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Approche intégrée à haut débit pour la caractérisation des agents cancérigènes non génotoxiques (NGTxC)

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Dedication

إلى مَنْ تَحَدُّوا الحَياةَ رُغْمَ صُعوبَتِها

إلى مَنْ عَجَزَ اليَأْسُ أَمَامَ إصْرارِهِمْ

إلى مَنْ يَسْتَحِقُ الحَياةَ عَلى هذِهِ اَلأَرْض

To those who have defied life despite its hardships

To those whom despair has faltered in face of their determination

To those who deserve life on this Earth

ما دامَ الأَمَلُ طَريقاً فَسَنَحياهْ

"As long as hope is a way, we'll find a way"

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Integrated high-throughput approaches for the characterization of non-genotoxic carcinogens

Table of content

Thesis valorization 21
1. Publications 23
2. Communication 24
3. Research grants and awards 25
Summary 27
List of Abbreviations 35
Introduction 41
1. Ancient toxicology 43
2. Chemical safety assessment 44
2.1. Poisons 44
2.2. Current CSA Framework 45
2.2.1. Hazard identification 46
2.2.2. Dose-response assessment 48
2.2.3. Exposure assessment 48
2.2.4. Risk characterization 49
2.3. CSA framework challenges 49
2.4. Limitations of animal-based CSA 51
3. Revolutionizing the chemical risk assessments 55
3.1. Regulatory decisions towards the phase-out of the use of experimental animals 55
3.2. Next Generation Risk Assessment 58
3.3. NAMs provide mechanistic evidence for toxicological endpoints 60
3.4. The Role of NAMs in toxicological Evaluation and safety assessment 62
3.5. Implementation and adoption of NAMs in carcinogenesity assessment 65
4. Cancer 66
4.1. Cancer burden and multifactorial etiology 66
4.2. Hallmarks of cancer 67
4.3. Chemical carcinogenesis 69
4.3.1. Initiation 70

4.3.2. Promotion 71
4.3.3. Progression 72
5. Carcinogens 73
5.1. Key Characteristics of Carcinogens 73
5.2. IARC carcinogen classification 75
5.3. Types of carcinogens 77
6. Genotoxic carcinogens 78
6.1. Definition 78
6.2. Genotoxicity evaluation of chemicals 79
6.3. Limitations of the current genotoxicity evaluation framework 84
6.4. Integration of alternative, NAM-based approaches for genotoxicity testing 85
7. Non-genotoxic carcinogens 87
7.1. Definition 87
7.2. NAMs-based NGTxC evaluation 91
7.2.1. Mechanistic-based strategies 91
7.2.2. High-throughput screening strategies 92
7.2.3. Transcriptomic strategies 93
7.3. Current tests in regulatory validation phase 95
8. Liver as a case study 98
8.1. Liver toxicity 98
8.2. Cellular structure of the liver 100
8.3. in vitro liver models 102
9. Advancing NGTxC hazard assessment: integration of PARC project 106
10. Key Challenges in NGTxC hazard assessment targeted in this thesis 108
Thesis objectives 110
References 113
Chapter I: "The art of chemical clasification, prioritization, and screening" 139
Preamble 141

Mechanistic toxicity profiling of chemicals: A comparative study in 2D and 3D HepaRG models using high-content analysis "Article" 145
Graphical abstract 147
Abstract 148
Highlights 149
Key words 149
1. Introduction 150
2. Materials and Methods 153
3. Results 159
4. Discussion 179
References 183
Authorship contribution 192
Chapter II "Decoding chemical toxicity:from cellular shadows to molecular signiture" 195
Preamble 197
Advancing genotoxicity assessment with transcriptomic biomarkers: From 2D to 3D HepaRG spheroid model "Article" 201
Graphical abstract 203
Abstract 204
Highlights 205
Key words 205
1. Introduction 206
2. Materials and Methods 210
3. Results 216
4. Discussion 221
References 225
Authorship contribution 233
Transcriptomic–based analysis to discriminate Non-Genotoxic Carcinogens from genotoxic carcinogens and non-carcinogens in a human HepaRG spheroid model: Mechanistic insights and biomarker identification "Article" 235

Graphical abstract 237
Abstract 238
Highlights 239
Key words 239
1. Introduction 240
2. Materials and Methods 242
3. Results and discussion 247
4. Conclusions and perspectives 265
References 267
Authorship contribution 275
Chapter III "Phynotypic footprints of chemical carcinogenes" 277
Revealing carcinogenic potential of chemicals: an in vitro evaluation using the Bhas42 cell transformation assay "Article" 279
Preamble 281
Graphical abstract 285
Abstract 286
Highlights 287
Key words 287
1. Introduction 288
2. Materials and methods 290
3. Results 297
4. Discussion 306
References 312
Authorship contribution 317
Conclusion and perspectives 319
Appendix 331
Appendix Int. 332
Introduction supplementary information 332

Appendix I 347
Chapter I supplementary information 347
Appendix II 351
Chapter II supplementary information 351
Appendix III 357
Chapter III supplementary informations 357
Appendix IV 363
Publicatoin *"Protocol"* 363
Appendix V 401
Poster presentations 401

Thesis valorization

1. Publications

1.1. Research articles

Saleh, M., Hogeveen, K., Fessard, V., & Le Hégarat, L. (2025). Mechanistic toxicity profiling of chemicals: A comparative study in 2D and 3D HepaRG models using high-content analysis. Toxicology, 519, 154280. Advance online publication. doi: 10.1016/j.tox.2025.154280

Saleh, M.; Thienpont, A.; De Clercq, G.; Hogeveen, K.; Fessard, V.; Mertens, B.; Le Hégarat, L. Advancing genotoxicity assessments with transcriptomic biomarkers: From 2D to 3D HepaRG spheroid model. ***In-preparation for submission***.

Saleh, M.; Honza, T.; Rundén-Pran, E.; Dusinska, M.; Hogeveen, K.; Fessard, V.; El Yamani, N.; Le Hégarat, L. *In vitro* evaluation of non-genotoxic carcinogenic potential using Bhas42 Cell Transformation Assay. ***In-preparation for submission***.

Saleh, M.; Léger, T.; Hogeveen, K.; Fessard, V.; Le Hégarat, L. Integrative high-throughput transcriptomic and clustering analysis to discriminate genotoxic and non-genotoxic effects of carcinogens in 3D HepaRG Spheroids. ***In-preparation***.

Saleh, M.; Honza, T.; Rundén-Pran, E.; Dusinska, M.; Hogeveen, K.; Fessard, V.; El Yamani, N.; Le Hégarat, L. *in vitro* mechanistic-based transcformics analysis in Bhas42 cell. ***In-data treatment process***.

1.2. Review articles

Audebert, M.; Azqueta, A.; Babica, P.; Benfenati, E.; Bortoli, S.; Bouwman, P.; Braeuning, A.; Burgdorf, T.; Coumoul, X.; Debizet, K.; Dusinska, M.; Ertych, N.; Fahrer, J.; Fetz, V.; Le Hégarat, L.; López De Cerain, A.; Heusinkveld, H.; Hogeveen, K.; Jacobs, M.; Luijten, M.; Raitano, G.; Recoules, C.; Rundén-Pran, E.; **Saleh, M.**; Sovadinova, I.; Stampar, M.; Thibol, L.; Tomkiewicz, C.; Vettorazzi, A.; Van De Water, B.; El Yamani, N.; Zegura, B.; Oelgeschläger, M. **(2023)**. New approach methodologies to facilitate and improve the hazard assessment of non-genotoxic carcinogens – A PARC project. *Frontiers in toxicology*, 5:1220998. doi:10.3389/ftox.2023.1220998

1.3. Protocols

Duclos, M.; Marzougui, Z.; **Saleh, M.**; Corlu, A.; Fromenty, B.; Massart, J.; Ferron., P.-J. Multi-cellular human liver organoids for modeling MASLD and DLI. doi: 10.1016/j.tiv.2023.105728

2. Communication

2.1. Oral presentations

Saleh, M.; Hogeveen, K.; Le Hégarat, L. *In vitro* based high-throughput approaches for the identification of non-gentotoxic carcinogens (NGTxC). *Journée Scientifique et Doctorales de l'Anses (JSDA 2025)*. Maisons-Alfort, France. **(2025, June 04-05)**.

Saleh, M.; Thienpont, A.; De Clercq, G.; Hogeveen, K.; Fessard, V.; Mertens, B.; Le Hégarat, L. *In vitro* high-throughput transcriptomic strategy accompanied by TGx-DDI & GENOMARK as powerful tools for genotoxicity evaluation in 3D HepaRG Spheroids. *53rd European Environmental Mutagenesis & Genomics Society Congress (EEMGS 2025)*. Bratislava, Slovakia. **(2025, June 02-05)**.

Saleh, M.; Hogeveen, K.; Le Hégarat, L. Carcinogens: Enemy of Humans & Mice How to save both of them? *Journées Scientifiques 2025 de l'École Doctorale Sciences de la Vie et de la Santé - Bretagne*. Saint-Brieuc, France. **(2025, April 24-25)**.

Saleh, M.; Hogeveen, K.; Fessard, V.; Le Hégarat, L. *In vitro* high-throughput methodologies for the characterization of NGTxC. *PARC WP. 5.2 annual meeting*. Madrid, Spain. **(2025, March 13-14)**.

Saleh, M.; Hogeveen, K.; Fessard, V.; Le Hégarat, L. *In vitro* based testing strategy for the identification of non-genotoxic carcinogens (NGTxC). *PARC WP. 5.2 annual meeting*. Berlin, Germany. **(2024, March 25-26)**.

2.2. Posters

Saleh, M.; Hogeveen, K.; Le Hégarat, L. *In vitro* based testing strategy for the identification of non-genotoxic carcinogens. *Journée Scientifique et Doctorales de l'Anses (JSDA 2024)*. Maisons-Alfort, France. **(2024, October 02-03)**.

Saleh, M.; Hogeveen, K.; Le Hégarat, L. *In vitro* based testing strategy for the identification of non-genotoxic carcinogens. *22nd International Congress of the European Society of Toxicology In Vitro (ESTIV 2024)*. Prague, Czech Republic. **(2024, June 03-06)**.

Saleh, M.; Porcq, C.; Hogeveen, K.; Le Hégarat, L. *In vitro* based testing strategy for the identification of non-genotoxic carcinogens (NGTxC). *Gen2Bio*. Saint-Malo, France. **(2024, March 14-15)**.

3. Research grants and awards

Jury Prize for the **Best Oral Presentation**
Journées Scientifiques 2025 de l'École Doctorale Sciences de la Vie et de la Santé – Bretagne
Saint-Brieuc, France **(2025)**

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Summary

Ensuring the safety of chemicals to which humans and the environment are continuously exposed remains one of the major challenges in **regulatory toxicology** and **public health**. Currently, thousands of chemicals are found and in use within various sectors such as consumer products, pharmaceuticals, food, industrial and others. The disparity between the accelerating proliferation of novel synthetic compounds and the throughput limitations of conventional safety paradigms has resulted in a significant evaluation gap; consequently, the toxicological profiles of most modern chemicals remain largely uncharacterized.

Among these thousands of new chemicals introduced each year, a significant portion could have the potential to cause cancer. **Cancer** represents a key public health issue. As per the World Health Organization (WHO's) cancer agency, the **International Agency for Research on Cancer (IARC)**, report (Global cancer burden growing, amidst mounting need for services, 2024), there is a notable increase in the global cancer burden worldwide. The report estimated, in 2022, 20 million new cancer cases with 9.7 million associated deaths worldwide. Hence, the cancer burden is predicted to increase to over 35 million cases globally by 2050.

Over time and to date, assessing the carcinogenic potential of substances has primarily relied on **long-term animal studies**. These studies have played a key role in the detection and the understanding of the biological pathways related to chemical carcinogenesis, and have significantly improved our comprehension of cancer biology. However, such approaches are questionable due to their limited relevance to humans and for ethical dilemmas. In addition, limitations relate to their high expenses and extended experimental time. Thus, such animal studies could not be used to rapidly assess the thousands of chemicals that are in rapid increase nowadays.

Carcinogens are generally classified based on their carcinogenicity potential into Genotoxic Carcinogens (GTxC) and Non-Genotoxic Carcinogens (NGTxC). Unlike the GTxC, there are no *in vitro* Organisation for Economic Co-operation and Development (OECD) approved methods to identify NGTxC. A battery of *in vitro* and *in vivo* tests is available to detect the genotoxic potential of chemicals. Then, if *in vitro* data are generally not sufficient to conclude about the genotoxicity potential of a chemical, subsequent *in vivo* positive results in a genotoxicity test classify the compound as potentially carcinogenic. However, in the absence of genotoxicity potential, the carcinogenicity potential of the chemical remains unknown in the

absence of long-term *in vivo* studies. Consequently, NGTxC might remain unidentified and therefore the risk associated with these potential carcinogens remains undetected. Besides, NGTxC induce cancer without a direct DNA interaction. These carcinogens have been shown to act as tumor promoters, endocrine-disrupters, immunosuppressants, receptor-mediators, epigenetic modifiers and others. Hence, the diversity in their **Mode of Actions (MoAs)**, and their tissue and species specificity, makes predicting their risk extremely challenging and predicting their carcinogenic potential difficult in the absence of human or animal data.

Therefore, the shift toward alternative approaches along with the progress in mechanistic toxicology highlights the need for mechanistic-based **New Approach Methodologies (NAMs)** to replace the traditional animal studies and to reduce the reliance on such studies in **Chemical Safety Assessments (CSA)**. By integrating an array of non-animal strategies, including predictive modeling and advanced cell-based assays, NAMs offers a more rapid and biologically relevant characterization of chemical hazards within the CSA framework.

Despite significant progress made to evaluate NGTxC using different human-relevant tissue models and high-throughput methodologies, a more advanced approach remains necessary and required to detect and identify the key MoA associated with NGTxCs. Therefore, as a part of the **European Partnership for the Assessment of Risks from Chemicals (PARC)**, which aims at developing *in vitro* strategies aligned with Integrated Approach to Testing and Assessment IATA/OECD to assess NGTxC and work towards their regulatory uptake. Our goal is to outline and evaluate a NAMs-based *in vitro* testing strategy capable to identify different NGTxC MoAs. To achieve this goal, and considering the liver as the primary target organ for many carcinogens due to its central role in xenobiotics metabolism, we applied multiple *in vitro* strategies coupled with human-hepatic HepaRG cell lines to identify different NGTxC MoAs both at cellular and molecular levels. Furthermore, Cell Transformation Assay (CTA) was applied in order to investigate their carcinogenic potential at the apical level. Different NGTxC chemicals with distinct MoAs were selected from the chemical list defined within the PARC consortium.

Accordingly, this work was conducted around three main *in vitro* testing strategies, presented through three chapters:

In the first chapter, High-Content cell imaging analysis in both 2D HepaRG cells and 3D HepaRG spheroids was applied with a focus on (i) the evaluation of diverse NGTxC MoAs at the cellular level such as cytotoxicity, mitochondrial dysfunction, oxidative stress, lipid

accumulation and inflammation, (ii) generating toxicity profiles for the selected tested chemical list to classify and prioritize chemicals tested, and (iii) comparing model sensitivity to determine the most suitable system for subsequent studies.

In the second chapter, high-throughput transcriptomic analysis was applied with the most sensitive cellular model selected (3D HepaRG spheroids). The focus of this chapter was: (i) to generate transcriptomic profiles for the tested chemicals, (ii) to determine the capacity of the GENOMARKS and TGx-DDI transcriptomic biomarkers to discriminate between GTxs and NGTxs based on profiles generated from the treated HepaRG 3D cells, (iii) to investigate if unsupervised clustering analysis applied to the sequenced whole genome of the treated HepaRG 3D model could successfully classify NGTxCs, and (iv) further to explore the key molecular events underlying NGTxC mechanisms.

In the third chapter, The Bhas42 CTA, currently under OECD validation, was employed to evaluate the applicability of this system in detecting potential human carcinogens. Specifically, it was used to assess the apical effects of chemicals by determining whether the cellular perturbations observed in Chapter have the potential of causing malignant transformation.

Overall, the strategies implemented in this study validated the applicability of specific NAMs for the detection, assessment, and classification of chemicals based on distinct NGTxC MoA. The data generated in this project contributed to the generation of *in vitro* data that addresses critical knowledge gaps in NGTxC identification. Furthermore, this project established a multi-tiered, integrated *in vitro* testing framework. This battery of assays demonstrates the utility of a weight-of-evidence (WoE) approach for the hazard characterization of human carcinogens. Despite these advancements, this work underscores the importance of enhancing bio-analytical complexity specifically by incorporating complex cellular systems. Hence, insuring advanced human physiological responses and improve translational relevance.

Résumé

Garantir la sécurité des produits chimiques auxquels les humains et l'environnement sont continuellement exposés reste l'un des principaux défis en toxicologie réglementaire et en santé publique. Actuellement, des milliers de produits chimiques sont découverts et utilisés dans divers secteurs tels que les produits de consommation, la pharmacie, l'alimentation, l'industrie et d'autres. L'écart entre la prolifération accélérée des nouveaux composés synthétiques et les limites de débit des paradigmes de sécurité conventionnels a entraîné un écart d'évaluation important ; Par conséquent, les profils toxicologiques de la plupart des produits chimiques modernes restent largement indéfinis.

Parmi ces milliers de nouveaux produits chimiques introduits chaque année, une part significative pourrait avoir le potentiel de provoquer le cancer. Le cancer représente un enjeu clé de santé publique. Selon le rapport de l'agence mondiale de la santé (WHO) et de l'Agence internationale de recherche sur le cancer (IARC), (Augmentation du fardeau mondial du cancer, face à un besoin croissant de services, 2024), on observe une augmentation notable du fardeau mondial du cancer. Le rapport a estimé, en 2022, 20 millions de nouveaux cas de cancer avec 9,7 millions de décès associés dans le monde. Ainsi, le fardeau du cancer devrait augmenter à plus de 35 millions de cas dans le monde d'ici 2050.

Au fil du temps et à ce jour, l'évaluation du potentiel cancérigène des substances s'est principalement appuyée sur des études animales à long terme. Ces études ont joué un rôle clé dans la détection et la compréhension des voies biologiques liées à la carcinogenèse chimique, et ont considérablement amélioré notre compréhension de la biologie du cancer. Cependant, de telles approches sont discutables en raison de leur pertinence limitée pour l'homme et de dilemmes éthiques. De plus, les limitations concernent leurs coûts élevés et leur temps d'expérimentation prolongé. Ainsi, de telles études animales ne pourraient pas être utilisées pour évaluer rapidement les milliers de produits chimiques qui augmentent rapidement de nos jours.

Les cancérogènes sont généralement classés en fonction de leur potentiel de cancérogénicité en cancérogènes génotoxiques (GTxC) et cancérogènes non gétoxiques (NGTxC). Contrairement au GTxC, il n'existe pas de méthodes *in vitro* approuvées par l'Organisation de coopération et de développement économiques (OCDE) pour identifier les NGTxC. Une batterie de tests *in vitro* et *in vivo* est disponible pour détecter le potentiel génotoxique des produits chimiques.

Ensuite, si les données *in vitro* ne suffisent généralement pas à conclure sur le potentiel de génotoxicité d'un produit chimique, les résultats positifs *in vivo* ultérieurs lors d'un test génotoxice classent le composé comme potentiellement cancérigène. Cependant, en l'absence de potentiel génotoxicité, le potentiel de cancérogénicité du produit chimique reste inconnu en l'absence d'études *in vivo* à long terme. Par conséquent, NGTxC pourrait rester non identifié et donc le risque associé à ces cancérogènes potentiels reste indétecté. De plus, NGTxC induit le cancer sans interaction directe avec l'ADN. Il a été démontré que ces agents cancérigènes agissent comme des promoteurs tumoraux, des perturbateurs endocriniens, des immunosuppresseurs, des médiateurs récepteurs, des modificateurs épigénétiques et d'autres encore. Ainsi, la diversité de leurs modes d'action (MoAs), ainsi que leur spécificité tissulaire et d'espèce, rend la prédiction de leur risque extrêmement difficile et la prévision de leur potentiel cancérigène difficile en l'absence de données humaines ou animales.

Ainsi, le passage vers des approches alternatives, ainsi que les progrès en toxicologie mécanistique, soulignent la nécessité de nouvelles méthodologies d'approche (NAM) basées sur le mécanisme pour remplacer les études animales traditionnelles et réduire la dépendance à ces études dans les évaluations de sécurité chimique (CSA). En intégrant une gamme de stratégies non animales, incluant la modélisation prédictive et les tests cellulaires avancés, les MAM offrent une caractérisation plus rapide et biologiquement pertinente des dangers chimiques dans le cadre de la CSA.

Malgré les progrès significatifs réalisés dans l'évaluation des NGTxC grâce à divers modèles tissulaires humains et à des méthodologies à haut débit, une approche plus avancée reste nécessaire pour détecter et identifier les MoA clés associés aux NGTxC. Dans le cadre du Partenariat européen pour l'évaluation des risques liés aux produits chimiques (PARC), qui vise à développer des stratégies *in vitro* alignées avec l'IATA/OCDE pour l'évaluation des NGTxC et leur adoption réglementaire, notre objectif est de définir et d'évaluer une stratégie de test *in vitro* basée sur les NAM, capable d'identifier différents MoA NGTxC.

En considérant le foie comme organe cible principal de nombreux cancérogènes en raison de son rôle central dans le métabolisme des xénobiotiques, nous avons appliqué plusieurs stratégies *in vitro* couplées à des lignées cellulaires humaines HepaRG afin d'identifier divers MoA NGTxC aux niveaux cellulaire et moléculaire. Un test de transformation cellulaire (CTA) a également été utilisé pour examiner leur potentiel cancérogène au niveau apical. Différents produits chimiques NGTxC présentant des MoA distincts ont été sélectionnés à partir de la liste

définie au sein du consortium PARC. En conséquence, ce travail s'est basé sur trois principales stratégies de test *in vitro*, présentées à travers trois chapitres :

Dans le premier chapitre, l'analyse d'imagerie cellulaire à haute teneur dans les cellules HepaRG 2D et les sphéroïdes HepaRG 3D a été appliquée, avec un accent sur (i) l'évaluation de divers MoA NGTxC au niveau cellulaire tels que la cytotoxicité, la dysfonction mitochondriale, le stress oxydatif, l'accumulation lipidique et l'inflammation, (ii) la génération de profils toxics pour la liste chimique testée sélectionnée afin de classifier et de prioriser les substances testées, et (iii) comparer la sensibilité des modèles afin de déterminer le système le plus adapté aux études ultérieures.

Dans le deuxième chapitre, une analyse transcriptomique à haut débit a été appliquée avec le modèle cellulaire le plus sensible sélectionné (sphéroïdes HepaRG 3D). L'objectif de ce chapitre était : (i) générer des profils transcriptomiques pour les substances testées, (ii) déterminer la capacité des biomarqueurs transcriptomiques GENOMARKS et TGx-DDI à discriminer entre GTxs et NGTxs à partir de profils générés à partir des cellules HepaRG 3D traitées, (iii) d'étudier si une analyse de clustering non supervisée appliquée au génome entier séquencé du modèle HepaRG 3D traité pouvait classifier avec succès les NGTxC, et (iv) d'explorer davantage les principaux événements moléculaires sous-jacents aux mécanismes NGTxC.

Dans le troisième chapitre, Le Bhas42 CTA, actuellement validé par l'OCDE, a été utilisé pour évaluer l'applicabilité de ce système à la détection de potentiels cancérogènes humains. Il a permis d'examiner si les perturbations cellulaires observées dans ce chapitre possèdent la capacité d'induire une transformation maligne. Dans l'ensemble, les stratégies mises en œuvre ont confirmé la pertinence de NAMs spécifiques pour détecter, évaluer et classer des substances chimiques selon des MoA NGTxC distincts. Les données générées ont contribué à combler des lacunes critiques dans l'identification des NGTxC en fournissant des résultats *in vitro* essentiels. Le projet a également établi un cadre intégré et multi-niveaux de tests *in vitro*, démontrant l'intérêt d'une approche fondée sur le poids des preuves (WoE) pour caractériser les dangers des cancérogènes humains. Malgré ces avancées, les résultats soulignent la nécessité d'accroître la complexité bio-analytique, notamment par l'intégration de systèmes cellulaires plus sophistiqués. Une telle évolution permettrait d'obtenir des réponses physiologiques humaines plus représentatives et d'améliorer la pertinence translationnelle des évaluations

List of Abbreviations

2DDG	2-deoxy-D-glucose
3D	Three-dimensional
3MCA	3 Methylcholanthrene
ABC	ATP-Binding Cassette
ACSL5	Acyl-CoA synthetase long-chain family member 5
ADI	Acceptable daily Intake
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AMP	Ampicillin Trihydrate
AOP	Adverse Outcome Pathway
AOPs	Adverse Outcome Pathways
gene	Adenomatous Polyposis Coli
Azox	Azoxystrobin
BaP	Benzo[a]pyrene
BMD	Benchmark Dose
BPO	Benzoyl Peroxide
CAA	intrahepatic cholangiocarcinoma
CAFF	Caffeine
CAS	Chemical Abstracts Service
CF	Clofibrate
CGA	Cell Growth Assay
COM	Committee on mutagenicity of chemicals in food, consumer products and the environment
CPA	Cyclophosphamide
CSA	Chemical Safety Assessments
CSR	Chemical safety report
CT	Chlorothalonil

CTA	Cell Transformation Assay
CYPs	Cytochrome P450 (enzymes)
D-man	D-mannitol
DCMU	Diuron
DDI	DNA damage Inducing
DEA	Diethanolamine
DEGs	Differentially expressed genes represented
DEHP	di(2-ethylhexyl) phthalate
DEM	Diethyl Maleate
DILI	drug-induced hepatotoxicity or drug-induced liver injury
DMEMF	Dulbecco's Modified Eagle's Medium / Ham's F12
DSBs	Double Strand Breaks
EFSA	European Food Safety Authority
EMS	ethyl methanesulfonate
EPA	Environmental Protection Agency
EU	European Union
EU PARC	Partnership for the Assessment of Risk from Chemicals
FDA	U.S. Food and Drug Administration
FICZ	6 Formylindolo[3,2-b]carbazole
GCx	Genotoxic
GD	Guidance Document
GJIC	Gap Junction Intercellular Communication
GO	Gene Ontology
GTxCs	Genotoxic carcinogens
HC	Hierarchical Clustering
HCA	High Content Analysis
HCC	Hepatocellular Carcinoma
HCI	High-Content Imaging

HCS	High-Content Screening
HR	Homologous Recombination
HTS	High-Throughput Screening
IARC	International Agency for Research on Cancer
IATA	Integrated Approaches for Testing and Assessment
IP	Indeno[1,2,3-cd]pyrene
iPSC	Induced Pluripotent Stem Cells
ITS	Integrated Testing Strategies
IVIVE	In Vitro to In Vivo Extrapolation
IWGT	International Workshops on Genotoxicity Testing
KCCs	Key Characteristics of Carcinogens
KE	Key Events
LLNA	Local Lymph Node Assay
LOAEL	Lowest Observed Adverse Effect Level
LRT	Likelihood Ratio Test
MEM	Minimum Essential Medium
MIE	Molecular Initiating Event
MLA	Mouse Lymphoma Assay
MLTK	*In vitro* Mouse Lymphoma TK Assay
MN	Micronucleus test
MoA	Mode of Action
MoSt	Modular Strategy of Testing and Assessment
NAMs	New Approach Methodologies
ND	No Data
NEs	Novel Entities
NGRA	Next Generation Risk Assessment
NGTxCs	Non-genotoxic carcinogens
NOAEL	No Observed Adverse Effect Level
NRC	National Research Council

NSCs	Nearest Shrunken Centroids
NTP	National Toxicology Program
OECD	Organization for Economic Co-operation and Development
ODE	Ordinary Differential Equation
PA	Lasiocarpine
PARC	European Partnership For The Assessment Of Risk From Chemicals
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PBK	Physiologically Based Kinetic
PFOA	Perfluorooctanoic acid
PHHs	Primary human hepatocytes
POD	Point-of-departure
PPARs	Peroxisome proliferator-activated receptors
qAOPs	Quantitative Adverse Outcome Pathways
qPCR	quantitative Polymerase Chain Reaction
REACH	Registration, Evaluation, Authorization, and Restriction of Chemicals
RF	Random Forest
RORα	Retinoic acid-related orphan receptor α
ROS	Reactive Oxygen Species
RT	Room Temperature
SHE	Syrian hamster embryo
SVM	Support Vector Machine
TAA	Thioacetamide
Tebu	Tebuconazole
TMRE	Tetramethylrhodamine Ethyl Ester
TPA	12 O Tetradecanoylphorbol 13 Acetate
ULA	Ultra-Low Attachment
USA	United States of America
WoE	Weight-of-evidence

Introduction

"State-of-the-Art"

1. Ancient toxicology

Observations from ancient toxicologists throughout history reflect a deep understanding of the danger from substances. Historically, any substance causing harmful effects was classified as a poison (Pappas et al., 1999).

The study and exploration of these poisons in ancient times laid the essential groundwork for the development of modern toxicology (Daugherty, 1995). Among the most notable historical events is the death of Socrates, who was executed by consuming hemlock— a plant-based poison (Bailey, 2018) (Figure Int. 1). This early recorded use of a toxin not only marks a pivotal moment in history but also illustrates the ancient attempts of researchers to comprehend how chemicals and substances affect the human body.



Figure Int. 1. Jacques-Louis David, 1787. Historical drawing of the death of Socrates, illustrating his final moment in 399 BCE as he accepts the cup of hemlock in adherence to Athenian law.

The foundational principle of toxicology articulated by Theophrastus Bombastus von Hobeneheim, known as Paracelsus (1493-1541) said: ‘‘All things are poison, and nothing is without poison; only the dose makes a thing not a poison.” (Grandjean, 2016). Likewise, Hippocrates (460-377 BCE), held the belief that “Everything in excess is opposed to nature.” and the body must be treated as a whole (Touwaide & Appetiti, 2015). In addition, early medical practitioners from ancient Egypt, Greece, and Rome have recognized early key principles in

toxicology related to dose-response, lethal potential of substances and others which still represent the scientific bases in modern toxicology (Nepovimova & Kuca, 2019).

Together, these historical statements and perspectives form a strong foundational principle in modern toxicology, and more specifically in the current bases of Chemical Safety Assessments (CSA).

2. Chemical safety assessment

2.1. Poisons

In addition to naturally occurring harmful substances, the rational development of novel chemicals or emerging products such as pharmaceuticals, cosmetics, agricultural, and others, has raised the questions in relation with their potential adverse outcomes (Chormare & Kumar, 2022; Alam et al., 2025).

Since 1950, global chemical production has increased about fiftyfold, a rapid escalation suggesting that humanity has likely surpassed the planetary capacity to safely produce, manage, and regulate novel substances (Persson et al., 2022). A recent global survey estimated that over 350,000 chemicals are currently registered for commercial use worldwide (Wang et al., 2020). Furthermore, as highlighted by Muir et al. (2023), both the annual volume of new substances and their proportion relative to total reported chemicals have increased significantly over time (Figure Int. 2). In addition, the amount of chemicals in use by humans are expected to double by 2030 (*UN Report*, 2019).

Newly synthesized substances, termed "Novel Entities" (NEs), include both entirely new chemical compounds and modified versions of existing ones. The detrimental impact of harmful NEs on human health is well-documented; evidence increasingly links these substances to endocrine disruption, neurotoxicity, carcinogenesis, immunosuppression, reproductive disorders, and systemic toxicity (Kemarau et al., 2025).

However, the inherent complexity of NEs, combined with the vast variability in human exposure throughout a lifetime, renders many traditional assessment methods insufficient for a comprehensive evaluation (EPA, 2014). Consequently, there is a critical need for a robust and comprehensive CSA to inform the development of more effective laws and regulations, ultimately improving both chemical production standards and public safety (ECHA, 2016; Pereira et al., 2022).

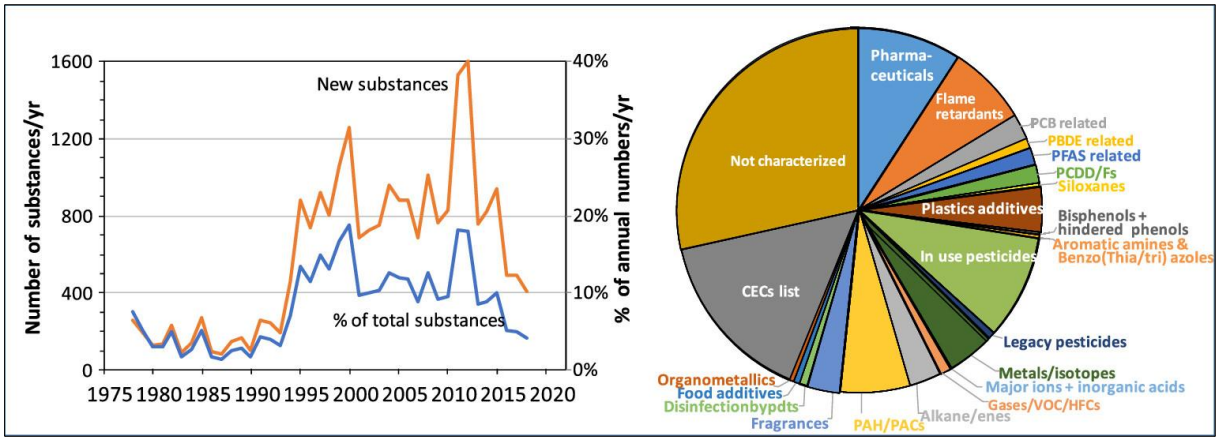

Figure Int. 2. Annual number and classification of newly reported chemical substances. Left figure: the number of new substances reported per year (orange line, left axis) and their percentage relative to total reported substances (blue line, right axis) from 1975 to 2020. Right chart: represents the distribution of newly identified substances according to their chemical classes, including pharmaceuticals, flame retardants, plastic additives, pesticides, and others as shown in the right chart (Muir et al., 2023).

2.2. Current CSA Framework

In regulatory toxicology, assessing chemical safety represents an important step in protecting human health and the environment from the harmful effects of chemicals and other substances (Brescia et al., 2023; Jacobs et al., 2024).

CSA is a structured process used in regulatory toxicology that provides an essential framework, based on different scientific evidence, to evaluate whether a chemical can be manufactured, imported, or used (Hasegawa et al., 2004). The CSA framework evaluates the potential adverse effects of chemicals on human health and the environment; hence, it guides regulatory actions and decisions regarding the safe use of chemicals in consumer products, industrial applications, pharmaceuticals, agriculture, and other sectors (Gebel et al., 2009). The Chemical Safety Report (CSR) that documents the CSA must be prepared above a certain threshold and under regulations such as the European Registration, Evaluation, Authorisation and Restriction of Chemicals (EU REACH) system to evaluate chemicals (Rudén & Hansson, 2010).

The scientific evidence of the CSA relies on structured principles of risk determination divided into four interconnected steps as shown in Figure Int. 3: 1) Hazard identification, 2) Hazard characterization, 3) Exposure Assessments, 4) Risk characterization (Sulsky et al., 2024).

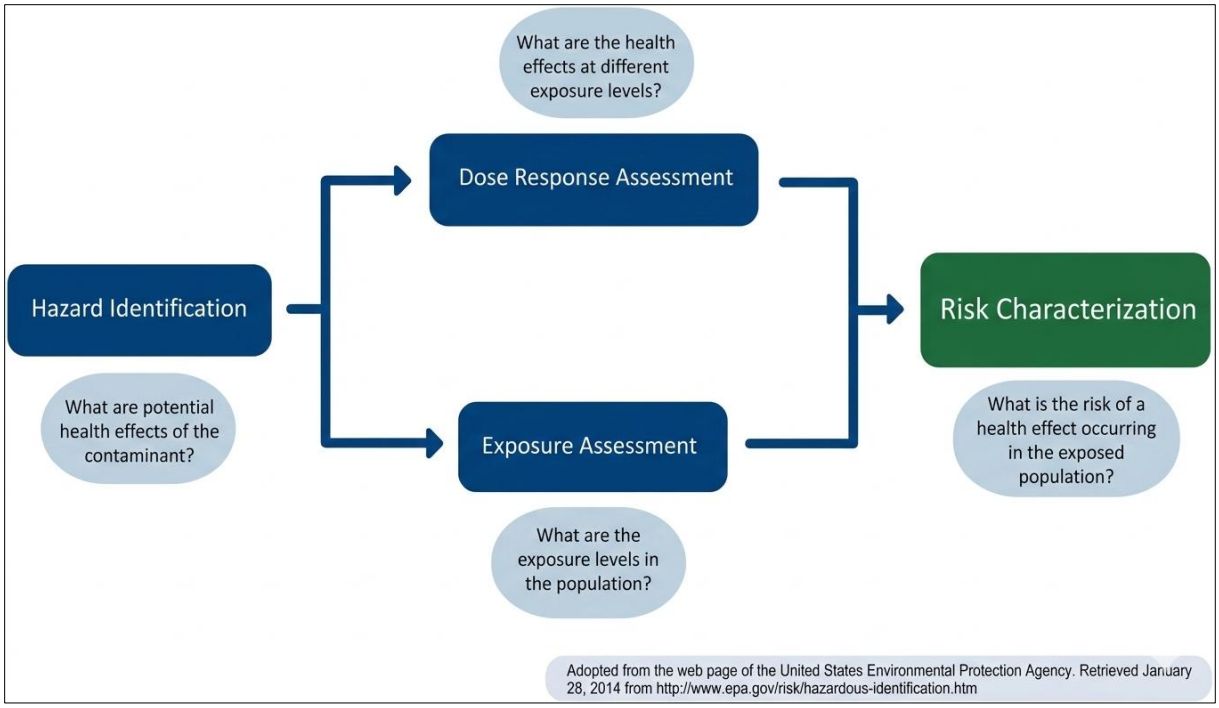

Figure Int. 3. The process of chemical risk assessment: Hazard identification, dose-response assessment (hazard characterization), exposure assessment, and risk characterization. (Food Packaging Forum, 2014).

2.2.1. Hazard identification

Hazard identification is the primary qualitative phase in evaluating the toxicological risks associated with chemical substances. This process integrates data from toxicological and epidemiological studies to characterize the specific nature of harm a chemical may induce (Gebel et al., 2009). The identification framework encompasses acute toxicity tests, which assess immediate effects following a single exposure (Qu et al., 2017), as well as sub-chronic and chronic tests that investigate the systemic consequences and organ damage resulting from prolonged or repeated exposure (Viluksela et al., 1997). Furthermore, evaluating potential carcinogenicity, genotoxicity, and reproductive or developmental toxicity is essential for establishing a comprehensive toxicity profile (Garralaga et al., 2022). This profile is further supported by critical data regarding toxicokinetics, structure-activity relationships, and the chemical's specific mode of action (Terry et al., 2016). While toxicological evaluations have historically relied on animal models as outlined in the OECD guidelines shown in Table Int. 1 recent scientific advancements have catalysed a significant transition. Current trends in toxicity profiling are shifting away from traditional *in vivo* studies toward innovative *in vitro* methodologies (EPA, 2017; Reddy et al., 2023), which will be discussed in further detail later in this chapter.

Table Int. 1. Regulatory test methods used for hazard identification in CSA.
This table compiles the principal toxicity evaluation assays currently accepted within regulatory frameworks, with reference to the corresponding OECD Test Guidelines or guidance.

Toxicological evaluations	Methods	Assay/Tools	Guideline document
Acute toxicity	*In vitro*	Cytotoxicity assays in cell lines	OECD TG 129
	In vivo	Oral, dermal, inhalation, LC_{50} testing in rodents	OECD TG 404, 403,420,423,425
Genotoxicity/Mutagenicity	*In vitro*	Ames test, micronucleus assay, chromosomal aberration	OECD TG 471, 487, 473
	In vivo	Micronucleus test, comet assay, transgenic rodent mutation	OECD TG 474, 488, 489
Carcinogenicity	*In vivo*	2-years rodent bioassay (rat/mouse)	OECD TG 451, 453
Repeated-dose toxicity	*In vivo*	28- or 90-day Oral, dermal, inhalation in rodents	OECD TG 407, 408, 410
Reproductive toxicity	*In vivo*	Two generation or extended one-generations studies in rats	OECD TG 416, 443
Developmental toxicity	*In vivo*	Parental developmental studies in rats or rabbits	OECD TG 414
Neurotoxicity	*In vivo*	Behavioral tests, neuropathology in rodents	OECD TG 424, 426
Toxicokinetic	*In vivo*	Blood, and tissue sampling in rodents	OECD TG 417
Skin/Eye Irritation & Corrosion	*In vivo*	Rabbit, and skin eye tests *(Restricted use)*	OECD TG 405
Sensitization	*In vivo*	Local Lymph Node Assay (LLNA), Guinea pig Maximization Test	OECD TG 406, 429

2.2.2. Dose-response assessment

The relationship between the magnitude of an identified hazard and its exposure intensity provides a critical quantitative description of the health risks associated with a chemical. This stage of the CSA, often referred to as Hazard Characterization, includes dose-response assessment to identify the thresholds below which exposure is unlikely to cause harm (Baert et al., 2009; Gebel et al., 2009).

In this phase, threshold values are utilized to rank the toxicological potency of substances. These values include: 1) the No Observed Adverse Effect Level (NOAEL), defined as the highest exposure level at which no statistically or biologically significant increase in the frequency or severity of adverse effects occurs; 2) the Lowest Observed Adverse Effect Level (LOAEL), which is the lowest tested dose where adverse effects are observed; and 3) Benchmark Dose (BMD) modeling, which identifies the dose causing a specific low-level change in a biological system (Faroon et al., 2017; Pouzou et al., 2020).

This potency evaluation integrates available toxicity data with mathematical modeling to establish safe exposure margins. For instance, linear or non-linear extrapolation models are employed to estimate low-dose risks for genotoxic and carcinogenic compounds, such as lifetime cancer risk from chronic exposure (Mutti, 2023). For non-genotoxic substances, NOAEL or LOAEL values derived from animal studies are used to establish health-based guidance levels, such as the Acceptable Daily Intake (ADI) or Reference Dose (RfD) (Renwick, 1999; Slikker et al., 2004).

These determined threshold values, generally referred to as the Point of Departure (POD), rely heavily on toxicological or epidemiological data. While BMD modeling is increasingly used to determine the POD for characterized health risks (Davis et al., 2011; Mendez et al., 2020), it is important to note that the selection of specific mathematical models can introduce uncertainty into the calculated POD values (Lu et al., 2025).

2.2.3. Exposure assessment

Exposure assessment involves quantifying the chemical intensity, source, frequency, duration, and its route through which human exposed to. This process in chemical safety assessment aims to provide an estimation of the actual and potential intake exposure via different routes such as environmental, occupational, internal, dietary (oral), or other pathways. As well, in addition to

the data provided by *in vivo* and epidemiological studies, this step requires predictive mathematical models or tools that allow the estimation of the amount of the substances absorbed into the body. This requires the integration of different data such as environmental monitoring data, and behavioral patterns, and the variability in exposure between populations to accounts the cumulative or aggregative effect of the exposure (Gebel et al., 2009).

2.2.4. Risk characterization

This step represents the concluding phase in the identification of the risk associated with the chemicals being assessed. Findings from hazard identification, dose-response evaluations, and exposure route estimations are synthesized to quantify the potential severity of the adverse health effects expected from exposure to a certain substance in a targeted population. This stage delivers a comprehensive overview of the potential health risk, incorporating both qualitative and quantitative data analysis (Gebel et al., 2009).

2.3. CSA framework challenges

Over the past five decades, regulatory framework guide and efforts have been focused on enabling the safe use of chemicals with a minimized risk to human health and the environment, and modern the risk assessment (Birnbaum et al., 2016). These efforts have aimed to identify gaps between conventional and emerging methodologies used in chemical risk evaluation, to move away from reliance on apical endpoints in animal toxicology studies, and to integrate new scientific approaches with high human relevance (Shao et al., 2024).

Despite advances made in recent years to restrict the use of animals in risk assessment, the current CSA still relies heavily on animal-based studies and epidemiological evidence (Worth & Berggren, 2025). The complete replacement of animal testing remains out of reach. As to date the progress achieved has been limited to the cosmetics sector (Walder et al., 2025).

However, validated non-animal alternative methods are still lacking for many toxicological applications, including systemic toxicology, chronic exposure, metabolism, and complex models capable of replacing full organ interactions. Furthermore, regulatory acceptance remains challenging (Juhasz et al., 2013).

Therefore, while regulatory frameworks have made strong efforts toward modern approaches to chemical safety, animal studies are still considered the regulatory backbone for evaluating

risks from new chemicals, pesticides, pharmaceuticals, consumer products, vaccines, and other substances (Chang & Grieder, 2024).

As outlined in a statistical report on the use of animals for scientific purposes in the Member States of the European Union (EU) and Norway during 2022 under Directive 2010/63/EU ("the Directive"), the total number of animals used in 2022 for research purposes, including regulatory activities, remains high. The report indicates that current methods used in CSA and regulatory frameworks still rely heavily on animal testing (Marinou & Dontas, 2023) (Figure Int. 4).

The structured CSA framework, which relies primarily on data from animal tests and human studies, is applied to evaluate a wide range of substance-related risks. For example, hazard identification within carcinogenicity assessment depends on human data on tumour incidence in exposed populations, in addition to animal data. However, such information is available for only a small percentage of chemicals and typically covers only specific types of tumour responses (Felter et al., 2021). This limitation applies not only to carcinogenicity but also to other toxicity endpoints. Moreover, such studies require long-term exposure in human populations before carcinogenic effects can be observed. Consequently, the characterization of carcinogenic potential still relies predominantly on 2-year animal bioassays conducted in rats and/or mice in accordance with OECD guidelines (Suarez-Torres et al., 2021).

Animal studies of shorter period (less than 2-years) have also been used in carcinogenic hazard characterization, including studies with transgenic rodents (e.g., *rasH2* or *p53* models), toxicogenomic analyses (mechanistic changes in gene expression), and assays detecting carcinogenic foci (lesions in target organs or tissues). However, these approaches still lack the regulatory acceptance (Storer et al., 2010; Furihata & Suzuki, 2023).

In addition to carcinogenicity assessment, genotoxicity evaluation is a critical component of CSA, given the role of genotoxic insults in cancer initiation. This evaluation integrates data from epidemiological studies, animal studies, and *in vitro* assays, some of which have achieved regulatory acceptance (Table Int. 1). Nevertheless, positive results in such tests typically trigger follow-up *in vivo* studies in animal models to assess the carcinogenic potential of the substance. In such cases, *in vivo* studies are used to confirm or refute the *in vitro* findings (Committee et al., 2021). Furthermore, dose–response characterization for tumour formation in carcinogenicity assessment relies mainly on data from animal cancer bioassays (Clewell et al., 2019). Exposure assessment, in turn, depends primarily on human exposure data.

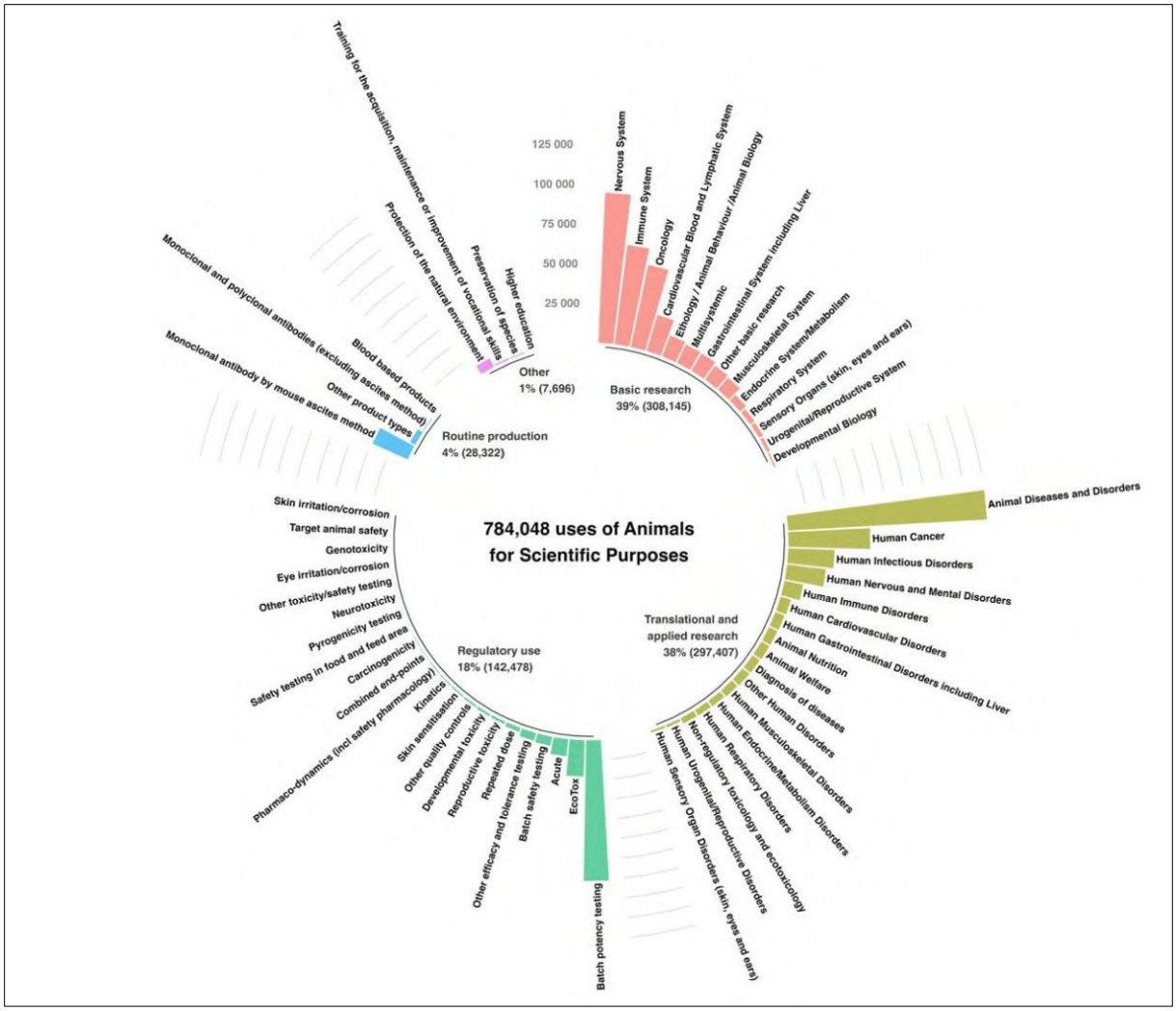

Figure Int. 4. Distribution of animal use for research and testing in 2022 across research sectors in the European Union. The figure illustrates the total of 784,048 uses of animals for scientific purposes in 2022, categorized by main research purpose. Basic research and translational and applied research represent the largest proportions, accounting for 39% and 38% of total animal use, respectively. Within these categories, human cancer research and oncology were among the fields with the highest numbers of animals used. Data are taken directly from the publicly available Summary Report on the Statistics on the Use of Animals for Scientific Purposes in the Member States of the European Union and Norway in 2022, published under Directive 2010/63/EU.

2.4. Limitations of animal-based CSA

Animal-based studies in toxicology and biomedical research face several fundamental limitations (Carusi, 2024) (Table Int. 2). Consequently, many drugs that show promise in animal models fail in human trials; only 10% of successful animal studies lead to clinical applications (Marshall et al., 2023).

Numerous studies have demonstrated poor concordance between animal and human data. For instance, a compound deemed safe in rodents might produce adverse effects in humans, and vice versa. This is particularly evident when investigating the toxic effects of chemicals or drugs

in rodents, which are often poor predictors of human toxicity (Atkins et al., 2020). Because of these physiological discrepancies, the risk of adverse effects in humans cannot be accurately predicted solely from animal studies.

This limitation also extends to carcinogenicity. Kobets et al. (2022) reported that exposure to acrylonitrile induced tumors in three different tissues as the forestomach, Zymbal's gland, and Harderian gland in rodent bioassays. However, the relevance of these tumors to humans is questionable, as extensive epidemiological data have not demonstrated a causal link between acrylonitrile and human cancer. This study highlights the complexity of assessing carcinogenic effects and emphasizes the need to integrate mechanistic data to inform regulatory decisions.

In addition to ethical concerns regarding the high number of animals required for traditional *in vivo* studies (Kiani et al., 2022) and the significant time investment involved, a critical shortcoming of animal testing is its limited translatability to human biology (Browne et al., 2024). Interspecies differences in physiology, metabolism, cellular and molecular responses represent significant challenges in accurately predicting chemical effects on humans (Clewell & Fuchsman, 2024).

Furthermore, many animal assays lack mechanistic insight and are not designed to reflect real-world exposure scenarios, such as low-dose exposure or the combined effects of multiple chemical exposures (Boberg et al., 2019). Consequently, despite the valuable data these studies may provide, substantial knowledge gaps remain.

On the other hand, animal studies provide a global understanding of various biological processes. Assessing chemical-associated risk within a complete biological system (the whole animal) provides insights into fundamental processes and disease mechanisms. This allows researchers to explore the complex relationships between genetics, physiology, cellular cross-talk, and disease progression (Barré-Sinoussi & Montagutelli, 2015).

However, the scientific, ethical, and predictive limitations of animal studies-upon which the current CSA framework relies heavily-have intensified the need for a paradigm shift toward New Approach Methodologies (NAMs) within the context of Next-Generation Risk Assessment (NGRA) (Knight, 2008; Frühwein & Paul, 2025).

Table Int. 2. Overview of the main limitations associated with the use of animal models in CSA.
This table presents the principal scientific and methodological limitations of animal-based approaches, providing for each limitation a concise description and an illustrative example of its functional consequences for risk assessment. The aim is to highlight how these constraints can affect the reliability, mechanistic relevance, and real-world applicability of CSA outcomes.

Limitation	Description	Example
Physiological differences	Differences in body and organs size. Microscopic anatomy of different organs. Metabolism differences. Differences in physiological parameters.	Differences in the anatomy of the respiratory tract and the localization of different enzymes in the nasal passages of rats could impact the metabolism of different inhaled chemicals. This may impact the ability of studies using rats to predict effects of chemicals on the respiratory system in humans (Stucki et al., 2024). Blood flow rates, organ sizes relative to the body mass, protein binding and drug detoxification rate could induce significant differences in chemical toxicity (Martinez et al., 2021).
Metabolism (toxicokinetic (TK)) differences	Differences in drug absorption, distribution, metabolism, and elimination, potentially resulting in different toxic responses. Metabolic activation or detoxification pathways may vary significantly.	Differences in cytochrome P450 (CYP) enzyme isoforms, that impact the activity of the enzymes such as CYP1A, -2C, -2D and -3A, thus impact the metabolism rate in different animal species compared to humans (Martignoni et al., 2006). Differences in phase II enzymes, for example Bisphenol A glucuronidation shows a much higher V_{max} in rat liver and intestine than humans (Godin et al., 2006). Species-specific differences in Aryl Hydrocarbon Receptor (AhR) ligand binding and gene activation, and AhR molecular structure between rats, mice, and humans that leads to differences in the downstream gene expression (Wilson & Bradfield, 2021). Differences in CAR/PXR activation that are responsible for CYPs expression such as (CYP2B, CYP3A, etc.), that affects how the drug is metabolized (Hammer et al., 2021).

Challenges in extrapolation	Limited transposability of the adverse effect of chemicals between rodents and humans.	Challenges in translating high-dose animal study outcomes to predict low-dose real-life human exposures (Chen et al., 2024). Use of a default 10-fold interspecies uncertainty factor (UF) and body surface area scaling for dose conversion. This default is conservative and sometimes insufficient or over-protective depending on the chemical and available TK/TD data (EPA 2014).
Adverse effect relevance	Species-specific effects that may mask or overestimate the human risk.	The differences between rodent species, strains, and genders (Lee et al., 2015). Further differences between rodents and human (Habert et al., 2014; Foreman et al., 2021).
Ethical concerns	High number of animals, as well as the animal-based tests themselves used in assessing chemical toxicity raises the moral question of animal welfare concerns.	Animal testing often involves pain, distress, and death (Persson et al., 2025). In addition, the high number of animals used from different sexes in each experiment (Kiani et al., 2022).
Scientific and practical	Relevance, reproducibility, cost, and time challenges.	Issues including limited statistical power due to small group sizes and low reproducibility of 60%-70% (Gottmann et al., 2001). In addition to the high-cost experimental work and low throughput.

3. Revolutionizing the chemical risk assessments

3.1. Regulatory decisions towards the phase-out of the use of experimental animals

Over the past several decades, both the European Union (EU) and the United States of America (USA) has taken steps towards reducing and ultimately replacing the use of animals in CSA. These efforts reflect a growing awareness of the ethical concerns and scientific limitations associated with the traditional animal-based studies.

The European Union implemented a phased approach to eliminating animal testing for cosmetics, with deadlines differentiated according to product type and the complexity of the toxicological endpoint. Under the Cosmetics Directive 76/768/EEC, animal testing of finished cosmetic products was banned in 2004, followed by a 2009 ban on testing cosmetic ingredients for endpoints with validated non-animal alternatives, such as skin and eye irritation. More complex systemic endpoints such as repeated-dose toxicity, reproductive toxicity, and toxicokinetics that lacked validated alternatives at that time, leading to a postponed marketing ban until 2013. These staggered deadlines were later consolidated into the Cosmetics Regulation No 1223/2009, which now serves as the governing legal framework. This stepwise regulatory strategy balanced scientific feasibility, consumer safety, and ethical considerations, driving the development of alternative methods while ensuring a controlled and progressive phase-out of animal testing in the EU cosmetics sector.

