New Approach Methodologies (NAMs) for Carcinogenicity Evaluation

Frederic Schorsch1, Mirjam Luijten2, Mark Cronin3, Heidrun Ellinger-Ziegelbauer4, Kelly Evans5, Joanne Elloway6

Affiliations

1. Frederic Schorsch, Regulatory Toxicology, Bayer SAS, Crop Science Division, Sophia Antipolis, France
2. Mirjam Luijten, Centre for Health Protection, National Institute for Public Health and the Environment, Bilthoven, NL
3. Mark Cronin, School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, UK
4. Heidrun Ellinger-Ziegelbauer, Investigative & Predictive Safety, Research & Development, Pharmaceuticals, Bayer AG, Wuppertal, Germany
5. Kelly Evans, Immune Safety, Clinical Pharmacology and Safety Sciences, Biopharmaceuticals R&D, AstraZeneca, Cambridge, UK
6. Joanne Elloway, Safety Innovation, Clinical Pharmacology and Safety Sciences, Biopharmaceuticals R&D, AstraZeneca, Cambridge, UK

The second session of the 2nd Joint BSTP/ESTP Toxicologic Pathology Congress, Manchester, UK, September 23–26, 2025, entitled "New Approach Methodologies for Carcinogenicity Evaluation," was dedicated to innovative strategies for assessing carcinogenic risks in various substances, particularly in drug development and agrochemicals. The two-year rodent cancer bioassay in two species (generally rats and mice) is currently the standard method for assessing the carcinogenic potential of agrochemicals for humans. However, this method has some weaknesses and is subject to ethical and scientific debate. Attempts to waive those studies have been proposed but more relevant methods using less or preferably no animals are still being sought.

The session featured five key presentations that explored cutting-edge methodologies aimed at improving the accuracy and reliability of carcinogenicity predictions. Below is a summary of each talk presented.

1. European Partnership for Alternative Approaches to Animal Testing (EPAA) Initiative on NAMs for Carcinogenicity of Agrochemicals

Prof Mirjam Luijten introduced the concept of New Approach Methodologies (NAMs). Although different definitions for NAMs are being used, there is general consensus that NAMs comprise innovative methods that can be used in regulatory safety testing of chemicals and pharmaceuticals. NAMs include *in silico* methods, ranging from well-

established tools like SARs (structure-activity relationships) to (upcoming) machine learning techniques, as well as *in vitro* test methods, which may range from simple 2D models to advanced, complex 3D models. Depending on the problem formulation and regulatory framework at hand, a combination of NAMs can either complement or even replace traditional animal testing. For regulatory assessment of more complex health effects such as carcinogenicity, an alternative approach that no longer relies on animal testing is more difficult to achieve. An interim solution may be to combine NAMs with short-term (i.e., 90-day) toxicity studies in experimental animals encompassing traditional endpoints and, e.g., omics readouts to increase mechanistic insight in the health effects induced by chemical treatment. Such an approach would already contribute to the 3Rs principle and could, in the shorter term, provide sufficient confidence to serve as the basis for deriving a Point of Departure (PoD). In addition, enhanced mechanistic insight could support further improvement of a NAM-based approach for carcinogenicity assessment.

The selection of methods to be used in a NAM-based approach should be guided by our knowledge of the Adverse Outcome Pathways (AOPs) relevant for carcinogenesis. AOPs constitute a mechanistic representation of critical toxicological effects over different layers of biological organization, starting with initial interaction of a chemical with a molecular target (Molecular Initiating Event (MIE)), leading via a series of Key Events (KE) to an Adverse Outcome (AO) at the individual or population level.[21 25]

