarized in Table 5.

Understanding the manner in which a chemical can cause cancer is important not only for identifying likely hazards to human health, but also for determining the quantitative risks to human health associated with specific magnitudes (and duration and frequencies) of exposures.<sup>49</sup> The existence of thresholds continues to be debated for exposure to chemicals that directly interact with DNA to cause mutations, which could then be carried forward in dividing cells.<sup>b</sup> In theory, there is sufficient knowledge of biological processes to support the existence of thresholds for such substances, but experimental demonstration is challenging. For unavoidable exposures to carcinogenic and genotoxic substances that occur naturally in food, the Scientific Committee for the EFSA has endorsed a threshold approach using a benchmark dose lower bound (BMDL<sub>10</sub>) as a point of departure for which a 10,000-fold composite uncertainty factor is added that includes the standard 100-fold interspecies and intraspecies uncertainty factor plus an additional 100-fold to account for uncertainties in human variability in cell cycle control as well as lack of a true human No Observed Adverse Effect Level (NOAEL).<sup>71</sup> Subsequent studies<sup>72</sup> have adopted this approach. Regardless of differing scientific opinions on thresholds for genotoxic and carcinogenic chemicals, there is (or should be) little disagreement within the regulatory science community, that there are thresholds for carcinogenesis for substances that induce receptor-mediated proliferation and for substances that induce regenerative proliferation caused by repeated cellular toxicity. Differentiating among these MOAs is then needed to

determine whether a threshold in the dose response is likely, which in turn informs the dose response method to use in the risk characterization.

One way to approach this differentiation is to use a recently developed causal analysis scoring approach for potential cancer MOAs,<sup>73</sup> which evolved from the World Health Organization International Programme on Chemical Safety (WHO/IPCS) MOA framework. The weight of evidence supporting a hypothesized MOA is assessed within the context of criteria inspired by the evolved Bradford Hill causal considerations<sup>14</sup> that have been operationalized for this method. The improved Quantitative Mode of Action (QMOA) confidence scoring approach (see Supplemental Material) employs a four criteria scoring approach: (1) Empirical Support for KEs; (2) Concordance of KEs and Key Event Relationships (KERs); (3) Essentiality; and (4) MOA Coherence. Dekant et al.<sup>74</sup> developed a similar approach for conducting a quantitative weight of evidence assessment of confidence in MOAs.

Qualitative or quantitative approaches for evaluating the evidence in support of alternative hypothesized MOAs provide a transparent, structured, and systematic system for evaluating a chemical dataset, including mechanistic data, using, for example, causal considerations for evaluating and integrating evidence with knowledge of the biological processes and pathways leading to cancer. Table 6 shows how the different chemical carcinogenesis MOAs, each with several unique key events, eventually lead to the common key events of proliferation, clonal expansion, and progression to cancer. Any novel hypothesized MOA can also be included in the analysis, as indicated in Table 6, provided the Molecular Initiating Events (MIEs) and the unique KEs can be articulated, and data are available for these.

Qualitative evaluations using the WHO/IPCS MOA framework for comparing alternative hypothesized MOAs have been the mainstay for a number of years by many scientists, and this approach continues to be valuable in differentiating amongst alternative plausible MOAs.<sup>75</sup> However, not all scientists or institutions conducting hazard and risk evaluations use such an approach; the IARC Monograph Programme, for example, does not use this approach. And even in those that do, quite often the opinions of data evaluators about the sufficiency of evidence in support of different plausible MOAs vary.

<sup>b</sup>For a series of papers expanding on the inappropriate use of a linear approach to cancer dose response assessment, see Calabrese and Selby.<sup>70</sup>

**Table 6. An improved approach for integrating evidence in a biological pathway construct – an example of an analysis template for organizing and analyzing data to compare alternative hypothesized mode of action (MOAs) for chemical carcinogenesis.**