In alignment with this, the European Union's REACH (Registration, Evaluation, Authorization, and Restriction of Chemical Substances), EC No. 1907/2006) strongly promotes the use of alternative methods for CSA. Similarly, the U.S. Environmental Protection Agency (EPA) has outlined plans in 2019 to phase out all animal testing, aiming in fully transition to alternative approaches for chemical and pesticide testing by 2035.

The table below outlines the different contribution adopted by both the EU and the USA from early animal welfare to the modern regulations targeting restrictions on animal use and the adoption of NAMs (Table Int. 3).

Table Int. 3. The European and American decisions regarding reducing traditional animal testing in CSA.
Each regulatory decision is presented with its beginning year, its key contribution, and the official link of each regulatory program.

Regulation	Year	Key Contribution	Official Link
EU			
Directive (86/609/EEC)	1986	First EU directive on animal protection in scientific research	Directive 86/609
Cosmetics directive (76/768/EEC) (7th Amendment 2003/15/EC)	2003	Introduced a phased ban on animal testing for cosmetics: testing ban on finished products (2004); testing ban on ingredients and marketing ban for endpoints with validated alternatives (2009); postponed marketing ban for complex systemic endpoints (repeated-dose toxicity, reproductive toxicity, toxicokinetics) until 2013.	Directive 76/768
REACH Regulation (EC No. 1907/2006)	2006	Promotes use of non-animal methods in chemical safety	REACH 1907/2006
Cosmetics Regulation (EC No 1223/2009)	2009	Fully replaced Directive 76/768/EEC; consolidated and maintained the animal-testing and marketing bans, including the final 2013 marketing ban for complex endpoints	Cosmetic regulation 1223/2009
Directive (2010/63/EU)	2010	Replaces 86/609/EEC, strengthens 3Rs and transparency	Directive 2010/63
EURL ECVAM Framework	ongoing	Supports validation and promotion of NAMs	ECVAM

USA			
Federal Food, Drug, and Cosmetic Act (FFDCA)	1938	Foundation of US drug and cosmetic safety law	Federal Food, Drug, and Cosmetic Act (FD&C Act) | FDA
Toxic Substances Control Act (TSCA)	1976	Governs industrial chemical safety	Summary of the Toxic Substances Control Act | US EPA
ICCVAM Authorization Act	2000	Requires agencies to consider ICCVAM methods	ICCVAM
TSCA Reform	2016	Mandates reducing vertebrate testing	The Frank R. Lautenberg Chemical Safety for the 21st Century Act | US EPA
EPA 2035 Elimination Plan	2019	Goal to end mammalian testing by 2035	US EPA
Modernization of Cosmetics Regulation Act (MoCRA)	2022	Updates cosmetics law, encourages NAMs	Modernization of Cosmetics Regulation Act of 2022 (MoCRA) | FDA

3.2. Next Generation Risk Assessment

Next Generation Risk Assessment (NGRA) is a modern hypothesis-based approach that relies on the integration of NAMs to assess chemical associated risk without the use of animal testing' (Schmeisser et al., 2023; Leso et al., 2025).

NAMs encompass a wide range of technologies, methodologies or approaches that can be used as alternatives to the animal testing methods in CSA, including *in silico*, *in chemico* assays, and *in vitro* biological models (Figure Int. 5) (Wambaugh et al., 2019; Schmeisser et al., 2023). Whether applied individually or in combination, these approaches aim to improve the characterization of chemical risk by relying more on human-relevant methods, ultimately enhancing the accuracy and relevance of CSA (Hoogstraaten et al., 2025).

The methodologies adapted and integrated within NAMs, known as *in vitro* alternatives, represent a diverse array of approaches including:

- *ex vivo* and *in vitro* assays with varying levels of complexity, from 2D cell cultures to tissue models and advanced systems like organ-on-chip (Nair & Weiskirchen, 2024).
- Computational tools, modeling techniques, and machine learning applications (Deng et al., 2023).
- High-throughput and High-content Screening/Analysis (HCS/HCA) data platforms (Harrill et al., 2019; Bundy et al., 2024).

The advancement and application of NAMs, whether in chemical prioritization, hazard screening, or risk assessment, are strongly aligned with the 3R's principle- aiming to replace, reduce, and refine the use of animals in traditional toxicological studies (Tannenbaum & Bennett, 2015; Sheng et al., 2025).

However, NAMs extend beyond simple *in vitro* alternatives; they can be combined with selected *in vivo* assays and clinical observations to strengthen toxicological evaluations. For example, integrating high-content omics analyses into short-term rodent studies enables the identification of perturbed molecular pathways, providing mechanistic insight while simultaneously reducing overall animal use and study duration. Such integrative applications of NAMs within CSA frameworks enhance both the mechanistic relevance and the predictive performance of hazard and risk evaluations (LaRocca et al., 2020; Lewis et al., 2020; Mitchell et al., 2025).

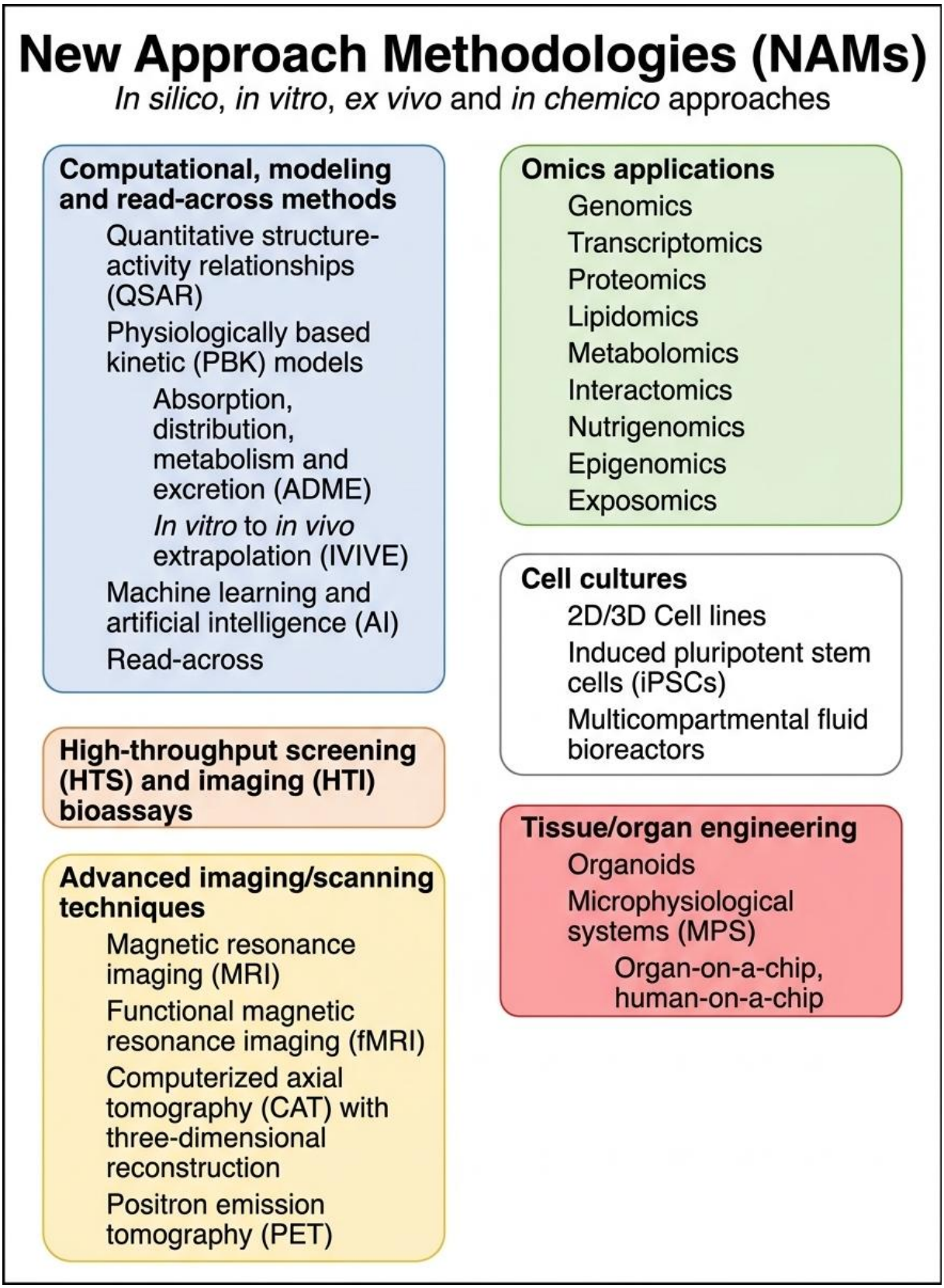

Figure Int. 5. Key methodological components and tools incorporated within the NAMs framework. The figure illustrates the principal experimental, and computational approaches that constitute NAM-based toxicological assessment (Schmeisser et al., 2023).

3.3. NAMs provide mechanistic evidence for toxicological endpoints

Unlike the traditional animal studies in regulatory toxicology, NAMs offer mechanistic evidence supporting the likelihood of an adverse outcome (Saarimäki et al., 2023). The integration of such mechanistic evidence through the Adverse Outcome Pathways (AOPs) with NAMs has accelerated the shift from descriptive toxicology toward a more predictive, and mechanistic-based science (Nymark et al., 2021).

The AOP framework provides structured, scientific-based, and computational approaches that can link the stressor (e.g., chemicals, viruses, or others) to an adverse outcome in an intact organism by outlining a sequence of key biological events that begins with a Molecular Initiating Event (MIE). As shown in Figure Int. 6, AOPs describe toxicity both as a single linear mechanism and as part of a broader interconnected network: Panel (A) presents a straightforward AOP beginning with a stressor such as SARS-CoV-2, which triggers a Molecular Initiating Event (ACE2 binding), leading through measurable Key Events (KEs) at the cellular level (e.g., IL-6 secretion), then tissue-level inflammation, and ultimately a relevant Adverse Outcome like acute respiratory distress; each arrow represents a KE Relationship showing how one biological change causally leads to the next. It's also illustrating the fact that multiple pathways can share the same KEs, branch into different mechanistic routes, or converge on common adverse outcomes as shown in panel (B). Hence, forming an AOP network that better reflects the complexity of real biological systems and helps scientists interpret diverse data streams and predict toxicity more accurately *in vitro*.

The identification of one or several KE in the biological pathway represents the building blocks of the AOP (Svingen et al., 2021). Non-animal methods or strategies supported by NAMs such as *in vitro*, *in chemico*, and *in silico*, are suited towards the different AOP KE of a biological pathway. Together, they can answer the adverse effect of a specific stressor.

A significant advancement during this period is the maturation of Quantitative Adverse Outcome Pathways (qAOPs), which transition the framework from qualitative descriptions toward predictive mathematical models. By leveraging systems biology and Bayesian approaches, qAOPs define the functional relationships between KEs, enabling the derivation of PoD directly from *in vitro* or *in silico* data (Jeong et al., 2025).

Building on this foundation, recent literature has introduced Ordinary Differential Equation (ODE)-based qAOP frameworks. Unlike static models, these explicitly capture the temporal dynamics of KE activation and incorporate biologically relevant feedback loops, offering a

more realistic representation of how molecular perturbations propagate through a biological system (Di Tillio & Beltman, 2024).

These dynamic models do more than just strengthen causal inference; they facilitate the exploration of population variability. By linking individual parameter differences such as metabolic rates or receptor sensitivity, to variations in the severity of an Adverse Outcome, these frameworks provide a robust tool for personalized risk assessment. Ultimately, these advances significantly enhance the predictive power and regulatory acceptance of NAM-derived evidence by providing a rigorous, time-resolved mechanistic narrative (Di Tillio & Beltman, 2024).

By organizing biological processes from the MIE to the final Adverse Outcome, AOPs provide the essential framework that allows high-throughput assays, "omics" platforms, and other NAM technologies to be translated into mechanistically-based, regulatory-relevant endpoints. This shift reduces reliance on animal testing while simultaneously improving human relevance. Together, these advances strengthen the weight of toxicological evidence, enhancing regulatory confidence and supporting faster, more accurate CSA.

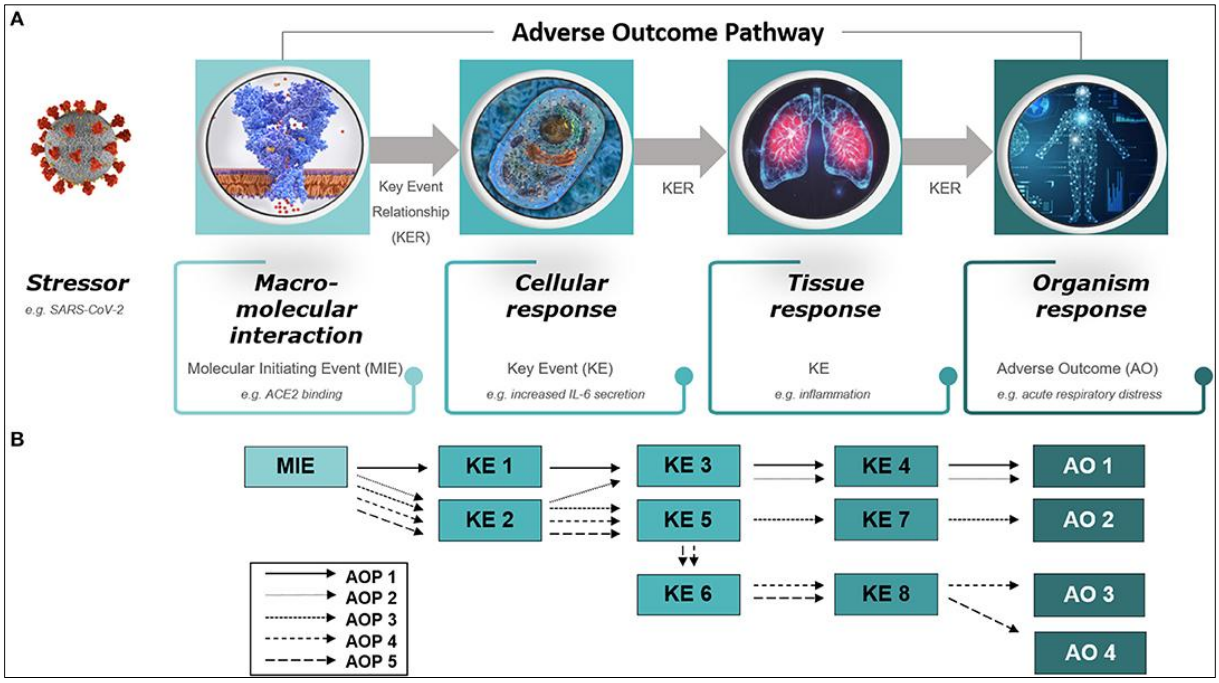

Figure Int. 6. Structural organisation of an AOP. (A) The AOP starts with a MIE followed by a series of KE at different biological levels. The diagram provides a virus as an example of a stressor. (B) Structure of an AOP network (Nymark et al., 2021).

3.4. The Role of NAMs in toxicological Evaluation and safety assessment

The integration of NAMs within CSA has accelerated significantly through the continuous development of innovative testing frameworks (Sewell et al., 2024). Recent progress highlights a shifting landscape where non-animal approaches are increasingly prioritized across various sectors (Reddy et al., 2023). A diverse range of *in silico* and *in vitro* methods, structured by the AOP framework, are being progressively incorporated into regulatory safety architectures. While this transition is occurring more gradually in some sectors compared to others, major regulatory bodies such as the U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) are actively implementing these modern risk assessment tools, particularly in the field of food safety (Reddy et al., 2023a).

The relatively slow incorporation of NAMs in certain areas is primarily attributed to a historical lack of validated and standardized models for systemic toxicity. However, there is a burgeoning interest in the validation of integrated systems that combine *in vitro* assays with computational modeling. Weight-of-Evidence (WoE) approaches and Integrated Testing Strategies (ITS) are increasingly leveraging these data to support robust hazard identification and risk assessment (Brescia et al., 2023).

Over the past two decades, NAMs have achieved notable success in evaluating specific endpoints, such as skin sensitization, an area largely spearheaded by the cosmetics industry (Table Int. 4). Despite these successes, many methods remain in the OECD validation pipeline. A persistent challenge lies in the interpretation of *in vitro* data to predict *in vivo* outcomes, as the inherent biological complexity of a whole organism is difficult to replicate in cell culture. Consequently, relying on a single *in vitro* model to predict complex adverse effects remains a significant limitation (Han et al., 2024).

To address these complexities, comprehensive frameworks have been developed to evaluate ingredient safety without animal testing, particularly within the cosmetics sector (Baltazar et al., 2020) (Figure Int. 7). These assessments typically begin with exposure estimations based on consumer practice data (Hall et al., 2007). These exposure parameters then serve as inputs for Physiologically Based Kinetic (PBK) models, which predict internal plasma concentrations resulting from realistic exposure scenarios (Baltazar et al., 2020). To identify potential biological targets, a battery of *in silico* and *in vitro* NAMs is employed, including receptor binding assays, high-throughput transcriptomics, cell stress panels, and genotoxicity-specific tools like the ToxTracker assay. These assays generate concentration-response data used to

determine the PoD, identifying the lowest concentration at which adverse biological activity is observed.

This integrated approach incorporates transparent documentation of uncertainties, such as *in vitro* metabolic limitations and the challenges of *in vitro-to-in vivo* extrapolation (IVIVE). Such case studies exemplify how the combination of exposure modeling, mechanistic insights, and a WoE approach can facilitate a fully integrated NGRA strategy. By providing human-relevant toxicity data for chemical safety decisions, these strategies align with evolving regulatory requirements and ethical standards (Barguilla et al., 2023).

This paradigm shift is further bolstered by policy initiatives that promote the use of NAMs, driven by both the ethical imperative to replace animal testing and the practical need for faster, more accurate safety evaluations.

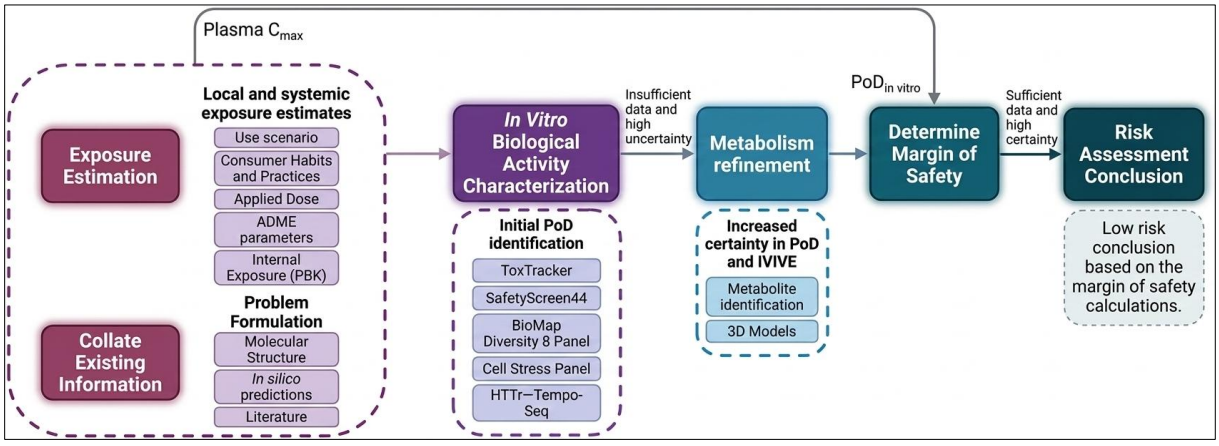

Figure Int. 7. The established NGRA framework for the safety evaluation of cosmetic consumer products. The workflow illustrates an integrated, non-animal approach to safety assessment as described by Baltazar et al. (2020). The process begins with Problem Formulation and Exposure Estimation, utilizing *in silico* tools and consumer habit data to determine internal exposure (PBK modeling). *In Vitro* biological activity characterization follows, employing a suite of assays (e.g., ToxTracker, BioMap, HTTr) to identify the initial PoD *in vitro* where high uncertainty or insufficient data exists, a metabolism refinement tier is triggered using 3D models and metabolite identification to increase certainty in IVIVE. The final risk assessment conclusion is derived by determining the Margin of Safety (MoS), comparing the predicted human plasma C_max against the PoD *in vitro*.

Table Int. 4. Overview of some OECD-validated guidelines/or guidance and predictive toxicological tools that supports the non-animal methodologies.
This table summarizes currently available *in vitro* assays modeling frameworks integrated into the OECD work plan. Highlighting the transition from traditional *in vivo* protocols to more mechanistic-based risk assessments.

Toxicological evaluations	Methods	Example of assay/Tools	OECD document
Genotoxicity	*In vitro*	genotoxicity batteries	OECD TG 471, 476, 490, 473
Carcinogenicity	*In vitro*	Cell transformation assays	OECD TG 214,231,238
Reproductive toxicity	*In vitro*	Sertoli cell function, aromatase inhibition, and leyding cell assay	OECD TG 456
Developmental Toxicity	*In vitro*	Embryonic stem cell test (EST), Zebrafish embryo test	OECD TG 236
Endocrine disruption	*In vitro*	Estrogen/androgen receptor binding and transactivation assays	OECD TG 455,457,458
Neurotoxicity	*In vitro*	Neuronal differentiation	OECD TG 377
Toxicokinetic	*In vitro*	Liver microsomes, hepatocytes and intestinal cell models	OECD TG 331
Eye irritation & Corrosion	*In vitro*	Reconstructed human epidermis (RhE) test	OECD TG 467, 431
Skin sensitization	*In vitro*	Keratinases, *in chemico* skin sensitization, *in vitro* skin sensitization	OECD TG 442D, 442C, 442E

3.5. Implementation and adoption of NAMs in carcinogenesity assessment

The integration of NAMs into CSA holds strong promise as human-relevant technologies advance, yet several persistent challenges continue to limit their full regulatory uptake (Oyetade et al., 2025). Many NAMs still lack the level of validation needed to demonstrate reliability and relevance for specific toxicological endpoints, and numerous *in vitro* systems do not yet fully replicate the complexity of *in vivo* physiology, making data translation difficult and reducing predictive confidence (Juhasz et al., 2013; Zagorski & Kaminski, 2023). Beyond these technical constraints, harmonized and validated guidelines for conducting and interpreting *in vitro* assays remain insufficient, and accurate *in vivo* predictions depend on standardized frameworks and robust validation datasets (González-García et al., 2015). Continued efforts are therefore required to standardize protocols and expand high-quality databases, particularly for challenging areas such as carcinogenicity (Sewell et al., 2024). To address these gaps, multiple standardization and validation initiatives are underway, including updates to OECD test guidelines, expanded data-sharing efforts, the development of comprehensive NAMs databases, coordinated European programs such as PARC, and strategic work plans from agencies like the U.S. EPA aimed at supporting regulatory acceptance (Schmeisser et al., 2023).

Although progress is gradual, regulatory integration of NAMs for carcinogenicity is advancing, reflecting the importance of assessing carcinogenic potential that represent a critical component of chemical safety evaluation (Cohen et al., 2019). Consequently, international initiatives, are strengthening the scientific foundation needed for regulatory use (Table Int. 5). As a result, demand continues to grow for non-animal approaches capable of identifying and characterizing substances with carcinogenic potential in humans (Jacobs et al., 2020).

Table Int. 5. Table summarizing the programs that support the integration of NAMs in carcinogenicity chemical safety assessment.

Program	Scope	NAM integration strategy
IATA/OECD	International framework for mechanistic, pathway-based toxicity assessment.	Integrates NAMs within AOP-based frameworks, combining *in vitro* data, read-across, and exposure modeling.
ToxCast / Tox21	High-throughput chemical screening to characterize biological activity profiles.	Uses large-scale *in vitro* assay batteries to map molecular targets and pathways linked to carcinogenesis.
EU PARC	European Partnership for the Assessment of Risks from Chemicals (advancing NGRA).	Promotes regulatory implementation of advanced NAMs targeting non-genotoxic carcinogenicity and mixture toxicity through human-relevant models.
EURION Cluster	EU research cluster developing innovative human-relevant toxicology tools.	Builds integrated NAM platforms combining omics, AI modeling, and advanced *in vitro* systems to detect endocrine disruption and non-genotoxic carcinogenic mechanisms.

4. Cancer

4.1. Cancer burden and multifactorial etiology

Cancer is a major public health concern, as its prevalence, incidence and mortality rates (e.g., age-standardised deaths per population) are increasing worldwide (Bray et al., 2018). According to the WHO's report, the global cancer burden is rising significantly worldwide, with an estimation 20 million new cancer cases and 9.7 million deaths reported in 2022. Based on these estimations, cancer cases are projected to increase by 77% to reach around 30 million new cancer diagnosis and 18.6 million deaths by 2050 (Luo & Smith, 2025). As illustrated in Figure Int. 8, based on estimates from the International Agency for Research on Cancer (IARC, 2024) a steady increase in both incidence and mortality, with projections indicating that cases will more than triple and deaths will nearly triple by 2050.

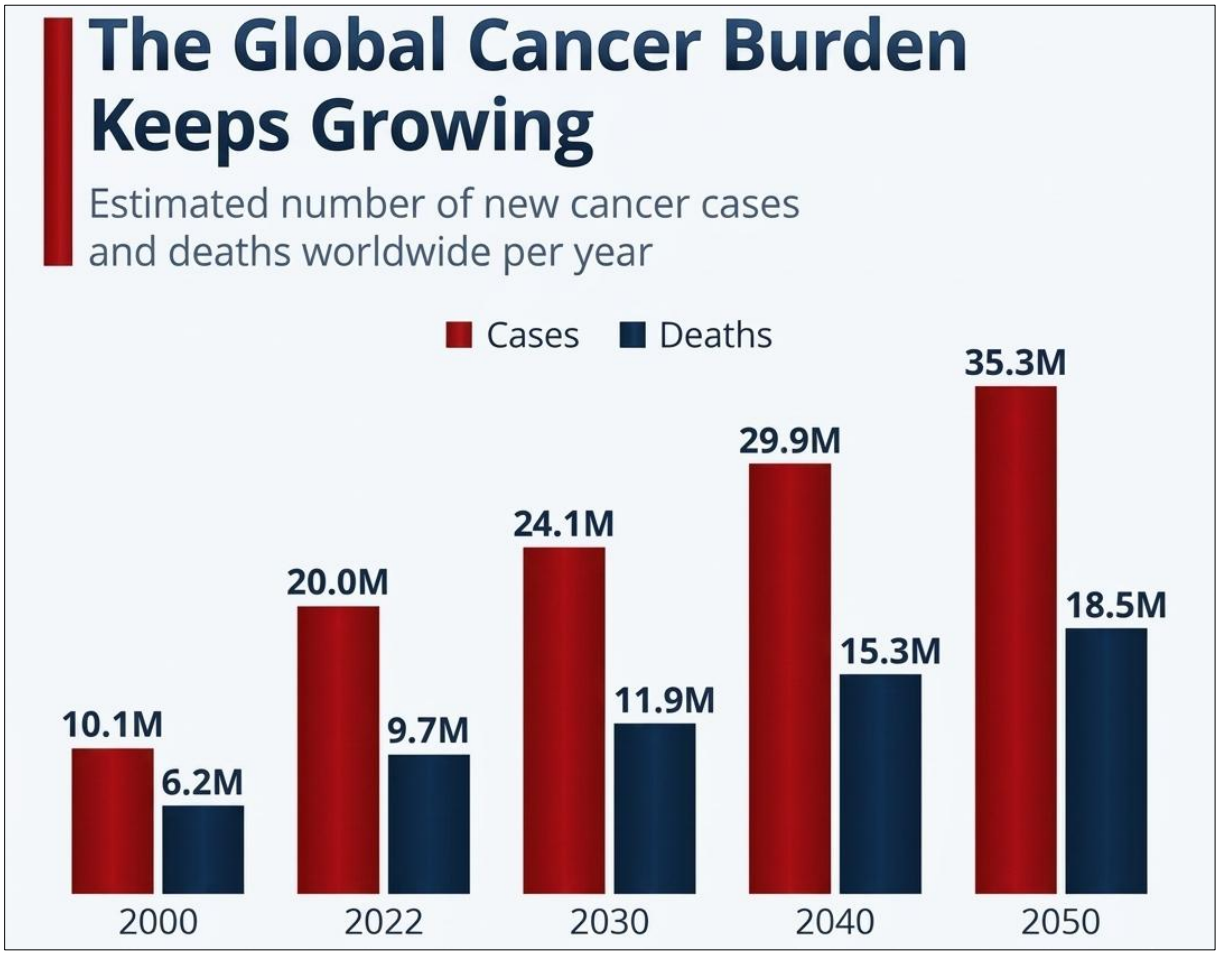

Figure Int. 8. The global cancer burden keeps growing worldwide. Estimated number of new cancer cases and deaths worldwide per year from 2000 to 2025 showing the estimated number of new cancer cases and deaths per year from 2000 to 2050. Data are based on estimates from IARC (2024) (Richter, 2025).

Cancer is a disease arising from the uncontrolled proliferation of transformed cells. It was described first by Hippocrates using the term ‘Karkinos’ defining it as the abnormal growth of a part of the body that is contrary to nature (Zheng et al., 2010; Hodgson et al., 2025).

To explain cancer development, it was hypothesized that gene mutations during uncontrolled and unchecked cell proliferation plays a key role (Sarkar et al., 2013). However, it was reported that these inherited mutations alone are not sufficient to induce cancer (Adjiri, 2017). Given that inherited mutations represent only about 5%-10% of all cancer cases (NCI, 2015). The remaining 90%-95% of cancer cases are largely attributed to other factors such as environmental exposure, dietary influences, and hormonal factors (Lynch et al., 1995). In addition, studies suggest that exposure to agents, which can alter the DNA, results in an initiated cell. These cells can later be further activated after the exposure of a non-mutagenic tumor-promoting agent, leading to tumor formation and cancer development. Given that, mutations are essential but not sufficient for cancer development (Balmain, 2020; Senft, 2024).

Cancer arises from a complex interplay between different endogenous and exogenous risk factors which disrupt normal cellular processes. Endogenous factors originate within the body including genetics (inherited genetic mutations), error in DNA replication, aging, hormonal imbalances, and others (Wu et al., 2018). On the other hand, exogenous risk factors involve external influences such as exposure to natural or anthropogenic environmental pollutants, lifestyle habits (e.g., tobacco use and alcohol consumption), and others (Silva et al., 2025). Recent epidemiological and experimental studies emphasize that both endogenous biological factors (genetics factors) and environmental exposures (particularly industrial pollutants) could represent a major global contributor to the rising cancer burden (Mbemi et al., 2020; Liu et al., 2024).

4.2. Hallmarks of cancer

The hallmarks of cancer is a concept that was originally outlined by Hanahan and Weinberg in 2000 to provide a clearer understanding of the complex, multistep process of tumor development (Hanahan & Weinberg, 2000). These hallmarks describe the functional capabilities required by normal cells that serve their ability to form malignant tumours. Thus, this concept provides a powerful, integrative framework to understand how normal cells evolve into malignant neoplasms and which mechanisms are implicated in the evolution of the malignancy (Hanahan, 2022). The recognition of this concept was driven by the recognition that human cancers emerge through the multistep process of carcinogenesis described earlier in

this chapter, thereby converting the complexity of cancerous cell phenotypes and genotypes into a provisional trait of underlying mechanisms (Hanahan & Weinberg, 2011).

Initially, six core hallmarks or biological capabilities were identified in tumour cells: sustaining proliferative singnaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Figure Int. 9). Each one of these six hallmarks represents a distinct capability core of the cells for tumour initiation and progression. However, over time, two additional hallmarks were recognized (Figure Int. 10, Left) and include dysregulated cellular metabolism and the ability to escape immune surveillance (Ravi et al., 2022). The addition of these two additional hallmarks reflects a deeper mechanistic understanding of tumour cells.

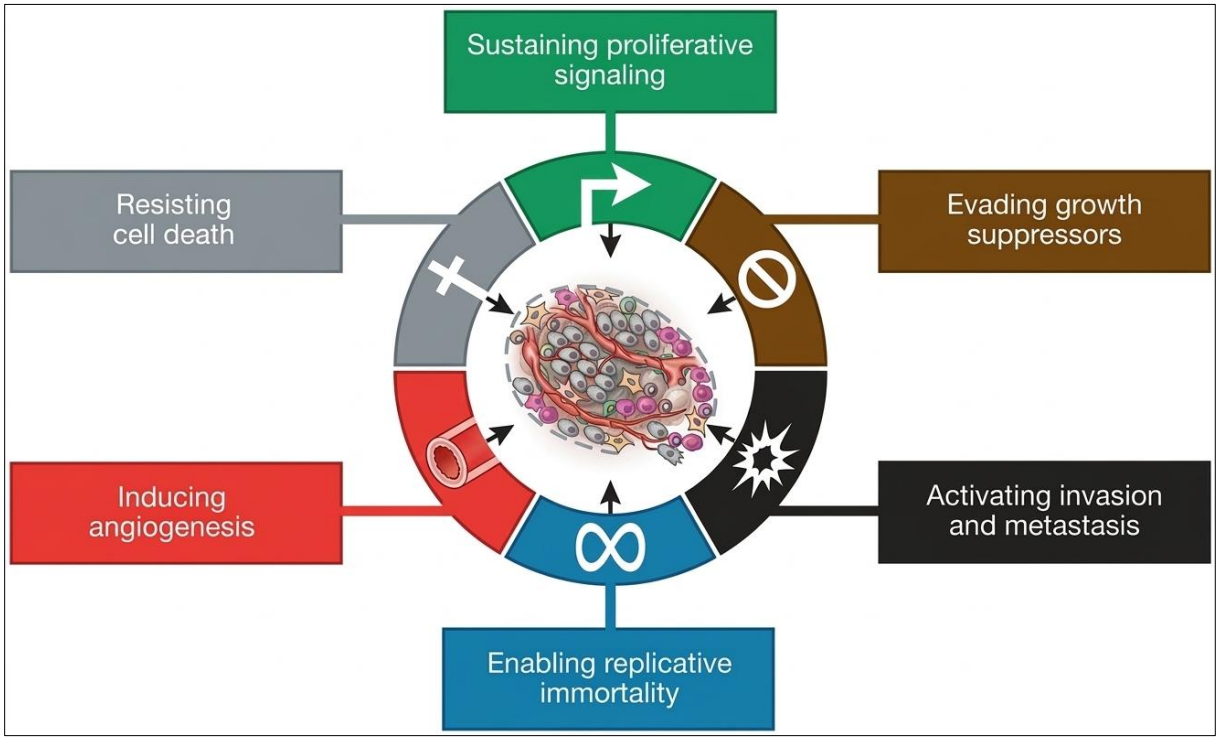

Figure Int. 9. Core Hallmarks of cancer or the biological capabilities identified in tumour cells (Hanahan & Weinberg, 2011).

As research advanced, the core traits of the original hallmarks alone did not fully encompass the complexities of cancer. Consequently, Hanahan and Weinberg introduced the concept of "enabling characteristics", which are the consequences of the neoplastic condition that provide means by which cancer cells and tumours can adopt the functional traits. These enabling characteristics reflect the molecular and cellular mechanisms through which specific hallmarks

are acquired, rather than the hallmarks themselves (Hanahan & Weinberg, 2011; Meirson et al., 2020).

As a result, two enabling characteristics represents by the genome instability and mutation, and tumor-promoting inflammation were added to the hallmarks of cancer, giving a total of ten hallmarks (Hanahan, 2022) (Figure Int. 10, Left).

In 2022, Hanahan and Weinberg expanded the ten hallmarks to better encompass the complexities of cancerous cells. They introduced four new hallmark capabilities: phenotypic plasticity and disrupted differentiation, non-mutational epigenetic reprogramming, polymorphic microbiomes, and senescent cells. These new hallmarks were added to highlight the importance of the non-genetic factors and the tumour microenvironment in tumorigenesis (Figure Int. 10, Right) (Hanahan, 2022).

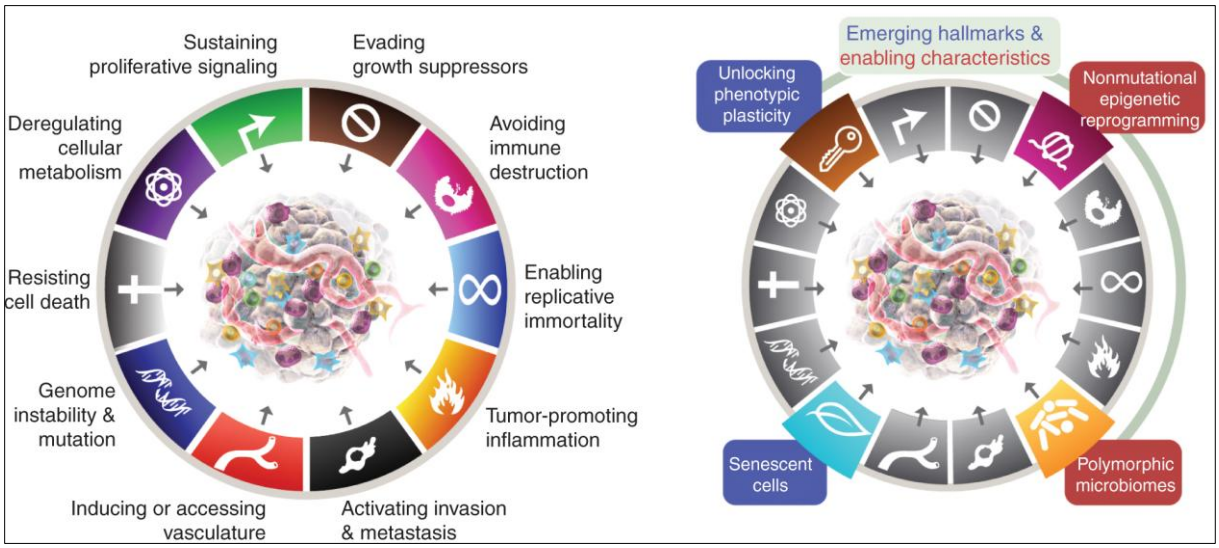

Figure Int. 10. Hallmarks of Cancer described by Hanahan and Weinberg. Left: Chart represents the eight hallmarks of cancer and the two enabling characteristics. Right: Chart represents the emerging hallmarks of cancer (Hanahan, 2022).

4.3. Chemical carcinogenesis

As described in section 4.2, exogenous factors such as exposure to different environmental pollutants and chemicals, represent an important contributor to cancer development in humans. Exposure to such chemicals may lead to cellular transformation and tumour formation through a process referred to as "chemical carcinogenesis" (Weston & Harris, 2003; Oliveira et al., 2007; Oliveira & Faustino-Rocha, 2025). The process by which chemicals are capable of inducing transformation, ultimately leading to cancer. Chemical carcinogenesis is a complex,

multi-step process divided into three distinct stages: initiation, promotion, and progression (Figure Int. 11).

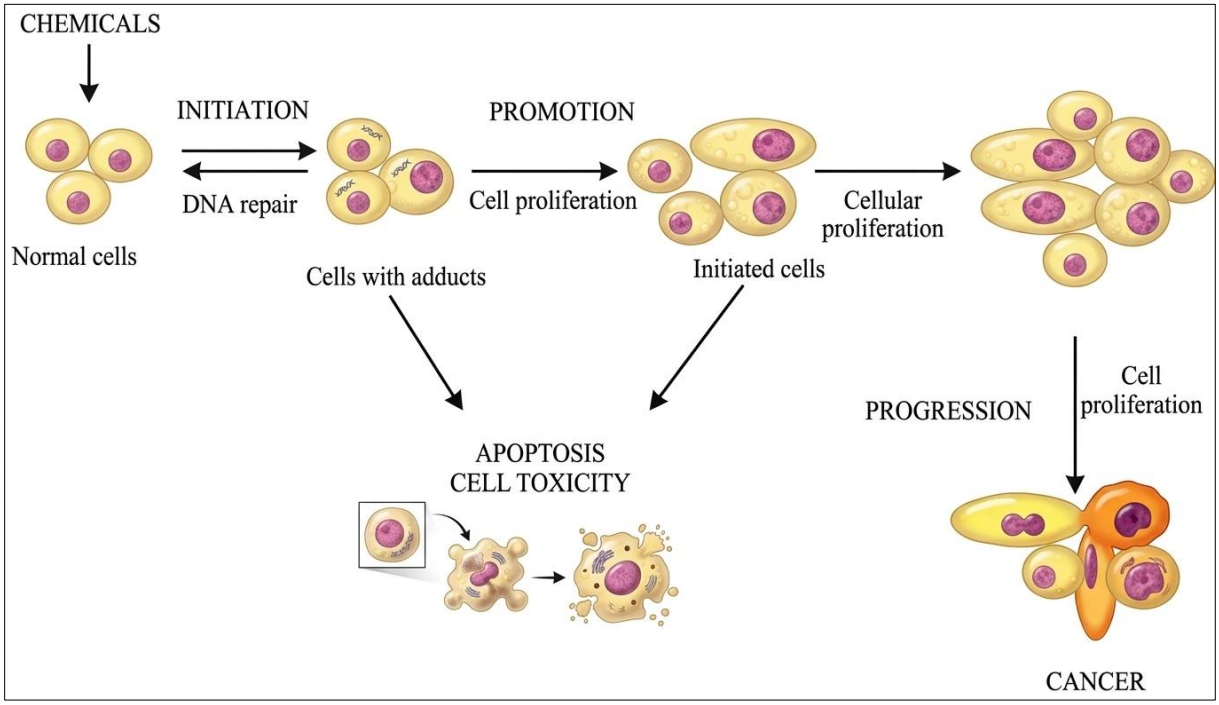

Figure Int. 11. The Multi-Stage process of Chemical Carcinogenesis. This diagram illustrates the sequential transition of normal cells into a malignant state through the three-stage process of initiation, promotion, and progression. The process begins when chemicals induce DNA damage (initiation), creating initiated cells that may either undergo DNA repair or succumb to apoptosis if the damage is too severe. If these cells survive and are exposed to promoting agents, they undergo rapid cell proliferation (promotion), forming a pre-neoplastic cluster. Finally, during progression, these cells acquire further genetic instability and permanent phenotypic changes, ultimately resulting in the uncontrolled growth and invasive characteristics of cancer. Adapted from Oliveira et al., 2007.

4.3.1. Initiation

Initiation is the first stage of chemical carcinogenesis, in which the cell's genetic material is subjected to changes caused by a mutagenic chemical agent (Stanfill et al., 2023; Baer-Dubowska & Szaefer, 2024). Mutagens are agents that induce genetic alterations, such as changes in DNA sequences, that lead to mutations. These mutations can occur through various mechanisms including base substitutions, insertions, or deletions, and may result in heritable changes.

However, not all mutagens have the potential to drive the malignant transformation of cells. In contrast, a subset of mutagens known as genotoxic carcinogens can not only cause mutations but also lead to other genetic damage, such as chromosomal fragmentation, crosslinking, and

strand breaks (Basu, 2018). These genomic alterations are caused through the direct interaction between the chemical agent (or its metabolites) and the genetic material. Such mutations commonly affect proto-oncogenes or tumor suppressor genes (Cadet & Douki, 2018).

At this stage, in response to genetic alteration, cells attempt to repair DNA damage through DNA repair systems or by activating apoptotic mechanisms to avoid tumor formation (Torgovnick & Schumacher, 2015). However, if cell division proceeds without repair, the damage becomes irreversible and is transmitted to daughter cells. This results in the conversion of normal cells into abnormal initiated cells (Chatterjee & Walker, 2017).

In 1914, Theodor Boveri proposed the Somatic Mutation Theory, which hypothesized that cancer arises from genetic alterations in normal somatic cells. He suggested this occurs due to errors in chromosome segregation or DNA replication during cell division; these errors could then trigger uncontrolled proliferation and give rise to malignant tumors (Hansford & Huntsman, 2014). This concept was followed by Karl-Heinrich Bauer's proposal that the accumulation of mutations causes cancer (Brücher & Jamall, 2016). These early observations established the principle that cancer is a genetic disease arising from the accumulation of mutations that alter cell proliferation and survival.

While spontaneous cell initiation can result from mutations caused by replication errors, it is less common than initiation induced by chemical agents (Koag et al., 2014; Brücher & Jamall, 2016). Although the initiation step does not always result in tumor formation, it represents a critical step in the multi-stage carcinogenesis process (Zhang et al., 2024).

4.3.2. Promotion

The promotion stage of chemical carcinogenesis represents an intermediate phase in which initiated cells undergo increased proliferation following exposure to substances known as tumor promoters (Siddiqui et al., 2015). These agents stimulate the expansion and division of cells that already harbor irreversible mutations, allowing additional mutations to accumulate and pushing the initiated cells toward malignant transformation.

Tumor promoters enhance tumor formation without directly interacting with DNA, acting instead through mechanisms such as chronic inflammation, oxidative stress, altered immune responses, and other pathways (Marlowe & Puga, 2010). However, some chemical promoters may indirectly damage DNA through oxidative processes or by modulating physiological

factors such as epigenetic regulation and mitogenic signalling (Lewis & Adams, 1987; Karakaidos et al., 2020).

The activity of tumor promoters depends on the specific molecular context of each tissue. Different tissues express distinct combinations of receptors, signalling molecules, and metabolic enzymes, which determine their responsiveness to a given promoter. As a result, the carcinogenic effects of these agents occur only in tissues that possess the appropriate molecular targets, enzyme systems, and microenvironmental conditions to support their action. For example, phorbol esters act through protein kinase C (PKC), and only tissues with active PKC isoforms involved in regulating cell proliferation such as the skin or liver respond strongly to these promoters. A well-known example is TPA (12-O-tetradecanoylphorbol-13-acetate), whose mode of action involves PKC disruption and which is a potent promoter of tumors in epidermal cells (Kawabe et al., 2013; Li & Zhao, 2013).

Initially, the effects of promotion may be reversible once exposure to the promoter ceases, due to reduced proliferation or the induction of apoptosis. However, repeated or prolonged exposure can lead to irreversible changes, thereby facilitating the transition from initiated cells to malignant ones (Pitot, 1993; Compton, 2020).

4.3.3. Progression

Progression represents the final, irreversible step in chemical carcinogenesis during which the malignant transformation occurs. This stage involves a series of molecular and cellular alterations leading to malignancy. In addition, cell proliferation during the progression stage is independent of the presence of stressor or stimulating agent (Oliveira et al., 2007).

Progression stage involves critical processes such as angiogenesis, tissue invasion, and metastasis. Throughout this phase, tumor cells undergo significant biochemical, metabolic, and morphological changes that promote their transformation from benign growths into malignant, aggressive cancers capable of spreading to distant sites. These alterations enhance tumor heterogeneity and promote resistance to therapy, marking the transition to advanced disease (Cazzato et al., 2024; Y. Li et al., 2025; Matsuoka & Yashiro, 2024).

5. Carcinogens

5.1. Key Characteristics of Carcinogens

Carcinogens are agents that can contribute to cancer development by inducing direct genetic alterations through initiation or by altering cellular pathways through promotion, leading to uncontrolled proliferation and tumor formation through the carcinogenesis process (Hill et al., 2024).

The work of Hanahan and Weinberg stimulated new perspectives on how carcinogens may drive the development of these hallmarks in cancerous cells, contributing to malignancy (Senga et al., 2024). While their work described the hallmarks of cancer, they did not describe the characteristics of carcinogens themselves. However, IARC developed a framework to organize mechanistic data for evaluating agents as potential human carcinogens (DeMarini, 2025).

This framework, referred to as the Key Characteristics of Carcinogens (KCCs), represents the chemical characteristics associated with hallmark properties that could be exhibited by agents having carcinogenic potential, based mainly on mechanistic evidence. Hence, the KCC concept provided a clear, guided framework for organizing the various mechanistic results observed with different carcinogens (Potera, 2016). Moreover, the KCC concept served not only for organizing mechanistic data but also to facilitate the mechanistic hazard identification of different carcinogens (Corvi et al., 2017).

The ten key characteristics included in the KCCs consist of (Table Int. 6):

1. The ability to act as an electrophile either directly or following metabolic activation;
2. Genotoxicity;
3. Alteration of DNA repair processes or induction of genomic instability;
4. Induction of epigenetic alterations;
5. Induction of oxidative stress;
6. Induction of chronic inflammation;
7. Immunosuppression;
8. Modulation of receptor-mediated effects;
9. Induction of cellular immortalization;
10. Alteration of cell proliferation, cell death, or nutrient supply.

From a regulatory point of view, the U.S. EPA, the National Toxicology Program (NTP), and the IARC have highlighted the importance of adopting a systematic approach for

carcinogenicity assessments (Rooney et al., 2014; Middleton et al., 2024). Similarly, the U.S. National Research Council (NRC) has stressed the need for transparent methods to identify, evaluate, and integrate evidence for carcinogen assessments and other human health hazard evaluations (Council et al., 2014).

Overall, the KCCs, together with the Hallmarks of Cancer concepts, emphasize the importance of mechanistic insights when assessing carcinogenic potential. Consequently, they were taken into account in the development of *in vitro* test batteries within NAMs.

Through the application of the KCC concept and assessing whether the evaluated chemical exhibits one or more of the ten KCCs of carcinogens, IARC can obtain critical information on the carcinogenic potential of a compound, even in the absence of definitive human or animal evidence (Rusyn & Wright, 2023). Hence, the integration of mechanistic data into the IARC classification framework provides biological relevance for the risk evaluation of chemicals, which complements traditional toxicological studies and aligns with the understanding of the hallmarks of cancer (Smith, 2025). For instance, Rusyn and Wright highlighted the use of the KCC framework in IARC monographs and found that mechanistic data played a significant role in the final decision and classification of several agents (Rusyn & Wright, 2023).

Table Int. 6. The 10 KCCs presented with an example of relevant evidence for each key characteristic. The table was taken directly from (Smith et al., 2016).

Characteristic	Examples of relevant evidence
1. Is electrophilic or can be metabolically activated	Parent compound or metabolite with an electrophilic structure (e.g., epoxide, quinone), formation of DNA and protein adducts
2. Is genotoxic	DNA damage (DNA strand breaks, DNA–protein cross-links, unscheduled DNA synthesis), intercalation, gene mutations, cytogenetic changes (e.g., chromosome aberrations, micronuclei)
3. Alters DNA repair or causes genomic instability	Alterations of DNA replication or repair (e.g., topoisomerase II, base-excision or double-strand break repair)
4. Induces epigenetic alterations	DNA methylation, histone modification, microRNA expression
5. Induces oxidative stress	Oxygen radicals, oxidative stress, oxidative damage to macromolecules (e.g., DNA, lipids)
6. Induces chronic inflammation	Elevated white blood cells, myeloperoxidase activity, altered cytokine and/or chemokine production
7. Is immunosuppressive	Decreased immunosurveillance, immune system dysfunction
8. Modulates receptor-mediated effects	Receptor in/activation (e.g., ER, PPAR, AhR) or modulation of endogenous ligands (including hormones)
9. Causes immortalization	Inhibition of senescence, cell transformation
10. Alters cell proliferation, cell death or nutrient supply	Increased proliferation, decreased apoptosis, changes in growth factors, energetics and signaling pathways related to cellular replication or cell cycle control, angiogenesis
Abbreviations: AhR, aryl hydrocarbon receptor; ER, estrogen receptor; PPAR, peroxisome proliferator–activated receptor. Any of the 10 characteristics in this table could interact with any other (e.g., oxidative stress, DNA damage, and chronic inflammation), which when combined provides stronger evidence for a cancer mechanism than would oxidative stress alone.	

5.2. IARC carcinogen classification

The IARC classifies agents based on their carcinogenic potential to humans. Evidence from epidemiological studies, long-term animal studies, and the integration of mechanistic data is used to evaluate the carcinogenic potential of chemicals in humans (Rusyn & Wright, 2024). This framework has been applied to the evaluation of diverse agents, including complex mixtures, supporting evidence-based decisions for public health and regulatory policy.

According to the IARC, agents are classified as: Group 1 (carcinogenic to humans), Group 2A (probably carcinogenic to humans), Group 2B (possibly carcinogenic to humans), and Group 3 (not classifiable as to their carcinogenicity to humans) (Figure Int. 12).

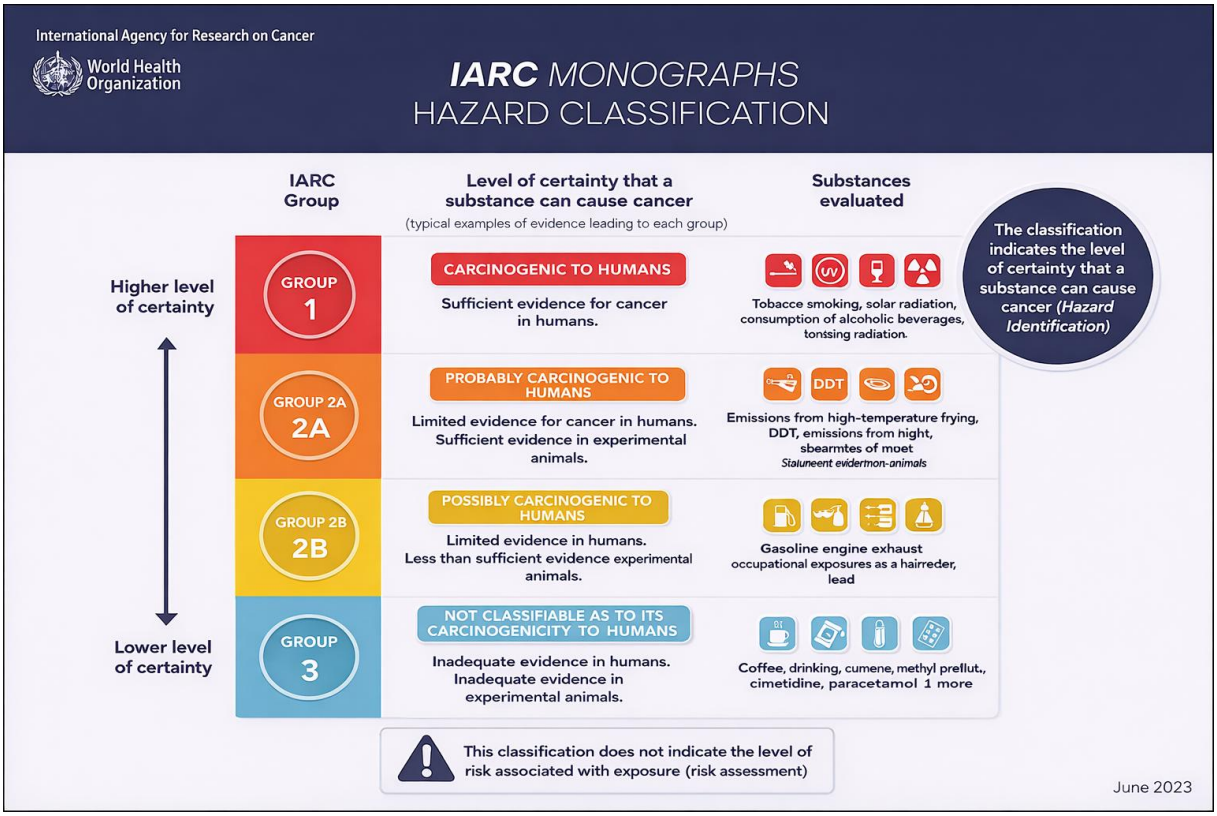

Figure Int. 12. IARC framework for chemical hazard classification. Different categories or groups used by the IARC monographs for the identification of carcinogenic hazard to humans. This classification of chemical carcinogens does not indicate the level of risk associated with exposure (IARC Monographs Hazard Classification, 2023).

IARC Monographs have long recognized that many human carcinogens share structural features such as electrophilicity and biological activities including genotoxicity. The implementation of the KCCs framework has proven to be a useful tool for carcinogen hazard identification, particularly because carcinogens may exhibit multiple distinct characteristics.

Consequently, a series of IARC workshops was convened to integrate the KCCs framework into carcinogenicity evaluations. The IARC Scientific Workshop on "Key Characteristics–Associated Endpoints for Evaluating Mechanistic Evidence of Carcinogenic Hazards," held on July 25–28, 2023, in Lyon, resulted in the incorporation of KCC-based mechanistic evidence into the evaluation process (DeMarini et al., 2025).

This workflow begins with a systematic review of existing monograph evidence, data analysis, and the development of structured outlines. This initial phase transitions into a critical discussion across multiple disciplines, including toxicology, epidemiology, cancer biology, and exposure science, to evaluate the KCs of carcinogens. The ultimate goal of this framework is the furtherance of the KC approach by interpreting associated biological end-points and incorporating modern data streams, such as high-throughput screening (HTS), omics, and *in silico* modeling. By integrating this diverse mechanistic evidence, the framework aims to address contemporary opportunities and challenges in identifying human carcinogens. (Figure Int. 13).