Prof Mirjam Luijten then presented the initiative funded by the European Partnership on Alternative Approaches to Animal Testing (EPAA) aimed at the development of a NAM-based approach for assessing the carcinogenicity of agrochemicals. The traditional 2-year carcinogenicity assays in rodents are under scrutiny regarding their accuracy, cost, and relevance regarding the number of animals used.[9 20 12] A mechanism-based, weight of evidence (WoE) approach requires enhanced understanding of the AOPs relevant for carcinogenesis in humans. The EPAA project is aimed at the identification of both AOPs to be included in a WoE-based approach and possible knowledge gaps. For this, over 400 unique agrochemicals were analysed, of which 170 agrochemicals could be categorized as non-genotoxic carcinogens.[11] In the analysis, only tumors that show a treatment-related tumor response were included. For two-thirds of the approximately 340 tumor cases, an underlying AOP or network of AOPs could be identified. Despite the wide variety of tumors in various organs distilled from the carcinogenicity studies, the number of identified AOPs was limited. This illustrates that the transition towards a WoE approach appears manageable. The AOPs involved in the remaining tumor cases remained undetermined. For these ‘unknowns’—representing 114 tumor cases related to 72 chemicals—patterns in tumor incidences and underlying AOPs were explored in a stepwise fashion. In brief, tumors suspected to have occurred due to excessive dosing were excluded and emphasis was placed on commonalities in tumor type and putative pre-neoplastic lesions across chemicals. The project team therefore also involved

multiple experienced pathologists. The analysis yielded additional AOPs that should be included in WoE-based carcinogenicity assessment. For some tumor cases, the underlying AOPs could not yet be unraveled (Bouwmeester et al., manuscript in preparation). The resulting overview of the AOP (networks) underlying non-genotoxic carcinogenicity of agrochemicals will greatly facilitate the selection of NAMs for the development of a WoE approach to carcinogenicity assessment.

2. In Silico Approaches to Predict Carcinogenicity

Prof Mark Cronin provided an overview of the history and advancements in *in silico* methodologies for predicting the carcinogenic potential of chemicals. He highlighted both real successes and the current limitations of these approaches.

The knowledge that chemical structure is fundamental to biological activity, and most notably its toxicity, has been apparent since the mid 19th Century. This has evolved into a number of techniques that can be used to predict hazard and exposure. The most usable methods have been formalised into computational tools for toxicity prediction which include, but are not limited to, (quantitative) structure-activity relationships (QSARs), structural alerts, read-across, machine learning and artificial intelligence (AI). These varied approaches have in common that they are attempting to relate some feature(s) of chemical structure and/or physico-chemical properties to toxicological activity. Such tools have been used routinely since the 1980s, with a particular growth in their use in the past three decades due to commercial pressures including the need to eliminate toxicity early, ethical concerns over the use of animals, as well as regulatory need and legislation.[18]

In silico, or computational, tools have a number of uses, ranging from identifying structures with potential toxicity, e.g., for screening and prioritisation of large inventories, or as part of a testing strategy, or weight of evidence to perform hazard identification of a substance. They are applied in specific regulatory situations such as ICH M7, EU REACH, Classification, Labelling and Packaging (CLP), as well as the prioritisation and screening of large inventory.[24] Computational models are also instrumental in dealing with new problems that may arise in toxicology, with the on-going issue of N-nitrosamines being one such example.[23]

Computational tools are used widely to assist in the prediction of effects relating to carcinogenicity. The full range of techniques can be utilised, starting with a knowledge of molecular (sub-)structures that are associated with electrophilic interactions with DNA. These so-called structural alerts build on the fundamental basis and investigation of carcinogenicity data by Ashby and Tennant.[1] More quantitative predictions may be achieved with quantitative structure-activity relationships (QSARs). The methodology underpinning QSARs has been extended to include machine learning methods which

can relate the hazardous effects of a large range of substances to aspects of their physico-chemical properties or structural attributes.

There are a variety of places in the drug development pipeline where computational tools may be applied. They have found particular use in screening of candidate molecules early in drug development. There are especially useful and widely applied in the identification of potent compounds, being largely successful at identifying DNA-reactive compounds such that they may be eliminated early. The reason for the success is due to the high level of understanding in the molecular initiating event (MIE) and the possibility of modelling interactions with DNA.