Mode of Action (MOA)	Unique Key Events			Common Key Events	
<b>Mutagenic (hypothesized non-threshold)</b>	KE #1 - Activation and formation of chemical-specific pro-mutagenic DNA adducts	KE #2 - Insufficient repair or mis-repair of pro-mutagenic DNA adducts	KE #3 - Early induced critical mutations driving cancer	KE #4 Proliferation, clonal expansion of mutant cells, and progression	KE #5 Cancer
<b>Cytotoxicity (threshold)</b>	KE #1 - Direct cytotoxicity or metabolic activation to reactive metabolite leading to cytotoxicity	KE #2 - Cytotoxicity in target tissues / cells	KE #3 - Sustained cytotoxicity with regenerative cell proliferation – with accompanying increase in spontaneous mutations		
<b>Receptor Mediated (threshold)</b>	KE #1 - Direct interaction, or metabolism to molecule that binds to/activates a cellular receptor	KE #2 - Interaction of the receptor in target tissues / cells	KE #3 - Cell proliferation stimulus – increase in spontaneous mutations		
<b>Immune Suppression (hypothesized threshold)</b>	KE #1 - Reduced humoral or cellular immune response	KE #2 - Reduced destruction of pre-cancer and cancer cells or increased opportunistic or latent infections	KE #3 - Survival of pre-cancer or cancer cells or increased inflammation due to infection or transcriptional reprogramming via oncogenic infection		
<b>Novel MOA</b>	KE #1 - (The initial interaction between a chemical and a biomolecule or biosystem)	KE #2 - (The biochemical or cellular response in the cell triggered by the initial event)	KE #3 - (The specific cellular or tissue response that initiates proliferation)		

One approach for using causal considerations for evaluating and scoring alternative hypothesized MOAs (modified from Becker et al., 2017) involves analyzing: 1) Empirical Evidence for Each KE, 2) Coherence (to be scored for each hypothesized MOA in toto), 3) Essentiality (to be scored for each hypothesized MOA in toto), and 4) Concordance (to be scored for each hypothesized MOA in toto). Additional details of the considerations risk assessors can use in applying this approach and example scoring criteria for Empirical Evidence, Coherence, Essentiality, and Concordance are included in Supplemental Material.

- Empirical Evidence for each KE is determined by evaluating the extent of evidence (i.e., number and quality of studies), dose response, and study consistency, repeatability, etc.
- Coherence (to be scored for each hypothesized MOA in toto, not for each KE) is determined by consideration of broader information to evaluate consistency with the proposed MOA, observed patterns for species, strain, sex, and target tissue are consistent with the MOA, evidence from structural and/or phenotypic analogs, etc.
- Essentiality (to be scored for each hypothesized MOA in toto, not for each KE) is determined by data showing the KE is required for the adverse effect to occur, such as blocking a KE or pathway results in blocking of the downstream KEs and/or adverse effect, or experimental modulation of the KE or pathway results in an expected modulation of downstream KEs or adverse effect.
- Concordance (to be scored for each hypothesized MOA in toto) is determined by examining the patterns of dose, incidence, severity, and temporal relationships for each of the key events and for the tumor response. Analysis of the pattern of data and evidence of the proposed relationships between KEs and KERs with respect to dose (e.g., KE1 occurs at a lower dose than a subsequent KE), incidence, severity, and temporal relationships (e.g., KE1 occurs earlier in time than a subsequent KE), etc.

Unfortunately, because guidance for determining when the evidence of an alternative MOA is sufficient to overcome the default position of low dose linearity is lacking, or has not been fully implemented in certain hazard and risk evaluation programs, it can seem that no matter how much scientific evidence is available in support of an alternative plausible MOA, an evaluator, or a program, using qualitative analysis approaches, still concludes, usually based on some data that is discordant with the alternative MOA, that the alternative MOA has not been conclusively proven, and therefore cancer risks need to be assumed to “conform with low dose linearity.”<sup>26</sup> To overcome this inappropriate use of a “default” an evaluation can use the WHO/IPCS MOA framework along with criteria to derive quantitative confidence scores for different degrees of causal evidence is necessary. Such an analysis facilitates side by side comparison of the extent of scientific evidence for different hypothesized cancer MOAs to the linear no-threshold (LNT) default MOA. We further note here that LNT MOA ignores decades of research that identified DNA repair mechanisms, cell cycle checkpoints, and driver gene mutations and instead assumes that even a single mutation can become fixed in the genome, resulting in cancer. Quantitative comparison provides clarity and confidence in determining the likely operative MOA. Examples of quantitative scoring approaches that use best available science and weight of

the evidence for comparing alternative MOAs and selecting the likely operative MOA include Becker et al.,<sup>63</sup> Dekant et al.,<sup>64</sup> and Kirman et al.<sup>76</sup>

Thus, the path forward we suggest is to de-emphasize empiricism and return to the basic principles of hypothesis formulation and testing. When evaluating mechanistic information to determine the likelihood that a chemical poses a carcinogenic risk to humans, an approach (once the systematic literature review is complete) is depicted in Figure 1, and described below:

- Create a table/template for each of the 4 distinct archetypal MOAs (Table 6). If a novel MOA is hypothesized, this too can and should be included in the table/template (see the last row in Table 6). Specific MIEs and KEs should be described for each postulated MOA (e.g., specific DNA adducts, cytotoxicity of a specific cell type in an organ system, specific receptor interaction (constitutive androstane receptor [CAR], peroxisome proliferator activated receptor alpha [PPAR-α], pregnane X receptor [PXR], estrogen receptor, etc.).
- For each Key Event, map the KCC or similar mechanistic assay (with relevant and reliable data) to each specific KE for each of the hypothesized MOA. If there is no data for a given MOA/KE pair, indicate such.