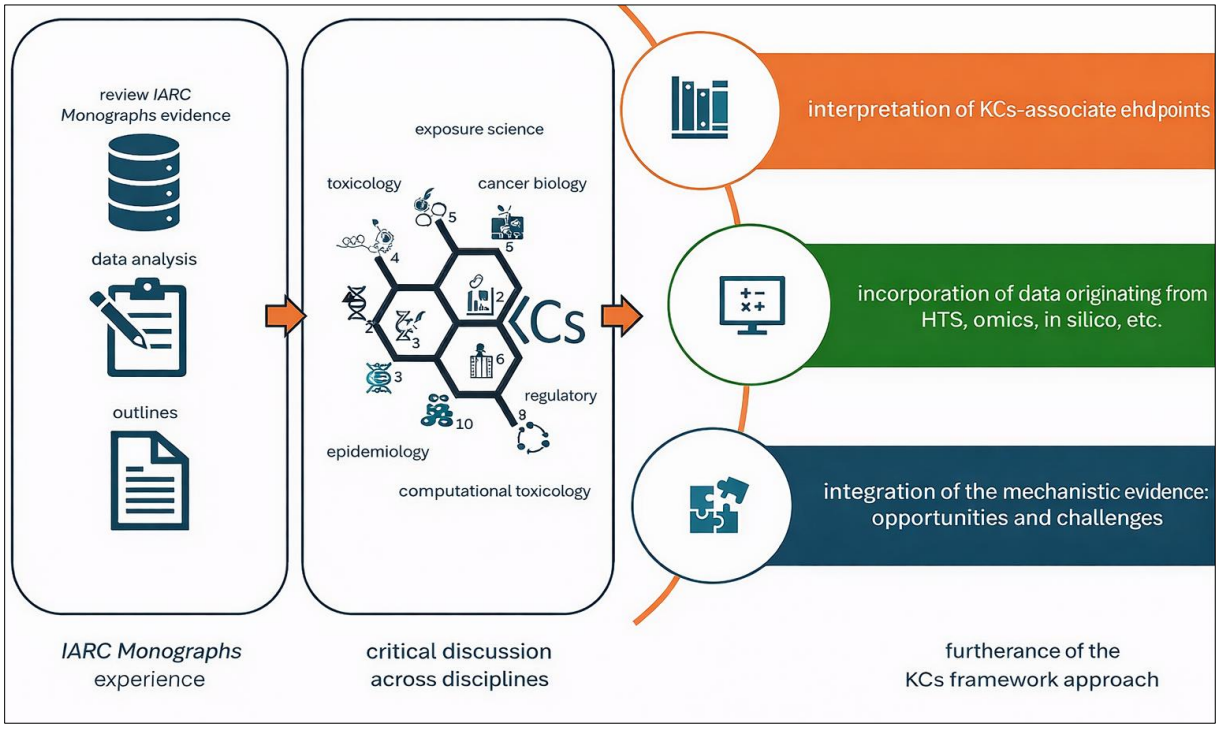

Figure Int. 13. Graphical presentation represents the integration of mechanistic-based approach including the KCCs concept in the classification and decision making of chemical exposure. Taken from the IARC Monographs Technical Report of the workshop held on July 25-28, 2023, in Lyon, France (DeMarini et al., 2025).

5.3. Types of carcinogens

Carcinogens can be generally divided into three types. Biological carcinogens such as viruses, bacteria, and parasites; physical carcinogens such as UV light, and radiation; and chemical carcinogens such as naturally occurring substances (e.g. mycotoxins), and anthropogenic substances (e.g. industrial, pharmaceuticals, cosmetics and others) (Das et al., 2020).

In addition, carcinogens are further divided into two main types based on their mode of carcinogenicity: (1) Genotoxic carcinogens (GTxCs), and (2) Non-genotoxic carcinogens (NGTxCs) (Hayashi, 1992**)** (Figure Int. 14).

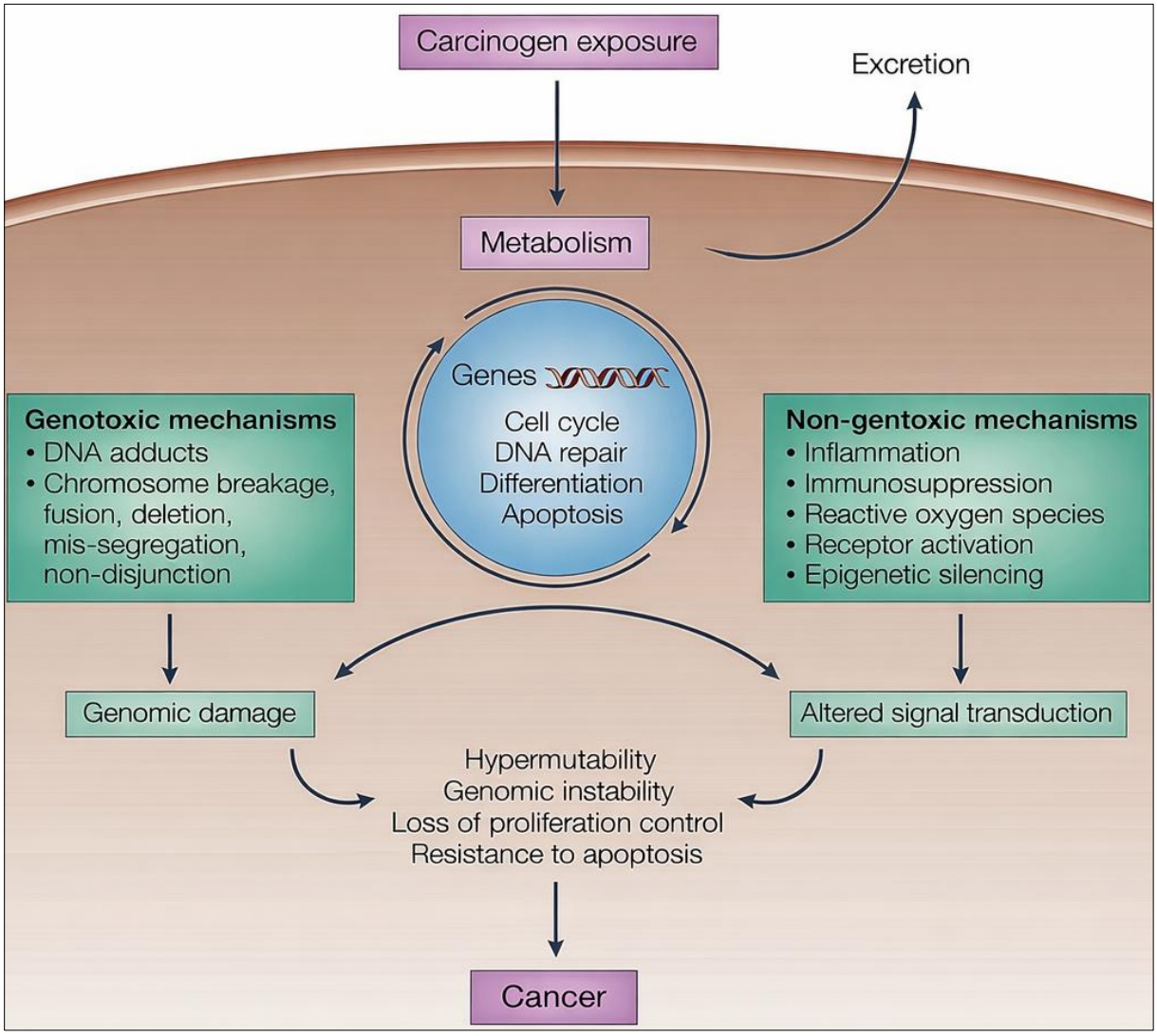

Figure Int. 14. Overview of the two main types of carcinogens, GTxCs and NGTxCs, and their mode of carcinogenicity (Anetor et al., 2012).

6. Genotoxic carcinogens

6.1. Definition

Genotoxicity has been defined as the capacity of any agent to induce or produce heritable changes in the genetic material of somatic or germinal cells (Phillips & Arlt, 2009). The toxicological significance of genotoxicity lies in its potential to cause cancer through the induction of mutations. Thus, GTxCs represent all agents whose physical or chemical properties make them capable of inducing cancer via DNA interactions.

Most GTxCs are electrophilic or nucleophilic, enabling them to interact directly, or following metabolic activation, with DNA through covalent bonding, resulting in DNA adduct formation. Around 95% of GTxCs require metabolic activation to convert the parent molecule into an electrophilic carcinogenic compound capable of reacting indirectly with the nucleophilic groups of DNA, RNA, and proteins. The remaining 5% are direct electrophilic alkylating agents that do not require metabolic activation (Cavalieri & Rogan, 2014) (Figure Int. 15).

DNA damage caused by GTxCs can occur in various forms, such as loss of DNA bases, formation of cross-links between DNA strands, and strand cleavage. Such adducts interfere with DNA polymerase during replication, leading to mutations that alter the genetic information carried by DNA. While DNA repair mechanisms can correct the original damage, mutations that escape repair or occur before repair can contribute to tumor formation (Chatterjee & Walker, 2017; J. Chen et al., 2024).

Cellular DNA damage caused by GTxCs triggers the activation of the tumour suppressor p53, which in turn can activate several protective processes, including DNA repair, cell cycle arrest, and apoptosis. Through these mechanisms, the initiation of programmed cell death and cell cycle modulation by the affected cells serves to maintain genomic integrity and prevent malignant transformation (Yadav et al., 2024).

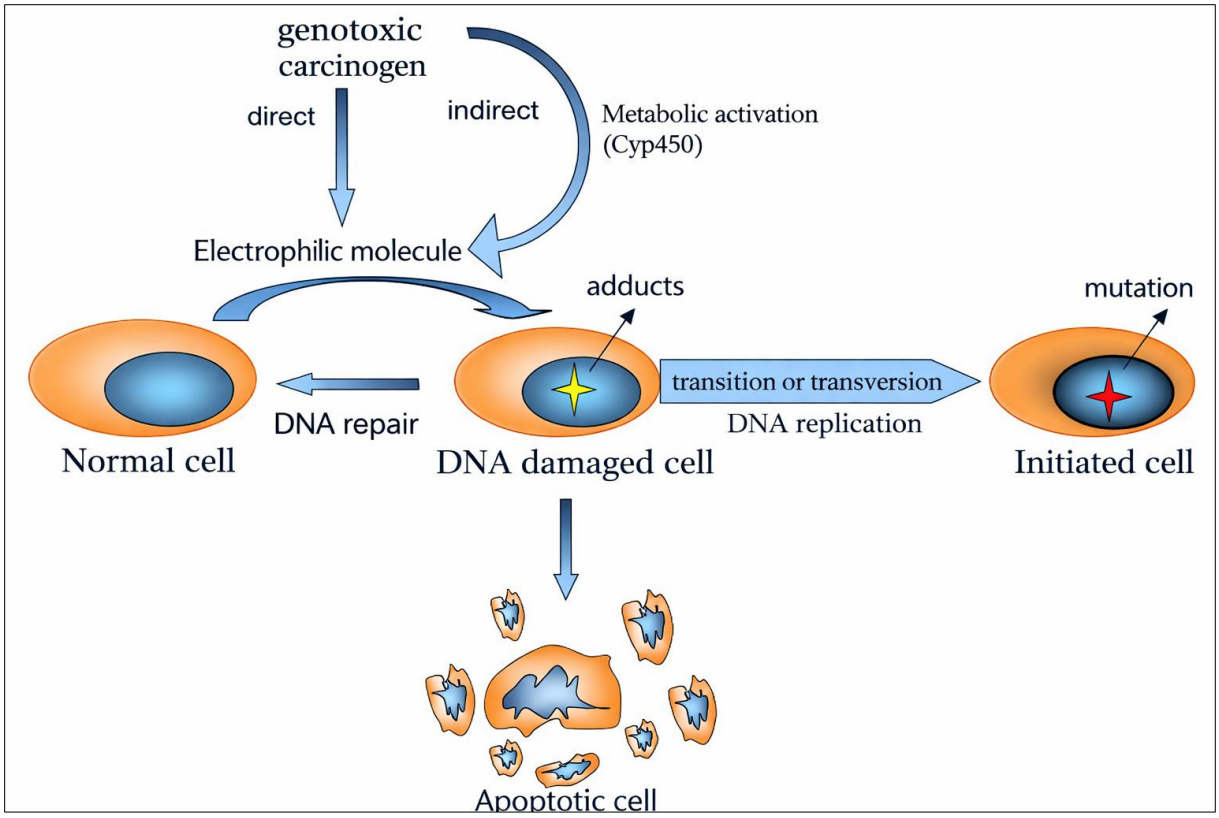

Figure Int. 15. Mechanism of genotoxic carcinogenesis in Tumor Initiation. The diagram illustrates the pathways of tumor formation through direct DNA interaction (active GTxC) or indirect DNA interaction requiring metabolic activation by the Cytochrome P450 (Cyp450) system. The process highlights the formation of DNA adducts, the role of DNA repair or apoptosis in preventing damage, and the fixation of mutations (transitions or transversions) during DNA replication, leading to the formation of an initiated cell (Pérez-Carreón & Meléndez-Zajgla, 2012).

6.2. Genotoxicity evaluation of chemicals

Various *in vitro* and *in vivo* assays have been validated and recommended by the OECD for evaluating genotoxicity and assessing the genotoxic potential of chemical (Table Int. 7).

These standardized test methods, outlined in OECD guidelines, are extensively used worldwide for regulatory safety evaluations of chemical substances. The broad application of these assays ensures reliable detection of genotoxicants across diverse chemical classes.

According to the standardized guidelines, the most common *in vitro* genotoxicity testing batteries consist of the Ames test, the micronucleus test (MN), and the mouse lymphoma assay (MLA) (Corvi & Madia, 2017). These tests serve as an initial screening tool to assess the genotoxic potential of chemicals. As the evaluation of genotoxic chemicals starts with these different *in vitro* tests, negative results across these assays typically lead to an overall conclusion of non-genotoxicity. However, when results are ambiguous, equivocal, or clearly positive in such tests, further *in vivo* genotoxicity assays become necessary to confirm the genotoxic potential of the tested chemicals under more physiologically relevant conditions.

A positive result in such *in vivo* tests indicates that the chemical could be considered genotoxic. The workflow of the decision-making process concerning the evaluation of genotoxicity used by the Committee on Mutagenicity (COM) is presented in detail in (Figure Int. 16)(Figure Int. 17).

In details, the two figures together illustrate a tiered, decision-based framework for interpreting *in vitro* mutagenicity data and determining whether a substance should be considered mutagenic, non-mutagenic, or insufficiently characterized. The Figure Int. 16 outlines the outcome of Stage 1 testing, where results from initial *in vitro* assays supported by Stage 0 information such as structure activity relationships, screening data, and physicochemical properties, are used to make a preliminary classification. A substance that is negative in all Stage 1 tests is considered not mutagenic, whereas a positive result in any Stage 1 assay leads to classification as an *in vitro* mutagen. If any Stage 1 result is equivocal, the available evidence is deemed insufficient, and further evaluation is required. Figure Int. 17 expands this logic to Stage 2 follow-up testing, which is triggered when Stage 1 results are unclear or positive. Here, a negative outcome in all appropriate Stage 2 assays supports the conclusion that the substance is not mutagenic *in vivo*, while a positive result in any Stage 2 test confirms that it should be considered an *in vitro* mutagen. Equivocal Stage 2 outcomes require a more nuanced, WoE evaluation that considers reproducibility, historical controls, mode of genotoxic action, and toxicokinetic information to determine whether additional testing is warranted. Together, the two figures depict a structured, stepwise approach that integrates multiple lines of evidence to guide regulatory decision-making on mutagenicity.

Table Int. 7. Standard regulatory genetic toxicity tests and OECD Guidelines.

This table summarizes the core battery of *in vitro* and *in vivo* assays used to evaluate the genotoxic potential of chemical substances, categorized by their specific endpoints and corresponding OECD TG.

Test	Type	Endpoint	OECD
Bacterial Reverse Mutation Test (Ames)	*In vitro*	Detects base-pair substitutions and frameshift mutagens.	TG 471
Mammalian Cell Gene Mutation (MLA/TK6)	*In vitro*	Forward mutations at thymidine kinase (TK), captures point mutations and small to large deletions	TG 490
Mammalian Cell Gene Mutation (Hprt/xprt)	*In vitro*	Forward mutations at HPRT (endogenous X-linked) and XPRT/gpt (autosomal transgene) loci.	TG 476
In vitro Mammalian Chromosomal Aberration	*In vitro*	Structural chromosomal aberrations (chromatid/chromosome breaks) clastogenicity.	TG 473
In vitro Mammalian Cell Micronucleus	*In vitro*	Detects clastogens (acentric fragments) and aneugens (spindle kinetochore dysfunction).	TG 487
Mammalian Erythrocyte Micronucleus	*In vivo*	Detects clastogens and aneugens in erythroblasts	TG 474
Mammalian Bone Marrow Chromosomal Aberration	*In vivo*	Structural chromosomal aberrations (chromosome/chromatid-type) in bone marrow metaphases of rodents; detects clastogenicity in hematopoietic tissue.	TG 475
Transgenic Rodent (TGR) Somatic & Germ Cell Gene Mutation	*In vivo*	Gene mutations in reporter transgenes (e.g., lacZ, cII, gpt) recovered from multiple tissues (somatic and/or germ cells).	TG 488
In vivo Mammalian Alkaline Comet Assay	*In vivo*	Primary DNA damage in target tissues such as single or double-strand breaks under alkaline conditions (pH>13).	TG 489

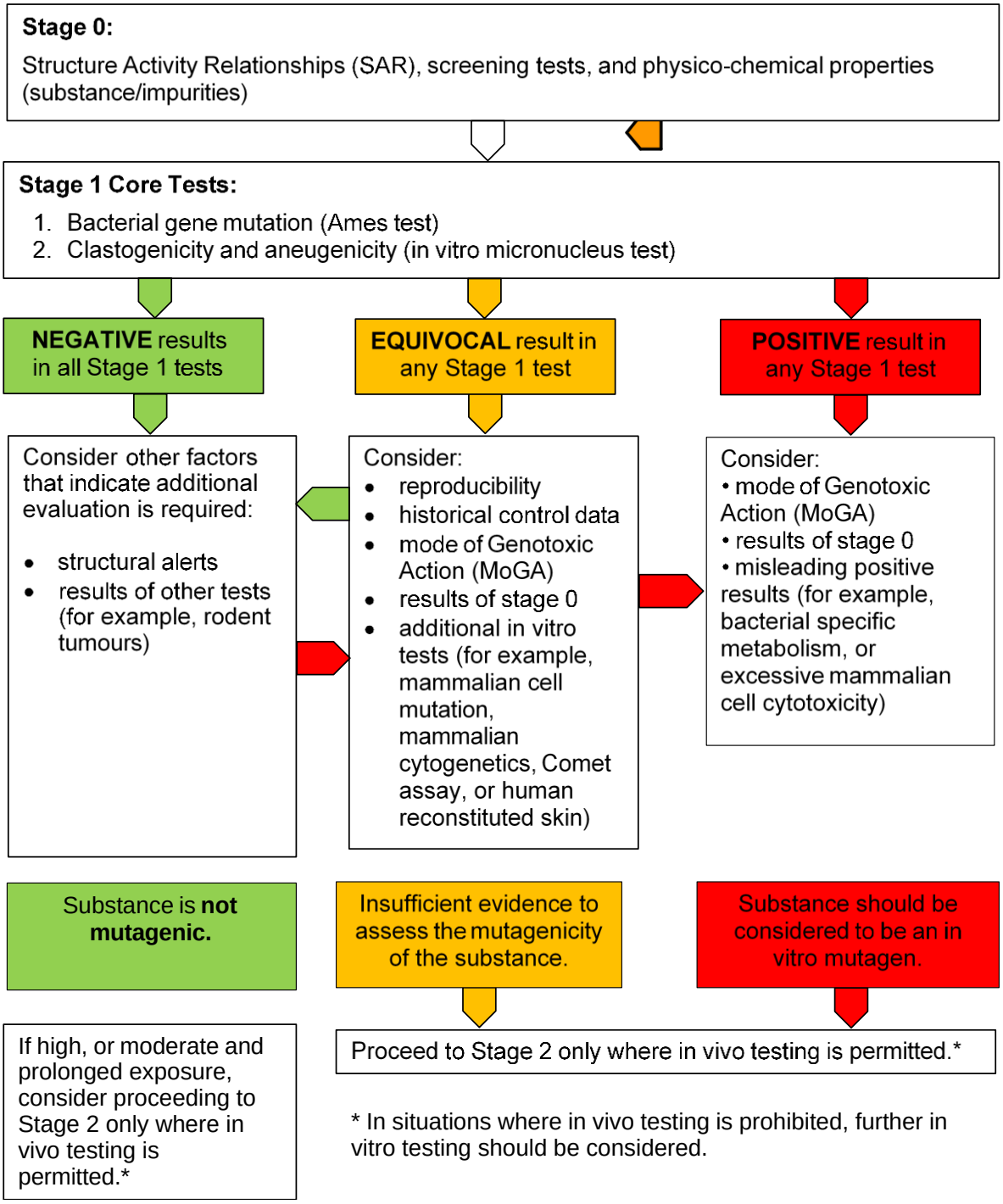

Figure Int. 16. Process and steps in the evaluation of genotoxicity. Chemical structure evaluation (stage 0) to *in vitro* stage tests. Taken directly from COM 'Guidance document on a strategy for genotoxicity testing of chemicals. Taken directly from COM 'Guidance document on a strategy for genotoxicity testing of chemicals.

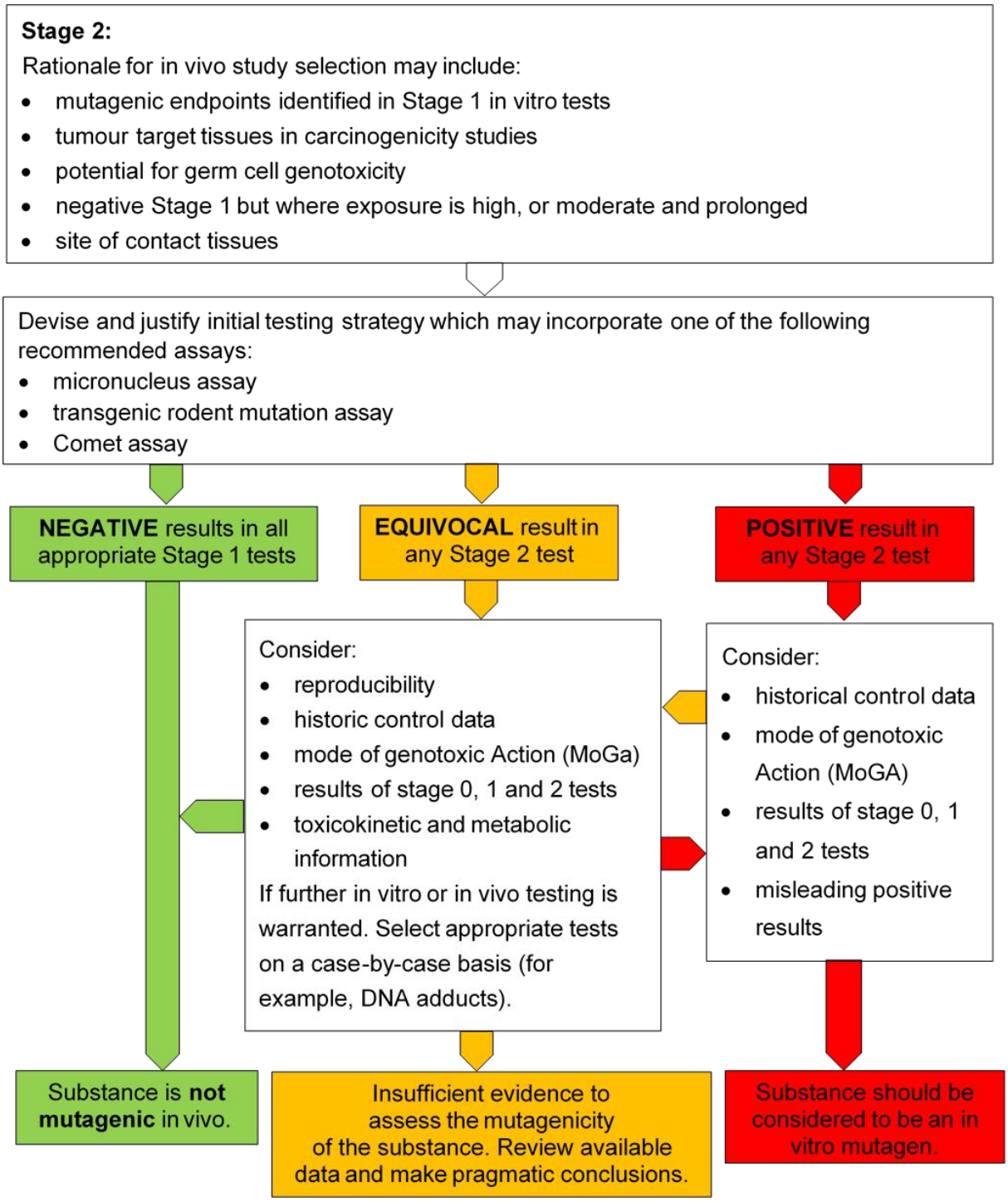

Figure Int. 17. Genotoxicity evaluation following *in vitro* evaluation. Taken directly from COM 'Guidance document on a strategy for genotoxicity testing of chemicals. Taken directly from COM 'Guidance document on a strategy for genotoxicity testing of chemicals.

6.3. Limitations of the current genotoxicity evaluation framework

The REACH program estimated that genotoxicity testing is expected to be the third most animal-consuming area of testing in the future (Stoykova, 2025). This is mainly due to the challenges associated with available test batteries used to evaluate the genotoxic potential of chemicals.

Challenges associated with current *in vitro* genotoxicity test batteries including the Ames test, MN test, or MLA assay, include the induction of both false positive and false negative responses. These inaccuracies lead to unnecessary, complementary *in vivo* tests (Doktorova, Ates, et al., 2014; Mišík et al., 2022). Additionally, the increasing number of industrial chemicals introduced each year raises the likelihood of assessing genotoxic potential through these complementary *in vivo* tests, thereby increasing the total number of animals used in genotoxicity evaluations.

The induction of false negative results is largely attributed to limited or artificial metabolic activation systems. Many standard cell lines, such as rodent lines (V79, CHL) and certain human lines (TK6 and HepG2) used in *in vitro* assays, lack comprehensive metabolic activity (Fowler et al., 2012). These cell lines depend on exogenous metabolic activation systems, such as rodent liver S9 fractions, which may not accurately reflect human metabolism and can introduce artifacts or unpredictable activation of the tested compounds (Kishino et al., 2019; Jurgelewicz et al., 2025).

Furthermore, common *in vitro* cell models, especially those derived from rodents, are often deficient in DNA repair pathways or tumor suppressor genes like p53, leading to irrelevant false positive signals (Mišík et al., 2022). Studies have shown that rodent cell lines (V79 and CHL) are more susceptible to misleading results compared to human lymphoblastoid TK6 cells and the human liver cell line HepG2. These findings suggest that a reduction in the frequency of misleading positive results can be achieved through the careful selection of mammalian cell types for testing (Fowler et al., 2012).

In addition, the high concentrations of chemicals used in *in vitro* tests and the high levels of cytotoxicity sometimes observed in mammalian assays can induce false positive results (Azqueta et al., 2022). High cytotoxicity levels can trigger oxidative stress, apoptosis, or non-specific cellular responses, which in turn cause DNA damage independent of a true genotoxic response.

The limitations of currently used *in vitro* mutagenicity and genotoxicity tests highlight the critical need for more human-relevant cell systems with enhanced metabolic competence, robust DNA repair capacity, and appropriate physiology. It is necessary to incorporate these criteria to reduce the incidence of false positive results and enhance predictivity, ultimately reducing unnecessary animal testing. Alongside more relevant human cell models, the integration of NAMs into *in vitro* genotoxicity testing could further improve evaluations and decrease animal use in this field (Chrz et al., 2020; Gutleb et al., 2025).

6.4. Integration of alternative, NAM-based approaches for genotoxicity testing

NAMs encompass innovative techniques and models designed to reduce animal use in CSA, and as such, they have played an important role in making genotoxicity testing more efficient and ethically acceptable. Diverse *in vitro* mechanistic-based testing strategies, techniques, and methods have been integrated into genotoxicity testing, including the use of advanced human-derived test systems, HTS, as well as computational tools (Gutleb et al., 2025).

Among the *in silico* methods applied to genotoxicity evaluation, QSAR methodology represents one of the assessment strategies implemented and integrated into genotoxicity prediction (Benigni & Bossa, 2019). Benigni & Bossa (2019) have documented that QSAR *in silico* models cover all the levels required by REACH to assess genotoxicity. Different QSAR algorithms and models have been used in combination with existing experimental data to predict the presence or absence of genotoxic potential (Vallés-Pardo et al., 2025). Moreover, these computational models serve in predicting the chemical structure through the physicochemical properties of the assessed chemicals, thus providing mechanistic insight into the relationship between chemical structure and its effects (Li et al., 2025).

Additional in vitro-based techniques are being used in predicting genotoxicity, such as transcriptomic biomarkers, including the recently described TGx-DDI and GENOMARK assays (Thienpont et al., 2024). These two assays represent the most widely used transcriptomic biomarkers, constituting a gene-set-based strategy to evaluate gene expression profiles of the tested chemicals and predict genotoxicity potential based on differential gene expression associated with genotoxicants (Li et al., 2025). Recent studies have shown a high classification accuracy for both biomarkers. For instance, chemicals classified with both biomarkers and tested in different cell lines such as 2D HepaRG cells and TK6 cells show high accuracy in classifying genotoxic chemicals (Thienpont et al., 2024). More detailed information will be

provided in upcoming chapters concerning the use of these biomarkers in evaluating genotoxicity.

An additional high-throughput method used in predicting genotoxicity is the *in vitro* CometChip assay. This assay relies on the same principles as the standard alkaline comet; however, it increases throughput by ~200-fold through the multi-well plate format, enabling simultaneous treatment and analysis of many samples at the same time (Boyadzhiev et al., 2022). This system has been used to successfully measure chemical-induced DNA damage in suspension and adherent mammalian cell cultures as well as in peripheral blood monocytes (Sykora, Chiari, et al., 2018).

Many other *in vitro* tests have been integrated into *in vitro* genotoxicity evaluation, such as the γH2AX/pH3 assay, which serves as a relatively high-throughput method compared to conventional assays such as the MN assay and chromosomal aberration tests. The quantification of γH2AX and pH3 biomarkers allows the detection of different types of genotoxic compounds in the same culture plate, resulting in significant time savings (Recoules et al., 2025). In addition, the *in vitro* mechanistic-based ToxTracker assay is also being used in genotoxicity assessment (Hendriks et al., 2024). This method is used to identify the specific cellular stress pathways activated by chemicals to assess genotoxicity, such as DNA damage, oxidative stress, p53-dependent pathways, and others (Hendriks et al., 2024).

Although various *in vitro* NAMs are available for genotoxicity assessment, none have regulatory acceptance and none have yet been incorporated into regulatory testing programs. In addition, the risk of false positives and misleading outcomes in the evaluation of certain genotoxicants using these integrated approaches remains challenging (Thienpont et al., 2024).

Therefore, further efforts are required to generate mechanistic-based data and conduct inter-laboratory validations to improve the predictive accuracy of chemical genotoxicity using NAMs-based methodologies.

7. Non-genotoxic carcinogens

7.1. Definition

As not all carcinogens act through genotoxic mechanisms, the agents capable of inducing cancer through mechanisms other than direct effects on DNA or the genetic machinery are referred to as NGTxC. According to the Integrated Approach to Testing and Assessment IATA/OECD expert group, "A non-genotoxic carcinogen has the potential to induce cancer without interacting directly with either DNA or the cellular apparatus involved in the preservation of the integrity of the genome" (Jacobs et al., 2020). Unlike GTxC, which share a common genotoxic carcinogenic potential, NGTxC can induce cancer through a variety of different mechanisms that can be quite diverse (Jacobs et al., 2016) (Figure Int. 18).

Mechanistically, NGTxCs act through a diverse array of cellular pathways that enhance tumor growth of already initiated cells and result in inducing a carcinogenic effect and tumour development (Williams, 2001, Ko et al., 2025). These pathways include, but are not limited to, tumor promotion, endocrine disruption, receptor-mediated signalling, immunosuppression, oxidative stress, peroxisome proliferation, regenerative proliferation following cytotoxicity, mitochondrial dysfunction, and inflammatory responses (Hernández et al., 2009; Colacci et al., 2025). In addition, Desaulniers et al. (2021) have reported that NGTxCs could act through epigenetic mechanisms involving different molecular processes, such as DNA methylation and histone modifications. These epigenome modifications can regulate gene transcription machinery, DNA replication, DNA repair systems, and genomic stability, and as a result, create a direct link between environmental contaminants and long-lasting alterations of gene expression patterns over generations (Desaulniers et al., 2021).

Although NGTxCs act through different mechanisms and cellular pathways, a recent study highlighted that increased cell proliferation could represent a KE associated with their role in carcinogenesis. This makes cell proliferation a unifying element in their MoA. Even though the pathways leading to that proliferation are numerous, increased cell proliferation could be due to a decrease in the rate of cell death. Thus, decreased apoptosis associated with an accumulation of cells increases the chance of spontaneous error events in cell replication. Overall, increased cell proliferation represents a KE in the assessment of non-genotoxic chemical carcinogenesis (Strupp et al., 2023).

The term non-genotoxic can be misleading. Although NGTxCs show no direct DNA-reactive activity, labelling them as non-genotoxic carcinogens may create a false impression that they are less harmful to humans, yet this is not true.

One NGTxC can act through multiple pathways, having different MoAs which are, for the most part, tissue- and species-specific (Srinivas et al., 2018). The different MoAs of NGTxCs involve complex regulatory networks, including interactions among chemicals, proteins, receptors, and signalling cascades, thereby complicating their characterization and hazard assessment (Hernández et al., 2009). Moreover, as NGTxCs are capable of inducing reactive oxygen species (ROS), epigenetic modifications, and chronic inflammation, they could induce indirect genomic instability through downstream processes that can cause oxidative DNA damage (Desaulniers et al., 2021; Jacobs et al., 2020; Veltman et al., 2023).

While the mechanisms involved in the activity of NGTxCs are diverse, they are closely interconnected with the fundamental hallmarks of cancer concept (Table Int. 8). These mechanisms modulate key biological processes, including sustained proliferative signalling, evasion of apoptosis, angiogenesis, and metastatic potential. Taken together, they illustrate how NGTxC MoAs contribute to carcinogenesis through pathways that do not depend on direct DNA damage but instead operate via persistent alterations in cellular signalling, tissue microenvironments, and homeostatic regulation. By perturbing these critical biological systems, NGTxCs drive tumor initiation and progression in ways that reflect both their mechanistic complexity and their strong alignment with established cancer biology (Audebert et al., 2023).

As NGTxCs are associated with a real danger to human health and the environment, there is an urgent need to develop NAMs for the identification of these types of carcinogens. Different WoE approaches have been developed based on NAMs such as high-throughput screening, transcriptomics, and others. However, in contrast to genotoxicity evaluation, no regulatory-accepted *in vitro* testing strategies for NGTxC assessment are available or recognized by regulatory agencies (Ouedraogo et al., 2025).

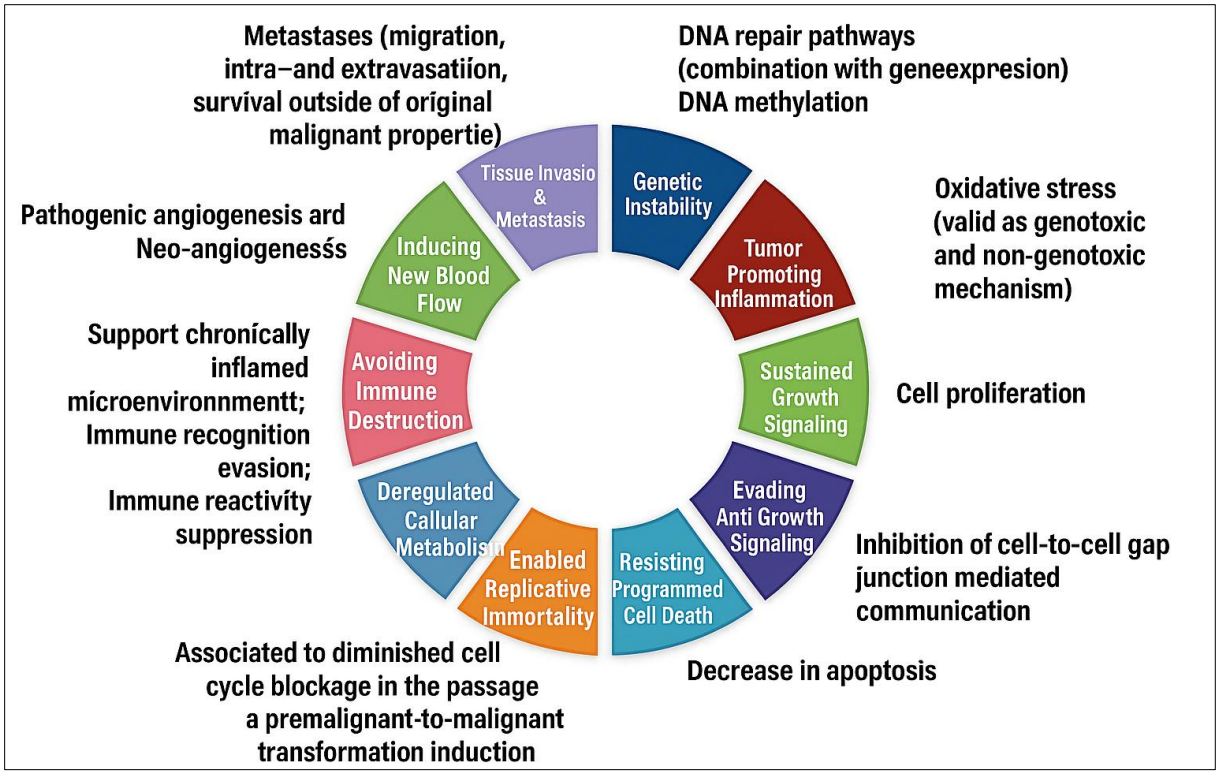

Figure Int. 18. NGTxC MoAs and their alignment with the Hallmarks of Cancer (Jacobs et al., 2016). This diagram integrates NGTxC MoA with the ten established Hallmarks of Cancer. Each coloured segment represents a biological capability essential for tumor development, while the accompanying descriptions illustrate how specific NGTxC-relevant mechanisms such as inflammation, metabolic disruption, immune evasion, altered cell communication, and replicative deregulation map onto the conceptual framework proposed by Jacobs et al. (2016).

Table Int. 8. Relationship between NGTxC MoA and Hallmarks of Cancer (Louekari & Jacobs, 2024).
This table summarizes major biological mechanisms associated with NGTxCs and maps them to the corresponding Hallmarks of Cancer. Each MoA reflects a biological mechanism through which NGTxCs can promote carcinogenesis without directly damaging DNA. These mechanisms include disruption of intercellular communication, imbalances in proliferation and apoptosis, immune modulation, chronic inflammation, oxidative stress, receptor-mediated signalling, and epigenetic alterations. Their alignment with specific cancer hallmarks highlights how NGTxCs contribute to tumor initiation and progression through persistent changes in cellular signalling, microenvironmental conditions, and regulatory homeostasis, consistent with contemporary carcinogenesis frameworks.

Key MoAs	Description	Hallmark of cancer	Reference
Inhibition of Gap Junction Intercellular Communication (GJIC)	Altered cellular communication and promoting carcinogenesis.	Sustaining proliferation signalling Evading growth suppressors	(Hernández et al., 2009)
Altered Cell Proliferation and Apoptosis Balance	Dysregulation of proliferation and programmed cell death.	Resisting cell death Evading growth suppressors Sustaining proliferation signalling	(Hanahan & Weinberg, 2011)
Immune System Modulation	Suppress or evade immune surveillance, allowing for tumor development.	Avoiding immune destruction	(Cavallo et al., 2011)
Chronic Inflammation	Persistent inflammation, which can promote tumor development.	Tumor promoting inflammation	(Mantovani et al., 2008)
Oxidative Stress Induction	ROS generation, leading to cellular damage and inflammation.	Sustaining proliferation signalling Resisting cell death Genome instability and mutation	(Sosa et al., 2013)
Receptor-Mediated Signalling	Activate nuclear or membrane receptors, leading to altered gene expression and cell proliferation.	Sustaining proliferation signalling Evading growth suppressors	(Hernández et al., 2009)
Epigenetic Modifications	DNA methylation, histone modification, leading to altered gene expression.	Epigenetic reprogramming Sustaining proliferation signalling Evading growth suppressors	(Feinberg et al., 2006)

7.2. NAMs-based NGTxC evaluation

7.2.1. Mechanistic-based strategies

In 2016, the OECD set up an expert group to develop an IATA for NGTxC (Louekari & Jacobs, 2024). The aim of this group was to bring together different scientific theories and approaches in order to create a practical framework for evaluating carcinogenic hazards associated with NGTxC. Their work was mainly based on the mechanistic insights of NGTxC, as it addresses the major hallmarks of cancer and biological KE associated with non-genotoxic MoA that may result in cell transformation and tumour formation. The testing and assessment strategy outlined by the IATA expert group recognized the need for a battery of validated methods to support the identification of NGTxC (Louekari & Jacobs, 2024). The strategy was developed based on the AOP framework mechanistic strategy, which organizes mechanistic understanding across different levels, including the molecular, cellular, and tissue levels. This approach was first used to trace the evolution of cancer models, which then served as the foundation for the overarching IATA/OECD for NGTxC (Jacobs et al., 2020).

For regulatory use, the IATA proposed a stepwise Modular Strategy of Testing and Assessment (MoSt) for NGTxC. The MoSt outlines how to reach conclusions in a sequential, module-based manner (Figure Int. 19). It provides structure to the assessment process, clarifying how and why conclusions can be drawn at each stage. This framework has been applied as an organizing principle to group assay blocks that test the events from early molecular-level to mid-level cellular responses and later tissue-level effects.

The MoSt framework suggests tests that could provide information relevant to NGTxC MoA and mechanistic effects (Louekari & Jacobs, 2024). It is organized into modules that can be applied sequentially:

1. **Module A** involves reviewing existing information and applying *in silico* tools, which can help in forming mechanistic hypotheses and guide the selection of the most relevant experimental tests.
2. **Modules B, C, D, and E** address distinct NGTxC MoAs, each linked to specific KEs in cancer development. These modules reflect current understanding of cancer biology and offer assays to detect relevant mechanisms.
3. **Module F** integrates all available evidence in a WoE evaluation, supporting prioritization and hazard identification.

The applicability of this modular strategy depends on the strength of the evidence available for each substance. Strong data supporting particular MoA can help direct and optimize subsequent testing steps within the MoSt framework in testing several carcinogens (Louekari & Jacobs, 2024).

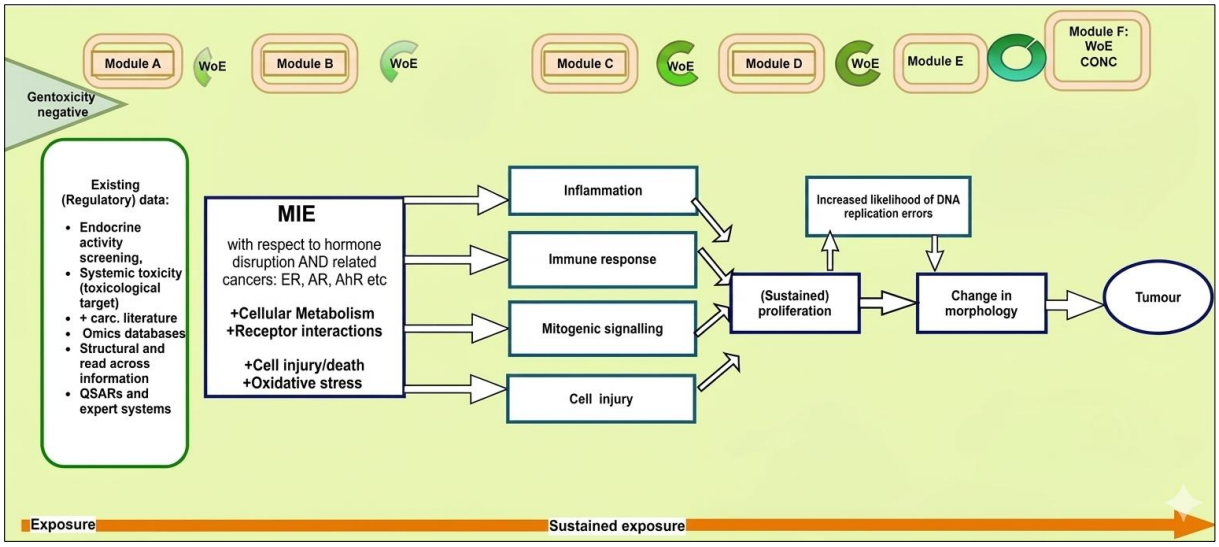

Figure Int. 19. The MoSt suggested by IATA/OECD for the testing and assessment of NGTxC (Louekari & Jacobs, 2024). The diagram illustrates a mechanistic progression starting from a substance confirmed as genotoxicity-negative, mapping its path toward tumor development. The process begins with the integration of existing (regulatory) Data to identify a MIE, such as receptor interactions (ER, AR, AhR) or metabolic disruption. These triggers lead to key biological responses including inflammation, immune response, mitogenic signalling, and cell injury. When exposure is sustained, these events drive sustained cell proliferation, which increases the likelihood of replication errors and change in morphology (Louekari & Jacobs 2024).

7.2.2. High-throughput screening strategies

The U.S. EPA ToxCast and Tox21 represent large public HTS programmes that generate standardized *in vitro* bioactivity data across a wide range of biological, cellular, and toxicological endpoints for thousands of chemicals. These programmes aim to shift chemical toxicity assessments toward NAMs-based approaches focused on the prioritization of chemicals (Jeong et al., 2022).

ToxCast/Tox21 HTS programmes are composed of different assay panels that provide quantitative data over a wide range of molecular targets and cellular pathways, such as receptor signalling, stress responses, transcriptional changes, mitochondrial function, and cell-cycle endpoints. Such information provided by these programmes can be linked to the KCC of carcinogens and their MIE. Therefore, ToxCast/Tox21 data are valuable for the identification

of mechanistic signatures associated with different NGTxC, which act through multiple molecular pathways (Bundy et al., 2024).

For instance, in vitro-based high-throughput imaging technology is also being used to test environmental chemicals as a NAMs-based strategy to identify carcinogenic chemicals. In a study conducted by Judson et al. (2010), chemical toxicity profiling was developed as an initial toxicity prioritization for 309 chemicals (mostly pesticides) in 467 assays across 9 technologies, including high-throughput cell-based assays, in multiple human primary cells, human cell lines, and primary rat hepatocytes (Judson et al., 2010). Their results showed that toxicity profiling through *in vitro* screening is a promising approach for the assessment of NGTxC activity.

Similarly, in a study conducted by Shah et al. (2021) to screen a panel of hepatotoxic chemicals using High Content Imaging (HCI) in PHH, their results suggest that HCI applied with PHH can provide detailed and quantitative data. When combined with IVIVE, these data show high potential for predicting hepatotoxicity (Shah et al., 2021).

Overall, HTS assays used to identify KCC associated with different chemicals could help fill data gaps, especially in the identification of hazard associated with different NGTxC. In addition, combining several mechanistic endpoints through these screening assays could play a significant role in improving the ability to distinguish between GTxC and NGTxC (Rigutto et al., 2025). However, the sensitivity of such assays and the lack of a whole-organism system highlight the need for more validated and integrated approaches before replacing long-term *in vivo* bioassays (Jeong et al., 2022).

7.2.3. Transcriptomic strategies

In addition, high-throughput transcriptomic approaches have been used to distinguish genotoxic GTxC from NGTxC. Transcriptomic approaches allow for the generation of signature global response profiles of a tested chemical at the molecular level. Oku et al. (2022) reported that, by applying transcriptomic profiling, they were able to identify key pathways and gene sets relevant to different NGTxC MoA, such as inflammation, immune response, cell injury, mitogenic signalling, and stress responses. However, these authors reported limitations regarding the reproducibility of such tests when applied to detect NGTxC, as well as inter-laboratory variations associated with these approaches.

Moreover, gene signatures can vary between studies or experimental conditions, which complicates their reliability. Thus, it is challenging to translate fold changes in gene expression or general response profiles into meaningful physiological and toxicological responses relevant to human exposure (Oku et al., 2022). These findings suggest that while transcriptomics could provide an important tool for generating mechanism-based data, relying solely on such assays could be challenging for interpreting the hazard associated with tested chemicals.

Furthermore, the selected *in vitro* cellular model can influence the overall performance of the applied transcriptomic analysis. For instance, since xenobiotic metabolism strongly influences chemical activity, metabolically active cellular models, such as HepaRG cells, could provide more accurate predictions of chemical toxicity through the evaluation of their gene profiles. A large number of cell lines used in the toxicological evaluation of chemicals lack sufficient xenobiotic metabolism and may miss certain chemicals for which biological activity depends on bioactivation. The use of such cell lines in NGTxC evaluation, therefore, results in less predictive power for identifying associated toxic effects (Doktorova et al., 2013). The integration of xenobiotic metabolism into *in vitro* high-throughput workflows is critical for mitigating the risk of false negatives in chemical hazard assessment. Jurgelewicz et al. (2025) demonstrated that conventional, metabolically deficient cell lines frequently underestimate the bioactivity of chemicals that require metabolic activation to engage cellular pathways, such as estrogen receptor signalling. By comparing metabolically competent models, like HepaRG cells, with standard non-metabolizing lines, the study revealed that functional metabolism significantly shifts both phenotypic responses and transcriptomic signatures. These findings highlight that metabolic bioactivation can transform seemingly inert compounds into potent endocrine disruptors, a phenomenon that traditional models fail to capture. Consequently, the authors advocate for the strategic selection of metabolically active cellular models to enhance the sensitivity and predictive power of toxicogenomic data, ensuring that HTS more accurately reflects the complex physiological realities of human exposure and risk (Jurgelewicz et al., 2025).

Although transcriptomics aids in the identification of response signatures and associated KE of different carcinogens at the molecular level for both GTxC and NGTxC, its application can be problematic. As changes in gene expression caused by NGTxC are often variable and tissue-specific due to their various MoAs, this can complicate their identification and classification through transcriptomic approaches (Doktorova et al., 2014).

Thus, transcriptomic approaches alone are not sufficient to be integrated into a validated regulatory framework for testing and assessing chemical carcinogens. Therefore, it has been suggested that transcriptomic approaches are more effective as part of an integrated testing and assessment framework that combines transcriptomic signatures with other functional assays and NAMs for evaluating NGTxC (Oku et al., 2022).

7.3. Current tests in regulatory validation phase

The Cell Transformation Assay (CTA) was recognized as one of the *in vitro* tests under consideration by regulatory bodies such as IATA/OECD (Jacobs et al., 2016). Originally, the CTA was developed to explore the multicellular process of carcinogenesis, in particular both the initiation and promotion steps. Soon, the CTA became an *in vitro* test providing WoE to support existing *in vitro* tests and knowledge about NGTxC (Colacci et al., 2023). The CTA measures the morphological changes of the treated cells, which provide important insight into tumour formation and the carcinogenic potential of the tested chemicals. *In vitro* CTA studies allow the characterization of various cellular alterations associated with the cell transformation process (Steinberg, 2017), including:

1. Acquisition of an infinite lifespan and immortalization through unlimited cell growth.
2. Cell morphology alterations.
3. Alterations in growth patterns through the multi-layered growth of cultured or treated cells.
4. Acquisition of autonomous growth through the ability to grow independently to form colonies or foci.

Despite these advantages, further mechanistic understanding is still missing to better understand the exact process of tumour formation with CTA tests (Colacci et al., 2023). Different CTA models using different types of cell lines or primary cells have been used. However, there are only three commonly used assays for testing the transformation potential of chemicals and carcinogens which include the Syrian hamster embryo (SHE) CTA, BALB/c3T3 CTA, and Bhas42 CTA.

Historically, chemically induced transformation studies in the context of the CTA were first introduced in 1963 (Figure Int. 20). Different CTA models using various types of cell lines or

primary cells have been used. However, there are only three most commonly used assays for testing the transformation potential of chemicals and carcinogens: the SHE CTA, the BALB/c 3T3 CTA, and the Bhas42 CTA.

As shown in Figure Int. 20, the first CTA method used was the SHE cell CTA (Groupe & Manaker, 1956). It has been successfully applied to the study of virus-induced cell transformation (Sachs & Medina, 1961). SHE cells are normal diploid primary cells isolated from the Syrian hamster embryo. As primary cells, SHE cells retain the ability to metabolize a wide range of xenobiotics, as shown in previous studies with indirect carcinogens requiring metabolic activation (Maire et al., 2012). SHE cells are stable, as they show low rates of spontaneous transformation. They can detect the early steps of transformation leading to malignancy and the formation of transformed colonies (Defendi & Lehman, 1965; Ahmadzai et al., 2012). However, the SHE cell assay is conducted within a variable pH range (6.7–7.3), which is not fully standardized and could impact normal xenobiotic biotransformation and affect how chemicals interact with DNA or cellular metabolism (LeBoeuf et al., 1996). In addition, SHE cells have a short lifespan and are associated with ethical concerns, as they are primary cells, along with many other limitations (Tsuda, 2003).

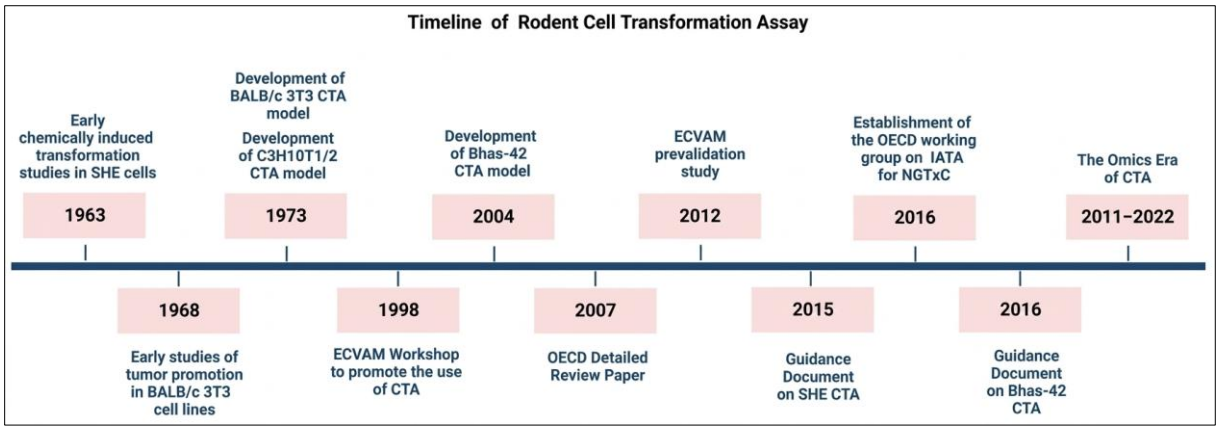

Figure Int. 20. Timeline of the development and regulatory validation of rodent CTAs. The chronology spans from early chemically induced transformation studies in SHE cells (1963) to the integration of high-throughput "Omics" technologies (2011–2022) (Colacci et al., 2023).

Other cell types have been established to offer additional advantages to the CTA assay and increase its efficiency and performance. The BALB/c 3T3 CTA model was the first cell line-based CTA assay used. BALB/c 3T3 originates from the clone A-31 of mouse whole-embryo fibroblasts derived from the BALB/c mouse strain (Mascolo et al., 2010) (Figure Int. 21). Like SHE cells, BALB/c 3T3 cells retain sufficient metabolic activity, CYP induction,

and Phase I and Phase II enzyme activity to support the metabolism of most carcinogens. Although different BALB/c 3T3 strains have been developed (Figure Int. 21), the BALB/c 3T3 clone A31-1-1 is the most widely used, as it has a relatively high susceptibility to transforming agents. In contrast to the SHE CTA assay, BALB/c 3T3 cell lines are able to detect not only initiators but also promoters (Maire et al., 2012). Mascolo et al. (2010) emphasized that BALB/c 3T3 cells are highly suitable for evaluating the transformation activity of both genotoxic and non-genotoxic chemicals, whether tested individually or in mixtures. However, this assay extends up to six weeks and requires 32 days to allow the transformed foci to grow to a sufficient size for analysis (Mascolo et al., 2010).

The utility of BALB/c 3T3 cells was further improved when they were transfected with an activated ras oncogene (v-Ha-ras), resulting in the establishment of a clone known as Bhas42, derived from BALB/c 3T3 clone A31-1-1 cells, which carries the ras oncogene (Figure Int. 21). The Bhas42 CTA was established by Sakai et al. (2010). The v-Ha-ras oncogene enhances the sensitivity of Bhas42 cells in transformation assays by acting as an initiating agent, thereby making the cells more susceptible to subsequent tumour-promoting stimuli. In addition, it allows the assay to identify carcinogens that may not be detected by traditional methods such as genotoxic carcinogens. Moreover, it promotes continuous growth, making transformed foci easier to detect. As Bhas42 cells are capable of detecting both initiators and promoters, the Bhas42 CTA assay is divided into two concurrent tests: the initiation and promotion assays. These cells show high sensitivity to the formation of foci, requiring up to 21 days in comparison with the BALB/c 3T3 CTA assay, which requires up to 31 days. Due to its enhanced sensitivity, it has been internationally recognized and recommended for the development of OECD guidelines (Sasaki et al., 2014).

The CTA assay has proven its ability to address one of the KEs and key data gaps in cell transformation and tumour formation, both for initiators and promoters. Changes in cell morphology represent a key point in adaptive sustained proliferation and hyperplasia that enable tumour formation (Colacci et al., 2023). However, this assay suffers from limitations related to non-automated transformed foci counting, as well as insufficient data and a limited number of chemicals evaluated with this assay (Sakai et al., 2010). Moreover, as stated earlier, the CTA assay does not provide mechanistic insights into the transformation of exposed cells.

Based on this, different transcriptomic approaches are being applied in combination with the Bhas42 CTA. Transcriptomic analyses applied to the Bhas42 CTA offer critical insights into

the molecular mechanisms underlying chemical-induced carcinogenesis. These approaches enable the identification of changes in gene expression over time, highlighting key pathways and biomarkers associated with transformation processes. By integrating transcriptomic data, mechanistic information can be used to better distinguish between genotoxic and non-genotoxic carcinogens, enhancing the predictive accuracy of *in vitro* assays for carcinogenic potential (Ohmori et al., 2022; Oku et al., 2022).

