International Agency for Research on Cancer

World Health Organization

IARC Monographs Programme

Key Characteristics-associated End-points for Evaluating Mechanistic Evidence of Carcinogenic Hazards

ey Characteristics of Carcinogens

IARC Monographs Technical Report

IARC, Lyon, France

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This publication contains the technical report drafted by the Working Group of the Key Characteristics-associated End-points for Evaluating Mechanistic Evidence of Carcinogenic Hazards Meeting held in Lyon in July 2023, which alone is responsible for the views expressed.

Acknowledgements

Since 1971, the *Monographs Programme* of the International Agency for Research on Cancer (IARC) has served as a compendium of global knowledge and expertise to identify the causes of human cancer. This Technical Report was planned by Dr. Federica Madia, Dr. Aline de Conti and Dr. Mary Schubauer-Berigan; it should serve as a valuable contribution to the ongoing understanding of mechanisms of carcinogenesis. This report reflects the collective expertise of committed independent participants in *Monographs* evaluations. In particular, IARC convened a group of 29 scientists from 8 countries for a scientific workshop held in Lyon in 2023, to discuss advances in mechanistic evidence for cancer hazard identification, including the reporting and interpretation of results within the Key Characteristic (KCs) framework (see list of contributors).

This project would not have been possible without the leadership of the Chair and Subgroup Chairs in guiding the scientific discussions, their ongoing contributions to the development of the report, and the active engagement and valuable feedback of all Workshop participants.

The authors are grateful to the IARC scientific Secretariat and to *IARC Monographs* programme colleagues Niree Kraushaar, Jennifer Nicholson, Solene Quennehen, Noëmi Joncour, Mathieu Rose, and Sandrine Ruiz for their invaluable support in managing the logistics aspects of the workshop and in the preparation of the Technical Report. The project was supported with funding from grant R01CA033193 of the US National Cancer Institute and the US National Institute of Environmental Health Sciences.

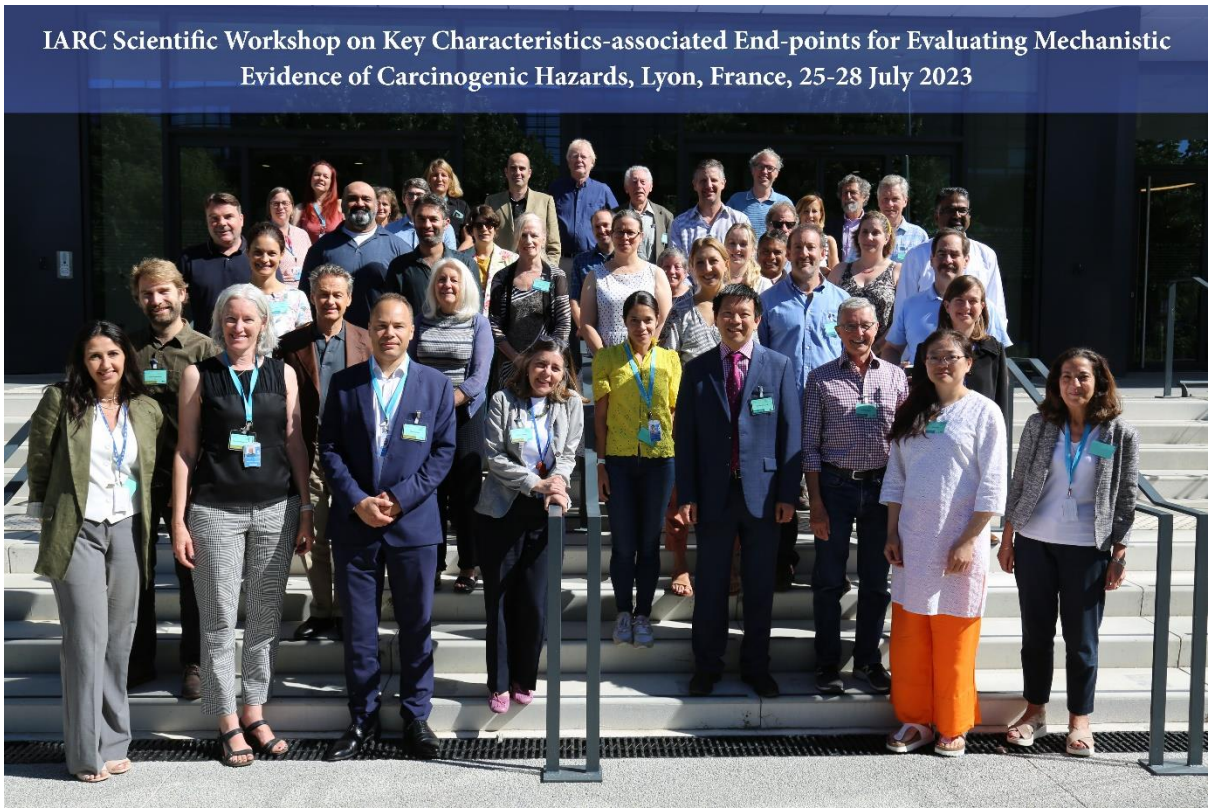
Summary

The implementation of the "10 key characteristics of carcinogens" (KCs) framework has significantly improved the way in which mechanistic evidence is evaluated in the *IARC Monographs*. The prominence of mechanistic evidence was recognized formally in the most recent update to the Preamble to the *IARC Monographs* in 2019. Since its introduction, the KCs framework has been applied to approximately one hundred agents evaluated in 23 volumes of the *IARC Monographs*. This framework has brought uniformity to *IARC Monographs* assessments and allowed a focused systematic review of the publicly available literature on mechanisms of carcinogens. Importantly, it has contributed to advancing the science by which mechanistic evidence is used to evaluate potential carcinogens.

Thus, it was considered timely to review aspects of how the KCs have been applied in past years, especially after the update to the Preamble, and to discuss suggested improvements to the process.

On 23–28 July 2023, 29 scientists from 8 countries met with IARC scientists in Lyon to discuss: (i) Interpretation and relevance of end-points forming the basis of the key characteristics of carcinogens; (ii) incorporation of data from high content and high throughput assays; and (iii) integration of mechanistic evidence as part of cancer hazard identification.

The workshop material reported in this Technical Report is expected to support future Working Groups of experts in the reporting and interpretation of results under the KCs framework of mechanistic evidence evaluation within the *IARC Monographs* or in other contexts.



List of participants of the Scientific Workshop

Working Group Members

Dinesh Barupal
Department of Environmental Medicine and Public Health, Icahn School of Medicine at Mount Sinai, USA[1]

Parveen Bhatti
BC Cancer Research Institute, University of British Columbia, Canada

Weihsueh A. Chiu *(Subgroup Chair)*
School of Veterinary Medicine and Biomedical Sciences, Texas A&M University, USA

Kevin P. Cross
Instem, USA[2]

David M. DeMarini *(Overall Chair)*
United States Environmental Protection Agency (*Scientist Emeritus*), USA

Eugenia Dogliotti
Istituto Superiore di Sanità, Department of Environment and Primary Prevention, Italy

Laure Dossus
Nutrition and Metabolism Branch, IARC, France

Jason M. Fritz *(attended remotely)*
United States Environmental Protection Agency, USA

Dori Germolec
Division of Translational Toxicology, National Institute of Environmental Health Sciences, USA

Akram Ghantous
Epigenomics and Mechanisms Branch, IARC, France

Maria Helena Guerra Andersen
The National Research Centre for the Working Environment, Copenhagen, Denmark

Kathryn Z. Guyton *(unable to attend)*
Board on Environmental Studies and Toxicology, National Academies of Sciences, Engineering, and Medicine, USA

William Gwinn *(Subgroup Chair)*
Division of Translational Toxicology, National Institute of Environmental Health Sciences, USA

Zdenko Herceg
Epigenomics and Mechanisms Branch, IARC, France

Jennifer Jinot *(unable to attend)*
United States Environmental Protection Agency (retired), USA

Michael Korenjak
Epigenomics and Mechanisms Branch, IARC, France

Sandra Perdomo
Genomic Epidemiology Branch, IARC, France

David H. Phillips
Department of Analytical, Environmental

[1] Dr Barupal reported having consulted for Brightseed Inc., and this work was determined not to involve competing interests for the topics covered by this *IARC Monographs* Scientific Workshop.

[2] Dr Cross reported being a salaried employee of Instem, whose work does not involve competing interests for the topics covered by this *IARC Monographs* Scientific Workshop.

& Forensic Sciences, King's College London, UK

Roger R. Reddel
Faculty of Medicine and Health, The University of Sydney, Australia

Brad Reisfeld *(Subgroup Chair)*
Department of Chemical and Biological Engineering, Colorado State University, USA

Nathaniel Rothman *(attended remotely)*
Division of Cancer Epidemiology and Genetics, National Cancer Institute, USA

Martin van den Berg
Department of Population Health, Utrecht University, The Netherlands

Roel Vermeulen
Institute for Risk Assessment Sciences, Utrecht University, The Netherlands

Paolo Vineis
School of Public Health, Imperial College, UK

Amy Wang
Division of Translational Toxicology, National Institute of Environmental Health Sciences, USA

Emily Watkins *(Subgroup Chair)*
Department of Life Sciences, University of Roehampton, UK

Maurice Whelan
Joint Research Centre (JRC), European Commission, Italy

Jiri Zavadil
Epigenomics and Mechanisms Branch, IARC, France

Lauren Zeise
Office of Environmental Health Hazard Assessment, California Environmental Protection Agency, USA

Invited Specialists

Dipak Panigrahy
Beth Israel Deaconess Medical Center, Harvard Medical School, USA[3]

Representatives of national and international health agencies

Johanna Berneron
Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES), France

Observers

Remi Bars
Bayer Crop Science, France[4]

Bette Meek
School of Epidemiology and Public Health, University of Ottawa, Canada

Bernard Stewart
Cancer Council Australia, Australia

[3] Dr Panigrahy reported receiving substantial personal consultancy fees from several law firms in connection with expert testimony for plaintiffs in cases related to radiation, continuous positive airway pressure (CPAP) machine, and chemicals (hexavalent chromium, arsenic, trichloroethylene, tetrachloroethylene, styrene) and cancer.

[4] Dr Bars reported to be employed by Bayer Crop Science and that he will receive support for travel and accommodation to attend this IARC Workshop from CropLife International, an international trade association of agrochemical companies.

Michael Wilde
Philosophy Department, University of Kent, UK

Jon Williamson
Philosophy Department, University of Kent, UK

Elisa Pasqual

Gabrielle Rigutto

Mathieu Rose

Mary K. Schubauer-Berigan *(Programme Head)*

Roland Wedekind

IARC Monographs Secretariat

Shirisha Chittiboyina

Danila Cuomo

Aline de Conti *(Co-Responsible Officer)*

Fatiha El Ghissassi

Caterina Facchin

John Kaldor

Federica Madia *(Responsible Officer)*

Administrative Assistance

Niree Kraushaar

Jennifer Nicholson

Solène Quennehen

Sandrine Ruiz

NOTE REGARDING CONFLICTS OF INTERESTS: Each participant first received a preliminary invitation with the request to complete and sign the IARC/WHO Declaration of Interests, which covers employment and consulting activities, individual and institutional research support, and other financial or non-financial interests (e.g. public statements and positions related to the subject of the meeting). Official invitations were extended after careful assessment of any declared interests that might constitute a conflict of interest. Pertinent and significant conflicts are disclosed here. Information about other potential conflicts that were not disclosed may be sent to the Head of the *IARC Monographs* programme at imo@iarc.who.int.

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The evolution of the key characteristics (KCs) of carcinogens and scope of the technical report

David M. DeMarini, Aline de Conti, Mary K. Schubauer-Berigan and Federica Madia

For more than 50 years the *Monographs Programme* of the International Agency for Research on Cancer (IARC) has convened expert Working Groups to evaluate evidence regarding preventable causes of human cancer. *IARC Monographs* reviews publicly available studies of cancer in humans, cancer in experimental animals, and mechanistic evidence. Considerations on mechanisms associated with carcinogenic exposures have evolved since early *Monographs* volumes up to the introduction of the framework of the key characteristics (KCs) of carcinogens (Smith et al., 2016), and its further implementation as an integral part of the *IARC Monographs* to evaluate mechanistic evidence of carcinogenic agents (IARC, 2019a).

(KC1) "is electrophilic or can be metabolically activated to an electrophile"
(KC2) "is genotoxic"
(KC3) "alters DNA repair or causes genomic instability"
(KC4) "induces epigenetic alterations"
(KC5) "induces oxidative stress"
(KC6) "induces chronic inflammation"
(KC7) "is immunosuppressive"
(KC8) "modulates receptor-mediated effects"
(KC9) "causes immortalization"
(KC10) "alters cell proliferation, cell death, or nutrient supply"

The characteristics of some classes of carcinogens had been identified decades prior to the inception of the KCs. For example, researchers identified structural differences between carcinogenic versus non-carcinogenic PAHs (Jerina et al., 1978) and the chemical moieties that distinguished many rodent carcinogens from non-carcinogens (Ashby and Tennant, 1991). For decades the *IARC Monographs* recognized that some structural features (electrophilic moieties) and biological activities (genotoxicity) of agents were characteristics of many human carcinogens.

The publication of the *Hallmarks of Cancer* by Hanahan and Weinberg (2000) and their extension and refinement a decade later (Hanahan and Weinberg, 2011) synthesized for the first time the enormous literature on cancer biology, resulting in the identification of the features of cancer cells. Their analyses initiated much discussion among cancer researchers about the features of cancer cells, the carcinogenic process, and carcinogenic mechanisms. Hanahan and Weinberg never discussed the characteristics of carcinogens, only the characteristics of cancer cells. However, their analysis prompted new thinking about how carcinogens might cause these characteristics of cancer cells.

In 2007, IARC convened an Advisory Group to develop a process for creating Volume 100 of the *Monographs*. This Volume was intended to update the IARC database for the Group 1 (known) human

carcinogens. It represented a chance to examine and raise questions using a reasonably comprehensive database from humans and experimental animals to identify possible hallmarks of carcinogens and carcinogenic mechanisms. Volume 100 was developed in six parts (IARC, 2012a, b, c, d, e, f) that reviewed the relevant information of all the ~100 Group 1 human carcinogens. As noted by Cogliano (2019), Volume 100 was viewed, at the time, as a bridge from the previous focus of IARC on cancer studies in humans and experimental animals to a future relying more on mechanistic data. Soon after the development of Volume 100 began, a publication listed a set of 15 "key events associated with carcinogenesis" (Guyton et al., 2009), which resulted also from discussions at a symposium on Predicting Chemical Carcinogenicity, Moving Beyond Batteries, held at the Environmental Mutagen Society Annual meeting in 2007 (Smith and Waters, 2007). These key events were exhibited by a diverse set of Group 1 and Group 2A carcinogens and served as an important first step towards identifying the key mechanisms associated with human carcinogens. During the four years of preparation of Volume 100, IARC and Working Group members of Volume 100 recognized the need for the systematic identification of the cancer sites observed in humans and those observed in experimental animals, as well as a listing of mechanistic events for human carcinogens (Cogliano, 2019). Thus, upon completion of Volume 100, IARC initiated a two-part Workshop on Tumour Site Concordance and Mechanisms of Carcinogenesis.

As result of the Workshop, the participants compiled a list of 24 toxicological end-points that were thought to be relevant to carcinogenesis (Al-Zoughool et al., 2019; Krewski et al., 2019a, b). However, the Workshop participants found that evaluating 24 toxicological end-points from the Volume 100 dataset was cumbersome and that many of the end-points could be combined. After some discussion, the list of 10 Key Characteristics of Carcinogens was proposed at the end of this 2012 Workshop.

In the following years, an IARC Scientific Publication (IARC, 2019b) and several other publications and Conference presentations reinforced within the scientific community the use and advantages of evaluating retrospectively the mechanistic data regarding potential carcinogenic agents and reported on progress in applying the KCs framework in cancer hazard identification (Guyton et al., 2009, 2018a, b; Gibbons et al., 2014; Smith, 2019; Wild et al., 2020; Samet et al., 2020).

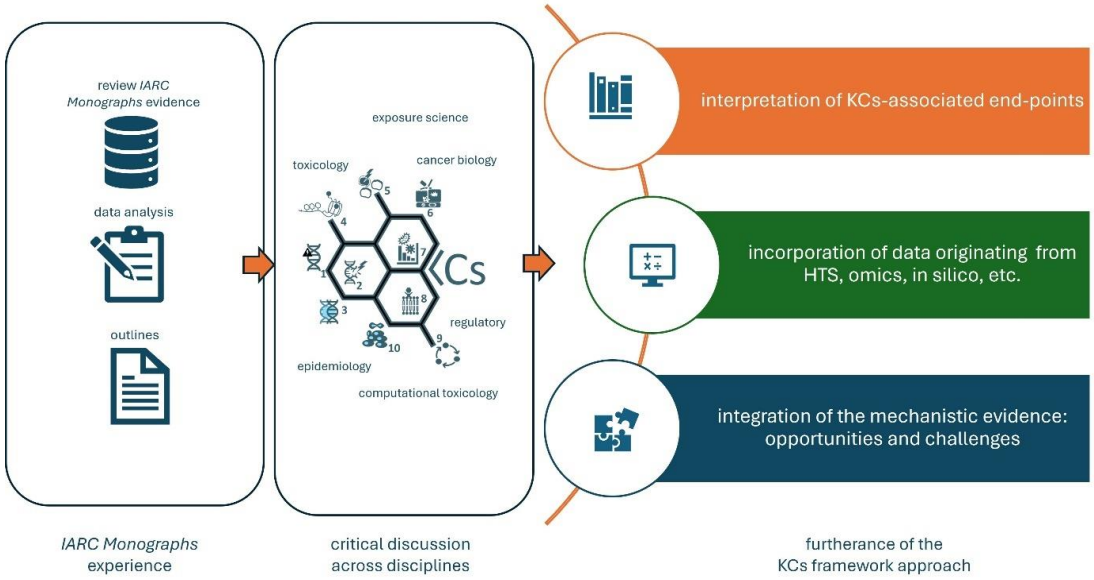
Currently, the 10 KCs provide the basis for a systematic and a rational approach to compiling and evaluating evidence on carcinogenic mechanisms of agents for the *IARC Monographs*, as well as in authoritative evaluations of carcinogens by the US EPA, the California EPA, and the US NTP, and in different other contexts (Ricker et al., 2024; Kay et al., 2024; Keller et al., 2023; Tice et al., 2021; Atwood et al., 2019).

The KCs framework is designed for a systematic review (retrospective analysis) of the published mechanistic literature, with procedures to minimize bias in the assessment to inform an evidence-based decision without an *a priori* hypothesis. To identify the mechanistic evidence of potential carcinogenic agents, *IARC Monographs* Working Group members who participate in the evaluations are asked to judge whether results associated with a wide range of end-points in several different systems are consistent and the overall mechanistic database is coherent by accessing the informativeness of the data. There is no requirement in the *IARC Monographs* Preamble (IARC, 2019) to establish the temporal, sequential or causal features of how a particular agent operates.

Since the implementation in 2014, the KCs framework has been shown to be a useful tool for cancer hazard identification and has been applied to a hundred agents. Considering such compelling experience, the *IARC Monographs* secretariat committed to a series of scientific workshops tackling several topics including the furtherance of the use of KCs. The insights gained from the application of this framework were discussed during an IARC Scientific Workshop on the "*Key Characteristics-associated End-points for Evaluating*

Mechanistic Evidence of Carcinogenic Hazards" held on July 25-28, 2023, in Lyon (DeMarini et al., 2025). The results of the discussions have been included in this technical report which provides expert opinions on three major themes:

1. Interpretation and relevance of end-points forming the basis of the KCs
2. Incorporation of data from high content and high throughput assays
3. Integration of the mechanistic evidence



Part I is dedicated to each of the 10 KCs. The experts were asked to reflect about: (*i*) the relevance of some of the KCs-associated end-points to assess the mechanistic evidence in cancer hazard identification and (*ii*) whether the strengths and limitations of the end-points differ among the test systems (exposed humans; human primary cells or tissues; or experimental systems *in vivo* or *in vitro*). Several considerations were described to establish the relevance of KCs-associated end-points, including each end-point's specificity, how well it explains the biological processes underlying the KC, and the extent to which the end-point has been associated with carcinogenesis, cancer risk, and/or the persistence of the alterations. According to the *IARC Monographs* Preamble, the available mechanistic evidence in exposed humans, human primary cells or tissues, and other experimental systems are first evaluated separately and then integrated forming the whole body of evidence. Considerations of the study quality and validity, including exposure assessment in mechanistic studies in humans, and reliability and sensitivity of experimental systems, are always taken into account as a part of the Working Group expert judgment. In this Technical Report, considerations regarding the test system in which the end-point is observed are described. These considerations are important because they may influence the informativeness of the result and the establishment of coherence of mechanistic data observed in molecular epidemiology studies, and in studies in experimental systems.

Part II includes opinions on the interpretation and incorporation of data from transcriptomics, metabolomics, mutational signatures, *in silico* data, and chemical high-throughput screening data (e.g. Tox21, including ToxCast) in the mechanistic evaluations. Considerations on the informativeness of the data generated by these methodologies are discussed in terms of the relevance to the KCs.

Part III focusses on how information described through the KCs framework can be integrated for the overall evaluation of carcinogenic hazard. Challenges of and opportunities for integration are discussed on the basis of examples of previous monographs.

The Preamble to the *IARC Monographs* describes the scientific principles and procedures to evaluate carcinogenic hazards, including those to evaluate mechanistic evidence, and guides the Working Group in conducting its carcinogenicity reviews. This Technical Report can be viewed as a source of scientific information built on the experience of agents previously evaluated with the KCs framework, and the expertise of the Workshop participants. As such, it is considered a useful tool to support and assist Working Group of experts in conducting mechanistic evidence evaluation within the *Monographs*, as described in the Preamble to the *IARC Monographs*, or in other contexts. Notably, the Working Group's expert judgment remains an essential part of the *IARC Monographs* evaluations.

The goal of this Technical Report is to facilitate the appropriate interpretation of the data relevant to the KCs for the mechanistic evidence evaluation, and thus to facilitate uniformity across assessments by the *IARC Monographs*. Nevertheless, as the science of mechanisms associated with cancer development continues to evolve, it is important to allow flexibility for incorporation of contemporaneous scientific concepts. Broader and consistent interpretation of the mechanistic evidence will further improve cancer hazard identification and thereby benefit public health.

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Part I. Interpretation and relevance of end-points forming the basis of the key characteristics of carcinogens

1. Key Characteristic 1: Is electrophilic or can be metabolically activated to electrophiles

David H. Phillips and David M. DeMarini

1.1 Introduction

As originally formulated by Smith et al. (2016), Key Characteristic 1 (KC1) is defined as "is electrophilic or can be metabolically activated to an electrophile". This recognises that some chemical carcinogens are direct-acting electrophiles, whereas others require conversion by enzymes either *in vitro* or within the host, a process termed metabolic activation. It was further recognised here and subsequently (Guyton et al., 2018; Smith et al., 2020b) that relevant evidence for this KC is that the parent compound or metabolite with an electrophilic structure can form DNA and/or protein adducts, with relevance to carcinogenicity. In addition, KC1 would also apply to an agent for which there was supportive *in silico* evidence that it is electrophilic. *In silico* prediction that an agent is mutagenic can be also supportive of evidence that the agent exhibits KC2 (genotoxicity) (see also Part 2).

1.2 Relevance of end-points

Historically, the hypothesis that carcinogens are electrophiles, or are metabolically activated to them, was an important development in the understanding of chemical carcinogenesis. Originally proposed by Miller (1970), it provided a unifying property of chemicals belonging to diverse classes and structures. However, it came at a time when the cellular target of carcinogens was unclear. Carcinogens had been shown to covalently modify DNA, RNA, and protein, and it was initially thought that protein was the key target, based on the "protein deletion" hypothesis (Pitot and Heidelberger, 1963). One reason that DNA was not thought relevant was that at first most carcinogens were not demonstrably mutagenic in standard assays. This changed when (a) Heinrich Malling (1971) showed that rat liver microsomes could activate dimethylnitrosamine to a mutagen in a suspension assay using Bruce Ames' *Salmonella* base-substitution strain TA1530; and (b) Ames et al. (1973) incorporated into the top agar both the bacteria and a crude microsome preparation of rat liver (S9) that had been developed by Garner et al. (1972), showing that many carcinogens were mutagenic after metabolic activation. This, coupled with the earlier demonstration by Brookes and Lawley (1964) that the level of binding in mouse skin of a series of PAHs to DNA (see Example 1 below), but not to RNA or protein, correlated with their carcinogenicity, shifted opinion towards DNA as the critical target of carcinogens and the consideration that cancer was a genetic disease.

However, it is now apparent that many biotransformations, including those that can be considered detoxication and not activation, involve transient formation of electrophiles (See Table 1a). For example, cytochrome P450s can add oxygen across C-C double bonds to form epoxides. Such species are reactive (i.e. electrophilic) but can be highly unstable and can undergo hydrolysis (either enzymically or spontaneously) to dihydrodiols. Such bioconversions take place within the extranuclear endoplasmic reticulum of cells such that these electrophiles do not survive for sufficient time to migrate through the

cytoplasm and enter the nucleus. In other cases (see Example 2 below) the electrophilic species may be reactive towards functional groups found only in proteins (e.g. thiols) and not in DNA.

Table 1a. End-points relevant to KC1: "is electrophilic or can be metabolically activated to an electrophile"

Category	End-point	Relevance	Comments	References
Untargeted methods	Electrophilic metabolites	Identifies metabolites by using liquid chromatography and mass spectroscopy	Can be considered especially important for the establishment of mechanistic class.	Yu et al. (2020a)
	DNA adducts	^{32}P-postlabelling: Detects bulky adducts due to differential mobility relative to un-adducted nucleotides	DNA adducts are usually considered an end-point of high relevance for KC1	Phillips et al. (2005)
		LC/MS: Identifies DNA adducts by liquid chromatograph and mass spectroscopy		Farmer & Singh (2008)
		Comet assay modified by using DNA synthesis inhibitors: Detects bulky adducts by trapping single-strand breaks (SSBs) formed during nucleotide excision repair, which repairs bulky adducts		Ngo et al. (2020)
	Protein adducts	LC/MS: Identifies protein adducts by liquid chromatograph and mass spectroscopy, or analytical chemical methods	Can be considered as supportive information of the reactivity of an agent	Guyton et al. (2018); Smith et al. (2020b)
	Skin sensitivity	Maximization test and Buehler test in guinea pigs*: In vivo* assays	Can be considered as supportive information of the potential reactivity of an agent	OECD (2022a)
		h-CLAT, U-SENS, IL- Luc, and GARD*: In vitro* assays		OECD (2023a)
In silico	Gene mutation and clastogenicity	GIST protocol: mutagenicity prediction	Can be considered as supportive evidence to inform KC1	Hasselgren et al. (2019)
		(Q)SAR models: skin sensitivity prediction	Can be considered as supportive evidence to inform KC1	Johnson et al. (2020)

LC/MS, Liquid chromatography–mass spectrometry; (Q)SAR, quantitative/qualitative structure activity relationships; GIST, genetic in silico protocol. Note: these are some the most relevant examples and not an exhaustive list of end-points.

Example 1. Polycyclic aromatic hydrocarbons

The first electrophilic metabolites identified were the so-called K-region epoxides (Sims and Grover, 1974), leading some to conclude that these were the ultimate forms of PAHs, after years in which there was disagreement over whether PAHs even required metabolic activation or were carcinogenic per se (e.g. by intercalation into DNA) (Arcos and Argus, 1968). However, despite being electrophilic, the K-region epoxides were barely carcinogenic, and they were not mutagenic. Subsequent studies showed that the DNA adducts formed by PAHs were more polar than those formed by K-region epoxides and that the ultimate carcinogenic form of benzo[a] pyrene (B[*a*]P, *Monographs* Volume 100F, IARC, 2012b) was not its K-

region epoxide but the bay-region diol-epoxide (Sims et al., 1974). The reason that K-region epoxides are not mutagenic despite being electrophilic is most likely linked to their rapid hydrolysis or further metabolism to K-region dihydrodiols. Thus, they do not survive long enough in biological milieu to reach and react with DNA. The bay-region diol-epoxide, on the other hand, is not rapidly metabolised by epoxide hydrolase to the tetrol and, thus, can bind covalently with cellular macromolecules, including DNA.

Example 2. Electrophiles may react with protein but not DNA

Isoeugenol (see *Monographs* Volume 134, IARC, 2024) has been described as a skin sensitizer. Evidence suggested a mechanism involving covalent modification of proteins of the skin. Natsch and Haupt (2013) reported that the protein modification did not require S9 activation and was likely the result of spontaneous oxidation. Melles et al. (2013) reported that isoeugenol forms quinones and quinone methides that are electrophilic and react with proteins. Ahn et al. (2020) demonstrated also that an electrophilic species, a dimeric 7,4'-oxyneolignan, resulted from photo-oxidation of isoeugenol and that it bound to thiol groups. However, isoeugenol has tested mostly negative in assays for genotoxicity, and it has not been found to form DNA adducts. In fact, thiol groups are abundant in proteins but are not found in DNA. Thus, in this case, the evidence for electrophilicity of isoeugenol is not relevant to either mutagenicity or carcinogenic potential.

1.3 Assessing the relevance of end-points in different test systems

Electrophilicity can be considered mainly as a chemical property of many agents, and a wide variety of systems can be used to detect it. Chemical assays involve detecting the reaction of the electrophile with a model nucleophile. Because cellular nucleophiles include nucleic acids and proteins, evidence for electrophilicity can include the formation of DNA or protein adducts *in vitro* or *in vivo* in experimental animals and in humans (See Table 1a). Although in the Preamble strong mechanistic evidence from studies in human primary cells are considered of high relevance and influence the overall classification, there is little evidence showing that electrophilicity, as a property, is more relevant to human carcinogenicity when measured in human primary cells or tissues versus in experimental systems *in vivo* or *in vitro.*

Along with chemical evidence for electrophilic metabolites, various assays/end-points can generate data that can be informative for KC1. These include DNA adducts determined by ^{32}P-postlabelling, Liquid chromatography and mass spectrometry (LC/MS) or comet assays modified to detect DNA adducts, as well as protein adducts determined by LC/MS or other analytical chemical methods. Prediction that an agent is electrophilic by *in silico* methods, as well as by skin sensitization assays, would provide supportive evidence that the agent is electrophilic (KC1) (Table 1a). In contrast, data showing the agent causes oxidative DNA damage by either LC/MS or other analytical chemical methods, or by a comet assay modified to detect oxidative DNA damage would be evidence that the agent causes oxidative stress (KC5).

1.4 Interpretation of results within the same database

An agent that is electrophilic based on its ability to form DNA or protein adducts or is predicted to be electrophilic by *in silico* calculations exhibits KC1. However, if an agent forms only protein adducts or has only *in silico* evidence for electrophilicity, these may be important descriptive chemical proprieties to be described when available but constitute weak evidence for KC1. In contrast, formation of DNA adducts is an end-point of high relevance for KC1.

Many agents that have exhibited evidence for KC1 have been found to exhibit *strong* mechanistic evidence of the key characteristics (See Annex 1).

These include 1-bromopropane; 3-chloro-2-methylpropene; hydrazine, *N*,*N*-dimethylformamide, pentachlorophenol; furfuryl alcohol; benzene; styrene and styrene-oxide; *o*-anisidine or *o*-anisidine hydrochloride; *o*-nitroanisole; aniline and aniline hydrochloride; acrolein; arecoline; crotonaldehyde; and methyleugenol (see Table 1b). Three of these agents exhibited strong evidence for KC1 in the absence of evidence for KC2 (1-bromopropane, *N*,*N*-dimethylformamide, and furfuryl alcohol).

Table 1b. Mechanistic evidence for agents with strong evidence for electrophilicity (KC1).

Agent	Group	KC1	KC2	KC3	KC4	KC5	KC6	KC7	KC8	KC9	KC10
1-Bromopropane	2B	√				√	√	√			
3-Chloro-2-methylpropene	2B	√	√								
Hydrazine	2A	√	√			√					√
N,*N*-Dimethylformamide	2A	√				√					√
Pentachlorophenol	1	√	√			√			√		√
Furfuryl alcohol	2B	√									
Benzene	1	√	√	√		√		√	√		√
Styrene, or styrene-7,8-oxide	2A	√	√						√		√
o-Anisidine, or *o*-anisidine HCl	2A	√	√								√
o-Nitroanisole	2A	√	√								√
Aniline, or aniline HCl	2A	√	√			√					√
Acrolein	2A	√	√	√		√	√	√			√
Arecoline	2B	√	√	√		√					
Crotonaldehyde	2B	√	√			√	√				
Methyleugenol	2A	√	√								√

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2. Key Characteristic 2: Is genotoxic

David M. DeMarini and David H. Phillips

2.1 Introduction

Genotoxicity includes both DNA damage and mutations, which are distinctly different (Shaughnessy and DeMarini, 2009). DNA damage may occur spontaneously due to errors of nucleic acid metabolism or be caused by endogenous or exogenous mutagens. DNA damage includes various types of lesions to the DNA involving either the phosphodiester backbone (the deoxyribose sugar moiety) or the nucleotides/bases. DNA damage typically does not change the sequence of nucleotides in DNA. Examples include lesions such as DNA adducts (a molecule bound covalently to the DNA), single-strand breaks (SSBs) or double-strand breaks (DSBs), DNA-DNA or DNA–protein crosslinks, and base damage (such as alkylation, methylation, or oxidation).

Mutations are heritable changes in the DNA sequence, and they are classifiable according to their genomic location. One category of mutation is gene mutation or point mutations, which are mutations within a gene and usually consist of base substitutions or small deletions, duplications, insertions, or inversions of nucleotides. Also included are frameshifts, which are the insertion or deletion of nucleotides that are not three or multiples of three. A second category of mutations consists of chromosomal mutations or multi-locus mutations. These are mutations that span more than one gene and are typically large deletions, duplications, insertions, or inversions. Agents that induce chromosomal mutations are also called clastogens. A third category is genomic mutation, exemplified by aneuploidy, which is the gain or loss of one or more chromosomes.

Mutagens typically make DNA damage (e.g. an adduct or strand break), which is sensed by the cell via the DNA damage response (DDR) system, and then the cell either repairs the damage or converts it into a mutation (a change in nucleotide sequence). Thus, mutagenesis is a cellular process, requiring enzymes and typically DNA replication to produce a change in DNA sequence. The distinction between DNA damage and mutation is critical to KC2 because DNA damage can be repaired, resulting in no mutation. Thus, DNA damage alone may not result in mutagenesis. In the end, it is mutation, i.e. a persistent and heritable change in the DNA sequence, that is most relevant to carcinogenesis

DNA adduct data are examples of DNA damage (KC2) and can occur as a result of electrophilic reactivity (KC1), which is the covalent binding of an agent to DNA. For purposes of data organization, DNA adducts (generated by ^{32}P-postlabelling, LC/MS analysis or modified comet assays) are described as an end-point relevant to electrophilicity (KC1). Data from the comet (not modified for DNA adduct detection), γ-H2AX, micronucleus, and chromosome aberration assays are types of DNA damage that are less clearly due to electrophilicity, and thus these data are placed into KC2 (Table 2). All mutagenicity data, whether generated *in vitro*, in experimental animals, or in humans, or predicted *in silico* are placed into KC2.

Table 2. End-points relevant to KC2: "is genotoxic"

Category	End-point	Relevance	Comments	References
DNA damage	DNA damage (other than DNA adducts)	Comet: Strand breaks and oxidized bases *in vitro* or in experimental animals	With modifications, the assay detects DNA damage involving BER, NER, and NHEJ, in various cell types.	OECD (2016e)
		γ-H2AX: Double-strand breaks	Can detect ds-breaks in various cell types.	Wu et al. (2022)
	Chromosome damage	Micronucleus: Chromosome breaks and aneuploidy	Can be done *in vitro* and *in vivo* in various cell types by various methods, including flow cytometry.	OECD (2023b)
		Chromosome aberrations: Cytologically detectable alterations: deletions, duplications, inversions, etc.	Can be done with standard staining or fluorescent staining (FISH).	OECD (2016d, e)
Mutagenicity	Gene mutation	*S. typhimurium* (Ames): Base substitutions and frameshifts	Strains have various genetic targets and DNA repair backgrounds but collectively detect all 6 base substitutionsand 2 types of frameshifts.	OECD (2020)
		E. coli WP2: Base substitution at an AT site	Strains have various DNA repair backgrounds and detect base substitutions at an AT site.	OECD (2020)
		Tk$^{-/-}$, *TK*$^{-/-}$, *Hprt* or *HPRT* mutant colonies, and large *Tk*$^{-/-}$ or *TK*$^{-/-}$ mutant colonies represent base substitutions, deletions, etc. within the gene	Large colonies typically represent mutations of various types within the gene; assay can be done in human TK6/*TK*$^{+/-}$ and mouse LY5178Y/*Tk*$^{+/-}$ cells.	OECD (2016a, b)
		Transgenic rodent: Base substitutions and deletions within the gene	Assays include Muta™ mouse, Big Blue® mouse and rat, *LacZ* plasmid mouse, and the *gpt*-delta mouse and rat. Selection by *gpt* of *gpt*-delta mutants detects gene mutations.	OECD (2022b)
		PIG-A, Pig-a: Base substitutions, frameshifts, and small deletions within the gene	Detects mutations in blood cells in rodents and humans *in vivo*.	OECD (2022c)
	Chromosomal mutation	*Tk*$^{-/-}$, *TK*$^{-/-}$, Small colonies represent multi-locus deletions in cell lines	Small colonies typically represent large multi-locus deletions. Assay in human TK6/*TK*$^{+/-}$ and mouse LY5178Y/*Tk*$^{+/-}$ cells lines.	OECD (2016b)
		Transgenic rodent: Multi-locus deletions and rearrangements	The delta-*gpt* mouse or rat permits detection of deletions and rearrangements due to DSBs when mutants are selected via Spi.	OECD (2022b)
	Genomic mutation	DNA sequence: Nucleotide-level analysis	All types of mutations in the genome	Alexandrov et al. (2020); COSMIC (2022)
	In silico	Genotoxicity structure-activity relationships (SAR)	Gene mutation and/or clastogenicity predictions can be used as supportive evidence to inform KC2	Landry et al. (2019)

BER, base-excision repair; DSBs, double-strand breaks; GSAR, genotoxicity structural alert relationship; NER, nucleotide-excision repair; NHEJ, non-homologous end-joining. Note: these are the most relevant examples and not an exhaustive list of end-points.

2.2 Relevance of end-points

2.2.1 DNA damage

As noted in above, DNA damage can occur from endogenous as well as exogenous processes. It involves a wide variety of alterations to DNA, including single-and double-strand breaks, DNA–protein crosslinks, UV-photoproducts, DNA-DNA crosslinks, DNA adducts, intercalation of molecules between bases, and various types of base modification (alkylation, methylation, oxidation, depurination and depyrimidination) (Shaughnessy and DeMarini, 2009). Table 2 lists the primary end-points relative to DNA damage. As noted above, DNA adducts are considered as relevant end-points of KC1.

The single-cell gel electrophoresis (SCGE) or comet assay is currently the primary assay for assessing a wide variety of DNA damage in cells *in vitro* and *in vivo* from almost any type of cell or organism (Collins et al., 2023; Gajski et al., 2019a, b, 2021). With various modifications, the assay can detect DNA damage involving three DNA repair pathways: base excision repair (BER), nucleotide excision repair (NER), and non-homologous end joining (NHEJ) (Ngo et al., 2020; Ge et al., 2021; Owiti et al., 2022). In particular, the assay can detect DNA strand breaks and alkali-labile sites (e.g. apurinic/apyrimidinic sites), alkylated and oxidized nucleobases, DNA-DNA crosslinks, UV-induced cyclobutane pyrimidine dimers, and some chemically induced DNA adducts (Collins et al., 2023). Most Group 1 carcinogens induce DNA damage that elicits these and additional DNA repair pathways (Krewski et al., 2019), making the findings provided by the comet assay highly relevant to KC2.

Visible chromosome aberrations are typically assessed in cytogenetic studies on stained, dead cells. Thus, it is unknown if the lesions observed under the microscope resulted in viable or dead cells. However, genetic assays that permitted the recovery of viable cells or organisms that contain multi-locus mutations (typically large deletions), such as mice mutant at the *d-se* region in the mouse specific-locus assay (Russell, 1951; Russell, 2004), mutants recovered at the *ad-3A* and/or *ad-3B* regions in the *Neurospora crassa ad-3* assay (de Serres and Malling, 1983), and small colonies of $Tk^{-/-}$ mutants recovered at the $Tk^{+/-}$ locus in the mouse lymphoma L5178Y/$Tk^{+/-}$ assay (Clive et al., 1972; Hozier et al., 1992) or the TK6/$TK^{+/-}$ assay permitted the use of the term chromosomal mutations to be applied to such large lesions. This is because the multi-locus lesions result in recoverable viable organisms or cells. Thus, depending on the assay used, the terminology varies, with DNA damage or clastogenicity being used to describe results obtained from cytogenetic assays (chromosome aberration assay or micronucleus assay), and chromosomal mutation and clastogenicity used to describe results obtained from assays permitting the recovery of viable cells or organisms containing multi-locus mutations (the mouse lymphoma $Tk^{+/-}$ or human TK6/$TK^{+/-}$ assays).

Although performed since the 1960s, chromosome aberration assays, which use a wide variety of methods, are no longer in general use for molecular epidemiology or human biomonitoring studies, having been replaced by the micronucleus assay. As discussed above, these cytogenetic assays are generally categorized as DNA damage or clastogen assays because the cells that are evaluated are dead, and it is unknown whether such cells would have been viable.

The micronucleus (MN) is the result of structural and numerical chromosome changes due either to chromosome breakage or to improper segregation of chromosomes (aneuploidy) (Heddle et al., 2011) (See Box 1). The assay can assess chromosomal mutation (chromosome breakage) and genomic mutation (aneuploidy) in a variety of cell types, including peripheral blood lymphocytes, as well as exfoliated cells from various organs, such as the oral cavity, nose, cervix, and bladder (Nersesyan et al., 2022). As with chromosome aberration assays, the micronucleus assay evaluates stained and dead cells; thus, it is not known whether such cells would have resulted in viable cells. Consequently, the micronucleus assay is categorized

as a DNA damage assay (KC2). The widespread adoption of the micronucleus assay during the past 20 years has caused it to largely replace classic chromosome aberration assays. The assay can be performed *in vivo* in rodents as described in OECD TG474 (OECD, 2016c) and *in vitro* as described by OECD TG487 (OECD, 2023b). Quality criteria for using the micronucleus assay in humans have been described in detail (Nersesyan et al., 2022).

BOX 1. Analysis of comet and micronuclei end-points in biomonitoring studies

Analysis of human biomonitoring comet data indicates that intra-individual variation in DNA repair capacities during a period of days to weeks is small, suggesting that reasonably reproducible results should be possible to be obtained from the same individual during that time period (Azqueta et al., 2019a, b). Among biomonitoring studies, a small variation by age was found in some data sets; however, no variation was found between males versus females or between smokers versus nonsmokers (Milić et al., 2021). Comet assay data can be obtained from exposed humans or from human primary cells or tissues, e.g. buccal, urothelial or blood cells. In addition, limited evidence indicates that comet data from experimental systems in vivo or in vitro may be reflective of data generated in human primary cells.

In a recent investigation conducted as part of the COMNET initiative (EU COST Action hCOMET) (Bonassi et al., 2021), the level of primary DNA damage, as assessed by the comet assay, was used to estimate the risks of overall mortality, cause-specific mortality, and cancer incidence in a cohort of over 2000 healthy individuals. Despite the limitations of this study, such as the small cohort size and the heterogeneity of the comet assay descriptors, the findings imply that the level of DNA damage as measured by the comet assay in healthy individuals could serve as a potential predictor for the risk of mortality and the development of non-communicable diseases, including cancer.

When analyzing data on MN frequency generated from biomonitoring studies, significant variability is observed across different studies primarily attributable to scoring issues (the coefficient of variation within these studies generally staying below 20%). Other factors to be considered include exposure assessment, quality of study design, sample size, dose–response, and confounding factors (e.g. age, gender, smoking and occupation), all of which may affect the outcome.

The micronucleus assay is highly relevant to human cancer because elevated frequencies of micronuclei in peripheral blood lymphocytes in humans are associated with increased risk for cancer (Bonassi et al., 2011). A set of reviews shows that elevated frequencies of micronuclei are associated with some specific cancers as well as a variety of other chronic diseases (Fenech et al., 2021). Micronuclei are now viewed as a fundamental feature of many disease processes (Fenech et al., 2016), and they are induced by a variety of environmental agents (Nersesyan et al., 2016).

In conclusion, the high relevance of micronucleus measured in exposed humans is beyond dispute. The species- and cell-specificity of DDR/DNA repair strategies should be taken into consideration when evaluating the strength of the evidence.

The detection of MN in humans with chronic exposure to carcinogens has led to the frequent use of this assay in biomonitoring studies. This assay is commonly used in human studies on various types of cells, including peripheral blood lymphocytes, buccal cells, and exfoliated urinary bladder cells. These cells are often selected because they can be easily collected from individuals and provide valuable information about genotoxic damage, making them suitable for assessing the impact of various exposures and environmental factors on human health. MN may originate from acentric chromosome fragments and/or whole chromosomes that are unable to engage with the mitotic spindle and/or fail to segregate properly to the daughter nuclei during anaphase. It is of note that recent advancements have revealed that MN serve as more than just biomarkers for DNA damage and aneuploidy. They can also induce chromosomal hypermutation (chemothripsis) and release pro-inflammatory DNA when disrupted (reviewed in Fenech et al., 2021). The

inflammatory consequences of MN formation and disruption provide an additional important explanation for the prospective association of MN not only with cancer but also with other inflammation-driven diseases (see also Box 1).

2.2.2 Mutations

Most cancers have mutations, primarily base substitutions (Vogelstein et al., 2013), in genes associated with the cancer process, and mutagenicity is among the most common characteristics of human carcinogens (Krewski et al., 2019). Among 86 Group 1 human carcinogens described by Krewski et al, 85% are genotoxic (Krewski et al., 2019), and most are mutagenic. Thus, mutagenicity is the most relevant component of KC2 and among the most studied of all the KCs with regard to carcinogenic mechanisms. Table 2 lists the primary end-points linked to mutagenesis, both gene and chromosomal, and a brief description of assays used to detect both classes of mutation is given below.

A relevant end-point for KC2 is represented by gene mutations as measured in bacterial systems, as assessed in the *Salmonella typhimurium* (Ames) or *Escherichia coli* mutagenicity assays. The Ames assay detects all six classes of base substitutions and two classes of frameshifts, using strains TA1535; TA1537 or TA97a or TA97; TA98; and TA100 (Mortelmans and Zeiger, 2000), and it has been used for a wide variety of purposes since its introduction by Bruce Ames in 1971 (Claxton et al., 2010). Certain oxidising mutagens, cross-linking agents, and hydrazines may be detected by *E. coli* WP2 strains *E. coli* WP2 uvrA, or *E. coli* WP2 uvrA (pKM101) or *S. typhimurium* TA102, which have an AT base pair at the primary reversion site.

A recent analysis has shown that just two strains, TA98 and TA100, detect 94% of all the known mutagens (> 20 000) detected by the various OECD-recommended bacterial assays (Cross and DeMarini, 2023), and the remaining 6% are detected by clastogenicity or chromosomal mutation assays (discussed below). Among the > 7000 compounds for which there were data in both strains, 32% required S9 to be mutagenic, 9% were mutagenic only without S9, and the remaining 59% were mutagenic both with and without S9. The analysis also showed that these strains detect gene mutagens having a wide variety of chemical structures, with most agents inducing primarily G to T mutations in TA100 and/or primarily the GC hotspot deletion in TA98 (Cross and DeMarini, 2023).

An earlier analysis showed that the other bacterial strains, including various strains of *E. coli* WP2 (Mortelmans and Riccio, 2000), were redundant with TA98 and TA00 (Williams et al., 2019). Thus, strains TA98 and TA100 of *Salmonella* are largely sufficient to screen for the ability of an agent to induce gene mutations. The extensive analysis by Williams et al. (2019) suggests that mammalian cell gene-mutation assays, such as the CHO/*Hprt*, and to some extent the mouse lymphoma *Tk* $^{+/-}$ or TK6/*Tk*$^{+/-}$ assays (OECD, test guidelines TG 490 and TG 476), are also redundant with the Ames assay. Indeed, these assays are generally no longer used for general screening for the ability of an agent to induce gene mutations.

Gene and chromosomal mutation can be detected (Lambert et al., 2005; Masumura et al., 2021) in transgenic rodents *in vivo* in almost any tissue, and there is some evidence showing tissue specificity for mutation induction that parallels tissue specificity of cancer induction (Lambert et al., 2005; Long et al., 2018) (see Table 2).

Gene mutations such as base substitutions, frameshifts, and small deletions in the phosphatidylinositol glycan class A gene (*Pig-a* or *PIG-A*) can be detected in rodents or in human bone marrow erythroid cells (Dertinger et al., 2021). More than 90 agents have been evaluated in the rodent assay (Shemansky et al., 2019), eclipsing the database available for transgenic rodent assays. However, the *Pig-a* or *PIG-A* assays are

limited to detecting mutations only in the bone marrow (which limits the assay's ability to detect mutagens requiring metabolic activation).

Beginning in the 1990s, DNA sequence analysis of various classes of cancer-associated genes (tumour-suppressor genes such as *TP53* and driver-mutation genes such as *KRAS*) in human tumours began identifying mutation spectra (mutational signatures) reflective of the mutation spectra of the agents associated epidemiologically with the tumours, where the mutation spectra of the agents had been determined previously in experimental systems (Dogliotti et al., 1998). Since then, various endogenous mechanisms of carcinogenesis and exogenous mutagen/carcinogen exposures associated with an array of tumour types have been shown to produce identifiable mutational signatures (Alexandrov et al., 2020; COSMIC, 2022). Some mutation spectra found in cancer-associated genes in human tumours are similar or even identical to those found in experimental systems, for bacteria, mammalian cells, or transgenic rodents exposed to the same agent associated with the human tumours (DeMarini, 2000; Kucab et al., 2019). Likewise, mutational signatures, which are mutation patterns across the entire genome, have been associated with specific exposures or mechanisms with specific types of cancers (Alexandrov et al., 2020), confirming the relevance of these mutation patterns in tumours linked epidemiologically to specific exposures (see also Part II). These types of data provide the strongest evidence possible that the agent is the likely cause of the tumour. (t should be noted that mutational spectra can reveal sites of DNA adduct formation, some which lead to mutations while others do not. Hence, not all DNA adduct-forming compounds may lead to mutations). Predictions of gene mutation and/or clastogenicity by *in silico* methods (Landry et al., 2019) are considered relevant for KC2.

2.3 Assessing the relevance of end-points in different test systems

2.3.1 DNA damage

The end-point measured by the comet assay is highly relevant because it can be assessed equally well in experimental systems *in vitro* and *in vivo*, also in exposed humans, and has been used extensively in biomonitoring studies, producing highly significant results in subjects with exposures to a diverse array of DNA-damaging agents (Azqueta et al., 2019b; Gajski et al., 2021; Milić et al., 2021).

The measurement of micronucleus is also highly relevant to human cancer because elevated frequencies of micronuclei in peripheral blood lymphocytes in humans are associated with increased risk for cancer (Bonassi et al., 2011) (see also Box 1 and Chapter 3). By extension, micronucleus data *in vitro* and in rodents have relevance to humans. A set of reviews shows that elevated frequencies of micronuclei are associated with some specific cancers as well as a variety of other chronic diseases (Fenech et al., 2021). Micronuclei are now viewed as a fundamental feature of many disease processes (Fenech et al., 2016), and they are induced by a variety of environmental agents (Nersesyan et al., 2016).

Of note, findings provided by the comet or micronucleus assay assessed in human studies are considered to inform also on potential alterations of the DDR.