In contrast, modelling non-genotoxic carcinogenicity has resulted in much less robust predictions. There are a number of reasons for the lower quality of non-carcinogenic models, but key amongst them are the number and complexity of mechanisms, lack of definitive MIEs on which to base a model, and the paucity of suitable data for modelling. Integrating such computational tools into carcinogenicity assessment should include an assessment of the uncertainty in the approaches and whether a prediction is acceptable on its own or will require other information, which may include "big" data from New Approach Methodologies (NAMs) and *in vivo* bioassays.

A potential solution to prediction of complex endpoints is the process of read-across, whereby similar structures are assumed to have similar activities. The utility of read-across has been demonstrated for carcinogenic substances, for instance where substances have common metabolic processes.[2] Justification may require information and variety of lines of evidence to provide an overall weight of evidence.

At the current time, there is no, or little, use of artificial intelligence beyond the development of global QSARs utilising machine learning (as noted above). There are a number of possibilities, however, for instance probabilistic approaches could integrate information from *in vitro* data that have been derived from Integrated Approaches to Testing and Assessment (IATA). This would be particularly valuable for some of the non-genotoxic mechanisms of carcinogenicity (an example of such an IATA was described by Jacobs et al. for breast cancer.[14] Although little explored, Large Language Models (LLMs) may ultimately assist in compiling information from pathology reports, forming summaries and identifying otherwise undiscovered trends and relationships.[16]

In summary, *in silico* approaches have the potential to assist in the identification of hazards relating to carcinogenicity. The approaches range from structural alerts, relating aspects of chemistry to toxicity up to machine learning approaches developing models from large datasets. Computational models work optimally when there is a mechanistic basis and have found success in predicting DNA-reactive genotoxicity. The complexity of non-genotoxic mechanisms of carcinogenicity has hindered their use to definitively replace the long-term carcinogenicity assay, but the possibility of integrating multiple lines of evidence should be investigated further to achieve this.

3. Genomics-Based NAMs for Classification of Carcinogens in Pharma

Dr Heidrun Ellinger-Ziegelbauer discussed the development of the use of genomics data for the prediction of carcinogenicity of pharmaceuticals. She outlined the evolution of transcriptomics data over the years and its potential for predicting long-term carcinogenicity from short-term animal studies. It is the objective of the Health and Environmental Sciences Institute (HESI) Carcinogenomics Working Group to use genomic signature and biomarkers for assessing tumorigenic potential, especially for rodent liver cancer.[5]

The primary outcomes of this long-term, ongoing effort were presented. Carcinogens can generally be assigned to two major categories, based on their overall mode of actions which has implications for their regulation:

(1) Genotoxic carcinogens (GCs) interact directly with DNA, mostly after being metabolized into the ultimate carcinogen, and are then mutagenic when the mutation is fixed by DNA replication leading to initiation of tumor formation. They are usually detected with *in vitro* and *in vivo* genotoxicity assays. Their dose response is assumed to be linear; thus a threshold dose is supposed to be absent. Recent investigations do suggest a threshold dose, e.g., due to repair mechanisms.[10]

(2) Non-genotoxic carcinogens (NGCs) do not directly interact with DNA. They act via multiple mechanisms including Nuclear Receptor activation (NR), immune suppression, hormonal perturbation, and induction of cellular damage leading to regenerative hyperplasia. They promote tumor formation by induction of cell proliferation or inhibition of apoptosis leading to clonal expansion of initiated cells. In general, they need to be present for prolonged times and at rather high doses to exert their promoting effect. They show non-linear dose responses and thus are associated with a threshold dose. Furthermore, they are mostly organ-specific and can be species-specific