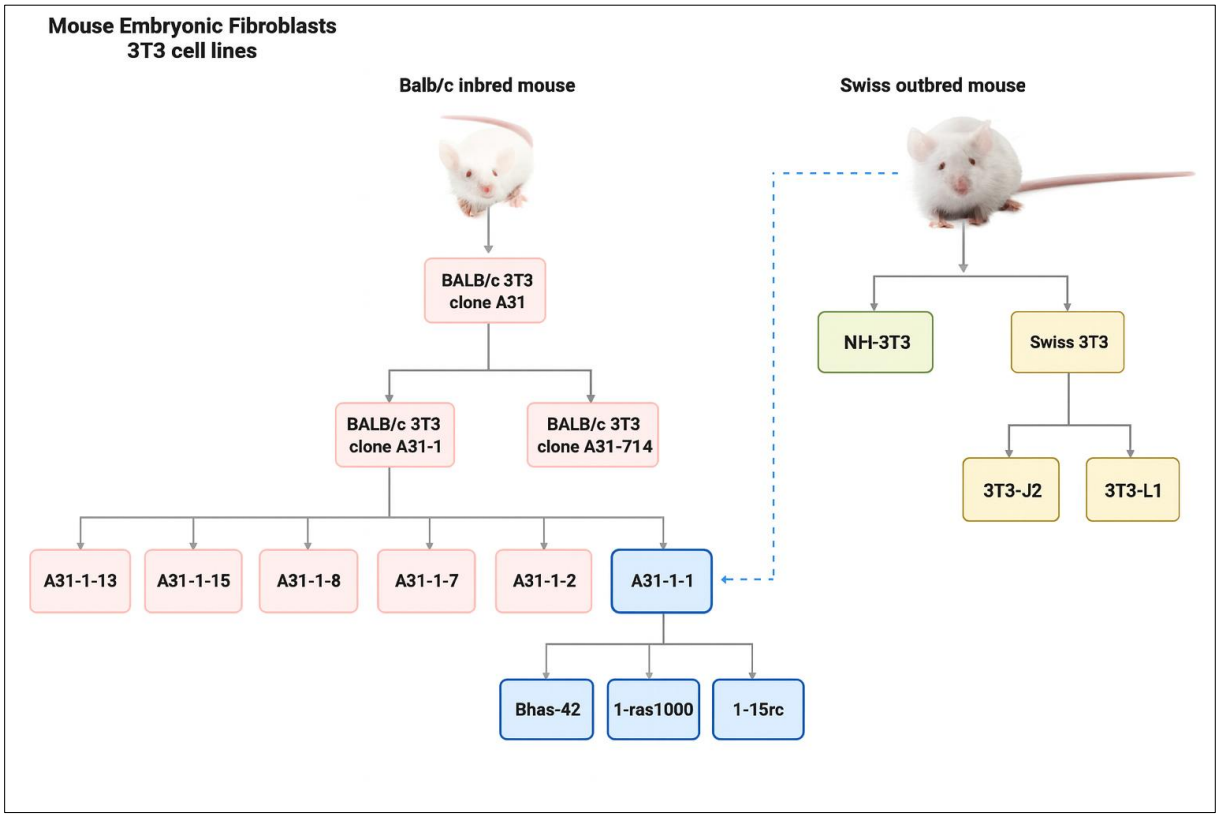

Figure Int. 21. Family tree that shows the origins of the cells used in cell line-based CTA assays (Colacci et al., 2023).

8. Liver as a case study

8.1. Liver toxicity

The liver represents a vital organ for human life. It plays a central role in drug metabolism and remains the primary organ for drug and xenobiotic metabolism through biotransformation and elimination via Phase I enzymes (primarily cytochrome P450 enzymes (CYPs)) and Phase II (conjugation) reactions, as well as through transporter-mediated uptake and excretion (Trefts et al., 2017). The liver plays a critical role in toxicity assessment, as it is a primary target for drug-induced toxicity. Drug-induced liver injury (DILI) and hepatotoxicity are leading causes

of acute or chronic liver failure (Thakkar et al., 2018). Liver damage caused by drugs and xenobiotics is a complex adverse effect (Skat-Rørdam et al., 2025). Such adverse effects can be influenced by several intrinsic factors, such as age, genetics (including polymorphisms in genes encoding CYPs and other drug-metabolizing enzymes), lifestyle (alcohol consumption, medications, and high-fat diets), and sex, in addition to the adverse effects of the xenobiotic itself through its intrinsic characteristics (e.g., electrophilicity) and exposure dose (Sahlman et al., 2020). This raises a significant challenge for clinicians and the pharmaceutical industry, as such injuries are often associated with serious consequences ranging from functional disruption of the liver to irreversible damage leading to cancer and death (Wang et al., 2025). Unlike direct DILI caused by xenobiotics that lead to acute liver failure, chronic DILI or prolonged drug-induced hepatotoxicity could potentially contribute to liver cancer. Chronic DILI may result in prolonged inflammation, fatty liver (excess fat in liver cells), fibrosis (scar tissue in the liver), and cirrhosis (irreversible scarring that disrupts liver function and structure), which in turn represents a major risk factor for Hepatocellular Carcinoma (HCC) (Safri et al., 2024).

Primary liver cancer originates in the liver itself and includes several types of cancer based on cell origin and pathological features, such as HCC, intrahepatic Cholangiocarcinoma (CAA), angiosarcoma, hemangiosarcoma, and hepatoblastoma. In contrast, secondary liver cancer refers to tumours that have spread (metastasized) to the liver from other parts of the body, such as the colon or pancreas. Among all types, HCC is the most prevalent, accounting for approximately 90% of primary liver cancer cases (Sharma et al., 2017; Yasir & Torbenson, 2019; Wang et al., 2021). HCC is the sixth most frequently diagnosed cancer and the third leading cause of cancer-related death worldwide (Pinazo-Bandera et al., 2022). In 2020, around 724,800 new cases of HCC were diagnosed globally, and approximately 664,000 deaths across 158 countries were attributed to HCC (Sung et al., 2021).

In addition, Heusinkveld et al. (2020) conducted a study with a total of 340 agrochemicals classified as GTxC, NGTxC, non-carcinogens, and others to investigate organ sensitivity and the specific target organ of the selected chemicals. Their results showed that the liver was the organ primarily targeted by most of the chemicals (around 107 of the total) compared with other tissues (Figure Int. 22). Moreover, 99 of the 107 chemicals targeting the liver tissue were categorized as chemicals associated with HCC. These data provide additional evidence that the liver is the primary target organ for most environmental contaminants, particularly carcinogens.

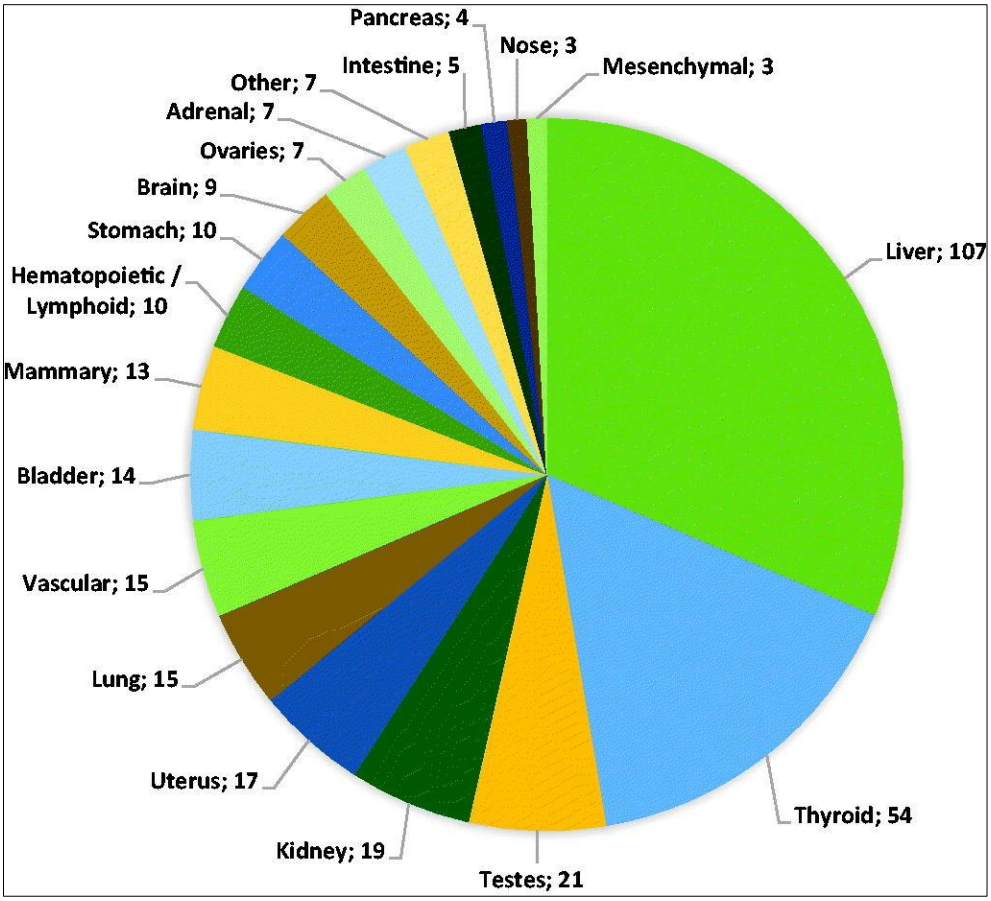

Figure Int. 22. Pie chart illustrates the number of chemicals targeting specific organs or tissues, based on a dataset of 340 agrochemicals classified as GTxC, NGTxC, non-carcinogens, and others. Each segment represents the number of chemicals associated with a particular organ or tissue. The liver is the most frequently targeted organ, with 107 chemicals, followed by the thyroid (54), testes (21), and kidney (19). These findings highlight organ sensitivity and the prevalence of HCC among chemical exposures (Heusinkveld et al., 2020).

8.2. Cellular structure of the liver

The liver is a dynamic, heterogeneous organ. It is composed of several cell types, including parenchymal hepatocytes and non-parenchymal cells such as stellate cells, Kupffer cells (immune cells), biliary epithelial cells (cholangiocytes), and liver endothelial cells. Each cell type plays a critical role and performs essential functions that regulate liver activity at multiple levels (Trefts et al., 2017).

The liver is organized into structural and functional units. The hepatic lobule represents the classical morphological unit of the liver and is characterized by a hexagonal structure located around a central vein, composed mainly of hepatocytes arranged in plates forming a hexagonal structure organized around the central vein (Figure Int. 23) (Figure Int. 24). The apical surface of each hepatocyte contributes to the formation of bile canaliculi, while the basal surface faces the sinusoids, which are highly permeable blood capillaries. The bile ducts collect bile produced

by the lobules, whereas the sinusoids supply nutrients to the lobules and collect substances arriving at the liver (Trefts et al., 2017).

Hepatocytes perform a wide range of essential metabolic, synthetic, and detoxifying functions. They are responsible for producing bile, plasma proteins, and clotting factors, as well as regulating carbohydrate, lipid, and protein metabolism. They also play a critical role in detoxifying harmful substances, processing drugs, and metabolizing waste products. Additionally, hepatocytes are highly regenerative, enabling the liver to recover from injury. Their dysfunction, however, underlies many forms of liver disease and contributes to the progression of hepatic pathology (Gong et al., 2022).

Cholangiocytes represent the second most common epithelial cell population, and these cells mainly contribute to bile secretion (Trefts et al., 2017; Oderberg & Goessling, 2023).

Kupffer cells are the resident macrophages of the liver responsible for eliminating pathogens and non-functional blood cells. They recognize the many pathogenic stimuli introduced through the portal circulation and act as the first line of immune defence by phagocytosing pathogens, cellular debris, and damaged erythrocytes. In addition, they secrete cytokines and other mediators that regulate inflammation, recruit immune cells, and influence liver regeneration. Kupffer cells also contribute to maintaining immune tolerance toward dietary and gut-derived antigens, thereby preventing excessive immune activation. However, in the setting of chronic liver injury, they can promote fibrosis by releasing pro-inflammatory and pro-fibrogenic factors that activate hepatic stellate cells (Trefts et al., 2017; Zhao et al., 2025).
Stellate cells are a versatile cell population that can exist in either a quiescent or an activated state. In their quiescent form, they primarily serve as vitamin A reservoirs stored within lipid droplets, although their additional functions in this state are not yet fully understood. Liver injury triggers their activation, during which stellate cells proliferate and gradually lose their vitamin A content. Once activated, they play a central role in collagen deposition and remodeling within the damaged liver. This fibrotic response promotes scar formation, which can ultimately progress to cirrhosis, a major contributor to end-stage liver disease (Yin et al., 2013; Trefts et al., 2017).

Finally, liver sinusoidal endothelial cells are a specialized endothelial population with unique characteristics. These cells form fenestrated sieve plates at the sinusoidal lumen (Trefts et al., 2017; Kaczara et al., 2024).

This specialized anatomy and cellular composition of the liver contribute to its function and to how the liver interacts with xenobiotics. In addition, these structural and compositional features reinforce the importance of the different liver cellular models used *in vitro* in toxicological assessments, and the relevance of the various cell lines employed in relation to NAMs-based CSA. In other words, do the different liver cell lines currently used *in vitro* accurately reflect real liver function and mimic the *in vivo* situation?

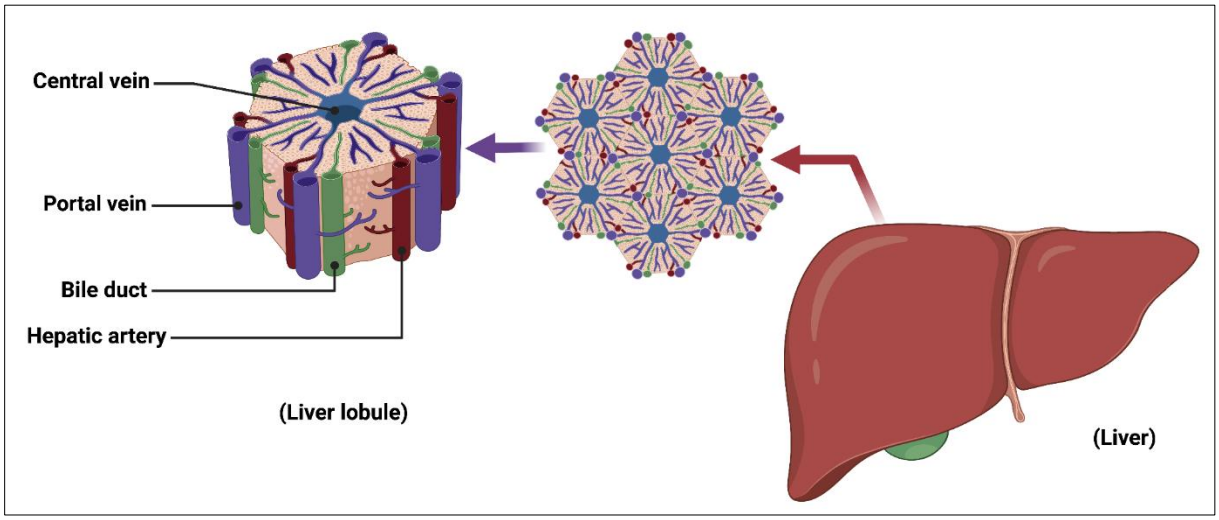

Figure Int. 23. The Liver Lobule: primary structural unit. BioRender. https://biorender.com

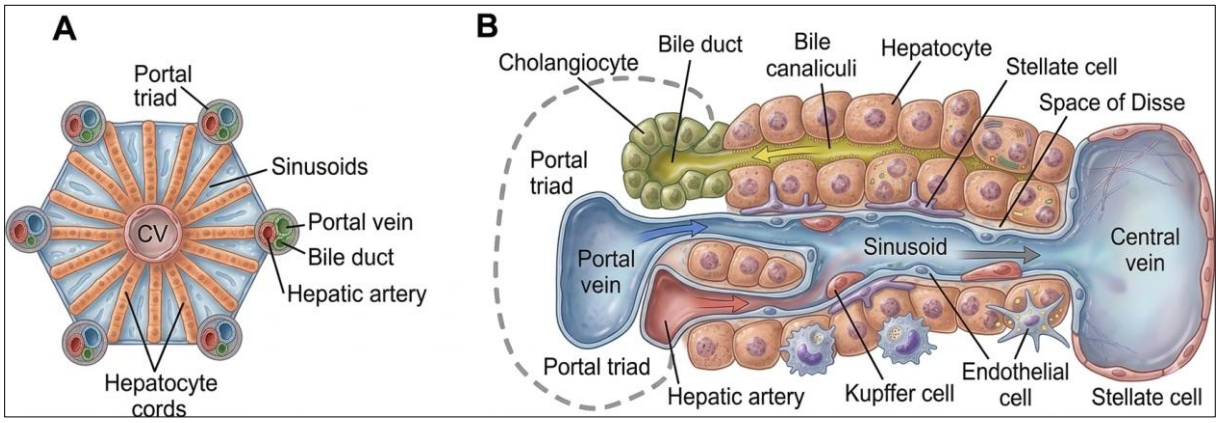

Figure Int. 24. Hepatic Lobule structure and its constituent cell populations (Rangel et al., 2022).

8.3. *in vitro* liver models

A variety of liver cell models are used in toxicological studies, as shown in Table Int. 9. PHHs represent the gold standard for liver cell culture; however, as they originate directly from patients, they are expensive and have limited availability (Han et al., 2021). In addition, inter-individual differences and the loss of liver functionality when cultured *in vitro* complicate

their use. Hence, different commercial hepatic cell lines have been introduced. Among others, HepG2, HepaRG, and other cell lines are shown in Table Int. 9.

HepaRG cells are bipotent progenitors and can differentiate into both hepatocyte-like and cholangiocyte-like cells while maintaining key features of mature human hepatocytes, such as the expression of phase I and phase II metabolic enzymes and transporters (Ranade et al., 2014). Other liver models, including HepG2 cells and stem cell-derived hepatocytes, present limitations such as low metabolic activity or complex and prolonged differentiation times (Gómez-Lechón et al., 2003; Behbahan et al., 2011). Given these challenges, HepaRG cells are increasingly used as an alternative to PHHs and HepG2 cells for *in vitro* hepatotoxicity testing (Nelson et al., 2017; Guo et al., 2024).

Moreover, advanced *in vitro* systems, including 3D spheroids, co-cultures with different cell types, and organ-on-chip platforms, have been developed to better replicate liver physiology (Wu et al., 2016) (Figure Int. 25). These systems represent an important advancement in liver models being integrated within NAMs-based *in vitro* alternative approaches, as they increase relevance to humans and thus contribute to better chemical evaluation and assessments (Telles-Silva et al., 2022).

In recent years, HepaRG 3D spheroids have gained more popularity compared to other 3D liver models. In comparison, HepG2 3D spheroids exhibit limitations such as low glutathione synthesis, inconsistent albumin secretion, and abnormal karyotypes. Similarly, other liver 3D models like PHH and iPSC-derived hepatocytes, although valuable, are associated with significant drawbacks and high costs. PHH cells are restricted by donor specificity, limited availability, and low expression of Glutathione-S-transferase P1. iPSC-derived hepatocytes require prolonged differentiation processes, which limits their practical application. In contrast, 3D HepaRG models offer notable advantages over both conventional 2D HepaRG monolayers and alternative 3D liver models. These HepaRG spheroids model demonstrate: (1) increased urea and albumin secretion, (2) enhanced activity of cytochrome P450 enzymes, (3) higher expression of both phase I and phase II metabolizing enzymes, (4) functional p53 signalling, (5) greater sensitivity to hepatotoxic chemicals, and (6) the ability to differentiate into both hepatocytes and biliary cells (Štampar & Žegura, 2024).

Despite these improvements, spheroid models still present challenges, including diffusion gradients, spheroid size, and the absence of the full cellular complexity of the hepatic microenvironment. More advanced systems, such as liver organoids derived from stem cells or

patient tissues, further recapitulate aspects of liver architecture and cellular diversity, making them valuable tools for disease modeling and toxicological. Nevertheless, organoid generation remains technically demanding, and the lack of vascularization can restrict nutrient and oxygen diffusion. Consequently, i*n vitro* liver models have undergone substantial refinement in to more accurately reproduce the structural and functional complexity of the human liver. Organ-on-chip technologies have emerged as promising platforms that incorporate microfluidic systems to mimic physiological flow, mechanical forces, and multicellular interactions characteristic of the liver sinusoid (Dalsbecker et al., 2022). These dynamic systems provide a more physiologically relevant microenvironment and improve the predictive capacity for drug-induced liver injury. However, their broader application is currently limited by high cost, technical complexity, and challenges related to standardization and scalability. Consequently, each model offers distinct advantages and limitations, and the selection of an appropriate system depends on the specific biological question under investigation (Mugaanyi et al., 2025).

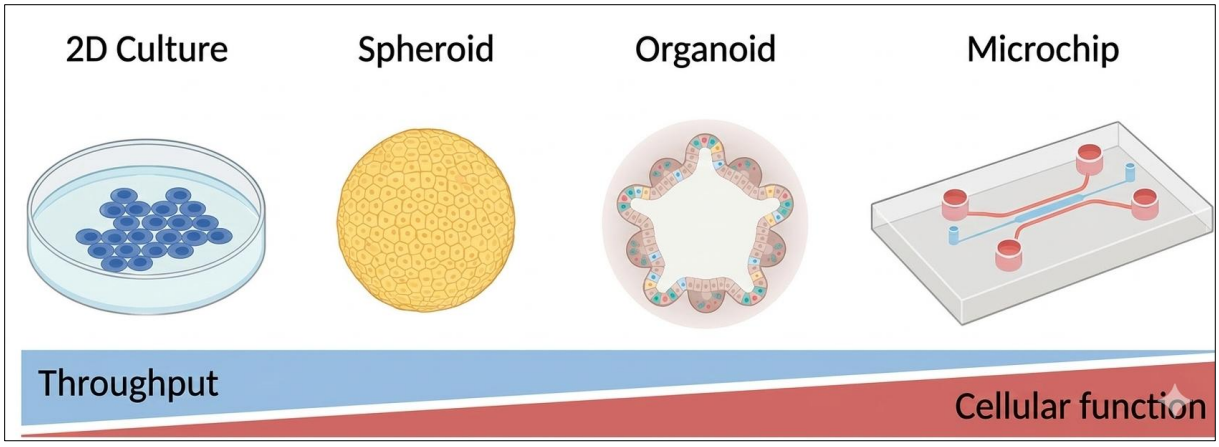

Figure Int. 25. *In vitro* liver models. The figure illustrates a comparison of throughput and cellular function, from high-throughput 2D cultures to high-functionality microfluidic "organ-on-a-chip" systems (Telles-Silva et al., 2022).

Table Int. 9. Summary of different *in vitro* liver cell models.
This table summarizes the origin, relevance to human liver, advantages, and limitations of commonly used *in vitro* liver cellular models.

Cell Model	Origin	Relevance to humans	Advantages	Limitations	References
HepG2	Human liver cancer cell line, derived from the liver tissue of the 15-year-old Caucasian male with a well-differentiated hepatocellular carcinoma.	Partially resembles human hepatocytes.	High proliferation rate, easy to culture, cost-effective, suitable for high-throughput screening.	Limited expression of liver-specific functions, lacks full cytochrome P450 (CYP) activity, not fully representative of *in vivo* hepatocytes.	(Aden et al., 1979 ; Xuan et al., 2016)
HepaRG	Isolated and established from a liver tumor called cholangiohepatocarcinoma of a patient suffering from chronic hepatitis C virus infection.	Closely mimics human hepatocytes	Full array of function of primary human hepatocytes, Formation of bile canaculi, having a strong capacity of self-renewal, High expression of CYP1A2, 2B6, 2C9, 2E1, 3A4 and nuclear receptors.	Complex differentiation protocol, potential for dedifferentiation over time, lack CYP2D6 activity .	(Gripon et al., 2002 ; Gupta et al., 2017); Hammour et al., 2022
Human Primary Hepatocytes (PHHs)	Isolated directly from human liver tissue.	highly relevant to human liver biology.	High fidelity to *in vivo* liver function, express all major CYP enzymes, retain native morphology and function of human liver.	Limited availability, donor variability, loss of function upon isolation, ethical concerns, limited proliferation capacity.	(Berry & Friend, 1969; Kaur et al., 2023)
iPSC-Derived Hepatocytes	Generated from induced pluripotent stem cells.	Potential to closely mimic human hepatocytes.	Unlimited source material, can model patient-specific diseases studies, can differentiate into hepatocyte-like cells.	Limited differentiation efficiency, immature phenotype, variability in function, difficult and requires an optimization of differentiation protocols.	(Takahashi & Yamanaka, 2006; Corbett & Duncan, 2019)

9. Advancing NGTxC hazard assessment: integration of PARC project

PARC[1] is a project funded by the EU under the Horizon Europe framework program (2021–2027) and builds on ongoing H2020 projects, including the ASPIS and EURION clusters, as well as activities by the OECD. It aims to enhance knowledge about the potential hazard of chemicals to better protect human health and the environment. With a particular emphasis on developing and validating NAMs, including *in vitro* and *in silico* strategies, for areas such as carcinogenicity and in particular non-genotoxic carcinogens, endocrine disruption, immunotoxicity, neurotoxicity, and metabolic disruption thereby reducing reliance on animal testing and closing data gaps in CSA.

The project is Coordinated by The French Agency for Food, Environmental and Occupational Health & Safety (ANSES), and is structured in into nine work packages addressing diverse activities including (among other things) hazard assessment, exposure, monitoring, methodology, and regulatory uptake. The Toxicology of contaminants unit at ANSES Fougères is part of Work Package 5 (WP5), which mainly focuses on chemical hazard assessment. This WP is divided into different tasks, Task 5.2.1 (NAMs for human health), a subcomponent dedicated to NGRA, particularly for NGTxCs.

Within PARC, work package 5.2.1, aims to advance the hazard assessment of NGTxCs through the development and integration of NAMs. The project brings together a consortium of European partners contributing complementary expertise in assay development to establish a comprehensive testing strategy for the identification and characterization of NGTxCs. A diverse panel of human-relevant NAMs is being implemented and integrated across the consortium, including *in vitro* and *in silico* approaches such as high-content imaging assays, transcriptomics, epigenetic analyses, receptor-mediated pathway assays, metabolomic profiling, and advanced cellular models including 3D spheroids, organoids, and zebrafish embryo systems. These models collectively address multiple key biological processes associated with carcinogenesis and are aligned with the framework of AOPs and the hallmarks of cancer. By systematically evaluating these complementary methodologies using well-characterized reference compounds, the project seeks to identify sensitive and mechanistically informative biomarkers of NGTxC activity and to determine how individual assays can be combined into an integrated testing strategy. Such integration is expected to improve hazard assessment by enhancing mechanistic

[1] https://www.eu-parc.eu/

understanding, increasing predictive capacity, and enabling the identification of relevant MoA without relying on long-term animal studies. WP5.2.1, aims to develop sensitive and reliable test batteries for identifying NGTxCs MoAs through the use of diverse *in vitro* testing approaches and systems. The defined strategies are expected to contribute to both OECD and EU CSA frameworks to identify NGTxC**.**

Within this framework, ANSES contributes by implementing high-content analysis, and transcriptomics approaches in liver cell models to quantify cellular processes associated with carcinogenesis, including oxidative stress, nuclear receptor translocation, epigenetic modifications, inflammatory responses, and mitochondrial metabolism. This multiparametric approach enables high-throughput evaluation of chemical effects at single-cell resolution and generates mechanistic data that contribute to the overall integration of NAMs within the PARC NGTxC assessment strategy. For further details regarding the methodological framework and consortium contributions within PARC work package 5, the full article is provided in (Appendix Int.)

10. Key Challenges in NGTxC hazard assessment targeted in this thesis

The identification and assessment of NGTxCs remain a major challenge within current CSA frameworks. As summarized in Table Int. 10, NGTxCs are characterized by substantial mechanistic diversity, acting through multiple biological pathways such as endocrine disruption, immune and inflammatory responses, epigenetic alterations, receptor-mediated signalling, and perturbations of tissue homeostasis. In contrast to genotoxic carcinogens, these mechanisms do not involve direct DNA damage and may operate through complex, tissue-specific processes that are not readily captured by conventional testing strategies.

Consequently, the detection of NGTxCs remains difficult within current regulatory workflows, which often rely on genotoxicity testing batteries followed by long-term rodent carcinogenicity studies. These approaches are not only resource-intensive and time-consuming but may also generate species-specific responses and high-dose artefacts that limit their relevance for human risk assessment. Moreover, the absence of validated mechanistic biomarkers and the limited availability of well-characterized NGTxC datasets further complicate the development of predictive and mechanistically informed assessment strategies.

Thus, the integration of NAMs represents a promising strategy to improve the identification and characterization of NGTxCs. NAMs offer the potential to capture mechanistic information at the molecular and cellular levels while enabling higher-throughput and more human-relevant testing approaches. However, their integration into carcinogenicity assessment frameworks requires the generation of robust mechanistic data and the evaluation of their ability to detect KE associated with NGTxC.

Table Int. 10. Summary of key challenges associated with the identification of NGTxC (Jacobs et al., 2020).

Challenge	Description	Reference
Mechanistic diversity	NGTxCs act via multiple pathways (e.g. hormonal disruption, epigenetic effects, immune/inflammation responses, receptor signalling, altered tissue homeostasis). With the absence of direct DNA interaction. In addition, one NGTxC can act through one or multiple of these different pathways. Thus, multiple assays are needed to assess their diverse effects.	(Audebert et al., 2023)
Tissue- and species-specific effects	Many NGTxC effects are tissue and or organ-specific, in addition, differ between rodents and humans.	(Luijten et al., 2020)
Detection gap	Standard regulatory carcinogenic assessment begins with genotoxicity test battery. when such test battery is negative, often no further carcinogenicity testing is done, so NGTxCs may remain unidentified.	(Jacobs et al., 2016)
Dependence on 2-year rodent bioassay and high dose artefacts	The long-term rodent carcinogenicity assay is time-consuming, expensive, and may produce rodent-specific tumour responses.	(Suarez-Torres et al., 2021)
Lack of validated biomarkers	The lack of the regulatory approved guideline of guidance to detect and identify NGTxC.	(Jacobs et al., 2020)
Dose-response / threshold uncertainty	Unlike GTxC, NGTxCs may have thresholds, however establishing exposure thresholds is difficult because mechanisms are linear.	(Nohmi, 2018)
Data gaps	Compared to genotoxic carcinogens, fewer well-characterized NGTxCs and mechanistic datasets exist.	(Audebert et al., 2023)

Thesis objectives

The present thesis aims to contribute to the international effort toward integrating NAMs for the identification of NGTxCs. This will be done by applying different NAM-based approaches to investigate their applicability and potential in identifying NGTxC-related MoAs. In particular, the work focuses on high-throughput screening methodologies, transcriptomic analyses, and the Bhas42 CTA, which is currently receiving increasing regulatory attention. Through these complementary approaches, the project seeks to generate mechanistic datasets that address several key challenges associated with NGTxC hazard assessment, including mechanistic diversity, the lack of validated biomarkers, and existing data gaps. By exploring KEs associated with NGTxCs at different biological levels (cellular, molecular, and apical) the thesis aims to improve the detection of relevant carcinogenic mechanisms while contributing to the development of an integrated *in vitro* framework for NGTxC identification and assessment. Ultimately, the findings generated in this work are expected to support the broader integration of NAM-based strategies into modern carcinogenicity testing and NGRA.

Building on the overall aim of this thesis is structured into three main objectives that address different biological levels:

Objective One:

The first objective, that will be addressed in Chapter One, is to evaluate the applicability of a multiparametric, high-content, high-throughput imaging strategy combined with a human-relevant *in vitro* cellular model to determine whether this approach can capture mechanisms associated with NGTxCs at the cellular level. These include markers of oxidative stress, mitochondrial dysfunction, lipid accumulation, cytotoxicity, and inflammatory responses.

The human-hepatic HepaRG cell line was selected for this purpose because of its metabolic competence and its ability to reproduce key functional characteristics of human hepatocytes, making it a suitable system for toxicological investigations. Both 2D monolayers and 3D spheroid configurations were used to explore how culture architecture influences the detection of NGTxC-related cellular perturbations.

A further component of this objective is the comparison of responses between the two HepaRG culture systems to assess potential differences in sensitivity and biological relevance.

Evaluating how 2D and 3D models respond to the same chemical exposures supports the selection of the most appropriate cellular system for subsequent chapters and helps determine the suitability of this NAM-based approach for detecting cellular perturbations linked to NGTxC mechanisms. Within this objective, we will be able to produce comprehensive cellular toxicity profiles for the tested chemicals, which could help in the selection of chemicals for the following strategies.

Objective Two:

The second objective is to apply a high-throughput transcriptomics approach using the most sensitive and responsive HepaRG cell model will be identified in Chapter 1. Objective sought to explore whether transcriptomic profiling could provide deeper mechanistic insights into the underlying signalling pathways induced by the selected chemicals.

Within this framework, the study aims to generate comprehensive gene expression profiles following chemical exposure in order to characterize the molecular perturbations associated with each compound. These transcriptomic datasets were then used to explore whether chemicals could be classified into genotoxic carcinogens, non-genotoxic carcinogens, or non-carcinogenic compounds based on their gene expression signatures.

In addition, the analysis aims to identify molecular KEs and pathways potentially associated with NGTxC mechanisms by examining the biological processes and singnaling pathways perturbed at the transcriptional level.

Overall, this chapter aims to evaluates the applicability of high-throughput transcriptomics as a NAM-based strategy for classifying chemicals and providing mechanistic insight at the molecular level, thereby strengthening the mechanistic understanding of NGTxC-related processes and supporting the development of integrated approaches for NGTxC identification.

Objective Three:

The third objective is to evaluate the applicability of the Bhas42 CTA as an *in vitro* approach for investigating the carcinogenic potential of NGTxCs. The Bhas42 CTA is currently under consideration within the OECD framework, with a guidance document available by regulatory agencies. This assay is particularly relevant for the detection of tumor-promoting activity, a mechanism commonly associated with NGTxC MoAs.

Within this context, the study in chapter three applies the Bhas42 CTA following the available standard guidance protocol in order to assess its potential for detecting carcinogenic effects induced by NGTxCs *in vitro*. Given that the assay is not yet fully validated for regulatory use, this work also aims to explore its practical applicability and to identify potential methodological considerations or improvements that could support its further development and regulatory integration.

To contribute to addressing existing data gaps, a selection of chemicals that had not previously been evaluated using the CTA was tested. By generating new transformation data for these compounds, this study seeks to expand the available evidence base for the assay and contribute to ongoing international efforts to integrate the Bhas42 CTA into NGTxC hazard assessment strategies.

References

Aden, D. P., Fogel, A., Plotkin, S., Damjanov, I., & Knowles, B. B. (1979). Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature*, *282*(5739), 615–616. https://doi.org/10.1038/282615a0

Adjiri, A. (2017). DNA mutations may not be the cause of cancer. *Oncology and Therapy*, *5*(1), 85–101. https://doi.org/10.1007/s40487-017-0047-1

Alam, M. S., Tahriri, F., & Chen, G. (2025). Trends, challenges, and research pathways in emerging contaminants: A comprehensive bibliometric analysis. *Integrated Environmental Assessment and Management*, *21*(3), 496–506. https://doi.org/10.1093/inteam/vjae036

Anetor, J. I., Anetor, G. O., Adeola, S., Esiaba, I., Anetor, J. I., Anetor, G. O., Adeola, S., & Esiaba, I. (2012). Chemical carcinogenesis: Risk factors, early detection and biomedical engineering. In *Biomedical science, engineering and technology*. IntechOpen. https://doi.org/10.5772/20209

Anthérieu, S., Chesné, C., Li, R., Guguen-Guillouzo, C., & Guillouzo, A. (2012). Optimization of the HepaRG cell model for drug metabolism and toxicity studies. *Toxicology in Vitro*, *26*(8), 1278–1285. https://doi.org/10.1016/j.tiv.2012.05.008

Atkins, J. T., George, G. C., Hess, K., Marcelo-Lewis, K. L., Yuan, Y., Borthakur, G., Khozin, S., LoRusso, P., & Hong, D. S. (2020). Pre-clinical animal models are poor predictors of human toxicities in phase 1 oncology clinical trials. *British Journal of Cancer*, *123*(10), 1496–1501. https://doi.org/10.1038/s41416-020-01033-x

Audebert, M., Assmann, A.-S., Azqueta, A., Babica, P., Benfenati, E., Bortoli, S., Bouwman, P., Braeuning, A., Burgdorf, T., Coumoul, X., Debizet, K., Dusinska, M., Ertych, N., Fahrer, J., Fetz, V., Le Hégarat, L., López de Cerain, A., Heusinkveld, H. J., Hogeveen, K., … Oelgeschläger, M. (2023). New approach methodologies to facilitate and improve the hazard assessment of non-genotoxic carcinogens—A PARC project. *Frontiers in Toxicology*, *5*, 1220998. https://doi.org/10.3389/ftox.2023.1220998

Baer-Dubowska, W., & Szaefer, H. (2024). From single DNA adducts measurement to DNA adductomics in molecular epidemiology of cancer. *Postepy Biochemii*, *70*(1), 52–56. https://doi.org/10.18388/pb.2021_509

Baert, K., Francois, K., Meulenaer, B. D., & Devlieghere, F. (2009). Risk assessment: A quantitative approach. In *Predictive modeling and risk assessment* (pp. 19–26). Springer. https://doi.org/10.1007/978-0-387-68776-6_2

Bailey, J. E. (2018). Socrates's last words to the physician god Asklepios: An ancient call for a healing ethos in civic life. *Cureus, 10*(12), e3789. https://doi.org/10.7759/cureus.3789

Balmain, A. (2020). The critical roles of somatic mutations and environmental tumor-promoting agents in cancer risk. *Nature Genetics, 52*(11), 1139–1143. https://doi.org/10.1038/s41588-020-00727-5

Baltazar, M. T., Cable, S., Carmichael, P. L., Cubberley, R., Cull, T., Delagrange, M., Dent, M. P., Hatherell, S., Houghton, J., Kukic, P., Li, H., Lee, M.-Y., Malcomber, S., Middleton, A. M., Moxon, T. E., Nathanail, A. V., Nicol, B., Pendlington, R., Reynolds, G., … Westmoreland, C. (2020). A next-generation risk assessment case study for coumarin in cosmetic products. *Toxicological Sciences, 176*(1), 236–252. https://doi.org/10.1093/toxsci/kfaa048

Barguilla, I., Maguer-Satta, V., Guyot, B., Pastor, S., Marcos, R., & Hernández, A. (2023). In vitro approaches to determine the potential carcinogenic risk of environmental pollutants. *International Journal of Molecular Sciences, 24*(9), 7851. https://doi.org/10.3390/ijms24097851

Barré-Sinoussi, F., & Montagutelli, X. (2015). Animal models are essential to biological research: Issues and perspectives. *Future Science OA, 1*(4), FSO63. https://doi.org/10.4155/fso.15.63

Behbahan, I. S., Duan, Y., Lam, A., Khoobyari, S., Ma, X., Ahuja, T. P., & Zern, M. A. (2011). New approaches in the differentiation of human embryonic stem cells and induced pluripotent stem cells toward hepatocytes. *Stem Cell Reviews and Reports, 7*(3), 748–759. https://doi.org/10.1007/s12015-010-9216-4

Berry, M. N., & Friend, D. S. (1969). High-yield preparation of isolated rat liver parenchymal cells: A biochemical and fine structural study. *The Journal of Cell Biology, 43*(3), 506–520. https://doi.org/10.1083/jcb.43.3.506

Birnbaum, L. S., Burke, T. A., & Jones, J. J. (2016). Informing 21st-century risk assessments with 21st-century science. *Environmental Health Perspectives, 124*(4), A60–A63. https://doi.org/10.1289/ehp.1511135

Boberg, J., Dybdahl, M., Petersen, A., Hass, U., Svingen, T., & Vinggaard, A. M. (2019). A pragmatic approach for human risk assessment of chemical mixtures. *Current Opinion in Toxicology, 15*, 1–7. https://doi.org/10.1016/j.cotox.2018.11.004

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians, 68*(6), 394–424. https://doi.org/10.3322/caac.21492

Brescia, S., Alexander-White, C., Li, H., & Cayley, A. (2023). Risk assessment in the 21st century: Where are we heading? *Toxicology Research, 12*(1), 1–11. https://doi.org/10.1093/toxres/tfac087

Browne, P., Paul Friedman, K., Boekelheide, K., & Thomas, R. S. (2024). Adverse effects in traditional and alternative toxicity tests. *Regulatory Toxicology and Pharmacology, 148*, 105579. https://doi.org/10.1016/j.yrtph.2024.105579

Brücher, B. L. D. M., & Jamall, I. S. (2016). Somatic mutation theory—Why it's wrong for most cancers. *Cellular Physiology and Biochemistry, 38*(5), 1663–1680. https://doi.org/10.1159/000443106

Bundy, J. L., Everett, L. J., Rogers, J. D., Nyffeler, J., Byrd, G., Culbreth, M., Haggard, D. E., Word, L. J., Chambers, B. A., Davidson-Fritz, S., Harris, F., Willis, C., Paul-Friedman, K., Shah, I., Judson, R., & Harrill, J. A. (2024). High-throughput transcriptomics screen of ToxCast chemicals in U-2 OS cells. *Toxicology and Applied Pharmacology, 491*, 117073. https://doi.org/10.1016/j.taap.2024.117073

Cadet, J., & Douki, T. (2018). Formation of UV-induced DNA damage contributing to skin cancer development. *Photochemical & Photobiological Sciences, 17*(12), 1816–1841. https://doi.org/10.1039/c7pp00395a

Carusi, A. (2024). Chemicals regulation and non-animal methods: Displacing the gold standard. *Wellcome Open Research, 9*, 167. https://doi.org/10.12688/wellcomeopenres.20581.2

Cavalieri, E., & Rogan, E. (2014). The molecular etiology and prevention of estrogen-initiated cancers. *Molecular Aspects of Medicine, 36*, 1–55. https://doi.org/10.1016/j.mam.2013.08.002

Cavallo, F., De Giovanni, C., Nanni, P., Forni, G., & Lollini, P.-L. (2011). 2011: The immune hallmarks of cancer. *Cancer Immunology, Immunotherapy, 60*(3), 319–326. https://doi.org/10.1007/s00262-010-0968-0

Cazzato, G., Ingravallo, G., & Ribatti, D. (2024). Angiogenesis still plays a crucial role in human melanoma progression. *Cancers, 16*(10), 1794. https://doi.org/10.3390/cancers16101794

Chang, M. C. J., & Grieder, F. B. (2024). The continued importance of animals in biomedical research. *Lab Animal, 53*(11), 295–297. https://doi.org/10.1038/s41684-024-01458-4

Chatterjee, N., & Walker, G. C. (2017). Mechanisms of DNA damage, repair, and mutagenesis. *Environmental and Molecular Mutagenesis, 58*(5), 235–263. https://doi.org/10.1002/em.22087

Chemical risk assessment | Food Packaging Forum. (2014). https://foodpackagingforum.org/resources/background-articles/chemical-risk-assessment

Chen, C., Lavezzi, S. M., & McDougall, D. (2024). The estimation and translation uncertainties in applying NOAEL to clinical dose escalation. *Clinical and Translational Science, 17*(6), e13831. https://doi.org/10.1111/cts.13831

Chen, J., Potlapalli, R., Quan, H., Chen, L., Xie, Y., Pouriyeh, S., Sakib, N., Liu, L., & Xie, Y. (2024). Exploring DNA damage and repair mechanisms: A review with computational insights. *BioTech*, *13*(1), 3. https://doi.org/10.3390/biotech13010003

Chormare, R., & Kumar, M. A. (2022). Environmental health and risk assessment metrics with special mention to biotransfer, bioaccumulation and biomagnification of environmental pollutants. *Chemosphere*, *302*, 134836. https://doi.org/10.1016/j.chemosphere.2022.134836

Clewell, H. J., & Fuchsman, P. C. (2024). Interspecies scaling of toxicity reference values in human health versus ecological risk assessments: A critical review. *Integrated Environmental Assessment and Management, 20*(3), 749–764. https://doi.org/10.1002/ieam.4842

Clewell, R. A., Thompson, C. M., & Clewell, H. J. (2019). Dose-dependence of chemical carcinogenicity: Biological mechanisms for thresholds and implications for risk assessment. *Chemico-Biological Interactions*, *301*, 112–127. https://doi.org/10.1016/j.cbi.2019.01.025

Cohen, S. M., Boobis, A. R., Dellarco, V. L., Doe, J. E., Fenner-Crisp, P. A., Moretto, A., Pastoor, T. P., Schoeny, R. S., Seed, J. G., & Wolf, D. C. (2019). Chemical carcinogenicity revisited 3: Risk assessment of carcinogenic potential based on the current state of knowledge of carcinogenesis in humans. *Regulatory Toxicology and Pharmacology, 103*, 100–105. https://doi.org/10.1016/j.yrtph.2019.01.017

Colacci, A., Corsini, E., & Jacobs, M. N. (2025). Addressing immune response dysfunction in an integrated approach for testing and assessment for non-genotoxic carcinogens in humans: A targeted analysis. *International Journal of Molecular Sciences, 26*(13), 6310. https://doi.org/10.3390/ijms26136310

Colacci, A., Corvi, R., Ohmori, K., Paparella, M., Serra, S., Da Rocha Carrico, I., Vasseur, P., & Jacobs, M. N. (2023). The cell transformation assay: A historical assessment of current knowledge of applications in an integrated approach to testing and assessment for non-genotoxic carcinogens. *International Journal of Molecular Sciences, 24*(6), 5659. https://doi.org/10.3390/ijms24065659

Committee (SC), More, S. J., Bampidis, V., Bragard, C., Halldorsson, T. I., Hernández-Jerez, A. F., Hougaard Bennekou, S., Koutsoumanis, K., Lambré, C., Machera, K., Naegeli, H., Nielsen, S. S., Schlatter, J., Schrenk, D., Turck, D., Younes, M., Aquilina, G., Bignami, M., Bolognesi, C., … Benford, D. (2021). Guidance on aneugenicity assessment. *EFSA Journal*, *19*(8), e06770. https://doi.org/10.2903/j.efsa.2021.6770

Compton, C. (2020). Cancer initiation, promotion, and progression and the acquisition of key behavioral traits. In *Cancer: The enemy from within* (pp. 25–48). Springer, Cham. https://doi.org/10.1007/978-3-030-40651-6_2

Corbett, J. L., & Duncan, S. A. (2019). iPSC-derived hepatocytes as a platform for disease modeling and drug discovery. *Frontiers in Medicine*, *6*, 265. https://doi.org/10.3389/fmed.2019.00265

Corvi, R., Madia, F., Guyton, K. Z., Kasper, P., Rudel, R., Colacci, A., Kleinjans, J., & Jennings, P. (2017). Moving forward in carcinogenicity assessment: Report of an EURL ECVAM/ESTIV workshop. *Toxicology in Vitro*, *45*, 278–286. https://doi.org/10.1016/j.tiv.2017.09.010

Council, N. R. (2014). *Review of EPA's Integrated Risk Information System (IRIS) process*. National Academies Press.

Dalsbecker, P., Beck Adiels, C., & Goksör, M. (2022). Liver-on-a-chip devices: The pros and cons of complexity. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *323*(3), G188–G204. https://doi.org/10.1152/ajpgi.00346.2021

Das, S., Kundu, M., Jena, B. C., & Mandal, M. (2020). Causes of cancer: Physical, chemical, biological carcinogens, and viruses. In S. C. Kundu & R. L. Reis (Eds.), *Biomaterials for 3D tumor modeling* (pp. 607–641). Elsevier. https://doi.org/10.1016/B978-0-12-818128-7.00025-3

Daugherty, C. G. (1995). The death of Socrates and the toxicology of hemlock. *Journal of Medical Biography*, *3*(3), 178–182. https://doi.org/10.1177/096777209500300310

Davis, J. A., Gift, J. S., & Zhao, Q. J. (2011). Introduction to benchmark dose methods and U.S. EPA's benchmark dose software (BMDS) version 2.1.1. *Toxicology and Applied Pharmacology*, *254*(2), 181–191. https://doi.org/10.1016/j.taap.2010.10.016

DeMarini, D. M. (2025). Origin story of IARC's 10 key characteristics of carcinogens. *Environmental and Molecular Mutagenesis*. Advance online publication. https://doi.org/10.1002/em.70028

DeMarini, D. M., Gwinn, W., Watkins, E., Reisfeld, B., Chiu, W. A., Zeise, L., Barupal, D., Bhatti, P., Cross, K., Dogliotti, E., Fritz, J. M., Germolec, D., Andersen, M. H. G.,

Guyton, K. Z., Jinot, J., Phillips, D. H., Reddel, R. R., Rothman, N., van den Berg, M., … Madia, F. (2025). IARC workshop on the key characteristics of carcinogens: Assessment of end points for evaluating mechanistic evidence of carcinogenic hazards. *Environmental Health Perspectives, 133*(2), 025001. https://doi.org/10.1289/EHP15389

Deng, S., Li, C., Cao, J., Cui, Z., Du, J., Fu, Z., Yang, H., & Chen, P. (2023). Organ-on-a-chip meets artificial intelligence in drug evaluation. *Theranostics, 13*(13), 4526–4558. https://doi.org/10.7150/thno.87266

Desaulniers, D., Vasseur, P., Jacobs, A., Aguila, M. C., Ertych, N., & Jacobs, M. N. (2021). Integration of epigenetic mechanisms into non-genotoxic carcinogenicity hazard assessment: Focus on DNA methylation and histone modifications. *International Journal of Molecular Sciences, 22*(20), 10969. https://doi.org/10.3390/ijms222010969

Di Tillio, F., & Beltman, J. B. (2024). Developing quantitative adverse outcome pathways: An ordinary differential equation-based computational framework. *Computational Toxicology, 32*, 100330. https://doi.org/10.1016/j.comtox.2024.100330

Doering, J. A., Dubiel, J., Stock, E., Collins, C. H., Frick, I., Johnson, H. M., Lowrey-Dufour, C. M., Miller, J. G. P., Xia, Z., Tomy, G. T., & Wiseman, S. (2024). A quantitative adverse outcome pathway for embryonic activation of the aryl hydrocarbon receptor of fishes by polycyclic aromatic hydrocarbons leading to decreased fecundity at adulthood. *Environmental Toxicology and Chemistry, 43*(10), 2145–2156. https://doi.org/10.1002/etc.5964

EURION cluster launches in Brussels | Improving identification of endocrine disruptors | EURION. Retrieved August 24, 2025, from https://eurion-cluster.eu/hello-world/

European Chemicals Agency. (2016). *Guidance on information requirements and chemical safety assessment: Chapter R.15: Consumer exposure assessment* (Version 3.0). ECHA. https://doi.org/10.823/940476

Faroon, O., Keith, S., Mumtaz, M., & Ruiz, P. (2017). Minimal risk level derivation for cadmium: Acute and intermediate duration exposures. *Journal of Experimental and Clinical Toxicology, 1*(1), 1–12. https://doi.org/10.14302/issn.2641-7669.ject-17-1725

Feinberg, A. P., Ohlsson, R., & Henikoff, S. (2006). The epigenetic progenitor origin of human cancer. *Nature Reviews Genetics, 7*(1), 21–33. https://doi.org/10.1038/nrg1748

Felter, S. P., Bhat, V. S., Botham, P. A., Bussard, D. A., Casey, W., Hayes, A. W., Hilton, G. M., Magurany, K. A., Sauer, U. G., & Ohanian, E. V. (2021). Assessing chemical carcinogenicity: Hazard identification, classification, and risk assessment. Insight from a Toxicology Forum state-of-the-science workshop. *Critical Reviews in Toxicology, 51*(8), 653–694. https://doi.org/10.1080/10408444.2021.2003295

Foreman, J. E., Koga, T., Kosyk, O., Kang, B.-H., Zhu, X., Cohen, S. M., Billy, L. J., Sharma, A. K., Amin, S., Gonzalez, F. J., Rusyn, I., & Peters, J. M. (2021). Species differences between mouse and human PPARα in modulating the hepatocarcinogenic effects of perinatal exposure to a high-affinity human PPARα agonist in mice. *Toxicological Sciences, 183*(1), 81–92. https://doi.org/10.1093/toxsci/kfab068

Froetschl, R., Corton, J. C., Li, H., Aubrecht, J., Auerbach, S. S., Caiment, F., Doktorova, T. Y., Fujita, Y., Jennen, D., Koyama, N., Meier, M. J., Mezencev, R., Recio, L., Suzuki, T., & Yauk, C. L. (2025). Consensus findings of an International Workshops on Genotoxicity Testing workshop on using transcriptomic biomarkers to predict genotoxicity. *Environmental and Molecular Mutagenesis*. Advance online publication. https://doi.org/10.1002/em.22645

Frühwein, H., & Paul, N. W. (2025). 'Lost in translation?' Animal research in the era of precision medicine. *Journal of Translational Medicine, 23*(1), 152. https://doi.org/10.1186/s12967-025-06084-3

Furihata, C., & Suzuki, T. (2023). Short-term in vivo testing to discriminate genotoxic carcinogens from non-genotoxic carcinogens and non-carcinogens using next-generation RNA sequencing, DNA microarray, and qPCR. *Genes and Environment, 45*(1), 7. https://doi.org/10.1186/s41021-023-00262-9

Garralaga, M. P., Lomba, L., Zuriaga, E., Santander, S., & Giner, B. (2022). Key properties for the toxicity classification of chemicals: A comparison of the REACH regulation and scientific studies trends. *Applied Sciences, 12*(22), 11171. https://doi.org/10.3390/app122211710

Gebel, T., Lechtenberg-Auffarth, E., & Guhe, C. (2009). About hazard and risk assessment: Regulatory approaches in assessing safety in the European Union chemicals legislation. *Reproductive Toxicology, 28*(2), 188–195. https://doi.org/10.1016/j.reprotox.2009.03.015

Godin, S. J., Scollon, E. J., Hughes, M. F., Potter, P. M., DeVito, M. J., & Ross, M. K. (2006). Species differences in the in vitro metabolism of deltamethrin and esfenvalerate: Differential oxidative and hydrolytic metabolism by humans and rats. *Drug Metabolism and Disposition, 34*(10), 1764–1771. https://doi.org/10.1124/dmd.106.010058

Gómez-Lechón, M. J., Donato, M. T., Castell, J. V., & Jover, R. (2003). Human hepatocytes as a tool for studying toxicity and drug metabolism. *Current Drug Metabolism, 4*(4), 292–312. https://doi.org/10.2174/1389200033489424

Gong, J., Tu, W., Liu, J., & Tian, D. (2022). Hepatocytes: A key role in liver inflammation. *Frontiers in Immunology, 13*, 1083780. https://doi.org/10.3389/fimmu.2022.1083780

González-García, I., Mangas-Sanjuán, V., Merino-Sanjuán, M., & Bermejo, M. (2015). In vitro-in vivo correlations: General concepts, methodologies and regulatory applications. *Drug Development and Industrial Pharmacy, 41*(12), 1935–1947. https://doi.org/10.3109/03639045.2015.1054833

Gottmann, E., Kramer, S., Pfahringer, B., & Helma, C. (2001). Data quality in predictive toxicology: Reproducibility of rodent carcinogenicity experiments. *Environmental Health Perspectives, 109*(5), 509–514. https://doi.org/10.1289/ehp.01109509

Grandjean, P. (2016). Paracelsus revisited: The dose concept in a complex world. *Basic & Clinical Pharmacology & Toxicology, 119*(2), 126–132. https://doi.org/10.1111/bcpt.12622

Gripon, P., Rumin, S., Urban, S., Le Seyec, J., Glaise, D., Cannie, I., Guyomard, C., Lucas, J., Trepo, C., & Guguen-Guillouzo, C. (2002). Infection of a human hepatoma cell line by hepatitis B virus. *Proceedings of the National Academy of Sciences, 99*(24), 15655–15660. https://doi.org/10.1073/pnas.232137699

Guo, X., Xu, H., & Seo, J.-E. (2024). Application of HepaRG cells for genotoxicity assessment: A review. *Journal of Environmental Science and Health, Part C, Toxicology and Carcinogenesis, 42*(3), 214–237. https://doi.org/10.1080/26896583.2024.2331956

Gupta, K., Song, Z., Tang, H., Fong, E. L. S., Ng, I. C., & Yu, H. (2017). Liver tissue engineering. In P. Ducheyne (Ed.), *Comprehensive biomaterials II* (pp. 491–512). Elsevier. https://doi.org/10.1016/B978-0-12-803581-8.10163-8

Gutleb, A. C., Murugadoss, S., Stępnik, M., SenGupta, T., El Yamani, N., Longhin, E. M., Olsen, A.-K. H., Wyrzykowska, E., Jagiello, K., Judzinska, B., Cambier, S., Honza, T., McFadden, E., Shaposhnikov, S., Puzyn, T., Serchi, T., Weber, P., Arnesdotter, E., Skakalova, V., … Dusinska, M. (2025). New approach methods (NAMs) for genotoxicity assessment of nano- and advanced materials; Advantages and challenges. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis, 904*, 503867. https://doi.org/10.1016/j.mrgentox.2025.503867

Guyton, K. Z., Rusyn, I., Chiu, W. A., Corpet, D. E., van den Berg, M., Ross, M. K., Christiani, D. C., Beland, F. A., & Smith, M. T. (2018). Application of the key characteristics of carcinogens in cancer hazard identification. *Carcinogenesis, 39*(4), 614–622. https://doi.org/10.1093/carcin/bgy031