2.3.2 Mutations

Mutations *in vivo* in rodents or humans might be viewed as an end-point of high relevance for KC2. In addition, positive *in vitro* data from mammalian cells of any type (human primary cells, human cell lines, or

non-human cell lines) or from bacterial systems are similarly relevant for KC2. This conclusion is based on analyses showing the redundancy of mammalian cell mutagenicity assays for gene mutation relative to the Ames assay (Williams et al., 2019). One of the exceptions to this would be that negative data in Ames would be overridden by positive data in a mammalian cell mutagenicity assay that also detects chromosomal (along with gene) mutations, such as the mouse lymphoma *Tk*$^{+/-}$ and TK6/*TK*$^{+/-}$ assays. Such data (negative in *Salmonella* but positive in these assays) would indicate that the agent acts solely as a chromosomal mutagen, which cannot be detected by the Ames assay or reflects a limitation of the biological system modelled in the *in vitro* assay (Williams et al., 2019).

Although the identification of mutagenicity in the Ames assay for carcinogenicity in rodents varies depending on the content searched (Zeiger, 2000), a positive result in the Ames mutagenicity assay is approximately 70% predictive of carcinogenicity in rodents, whereas a negative result in *Salmonella* is approximately 50% predictive of carcinogenicity in rodents (Zeiger, 1987, 1998). The addition of data from other genetic toxicology assays to that from the Ames mutagenicity assay does not improve predictivity for rodent carcinogenicity (Zeiger, 1998). Two cancer case–control studies in humans have shown that urinary mutagenicity assessed by the Ames mutagenicity assay in non-smokers is highly predictive for colorectal adenoma (odds ratio 2.4; 95% confidence interval (CI), 1.1-5.1) (Peters et al., 2003) and bladder cancer (odds ratio 3.8; 95% CI, 1.3-11.2) (Wong et al., 2024).

2.4 Interpretation of results within the same database

2.4.1 DNA damage

DNA damage findings provided from only the comet assay *in vitro* or in rodents or in humans should be interpreted cautiously for KC2 because much of DNA damage can be repaired and, thus, may not result in a mutation playing a causal mechanistic role in carcinogenesis. In contrast, elevated levels of DNA damage as determined by the micronucleus assay, especially when observed in experimental systems *in vivo* and exposed humans, is considered a more relevant end-point and a risk factor for cancer (Bonassi et al., 2011) and other diseases (Fenech et al., 2021), and such data are of high relevance for KC2 (see Section 2.3). An analysis of data from various genotoxicity assays for their predictivity for rodent carcinogenicity showed that the addition of data from other assays to those from the Ames mutagenicity assay did not improve predictivity for rodent carcinogenicity based on the Ames data alone (Zeiger, 1998). Thus, positive results from assays that detect DNA damage would complement positive mutagenicity data, but negative DNA damage data would not diminish the relevance of positive mutagenicity data.

2.4.2 Mutations

As discussed above, mixed results in a variety of mutagenicity or genotoxicity assays must be interpreted carefully, based on what each assay detects. For example, an agent may give a variety of results in various strains of *Salmonella* with and without S9. However, those mixed results may simply reflect the mutational and metabolic specificity of the agent. Likewise, agents may be negative in *Salmonella* but positive in mammalian cell *Tk*$^{+/-}$ or *TK*$^{+/-}$ assays as well as in the micronucleus or comet assays, all of which detect DNA damage and/or chromosomal mutation, which cannot be detected by the Ames assay. Reviews and meta-analyses of micronucleus studies in humans have found mixed results, but these can result from

procedural differences between laboratories (Fenech et al., 2021). Interlaboratory reproducibility is also an issue for Ames tests or other assays.

Table 2 Shows the primary end-points and assays that are currently used to detect various types of DNA damage and mutation. These are largely established assays that have been in use for decades and for which there are compelling data showing their relevance to carcinogenic mechanisms. Because mutation, which is considered a causal mechanism of carcinogenesis, is frequently preceded by DNA damage, the assays and end-points listed here are highly relevant to carcinogenic mechanisms.

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3. Key Characteristic 3: Alters DNA repair or causes genomic instability

Eugenia Dogliotti

3.1 Introduction

Living organisms are continuously exposed to a variety of DNA-damaging agents that have the potential to compromise the integrity of their genome. When a cell detects DNA damage, it initiates a series of events designed to remove or tolerate this damage to ensure the organism's overall survival. This orchestrated response is known as the DNA damage response (DDR). It includes the activation of cell-cycle checkpoints, DNA repair pathways, and various signalling cascades to coordinate the repair process and prevent the propagation of damaged DNA (Harper and Elledge, 2007). DNA repair is an integrated component of this response and plays a key role in preserving the stability of the genome (Groelly et al., 2023).

It has been known since the 1960s that rare inherited mutations in DNA repair genes increase the risk of cancer (Cleaver, 1968; Dogliotti and Bignami, 2019). In relation to the general population, increasing evidence suggests that mutational patterns observed in the most prevalent types of cancer result from the combined influence of DNA damage and impaired DNA maintenance processes (Alexandrov et al., 2020). For instance, several base substitution signatures in common cancers show transcriptional strand bias, which may be attributable to efficient repair of the transcribed strands (transcription-coupled repair, TCR). The likely DNA-damaging IARC Group 1 agents were identified through their mutational fingerprint as tobacco mutagens (Volume 100E), UV light (Volume 100D), aristolochic acid (Volume 100A), aflatoxins (Volume 100F), and chemotherapeutic agents (Volume 100A) (IARC, 2012a, b, c, d). Signatures of altered DNA repair mechanism, such as those due to inactivating germline or somatic mutations in the base excision repair (BER) gene MUTYH or to defective DNA mismatch repair (MMR), are also often present particularly in gastric and ovarian cancers. Also very frequent is the signature of background mutagenesis by APOBEC3B (Chan et al., 2015). We may conclude that there is strong evidence that exogenous and endogenous exposures together with defective genome surveillance mechanisms are associated with cancer.

A defect in DDR or DNA repair is normally considered as highly relevant, but its activation is not. Though beneficial by protecting against DNA damage, long-term activation of the DDR/DNA repair machinery could also contribute to persistent inflammation, through mechanisms such as the induction of cellular senescence. Senescence is a potential strategy for cells to avoid malignant transformation, but it can also promote cancer development by altering the cellular microenvironment through a senescence-associated secretory phenotype (SASP) (Mathon and Lloyd., 2001; Campisi and d'Adda di Fagagna, 2007).

Carcinogens may alter DNA replication, DDR and DNA repair via different mechanisms by:

- **Induction of extensive DNA damage**: most carcinogens can overwhelm the cellular DNA repair and DDR systems. The overload can result in the accumulation of DNA damage that remains unrepaired.

- **Direct inhibition of DNA repair/DDR enzymes**: alteration of enzyme activity. For example, the IARC Group 1 carcinogen inorganic arsenic (Volume 100C) can interact with both thiol groups and zinc finger domains in DNA repair enzymes, leading to disruption of the enzymes' structure and function, thus increasing the likelihood of accumulation of DNA damage.

- **DNA modifications**: e.g. bulky or distorting adducts, DNA–DNA or DNA–protein cross-links and clustered DNA lesions. If not resulting in cell death, these modifications can prevent repair enzymes from recognizing and fixing the damaged sites effectively. Finally, carcinogens can induce changes in the transcriptional profile of exposed cells, leading to alterations in the expression of multiple genes, including DNA repair/DDR genes.

The interference with DNA repair and DDR pathways may lead to genomic instability (Aguilera and García-Muse, 2013) that is a common feature of cancer. Genomic instability includes small structure variations such as increased frequencies of base-pair mutation, microsatellite instability (MSI), as well as significant structure variation such as chromosome number or structure changes, which is also called chromosome instability (CIN) (reviewed in Al-Sohaily et al. 2012; Roschke and Kirsch, 2010; Yao and Dai, 2014). These events are mainly mediated by double-strand breaks (DSBs) or single-strand gaps: cancer cells often exhibit an increased burden of DSB compared to healthy cells, primarily due to acquired deficiencies in DDR mechanisms. The induction and repair of DSB are therefore highly relevant for the identification of potential carcinogens (reviewed in Jeggo and Löbrich, 2015).

3.2 Relevance of endpoints

3.2.1 DNA repair/DDR

The primary end-points describing alteration of DNA repair and DDR are the activity of specific DNA repair enzymes, alteration of the expression of specific genes or proteins expression, or phenotypic end-points such as repair of single-strand breaks (SSBs) or DSBs and repair of transcription-blocking lesions, or finally measurement of genomic instability, including chromosome instability, telomere length, microsatellite instability and others. These end-points can be either measured *in vitro*, including cellular extracts, or *in vivo*, including in exposed humans. The induction and repair of DSB is considered a relevant end-point in the identification of carcinogens and a marker of cancer risk; a distinct section is included to describe the assays specifically designed for detecting DSB (see Table 3).

DNA repair/DDR Enzymes

The activity of individual enzymes can be measured in cell extracts (cell-free *in vitro* system*)* with a high degree of precision and a reasonable degree of reproducibility using defined synthetic DNA substrates. The identification of the specific step within a repair pathway that is altered is feasible by reconstructing a DNA repair pathway using modified oligonucleotides containing the original DNA lesion and its repair intermediates (e.g. BER as discussed in Frosina et al., 2006). However, the relevance of findings from the cell-free systems may be limited. First, the use of synthetic substrates in all the extract-based approaches does not fully replicate the complex DNA microenvironment of a damaged live cell. In addition, these assays may overlook coordination defects within the pathway, such as pathway imbalances, or fail to detect deficiencies in non-enzymatic components, such as accessory proteins that act as scaffold proteins. Nonetheless, these systems retain their significance when studying the mechanism of action of carcinogens. This is exemplified by the use of synthetic oligonucleotides containing a single 8-oxoguanine to test 8-oxoguanine DNA glycosylase 1 (OGG1) repair activity of cell extracts. Carcinogens often induce the oxidation of DNA bases, and the inhibition of OGG1 can result in various outcomes related to DNA repair, genomic stability, and cellular function (Evans et al., 2004) (see also Chapter 5 of Part I). Another example involves the inhibition of the activity of adenosine diphosphate poly-ribose polymerase-1 (PARP1) by the

Group 1 carcinogen inorganic arsenic and its metabolites, which have been shown to interact with the zinc finger motif of PARP1, resulting in increased formation of SSBs and DSBs in cultured cells (reviewed by Tam et al., 2020).

Table 3. End-points relevant to KC3: "Alters DNA repair or causes genomic instability"

Category	End-point	Relevance	Comments	References
DNA repair/DDR	Repair of DNA lesions present on synthetic DNA *Measured by cell extract-based assays*	Activity of DNA repair/DDR enzymes	The assay can detect the efficiency of both single- and multi-step pathways. Main limitation: the use of a synthetic substrate. Can be done with extracts from almost all types of cells.	Frosina et al., 1999
	Transcriptomics, proteomics, SNPs *Measured by genotypic assays*	Gene expression, protein activity, genetic variations	Main limitations: the relevant gene/s may not be expressed or may be inactive; the functional consequences of a SNP are often unknown. Significant advancements with the integrated omics analysis.	Sherill-Rofe et al., 2022; Kratz et al., 2023
	UDS; *Measured by phenotypic assays*	Measures DNA repair synthesis	Identifies substances inducing "long-patch repair". Can be done with almost all types of cells including mixed cell populations. Limits of sensitivity.	OECD, 1997
	Phenotypic assays: Comet assay[a]	Repair of SSB and DSBs	Frequently used in biomonitoring studies in various types of human cells. Modifications, such as the challenge assay, the in vitro comet and the comet chip, allow investigation of the efficiency of specific or multiple DNA repair pathways.	Banerjee et al., 2008; Azqueta et al., 2020; Ge et al., 2021
	Phenotypic assays: Host-cell reactivation assay	Repair of transcription-blocking lesions	Used in biomonitoring studies in various types of human cells. Limitations: plasmid DNA is the substrate; measures the synthesis of a protein coded by a plasmid reporter gene. Flow-cytometric HCR allows measuring repair capacity in multiple pathways	Nagel et al., 2014
	DSB repair assays: Pulse field gel electrophoresis	Measure DSBs induction and repair	Quantification of DSB across a broad size range. Limitations: labour-intensive, technical requirements, low sensitivity	Lopez-Canovas et al. 2019
	DSB repair assays: Neutral comet assay		Inadequate standardization, inability to offer quantitative data	
	DSB repair assays: γ-H2AX		Can detect DSB in almost all types of cells including human cells. High sensitivity. Co-staining with 53BP1 required to unequivocally identify DSB.	Löbrich et al., 2010.
	DSB repair assays: Reporter assays		Provide a direct evaluation of DSBR capacity in live cells. Reporter gene previously introduced into the cells through transfection or direct induction into DNA of a DSB.	Gunn and Stark 2012; van de Kooij and van Attikum, 2022
	DSB repair assays: High-throughput assays for DSB	Analysis of cell response to DSBs	Single cell network profile to measure activation of DDR, biochip multiplex	Rosen et al., 2014; Tatin et al., 2022

Table 3. End-points relevant to KC3: "Alters DNA repair or causes genomic instability"

Category	End-point	Relevance	Comments	References
			cell-free assays to measure HR and NHEJ	
Genomic instability	Chromosome instability: Micronucleus[a]	Chromosome breaks and aneuploidy	Frequently used in biomonitoring studies in various types of human cells.	Fenech et al., 2021
	Chromosome instability: Telomere length[b]	Measurement of telomere length	Alteration of telomere length is often associated with chromosome instability.	Lai et al., 2018
	Genome instability: Microsatellite instability	Expansion or contraction of repetitive DNA sequences	A few studies document the induction of MSI by xenobiotics.	Li et al., 2020a
	Genome instability: Flow cytometry	Detection of cell ploidy	Can be combined with FISH to assess genomic instability at the chromosome level.	Torres-Ruiz et al., 2021
	Genome instability: Karyotyping (spectral karyotyping)	Analysis of the complete set of chromosomes	Detection of inter-/intra-chromosomal translocations	Anguiano et al., 2012
	Genome instability: Array of comparative genomic hybridization	Detection of chromosomal abnormalities	Can identify chromosomal gain and losses. Uses microarrays to detect CNV with higher resolution.	Vissers et al., 2003

CNV, copy number variations; DDR, DNA damage response; DSB, double-strand break; DSBR, DSB repair; FISH, fluorescence in situ hybridization; HCR, host-cell reactivation; HR, homologous recombination; MSI, microsatellite instability; NHEJ, non-homologous end-joining; SNP, single nucleotide polymorphism; SSB, single-strand break; UDS, unscheduled DNA synthesis.

[a] End-points reported in KC2; bEnd-point relevant to KC9. Note: these are the most relevant examples and not an exhaustive list of end-points.

Phenotypic end-points

These assays that are conducted in living cells are currently the most relevant tool to investigate DNA repair/DDR capacity. Indirect measurements of DNA repair/DDR capacity by transcriptomics (Magkoufopoulou et al., 2012), proteomics (von Stechow and Olsen, 2017), and single nucleotide polymorphisms (SNPs) screening (Niazi et al., 2021) are less informative because the relevant gene may not be expressed, or the gene product may be inactive. Of interest are the recent developments in integrated methods for the analysis of transcriptomics and proteomics that have been successfully applied to identify the mechanisms of dysregulation of DNA repair/DDR in response to DNA damage (Sherill-Rofe et al., 2022; Kratz et al., 2023) (see Section 2.3, Chapter 2, Part I)

The unscheduled DNA synthesis (UDS) assay is used to assess a cell's ability to perform global nucleotide excision repair (NER) (Kelly and Latimer, 2005). When [^{3}H]thymine is included in a culture medium containing damaged cells, their DNA is tritiated throughout the cell cycle unlike unirradiated cells, which incorporate [^{3}H]thymine into DNA only in S phase. UDS is then detected by autoradiography. Even though UV lesions were originally discovered to evoke UDS, it is now known that many other carcinogenic compounds elicit UDS as well. Nonetheless, the ability to detect a UDS response relies on the quantity of DNA bases that are excised and replaced at the damaged site. As a result, the UDS test is particularly useful in identifying substance-induced "long-patch repair," which involves the excision and replacement of a larger segment of DNA (approximately 20–30 bases). It can be applied to almost all cellular systems and

can be particularly valuable in mixed cell populations in which cells cannot be physically separated but can be visually differentiated. However, the limits of sensitivity are such that the current assay may be unable to detect a low level of DNA damage induced by some weakly genotoxic agents, and as such have reduced its use. A validated OECD test guideline TG486, describing UDS *in vivo*, is available (OECD, 1997).

The repair kinetics of SSB and DSB and alkali-labile sites (such as apurinic/apyrimidinic sites) can be measured by specific modified versions of comet assay, which can detect various forms of DNA damage by using lesion-specific endonucleases and measuring the efficiency of different DNA repair pathways (Azqueta et al., 2014).

In biomonitoring studies, a modified comet assay can be employed to assess the repair capability of cells using a specific approach, the challenge assay. In this method, DNA damage is experimentally induced in cells, typically lymphocytes, obtained from donors, and the comet assay is subsequently used to evaluate the DNA repair capacity. The challenging agents used in this assay are ionizing radiation or bleomycin, which induce a variety of lesions (oxidative base damage, DNA SSB and DSB, thus enabling the investigation of the BER and DSB repair capacity in the donors. This approach has been used in various studies to compare the DNA repair capacity between cancer patients and controls or people exposed to environmental contaminants versus unexposed subjects. This assay has been recently performed also in salivary leucocytes (Fernández-Bertólez et al., 2022). Although the challenge comet assay is a useful tool to identify susceptible individuals, and to identify potentially harmful environmental exposures (Banerjee et al., 2008), it is particularly sensitive to day-to-day individual variability; therefore, it lacks consistent reproducibility. The mutagen sensitivity assay, a variant of this assay, measures the DNA repair capacity indirectly via induction of bleomycin-induced chromatid breaks (Hsu et al., 1989).

To streamline biomonitoring trials that require simultaneous processing of multiple samples, a novel method has been developed for assessing BER or NER in cells, the comet-based *in vitro* DNA repair assay. This approach utilizes the alkaline comet assay in an *in vitro* assay, wherein cell extracts containing repair enzymes are incubated with DNA nucleoids containing a specific lesion (Azqueta et al., 2020). The breaks induced at the site of the lesion in the substrate are then measured using the alkaline comet assay. The ability of the cell extracts to carry out the incision is considered a rate-limiting step in the repair process and serves as an indicator of the DNA repair activity of the cells. The assay's critical elements include the abundance of lesions within the substrate nucleoid, which must surpass a certain threshold for the extract to function, as well as the lesions' specificity. Additionally, the length of the incubation period is vital for distinguishing levels of repair activity across different extracts. The application of the comet-based *in vitro* DNA repair assay is rare.

To overcome the problem of looking at single DNA repair pathway, the Comet-Chip assay has been recently developed to assess repair kinetics across several classes of DNA damage, using cells trapped in agarose in 96-well format. The platform can address the three major DNA repair pathways, namely BER, NER, and NHEJ in living cells (Ge et al., 2021).

The capacity of cells to repair DNA damage that blocks the transcription of a transiently transfected reporter gene can also be assessed by the host cell reactivation (HCR) assay. Initially, this assay was employed in molecular epidemiology studies to establish a correlation between NER capacity and cancer risk, transfecting human lymphocytes with a plasmid containing the chloramphenicol acetyltransferase (CAT) reporter gene (Athas et al., 1991). Subsequently, a modified version of the HCR assay was developed, which involves nucleofection of the plasmid into target cells. This modified version demonstrated high sensitivity, reliability and reproducibility (Mendez et al., 2011).

Although the HCR assay is relatively fast and simple, it has several limitations. It relies on a non-genomic DNA substrate and depends on the synthesis of a protein as a reporter for successful DNA repair. Hence, any changes in protein synthesis, regardless of their impact on DNA repair capacity, can influence the outcomes of the assay. Furthermore, HCR assays are generally unable to detect repair of DNA lesions that do not impede the progression of RNA polymerase, thus limiting their detection to the TCR pathway, which is a sub pathway of NER. Assessing DNA repair capacity using the HCR assay is further constrained by the need for separate experiments to measure repair capacity in multiple pathways or at different levels of DNA damage. To address this limitation, a fluorescence-based multiplex flow-cytometric host cell reactivation assay (FM-HCR) has been developed (Nagel et al., 2014). The FM-HCR enables the measurement of the repair efficiency of plasmid reporters bearing various types or doses of DNA damage, by nucleotide excision, mismatch, base excision, nonhomologous end joining (NHEJ), homologous recombination (HR), and methyl-guanine methyltransferase (O^6-methyl-guanine-DNA methyltransferase). Finally, although the assay allows high-throughput analysis, the issues of inter- and intra-individual variation still need to be addressed adequately, and the assay is not standardized.

Double strand break repair

DSBs are the most cytotoxic DNA lesions. Their detection, signalling, and repair require an active response to DNA damage. The signature of defective DSB repair has been identified in several cancers (Alexandrov et al., 2020). DSB repair (DSBR) can be assessed through various methods, including indirect techniques such as pulse field gel electrophoresis (PFGE), the neutral comet assay, and immunofluorescence (IF). These techniques evaluate DSBR capacity by measuring the decrease in DSB levels over time after exposure to a DNA-damaging agent. Alternatively, cell-free assays and reporter-based methods directly track the repair of artificial DNA substrates (Tatin et al., 2021). Each approach has its own strengths and limitations. Despite significant efforts, there is currently no ideal method for quantifying DSBR capacity.

PFGE using an alternating cross field allows separation of fragments longer than 50 kb, thus providing a tool for DSB detection. However, the application of PFGE in DSBR studies has diminished in the past twenty years. This decline can be attributed to the emergence of alternative methods and inherent limitations, notably its limited sensitivity in detecting DSB at biologically relevant doses (Lopez-Canovas et al., 2019).

The neutral comet assay also has limitations, including inadequate standardization and the inability to offer quantitative data. In addition, when the comet assay is conducted under neutral conditions, it can convert lesions such as modified bases into DNA strand breaks. Consequently, this conversion can result in biased estimations of DSBR capacity.

Early steps in the damage response can be considered relevant end-points and can be assessed by staining the factors that gather within structures called foci using specific antibodies, such as the analysis of γH2AX foci loss by immunofluorescence (IF). DSBR by IF is detectable at low dose exposures (e.g. radiations around 1 mGy) differently from PFGE and the neutral comet assays that require relatively high doses of chemical or ionizing radiation exposure. This is particularly relevant as it allows for the study of chronic low-dose exposures. Despite the high sensitivity of IF assays and their applicability to a large panel of cells and tissues, it should be noted that the one-to-one correlation between DSB induction and γ-H2AX foci has never been entirely confirmed, and DNA damage detected by IF can differ from DSB, usually because of cellular mechanisms indirectly connected to DSB processing. Co-staining with other markers such as 53BP1 may be required to correctly identify DSBs (Löbrich et al., 2010).

DSBR capacity can be also measured in damaged plasmid DNA incubated with cell extracts *in vitro*. However, a significant drawback of these direct assays is their limited ability to replicate the complexity of

naturally occurring DSB. It is worth noting that the repair processes observed in plasmids may not accurately reflect the actual DSBR that occurs in genomic DNA.

A direct evaluation of DSBR capacity in live cells is also provided by measuring the restoration of the expression of a reporter gene that was previously introduced into the cells through transfection (Gunn and Stark, 2012). Since the end-point is measured in living cells, it better mirrors the repair events occurring on the genomic DNA when compared to cell-free extracts. When transfected, plasmids are complexed into functional nucleosomal structures that undergo histone modification in response to DNA damage, offering a more realistic representation of *in vivo* DSBR capacity. However, a potential limitation is the reduced diversity of the DSB models on which they rely. To overcome this problem, recent developments directly induce DSB in genomic DNA instead of relying on plasmids (van de Kooij and van Attikum, 2022).

The activation of DDR proteins (e.g. p-H2AX, p-ATM, p-DNA-PKcs, p-53BP1, p-RPA2/32, p-BRCA1, p-p53 and p21) induced by genotoxins is measured by flow cytometry-based single-cell network profiling (SCNP). Both NHEJ and HR pathways can be examined by measuring changes in intracellular readouts (including p-H2AX, p-ATM, p-DNA-PKcs, p-53BP1, p-RPA2/32, p-BRCA1, p-p53 and p21) in response to exposure to mechanistically distinct genotoxins (Rosen et al., 2014). A biochip multiplex cell-free assay has been developed to measure functional aspects of DSBR. A new enzymatic cell-free DSB repair assay (NEXT-SPOT) can simultaneously characterize strand invasion, end-joining, and polymerase activities of the DSBR machinery (Tatin et al., 2022). The assay quantifies the incorporation of different markers on two plasmid templates immobilized on a biochip, the combination of which provides information on several DSB-specific repair steps.

3.2.2 Genomic instability

Genomic instability encompasses various end-points, such as chromosomal aberrations, sister chromatid exchange (SCE), micronucleus (MN) formation and aneuploidy detection, that are considered within KC2 (Chapter 2) but also karyotyping, comparative genome hybridization, MSI, chromosomal instability, and alteration of telomere length and whole-genome sequencing (WGS).

The MN formation in biomonitoring studies reveals a type of genetic damage that may generate genetic instability (see Box 1 and Chapter 2, Part I).

Assay for measuring telomere length

xposure to carcinogens can result in increased DNA damage and replication stress. Telomeres, which are the protective caps at the ends of chromosomes, are sensitive to such damage. If the DNA damage and replication stress are persistent and severe, they can lead to changes in telomere length and to genomic instability. There are several methods for measuring telomere length, including Southern blot analysis, quantitative polymerase chain reaction (qPCR) and more recent techniques like quantitative fluorescence in situ hybridization (qFISH) and flow cytometry. Both shorter and longer telomeres have been linked to an increased risk of cancer (reviewed in Tsatsakis et al., 2023). Notably, two studies (Mitro et al., 2016; Shin et al., 2010) have found connections between exposure to polychlorinated biphenyls (PCBs) and the length of telomeres in leukocytes. These findings have been further corroborated by the discovery of associations between elevated levels of various specific categories of persistent organic pollutants (POPs), including PCBs, and longer telomeres in blood leukocytes (Scinicariello and Buser, 2015). Telomere length is also discussed as an end-point relevant for KC9 (see Section 9.1, Chapter 9, Part I).

Microsatellite instability

Microsatellite instability (MSI) arises due to defects in mismatch repair (MMR) genes such as MSH, MLH, and PMS. The expression profile of MMR genes correlates with the occurrence of MSI.

The standard approach for detecting MSI involves amplifying microsatellite regions using PCR and quantifying the lengths of PCR products by electrophoresis, employing autoradiography, silver staining, or fluorescent methods. Next-generation DNA sequencing (NGS) is also employed to detect MSI. Because mutant MMR genes lead to the production of mutant MMR proteins, immunohistochemistry (IHC) using mutation-specific monoclonal antibodies can be applied (reviewed in Li et al., 2020a). There are a few studies that document the induction of MSI by xenobiotics. An example is the case of human colorectal cancer cells exposed to sublethal doses of cadmium or arsenite, resulting in increased frequency of MSI. This increase is linked to the generation of oxidative stress (Wu et al., 2017).

Chromosome instability

Chromosome segregation errors lead to aneuploidy, a state of abnormal chromosome numbers, and a persistently high rate of chromosome segregation errors causes the related phenomenon of whole chromosomal instability (CIN) (Thompson et al., 2010; Godek and Compton, 2018). Various methods have been devised to assess CIN, some of which are outlined below.

Flow cytometry is a widely applicable technique used to detect cellular ploidy and assess cell cycle distribution using fluorescent dyes that bind to DNA in a stoichiometric manner. However, flow cytometry does not provide information about other levels of genome instability. It can be combined with fluorescence in situ hybridization (FISH) to investigate specific chromosomal abnormalities and assess genomic instability at the chromosome level (Torres-Ruiz et al., 2021). Karyotyping is a more informative method that involves staining metaphase spreads with a DNA-binding dye, which intercalates into specific DNA regions of chromosomes, resulting in a distinct banding pattern for each chromosome. Spectral karyotyping, a multicoloured whole-chromosome painting assay using FISH probes, allows visualization of each chromosome, allowing the detection of global changes in chromosomes but preventing the evaluation of alterations at the sequence level (Anguiano et al., 2012). Array of comparative genomic hybridization (aCGH) is a technique that involves the quantitative detection and visualization of chromosomal alterations in multiple cells (Vissers et al., 2003). By comparing the sample DNA to a normal reference genome, aCGH can identify gains, losses, amplifications and loss-of-heterozygosity, revealing unbalanced chromosomal abnormalities related to changes in copy number. However, aCGH is unable to differentiate reciprocal translocations, inversions, or somatic mutations. SNP arrays, which are also hybridization-based, utilize fragmented nucleic acid sequences labelled with fluorescent dyes that bind to immobilized, allele-specific oligonucleotide probes (LaFramboise, 2009). For comprehensive and informative analysis of nucleotide mutations in coding, non-coding and unannotated regions, whole genome sequencing (WGS) is considered the most suitable method. WGS can also identify larger genomic rearrangements commonly found in cancer, such as copy number variations (CNV), insertions and translocations (Hehir-Kwa et al., 2015). NGS systems enable efficient and accurate analysis at high throughput while reducing sequencing costs. Additionally, sequencing the transcriptome (RNA-seq) allows the discovery of specific genetic aberrations like gene fusions (Weissbein et al., 2016), potentially replacing microarrays in the future. Advances in NGS and whole-genome amplification have already made it possible to perform single-cell sequencing. Single-cell genomic DNA sequencing to determine chromosome copy numbers is not yet routine due to cost considerations, but it holds promise as a future technology.

There is a substantial body of evidence implicating DNA damage-induced genome instability as a significant contributor to cancer. Recent research efforts have introduced the era of studying DNA damage and repair at a nucleotide resolution on a genome-wide scale, referred to as the "breakome" era (Rybin et al., 2021; Saayman and Esashi, 2022). These new comprehensive technologies will undoubtedly enhance our understanding of how impaired DDR and repair mechanisms contribute to the development of cancer. However, to date the application of these techniques has primarily focused on investigating the molecular features of tumours. Further research should validate these genomic end-points and ensure their relevance by testing a wide variety of xenobiotics for the purpose of understanding their mechanism of action and establishing their safety.

3.3 Assessing the relevance of end-points in different test systems

DNA repair capacity exhibits differences both between species and within different cell types. In general, mice that live for a short length of time (2 yrs vs 70 yrs for humans) are less dependent on mechanisms supporting genome maintenance and integrity. Accordingly, mutation rates are higher in mice than in humans (Milholland et al., 2017), and expression of DNA repair genes is lower. For instance, comparison of transcriptomes of liver (MacRae et al., 2015) showed statistically significant upregulation of several DNA repair signalling pathways (including core genes) in long-lived species, humans and naked mole-rats, compared with short-lived mice. In addition, organ and cell-type specificity have been described. There are examples of cell-type specificity in the response to DNA damage (for instance human primary keratinocytes vs fibroblasts) (D'Errico et al., 2007). In addition, Vougioukalaki et al. (2022) have shown that organs employ different genome maintenance strategies. For instance, intestine with short lifespan favours apoptosis of damaged cells thus limiting the time for damage accumulation and repair, liver having a low renewal rate depends more on DNA repair and particularly on repair of the transcribed compartment and finally, the haematopoietic system, with intermediate self-renewal, mainly uses replication-linked mechanisms, apoptosis and senescence.

DNA damage repair as detected by the comet assay in biomonitoring studies is an end-point of high relevance (see also Box 1) (Azqueta et al., 2019b, 2020; Gajski et al., 2021; Milić et al., 2021). An examination of the chemical exposures evaluated in biomonitoring studies using the comet assay indicates that many of these chemicals were first shown to induce DNA damage detectable by the comet assay in mammalian cell lines (Milić et al., 2021).

3.4 Interpretation of results within the same database

Currently, the assessment of the cellular DNA repair phenotype serves as the primary method for measuring changes in DNA repair/DDR. Specific facets of the DNA repair phenotype, such as the efficiency and fidelity of DNA repair mechanisms, can be assessed by different end-points. For instance, the extent of DNA damage and the efficiency of repair can be evaluated using the comet assay; DSB formation and repair dynamics can be measured by the host-cell reactivation assay or by monitoring γ-H2AX levels, a marker of DSB. Importantly, alteration of DNA repair/DDR is expected to compromise genome stability leading to heightened sensitivity to agents that cause DNA damage and increased mutation frequency.

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4. Key Characteristic 4: Induces epigenetic alterations

Parveen Bhatti, Zdenko Herceg, and Akram Ghantous

4.1 Introduction

By affecting chromatin structure and gene expression, epigenetic dysregulation is critical to the development of virtually all human cancers, occurring progressively throughout the multistep process of tumorigenesis. Described as the interface between the genome and the environment, epigenetic modifications occur in response to environmental stressors and are plausible mechanisms by which environmental factors contribute to carcinogenesis (Herceg and Vaissière, 2011; Herceg et al., 2018). Major epigenetic mechanisms include DNA methylation, histone modifications, and noncoding RNAs, deregulation (see Table 4) of which have been associated with a range of known carcinogens, including chemical, physical and biological agents (Herceg et al., 2013; Chung and Herceg, 2020).

Table 4. End-points relevant to KC4: "induces epigenetic alterations"

Category	End-point	Relevance	Comment	Reference
DNA methylation	Global DNA hypomethylation	↑ oncogene expression ↑genomic instability	Associated with carcinogenesis Assays include HPLC and pyrosequencing	Grønbaek et al., 2007; Joyce et al., 2016
	Tumour suppressor gene promoter hypermethylation	↓tumour suppressor gene expression	Associated with carcinogenesis Assays include microarrays and next generation sequencing	Ando et al., 2019
	Erosion of or shift in CpG island methylation boundary	↑ oncogene expression ↓tumour suppressor gene expression	Associated with carcinogenesis Assays include microarrays and next generation sequencing	Hansen et al., 2011
	Methylation clock	↑ biological age	May be associated with increased risk of cancer Assays include microarrays	Ambatipudi et al., 2017; Chen et al., 2022
Histone post-translational modifications	Histone methylation, acetylation, ubiquitination, phosphorylation	Altered gene transcription, Impaired DNA repair	Implicated in carcinogenesis Assays include Chromatin Immunoprecipitation Sequencing (ChIP-Seq)	Murr et al., 2006; Greer and Shi, 2012; Zhao and Shilatifard, 2019; Komar and Juszczynski, 2020
microRNAs	micro-RNA dysregulation	Disruption of cell proliferation, differentiation and apoptosis	Implicated in carcinogenesis Assays include RNA sequencing	Krutovskikh and Herceg; 2010; Yang et al., 2020b

ChIP-Seq, Chromatin immuno precipitation-sequencing; HPLC: High-performance liquid chromatography. Note: these are some key examples and not an exhaustive list of end-points.

4.2 Relevance of end-points

4.2.1 DNA Methylation

DNA methylation is the most widely studied and best characterized epigenetic mechanism. DNA methylation plays important roles in critical cellular processes, including gene transcription regulation, genomic imprinting, and chromosome stability (Jones, 2012). Two forms of aberrant DNA methylation are found in virtually all cancers: global hypomethylation (resulting from a global loss of 5-methyl-cytosine) and gene-specific hypermethylation (an unscheduled gain of methylation in a CpG island (CGI) associated with the promoter of specific genes) (Baylin and Jones, 2016; Jones et al., 2019).

Hypomethylation of CpG sites may lead to the upregulation of oncogenes, though the primary carcinogenic effect seems to be a loss of genomic stability leading to elevated mutation rates (Grønbaek et al., 2007). Measures of global DNA hypomethylation may not only be markers of cancer prognosis (Li et al., 2014) but may also serve as markers of future cancer risk (Joyce et al., 2016). Overall, global DNA hypomethylation can be considered an end-point of relevance for KC4, particularly if the hypomethylation is confirmed by multiple measurement methods.

High performance liquid chromatography (HPLC) methods have long been considered the gold standard assay for measuring global DNA methylation levels (Kurdyukov and Bullock, 2016), though the large amounts of DNA required and complexities around assay optimization have made the method impractical for larger scale studies (Lisanti et al., 2013). Assessing methylation levels using pyrosequencing of repetitive DNA elements such as LINE-1 and Alu has been used as an alternative approach (Crary-Dooley et al., 2017), demonstrating reasonable levels of correlation with HPLC methods (Lisanti et al., 2013).

Hypermethylation of CpG islands within genes, which tend to be unmethylated in normal cells, has been observed in cancer (Baylin and Jones, 2016). When occurring in the promoter regions of genes, this change is generally associated with transcriptional silencing (Weber et al., 2007; Lister et al., 2009), which can influence carcinogenesis by reducing expression of tumour suppressor genes (Ando et al., 2019). However, the ultimate impact of methylation on expression of any specific gene will be context dependent. For example, increased methylation may lead to increased expression levels in genes that are enriched for methyl-sensitive transcriptional repressors. As such, altered methylation patterns that are directly linked to reduced expression of tumour suppressor genes or increased expression of oncogenes are end-points of relevance for KC4.

Microarrays have been extensively used to measure methylation at hundreds of thousands of pre-selected CpG sites across the genome. Limitations of microarray approaches include the need for a priori knowledge of the genome or genomic features, and cross-hybridization between similar sequences (Hurd and Nelson, 2009). High-coverage next generation sequencing (NGS) does not suffer from such limitations and offers more comprehensive coverage of methylation across the genome, but NGS-based methods have relatively higher costs, do not often cover the same methylation sites reproducibly across all tested samples, and are associated with important challenges for downstream bioinformatic analyses (Arora and Tollefsbol, 2021).

Aging is correlated with changes in methylation (Fraga and Esteller, 2007), and these age-related changes have been hypothesized as drivers of chronic disease, including cancer (Issa, 1999). In fact, methylation changes occurring at specific sites across the genome, as measured in normal tissues, can be used as ‘epigenetic clocks’ to accurately predict an individual’s chronological age (Horvath, 2013; Bell et al., 2019). Those with greater epigenetically predicted ages than their chronological ages are hypothesized

to suffer from biological age acceleration, putting them at increased risk of age-related chronic diseases such as cancer (Ambatipudi et al., 2017; Chen et al., 2022). The association between accelerated epigenetic aging and cancer is, however, influenced by factors such as the type of epigenetic clock used, the study design, and the cancer type under consideration (Morales Berstein et al., 2022). Therefore, the relevance of accelerated epigenetic aging as an end-point for KC4 is context dependent.

4.2.2 Histone modification

As with DNA methylation, dysregulation of histone modifications can contribute to carcinogenesis by impacting gene expression (i.e. increased expression of oncogenes and repression of tumour suppressor genes). Histone modifications also play a key role in DDR by facilitating the access of repair proteins to DNA breaks (Hunt et al., 2013). There are four core histones (H3, H4, H2A and H2B), and transcriptional activation and repression are controlled through modifications of these histones, primarily methylation, acetylation, ubiquitination and phosphorylation (Zhao and Shilatifard, 2019; Vanzan et al., 2023). The aberrant histone modification patterns typically observed in cancer appear to be driven by mutations in the genes encoding proteins that directly regulate the epigenome (Plass et al., 2013; Yang et al., 2022), and it has been estimated that half of all cancers harbour mutations in these enzymes (Flavahan et al., 2017). These discoveries have stimulated research on the role of epigenome regulating genes as "epigenetic drivers" in carcinogenesis (Halaburkova et al., 2020).

Histone methylation modifies gene transcription by impacting chromatin architecture, recruiting transcription factors, interacting with initiation and elongation factors, and affecting RNA processing. However, the specific effect on transcription depends on the degree of methylation (mono-, di-, or trimethylated) and the specific location of the methylated arginine or lysine residues on a histone (Greer and Shi, 2012). Even then, there are examples where the same degree of methylation at a specific histone residue is associated with repressed transcription rather than transcriptional activation. SETDA1, a methyltransferase responsible for methylating H3, has been implicated in carcinogenesis at multiple cancer sites (Salz et al., 2015; Yang et al., 2020a; Kang et al., 2021; Wu et al., 2021), likely through increasing the expression of oncogenes.

Positively charged lysine histone residues interact with negatively charged nucleosomal DNA to produce a closed chromatin structure. Acetylation neutralizes the positive charge of lysine, contributing to a more open chromatin state, which enables transcription factor binding and promotes transcriptional activity (Greer and Shi, 2012; Nitsch et al., 2021). Global changes to acetylation, in particular acetylation of a lysine residue on H4, have been observed to occur early and accumulate as carcinogenesis progresses (Fraga et al., 2005).

Histone ubiquitination is also associated with modulation of transcriptional activity by affecting chromatic structure and signalling for other histone modifications (Zhao and Shilatifard, 2019). Ubiquitination commonly occurs on H2A and H2B, where H2A ubiquitination is typically associated with gene silencing, and H2B ubiquitination is typically associated with transcriptional activation (Cao and Yan, 2012). Reduced H2B ubiquitination, in particular, has been reported in cancer cells, but the mechanism by which this contributes to carcinogenesis is not well understood (Zhou et al., 2021).

By adding a negative charge to the histone, phosphorylation results in a more open chromatin conformation and is, therefore, associated with transcriptional activation. H3S10P (phosphorylation of the 10th serine residue of H3) has been observed to play a role in the initiation and progression of cancer and has been associated with poor prognosis of multiple cancers (Komar and Juszczynski, 2020). Overall,

histone modifications that have been directly linked to impaired DNA repair and/or with alterations in expression of genes tied to carcinogenesis are relevant end-points for KC4.

A wide range of methods is available for analyses of histone modifications, including site-specific analysis of histone marks, Chromatin Immunoprecipitation Sequencing (ChIP-Seq) and modified ChIP-based methods (Halabian et al., 2021). ChIP has been the preferred method for genome-wide characterization of histone modifications (O'Geen et al., 2011).

4.2.3 Non-coding RNAs

Non-coding RNAs (ncRNAs) are untranslated transcripts that regulate gene expression through interactions with DNA, mRNAs, and proteins (Esteller, 2011). Subcategories of ncRNAs include micro-RNAs (miRNAs), piwi-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), and long noncoding RNAs (lncRNAs). Multiple studies have revealed the role of ncRNAs in the regulation of cellular processes and signalling pathways, including those involved in tumour initiation and progression (Krutovskikh and Herceg, 2010; Yang et al., 2020b). In the context of cancer, miRNAs have been the most extensively studied. Given the involvement of miRNAs in regulation of proliferation, differentiation, and apoptosis, it is not surprising that miRNA dysregulation contributes to carcinogenesis. There is growing evidence, however, that other categories of ncRNAs may be involved in carcinogenesis as well (Huarte, 2015). For example, profiles of transcribed ultra-conserved noncoding RNAs (T-UCR), a subclass of lncRNAs, have been found to be altered in human cancers in a cancer type-specific manner (Calin et al., 2007). While dysregulated miRNAs with established regulatory roles in proliferation, differentiation, and/or apoptosis are relevant end-points for KC4, the relevance of other ncRNAs remains uncertain.

There are various well-established microarrays for measuring thousands of specific miRNAs and lncRNAs (Grillone et al., 2020). RNA sequencing has allowed for detection and quantification of all the various classes of ncRNAs, though quantification of lncRNAs may require hundreds of read counts due to the relatively low abundance of lncRNAs.

4.3 Assessing the relevance of end-points in surrogate tissues

Many epigenetic cancer studies have relied on human tumour tissues in which epigenetic changes are ubiquitous. While their ubiquitous nature may indicate that epigenetic changes are drivers of carcinogenesis, like other types of molecular changes detectable in tumour tissues, these alterations could also be a result of the cancer itself rather than its cause (i.e. reverse causality). Moreover, some types of human tissues, such as brain, are highly inaccessible in living beings for downstream epigenetic profiling. Alternative sources of data are, hence, needed. Several studies have demonstrated that epigenetic changes associated with cancer risk or with carcinogenic exposures can be detected in non-malignant forms of the target tissues of interest (such as normal tissues and negative surgical margins) (Woo et al., 2018) or in surrogate tissues (e.g. peripheral blood and saliva) (Ambatipudi et al., 2016a; Awada et al., 2021), paving the way for the discovery of novel biomarkers of exposure and risk-stratification (van der Pol and Mouliere, 2019; Brito-Rocha et al., 2023).

Though epigenetic signatures are cell- and tissue-specific, there is evidence supporting the use of surrogate tissues as proxies for tissue-specific epigenetic impacts. For example, imprinted genes tend to exhibit similar methylation levels across various tissue types within the same organism. This is partly

because these genes escape the wave of demethylation that resets the epigenome during embryogenesis (Zeng and Chen, 2019). Similarly, genes known as metastable epialleles exhibit low intra-individual methylation differences (i.e. across tissues within the same subject) but high inter-individual differences (i.e. functioning as epigenetic SNPs) due to epigenetic modifications that are established during early development (Van Baak et al., 2018). The earlier that methylation of metastable epialleles occurs during embryonic development, the less variation that occurs across different tissue types in the organism. As such, methylation levels of imprinted genes and metastable epialleles that are similarly deregulated in surrogate and target tissues could reflect an *in utero* origin. Imprinted genes and metastable epialleles can act as sensitive molecular sensors of exposure and play key roles in carcinogenesis (Van Baak et al., 2018).

Some epigenetic aberrations could still show inter-tissue correlation even though they do not relate to the epigenetic clock or to specific gene families. Conceptually, some epigenetic changes occur in progenitor cells, especially during embryogenesis, hence, spreading over several cell types and cell generations. These epigenetic alterations may result in constitutional "epimutations" throughout the body that are not simply determined by tissue-specific epigenetic patterns (Herceg et al., 2022; Hitchins, 2015).

It has been observed that several DNA methylation sites show substantial blood-brain correlation (Edgar et al., 2017; Nishitani et al., 2023). For such CpG sites to be informative, however, methylation levels at these sites need to also vary across individuals. Approximately 10% of CpGs on the HM450 array were found to fulfil this criterion (correlation threshold ranging between 0.3 to 0.4) (Edgar et al., 2017). A growing number of databases across several tissue types, including saliva and buccal samples (Nishitani et al., 2023), will help provide a basis for better interpretation of DNA methylation results when measured in surrogate tissues. Overall, DNA methylation alterations detectable in surrogate tissues are relevant end-points for KC4 when they occur in imprinted genes and/or have been correlated with methylation alterations at the cancer site of interest.

4.4 Interpretation of epigenomic result data

Despite the remarkable progress in epigenetics and a growing recognition of the importance of epigenetic mechanisms in carcinogenesis, challenges remain related to the incorporation of epigenetic data into carcinogen identification and evaluation. One of the major challenges is the definition of "normal" epigenomes for all tissue and cell lineages (Herceg et al., 2013; Stueve et al., 2016; Tonge and Gant, 2016). Epigenome patterns are highly tissue- and cell type-specific, with significant variability within and between tissue types, populations, and age categories, making defining reference epigenomes crucial (Herceg et al., 2013; Marczylo et al., 2016). These challenges have been partly addressed through methods for the correction of cell-composition effects (Houseman et al., 2016; McGregor et al., 2016) and the creation of high resolution cell type-specific epigenomic maps, including the Roadmap Epigenomics Program (Kundaje et al., 2015; Satterlee et al., 2019), the BLUEPRINT projects (Bock et al., 2016; Fernández et al., 2016), ENCODE project (Snyder et al., 2020), the 4D Nucleome Project (Dekker et al., 2017), and the International Human Epigenome Consortium (Bujold et al., 2016). In addition, the Toxicant Exposures and Responses by Genomic and Epigenomic Regulators of Transcription (TaRGET) program by the National Institute of Environmental Health Sciences offers a valuable foundation for high-resolution epigenomic, chromatin accessibility, and transcriptomic data generated via experimental *in vivo* models (Wang et al., 2018). These resources permit an enhanced understanding of the influence of established and suspected environmental carcinogens on the epigenome, as well as an improved evaluation of epigenetic marks in surrogate tissues.

Recent emergence of powerful technologies in epigenomics, including application of next-generation bead arrays and massively parallel sequencing technologies, has tremendously accelerated epigenomic research and opened new perspectives for investigating epigenomic deregulation in carcinogen testing. The wide range of methods for epigenome profiling differ in their sensitivity, throughput, and coverage (Umer and Herceg, 2013). The method of choice is guided by the type of samples, desired level of information, and cost. The most common methods include DNA methylation arrays (Pidsley et al., 2016), reduced-representation bisulfite sequencing (RRBS) (Meissner et al., 2005), whole-genome methylome analysis by bisulfite sequencing (Urich, Nery et al., 2015) and histone mark analysis by ChIP-seq (Schmidl et al., 2015). Epigenome-wide methods provide high-coverage and high-resolution information for epigenetic marks, and in turn they do not require a priori knowledge of potential genomic targets, thus facilitating the discovery of new alterations (including "signatures") in the epigenome. However certain epigenome-wide assays (such as NGS-based RRBS assay) may not be sufficiently sensitive (often due to missing values and insufficient read depth) for detecting subtle changes induced by environmental exposures in samples with heterogeneous cell populations.

Some of these issues could be addressed by the development of single-cell epigenomics assays that permit generation of state-resolved epigenome maps, thereby enhancing our ability to interpret the impact of exposures on the epigenome and cellular phenotypes in heterogeneous tissues (Gaulton et al., 2023). Recent advances in tissue spatial technologies, including spatial epigenomics/transcriptomics, have generated powerful tools for high-resolution profiling of epigenomic states in the tissue context (Zhang, Deng et al., 2023). One issue with most of these methods is that they are not cost-effective nor amenable to miniaturization for a simultaneous screening of large libraries of compounds in the context of carcinogen testing (Chung and Herceg, 2020). However, with further technological advances and improvement in cost–effectiveness, it should be possible to develop scalable epigenomic screens (Rasoulpour et al., 2011).

Another challenge associated with interpretation of epigenomic data is that phenotypic traits associated with epigenome alterations induced by exposures can become visible only years after the initial exposure. This is a particular issue when the exposure occurs *in utero*, a putative window of vulnerability that is commonly incompatible with human testing (Ghantous et al., 2015; Barouki et al., 2018; Sexton-Oates et al., 2020).

The different types of epigenetic modifications are intimately connected and typically operate in a self-reinforcing manner in the regulation of gene transcription and other cellular processes (Vaissière et al., 2008). As such, observing multiple epigenetic modifications that are consistent in effect should be considered as highly relevant evidence for KC4.

4.5 Persistence over time of epigenetic end-points

There has been relatively little work to examine the reversibility or irreversibility of epigenetic modifications over time. Persistence of these modifications may have implications for the ultimate impact of these modifications on cancer development (i.e. short-lived changes may be less likely to influence carcinogenesis). As such, studies showing persistence of epigenetic alterations over time may add informativeness to KC4.

Recent studies have revealed several robust epigenetic markers of environmental/lifestyle factors, including DNA methylation markers of tobacco smoking (Ambatipudi et al., 2016a; Joubert et al., 2016), alcohol consumption, and body mass index (McCartney et al., 2018), and these markers have been proposed

as alternatives to self-reported measurements. In a study of 92 participants with blood samples collected six years apart, 17% of the CpG sites measured by Illumina's Infinium HumanMethylation450 (HM450) array were found to have an interclass correlation coefficient (ICC) > 0.50 (Flanagan et al., 2015). Four CpG sites previously associated with smoking were found to be significantly associated with smoking in this study at both time points. In another larger scale study examining 37 CpG sites associated with current smoking, a higher degree of correlation was observed among these CpG sites in DNA from repeat blood samples collected approximately six or 10 years apart than the rest of the CpG sites captured by HM450 (Shah et al., 2014).