A major target organ for chemical induced cancerogenesis is the liver, which is well described in rats due to the performance of the so-called 2-year cancer bioassay in the context of chemical risk assessment. For an initial investigation whether short term studies combined with molecular omics methods may be able to classify compounds with respect to later liver tumor induction, liver gene expression profiles were analyzed after up to 14-day treatment of rats with representative rodent hepatocarcinogens, including GCs and NGCs at carcinogenic doses, in comparison to non-carcinogens.[8] Histopathological findings supported the dose selection, featuring weak apoptosis and/or necrosis and inflammation for GCs, and early or regenerative hyperplasia for NGCs. For functional interpretation of the deregulated genes to increase mechanistic insight, the genes were assigned to a biochemical category describing its main biochemical, cellular or systemic function. Then a “Tox” category for each gene was

defined using the biochemical category, the direction of deregulation, and available information upon upstream pathways and transcriptional regulators. This revealed that GCs induce a DNA damage response, characterized by upregulation of target genes of the transcription factor p53.

In comparison, NGCs affect various pathways, such as Nuclear Receptor (NR) activation, cell proliferation, and oxidative stress responses. These pathways may all contribute to the NGC Mode of Action (MOA) if they support survival or proliferation of mutated cells or induce mutations themselves via generation of reactive species. Thus, gene expression profiles representing certain pathways or functions may contribute to characterization of a compound's carcinogenic potential after short time treatment of rodents. Since different NGCs are associated with different MoAs, distinct gene expression signatures representing major MoAs are needed. This lends itself to the AOP concept with signatures representing well defined MIEs or KEs as way forward. The publication of extensive transcriptomics data sets representing liver gene expression profiles after short-term treatment of rats with GCs, NGCs and other chemicals then made engagement into development of such signatures possible. This was taken up by the Carcinogenomics Project (https://hesiglobal.org/emerging-systems-toxicology-for-assessment-of-risk-committee/). The major objectives of this project are (1) determination of gene expression signatures representing major MIE/KEs for rat carcinogens on (a) a general mechanistic level, associated with (b) signature induction thresholds based on known carcinogenic doses, and (2) identification of cancer driver gene mutations in few mutated cells only within a pool of cells without DNA sequence mutations, before visible tumor appearance, employing highly sensitive sequencing methods with very low error rates, also called error corrected Next Generation Sequencing (ecNGS).[5]

With respect to objective 1, the different carcinogens used for generating the available short term rat liver transcriptomics data (TG-GATEs, DrugMatrix, IMI Marcar), were assigned to the different MIEs, gene expression data of the corresponding samples were compiled and quality controlled and analyzed by several experts using their usual workflows. This yielded several overlapping gene expression signatures per MIE (a list of genes associated with a certain expression pattern). Studies with rats with either p53 or any of the NRs knocked out and treated with three reference compounds each are being used to confirm and adapt signatures derived from WT rats.

Investigations with respect to objective (2) “cancer driver mutation detection” are ongoing in collaboration with the HESI Genetic Toxicology Technical Committee (GTTC).[26 17 22]

With respect to detection and characterization of carcinogens using *in vitro* models, which is the goal for NAM approaches, more research appears necessary. It is to be noted that for GC, well-established *in vitro* models are available. For subtopics like

characterization of compounds which are AMES-neg but potentially false-positive in chromosome damage assays, HESI has developed an *in vitro* transcriptomic biomarker to predict the probability that an agent is DNA Damage-Inducing (DDI) or non-DDI.[15] This approach is currently undergoing biomarker qualification with the FDA.

Concerning NGCs, simple *in vitro* models will not suffice to cover the various MoAs employed by NGCs to induce cancer, as exemplified by the comparison of 3 GCs and 3 NGCs applied to both a 2D primary rat hepatocyte model and to rats *in vivo*.[7] On the basis of the AOP concept, insights gained from investigations of *in vivo* studies employing omics methods in addition to traditional parameters, like the development of NR-MIE gene expression signatures mentioned above, could be used to develop *in vitro* models for specific single NGC MoAs.