Habert, R., Muczynski, V., Grisin, T., Moison, D., Messiaen, S., Frydman, R., Benachi, A., Delbes, G., Lambrot, R., Lehraiki, A., N'Tumba-Byn, T., Guerquin, M.-J., Levacher, C., Rouiller-Fabre, V., & Livera, G. (2014). Concerns about the widespread use of rodent models for human risk assessments of endocrine disruptors. *Reproduction, 147*(4), R119–R138. https://doi.org/10.1530/REP-13-0497

Hall, B., Tozer, S., Safford, B., Coroama, M., Steiling, W., Leneveu-Duchemin, M. C., McNamara, C., & Gibney, M. (2007). European consumer exposure to cosmetic products, a framework for conducting population exposure assessments. *Food and Chemical Toxicology*, *45*(11), 2097–2108. https://doi.org/10.1016/j.fct.2007.06.017

Hammer, H., Schmidt, F., Marx-Stoelting, P., Pötz, O., & Braeuning, A. (2021). Cross-species analysis of hepatic cytochrome P450 and transport protein expression. *Archives of Toxicology*, *95*(1), 117–133. https://doi.org/10.1007/s00204-020-02939-4

Han, B., Mo, H., Svarovskaia, E., & Mateo, R. (2021). A primary human hepatocyte/hepatic stellate cell co-culture system for improved in vitro HBV replication. *Virology*, *559*, 40–45. https://doi.org/10.1016/j.virol.2021.03.012

Han, P., Li, X., Yang, J., Zhang, Y., & Chen, J. (2024). Advancing toxicity predictions: A review on in vitro to in vivo extrapolation in next-generation risk assessment. *Environment & Health*, *2*(7), 499–513. https://doi.org/10.1021/envhealth.4c00043

Hanahan, D. (2022). Hallmarks of cancer: New dimensions. *Cancer Discovery*, *12*(1), 31–46. https://doi.org/10.1158/2159-8290.CD-21-1059

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, *100*(1), 57–70. https://doi.org/10.1016/S0092-8674(00)81683-9

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, *144*(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Hansford, S., & Huntsman, D. G. (2014). Boveri at 100: Theodor Boveri and genetic predisposition to cancer. *The Journal of Pathology*, *234*(2), 142–145. https://doi.org/10.1002/path.4414

Harrill, J., Shah, I., Setzer, R. W., Haggard, D., Auerbach, S., Judson, R., & Thomas, R. S. (2019). Considerations for strategic use of high-throughput transcriptomics chemical screening data in regulatory decisions. *Current Opinion in Toxicology*, *15*, 64–75. https://doi.org/10.1016/j.cotox.2019.05.004

Hasegawa, R., Koizumi, M., & Hirose, A. (2004). Principles of risk assessment for determining the safety of chemicals: Recent assessment of residual solvents in drugs and di(2-ethylhexyl) phthalate. *Congenital Anomalies*, *44*(2), 51–59. https://doi.org/10.1111/j.1741-4520.2004.00009.x

Hayashi, Y. (1992). Overview of genotoxic carcinogens and non-genotoxic carcinogens. *Experimental and Toxicologic Pathology*, *44*(8), 465–471. https://doi.org/10.1016/S0940-2993(11)80159-4

Hernández, L. G., van Steeg, H., Luijten, M., & van Benthem, J. (2009). Mechanisms of non-genotoxic carcinogens and importance of a weight of evidence approach. *Mutation Research/Reviews in Mutation Research, 682*(2–3), 94–109. https://doi.org/10.1016/j.mrrev.2009.07.002

Heusinkveld, H., Braakhuis, H., Gommans, R., Botham, P., Corvaro, M., van der Laan, J. W., Lewis, D., Madia, F., Manou, I., Schorsch, F., Wolterink, G., Woutersen, R., Corvi, R., Mehta, J., & Luijten, M. (2020). Towards a mechanism-based approach for the prediction of nongenotoxic carcinogenic potential of agrochemicals. *Critical Reviews in Toxicology, 50*(9), 725–739. https://doi.org/10.1080/10408444.2020.1841732

Hill, W., Weeden, C. E., & Swanton, C. (2024). Tumor promoters and opportunities for molecular cancer prevention. *Cancer Discovery, 14*(7), 1154–1160. https://doi.org/10.1158/2159-8290.CD-24-0128

Hodgson, S. V., Foulkes, W. D., Maher, E. R., & Turnbull, C. (2025). Inherited susceptibility to cancer: Past, present and future. *Annals of Human Genetics, 89*(5), 354–365. https://doi.org/10.1111/ahg.70013

Hoogstraaten, M. J., Vriend, J., de Leeuw, V. C., Negro, S. O., Moors, E. H. M., Kienhuis, A. S., & Hoekman, J. (2025). Animal-free safety assessment of chemicals: An innovation system perspective. *Archives of Toxicology, 99*(1), 43–56. https://doi.org/10.1007/s00204-024-03878-0

IARC Monographs hazard classification. Retrieved August 31, 2025, from https://www.iarc.who.int/infographics/iarc-monographs-classification

IARC Publications website—IARC monographs on the identification of carcinogenic hazards to humans. (n.d.). Retrieved September 7, 2025, from https://publications.iarc.who.int/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans

Integrated approaches to testing and assessment (IATA). OECD. Retrieved July 13, 2025, from https://www.oecd.org/en/topics/sub-issues/assessment-of-chemicals/integrated-approaches-to-testing-and-assessment.html

Jacobs, M. N., Colacci, A., Corvi, R., Vaccari, M., Aguila, M. C., Corvaro, M., Delrue, N., Desaulniers, D., Ertych, N., Jacobs, A., Luijten, M., Madia, F., Nishikawa, A., Ogawa, K., Ohmori, K., Paparella, M., Sharma, A. K., & Vasseur, P. (2020). 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. *Archives of Toxicology, 94*(8), 2899–2923. https://doi.org/10.1007/s00204-020-02784-5

Jacobs, M. N., Colacci, A., Louekari, K., Luijten, M., Hakkert, B. C., Paparella, M., & Vasseur, P. (2016). International regulatory needs for development of an IATA for non-genotoxic carcinogenic chemical substances. *ALTEX*, *33*(4), 359–392. https://doi.org/10.14573/altex.1601201

Jacobs, M. N., Hoffmann, S., Hollnagel, H. M., Kern, P., Kolle, S. N., Natsch, A., & Landsiedel, R. (2024). Avoiding a reproducibility crisis in regulatory toxicology—On the fundamental role of ring trials. *Archives of Toxicology*, *98*(7), 2047–2063. https://doi.org/10.1007/s00204-024-03736-z

Jaylet, T., Coustillet, T., Smith, N. M., Viviani, B., Lindeman, B., Vergauwen, L., Myhre, O., Yarar, N., Gostner, J. M., Monfort-Lanzas, P., Jornod, F., Holbech, H., Coumoul, X., Sarigiannis, D. A., Antczak, P., Bal-Price, A., Fritsche, E., Kuchovska, E., Stratidakis, A. K., … Audouze, K. (2024). Comprehensive mapping of the AOP-Wiki database: Identifying biological and disease gaps. *Frontiers in Toxicology*, *6*, 1285768. https://doi.org/10.3389/ftox.2024.1285768

Jeong, J., Gasparyan, M., & Choi, J. (2025). Advancing the quantitative understanding of adverse outcome pathways: Current status, methodologies, and future directions. *Environmental Toxicology and Chemistry*, *44*(3), 614–623. https://doi.org/10.1093/etojnl/vgae063

Juhasz, A. L., Basta, N. T., & Smith, E. (2013). What is required for the validation of in vitro assays for predicting contaminant relative bioavailability? Considerations and criteria. *Environmental Pollution*, *180*, 372–375. https://doi.org/10.1016/j.envpol.2013.05.008

Jurgelewicz, A., Breaux, K., Willis, C. M., Harris, F. R., Byrd, G., Witten, J., Haggard, D. E., Deisenroth, C., & Harrill, J. A. (2025). Assessing the impact of in vitro xenobiotic metabolism on estrogenic chemical bioactivity in high-throughput profiling assays. *Toxicology*, *517*, 154215. https://doi.org/10.1016/j.tox.2025.154215

Kaczara, P., Czyzynska-Cichon, I., Kus, E., Kurpinska, A., Olkowicz, M., Wojnar-Lason, K., Pacia, M. Z., Lytvynenko, O., Baes, M., & Chlopicki, S. (2024). Liver sinusoidal endothelial cells rely on oxidative phosphorylation but avoid processing long-chain fatty acids in their mitochondria. *Cellular & Molecular Biology Letters*, *29*(1), 67. https://doi.org/10.1186/s11658-024-00584-8

Karakaidos, P., Karagiannis, D., & Rampias, T. (2020). Resolving DNA damage: Epigenetic regulation of DNA repair. *Molecules*, *25*(11), 2496. https://doi.org/10.3390/molecules25112496

Kaur, I., Vasudevan, A., Rawal, P., Tripathi, D. M., Ramakrishna, S., Kaur, S., & Sarin, S. K. (2023). Primary hepatocyte isolation and cultures: Technical aspects, challenges and

advancements. *Bioengineering*, *10*(2), 131. https://doi.org/10.3390/bioengineering10020131

Kawabe, M., Urano, K., Suguro, M., Numano, T., Taguchi, F., Tsutsumi, H., & Furukawa, F. (2013). Tumor promotion by 12-O-tetradecanoylphorbol-13-acetate in an ultra-short-term skin carcinogenesis bioassay using rasH2 mice. *Veterinary Pathology*, *50*(5), 903–908. https://doi.org/10.1177/0300985813486811

Kemarau, R. A., Sakawi, Z., Eboy, O. V., Wan Mohd Jaafar, W. S., Sa'adi, Z., & Anak Suab, S. (2025). Impacts of breaching planetary boundaries on human health: Current and future threats. *GeoHealth*, *9*(6), e2024GH001107. https://doi.org/10.1029/2024GH001107

Kiani, A. K., Pheby, D., Henehan, G., Brown, R., Sieving, P., Sykora, P., Marks, R., Falsini, B., Capodicasa, N., Miertus, S., Lorusso, L., Dondossola, D., Tartaglia, G. M., Ergoren, M. C., Dundar, M., Michelini, S., Malacarne, D., Bonetti, G., Dautaj, A., … Bertelli, M. (2022). Ethical considerations regarding animal experimentation. *Journal of Preventive Medicine and Hygiene*, *63*(2, Suppl. 3), E475–E483. https://doi.org/10.15167/2421-4248/jpmh2022.63.2S3.2768

Knight, A. (2008). Non-animal methodologies within biomedical research and toxicity testing. *ALTEX*, *25*(3), 213–231. https://doi.org/10.14573/altex.2008.3.213

Ko, M. Y., Min, E., Kim, M., Park, H., Jang, S., Kim, Y., Lee, B. S., Hyun, S. A., & Ka, M. (2025). Non-genotoxic carcinogens (TPA and mezerein) activate tumourous transformation through miR let-7-mediated Hmga2 expression in Bhas42 cells. *Environmental Epigenetics*, *11*(1), dvaf005. https://doi.org/10.1093/eep/dvaf005

Koag, M.-C., Nam, K., & Lee, S. (2014). The spontaneous replication error and the mismatch discrimination mechanisms of human DNA polymerase β. *Nucleic Acids Research*, *42*(17), 11233–11245. https://doi.org/10.1093/nar/gku789

Kobets, T., Iatropoulos, M. J., & Williams, G. M. (2022). Acrylonitrile induction of rodent neoplasia: Potential mechanism of action and relevance to humans. *Toxicology Research and Application*, *6*, 23978473211055363. https://doi.org/10.1177/23978473211055363

LaRocca, J., Costa, E., Sriram, S., Hannas, B. R., & Johnson, K. J. (2020). Short-term toxicogenomics as an alternative approach to chronic in vivo studies for derivation of points of departure: A case study in the rat with a triazole fungicide. *Regulatory Toxicology and Pharmacology*, *113*, 104655. https://doi.org/10.1016/j.yrtph.2020.104655

Lee, J., Prokopec, S. D., Watson, J. D., Sun, R. X., Pohjanvirta, R., & Boutros, P. C. (2015). Male and female mice show significant differences in hepatic transcriptomic response to

2,3,7,8-tetrachlorodibenzo-p-dioxin. *BMC Genomics*, *16*(1), 625. https://doi.org/10.1186/s12864-015-1840-6

Leso, V., Nowack, B., Karakoltzidis, A., Nikiforou, F., Karakitsios, S., Sarigiannis, D., & Iavicoli, I. (2025). Next generation risk assessment and new approach methodologies for safe and sustainable by design chemicals and materials: Perspectives and challenges for occupational health. *Toxicology*, *517*, 154211. https://doi.org/10.1016/j.tox.2025.154211

Lewis, J. G., & Adams, D. O. (1987). Inflammation, oxidative DNA damage, and carcinogenesis. *Environmental Health Perspectives*, *76*, 19–27. https://doi.org/10.1289/ehp.877619

Lewis, R. W., Hill, T., & Corton, J. C. (2020). A set of six gene expression biomarkers and their thresholds identify rat liver tumorigens in short-term assays. *Toxicology*, *443*, 152547. https://doi.org/10.1016/j.tox.2020.152547

Li, H.-H., Aubrecht, J., Doktorova, T. Y., et al. (2025). Review of transcriptomic biomarkers that predict in vitro genotoxicity in human cell lines. *Environmental and Molecular Mutagenesis*. Advance online publication. https://doi.org/10.1002/em.70004

Li, W., & Zhao, Y. (2013). Withaferin A suppresses tumor promoter 12-O-tetradecanoylphorbol 13-acetate-induced decreases in isocitrate dehydrogenase 1 activity and mitochondrial function in skin epidermal JB6 cells. *Cancer Science*, *104*(2), 143–148. https://doi.org/10.1111/cas.12051

Li, Y., Liu, F., Cai, Q., Deng, L., Ouyang, Q., Zhang, X. H.-F., & Zheng, J. (2025). Invasion and metastasis in cancer: Molecular insights and therapeutic targets. *Signal Transduction and Targeted Therapy*, *10*, 57. https://doi.org/10.1038/s41392-025-02148-4

Liu, J., Gan, T., Hu, W., & Li, Y. (2024). Current status and perspectives in environmental oncology. *Chronic Diseases and Translational Medicine*, *10*(4), 293–301. https://doi.org/10.1002/cdt3.148

Louekari, K., & Jacobs, M. N. (2024). A modular strategy for the testing and assessment of non-genotoxic carcinogens. *Archives of Toxicology*, *98*(8), 2463–2485. https://doi.org/10.1007/s00204-024-03753-y

Lu, E.-H., Ford, L. C., Rusyn, I., & Chiu, W. A. (2025). Reducing uncertainty in dose-response assessments by incorporating Bayesian benchmark dose modeling and in vitro data on population variability. *Risk Analysis*, *45*(2), 457–472. https://doi.org/10.1111/risa.17451

Luijten, M., Corvi, R., Mehta, J., Corvaro, M., Delrue, N., Felter, S., Haas, B., Hewitt, N. J., Hilton, G., Holmes, T., Jacobs, M. N., Jacobs, A., Lamplmair, F., Lewis, D., Madia, F., Manou, I., Melching-Kollmuss, S., Schorsch, F., Schütte, K., … Braakhuis, H. (2020). A

comprehensive view on mechanistic approaches for cancer risk assessment of non-genotoxic agrochemicals. *Regulatory Toxicology and Pharmacology, 118*, 104789. https://doi.org/10.1016/j.yrtph.2020.104789

Luo, Q., & Smith, D. P. (2025). Global cancer burden: Progress, projections, and challenges. *The Lancet, 406*(10512), 1536–1537. https://doi.org/10.1016/S0140-6736(25)01570-3

Lynch, H. T., Fusaro, R. M., & Lynch, J. (1995). Hereditary cancer in adults. *Cancer Detection and Prevention, 19*(3), 219–233.

Mantovani, A., Allavena, P., Sica, A., & Balkwill, F. (2008). Cancer-related inflammation. *Nature, 454*(7203), 436–444. https://doi.org/10.1038/nature07205

Marinou, K. A., & Dontas, I. A. (2023). European Union legislation for the welfare of animals used for scientific purposes: Areas identified for further discussion. *Animals, 13*(14), 2367. https://doi.org/10.3390/ani13142367

Marlowe, J., & Puga, A. (2010). Novel AHR interactions. In C. A. McQueen (Ed.), *Comprehensive toxicology* (2nd ed., pp. 93–115). Elsevier. https://doi.org/10.1016/B978-0-08-046884-6.00207-4

Marone, P. A., Hall, W. C., & Hayes, A. W. (2014). Reassessing the two-year rodent carcinogenicity bioassay: A review of the applicability to human risk and current perspectives. *Regulatory Toxicology and Pharmacology, 68*(1), 108–118. https://doi.org/10.1016/j.yrtph.2013.11.011

Marshall, L. J., Bailey, J., Cassotta, M., Herrmann, K., & Pistollato, F. (2023). Poor translatability of biomedical research using animals—A narrative review. *Alternatives to Laboratory Animals, 51*(2), 102–135. https://doi.org/10.1177/02611929231157756

Martignoni, M., Groothuis, G. M. M., & de Kanter, R. (2006). Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opinion on Drug Metabolism & Toxicology, 2*(6), 875–894. https://doi.org/10.1517/17425255.2.6.875

Martinez, M. N., Mochel, J. P., Neuhoff, S., & Pade, D. (2021). Comparison of canine and human physiological factors: Understanding interspecies differences that impact drug pharmacokinetics. *The AAPS Journal, 23*(3), 1–16. https://doi.org/10.1208/s12248-021-00590-0

Matsuoka, T., & Yashiro, M. (2024). Molecular mechanism for malignant progression of gastric cancer within the tumor microenvironment. *International Journal of Molecular Sciences, 25*(21), 11735. https://doi.org/10.3390/ijms252111735

Mbemi, A., Khanna, S., Njiki, S., Yedjou, C. G., & Tchounwou, P. B. (2020). Impact of gene-environment interactions on cancer development. *International Journal of Environmental Research and Public Health, 17*(21), 8089. https://doi.org/10.3390/ijerph17218089

Meirson, T., Gil-Henn, H., & Samson, A. O. (2020). Invasion and metastasis: The elusive hallmark of cancer. *Oncogene, 39*(9), 2024–2026. https://doi.org/10.1038/s41388-019-1110-1

Mendez, W., Shao, K., Lee, J. S., Cote, I., Druwe, I. L., Davis, A., & Gift, J. S. (2020). Model averaging methods for the evaluation of dose-response model uncertainty when assessing the suitability of studies for estimating risk. *Environment International, 143*, 105857. https://doi.org/10.1016/j.envint.2020.105857

Middleton, D. R. S., Wedekind, R., Hall, A. L., Suonio, E., DeBono, N., Vermeulen, R., Fritschi, L., Stewart, P. A., & Schubauer-Berigan, M. K. (2024). Critical reviews of exposure assessment in carcinogenic hazard identification: The IARC Monographs experience. *Occupational and Environmental Medicine, 81*(7), 366–372. https://doi.org/10.1136/oemed-2023-109277

Mitchell, C. A., Wehmas, L., Rouquié, D., Caiment, F., Currie, R. A., & Crute, C. E. (2025). Navigating complexity in modern toxicology: The role of omics in short-term in vivo studies. *Toxicological Sciences, 208*(1), 32–37. https://doi.org/10.1093/toxsci/kfaf120

Mugaanyi, J., Huang, J., Fang, J., Musinguzi, A., Lu, C., & Chen, Z. (2025). Developments and applications of liver-on-a-chip technology-current status and future prospects. *Biomedicines, 13*(6), 1272. https://doi.org/10.3390/biomedicines13061272

Muir, D. C. G., Getzinger, G. J., McBride, M., & Ferguson, P. L. (2023). How many chemicals in commerce have been analyzed in environmental media? A 50 year bibliometric analysis. *Environmental Science & Technology, 57*(25), 9119–9129. https://doi.org/10.1021/acs.est.2c09353

Mutti, A. (2023). The linear non-threshold extrapolation of dose-response curves is a challenge for managing the risk associated with occupational exposure to carcinogenic agents. *La Medicina del Lavoro, 114*(1), e2023001. https://doi.org/10.23749/mdl.v114i1.14176

Nair, D. G., & Weiskirchen, R. (2024). Advanced in vitro models for preclinical drug safety: Recent progress and prospects. *Current Issues in Molecular Biology, 47*(1), 7. https://doi.org/10.3390/cimb47010007

Nelson, L. J., Morgan, K., Treskes, P., Samuel, K., Henderson, C. J., LeBled, C., Homer, N., Grant, M. H., Hayes, P. C., & Plevris, J. N. (2017). Human hepatic HepaRG cells maintain an organotypic phenotype with high intrinsic CYP450 activity/metabolism and significantly outperform standard HepG2/C3A cells for pharmaceutical and therapeutic

applications. *Basic & Clinical Pharmacology & Toxicology*, *120*(1), 30–37. https://doi.org/10.1111/bcpt.12631

Nepovimova, E., & Kuca, K. (2019). The history of poisoning: From ancient times until modern era. *Archives of Toxicology*, *93*(1), 11–24. https://doi.org/10.1007/s00204-018-2290-0

Nohmi, T. (2018). Thresholds of genotoxic and non-genotoxic carcinogens. *Toxicological Research*, *34*(4), 281–290. https://doi.org/10.5487/TR.2018.34.4.281

Nymark, P., Sachana, M., Leite, S. B., Sund, J., Krebs, C. E., Sullivan, K., Edwards, S., Viviani, L., Willett, C., Landesmann, B., & Wittwehr, C. (2021). Systematic organization of COVID-19 data supported by the adverse outcome pathway framework. *Frontiers in Public Health*, *9*, 638605. https://doi.org/10.3389/fpubh.2021.638605

Oderberg, I. M., & Goessling, W. (2023). Biliary epithelial cells are facultative liver stem cells during liver regeneration in adult zebrafish. *JCI Insight*, *8*(1), e163929. https://doi.org/10.1172/jci.insight.163929

Oliveira, P. A., & Faustino-Rocha, A. I. (2025). Chemical carcinogens. In N. Rezaei (Ed.), *Cancer and surrounding environment: Handbook of cancer and immunology* (Vol. 3). Springer. https://doi.org/10.1007/978-3-032-00761-2_121

Oliveira, P. A., Colaço, A., Chaves, R., Guedes-Pinto, H., De-La-Cruz, P. L. F., & Lopes, C. (2007). Chemical carcinogenesis. *Anais da Academia Brasileira de Ciências*, *79*(4), 593–616. https://doi.org/10.1590/s0001-37652007000400004

Ouedraogo, G., Alépée, N., Tan, B., & Roper, C. S. (2025). A call to action: Advancing new approach methodologies (NAMs) in regulatory toxicology through a unified framework for validation and acceptance. *Regulatory Toxicology and Pharmacology*, *162*, 105904. https://doi.org/10.1016/j.yrtph.2025.105904

Oyetade, O., Allen, D. G., Carder, J., Farley-Dawson, E. A., Reinke, E., Marko, S., Kleinstreuer, N. C., & Hogberg, H. T. (2025). Ways to broaden the awareness, consideration and adoption of new approach methodologies (NAMs). *ALTEX - Alternatives to Animal Experimentation*. Advance online publication. https://doi.org/10.14573/altex.2505281

Pappas, A. A., Massoll, N. A., & Cannon, D. J. (1999). Toxicology: Past, present, and future. *Annals of Clinical & Laboratory Science*, *29*(4), 253–262.

Partnership for the Assessment of Risks from Chemicals | PARC. (2025, July 1). https://www.eu-parc.eu/

Pereira, M., Macmillan, D. S., Willett, C., & Seidle, T. (2022). REACHing for solutions: Essential revisions to the EU chemicals regulation to modernise safety assessment. *Regulatory Toxicology and Pharmacology, 136*, 105278. https://doi.org/10.1016/j.yrtph.2022.105278

Pérez-Carreón, J., & Meléndez-Zajgla, J. (2012). In vitro and in vivo models for cancer research. *Molecular Oncology Principles and Recent Advances*, 148–162. https://doi.org/10.2174/978160805016111201010148

Persson, K., Rodriguez Perez, C., Louis-Maerten, E., Müller, N., & Shaw, D. (2025). "Killing in the name of 3R?" The ethics of death in animal research. *Journal of Agricultural and Environmental Ethics, 38*(1), 1–18. https://doi.org/10.1007/s10806-024-09936-y

Persson, L., Carney Almroth, B. M., Collins, C. D., Cornell, S., de Wit, C. A., Diamond, M. L., Fantke, P., Hassellöv, M., MacLeod, M., Ryberg, M. W., Søgaard Jørgensen, P., Villarrubia-Gómez, P., Wang, Z., & Hauschild, M. Z. (2022). Outside the safe operating space of the planetary boundary for novel entities. *Environmental Science & Technology, 56*(3), 1510–1521. https://doi.org/10.1021/acs.est.1c04158

Pham, L. L., Sheffield, T. Y., Pradeep, P., Brown, J., Haggard, D. E., Wambaugh, J., Judson, R. S., & Paul Friedman, K. (2019). Estimating uncertainty in the context of new approach methodologies for potential use in chemical safety evaluation. *Current Opinion in Toxicology, 15*, 40–47. https://doi.org/10.1016/j.cotox.2019.04.001

Phillips, D. H., & Arlt, V. M. (2009). Genotoxicity: Damage to DNA and its consequences. *EXS, 99*, 87–110. https://doi.org/10.1007/978-3-7643-8336-7_4

Pinazo-Bandera, J. M., García-Cortés, M., Segovia-Zafra, A., Lucena, M. I., & Andrade, R. J. (2022). Recreational drugs and the risk of hepatocellular carcinoma. *Cancers, 14*(21), 5395. https://doi.org/10.3390/cancers14215395

Pitot, H. C. (1993). The molecular biology of carcinogenesis. *Cancer, 72*(S3), 962–970. https://doi.org/10.1002/1097-0142(19930801)72:3+<962::AID-CNCR2820721303>3.0.CO;2-H

Potera, C. (2016). Making sense of carcinogens: A new method for navigating mechanistic data. *Environmental Health Perspectives, 124*(6), A113. https://doi.org/10.1289/ehp.124-A113

Pouzou, J. G., Kissel, J., Yost, M. G., Fenske, R. A., & Cullen, A. C. (2020). Use of benchmark dose models in risk assessment for occupational handlers of eight pesticides used in pome fruit production. *Regulatory Toxicology and Pharmacology, 110*, 104504. https://doi.org/10.1016/j.yrtph.2019.104504

Qu, X., Xu, K., Zhao, C., Song, X., Li, J., Li, L., Nie, W., Bao, H., Wang, J., Niu, F., & Li, J. (2017). Genotoxicity and acute and subchronic toxicity studies of a bioactive polyoxometalate in Wistar rats. *BMC Pharmacology & Toxicology, 18*, 26. https://doi.org/10.1186/s40360-017-0133-x

Ranade, A. R., Wilson, M. S., McClanahan, A. M., & Ball, A. J. (2014). High content imaging and analysis enable quantitative in situ assessment of CYP3A4 using cryopreserved differentiated HepaRG cells. *Journal of Toxicology, 2014*(1), 291054. https://doi.org/10.1155/2014/291054

Rangel, G., Leksomboon, R., & Wanram, S. (2022). Impact of *Plasmodium falciparum* malaria on liver and red blood cell. *International Journal of Gastroenterology, Hepatology and Endoscopy, 1*(1). https://doi.org/10.51521/IJGHE.2022.1104

Ravi, S., Arakelyan, J., Xu, W., Stauber, R., Wang, C.-C. I., Papyan, R., Ghazaryan, N., & Pereira, R. M. (2022). An update to hallmarks of cancer. *Cureus, 14*(5), e24803. https://doi.org/10.7759/cureus.24803

Reddy, N., Lynch, B., Gujral, J., & Karnik, K. (2023a). Alternatives to animal testing in toxicity testing: Current status and future perspectives in food safety assessments. *Food and Chemical Toxicology, 179*, 113944. https://doi.org/10.1016/j.fct.2023.113944

Reddy, N., Lynch, B., Gujral, J., & Karnik, K. (2023b). Regulatory landscape of alternatives to animal testing in food safety evaluations with a focus on the western world. *Regulatory Toxicology and Pharmacology, 143*, 105470. https://doi.org/10.1016/j.yrtph.2023.105470

Renwick, A. G. (1999). Exposure estimation, toxicological requirements and risk assessment. In *International food safety handbook*. Routledge.

Richter, F. (2025, February 4). *Infographic: The global cancer burden keeps growing*. Statista Daily Data. https://www.statista.com/chart/20706/new-cancer-cases-per-year

Rooney, A. A., Boyles, A. L., Wolfe, M. S., Bucher, J. R., & Thayer, K. A. (2014). Systematic review and evidence integration for literature-based environmental health science assessments. *Environmental Health Perspectives, 122*(7), 711–718. https://doi.org/10.1289/ehp.1307972

Rudén, C., & Hansson, S. O. (2010). Registration, evaluation, and authorization of chemicals (REACH) is but the first step–How far will it take us? Six further steps to improve the European chemicals legislation. *Environmental Health Perspectives, 118*(1), 6–10. https://doi.org/10.1289/ehp.0901157

Rusyn, I., & Wright, F. A. (2023). *Ten years of using key characteristics of human carcinogens to organize and evaluate mechanistic evidence in IARC Monographs on the Identification of Carcinogenic Hazards to Humans: Patterns and associations* [Preprint]. bioRxiv. https://doi.org/10.1101/2023.07.11.548354

Rusyn, I., & Wright, F. A. (2024). Ten years of using key characteristics of human carcinogens to organize and evaluate mechanistic evidence in IARC Monographs on the identification of carcinogenic hazards to humans: Patterns and associations. *Toxicological Sciences, 198*(1), 141–154. https://doi.org/10.1093/toxsci/kfad134

Saarimäki, L. A., Morikka, J., Pavel, A., Korpilähde, S., Del Giudice, G., Federico, A., Fratello, M., Serra, A., & Greco, D. (2023). Toxicogenomics data for chemical safety assessment and development of new approach methodologies: An adverse outcome pathway-based approach. *Advanced Science, 10*(2), e2203984. https://doi.org/10.1002/advs.202203984

Sahlman, P., Nissinen, M., Puukka, P., Jula, A., Salomaa, V., Männistö, S., Lundqvist, A., Valsta, L., Perola, M., Färkkilä, M., & Åberg, F. (2020). Genetic and lifestyle risk factors for advanced liver disease among men and women. *Journal of Gastroenterology and Hepatology, 35*(2), 291–298. https://doi.org/10.1111/jgh.14770

Sarkar, S., Horn, G., Moulton, K., Oza, A., Byler, S., Kokolus, S., & Longacre, M. (2013). Cancer development, progression, and therapy: An epigenetic overview. *International Journal of Molecular Sciences, 14*(10), 21087–21113. https://doi.org/10.3390/ijms141021087

Schmeisser, S., Miccoli, A., von Bergen, M., Berggren, E., Braeuning, A., Busch, W., Desaintes, C., Gourmelon, A., Grafström, R., Harrill, J., Hartung, T., Herzler, M., Kass, G. E. N., Kleinstreuer, N., Leist, M., Luijten, M., Marx-Stoelting, P., Poetz, O., van Ravenzwaay, B., … Tralau, T. (2023). New approach methodologies in human regulatory toxicology—Not if, but how and when! *Environment International, 178*, 108082. https://doi.org/10.1016/j.envint.2023.108082

Senft, D. (2024). No mutation, tumour initiation. *Nature Reviews Cancer, 24*(7), 446. https://doi.org/10.1038/s41568-024-00711-9

Senga, S. S., Bisson, W. H., & Colacci, A. (2024). Key characteristics of carcinogens meet hallmarks for prevention-cutting the Gordian knot. *Frontiers in Oncology, 14*, 1420687. https://doi.org/10.3389/fonc.2024.1420687

Sewell, F., Alexander-White, C., Brescia, S., Currie, R. A., Roberts, R., Roper, C., Vickers, C., Westmoreland, C., & Kimber, I. (2024). New approach methodologies (NAMs): Identifying and overcoming hurdles to accelerated adoption. *Toxicology Research, 13*(2), tfae044. https://doi.org/10.1093/toxres/tfae044

Shao, K., Ji, C., & Gadagbui, B. (2024). Editorial: Next generation chemical risk assessment: Integration of advances in toxicology, biology and computation. *Frontiers in Toxicology*, *6*, 1440229. https://doi.org/10.3389/ftox.2024.1440229

Sharma, D., Subbarao, G., & Saxena, R. (2017). Hepatoblastoma. *Seminars in Diagnostic Pathology*, *34*(2), 192–200. https://doi.org/10.1053/j.semdp.2016.12.015

Sheng, Q. S., Liu, B., Wang, X., Hua, L., Zhao, S. C., Sun, X. Z., Li, M. Y., Zhang, X. Y., Wang, J. X., & Hu, P. L. (2025). Revolutionizing toxicological risk assessment: Integrative advances in new approach methodologies (NAMs) and precision toxicology. *Archives of Toxicology*, *99*(12), 4697–4707. https://doi.org/10.1007/s00204-025-04169-y

Siddiqui, I. A., Sanna, V., Ahmad, N., Sechi, M., & Mukhtar, H. (2015). Resveratrol nanoformulation for cancer prevention and therapy. *Annals of the New York Academy of Sciences*, *1348*(1), 20–31. https://doi.org/10.1111/nyas.12811

Silva, D. B. da, Pianovski, M. A. D., & Carvalho Filho, N. P. de. (2025). Environmental pollution and cancer. *Jornal de Pediatria*, *101*, S18–S26. https://doi.org/10.1016/j.jped.2024.09.004

Skat-Rørdam, J., Lykkesfeldt, J., Gluud, L. L., & Tveden-Nyborg, P. (2025). Mechanisms of drug induced liver injury. *Cellular and Molecular Life Sciences*, *82*(1), 213. https://doi.org/10.1007/s00018-025-05744-3

Slikker, W., Andersen, M. E., Bogdanffy, M. S., Bus, J. S., Cohen, S. D., Conolly, R. B., David, R. M., Doerrer, N. G., Dorman, D. C., Gaylor, D. W., Hattis, D., Rogers, J. M., Woodrow Setzer, R., Swenberg, J. A., & Wallace, K. (2004). Dose-dependent transitions in mechanisms of toxicity. *Toxicology and Applied Pharmacology*, *201*(3), 203–225. https://doi.org/10.1016/j.taap.2004.06.019

Smith, M. T. (2025). The key characteristics concept. *Current Opinion in Toxicology*, *41*, 100515. https://doi.org/10.1016/j.cotox.2024.100515

Smith, M. T., Guyton, K. Z., Gibbons, C. F., Fritz, J. M., Portier, C. J., Rusyn, I., DeMarini, D. M., Caldwell, J. C., Kavlock, R. J., Lambert, P. F., Hecht, S. S., Bucher, J. R., Stewart, B. W., Baan, R. A., Cogliano, V. J., & Straif, K. (2016). Key characteristics of carcinogens as a basis for organizing data on mechanisms of carcinogenesis. *Environmental Health Perspectives*, *124*(6), 713–721. https://doi.org/10.1289/ehp.1509912

Sosa, V., Moliné, T., Somoza, R., Paciucci, R., Kondoh, H., & LLeonart, M. E. (2013). Oxidative stress and cancer: An overview. *Ageing Research Reviews*, *12*(1), 376–390. https://doi.org/10.1016/j.arr.2012.10.004

Štampar, M., & Žegura, B. (2024). In vitro hepatic 3D cell models and their application in genetic toxicology: A systematic review. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, *900*, 503835. https://doi.org/10.1016/j.mrgentox.2024.503835

Stanfill, S. B., Hecht, S. S., Joerger, A. C., González, P. J., Maia, L. B., Rivas, M. G., Moura, J. J. G., Gupta, A. K., Le Brun, N. E., Crack, J. C., Hainaut, P., Sparacino-Watkins, C., Tyx, R. E., Pillai, S. D., Zaatari, G. S., Henley, S. J., Blount, B. C., Watson, C. H., Kaina, B., & Mehrotra, R. (2023). From cultivation to cancer: Formation of N-nitrosamines and other carcinogens in smokeless tobacco and their mutagenic implications. *Critical Reviews in Toxicology*, *53*(10), 658–701. https://doi.org/10.1080/10408444.2023.2264327

Storer, R. D., Sistare, F. D., Reddy, M. V., & DeGeorge, J. J. (2010). An industry perspective on the utility of short-term carcinogenicity testing in transgenic mice in pharmaceutical development. *Toxicologic Pathology*, *38*(1), 51–61. https://doi.org/10.1177/0192623309351718

Stucki, A. O., Sauer, U. G., Allen, D. G., Kleinstreuer, N. C., Perron, M. M., Yozzo, K. L., Lowit, A. B., & Clippinger, A. J. (2024). Differences in the anatomy and physiology of the human and rat respiratory tracts and impact on toxicological assessments. *Regulatory Toxicology and Pharmacology*, *150*, 105648. https://doi.org/10.1016/j.yrtph.2024.105648

Suarez-Torres, J. D., Orozco, C. A., & Ciangherotti, C. E. (2021). The 2-year rodent bioassay in drug and chemical carcinogenicity testing: Performance, utility, and configuration for cancer hazard identification. *Journal of Pharmacological and Toxicological Methods*, *110*, 107070. https://doi.org/10.1016/j.vascn.2021.107070

Sulsky, S. I., Greene, T., & Gentry, P. R. (2024). A framework for integrating evidence to assess hazards and risk. *Critical Reviews in Toxicology*, *54*(5), 315–329. https://doi.org/10.1080/10408444.2024.2342447

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *71*(3), 209–249. https://doi.org/10.3322/caac.21660

Svingen, T., Villeneuve, D. L., Knapen, D., Panagiotou, E. M., Draskau, M. K., Damdimopoulou, P., & O'Brien, J. M. (2021). A pragmatic approach to adverse outcome pathway development and evaluation. *Toxicological Sciences*, *184*(2), 183–190. https://doi.org/10.1093/toxsci/kfab113

Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, *126*(4), 663–676. https://doi.org/10.1016/j.cell.2006.07.024

Tannenbaum, J., & Bennett, B. T. (2015). Russell and Burch's 3Rs then and now: The need for clarity in definition and purpose. *Journal of the American Association for Laboratory Animal Science*, *54*(2), 120–132.

Telles-Silva, K. A., Pacheco, L., Komatsu, S., Chianca, F., Caires-Júnior, L. C., Araujo, B. H. S., Goulart, E., & Zatz, M. (2022). Applied hepatic bioengineering: Modeling the human liver using organoid and liver-on-a-chip technologies. *Frontiers in Bioengineering and Biotechnology*, *10*, 845360. https://doi.org/10.3389/fbioe.2022.845360

Terry, C., Hays, S., McCoy, A. T., McFadden, L. G., Aggarwal, M., Rasoulpour, R. J., & Juberg, D. R. (2016). Implementing a framework for integrating toxicokinetics into human health risk assessment for agrochemicals. *Regulatory Toxicology and Pharmacology*, *75*, 89–104. https://doi.org/10.1016/j.yrtph.2015.10.003

The genetics of cancer. (2015). National Cancer Institute. https://www.cancer.gov/about-cancer/causes-prevention/genetics

Torgovnick, A., & Schumacher, B. (2015). DNA repair mechanisms in cancer development and therapy. *Frontiers in Genetics*, *6*, 157. https://doi.org/10.3389/fgene.2015.00157

Touwaide, A., & Appetiti, E. (2015). Food and medicines in the Mediterranean tradition. A systematic analysis of the earliest extant body of textual evidence. *Journal of Ethnopharmacology*, *167*, 11–29. https://doi.org/10.1016/j.jep.2014.10.035

Trefts, E., Gannon, M., & Wasserman, D. H. (2017). The liver. *Current Biology*, *27*(21), R1147–R1151. https://doi.org/10.1016/j.cub.2017.09.019

U.S. Environmental Protection Agency. (2014). *Next generation risk assessment: Incorporation of recent advances in molecular, computational, and systems biology* (Final Report). https://assessments.epa.gov/risk/document/&deid%3D286690

U.S. Environmental Protection Agency. (2015,). *Toxicology testing in the 21st century (Tox21)*. https://www.epa.gov/chemical-research/toxicology-testing-21st-century-tox21

U.S. Environmental Protection Agency. (2017). *Alternative test methods and strategies to reduce vertebrate animal testing*. https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/alternative-test-methods-and-strategies-reduce

UN Environment Programme. (2019). *UN report: Urgent action needed to tackle chemical pollution as global production is set to double by 2030*. https://www.unep.org/news-and-stories/press-release/un-report-urgent-action-needed-tackle-chemical-pollution-global

Veltman, C. H. J., Pennings, J. L. A., van de Water, B., & Luijten, M. (2023). An adverse outcome pathway network for chemically induced oxidative stress leading to (non)genotoxic carcinogenesis. *Chemical Research in Toxicology, 36*(6), 805–817. https://doi.org/10.1021/acs.chemrestox.2c00396

Viluksela, M., Stahl, B. U., Birnbaum, L. S., Schramm, K. W., Kettrup, A., & Rozman, K. K. (1997). Subchronic/chronic toxicity of 1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin (HpCDD) in rats. Part I. Design, general observations, hematology, and liver concentrations. *Toxicology and Applied Pharmacology, 146*(2), 207–216. https://doi.org/10.1006/taap.1997.8239

Walder, L., Pallocca, G., Bastos, L. F., Beekhuijzen, M., Busquet, F., Constantino, H., Corvaro, M., Courtot, L., Escher, B., Fernandez, R., Gougeon, E., Hansell, L., Herzler, M., Holden, L., Hornek-Gausterer, R., Irizar, A., Kandarova, H., Kern, P., Kolle, S., … Baines, J. (2025). EU roadmap for phasing out animal testing for chemical safety assessments: Recommendations from a multi-stakeholder roundtable. *ALTEX, 42*(3), 435–450. https://doi.org/10.14573/altex.2503241

Wambaugh, J. F., Bare, J. C., Carignan, C. C., Dionisio, K. L., Dodson, R. E., Jolliet, O., Liu, X., Meyer, D. E., Newton, S. R., Phillips, K. A., Price, P. S., Ring, C. L., Shin, H.-M., Sobus, J. R., Tal, T., Ulrich, E. M., Vallero, D. A., Wetmore, B. A., & Isaacs, K. K. (2019). New approach methodologies for exposure science. *Current Opinion in Toxicology, 15*, 76–92. https://doi.org/10.1016/j.cotox.2019.07.001

Wang, L., Shao, Z., Wang, X., Lu, W., & Sun, H. (2025). Xenobiotic-induced liver injury: Molecular mechanisms and disease progression. *Ecotoxicology and Environmental Safety, 303*, 118854. https://doi.org/10.1016/j.ecoenv.2025.118854

Wang, Z. G., He, Z. Y., Chen, Y. Y., Gao, H., & Du, X. L. (2021). Incidence and survival outcomes of secondary liver cancer: A Surveillance Epidemiology and End Results database analysis. *Translational Cancer Research, 10*(3), 1273–1283. https://doi.org/10.21037/tcr-20-3319

Wang, Z., Walker, G. W., Muir, D. C. G., & Nagatani-Yoshida, K. (2020). Toward a global understanding of chemical pollution: A first comprehensive analysis of national and regional chemical inventories. *Environmental Science & Technology, 54*(5), 2575–2584. https://doi.org/10.1021/acs.est.9b06379

Weston, A., & Harris, C. C. (2003). Chemical carcinogenesis. In D. W. Kufe, R. E. Pollock, & R. R. Weichselbaum (Eds.), *Holland-Frei cancer medicine* (6th ed.). BC Decker. https://www.ncbi.nlm.nih.gov/books/NBK13216/

Wilson, R. H., & Bradfield, C. A. (2021). Rodent genetic models of Ah receptor signaling. *Drug Metabolism Reviews*, *53*(3), 350–374. https://doi.org/10.1080/03602532.2021.1955916

World Health Organization. (2014). *Guidance for applying quantitative data to develop data-derived extrapolation factors for interspecies and intraspecies extrapolation*. WHO Press.

Worth, A. P., & Berggren, E. (2025). A twin transition in regulatory toxicology: Moving toward Chemicals 2.0 and phasing out animal testing. *Toxicological Sciences*, *203*(2), 160–165. https://doi.org/10.1093/toxsci/kfae130

Wu, S., Zhu, W., Thompson, P., & Hannun, Y. A. (2018). Evaluating intrinsic and non-intrinsic cancer risk factors. *Nature Communications*, *9*(1), 3490. https://doi.org/10.1038/s41467-018-05467-z

Wu, Y., Geng, X., Wang, J., Miao, Y., Lu, Y., & Li, B. (2016). The HepaRG cell line, a superior in vitro model to L-02, HepG2 and hiHeps cell lines for assessing drug-induced liver injury. *Cell Biology and Toxicology*, *32*(1), 37–59. https://doi.org/10.1007/s10565-016-9316-2

Xuan, J., Chen, S., Ning, B., Tolleson, W. H., & Guo, L. (2016). Development of HepG2-derived cells expressing cytochrome P450s for assessing metabolism-associated drug-induced liver toxicity. *Chemico-Biological Interactions*, *255*, 63–73. https://doi.org/10.1016/j.cbi.2015.10.009

Yadav, R., Mahajan, S., Singh, H., Mehra, N. K., Madan, J., Doijad, N., Singh, P. K., & Guru, S. K. (2024). Emerging in vitro and in vivo models: Hope for the better understanding of cancer progression and treatment. *Advanced Biology*, *8*(6), e2300487. https://doi.org/10.1002/adbi.202300487

Yasir, S., & Torbenson, M. S. (2019). Angiosarcoma of the liver: Clinicopathologic features and morphologic patterns. *The American Journal of Surgical Pathology*, *43*(5), 581–590. https://doi.org/10.1097/PAS.0000000000001228

Yin, C., Evason, K. J., Asahina, K., & Stainier, D. Y. R. (2013). Hepatic stellate cells in liver development, regeneration, and cancer. *The Journal of Clinical Investigation*, *123*(5), 1902–1910. https://doi.org/10.1172/JCI66369

Zagorski, J. W., & Kaminski, N. E. (2023). When the weight of evidence does not weigh enough: EFSA's draft scientific opinion on BPA. *Toxicological Sciences, 193*(2), 115–118. https://doi.org/10.1093/toxsci/kfad034

Zhang, S., Xiao, X., Yi, Y., Wang, X., Zhu, L., Shen, Y., Lin, D., & Wu, C. (2024). Tumor initiation and early tumorigenesis: Molecular mechanisms and interventional targets. *Signal Transduction and Targeted Therapy, 9*(1), 149. https://doi.org/10.1038/s41392-024-01848-7

Zhao, Y., Zhao, S., Liu, S., Ye, W., & Chen, W.-D. (2025). Kupffer cells, the limelight in the liver regeneration. *International Immunopharmacology, 146*, 113808. https://doi.org/10.1016/j.intimp.2024.113808

Zheng, Q., Nan, K., & Zheng, H. (2010). Hippocrates' viewpoint on cancer. *Zhonghua Yi Shi Za Zhi, 40*(4), 234–236.

Chapter I

"The art of chemical classification, prioritization, and screening"

Preamble

The classification and prioritization of chemicals is a crucial step in assessing chemical toxicity. It provides the foundation for more efficient risk-evaluation strategies and serves as an initial step in toxicity screening. By organizing chemicals according to their toxicity profiles, and their MoAs, researchers can streamline testing workflows. Such classification reduces redundancy and optimizes the use of time and resources, ultimately enabling more cost-effective and targeted experimental designs.

Because chemicals can trigger different cellular mechanisms depending on the specific cell type or *in vitro* model used, selecting an appropriate cellular system is essential for accurately capturing chemical-associated toxicity. The choice of model ranging from simple 2D monolayers to more complex 3D spheroids can strongly influence the sensitivity, relevance, and predictive value of chemical toxicity assessments. Integrating a chemical-classification strategy with the selection of human-relevant, sensitive models therefore enhances the reliability of toxicity evaluations and contributes to a robust framework for decision-making in CSA and regulatory toxicology.

In this context, in this chapter a NAM-based strategy was employed to generate mechanistic toxicity profiles at the cellular level for a selected panel of hepatotoxic chemicals from the PARC list. These profiles were generated using a high-throughput, high-content screening approach in both 2D HepaRG monolayers and the more physiologically relevant 3D HepaRG spheroid models. Various endpoints were analyzed, including cytotoxicity, mitochondrial dysfunction, oxidative stress, lipid accumulation, and inflammation. Additionally, several statistical tools were applied to the data generated for each endpoint and model to evaluate and compare the sensitivity of the different systems to the chemicals tested in this chapter.

Overall, generating toxicity profiles for a panel of chemicals based on *in vitro* mechanistic endpoints enables a rapid, efficient, and human-relevant approach to chemical classification and prioritization. This supports the identification of high-priority compounds for further investigation and strengthens evidence-based decision-making. Consequently, the NAM-based strategy presented in this chapter provides important preliminary information on both the sensitivity of cellular models to different NGTxC mechanisms and the classification of chemicals according to their toxicity profiles, forming a foundation for the more comprehensive analyses discussed in the following chapters.

Préambule

La classification et la priorisation des substances chimiques constituent une étape essentielle dans l'évaluation de leur toxicité. Elles fournissent la base de stratégies d'évaluation des risques plus efficaces et représentent une première étape du criblage toxicologique. En organisant les substances selon leurs profils de toxicité et leurs MoAs, les chercheurs peuvent rationaliser les flux de travail, réduire les redondances et optimiser l'utilisation du temps et des ressources, permettant ainsi des conceptions expérimentales plus ciblées et rentables.

Comme les substances chimiques peuvent déclencher différents mécanismes cellulaires selon le type cellulaire ou le modèle *in vitro* utilisé, le choix d'un système approprié est crucial pour caractériser avec précision la toxicité. Le modèle retenu de simples monocouches 2D à des sphéroïdes 3D plus complexes influence fortement la sensibilité, la pertinence et la valeur prédictive des évaluations. L'intégration d'une stratégie de classification chimique avec des modèles sensibles et pertinents pour l'humain améliore donc la fiabilité des évaluations et contribue à un cadre solide pour la prise de décision en CSA et en toxicologie réglementaire.

Dans ce contexte, ce chapitre a appliqué une stratégie fondée sur les NAMs afin de générer des profils mécanistiques de toxicité pour un panel de substances hépatotoxiques issues de la liste PARC. Ces profils ont été obtenus grâce à un criblage à haut débit et à haut contenu, dans des monocouches 2D HepaRG et des sphéroïdes 3D HepaRG plus proches de la physiologie humaine. Divers paramètres ont été analysés, notamment la cytotoxicité, la dysfonction mitochondriale, le stress oxydant, l'accumulation lipidique et l'inflammation. Plusieurs outils statistiques ont ensuite été appliqués pour évaluer et comparer la sensibilité des différents modèles aux substances testées. Classification des substances selon leurs profils de toxicité, constituant ainsi une base pour les analyses plus approfondies développées dans les chapitres suivants.

Globalement, la génération de profils de toxicité à partir d'endpoints mécanistiques *in vitro* constitue une approche rapide, efficace et plus pertinente pour l'humain en matière de classification et de priorisation des substances chimiques. Elle soutient l'identification de composés prioritaires pour des investigations approfondies et renforce la prise de décision fondée sur des données probantes. Ainsi, la stratégie NAMs présentée dans ce chapitre fournit des informations préliminaires essentielles sur la sensibilité des modèles cellulaires aux mécanismes NGTxC et sur la classification des substances selon leurs profils toxicologiques, constituant une base pour les analyses approfondies des chapitres suivants.

Mechanistic toxicity profiling of chemicals: A comparative study in 2D and 3D HepaRG models using high-content analysis

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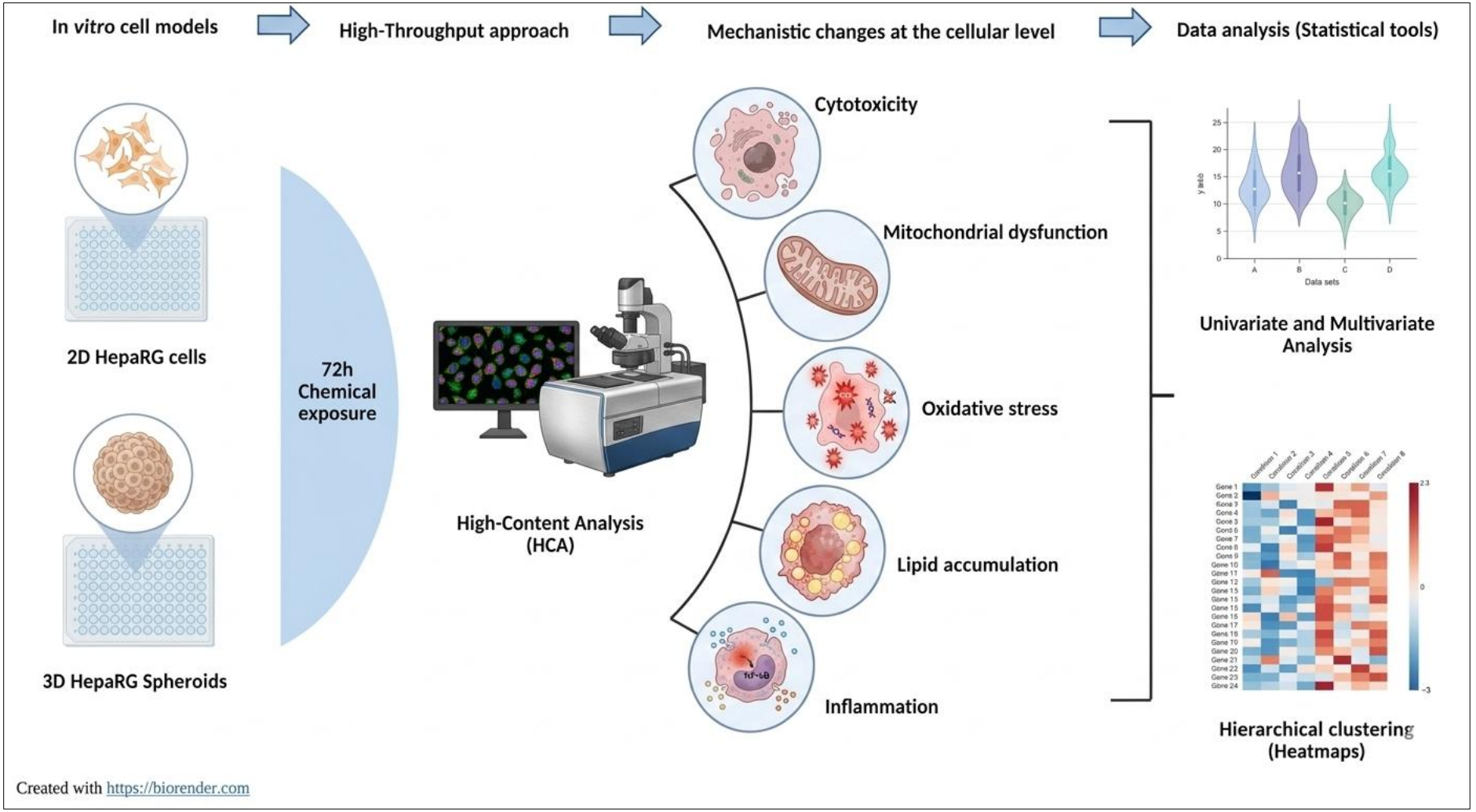
Graphical abstract



Abstract

Humans and the environment are exposed to a diverse range of chemicals, many of which are introduced through the human activities. This growing chemical burden has given rise to concerns related to their potential adverse effects. Traditionally, chemical toxicity evaluation has relied on animal tests that are time consuming, expensive, pose ethical concerns, and may not be directly extrapolated to humans. This highlights the urgent need to develop New Approach Methodologies (NAMs) to evaluate chemical- associated toxicity. Since the liver is a primary target organ for xenobiotics, our objective is to use both 2D and 3D HepaRG models combined with High-Content Analysis (HCA) for chemical mechanistic toxicity profiling. HepaRG models were exposed to eight chemicals (Chlorothalonil, Tebuconazole, Thioacetamide, Benzoyl peroxide, Diethyl maleate, clofibrate, indeno[1,2,3-cd]pyrene, 3-methylcholanthrene) for 72h with daily media renewal. Cellular endpoints including cytotoxicity, oxidative stress, mitochondrial dysfunction, lipid accumulation and the pro-inflammatory response were quantified. Additionally, four chemicals were added in this study to test chemicals having different MOA and to demonstrate the sensitivity of the 3D HepaRG spheroid model (FICZ, Diuron, 12-O-Tetradecanoylphorbol-13-acetate, Azoxystrobin). Our findings revealed that 3D HepaRG cells exhibited more pronounced positive responses to chemical treatment compared to the 2D HepaRG cells. In addition to the chemical-specific toxicity profiling generated by HCA, we identified correlations between different cellular alterations such as mitochondrial dysfunction, dysregulated lipid metabolism and oxidative stress in the treated 3D HepaRG spheroids. Overall, this approach proposed in this study provides a rapid, human-relevant *in vitro* method that enhances the predictive power of NAMs for chemical associated toxicity.

Highlights

- The applicability of NAMs-based HCA in evaluating chemical-associated toxicity
- HepaRG spheroids as a sensitive model in predicting chemical-associated toxicity
- Possible correlation between different mechanistic changes at the cellular level
- Employing multiple statistical models to analyse HCA-generated data

Key words

New Approach Methodologies (NAMs), Cellular mechanistic approach, Liver toxicity, oxidative stress, mitochondrial dysfunction, lipid accumulation, Inflammation.