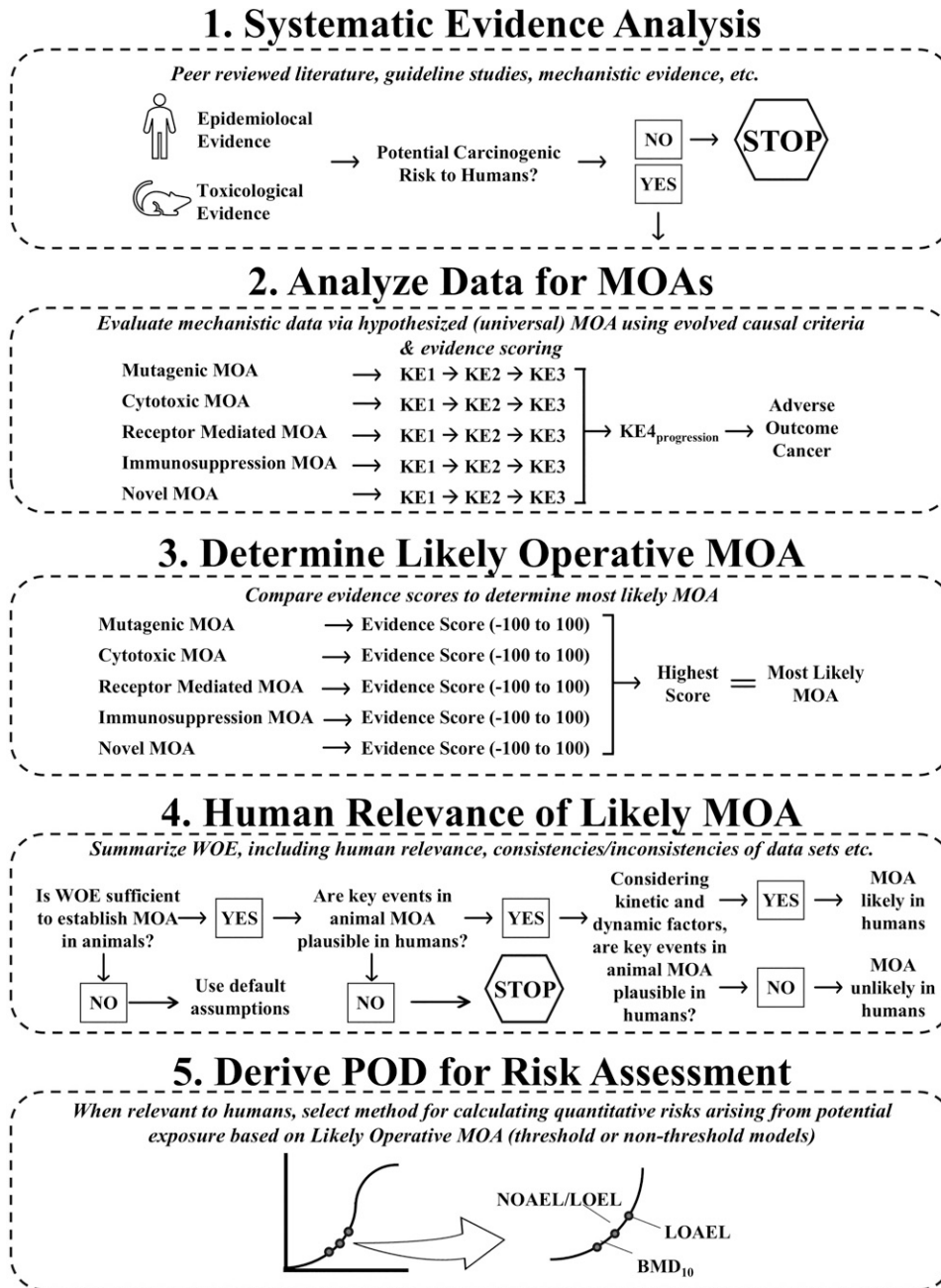


Figure 1. Representation of steps in the hypothesis-testing approach using archetypal cancer mode of actions (MOAs) to determine the likely operative MOA for hazard and risk assessment.