4. Advanced Cellular Models for Carcinogenicity Assessment

Dr Kelly Evans (AstraZeneca) focused on the challenges associated with traditional pre-clinical drug safety models, which often do not accurately represent human physiology. Many current *in vitro* pre-clinical safety models utilise either 2D-cell cultures or cancer cell lines, neither of which accurately represent the patient population due to their simplified nature or cancer background. Therefore, there is often a translational disconnect between safety findings found in a dish and those observed in animal studies or in the clinic.

In the context of complex, multi-stage processes such as carcinogenicity, there is a clear need for more human-relevant, advanced cell models that can better recapitulate the complex structure and mechanisms of human tissues.

To this end, advanced human cell models, including organoids and organ-on-a-chip systems, are currently under development to help overcome these issues. Due to their three-dimensional arrangement and increased complexity through inclusion of multiple cell types, they provide the opportunity to assess carcinogenicity within a physiologically relevant, human-appropriate system. Such systems have the advantage of being generally long-lived, allowing assessment of repeat dosing or carcinogenicity that may only be detected over an extended period.[13 6 4]

Kelly Evans used the complexity of evaluating the safety of cell-based therapies to present new models and methodologies. Multicellular organoids could hold promise in providing material for organ transplantation, because of their unique potential for tissue repair. A model of bone marrow representing the possibility to test the immune response was presented. In addition, as data from human trials continue to expand, there is also scope for a significant impact of computational and machine learning models to make predictions of key parameters involved in immune adverse events.[3]

5. Identifying Potential Carcinogenicity Risks through Innovative Genetic Toxicology Methods and Target Safety Assessments

Dr Joanne Elloway highlighted the pivotal role of target safety assessments, along with advancements in genetic toxicology and sequencing in predicting carcinogenicity during drug development.

Target safety reviews for carcinogenicity prediction focus on evaluating compounds for potential cancer risks early in development by reviewing biology of the target, expression profiles, pathway roles (e.g., cell cycle, DNA repair), and human genetics. These reviews assess biological pathways and molecular targets to identify potential oncogenic pathways and genetic markers, utilising published and data repositories.

Error-corrected Next Generation Sequencing (ecNGS) is rapidly emerging as a valuable, highly sensitive and accurate method for detecting and characterizing mutations in any cell type, tissue or organism from which DNA can be isolated. EcNGS is under extensive investigation for the use in genetic toxicology and has been applied to experiments assessing mutagenicity and carcinogenicity to quantify drug-/chemical-induced mutations and mutational spectra associated with cancer risk. It has potential applications in genotoxicity assessment as a new readout for traditional models, for mutagenesis studies in 3D organotypic cultures, and for detecting off-target effects of gene editing tools.[19]

These methodologies may be conducted without animal testing, thereby facilitating early identification of carcinogenic risks.

Author Contributions

The authors are solely responsible for the contents and drafting of this paper. All of them have contributed to their respective talks and summaries, except Frédéric Schorsch who chaired the organization of the session and assembled those proceedings.

Declaration of Conflicting Interests

The opinions expressed in this document reflect only the author's view. The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. Dr Heidrun Ellinger-Ziegelbauer and Frederic Schorsch are employees of Bayer AG and may hold stock in the company. Dr Kelly Evans and Dr Joanne Elloway are employees of AstraZeneca Pharmaceuticals LP and may hold stock in the company.