It is possible that the stability of epigenetic modifications can be traced back to underlying mutations in epigenetic enzymes that are responsible for continuously writing or editing these epigenetic modifications. In mammals, DNA methylation is catalysed by enzymes in the DNA methyltransferase (DNMT) family. Mutations in DNMTs, especially DNMT3A, are elevated in many tumours, particularly in acute myeloid leukaemia (AML) in which DNMT3A mutations are observed in ~20% of cases (Han et al., 2019; Park et al., 2020). Mutations in the DNA hydroxymethylase TET2, which catalyses the conversion of 5-methyl-cytosine to 5-hydroxymethyl-cytosine, have also been observed in various diseases, especially AML and myelodysplastic syndromes (Han et al., 2019). As such, mutations that disrupt the function of epigenetic enzymes are relevant end-points for KC4.

Other types of epigenetic aberrations could have systemic effects on the organism even though they occur across the lifetime rather than at a specific time window. For example, a gradual accumulation of epigenetic changes over time (known as epigenetic drift), as induced by environmental hits and/or stochastic events, may show remarkable consistency across human tissues and contribute to cancer risk. Epigenetic drift can be captured, at least partly, by the epigenetic clock (Horvath & Raj, 2018; Zhang et al., 2019a). Beyond its functioning as a clock, DNA methylation measures of aging have also been used to track the accumulation of mutations, clinical outcomes, and cancer risk (Ambatipudi et al., 2017; Yu et al., 2020b). Altogether, metrics of epigenetic drift represent powerful toolkits for epidemiologic research to understand the mechanisms underlying the effects of biological aging, lifetime exposure, and/or stochastic events on cancer development (Herceg et al., 2022).

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5. Key Characteristic 5: Induces oxidative stress

Emily Watkins and Eugenia Dogliotti

5.1 Introduction

Smith et al., (2016; 2020b) described examples of relevant end-points for the key characteristic of carcinogens "induces oxidative stress" including "Oxygen radicals, oxidative stress, oxidative damage to macromolecules (e.g. DNA, lipids)". Oxidative stress occurs when there is an imbalance between antioxidants and oxidants: reactive oxygen species (ROS)/ reactive nitrogen species (RNS), favouring the latter, which leads to disruption of redox signalling and/or molecular damage (Sies and Jones, 2007; Smith et al., 2016). Elevated ROS/RNS can cause DNA damage, leading to genotoxicity (KC2) and altering DNA repair (KC3); they are also associated with chronic inflammation (KC6), and can alter cell proliferation (KC10) (Smith et al., 2020b). These effects can be associated with initiation or promotion of carcinogenesis (Valko et al., 2007; Guyton et al., 2018; Hayes et al., 2020). Numerous sources result in the formation of ROS and RNS, including endogenous sources such as metabolism, immune response and inflammation, and exogenous sources such as radiation, chemicals, and drugs (Valko et al., 2007; Kryston et al., 2011; Di Meo et al., 2016; Hayes et al., 2020). As such, end-points associated with oxidative stress include assessment of the redox balance or markers of oxidative damage to DNA, lipids, and proteins. In the previous *IARC Monographs* Volumes 112 – 134, oxidative stress was evaluated as "consistent and coherent" for 23 agents; 21 of these agents also exhibited "consistent and coherent" evidence for one or more other KCs (See Annex 1). The *Monographs* Preamble recommends that, because non-carcinogens can also induce oxidative stress, this key characteristic should be interpreted with caution unless it is found in combination with other KCs. This section describes some of the key end-points and considerations on how to assess their informativeness. This section does not provide a comprehensive background for all end-points that could be associated with oxidative stress or all the different assessment techniques, and additional information can be found in other peer-reviewed publications (Di Meo et al., 2016; Marrocco et al., 2017; Katerji et al., 2019; Hayes et al., 2020; Nilsson and Liu, 2020; Chao et al., 2021; Pisoschi et al., 2021; Murphy et al., 2022).

5.2 Relevance of end-points

5.2.1 Oxidative Damage to Nucleic Acids

Oxidative damages caused by ROS to DNA, lipids, and proteins are considered end-points of relevance to KC5, because they serve as indirect measurements of oxidative stress (Katerji et al., 2019) (see Table 5). An adverse outcome pathway (AOP) has been developed (Cho et al., 2022) that links oxidative damage to DNA to two adverse outcomes, gene mutations and chromosomal aberrations. DNA strand breaks generated during oxidative stress can be identified using the comet assay (Katerji et al., 2019). Comet assays modified with enzymes, such as endonuclease III to recognize oxidised pyrimidines, or formamidopyrimidine DNA glycosylase (FPG) or human 8-oxoguanine DNA glycosylase 1 (hOGG1) to detect oxidised purines, can be used specifically to identify strand breaks resulting from oxidative damage (Collins, 2009). FPG is the most widely used modification (Azqueta et al., 2019b). The Comet assay represents primary an end-point relevant for the KC2 "is genotoxic," (see Section 2.2, Chapter 2, Part I); however, the inclusion of specific

modifications to the assay, such as the inclusion of FPG and endonuclease III enzymes, will feature the assay as representing an end-point for KC5 "induces oxidative stress".

Table 5. End-points relevant for KC5: "induces oxidative stress"

Category	End-point	Relevance	Comment	Reference
Oxidative damage to DNA	DNA single strand breaks (identified via FPG, hOGG1 or Endo III modified comet assays) Oxidative clustered DNA lesions 8-oxoGua 8-oxodG TG	↑ can be associated with oxidative stress	Adverse outcome pathway links oxidative damage to DNA to mutations and chromosomal aberrations. Oxidative damage to DNA is therefore considered to be the most relevant end-point for KC5. DNA damage will reduce with increasing time post exposure due to DNA repair, cell turnover and apoptosis. The exact source of biomarkers (eg. 8-oxoGua/8-oxodG) is unclear when measured in urine. Methods should include techniques to reduce artefactual damage to DNA Different methods of damage identification are not directly comparable	Evans et al. (2010); Kryston et al. (2011); Collins (2014); Azqueta et al. (2019b); Katerji et al. (2019); Cho et al. (2022)
Oxidative damage to other nucleic acids	Mitochondrial DNA damage – increased strand breaks and copy number Precursor 2'-deoxyribonucleotide and ribonucleotide pool DNA damage RNA damage – 8-oxoGua	↑ can be associated with oxidative stress	Underpinning mechanism between mitochondrial DNA copy number; association with cancer needs further investigation. These end-points are not currently commonly reported. Advancements in measurement techniques may improve evidence availability and understanding of mechanisms in the future.	Kong & Lin (2010); Hofmann et al. (2014); Chao et al. (2021)
Oxidative damage to lipids	MDA 4-HNE 8-iso-PGF2α CD LOOH	↑ can be associated with oxidative stress	High relevance for KC5. HPLC or mass spectrometry analysis for MDA is more specific and reliable than the TBARS method. Artifact of MDA may develop over time Mass spectroscopy techniques currently considered gold standard measurement of 8-iso-PGF2α	Tsikas (2017); Ito et al. (2019); Katerji et al. (2019); Mas-Bargues et al. (2021)
Oxidative damage to proteins	AOPP AGE PC	↑ can be associated with oxidative stress	High relevance for KC5. When HOCl is primary oxidizing species, measurement of protein oxidation is recommended.	Shacter (2000); Dalle-Donne et al. (2003); Fedorova et al. (2014); Katerji et al. (2019)

Table 5. End-points relevant for KC5: "induces oxidative stress"

Category	End-point	Relevance	Comment	Reference
ROS / RNS	H_2O_2 OH^- ROO^- O_2^- R-OOH	↑ can be associated with oxidative stress	Should be interpreted in consideration of antioxidant findings, presence of ROS does not necessarily result in oxidative stress. ROS may be elevated in cell culture	Halliwell (2014) ; Katerji et al. (2019); Murphy et al. (2022)
Antioxidants	SOD CAT GPx GST Nrf2 GSH GSSG Vitamin A Vitamin C Vitamin E TAC	↓ can be associated with loss of protective capacity or minimal ROS/RNS stimulus ↑ can be associated with an ROS/RNS stimulus and a protective mechanism against oxidative stress	Should be interpreted in consideration of ROS/RNS findings. Multiple TAC measurement techniques. Comparison between them is not recommended due to varying levels of agreement.	Weydert & Cullen (2010); Lee et al. (2017); Katerji et al. (2019)

8-oxoGua, 8-oxoguanine; 8-oxodG, 8-Hydroxy-2'-deoxyguanosine; 4-HNE, 4-hydroxynonenal; 8-iso-PGF2α, 8-isoprostaglandin F2α; AGE, advanced glycation end products; AOPP, advanced oxidative protein products; CAT, catalase; CD, conjugated dienes; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, glutathione disulphide; GST, glutathione-s-transferase; H2O2, hydrogen peroxide; HOCl, hypochlorous acid; LOOH, lipid hydroperoxides; MDA, malondialdehyde; Nrf2, nuclear factor erythroid 2-related factor 2; O2-, superoxide molecule; OH-, hydroxyl radicals; PC, protein carbonyl; RNS, reactive nitrogen species; ROO-, peroxyl radicals; R-OOH, hydroperoxides ; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant status; TBARS, thiobarbituric acid-reactive substances; TG, thymidine glycol. Note: these are some key examples and not an exhaustive list of end-points.

Modified comet assays together with specialised techniques for double-strand break (DSB) detection (discussed in more detail in Cahapter 1) can also be used to measure oxidative clustered DNA lesions (OCDL) (Sutherland et al., 2003; Georgakilas, 2008; Georgakilas et al., 2010). OCDL refers to the presence of numerous occurrences of oxidative damage to DNA, such as base modifications, strand breaks, and modifications to the sugar backbone, all within a short DNA segment of up to two helical turns (Georgakilas, 2008; Kryston et al., 2011). When two nearby single-strand breaks (SSBs) arise on opposing strands, it can result in the formation of a DSB, which is recognized as one of the most serious forms of DNA damage. Repair of OCDL has been noted to be more challenging than single DNA lesion repair, suggesting that OCDL are highly significant for genomic instability and mutagenesis (Georgakilas, 2008; Kryston et al., 2011; Georgakilas et al., 2013).

8-oxoguanine (8-oxoGua), also referred to as 8-oxo-7,8-dihydroguanine, 8-hydroxyguanine, 8-oxoG, or 8-OHG, is the guanine oxidation product at the C8 position and is one of the most common products of oxidative damage to DNA. It is therefore frequently used as an indirect biomarker of oxidative stress (Kryston et al., 2011; Chiorcea-Paquim, 2022). 8-oxo-2′-deoxyguanosine (8-oxodG), also known as 8-hydroxy-2′-deoxyguanosine, 8-OHdG, or 8-oxo-7,8-dihydro-2′-deoxyguanosine, results from oxidative damage to 2′-deoxyguanosine, and is consequently another relevant end-point associated with oxidative damage to DNA (Wu et al., 2004; Katerji et al., 2019; Qing et al., 2019). Terminology for these biomarkers can vary across publications, and it is recommended to use the same terminology during KC5 evaluations, with advice outlined by Cooke et al., (2010) and Chao et al., (2021).

While 8-oxoGua and 8-oxodG are the primary lesions studied (Cooke et al., 2002; Valavanidis et al, 2009), thymidine glycol (TG) may be a more relevant end-point for oxidative damage to DNA, as thymidine is not incorporated into RNA (Katerji et al., 2019). TG is the oxidation product of damage to thymidine caused by hydroxyl radicals (Lowe et al., 2013). Both 8-oxodG and TG levels are selected as end-points of higher relevance to oxidative stress and have been identified in association with cancer (Kryston et al., 2011).

Damage to other nucleic acids has become a new focus in oxidative stress research. Other nucleic acids in this instance refers to mitochondrial DNA, precursor 2′-deoxyribonucleotide and ribonucleotide pools, and RNA (Chao et al., 2021). Mitochondrial DNA (mtDNA) is vulnerable to oxidatively generated damage, because of its proximity to sources of ROS production, fewer protective histones, and lack of some DNA repair mechanisms (Yakes and Van Houten, 1997; Muftuoglu et al., 2014; Chao et al., 2021). It has been shown that oxidative stress can lead to the degradation of mtDNA by inducing strand breaks and abasic sites, and that inhibition of base excision repair enhances these effects (Shokolenko et al., 2009). Increased degradation reduces mtDNA content, which is reported to increase metastasis (Chandra and Singh, 2011). However, it should be noted that the main cause of mtDNA mutations is debated, with both oxidative stress and spontaneous replication errors as suggested contributors (Liu et al., 2003; Kauppila et al., 2018; Filograna et al., 2021). Both mtDNA mutations and alteration of mtDNA copy number have been associated with numerous cancer types (Chatterjee, Mambo and Sidransky, 2006; Chandra and Singh, 2011). Both increased and decreased mtDNA copy number have been reported to be associated with cancer (Chatterjee, Dasgupta and Sidransky, 2011; Hofmann et al., 2014; Castellani et al., 2020), with mutation location, cancer type and progression potentially important in the orientation of the association (Castellani et al., 2020). It is hypothesized that mtDNA copy number may increase as a compensation for mtDNA damage. Studies suggesting a correlation between the two have employed prospective study designs to avoid potential bias from reverse causation (Hosgood et al., 2010; Lynch et al., 2011; Hofmann et al., 2014; Kim et al., 2015). Analysis of mtDNA can be performed using PCR, as outlined by Chao et al. (2021) with consideration of contributing factors discussed by Picard (2021). While mtDNA copy number may become an informative end-point, further research is required into the underpinning mechanisms regarding its association with carcinogenesis (Castellani et al., 2020). Inclusion of mtDNA copy number as a relevant end-point to KC5 could be appropriate with careful consideration regarding study design and mechanistic pathways involved.

Precursor nucleic acid and ribonucleotide pools are also targets for ROS due to their structure, location, and reduced repair capacity in comparison to DNA (Chao et al., 2021). Oxidation of nucleic acid within these pools can result in misincorporation of oxidised nucleobases into both nuclear and mitochondrial DNA (Smith et al., 2020a), consequently leading to strand breaks, cell death, and mutations (Chao et al., 2021). Sanitation of pools by enzymes such as MTH1 can prevent misincorporation (Rai, 2010; Rai and Sobol, 2019). Primary focus has been on the oxidation of dGTP resulting in misincorporation of 8-oxodGTP, although currently this does not commonly feature in assessments of oxidative stress generation.

RNA is more vulnerable to oxidatively generated damage than are DNA, lipids, and proteins (Kong and Lin, 2010; Chao et al., 2021). 8-oxoGua is the most prevalent oxidized base in RNA (Kong and Lin, 2010). Current evidence indicates that mRNAs and rRNA are more susceptible than DNA to oxidatively generated damage (Kong and Lin, 2010; Chao et al., 2021). The detailed mechanisms for repair of oxidative damage to RNA are unclear, and pathways commonly seen in DNA repair have not been reported in RNA; however, it has been suggested that oxidative demethylases are involved (Kong and Lin, 2010; Yan and Zaher, 2019; Chao et al., 2021). It has also been noted that oxidative damage to RNA may alter protein synthesis (Kong and Lin, 2010; Yan and Zaher, 2019). Research into the consequences of oxidative damage to RNA has primarily focused on degenerative neurological disorders (Poulsen et al., 2012), although a link between

oxidative damage to RNS and tumour progression has been suggested from in vitro models only (D'Souza et al., 2020; Li et al., 2020b)

Oxidative damage to these other types of nucleic acid has been seldom reported due to the large sample quantity required and the available analytical techniques. However, as sensitivity of analysis methods improves and advancements are made in genome mapping and sequencing in relation to oxidative damage (Chao et al., 2021), it is suggested that these end-points could be considered relevant to KC5 in the future.

5.2.2 Oxidative damage to proteins and lipids

Oxidative stress can cause lipid peroxidation, damaging lipoproteins and membrane lipid bilayers (Katerji et al., 2019; Pisoschi et al., 2021). Relevant end-points for lipid peroxidation may be concentrations of lipid peroxides themselves or end products of lipid peroxidation (Ito et al., 2019). Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are commonly determined aldehydes, with MDA the most frequently used to measure oxidative stress (Marrocco et al., 2017; Katerji et al., 2019; Mas-Bargues et al., 2021). MDA is mutagenic, inducing DNA insertions, deletions, and base pair substitutions (Niedernhofer *et al.*, 2003; Nilsson and Liu, 2020). Other key end-points relevant to lipid peroxidation are 8-isoprostaglandin F2α (8-iso-PGF2α), conjugated dienes (CD), and lipid hydroperoxides (LOOH) (Katerji et al., 2019).

Oxidative damage to proteins can be detected via multiple end-points, including advanced oxidative protein products (AOPP), advanced glycation end products (AGE), and protein carbonyls (PCs). Shacter (2000) and Dalle-Donne et al., (2003) provide a considered discussion of the advantages and disadvantages of using oxidative damage to proteins as a marker of oxidative stress, highlighting that due to the unique biological function of proteins, modifications can provide information on the type of oxidant involved and the functional consequences. Recommendations are also made for the use of protein markers when hypochlorous acid is the predominant ROS, as this ROS results in limited modifications to DNA or lipids. PCs are the most commonly used marker of oxidative modification of proteins (Dalle-Donne et al., 2003). PCs are generated due to the oxidation of amino acid side chains and cleavage of protein backbones by ROS (Fedorova et al., 2014; Katerji et al., 2019). However, PCs can be generated for a range of ROS and therefore do not provide information regarding the exact source of the oxidative stress, although detection of PCs may be reflective of more severe oxidative stress (Shacter, 2000; Dalle-Donne et al., 2003).

5.2.3 Antioxidant and ROS balance

ROS and RNS production can be assessed directly by measuring the levels of hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), peroxyl radicals (ROO^-), superoxide molecules (O_2^-), and hydroperoxides (R-OOH) (see Table 5). Antioxidants play a key role in reducing oxidative stress via limiting the formation of ROS/RNS, detoxifying the reactive metabolites generated, aiding in repair of oxidatively damaged DNA, proteins or lipids, or exploiting adaptive mechanisms (Gaté et al., 1999; Halliwell, 2007; Pisoschi et al., 2021). Both enzymatic and nonenzymatic antioxidants act as antioxidant markers, although enzyme antioxidants play the main role in antioxidant defence (Sies, 2015). Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione s-transferase, all considered relevant end-points to KC5. Gene expression encoding for enzymatic antioxidants can be regulated by Nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 is a measurable transcription factor and a relevant end-point for KC5, that when stimulated by oxidative stress can stabilize and accumulate, and can upregulate genes which result in increased expression of antioxidant enzymes (Osburn and Kensler, 2008;

Nguyen et al., 2009; Lee and Hu, 2020). Non-enzymatic antioxidants are also relevant end-points to KC5, including glutathione – reduced as GSH or oxidised as glutathione disulphide (GSSG), retinoids and carotenoids (Vitamin A), ascorbic acid (Vitamin C), and α-tocopherol (Vitamin E) (Valko et al., 2007; Pizzino et al., 2017; Katerji et al., 2019). Total antioxidant status (TAC) can also be determined, providing a less time-consuming assessment of antioxidant status, as individual measurement of multiple antioxidant markers is not required (Katerji et al., 2019), although it has been highlighted that this may not provide helpful information on the state of the organism (Sies, 2015) and therefore could be considered a less-relevant end-point for KC5.

5.3 Assessing the relevance of end-points in different test systems

5.3.1 Oxidative Damage to DNA

As discussed also in Chapters 2 and 3, the comet assay is frequently used in biomonitoring studies to measure oxidative damage to DNA in exposed humans. Oxidative damage to DNA from *in vitro* or animal models is also relevant for KC5 evaluation, although extrapolation from animal to human results has not been systematically reviewed. A suspension of single cells is required for comet assay performance, with peripheral blood mononuclear (PBMN) cells commonly used for human biomonitoring (Collins, 2014; Collins et al., 2023). Frozen whole blood and frozen isolated leukocytes samples can be used for analysis (Collins, 2014; Collins et al., 2023). Due to DNA repair, cell turnover, damaged cell apoptosis or necrosis, DNA damage will be reduced with increasing time post exposure (Albertini et al., 2000; Valverde and Rojas, 2009). Data from exposed humans may therefore reflect the response to DNA damage, as described in KC3 (Chapter 3). Optimal sample timing is suggested as either during chronic exposure or within a few hours after exposure ceases (Albertini et al., 2000; Valverde and Rojas, 2009). Control of inter-assay variation should also be considered, with studies reporting incorporation of samples from different exposure groups into the same experiment or use of assay control samples in biomonitoring to standardise results (Azqueta et al., 2019b; Collins et al., 2023) viewed as more informative. Collins (2014), Azqueta et al. (2019b) and Collins et al. (2023) provide a detailed review of the comet assay method with protocol suggestions for improved validity, while Sutherland et al. (2003) and Georgakilas et al. (2010) focus on OCDL measurement techniques.

8-oxoGua, 8-oxodG and TG, can be assessed with high performance liquid chromatography (HPLC), liquid chromatography/tandem mass spectrometry (LC-MS/MS), immunohistochemical analysis and enzyme assays (Guetens et al., 2002; Cooke et al., 2008; Kryston et al., 2011; Katerji et al., 2019; Qing et al., 2019; Chao et al., 2021). Sample types can include blood, urine and tissue. The source of urinary 8-oxoGua/8-oxodG has been addressed in several studies (Chao et al., 2008; Cooke et al., 2009; Evans et al., 2010). Regarding 8-oxoGua, it has been suggested that its presence in urine primarily stems from its cleavage by OGG1 through the process of base excision repair, while the nucleotide pool has been proposed as a significant contributor to extracellular 8-oxodG levels (Cooke et al., 2002, 2009; Chiorcea-Paquim, 2022). In addition, the levels of 8-oxoGua/8oxodG in urine may be affected by several confounding factors like smoking habits, age, diet and sex (Zanolin et al., 2015). Accounting for these confounding factors is crucial when interpreting results. Analysing oxidative damage in nuclear DNA leaves no doubt that oxidative stress has altered the cellular genetic material (Wu et al., 2004). Urine samples may be corrected in different ways, for example corrected for creatinine or expressed relative to body weight and urine volume, hampering comparison of results (Cooke et al., 2002). The length of time that samples were stored should be considered, with urine samples levels of 8-oxodG measured by mass spectrometry suggested to be stable up to 1 year

when frozen at −80 °C, but not over a 2-year period (Cooke et al., 2002). Alternatively, 8-oxodG has also been reported to be stable across a 15-year time period when measured from urine stored at −20 °C via HPLC (Loft et al., 2006). Mass spectrometric methods using isotopically labelled internal standards may control for sample loss (Cooke et al., 2002). The measurement of TG may act as a more appropriate end-point in tissues because TG is not rapidly excised and excreted (Katerji et al., 2019). As a result of DNA extraction and preparation, artefactual DNA damage may occur, with rates differing between methods (Wu et al., 2004; Valavanidis et al., 2009; Kryston et al., 2011). Techniques that reduce artefactual DNA have been reported on previously (Ravanat et al., 2002; Chao et al., (2008, 2021). It is suggested that assays and sample type should be considered when evaluating if an agent induces oxidative stress. Despite the use of both 8-oxodG and DNA strand breaks identified via the modified comet assay as markers of oxidative damage to DNA, strong association between the methods has not been noted, and conflicting findings are sometimes reported (Gedik and Collins, 2005; Watters et al., 2009), complicating comparison of findings between assays.

5.3.2 Oxidative Damage to Proteins and Lipids

Determination of MDA by HPLC or mass spectrometry provides more specific, reproducible, and reliable findings in comparison to the thiobarbituric acid-reactive substances (TBARS) method (Marrocco et al., 2017; Katerji et al., 2019; Mas-Bargues et al., 2021). MDA can be measured from a range of samples, including serum, plasma, and cultured cells and tissues (Mas-Bargues et al., 2021). Variations in plasma and serum MDA because of storage time indicate that artefactual formation of MDA over time may occur. Therefore, samples should be analysed as soon as possible (Tsikas et al., 2016). Studies reporting variations in sample analysis time, for instance, pre- and post- agent exposure samples taken months apart yet analysed together at the end of a project, should be interpreted with caution (Tsikas et al., 2016). However, storage time did not affect results from urine samples (Tsikas, 2017). Anticoagulants used for plasma storage should also be reviewed because EDTA may also increase ex vivo MDA; thus, the use of heparinized plasma samples or serum samples may be advised (Tsikas et al., 2016; Mas-Bargues et al., 2021). Timing of sample collection within a day for MDA and 8-iso-PGF2α does not seem of paramount importance, with no circadian rhythm apparent (Tsikas et al., 2016).

8-iso-PGF2α are unaffected by dietary lipids and can therefore provide an estimate of total body production from samples of biological fluid or exhaled breath condensate, whereas tissue sample analysis can quantify localized lipid peroxidation (Richelle et al., 2001; Marrocco et al., 2017). Mass spectroscopy techniques are the gold standard measurement of 8-iso-PGF2α (Mas-Bargues et al., 2021), although radioimmunoassay and enzyme linked assays are available (Graille et al., 2020).

Protein carbonyls can be analysed via numerous methods including HPLC, gel electrophoresis, mass spectrometry, and enzyme linked immunosorbent assays (Dalle-Donne et al., 2003; Fedorova et al., 2014). PC can be measured from a range of samples, such as lung aspirates, plasma, serum, tissue, and cell culture extracts (Dalle-Donne et al., 2003). Degradation of oxidised proteins occurs between hours and a day, compared to the detoxification of lipid peroxidation products, which can occur within minutes (Dalle-Donne et al., 2003). Timing of sample collection should be considered for PC, with peak levels noted 4 h post cessation of exercise, and elevations present from 30 min to 8 h post (Michailidis et al., 2007).

5.3.3 Antioxidant and ROS balance

Numerous methods exist for measuring end-points associated with the redox balance, including, but not limited to, colorimetric assays, activity gels, electron paramagnetic resonance, immunohistochemical, and immunofluorescence analysis (Weydert and Cullen, 2010; Mrakic-Sposta et al., 2012; Lee et al., 2017; Murphy et al., 2022). There are multiple TAC measurement techniques with varying levels of agreement among them (Lee et al., 2017); thus, comparison of values performed by differing techniques would not be advised. A range of sample types can be relevant, depending on the marker and method of interest; these may include urine, saliva, whole blood, plasma, serum, cultured cells, and tissue (Michailidis et al., 2007; Weydert and Cullen, 2010; Lee et al., 2017). Cell culture conditions promote oxidative stress, which may alter proliferation, cause adaptation, or lead to senescence or cell death. ROS measured in cell cultures may therefore be elevated in comparison to *in vivo* scenarios (Halliwell, 2014; Murphy et al., 2022). Good practice recommendations and guidance for measurement of ROS are outlined by Halliwell (2014) and Murphy et al. (2022). Consequently, measurement of ROS from *in vitro* systems should be carefully evaluated to determine end-point relevance to identify if an agent exhibits KC5.

Identification of antioxidant end-points may be affected by sample timing. For instance, following physical activity in humans, CAT was greatest one hour post activity, whereas TAC, GSH and GSSG were greatest two hours post activity (Michailidis et al., 2007). In an animal model, exposure of mice to e-cigarette aerosols resulted in reduced GSH:GSSG ratio and GPx activity after 2 weeks; however, 4 weeks of exposure were required for reduced CAT to also be detected (Alzoubi et al., 2022). Caution should therefore be applied when comparing studies with different sampling times, and if limited time points are available, then the lack of change in antioxidant end-points should not necessarily be interpreted as no antioxidant response. ROS typically have short lifespans of nanoseconds to minutes (Katerji et al., 2019; Murphy et al., 2022) and consequently may not accumulate sufficiently to enable detection (Ito et al., 2019).

5.4 Interpretation of results within the same database

Given the wide variety of end-points, detection techniques, and mechanistic pathways associated with KC5, the presence of mixed results is likely, and the interpretation of the findings between end-points may be difficult (Katerji et al., 2019). Previous monographs on agents with substantial evidence for oxidative stress typically demonstrated significant alterations in numerous end-points, across multiple tissue sites having clear association with the agent. Most of this evidence was provided from experimental systems and human primary cells and tissues.

For 11 agents, from *IARC Monographs* Volumes 112 – 134, the evidence for oxidative stress was considered relevant to humans, or it was considered as "consistent and coherent" for KC5 in exposed humans (See Annex 1):

vol 112: Diazinon, glyphosate, malathion
vol 113:2,4-Dichlorophenoxyacetic acid, 4,4'-dichlorodiphenyltrichloroethane, lindane
vol 115: N,N-Dimethylformamide, tetrabromobisphenol A
vol 117: Pentachlorophenol
vol 120: Benzene
vol 132: Occupational exposure as firefighter

Due to the multifaceted causes of oxidative stress in humans and potential negative health consequences, controlled human trials with high quality study designs can be scarce.

In scenarios of varied results across several oxidative stress end-points, higher relevance should be placed on direct markers of oxidative damage to DNA owing to its stronger association with carcinogenesis. However, it should be noted that the effect of DNA lesions in relation to the carcinogenic process is dependent on various factors, including the gene location and the effectiveness of DNA repair mechanisms. DNA lesions may be removed before DNA replication, therefore oxidative damage to DNA, as detected by the modified comet assay, does not necessarily provide a direct link to cancer prevalence (Azqueta et al., 2019b). End-points with higher relevance may also include oxidative damage to proteins or lipids.

Interpretation of antioxidant marker levels can be complex; for example, low levels of antioxidants may indicate a loss of protective capacity or minimal ROS stimulus. Alternatively, elevated antioxidant levels may suggest an adaptive response to oxidative stress (Katerji et al., 2019) and, as such, a protective mechanism against oxidative damage. Increased activation of Nrf2 can be synonymous with a sustained antioxidant defence (Osburn and Kensler, 2008; Nguyen et al., 2009; Lee and Hu, 2020). Increased ROS may not necessarily be representative of oxidative stress if an antioxidant response occurs. Moreover, low to moderate levels of ROS and RNS can have beneficial effects, functioning in cellular signalling systems and mediating immune responses (Halliwell, 2007; Valko et al., 2007; Powers et al., 2010; Zuo et al., 2015). However, the concentrations at which ROS and RNS shift from beneficial functions to detrimental are not yet established (Di Meo et al., 2016). Consequently, increases of ROS/RNS and antioxidant markers should be interpreted in relation to the redox balance. Increase in an antioxidant marker alone with no corresponding alteration in ROS/RNS should be considered of limited relevance when interpreting evidence for KC5.

Study design should be evaluated for appropriateness of sample timing and analysis suitability when determining the relevance of evidence for KC5. Particular attention should also be given to the control of extraneous variables and isolation of exposure agent of interest when considering the strength of the findings. Given the variety of oxidative stress stimuli, findings with measurable association with exposure to the agent of interest should be considered as the most informative. Studies that note amelioration of oxidative stress markers after intervention with an antioxidant may also be viewed as particularly informative. As highlighted by Smith et al., (2016), it is important to note that oxidative stress is not unique to carcinogenesis, being also associated with numerous other health conditions (Valko et al., 2007; Frijhoff et al., 2015; Sies, 2015; Pizzino et al., 2017). Consequently, and in agreement with Guyton et al., (2018), the consideration of evidence for this KC alone, without evidence for other associated KCs, should be interpreted with caution.

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6. Key Characteristic 6: Induces Chronic Inflammation

William Gwinn

6.1 Introduction

Inflammation can be characterized as acute or chronic and can occur systemically or locally in specific tissues/organs. Whether it is acute or chronic can depend on several factors such as the length of exposure (or infection) as well as the nature and duration of the response (including the types of cells and proinflammatory biomarkers/mediators involved). Acute inflammation may be triggered by various "stressors", such as environmental pollutants (e.g. cigarette smoke and diesel exhaust), infectious agents (e.g. viruses and bacteria), injury (e.g. surgical trauma), food factors (e.g. red meat), or immunological (e.g. autoimmune) complexes (Serhan and Levy, 2018; Singh et al., 2019). In healthy individuals, acute inflammatory responses are typically self-limited and classically divided into two distinct and active phases of inflammation: initiation and resolution (Serhan, 2014; Serhan and Levy, 2018). The ideal outcome of acute inflammation is resolution (Serhan, 2014; Panigrahy et al., 2021); however, the failure of resolution results in the progression to chronic inflammation (Panigrahy et al., 2021) which may result from a response over time to a repeated or persistent inflammatory trigger (Fishbein et al., 2021). Unresolved acute inflammation progresses to chronic inflammation in various diseases, including cancer, over a prolonged time frame of weeks to months to years (Fishbein et al., 2021), and unresolved inflammation (i.e. chronic) is a hallmark of many cancers (Mantovani et al., 2008; Hanahan and Weinberg, 2011).

Chronic (unresolved) inflammation pertains to Key Characteristic (KC) 6 – "Induces chronic inflammation," but with regards to the evaluation of mechanistic evidence for cancer hazard assessment, it can be challenging to conclusively determine if an exposure induces chronic inflammation (as opposed to acute inflammation or an immune response not resulting in inflammation). For those agents with an evaluation of "strong" mechanistic evidence from Volumes 112 to 135, chronic inflammation has been shown to be a characteristic of 2 of 7 (30%) Group 1 carcinogens (welding fumes and occupational exposure as a firefighter) and 7 of 24 (30%) Group 2A agents (including, for example, diazinon, 4,4'-dichlorodiphenyltrichloroethane (DDT), tetrabromobisphenol A, aniline, acrolein, cobalt metal, trivalent antimony, and 2-bromopropane) (See Annex 1).

For the purposes of this review of the most relevant end-points for KC6, inflammation has been broadly divided into two essential components: 1) the cellular inflammatory response and 2) the non-cellular inflammatory response (e.g. involving expressed/secreted proinflammatory biomarkers, like cytokines and chemokines). Immune modulation/activation plays a critical role as a driver of proinflammatory responses to various exposures (or infections); therefore, indicators of immune modulation/activation are also included for each component.

6.2 Relevance of end-points

6.2.1 Cellular inflammatory response

For the cellular response, the most relevant end-points pertain to the identification of increased inflammatory/immune cells (e.g. leukocytes such as neutrophils, lymphocytes and macrophages) locally in

specific tissues/organs or systemically in peripheral blood (see Table 6). These cells can also be identified in lavage fluid collected from tissue compartments such as bronchioalveolar lavage fluid (BALF), which contains cellular and humoral components of the lung microenvironment (Vaguliene, et al., 2013), or peritoneal fluid from the peritoneum/abdominal cavity. Typically, the assays used for the evaluation and diagnosis of the cellular response are histopathology (tissue), haematology (blood), and flow cytometry (applicable to both tissue and blood). The cell type (i.e. subset of leukocyte) involved can be indicative of an acute or chronic (cellular) inflammatory response.

Table 6. Relevant end-points for KC6: "induces chronic inflammation"

Category	End-points	Relevance	Comments	References
Cellular inflammatory response				
Increased inflammatory/immune cells[a] in peripheral blood and/or tissue	Leukocytes (e.g. neutrophils, lymphocytes, macrophages, eosinophils, basophils, plasma cells, NK cells)	↑ Acute or chronic inflammation	Early presence of granulocytes (e.g. neutrophils) within tissue typically a hallmark of acute inflammation; Later or delayed appearance of monocytes/activated macrophages, plasma cells, and/or lymphocytes more indicative of chronic inflammation; Neutrophils also involved in chronic inflammation; Giant cells (e.g. in lung) indicative of chronic inflammation.	Pahwa & Jialal, 2019; Dutta et al., 2020
Inflammatory disease	Colitis/IBD, pancreatitis, hepatitis (from viral (Hepatitis B or C) or non-viral infections), gastritis (e.g. Helicobacter pylori infection), esophagitis (e.g. Barrett's oesophagus), bronchitis, cervicitis (from human HPV infection), prostatitis, asbestosis, bile duct inflammation (primary sclerosing cholangitis or cholecystitis) (e.g. liver fluke infection), bladder inflammation (e.g. Schistosoma haematobium infection)	↑ Acute or chronic inflammation	Some chronic inflammatory diseases associated with cancer (e.g. colitis/IBD and colorectal cancer).	Shah & Itzkowitz, 2022; Aggarwal et al., 2006; Perletti et al., 2010; Podgórska et al., 2018; Bonnans et al., 2014; Zhang et al., 2017; Honeycutt et al., 2014; Brindley et al., 2021
Non-cellular inflammatory response				
Proinflammatory biomarkers[b] in peripheral blood and/or tissue	Cytokines and chemokines (e.g. IL-6, IL-1α or β, IL-8, IL-17, TNF-α, MCP-1) Involves cytokine and chemokine receptor interactions	↑ Acute or chronic inflammation	Increased circulating IL-6 and IL-8 associated with risk of lung cancer (colon cancer for IL-6).	Brenner et al. 2017; Michels et al. 2021; Yanagawa et al. 1995; Kakourou et al., 2015

Table 6. Relevant end-points for KC6: "induces chronic inflammation"

Category	End-points	Relevance	Comments	References
			Carcinogenic benzene metabolites stimulate proinflammatory cytokines (TNF-α and IL-6), chemokines and Th2 cytokines (IL-4 and IL-5) and reduce the anti-inflammatory cytokine IL-10.	Gillis et al., 2007
			HPV triggers increased proinflammatory cytokines (IL-1, IL-6, IL-17, TGF-β and TNF-α) and NF-kβ in breast cancer.	Khodabandehlou et al., 2019
			Proinflammatory mediators related to pre-cancerous conditions (e.g. reflux esophagitis, Barrett's oesophagus, or colitis) include increases in COX-2 (KC5), IL-6, IL-8, IL-1β, IL-10 and TNF-α; loss of TGF-β signalling; and activation of NF-kB.	Hong et al., 2010; Polytarchou et al., 2015
			Asbestos stimulates IFN-α, EOTAXIN and RANTES in exposed workers and IL-12(p40), IL-3, IL-1α, MCP-3, NGF-β, TNF-β and RANTES in malignant mesothelioma patients; Surrogate biomarkers of systemic inflammation in serum of exposed workers include increased FGF-β and VEGF (KC 10), RANTES, CXCL10 (IP-10), CLEC11A (SCGF-b), CCL27 (CTACK), EOTAXIN, IL-5 and IL-6.	Comar et al., 2014; Comar et al., 2016
			Asbestos stimulates proinflammatory adipokines (e.g. MCP-1) and inhibits anti-inflammatory adipokines (e.g. adiponectin); Dysregulated adipokines promote asbestos-induced mesothelioma carcinogenesis.	Chew et al., 2014
			Biomarkers associated with inflammation in intestinal tumour tissue of pre-clinical animal models of colorectal cancer include proinflammatory cytokines (TNF-α, IFN-λ, IL-1β, IL-6, IL-17 and IL-23), inflammation-signalling pathways (activation of the NLRP3 inflammasome and TLR4 pathways such as MyD88 and p38 phosphorylation) and inflammation-associated cell surface markers (AP-1, CD11b and F4/80).	Bay et al., 2017; de Carvalho et al., 2019; Chang et al., 2022; Silveira et al., 2020; Fukata et al., 2011

Table 6. Relevant end-points for KC6: "induces chronic inflammation"

Category	End-points	Relevance	Comments	References
			Serum levels of IL-6, IL-10, IL-5, IL-4, IFN-λ and GM-CSF increased during preneoplastic stages of colon carcinogenesis in mice; Inflammatory response induced by NDEA includes the upregulation of chemokines such as CCL2, CCL5 and CXCL9 in murine models.	Mentor-Marcel et al., 2009; Schneider et al., 2012
	Acute phase proteins (e.g. CRP, fibrinogen)	↑ Acute or chronic inflammation	Elevated levels of CRP and fibrinogen in peripheral blood associated with risk for multiple types of cancer including lung and colorectal.	Zhou et al. 2012; Yanagawa et al. 1995; Bu et al. 2023; Michels et al. 2021
	NLR, SII, PLR	↑ Acute or chronic inflammation	Elevated levels in peripheral blood associated with risk for multiple cancers.	Nøst et al., 2021; Gago-Dominguez et al., 2020
	HMGB1	↑ Acute or chronic inflammation	Endogenous ligand of TLR4 released by dead cells that can stimulate inflammatory/immune response via multiple surface receptors including RAGE and TLR4; RAGE signalling stimulates inflammation-driven cancers such as colorectal; Proinflammatory biomarker (along with COX-2) in AOM/DSS colitis-associated colorectal cancer model.	Mittal et al., 2010; Wang et al., 2021; Yan et al., 2013; Turovskaya et al., 2008; Wang et al., 2021
			Potential biomarker of asbestos exposure and early indicator of mesothelioma.	Zolondick et al., 2021
	Lipid-derived autocoids	↑ Acute or chronic inflammation	Biosynthesized from polyunsaturated FA precursors; Regulate the initiation (e.g. prostaglandins, thromboxanes, leukotrienes) and resolution (e.g. resolvins and lipoxins) of inflammation.	Fishbein et al., 2021; Panigrahy et al., 2021; Fredman and Serhan, 2024
			Acute inflammation initiates the release of proinflammatory eicosanoids that can lead to "eicosanoid storm" driving proinflammatory cytokine production.	Serhan, 2014; Dennis & Norris, 2015

[a] Although the presence of specific cell types can help determine if an inflammatory response is acute or chronic, it is also important to put these cellular changes (in blood and/or tissue) in the context of exposure duration/persistence or time after exposure.
[b] Many of these proinflammatory biomarkers can be identified in acute or chronic inflammatory responses; therefore, as with the cellular response, it is important to put these non-cellular changes (in blood and/or tissue) in the context of exposure duration/persistence or time after exposure. Note, these are some key examples and not an exhaustive list of end-points (specific examples are also described under the "Comments").

Tissue histopathology can allow visualization and assessment of the cellular inflammatory response via routine haematoxylin and eosin (HandE) staining and immunohistochemical analyses of, for example, inflammatory/immune cells. Such cells include total leukocytes (CD45), macrophages (F4/80 and CD68), neutrophils (Gr1), T lymphocytes (CD3, helper-CD4 and cytotoxic-CD8), B lymphocytes (CD19 and CD20), and NK cells (CD335), as well as markers of cell proliferation (Ki-67) and the tissue fibrotic response (Masson's trichrome staining of collagen) (Rahman et al., 2017). BALF can be collected and evaluated for the presence of various inflammatory/immune cells such as macrophages, neutrophils, lymphocytes, or eosinophils at various timepoints, and the inflammatory response in the lung can be assessed histologically by the extent of cellular infiltration as well as bronchioloalveolar hypertrophy and pulmonary (interstitial/alveolar) oedema (Viegas et al., 2022). While histopathology allows for the spatial visualization of the inflammatory response, the multitude of inflammatory/immune cells along with proinflammatory biomarkers (see Section 6.2 of this Chapter – non-cellular inflammatory response) can be more precisely measured using techniques such as flow cytometry and reverse transcription-polymerase chain reaction (RT–PCR). These tools complement each other to characterize the qualitative and quantitative (cellular and non-cellular) inflammatory response.

Inflammatory/immune cells may persist in tissue over time or be cleared quickly. The early presence of infiltrating granulocytes (e.g. neutrophils) within a tissue is typically a hallmark of acute inflammation; whereas the later or delayed appearance of monocytes/activated macrophages, plasma cells, and/or lymphocytes are more indicative of chronic inflammation (Pahwa and Jialal, 2019). However, neutrophils can also play a pivotal role in chronic inflammation (Soehnlein et al., 2017). Another feature of chronic inflammation is the presence of giant cells in tissue (e.g. lung), which consist of fused macrophages (Dutta et al., 2020). These histological changes can be diagnosed by a pathologist as, for example, "chronic active inflammation"; however, this involves some level of subjectivity on the part of the pathologist. In addition, specific leukocyte subsets can be measured in peripheral blood and tissue by haematology/complete blood count (CBC) with white blood cell (WBC) differentials and/or flow cytometry. Although the presence of specific cell types can help determine if an inflammatory response is acute or chronic, it is also important to put these cellular changes (in tissue or blood) in the context of exposure duration/persistence or time after exposure.

Chronic inflammation in tissues/organs other than the cancer site can be considered relevant evidence for KC6, but more confidence/weight (in terms of a potential association with cancer) should be given to evidence of chronic inflammation concordant with the cancer/tumour site, and chronic inflammatory diseases (see Table 6) could be included as end-points under KC6 with an emphasis on those having known associations with cancer. Although this primarily applies to cellular inflammation, non-cellular inflammatory components (see Section 6.2 of this Chapter) may also be present within the inflamed site. A good example is colitis/inflammatory bowel disease (IBD) and colorectal cancer (Shah and Itzkowitz, 2022). Other examples of chronic tissue/organ inflammation (including infections) with strong associations with cancer are pancreatitis, hepatitis (from viral (Hepatitis B or C) or non-viral infections), gastritis (e.g. *Helicobacter pylori* infection), esophagitis (e.g. Barrett's oesophagus), bronchitis, cervicitis (from human papilloma virus (HPV) infection), prostatitis, asbestosis, bile duct inflammation (primary sclerosing cholangitis or cholecystitis) (e.g. liver fluke infection) and bladder inflammation (e.g. *Schistosoma haematobium* infection) (Aggarwal et al., 2006; Perletti et al., 2010; Bonnans et al., 2014; Honeycutt et al., 2014; Zhang et al., 2017; Podgórska et al., 2018; Brindley et al., 2021). However, it is often challenging to determine if chronic inflammation within a cancer/tumour site is associated with the cancer outcome or is simply a non-specific effect. Some agents are potent inducers of chronic inflammation within specific tissues but do not cause cancer within the same tissues. For instance, pneumoconiosis of the lung is an indicator of

a chronic inflammatory and progressive fibrotic response (Qi et al., 2021), as observed in exposed humans and experimental systems *in vivo* for trivalent antimony (IARC, 2023). Pneumoconiosis is not necessary but can be potentially associated with lung cancer (Samet, 2000; Abu Qubo et al., 2022). In contrast, decades of chronic inflammation in the pleural microenvironment from asbestos in exposed humans is thought to initiate and promote malignant pleural mesothelioma (MPM) (Severson et al., 2020).

6.2.2. Non-cellular inflammatory response

For the non-cellular response, the emphasis should be on the measurement of circulating and cell/tissue-derived (expressed/secreted) proinflammatory biomarkers/mediators (see Table 6). The most relevant end-points pertain to the identification of increased inflammatory cytokines or chemokines (e.g. tumour necrosis factor (TNF), interleukin (IL)-1α/β, IL-6, or IL-8), their associated receptors (including soluble forms, e.g. sIL-1Rα), and other critical biomarkers of systemic inflammation like acute phase proteins (e.g. C-reactive protein (CRP) and fibrinogen), but it is difficult to establish an association of these biomarkers/mediators with cancer, especially in exposed humans (epidemiological studies) (Allin et al., 2016; Brenner et al., 2014). Elevated levels of CRP and fibrinogen in peripheral blood have been associated with an increased risk for multiple types of cancer including lung (Zhou et al. 2012; Michels et al. 2021; Yanagawa et al. 1995) and colorectal (Bu et al. 2023). Other relevant biomarkers of neutrophilic inflammation are the formation/release of neutrophil extracellular traps (NETs) by neutrophils (which can exacerbate inflammatory tissue damage) (Castanheira and Kubes, 2019) and the neutrophil to lymphocyte ratio (NLR) in peripheral blood as a measurement of systemic (neutrophilic) inflammation. The NLR and additional biomarkers of systemic inflammation in peripheral blood like the systemic immune-inflammation index (SII) and platelet-to-lymphocyte ratio (PLR) have been positively associated with an increased risk for multiple cancers (Gago-Dominguez et al., 2020; Nøst et al., 2021). Altered platelet measurements have also been shown to be a biomarker of low-grade inflammation in healthy children exposed to polycyclic aromatic hydrocarbons (PAHs) (Dai et al., 2018).

In addition, there are many relevant (non-cellular) biomarkers/indicators of immune modulation/activation that can be linked to proinflammatory responses. These end-points include measurements of cell surface activation/differentiation markers including toll-like receptors (TLRs) and adhesion molecules on leukocytes (e.g. on lymphocytes (CD4-Th1 versus -Th2 versus -Th17 versus CD8-cytotoxic) and antigen-presenting cells (APCs) such as macrophages (M1 versus M2) and dendritic cells), activation of the NOD-like receptor protein 3 (NLRP3) inflammasome, and activation of transcription factors (e.g. nuclear factor kappa light chain enhancer of activated B-cells (NF-κB) and JAK/STAT) and other proinflammatory signalling/molecular pathways like PI3K/Akt/mTOR, Wnt/β-catenin, and transforming growth factor (TGF)-β/Smad (Sachi Das et al., 2022). Macrophage differentiation (polarization), which can be identified by alterations in the expression of specific (CD and CCR) markers on the cell surface, may also be an indicator of proinflammatory and anti-resolution responses if increased M1 macrophages (CD11c and CCR7 expression) and decreased M2 macrophages (CD163 and CD206 expression) are present (Chang et al., 2022). End-points pertaining to skin sensitization/allergic (including mast cell) responses as hypersensitivity reactions have been reviewed for some agents (i.e. isoeugenol, IARC Group 3, Volume 134) evaluated in the *IARC Monographs*. However, they are considered not informative as to cancer hazard identification.

These proinflammatory biomarkers and indicators of immune activation can be identified in peripheral blood (serum or plasma) or lavage fluid (e.g. BALF), or expressed by specific cells/tissues. mRNA and protein levels can be measured using approaches such as RT–PCR or microarray/transcriptomic analyses

for mRNA and ELISA (including microfluidics-based ELLA), ELISpot, multiplex assay/cytokine array (e.g. Luminex technology), Western blot, or proteomic/secretomic analyses for protein and liquid chromatography/mass spectrometry (LC/MS) for lipid autacoids (e.g. oxylipins). Many of these biomarkers/indicators can be identified in acute or chronic inflammatory responses; therefore, as with the cellular response (see Section 6.2.1 of this Chapter), it is important to put these non-cellular changes (in blood or cells/tissue) in the context of exposure duration/persistence or time after exposure. For instance, there should be more confidence that a specific biomarker (e.g. cytokine) is indicative of a chronic inflammatory response if it is elevated (in blood and/or tissue) after a prolonged (weeks to months to years) exposure duration (with persistent or repeated exposure) or time after exposure.

Inflammatory cytokines can be proinflammatory (e.g. IL-1α/β, IL-4, IL-6, IL-12, IL-15, IL- 17, IL-23, TNF-α, and interferon (IFN)-α) or anti-inflammatory (e.g. IL-10 and TGF-β) (Pak et al., 2019). There are also proinflammatory chemokines (chemotactic cytokines) such as IL-8 and monocyte chemoattractant protein (MCP)-1. Subclinical systemic inflammation in exposed humans can be measured by serum CRP and immune responsiveness ex vivo via stimulated (e.g. with lipopolysaccharide) or unstimulated production of IL-1β, IL-6, and TNF-α by peripheral blood-derived leukocytes (Karvonen et al., 2018). Biomarkers of the inflammatory response induced by urban air pollution include a significant increase in the levels of anti-intercellular adhesion molecule (ICAM), IL-1β, and TNF-α in the polluted group compared to the filtered air control group (de Oliveira Alves et al., 2020). The proinflammatory response in lymphocytes and lung cells of humans after exposure to traffic-related particles includes increased expression of cytokine/chemokine mRNA (IL-6 and IL-8), Clara cell protein (CC16), lung surfactant protein-A (SP-A), and oxidative damage to DNA as measured by levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) (interaction with KC 5) (Vattanasit et al., 2014). Gene and protein expression profiling demonstrated that metabolites of the carcinogen benzene stimulate the production of proinflammatory cytokines (TNF-α and IL-6), chemokines, and Th2 cytokines (IL-4 and IL-5) and reduce the production of the anti-inflammatory cytokine IL-10 (Gillis et al., 2007).