- Evaluate the data for each KE in each MOA, using a quantitative scoring system based on causal analyses.<sup>c</sup> Data evaluation is key. It is not simply determining if a substance is positive in an assay, but must include, for example,

<sup>c</sup>For example, the modified approach of Becker et al.<sup>73</sup> summarized in text at the bottom of Table 6, or the methods of Dekant et al.<sup>74</sup> or Kirman et al.<sup>76</sup> Additional details of the considerations that risk assessors can use in applying the modified approach of Becker et al.<sup>73</sup> are included in Supplemental Materials.

consideration of the validation status of the assay (sensitivity, specificity, reproducibility, and domain of applicability), the characteristics and slope of dose response curves for each assay in each KE, and, importantly, whether response is specific or nonspecific (such as the cytotoxicity burst phenomenon).<sup>77</sup>

- Analyze the quantitative results for each hypothesized MOA. The MOA having the largest positive score indicates the likely operative MOA responsible for inducing cancer.

- Evaluate the human relevance of the likely operative MOA to assess whether the animal MOA and kinetic and dynamic factors are plausible in humans.
- If the tumor site or the likely MOA is not relevant to humans it would generally be considered not applicable to human health risk assessment.
- Based on the determination of the likely operative MOA and human relevance, select the method for quantifying the potential hazard and risk at the level of exposures humans experience.
- As noted, threshold models are appropriate for receptor-mediated MOAs and for cytotoxicity MOAs.
- If evidence is insufficient to establish a likely operative MOA, then default assumptions would generally be used.

While it may appear to be a simple exercise on its face, aligning or mapping the current set of KCCs to Key Events within these four MOAs is not straightforward. As we considered approaches to actualize the diagram in Figure 1, it became apparent that analyses have to be at the level of each specific assay and each level of biological organization, not simply at the “named characteristic level.” As discussed in detail by Meek and Wikoff,<sup>30</sup> a given KCC typically agglomerates many assays and many endpoints and these need to be disaggregated to be able to conduct MOA hypothesis testing as described in Table 6. For example, assays for KCC #3 “alters DNA repair or causes genomic instability” need to be explicitly segregated into assays that align with early Key Events, such as “insufficient repair or misrepair of pro-mutagenic DNA adducts” and late Key Events – such as “genomic instability” – that are likely common to all cancers.

In other words, several of the KCCs are endpoint agnostic. As noted above, key characteristics are not necessarily unique nor characteristics of any one adverse outcome. In fact, many are general biological responses associated with many different mechanisms of toxicity, diseases, and aging. For example, many different responses in different cells may ensue from, for example, oxidative stress, or chronic inflammation, or immunosuppression, or modulating receptor effects, or altering cell proliferation. Thus, integrating these endpoint-agnostic KCCs into an approach that describes a sequence of biological events postulated to occur during carcinogenesis, necessitates an understanding of the underlying relationships. From this perspective, the National Academies of Sciences, Engineering, and Medicine<sup>78</sup> may have complicated the regulatory science community’s discourse by paraphrasing Smith et al.<sup>37</sup> in stating, the KCC approach “... avoids a narrow focus on specific pathways and hypotheses and provides for a broad, holistic consideration of the mechanistic evidence.” Why? Because hypothesis testing is the core principle of the scientific method. Understanding how any particular KCC fits within the framework of a cancer hazard identification analysis necessitates understanding how it relates to the key events and sequence of biological changes that lead to cancer and not just any endpoint. To do so, we contend testing hypotheses using a causative biological pathway framework, such as the evolved MOA/AOP framework depicted in Figure 1 and illustrated in Table 6. Thus, the path forward we suggest is to de-emphasize

empiricism and return to the basic principles of hypothesis formulation and testing that explicitly addresses the causal question.

## 5. DISCUSSION

One might think that application of the KCCs to cancer hazard identification is a robust new approach to organizing and evaluating the mechanistic data that avoids the need to identify specific pathways and hypotheses. From one vantage point, this is correct – because KCCs are based on empirical observations of characteristics associated with known carcinogens, they can provide a survey and agglomeration of the mechanistic literature. Yet, as Tables 3 and 4 show, as much as 50% of classified carcinogens do not exhibit KCCs, while other chemicals not known to evoke cancer often do. Furthermore, across the sets of KCCs (cardiovascular toxicants, endocrine disrupting chemicals, female reproductive toxicants, hepatotoxicants, immunotoxicants, male reproductive toxicants, and aging) there’s considerable redundancy as shown in Table 2, indicating many other toxicities unrelated to cancer may be mediated by similar toxicity pathways and biological responses.