1. Introduction

Chemical pollution poses significant risks to both human health and the environment. While initial studies of chemical impacts has first mainly concerned naturally occurring substances as well as manufactured chemicals, it has been recently extended the novel entities (NEs)- a term used to describe the new chemicals or the modified forms of existing substances (Persson et al., 2022). A recent global survey estimated over 350,000 of novel chemicals and substances had been introduced through the human activities (Wang et al., 2020). Given the rapid increase in the number of novel chemicals and their potential to cause harm effects, there is an urgent need to shift towards mechanistic toxicology guided by New Approach Methodologies (NAMs). These approaches provide more ethical, efficient, and potentially human-relevant alternatives (Silva & Tamburic, 2022; Li et al., 2024).

Traditionally, the potential adverse effects of chemicals for humans have been evaluated using *in vivo* animal testing, mainly in mammalian species such as rodents (Sewell et al., 2017). However, animal studies face various challenges such as high cost, long experimental duration, limited human relevance due to interspecies differences, and growing ethical concerns (Kiani et al., 2022). Therefore, the reduction and replacement of animal testing in regulatory toxicology, that started more than half century ago with the 3R principle (Grimm et al., 2023), has recently accelerated worldwide. Over the past several decades, regulatory agencies in both the European Union (EU) and the United States of America (USA) have strongly questioned the place of animal testing in chemical and product safety assessments. The European Directive 76/768/EEC (EU, 2009) introduced the ban of animal testing for finished cosmetic products on ethical grounds (*EU Cosmetic Regulations | Directive 76/768/EEC, New EU Cosmetic Products Regulation (EC) No 1223/2009 and REACH | CIRS*). Moreover, the European Union's REACH *(Registration, Evaluation, Authorisation, and Restriction of Chemical Substances, EC No. 1907/2006)* strongly supports the use of alternative methods in chemical assessments (Taylor, 2018). More recently, the US Environmental Protection Agency (US-EPA) has outlined plans to progressively eliminate all animal testing, and to commit the alternative methods used in chemical and pesticide testing by 2035 (*U.S. EPA to Eliminate All Mammal Testing by 2035*). Hence, the development of non-animal methods not only for toxicology research but also for chemical hazard assessment is vital to support regulation (Tarazona et al., 2025).

A large panel of NAMs including *in silico* (computational), *in chemico* (analytical), and *in vitro* (cell-based, *ex vivo*, organoids) methods are currently being developed to improve efficiency,

and more human-relevance (Hoogstraaten et al., 2025). Therefore, in the context of integrated approaches for testing and assessment (IATA), NAMs are primary designed to utilize human-relevant models and technologies to better characterize the potential toxic effects of chemicals to human health (Escher et al., 2022). *In vitro* bioassays combined with High-Throughput Screening, such as High-Content analysis and omics enable rapid and comprehensive chemical classification, profiling and prioritization (Nyffeler et al., 2020; Paul Friedman et al., 2025). Furthermore, the mechanistic approach of toxicological assessment is based on the concept of Adverse Outcome Pathways (AOPs), which are designed to elucidate the cellular and molecular mechanisms underlying chemical toxicity (Jin et al., 2022; Veltman et al., 2025). AOPs provide information about the key events that occur along a toxicological pathway, from the initial molecular interaction to the adverse outcome at the organism level. As such, AOPs support the development and application of NAMs, to replace animal testing in Chemical Safety Assessment (CSA) (Groh et al., 2015; Wambaugh et al., 2019; Sewell et al., 2024).

As liver represents one of the major organs targeted by xenobiotics, the evaluation of hepatotoxicity represents a major focus in CSA (Pridgeon et al., 2018). Commonly known hepatotoxicants affect different biological functions at the cellular level such as oxidative stress, mitochondrial dysfunction, changes in lipid metabolism and inflammation (Rusyn et al., 2021). Gaining a deeper understanding of these mechanisms at the cell level is essential for improving the prediction and assessment of chemical toxicity (Perkins et al., 2019). Various hepatic cell models ranging from primary human hepatocytes (PHHs) to human cell lines and advanced systems such as 3D spheroids, co-cultures, and organ-on-chips have been developed (Wu et al., 2016; Kim et al., 2025). In recent years, the human hepatic HepaRG cell line has proven to be a relevant model for chemical safety evaluation (Guo et al., 2024). Indeed, as bipotent progenitors, they have the capacity to differentiate into hepatocytes and cholangiocyte-like cells, and retain characteristics similar to mature human hepatocytes, including expression of phase I and phase II metabolic enzymes and transporters (Anthérieu et al., 2012; Ranade et al., 2014). Other liver cell models such as PHHs, HepG2 cell line, and stem cell-derived hepatocytes have shown some limitations, including short life span, poor metabolic activities and prolonged differentiation respectively (Gómez-Lechón et al., 2003; Behbahan et al., 2011). In light of these limitations, HepaRG cells are being increasingly adopted as an alternative to PHHs and HepG2 cells for *in vitro* hepatotoxicity studies (Guillouzo et al., 2007; Nelson et al., 2017; Guo et al., 2024). An increasing number of studies that examined various toxicological endpoints including genotoxicity, have been conducted in 3D HepaRG models (Mueller et al.

2014; Mandon et al., 2019; Barranger & Le Hégarat, 2022; Varet et al., 2024). These studies demonstrated that 3D HepaRG spheroids represent a promising, sensitive, and physiologically relevant *in vitro* model for chemicals toxicity assessment. In fact, the 3D architecture of HepaRG spheroids mimics more closely *in vivo* complexity, due to enhanced cell-cell interactions and spatial organisation (Kammerer, 2021).

HCA has been successfully applied to 2D HepaRG cells to investigate adverse cellular changes for early-stage chemical safety assessment (Hussain et al., 2020) and a rapid screening to explore the mechanisms induced in chemical- toxicity could be set up (Joossens et al., 2019; Li et al., 2024). In contrast, the application of HCA to assess chemical toxicity remains limited in 3D HepaRG spheroids (Zhou et al., 2019) and has been challenging to be adapted to 3D PHHs (Takayama et al., 2013; Yang et al. 2023)

In this study, as part of the European Partnership for the Assessment of Risk from Chemicals (PARC) project (Audebert et al., 2023), our objective is to investigate the utility of HCA in 2D and 3D HepaRG cell model as a high-throughput *in vitro* strategy for chemical toxicity evaluations. A panel of mechanistically relevant endpoints including cell viability, mitochondrial dysfunction, oxidative stress, disruption in lipid accumulation, and pro-inflammatory response was quantified to evaluate the chemical toxicity in 2D and 3D HepaRG models at the cellular level. In addition, to determine the sensitivity, predictive performance, and toxicity profiling of the tested chemicals, a comparative analysis was performed in both HepaRG models. To further understand the intricacy of the various responses and improve the translational relevance of *in vitro* platforms, Heatmaps and multivariate statistical techniques employing PCA were utilized to identify correlations among cellular mechanisms.

2. Materials and Methods

2.1. 2D HepaRG cell culture

The human hepatic cell line HepaRG was used at passage numbers between 15-18. Cells were cultured as previously described (Barranger & Le Hégarat, 2022) (Dubreil et al., 2024). The cells were purchased form Biopredic International and cultured in Williams E medium + Glutamax^{TM}-I (Gibco 32551-087) supplemented with 10% FBS FetalClone II (Cytiva SH30066.03), penicillin 5000 units/mL / streptomycin 5000 µg/mL (Gibco 15070-063), 5 mg/mL insulin (Sigma I2643-50MG), and 18 mg/mL hydrocortisone hemisuccinate (Upjohn). In brief, cells were seeded in 75 cm^2 flasks (VWR 3290) at a density of $20x10^3/cm^2$ and incubated at 37 °C with 5% CO_2. After 2 weeks in culture, cells were dissociated with TryplE 1X (Thermo Fisher Scientific - REF 12563-011) and seeded in 75 cm^2 flasks at a density of $27x10^3/cm^2$. For differentiation, $1x10^4$ cells/well were seeded in 96-well plates (Sigma CORNING 3599). After 2 weeks of culture, HepaRG cells were incubated with medium supplemented with DMSO (Sigma D4540) at increasing concentrations (1%, 1.5%, and 1.7%) which was renewed every 2 days in the first week of differentiation. Afterwards, HepaRG cells were cultured in medium supplemented with 1.7% DMSO. Medium was changed every 2 days during the second week of differentiation (Figure C.I 1).

2.2. 3D HepaRG spheroid culture

Spheroid formation was performed as previously described (Mandon et al., 2019). After 14 days of differentiation in flasks, cells were trypsinized, and the resulting cell suspension was seeded in 96-well Ultra-Low Attachment (ULA) plates (Sigma, France - CORNING REF 4520) at a density of 2,000 cells/Spheroid. Medium was replaced every 2 days. Spheroids were treated 7 days after seeding (Figure C.I 1).

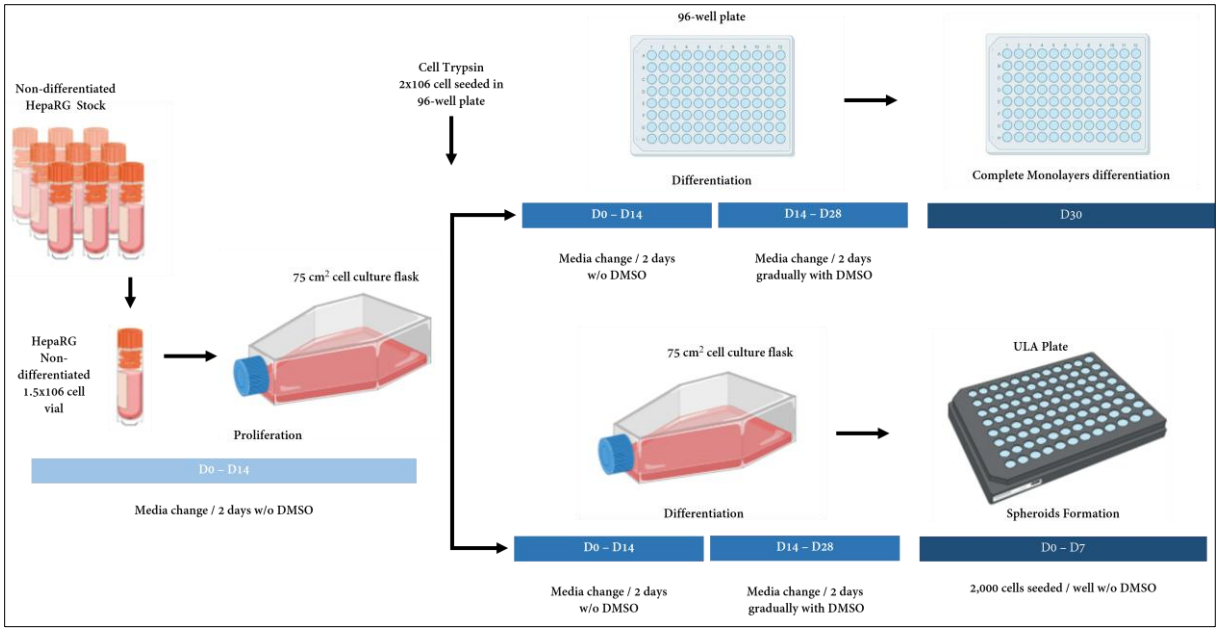


Figure C.I 1. 2D HepaRG monolayer and 3D HepaRG spheroid culture protocol. Created with https://biorender.com.

2.3. Chemicals

The different chemicals used in this study are listed in (Table C.I 1), with their uses and, information on reported cellular toxicity. All chemicals were supplied from Sigma (Saint-Quentin-Fallavier, France) except CT, Tebu, 3MCA, and IP that were supplied from LGC (Molsheim, France). Eight different chemicals were tested in both 2D and 3D HepaRG models. In addition, four chemicals (DCMU, FICZ, TPA, Azox) were tested only in the 3D HepaRG model.

Six concentrations were tested for each chemical. The highest concentrations used in this study, based on the published toxicity data available or/and solvent solubility, are shown in (Table C.I 1).

Table C.I 1. Set of chemicals tested in this study.

Chemicals tested with both models					
Chemical	CAS	Uses	Cellular toxicity	Reference	Highest tested concentration
Chlorothalonil (CT)	1897-45-6	Fungicide	Oxidative stress	(Suzuki et al., 2004)	100 µM
Tebuconazole (Tebu)	107534-96-3	Fungicide	Oxidative stress, Lipid accumulation	(Ku et al., 2021), (Kwon et al., 2021)	200 µM
Thioacetamid (TAA)	62-55-5	Industrial applications	Oxidative stress, necrosis, fibrosis	(Ezhilarasan, 2023), (Amali et al., 2006)	10000 µM
Benzoyl peroxide (BPO)	94-36-0	Acne treatment	Oxidative stress, Lipid metabolism disruption	(Matin et al., 2025), (Burkhart et al., 2000)	200 µM
Diethyl maleate (DEM)	141-05-9	Glutathione-depleting agent	Oxidative stress	(Stevens & Anders, 1981)	200 µM
Clofibrate (CF)	637-07-0	Pharmaceutical drug	Mitochondrial dysfunction, Oxidative stress, Lipid metabolism disruption	(Qu et al., 2001), (Zhao et al., 2023)	200 µM
indeno[1,2,3-cd]pyrene (IP)	193-39-5	Environmental pollutant (PAH)	DNA adduct formation, genotoxicity	(Cunha & Fernandes, 2020)	6.25 µM
3-methylcholanthrene (3MCA)	56-49-5	Chemical carcinogen	Oxidative stress, Lipid metabolism disruption, Genotoxicity.	(Jin et al., 2014), (Ishidate et al., 1982)	6.25 µM
Chemicals tested with 3D HepaRG spheroids					
Chemical	CAS	Uses	Cellular toxicity	Reference	Highest tested concentration
Diuron (DCMU)	330-54-1	Herbicide	Mitochondrial dysfunction, oxidative stress, Lipid metabolism disruption, genotoxicity.	(Mohammed et al., 2020), (Lima et al., 2022)	100 µM
FICZ	172922-91-7	AhR-agonist	Oxidative stress, aryl hydrocarbon receptor (AhR) activation	(Wincent et al., 2016)	1.56 µM
12-O-Tetradecanoylphorbol-13-acetate (TPA)	16561-29-8	Tumour-promoter	Lipid metabolism disruption, Protein Kinase C (PKC) activation	(Agarwal et al., 1994), (Cabot et al., 1980)	100 µM
Azoxystrobin (Azox)	131860-33-8	Fungicide	Mitochondrial dysfunction, oxidative stress, Lipid metabolism disruption	(Han et al., 2016), (Gao et al., 2014)	100 µM

2.4. Chemical exposure

All chemicals were prepared in DMSO (Sigma D4540). Both cell models were treated with serial dilutions of chemicals prepared in treatment media containing 1% DMSO and 2% FBS. Both 2D and 3D models were treated for 72h with daily renewal of cell culture media containing treatment. Two technical replicates were analysed for all the tested chemical conditions, and all conditions were repeated independently three times.

2.5. Cytotoxicity assessment

The cytotoxicity of chemicals in 2D HepaRG cells was calculated from the number of cells quantified by DAPI staining with the HCA software as 'object number'.

For 3D spheroids, viability was assessed for each spheroid using CellTiter-Glo 3D Cell Viability kit (Promega G9682), according to the manufacturer's instructions. ATP luminescence was measured using a FLUOstar Optima Microplate reader (BMG Labtech, Champigny-sur-Marne, France). Relative ATP levels were calculated using the following formula:

Relative ATP level (%) = [Blank corrected raw data (sample) / Blank corrected raw data (control)] x 100

2.6. Mitochondrial membrane potential (ΔΨm) assay

Following 72h treatment with chemicals, 2D and 3D HepaRG models were incubated for 30 minutes with Tetramethylrhodamine ethyl ester (TMRE) (Sigma 87917) and Hoechst 33342 (Sigma H3570) (150 nM TMRE / 3 µM Hoechst and 80 nM TMRE / 1 µM Hoechst for 2D and 3D cultures respectively). Cells were washed 3 times with serum-free medium before image acquisition and analysis using the Target Activation Bioapplication from HCS Studio software.

2.7. Oxidative stress assay

ROS levels in 2D and 3D HepaRG models were quantified after 72h chemical treatment using H_2DCFDA fluorescent probe (Thermo Fisher C6827). Solutions were prepared in media as 10 µM H_2DCFDA / 3 µM Hoechst 33342 and 8 µM H_2DCFDA / 1 nM Hoechst 33342. After 2 hours incubation, cells were washed 3 times with PBS before analysis by Target Activation Bioapplication.

2.8. Neutral lipid accumulation assay

After 72h treatment, 2D HepaRG cells were fixed with 4% formaldehyde (Thermo Fisher Scientific 28908) for 15 min at room temperature. Following a one hour with blocking solution (1x PBS, 0.05% Tween-20, 1% BSA), plates were incubated with staining solutions prepared in PBS (BODIPY (Cayman chemical company 25892) 5 µM / 3 µM DAPI (Sigma D9542)) and incubated for 1 hour at room temperature. Cells were washed 3 times with PBS before quantification with the Compartmental analysis BioApplication of the HCS Studio software.

For 3D models, after a 30 min incubation at 37 °C with BODIPY 5 µM / 1 µM Hoechst 33342 prepared in PBS, cells were washed 3 times with PBS before screening with Target activation protocol.

2.9. NF-κB translocation assay

After cell fixation as described above, 2D HepaRG cells were incubated with 1:1000 Rb anti-NF-κB primary antibody (Abcam AB16502) overnight. A goat anti-Rabbit IgG secondary antibody Alexa fluor 549 (1:2000) (Abcam AB150116) was added for 2 hours at room temperature (RT). Cells were then washed 3 times and stained with. DAPI for 5 minutes at RT. Cells washed 3 times with PBS prior to image acquisition and analysis with the Compartmental Analysis Bioapplication of the HCS Studio software. This protocol was not applicable to 3D HepaRG spheroids.

2.10. High-Content Analysis

An Arrayscan VTI automated microscope associated with the provided BioApplication software (Thermo Scientific, Waltham, MA, USA) were used to quantify the fluorescence from images using Target Activation or Compartmental Analysis modules for live cell or non-live cell protocols respectively. Images from six fields per well at 20x magnification were analysed to quantify the fluorescence of each marker in 2D HepaRG cells. Fluorescence average intensities of DAPI and Hoechst 33342 were quantified from Nuclei. TMRE, H_2DCFDA, and BODIPY were quantified from the fluorescence found in cytoplasm. Nuclear and cytoplasmic average intensities of NF-κB were quantified to calculate the ratio.

For 3D spheroids, images from one object in one field (one spheroid/well) at 4x magnification were analysed. Total intensities were quantified from the entire fluorescence using the Target

Activation module. Spheroid areas were determined with the software by measuring the total pixels in the segmented object.

2.11. Statistical analysis

Intensities of the quantified markers from HCA images for both models treated with the chemicals were normalized to the solvent negative control to calculate the fold change of each biological replicate. Fold changes, dose responses, and relative ATP levels were analysed relative to the negative control in each treatment condition using a one-way ANOVA, followed by a Dunnett's post-hoc test using GraphPad prism (version 9.5.1). Data are presented as means ± SEM with ANOVA and Dunnett's post-hoc tests p-values presented as: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Heatmaps coupled to Dendrogram Hierarchical Clustering Analysis was performed using R software version 3.3.3 ("ComplexHeatmap", "dendsort" and "circlize" packages), adapted from (Ghosson et al., 2024). To equilibrate fold change weights, and to centre values equal to one at zero, a Log2 transformation was applied. Mean values of the three transformed biological replicate values were used to generate the heatmaps. To avoid infinite values of the data equal to zero, a pseudocount of +1E-10 was applied on all the fold change dataset prior the Log2 transformation. The applied hierarchical clustering on heatmap rows (parameters) was done using the Ward algorithm. The applied distance measuring is Euclidean. Each column corresponds to a specific chemical–concentration combination, grouped and colour-coded by compound type as shown in the legend. The intensity scale ranges from dark to light blue (low response), and light to dark red (high response), reflecting the variation in cellular responses. Scale centre values have a white colour intensity. In addition, the end points (in rows) for both models were hierarchically clustered.

PCA Analysis was done on MetaboAnalyst 6.0 web-based platform [MetaboAnalyst]. The transformed data used in the heatmaps were used as well in this analysis.

3. Results

3.1. Mechanistic cellular changes in 2D HepaRG cells

3.1.1. Cytotoxicity

Cell viability was measured in 2D HepaRG cells using HCA based on automated cell counting. Following the 72h repetitive treatment, a significant decrease in cell count was observed in 2D HepaRG cells treated with CT, Tebu, and 3MCA while no effect in cell count was observed with TAA, BPO, DEM, CF, IP (Figure C.I 2).

3.1.2. Effects on mitochondrial membrane potential (ΔΨm)

A significant concentration-dependent decrease in mitochondrial membrane potential (ΔΨm) was observed in 2D HepaRG cells treated with CT, concomitantly to a decrease of cell viability (Figure C.I 3). However, a significant increase in the ΔΨm was observed following treatment of 2D HepaRG cells with Tebu, IP, and 3MCA treatment (Figure C.I 3 A).

3.1.3. Oxidative stress

A 72h treatment of 2D HepaRG cells with 3MCA resulted in a statistically significant increase in levels of reactive oxygen species (ROS). In addition, although not statistically significant, a slight increase of ROS levels was observed with Tebu, and TAA at the highest concentration tested. No changes in ROS levels were observed in 2D HepaRG cells treated with the other chemicals tested (Figure C.I 4).

3.1.4. Neutral lipid accumulation

A statistically significant increase in neutral lipid accumulation were observed in 2D HepaRG cells following treatment with Tebu at concentrations of 25 and 50 µM following 72h repetitive treatment (Figure C.I 5). No observed increase or decrease in the neutral lipid accumulation in 2D HepaRG cells treated with CT, TAA, BPO, DEM, CF, IP, 3MCA (Figure C.I 5).

3.1.5. Nuclear translocation of NF-κB in 2D HepaRG cells

Nuclear translocation of NF-κB was quantified as a marker of the induction of a pro-inflammatory response. Translocation was measured by calculating the ratio of NF-κB intensities between the nucleus and cytoplasm. A statistically significant increase in NF-κB translocation in 2D HepaRG cells was only induced by Tebu and 3MCA treatments after 72h as shown in (Figure C.I 6).

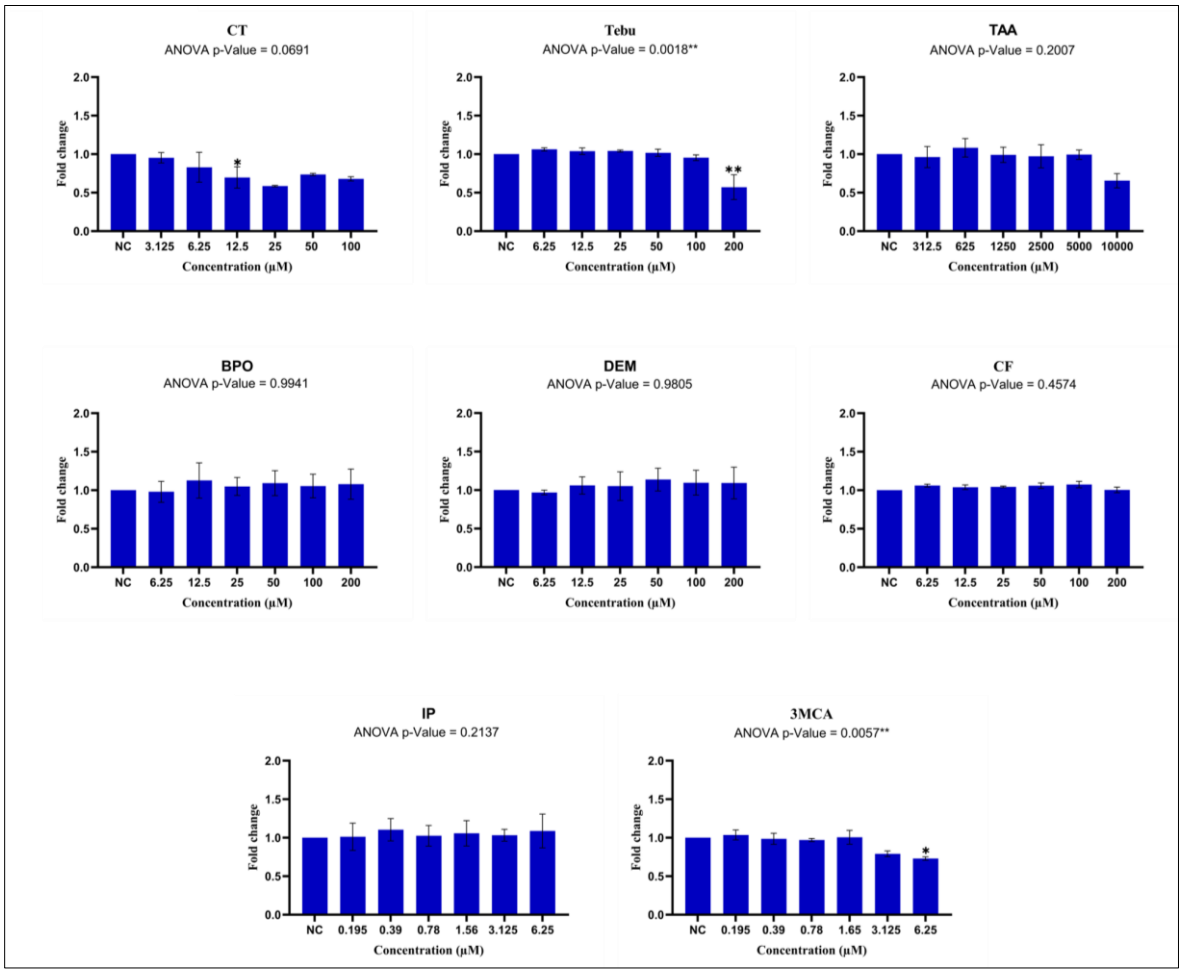


Figure C.I 2. Cell Count following 72h repeated treatments with the selected chemicals in 2D HepaRG cells. Chemicals tested in both 2D and 3D HepaRG cells: CT, Tebu, TAA, BPO, DEM, CF, IP, and 3MCA. Data are presented as means ± SEM with ANOVA and Dunnett's post-hoc tests p-values presented as: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

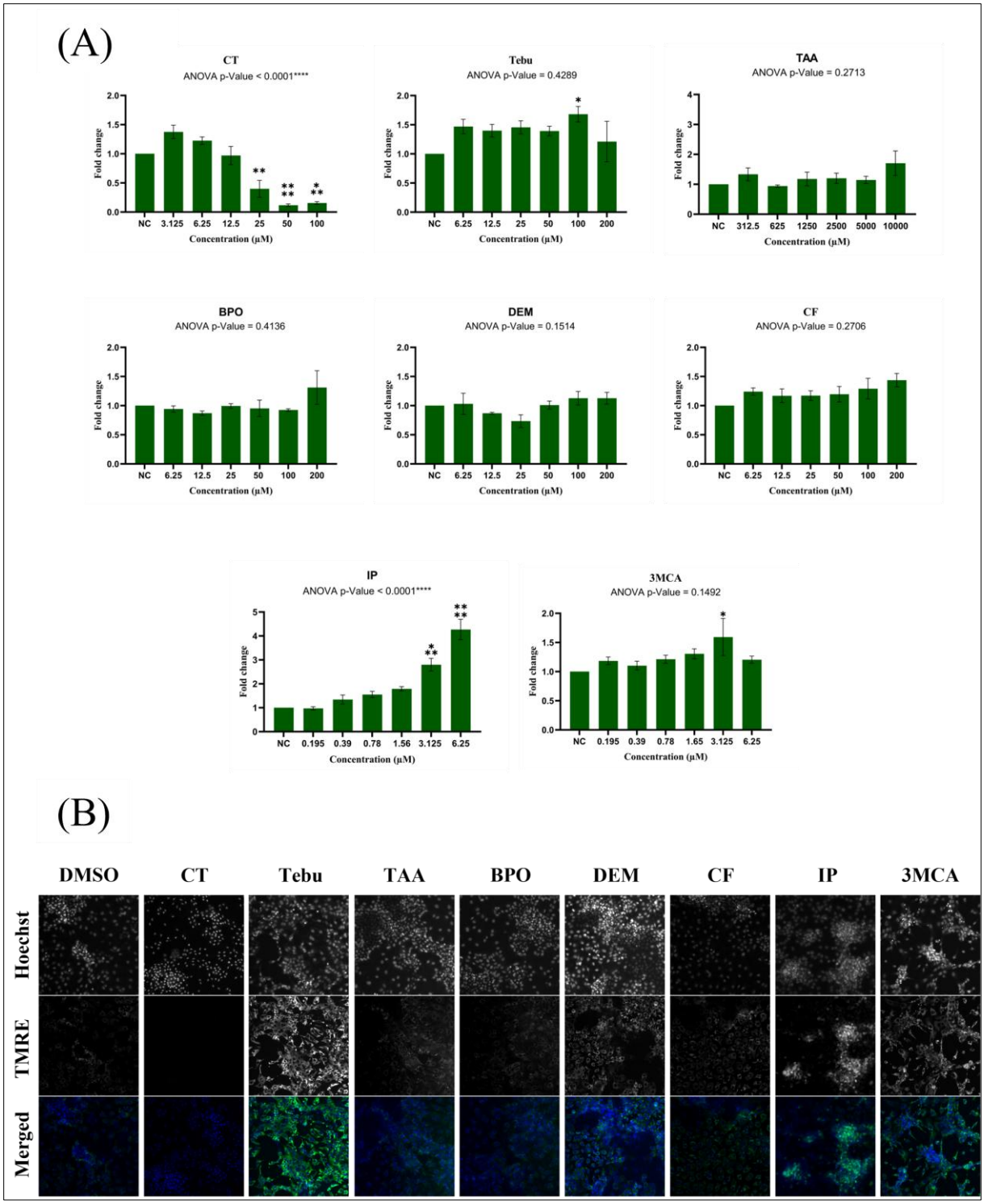


Figure C.I 3. Effects on mitochondrial membrane potential induced by the tested chemicals in 2D HepaRG cells and quantified by TMRE fluorescence. (A): TMRE fluorescence following a 72h repetitive treatment with CT, Tebu, TAA, BPO, DEM, CF, IP, and 3MCA. (B): Representative images of the TMRE fluorescence in HepaRG cells treated with CT (25 µM), Tebu (100 µM), TAA (10000 µM), BPO (200 µM), DEM (200 µM), CF (200 µM), IP (3.125 µM), 3MCA (3.125 µM), giving rise to a statistically significant response. Data are presented as means ± SEM with ANOVA and Dunnett's post-hoc tests p-values presented as: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

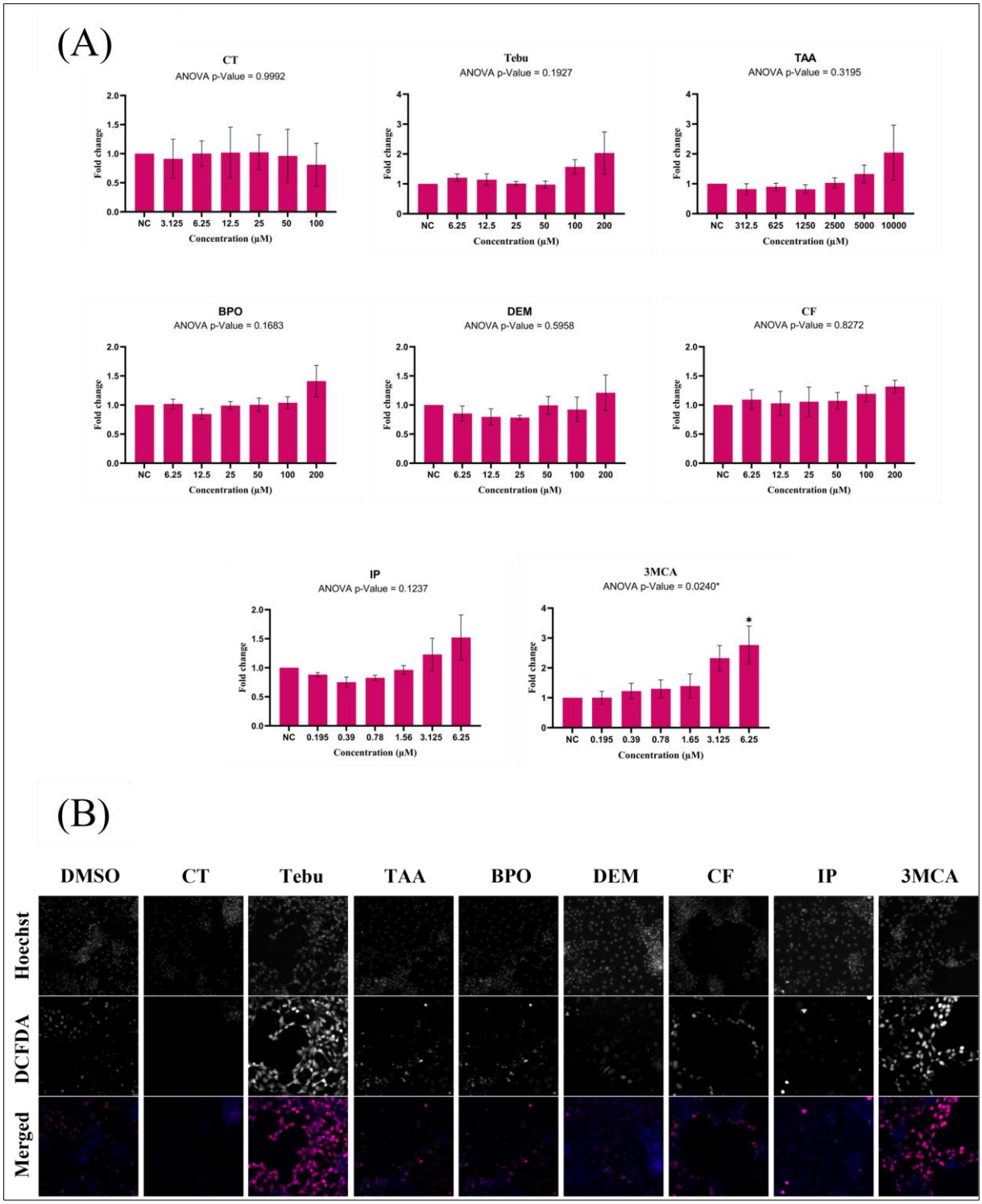


Figure C.I 4. Oxidative stress induced by the tested chemicals on 2D HepaRG cells quantified by H_2DCFDA fluorescence. (A): Quantification of ROS accumulation after 72h repetitive treatment with CT, Tebu, TAA, BPO, DEM, CF, IP, and 3MCA. (B): Representative images of the mitochondrial dysfunction at the highest tested concentration of each chemical. Data are presented as means ± SEM with ANOVA and Dunnett's post-hoc tests p-values presented as: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

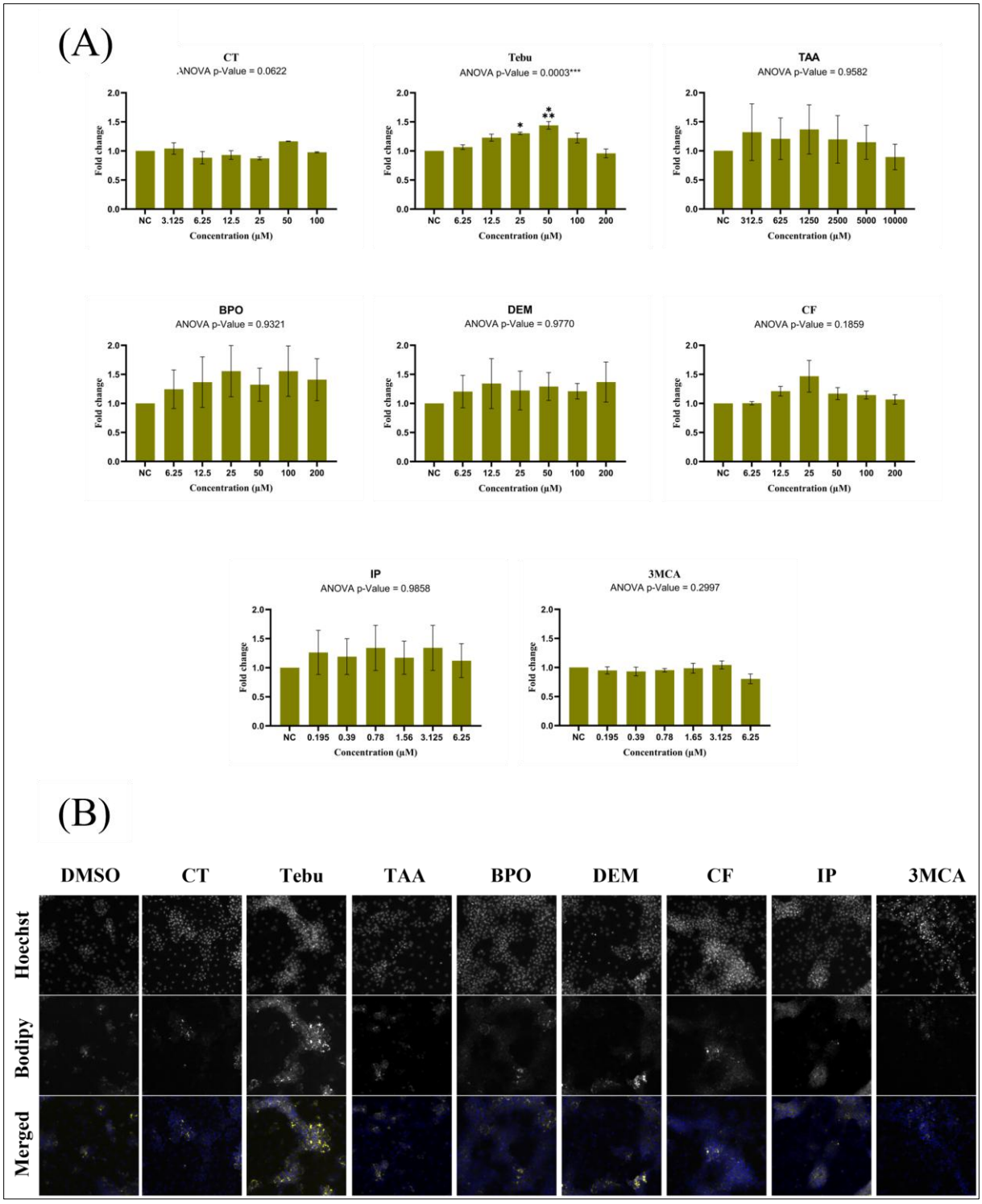


Figure C.I 5. Neutral lipid accumulation following treatment with the tested chemicals in 2D HepaRG cells quantified by BODIPY fluorescent probe. (A): Quantification of accumulated lipids following 72h repetitive treatment with CT, Tebu, TAA, BPO, DEM, CF, IP, and 3MCA. (B): Representative images of the BODIPY fluorescence at the highest tested concentration of each chemical. Data are presented as means ± SEM with ANOVA and Dunnett's post-hoc tests p-values presented as: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

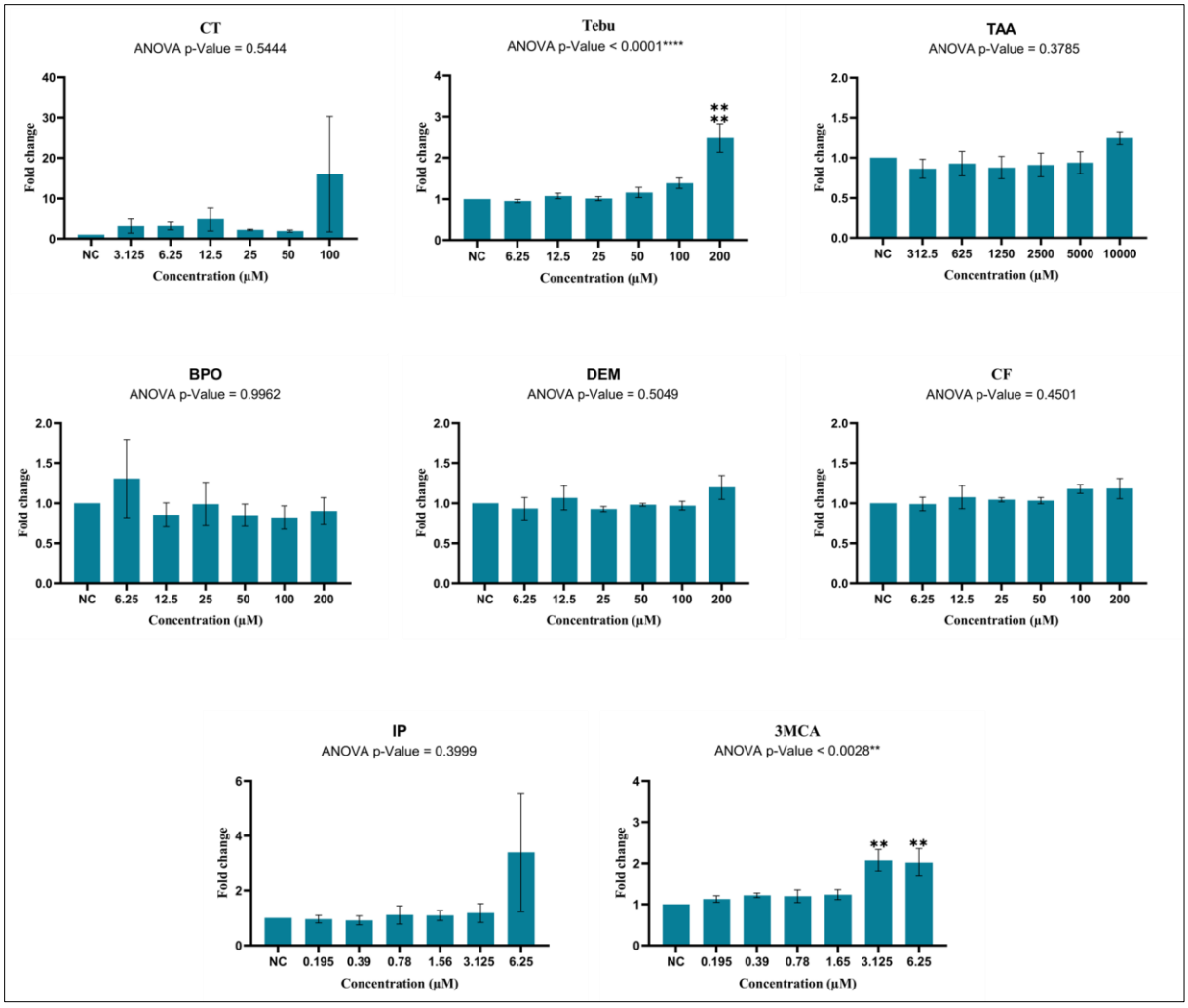


Figure C.I 6. Effect of the tested chemicals on nuclear translocation of NFkB in 2D HepaRG cells. NFκB translocation following a 72h repetitive treatment with CT, Tebu, TAA, BPO, DEM, CF, IP, and 3MCA. Data are presented as means ± SEM with ANOVA and Dunnett's post-hoc tests p-values presented as: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

3.2. Mechanistic cellular changes in 3D HepaRG spheroids

3.2.1. Cytotoxicity

Following a 72h repetitive treatment with the selected chemicals, cell viability of 3D spheroids was measured by determining the cellular ATP levels. As shown in (Figure C.I 7 A), significant concentration-dependent decreases in ATP levels were observed following treatments with CT, Tebu, and 3MCA. Decreased ATP levels were also observed in HepaRG spheroids treated for 72h with TAA, TPA, and Azox, although these decreases were not statistically significant (Figure C.I 7 A). No significant changes in ATP levels were observed in spheroids treated with BPO, DEM, CF, IP, DCMU, and FICZ, as shown in (Figure C.I 7 B).

Treatment with CT, Tebu, TPA, and Azox resulted in a decrease in cell viability which was associated with a concentration-dependent increase in 3D spheroid area (Figure C.I 8 A). In contrast, toxic concentrations of 3MCA did not affect the spheroid area (Figure C.I 8 A). DEM and DCMU treatments slightly affected spheroid area, but only at the highest tested concentration. Finally, no significant change of spheroid area was observed with BPO, CF, IP and FICZ treatments (Figure C.I 8 B).

3.2.2. Effects on mitochondrial membrane potential (ΔΨm) in 3D HepaRG spheroids

Treatments with Tebu and 3MCA resulted in a significant decrease in ΔΨm in 3D HepaRG spheroids. In contrast, exposure to BPO, CF, as well as IP led to a significant increase in TMRE fluorescence compared to the negative control (Figure C.I 9 A-B). Furthermore, following a 72h treatment of HepaRG spheroids with the additional chemicals tested only in the 3D model a significant increase in ΔΨm was only observed with DCMU (Figure C.I 9 C-D).

3.2.3. Oxidative stress

Cellular ROS levels were increased in 3D HepaRG spheroids following treatment with CT, Tebu, TAA, DEM, 3MCA, and Azox (Figure C.I 10 A-D).

3.2.4. Neutral lipid accumulation

Nearly all chemicals tested in 3D HepaRG spheroids resulted in an alteration in the neutral lipid content, either significantly increasing or decreasing the lipid levels compared to the negative control (Figure C.I 11 A-D). 72h treatments of HepaRG spheroids with CT, Tebu, TAA, DEM, CF, 3MCA, DCMU, TPA, and Azox resulted in significant increases in lipid levels whereas treatments with BPO, IP, and FICZ, induced a significant decrease in the neutral lipid content compared to negative control (Figure C.I 11 A-D).

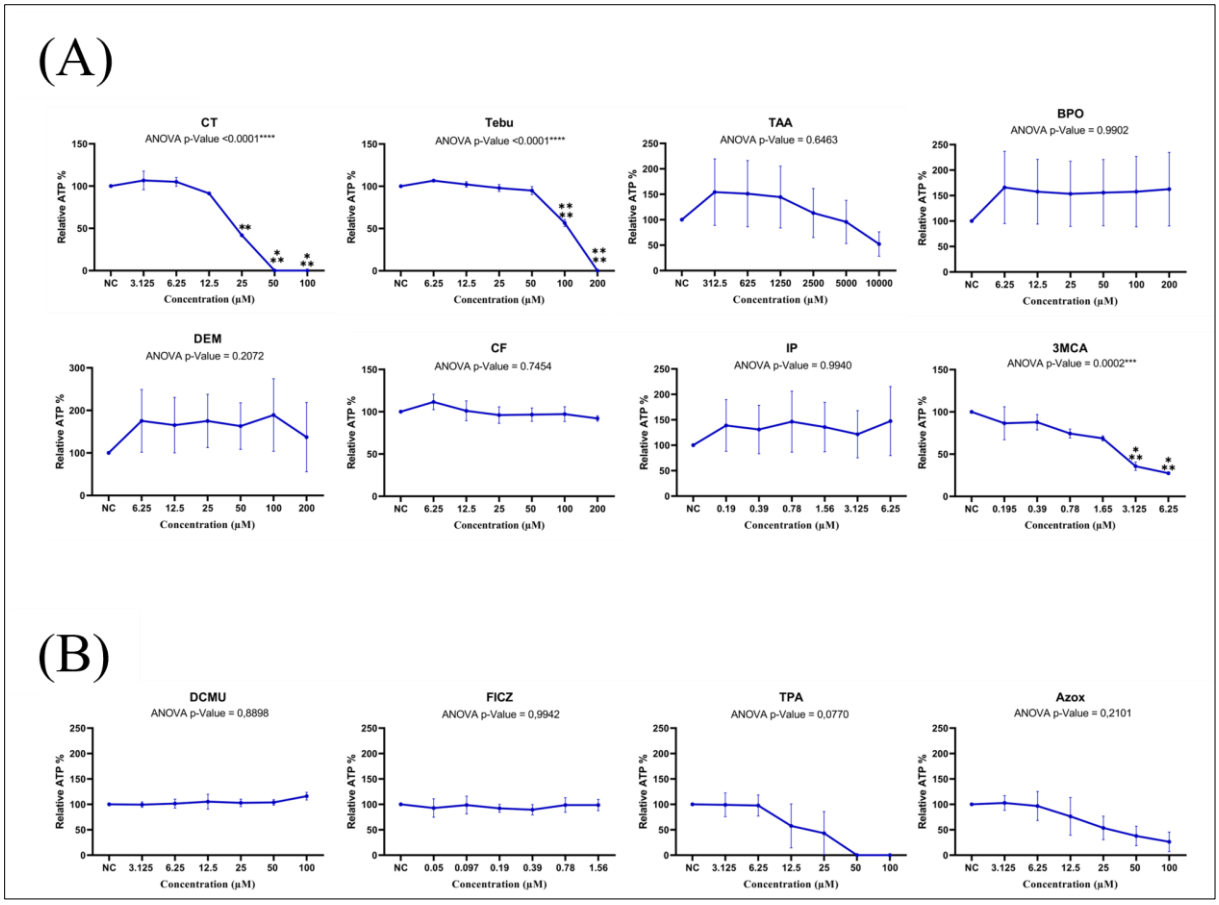


Figure C.I 7. Relative ATP levels following 72h repetitive treatments with the selected chemicals in 3D HepaRG spheroids. (A): Chemicals tested in both 2D and 3D HepaRG cells, CT, Tebu, TAA, BPO, DEM, CF, IP, and 3MCA. (B): Chemicals tested only in 3D HepaRG spheroids, DCMU, FICZ, TPA, and Azox. Data are presented as means ± SEM with ANOVA and Dunnett's post-hoc tests p-values presented as: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

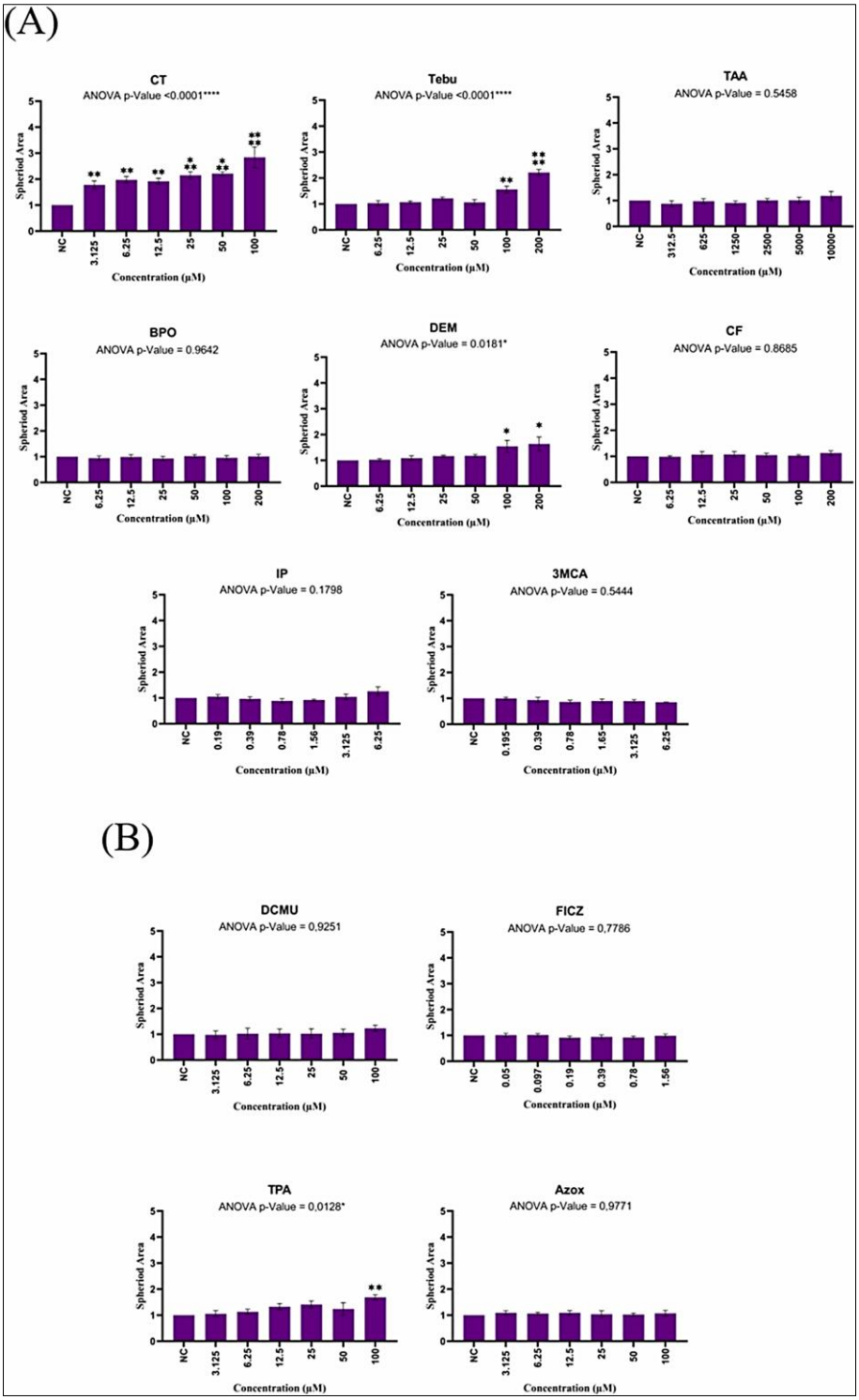


Figure C.I 8. Changes in spheroid area following 72h repetitive treatments with chemicals. (A): Chemicals tested in both 2D and 3D HepaRG cells, CT, Tebu, TAA, BPO, DEM, CF, IP, and 3MCA. (B): Chemicals tested only in 3D HepaRG spheroids, DCMU, FICZ, TPA, and Azox. Data are presented as means ± SEM with ANOVA and Dunnett's post-hoc tests p-values presented as: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

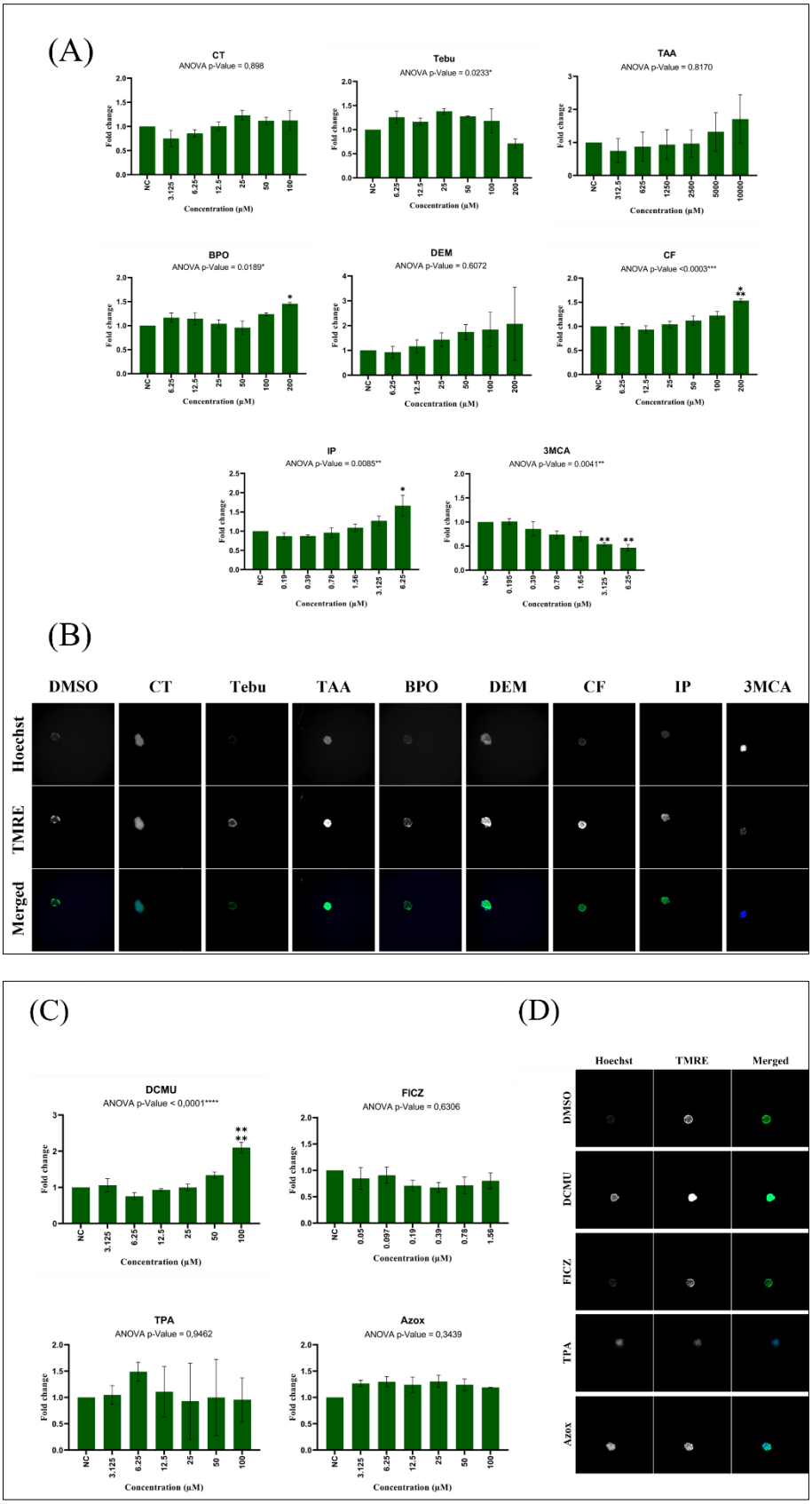


Figure C.I 9. Effects of the tested chemicals on mitochondrial membrane potential in HepaRG cells. (A): Quantification of mitochondrial membrane potential after 72h repetitive treatment with CT, Tebu, TAA, BPO, DEM, CF, IP, and 3MCA in 2D HepaRG cells. (B): Representative images of the TMRE fluorescence at the highest tested concentration of the chemicals. (C): Quantification of the TMRE fluorescence after 72h repetitive

treatment with Diuron (DCMU), FICZ, TPA, Azox. (D): Representative images of TMRE fluorescence at the highest tested concentration of the tested chemicals in 3D HepaRG spheroids. Data are presented as means ± SEM with ANOVA and Dunnett's post-hoc tests p-values presented as: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