Some cytokines/chemokines are better correlates to specific types of cancer. For example, pre-diagnosed increased circulating levels of IL-6 and IL-8 are associated with increased risk of lung cancer (Brenner et al. 2017; Michels et al. 2021; Yanagawa et al. 1995) and of colon cancer in the case of IL-6 (Kakourou et al., 2015). IL-6 is considered one of the better biomarkers available due to its stability in plasma and serum (Gong et al., 2019a). The presence of HPV triggers increased expression of proinflammatory cytokines (IL-1, IL-6, IL-17, TGF-β, and TNF-α) and NF-kβ in breast cancer (Khodabandehlou et al., 2019). A proinflammatory host genotype with a cytokine genetic profile of IL-1β, IL-1RN (antagonist), IL-10, and TNF-α was shown to increase the risk of non-cardia gastric adenocarcinoma but not of cardia or oesophageal cancers (El-Omar et al., 2003). Furthermore, Luminex technology was used to evaluate 64 circulating proinflammatory biomarkers in plasma from individuals at risk for gastric and oesophageal cancers: soluble epidermal growth factor receptor (sEGFR) (KC10) and the cytokine thymic stromal lymphopoietin (TSLP) were minimally associated with gastric cancer risk, whereas CRP, CXCL11/ITAC, and CCL15/MIP1D were associated with oesophageal cancer (Camargo et al., 2019). Proinflammatory mediators related to pre-cancerous conditions (e.g. reflux esophagitis, Barrett's oesophagus, or colitis) include increases in cyclooxygenase (COX)-2 (KC5), IL-6, IL-8, IL-1β, IL-10, and TNF-α; loss of TGF-β signalling; and activation of NF-kB (Hong et al., 2010; Polytarchou et al., 2015)

Asbestosis has been shown to induce NLRP3 inflammasome activation resulting in cytokine secretion by inflammatory cells, which, along with production of reactive oxygen species (ROS) (KC5), is a hallmark of asbestos exposure (Yang et al., 2010; Benvenuto et al., 2016). Based on Luminex analysis of serum, asbestos stimulated production of cytokines and chemokines IFN-α, EOTAXIN, and RANTES in exposed

workers and IL-12(p40), IL-3, IL-1α, MCP-3, nerve growth factor (NGF)-β, TNF-β, and RANTES in malignant mesothelioma patients compared to healthy controls (Comar et al., 2014). In another study of asbestos-exposed workers, surrogate biomarkers of systemic inflammation in serum included increased levels of human fibroblast growth factor (FGF)-β and vascular endothelial growth factor (VEGF) (KC10), RANTES, CXCL10 (IP-10), CLEC11A (SCGF-b), CCL27 (CTACK), EOTAXIN, IL-5, and IL-6 compared to controls (Comar et al., 2016). Asbestos also stimulates proinflammatory adipokines (e.g. MCP-1) and inhibits secretion of anti-inflammatory adipokines (e.g. adiponectin), and dysregulated adipokine production by adipose tissue is thought to promote asbestos-induced mesothelioma carcinogenesis (Chew et al., 2014).

Biomarkers associated with inflammation in intestinal tumour tissue of pre-clinical animal models of colorectal cancer (e.g. azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced colitis-associated and N-nitrosodiethylamine (NDEA)-induced) include proinflammatory cytokines (TNF-α, IFN-λ, IL-1β, IL-6, IL-17, and IL-23), inflammation-signalling pathways (activation of the NLRP3 inflammasome and TLR4 pathways such as MyD88 and p38 phosphorylation), and inflammation-associated cell surface markers (activator protein (AP)-1, CD11b, and F4/80) (Bay et al., 2017; de Carvalho et al., 2019; Silveira et al., 2020; Chang et al., 2022). Other studies have also shown that activation of TLR4 stimulates a proinflammatory response that leads to colitis-associated cancers (Fukata et al., 2011). Serum levels of IL-6, IL-10, IL-5, IL-4, IFN-λ, and granulocyte macrophage-colony stimulating factor (GM-CSF) were shown to be increased during preneoplastic stages of colon carcinogenesis in mice (Mentor-Marcel et al., 2009). The inflammatory response induced by carcinogens such as NDEA include the upregulation of chemokines such as CCL2, CCL5, and CXCL9 in murine models (Schneider et al., 2012). The transcription factor STAT5 exhibits a pro-tumorigenic role in nitrosamine (e.g. NDEA)-induced cancer (Kaltenecker et al., 2019), and STAT6 plays a key role in the inflammatory response in colitis-associated colon cancer via the induction of immune cell infiltration and epithelial cell proliferation (KC10) (Leon-Cabrera et al., 2017). Carcinogenic bacteria such as *Bacteroides fragilis* can stimulate a myeloid cell-dependent inflammatory response via STAT3 signalling resulting in NF-κB activation in colon carcinogenesis (Chung et al., 2018).

High mobility group box-1 protein (HMGB1) is an endogenous ligand of TLR4 released by dead cells that can stimulate an inflammatory response (Mittal et al., 2010; Wang et al., 2021). HMGB1 can activate immune cells via multiple surface receptors including RAGE (receptor for advanced glycation end products) and TLR4 (Yan et al., 2013), and RAGE signalling stimulates inflammation-driven cancers such as colorectal and the associated colitis (Turovskaya et al., 2008). Proinflammatory biomarkers in the AOM/DSS colitis-associated colorectal cancer model include high levels of COX-2 (KC5) and HMGB1, which trigger inflammation and oxidative damage to DNA (8-nitroG and 8-oxodG) (KC5) (Wang et al., 2021). HMGB1 is also a potential biomarker of asbestos exposure and early indicator of mesothelioma (Zolondick et al., 2021).

Lipid-derived autacoid mediators are biosynthesized from polyunsaturated fatty acid precursors by cellular enzymes and are critical regulators of the initiation and resolution of inflammation (Wang and Dubois, 2010; Serhan, 2014). Bioactive lipids may initiate (e.g. prostaglandins, thromboxanes, and leukotrienes) or terminate inflammation or induce resolution (e.g. specialized pro-resolving lipid mediators (SPMs) such as resolvins and lipoxins) (Greene et al., 2011). Acute inflammation initiates the release of proinflammatory eicosanoids that, when uncontrolled, lead to an "eicosanoid storm" that drives proinflammatory cytokine production (Serhan, 2014; Dennis and Norris, 2015). Humans exhibit a differential resolution response to inflammatory insults (Morris et al., 2010), which may explain why some individuals are more susceptible to the pro-tumorigenic activity of certain environmental chemicals. Proinflammatory eicosanoid enzymes upstream of cytokines include COX and lipoxygenase (LOX) (KC 5),

cytochrome p450 (CYP450), and soluble epoxide hydrolase (sEH) (Imig and Hammock, 2009; Wang and Dubois, 2010).

6.3 Assessing the relevance of end-points in different test systems

6.3.1 Cellular inflammatory response

The end-points for the cellular inflammatory response are most applicable "*in vivo*" either in exposed humans or experimental systems, although histologic evaluations are often limited in humans (Smith et al., 2020b), and the strength of the evidence for the cellular response should largely depend on the strength of the *in vivo* data (in exposed humans or animal models). This is because these cellular end-points are difficult to model *in vitro* (using human primary cells or experimental systems *in vitro*) with regards to chronic inflammation, owing to the complexity of the response (e.g. cellular recruitment) and the extended exposure time (or time after exposure) in culture required to achieve chronicity (as most *in vitro* assays are short-term in nature) (Smith et al., 2020b). Thus, under most circumstances, the strength of evidence for the cellular (chronic) response should not rely on *in vitro* data alone. Some new approach methodologies (NAMs) are trying to recapitulate the extravasation of leukocytes from the circulation (systemic/vascular (endothelial) compartment) into tissue (e.g. microphysiological systems (MPS) like tissue/organ-on-a-chip technology), but the length of exposure/culture time often remains a challenge with these NAMs.

6.3.2 Non-cellular inflammatory response

As with the cellular response (see Section 6.3.1 of this Chapter), the end-points for the non-cellular inflammatory response are most applicable "*in vivo*" either in exposed humans or experimental systems, and the strength of the evidence for the non-cellular response should largely depend on the strength of the *in vivo* data (in exposed humans or animal models). Nevertheless, alterations in many of the specific proinflammatory biomarkers can also be effectively measured in experimental systems *in vitro* (including human primary cells) to provide complimentary/supporting evidence to the *in vivo* findings. However, as was the case with the cellular response, these biomarker end-points are difficult to model *in vitro* with regard to chronic inflammation, owing to the complexity of the response (e.g. signalling pathways involved) and the extended exposure time (or time after exposure) in culture required to achieve chronicity (Smith et al., 2020b). Thus, under most circumstances the strength of evidence for the non-cellular (chronic) response should not rely on *in vitro* data alone. There are some "long-term" *in vitro* assays that have attempted to better model chronic inflammation. For example, in one assay, prolonged (60-week) treatment of mouse colonic (3D) organoids *in vitro* with a mixture of cytokines (TNF-α, IL-1β, and IL-6) was able to induce cell transformation (KC 9) (Hibiya et al., 2017). Overall, it is important to emphasize that it is possible but very difficult to make *in vitro* co-culture models that reflect the complexity of the immune/inflammatory response (both cellular and non-cellular) *in vivo* which impacts their reliability for the assessment of chronic inflammation.

For *in vivo* data in exposed humans (e.g. in occupational firefighters –Volume 132), persistent (repeated) acute exposures resulting in acute exacerbations in inflammatory responses over time (e.g. increased inflammatory/immune cells (see Sections 6.2.1 and 6.3.1 of this Chapter) and/or proinflammatory biomarkers such as cytokines/chemokines in peripheral blood) can be considered to provide relevant evidence of chronic inflammation. This relates to the fact that end-points used to measure chronic

inflammation may be clear in experimental systems *in vivo* (animal models), but it is unrealistic to expect the same end-points in exposed humans.

Past *IARC Monographs* Working Groups have debated the utility of acute/short-term *in vitro* and *in vivo* assays to identify biomarkers or other indicators of inflammation/immune activation that could be linked to chronic inflammation (and thus might be relevant to KC6 and cancer hazard assessment). Many studies (in exposed humans and experimental systems *in vivo* or *in vitro*) report acute inflammation/immune activation (proinflammatory signalling, etc.), which may or may not be critically tied to "downstream" chronic inflammation. These data can be reported as complimentary/supporting findings, but in the absence of "chronic" evidence, acute evidence alone is not sufficient evidence for KC6. If a substantial evidence base for acute inflammation/immune activation exists, a summary of the data should be provided, but in-depth study details and discussion should not be a focus. In *IARC Monographs* Volume 131 for cobalt (IARC, 2023), the evidence for acute immune activation/proinflammatory signalling in exposed humans and human cells *in vitro* were reported as supporting data (*in vitro* data in experimental systems were not reported) but was not considered as significant evidence for KC6 (which was based on the data from studies in experimental systems *in vivo*), as there was no way to conclusively link these acute data to chronic inflammatory responses.

6.4 Interpretation of results within the same database

Whilst reviewing the evidence of chronic inflammation, it may be possible to put more weight on the evidence derived from studies in animal models with repeated dosing because of the possibility to examine cellular (see Sections 2.1 and 3.1 of this Chapter) and non-cellular (see Sections 2.2 and 3.2 of this Chapter) inflammatory responses in blood and multiple (target and non-target) tissues across several timepoints and exposure concentrations in a well conducted *in vivo* animal study with appropriate control groups then other models after single exposure. As already mentioned in Section 6.3.1 of this Chapter, histologic evaluations are often limited in humans, whereas numerous tissues (and timepoints) can be evaluated in animal studies. However, this will depend on an evaluation and determination by the Working Group of the quality of the studies in exposed humans and experimental systems *in vivo*. Historically, informative mechanistic evidence for KC6 has largely been based on chronic bioassays in rodents.

In addition, it is ideal when alterations in cellular and non-cellular responses both correlate (e.g. increased IL-8 and neutrophils in BALF/lung tissue), but evidence for a cellular response (in exposed humans or experimental systems *in vivo*) (see Sections 6.2.1 and 6.3.1 of this Chapter) is considered more relevant for KC6 than that for a non-cellular response (see Sections 6.2.2 and 6.3.2 of this Chapter).

In terms of the relevant and informative mechanistic evidence for KC6, there should be evidence that the agent induces increased inflammatory responses (inflammatory cells and/or proinflammatory biomarkers) systemically in blood and/or locally in target tissue(s), preferably the cancer site(s), of exposed humans and/or experimental systems *in vivo*. In addition, these responses should be present after a prolonged (weeks to months to years) exposure duration (with persistent or repeated exposure) or length of time after exposure. Due to a reduction in chronic toxicity (carcinogenicity) testing in animals (e.g. 2-year bioassays in rodents) that is happening in the field, there is a current and future need for novel/alternative approaches (e.g. NAMs) to identify mechanistic evidence relevant to KC6 in exposed humans and experimental systems *in vivo* (involving relatively shorter-term studies) and *in vitro*.

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7. Key Characteristic 7: Is Immunosuppressive

Dori Germolec and Parveen Bhatti

7.1 Introduction

Immune suppression, in the context of cancer, is a reduction in the immune system's ability to respond effectively to pathogen- or tumour-associated antigens. The immune system carries out constant surveillance to determine whether or not to initiate an immune response. Immunity can be viewed as a careful balance between response initiation, resolution, and no response to achieve homeostasis. There is considerable evidence from patients with congenital immunodeficiencies (Tangye et al. 2020; Lewandowicz-Uszyńska et al., 2021), virally induced immunodeficiencies (e.g. human immunodeficiency virus (HIV)-mediated) (Spano et al., 2008; Guiguet et al., 2009; José and Brown, 2016), and from therapeutically induced immunodeficiencies (e.g. transplant rejection prevention) (Penn, 2000; Mueller, 2008) that immunosuppression increases the risk of certain types of cancer (Kasiske et al. 2004). Immune deficiencies may occur in innate or adaptive immune responses as well as the soluble components that support the communication between cells and their effector functions.

7.2 Relevance of end-points

7.2.1 Altered haematopoiesis

Adverse effects on haematopoietic stem cells have far-reaching consequences and can completely abrogate the immune response (Table 7). Cell proliferation plays an integral role in the immune response, and decreases in the proliferation of haematopoietic stem and progenitor cells (HSPC) can result in depletion of entire lineages of immune cells (e.g. neutropenia, lymphopenia) (Kurtin, 2012). Myelotoxicity resulting in neutropenia is the most common dose-limiting toxicity in cancer therapy with classical chemotherapeutic agents or radiation (Kurtin, 2012). However, it is unclear how frequently leukopenia occurs in the absence of therapeutic sequelae or how useful this would be as a biomarker for potential carcinogenic agents. The solvent benzene, a frequent soil and groundwater contaminant, is one example of a known non-therapeutic myelotoxic agent (Snyder, 2012; IARC, 2018a). Through multiple genetic and epigenetic alterations, benzene induces abnormalities and apoptosis of HSCs and stromal cells, thereby altering cell proliferation and differentiation, leading to haematotoxicity, aplastic anaemia, and leukaemia (Snyder, 2012; IARC, 2018a).

Table 7. End-points relevant to KC7: "is immunosuppressive"

Category	End-point	Relevance	Comment	Reference
Altered Haematopoiesis	Cytotoxicity (myelotoxicity, neutropenia, lymphopenia)	Deletion of stem cells that populate the immune system. Altered numbers of circulating leukocytes	Compounds capable of damaging or destroying bone marrow cells or depleting specific cell populations will be immunotoxic.	Luster et al., 1992; Pessina et al., 2003; Pfortmueller et al., 2017
Innate Immune System	Measurement of soluble mediators (cytokines, chemokines, growth factors); Levels in tissues or cell culture	Generally, ↓ when immune suppression is present but can vary with the specific cytokine and the immune response that it supports	Can be measured in the human whole blood cytokine release assay using disease relevant antigens to stimulate the immune response *ex vivo* in cells obtained from animal studies or cultured cells. For innate immunity, relevant cytokines could include TNFa, IL-1 α and β, IL-6, IL-8 and IL-12, which are considered proinflammatory and overlap with KC6.	Lebrec et al., 1995; Langezaal et al., 2002; Liu et al., 2021
Innate Immune System	Natural killer cell activity	↓ can result in immune suppression and increased tumour burden	↓ NK cells associated with increased tumour burden in animal models. Can be measured in human whole blood or in *ex vivo* assays. Required in some regulatory testing panels.	Luster et al., 1992; Lebrec et al., 1995
Innate Immune System	Macrophage/neutrophil function	↓ can result in immune suppression	While more relevant to infectious disease ↓in phagocytosis or respiratory burst may impair the ability to clear tumour cells. Can be measured *ex vivo* in human peripheral blood cells, isolated cells from laboratory animals, or in relevant cells lines using flow cytometry and fluorescent materials.	Luster et al., 1992; Lebrec et al., 1995; Pfortmueller et al., 2017
Adaptive Immune System	Measurement of soluble mediators (cytokines, chemokines, growth factors); Levels in tissues or cell culture	Generally, ↓ when immune suppression is present but can vary with the specific cytokine and the immune response that it supports	Can be measured in the human whole blood cytokine release assay using disease relevant antigens to stimulate the immune response *ex vivo* in cells obtained from animal studies or cultured cells. For adaptive immunity, relevant cytokines could include IL-4, IL-10, IL-13, IL-17, TGF β, which promote clonal expansion of specific lymphocyte populations or regulate proinflammatory responses.	Luster et al., 1992; Lebrec et al., 1995; Langezaal et al., 2002; Liu et al., 2021
Adaptive Immune System	Lymphocyte proliferation	↓ when immune suppression is present. Critical for the clonal expansion of tumour-specific B and T lymphocytes.	Can be measured in the human whole blood cytokine release assay using disease relevant antigens to stimulate the immune response, *ex vivo* in cells obtained from animal studies or cultured cells.	Luster et al., 1992; Lebrec et at 1995; Langezaal et al., 2002

Table 7. End-points relevant to KC7: "is immunosuppressive"

Category	End-point	Relevance	Comment	Reference
Adaptive Immune System	Cytotoxic T lymphocyte activity	↓ can result in immune suppression; Important in the killing of virus infected cells and related tumours.	CTL can be identified in whole blood and function assessed in ex vivo assays. Can measure antigen specific CTL in lung and spleen of animal models	Luster et al., 1992; Lebrec. et al., 1995
Adaptive Immune System	T dependent antibody response	↓ can result in immune suppression; Measurement of immunoglobulin titres following vaccination in humans.	Primary and secondary (recall) responses following vaccination can be measured in human blood. Used in several epidemiology studies in conjunction with childhood or annual vaccinations. Most predictive single test in animal models. Required in some regulatory testing panels	Luster et al., 1992; Lebrec. et al., 1995; Timmermann et al. 2022

CTL, cytotoxic T lymphocytes activity; IL, interleukin; NK, natural killer; TGF, tumour growth factor; TNF, tumour necrosis factor. Note: these are some key examples and not an exhaustive list of end-points.

7.2.2 Innate immune response

The cells that are involved in detecting a threat are those belonging to the innate arm of the immune response. The acute inflammation phase of the innate response recognizes and attempts to eliminate pathogens, damaged or infected cells, and foreign proteins. Cells that belong to the innate arm of the immune system, such as macrophages and neutrophils, express receptors that specifically recognize tumour-specific antigens and pathogen-specific patterns of proteins and lipids on bacteria, virally infected cells, or cancer cells (Szczykutowicz, 2023). These innate cells release cytotoxic proteins, cytokines, and chemokines to stimulate other immune cells or to recruit them to specific tissues for resolution and repair. Macrophages and dendritic cells (DCs) also serve as antigen presenting cells (APCs), acting as a bridge between the innate and adaptive arms of the immune response. Alterations in the function of these cells may be indicative of immune suppression, and assessment of relevant end-points such as cytokine secretion and antigen presentation would provide information to support the use of KC7 to provide evidence of cancer risk (Germolec et al. 2022). Innate lymphoid cells are not restricted to respond to specific antigens and play a critical role in early anti-tumour responses, in part through production of cytokines and proinflammatory proteins (Jacquelot et al. 2022). Natural killer (NK) cells rapidly respond following viral infections and mediate tumour cell killing in a major histocompatibility complex (MHC)-unrestricted manner. The latter function is important, because some tumour cells lose the surface antigens that are needed to recognize non-self, thereby escaping immune surveillance (Ponce 2018). In humans, higher levels of *ex vivo* NK cell activity have been associated with reduced cancer risk (Imai et al. 2000), and evidence from studies in rodents and non-human primates suggests that reduction in the numbers or function of NK cells increases cancer risk (Goyos et al., 2019). Evidence of alterations in NK cell function and numbers would be of high relevance in the assessment of immune suppression (Luster et al. 1992; Germolec et at 2022). During acute inflammation, innate immune responses are tumoricidal, and suppression of this aspect of immune function can lead to uncontrolled tumour growth.

7.2.3. Adaptive immune response

The adaptive immune response includes T- and B-cells, which express cell-surface receptors that recognize antigenic epitopes. Antigen-specific T-cells and B-cells then undergo robust proliferation, known as clonal expansion, to ensure that a large population of cells is present to react to perceived threats. When lymphocyte proliferation is inhibited, immunosuppression is a common outcome. Specialized subsets of T-cells aid in the immune response by recruiting or activating other immune cells (Th1 or Th2 cells), by serving as effectors to kill tumour cells (cytotoxic T-cells, CTL), or by producing cytokines that recruit innate immune cells and regulatory T-cells (Tregs) which help to control the response and restore homeostasis (Th17; Ou et al. 2023). CTLs are thought to be the most important effector in the anti-tumour response. Antigen presenting cells prime naïve T-cells, and in the presence of appropriate co-stimulatory signals and cytokines, the T-cells differentiate into CTLs that can migrate to the site of a tumour to effect cytotoxicity (Barry and Bleackley, 2002). Tregs circulate in low numbers relative to other T-cell populations but are frequently identified as tumour infiltrating lymphocytes, suggesting that they are important in anti-tumour responses (Tay et al., 2023). The role of B-cells in the anti-tumour response has recently been given increased attention, as B-cells have been identified in high numbers in specific types of tumours (Yuen et al., 2016; Kinker et al. 2021). The primary role of B-cells is to produce antibodies, which as part of the anti-tumour response can opsonize cancer cells to facilitate antigen presentation, initiate cytolysis of infected cells through activation of the complement cascade, or enhance the actions of NK cells to promote antibody-dependent cellular cytotoxicity (Yuen et al., 2016). Aryl hydrocarbon receptor ligands, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, are some of the most potent immunosuppressants in experimental models and have been shown to modulate nearly all aspects of adaptive immune function (Germolec et al. 2022).

7.3 Assessing the relevance of end-points in different test systems

Cross-sectional and cohort epidemiology studies or clinical trials that report information on overall immune function such as changes in infectious disease incidence and/or reduced vaccine responses should be emphasized when available and can provide highly informative evidence for immunosuppression. Immunotoxicology studies in humans, which generally consist of quantifying cell populations, antibody levels or soluble mediators in peripheral blood, have the advantage of relevant exposure scenarios, diversity of subjects and opportunities to evaluate susceptible populations (DeWitt et al. 2016). However, these studies can also be limited in value because of design constraints and a lack of quality data on the intensity and duration of exposure. The relationship between suppression of the functional immune measures described below and clinical disease remains uncertain, although the association between immune suppression and certain cancers is strong (Luster et al. 1992.; DeWitt et al. 2016; Tangye et al. 2020; Lewandowicz-Uszyńska et al., 2021; Penn, 2000; Mueller, 2008; Kasiske et al. 2004). The continued accumulation of data on functional immune end-points in the human population (e.g. antibody titres and vaccine efficacy) should allow for better prediction as to how these end-points relate to the development of neoplastic disease.

In humans and experimental animals, effects on haematopoiesis can be evaluated through immunophenotyping, which quantitates specific cell surface markers on circulating leukocyte populations (i.e. leukocytopenia/leukocytosis, granulocytopenia/granulocytosis, or lymphopenia/lymphocytosis). An assessment of haematotoxicity is required for the approval of drugs, chemicals, and food additives (Pessina et al. 2009). Clinical information on depletion of specific cell lineages would be a relevant end-point for immune suppression, particularly in humans where other end-points may not be available. Cytotoxicity to cultured primary human leukocytes *ex vivo* and/or cell lines *in vitro* when combined with data from

experimental systems *in vivo* has been considered to provide suggestive evidence of immune suppression in the assessment of benzene (IARC, 2018a). Commercially available high-throughput platforms can be used to measure myelotoxicity, and *ex vivo*/*in vitro* data from primary human cells and/or cell lines used in the safety assessment of novel therapeutics can include cytotoxicity or colony forming unit assays which interrogate effects on specific cell-linages (Pessina et al., 2009; Haglund et al. 2010). For *in vitro* assessments of immunotoxicity, tiered approaches have been suggested, with myelotoxicity as the first step (Gennari et al. 2005; Corsini and Roggen, 2009).

Human peripheral blood mononuclear cells (PBMCs) can be used to investigate both innate and adaptive immune function. With the use of methods that assess multiple end-points from a single donor, the ability to integrate evaluation of complementary end-points (e.g. cell number and function) in human primary cells has been greatly enhanced. In the human whole blood assay, cultured cells can be stimulated with non-specific (LPS), or specific antigens, and the secretion of cytokines, cell proliferation, NK and CTL cell activity can be measured (Langezaal et al. 2002; Lebrec et al. 2016). Because these cultures contain multiple cell types and allow for the secretion of soluble mediators, they capture at least some of the physiologic interaction of the *in vivo* response. Immunophenotyping that assesses the major leukocyte subpopulations and quantification of cell surface and activation markers can also be used as a biomarker of immune suppression in exposed populations (Vogt, 1991) or experimental animals (Luster et al. 1988). Circulating cytokine levels or measurement of cytokines from target tissues, such as bronchoalveolar lavage fluid, can also provide information on alterations in soluble mediators that may be indicative of immune suppression. Measurement of tumour-relevant end-points such as the secretion of TNF α, IL-1α/βand IL-6 by specific populations of inflammatory cells or inflammatory mediators that serve as direct effectors such as superoxide and myeloperoxidase can also provide specific information on the ability to mount and anti-tumour response.

While primary cells are the most clinically relevant, human cell lines can serve as surrogates to assess immune responses *in vitro* and provide data on chemical-induced alterations in immune function (Gennari et al., 2005). With the increased use of alternatives to animal methods, several microphysiological systems or multiple cell-based assays have been developed to model the human immune system (Polini et al. 2019; Ramadan et al. 2023; Chernyavska et al. 2023), but the use of these methods has been limited.

The use of tiered testing strategies to screen for alterations in immune function using laboratory animals has often provided the most compelling evidence of the immunotoxicity of xenobiotics (Luster et al. 1988). These testing panels have been designed to provide a comprehensive assessment of immune function by evaluating innate and adaptive immunity, with an emphasis on assays that require cellular interaction and coordinated responses.

The assessment of NK cell numbers and function in laboratory rodents is routinely included in tiered screening and has been identified by regulatory agencies as a mechanism to assess cancer risk because it provides information relevant to immune surveillance (Goyos et al., 2019). Suppression of NK cell activity in cultured human PBMCs contributed to the mechanistic evidence identifying dichlorodiphenyltrichloroethane as *probably carcinogenic to humans* (Group 2A) (IARC, 2017). In laboratory animals, the assay is performed using either splenic or peripheral blood mononuclear cells and measures lytic activity against tumour targets in a non-MHC restricted manner.

Among the various functional immune tests, the T-dependent antibody response (TDAR) is the consensus choice for identification of immunotoxicity hazards in most, if not all, regulatory guidelines for assessment of immunotoxicity (Lebrec et al. 2014). A successful TDAR requires sequential activation of multiple aspects of the immune response, including (but not limited to) antigen presentation by innate cells, secretion of soluble mediators for cell growth and differentiation by Th cells, and clonal expansion of antigen

specific B-cells. In humans, the TDAR can be measured in response to common vaccines such as tetanus and diphtheria, and alterations in vaccine titres have been used to demonstrate immune suppression in humans following exposure to perfluorinated alkylated substances (Grandjean et al. 2017; Timmermann et al. 2020) and polychlorinated biphenyls (Heilmann et al. 2006). In most instances, the responses measured in humans are secondary (IgG), but if antibody titres following the initial administration of early childhood vaccines or novel antigens (e.g. SARS-COV-2) are assessed, a primary response (IgM), similar to what is observed in most laboratory animal studies, can be evaluated. In laboratory rodents, T-dependent and T-independent antibody responses can be evaluated using a variety of antigens (sheep erythrocytes, keyhole limpet haemocyanin) and techniques by quantifying numbers of antibody secreting B-cells or through the measurement of antigen specific immunoglobulins (Watson et al. 2021). Although these assays do not commonly use antigens that would reflect anti-tumour activity, they have been shown to be highly predictive in identifying immunosuppressive compounds that alter disease resistance, particularly when combined with one or more additional tests (Luster et al., 1992, 1993).

Cytotoxic T-lymphocyte activity is measured similarly to that of NK cell cytotoxicity. In humans, circulating peripheral blood leukocytes can be cultured ex vivo in the presence of viral or tumour antigens to stimulate the production of CTL, and lytic activity of these cells can be measured using a variety of techniques. In this model, supernatants can be collected to examine effects on soluble mediators that support proliferation and differentiation of the cells. In laboratory animals, inoculation of tumour or virally infected target cells can be used to simulate the natural course of exposure to pathogenic cells. Subsequently, effector cells from the spleen or lung (depending on route of exposure) can be harvested to assess cytolytic activity. The major advantage of this method is that when training of the CTL population occurs *in vivo*, it is in the context of chemical exposure and thus reflects the effects of all the components in the response. *In vitro* techniques using spleen or pulmonary cells from experimental animals are similar to those described for human cells, and, depending on the target cells or antigens used, may provide data directly relevant to human cancer.

Cell proliferation is one of the end-points that can be measured in the human whole blood cytokine release assay. Lymphocyte proliferation can also be measured in cultured human cells as a single end-point, and when relevant antigens such as anti-CD3 or viral peptides are used (rather than non-specific stimulants such as Lipopolysaccharide, Pokeweed mitogen or Conconavalin A) can be an important tool for identifying and comparing the relative potencies of immunosuppressive agents (Hartung and Corsini, 2013). Changes in cell proliferation can provide supportive evidence of cellular targets when used in a weight-of-evidence approach; however, it should be noted that in laboratory rodent studies, cell proliferation as a stand-alone end-point was a relatively poor predictor of immune suppression (Luster et al. 1992).

Finally, clinical and anatomic pathology end-points can flag potential immunotoxicity. As noted above, assessment of circulating leukocyte numbers and populations shifts can provide information on changes in immune cell populations. Shifts in cell recruitment to specific tissues may be observed following histological evaluation and may be indicative of altered cytokine or chemokine signalling and potential immune suppression; these are likely most important in assessment of chronic inflammation or changes in adaptive immunity. Thymic atrophy is a hallmark of stress in laboratory animals but may also be indicative of immune suppression (Elmore, 2018). Similarly, splenic or lymph node atrophy may occur following exposure to carcinogens such as N,N-dimethyl-*p*-toluidine (NTP 2012). Assessment of the specific anatomic compartments in the thymus (cortical and medullary zones), spleen (red pulp and white pulp including periarteriolar sheaths, germinal centres and marginal zones) and lymph nodes (cortical area with B-cell lymphoid follicles, T-cell containing paracortical area and medulla) from experimental toxicology studies may provide highly specific information on immune targets (Elmore, 2018).

7.4 Interpretation of the results within the same database

The information on different end-points across multiple methods should be used to interrogate the potential for a chemical to cause immunosuppression or other deleterious effects on the immune system. With the use of assays that can assess multiple end-points in a single donor, information in human primary cells may be now more accessible and exhaustive. However, except for their use to evaluate potential hazards for therapeutic agents, this approach has not been widely employed.

End-points that measure overall immune system function, such as: altered vaccine responses or changes in infectious disease incidence in exposed humans, or modulation of disease resistance in laboratory animals, are considered to provide the most informative evidence for a mechanistic role of immune suppression as for example in the evaluation of PFOA and PFOS (Groups 1 and 2B respectively, Volume 135). In another recent *IARC Monographs* volume, multiple studies that demonstrated increased self-reported infections were considered along with a large number of reports in experimental animals indicating suppression of tumour clearance, humoral- and cell-mediated immune function, changes in cytokine secretion and other measures as providing strong mechanistic evidence for the carcinogenicity of night shift work (Group 2A, Volume 124, IARC, 2020). There, various results were obtained from several observational end-points but were more difficult to interpret owing to the varying collection or the experimental conditions.

Normally, functional end-points that evaluate integrated responses should be considered as more predictive than quantification of cell populations. Thus, in some instances, data from laboratory rodent studies measuring outcomes associated with disease resistance may be more informative for cancer hazard evaluation than data from observational studies in exposed humans, human primary cells or cell lines.

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8. Key Characteristic 8: Modulates Receptor-Mediated Effects

Martin van den Berg and Maria Helena Guerra Andersen

8.1 Introduction

Many carcinogens exhibit receptor-mediated effects, as in the case of menopausal hormonal treatments or hormonal contraceptives, where evidence points to receptor mediation as a necessary mechanism for the agent's carcinogenicity (IARC, 2012a). Nevertheless, the mediated effects of receptor activation and cancer have complex pathways, often redundant or with dual roles, and much remains largely unknown.

Carcinogenic chemicals have been often described with the ability to act as ligands to nuclear receptor proteins, namely as agonists (Fig 8.1). Agonistic chemicals can activate or deactivate nuclear receptor (NR)-mediated processes, which includes their transportation and binding to the DNA-responsive element. Once bound, these agonists can act as transcription factors, inducing the process of RNA transcription. Subsequently, proteins are synthesized, leading to the (de)activation of biological responses within the cell. Many nuclear receptor proteins have known endogenous ligands (e.g. oestrogens and androgens), but for several of these nuclear receptors the natural ligands remain unknown, e.g. the aryl hydrocarbon receptor (AhR). The most common event after binding to a nuclear receptor relevant for carcinogenicity is cell proliferation, but this can also lead to apoptosis or induction of metabolism that activates xenobiotics into procarcinogens (Kim and Cheng, 2013). It is also important to note that for many well-known NRs there is a significant promiscuity with respect to ligand binding and differences in binding affinity. Due to promiscuity of ligands, the relationship with cancer, either with agonistic or antagonistic properties, can form the basis of the mechanism of action for carcinogenicity. Upon binding of an agonist to a nuclear receptor protein, a dimerization process often occurs, and various co-factors contribute to the activation process and transport the agonist into the nucleus before binding to DNA. This could be either homo- or heterodimerization, and this general process is illustrated in Fig 8.1 (Gangwar et al., 2022).

The KCs framework has been used to evaluate the mechanistic evidence since *Monographs* Volume 112. Of the 98 agents included in Volumes 112 to 135, 10 were reported to have consistent and coherent evidence for KC8 (Fig 8.2) (See Annex 1).

In this chapter, several NRs and their possible role in carcinogenesis are further described. However, it should be noted that the selected NRs are in no way complete but are merely used to illustrate the role of NRs as KC8 in initiating and mediating carcinogenic processes. The 10 agents for which evidence of KC8 was evaluated to be consistent and coherent were malathion, DDT, tetrabromobisphenol A, pentachlorophenol, 3,3′,4,4′-tetrachloroazobenzene, benzene, styrene, occupational exposure as firefighter, PFOA, and PFOS. For 10 additional agents, the evidence was moderate: lindane, 1-bromopropane, 2-mercaptobenzothiazole and dieldrin; or suggestive: night shift work, 1,1,1-trichloroethane, cobalt metal, soluble cobalt(II) salts, cobalt (II,III) oxide, and cobalt (II) oxide. These conclusions were based on disruption pathways mainly involving NRs (i.e. thyroid, aryl hydrocarbon, estrogen, androgen, progesterone and peroxisome proliferator-activated receptors), but also prolactin receptor and other pathways including hypothalamic-pituitary-gonadal axis interaction and cholinergic neurotransmission (see Fig 8.2 and Tables 8a and 8b).

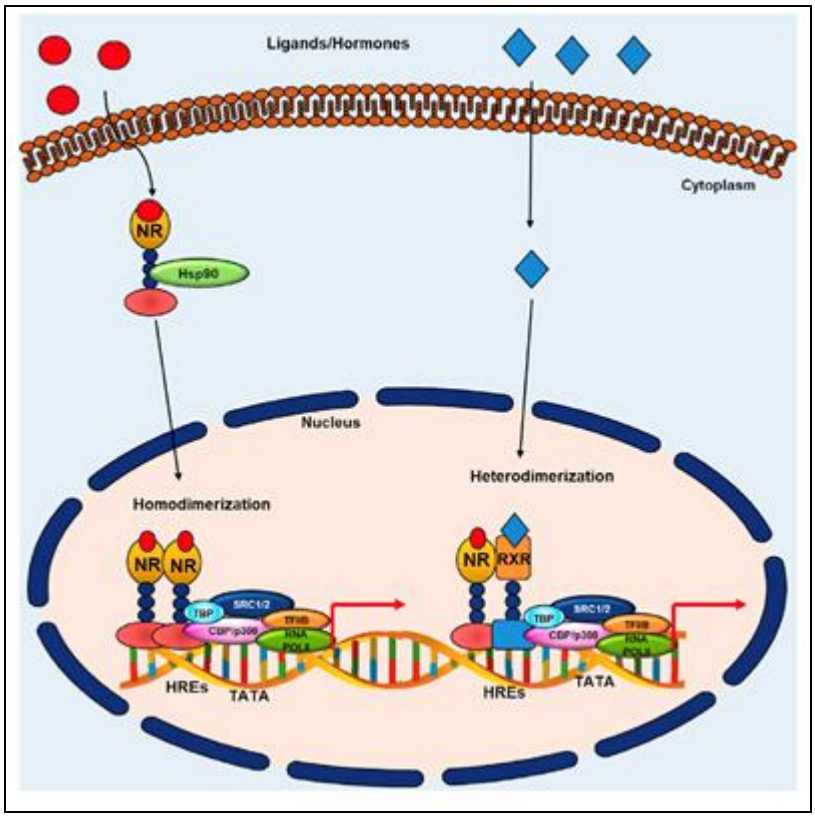

Figure 8.1 Dimerization process. General process of homo- and heterodimerization of nuclear receptors with binding to the DNA, and cofactors involved with DNA binding and activation (Gangwar et al. 2022).

8.2 Relevance of end-points

8.2.1 Selected nuclear receptors and membrane receptors that can play a role in carcinogenesis

Estrogen receptors

Among the many nuclear receptors (NRs) that have been studied in relation to carcinogenicity (Table 8a), the estrogen receptors are clearly best studied (Dhiman et al., 2018). This originates from the fact that the estrogen receptor α (ERα) plays a significant role in breast and ovary tumours, among others. It is generally recognized that binding of ERα to the DNA binding elements and subsequent mRNA expression stimulates cell proliferation and can initiate tumour formation (Jia et al, 2015). This process has been well described in relation to oncogenesis, but it is also of major importance for many reproductive and developing process in mammalian species, including humans (see Box 2). Treatment with estrogen receptor antagonists like tamoxifen has been highly successful, e.g. in certain breast cancer patients. Binding of these estrogen antagonists may reduce the risk of recurrence of estrogen-responsive tumours. However, these can also simultaneously cause pronounced disturbance of other essential endocrine processes in which ERα plays a distinct role.

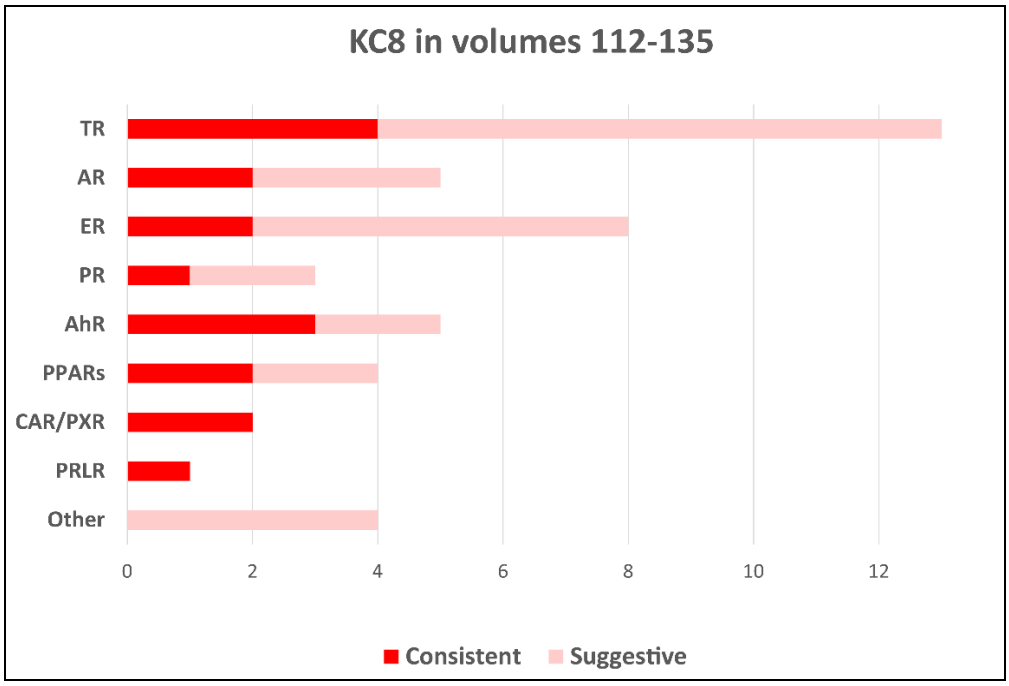

Figure 8.2. Receptor disruption pathways leading to the KC8 evidence evaluation as consistent and coherent, moderate, or suggestive in *Monographs* Volumes 112 to 135. The figure depicts the number of agents for which evidence was found to be consistent and coherent or suggestive for pathways involving the thyroid receptor (TR), the androgen receptor (AR), the estrogen receptor (ER), the progesterone receptor (PR), the AhR, the peroxisome proliferator-activated receptors (PPARs), the constitutive androstane receptor and pregnane X receptor (CAR/PXR), the prolactin receptor (PRLR) and other pathways (neurotoxicity through the inhibition of acetylcholinesterase, and hypothalamic-pituitary-gonadal and -adrenal axis interaction) (n=22 agents).

The estrogen β receptor (ERβ), unlike the ERα, has a clearly different role in oncogenesis involving both anti-ERα and tumour-suppressor functions. Thus, specific binding to ERβ should be considered more as an anti-carcinogenic effect. In addition, the actual function of ERβ in endocrine or other physiological process remains largely unknown (Huang et al., 2015).

One aspect regarding binding of chemicals to ERα has been the endocrine disrupting process in humans and wildlife, which has been associated with both tumour formation and dysregulation of reproduction and developmental processes in early life stages (see Box 2). Although much attention has been given to the endocrine disturbing process via the estrogen receptor, it should be noted that binding of a chemical to ERα by itself does not necessarily trigger a tumour initiating process *per se*. Nevertheless, such binding to ERα when observed in conjunction with other information regarding carcinogenic processes (as proliferation, steroidogenic enzymes modulation and cross-talks) is of high relevance to KC8 as a possible underlying mechanism of tumour formation.

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Table 8a End-points relevant to KC8: "modulates receptor-mediated effects"

Category	End-point	Relevance	Comment	References
Functional assays	Physiological responses of receptor activation	Assessing physiological responses to receptor agonists/antagonists, such as cell proliferation, differentiation, or changes in metabolic activity Provide insights into the functional consequences of receptor activation or inhibition associated with an agent exposure; Can be used to explain both carcinogenicity as well as anti-carcinogenicity.	Generally *in vitro*, but *in vivo* also possible in some cases	Lambert et al. 2006
Hormone levels	Circulating levels of receptor binding hormones	Measuring circulating levels of related hormones provides information about disruption in hormonal levels and the overall receptor-signalling environment associated with an agent exposure; Can be used to explain sex dependent tumour formation.	Suitable for assessing abnormal or altered levels in exposed humans or experimental systems *in vivo*	Key et al., 2011
Expression levels	Receptor expression and expression of genes regulated by the receptor and downstream signalling proteins	Changes in the expression levels and phosphorylation status of receptors and downstream signalling proteins can provide information about the consequences of receptor activation and the modulation of signalling pathways associated with an exposure agent; Changes in mRNA levels of target genes can indicate the activation or repression of receptor-mediated transcription; Can be used to elucidate pathways of chemical (anti-)carcinogenesis.	Suitable for assessing molecular changes occurring at the receptor, target gene, and transcription levels using *in vitro* and *in vivo* models, but also in exposed humans	Özturan et al., 2022; Aranda et al. 2009
Receptor activity	Reporter gene assays	Using a construct of a reporter gene under the control of the receptor responsive elements; When the receptor is activated and binds to these elements, it induces the expression of the reporter gene, allowing monitoring of the activation of specific receptor pathways; Especially relevant when involved in known carcinogenic pathways.	Generally *in vitro*	Steinberg et al., 2017

Table 8b. Evaluation of data associated with KC8 in *Monographs* Volumes 112 to 135[a]

Agent (Volume)	TR	AR	ER	PR	AhR	PPAR	CAR/PXR	PRLR	other	Human cancer site	Group	Cancer in humans	Cancer in experimental animals	Mechanistic evidence
Lindane (113)										NHL	1	Sufficient	Sufficient	Strong
Pentachlorophenol (117)										NHL		Sufficient	Sufficient	Strong
Benzene (120)										AML (NHL, CLL, multiple myeloma, CML, lung)		Sufficient	Sufficient	Strong
Occupational exposure as a firefighter (132)										mesothelioma, bladder (colon, prostate, testicular, melanoma and NHL)		Sufficient	Inadequate	Strong
PFOA (135)										(renal and testis)		Limited	Sufficient	Strong
Malathion (112)										(NHL, prostate)	2A	Limited	Sufficient	Strong
DDT (113)										(NHL, liver, testis)		Limited	Sufficient	Strong
2-Mercaptobenzothiazole (115)										(bladder)		Limited	Sufficient	Limited
Tetrabromobisphenol A (115)												Inadequate	Sufficient	Strong
3,3′,4,4′-Tetrachloroazobenzene (117)												Inadequate	Sufficient	Strong
Dieldrin (117)										(breast)		Limited	Sufficient	Limited
Night shift work (124)										(breast, prostate, colon and rectum)		Limited	Sufficient	Strong
Styrene (121)										(hematolymphoid malignancies)		Limited	Sufficient	Strong
1,1,1-Trichloroethane (130)										(myeloma)		Limited	Sufficient	Limited
Cobalt metal (131)												Inadequate	Sufficient	Strong
Soluble cobalt(II) salts (131)												Inadequate	Sufficient	Strong
2-Bromopropane (133)												Inadequate	Sufficient	Strong
1-Bromopropane (115)											2B	Inadequate	Sufficient	Strong
Cobalt (II) oxide (131)												Inadequate	Sufficient	Limited
Anthracene (134)												Inadequate	Sufficient	Limited
PFOS (135)												Inadequate	Limited	Strong
Cobalt (II, III) oxide (131)											3	Inadequate	Inadequate	Limited

AhR, aryl hydrocarbon receptor; AML, acute myeloid leukemia; AR, androgen receptor; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; CAR/PXR, constitutive androstane receptor and pregnane X receptor; ER, estrogen receptor; NHL, Non-Hodgkin lymphoma; PR, progesterone receptor; PPARs, peroxisome proliferator-activated receptors; PRLR, prolactin receptor; TR, thyroid receptor.

[a]The table includes the mechanistic evidence for the various receptor pathways and relevant cancer sites on which the evidence for cancer in humans was based, and the overall evaluation of each agent. Red colour when the evidence was considered consistent and coherent; Pink colour when the evidence was considered suggestive. Cancer sites in parentheses when associations were noted with limited evidence for cancer type in humans.

BOX 2. Relationship between sex steroid nuclear receptors (NRs) and steroidogenic enzymes

It is crucial to recognize that, while not considered nuclear or membrane receptors, many steroidogenic enzymes produce sex steroid hormones, the natural ligands of the ER, AR, or PR. These enzymes are found in various tissues throughout the body, including the ovaries, testes, adrenal glands, and certain parts of the brain, and play a vital role in numerous bodily functions, including growth and development, reproduction, and metabolism. However, it has also been established that either excessive or insufficient expression of these steroidogenic enzymes and their respective hormones can lead to a range of health problems, including several forms of cancer in which sex steroid hormones play a critical role (Luu-The, 2013).

Estrogen, a key sex hormone, is implicated in the development and progression of many breast tumours. Steroidogenic enzymes involved in estrogen production, such as aromatase (CYP19) and 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1), are often overexpressed in breast cancer cells. Both steroidogenic enzymes are involved in the conversions of androgens to estrogens and the conversion of estrone (E1) to estradiol (E2), the latter being the most potent form of estrogen. Notably, overexpression of aromatase and 17β-HSD1 in breast cancer cells can lead to elevated estrogen levels, which, through ERα, can promote cell growth and proliferation of breast tumour cells (Africander and Storbeck, 2018).

Other steroidogenic enzymes producing androgens play a role in the development and progression of prostate cancer. In particular, the 5α-reductase enzyme converts testosterone to the more potent dihydrotestosterone (DHT) and is often overexpressed in prostate cancer cells. Through binding to the AR, cell growth and proliferation can be initiated in prostate tumour cells (Sharifi and Auchus, 2012).

Additionally, the activity of steroidogenic enzymes involved in estrogen and progesterone production is crucial for the development and progression of endometrial cancer. It has been demonstrated that aromatase and 17β-HSD1 are frequently overexpressed in endometrial cancer cells (Plaza-Parrochia et al., 2017; Zhao et al., 2016).

Clearly, there are several similarities between the roles of steroidogenic enzymes in breast, prostate, and endometrial cancer. In all three cancers, overexpression of steroidogenic enzymes can lead to increased levels of sex hormones, which, through binding to sex hormone NRs, can promote cell growth and proliferation. Therefore, it can be concluded that modulation of sex steroidogenic enzymes should always be considered in conjunction with the role of ER, AR, and PR, which can influence both carcinogenesis and anti-carcinogenesis.

Androgen receptor

The role of the androgen receptor is best known in the carcinogenesis of prostate tumours. Treatment with an anti-androgen such as flutamide is a well-known therapeutic to reduce the recurrence of these tumours. In addition to the role of the androgen receptor in the formation of prostate tumours, there is increasing evidence that estrogen receptors may also be involved in the carcinogenesis of this male gland. It has been found that ERα has been upregulated in high-grade prostatic neoplasia, which may indicate that estrogens stimulate this type of tumour, via the ERα. In addition, it was reported that there was a reduction of ERβ expression in prostate tumours, which could indicate a loss of tumour suppression in the prostate (Bonkhoff, 2018). Thus, it may very well be that, in the case of prostate cancer and the role of the androgen receptor, there is a complex interplay occurring between this nuclear receptor and both estrogen receptors. Binding of a chemical to the androgen receptor may lead to an oncogenic process, e.g. in the prostate, and situations when this is observed in combination with other physiological information (namely, upregulated ERα and downregulated ERß), are of higher relevance to KC8.

Progesterone receptor

In contrast to estrogens and the estrogen receptors, the role of the progesterone receptor (PR) and progestins is less well known. Both *in vivo* and *in vitro* studies indicate that the PR and progestins are implicated in the carcinogenesis of breast cancer, but epidemiological studies do not convincingly

demonstrate such a relationship. From a mechanistic point of view, the role of the PR and associated transcription processes is difficult to discern from that of the Erα, because DNA binding and transcription processes are interlinked. It has also been suggested that PR and progestins can stimulate breast tissue growth and that cancer progression could be stimulated by the PR and progestins. In breast cancer, the cell proliferative effect by PR is mainly mediated via PRβ (Trabert et al., 2020). Moreover, it has been established that progesterone and the PR also have a role in antagonizing estrogen-stimulated effects on endometrial, ovarian and breast cancer (Kim et al., 2013). Thus, based on current knowledge, it can be concluded that the PR and progestins play a role in hormonal carcinogenesis, possibly through antagonistic driven effects on Erα-stimulated mitosis. Therefore, the possible role of the PR should be considered in both carcinogenic as well as anti-carcinogenic effects when evaluating a chemical for which interaction with the PR has been established.