Funding

The authors were all employed during the preparation and review of the manuscript, but no funding was provided from the respective employers for the preparation of this manuscript. Prof Mirjam Luijten receives funding from the European Partnership for Alternative Approaches to animal testing (EPAA, Brussels, Belgium) for the research presented. Prof Mark Cronin receives funding from the European Union's Horizon 2020 Research and Innovation programme under Grant Agreement No. 964537 (RISK-HUNT3R), and he is part of the ASPIS cluster and the QUANTUM-TOX – Revolutionizing Computational Toxicology with Electronic Structure Descriptors and Artificial Intelligence (QUANTUM-TOX) HORIZON-EIC-2023-PATHFINDEROPEN-01 Project number: 101130724. The other author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iDs

- Frederic Schorsch https://orcid.org/0000-0001-7825-3823
- Mirjam Luijten https://orcid.org/0000-0002-5277-1443
- Mark Cronin https://orcid.org/0000-0002-6207-4158
- Heidrun Ellinger-Ziegelbauer https://orcid.org/0000-0003-0247-4210
- Kelly Evans https://orcid.org/0000-0002-8276-1211
- Joanne Elloway https://orcid.org/0000-0002-1857-5887

References

1. **Ashby J, Tennant RW.** Chemical structure, Salmonella mutagenicity and extent of carcinogenicity as indicators of genotoxic carcinogenesis among 222 chemicals tested in rodents. *Mutat Res*. 1988;204:17–115. PMID: 3277047. https://doi.org/10.1016/0165-1218(88)90114-0

2. **Atwood ST, et al.** New perspectives for cancer hazard evaluation by the report on carcinogens: a case study using read-across methods in the evaluation of haloacetic acids found as water disinfection by-products. *Environ Health Perspect*. 2019;127:125003. PMID: 31854200. https://doi.org/10.1289/ehp5672

3. **Bates S, et al.** Immune safety challenges facing the preclinical assessment and clinical progression of cell therapies. *Drug Discov Today*. 2024;29(12). PMID: 39521331 https://doi.org/10.1016/j.drudis.2024.104239

4. **Cairns J., et al.** Optimal experimental design for efficient toxicity testing in microphysiological systems: A bone marrow application. *Front. Pharmacol.* 2023;14:1142581. https://doi.org/10.3389/fphar.2023.1142581

5. **Corton JC, et al.** A collaborative initiative to establish genomic biomarkers for assessing tumorigenic potential to reduce reliance on conventional rodent carcinogenicity studies. *Toxicol Sci*. 2022;188(1):4–16. https://doi.org/10.1093/toxsci/kfac041

6. **David R. et al.** Three dimensional and microphysiological bone marrow models detect in vivo positive compounds. *Sci Rep.* 2021 Nov 9;11:21959. PMID: 34754012 https://doi.org/10.1038/s41598-021-01400-5

7. **Doktorova TY, et al.** Comparison of hepatocarcinogen-induced gene expression profiles in conventional primary rat hepatocytes with in vivo rat liver. *Arch Toxicol*. 2012;86:1399–1411. PMID: 22484513. doi: 10.1007/s00204-012-0847-x

8. **Ellinger-Ziegelbauer H, et al.** Prediction of a carcinogenic potential of rat hepatocarcinogens using toxicogenomics analysis of short-term in vivo studies. *Mutat Res*. 2008;637:23–39. PMID: 17689568. DOI: 10.1016/j.mrfmmm.2007.06.010

9. **Gottmann, E., et al.** Data quality in predictive toxicology: reproducibility of rodent carcinogenicity experiments. *Environ Health Perspect*. 2001;109(5):509–514. PMID: 11401763. https://doi.org/10.1289/ehp.01109509

10. **Hartwig A, et al.** Mode of action-based risk assessment of genotoxic carcinogens. *Arch Toxicol*. 2020;94:1787–1877. PMID: 32542409. https://doi.org/10.1007/s00204-020-02733-2

11. **Heusinkveld HJ, et al.** Towards a mechanism-based approach for the prediction of nongenotoxic carcinogenic potential of agrochemicals. *Crit Rev Toxicol*. 2020;50(9):725–739. PMID: 33236972. https://doi.org/10.1080/10408444.2020.1841732

12. **Hendriksen, C.F.M.** The Ethics of Research Involving Animals: A Review of the Nuffield Council on Bioethics Report from a Three Rs Perspective. *Altern Lab Anim*. 2005;33(6):659–662. PMID: 16419361 https://doi.org/10.1177/026119290503300604