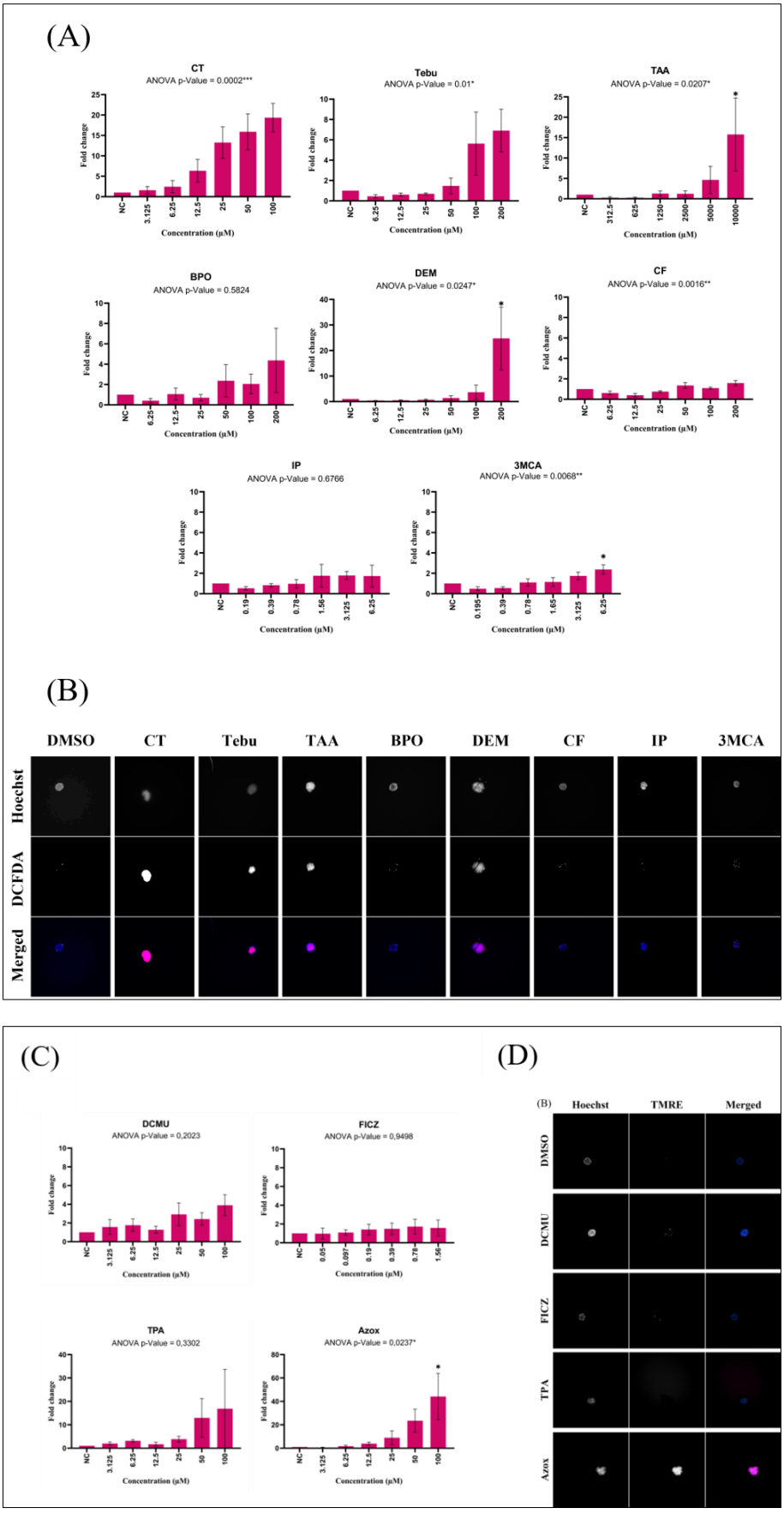


Figure C.I 10. Impact of the tested chemicals on oxidative stress in 3D HepaRG spheroids. *(*A): Quantification of the accumulated ROS after 72h repetitive treatment with CT, Tebu, TAA, BPO, DEM, CF, IP, and 3MCA in 3D

spheroids. (B): Representative images of ROS accumulation at the highest tested concentration of each chemical. (C): Quantification of the accumulated ROS after 72h repetitive treatment with DCMU, FICZ, TPA, and Azox. (D): Representative images of ROS accumulation at the highest tested concentration of the tested chemical in 3D HepaRG spheroids. Data are presented as means ± SEM with ANOVA and Dunnett's post-hoc tests p-values presented as: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

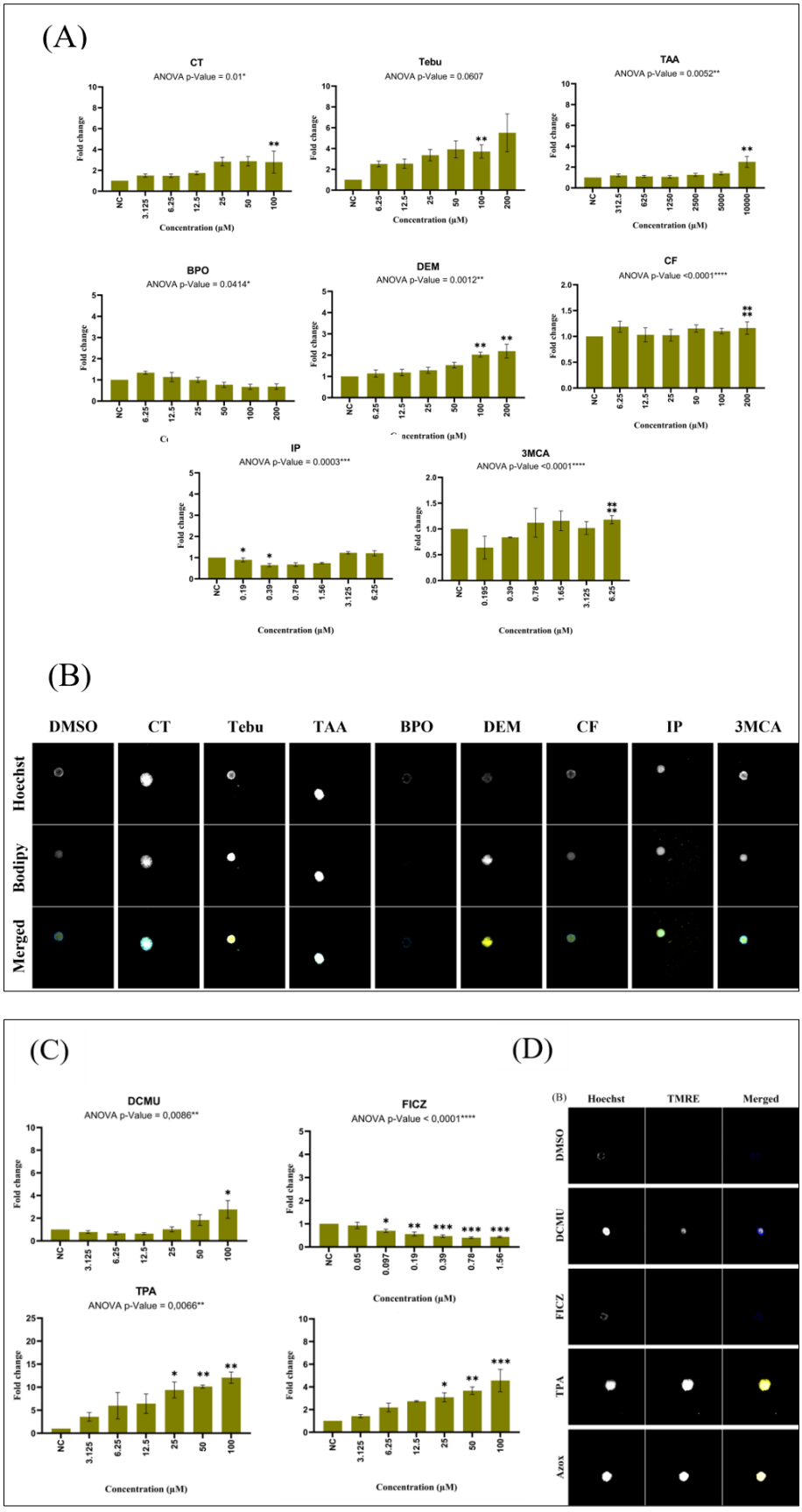


Figure C.I 11. Effect of the tested chemicals on neutral lipid content in 3D HepaRG spheroids. (A): Quantification of the accumulated lipids after 72h repetitive treatment with CT, Tebu, TAA, BPO, DEM, CF, IP, and 3MCA. (B): Representative images of lipid content at the highest tested concentration of the tested chemicals in both models.

(C): Quantification of the lipid accumulation after 72h repetitive treatment with DCMU, FICZ, TPA, Azox. (D): Representative images of lipid content at the highest tested concentration of the tested chemicals in 3D HepaRG spheroids. Data are presented as means ± SEM with ANOVA and Dunnett's post-hoc tests p-values presented as: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

4. Cellular response profiling

Cellular response profiles for each tested chemical were generated for 2D (Figure C.I 12) and 3D HepaRG models (Figure C.I 13) (Figure C.I 14).

Intensity colour changes were clearly observed with a higher change (increased or decreased) in the cellular toxicity profiles of the different chemicals tested with 3D spheroids model shown in (Figure C.I 13). compared to the intensity changes for the same chemicals tested in 2D HepaRG cells shown in (Figure C.I 12). In (Figure C.I 14), the cellular profiles of the additional chemical list tested in the 3D HepaRG model shows as well different changes with the cellular toxicity responses tested especially the lipid accumulation. In addition, the hierarchical clustering of different endpoints in 2D HepaRG cells shown in (Figure C.I 12). represent different system compared to the clustering system in the 3D HepaRG models shown in (Figure C.I 13) (Figure C.I 14).

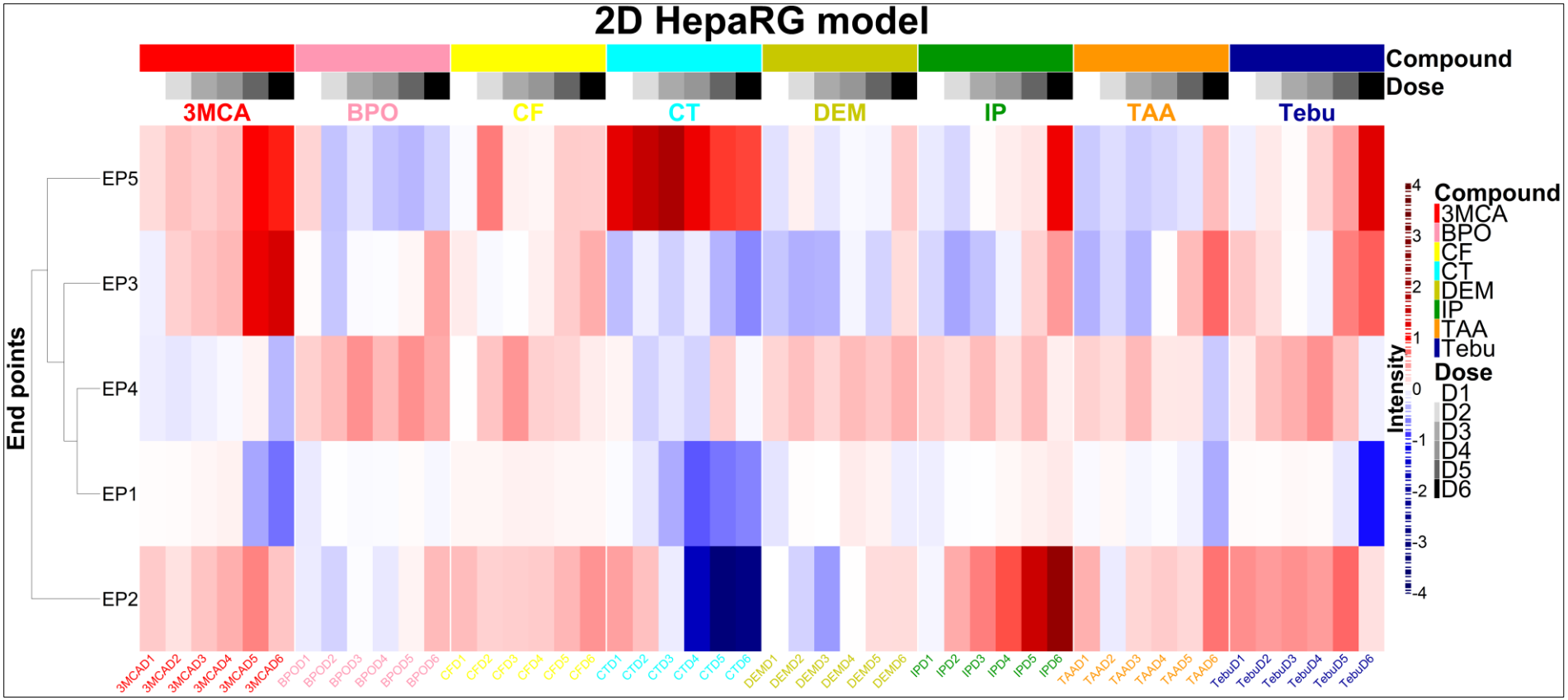


Figure C.I 12. Heatmaps illustrating the quantified intensities across five different cellular toxicity responses tested in 2D HepaRG cells (EP1–EP5) after exposure to chemicals tested (3MCA, BPO, CF, CT, DEM, IP, TAA, Tebu) using a wide concentration range. In 2D HepaRG Heatmaps: EP1: Cytotoxicity, EP2: Mitochondrial dysfunction, EP3: Oxidative stress, EP4: Lipid accumulation, EP5: Inflammation. Each column corresponds to a specific chemical–concentration combination, grouped and color-coded by compound type as shown in the legend. The intensity scale ranges from dark to light blue (low response), and light to dark red (high response), reflecting the variation in cellular responses. Scale centre values have a white colour intensity. In addition, the end points (in rows) were hierarchically clustered.

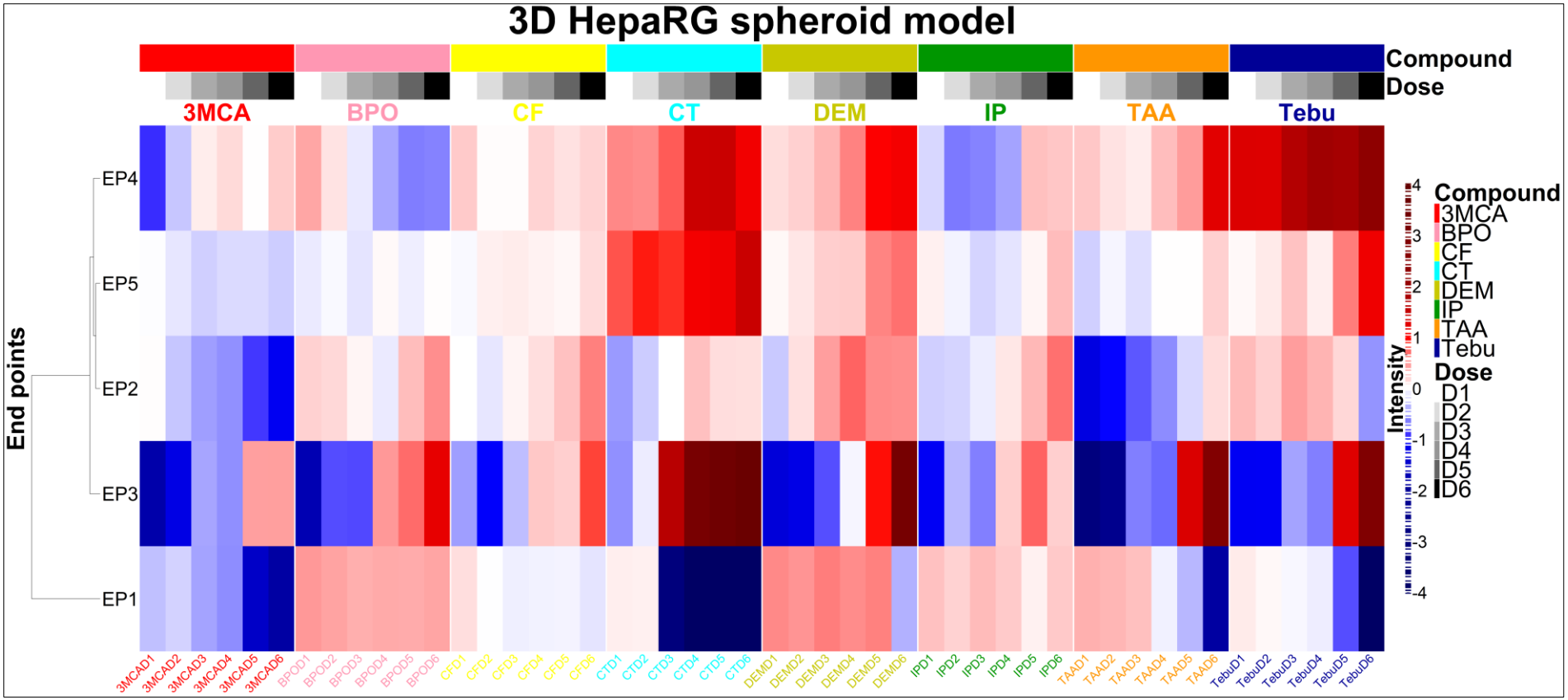


Figure C.I 13. Heatmaps illustrating the quantified intensities across five different cellular toxicity responses tested in 3D HepaRG cells (EP1–EP5) after exposure to chemicals tested (3MCA, BPO, CF, CT, DEM, IP, TAA, Tebu) using a wide concentration range. In 3D HepaRG Heatmaps: EP1: Cytotoxicity, EP2: Mitochondrial dysfunction, EP3: Oxidative stress, EP4: Lipid accumulation, EP5: Spheroid Area. Each column corresponds to a specific chemical–concentration combination, grouped and color-coded by compound type as shown in the legend. The intensity scale ranges from dark to light blue (low response), and light to dark red (high response), reflecting the variation in cellular responses. Scale centre values have a white colour intensity. In addition, the end points (in rows) were hierarchically clustered.

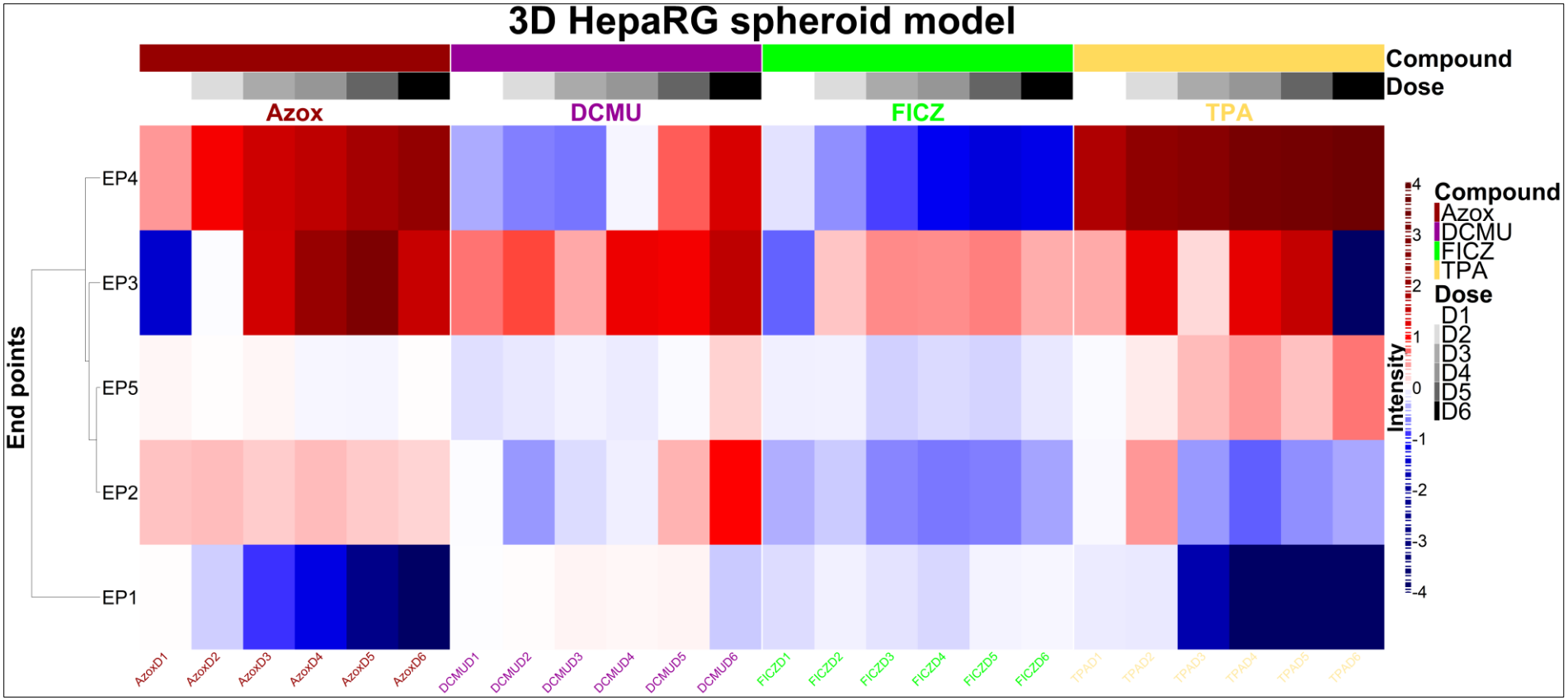


Figure C.I 14. Heatmaps illustrating the quantified intensities across five different cellular toxicity responses tested in 3D HepaRG cells (EP1–EP5) after exposure to chemicals tested (Azox, DCMU, FICZ, TPA) using a wide concentration range. In 3D HepaRG Heatmaps: EP1: Cytotoxicity, EP2: Mitochondrial dysfunction, EP3: Oxidative stress, EP4: Lipid accumulation, EP5: Spheroid Area. Each column corresponds to a specific chemical–concentration combination, grouped and color-coded by compound type as shown in the legend. The intensity scale ranges from dark to light blue (low response), and light to dark red (high response), reflecting the variation in cellular responses. Scale centre values have a white colour intensity. In addition, the end points (in rows) were hierarchically clustered.

5. Multi variant dose-response analysis

After univariant comparisons between the tested chemicals in both 2D and 3D models (CT, Tebu, TAA, BPO, DEM, CF, IP, 3MCA) using one-way ANOVA followed by a Dunnett's post-hoc test, a multivariant test using the classical Principal Component Analysis (PCA) was done on the chemicals tested taking into account the different endpoints screened in each model following a 72h treatment with CT, Tebu, 3MCA, and CF. The chemicals that showed significant responses in both cell models were evaluated by PCA as shown in (Figure C.I 15 A-B). The PCA plots revealed a clear concentration-dependent clustering of the tested chemicals in the 3D HepaRG model treated with CT, Tebu, 3MCA, and CF compared to the 2D HepaRG model. Medium and high-concentration treatments showed progressively greater separation along the principal components in the 3D HepaRG model, indicating a significant shift in the response profiles at higher concentrations (Figure C.I 15).

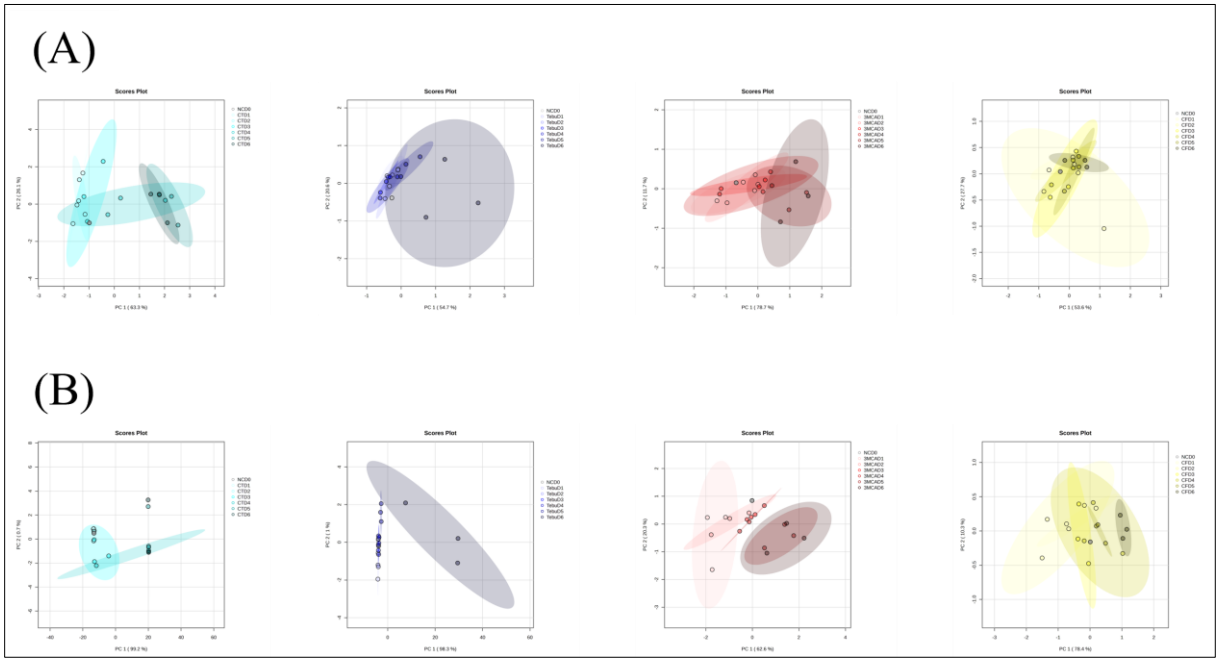


Figure C.I 15. Principle Component Analysis (PCA) following the 72h treatments with CT, Tebu, 3MCA, and CF in both HepaRG models. (A) PCA plots with 2D HepaRG cells. (B) PCA plots with 3D HepaRG spheroids.

4. Discussion

To support NAM-based CSA, in particular for chemical hazard identification and characterization, a thorough evaluation of the usefulness of *in vitro* methodologies to provide predictive, human-relevant data is required. In this context, we have employed a high-throughput imaging technology to investigate a comprehensive panel of toxic endpoints in human-relevant hepatic models. Cytotoxicity, mitochondrial dysfunction, oxidative stress, lipid content, and the pro-inflammatory response were evaluated and compared in both 2D and 3D HepaRG cell models. A diverse set of chemicals (pesticides, mycotoxins, and synthetic compounds) was selected based on their known cellular mechanisms of cytotoxicity **(**Table C.I 1).

In the first part of this study, we identified multi-parametric toxicity effects of eight different chemicals (CT, Tebu, TAA, BPO, DEM, CF, IP, and 3MCA) in both 2D and 3D HepaRG models using an HCA-based approach. The results clearly showed a higher amplitude of change at different levels of cellular toxicity (oxidative stress, mitochondrial dysfunction, and in neutral lipid accumulation) in the 3D model compared to 2D HepaRG cells, based on the univariant data analysis with the application of one-way ANOVA followed by Dunnett's post-hoc statistic tests. Regarding these findings, the results demonstrate that compared to 2D HepaRG cells, the 3D HepaRG spheroid model shows greater sensitivity in the detection of the cellular responses following a 72h exposure to the chemicals evaluated in this study (Table APP. I 1). It should be noted that, negative control reference compounds (e.g. D-mannitol) tested with all the cytotoxic parameters applied in this study showed no significant responses (data not shown).

In addition, as statistical models represent an important application for scientific data analysis (Kim et al., 2024), different statistical methods were applied to analyse the large dataset generated by HCA. Global response-profiles of each chemical tested were visualized through heatmaps, while multivariate statistical analysis using PCA was employed to discern patterns and clusters. These statistical approaches revealed more pronounced changes in 3D HepaRG spheroids and exhibited greater sensitivity as evidenced by clearer dose-response clustering patterns than those observed in 2D models.

In agreement with our findings in this study, recent studies (Yang et al. 2023; Drees et al., 2025) have demonstrated increased sensitivity of 3D HepaRG spheroids in detecting hepatotoxicants, when compared to 2D models. Our findings are consistent with the work conducted by Mueller et al. (2014) and Zhang et al., (2020) have shown that 3D HepaRG spheroids are well suited for

the repeated-dose toxicity studies. Additionally, recent study revealed that using 3D HepaRG models in high-throughput genotoxicity assays as well showed a high sensitivity and predictivity (Seo et al., 2022).

As previously reported, 3D HepaRG models show greater sensitivity compared to 2D HepaRG cells, and this difference could possibly be due to their higher similarity to the complex tissue-like architecture observed *in vivo*, and greater physiological relevance (Ramaiahgari et al., 2017). A study by Gunness et al. (2013) found that the production rates of albumin and glucose, as well as CYP2E1 activity, were significantly higher in 3D HepaRG models when compared to 2D cultures (Gunness et al., 2013). Moreover, Leite et al. (2012) showed the formation of tissue-like arrangements in 3D HepaRG spheroids, including bile canaliculi–like structures and polar distribution of transporters (Leite et al., 2012; Yang et al., 2023; Drees et al., 2025). In addition, 3D HepaRG spheroids maintain higher expression levels of liver-specific markers and drug-metabolizing enzymes which are critical criteria for accurate toxicity testing. In the second part of this study, we screened the toxicological events induced by four additional chemicals (DEMU, FICZ, TPA, and Azox) in the 3D HepaRG model. These chemicals were added to assess the sensitivity of 3D HepaRG spheroids on a larger set of chemicals with known effects on cellular toxicity pathways. It also served as a proof of concept of the applicability of HCA, in combination with 3D liver spheroids, to screen a variety of toxicants having different cellular effects (Table APP. I 2). Indeed, in our study, these chemicals induced significant effects on lipid accumulation in 3D HepaRG spheroids in response to a 72h treatment, which is in agreement with published literature concerning the effects of these compounds on hepatic lipid accumulation. In addition to the ability of 3D HepaRG spheroids to detect effects on lipid accumulation, our results confirm that, when compared to 2D models, the 3D HepaRG model is more sensitive in its capacity to predict toxicity of compounds having known cellular toxicity effects.

Among the screened chemicals, cytotoxicity was observed following treatment with CT, Tebu and 3MCA in both models. While significant cellular toxicity was observed, the minimal concentration at which a statistically significant effect on mechanistic endpoints was obtained induced less than 50% cell mortality. The detection of cellular responses and changes in our study started at non-cytotoxic concentrations, providing an early biological response prior to the initiation of cytotoxicity. This minimal level of cytotoxicity is important to take into consideration in order to avoid attributing mechanistic biological responses to non-specific

effects observed at cytotoxic concentrations. In addition, this concept is in line with the IC50/Cmax safety margin commonly used in toxicological testing and drug safety evaluation (Lehmann et al., 2018).

One of the main advantages of the approach applied in this study relies in the valuable predictivity of HCA that has been widely employed for mechanistic investigations, particularly in the prioritization of safety and assessment of chemicals (Liu et al., 2022). Consequently, we studied the potential correlations between the different cellular responses detected in the 3D HepaRG model. Within the tested cellular responses, two major changes were observed: (1) a high sensitivity towards the effects on neutral lipid accumulation in 3D HepaRG spheroids, which were detected with almost all the tested chemicals, with the exception of BPO, IP and FICZ that showed a decrease in lipid accumulation, and (2) a disrupted mitochondrial function (ATP or ΔΨm) in conjugation with the neutral lipid accumulation in most of the chemicals tested with the exception of TAA, DEM, and AZOX. Many studies have explored the relationship between disrupted mitochondrial membrane potential and lipid accumulation in HepaRG cells. Nakamura et al., (2024) demonstrated that the hepatotoxicant Triclocarban induces lipid droplet accumulation via a disrupted mitochondrial dysfunction through the inhibition of mitochondrial fatty acid oxidation. Further, (Wajner & Amaral, 2016) reported the disruption in mitochondrial membrane potential lead to mitochondrial dysfunction that could affect fatty acid accumulation and lead to accumulated lipid droplets in hepatic cells. These findings also support the relation between lipid accumulation through mitochondrial disruption mechanisms leading to lipotoxicity which could result in an increase in ROS as reported in (Lee et al., 2013). Furthermore, the observed increase in mitochondrial membrane potential, known as mitochondrial hyperpolarization with 3D HepaRG spheroids treated with Tebu, BPO, CF, IP, 3MCA, and DCMU, that could be observed with some contaminants. For instance, it has been reported by Hardonnière, *et al* (2016) exposure to compounds like BaP results in increase mitochondrial membrane potential as an early stress response to increase the ATP production, supporting energy-demanding process. Hence, acts as protective adaptation to mitochondrial dysfunction or increased ROS in some cell lines (Hardonnière et al., 2016). A similar mechanism could be suggested based on our results with CT, Tebu, and 3MCA. However, these tested endpoints and relation between the mitochondrial dysfunction and lipid accumulation was not applicable to all chemicals tested with 2D HepaRG cells and this might be due to its lower sensitivity compared to the 3D HepaRG spheroids, and this could possibly be explained

by the enhanced expression of genes related to lipid metabolism in the 2D model (Takahashi et al., 2015).

Some studies have reported that the decrease in the neutral lipid accumulation could be an indication to an early toxicity rather a disrupted lipid metabolism (Alza et al., 2021). In that regard, it is appreciated to go further in the analysis at the molecular levels using Multiomics that could justify the cellular changes regarding chemical–specific effects (Mesnage et al., 2018; Kreuzer et al., 2020). As well as investigates chemical-specific responses at the molecular level.

Despite promising findings, several limitations of the current study must be acknowledged. While the pro-inflammatory response could be assessed with the 2D HepaRG cells in this study through NFκB translocation, we were not be able to detect signals while assessing the pro-inflammatory response markers in 3D HepaRG spheroids due to the low number of cells/well in the ULA-system. Although the 3D HepaRG model might offer significant potential for assessing chronic toxicity due to their long-term functionality, future improvements to this model could be proposed, including the use of a multicellular spheroid models cocultured with nonparenchymal cells, which may enhance the model complexity and enable the evaluation of immune-mediated toxicity and other safety assessments (Bronsard et al., 2024).

In conclusion, this study presents a NAM-based approach that enables the rapid mechanistically informative screening of chemical-induced cellular toxicity using the human-relevant HepaRG cell line. By employing 3D HepaRG spheroids, the model achieves greater physiological complexity, enhancing its relevance for *in vitro* toxicity assessment. Moreover, the integration of HCA allowed for detailed mechanistic insights at the cellular level as it allows the screening of multiple parameters/endpoints at the same time and in the same cells. Overall, this approach offers a robust, rapid, and human-relevant platform that significantly strengthens the utility of NAMs for identifying chemical toxicity risk *in vitro*. The use of 3D HepaRG spheroids thus offers a high predictivity and sensitivity for chemical classification and prioritization.

References

Agarwal, R., Katiyar, S. K., Lundgren, D. W., & Mukhtar, H. (1994). Inhibitory effect of silymarin, an anti-hepatotoxic flavonoid, on 12-O-tetradecanoylphorbol-13-acetate-induced epidermal ornithine decarboxylase activity and mRNA in SENCAR mice. *Carcinogenesis, 15*(6), 1099–1103. https://doi.org/10.1093/carcin/15.6.1099

Alza, N. P., Conde, M. A., Scodelaro-Bilbao, P. G., & Salvador, G. A. (2021). Neutral lipids as early biomarkers of cellular fate: The case of α-synuclein overexpression. *Cell Death & Disease, 12*(1), 52. https://doi.org/10.1038/s41419-020-03254-7

Amali, A. A., Rekha, R. D., Lin, C. J.-F., Wang, W.-L., Gong, H.-Y., Her, G.-M., & Wu, J.-L. (2006). Thioacetamide induced liver damage in zebrafish embryo as a disease model for steatohepatitis. *Journal of Biomedical Science, 13*(2), 225–232. https://doi.org/10.1007/s11373-005-9055-5

Anthérieu, S., Chesné, C., Li, R., Guguen-Guillouzo, C., & Guillouzo, A. (2012). Optimization of the HepaRG cell model for drug metabolism and toxicity studies. *Toxicology in Vitro, 26*(8), 1278–1285. https://doi.org/10.1016/j.tiv.2012.05.008

Audebert, M., Assmann, A.-S., Azqueta, A., Babica, P., Benfenati, E., Bortoli, S., Bouwman, P., Braeuning, A., Burgdorf, T., Coumoul, X., Debizet, K., Dusinska, M., Ertych, N., Fahrer, J., Fetz, V., Le Hégarat, L., López de Cerain, A., Heusinkveld, H. J., Hogeveen, K., … Oelgeschläger, M. (2023). New approach methodologies to facilitate and improve the hazard assessment of non-genotoxic carcinogens—A PARC project. *Frontiers in Toxicology, 5*, 1220998. https://doi.org/10.3389/ftox.2023.1220998

Barranger, A., & Le Hégarat, L. (2022). Towards better prediction of xenobiotic genotoxicity: CometChip technology coupled with a 3D model of HepaRG human liver cells. *Archives of Toxicology, 96*(7), 2087–2095. https://doi.org/10.1007/s00204-022-03292-4

Behbahan, I. S., Duan, Y., Lam, A., Khoobyari, S., Ma, X., Ahuja, T. P., & Zern, M. A. (2011). New approaches in the differentiation of human embryonic stem cells and induced pluripotent stem cells toward hepatocytes. *Stem Cell Reviews and Reports, 7*(3), 748–759. https://doi.org/10.1007/s12015-010-9216-4

Bronsard, J., Savary, C., Massart, J., Viel, R., Moutaux, L., Catheline, D., Rioux, V., Clement, B., Corlu, A., Fromenty, B., & Ferron, P. J. (2024). 3D multi-cell-type liver organoids: A new model of non-alcoholic fatty liver disease for drug safety assessments. *Toxicology in Vitro, 94*, 105728. https://doi.org/10.1016/j.tiv.2023.105728

Burkhart, C. G., Butcher, C., Burkhart, C. N., & Lehmann, P. (2000). Effects of benzoyl peroxide on lipogenesis in sebaceous glands using an animal model. *Journal of Cutaneous Medicine and Surgery, 4*(3), 138–141. https://doi.org/10.1177/120347540000400305

Cabot, M. C., Welsh, C. J., Callaham, M. F., & Huberman, E. (1980). Alterations in lipid metabolism induced by 12-O-tetradecanoylphorbol-13-acetate in differentiating human myeloid leukemia cells. *Cancer Research*, *40*(10), 3674–3679.

Cunha, S. C., & Fernandes, J. O. (2020). Application in food analysis. In C. F. Poole (Ed.), *Liquid-phase extraction* (pp. 643–665). Elsevier. https://doi.org/10.1016/B978-0-12-816911-7.00021-9

Drees, A., Nassiri, V., Tabernilla, A., Serroyen, J., Gustin, E., dos Santos Rodrigues, B., Moss, D. M., De Smedt, A., Vinken, M., Van Goethem, F., & Sanz-Serrano, J. (2025). Optimization of the drug-induced cholestasis index based on advanced modeling for predicting liver toxicity. *Toxicology*, *514*, 154119. https://doi.org/10.1016/j.tox.2025.154119

Dubreil, E., Darney, K., Delignette-Muller, M.-L., Barranger, A., Huet, S., Hogeveen, K., Léger, T., Fessard, V., & Hégarat, L. L. (2024). Modeling HepaRG metabolome responses to pyrrolizidine alkaloid exposure for insight into points of departure and modes of action. *Journal of Hazardous Materials*, *474*, 134721. https://doi.org/10.1016/j.jhazmat.2024.134721

Escher, S. E., Aguayo-Orozco, A., Benfenati, E., Bitsch, A., Braunbeck, T., Brotzmann, K., Bois, F., van der Burg, B., Castel, J., Exner, T., Gadaleta, D., Gardner, I., Goldmann, D., Hatley, O., Golbamaki, N., Graepel, R., Jennings, P., Limonciel, A., Long, A., … Fisher, C. (2022). Integrate mechanistic evidence from new approach methodologies (NAMs) into a read-across assessment to characterise trends in shared mode of action. *Toxicology in Vitro*, *79*, 105269. https://doi.org/10.1016/j.tiv.2021.105269

EU cosmetic regulations | Directive 76/768/EEC, New EU Cosmetic Products Regulation (EC) No 1223/2009 and REACH. (2025), from https://www.cirs-reach.com/Cosmetics_Registration/eu_cosmetics_directive_cosmetics_registration.html

Ezhilarasan, D. (2023). Molecular mechanisms in thioacetamide-induced acute and chronic liver injury models. *Environmental Toxicology and Pharmacology*, *99*, 104093. https://doi.org/10.1016/j.etap.2023.104093

Gao, A.-H., Fu, Y.-Y., Zhang, K.-Z., Zhang, M., Jiang, H.-W., Fan, L.-X., Nan, F.-J., Yuan, C.-G., Li, J., Zhou, Y.-B., & Li, J.-Y. (2014). Azoxystrobin, a mitochondrial complex III Qo site inhibitor, exerts beneficial metabolic effects in vivo and in vitro. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1840*(7), 2212–2221. https://doi.org/10.1016/j.bbagen.2014.04.002

Ghosson, H., Raviglione, D., Bertrand, C., & Salvia, M.-V. (2024). LC–HRMS-driven computational toolbox to assess extraction protocols dedicated to untargeted analysis: How to ease analyzing pesticide-contaminated soils? *Analytical Chemistry*, *96*(7), 2810–2821. https://doi.org/10.1021/acs.analchem.3c03242

Gómez-Lechón, M. J., Donato, M. T., Castell, J. V., & Jover, R. (2003). Human hepatocytes as a tool for studying toxicity and drug metabolism. *Current Drug Metabolism, 4*(4), 292–312. https://doi.org/10.2174/1389200033489424

Grimm, H., Biller-Andorno, N., Buch, T., Dahlhoff, M., Davies, G., Cederroth, C. R., Maissen, O., Lukas, W., Passini, E., Törnqvist, E., Olsson, I. A. S., & Sandström, J. (2023). Advancing the 3Rs: Innovation, implementation, ethics and society. *Frontiers in Veterinary Science, 10*, 1185706. https://doi.org/10.3389/fvets.2023.1185706

Groh, K. J., Carvalho, R. N., Chipman, J. K., Denslow, N. D., Halder, M., Murphy, C. A., Roelofs, D., Rolaki, A., Schirmer, K., & Watanabe, K. H. (2015). Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: I. Challenges and research needs in ecotoxicology. *Chemosphere, 120*, 764–777. https://doi.org/10.1016/j.chemosphere.2014.09.068

Guillouzo, A., Corlu, A., Aninat, C., Glaise, D., Morel, F., & Guguen-Guillouzo, C. (2007). The human hepatoma HepaRG cells: A highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chemico-Biological Interactions, 168*(1), 66–73. https://doi.org/10.1016/j.cbi.2006.12.003

Gunness, P., Mueller, D., Shevchenko, V., Heinzle, E., Ingelman-Sundberg, M., & Noor, F. (2013). 3D organotypic cultures of human HepaRG cells: A tool for in vitro toxicity studies. *Toxicological Sciences, 133*(1), 67–78. https://doi.org/10.1093/toxsci/kft021

Guo, X., Xu, H., & Seo, J.-E. (2024). Application of HepaRG cells for genotoxicity assessment: A review. *Journal of Environmental Science and Health, Part C: Toxicology and Carcinogenesis, 42*(3), 214–237. https://doi.org/10.1080/26896583.2024.2331956

Han, Y., Liu, T., Wang, J., Wang, J., Zhang, C., & Zhu, L. (2016). Genotoxicity and oxidative stress induced by the fungicide azoxystrobin in zebrafish (*Danio rerio*) livers. *Pesticide Biochemistry and Physiology, 133*, 13–19. https://doi.org/10.1016/j.pestbp.2016.03.011

Hardonnière, K., Saunier, E., Lemarié, A., Fernier, M., Gallais, I., Héliès-Toussaint, C., Mograbi, B., Antonio, S., Bénit, P., Rustin, P., Janin, M., Habarou, F., Ottolenghi, C., Lavault, M.-T., Benelli, C., Sergent, O., Huc, L., Bortoli, S., & Lagadic-Gossmann, D. (2016). The environmental carcinogen benzo[a]pyrene induces a Warburg-like metabolic reprogramming dependent on NHE1 and associated with cell survival. *Scientific Reports, 6*(1), 30776. https://doi.org/10.1038/srep30776

Hoogstraaten, M. J., Vriend, J., de Leeuw, V. C., Negro, S. O., Moors, E. H. M., Kienhuis, A. S., & Hoekman, J. (2025). Animal-free safety assessment of chemicals: An innovation system perspective. *Archives of Toxicology, 99*(1), 43–56. https://doi.org/10.1007/s00204-024-03878-0

Hussain, F., Basu, S., Heng, J. J. H., Loo, L.-H., & Zink, D. (2020). Predicting direct hepatocyte toxicity in humans by combining high-throughput imaging of HepaRG cells and machine learning-based phenotypic profiling. *Archives of Toxicology, 94*(8), 2749–2767. https://doi.org/10.1007/s00204-020-02778-3

Ishidate, K., Tsuruoka, M., & Nakazawa, Y. (1982). Induction of choline kinase by polycyclic aromatic hydrocarbons in rat liver. II. Its relation to net phosphatidylcholine biosynthesis. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism, 713*(1), 103–111. https://doi.org/10.1016/0005-2760(82)90172-2

Jin, Y., Miao, W., Lin, X., Pan, X., Ye, Y., Xu, M., & Fu, Z. (2014). Acute exposure to 3-methylcholanthrene induces hepatic oxidative stress via activation of the Nrf2/ARE signaling pathway in mice. *Environmental Toxicology, 29*(12), 1399–1408. https://doi.org/10.1002/tox.21870

Jin, Y., Qi, G., Feng, M., & Yu, D. (2022). The path via pathway-based approaches towards safety assessment: A concise review. *Toxicology and Applied Pharmacology, 452*, 116195. https://doi.org/10.1016/j.taap.2022.116195

Joossens, E., Macko, P., Palosaari, T., Gerloff, K., Ojea-Jiménez, I., Gilliland, D., Novak, J., Fortaner Torrent, S., Gineste, J.-M., Römer, I., Briffa, S. M., Valsami-Jones, E., Lynch, I., & Whelan, M. (2019). A high throughput imaging database of toxicological effects of nanomaterials tested on HepaRG cells. *Scientific Data, 6*(1), 46. https://doi.org/10.1038/s41597-019-0053-2

Kammerer, S. (2021). Three-dimensional liver culture systems to maintain primary hepatic properties for toxicological analysis in vitro. *International Journal of Molecular Sciences, 22*(19), 10214. https://doi.org/10.3390/ijms221910214

Kiani, A. K., Pheby, D., Henehan, G., Brown, R., Sieving, P., Sykora, P., Marks, R., Falsini, B., Capodicasa, N., Miertus, S., Lorusso, L., Dondossola, D., Tartaglia, G. M., Ergoren, M. C., Dundar, M., Michelini, S., Malacarne, D., Bonetti, G., Dautaj, A., … Bertelli, M. (2022). Ethical considerations regarding animal experimentation. *Journal of Preventive Medicine and Hygiene, 63*(2S3), E483–E494. https://doi.org/10.15167/2421-4248/jpmh2022.63.2S3.2768

Kim, J., Kim, D. H., & Kwak, S. G. (2024). Comprehensive guidelines for appropriate statistical analysis methods in research. *Korean Journal of Anesthesiology, 77*(5), 503–517. https://doi.org/10.4097/kja.24016

Kim, Y., Kim, H., & Kim, Y. (2025). Advancing hepatotoxicity assessment: Current advances and future directions. *Toxicological Research, 41*(4), 303–323. https://doi.org/10.1007/s43188-025-00289-w

Kreuzer, K., Böhmert, L., Alhalabi, D., Buhrke, T., Lampen, A., & Braeuning, A. (2020). Identification of a transcriptomic signature of food-relevant genotoxins in human HepaRG hepatocarcinoma cells. *Food and Chemical Toxicology, 140*, 111297. https://doi.org/10.1016/j.fct.2020.111297

Ku, T., Zhou, M., Hou, Y., Xie, Y., Li, G., & Sang, N. (2021). Tebuconazole induces liver injury coupled with ROS-mediated hepatic metabolism disorder. *Ecotoxicology and Environmental Safety, 220*, 112309. https://doi.org/10.1016/j.ecoenv.2021.112309

Kwon, H.-C., Kim, D.-H., Jeong, C.-H., Kim, Y.-J., Han, J.-H., Lim, S.-J., Shin, D.-M., Kim, D.-W., & Han, S.-G. (2021). Tebuconazole fungicide induces lipid accumulation and oxidative stress in HepG2 cells. *Foods, 10*(10), 2242. https://doi.org/10.3390/foods10102242

Lee, S.-J., Zhang, J., Choi, A. M. K., & Kim, H. P. (2013). Mitochondrial dysfunction induces formation of lipid droplets as a generalized response to stress. *Oxidative Medicine and Cellular Longevity, 2013*, 327167. https://doi.org/10.1155/2013/327167

Lehmann, D. F., Eggleston, W. D., & Wang, D. (2018). Validation and clinical utility of the hERG IC50:Cmax ratio to determine the risk of drug-induced torsades de pointes: A meta-analysis. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 38*(3), 341–348. https://doi.org/10.1002/phar.2087

Leite, S. B., Wilk-Zasadna, I., Zaldivar, J. M., Airola, E., Reis-Fernandes, M. A., Mennecozzi, M., Guguen-Guillouzo, C., Chesne, C., Guillou, C., Alves, P. M., & Coecke, S. (2012). Three-dimensional HepaRG model as an attractive tool for toxicity testing. *Toxicological Sciences, 130*(1), 106–116. https://doi.org/10.1093/toxsci/kfs232

Li, X., Shen, X., Jiang, W., Xi, Y., & Li, S. (2024). Comprehensive review of emerging contaminants: Detection technologies, environmental impact, and management strategies. *Ecotoxicology and Environmental Safety, 278*, 116420. https://doi.org/10.1016/j.ecoenv.2024.116420

Li, Y., Bowling, A. J., Lehman, A., Johnson, K., Pence, H. E., Breitweiser, L. A., Sherer, E., LaRocca, J., & Chen, W. (2024). High-throughput image-based assay for identifying in vitro hepatocyte microtubule disruption. *Journal of Agricultural and Food Chemistry, 72*(39), 21804–21819. https://doi.org/10.1021/acs.jafc.4c04969

Lima, T. R. R., de Oliveira Lima, E., Delafiori, J., Ramos Catharino, R., Viana de Camargo, J. L., & Pereira, L. C. (2022). Molecular signatures associated with diuron exposure on rat urothelial mitochondria. *Toxicology Mechanisms and Methods, 32*(8), 628–635. https://doi.org/10.1080/15376516.2022.2062271

Liu, J., Li, R., Zhang, T., Xue, R., Li, T., Li, Z., Zhuang, X., Wang, Q., Chen, Y. A., Dong, J., Zhang, Y., & Wang, Y. (2022). High-content imaging of human hepatic spheroids for

researching the mechanism of duloxetine-induced hepatotoxicity. *Cell Death & Disease*, *13*(8), 669. https://doi.org/10.1038/s41419-022-05042-x

Mandon, M., Huet, S., Dubreil, E., Fessard, V., & Le Hégarat, L. (2019). Three-dimensional HepaRG spheroids as a liver model to study human genotoxicity in vitro with the single cell gel electrophoresis assay. *Scientific Reports*, *9*(1), 10548. https://doi.org/10.1038/s41598-019-47114-7

Matin, T., Patel, P., & Goodman, M. B. (2025). *Benzoyl peroxide*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK537220/

Mesnage, R., Biserni, M., Balu, S., Frainay, C., Poupin, N., Jourdan, F., Wozniak, E., Xenakis, T., Mein, C. A., & Antoniou, M. N. (2018). Integrated transcriptomics and metabolomics reveal signatures of lipid metabolism dysregulation in HepaRG liver cells exposed to PCB 126. *Archives of Toxicology*, *92*(8), 2533–2547. https://doi.org/10.1007/s00204-018-2235-7

Mohammed, A. M., Huovinen, M., & Vähäkangas, K. H. (2020). Toxicity of diuron metabolites in human cells. *Environmental Toxicology and Pharmacology*, *78*, 103409. https://doi.org/10.1016/j.etap.2020.103409

Mueller, D., Krämer, L., Hoffmann, E., Klein, S., & Noor, F. (2014). 3D organotypic HepaRG cultures as in vitro model for acute and repeated dose toxicity studies. *Toxicology in Vitro*, *28*(1), 104–112. https://doi.org/10.1016/j.tiv.2013.06.024

Nakamura, H., Matsui, T., & Shinozawa, T. (2024). Triclocarban induces lipid droplet accumulation and oxidative stress responses by inhibiting mitochondrial fatty acid oxidation in HepaRG cells. *Toxicology Letters*, *396*, 11–18. https://doi.org/10.1016/j.toxlet.2024.04.002

Nelson, L. J., Morgan, K., Treskes, P., Samuel, K., Henderson, C. J., LeBled, C., Homer, N., Grant, M. H., Hayes, P. C., & Plevris, J. N. (2017). Human hepatic HepaRG cells maintain an organotypic phenotype with high intrinsic CYP450 activity/metabolism and significantly outperform standard HepG2/C3A cells for pharmaceutical and therapeutic applications. *Basic & Clinical Pharmacology & Toxicology*, *120*(1), 30–37. https://doi.org/10.1111/bcpt.12631

Nyffeler, J., Willis, C., Lougee, R., Richard, A., Paul-Friedman, K., & Harrill, J. A. (2020). Bioactivity screening of environmental chemicals using imaging-based high-throughput phenotypic profiling. *Toxicology and Applied Pharmacology*, *389*, 114876. https://doi.org/10.1016/j.taap.2019.114876

Paul Friedman, K., Thomas, R. S., Wambaugh, J. F., Harrill, J. A., Judson, R. S., Shafer, T. J., Williams, A. J., Lee, J.-Y. J., Loo, L.-H., Gagné, M., Long, A. S., Barton-Maclaren, T. S., Whelan, M., Bouhifd, M., Rasenberg, M., Simanainen, U., & Sobanski, T. (2025).