Aryl hydrocarbon receptor

Besides the ERs, without any doubt the AhR is the best studied nuclear receptor. The AhR is expressed in many mammalian tissues, and its role in many physiological functions has been well described, including reproduction, development and in the pathophysiology of cancer. In human and mammalian tumour cells, the AhR is expressed, and its binding to DNA and transcription processes have been described in detail. Experimental studies indicate a dual role of the AhR in oncogenesis that involves both anti-carcinogenicity as well as carcinogenicity. It has been found that chronically active AhR plays a role in the tumour cell invasion, migration, and survival in addition to observed changes in stem cell characteristics. In many ERα-mediated hormonal tumour initiations, the AhR acts with an antagonistic role, which is explained by the negative crosstalk that occurs between the AhR and ERα DNA binding elements. In contrast, the AhR has also been implicated in pro-oncogenic effects in liver and stomach cancer (Safe, Lee, and Jin 2013; Safe, Cheng, and Jin 2017; Wang et al. 2020a). It should be noted that the binding of chemicals to the AhR is presently best known from that of a wide range of xenobiotics. One of the thoroughly studied transcription processes of the AhR is the induction of CYP1A1. Induction of this enzyme has a distinct role in the formation of genotoxic metabolites, e.g. with polyaromatic hydrocarbons (PAHs) and, as such, shows a clear relationship with pro-oncogenic properties of these compounds (Wang et al. 2020a). Notably, AhR showed stronger reactivity for dioxins and lower reactivity for some endogenous ligands in murine versus human AhR, and there were also with differences in transcriptional profiles (Flaveny and Perdew 2009); such interspecies differences should be taken into account (Wang et al. 2020a). Evidence invoked in IARC monographs has included end-points of the induction of cytochrome P450s (namely the levels of downstream CYP1 enzymes), increased rodent liver weights and thymic atrophy, decreased circulating thyroxine levels, comparisons between transgenic mice effects, agonistic effects, and metabolic enzyme activity modified by genotype. Overall, it must be concluded that in order to establish the relevance to KC8, binding of chemical to the AhR should be associated with additional (pro-)oncogenic effects, because binding of a chemical alone can also lead to anti-oncogenic effects (Safe et al., 2017).

PPAR receptors

The possible pro-oncogenic role of different PPARs is at present equivocal and, in fact, multiple studies with different PPAR receptors have instead indicated an anti-carcinogenic effect (Dhaini and Daher 2019). With respect to an anti-oncogenic role of PPARγ, it has been shown that it can regulate genes that are involved with cell cycle and differentiation. It has also been found that activation of PPARγ has anti-cell proliferative properties for several different tumour cells, including liposarcoma, breast adenocarcinoma, prostate carcinoma, colorectal carcinoma, non-small-cell lung carcinoma, pancreatic carcinoma, bladder cancer, gastric carcinoma, and glial tumours of the brain (Tyagi et al. 2011). The underlying mechanism of

anti-oncogenic properties of PPARγ has recently been studied in detail, and this receptor has been shown to have a role in lipid and glucose metabolism, and inhibition of inflammation and immune responses, while also suppression of cell proliferation and induction of cell differentiation (Chi et al. 2021). These combined processes are suggested to have an auxiliary function in some of the anti-carcinogenic properties that have been related to PPARγ. However, it has also been found that PPARγ may promote tumorigenesis via increase of intercellular adhesion and inhibition of apoptosis. Taken together, these mechanisms could explain the equivocal properties of PPARγ in (anti) tumorigenesis (Chi et al. 2021). With regard to breast cancer, the role of PPARs should certainly be considered further. In addition, there is a distinct link between PPAR gene–environment interactions, in which both genetic polymorphism as well as type of experimental exposure can be influential (Dhaini and Daher 2019). The role of PPARα has been suggested in rodent hepatocarcinogenicity, and the extent to which this possible oncogenic role has human relevance has been discussed. Moreover, there are experimental indications that hepatocarcinogenesis in rodents can also develop independently from PPARα (Guyton et al. 2009). Thus, for chemicals that show simultaneous binding to PPARs as well as oncogenic effects, a careful examination should be included of the underlying mechanisms to establish the role of this receptor in the observed carcinogenic effects.

Thyroid hormone receptor

It has clearly been shown in experimental studies that thyroid hormone receptors (TRs) can play an important role in tumour progression. However, no unequivocal role of TRs in humans has been shown thus far. The TRs have an endogenous role in normal cell growth and differentiation, but in several human cancer cells TRs also act as growth suppressors (Aranda et al. 2009). In fact, it has been observed that TRs are frequently mutated, or their expression is reduced in human cancer cells, which may lead to possible oncogenic processes in which the tumour supressing activity of TRs may be diminished. *In vitro* studies with pituitary cell lines, as well as estrogen-responsive breast and prostate cell lines, have shown that thyroid hormones induce cell proliferation, hence, it is reasonable to anticipate that TRs may indeed play a role (Aranda et al. 2009). Overall, it can be concluded that loss of normal function or mutations in TRs can be involved in human carcinogenesis, progression, and metastasis, but further experimental studies should evaluate the possible role of TRs (Kim and Cheng 2013). Thus, changes in normal thyroid hormone functions and TRs in conjunction with observed carcinogenic effects of the agent should be further evaluated in relation to the possible underlying role of TRs.

Membrane receptors

Besides intracellular nuclear receptors, multiple membrane proteins play a pivotal role in the formation and progression of cancer. Overexpressed membrane receptors (MRs) can be considered a hallmark of cancer cells and are increasingly targeted in cancer therapies. It has been well established that MRs can significantly facilitate interactions between tumour cells and the tumour microenvironment (Kampen 2011)

One of the well-studied MRs is the epidermal growth factor receptor (EGFR), which has a physiological role in regulating the development and homeostasis of epithelial tissues. However, in pathological conditions, among others in lung and breast cancer and glioblastoma, the EGFR becomes a driver of tumorigenesis (Sigismund et al., 2018). Aberrant activation of the EGFR by, for example, epiregulin (EREG) has been found to be a significant contributor to various types of tumours. Elevated levels of EREG in cancer cells primarily activate EGFR signalling pathways, thereby promoting cancer progression. Furthermore, inappropriate EGFR activation in cancer can result from various mechanisms, including genomic amplification, point mutations, transcriptional upregulation, or increased ligand production. These abnormalities contribute to the initiation and progression of cancer in multiple tissues. Thus, overexpression

of the EGFR may be considered a relevant end-point for KC8, as it is a common feature in various cancers, including lung, colorectal, and breast cancer (Cheng et al. 2021; Sigismund et al., 2018).

Another important MR in cancer, that might be a relevant end-point for KC8, is the human epidermal growth factor receptor 2 (HER2), a member of the tyrosine kinase receptors. Approximately 20% of breast cancers exhibit HER2 overexpression, leading to more aggressive disease and a poorer prognosis. Heterodimerization of HER2 with other members of the EGFR family, often resulting from HER2 overexpression, leads to autophosphorylation of tyrosine residues and the activation of multiple signalling pathways, contributing to cellular proliferation and tumorigenesis (Oh and Bang 2020).

The vascular endothelial growth factor receptor (VEGFR) is crucial in angiogenesis (relevant end-point also for KC10), which is also an essential process for tumour growth and metastasis. VEGF, particularly VEGF-A, binds to VEGFR2 and triggers a cascade of events promoting cell survival, proliferation, and migration. Additionally, VEGF exhibits immune-regulatory properties that suppress immune cell antitumour activity, making it a key player in tumour angiogenesis (Ghalehbandi et al. 2023). The platelet-derived growth factor receptor (PDGFR) is another essential MR in cancer, and it should be considered as a possibly relevant end-point for KC8. The PDGFR is involved in cell growth and proliferation and is overexpressed in various cancer types, such as glioblastoma, sarcoma, and colorectal cancer. As a result, the PDGFR pathway is crucial for the growth and spread of several cancers. PDGFs and their receptors are often significantly expressed in malignant tumour cells and various organs. The PDGF/PDFGR pathway stimulates tumour cells in an autocrine or paracrine manner, thereby promoting tumour growth, invasion, angiogenesis, and migration (Pandey et al. 2023).

8.3 Interpretation of results: nuclear and membrane receptors

The well-known NRs, estrogen, androgen, progesterone, arylhydrocarbon, PPAR, and thryroid hormone receptors, were reviewed to determine their role in initiating an oncogenic mechanism of action. These receptors are by no means the only ones with some role in oncogenic processes. In this context, other NRs, such as nuclear orphan receptors, should also be considered in IARC's evaluation processes of carcinogenic properties of xenobiotics (Safe et al. 2014; Mohan et al. 2012). To establish relevance of end-points for KC8, some factors warrant consideration, namely, the association of the receptor binding by itself to established carcinogenic cellular and pathological effects, including tissue and species specificity. For example, the anti-estrogenicity initiated by the AhR and anti-cell proliferative properties of ERβ have been well described (Safe et al., 2017; Huang et al., 2015). In addition, MRs like EGFR, HER2, VEGFR, and PDGFR, and others also play crucial roles in tumour development and progression. When overexpression and dysregulation of these MRs and the role of their natural ligands is associated with exposure to exogenous agents, these MRs may be considered relevant end-points for KC8. For KC8, NRs as well as MRs retain an essential role in IARC's evaluations of carcinogenicity, because there is ample evidence that many of these receptors are a key factor in initiating a molecular event that will eventually lead to carcinogenicity. However, the ligand-binding process to a NR or MR followed by DNA binding and, consequently, mRNA transcription in conjunction with downstream cellular processes such as cell proliferation, apoptosis, and changes in cell cycle arrest is of greater relevance.

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9. Key Characteristic 9: Causes Immortalization

Roger Reddel and Jason Fritz

9.1 Introduction

Immortalization, which is the acquisition of unlimited replicative capacity, is a hallmark of cancer and major contributor to oncogenesis (Hanahan and Weinberg, 2000), and it occurs in human cells via a multistep process. Human somatic cells have multiple barriers to immortalization, and its occurrence therefore requires a series of genetic events, not all of which are known or fully understood (Reddel, 2000). The first barrier to immortalization is senescence, which may be replicative or stress-induced (Fig 9.1, panel 1). This is followed by an escape due to inactivation of the p53 and pRB/$p16^{INK4a}$ pathways (Reddel, 2000), but this restoration of proliferative capacity is limited: eventually these cells enter a state referred to as crisis characterized by telomere dysfunction and extensive autophagic cell death (Nassour et al., 2019). Survival and immortalization of human cells following crisis is achieved via activation or upregulation of a telomere length maintenance mechanism (TMM) – either telomerase or alternative lengthening of telomeres (ALT) (Colgin and Reddel, 1999). While tumorigenesis can result from the immortalization of somatic human cells, immortalization per se is insufficient for tumorigenesis; other phenotypic traits have been described to contribute to neoplastic transformation in addition to immortalization, including a loss of cellular differentiation and proliferative regulation (LeBoeuf et al., 1999). For example, in *in vitro* models of human cellular immortalization, additional pro-oncogenic (epi)genetic changes such as overexpression of a *RAS* oncogene (which can facilitate insensitivity to growth-inhibition as well as directly stimulate cellular proliferation) were required to render human cells tumorigenic in immunocompromised mice (Amstad et al., 1988).

9.2 Relevance of end-points

9.2.1 Senescence and temporary escape

Replicative senescence refers to the cell cycle exit, which is normally permanent, that occurs when proliferating cells reach a cumulative population doubling level (Hayflick, 1965) often referred to as the "Hayflick limit." Cell cycle withdrawal is thought to be due to replication-dependent telomere shortening (Olovnikov, 1973), which results in DDR signalling (d'Adda di Fagagna et al., 2003) (see also Chapter 3). Stress-induced senescence has a range of causes including overactive oncogene signalling, viral infection, and oxidative stress (Reddel, 1998) (See Fig 9.1).

Universal markers of human cell senescence have not yet been identified, but in addition to permanent withdrawal from the cell cycle and the associated changes in p53 and pRB/p16INK4a (p16; CDKN2A) signalling (Campisi, 2001), which may be key indicators of senescence, other features of senescent cells may include characteristic morphological changes (Hayflick, 1965), upregulation of β-galactosidase activity (Dimri et al., 1995), the senescence-associated secretory phenotype (SASP) which is essentially an inflammatory response (Rodier et al., 2009), and senescence-associated heterochromatic foci (SAHF) (Narita et al., 2003). The PI3K/mTOR/FoxO pathway is involved in aspects of the senescent phenotype, including increased cell mass, autophagy, and cell survival (Zhang et al., 2000; Carroll et al., 2017).

Although senescence protects against the continued proliferation of cells that are damaged (e.g. by excessively short telomeres or with activated oncogenes), SASP is potentially pro-tumorigenic which has led to speculation that senescence evolved for some purpose other than tumour suppression, such as protection against viral infection (Reddel, 2010).

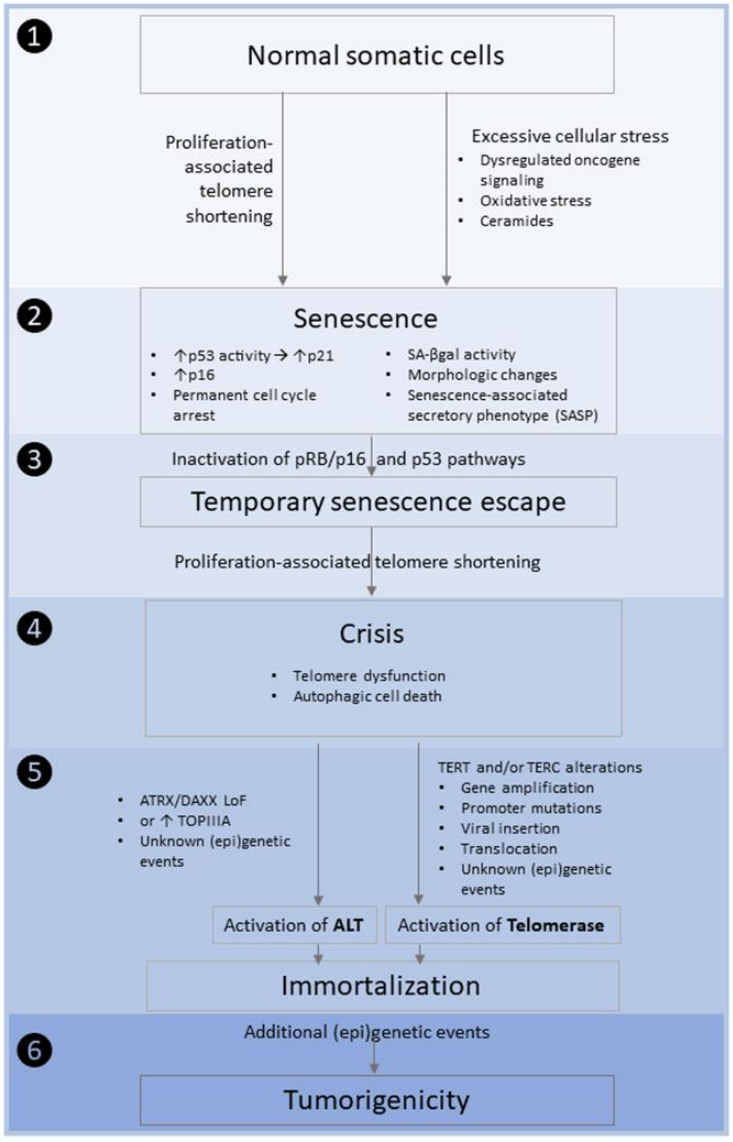

Figure 9.1 Molecular changes required for immortalization of normal human somatic cells. p16: p16INK4a, CDKN2A; p21: p21WAF1/CIP1, CDKN1; SA-βgal: senescence-associated -galactosidase; pRB, retinoblastoma protein; LoF, loss of function; TERT, telomerase reverse transcriptase; TERC, telomerase RNA component; ALT, alternative lengthening of telomeres.

In *in vitro* human model systems of immortalization, escape from senescence occurs due to inactivation of the p53 and pRB/p16^{INK4a} pathways (Reddel, 2000). This may be induced by mutations in the *TP53* and *CDKN2A* genes, epigenetic changes that silence expression of *CDKN2A*, or by viral oncoproteins that

inactivate p53 and pRB (and the pRB-related proteins, p107 and p130). For example, the high-risk (i.e. oncogenic) human papillomaviruses (HPV) encode E6 and E7 proteins that degrade p53 and pRB, respectively. Alterations of these tumour suppressors contribute to other hallmarks of cancer including insensitivity to anti-growth signals, evading programmed cell death, deregulated metabolism, and genome instability. The escape from senescence that occurs by these means is only temporary: cells in which the p53 and pRB/p16 pathways are inactivated proliferate for a limited number of population doublings during which additional telomere shortening occurs, and the cells eventually enter a state referred to as crisis through telomere-mediated activation of the innate immune signalling pathway (Nassour et al., 2023).

9.2.2 Immortalization and Tumorigenicity

Crisis is characterized by telomere dysfunction and extensive autophagic cell death (Nassour et al., 2019), and human cells escape from crisis at a very low frequency. For example, escape from crisis occurs in only about 1–3 in 10^7 human fibroblasts infected with simian virus 40 (SV40) (Huschtscha and Holliday, 1983; Shay et al., 1993) which encodes an oncoprotein (SV40 large T antigen) that binds and inactivates p53 and the pRB protein family. Without any known exceptions, immortalization of human cells in model systems has been accompanied by upregulation of a telomere length maintenance mechanism (TMM) – either telomerase or ALT (Colgin and Reddel, 1999) (see Fig 9.1).

Telomerase is a ribonucleoprotein enzyme that synthesizes telomeric DNA by reverse transcription and thereby counteracts the telomere shortening that normally accompanies replication of the genome. Its catalytic core consists of a reverse transcriptase protein subunit, TERT, and an RNA template molecule, TERC (Bryan and Cech, 1999). In most somatic human cells, telomerase activity is undetectable or present at a low level that is insufficient to prevent telomere shortening (see Box 3). The genes for both catalytic subunits are haploinsufficient, and their products are present in rate-limiting amounts. Increased telomerase enzymatic activity therefore requires upregulation of both TERT and TERC (Cairney and Keith, 2008). "Activation" of telomerase in cancer (i.e. upregulation of telomerase activity to a level that is sufficient to prevent telomere shortening) may be associated with amplification of the TERT and/or TERC genes (Cao et al., 2008). TERT gene expression may also be increased by mutations at specific locations in its promoter region (the most common recurrent non-coding mutations in cancer), which create ETS family transcription factor binding sites, altered promoter methylation, altered transcriptional control (e.g. by the product of the MYC gene), structural rearrangements, insertion of viral enhancers upstream of the gene, and post-transcriptional changes in TERT mRNA (Yuan et al., 2019). Most oncogenic viruses, including HPV, Epstein-Barr Virus (EBV), Kaposi's sarcoma-associated herpesvirus (HHV-8), hepatitis B and C viruses, and human T-cell leukaemia virus-1 (HTLV-1) (Tornesello et al., 2022) – despite being very different types of viruses – encode proteins that participate in transcriptional upregulation of the TERT gene. However, in some cancers the mechanism underlying telomerase activation remains unknown. TERT upregulation is claimed to have oncogenic effects well beyond its role in replicative immortality, including in enhancement of stemness, epithelial-mesenchymal transition, survival signalling, growth signalling, and angiogenesis (Low and Tergaonkar, 2013; Yuan et al., 2019).

Unlike the reverse transcriptase telomerase, ALT is a recombination-dependent DNA repair mechanism that uses telomeric DNA as a template for de novo synthesis of telomeric DNA to counteract the telomeric shortening that accompanies replication (Dunham et al., 2000). Similar to telomerase, it is possible that ALT activity occurs in normal somatic cells at a very low level that is insufficient to prevent telomere shortening. Activation of ALT in cancer may be associated with inactivating mutations in ATRX or DAXX genes (Heaphy et al., 2011a) whose protein products form a heterodimer that is involved in remodelling of

repetitive chromatin (Dyer et al., 2017). Loss of ATRX/DAXX function is insufficient for activation of ALT, and the additional events are currently unknown but may be epigenetic. In ALT-activated cancers with wild-type ATRX/DAXX, overexpression of TOPIIIA may occur (de Nonneville et al., 2022).

BOX 3. Increased telomere length

Long telomeres have been associated with numerous human cancers, including NSCLC, glioma, chronic lymphocytic leukaemia, melanoma (reviewed in McNally et al. (2019)), thyroid cancer, and osteoscarcoma (reviewed in Chen et al., 2023), while not in non-melanoma skin cancers (Caini et al., 2015), and inversely associated with gastrointestinal cancer risks (Giaccherini et al., 2021; Gao et al., 2023). For some human neoplasms, different associations were observed in tumour versus peripheral tissues: decreased survival was associated with shorter telomeres in peripheral blood leukocytes (PBLs) and longer telomeres in colorectal cancers (Pauleck et al., 2023); while increased cancer risk was associated with shorter telomeres in prostate stroma and epithelium, but negatively associated with shorter telomeres in PBLs (Hu et al., 2019); and better outcomes were associated longer telomeres in breast cancer tissues, but not PBLs (Ennour-Idrissi et al., 2017). Other non-cancer proliferative disorders such as metabolic syndrome have been associated with longer telomeres, while an inverse association was observed for idiopathic pulmonary fibrosis (reviewed in Chen et al. (2023)). Longer telomeres have also been associated with environmental and/or lifestyle factors such as increased physical activity, better sleep habits, and less or no smoking (reviewed in Astuti et al. (2017); Barragán et al., (2021)), observations which further complicate associations between telomere length and human cancers as these factors are also independently associated with reduced cancer risk.

As noted previously, while some TMM-negative cancer cells with long telomeres have been reported to replicate in vitro for a substantial number of PDs (Dagg et al., 2017) and form lethal tumours (Dagg et al., 2017, reviewed in McNally et al. (2019)), this suggests that telomere length together with tumorigenicity are not sufficient evidence of immortalization. Given that some cells can undergo extensive proliferation in the absence of a TMM because the starting length of their telomeres is long, there may be circumstances where it would be of interest to test for agents that result in increased telomere length in the absence of a TMM. There is evidence that germline mutations associated with long telomeres are a risk factor for cancer (reviewed in Liu et al. (2018) and McNally et al. (2019)), but although agents such as polychlorinated biphenyls have been reported to be associated with increased peripheral blood leukocyte telomere length (O'Sullivan et al., 2014; Scinicariello & Buser, 2015), it is not clear whether this is due to TERT transactivation via increased MYC oncogene activity. At present, there do not appear to be any well-documented examples of carcinogens where TMM-independent telomere lengthening is a mechanism of action.

Some cancers do not exhibit activation of telomerase or ALT, and cell culture studies have shown that TMM-negative cancer cells with long telomeres are able to proliferate for very large numbers of population doublings while undergoing continuous telomere shortening (Dagg et al., 2017; Viceconte et al., 2017). Cancer cells with this "ever shorter telomeres" (EST) phenotype can produce tumours that may be lethal (Dagg et al., 2017). A recent study of neuroblastomas has found evidence that TMM-negative tumours have undergone less clonal evolution than those with an activated TMM, which is consistent with them having undergone fewer population doublings and hence less telomere shortening and no selection pressure to activate a TMM (Körber et al., 2023). Conversely, immortalization alone is insufficient for tumorigenesis, as other traits including insensitivity to growth-inhibition, loss of cellular differentiation, and proliferative dysregulation contribute to neoplastic growth in both human (Amstad et al., 1988; LeBoeuf et al., 1999) and murine cells (May et al., 2005) (see also Box 3).

9.3 Assessing the relevance of end-points in different test systems

Experimentally, transduction of supposedly normal cells with TERT expression constructs is able to bypass senescence and result in immortalization of cells without any apparent crisis, but the first reports of this outcome involved the use of cells that have an unusually weak ability to upregulate p16 (Bodnar et al., 1998; Vaziri and Benchimol, 1998), and in other experiments immortalization by TERT overexpression was accompanied by spontaneous loss of p16 expression and p53 mutation (Kiyono et al., 1998; Noble et al., 2004).

An agent that appeared to be able to cause immortalization directly is Epstein-Barr virus (EBV) (Group 1, *Monographs* Volume 100B), which produces a profound increase in the proliferative capacity of human lymphocytes. However, careful study of the proliferation of lymphocytes following EBV infection showed that the lymphocytes cease proliferating at around 160 population doublings unless they undergo activation of telomerase (Sugimoto et al., 2004), which suggests that EBV infected cells may be able to escape from senescence but may not be able to avoid crisis. Similarly, oncogenic HPVs encode proteins that enable cells to bypass senescence and to transactivate TERT, but additional genetic events are required for immortalization (DeSilva et al., 1994). To assay for the ability to cause the critical last steps in immortalization, which involve activation of a TMM (i.e. telomerase and/or ALT), cells that have escaped from senescence (Fig 9.1, panel 2), e.g. by expression of viral oncoproteins that inactivate the p53 and pRB pathways (Fig 9.1, panel 3), could be used. Cells that escape from crisis (Fig 9.1, panel 4) usually become immortalized (Fig 9.1, panel 5), so some immortalization assays involve scoring the number of colonies of proliferating cells emerging from a background of culture crisis; accuracy of these assays is limited by the extent to which cells emerging from crisis are able to seed multiple colonies. Various assays and end-points have been investigated to gain insight into the potential for exposures to induce functional immortality (see Table 9); the relevance and limitations of several such measures are described below.

Table 9. End-points relevant to KC9: "causes immortalization"

Category	End-point	Relevance	Comments [a,b]	Reference
Senescence	↑ B-galactosidase, CDKN2A, CDKN1A, TP53 activity	↓ cell cycle progression, ↑ protein activity, expression	Step 2 Limitations associated with lack of information on sustained proliferative capacity	Campisi, 2001; Hayflick, 1965; Dimri et al., 1995
Senescence escape	Changes in p53 and/or pRB/p16^{INK4a} expression levels	Inferred p53 and/or pRB/p16^{INK4a} inactivation, escape from growth inhibition	Step 3 Limitations associated with lack of functional consequence measured	Yasaei et al., (2013); Thomas et al., (2022)
	p53 and/or pRB/p16^{INK4a} inactivation	↑ cell cycle progression, associated with viral protein expression or CTA model system (e.g. SHD cells)	Step 3 Some limitations due to functional consequence measured	Carnero et al., (2015); DeCaprio (2021)
Telomerase	↑ telomerase activity by TRAP	Counteracts telomere shortening, permitting continuous proliferation	Step 5 Strengthen: Direct measure of ↑ telomerase activity	Kim et al., (1994); Mender and Shay (2015)
	↑ TERT and TERC expression	Haplo-insufficient mediators of telomerase activity	Step 5 Some limitations due to inferred measure of ↑ telomerase activity	Carnero et al., (2015)

Table 9. End-points relevant to KC9: "causes immortalization"

Category	End-point	Relevance	Comments [a,b]	Reference
	Cellular morphology with ↑ Sox2/Cdx2 gene expression	Stemness, iPSC capability and inferred immortalization	Step 5 Some limitations due to associations with ↑ telomerase activity	Wakao et al., (2012)
	↑ Sox2/Cdx2 gene expression	Stemness, iPSC capability and inferred immortalization	Step 5 Limitations due to expression alone not predicting ↑ telomerase activity	Noureen et al., (2021)
Alternative lengthening of telomeres (ALT)	Partially single-stranded circles of telomeric DNA	C-circle assay can be used to detect a phenotypic marker of ALT activity	Step 5 Strengthen: direct measure of ↑ activity of TMM	Yasaei et al., (2013)
	Telomeric ssDNA	ALT-FISH		Frank et al., (2022)
	APB, ATSA	APB, ATSA		Yeager et al., (1999); Zhang et al., (2019b)
	Ultrabright telomeres			Heaphy et al., (2011b)
	MiDAS			Min et al., (2017)
ALT or Telomerase	↑ telomere length	Phenotypic, indirect evidence of possible TMM activity	Step 5 Some limitations due to tissue specificity of effect, other explanatory factors	Liu et al., (2018); McNally, et al., (2019)
Immortalization	Indefinite proliferative capacity with consistent telomere length	Continuous proliferation permits neoplastic expansion	Step 5 Strengthen: phenotypic measures defined as immortalization, but experimentally labour-intensive	Bryan and Reddel, (1994)
Tumorigenicity	↑ response in CTA	Morphological transformation, phenotypic expression of neoplastic characteristics	Step 6 Some limitations due to numerous other contributory factors, no measure of TMM	OECD (2015, 2017)

ALT, alternative lengthening of telomeres; APBs, ALT-associated PML (promyelocytic leukaemia) bodies; ATSA, ALT telomere synthesis in APBs; CTA, cell transformation assay; iPSC, induced pluripotent stem cell; MiDAS, synthesis of telomeric DNA in mitosis; SHD, Syrian hamster dermal fibroblast; ssDNA, single-stranded DNA; TERC, telomerase RNA component; TERT, telomerase reverse transcriptase; TMM, telomere length maintenance mechanism; TRAP, telomerase repeated amplification protocol

[a] Steps correspond to Fig 9.1: Molecular changes required for immortalization of normal human somatic cells. Step 1: Normal somatic cells; Step 2: Senescence; Step 3: Temporary senescence escape; Step 4: Crisis; Step 5: Immortalization via activation of telomerase and/or ALT; Step 6: Tumorigenicity.

[b]The data described potentially contribute to the overall weight of evidence (WoE) determination for KC9 – Causes Immortalization.

9.3.1 Immortalization assays

Narrowly defined, a human cell immortalization assay uses normal human cells and tests whether an agent can cause immortalization, a process that usually takes many months of cell culture. To be certain that immortalization has occurred it is necessary to continue serial passaging for a sufficient number of population doublings (PDs) to be reasonably certain that indefinite proliferative capacity has been acquired

and that telomere length is being maintained. The required number of PDs is context-dependent: for EBV-induced immortalization of lymphocytes 200 PDs s required, but in other contexts 100 PDs after emergence from crisis may be sufficient.

As will be evident from their duration and labour-intensiveness, immortalization assays are currently not suitable as a screen for carcinogenicity, and they are best reserved for studying agents where priming cells for immortalization is suspected to be an important aspect of their mechanism of action. This applies especially to certain classes of virus, but the immortalization assays need to be complemented by epidemiological evidence to conclude that such agents are human carcinogens. For example, SV40 early region genes have been extensively studied for their ability to inactivate p53 and proteins of the pRB family, and thereby permit escape from senescence, followed by rare cells escaping from crisis and becoming immortalized by additional genetic events (Shay et al., 1993; Bryan and Reddel, 1994), but definitive evidence that SV40 is a human carcinogen is lacking (Group 3, *Monographs* Volume 104, IARC, 2013; Rotondo et al., 2019).

A surrogate end-point for immortalization assays is detection of robust levels of telomerase activity or markers of ALT. Telomerase activity is usually detected by a version of the TRAP assay (Kim et al., 1994). Somatic cells reprogrammed by forced expression of Oct3/4, Sox2, c-Myc, and Klf4 may become induced pluripotent stem cells (iPSC) (Takahashi and Yamanaka, 2006), which are functionally immortal owing to expression of telomerase activity (Huang et al., 2014). iPSC can be identified accurately by a combination of morphologic parameters and expression of endogenous Sox2 and Cdx2 (Wakao et al., 2012), which in this context could therefore be used to infer the presence of telomerase activity. However, although in other contexts telomerase expression correlates with stemness, expression of the Sox2 and Cdx2 genes has not been found to be useful for predicting telomerase activity (Noureen et al., 2021).

Activation of ALT can be detected by the presence of various phenotypic markers, especially the C-circle assay (Henson et al., 2009) that detects by isothermal amplification the presence of partially single-stranded circles of telomeric DNA (which has the sequence 5′TTAGGG3′) in which the C-rich strand is intact, and the G-rich strand is gapped. Depending on the context, assays for ALT-associated PML bodies (APBs) (Yeager et al., 1999), ultrabright telomeres (Heaphy et al., 2011b), single-stranded telomeric DNA (ALT-FISH) (Frank et al., 2022), synthesis of telomeric DNA in mitosis (MiDAS) (Min et al., 2017), or ALT telomere synthesis in APBs (ATSA) (Zhang et al., 2019b) may also be useful.

Viruses that contribute to immortalization by expression of oncoproteins that, for example, interfere with p53 and/or the pRB pathway, or transcriptionally activate TERT exert effects that do not appear to be covered by other KCs. The mode of interaction of viral oncoproteins with the p53 and pRB pathways is complex, and appropriate assays are therefore challenging to design. For example, SV40 large T antigen results in a large increase in p53 protein levels (Thomas et al., 1983), whereas high-risk HPV E6 proteins degrade p53 protein resulting in very low levels (Scheffner et al., 1990). SV40 large T antigen also sequesters non-phosphorylated pRB, p107, and p130 proteins by binding to its LXCXE motif and thereby causes functional inactivation of the cell cycle inhibitory effects of these proteins without a major change to their levels (Ludlow et al., 1989), whereas high-risk E7 proteins bind and degrade pRB (Dyson et al., 1989). Merkel Cell Polyomavirus (MCPyV) encodes a large T antigen that does not bind or inactivate p53, but it does bind to pRB via its LXCXE motif and inactivate it (DeCaprio, 2021).

Readouts of the functional consequences of interference with these key pathways may therefore be the most appropriate assays, and key to understanding whether the molecular effect(s) reported is in fact associated phenotypically with immortalization. For example, detecting the combination of upregulated $p16^{INK4a}$ and upregulated E2F-mediated cell cycle effects may indicate interference with function of the pRB

protein family. The p53 protein is involved in such a wide range of cellular functions (Thomas et al., 2022) that it is difficult to assay for all potential downstream consequences of interference with its activity, but suitable assays could, for example, detect reduced ability to upregulate p21 protein in response to DNA damage (el-Deiry et al., 1993) or a larger range of downstream effects (Andreotti et al., 2011).

Ideally, assays to detect agents that upregulate telomerase activity would address both the TERT and TERC components of the enzymes catalytic core (Cairney and Keith, 2008). TERT-reporter assays have been described (Carnero et al., 2015), and normal cell strains with reporters knocked-in to the TERT locus would be very useful for this purpose. The C-circle assay could be used to detect agents that induce ALT activity (Yasaei et al., 2013).

9.3.2 Cell transformation

Cell transformation assays (CTAs), which have previously been regarded as a surrogate for immortalization when evaluated as part of a multicomponent carcinogenicity assessment, more directly test the dysregulation of proliferation as an aspect of myriad pro-tumorigenic properties which suspect carcinogens may confer upon on cells (Fig 9.1, panel 6,) versus evaluation of immortality per se. CTAs rely upon morphological transformation as a phenotypic readout of onco-transformation, which can result from a variety of (epi)genetic as well as other mechanisms (summarized in OECD, 2007) and are associated with cells exhibiting neoplastic potential (Barrett and Ts'o, 1978; Kakunaga and Yamasaki, 1985), including the ability to induce tumours in susceptible animals (Berwald and Sachs, 1963; Newbold et al., 1982; Elias et al., 1989; Sasaki et al., 2015). As with many aspects of multistage tumorigenesis *in vivo*, the precise molecular mechanisms involved in cell transformation *in vitro* are only partially understood, but evidence supports (epi)genetic involvement in alteration of cell cycle control, genomic stability, proliferation, and differentiation (LeBoeuf et al., 1999). Several phenotypic stages have been described in cellular transformation, including: (a) loss of cellular differentiation; (b) acquisition of immortality related to an apparently unlimited lifespan and genetic instability; (c) dysregulation of proliferation associated with tumorigenic phenotypes; and (d) neoplastic growth upon implantation *in vivo* (LeBoeuf et al., 1999).

Cells commonly used in CTAs historically included rodent BALB/3T3 and C3H/10T1/2 fibroblasts (reviewed in OECD, 2007; Vasseur and Lasne, 2012), SHE (reviewed in OECD, 2015), and more recently Bhas 42 cells (reviewed in OECD, 2017), derived by stable transfection of multiple Ha-*ras* copies into BALB/3T3 A31–1–1 fibroblasts and considered to be initiated (Sasaki et al., 1988). Transformation in CTAs has been induced by human c-Ha-*ras* plus *myc* genes (Land et al., 1983), chemical agents like TPA known to induce *myc* expression (Hsiao et al., 1984; Müller et al., 1984), and human c-Ha-*ras* plus polyoma virus middle-T genes (Ruley, 1983), and other chemical carcinogens (Newbold and Overell, 1983) in primary cells. While *RAS* family genes are frequently manipulated, not all mechanisms leading to cell transformation involve mutations or overexpression of *RAS* genes: other (epi)genetic alterations are also associated with cell transformation (Hahn and Weinberg, 2002; Futscher, 2013; Yasaei et al., 2013), including some historically associated with immortalization (Newbold et al., 1982, Stepanenko and Kavsan, 2012).

CTAs using rodent cell cultures where the p53 pathway and/or the pRB/p16^{INK4a} pathway is the sole barrier that needs to be bypassed to achieve immortalization allow the assessment of p53 and/or pRB/p16^{INK4a} inactivation in these cells (Carnero et al., (2015)). For example, Syrian hamster dermal fibroblast (SHD) cells undergo stress-induced, but not replicative, senescence. SHD cultures treated with ionizing radiation (IR), benzo(a)pyrene (BaP), or nickel escape from senescence by inactivation of the p53

and INK4A/INK4B pathways by gene deletion (IR), point mutation (BaP), or epigenetic silencing (nickel) (Yasaei et al., 2013).

9.4 Interpretation of results within the same database

Immortalization is an important component of the oncogenic process across species, as well as a hallmark of cancer in humans (Hanahan and Weinberg, 2000). The molecular pathways to immortalization involve many (epi)genetic changes, and many of these changes (including mutations in the TP53 and pRB/$p16^{INK4a}$ pathways which are key to escape from senescence) have major implications for other aspects of oncogenesis, and as such are facilitated by other KCs such as mutagenicity, clastogenicity, etc. Moreover, there are not many carcinogens that are known to be directly immortalizing agents in human somatic cells. Human cell immortalization assays are very time-consuming and are unlikely to be identified in the literature, except where there is other evidence that this may be part of the mechanism of action of an agent being evaluated for carcinogenicity, such as for viruses endemic in human populations.

CTAs, conversely, can provide a phenotypic readout of carcinogenic potential after a few weeks of exposure, but owing to the potential involvement of nearly all other KCs there is rarely evidence linking any effects observed specifically to immortalization and KC9 per se. Moreover, CTAs are typically performed in rodent cells, including hamster embryos and mouse fibroblasts, which may achieve immortalization more easily than human somatic cells progressing through the multiple steps outlined above and in Fig 9.1 (Takahashi and Yamanaka, 2006). It is worth noting that the complexity of the end-points available to evaluate KC9 makes it difficult to provide strong mechanistic evidence for this KC. *Monographs* Volumes 112–135 did not include any viral agents and identified a dearth of evidence available to support evaluation of KC9 aside from transformation assays and similar end-points, including occasionally in human cell lines *in vitro* (e.g. Volume 131 for cobalt metal). Therefore, the more frequent challenge for Working Groups conducting the evaluation of KC9 appears to be a general lack of evidence directly and specifically pertaining to the multistep process of immortalization in human cells for the agent(s) of interest.

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10. Key Characteristic 10: Alters cell proliferation, cell death, or nutrient supply

Jason Fritz

10.1 Introduction

As a disease characterized by proliferation dysregulated at many levels, cancer is fundamentally driven by an imbalance between cellular proliferation and regulated cell death (Smith et al., 2016; Hanahan and Weinberg, 2000). Resulting scenarios may include sustained replication, increased potential for low-fidelity repair of DNA damage leading to cancer-permissive mutations in replicating cells, and the ability to escape normal cell-cycle control checkpoints, eventually permitting evasion of apoptosis, senescence (see KC9 Chapter 9), or other terminal programming. As energetic demands increase, cancer cells may increasingly rely on glycolysis despite aerobic conditions and stimulate augmented nutrient delivery. Alternatively, necrosis resulting from nutrient deficiency would trigger an inflammatory response, which could be polarized to support neoplastic expansion (Hanahan and Weinberg, 2011). Thus, agents that stimulate cellular sufficiency and angiogenesis may promote tumourigenesis, as well as those that tilt the balance between proliferation and death towards dysregulated cell proliferation (Baan et al., 2019; Smith et al., 2020b). Increasingly, evaluating results in the context of local microenvironment effects becomes crucial, and careful attention to the strengths and limitations of experimental model systems is advisable. For example, while elevated cytotoxicity or regulated cell death observed in single cell cultures *in vitro* may not independently constitute evidence relevant to KC10, the potential for compensatory cellular proliferation in the context of field effects arising from apoptosis and tissue damage in whole organisms or complex culture systems may be highly relevant (Diwanji and Bergmann, 2019; Smith et al., 2020b).

10.2 Relevance of end-points

10.2.1 Alters cell proliferation

A diverse array of techniques has been employed to evaluate cell proliferation in tissue cultures *in vitro* and in whole organisms *in vivo*. Those outcomes most frequently encountered in evidence streams pertinent to cancer hazard identification are briefly described below, generally categorized as measurements of cellular division, viability, motility, and colony formation assays. Generally, direct measurements more closely related to the apical outcome are of higher relevance to KC10 (e.g. increases in the number or proportion of cells successfully completing mitosis), and provide greater support in an overall strength of evidence evaluation, compared with outcomes that are indirect, may have multiple interpretations, or are otherwise several steps removed from cell enumeration, such as changes in expression of individual cycle cell regulating proteins, incorporation of labelled nucleotides, or mitochondrial activity (see Table 10).

Table 10. End-points relevant to KC10: "alters cell proliferation, cell death, and nutrient supply"

Category	End-point	Relevance	Comments[a]	References
Cell Proliferation				
Cellular division	↑ number of viable cells in vivo or in vitro, including evidence of proliferative histology	↑ cells completing division	Strengthen: direct assessment (e.g. Hemacytometer counting, histology, or microscopy enumeration); use of vital dyes (e.g. Trypan blue) to differentiate live from dying cells increases confidence.	Wiepz et al. (2006); Maronpot et al., (2004); Boorman et al., (1994)
Cellular division	↑ expression of relevant markers (e.g. Ki-67, PCNA) in vivo or in vitro	↑ expression of protein markers of cell cycle progression is associated with cell division	Strengthen: use of well documented albeit indirect markers; expression may not reflect cell division but does indicate proliferative response. Expression may exhibit cell- and tissue-specificity.	Beresford et al. (2006); Wiepz et al. (2006)
Cellular division	↑ nucleotide incorporation in DNA-synthesizing cells (e.g. BrdU/EdU, 3H-thymidine) in vivo or in vitro	↑ incorporation of labelled nucleotide content in cells is associated with cell division	Some limitations due to indirect markers, and potential for contribution from DNA damage and repair (KCs 2, 3), possibly resulting from oxidative stress, and/or chronic inflammation (KCs 5, 6).	Riccardi et al. (1988); Wiepz et al. (2006); Salic and Mitchison (2008); Mead and Lefebvre (2014)
Cellular division by cell cycle analysis	Evaluation of DNA content to determine cells in G0/1, S, and G2/M phases (by flow cytometry); ↑ cyclins A, D, E, or B1 versus DNA content; vital dye quantification ex vivo or in vitro	↑ cell cycle progression and mitosis	Some limitations due to significant variety in analytical flow cytometric approaches, where multivariate analysis provides stronger evidence compared with evaluation of DNA content in isolation.	Riccardi et al. (1988); Pozarowski and Darzynkiewicz (2004); Matson and Cook (2017)
Cellular viability	↑ mitochondrial activity (e.g. colorimetric evaluation of formazan products from tetrazolium dyes) in vitro	↑ production of formazan products can be associated with increased cell numbers	Limitations due to indirect marker, as mitochondrial activity can change independently from changes in cell cycle progression and proliferation. Recommended as a supporting line of evidence.	Marshall et al. (1995); Bruggisser et al. (2002); Quent et al. (2010); Müller et al. (2018)
Cellular motility	↑ movement, migration, invasion in vitro (e.g. Boyden chamber); metastasis and/or EMT in vivo	↑ cell numbers associated with changes in mobility may also reflect alterations in proliferation	Limitations due difficulty attributing changes in cell numbers to proliferation per se; recommend careful attention to experimental design and relevant controls.	Mittal (2018); Kramer et al. (2013); Albuquerque et al. (2021); Choi et al., (2022)
Colony formation	↑ cell number and/or changes in colony growth morphology by microscopic evaluation (e.g. CTA; soft agar, ECM, or ultra-low adherence matrix) in vitro	Conditions which inhibit growth of non-neoplastic cells can provide insight relevant to loss of proliferative regulation	Some limitations due to direct evaluation of colony growth, including cytology; recommend careful attention to experimental, design, cytotoxicity, and relevant controls.	Kakunaga and Yamasaki (1985); Creton et al. (2012); Borowicz et al. (2014); Choi et al. (2022)
Colony formation	↑ tumour formation in vivo following in vitro exposure of non-neoplastic cells	Direct assessment of acquisition of neoplastic potential	Strengthen direct evaluation; sufficient information must be available to support increased proliferative capacity after relevant exposure(s) in vitro.	Newbold et al. (1982); Parida et al. (2021); Yu et al. (2021)

Table 10. End-points relevant to KC10: "alters cell proliferation, cell death, and nutrient supply"

Category	End-point	Relevance	Comments[a]	References
Cell Death				
Apoptosis	↓ programmed cell death from intrinsic or extrinsic apoptosis (e.g. by TUNEL staining, Annexin-V, PARP1 cleavage, flow cytometry) ex vivo or in vitro	Evasion of apoptosis signalling is associated with dysregulated proliferation	Some limitations due to variety in analytical approaches, where multiple end-points measured and/or multivariate analysis provide stronger evidence.	Pozarowski et al. (2004); Wlodkowic et al. (2011)
Apoptosis	Changes in expression or activity of pro- and anti-apoptotic factors in vivo or in vitro	Emerging biomarkers in apoptotic signalling pathway may indicate resistance	Limitations due to indirect markers, where more specific information linking biomarkers to apical outcomes may not be available.	Wong (2011) ; Messmer et al. (2019) ; Sugiura et al. (2021)
Other cell death	Changes in other regulated cell death pathways including autophagy, necroptosis, pyroptosis, cuproptosis, ferroptosis, ex vivo or in vitro	Cell death can result from numerous pathways aside from classical apoptosis, which is relevant to proliferation regulation	Some limitations may be associated with the relevance of specific end-points that are highly context-dependent, and evidence from complex multicellular systems is likely to be most informative.	Mohammad et al. (2015) ; Su et al. (2015) ; Messmer et al. (2019) ; Tong et al. (2022)
Nutrient Supply				
Neo-angiogenesis	↑ capillary volume, size, or permeability (e.g. by factor VIII stains, MVD and HGP, vascular network or plug) in vivo	↑ in either existing vascularization or de novo formation	Some limitations due to MVD specifically has prognostic value in several human cancers, but it is recommended to evaluate in combination with HGP or appropriate surrogate markers.	Miyagami et al., (1987) ; Nowak-Sliwinska et al. (2018)
Neo-angiogenesis	↑ endothelial cell proliferation, migration, vessel formation (e.g. Transwell® cell invasion, aortic ring assay, CAM assay) in vivo, ex vivo or in vitro	↑ in either existing vascularization or de novo formation	Some limitation may be associated with the fact that aortic ring assay reproduces mechanisms that are essential for the regulation of the angiogenic process, and CAM is a very informative technique, but other systems may only inform specific aspects of angiogenesis (e.g. endothelial cell migration).	Nowak-Sliwinska et al. (2018)
Glycolytic Shift	↑ glucose update and metabolism (e.g. analysing 18F-FDG PET-CT) in vivo; ↑ cellular respiration and acidification (e.g. Seahorse XF assay) ex vivo or in vitro	Changes in cellular energetics consistent with observations in numerous human cancers	Limitations due to indirect markers of changes which may reflect cellular proliferation, death, or nutrient availability; Seahorse end-points require careful attention to experimental design.	Viana et al. (2019); Duraj et al. (2021)

18F-FDG PET-CT, 8-fludeoxyglucose positron emission computed tomography; BrdU, 5′-bromo-2′-deoxyuridine; CAM, chick embryo chorioallantoic membrane; CTA, cell transformation assay; ECM, extracellular matrix; EdU, 5-ethynyl-2′-deoxyuridine; EMT, epithelial mesenchymal transition; FDG, F-18-fluorodeoxyglucose; HGP, histopathological growth patterns; MVD, micro vessel density; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; PARP1, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labelling.

[a] Potential contribution of data of this type to the overall strength of evidence for KC10 – Alter cell proliferation, cell death, or nutrient supply

Directly counting cells has been a longstanding method of determining differences in cellular proliferation, and numerous techniques using various dyes (e.g. Trypan blue) have been described to facilitate the experimental enumeration of living versus dying cells (Wiepz et al., 2006). In addition to cell counting, measuring proteins expressed specifically in cells progressing through division such as Ki-67 and proliferating cell nuclear antigen (PCNA) have also been well documented as reliable proxies in numerous tissue types and cancers (Gerdes, 1990; Beresford et al., 2006). Together, these techniques are generally considered to be gold standards for determining impacts to cellular proliferation and can be key to strength-of-evidence determinations. Labelling DNA with ^{3}H-thymidine or in recent years BrdU/EdU, for example, has also been used as a stand-alone technique for assessing cell division by measuring increases in proportions of cells containing label compounds, which are presumably in S-phase before progressing through mitosis (Salic and Mitchison, 2008; Mead and Lefebvre, 2014). However, incorporation of marker DNA analogues is not indicative of cells undergoing S-phase progression, *per se*, but DNA synthesis, and some markers may impact DNA stability and cell cycle themselves, in addition to eliciting cytotoxic effects (Taupin, 2007). As such, there is potential for labelled DNA to be incorporated into some cells during DNA repair and maintenance independent from S-phase progression, possibly resulting from genotoxicity (KC2) and DNA repair pathway activation (KC3), which could decrease confidence that this end-point is solely indicative of cellular proliferation when evaluated in isolation (Wiepz et al., 2006; Goldsworthy et al., 1993). However, when evaluated in the presence of other relevant information, DNA analogue incorporation can provide considerable support to evidentiary determinations, as well as useful mechanistic insight.

Flow cytometry permits bivariate or higher analytical approaches for evaluating cell cycle progression and cellular division, in which cellular DNA can be labelled (e.g. by propidium iodide [PI], or 4',6'-diamidino-2-phenylindole (DAPI)), or cell populations with integrated DNA labels analysed, and compared with the expression of proliferation-associated protein markers, such as the cell-cycle regulating cyclins (e.g. A, D, E, or B1) or other protein markers discussed above (e.g. Ki-67, PCNA) (Riccardi et al., 1988; Pozarowski and Darzynkiewicz, 2004). These multiple lines of investigation allow the evaluation of DNA or DNA-label content in the fraction of cells by major phase of cell cycle (e.g. G, S, and M phases), as well as comparison with expression level of proliferation-associated proteins, which can greatly increase confidence in the interpretation of the results; in particular, multivariate analysis of flow cytometric data can provide considerable support when appropriately conducted.

Cellular viability is frequently evaluated to gain information on the number of metabolically active cells in various tissue culture conditions, and these assays are based upon the activity of biochemical markers, which can then be associated with cell number in a manner dependent upon the specific maker, the cell types evaluated, and the culturing conditions (Präbst et al., 2017). Markers commonly evaluated include resazurin or tetrazolium reagents, which in the presence of NADH- or NADPH-reducing agents (i.e. produced by mitochondrial activity) form products measured using colorimetric or fluorometric assays, as well as ATP, which can measured using a luminogenic assay (Riss et al., 2013). As these assays are reasonably inexpensive, technically straightforward, and amenable to high-throughput or multi-well format analysis, relevant data of this type may be highly abundant. However, in the context of determining changes in cellular proliferation, it is crucial to understand that these end-points use metabolic activity as a surrogate marker for changes in cell division. Therefore, changes in metabolic activity (or more specifically mitochondrial function) should be interpreted as changes in "cellular viability" which are possibly independent from alterations in cell number. The reduction of tetrazolium reagents is known to be: impacted by various alterations in the tissue culture environment, including glucose levels and pH of the medium, (Marshall et al., 1995); caused by direct interaction with some experimental agents, resulting in an artificially increased viability signal even in the context of decreased cell numbers enumerated by the more direct methods

discussed above (Bruggisser et al., 2002); and attenuated by compounds such as the hypoxia mimetic agents including deferoxamine and $CoCl_2$, resulting in an artificially decreased viability signal (Müller et al., 2018). Specifically in human primary cells and cancer cell lines, viability assays tended to overestimate cell numbers when metabolic activity or "cellular viability" was compared with DNA content markers (Quent et al., 2010). Therefore, significant caution should be exercised when interpreting viability assays as an independent measure of cellular proliferation, in which circumstance this line of evidence should be considered of limited informativeness for KC10.