Translating the information embedded in the KCCs to the approach depicted in Figure 1 is not a straightforward process. Obviously, just counting the number of positives in each KCC assay would not allow a ready integration with MOAs or AOPs. For example, counting the number of positive assays for oxidative stress with high doses of oxygen will give uniformly positive findings. Without some criteria, such as the dose at which KCCs occur, however, one cannot predict strong evidence of cancer classification by counting positive checks. Correspondingly, it is difficult to integrate KCCs into the existing, and well accepted, MOA/AOP frameworks without better defined criteria for strong evidence.

The proposed way forward, as shown in Figure 1 and Table 6, has several advantages. It is based on the well-established and internationally used MOA/AOP framework, on which numerous risk assessments have been conducted and risk management decisions have been made. This framework is also embedded in the risk assessment guidelines of several authorities (e.g.,<sup>7,26,29,41</sup>) and is taught in numerous risk assessment courses to international groups (e.g., Toxicology Excellence for Risk Assessment [TERA] boot camps).

As noted earlier, the original purpose of KCCs was to organize data for mechanistic evaluation, and it was originally postulated that KCCs could provide a more holistic alternative to what some viewed as a narrow focus on pathways and hypotheses.<sup>37</sup> However, instead of improving mechanistic evidence integration, KCCs can obfuscate mechanistic data analysis and integration, since KCCs can be nonspecific and require disaggregation.<sup>30</sup> Our solution to this problem is the framework described in Figure 1 and illustrated in Tables 5 and 6, which is consistent with existing regulatory frameworks to organize and evaluate mechanistic information for the purpose of risk assessment and allow (but do not require) use of KCCs. This is essential to informing subsequent risk management decisions. Table 6 applies the scientific method of hypothesis testing into an actionable

construct on how one can go about evaluating differing hypothesized cancer MOAs for a specific agent.

The KCCs have generated important discussions on arranging mechanistic information on cancer hazard into manageable packets. However, we note that if used as a stand-alone, the KCCs fall short of objective, best available science standards for evaluating the potential of a chemical to pose a carcinogenic hazard to humans. We have therefore looked beyond KCCs by advancing this archetypal MOA-based evidence system for hypothesis testing.

In our approach, considering the potential MOAs and the potency of those MOAs are paramount and KCCs are optional. Crucially, our approach goes beyond binary considerations of whether a chemical is a carcinogen or noncarcinogen to more fully embrace the complexity of cancer causation, including the conditions under which a chemical exhibits carcinogenic behavior and the conditions in which it does not.<sup>42</sup> Future work in this area needs to be acknowledged and, more importantly, be integrated into the extensive international work on MOAs and AOPs for ongoing cancer risk assessments.

To go beyond the LNT default, systematic hypothesis testing using the four archetypal MOAs from Table 5, along with quantitative evidence evaluation procedures, as presented herein, allows all of evidence to be evaluated on an equal basis to identify the likely operative carcinogenic MOA for determining the potential cancer risk of a chemical to humans. The evidentiary value in documenting the most likely MOA from either qualitative or quantitative comparative analysis of alternative plausible MOAs has been repeatedly demonstrated.<sup>13,14,36,48,73,75</sup> This approach is scientifically robust and sufficiently broad to incorporate the advancements in knowledge gained on how chemicals interact with biological systems at the molecular, cellular, organ, and organism level to cause cancer in humans and is consistent with all risk-based regulatory frameworks. It is also compatible with previously published frameworks for assessing carcinogenicity.<sup>36</sup>

While a number of case examples of comparative analysis of alternative plausible MOA's can be found in the literature,<sup>13,14,36,48,73,75</sup> additional case studies employing quantitative scientific evidence scoring approaches are needed. Moreover, the systematic and thorough comparative analysis of alternative plausible MOA's, as proposed herein, should not be characterized as "a narrow focus on specific pathways and hypotheses" but rather as a scientifically robust method to broadly evaluate mechanistic evidence using the most up to date understanding of biological pathways, dosimetry, and the sequence of causative steps in the processes of chemical carcinogenesis. We expect that incorporating this methodology into hazard and risk evaluations will improve the scientific basis for determining the conditions under which a substance may or may not exhibit carcinogenic behavior, and result in more reliable predictions and in the development of and accurate quantitative estimates of risks to humans from differing levels of exposure.

#### ■ AUTHOR CONTRIBUTIONS

All authors contributed to conceptualization, writing, reviewing, and editing the manuscript. R. Becker developed Table 2. M. Dourson and C. Onyema

contributed equally to the analysis summarized in Tables 3 and 4, and J. Ryman validated the results. R. Becker and J. Ryman contributed equally to the development of Figure 1 and Tables 5 and 6.

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#### ■ SUPPLEMENTARY DATA

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