13. **Herms A, et al.** Self-sustaining long-term 3D epithelioid cultures reveal drivers of clonal expansion in esophageal epithelium. *Nature Genetics*. 2024;56:2158–2173. https://doi.org/10.1038/s41588-024-01875-8

14. **Jacobs MN, et al.** Chemical carcinogen safety testing: OECD expert group international consensus on the development of an integrated approach for the testing and assessment of chemical non-genotoxic carcinogens. *Arch Toxicol*. 2020;94(8):2899–2923. PMID: 32594184 https://doi.org/10.1007/s00204-020-02784-5

15. **Li HH, et al.** TGx-DDI, a transcriptomic biomarker for genotoxicity hazard assessment of pharmaceuticals and environmental chemicals. *Front Big Data*. 2019;2:36. PMID: 33693359 https://doi.org/10.3389/fdata.2019.00036

16. **Lu J, et al** Large language models and their applications in drug discovery and development: A primer. *Clin Transl Sci.* 2025; 18:e70205 PMID: 40208836 https://doi.org/10.1111/cts.70205

17. **Lynch AM, et al.** Next Generation Sequencing Workshop at the Royal Society of Medicine (London, May 2022): how genomics is on the path to modernizing genetic toxicology. *Mutagenesis*. 2023;38(4):192–200. PMID: 37300447. https://doi.org/10.1093/mutage/gead012

18. **Madden JC, et al.** A review of In Silico Tools as alternatives to animal testing: principles, resources and applications. *Altern Lab Anim*. 2020;48(4):146–172. PMID: 33119417. https://doi.org/10.1177/0261192920965977

19. **Marchetti F, et al.** Error-corrected next generation sequencing – Promises and challenges for genotoxicity and cancer risk assessment. *Mutat Res*. 2023;792:108466. PMID: 37643677. https://doi.org/10.1016/j.mrrev.2023.108466

20. **Marone, P.A., W.C. Hall, and A.W. Hayes.** Reassessing the two-year rodent carcinogenicity bioassay: a review of the applicability to human risk and current perspectives. *Regul Toxicol Pharmacol*. 2014;68(1):108–18. PMID: 24287155 https://doi.org/10.1016/j.yrtph.2013.11.011

21. **OECD (2018).** Users' Handbook supplement to the Guidance Document for developing and assessing Adverse Outcome Pathways. OECD Series on Adverse Outcome Pathways, No. 1, OECD Publishing, Paris. http://dx.doi.org/10.1787/5jlv1m9d1g32-en

22. **Parsons BL.** Clonal expansion of cancer driver gene mutants investigated using advanced sequencing technologies. *Mutat Res*. 2024;794:1085145. PMID: 39369952. https://doi.org/10.1016/j.mrrev.2024.108514

23. **Schieferdecker S and Vock E.** Quantum Chemical Evaluation and QSAR modeling of N-Nitrosamine carcinogenicity. *Chem Res Toxicol*. 2025;38(2):325–339. PMID: 39915909. https://doi.org/10.1021/acs.chemrestox.4c00476

24. **Tice RR, et al.** *In Silico* approaches in carcinogenicity hazard assessment: current status and future needs. *Comput Toxicol*. 2021;20:100191. PMID: 35368437. https://doi.org/10.1016/j.comtox.2021.100191

25. **Vinken M, et al.** Adverse outcome pathways: a concise introduction for toxicologists. *Arch Toxicol*. 2017;91(11):3697–3707. PMID: 28660287. https://doi.org/10.1007/s00204-017-2020-z

26. **Zhang Y, et al.** Transferability, reproducibility and sensitivity of mutation quantification by duplex sequencing. *Environ Mol Mutagen*. 2025;66(6–7):311–326. PMID: 40586373. https://doi.org/10.1002/em.70020