Changes in cellular motility, assessed by migration or invasion across a Transwell® insert or "Boyden chamber" assay *in vitro*, or by evaluation of metastasis *in vivo*, as well as acquisition of a mesenchymal phenotype in typically non-migratory epithelial cells (i.e. epithelial to mesenchymal transition (EMT)), may also provide some information regarding proliferative potential in response to exposures of interest. The capacity to migrate into the vasculature, extravasate, invade, and successfully colonize tissue involves triggering alterations in cellular proliferation as part of a larger set of diverse characteristics of metastasis (Kramer et al., 2013; Baan et al., 2019). Altered cell motility and invasion may not be directly linked with altered cellular proliferation per se, and particularly so in experimental models; despite this, in human patients metastasis is the primary overall cause of cancer morbidity (Seyfried and Huysentruyt, 2013), and is strongly associated with mortality in numerous cancers including lung (Romaszko and Doboszyńska, 2018), breast (Park et al., 2022), prostate (Berish et al., 2018), and brain (Corti et al., 2022). While understanding metastasis is a crucial component of human cancer, experimental evaluations of various individual facets of metastatic characteristics such as increased mobility, invasion through a basement membrane, cytokinesis, and adoption of an EMT phenotype (or lack therefore) are not sufficient support for strong confidence relating to altered cellular proliferation in the absence of more direct lines of evidence (Mittal, 2018), such as those discussed above. Careful attention should be paid to the specifics of the experimental design, such as the inclusion of appropriate controls to differentiate increased cellular motility versus division, as agents may affect the migration and/or invasion stages of metastasis *in vitro* (reviewed in Albuquerque et al., 2021), in a manner independent from effects on proliferation, e.g. due to selective stimulation of Rho family GTPases and changes in collective cell signalling resulting from secreted and contact-mediated signals (reviewed in Mayor and Etienne-Manneville, 2016).

Cell transformation assays (CTAs) are used to determine the proliferative capacity of cells *in vitro* when cultured under conditions designed to be growth-restrictive for non-neoplastic cells, typically due to modification of tissue culture surfaces to limit cellular adherence, traditionally by incorporating soft agar or more recently with ultra-low adherence treatment of tissue culture plastics (Creton et al., 2012). Although guidelines have been proposed specifically for SHE (OECD, 2015) and Bhas-42 (OECD, 2017) CTAs (refer to Chapter 9 on KC9 for more discussion), evaluating changes in colony morphology and attributing them to changes in proliferative capacity resulting from neoplastic transformation can be highly subjective, and careful attention to experimental protocols, including evaluation of concomitant cytotoxicity as well as positive and negative controls, is recommended (Choi et al., 2022).

One extension of the CTA, which could provide strong support for alterations in cell proliferation, is the evaluation of tumour formation from installation of cells *in vivo* following exposure during a CTA *in vitro*, and acquisition of neoplastic growth potential compared with unexposed cells (Newbold et al., 1982). This in effect allows for the simultaneous examination of numerous mechanistic pathways relevant to cancer proliferation (Borowicz et al., 2014; Parida et al., 2021), and when performed along with the appropriate experimental controls, can provide convincing evidence.

10.2.2 Alters cell death

Many of the experimental techniques described above in the context of estimating cellular proliferation are also commonly applied to evaluate the rate and/or mechanism of co-occurring cell death, and it is in this context of impact to neoplastic proliferation that effects on regulated or unregulated cell death are most informative. For example, cell counting techniques will typically employ a dye (e.g. Trypan blue) to facilitate the estimation of both live and dead or dying cells (Phelan and Lawler, 2001). Using various markers and sorting approaches, flow cytometry analysis can determine the proportion of apoptotic and necrotic cells in addition to those living cells in various stages of cell division (Pozarowski et al., 2004), while numerous other experimental approaches employ various combinations of DNA markers, vital dyes, and viability markers to allow experimenters to estimate the fractions of live versus dead or dying cells (Pfeffer and Fliesler, 2017), with some limitations (Zhou et al., 2011). These methods continue to evolve, including the increased application of machine learning to the analytical pipeline (Hu et al., 2022).

In addition to being one of the first mechanisms of regulated cell death described (reviewed in Kaczanowski, 2016), apoptosis is commonly evaluated in the context of cancer hazard identification, as resistance to cell death is a hallmark of cancer cells described by Hanahan and Weinberg (2000), along with sustained proliferative signalling and angiogenesis. Cells undergoing regulated cell death via extrinsic or intrinsic apoptosis (reviewed in Wong, 2011) can be characterized by numerous morphological and biochemical features, which vary depending on the mechanism and timing of induction, cell type, and the observational period during which the process is measured (Wlodkowic et al., 2011). Because many defining characteristics of apoptosis can be evaluated by flow and image cytometry, cytometric methods have generally been considered a gold standard for the evaluation of regulated cell death (Pozarowski et al., 2004), and, as above for cell proliferation, multivariate analysis can be key to strength-of-evidence evaluations. While resistance to apoptosis-induced cell death is a characteristic of human cancers, apoptotic cells may release mitogenic proteins, which can trigger proliferative responses in neighbouring cells via apoptosis-induced compensatory proliferation (reviewed in Moreno-Celis et al., 2022). Therefore, in addition to evaluating apoptotic resistance, increases in regulated cell death could also be informative for cancer hazard characterization if potentially associated with attendant increases in proliferation, which would depend upon the experimental systems involved (e.g. whole organisms), cellular context, and availability of other lines of investigation. While increased cell death would typically not be considered a relevant outcome for KC10 when evaluated independently, it could provide additional support when interpreted in the context of other more directly relevant lines of evidence discussed above.

The molecular mechanisms regulating apoptosis are complex, including roles for plasma and mitochondrial membrane receptors, as well as myriad intracellular mediators including the numerous Bcl-2 protein family (reviewed in Mohammad et al., 2015); changes in expression or activity can occur at any point along these pathways, which may facilitate resistance to regulated cell death (Wong, 2011). Increasingly, evidence is accumulating that well-described cellular mediators of proliferation such as ERK1/2 may also have a central role in facilitating apoptosis via either the extrinsic or intrinsic pathways (Sugiura et al., 2021). Because of this complexity, interpretation of changes in pro- or anti- apoptotic factors or emerging biomarkers should be carefully evaluated in the context of other impacts on cell function, including evidence of concomitant cell division and death. When evaluated independently from more apical end-points, changes in such mediators would be of limited informativeness.

Other mechanisms have been described more recently that may result in regulated cell death, including autophagy, necroptosis (reviewed in Messmer et al., 2019; Mohammad et al., 2015; Su et al., 2015), pyroptosis, ferroptosis (reviewed in Tong et al., 2022; Zhang and Liu, 2022), and cuproptosis (Tsvetkov et

al., 2022). Autophagy is a conserved catabolic process in which complexes of proteins and organelles are packaged for degradation to regenerate other intracellular structures. While this serves as a cellular strategy for survival when stressed, excess autophagy may also ultimately result in cell death (Su et al., 2015). Necroptosis is morphologically similar to necrosis but occurs via a unique mechanism of regulated cell death not involving caspase activation, and as such is independent from apoptosis. Pyroptosis is characterized by cell lysis following the formation of membrane pores and subsequent release of pro-inflammatory cell contents (reviewed by Zhang and Liu, 2022). Ferroptosis was initially described by Dixon et al., (2012) and is a distinct mechanism of regulated cell death, arising from the reactive oxygen species (ROS) imbalance triggered by excessive and/or dysregulated iron content. Interestingly, recent evidence indicates that cytotoxic CD8 T-cells may elicit anti-tumour activity via ferroptosis and pyroptosis, suggesting the potential involvement these regulated cell death pathways in antitumour immunity and immunogenic cell death (Tang et al., 2020). Cuproptosis was recently described by Tsvetkov et al., (2022) as a novel mechanism of regulated cell death dependent on mitochondrial respiration, triggered by direct binding of copper to lipoylated components of the tricarboxylic acid cycle, resulting in protein aggregation, iron-sulfur cluster protein loss, and ultimately cell death. As indicated by the various mechanisms described above, the field of cell death regulation is evolving rapidly, with recent interest focused specifically on the interactions between specific mechanisms of regulated cell death and immune responses in the tumour microenvironment. Because of this, relevant information should be carefully considered in the context of appropriate experimental controls, relevance of the experimental systems to human carcinogenesis, and other lines of evidence available for the exposures of interest.

10.2.3 Alters tissue nutrient supply

As a result of net increases in proliferation rates, neoplastic cells can quickly outgrow the capacity of existing tissue vasculature (see discussions in Baan et al., 2019). Angiogenesis, which is the recruitment of new and generally less coherent vasculature, is essential to providing an increased nutrient supply (Smith et al., 2016). The process of angiogenesis involves multiple discrete steps that can be individually quantified by an increasing number of bioassays, each with specific advantages and limitations (reviewed in Dudley and Griffioen, 2023). Consensus guidelines have been published highlighting critical aspects for the proper interpretation of this evidence, covering dozens of end-points and across *in vivo*, *ex vivo*, and *in vitro* bioassays (Nowak-Sliwinska et al., 2018). While a few examples are illustrated in Table 10, these guideline recommendations should be considered when evaluating the strength of the available evidence for induction of angiogenesis, due to the potential variety and complexity of these systems.

Neoplastic cellular metabolism may shift towards glycolysis (i.e. the Warburg effect), increasing lactate production to satisfy energic needs despite sufficient oxygen supply for aerobic respiration, and increasing activity of the pentose phosphate pathway to provide necessary building blocks for replication (Yang, et al., 2019). Aggressive tumours frequently rely on anaerobic glycolysis *in vivo* (reviewed in Kaczanowski, 2016), which can lead to characteristic alterations in tumour cell metabolic machinery including upregulation of hypoxia inducible factor-1α (HIF-1α) (reviewed by Kierans and Taylor, 2021; Kroemer and Pouyssegur, 2008), resulting in increased neoplastic cell proliferation and evasion of regulated cell death (Icard et al., 2018) (See Box 4). The stimulating effects of the glycolytic shift on neoplastic growth can be reversed to some extent by dietary modification (Viana et al., 2019), suggesting that systemic nutrient supply may affect tumour energetics locally. While numerous techniques have been used to evaluate tumour cell energetics, the Seahorse XF system appears to enjoy widespread adoption, although careful attention to experimental

design and caution in interpretation of results from such end-points is recommended (reviewed in Duraj et al., 2021).

BOX. 4 Metabolic alterations: a case study for considering end-points associated with systemic changes in the metabolism as informative for the KCs

Impacts to nutrient supply (as part of the KC10 evidence) have historically focused on cellular energetics and local changes in the cancer microenvironment; however, the relevance of systemic changes in metabolism resulting from endocrinopathies, such as metabolic syndrome that describes metabolic dysfunctions typically associated with obesity and type II diabetes (Esposito et al., 2012; Karra, et al., 2022), or also metabolic alterations caused directly by occupational or environmental exposures, has become a topic of increasing interest, as they may be associated with cancer hazard.

Metabolic alterations, induced by several exposures, can be associated with a variety of mechanisms that can potentially favor neoplastic formation. Data on metabolic alterations induced by carcinogenic agents have not yet been systematically evaluated within the *IARC Monographs*; however, this type of information has been reported for several agents. For example, alterations of adipocytes cycle, hyperadiposity, hyperlipidemia, glucose intolerance and toxicity, insulin toxicity and resistance, perturbations of specific receptors have been reported as relevant to carcinogenesis. Such metabolic alterations and other relevant end-points can be supportive of the evidence related to KC10, as well as other KCs such as KC2, KC4, KC5, KC6, KC7, and KC8. The *IARC Handbooks of Cancer Prevention* Volume 16, which evaluated "absence of excess body fatness" (Micucci et al., 2016; IARC, 2018b) noted several metabolic alterations causally linked with obesity and their relevance for cancer.

Chronic elevations of blood glucose or "glucose toxicity" can cause numerous dysfunctions, including oxidative stress (KC5) in various tissues. Correspondingly, "insulin toxicity" and insulin resistance, can be associated with elevated levels of insulin, thus inhibiting lipolysis while stimulating liponeogenesis (reviewed in Kolb et al., 2020). Insulin resistance can favour a hyperproduction of insulin and IGF-1, which can have potent mitogenic effects on tumour cells, with a series of downstream effects leading to increased angiogenesis, reducing apoptosis (KC10) (Iyengar et al., 2016; Battelli et al., 2019; Belladelli et al., 2022). Insulin resistance is also associated with altered glucose-6-phosphate dehydrogenase (G6PD) activity, which generates NADPH as well as nucleotide and aromatic amino acid precursors necessary for sustained proliferation (KC10) (Yang et al., 2019). In a variety of tissues, insulin receptor (IR) activation stimulates cell signaling growth pathways including RAS-RAF-MEK-ERK, Src, and PI3K/Akt, and enhances G6PD activity, as well as suppression of autophagy via mechanistic target of rapamycin complex 1 (mTORC1) activation (KC10); all contributing to proliferative stimulation (KC10). Furthermore, the modulation of growth factor signaling via insulin (KC10) and estrogen receptor activation (KC8), as well as increased aromatase expression (KC8), can stimulate downstream signaling pathways, potentially impacting cell proliferation and death regulation (KC10). Perturbations of VEGF (KC10) and its downstream mediators potentially impact vasculature structure and angiogenesis (KC10). Chronic inflammation (KC6), particularly in adipocytes, can involve several pathways. Emerging evidence may also support a role for epigenetic reprogramming (KC4), immunosuppression (KC7) (reviewed in Garcia et al., 2023) and oxidative stress (KC5) (reviewed in Baan et al., 2019; Battelli et al., 2019).

At the Workshop, relevant end-points associated with metabolic alterations (i.e. insulin level, glucose imbalance, adiponectin alterations, triglycerides alterations, among others), altered by agents evaluated within the *IARC Monographs,* were discussed as potentially relevant to the KCs.

Based on the above, and the increasing knowledge on the potential role of metabolic dysregulation in carcinogenesis, especially from emerging assays, such as metabolomics, the Working Group considered that this type of information is an important component of the mechanistic evidence that would add value for cancer hazard identification. Therefore, efforts have been put in place by the IARC Secretariat to elaborate a more systematic tool to screen, organize and evaluate the literature associated with metabolic alterations and thus facilitate its inclusion in the evaluation of potential carcinogens.

10.3. Assessing the relevance of end-points in different test systems

Elevated rates of tumour cell proliferation have both diagnostic importance and independent prognostic value (reviewed by Gerdes, 1990; Niotis et al., 2018; Yang et al., 2018), which has been extensively characterized by evaluation of Ki-67, PCNA, or minichromosome maintenance family (MCM) levels as standard markers of this phenomenon in numerous human cancers (reviewed in Juríková et al.; 2016; Wang et al., 2020b). Evidence for preneoplastic lesions specifically in human populations with relevant exposures may be limited; however, available evidence from experimental systems *in vivo* may also provide key support for evaluation of KC10, and particularly if evidence supports the emergence of proliferative lesions preceding the onset of neoplasia in similar tissues (within the species) as a function of increasing dose and duration of exposure. Although tissue and organ-specific considerations exist, proliferative lesions in epithelial tissues can be generally described on continuum of increasing severity and/or relationship to cancer: hyperplasia can be reactive/diffuse or focal, with focal or atypical hyperplastic responses typically considered to be potentially preneoplastic; metaplasia is generally considered a protective tissue response to sustained injury potentially resulting from various factors including caustic exposures, oxidative stress (KC5), inflammation (KC6), or cell death, and while a more severe effect than hyperplasia (i.e. considered irreversible), may not be specifically linked to neoplastic progression; dysplasia, however, is a dedicated, pre-neoplastic lesion, and would contribute the strongest support as evidence of a sustained, cancer-proximal proliferative effect.

While proliferative lesions could provide the strongest evidence when identified in similar tissues as those evolving tumours within a human population or experimental system, Baan et al., (2019) report that tumour site concordance among experimental systems and humans should not be regarded as a general principle for evaluation, meaning that concordance is important to consider when there is evidence supporting it, but that lack of site concordance across organisms should not be weighed negatively during the strength of evidence evaluation. This consideration also applies to the evaluation of proliferative lesions. There are a variety of factors that may be involved in the appearance of tumours or lesions in different tissues in a species- and/or exposure- specific manner, including differences in anatomy, physiology and metabolic enzyme expression, agent distribution and tissue dosimetry, as well as immune system function, and cell or tissue-specific expression of oncogenic pathways across species (e.g. see discussions in Baan et al., 2019; Maronpot et al., 2004; Boorman et al., 1994). Acknowledging this lack of *a priori* requirement for tissue concordance, there occasionally emerge specific tissues in selected experimental models that may be regarded as not relevant to human cancer risk due to high background tumour emergence rates and nonhuman-specific biology, such as Leydig cell tumours and mononuclear cell leukaemia in F344 rats (Maronpot et al., 2016).

Aggressive human cancers with a high proliferation rate are also, perhaps paradoxically, generally associated with considerable constitutive levels of apoptosis (reviewed in Diwanji and Bergmann, 2019). Apoptosis in the tumour population may condition the local microenvironment, engendering pro-oncogenic effects which have the potential to promote cell survival, apoptosis-induced compensatory proliferation, and therapeutic resistance, in part via activation of innate immune cells and simulation of ROS production (reviewed in Morana et al., 2022). While excessive ROS may yet be cytotoxic and induce ferroptosis (Battaglia et al., 2020), along with apoptosis this regulated cell death may suppress tumorigenesis by removal of some malignant cells, while contemporaneously promoting tumorigenesis through curation of a more favourable microenvironment (reviewed in Morana et al., 2022; Diwanji and Bergmann, 2019). Metabolic dysregulation in cancer cells may be both the cause and consequence of modifications in stromal cells of the local microenvironment including but not limited to the vasculature, and the potential impacts specifically

on metastases are still an area of concerted investigation (reviewed by Martínez-Reyes and Chandel, 2021). As with the above considerations for evaluating proliferation, the most supportive evidence for alterations in cell death and/or nutrient supply would be from intact organisms, and where the outcomes were reported in similar biological contexts as those associated either with emergence of neoplasms, or with other proliferative effects, i.e. multiple lines of investigation and coherence of the evidence greatly increase confidence in the evaluation. Other *in vivo* evidence streams could be supportive, to a degree depending upon the context of the responses and the sparsity of relevant evidence, with evidence from *in vitro* and particularly immortalized cell systems informative to a lesser extent.

10.4 Interpretation of results within the same database

In situations where a significant volume of relevant study information has been identified, evaluation of the strength of evidence should include careful consideration of biological system similarity or relevance, for example along the lines of organ, system, tissue, and cellular origin, as well as duration, route, species, and sex. In addition to increased reliance on those most informative or key outcomes described above, the context of the response in the experimental system as well as directionality may be central to understand whether results within a category of end-points are truly mixed, i.e. indicating evidence of induction, suppression and no effect, or instead stratified along a biologically rational hierarchy. For example, in intact organisms, increases in both apoptosis and cellular proliferation rates may be evident at the organ or tissue level, while finer delineation may reveal separation of effects in discrete cellular subpopulations (e.g. see discussion in Diwanji and Bergmann, 2019), which could have independent support from end-points reported in tissue culture systems *in vitro*. Also, consideration for different potential ontogenies of cancer could provide a helpful conceptual framework in cases where carcinogenic field effects may result in cytotoxicity driving regenerative proliferation in similar cells and/or vascular remodelling and growth dysregulation in nearby stroma (reviewed in Baker, 2014).

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Part II. Incorporation of data from high content and high throughput assays

1. Transcriptomics

Michael Korenjak and Jiri Zavadil

1.1 Introduction

Transcriptomics, which measures global gene expression changes in a cell, tissue, or organism, includes a collection of various technical approaches with well-established applications in medical and life sciences, including cancer hazard identification and risk assessment. Standard transcriptomics approaches are based on either microarray technologies or Next-Generation Sequencing (NGS). The former offers proven and more standardized data analysis strategies, as well as relatively low costs for large-scale study designs; however, microarray-based technologies are limited to a fixed probe design (and therefore to known genes and isoforms) and provide a restricted dynamic range of readings. RNA-sequencing (RNA-seq) overcomes these limitations, improves the detection of weakly expressed genes (Wang et al., 2014), and depending on the experimental design (e.g. 3′-Tag vs standard RNA-seq, single- vs paired-end sequencing, sequencing depth), offers applications ranging from basic gene expression profiling to total RNA-seq, including analysis of non-coding RNAs and novel isoform discovery.

The success of transcriptomics technologies and their pertinence for clinical and regulatory applications have spurred the need for a thorough understanding of the effects of study design, platform, and data analysis differences on the results. Dedicated research projects, most notably the MicroArray Quality Control (MAQC) project (Shi et al., 2006), carried out systematic and analytical analyses of many of these key parameters. For instance, the work showed high intra- and inter-platform concordance for gene expression measurements (Shi et al., 2006; Guo et al., 2006). For the Sequencing Quality Control (SEQC or MAQC-III) project, the analyses were expanded to include RNA-seq data, revealing consistent gene expression readings for different RNA-seq platforms (SEQC/MAQC-III Consortium, 2014; Li et al., 2014), and generally, between RNA-seq and microarray data (SEQC/MAQC-III Consortium, 2014). However, differences between microarray and sequencing data were observed for chemical treatments with complex modes of action and for weakly expressed genes, for which RNA-seq applications were better suited (Wang et al., 2014). This overall concordance between different technological platforms is of considerable importance for the comparability of transcriptomics data sets in the context of systematic literature reviews, given that gene expression studies were originally solely based on microarrays and are now mostly carried out using NGS approaches. Nonetheless, the MAQC/SEQC project also revealed performance differences between the technologies, some of which will be discussed in more detail.

The use of transcriptomics data holds substantial benefits for cancer hazard and risk assessment. Being a genome-wide readout, they enable an unbiased view of gene expression changes in response to exposure to an agent. These data can provide comprehensive information regarding the underlying mode(s) of action of a given exposure, which may in turn facilitate the association with the key characteristics of carcinogens (KCs). As a result, transcriptomics studies contribute to the mechanistic evidence for cancer hazard identification by the *IARC Monographs*, similarly to other assays or end-points currently used during the evaluation process. An inherent limitation of findings from data-rich technologies, including transcriptomics, is their dependence on the quality of the study design and the applied data processing pipelines, which need to be considered when selecting transcriptomics studies and data for cancer hazard identification. Partly due to the popularity of transcriptomics studies, numerous data analysis tools and pipelines have been developed,

which results in a lack of uniformity of analyses between studies, despite certain recommended standards. While large consortium-based studies follow strict study design and data analysis guidelines, individual research studies are not bound to these standards. As part of this Chapter on the use of transcriptomics data for cancer hazard identification, we will discuss (i) study quality criteria, including study design; (ii) considerations for association with KCs; and (iii) the development of transcriptomics-based biomarkers.

1.2. Criteria to assess the quality of transcriptomic studies

Experts participating in *Monographs* meetings and assessing transcriptomics data are expected to select studies based on their quality and information content with respect to the KCs. To identify the most relevant transcriptomics studies, the study quality should be assessed as informed in the *IARC Monographs* Preamble and in the Instructions for Authors. In the following section, additional criteria associated with transcriptomics assays that should be considered will be discussed.

1.2.1 Study design parameters

The general study design can either be based on whole transcriptomics analysis or targeted transcriptomics, which focus on specific gene sets representing selected biological processes. The former characterizes gene expression changes without prior knowledge, and in an unbiased (hypothesis-free) way, potentially leading to unexpected, data driven research discoveries, while the latter identifies changes in a hypothesis-based manner.

Relevance of test system

As outlined in the Preamble – Instructions for Authors, studies in exposed humans have higher relevance for *Monographs* evaluations. However, transcriptomics studies in exposed humans are often characterized by mixed exposures and limited exposure assessment information, requiring close collaboration with experts on exposure characterization for the selection of studies and the interpretation of findings.

Transcriptomics analyses have been extensively applied in the context of experimental studies *in vivo* and *in vitro* to help inform cancer hazard identification and risk assessment (Ganter et al., 2005; Harrill et al., 2021; Igarashi et al., 2015; Lamb et al., 2006; Uehara et al., 2010). In the future, the *in vitro* cell-based studies are expected to gain further relevance due to the implementation of the 3R principles (Replacement, Reduction, Refinement) aimed at avoiding animal experimentation. This transition is also prompted by the continuous improvement of cell-based exposure systems, which takes into account human primary cell context, multicell type co-cultures, and 3D culture models. However, systematic multi cell type comparison of gene expression changes observed upon exposure to the same compounds *in vitro*, in primary rat hepatocytes, as opposed to *in vivo*, in rat liver tissue, revealed surprisingly little overlap of altered biological pathways in the two model systems (Luijten et al., 2021; McMullen et al., 2019). These differences are likely due to variations in metabolism, microenvironment, or hormone levels, among other factors, and concordant alterations observed *in vitro* and *in vivo* have been proposed to provide the most reliable conclusion concerning altered biological processes (Luijten et al., 2021). Correction for systematic, stress-correlated but chemical-independent gene expression changes in the model systems is also being explored as a potential strategy to pinpoint relevant alterations (McMullen et al., 2019).

Sample type

The use of fresh or fresh-frozen samples is preferable over formalin-fixed paraffin-embedded (FFPE) samples for transcriptomics analyses, owing to the damage introduced to RNA by fixation and storage. However, FFPE tissues are frequently the only available sample type in clinical and epidemiological studies, and in long-term animal bioassays. As a general rule, the quality of RNA used for transcriptomics analyses needs to be carefully controlled, especially when using FFPE samples. Different metrics, such as the RNA integrity number (RIN) or the percentage of RNA fragments > 200 nucleotides (DV200), have been established for this purpose. For FFPE samples, mRNA enrichment using hybridization-based capture of coding regions or depletion of rRNA were better suited than methods using poly-A enrichment, resulting in higher overlap of differentially expressed genes and pathways when compared to matched frozen samples (Webster et al., 2015). Poly-A enrichment relies on intact poly-A tails, which are adversely affected by formalin fixation (Farragher et al., 2008; Klopfleisch et al., 2011). Interestingly, the fixation time had a limited effect on the results, as did the storage time of FFPE tissues (Webster et al., 2015). Comparison of differentially expressed genes from FFPE and matched frozen samples revealed a higher proportion of intronic and intergenic reads as well as mitochondrial and long non-coding transcripts in FFPE samples (Jacobsen et al., 2023). Moreover, FFPE-specific enrichment and depletion of certain biological pathways has been observed (Newton et al., 2020). However, good overall correlation between protein-coding transcripts in fresh, frozen and FFPE samples has been reported (Bossel Ben-Moshe et al., 2018; Jacobsen et al., 2023; Newton et al., 2020). New RNA-seq approaches avoid cDNA synthesis (Geiss et al., 2008; Girard et al., 2016; Yeakley et al., 2017), which is known to be impacted by fixation time, and will further improve the reliability of transcriptomics on FFPE-derived samples. Originally developed as targeted approaches, these methods are applicable to the entire transcriptome and have been shown to perform well on decades-old, archived FFPE samples (Trejo et al., 2019).

Sample number

An important consideration for transcriptomics studies concerns the statistical power for detecting differentially expressed genes. This power is defined not only by the number of samples and replicates analysed, but also by sequencing depth in the case of RNA-seq studies. The number of replicate samples required depends on the technical variability of the transcriptomics approach and the biological variation of the study system, and it is, therefore, difficult to define general recommendations. Nevertheless, certain general considerations are important to keep in mind. The appropriate sample number for achieving sufficient power is closely linked to the type of sample collection, with unpaired samples requiring larger sample numbers than paired ones (e.g. tumour-normal pairs) (Ching et al., 2014). Similarly, the effect sizes (fold-change) of differential expression influence the required sample numbers, to the extent that current tools for estimating sample number requirements for transcriptomics studies gave highly inconsistent results depending on the effect size (Poplawski and Binder, 2018). In general, required replicate numbers are lower for defined experimental studies than for investigations in exposed human populations. A study that focused on experimentally generated data proposed 6 samples (biological replicates) to capture the main differences in expression between paired samples, while 12 samples were required to detect the majority of differentially expressed genes (Schurch et al., 2016). However, the Encyclopedia of DNA Elements (ENCODE) project, recommends two or more biological replicates for RNA-seq studies (ENCODE, 2023). In line with this recommendation, three replicates per condition are considered the general consensus for experimental studies, providing a compromise between power and experimental cost. For RNA-seq applications, increased sequencing depth positively influenced the number of detected genes, thereby extending the analysis to weakly expressed genes (SEQC/MAQC-III Consortium, 2014), but it had limited impact on the power to detect differentially expressed genes. Beyond 10 million aligned reads, increasing the number of biological replicates proved more beneficial (Ching et al., 2014; Robles et al., 2012).

Exposure strategy

The biological relevance of selected exposure concentrations has implications for the interpretation of results, such as the distinction of direct and indirect effects of an exposure (e.g. exposure-induced cytotoxicity). Systematic, large-scale toxicogenomics efforts based on transcriptomics analyses, therefore, incorporate single and repeat dosing, as well as multiple test doses in the study design (Chen et al., 2012). This strategy has been employed for some of the most comprehensive toxicogenomics databases covering hundreds of test compounds, such as the Japanese TG-GATEs project (Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System) (Uehara et al., 2010) or DrugMatrix (Ganter et al., 2005). The estimation of benchmark dose levels (BMDL), a key concept in human health risk assessment, is based on dose–response measurements and has been adapted to transcriptomics data. Transcriptional BMDLs, derived from short-term animal bioassays, can be linked to biological processes, including pathways and gene ontologies, and their implementation in the context of cancer risk assessment revealed concordance with traditional end-points (Bhat et al., 2013; Thomas et al., 2007; Thomas et al., 2011; Thomas et al., 2012; Thomas et al., 2013). For single-dose experiments, strong biological relevance of the selected dose and rigorous control of adverse phenotypes, which can result in unwanted secondary gene expression changes, are crucial for including the findings in cancer hazard identification approaches. As for the multiple dosing design, time-course experiments provide valuable information for differentiating direct from indirect exposure effects on gene expression changes in experimental studies, and they have been applied in large-scale toxicogenomics projects (Ganter et al., 2005; Uehara et al., 2010).

1.2.2 Sequencing/microarray quality parameters

The quality control of raw transcriptomics data, from microarrays or RNA-seq, provides important information on the samples and the technical quality of the data acquisition approach. For microarray data, this includes parameters such as the signal intensity of hybridization controls, signal-to-noise ratios (background), percentage of genes present (expressed) and cRNA transcript integrity (ratio 3′ to 5′ probe sets for defined housekeeping genes). For RNA-seq data, among others, it includes a defined sequencing quality (Q) score (Q30 corresponding to the percentage of bases with an inferred base call accuracy > 99.9%), read distribution between samples sequenced as part of the same run, GC content and duplicate read numbers. The percentage of duplicate reads depends on the experiment, but the levels should be similar for samples within an experiment, with variations of more than 30% having been proposed as a general cut-off (Conesa et al., 2016).

1.2.3 Data pre-processing criteria

After assessment of the raw data quality, the steps of data pre-processing include various additional quality control checkpoints for RNA-seq experiments (Corchete et al., 2020). As part of aligning sequencing reads to the genome, the percentage of mapped reads provides an important quality control parameter. For standard RNA-seq, a mapping percentage on the genome of 70–90% is expected (Dobin et al., 2013), whereas it should be slightly lower when mapped on the transcriptome owing to the presence of reads corresponding to unannotated transcripts. As an additional quality control step, principal component analysis (PCA) is recommended to project the relationship between samples and identify potential outliers.

A critical requirement to ensure the comparability of gene expression data is data normalization, within and across samples. To compare expression between samples, transcript length and sequencing depth need to be taken into account, as longer transcripts will accumulate more reads. This within-sample normalization

calculates the reads per kilobase of transcript per million reads (RPKM) or fragments per kilobase of transcript per million reads (FPKM), which designate the relative presence of a transcript amid all sequenced transcripts (Mortazavi et al., 2008). However, RPKM/FPKM-based normalization is less suited for comparing samples with uneven distribution of transcripts, for example due to the presence of highly and differentially expressed transcripts. Normalization methods based on compensating expression levels of non-differentially expressed genes take such profiles into account (Evans et al., 2018). Some of the most commonly applied differential expression pipelines, such as DESeq2, edgeR or cuffdiff2, use this normalization strategy. In certain instances, limited study designs involving two samples and few replicates may require more basic normalization methods based on Poisson distributions, for example (Conesa et al., 2016). Numerous normalization tools exist for RNA-seq data, often based on very similar statistics, and have been systematically reviewed with respect to the frequency of their use in the literature (Verheijen et al., 2022).

1.2.4 Differential analysis

The MAQC/SEQC consortium provided important insight regarding the presence of false positives in unfiltered differential expression data from microarrays and RNA-seq. Filtering of differential genes using a $\log_2$ fold-change > 1 significantly reduced the number of false positives for microarray analysis and has been recommended by the MAQC project (Shi et al., 2006). With respect to RNA-seq data, filtering of differentially expressed genes by p-value, fold-change and expression level (removal of the lowest third of genes) efficiently resolved the high false positive rates (SEQC/MAQC-III Consortium, 2014). Specific recommendations frequently suggest a 5% false discovery rate (FDR) and a $\log_2$ fold-change between 1 and 2 (Verheijen et al., 2022), although it is increasingly acknowledged that these fold-change cut-offs might be reconsidered, as smaller fold-changes occurring in several genes of a biological pathway in a concerted manner can be highly relevant. These patterns are taken into account by gene set enrichment analysis-based functional classification tools (Subramanian et al., 2005) The general lack of uniformity in data analysis is a common problem in transcriptomics, especially RNA-seq, studies. Particularly risk and hazard assessment for regulatory applications would benefit from common guidelines for processing transcriptomics data. In an attempt to provide such guidelines, the Omics data analysis framework for regulatory applications (R-ODAF) has recently been proposed (Verheijen et al., 2022). Key criteria for sample inclusion are based on total read count (> 5 million), sequencing quality (Q30 > 70%, difference of genomic coverage of data above Q30 between forward and reverse reads < 25%), genome alignment (> 70%) and absence of outliers (< 20% variance in PCA). Before differential analysis, a filtering step is recommended to remove genes for which less than 75% of replicates are expressed at least 1 count per million in any of the experimental conditions. Filtering recommendations after differential analysis include a 0.01 FDR threshold and steps to eliminate genes with excessive signal in one sample of the condition or one replicate of a sample. Although the application of the proposed analysis framework will have to be evaluated in the future, transcriptomics data from large consortia that apply uniform data analysis strategies or re-analysis of existing data from distinct studies remain the best options for the wide-ranging use of transcriptomics data in cancer hazard identification. When used to support findings from established assays for toxicity end-points, transcriptomics data derived using the general data analysis standards discussed above likely offer sufficient quality.

1.3. Incorporation of transcriptomics data into KCs

Differential gene expression data derived from transcriptomics studies provide useful insight regarding the differences between test conditions, such as exposed versus non-exposed or cancer versus normal. However, to identify and understand the complex biological mechanisms controlled by the underlying gene expression changes, it is necessary to organize the large number of differentially expressed genes into a manageable number of biological processes or pathways. This strategy takes advantage of highly curated gene lists annotated with respect to their biological function, and it is an essential step for the interpretation of transcriptomics data, including the potential association with KCs.

Pathway analysis can be divided into two main categories, non-topology-based and topology-based approaches. Non-topology-based methods are based on gene sets that are merely grouped together due their involvement in the same process or pathway, without considering hierarchies, directional signalling or specific roles of genes within the pathway. Topology-based methods, on the other hand, take this information, when available, into account.

Among non-topology-based approaches, overrepresentation analysis (ORA) assesses the over-/underrepresentation of genes from an input list (i.e. differentially expressed genes) among the genes assigned to a pathway, by comparing the input to a background list. In contrast to ORA, functional class scoring methods (FCS) do not require differentially expressed genes (selected by an arbitrary cut-off) as input but consider the entire data set. By analysing differential expression of individual genes and subsequent grouping of all genes in a pathway to generate a pathway-level statistic, subtle gene expression changes occurring in a concerted manner within a pathway are also being captured.

Topology-based approaches are based on the same steps as FCS methods, but they take pathway structure into account when determining the statistics at the level of individual genes. This was originally based on the similarity between each gene pair in a pathway (Rahnenführer et al., 2004). It was later extended to specific determinants of pathway topology, such as types of interactions, gene expression changes and hierarchy of genes within the pathway (Draghici et al., 2007), as well as to provide information on central hub genes in the pathways (Gu et al., 2012; Ibrahim et al., 2012). Upstream regulator analysis, as applied in the Ingenuity Pathway Analysis (IPA), takes the relationship between transcription factors, miRNAs, kinases or chemicals, and their target genes into account (Krämer et al., 2014). The analysis can be further extended to link upstream regulators operating in the same signalling pathway. Furthermore, powerful methods of deconvolution of transcriptomic data emerged recently, dissecting the cell-type specific gene expression profiles and associated pathways in bulk transcriptomic data generated from mixed cell populations or heterogeneous tissues (Luca et al., 2021)

Many different pathway analysis tools have been introduced and are commonly used to interpret transcriptomics data sets. Benchmarking of pathway analysis methods revealed that all approaches have limitations, but topology-based methods appear to be somewhat superior to the non-topology-based ones (Nguyen et al., 2019). In addition, most methods report a considerable number of false positive pathways, which are frequently characterized by a small size (< 50 genes).

The knowledge bases underlying pathway analysis include gene lists defining processes and pathways that can be mapped to most KCs. As many other pathway collections, the Kyoto Encyclopedia of Genes and Genomes (KEGG) knowledge base (Kanehisa and Goto, 2000), one of the most commonly used databases, contains pathways that can be easily associated with "Alters DNA repair or causes genomic instability" (KC3), "Induces epigenetic alterations" (KC4), "Induces oxidative stress" (KC5), "Induces chronic inflammation" (KC6), "Is immunosuppressive" (KC7), "Modulates receptor-mediated effects" (KC8), and "Alters cell proliferation, cell death or nutrient supply" (KC10). It is, however, important to keep in mind

that pathways are often interconnected; for example, increased cell proliferation affects the accumulation of DNA damage and energy metabolism. Moreover, the induction of oxidative stress can activate various signalling pathways and transcription factors linked to inflammation and cell proliferation and it induces DNA damage, mostly through the formation of 8-hydroxydeoxyguanosine (8-OHdG) DNA adducts (Arfin et al., 2021). Additional considerations to keep in mind when relating transcriptomics-based pathway analysis to KCs include the indirect association that gene expression profiles provide for biological processes (e.g. transcriptional deregulation of epigenetic regulators are not proof of corresponding modifications), and the reality that the annotation of pathways is likely not complete and possibly in part incorrect. In addition, not all the KCs may be assessed by transcriptomics-based pathways to the same extent.

To use transcriptomics data as supportive evidence in *IARC Monographs* evaluations, it is recommended that the gene expression changes be associated with specific KCs. To establish this association, the Working Group should be able to map the enriched pathways (or genes) to the KC, based on the known association of the transcripts with a biological process underlying the KC.

1.4. Development of biomarkers from transcriptomics data

Transcriptomics studies provide abundant information on deregulated genes and pathways between conditions; however, converting this information into reliable biomarkers indicative of a KC remains challenging. Gene expression profiles of different exposures that share the same mode of action can be rather diverse, and the challenge lies in identifying the key gene expression responses common to a specific mechanism. Moreover, indirect exposure effects frequently complicate the association between exposure and mode of action.

The Toxicogenomic-DNA Damage Inducing (TGx-DDI) biomarker is a prime example for a genotoxicity biomarker derived from transcriptomics data. It has been developed to address the high percentage of compounds testing positive in standard *in vitro* genotoxicity assays as part of chemical and drug safety evaluation, many of which do not hold true in *in vivo* follow-up studies (Snyder and Green, 2001). The TGx-DDI biomarker is based on specific transcriptional responses to genotoxic stress as opposed to those induced by non-genotoxic stress. In addition, transcriptional activation of immediate early DNA damage response genes, gene expression analysis 4 h post-treatment, and control for treatment cytotoxicity limits the contribution of secondary (indirect) effects, which contributes to genotoxicity in standard *in vitro* mutation and chromosomal damage assays.

Li et al. used controlled exposure to known genotoxic and non-genotoxic agents in TK6 cells to identify a gene expression classifier for genotoxicity testing (Li et al., 2015). The discovery phase included 28 exposures, and the transcriptional response was measured using DNA microarrays. Based on this analysis, a set of 65 transcripts (64 genes), mostly involved in cell cycle regulation and p53 signalling, was extracted. The gene panel correctly distinguished genotoxic from non-genotoxic compounds in case studies to validate the biomarker (Buick et al., 2015; Li et al., 2015) and in a publicly available gene expression data set of controlled exposure experiments in HepaRG cells (Buick et al., 2015). In a follow-up study comprising 45 exposures, the classifier showed very good performance in differentiating genotoxic from non-genotoxic agents and in identifying false-positive results from chromosomal damage assays, of which 90% were correctly categorized as non-genotoxic by the TGx-DDI biomarker (Li et al., 2017). Moreover, the gene panel showed high sensitivity and specificity after switching from the original microarray-based readout to

a standard quantitative RT–PCR platform (Cho et al., 2019) and to the NanoString technology (Li et al., 2017), which is amenable to high-throughput screening. More recent work has focused on standardizing the use of the TGx-DDI biomarker in "near-normal," metabolically competent HepaRG cells, using high-throughput compatible sequencing-based expression analysis, and combining the mechanistic data with standard *in vitro* genotoxicity assays that have a similar potential to scale up the throughput of genetic toxicology testing (Buick et al., 2020, 2021).

Summary of suggested practices for study selection and incorporation in *Monographs* evaluations

- Evaluate the study design
  - Relevance of the organism/model system,
  - Sample quality & number (power of the study)
  - Exposure strategy and applied concentrations (for experimental studies)
- Assess the data quality
  - Microarray- or RNA-seq-specific criteria
  - Data pre-processing standards
  - Quality of differential analysis
- Incorporation of transcriptomics data with KCs
- Assess the association of enriched pathways (or genes) with the KC, based on the known association with an underlying biological process.

1.5 Conclusion & future directions

Transcriptomics approaches are well established, show high inter-platform concordance, and provide invaluable information for assessing the mode(s) of action of cancer risk factors and ensuing effects (Meier et al., 2025). Depending on study design, and relevance of the data with respect to the KCs, experts are encouraged to take advantage of this resource and select the most relevant studies to incorporate them in *Monographs* evaluations. One of the main conclusions of the Workshop held at IARC was that systematically connecting deregulated genes and pathways with the KCs was a major challenge, and innovative mapping strategies need to be developed to ensure a more effective use of transcriptomics data in cancer hazard identification. In the future, incorporating data from high-quality, standardized public data repositories into *Monographs'* evaluations would be desirable, but this project would require dedicated resources for data extraction and processing.

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2. Metabolomics

Dinesh Barupal

2.1 Introduction

Metabolomics (Panyard et al., 2022) is an analytical approach to measure multiple metabolites in an organism under a genetic or environmental stress (Chen et al., 2022; Maitre et al., 2022; Sen et al., 2022; Surendran et al., 2022; Novotny et al., 2023) to study changes in metabolic pathways and reactions. A metabolite can be defined as a small molecule chemical (< 1500 Da) that is generated by enzymatic, non-enzymatic and gut-microbiota transformations of endogenous or exogenous substrates inside an organism's body (Noronha et al., 2019; Bansal et al., 2022; Grahnemo et al., 2023). Up to ~10% (~3000) of genes in the human genome encode proteins with enzymatic activities (NCBI, n.d). There are ~2500 known metabolites for the human body that are linked with at least one known endogenous reaction in biochemical databases (Caspi et al., 2020) such as MetaCyc (Caspi et al., 2020), Reactome (Gillespie et al., 2022) or KEGG (Kanehisa et al., 2023).

A transformation reaction has substrate(s) and product(s) (Fig 2.1), which may be detected by metabolomics assays in a biological sample. The biological effects of a chemical (or its metabolites), depend on the chemical's availability as an intracellular substrate and the genetic and epigenetic regulation of the involved enzymes in the transformation processes that can change the substrate availability (Fig 2.1) (Hecht et al., 2016; Hecht and Hatsukami, 2022). Metabolites informative for KCs of carcinogens are often observed in these metabolomics data sets, suggesting that specific metabolites can be used as end-points to support the evaluation for cancer hazard identification.

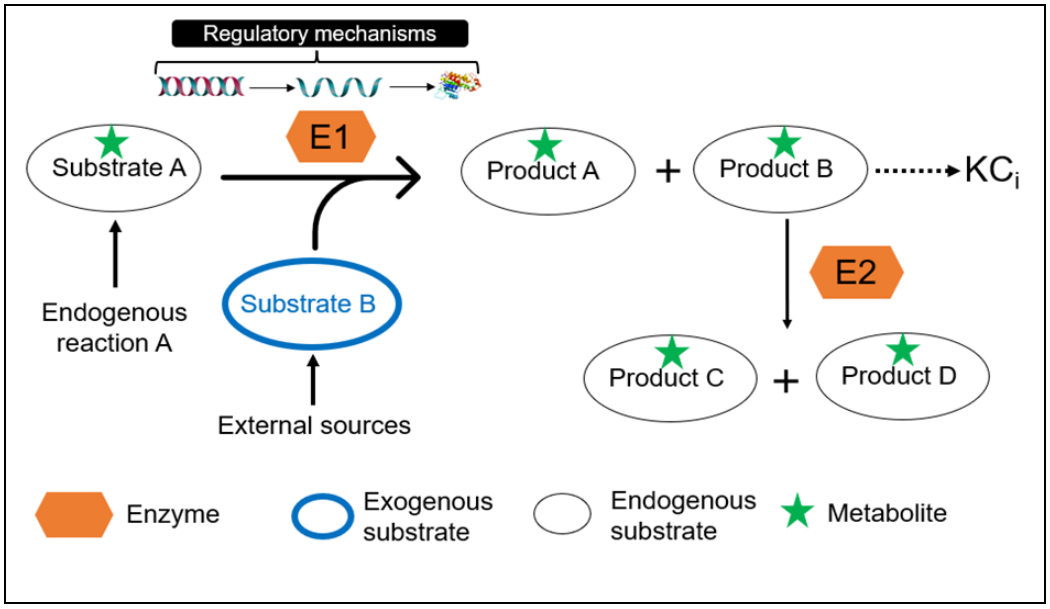

Figure 2.1. A schematic of biochemical reactions. Stars show the chemical structures that are metabolites. For example, metabolic activation of heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) by cytochromes P450 (CYP) 1A1 and 1A2 enzymes.

2.2. Criteria to assess quality of metabolomics studies

High-quality metabolomics data sets are regularly available for studies in exposed humans and in experimental systems *in vivo* and *in vitro*. (Metabolomics Workbench, n.d.). Publications originating from these data sets may have already reported metabolites and their pathways that are relevant to KCs. While accessing these data sets, quality control, compound annotation coverage, and biases during data generation and analysis need to be carefully evaluated so that false associations as well as resource utilization can be minimized. *IARC Monographs* Working Groups may use these suggestions to screen for metabolomics reports and data sets, and they may also plan to analyse the data set whenever required.

Experts participating in *Monographs* meetings and assessing metabolomics studies are expected to select the most relevant studies based on study quality and information content with respect to the KCs. In the following section we will discuss important general considerations and quality control parameters that can be applied to select metabolomics studies for *Monographs* evaluations. To identify the most relevant metabolomics studies, the study quality should be assessed as informed by the *IARC Monographs* Preamble and in Instructions to Authors. In addition, the study quality evaluation criteria associated with metabolomics assays should be considered.

Metabolomics assays can be grouped into two categories – targeted and untargeted. A targeted assay requires a predefined list of analytes such as oxylipins, steroid hormones, or primary metabolites and the optimized protocols for sample preparation and data collection (St John-Williams et al., 2017), whereas an untargeted metabolomics assay aims to impartially detect all the chemical structures present in a sample (Guida et al., 2021). Mass spectrometry and Nucleic Magnetic Resonance instruments are the most commonly used techniques in metabolomics. Exclusion criteria for metabolomics data collected using a targeted assay can be the sample size, measured metabolite's relevance to KCs, and missing exposure assessment or outcome data. However, untargeted metabolomics assays differ greatly in terms of the coverage, annotation rates, and data quality (Barupal and Fiehn, 2017). Metabolite annotation, in parallel to the gene annotation, is a computational process to match a segment of raw instrument data, for example mass, retention time, or fragmentation spectra with the chemical information such as structure, chemical class, or pathway that is catalogued in a reference database. The annotation process is specific to only untargeted metabolomic assays, and studies that do not report any annotated metabolites may be considered less relevant or can be excluded. The annotation quality varies by metabolomics laboratories and can significantly influence the biological interpretation depending on which annotation criteria were used. Most importantly, for untargeted data to be used as reliable evidence in an *IARC Monographs* evaluation, the metabolite annotation must be supported by accurate mass, chromatographic retention time, and fragmentation spectra collected for an authentic reference standard of chemical compounds by the metabolomics laboratory. For human epidemiological studies using blood and urine specimens, statistical models should have been adjusted minimally for age, sex, BMI, drug usage, and dietary factors. Analytical and computational steps for generating untargeted metabolomics data must be evaluated for chemical class biases, batch effects, data normalization, pathway analysis, and literature biases during interpretation.

Several currently available metabolomics assays can generate data matrices with over 1500 small molecules for a human blood specimen (Metabolomics Workbench, n.d.; Ding et al., 2021; Fromentin et al., 2022). While interpreting such comprehensive blood metabolomics data sets in the context of exposure biology and toxicity mechanisms, careful attention should be paid to sources and origins of small molecules if they originated from endogenous reactions or from external sources (Barupal and Fiehn, 2019; Sevelsted et al., 2022; Xu et al., 2023). About 90% of the detected small molecules are endogenous metabolites such as lipids, amino acids, modified nucleosides, and hormones, and the rest are exogenous chemicals such as

drugs and dietary or environmental chemicals (Metabolomics Workbench, n.d; Ding et al., 2021; Fromentin et al., 2022; Xu et al., 2023).

Working Group members could access the metabolomics data matrices to review the quality of data and metabolite annotation. Metabolomics data tables along with sample metadata can be obtained from well-established metabolomics repositories, including EBI Metabolights (Haug et al., 2020), Metabolomics WorkBench (Sud et al., 2016), Massive UCSD (MassIVE, n.d.), iProX (Ma et al., 2019) and OMIX (Xue et al., 2022) databases, as well as generic repositories such as Zenodo.org. The metabolomics data matrices are often available in the supplementary section of the published articles. For larger human epidemiological studies, data can be accessed through an approval process, for example TopMed data sets accessed using the NCBI dbGap approval. While data sets originating from peer-reviewed papers should be prioritized, curated, freely available, and well-maintained data sets by large consortia such as NIEHS HHEAR, EU Exposomics, TopMed, or NIH Precision Nutrition programmes could be directly used for a KC-oriented re-analysis.

2.3. Incorporation of metabolomics data as supporting evidence for the KCs

Metabolism is a fundamental biological process that produces building blocks, such as lipids and nucleotides, and energy equivalents, such as ATPs, that are essential for a normal cellular growth (Bergers and Fendt, 2021; Schmidt et al., 2021; Finley 2023; Rattigan et al., 2023). Metabolic reprogramming is a hallmark of rapidly growing cells, including cancer cells, which heavily relies on glycolysis for ATP generation from glucose (Martinez-Reyes and Chandel, 2021; Finley, 2023), also known as the Warburg effect. The regulatory mechanisms that support metabolic programming in cancer cells have been extensively investigated using metabolomics (Schmidt et al., 2021), multiomics (Wang et al., 2021a; Ravi et al., 2022), and pharmacological studies (Gwynne et al., 2022; Ravi et al., 2022; Stine et al., 2022; Weiss et al., 2022). It is hypothesized that exposure to hazardous agents may modulate this metabolic reprogramming by interacting with regulatory and signalling cascades, with or without modifying the DNA sequence (Vives-Usano et al., 2020; Torres et al., 2021; Guo et al., 2022a; Sen et al., 2022; Zhu et al., 2023). In metabolomics data generated from human blood or tissue biospecimens, it is possible to identify signatures of these metabolic changes, including individual metabolites, pathways, or metabolite classes. Metabolites altered in response to a potential carcinogenic exposure may be relevant to the mechanistic evidence for *IARC Monographs* evaluations.

For the purpose of using metabolomics alterations as supportive evidence in *IARC Monographs* evaluations, it is recommended that metabolomics data be associated with specific KCs. To perform this association, the Working Group can map the metabolite(s) to the KC, considering the association of the metabolite(s) to the biological process underlying the KCs. Some key examples of such metabolites and related pathways are highlighted in the following subsection. It is recommended that results from published metabolomics studies are organized and interpreted according to the relevance of individual or a group of metabolites for KCs.

KC1: Is electrophilic or can be metabolically activated to an electrophile; KC2: Is genotoxic; KC3: Alters DNA repair or causes genomic instability – DNA alterations

A chemical exposure or its reactive metabolite can interact with DNA bases, generating DNA adducts that can be observed by metabolomics assays in human biospecimens (Wilson et al., 2019).

For example, vinyl chloride's DNA adduct N^2-ethenoguanine, nitrosamines' adduct 4-hydroxy-1-(3-pyridyl)-1-butanone (4-HPB)-DNA adduct, and aldehyde's adducts N^2-ethylidene-deoxyguanosine and N^2-hydroxymethyl-deoxyguanosine (Dingler et al., 2020) can be detected.

Reactive oxygen or nitrogen species (ROS/RNS) can damage DNA by oxidation and form oxidative DNA adducts such as 8-oxo-7,8-dihydroguanine, 8-oxo-7,8-dihydroadenine, 5,6-dihydroxy-5,6-dihydrothymine or 5-hydroxy-methyluracil (Hwa Yun et al., 2020; Dannenberg et al., 2022).

Benzopyrene, a polycyclic aromatic hydrocarbon (PAH), is metabolized to 7,8-epoxide, 7,8-diol, and finally to 7,8-diol-9,10-epoxide, which reacts with deoxyguanosine in DNA. PAH exposure biomarkers such as phenanthrene tetrol can be detected in urine samples to confirm the DNA modification (Hatsukami et al., 2018). Furthermore, specialized DNA adductomics assays can comprehensively screen for DNA adducts by monitoring the loss of specific fragments in the untargeted LC-HR-MS/MS data sets (Liu and Wang, 2015; You and Wang 2016; Dingler et al., 2020; Hwa Yun et al., 2020). Untargeted metabolomics and adductomics data sets in which these adducts have been reported can be prioritized for cancer hazard evaluations.

KC4: Induces epigenetic alterations

The correlations among epigenetics and metabolomics signatures have been observed across several cancer types (Gopi and Kidder, 2021; Luo et al., 2021), particularly in reference to the one-carbon metabolism (Ducker et al., 2017; Gao et al., 2019a; Thakur and Chen, 2019; Dai et al., 2020). This pathway provides the methyl group that is require for the methylation reaction in epigenetic re-programming (Cornacchia et al., 2019; Annibal et al., 2021; Lee et al., 2021); therefore, the corresponding enzymes are overexpressed in many cancers to support the use of serine and methionine amino acids for the one-carbon metabolism (Gao et al., 2019b; Ramalingam et al., 2021). Furthermore, N6-methyladenosine(m6A) methylation, the most prevalent mRNA modification, has been shown to induce metabolic reprogramming in cancer and to generate immunosuppressive metabolites (Zhang et al., 2022). Notably, multiomics investigations in the TCGA (Li et al., 2023) and other cancer cohorts have identified the metabolic pathways and the epigenetic signatures that are jointly associated with advanced cancer types.

KC5: Induces oxidative stress

Reactive oxygenated species (ROS) stress is a well-recognized process involved in the toxicity of several chemical agents (Spinelli and Haigis, 2018). ROS can damage cellular molecules, generating reactive products that can interact and interfere with normal signalling pathways. Redox-neutralizing compounds such as NADP+, NAD+, and glutathione disulfide (GSSG) and their oxidized and reduced forms are an indicator of internal ROS state. Glutathione and lipid metabolism are often affected by ROS stress. The ROS-related products and related metabolites can be observed in metabolomics data sets. Ubiquitous pollutants such as $PM_{2.5}$ exposure (Turner et al., 2020) can disturb glutathione metabolism and induce ROS stress in pulmonary cells (Yue et al., 2019). Oversupply of fatty acids by diet or endogenous metabolism can cause ROS increase by the β and omega-oxidation reactions. 4-Hydroxynonenal and malondialdehyde are examples of lipid peroxidation biomarkers (Cui et al., 2021), which can be detected in human biospecimens after an exposure to industrial chemicals such as trichloroethylene or trichloroacetic acid (Wang et al., 2019). Advanced lipoxidation end-products and advanced glycation end products have been associated with a disturbed hepatic lipid metabolism. These metabolic pathways and reactive products can be induced by multiple chemical exposures, supporting the application of metabolomics assays in understanding ROS stress mechanisms in carcinogenesis.

KC6 Induces chronic inflammation

Several derivatives of unsaturated fatty acids (i.e. arachidonic acid or linoleic acid) are inflammation mediators (Dennis and Norris, 2015). Cyclooxygenase (COX) and lipoxygenase (LOX) enzymes mainly produce these mediators (Dennis and Norris, 2015). Eicosanoids include prostaglandins, leukotrienes and isoprostanes, and represent the main class of pro-inflammatory lipid species (Dennis and Norris, 2015). Environmental exposures have been associated with elevated oxylipins (Fishbein et al., 2020). In addition to fatty acid metabolism, arginine and kynurenine metabolism have been shown to correlate with proinflammatory cytokines (Xiao et al., 2021). These metabolites are readily reported in metabolomics data sets (Metabolomics Workbench, n.d.) and can be used as biomarkers for chronic inflammation.

KC7: Is immunosuppressive

Endogenous metabolites have been shown to regulate immune response pathways. Kynurenine, glutamine, 2-HG, and itaconate and lactate interreact with aryl hydrocarbon receptor and the lactate receptor and exert immunosuppressive effects by promoting the differentiation of immunosuppressive t-regulatory cells and lowering the chemokine production (Ivanov and Anderson, 2013). Indoleamine 2,3-dioxygenses (Liu et al., 2018) and kynurenine-3-monooxygenase enzymes (Shi et al., 2022a) are overexpressed in tumour cells to produce the immunosuppressive metabolite kynurenine (Minhas et al., 2019). Several gut microbiota metabolites including 1H-indole-3-carboxaldehyde, kynurenic acid, butyrate, and propionate are also immunosuppressive and can provide long-term radioprotection and increase t-regulatory cells (Guo et al., 2020). A chemical exposure that increases the generation of these metabolites may be contributing the creation of a pro-tumour environment by immunosuppression effects. T-regulatory cells can be promoted by many factors, including the arginase enzyme, which generate polyamines that inhibit the indoleamine 2,3-dioxygenses. These biochemical links suggest a close dependence of tumour on the metabolism of tryptophan and kynurenine to create an immunosuppressive tumour microenvironment (Hargadon, 2020). Many metabolites that are needed for T-cell activation are depleted from the microenvironment and are replaced with metabolites that different t-cell into t-regulatory cells, which are immunosuppressive.

KC8: Modulates receptor-mediated effects

G protein-coupled receptors (GPCRs) are frequently targeted by FDA-approved drugs. These receptors can be activated by a range of endogenous metabolites, including carboxylic acids, triglyceride, bile acids, hormones, and neurotransmitters (Chen et al., 2023).

KC9: Causes immortalization

Metabolism of nucleotides (monomers of nucleic acids) is a key pathway for supporting the cell immortalization (Burton and Gewurz, 2022). Immortalized cancer cells have been shown to depend on purine metabolism via adenosine deaminase enzyme (Lamontagne et al., 2021). This pathway along with pentose phosphate pathway (De Falco et al., 2023) seems to play a critical role in the early state of cell immortalization (Hafez et al., 2017). MTHFD2 deficiency in immortalized cancer cells is also linked with the accumulation of purine metabolism intermediates (Ducker et al., 2016; Sugiura et al., 2022). A specific metabolic signature that represents a disturbed nucleotide metabolism can be used to link a chemical exposure with the cell immortalization mechanisms.

KC10: Alters cell proliferation, cell death, or nutrient supply

A sustained tumour growth depends on a proper supply of amino acids, nucleotides, and lipid substrates for synthesizing cell membranes, proteins, and DNA and RNA molecules (Xiao et al., 2022). Both de novo and salvage pathways are used to fuel the substrate supply (Shi et al., 2022b). Abnormal lipid metabolism is

a hallmark of most tumours to generate longer and desaturated fatty acids, which are needed to support rapid proliferation (Zhu and Thompson, 2019; Krug et al., 2020; Bergers and Fendt, 2021). Tumours rely on Stearoyl-CoA desaturase enzyme to generate desaturated fatty acids as well as to use glucose and glutamine, the most abundant carbon substrates for fatty acid synthesis (Zhu and Thompson, 2019; Krug et al., 2020; Bergers and Fendt, 2021). The sphingolipid metabolite sphingosine-1-phosphate and polyamines (spermine, putrescine and spermidine) are multifunctional signalling molecules and have been shown to promote cell proliferation (Casero et al., 2018; Nagahashi et al., 2018; Ogretmen, 2018; Cartier and Hla, 2019). By contrast, Ceramide lipids are pro-apoptotic (Dadsena et al., 2019; Choi et al., 2021) and can cause autophagic cell death, and tumour cells may reduce the levels of ceramides during metabolic reprogramming.

2.4. Conclusion and future directions

Metabolomics data sets can offer supporting evidence for biological processes that are relevant for KCs. These data sets are often collected for toxicology and human epidemiological studies, and their quality has substantially improved over the past decade. Analysis of metabolomics data sets using diverse and complementary bioinformatics approaches (Barupal et al., 2018), data integration methodologies, and knowledge mapping may contribute significantly to the expanding use of KCs for evaluating cancer hazard potentials of prioritized agents. As summarized in Section 2.3, metabolites from various metabolic pathways can be linked with KCs. A list of metabolites, pathways, and Gene Ontology (GO) terms that are relevant for KCs can be curated and maintained by the *IARC Monographs* Secretariat, which can help in selecting relevant publications (Fig 2.2).

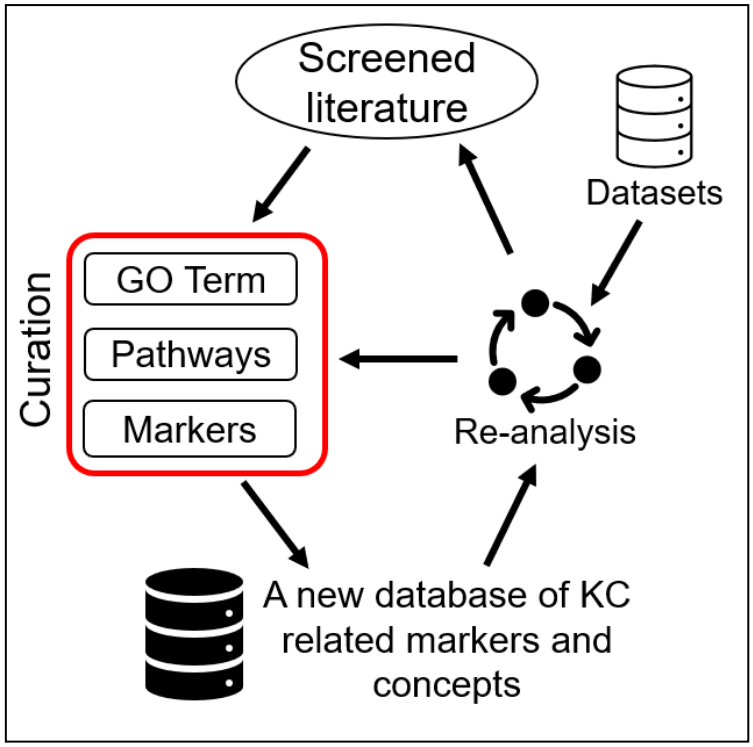

Figure 2.2 Continuous integration and evolution of a KC concept database by literature and omics data fusion

These interpretations can be tested in a metabolomics data set using several bioinformatics methods. The first step is to categorize metabolites into relevant groups. Conventionally, pathway maps are often used for this purpose. More recent approaches can use chemical classes (Barupal and Fiehn, 2017) and GO terms (Mahajan et al., 2024) to cover all metabolites and biological processes that are not yet catalogued in pathway databases. Using frequency (hypergeometric test) or distribution comparison (Kolmogorov–Smirnov test) statistics, the groups that are significantly associated with an exposure or disease stage can be identified. In multiomics data sets, GO terms can be used as a backbone to integrate the data and find metabolic processes that are linked with GO terms related to each KC. There is a need to develop a curated list of GO terms linked with each KC, which can subsequently be used as a guideline for filtering the GO terms in multiomics investigations. Known biomarkers for KCs, for example cytokines for chronic inflammation (KC6), can be used for identifying metabolites and their groups that are relevant for inflammatory pathways. Individual metabolites and their GO terms found to be associated with chemical exposures should be checked in the lists of KC related metabolites and GO terms.

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3. Considerations for interpretation of mutational signature studies

Sandra Perdomo, Michael Korenjak, and Jiri Zavadil

3.1 Introduction

Mutational signature analyses are increasingly applied to genomic studies because of their ability to reveal environmental and endogenous mutagenic mechanisms that are operative in cells (Alexandrov et al., 2013a; 2020; Alexandrov and Stratton, 2014; Hollstein et al., 2017; Degasperi et al., 2022).

Somatic mutations in cells are caused by mutational processes induced by both exogenous and endogenous factors that operate in the cell lineage between the fertilized egg and the development of a cancer cell (Stratton et al., 2009). Sequencing the genomes of cancer or non-cancer somatic cells allows the identification of somatic mutations that are the result of DNA damage that occurred in the past. Therefore, the analysis of somatic mutations can be indirectly used to understand DNA damage and repair processes, as these activities determine many of the acquired mutations. Each mutational process may involve components of DNA damage or modification, DNA repair, and DNA replication (which may be normal or abnormal) and generates a characteristic mutation profile that contributes to the overall mixed mutation spectrum in a given cell or tissue. Each process-specific mutation profile can then be expressed mathematically as a unique mutational signature. Mutational signatures can be based on base substitutions (single (SBS) and doublet (DBS)), small insertions and deletions (indels (ID)), structural genomic rearrangements (SV) and chromosome copy-number (CN) changes (Alexandrov et al., 2020).

The rapid development of next-generation sequencing (NGS) technologies introduced in the mid-2000s enables the sequencing of large numbers of cancer genomes with high accuracy at a relatively low cost (Goodwin et al., 2016). These technology advances drove the coordinated efforts of the International Cancer Genome Consortium (ICGC) (Aaltonen et al., 2020) and The Cancer Genome Atlas (TCGA) (Tomczak et al., 2015) to generate large-scale data sets that characterized the genomic landscapes of cancers in thousands of patients from more than 40 tumour types. These data sets, now publicly available, provided sufficient power to identify and characterize a large number of mutational processes operating across human cancer. Additional mutational signatures have been extracted and the existing ones validated with the analysis of over 12 000 whole-genome–sequenced cancers collected prospectively as part of the United Kingdom National Health Service (NHS) for the 100 000 Genomes Project (Degasperi et al., 2022).

This chapter summarizes the opportunities, practical considerations, and caveats that should be considered when evaluating studies involving mutational signature analyses in the context of cancer hazard identification.

3.2. Evaluation of the study design and experimental models

The association of mutational signatures with specific mutational processes and cancer risk factors relies on studies and experimental work conducted in different designs. Most of the genomic studies are based on biological samples from patients diagnosed with cancer. Cancer genomics studies have successfully been used to extract the catalogue of mutational signatures active in cancer (Alexandrov et al., 2020). They rely on large sample numbers and commonly lack relevant exposure information needed to link the extracted signatures to specific mutagenic agents or processes. This association has successfully been established for

several mutational signatures with the help of controlled *in vitro* and animal exposure models (Melki et al., 2020), as well as dedicated mechanistic studies using epidemiological collections (Drost et al., 2017; Huang et al., 2017). More recently, large-scale international studies have started to harness epidemiological collections with exposure information for mutational signature analysis (Moody et al., 2021). Study designs frequently include primary tumours and, to a lesser extent, analyses of metastatic tumours (Priestley et al., 2019; Degasperi et al., 2022; Martínez-Jiménez et al., 2023). However, the interpretation of the presence of mutational signatures using these different designs may vary, as primary tumours reflect the original mutational processes contributing to tumorigenesis, including plausible causal exposures, while mutational analyses in metastatic cancer might reflect additional mechanisms derived from both therapeutic interventions and the endogenous changes in the evolution of the tumour as the carcinogenic process progresses (Pich et al., 2019).

Gaps in understanding the early events in the carcinogenic process, in combination with advances in sequencing technologies, have shifted some of the focus towards studies examining mutational processes in non-tumoral human tissues, including, skin, liver, lung, oesophagus, bladder, colon, and endometrium (Martincorena and Campbell, 2015; Martincorena et al., 2018; Moore et al., 2020; Yoshida et al., 2020; Kakiuchi and Ogawa, 2021). The confirmed presence of mutational signatures in non-cancerous tissues creates potential opportunities to refine both pertinent cancer prevention interventions and more timely early detection strategies (Balmain, 2023). The evaluation of mutational signatures in non-tumour tissues requires the use of high-depth and error-corrected sequencing techniques allowing for accurate identification of mutations with low variant allele frequency (explained in Section 2.2, Chapter 2, Part I).

Most of our current knowledge of the causal role that exposures play in inducing cancer has come from experimental model systems used to characterize mutational signatures induced by both exogenous factors and endogenous processes. Exposure assays are usually performed in cell cultures (Zavadil and Rozen, 2019), including tissue specific organoids (Drost et al., 2017), in *Caenorhabditis elegans* models (Meier et al., 2020), and in rodent models (Riva et al., 2020), or in multiple systems simultaneously (Huang et al., 2017). Each model system contributes singular advantages but also has experimental restrictions for generating evidence of the causes or processes associated with a particular mutational signature (Melki et al., 2020). Experimental protocols using cell lines can be highly standardized. Key advantages of cell-line models are (i) the capacity to evaluate systematically, mutational patterns associated with a broad selection of environmental or therapeutic mutagens, generated under highly controlled conditions (Kucab et al., 2019); and (ii) their shorter duration compared to standard 2-year animal bioassays. However, the experiments still take several months, owing to the required subcloning steps for making the cells amenable to massively parallel sequencing-based mutation analysis. Moreover, most cell lines have important metabolic limitations, and cancer cell lines are often physiologically highly abnormal and harbour genetic alterations that result in high intrinsic mutation rates and characteristic profiles that can interfere with the exposure-specific effects under investigation (Koh et al., 2020). Immortalized cell lines derived from normal (non-cancerous) tissue represent less-transformed alternatives that are being exploited (Severson et al., 2014; Boot et al., 2018). Nevertheless, time-matched, untreated control cells are an essential element in the study design when relying on all cell-based exposure models (Kucab et al., 2019). In contrast to cell models, rodent models may be exposed to carcinogens or cancer-promoting agents over months or years, similar to environmental exposures in humans, and thus provide a better representation of the long-term carcinogenic activities of chemicals (Bucher, 2002). Important caveats include potential metabolic and DNA repair differences between rodents and humans that could affect the profiles of mutational signatures, as well as the duration and high cost of animal cancer bioassays.

Environmental exposure compounds that react directly with DNA frequently must be metabolically activated to form DNA adducts (see Chapter 1, Part 1). This important requirement needs to be considered

for the design of chemical exposure/mutational signature studies in cell lines, many of which lack key metabolic competence. Different strategies have been devised to overcome this caveat. First, experiments can be carried out in a metabolically active cell type, such as liver cells (Huang et al., 2017). Second, an exogenous metabolization system containing a mixture of phase I and phase II enzymes involved in drug metabolism (liver fraction S9 mix) can be used. The cells can either be simultaneously exposed to a chemical and the S9 mix, along with required co-factors, a strategy successfully applied for large-scale mutational signature screening (Kucab et al., 2019), or the chemicals can be pre-activated by the S9 mix before exposure of the cells (Kucab et al., 2019; Shao et al., 2020). Third, chemically stable reactive metabolites of compounds of interest are commonly being used for studies involving characterization of DNA damage and mutational signatures, to circumvent the metabolic limitations of cells (Upadhyaya et al., 2006; Zhivagui et al., 2019). These metabolites can either directly react with DNA or they are used as stable precursors that are activated by common enzymes expressed in all cells.

3.3. Quality assessment of the mutational signature analysis

Assessing the quality of the evidence generated by most of the genomic studies, including mutational signature analyses, requires a close evaluation of the quality and restrictions of the types of samples used and the sequencing methodology implemented.

3.3.1 Sample type

Most of the studies performed using human or animal tissues use either frozen tissue or archived specimens (formalin fixed paraffin embedded tissue, FFPE). Mutational signature analysis on FFPE material is more problematic because of the artefactual mutations induced by formalin fixation. For some studies, especially in the context of animal bioassays, simultaneous sequencing of exposure-associated and spontaneously formed tumours from the same study and tissue can be used as a strategy to minimize artefactual mutation patterns. Moreover, many of these artefacts have been already catalogued, and algorithms are being developed to identify and correct artefacts induced by formalin fixation (Alexandrov et al., 2020; Guo et al., 2022b; Basyuni et al., 2024; Chavanel et al., 2024).

More recent studies have begun analysing non-invasive sources of DNA such as cell free (cfDNA) to identify mutational signatures for possible translational application in cancer early detection and precision diagnosis. Initial studies have identified mutational signatures in cfDNA in plasma from colorectal, gastric, non-small cell lung, ovarian, and breast cancer patients (Wan et al., 2022) and in urine from upper tract urothelial carcinoma patients (Lu et al., 2020). The fragmented nature of cfDNA, however, restricts the identification of mutational signatures to those based on single base substitutions (SBS).

3.3.2 Sequencing technology

The use of different sequencing technologies can result in differences in the mutational patterns observed in samples and affect the mutational signatures extracted from these patterns (Alexandrov et al., 2020). Different NGS technologies have inherent sequencing biases and can cause technical artefacts specific to the experimental preparation protocol or to a sequencing platform. Both biases and artefacts can impact the reliable identification of mutational signatures. Therefore, it is important to evaluate key sequencing metrics to determine the robustness of sequencing methodologies used. This includes the *use of standard* vs *error-*

corrected NGS methodologies, and the number of sequencing reads and depth according to the experimental model (i.e. normal tissue versus tumours) and the technique used (whole exome sequencing (WES) or whole genome sequencing (WGS)).

Mutational signature analysis has predominantly used cancer WES (representing ~1–3% of the human genome footprint). However, the many-fold-greater numbers of somatic mutations present in whole genomes provide substantially increased power for signature characterization, enabling a better separation of partially correlated signatures and the extraction of signatures that contribute relatively small numbers of mutations (Abbasi and Alexandrov, 2021). Signatures of base substitutions and some indels can be derived, but at much lower resolution, from WES data. Overall, tumour types with high numbers of mutations have a larger fraction of samples showing consistent mutational profiles generated based on WES or WGS data. However, mutational signatures with flat profiles such as SBS3 (attributed to defective homologous recombination (Nik-Zainal et al., 2012a, b), SBS5 (attributed to clock-like ageing processes) or SBS40 (unknown aetiology (Alexandrov et al., 2013a, 2020), and signatures based on a lower mutational burden (i.e, SBS18, attributed to reactive oxygen species (Alexandrov et al., 2013a) can be detected with much higher resolution from WGS data. Surprisingly, this is also the case for several signatures generally attributed to hyper-mutation, which now can be also detected at lower mutational burden in WGS data. Genome rearrangements and chromosome copy-number signatures can be derived only from WGS data. New sequencing technologies have been developed in the last years to overcome the limitations of traditional WES or WGS. For instance, error-corrected sequencing has been developed as a sensitive technique that accurately identifies both clonal and non-clonal mutations with low variant allele frequency, making it suitable for the study of normal tissues. However, it can generate end-repair artefacts produced by the experimental procedures that should be identified and removed before mutational signature extraction (Schmitt et al., 2012; Krimmel et al., 2016; Abascal et al., 2021; Abbasi and Alexandrov, 2021). In the past few years, targeted and genome-scale error-corrected sequencing approaches have been used to successfully characterize mutation spectra and mutational signatures of cancer risk factors at very low mutational frequencies ($< 1X\ 10^{-7}$ per nucleotide) in non-clonal tissue samples and cell cultures (Valentine et al., 2020; Abascal et al., 2021; Wang et al., 2021b; LeBlanc et al., 2022; Zhivagui et al., 2023). Advances in single cell sequencing technologies have also allowed researchers to capture a better resolution of mutational processes operative at a single-cell level, eliminating the heterogeneity of mutational processes prevalent at the bulk tissue level. Currently, the methodologies of extraction and validation of mutational signatures at the single-cell level have not been standardized because of the large number of whole-genome and transcriptome amplification artefacts and the variability of sequencing depth in the assays. Finally, cost-efficient sequencing technologies based on targeted panels, although commonly used in the clinic, only allow identification of single base substitution signatures in highly mutated samples. The assignment of mutational signatures to a single sample is much more limited, owing to the overall low mutation burden in targeted sequencing.

3.3.3 Mutational signature identification

Evaluation of methods used for exposure estimation (also termed as *contribution of* a signature or *activity of* a signature) have evolved in parallel to sequencing technologies. Various tools for calling mutations and computational frameworks for extracting mutational signatures have been applied to different tissue types and sequencing platforms (Abascal et al., 2021). It becomes essential then to evaluate the methodology of signature extraction and/or assignment accounting for the technical limitations and assumptions of each method (Islam et al., 2022; Wu et al., 2022). Identification of mutational signatures is more difficult when cancers show multiple signatures each having a small contribution (Islam and Alexandrov, 2021). Detection

methods based on probabilistic models, especially EMu and bayesNMF (non-negative matrix factorization), have in general better performance than NMF-based methods (Omichessan et al., 2019). It is rather important to indicate the level of certitude when a signature is assigned (Huang et al., 2018; Senkin, 2021) and if strand bias (transcriptional strand asymmetry in the mutation called) was included in the final analysis.

Many other parameters independent of the signature extraction and assignment process can impact the specificity and sensitivity for detecting a particular mutational signature in cancer genomes. These parameters include (Alexandrov et al., 2013b; Islam et al., 2022): (i) the number of cancer genomes analysed, as the number of genomes required to identify all active signatures increases exponentially with the total number of operative signatures; (ii) the degree of similarity between different operative mutational signatures; (iii) the total number of operative signatures; (iv) the strength of an exposure resulting in a mutational signature, because it can impact the discovery of other signatures present at low levels (less than 5% contribution) across a set of cancer genomes; (v) the overall tumour mutational burden (TMB) of individual samples (a high TMB allows for reliable signature extraction); (vi) the number of sequence contexts per mutation type (i.e. include one or the two bases in the immediate 5′ and 3′ sequence context of the mutated base/s) used to examine the operative signatures. More contexts can aid in the discovery of additional signatures but could limit the ability to robustly identify individual mutational signatures, as fewer mutations exist per context.

3.4. Assessment of relevance, interpretation of results, and association with Key Characteristics of Carcinogens (KCs)

Understanding the mechanisms underlying the mutational signature formation can support the evidence regarding the KCs for a particular agent. In this context, mutational signatures can help assess the contribution of an agent to three KCs: *KC2 (is genotoxic), KC3 (alters DNA repair or causes genomic instability), and KC5 (induces oxidative stress)*

A mutational signature is the outcome of a mutagenic process caused by DNA damage that is subsequently resolved by a DNA repair mechanism and/or the cell replicative machinery. However, this multistep process creates more mechanistic nuances when it comes to the interpretation of mutational signature evidence for evaluation of potentially carcinogenic compounds. For instance, a mutational signature can form as a consequence of a direct (genotoxicity – *KC2*) or indirect process(es) (oxidative stress/damage – *KC5*; altered DNA repair, deregulated endogenous mutagenic enzymatic activities, or genomic instability – *KC2, KC3*). Some mutational signatures clearly reflect the primary DNA damage caused by DNA adducts, such as alkylating agents causing *G: O6-meG adducts, which result in mutational signatures characterized predominantly by C:G > T:A transitions in specific trinucleotide contexts. Yet other DNA adducts at some sites do not result in mutations. Hence, for (potentially) direct-acting chemicals, prior knowledge of compound-specific DNA adducts or simultaneous adduct analyses help strengthen the link with a characteristic mutational signature. In some instances, however, one or multiple mutational signatures can be formed as a result of impaired DNA repair mechanisms involved in resolving DNA damage rather than from the direct effect of an exposure. This relationship has been studied in detail in the context of exposure studies in DNA damage deficient genetic backgrounds in *C. elegans* (Meier et al., 2014; Volkova et al., 2020). Therefore, the interaction between DNA damage and DNA repair underlying the mutational signature formation can be defined by the following formula:

Mutational Signature = DNA damage – DNA repair mechanism(s).

Additional information on genomic and epigenomic contexts, which include chromatin structure, replication timing, and transcriptional and replication strand biases, can help to elucidate the mechanisms of DNA damage and DNA repair processes associated with a particular mutational signature (Haradhvala, Polak et al. 2016; Coleman and De, 2018; Tate et al., 2019).

Mutational signatures linked to molecular mechanisms of DNA damage and repair associated with *KC3* are also valuable to understand tumour biological processes that are clinically relevant. For instance, cancers presenting homologous recombination deficiency (HRD) owing to abnormal double strand break repair are characterized by a range of mutational signatures including a flat base substitution profile, with some general prominence of C:G > G:C transversions (SBS3), small indels (ID6), mutations at TC dinucleotides (DBS13), and genome rearrangements (CN17, SV3) (Nik-Zainal et al., 2012a). These cancers respond to therapies targeting this DNA defect, including poly-ADP ribose polymerase inhibitors (PARPi) and platinum chemotherapy (Lord and Ashworth, 2017). Another example is tumours characterized by defective DNA mismatch repair and microsatellite instability (MSI), which present MSI associated signatures: SBS14, SBS15, SBS20, SBS21, SBS26, and SBS44 and can be candidate targets for immunotherapy (Le, Uram et al. 2015).

Experimental validation of signatures linked to specific exposures in human sample collections adds valuable information to establish causality (Koh et al., 2021). An increasing number of signatures of different mutation classes is being reported, and correlations are being drawn with various exposures and /or endogenous factors. Therefore, it is fundamental to gain a mechanistic understanding of how mutational signatures arise through experimental exploration. This can be done through classic genotoxicity screens, the generation of mutational signature catalogues of environmental agents using cells or animal bioassays (Olivier et al., 2017; Kucab et al., 2019; Riva et al., 2020) and gene editing assays using cell lines, organoids, and *in vivo* models (Drost et al., 2017; Meier et al., 2018; Zou et al., 2018, 2021). It is imperative to also assess caveats of experimental signature validation strategies and of the available evidence. For example, several important cancer risk factors are complex chemical mixtures, while experimental studies generally characterize mutational signatures of individual compounds, and it can be very challenging to infer the contribution of specific chemicals to signatures associated with complex exposures *in vivo*.

A recent example shows how experimental evidence could elucidate the genotoxic (*KC2*) effect of a secondary metabolite secreted by certain strains of bacteria in human colon tissue and colorectal cancer. In 2019, a particular mutational signature (designated SBSA) characterized by T:A > C:G mutations in A**T**A, A**T**T and T**T**T trinucleotide contexts, and T:A > G:C mutations at T**T**T, was described in colorectal cancers and healthy colorectal epithelial cells (Lee-Six et al., 2019). SBSA mutational burden correlated closely with that of another indel mutational signature (IDA), in which single T deletions in short runs of T bases (with a modal average of four) predominate, leading to the suggestion that these two signatures are a result of the same underlying mutational process. The pattern of mutations suggested an extrinsic, locally acting and unevenly distributed mutagenic insult as the potential cause. A year later, a new study using human intestinal organoids subjected to repeated luminal microinjections of colibactin-producing *pks+ Escherichia coli* showed a unique substitution signature (SBS88) characterized by T:A > A:T and T:A > C:G mutations particularly at A**T**A, A**T**T and T**T**T motifs as well as T:A > G:C mutations at T**T**T, and an ID signature (ID18) featuring single A or T deletions at poly(dA:dT) tracts (Pleguezuelos-Manzano et al., 2020). These findings explained the mutagenic impact of the DNA alkylating effects of colibactin described in 2019 (Wilson et al., 2019) and they became additionally validated by molecular dynamics simulations and experimental data from an independent study showing enrichment of colibactin-induced damage at (A+T)-rich hexameric sequence motifs (Dziubańska-Kusibab et al., 2020). Additional findings related to bacterial infection and oral carcinogenesis confirmed the presence of this signature in other mucosal tissues (Boot et

al., 2020). Together, the experimental data provided strong evidence of mutational signatures caused by colibactin, a genotoxic compound secreted by *pks+ Escherichia coli* (Díaz-Gay et al., 2025).

3.5. Case Studies

The following case studies have been selected to describe mutational signatures associated with different directly and indirectly acting exposures (or biological processes), and to exemplify the diversity of possible associations found in the literature:

3.5.1 Case study 1

Mutational signatures originate from directly DNA damaging environmental mutagens. Three mutational signatures SBS7, DBS1 and ID13 (See Fig 3.1) are characterized by transition mutations and indel profiles in DNA reflecting the formation of cyclobutane pyrimidine dimers or 6–4 photoproducts directly triggered by ultraviolet radiation (Hayward et al., 2017). A large amount of sequencing data from cancers of the skin (melanoma, basal, and squamous cell carcinoma) directly exposed to sun has now been analysed allowing SBS7 to be split into four distinct components: SBS7a/SBS7b/SBS7c/SBS7d.

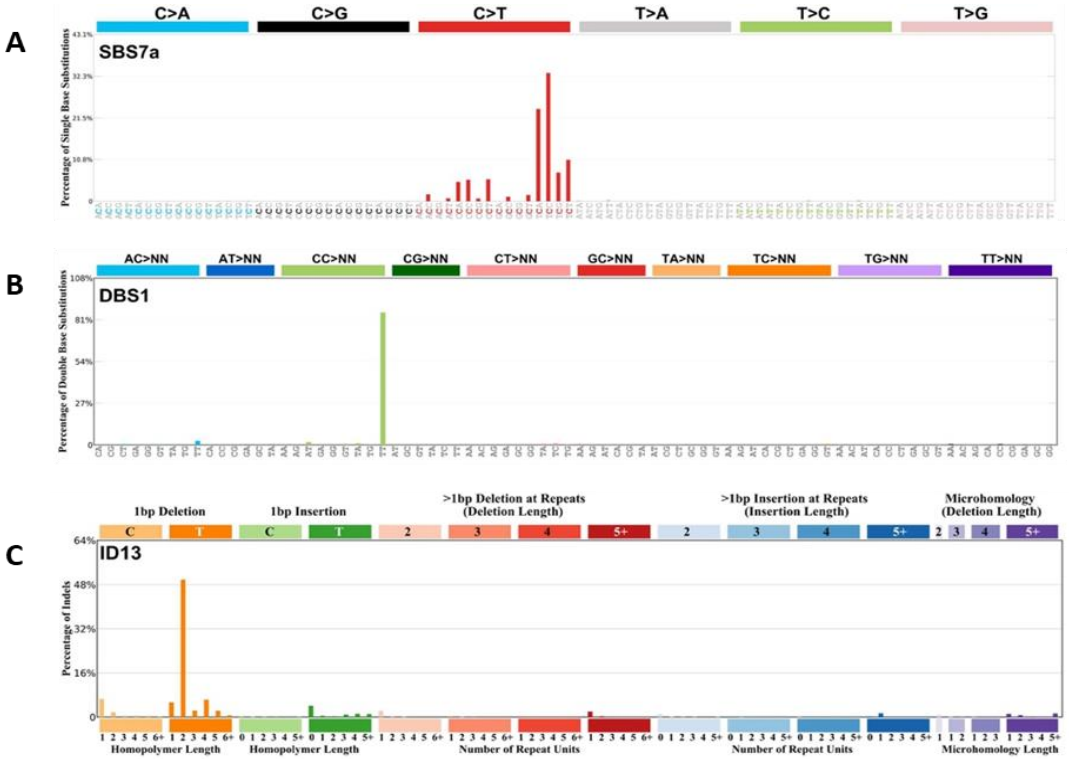

Figure 3.1. Mutational signatures originate from direct environmental mutagens. A. SBS7a is composed of C:G > T:A substitutions at TpC dinucleotides probably due to repair of 6-4-photoproducts; B. DBS1 shows directly more CC > TT mutations on the untranscribed strands of genes indicative of damage to cytosine and repair by transcription coupled nucleotide excision repair; C. ID13 mutational signature. Taken together SBS7, DBS1 and ID13 mutational signatures correlate with each other, and it strongly suggests they are all caused by *UV light* exposure.

- SBS7a is composed of C:G > T:A substitutions at TpC dinucleotides probably owing to repair of 6-4-photoproducts.
- SBS7b involves C:G > T:A substitutions at CpC and TpC, characteristic of cyclobutane pyrimidine dimers.
- SBS7c presents high levels of T:A > C:G and T:A > A:T mutations, potentially caused by indirect DNA damage after ultraviolet radiation.
- SBS7d involves C:G > T:A and T:A > C:G substitutions.
- DBS1 shows more CC > TT mutations on the untranscribed strands of genes indicative of damage to cytosine and repair by transcription coupled nucleotide excision repair.
- ID13 is characterized by T deletions of TT dinucleotides.

Taken together, the presence of SBS7, DBS1, and ID13 mutational signatures in tumour samples is intercorrelated; they are almost exclusively found in skin cancer, and the mechanistic evidence strongly suggests they are all caused by UV light exposure.

3.5.2 Case study 2

Mutational signatures can form as an indirect consequence of multiple potential processes. For example, SBS17 (Alexandrov et al., 2013a) is predominantly characterized by T:A > G:C transversions at NTT and T:A > A:T and T:A > C:G transitions at CTT sequence contexts. A larger number of analysed samples later allowed separating SBS17 into two distinct components: SBS17a and SBS17b (see Fig 3.2) (Alexandrov et al., 2020).

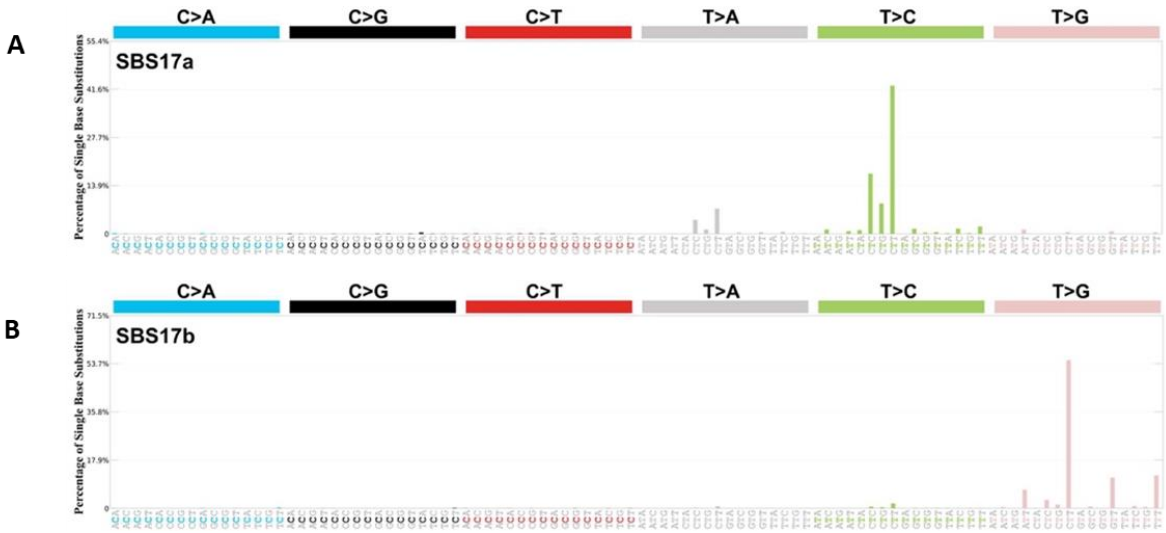

Figure 3.2. Mutational signatures can form as an indirect consequence of multiple potential processes. SBS17 is predominantly, but not exclusively, found in stomach and oesophageal adenocarcinoma. It has been split into signatures SBS17a (A) and SBS17b (B).

The analytical and experimental evidence that has been collected for this signature has shown that:

- SBS17 is present both at low and high mutational densities in oesophageal and stomach cancers. Gastric acid exposure was suggested as a potential cause (Dvorak et al., 2007; Dulak et al., 2013; Nones et al., 2014).
- SBS17 has been detected in metastatic cancers and human small intestinal organoid cultures treated with 5-fluorouracil, a second suggested cause (Christensen et al., 2019).
- SBS17 has also been observed in untreated breast cancers (Nik-Zainal et al., 2016) and in clones from untreated mouse embryonic fibroblasts (Nik-Zainal et al., 2015; Milholland et al., 2017; Zhivagui et al., 2019).
- *In vitro* experiments demonstrated that oesophageal tissues and cell lines exposed to bile/gastric acid will increase levels of free 8-oxo-dGTP, a product of oxidative DNA damage that can mispair with A, leading to T:A > G:C transversion mutations (Dvorak et a., 2007).

In summary, the current data suggest that the T:A > C:G, T:A > A:T and T:A > G:C mutations of SBS17a and SBS17b are possibly byproducts of oxidative damage in the free nucleotide pool, which may be secondary to exposure to bile or gastric acid or 5-fluorouracil. However, further experimental studies are required to validate this hypothesis.

3.5.3 Case study 3

Convergence, multiple compounds can result in highly similar mutational signatures.

There is experimental evidence showing that cell lines exposed to dibenzo[a,l]pyrene (DBP) and dibenzo[a,l]pyrene diol-epoxide (DBPDE), both components of tobacco smoke, and cells exposed to aristolochic acid I (AAI), a phytochemical associated with urothelial cancer, exhibit remarkably similar mutational profiles (see Fig 3.3A) (Kucab et al., 2019). The similarity of peaks of the T:A > A:T component between these different compounds reflects a common process of adduct formation at adenine residues (N^6-adenine). In AAI, this transversion mutation accounted for 83% of the signature, whereas for DBP and DBPDE it amounted to 53–70% of the total mutations.

Mouse models exposed to using 7,12-dimethylbenz[a]anthracene (DMBA) to induce skin cancer have also reported an enrichment of T:A > A:T signature mutations similar to those induced by AAI and contributing > 60% of all detected mutations (see Fig 3.3B)(McCreery et al., 2015; Nassar et al., 2015). Cell-based model systems exposed to glycidamide, the reactive metabolite of acrylamide, revealed a new mutational signature with a very similar T:A > A:T mutation pattern, accompanied by C:G > A:T transversions and T:A > C:G transitions, all marked by significant transcription strand bias (Zhivagui et al., 2019). A more recent analysis of over 12 000 human whole genomes sequenced as part of the 100K Genomes project (Degasperi et al., 2022) showed signatures presenting T:A > A:T mutations, with similarities to AAI-associated signatures in kidney, colon and liver cancer cases, without documented exposure to AAI. (Ng et al., 2021; Degasperi et al., 2022). In conclusion, example 3 shows how DNA adducts of different chemicals from unrelated exposures can leave similar mutagenic footprints.

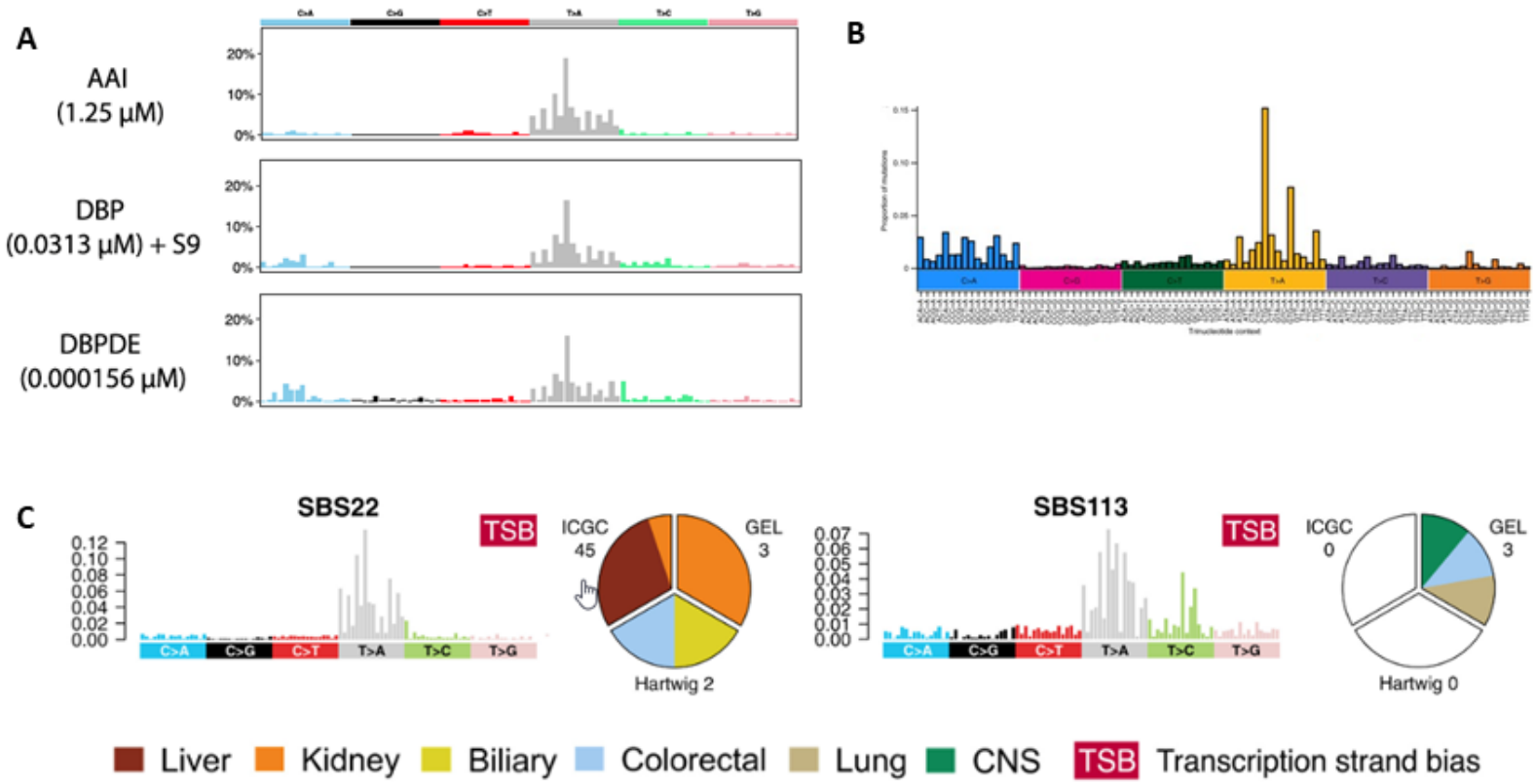

Figure 3.3. Convergence, multiple compounds can result in highly similar mutational signatures. A. The similarity of peaks of the T:A > A:T component induced by different compounds reflects a common process of adduct formation at adenine residues in iPSC cells (Kucab et al., 2019). In AAI, this transversion mutation accounted for 83% of the signature, whereas for DBP and DBPDE it amounted to 53–70% of the total mutations; **B.** Mouse models using 9,10-dimethyl-1,2-benzanthracene (DMBA) to induce skin cancer and study the tumour progression have also reported an enrichment of T:A > A:T signature mutations similar to AAI-induced ones, and contributing to > 60% of all detected mutations (McCreery et al., 2015; Nassar et al., 2015); **C.** A more recent analysis of over 12 000 human whole genomes sequenced as part of the 100K Genomes project (Degasperi et al., 2022) showed a signature SBS113 presenting T:A > A:T mutations in central nervous system (CNS), colorectal and lung tumors of cases without documented exposure to AAI, with similarities to AAI-associated signature SBS22 in the liver, kidney, biliary, and colorectal tumors.

3.6. Conclusion & future directions

In the past 15 years, evaluations of genotoxic compounds began including information on patterns of DNA damage associated with *TP53* mutations and caused by certain compounds such as aristolochic acid and cyclophosphamide. (IARC, 2012a). The recent development of mutational signature studies has provided valuable support for different KCs (KC2, KC3, KC5), and their genome-scale nature has considerable advantages over classic genotoxicity readouts. The association of specific compounds and mutational processes with individual mutational signatures requires sample collections with extensive exposure information, validation in controlled, experimental systems, or mechanistic data from other assays. Mutational signature analyses should be viewed as supportive data and used complementarily to other assays as part of the mechanistic evidence evaluations for the *IARC Monographs*.

With the advancements of NGS and an increasing focus on the utilization of large epidemiological sample collections with exposure information, mutational signature analysis is likely to mature and become increasingly relevant in the future, as part of the evaluation process for KCs and cancer hazard identification. Evaluation of studies presenting evidence of mutational signatures associated with specific exposures requires a comprehensive understanding of the mechanisms behind the generation of mutational signatures, including the different models used and the technical aspects of the study conduct and the analysis and interpretation of data, as described above.

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4. Data from high-throughput screening assays

Brad Reisfeld and Weihsueh Chiu

4.1 Introduction

To date, the sole source of high-throughput screening (HTS) data used in *IARC Monographs* evaluations is the United States Environmental Protection Agency's ToxCast and Tox21 database (Richard et al. 2016). Henceforth, this database will be referred to simply as 'ToxCast'. As of v3.5, which was released in August 2022, ToxCast contained the detailed results and summary analyses for over 2200 high-throughput assays related to molecular targets and cellular responses for over 9400 unique substances (mostly not requiring metabolic activation for activity). For each compound–assay pair, a variety of information is available (US EPA, 2018), including the concentration at which 50% of maximum activity is observed (AC_{50}), parameters associated with dose response models fit to the data, a resulting indicator of whether the assay was active or inactive via a 'hit call', and diagnostics flags to help interpret the validity of the results.

Starting in Volume 110, IARC Working Groups included ToxCast HTS data to supplement other mechanistic evidence by creating a mapping of certain of the assays to relevant key characteristics of carcinogens (KCs) (Chiu et al. 2018). Since that time, the mapping has been updated for *Monographs* Volumes 123 and 130 to reflect changes to the ToxCast database and new information regarding the relationship of some assay end-points to the KCs. The current version includes almost 300 assays collectively mapping to seven of the ten KCs (KC1: is electrophilic or can be metabolically activated to an electrophile; KC2: is genotoxic; KC4: induces epigenetic alterations; KC5: induces oxidative stress; KC6: induces chronic inflammation; KC8: modulates receptor-mediated effects; KC10: alters cell proliferation, cell death, or nutrient supply). Table 4.1 summarizes how ToxCast data were incorporated in *Monographs* Volumes 110-131.

4.2 Procedures for accessing ToxCast data pertinent to the KCs

ToxCast data can be accessed through the CompTox Chemicals Dashboard (Williams et al. 2017; US EPA, 2023a) using an appropriate identifier for the compound(s) of interest. These results may also be obtained by using the analysis pipeline and database provided by the US EPA (2023b). Because the database changes over time, it is important that the date of download be documented, and the results supplied as supplementary materials for the monograph.

Table 4.1 – Use of ToxCast data in *IARC Monographs* Volumes 110 – 131

Vol.	Agents with ToxCast data included in the Volume	Narrative	Table of active/ inactive results by KC	ToxPI analysis across agents	Inclusion in the Summary (5.4)	Supplemental Material
110	perfluorooctanoic acid (PFOA)	Yes*	No	No	No	No
111	none					
112	malathion, parathion, diazinon, tetrachlorvinphos (including metabolites)	Yes	No	Yes, by KC	Parathion only	Yes
113	2,4-D, lindane, DDT (including isomers and metabolites)	Yes	No	Yes, by KC	Yes	Yes
114	none					
115	1-bromopropane, 2-mercaptobenzothiazole, N,N-di-methylformamide, hydrazine sulfate, tetrabromobisphenol A	Yes, by KC	No	No	Yes	Yes
116	none					
117	pentachlorophenol, 2,4,6- trichlorophenol, 3,3',4,4'-tetrachloroazobenzene, aldrin and dieldrin	Yes	Yes, by sub-category within KC	Yes, by KC	Yes	Yes
118	none					
119	1-tert-butoxypropan-2-ol, β-myrcene, furfuryl alcohol, melamine, pyridine, tetrahydrofuran	Yes	No	No	No	Yes
120	benzene metabolites phenol, catechol, hydroquinone, benzoquinone	Yes	No	Yes, by KC and across KCs, and by assay category	Yes	Yes
121	styrene, styrene-7,8-oxide, styrene glycol, 2-phenylethanol, quinoline	Yes	Yes	No	Yes - but largely null/inactive	Yes
122	isobutyl nitrite, β-picoline, methyl acrylate, ethyl acrylate, 2-ethylhexyl acrylate, trimethylolpropane triacrylate	Yes	Yes	No	No	Yes
123	2-chloronitrobenzene, 4-chloronitrobenzene, 1,4-dichloro-2-nitrobenzene, 2,4-dichloro-1-nitrobenzene, 2-amino-4-chlorophenol, o-phenylenediamine dihydrochloride, ortho-phenylenediamine, N,N-dimethylacetamide, 2-chloroaniline, 4-chloroaniline, p-nitrophenol, acetamide	Yes	Yes	No	No	Yes
124	none					
125	allyl chloride, 1-butyl glycidyl ether, 4-chlorobenzotrifluoride, glycidyl methacrylate and glycidol	No	Yes	No	Yes - but largely null/inactive	No
126	none					
127	o-anisidine, aniline and aniline hydrochloride, cupferron	No	Yes	No	Yes - but largely null/inactive	No
128	acrolein, arecoline	No	Yes	No	Yes - but largely null/inactive	No
129	gentian violet, malachite green chloride, malachite green oxalate, and leucomalachite green	Yes	Yes	No	No	Yes
130	1,1,1-trichloroethane, 1,2-diphenylhydrazine, diphenylamine, and isophorone.	Yes	Yes, in suppl. material	No	Yes - but largely null/inactive	Yes
131	none					

Focused only on nuclear receptors, comparing AC50s with "prototypical" activators.

Outside of the CompTox Dashboard, ToxCast and Tox21 data have been incorporated into several informative resources that can be useful in gaining additional insights into the assay results. These include Bioplanet (https://tripod.nih.gov/bioplanet), which provides a means for interactive exploration of biological pathways and their connections related to the assays of interest; the Tox21 Activity Profiler (https://sandbox.ntp.niehs.nih.gov/tox21-activity-browser/), which provides a way for users to apply a series of flexible filters to examine details of the underlying assay results and various model-derived measures; and the Integrated Chemical Assessment suite of tools (https://ice.ntp.niehs.nih.gov/), which includes utilities to examine concentration response curves and conduct IVIVE and PBPK modelling based on the underlying chemical data and assays. Additionally, the Tox21 Sample Quality Control viewer (https://tripod.nih.gov/tox/samples) provides information about evaluating the tested substance's constituents and purity.

Additionally, members of the *IARC Monographs* Secretariat recently created the open-source software tool *kc-hits* (Reisfeld et al. 2022) (Fig 4.1 and Fig 4.2) to facilitate the process of summarizing, analysing, and presenting the ToxCast data relevant to the KCs for any chemical within the database. Because of its convenience and the uniformity of the results across agents, it is recommended that this software be used by Working Group members as the first step in characterizing the ToxCast data pertinent to the KCs. Moreover, it provides output files in terms of activity, AC50 values, and any flags in a uniform format that can be retained as supplementary materials for the monograph.

When accessing ToxCast data, it is important not only to consider the agent under evaluation, including different chemical forms and isomers, but also any metabolites, as metabolic activation is not fully covered across the assays. For instance, in *Monographs* Volume 110, data from both perfluorooctanoic acid as well as its salt, ammonium perfluorooctanoate, were included. In Volume 113, both isomers p-p'-DDT and o,p-DDT were included, in addition to metabolites p,p'-DDD and p,p'-DDE. Input into agents to include when accessing ToxCast data can be obtained from Working Group members in Subgroup 1 (related to Identification of the Agent) as well as other members from Subgroup 4 (related to metabolism).

4.3 Approaches to summarizing ToxCast assay results

As shown in Table 4.1**,** there are multiple approaches to summarizing ToxCast data, including narratives, tables of active/inactive assays by KC, and ToxPI rankings and visualizations making comparisons across agents. Additionally, in only 5 out of 15 agents were informative conclusions from the analysis of ToxCast included in the Summary Evaluation. In 5 cases, it was mentioned in the Summary but concluded to null, inactive, and/or uninformative.

Moving forward, starting in Volume 130, the kc-hits software has made it easier to produce tabular summaries of active/inactive calls by KC. Additionally, these also facilitate developing narrative descriptions of the results. When there are many active assays, it can be useful to further divide the table by sub-categories within a KC, and for the narrative to be divided into subsections by KC. The ToxPI ranking and visualization approach has not been used since Volume 120, but this may reflect the familiarity of the Working Group members with ToxPI approach, rather than the inherent utility of this approach (Fig 4.2A and B). In order to make this a routine part of the analysis, it is likely that additional specialized software, perhaps as an extension to kc-hits, would require development.

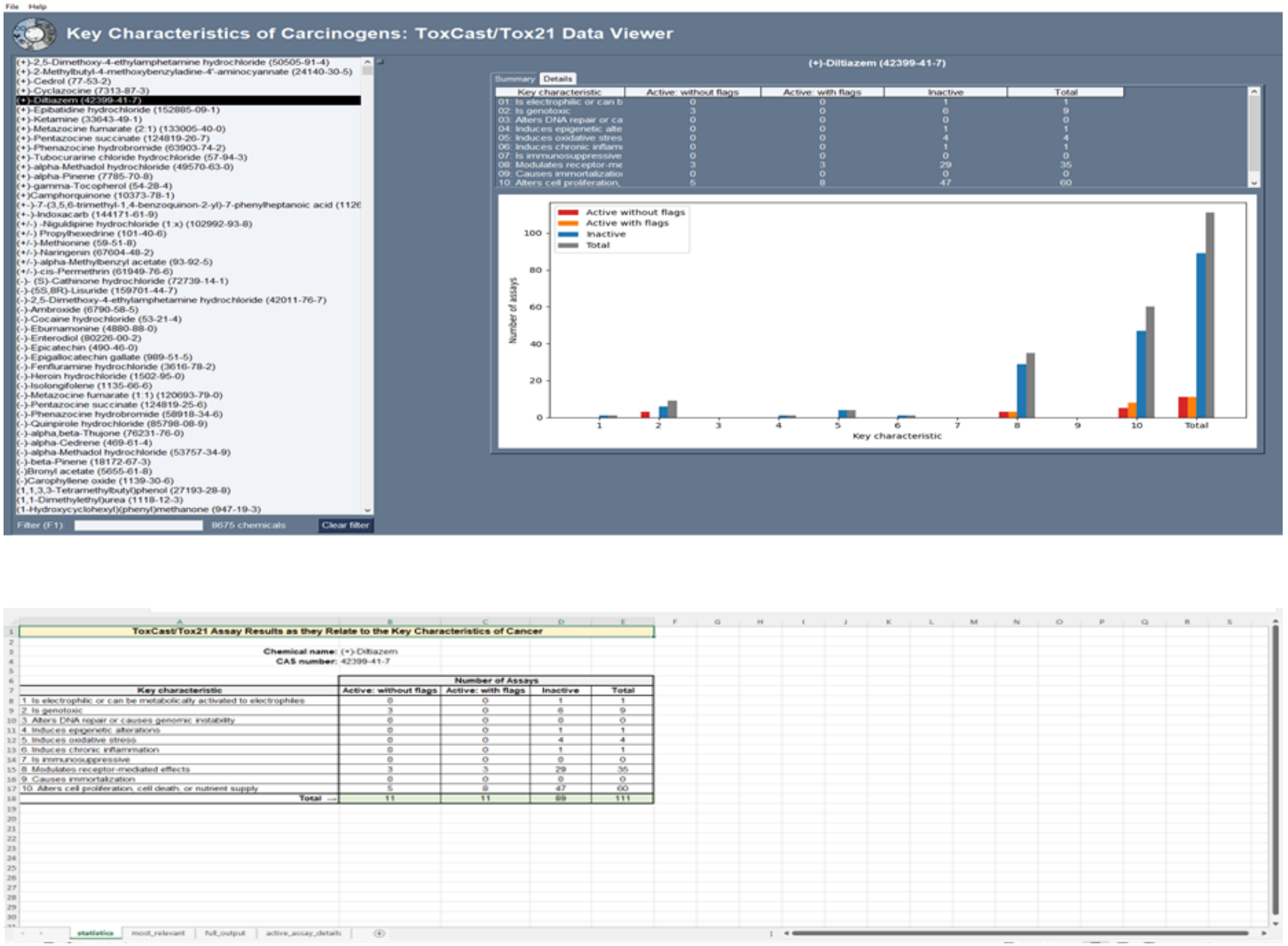

Figure 4.1. Kc-hits Software. Chemical selection (upper panel); and Saved workbook as a summary of statistics related to active assays (lower panel).

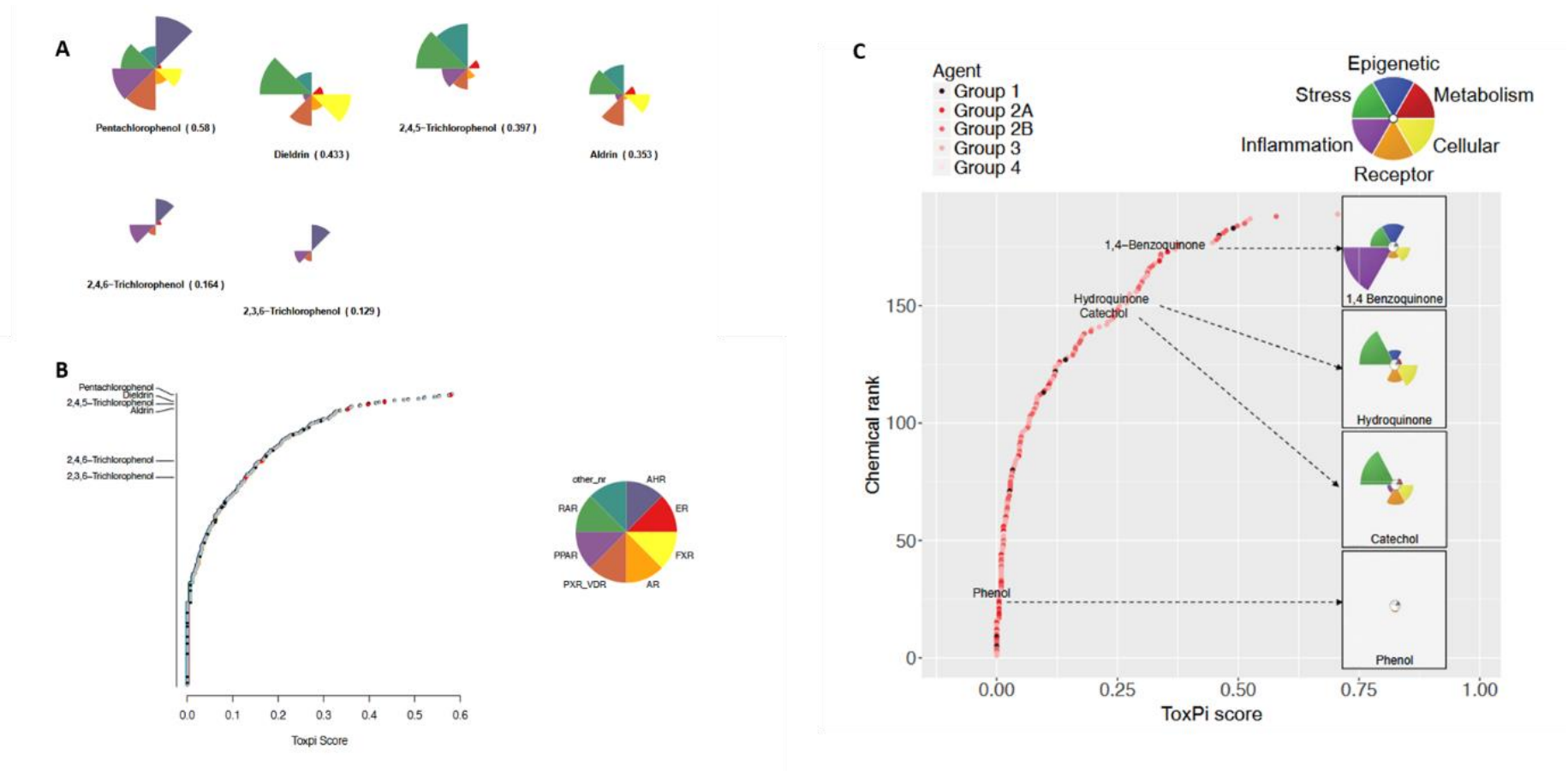

Figure 4.2. Examples of ToxPI visualizations. A and B, ToxPI chemicals ranking using ToxCast assay data results and mapped to modulation of receptor-mediated effects (KC8) (from Fig 4.7 of Volume 117; IARC, 2018); and C, benzene metabolites ranking to six-different KCs (from Fig 4.2 of Volume 120; IARC, 2019).

Of importance in evaluating results produced by kc-hits and ToxPI is the fact that all active assays are considered to be of equal importance in terms of their relevance to the KC to which they are mapped. This is a current deficiency in the assay mappings.

4.4 Procedures for assessing the strength of the ToxCast assay results

Individual assay features are available in abbreviated form through hyperlinks in any workbook saved in *kc-hits* and in detailed form through the assay documentation available from the US EPA (US EPA, 2018).

4.4.1 Items to consider

It is recommended that the following be evaluated when considering the results from individual assays: the AC_{50}, the levels of chemicals used in the assay relative to their biological relevance, the assay methods (e.g. cell line) and their appropriateness for the KC, the potential for cytotoxicity. With regard to all of the assays taken in aggregate, it is recommended that several factors be considered: the particular assays that were active (i.e. those with positive hits calls), the number of positive hit calls, and the relative number of positive hit calls per KC. The AC50s for assay end-points related to viability may be useful in evaluating whether cytotoxicity could be affecting the activity hit calls for the intended KC target – e.g. if the viability and KC target AC50s are in a similar range.

4.5 Conclusions and future directions

To address the limitations noted previously, it is recommended that efforts be undertaken to improve the coverage of the ToxCast data to KC mappings, develop a weighting factor for each assay in the mapping that indicates its relative biological evidence to the KC, update and re-evaluate the existing mappings based on machine learning and artificial intelligence-based methods, and specify a set of potential new assays to increase the coverage of ToxCast, to bolster coverage of the seven already-covered KCs and provide coverage of the remaining three KCs.

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5. Data from in silico assays

Kevin Cross

5.1 Introduction

In silico toxicology uses computational models, based upon different methodologies, e.g. statistical machine-learning (artificial intelligence (AI/ML) methods) and expert rule-based (alert) systems to predict the toxicity of a chemical from its molecular structure and chemical and biological properties.

Many computational models focus on the prediction of assay end-points forming the evidence base for the KCs in exposed humans and experimental systems and can be similarly assessed according to end-point relevance, validity, and reliability of the biological assays upon which they are built in addition to predictive performance and fitness-for-purpose of the individual models. For example, quantitative structure–activity relationship (Q)SAR modelling predicted the mutagenic potential of cupferron (IARC, 2021).

5.2 Application of the in silico predictions to the KCs

The *in silico* methods able to support assessment of each of the KCs have been effectively described recently by Tice et al. (2021). For each test method available, its OECD test guideline, end-point, and available *in silico* methods are described for each KC. Much of the following description is largely summarized from the Tice paper, where additional detail is available.

KC1: is electrophilic or can be metabolically activated, and KC2: is genotoxic

The two KCs are highly interrelated, focusing primarily on creation of DNA mutations resulting from electrophilic interactions. Ashby and Tennant (Ashby and Tennant, 1988) identified a general set of reactive chemical features capable of covalently binding to DNA, which were subsequently refined to predict electrophilicity as a set of structural alerts for genotoxicity based upon electrophilic mechanisms (Benigni and Bossa, 2011; Enoch and Cronin, 2010; LoPachin et al., 2019; Sakanyan, 2018). They can now routinely be applied computationally using multiple software tools. However, relying on the identification of such reaction mechanisms alone led to highly over-predictive results, with a large percentage of false positives (Schwöbel et al., 2011). Consequently, "expert" genotoxic alerts were developed for which the steric and electronic effects, as well as global physicochemical properties of a chemical, were considered and established through both theory and observation. Although prediction of electrophilicity did not initially rely upon end-point data, the qualification of alerts using experimental data for bacterial mutation (in particular) improved their performance over time, making them ultimately suitable for regulatory use (Benigni et al., 2020; Landry et al., 2019; ICH, 2017). Several stand-alone *in silico* models are also available for prediction of both rodent and human metabolism (Cruciani et al., 2005; Djoumbou-Feunang et al., 2019; Tyzack and Kirchmair, 2019).

Genotoxicity resulting from mutagenicity and DNA damage is the best-understood KC with the largest number of regulatory assays and by far the largest amount of data available, primarily owing to regulatory use of the Ames assay over several decades (Benigni and Bossa, 2011; Tice et al., 2021). As a result, KC2 is also the most common KC used when assessing human carcinogens (Krewski et al., 2019). High performing, statistically based *in silico* models using large bacterial mutation data training sets have been developed and accepted for regulatory use (Landry et al., 2019; Ahlberg et al., 2016; Bassan et al., 2024).

The extremely large amount of assay data, coupled with a well-known and relatively small set of mechanisms based primarily on electrophilicity, as well as long maturation has made these models the most successful predictors of carcinogenicity among all *in silico* approaches developed to date. Recent external model validation performance by the U.S. FDA of a set of 388 drug impurities representing proprietary pharmaceutical chemical space using two mature, commercial models showed 67-87% sensitivity, and 91-95% specificity and were able to predict 96% of all compounds (Landry et al., 2019).

Initially, consensus predictions from applying (Q)SAR statistical models and expert genotoxicity alerts were combined to support prediction of mutagenicity for the ICH M7 guideline. Subsequently, a more comprehensive approach to support genotoxicity prediction was developed through establishing a Genetox *In silico* Toxicology Protocol (GIST) for hazard prediction (Hasselgren et al., 2019). This protocol expanded *in silico* predictions to consider both mutagenicity and clastogenicity, integrating both experimental data and *in silico* predictions covering both *in vitro* and *in vivo* test methods for a defined series of relevant toxicological effects or mechanisms. The protocol follows the methodology outlined for *in silico* protocols as specified in Myatt et al. (2018).

This methodology defines a means of identifying the assays relevant to predicting the end-point and shows how different assays may provide a unique aspect of the assessment, as well as those that could be optionally replaced by another test method. In the GIST protocol, clastogenicity can be assessed by data (or predictions) from either *in vivo* or an *in vitro* test method. Each test method could optionally be replaced with an *in silico* prediction. The reliability of an assessment of each test method is individually calculated and is dependent on an objective determination of the quality of the data or prediction, with predictions usually being categorized as less reliable than actual experimental data (Johnson et al., 2022). After each test method is assessed and its reliability calculated, it may be combined with assessments from other test methods to establish an assessment for a higher-level effect (including its confidence).

In the GIST protocol (see also Table 1 in Part I) it is apparent that mutation effects can be determined from either bacterial mutation or mouse lymphoma data or predictions. *In vitro* clastogenicity effects can be determined from *in vitro* chromosome aberration or from *in vitro* micronucleus or mouse lymphoma data or predictions, whereas *in vivo* clastogenicity effects can be determined from *in vivo* chromosome aberration or *in vitro* micronucleus data or predictions. The assessment of a higher-level effect considers the assessment of each input test method assessment and its established reliability value. Expert judgement is used to establish the confidence of this assessment. The hierarchy of higher-level effects is then tracked to ultimately establish an assessment and confidence for the end-point; here using the effects of *in vitro* mutagenicity and clastogenicity to establish an assessment for genotoxicity. Using *in silico* protocols, toxicological assessments can be performed and evaluated in a consistent, reproducible, and well-documented manner across industries and regulatory bodies to support wider uptake and acceptance of the approaches. This methodology can be extended to support other end-points, like carcinogenicity, once all of the effects and mechanisms, along with test methods for measuring them have been established.

KC3: Alters DNA repair or causes genomic instability

Assays to detect DNA damage can be used to assess KC3. There are several test methods available, although not all have corresponding *in silico* models for prediction, primarily owing to insufficient quantity of available data for modelling. Consequently, the performance of these models varies significantly depending on the compound classes being predicted; i.e. the coverage of chemical space by these models is more limited. *In silico* statistical models and/or expert alerts are available for mouse lymphoma, *in vitro* and *in vivo* micronucleus, *in vitro* and *in vivo* chromosome aberrations, and sister chromatid exchange (Yoo et al., 2020; Hsu et al., 2018; Matthews et al., 2006; Pradeep et al., 2021).

KC4: induces epigenetic alterations

Epigenetic mechanisms are considered important for assessment of non-genotoxic carcinogens (Jacobs et al., 2016), and carcinogenicity alerts have been developed on the basis of known mechanisms (Woo and Lai, 2009; Benigni et al., 2013). However, the lack of *in vivo* test methods to study epigenetics has resulted in a lack of *in silico* models based upon assay data. Instead, computational approaches have focused on the identification of epigenetic drug targets leading to mis-regulation (Lu et al., 2018). See Chapter 4, Part I, on epigenomics.

KC5: induces oxidative stress

Several assays are available to measure ROS formation, oxidative DNA damage, and antioxidant response (Woolley et al., 2013; Tice et al., 2000). (Q)SAR models have been developed to predict oxidative damage by training on bacterial mutation *Salmonella* strains specific to oxidative damage (Patrineli et al., 1996; Wilcox et al., 1990). Expert rule-based systems and quantum mechanical chemical models have been developed to help predict ROS formation (Mekenyan et al., 1996). Models based on toxicogenomic datasets have been developed to predict antioxidant response (Kim et al., 2016) as well as quantum mechanical models (Williamson et al., 2012).

KC6: induces chronic inflammation

In silico methodologies do not exist for predicting tissue inflammation, although there are phenotypic *in vitro* assays available such as Tox21 that can detect changes in regulation of pathways associated with chronic inflammation (Chiu et al., 2018) (see Section 2.2, Chapter 2, Part II). The complexity of the mechanisms linking chronic inflammation and cancer (see Chapter 6, Part I), and the lack of specific initiating events, has hindered model development.

KC7: is immunosuppressive

In silico methodologies do not exist for predicting immunosuppression, although some experimental methods for assessing immunosuppression have been documented (US FDA, 2020). The lack of data and understanding of mechanisms has hindered model development.

KC8: modulates receptor-mediated effects

Several endocrine activity nuclear receptor assays have provided data for building *in silico* methods modelling endocrine receptor and androgen receptor modulation (Judson et al., 2015). This includes development of an *in silico* protocol of (Q)SAR models for endocrine disruption, read-across, molecular docking, and virtual screening (Cotterill et al., 2019; Garcia-Serna et al., 2015; Porta et al., 2016). These models are currently being used for *in silico* hazard identification in occupational safety, labelling, and transportation (6-pack).

KC9: causes immortalization

Genotoxic compounds may cause immortalization via point mutations and deletions, whereas non-genotoxic carcinogens may cause immortalization via epigenetic mechanisms. (Q)SAR models training on Syrian hamster embryo (SHE) *in vitro* cell transformation assays have been developed for predicting cell transformation (Guan et al., 2018; Matthews et al., 2006) though are not used in a regulatory capacity due to the lack of mechanistic understanding.

KC10: alters cell proliferation, cell death, or nutrient supply

In silico methodologies do not exist for globally predicting the ability of chemicals to alter cell proliferation, cell death, or nutrient supply. However, some local (Q)SARs have been developed for prediction of specific classes of chemicals of importance for advancing pharmaceutical drug candidates (Gao et al., 2012; Zanni et al., 2015; Lakhlili et al., 2016).

During the Workshop, application of (Q)SAR models and alerts supporting KC1, KC2, and KC8 were discussed; however, it was acknowledged that direct prediction of human carcinogenicity as an overall end-point is not currently feasible.

5.3 Best practice of in silico predictions

Development of best practices (Myatt et al., 2022), methods for integrating model prediction results for multiple assay end-points with existing experimental data into decision support systems (Hasselgren et al., 2019), and establishment of measures of uncertainty and confidence levels (Johnson et al., 2022) have increased their regulatory acceptance. The Working Group agreed to some acceptance criteria for using results from computational models in IARC cancer hazard identification. In general, high sensitivity (the ability to predict toxic compounds) and high negative predictivity (the ability to avoid false negatives) are desirable, emphasizing the probability of possible positive outcomes and the robustness of non-carcinogenic outcomes, while being less concerned with false positive predictions.

Assessment of (Q)SAR model validity is required, along with consideration of reported results. These factors are related to model construction. To meet OECD guideline standards (OECD, 2007), models are required to have: 1) a defined end-point, 2) an unambiguous algorithm, 3) a defined domain of applicability, 4) an appropriate measure of goodness-of-fit robustness and predictivity, and 5) mechanistic interpretation, if possible, where the suitability of using a reported outcome depends on its original intent. Individual raw prediction results should be available and targeted to support one or more end-points for a specific KC. A recent OECD guideline for assessment of (Q)SAR prediction results for regulatory use includes: 1) correctness of model inputs, 2) the compound being within the applicability domain of the model, 3) acceptable predictive performance for the compound of interest, on the basis of a performance metric (i.e. probability of being carcinogenic), and 4) the model's fitness-for-purpose, that is, its ability to provide specific supporting evidence for carcinogenicity, by itself or in conjunction with other models or explanatory data in a protocol for an end-point assessment. These criteria imply that model prediction results must be robust, transparent, explainable (both computationally and based upon underlying mechanism), and repeatable.

5.4 Conclusions and future directions

The lack of standardized assays for measuring some of the KCs-associated end-points and the subsequent lack of robust experimental data have hindered the development of *in silico* models to support the KC approach to assessing carcinogenicity. For all KCs except KC1 and KC2, the complex causal relationship to carcinogenesis and its limited understanding restricts the applicability of robust models to support them. This is particularly true for non-genotoxic carcinogens where low doses and extended durations are important considerations.

However, integration of KC data from various toxicological studies into carcinogenicity hazard assessments is still useful. The development and use of *in silico* models will continue to maximize exploitation of existing data, identify data gaps in mechanistic understanding and *in silico* protocols can serve as a framework for representing and organizing existing mechanistic knowledge. The pursuit and integration of robust *in silico* models and experimental data into a single *in silico* carcinogenicity protocol remains an ongoing process with its promise of providing a more comprehensive mechanistic understanding of cancer and its development.

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Part III. Integration of the mechanistic evidence: opportunities and challenges

1. Interpretation of evidence for key characteristics of carcinogens from studies in exposed humans

Parveen Bhatti, Nathaniel Rothman, Paolo Vineis, and Roel Vermeulen

1.1 Introduction

The interpretation and synthesis of evidence from mechanistic studies in humans is an important part of the *IARC Monographs* evaluations. In addition to the considerations described in Part 1 related to strengths and limitations of KCs-associated end-points, there are multiple features of mechanistic studies in exposed humans that require evaluation. Here we summarize key features and describe strategies on how to account for such features when evaluating evidence for the KCs.

1.2 Relevant features to study mechanisms in exposed humans

Study design

Most studies of exposed humans have tended to be cross-sectional in design, with the exposure and end-points assessed concurrently. While prospective studies, with exposure distinctly occurring before collection of the biospecimen in which end-points are to be measured, are needed for assessment of causality, such studies are not always possible, particularly if they involve participants with ongoing exposures. This is less of an issue if impacts on the end-points are short-lived. In such circumstances, timing of biospecimen collection relative to the exposure is an important issue, not only because of temporal dissipation of effects but also due to the potential for circadian variation in the baseline levels of the end-points. Studies would have, ideally, collected samples within the same time frames for all participants, in order to mitigate the impacts of these issues.

Sample size and multiple comparisons

Relative to studies of cancer endpoints, mechanistic studies of exposed humans tend to have much smaller sample sizes, with limited statistical power, while often evaluating multiple end-points. In the case of studies using high-dimensional omics data, studies may be evaluating end-points numbering in the thousands to hundreds of thousands. Small sample sizes and multiple comparisons contribute to increased probabilities of false positive findings (see also Chapters 1 and 2 of Part II).

Post-hoc power calculations should be avoided when assessing whether a study was sufficiently powered to detect associations of interest (Hoenig and Heisey, 2001). Power can, instead, be judged by examining the width of confidence intervals that have been presented for study associations. The false positive report probability (FPRP) may be a helpful tool with which to assess the likelihood that limited statistical power contributed to false positive associations. In addition to statistical power, the FPRP depends on the observed p-value as well as the prior probability that the association under investigation is real (Wacholder et al., 2004).

If the selection of multiple end-points for a study is based on a compelling *a priori* hypothesis (e.g. related end-points relevant to a single KC), adjustment for multiple comparisons may be less of an issue. Appropriate multiple comparisons adjustment is more of a concern in the context of exploratory studies,

particularly those evaluating omics data. While Bonferroni adjustment is ideal, controlling for the false discovery rate has proven a more tenable strategy, though the thresholds selected often range from 5 to 20%, and justification for the selection of a specific threshold is seldom provided.

Biases

As with observational studies of cancer endpoints, careful consideration of the potential impacts of confounding on associations between exposures and end-points of interest is necessary. A review of the scientific literature can help identify factors that have been associated with the end-points of interest and may be acting as potential confounders of reported associations.

Humans are seldom exposed to agents in isolation. Thus, the potential impact of confounding by co-exposures on end-points of interest is an important consideration. If information about potential co-exposures is not provided by the study report, consultation with experts in exposure assessment can help identify typical co-exposures that may be acting as confounders.

A particular concern with human mechanistic studies is the potential for publication bias. Omics studies aside, mechanistic studies of exposed humans are likely to generate data on dozens of end-points but only publish on the biomarkers demonstrating statistically significant associations. Thus, review of the evidence for KCs may be skewed by the lack of published null results.

Measurement of end-points

Human mechanistic studies usually rely on the collection of minimally invasive samples (e.g. blood and urine) for biomarker measurement. The suitability of a sample type is KC- and biomarker-dependent. For example, genotoxicity (KC2)-associated end-points are often measured in blood cells or urine. In measuring biomarkers of inflammation (KC6) and immunosuppression (KC7), blood is likely to be a suitable biospecimen. The marker of oxidative DNA damage (KC5), 8-hydroxydeoxyguanosine, is excreted in urine, and, as such, can be treated as a systemic measure of oxidative DNA damage. On the other hand, DNA methylation (KC4) is generally tissue-specific, meaning that measures in blood may not necessarily reflect measures in other tissues of the body. Regardless of the specific end-point measured across the various KCs, study reports should present quality-control data with which to assess the quality of the generated end-point data. It is worth noting that in several *Monographs* Volumes, end-points of disease outcomes have been also considered in supporting the evidence for specific KCs. These disease-associated end-points are informative when representing outcomes associated with the KCs, and intermediate phenotypes are associated with carcinogenesis. Some examples include fibrosis, pneumoconiosis, reduced lung function parameters, or bronchial hyperreactivity as end-points for chronic inflammation (KC6); reduced response to vaccines, augmentation of infections in children, or modification of immune cell populations as end-points for immunosuppression (KC7); and actinic keratosis as an end-point of abnormal cell proliferation (KC10).

1.3 Conclusions & future directions

Considering the that mechanistic studies in exposed humans can play a crucial role in establishing the carcinogenicity of agents, a collective effort and focus is mandatory in the improving their quality. The potential contributions of publication bias to the mechanistic evidence being evaluated will require critical consideration to help ensure that appropriate conclusions are being reached. The challenges that we outlined highlight the value of establishing associations with multiple mechanistic end-points across studies in humans and in experimental systems when evaluating the potential carcinogenicity of an agent.

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2. Integration of the evidence across the Key Characteristics

Amy Wang, Weihsueh Chiu, Lauren Zeise, Kathryn Z Guyton, and Maurice Whelan

2.1 Introduction

An agent can exhibit evidence for a KC through alterations in one or multiple end-points, however, one end-point might be associated with multiple KCs (e.g. 8-oxo-7,8-dihydroguanine (8-oxoG) being associated with KC2 and KC5).

For end-points that might be associated with multiple KCs, the assignment to a specific KC depends on the totality of the evidence that is evaluated. Importantly, even with some variances, the relevant mechanistic evidence is evaluated as a whole, rather than a few independent end-points. Based on the end-points associated with each KC, KCs have one-to-many and many-to-one relationships with each other (Fig 3.1, for interactive figure, see https://public.flourish.studio/visualisation/17399594/).

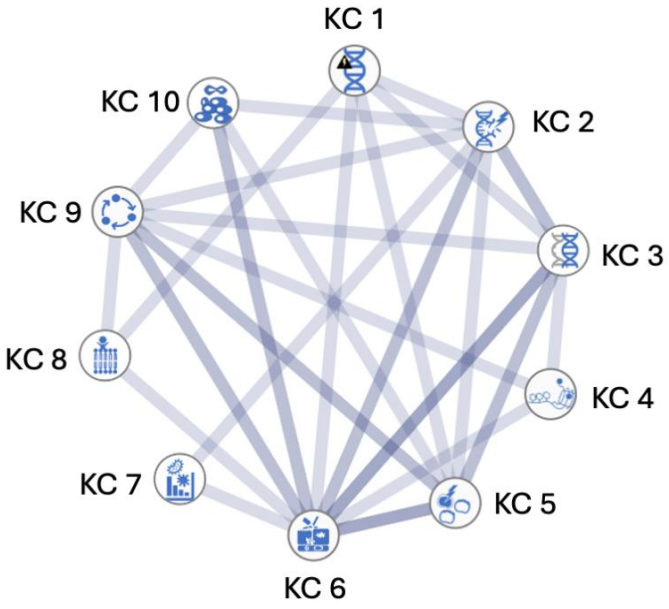

Figure 3.1 Interrelationship between KCs. Grey lines connect two associated KCs. Darker grey lines indicate the influence can be bidirectional. For instance, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (KC5) can induce the release of proinflammatory cytokines (KC6), and activated macrophages (KC6) can trigger the production of ROS and RNS (KC5). Such bidirectional influences don't need to be based on the same set of events or end-points.

The relationship could arise from the same end-points relevant to multiple KCs as well as one end-point (relevant to one KC) influencing another end-point (relevant to another KC), which does not need to be a causal relationship.

The strength of the mechanistic evidence is expressed as the strength of evidence in each KC, because it serves as logical grouping method. As described previously, evidence for a group of KCs displayed by an agent can strengthen the mechanistic conclusions or the likelihood of an agent to be a carcinogen (Smith et

al., 2016; IARC, 2019). The strength of mechanistic evidence can be increased by the coherence or known relationship of the data across multiple end-points within a KC, or end-points associated with multiple KCs.

The discussion on the potential interrelationships among KCs reported below is a collection of experience and knowledge from the experts attending the Workshop. These are not intended to be a comprehensive list. The discussion is organized by the order of the KCs, as presented in the *IARC Monographs* Preamble (IARC, 2019).

2.2 Examples of evidence integration for the KCs

Integration of the evidence among KC1, KC2, KC3 and KC5

KC1, KC2, and KC3 have a strong interrelationship, as end-points associated with them can be a part of a continuum. Agents that are electrophilic (KC1) can form DNA adducts, which may then induce replication errors (KC3). Agents that are genotoxic can cause DNA damage (KC2). Agents that induce oxidative stress (KC5) can result in oxidative damage to DNA (KC2), which is the most relevant outcome for evaluating KC5 compared with non-specific measures of oxidative damage. Comet assays modified with enzymes such as endonuclease III to recognize oxidised pyrimidines, or formamidopyrimidine DNA glycosylase (FPG) or human 8-oxoguanine DNA glycosylase 1 (hOGG1) to detect oxidised purines, can be used specifically to identify strand breaks resulting from oxidative damage (Collins, 2009). FPG is the most widely used modification (Azqueta et al., 2019). Comet assays showing genotoxicity will likely feature as an end-point for KC2 "is genotoxic;" however, focus in KC5 "induces oxidative stress" should be on methods specific to oxidative stress, such as FPG and endonuclease III outcomes.

All of these DNA alterations can be mis-repaired or unrepaired, effects that are enhanced by agents exhibiting KC3, possibly leading to mutations (KC2) that can lead or further contribute to the development of cancer. The analysis of KC3 in the *Monographs* reveals that only a small number of agents had evidence of inducing alterations of DNA repair mechanisms or of genomic instability. For example, even among Group 1 agents, only 23 out of 86 exhibited some evidence of KC3, while 85 out of 86 show evidence of KC2 (DeMarini et al., 2025). In the most recent analysis of the mechanistic evidence described using the KCs framework in *Monographs* Volumes 112 to 132 (DeMarini et al., 2025), no studies were retrieved for KC3 for 28 out of the 89 agents analysed (including agents classified into each of the four Groups), and only three agents - benzene, acrolein, and arecoline - showed "consistent and coherent evidence" of KC3. Evidence for end-points associated with KC3 may be underestimated. It is true that the literature is quite specific, and some of the end-points can be inferred indirectly through evidence of KC2. Additionally, some other end-points such as microsatellite instability (MSI) alterations or copy number variations are rarely measured in *in vitro* and *in vivo* assays following exposure to different agents. It is expected that similar but indirect effects may be classified under KC2 because of the strong association between genotoxicity and alterations in DNA repair or genomic instability. This does not pose a problem for identifying a carcinogen, but it may affect the strength-of-evidence determination, if the number of positive KCs per agent is considered important.

Besides the associations mentioned above, KC1, KC2, and KC3 can be associated with other KCs. For example, increased frequencies of micronuclei (KC2) are associated with auto-immune disease, thus potentially causing inflammation and/or immune dysregulation (KC6 and KC7) and oxidative damage (KC5) (Kirsch-Volders et al., 2020). KC3 is also associated with KC4 and KC6, because epigenetic changes (KC4), oncogene-driven replication stress, and chronic inflammation (KC6) have the potential to disrupt the

stability of the genome (KC3). Given these relationships, consistent and coherent evidence for an agent across these KCs would provide strong mechanistic evidence for the carcinogenicity of the agent.

Integration of the evidence among KC4 and other KCs

KC4 covers various processes that alter the gene expression without altering the gene sequence, and KC4's effects depend on the type of epigenetic change and the location of such change. KC4 has the potential to be associated with all other KCs. For example, hypomethylation of the promoter region of a receptor gene would increase the expression of this receptor (KC8). Some of the better-studied KC4 associations with other KCs are with KC6 and/or KC10. Epigenetic alterations such as dysregulated DNA hypermethylation (KC4) are often associated with chronic inflammation (KC6), as found in non-cancerous tissues of patients with inflammation-associated cancers (Touati, 2010). Proinflammatory cytokines (like TNF, IL-1α, and IL-1β) can reduce the activities of microRNAs (KC4) which enhance inflammation-associated tumourigenesis of the colon (Kaltenmeier et al., 2021; Yoshikawa et al., 2017). The proinflammatory carcinogenic response to cigarette smoke occurs from epigenetic (KC4) as well as pro-angiogenic and cell proliferative (KC10) effects in the lung, such as blood vessel formation (Balansky et al., 2018; Seiler et al., 2020).

Integration of the evidence among KC6 and KC7 with other KCs

KC6 covers not only the classical chronic inflammation responses but also persistence and long-term re-occurrence of acute inflammation (e.g. from repeated injury), as reported in interpretation of data for occupational exposure as a firefighter (Volume 132). KC6 is associated with at least KC2, KC3, KC4, KC5, KC7, KC9, and KC10. The interaction of chronic proinflammatory responses (both cellular and non-cellular) with other KCs can increase confidence that KC6 caused by an agent (or infection) is relevant to carcinogenesis.

Interactions of KC6 with KC2, KC5, and/or KC10 may be described by the interaction of end-points relevant for the non-cellular inflammatory response. Surrogate markers of neutrophilic activity/inflammation include the production of reactive oxygen species (ROS) and myeloperoxidase (MPO) (relevant for KC5) and neutrophil elastase (NE), for instance, in the peripheral blood (systemic) and bronchioalveolar lavage fluid (local) of lung cancer patients; and increased local and systemic neutrophilic inflammation can distinguish lung cancer patients from healthy people or those with chronic obstructive pulmonary disease (COPD) (Vaguliene et al., 2013). Carcinogen-stimulated neutrophils and activated macrophages can trigger production of ROS and reactive nitrogen species (RNS) to cause oxidative damage to DNA (relevant to KC5), impair DNA repair (relevant to KC3), or cause tissue injury/regeneration (relevant to KC10), leading to cancer (Moalli et al., 1987; Beaver et al., 2009). Carcinogens such as arsenic and lead disrupt the normal function of inflammatory cells such as macrophages (Sengupta & Bishayi, 2002), and carcinogen-stimulated macrophages can produce pro-angiogenic growth factors to induce angiogenesis and cell proliferation (relevant to KC10) (Beaver et al., 2009; Fishbein et al., 2020). Increased ROS and RNS (relevant to KC5) can also induce the release of proinflammatory cytokines (Amara et al., 2016). The proinflammatory carcinogenic response (e.g. to tobacco carcinogens, ionizing radiation, urban air pollution, silica and carbon black particles, or combustion exhaust particles) occurs from DNA damage caused directly via formation of DNA adducts/mutagenic effects (relevant to KC1 and KC2) or indirectly via oxidative stress mediators such as ROS (KC5) as well as from impaired DNA repair/genomic instability (relevant to KC3) and altered cell death/proliferation (relevant to KC10) (Borm & Driscoll, 1996; Gordon et al., 2013; Proctor et al., 2014; Benvenuto et al., 2016; Øvrevik et al., 2017; Borm et al., 2018; de Oliveira Alves et al., 2020; Helm & Rudel, 2020; Nakamura, 2021). Prostate cancer is associated with an increased release of proinflammatory and proangiogenic factors such as NF-kB, VEGF (relevant to KC10), TNF-α as well as *i*NOS and COX-2 (relevant to KC5) along with monocyte infiltration (Narayanan et al., 2009).

Chronic inflammation (KC6) results in the early loss of DDR genes (relevant to KC3) and the increase of MSI (relevant to KC3), leading to colitis-associated colon cancer (Sharp et al., 2018). Pulmonary inflammation induced by carcinogenic air pollutants (e.g. polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene) leads to DNA damage via genotoxic mechanisms from covalent benzo[a]pyrene-DNA adduct formation in the lung (relevant to KC1 and KC2) (Arlt et al., 2015).

The proinflammatory response to asbestos in mesothelioma is related to its action to induce immunosuppression (KC7) via functional alterations with a decrease in T helper cells, natural killer (NK) cells, and cytotoxic T lymphocytes (CTLs) (Nishimura et al., 2015; Benvenuto et al., 2016). NLRP3 inflammasome activation can also inhibit the host-protective NK cell anti-tumour response (relevant to KC7) in methylcholanthrene-induced carcinogenesis (Chow et al., 2012). M2 macrophages can be immunosuppressive by inhibiting the cytotoxic activity of T-cells and NK cells (relevant to KC7) (Noy & Pollard, 2014), and M2 macrophage polarization is involved in inflammation-associated carcinogenesis induced by fibres and asbestos (Larson et al., 2016; Napolitano et al., 2016). Chronic inflammation can create an immunosuppressive tumour microenvironment (relevant to KC7), consisting of myeloid-derived suppressor cells (MDSC), regulatory T-cells (via T-cell exhaustion), type 2-polarized tumour-associated macrophages (TAMs), programmed cell death (PD)-1+ TAMs, and increased PD-1/PD-ligand (L)1 in lung cancer (Narayanapillai et al., 2020; Liu et al., 2021), and PD-L1 exhibits a negative regulatory function in macrophages that contributes to the immunosuppressive tumour microenvironment (Wen et al., 2022). Proinflammatory cytokines such as TNF-α increase PD-LI expression by infiltrated macrophages to stimulate lung tumour growth (Wen et al., 2022).

The relevant end-points between immune functions (KC7) can be distinct or overlapping, and when immune dysregulation occurs, immune suppression can result in chronic inflammation (KC6) owing to uncontrolled infection, autoimmunity, or impairment of specific regulatory function. The paradox of the immune system in cancer is that chronic inflammation can facilitate tumorigenesis, but targeted activation of immune system components can be therapeutic; suppression of the ability to activate these components can also promote tumour development (Khatami, 2008; Ponce, 2018).

Inflammation from NF-kB activation (relevant to KC6) induces malignant cell transformation (relevant to KC9) via oncogenes such as *RAS* (Natarajan et al., 2014). Some examples include arsenic and hexavalent chromium. Arsenic stimulates ROS (KC5), which then activates proinflammatory pathways (KC6) leading to cell transformation (KC9) in urinary bladder cancer and other tumour types (Singhal et al., 2022). Carcinogen-induced cell immortalization (KC9), as in the case of hexavalent chromium, can be mediated, in part, by an inflammatory response via the expression of TNF-α and NF-kB-p65, as well as COX-2 and the generation of ROS (KC5) (Roy et al., 2016).

A wounding response is a risk factor for early cancer development via the induction of both inflammation (KC6) and cell proliferation (KC10) even in the absence of genetic damage (KC2) (Hayes et al., 2011). The cytotoxicity of carcinogens and the resulting tissue damage/injury may lead to inflammation (KC6) (Bogen, 2019, 2023; Fishbein et al., 2020, 2021). For instance, apoptotic cell death can activate the "Phoenix Rising" pathway to stimulate inflammation, wound healing, and tissue regeneration (KC10) leading to inflammation-driven cancers (Zhao et al., 2018). Cell death induced by carcinogens can also result in tumour growth by stimulating a macrophage-derived eicosanoid and cytokine storm (Sulciner et al., 2018; Chang et al., 2019; Gartung et al., 2019; Fishbein et al., 2020). The proinflammatory response of nitrosamines (e.g. N-nitrosodimethylamine, NDMA) acts by increasing cell proliferation-related proteins (KC10) such as E2F1 and Ki-67, as well as the cancer-related protein cytokeratin 19 (Kim et al., 2019).

Integration of the evidence among KC8 and other KCs

KC8 covers not only the interactions with receptors (e.g. expression, binding, activation/inactivation) but also a wide range of factors influencing receptor-mediated effects, such as hormone synthesis and transportation. KC8 is not restricted by the receptor location (e.g. nuclear, membrane) or ligand families (e.g. tyrosine, serine, cytokines (relevant to KC6, KC7), if the change leads to carcinogenesis. For example, Tris(2-chloroisopropyl)phosphate (TCPP) can activate AhR, which induces CYP1A1 leading to excessive generation of ROS (KC5) (Vogel et al., 2020). Agents that modulate receptor-mediated effects (KC8) can stimulate cell proliferation (KC10) when the activation of growth pathway receptors are triggered (e.g. estrogen, androgen, and progesterone receptors and growth factor receptors such as EGFR and ERBB2) (KC8), although this is likely to occur in a cell-type and tissue-specific manner.

Integration of the evidence among KC9 and other KCs

KC9 can be associated with KC6, as reported above, likewise both epigenetic alterations (relevant to KC4) and genetic changes (relevant to KC2), especially point mutations and genomic rearrangements, can lead to compensation of telomere loss that normally occurs with each cell division. Therefore, an immortalization assay and cell transformation assays (CTAs), while critically important for identifying the (epi)genetic changes involved in immortalization, are likely to be accompanied by evidence of KC2, KC3, and/or KC4.

Agents that cause immortalization (KC9) could permit escape from cellular senescence programming, via the activation of alternative lengthening of telomeres (ALT) or telomerase, and permit cell proliferation (KC10) unlimited by molecular cell cycle control mechanisms operating in non-neoplastic cells (e.g. pRb/p16 or p53), or telomere length (e.g. the Hayflick limit).

Integration of the evidence among KC10 and other KCs

KC10 covers increased (but not decreased) cell proliferation, decreased (but not increased) cell death, and altered nutrient supply, which includes increased angiogenesis.

Following exposure to agents that may be genotoxic (KC2), resulting oncogenic mutations permit augmentation of and/or dysregulated proliferation, attenuation, or stimulation of regulated cell death, and secretion of microenvironment-modifying enzymes, cytokines, chemokines, and vascular growth factors, which can then contribute to altered cellular proliferation as well tissue nutrient supply. Agents that increase ROS or RNS (relevant to KC5) can stimulate cellular proliferation in a concentration and cell-type dependent manner (e.g. via EGFR-MEK/MAPK activation); decrease apoptotic signalling (e.g. via Src, NF-kB, and PI3KCA/Akt1 activation) (KC10) or increase cell death via ferroptosis; and stimulate cellular motility (e.g. via Met overexpression, and Rho–Akt1 interaction), tissue invasion and metastasis (e.g. via MMP secretion breaking down local basement membrane matrix components), and angiogenesis (e.g. VEGF release, increased vasodilation, and endothelial cell chemotaxis).

Cell proliferation, along with acute and chronic inflammation themselves, can stimulate chronic inflammation in local tissue stem and progenitor cells, leading to cancer development after a prolonged latency (e.g. *Helicobacter pylori* infection of gastric tissue, persistent hepatitis B infection in the liver). Immune mediators of chronic inflammation (e.g. EGF, TGF) (relevant to KC6) can also directly alter epithelial cell proliferation and cell death, and can affect tissue nutrient supply (relevant to KC10).

2.3 Conclusions & future directions

There is no requirement in the *Monographs* Preamble to establish the temporal, sequential or causal features of how a particular agent operates. As such, each KC can be seen as independent with a distinct role, because the conclusion that an agent exhibits a KC means that this agent has chemical or biological properties similar to those of one or more agents already established to be carcinogenic to humans (Group 1). Nonetheless, as illustrated by numerous examples, above, linkages across KCs can further strengthen the overall biological plausibility of carcinogenicity when there is coherence across multiple end-points from multiple KCs. Such coherence can be based on "established" causal relationships between end-points, such as within a plausible model of carcinogenesis.

In this way, the empirically based KCs, which are derived from data on known human carcinogenic agents, complement other ways of organizing mechanistic data, such as Mode-of-Action and Adverse Outcome Pathways, which are established through a hypothesis-driven process. However, only in limited circumstances have there been published scientific literature specifically testing hypotheses regarding causal relationships among mechanistic events.

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Annex 1

Overall database of *IARC Monographs* agents (vol 112-135) evaluated with the KCs framework, in supplementary material of DeMarini et al 2025.

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