Integration of new approach methods for the assessment of data-poor chemicals. *Toxicological Sciences*, *205*(1), 74–105. https://doi.org/10.1093/toxsci/kfaf019

Perkins, E. J., Ashauer, R., Burgoon, L., Conolly, R., Landesmann, B., Mackay, C., Murphy, C. A., Pollesch, N., Wheeler, J. R., Zupanic, A., & Scholz, S. (2019). Building and applying quantitative adverse outcome pathway models for chemical hazard and risk assessment. *Environmental Toxicology and Chemistry*, *38*(9), 1850–1865. https://doi.org/10.1002/etc.4505

Persson, L., Carney Almroth, B. M., Collins, C. D., Cornell, S., De Wit, C. A., Diamond, M. L., Fantke, P., Hassellöv, M., MacLeod, M., Ryberg, M. W., Søgaard Jørgensen, P., Villarrubia-Gómez, P., Wang, Z., & Hauschild, M. Z. (2022). Outside the safe operating space of the planetary boundary for novel entities. *Environmental Science & Technology*, *56*(3), 1510–1521. https://doi.org/10.1021/acs.est.1c04158

Pridgeon, C. S., Schlott, C., Wong, M. W., Heringa, M. B., Heckel, T., Leedale, J., Launay, L., Gryshkova, V., Przyborski, S., Bearon, R. N., Wilkinson, E. L., Ansari, T., Greenman, J., Hendriks, D. F. G., Gibbs, S., Sidaway, J., Sison-Young, R. L., Walker, P., Cross, M. J., … Goldring, C. E. P. (2018). Innovative organotypic in vitro models for safety assessment: Aligning with regulatory requirements and understanding models of the heart, skin, and liver as paradigms. *Archives of Toxicology*, *92*(2), 557–569. https://doi.org/10.1007/s00204-018-2152-9

Qu, B., Li, Q.-T., Wong, K. P., Tan, T. M. C., & Halliwell, B. (2001). Mechanism of clofibrate hepatotoxicity: Mitochondrial damage and oxidative stress in hepatocytes. *Free Radical Biology and Medicine*, *31*(5), 659–669. https://doi.org/10.1016/S0891-5849(01)00632-3

Ramaiahgari, S. C., Waidyanatha, S., Dixon, D., DeVito, M. J., Paules, R. S., & Ferguson, S. S. (2017). Three-dimensional (3D) HepaRG spheroid model with physiologically relevant xenobiotic metabolism competence and hepatocyte functionality for liver toxicity screening. *Toxicological Sciences*, *159*(1), 124–136. https://doi.org/10.1093/toxsci/kfx122

Ranade, A. R., Wilson, M. S., McClanahan, A. M., & Ball, A. J. (2014). High content imaging and analysis enable quantitative in situ assessment of CYP3A4 using cryopreserved differentiated HepaRG cells. *Journal of Toxicology*, *2014*, 291054. https://doi.org/10.1155/2014/291054

Rusyn, I., Arzuaga, X., Cattley, R. C., Christopher Corton, J., Ferguson, S. S., Godoy, P., Guyton, K. Z., Kaplowitz, N., Khetani, S. R., Roberts, R., Roth, R. A., & Smith, M. T. (2021). Key characteristics of human hepatotoxicants as a basis for identification and characterization of the causes of liver toxicity. *Hepatology*, *74*(6), 3486–3496. https://doi.org/10.1002/hep.31999

Seo, J.-E., He, X., Muskhelishvili, L., Malhi, P., Mei, N., Manjanatha, M., Bryant, M., Zhou, T., Robison, T., & Guo, X. (2022). Evaluation of an in vitro three-dimensional HepaRG spheroid model for genotoxicity testing using the high-throughput CometChip platform. *ALTEX, 39*(4), 583–604. https://doi.org/10.14573/altex.2201121

Sewell, F., Alexander-White, C., Brescia, S., Currie, R. A., Roberts, R., Roper, C., Vickers, C., Westmoreland, C., & Kimber, I. (2024). New approach methodologies (NAMs): Identifying and overcoming hurdles to accelerated adoption. *Toxicology Research, 13*(2), tfae044. https://doi.org/10.1093/toxres/tfae044

Sewell, F., Doe, J., Gellatly, N., Ragan, I., & Burden, N. (2017). Steps towards the international regulatory acceptance of non-animal methodology in safety assessment. *Regulatory Toxicology and Pharmacology, 89*, 50–56. https://doi.org/10.1016/j.yrtph.2017.07.001

Silva, R. J., & Tamburic, S. (2022). A state-of-the-art review on the alternatives to animal testing for the safety assessment of cosmetics. *Cosmetics*, *9*(5), 90. https://doi.org/10.3390/cosmetics9050090

Stevens, J. L., & Anders, M. W. (1981). Effect of cysteine, diethyl maleate, and phenobarbital treatments on the hepatotoxicity of [1H]- and [2H]chloroform. *Chemico-Biological Interactions*, *37*(1), 207–217. https://doi.org/10.1016/0009-2797(81)90178-2

Suzuki, T., Nojiri, H., Isono, H., & Ochi, T. (2004). Oxidative damages in isolated rat hepatocytes treated with the organochlorine fungicides captan, dichlofluanid and chlorothalonil. *Toxicology*, *204*(2–3), 97–107. https://doi.org/10.1016/j.tox.2004.06.025

Takahashi, Y., Hori, Y., Yamamoto, T., Urashima, T., Ohara, Y., & Tanaka, H. (2015). 3D spheroid cultures improve the metabolic gene expression profiles of HepaRG cells. *Bioscience Reports, 35*(3), e00208. https://doi.org/10.1042/BSR20150034

Takayama, K., Kawabata, K., Nagamoto, Y., Kishimoto, K., Tashiro, K., Sakurai, F., Tachibana, M., Kanda, K., Hayakawa, T., Furue, M. K., & Mizuguchi, H. (2013). 3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing. *Biomaterials, 34*(7), 1781–1789. https://doi.org/10.1016/j.biomaterials.2012.11.029

Tarazona, J. V., Fernandez-Agudo, A., Adamovsky, O., Baccaro, M., Burden, N., Campos, B., Hidding, B., Jenner, K., John, D., Lacasse, K., Lillicrap, A., Lyon, D., Maynard, S. K., Ott, A., Poulsen, V., Rasenberg, M., Schutte, K., Sobanska, M., & Wheeler, J. R. (2025). Use of alternatives to animal testing for environmental safety assessment (ESA): Report from the 2023 EPAA partners' forum. *Regulatory Toxicology and Pharmacology, 156*, 105774. https://doi.org/10.1016/j.yrtph.2025.105774

Taylor, K. (2018). Ten years of REACH—An animal protection perspective. *Alternatives to Laboratory Animals, 46*(6), 347–373. https://doi.org/10.1177/026119291804600610

U.S. EPA to eliminate all mammal testing by 2035. Science. Retrieved 2025, from https://www.science.org/content/article/us-epa-eliminate-all-mammal-testing-2035

Varet, J., Barranger, A., Crochet, C., Huet, S., Hogeveen, K., Le Hégarat, L., & Fessard, V. (2024). New methodological developments for testing the in vitro genotoxicity of nanomaterials: Comparison of 2D and 3D HepaRG liver cell models and classical and high throughput comet assay formats. *Chemosphere*, *350*, 140975. https://doi.org/10.1016/j.chemosphere.2023.140975

Veltman, C. H. J., van der Ven, L. T. M., Menegola, E., & Luijten, M. (2025). A pragmatic workflow for human relevance assessment of toxicological pathways and associated new approach methodologies. *Regulatory Toxicology and Pharmacology*, *160*, 105828. https://doi.org/10.1016/j.yrtph.2025.105828

Wajner, M., & Amaral, A. U. (2016). Mitochondrial dysfunction in fatty acid oxidation disorders: Insights from human and animal studies. *Bioscience Reports*, *36*(1), e00281. https://doi.org/10.1042/BSR20150240

Wambaugh, J. F., Bare, J. C., Carignan, C. C., Dionisio, K. L., Dodson, R. E., Jolliet, O., Liu, X., Meyer, D. E., Newton, S. R., Phillips, K. A., Price, P. S., Ring, C. L., Shin, H.-M., Sobus, J. R., Tal, T., Ulrich, E. M., Vallero, D. A., Wetmore, B. A., & Isaacs, K. K. (2019). New approach methodologies for exposure science. *Current Opinion in Toxicology*, *15*, 76–92. https://doi.org/10.1016/j.cotox.2019.07.001

Wang, Z., Walker, G. W., Muir, D. C. G., & Nagatani-Yoshida, K. (2020). Toward a global understanding of chemical pollution: A first comprehensive analysis of national and regional chemical inventories. *Environmental Science & Technology*, *54*(5), 2575–2584. https://doi.org/10.1021/acs.est.9b06379

Wincent, E., Kubota, A., Timme-Laragy, A., Jönsson, M. E., Hahn, M. E., & Stegeman, J. J. (2016). Biological effects of 6-formylindolo[3,2-b]carbazole (FICZ) in vivo are enhanced by loss of CYP1A function in an Ahr2-dependent manner. *Biochemical Pharmacology*, *110–111*, 117–129. https://doi.org/10.1016/j.bcp.2016.04.012

Yang, S., Ooka, M., Margolis, R. J., & Xia, M. (2023). Liver three-dimensional cellular models for high-throughput chemical testing. *Cell Reports Methods*, *3*(3), 100432. https://doi.org/10.1016/j.crmeth.2023.100432

Zhang, C., Zhang, Q., Li, J., Yu, L., Li, F., Li, W., Li, Y., Peng, H., Zhao, J., Carmichael, P. L., Wang, Y., Peng, S., & Guo, J. (2020). Integration of in vitro data from three dimensionally cultured HepaRG cells and physiologically based pharmacokinetic modeling for assessment of acetaminophen hepatotoxicity. *Regulatory Toxicology and Pharmacology*, *114*, 104661. https://doi.org/10.1016/j.yrtph.2020.104661

Zhao, J., Pike, B., Huang, J., Feng, Z., Odle, J., & Lin, X. (2023). Effects of medium chain triglycerides on hepatic fatty acid oxidation in clofibrate-fed newborn piglets. *Animal Nutrition, 12*, 334–344. https://doi.org/10.1016/j.aninu.2022.12.001

Zhou, Y., Shen, J. X., & Lauschke, V. M. (2019). Comprehensive evaluation of organotypic and microphysiological liver models for prediction of drug-induced liver injury. *Frontiers in Pharmacology, 10*, 1093. https://doi.org/10.3389/fphar.2019.01093

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Chapter II

"Decoding chemical toxicity: from cellular shadows to molecular signatures"

Preamble

The application of transcriptomic analysis in evaluating chemical toxicity serves as a bridge between broad cellular-level changes and more detailed molecular insights. By capturing global shifts in gene expression, this approach provides a robust framework for classifying chemicals based on toxic effects reflected in the underlying signalling pathways involved in their adverse outcomes. Furthermore, integrating transcriptomic data with cellular-level studies enhances both the sensitivity and specificity of toxicity assessments. This enables the identification of early-response biomarkers, predictive factors, and mechanistic endpoints associated with the adverse effects of the tested chemicals, thereby improving our understanding of NGTxC MoAs.

Building on the findings of Chapter One which focused on cellular-level toxicity screening and the classification of chemicals based on their toxicity profiles, the more sensitive *in vitro* cellular model was selected for deeper mechanistic evaluation of the tested chemicals at the molecular level. In this chapter, a transcriptomic approach was applied to generate gene expression profiles of 3D HepaRG cells treated with three distinct groups of chemicals: (i) genotoxic compounds (GTx), (ii) non-genotoxic non-carcinogenic compounds (NGTx), and (iii) non-genotoxic carcinogens (NGTxC). This approach enabled a closer molecular investigation of the selected chemical panel, going beyond cellular-level changes to explore transcriptional signatures and signalling pathways.

In the first part of this chapter, genotoxicity-related transcriptomic biomarkers (TGX-DDI and GENOMARK) were applied to evaluate the ability of this approach to distinguish GTx from NGTxC. As these biomarkers have already been validated in various human cell models, including TK6 and 2D HepaRG cells, we aimed to determine whether they could also be applied to transcriptomic profiles generated from treated 3D HepaRG cells, which are considered a more relevant and predictive human metabolically competent *in vitro* model.

Moreover, since NGTxCs do not directly interact with DNA but act through diverse MoAs, identifying specific genes and pathways involved in their adverse effects could provide valuable insights into how NGTxCs function and exert toxicity, particularly in the liver, which is central to this research. Therefore, in the second part of this chapter, we applied unsupervised clustering and gene ontology enrichment analysis to assess whether this approach could classify and distinguish NGTxC from GTx and NGTx *in vitro*, and to identify key molecular events or molecular pathways altered during non-genotoxic carcinogenic processes.

Préambule

L'application de l'analyse transcriptomique dans l'évaluation de la toxicité chimique établit un lien entre les modifications globales observées au niveau cellulaire et les informations détaillées au niveau moléculaire. En capturant les variations d'expression génique, cette approche fournit un cadre robuste pour classifier les substances selon leurs effets toxiques et les voies de signalisation sous-jacentes. L'intégration de données transcriptomiques aux études cellulaires améliore la sensibilité et la spécificité des évaluations, permettant d'identifier des biomarqueurs précoces, des facteurs prédictifs et des points d'ancrage mécanistiques associés aux effets indésirables, et d'affiner la compréhension des modes d'action des NGTxC.

En s'appuyant sur les résultats du premier chapitre, consacré au criblage cellulaire et à la classification des substances selon leurs profils toxiques, le modèle *in vitro* le plus sensible a été retenu pour une analyse mécanistique approfondie. Une approche transcriptomique a ainsi été appliquée à des sphéroïdes HepaRG 3D traités avec trois groupes de substances : (i) des composés génotoxiques (GTx), (ii) des composés non génotoxiques et non cancérogènes (NGTx) et (iii) des composés non génotoxiques cancérogènes (NGTxC). Cette stratégie a permis d'examiner les signatures transcriptionnelles et les voies de signalisation au-delà des seules altérations cellulaires.

Dans la première partie du chapitre, des biomarqueurs transcriptomiques de génotoxicité (TGX DDI et GENOMARK) ont été utilisés pour évaluer leur capacité à distinguer les GTx des NGTxC. Déjà validés dans divers modèles humains, ces biomarqueurs ont été testés sur les profils issus des HepaRG 3D, un modèle métaboliquement compétent et plus prédictif. Dans la seconde partie, l'analyse de clustering non supervisée et l'enrichissement en ontologie génique ont été appliqués pour déterminer si ces approches permettent de classifier les NGTxC par rapport aux GTx et NGTx, et d'identifier des gènes, événements moléculaires ou voies altérées impliqués dans les processus cancérogènes non génotoxiques, en particulier au niveau hépatique.

Advancing genotoxicity assessment with transcriptomic biomarkers: From 2D to 3D HepaRG spheroid model

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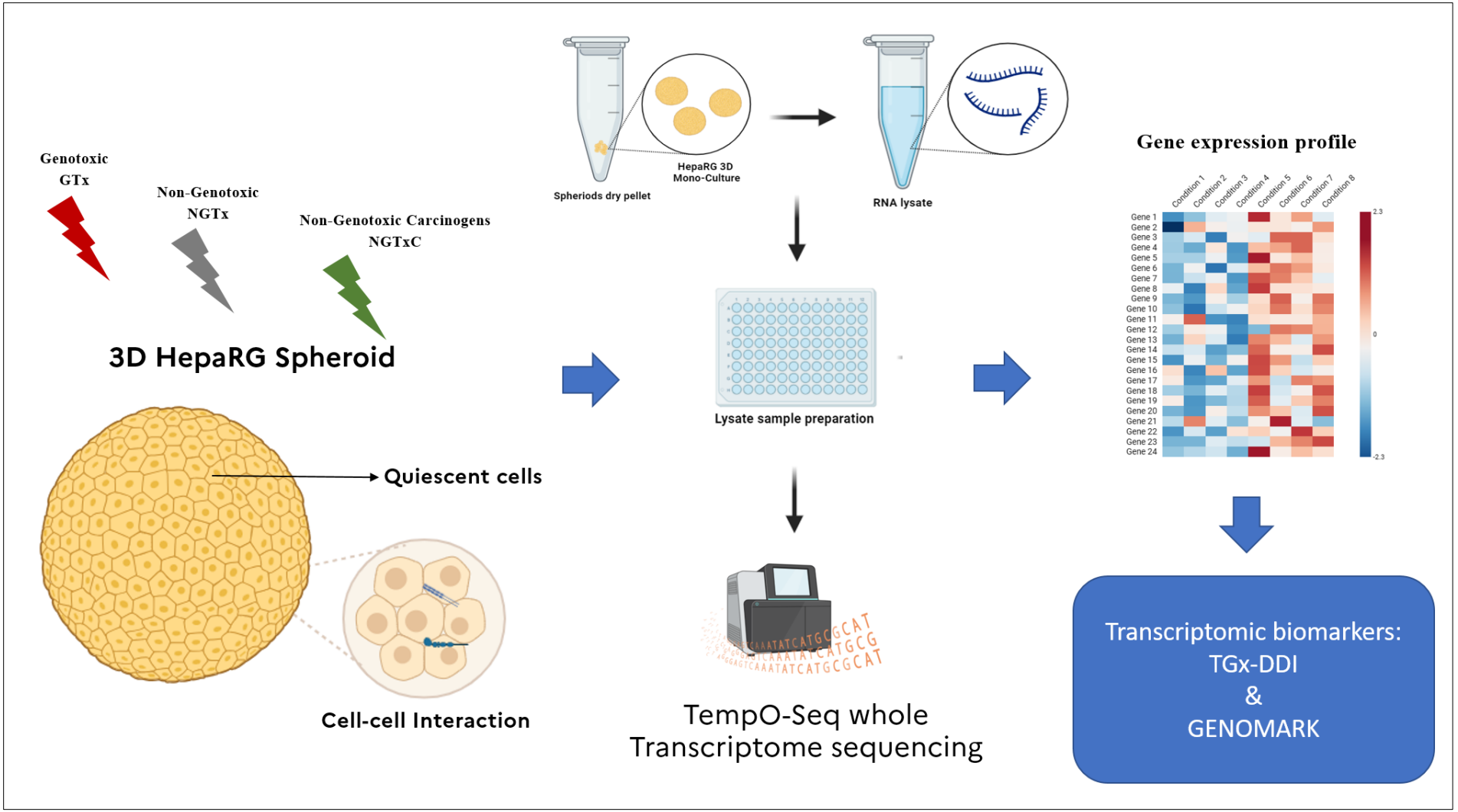

Abstract

The development of New Approach Methodologies (NAMs) for genotoxicity testing is needed to support the transition towards animal-free human-relevant chemical genotoxicity assessment. In this context, we investigated the predictive power of two transcriptomic biomarkers developed in 2D cell systems (GENOMARK and TGx-DDI) in metabolically active 3D HepaRG spheroids to classify chemicals as genotoxic (GTx) or non-genotoxic (NGTx). Human-hepatic HepaRG spheroids were treated for 72h with three different groups of chemicals: A) five genotoxic chemicals: lasiocarpine, cyclophosphamide, ethyl methanesulfonate, aflatoxin B1, benzo[a]pyrene; B) five non-genotoxic non-carcinogens: aflatoxin B2, 2-deoxy-D-glucose, D-mannitol, caffeine, ampicillin trihydrate; and C) five non-genotoxic carcinogens: perfluorooctanoic acid, diethanolamine, thioacetamide, phorbol-12-myristate-13-acetate, di(2-ethylhexyl) phthalate. Gene expression data from the treated HepaRG spheroids were collected using the high-throughput TempO-SeqTM technology and analysed with the transcriptomic biomarkers GENOMARK and TGx-DDI. Results with both biomarkers generally aligned with existing knowledge for all three groups of tested chemicals, effectively distinguishing genotoxic from non-genotoxic chemicals. The results of this study indicate that combining the 3D HepaRG spheroid model with transcriptomic biomarkers offers a promising, human-relevant approach to complement traditional *in vitro* genotoxicity tests.

Highlights

- 3D HepaRG spheroids were exposed to 15 chemicals for genotoxicity assessment.
- Transcriptomics data were collected and analyzed with the TGx-DDI and GENOMARK biomarkers for genotoxicity.
- Genotoxicity classifications by both biomarkers aligned well with existing knowledge.
- These results demonstrate the applicability of both transcriptomic biomarkers in a 3D HepaRG model.

Key words

New Approach Methodologies (NAMs), TGx-DDI, GENOMARK, High-Throughput Sequencing, TempO-Seq^{TM}, Genotoxic (GTx), Non-Genotoxic (NGTx), Non-Genotoxic Carcinogens (NGTxC).

1. Introduction

Genotoxicity assessment is a critical element in Chemical Safety Assessment (CSA), as changes to the genetic material can lead to heritable diseases and various other severe health outcomes, including cancer, depending on the specific cells and tissues involved (Turkez et al., 2017). Therefore, genotoxicity testing is required by regulatory agencies worldwide (Auletta et al., 1993; Sachse et al., 2026).

Currently, *in vitro* testing batteries are available to evaluate the genotoxic potential of chemicals (Cartus and Schrenk, 2017). These testing batteries are designed to identify both gene mutations and chromosomal alterations using internationally recognized methods. Gene mutations are assessed with assays such as the bacterial reverse gene mutation test (also referred to as the 'Ames test'), and mammalian gene mutation assays, including the Thymidine Kinase gene assays (Mouse Lymphoma Assay [MLA] and TK6 assay) as well as assays targeting the Hprt and Xprt genes (Chen et al., 2022). Chromosomal alterations are typically detected using the *in vitro* chromosome aberration assay or the *in vitro* micronucleus (MN) assay. In recent years, the *in vitro* MN test has been preferred over the *in vitro* chromosome aberration assay because it can detect both structural and numerical chromosome aberrations (Zeller et al., 2018). Testing batteries that combine *in vitro* tests for gene mutations and chromosomal modifications are considered appropriate for identifying potential *in vivo* genotoxicants and/or carcinogens (Nesslany, 2017). Specifically, it has been shown that a combination of the Ames test and the *in vitro* MN assay can effectively detect most genotoxins (Kirkland et al., 2005; 2007). Lately, Luijten et al., (2024) demonstrated that a mammalian *in vitro* chromosomal damage test together with a mammalian cell gene mutation test provides strong predictive weight-of-evidence (WoE) for evaluating the genotoxic hazard of a substance, and that this combination improves the specificity of the test battery (Luijten et al., 2024). However, in most regulatory settings, positive *in vitro* results need to be followed up with an adequate *in vivo* test to confirm the *in vitro g*enotoxic effect in a more complex *in vivo* setting (Fortin et al., 2023). Consequently, most current genotoxicity testing strategies still rely on animal testing. To address this, significant efforts are ongoing to develop advanced cell-based *in vitro* New Approach Methodologies (NAMs) that can reduce, and ultimately even exclude, the need for *in vivo* testing.

In general, these methods aim to improve genotoxicity prediction by collecting more mechanistic information using human-relevant cell lines, thereby enhancing CSA (Fortin et al., 2023; Menz et al., 2023; Luijten et al., 2021). An example includes the γH2AX/pH3 method.

γH2AX serves as an early marker for DNA double-strand breaks (Li et al., 2019) and can be detected in many different 2D and 3D cell systems. Moreover, when combined with the pH3 biomarkers, the biomarker allows to distinguish between clastogens and aneugens. Another example is the ToxTracker assay that identifies specific cellular stress pathways activated by chemicals to evaluate genotoxicity (Hendriks et al., 2012). Additionally, high-throughput transcriptomic approaches are being integrated into NAMs to evaluate the genotoxic potential of chemicals (Buick et al., 2021). To facilitate the analysis of large transcriptomics datasets, selected sets of biomarker genes for genotoxicity have been identified *in vitro* in human-relevant cell lines (Li et al., 2025). By analysing the chemical-induced expression profiles of these genes using machine learning methods, the *in vitro* transcriptomic biomarkers allow the evaluation of DNA damage caused by different chemicals (Li et al., 2025).

In 2022, the International Workshops on Genotoxicity Testing (IWGT) convened a workshop to assess advancements in the development and application of transcriptomic biomarkers for genotoxicity testing. The working group identified several promising biomarker candidates, with three standing out due to their independent development (TGx-DDI, GENOMARK, and MU2012). Among these, TGx-DDI and GENOMARK are the most widely applied in genotoxicity testing (Li et al., 2025). In addition, Health Canada's GeneTox21 program, which aims to modernize genotoxicity testing by integrating transcriptomic approaches, utilized the TGx-DDI biomarker and proposed its integration into current genotoxicity hazard assessments (Fortin et al., 2023).

The TGx-DDI biomarker consists of a 64-gene set derived from TK-6 cells exposed to different genotoxic substances for a short exposure time (3h) (Barutcu, 2024). The TGx-DDI gene set signature has demonstrated strong reliability in distinguishing genotoxic agents from non-genotoxic agents (Allemang et al., 2021; Chen et al., 2022) using different cell lines (including HepaRG cells) and gene expression technologies (Cho et al. 2019; Buick et al. 2020; 2021). Similarly, the GENOMARK transcriptomic biomarker includes 84 genes selected from human 2D HepaRG cells exposed to various compounds, including genotoxic and non-genotoxic compounds, for a longer exposure time (72h) (Thienpont 2024). GENOMARK has also shown good predictivity and accuracy in distinguishing genotoxic from non-genotoxic substances (Ates et al., 2018; Thienpont, Cho, Andrew Williams, et al., 2023). Interestingly, Thienpont et al., (2024) demonstrated that combining both the TGx-DDI and GENOMARK biomarkers on transcriptomic data generated by the high-throughput TempO-Seq^{TM} sequencing technology in 2D HepaRG cells can improve chemical genotoxicity assessment (Thienpont et al., 2024).

These findings support the integration of the transcriptomic biomarkers into regulatory frameworks and standard genotoxicity testing strategies (Froetschl et al., 2025).

TGx-DDI has been extensively validated for the classification of DNA damage–inducing chemicals in several *in vitro* systems; however, its application in advanced three-dimensional (3D) liver models remains limited. Most studies have relied on conventional two-dimensional cell systems, particularly TK6 cells and HepaRG cells, where the biomarker was originally developed and validated. Only a few investigations have explored the use of TGx-DDI in more physiologically relevant 3D liver models. For example, TGx-DDI analysis has been applied to primary human hepatocytes (PHH) exposed to per- and polyfluoroalkyl substances, demonstrating the potential of transcriptomic biomarkers to evaluate genotoxic mechanisms in complex liver systems (Wang et al., 2025). Nevertheless, the broader applicability of transcriptomic biomarkers in 3D hepatic models is still underexplored. In addition, GENOMARK transcriptomic biomarker have been predominantly implemented in 2D *in vitro* platforms, without any reported evaluation in 3D liver systems such as spheroids or organoid-based models. Thus, expanding the application of these transcriptomic approaches to advanced 3D hepatic models may enhance their physiological relevance and improve mechanistic understanding of chemical-induced genotoxicity.

Human hepatic HepaRG cells have become an important model for genotoxicity assessment, owing to their ability to detect a broad spectrum of genotoxicants, including compounds requiring metabolic activation (Pridgeon et al., 2018; Seo et al., 2019). Compared with other hepatic cell lines, HepaRG cells exhibit enhanced physiological and metabolic competence, characterized by high expression of key Cytochrome P450 (CYP) enzymes, functional activation of nuclear receptors, and a bipotent differentiation capacity that enables the formation of both hepatocyte- and cholangiocyte-like cells (Anthérieu et al., 2012; Ranade et al., 2014; Guo et al., 2024). These features underpin their growing relevance in chemical hazard evaluation and mechanistic toxicology.

In recent years, the development of three-dimensional (3D) HepaRG spheroid models has gained momentum. Although 3D cultures are associated with higher costs and increased technical complexity, they offer several advantages over traditional 2D monolayers. Notably, 3D HepaRG spheroids display improved differentiation stability, enhanced metabolic functionality, and more physiologically relevant cellular architecture (Rabiet et al., 2024). Consistent with these properties, 3D spheroids often exhibit more pronounced cellular responses to chemical exposure than 2D cultures (Saleh et al., 2025).

Their suitability for high-throughput genotoxicity testing has also been demonstrated. For example, in the CometChip assay, 3D HepaRG spheroids showed greater sensitivity to genotoxicants, detecting effects at lower benchmark doses than 2D monolayers (Seo et al., 2022). Similarly, in a high-throughput flow cytometry-based micronucleus assay evaluating 34 compounds, 3D HepaRG spheroids outperformed 2D cultures in identifying indirect-acting genotoxicants and carcinogens requiring metabolic activation (Seo et al., 2023). When compared with other hepatic 3D models, such as HepG2 spheroids or primary human PHH, 3D HepaRG spheroids also demonstrate greater adaptability to high-throughput platforms (Quintás et al., 2021; Guo et al., 2024).

By contrast, several limitations have been reported for alternative hepatic 3D systems (Štampar and Žegura, 2024). HepG2 spheroids exhibit low glutathione synthesis and inconsistent albumin secretion, while PHH-based models suffer from donor-to-donor variability and low glutathione-S-transferase P1 expression. Moreover, both PHH-derived spheroids and induced pluripotent stem cell (iPSC)-derived hepatocytes remain costly and labor-intensive, largely due to prolonged and complex differentiation protocols (Štampar and Žegura, 2024).

Taken together, these findings support 3D HepaRG spheroids as a promising and human-relevant *in vitro* liver model for the assessment of genotoxicity. Building on these advantages, we applied an *in vitro* high-throughput transcriptomic methodology to investigate the applicability and predictive power of the two transcriptomic biomarkers for detecting the genotoxic potential of chemicals when combined with the more complex 3D HepaRG spheroid model.

2. Materials and Methods

2.1. HepaRG Cell Culture

The human hepatic HepaRG cell line was purchased from Biopredic International (Rennes, France) and cultured as previously described (Saleh et al., 2025). Briefly, HepaRG cells were cultured in Williams E medium 1X + GlutamaxTM-I (Gibco, Noisy le Grand, France) supplemented with 10% FBS FetalClone II (Cytiva, Saint Germain en Laye, France), Penicillin 5000 units/mL / Streptomycin 5000 µg/mL (Gibco, Noisy le Grand, France), 5 µg/mL insulin (Sigma, Saint Quentin Fallavier, France), and 18 µg/mL Hydrocortisone hemisuccinate (Upjohn, Paris, France). HepaRG cells between passages 15-18 were seeded in 75cm^2 flasks (VWR, Rosny sous Bois, France) at a density of 20x10^3/cm^2 and incubated at 37 °C with 5% CO_2. Cell dissociation with TryplE 1X (Thermo Fisher Scientific, Waltham, USA) was performed after 2 weeks in 75cm^2 flasks at a density of 27x10^3/cm^2 cells to start differentiation with DMSO (Sigma, Saint Quentin Fallavier, France).

2.2. HepaRG in a 3D culture system

As previously described by Saleh et al. (2025), after 14 days of differentiation in 1.7 % DMSO (Figure C.II-A 1), cells were trypsinized, and seeded in a 96-well Ultra-low attachment (ULA) Plates (Sigma, France - CORNING REF 4520) at a density of 2,000 cells/well. Fresh media without DMSO was replaced every 2 days. Spheroids were ready for chemical exposure one week after seeding (Saleh et al. 2025).

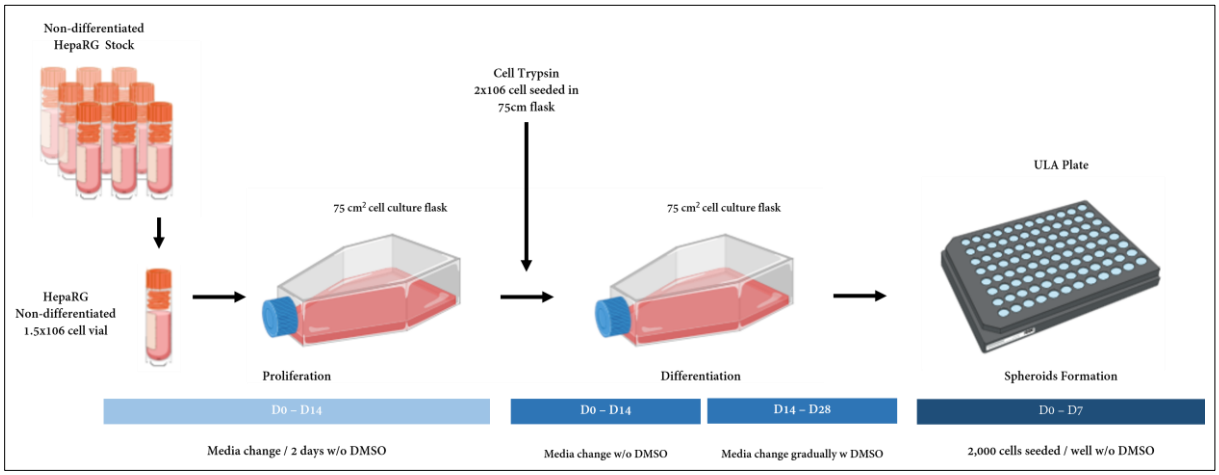

Figure C.II-A 1. 3D HepaRG spheroid culture protocol (Saleh et al., 2025).

2.3. Chemical selection

Fifteen chemicals were selected in this study based on their known genotoxic effects (Table C.II-A 1**)**. Five different known Genotoxic (GTx) chemicals: lasiocarpine (PA), cyclophosphamide (CPA), ethyl methanesulfonate (EMS), aflatoxin B1 (AFB1), and benzo[a]pyrene (B[a]P); five Non-Genotoxic (NGTx) chemicals: aflatoxin B2 (AFB2) 2-deoxy-D-glucose (2DDG), D-mannitol, caffeine, and ampicillin trihydrate; and five Non-Genotoxic Carcinogens (NGTxC): perfluorooctanoic acid (PFOA), diethanolamine (DEA), thioacetamide (TAA), phorbol-12-myristate-13-acetate (TPA), and di(2-ethylhexyl) phthalate (DEHP)). All chemical solutions were prepared in 1% DMSO (Sigma, Saint Quentin Fallavier, France). Chemical treatments were performed in serum-free media. All chemicals were purchased from Sigma (Saint-Quentin-Fallavier, France) except PA, which was from Phytoplant Research S.L. (Córdoba, Spain).

2.4. Cell viability assay

A pre-cytotoxicity assay was performed for all the selected chemicals to determine the concentrations for the transcriptomic experiments. For the GTx and NGTxC chemicals, six concentrations were selected for the pre-cytotoxicity assay based on a previously published toxicity range in 3D HepaRG spheroids with the same assay in literatures. For the NGTx chemicals, the six different concentrations were selected starting from the highest soluble concentrations (not more than 10mM) were selected (APP). After 72h repeated dose treatment, cytotoxicity was assessed using the CellTiter-Glo 3D Cell Viability kit (Promega G9682), according to the manufacturer's instructions. One spheroid per condition was used for the cytotoxicity measurements. ATP luminescence was measured using a FLUOstar Optima microplate reader (BMG Labtech, Champigny-sur-Marne, France). Blank-corrected raw data were used to calculate the relative cell viability using the following formula:

Relative ATP level (%) = [Blank corrected raw data (sample) / Blank corrected raw data (control)] x 100

Next, IC_{10}, IC_{50}, and IC_{90} values of each chemical were calculated based on the relative ATP values using GraphPad prism (version 9.5.1). In addition, concurrent cytotoxicity assays were conducted with the selected IC_{10} and $IC_{10/2}$, as described above, to assess cytotoxicity across all conditions tested in the transcriptomics study (Table C.II-A 1).

Table C.II-A 1. Chemicals selected for this study. The table presents the full name of the chemicals and the abbreviation, the CAS number, the Mode of Action (MoA), previously published *in vitro* genotoxicity information collected with the Ames test and the micronucleus test (MN), previously published *in vivo* genotoxicity information, previously published data obtained with GENOMARK/TGx-DDI in the 2D HepaRG cell system and the concentrations tested in this study. (Δ): Additional tested concentrations, (ND): no data, (N/A): not applicable.

Chemicals	CAS	MoA	*In vitro*		*In vivo*	Genomark/ TGx-DDI 2D HepaRG	Concentrations (µM)		
			Ames	MN			IC_{10}	$IC_{10/2}$	Δ
GTx									
Ethyl methanesulfonate (EMS)	62-50-0	Hepatotoxic Alkylation	+ (Lim et al., 2025)	+ (Lim et al., 2025)	+ Rodent Boiassay (Zhu et al., 2022)	+ (Thienpont et al., 2024)	1000	500	N/A
Aflatoxin B1 (AFB1)	1162-65-8	Hepatotoxic DNA Adducts	+ (Wong & Hsieh, 1976)	+ (Marchese et al., 2018)	+ *in vivo* micronucleus and comet assay in Rats (Corcuera et al., 2015)	+ (Thienpont et al., 2024)	0.1	0.05	N/A
Benzo[a]pyrene (BaP)	50-32-8	Hepatotoxic DNA adducts	+ (Phillipson & Ioannides, 1989)	+ (Lim et al., 2025)	+ *in vivo* micronucleus in mouse (Long et al., 2016)	+ (Buick et al., 2021) *Unpublished data + GENOMARK	3.6	1.8	5
Cyclophosphamide (CPA)	6055-19-2	Hepatotoxic Alkylation	+ (Anderson et al., 1995a)	+ (Anderson et al. 1995b) (Yadav et al., 2023)	+ *in vivo* micronucleus in mouse (Krishna et al., 1995)	+ (Thienpont et al., 2024)	2000	1000	N/A
Lasiocarpine (PA)	303-34-4	Hepatotoxic Alkylation	+ (Yamanaka et al., 1979)	+ (Li et al., 2020)	+ *in vivo* rat (Xia et al., 2006)	ND	0.5	0.25	N/A

NGTx									
Caffeine (CAFF)	58-08-2	Not hepatotoxic	-	-	-	ND	5000	2000	N/A
Ampicillin trihydrate (AMP)	7177-48-2	Not hepatotoxic	-	-	-	- (Thienpont et al., 2023 ; Buick et al., 2020)	10000	N/A	N/A
Aflatoxin B2 (AFB2)	7220-81-7	Unknown Hepatotoxicity	ND	ND	ND	ND	15	7.5	10
2-Deoxy-D-glucose (2DDG)	154-17-6	Not hepatotoxic	-	-	-	- (Thienpont et al., 2023 ; Buick et al., 2020)	10000	N/A	N/A
D-mannitol (D-man)	69-65-8	Not hepatotoxic	-	-	-	ND	10000	N/A	N/A
NGTxC									
Di(2-ethylhexyl) phthalate (DEHP)	117-81-7	Hepatotoxic PPARα nuclear receptor activation	-	-	-	ND	200	100	N/A
Thioacetamide (TAA)	62-55-5	Hepatotoxic Oxidative stress	-	-	-	ND	7000	3500	N/A
Diethanolamine (DEA)	111-42-2	Hepatotoxic Oxidative stress Phospholipid metabolism	-	-	-	ND	200	100	N/A
Perfluorooctanoic acid (PFOA)	3825-26-1	Hepatotoxic Oxidative stress Mitochondrial activity	-	-	-	ND	40	20	N/A
12-O-Tetradecanoylphorbol -13-acetate (TPA)	16561-29-8	Hepatotoxic Promoting cell proliferation	-	-	-	ND	20	10	5

2.5. Chemical exposure

Based on the results of the pre-cytotoxicity tests, concentrations equivalent to the IC_{10} and $IC_{10/2}$ were selected, except for 3 out of the 5 NGTx chemicals (2DDG, MAN, and D-MAN) for which only a single concentration equivalent to the IC_{10} was tested. Moreover, an additional concentration was tested for B[a]P and AFB2 as the pre-cytotoxicity results differed from previously collected in-house or literature data, resulting in a total of 3 concentrations for these two compounds (Table C.II-A 1). Moreover, since the IC_{50} of TPA was close to IC_{10} (Figure APP. II 3), an additional concentration (5µM) was also included in this study.

Spheroids were treated with the selected concentrations of the chemicals for 72h, with media being replaced every 24h. After exposure (Table C.II-A 1), fifty-five treated spheroids per condition were collected in an eppendorf tube and centrifuged for 3 minutes at 500g. After washing twice with PBS, spheroids were stored as a dry pellet at -80°C before RNA-lysate preparation. As previously mentioned, concurrent cytotoxicity measurements were performed for one spheroid/tested concentration. Moreover, this experimental design was repeated independently 3 times using different HepaRG cell batches.

2.6. RNA-Lysate preparation

BioSpyder Enhanced 2X Lysis Buffer (P/N 201247) was diluted with PBS and used as a 1X solution to lyse 35 spheroids/condition and prepare the RNA-isolated lysate following the TempO-Seq™ Assay user guide. In brief, spheroids were incubated for 10 minutes with the lysis buffer at 37 °C. The lysate samples were shipped on dry ice to Bioclavis (Glasgow, UK), a spinoff company of BioSpyder Technologies.

2.7. TempO-Seq™ Assay

Gene expression profiles of the spheroids treated with the controls or chemicals were determined using the high-throughput TempO-Seq™ technology. The Human whole transcriptome (v2.1) was used as probe set along with the TempO-seq™ Human whole transcriptome reagent kit to prepare libraries in 96-well plate format. One negative control (1X TempO-seq™ Lysis buffer only) and two positive qPCR assay controls (Human Reference Total RNA) (Brain total RNA) were used as four replicates in the analysis. Briefly, RNA-lysate samples were hybridized under a temperature gradient to the whole transcriptome probe pool through the complementary sequence between mRNA and primer binding sites. After

hybridization, the unbound probes were removed through nuclease digestion. The amplification templates of the bound probes were then amplified using a primer set (single-plex PCR reaction, with a single primer pair for each sample) PCR reaction to add sample-specific tags and sequencing adapters. The amplified libraries were then sequenced using an Illumina NextSeq 2000 system, yielding a median read depth of 6 million reads per sample. This TempO-Seq™ sequencing protocol ensures high specificity and sensitivity as described in detail by Yeakley et al. (2017).

2.8. Transcriptomic biomarkers-based chemical classification

The gene expression profiles of the tested chemicals were analysed using the bioinformatics tools of the GENOMARK and TGx-DDI biomarkers as described previously in Thienpont et al. 2024. In brief, gene expression data generated by the TempO-Seq™ technology were transformed into log2 values. For both biomarkers, genes having multiple probe IDs were summed prior to the log transformation. For GENOMARK, the support vector machine (SVM) using R packages e1071 and caret, and the Random Forest (RF) algorithm using RF package in R (version 4.0.4) were used to compute prediction scores. The prediction scores are values between 0 and 1, and can lead to three different predictions: positive if the score is > 0.55, negative if the score is < 0.45 and equivocal if the score is between 0.55 and 0.45. When both algorithms returned a score > 0.55, the samples are classified as genotoxic. When both algorithms returned a score < 0.45, the sample is classified as non-genotoxic. If one of the two prediction scores is between 0.45 and 0.55, the prediction is set as inconclusive for the sample. Finally, if the two methods returned a different prediction, the sample is also considered as Inconclusive. For TGx-DDI, the Nearest Shrunken Centroids (NSCs) method, implemented in the TGx-DDI classification tool (TGx-DDI Biomarker for DNA Damage Classification), was conducted to classify the samples. The method returns two NSC probabilities; a DNA Damage Inducing (DDI) probability and a Non-DNA Damage Inducing probability (NDDI). The sample was classified as DDI when the NSC analysis yielded a higher DDI probability than the NDDI probability. In the opposite case, the sample was classified as NDDI.

3. Results

3.1. Cytotoxicity

Pre-cytotoxicity evaluations were performed for all the selected chemicals to determine the concentrations corresponding to the IC_{10} and $IC_{10/2}$ (Figure APP. II 1) (Figure APP. II 2) (Figure APP. II 3). In addition, concurrent cytotoxicity evaluations were conducted together with the transcriptomics study to ensure that no severe cytotoxic effects were present. The results of the former showed that all treatment conditions tested with 3D HepaRG spheroids for a 72h repetitive treatment exhibited no more than 30% associated cytotoxicity, except for the highest concentrations tested with B[a]P (10 µM) and TPA (20 µM) (Figure C.II-A 2).

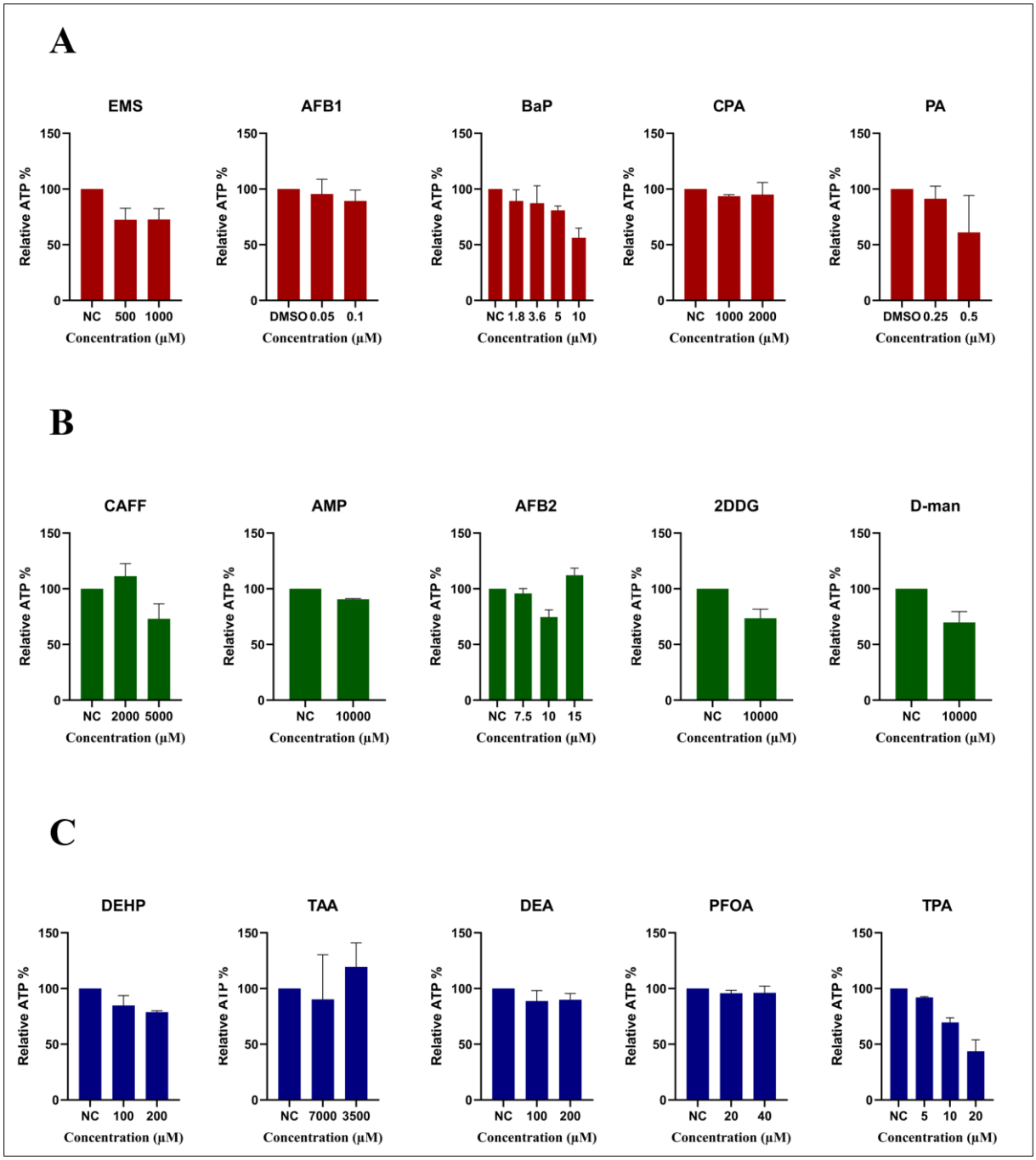

Figure C.II-A 2. Concurrent cytotoxicity check to the selected tested concentration of the principal study. Relative ATP levels following 72 h repetitive treatments with the selected concentrations of each tested chemical in 3D HepaRG spheroids. (A): Genotoxic chemicals (GTx) selected EMS, AFB1, BaP, CPA, and PA. (B): Non-Genotoxic chemicals (NGTx) selected CAFF, AMP, AFB2, 2DDG, and D-man. (C): Non-Genotoxic Carcinogens (NGTxC) chemicals selected DEHP, TAA, DEA, PFOA, and TPA. Data presented as means (3 independent biological replicates) ± SEM.

3.2. Transcriptomic biomarker-based classification results

A result is considered as positive with TGx-DDI if it is classified as DDI in the statistical prediction model used. The results demonstrated that the TGx-DDI transcriptomic biomarker correctly classified all GTx chemicals as DDI chemicals (Table C.II-A 2). EMS and B[a]P were classified as DDI only at the highest concentration tested. Among the NGTx chemicals, only AFB2 was classified as DDI. In addition, the highest concentration tested for CAFF, corresponding to 5000 µM, also resulted in a classification as DDI with the TGx-DDI biomarker. Moreover, all chemicals in the NGTxC group were classified as non-DDI, with the exception of TPA, which was classified as DDI at a cytotoxic concentration close to the IC_{50} of 20 µM (Table C.II-A 2).

The GENOMARK biomarker classifies chemicals as either positive or negative when both statistical predictive models give similar prediction scores, i.e., two scores > 0.55 or two scores < 0.45, respectively. A sample is classified as inconclusive when there are different calls between the SVM and RF prediction models, or when at least one of the two models returns an equivocal prediction score (between 0.45 and 0.55). The GENOMARK biomarker correctly classified all GTx chemicals as positive (Table C.II-A 2). Like for TGx-DDI, EMS and B[a]P were classified as DDI only at the highest concentration tested. Among the NGTx chemicals, only AFB2 was classified as DDI. However, CAFF, 2DDG and AMP (NGTx) showed inconclusive results at the concentrations tested. It should be noted that the classification as inconclusive was mostly based on an inconclusive result in one of the two prediction models. NGTxC chemicals were classified negative with GENOMARK, with the exception of TPA at a toxic concentration near the IC_{50} (20 µM) (Table C.II-A 2). Inconclusive results were obtained for the highest concentrations of DEHP and DEA.

Table C.II-A 2. Overview of the TGx-DDI and GENOMARK classification results based on gene-expression profiles from 3D HepaRG spheroids obtained with TempO-Seq™ and analysed with the different statistical tools. Red represents the positive results, Green represents the negative results, and yellow represents the inconclusive results (GENOMARK only). (*): represents toxic concentrations.

Biomarker			GENOMARK			TGx-DDI	
Chemical	Condition	Concentration µM	RF_GTX	SVM_GTX	Prediction	DDI_probability	Prediction
GTx							
EMS	C1	1000	0,58	0,75	POSITIVE	1	DDI
	C2	500	0,33	0,21	NEGATIVE	0,0000196	NDDI
CPA	C1	2000	0,85	0,98	POSITIVE	1	DDI
	C2	1000	0,56	0,55	POSITIVE	1	DDI
AFB1	C1	0,1	0,61	0,82	POSITIVE	1	DDI
	C2	0,05	0,45	0,23	INCONCLUSIVE	1	DDI
B[a]P	C1	5	0,94	0,99	POSITIVE	1	DDI
	C2	3,6	0,37	0,09	NEGATIVE	8,66E-29	NDDI
	C3	1,8	0,28	0,02	NEGATIVE	5,05E-34	NDDI
PA	C1	0,5	0,84	0,98	POSITIVE	1	DDI
	C2	0,25	0,62	0,62	POSITIVE	1	DDI
NGTxs							
MAN	C1	10000	0,40	0,11	NEGATIVE	1,78E-56	NDDI
CAF	C1	5000	0,66	0,45	INCONCLUSIVE	1	DDI
	C2	2000	0,48	0,03	INCONCLUSIVE	1,01E-32	NDDI
AMP	C1	10000	0,47	0,30	INCONCLUSIVE	8,94E-36	NDDI
AFB2	C1	15	0,72	0,74	POSITIVE	1	DDI
	C2	10	0,55	0,31	INCONCLUSIVE	1	DDI
	C3	7,5	0,59	0,26	INCONCLUSIVE	1	DDI
2DDG	C1	10000	0,48	0,48	INCONCLUSIVE	3,38E-36	NDDI
NGTxCs							
PFOA	C1	40	0,35	0,12	NEGATIVE	4,5E-59	NDDI
	C2	20	0,29	0,08	NEGATIVE	1,99E-60	NDDI
DEA	C1	200	0,50	0,31	INCONCLUSIVE	1,64E-59	NDDI
	C2	100	0,41	0,22	NEGATIVE	1,27E-35	NDDI
DHEP	C1	200	0,41	0,55	INCONCLUSIVE	3,89E-37	NDDI
	C2	100	0,30	0,05	NEGATIVE	3,75E-32	NDDI
TAA	C1	7000	0,44	0,10	NEGATIVE	1,66E-34	NDDI
	C2	3500	0,25	0,04	NEGATIVE	2,54E-43	NDDI
TPA	C1	20*	0,72	0,57	POSITIVE*	1	DDI*
	C2	10	0,24	0,01	NEGATIVE	1E-79	NDDI
	C3	5	0,31	0,01	NEGATIVE	1,43E-78	NDDI

3.3. Overall classification with both biomarkers

Results of both biomarkers were combined to obtain an overall conclusion on the genotoxic potential of the tested chemicals. Chemicals were considered genotoxic if they were classified as inconclusive or positive by GENOMARK and as DDI by TGx-DDI. Chemicals were considered to be non-genotoxic if they were classified as negative or inconclusive by GENOMARK and as NDDI by TGx-DDI. If both biomarkers returned contradictory results (negative by GENOMARK and DDI by TGx-DDI, or positive by GENOMARK and NDDI by TGx-DDI), the chemical was considered equivocal. The classification of chemicals as GTx or NGTx with both biomarkers showed high accuracy, especially with the GTx and NGTxC group of chemicals (Table C.II-A 3).

Table C.II-A 3. Summary of classification results with both applied biomarkers, TGx-DDI and GENOMARK.

Chemicals	GENOMARK	TGx-DDI	Overall conclusion
GTx			
EMS	Positive	DDI	GTx
AFB1	Positive	DDI	GTx
B[a]P	Positive	DDI	GTx
CPA	Positive	DDI	GTx
PA	Positive	DDI	GTx
NGTxs			
CAFF	INCONCLUSIVE	DDI	GTx
AMP	INCONCLUSIVE	NDDI	NGTx
AFB2	Positive	DDI	GTx
2DDG	INCONCLUSIVE	NDDI	NGTx
D-man	Negative	NDDI	NGTx
NGTxCs			
DEHP	INCONCLUSIVE	NDDI	NGTx
TAA	Negative	NDDI	NGTx
DEA	INCONCLUSIVE	NDDI	NGTx
PFOA	Negative	NDDI	NGTx
TPA	Negative	NDDI	NGTx

4. Discussion

Transcriptomic biomarkers are gene set-based NAMs that aim to improve *in vitro* genotoxicity evaluation. TGx-DDI and GENOMARK are among the most widely used transcriptomic biomarkers for genotoxicity. They have been successfully applied, both qualitatively and quantitatively, to various gene expression datasets collected for GTx and NGTx chemicals in 2D HepaRG cells, one of the most widely used cell models in chemical hazard identification (Guo et al., 2024). However, TGx-DDI has been evaluated in only a few physiologically relevant 3D liver models (e.g. PHH), and its broader application in such models remains limited. GENOMARK, in contrast, has been used exclusively in 2D *in vitro* systems, with no reported implementation in 3D liver spheroids model such as the 3D HepaRG. In this context, we conducted a qualitative *in vitro* genotoxicity test using both TGx-DDI and GENOMARK transcriptomic biomarkers in 3D HepaRG spheroids. Gene expression data were obtained using the TempO-Seq^{TM} targeted sequencing approach, for which its applicability with both biomarkers has previously been demonstrated (Thienpont et al., 2024).

All GTx chemicals tested in our study were correctly classified as DDI and GTx with TGx-DDI and GENOMARK, respectively. Although TGx-DDI and GENOMARK share only two genes (Bruin et al., 2003; Carvajal et al., 2012), they can both classify chemicals with known genotoxic effects with high accuracy using gene expression data collected in a 3D HepaRG test system. The TGx-DDI biomarker has been developed using gene expression data from cells exposed to DNA-damaging agents for a short time period (Corton et al., 2018). In contrast, the GENOMARK biomarker is based on data from both compounds that cause direct DNA damage, such as DNA strand breaks, cross-linking DNA adducts, and bulky DNA adducts, and indirect DNA damage (Li et al., 2025). It should be noted that all the GTx chemicals (or their metabolites) tested in our study interact directly with the DNA. For instance, EMS is an alkylating agent that directly damages DNA by transferring ethyl groups to nucleophilic sites, leading to mutations (Pool et al., 1988; Jacociunas et al., 2012). In addition, CPA, AFB1, B[a]P, and PA damage DNA through their metabolites that strongly bind to the DNA and cause DNA and chromosomal damage (Ebmeyer et al. 2019; Chen and Liu 2019). Additional tests with, for example, aneugens are needed to assess how both biomarkers perform for compounds that do not directly interact with the DNA.

Both biomarkers classified B[a]P and EMS only as genotoxic at the highest concentration tested. This indicates that B[a]P at 1.6 µM and 1.8 µM, and EMS at 500 µM, may be too low to induce detectable genotoxic effects, highlighting the importance of testing at sufficiently

high concentrations to initiate their genotoxic potential (Kopp et al., 2020). However, as with other genotoxicity assays in mammalian cells, care should be taken that the tested concentrations do not induce excessive cytotoxicity (i.e. below 50%) as such high toxicity levels may lead to non-specific effects.

For AFB1, an inconclusive result was obtained with the GENOMARK biomarker at 0.05 µM, whereas this concentration was already classified as DDI by the TGx-DDI biomarker. This finding is in line with the study by Thienpont et al., (2024), which reported that due to the underlying classification criteria, TGx-DDI generally classifies genotoxic agents as DDI at lower concentrations than GENOMARK (Thienpont et al., 2024; Li et al., 2025).

In contrast, several inconclusive results were obtained with the GENOMARK biomarker for NGTx chemicals that were classified as non-DDI by TGx-DDI. More specifically, CAFF, AMP and 2DDG were classified as inconclusive with GENOMARK. However, it should be noted that these chemicals were tested at very high concentrations (i.e. CAFF at 2 and 5 mM and AMP and 2DDG both at 10 mM). Importantly, except for the highest concentration of CAFF, the probabilities of the two prediction models were always below 0.5 and the classification as inconclusive was due to a prediction score between 0.55 and 0.45 in one or two of the prediction models. Moreover, all concentrations of AMP and 2DDG were classified as NDDI by TGx-DDI. For this reason, the overall conclusion for AMP and 2DDG was non-genotoxic. In contrast, the highest concentration of CAFF was also classified as DDI by TGx-DDI, indicating that at this concentration, some genes related to genotoxicity may be activated.

Interestingly, AFB2 was overall classified as genotoxic, given that the highest concentration was positive in GENOMARK, and all concentrations were classified as DDI by TGx-DDI. AFB2 was included in the NGTx group based on its negative *in vitro* genotoxicity in the γH2AX In-Cell Western assay in a study conducted by (Theumer et al., 2018). However, some previous studies have shown that AFB2 binds to DNA and histones in rat liver *in vivo*, disturbing normal cellular processes (Groopman et al., 1981). Additionally, liver enzymes can metabolically activate AFB2, producing reactive intermediates, including AFB1. As shown in this study, AFB1 was classified as overall genotoxic and could possibly contribute to the genotoxic effects of AFB2 (Roebuck et al., 1978). Both these mechanisms could explain the DNA-damaging potential of AFB2 observed in this study. The discrepancies between our findings and the previously reported negative results might be due to the cellular test system used. Indeed, for this study, 3D HepaRG spheroids were used, whereas most of the previous *in vitro* studies with AFB2 were performed in 2D cell systems (e.g. HepG2) (Theumer et al., 2018). The 3D HepaRG

spheroid model might more closely reflect the human situation due to their increased complexity. It should also be noted that AFB1 exhibited genotoxicity at much lower concentrations (0.05 µM) compared to AFB2 (7.5 µM). This raises the possibility that the positive results observed with AFB2 may be due to contamination of the AFB2 standard with AFB1. Given that the AFB2 standard has a reported purity of 99%, the remaining 1% impurity could contain a fraction of AFB1, potentially accounting for the positive genotoxicity result. Supplementary analyses, e.g. a differential gene expression analysis, could be applied to the dataset to obtain more insight into the relative expression of biomarker genes in samples leading to unexpected results such as for AFB2. Moreover, pathway analyses, such as Gene Set Enrichment Analysis (Subramanian, 2005; Mootha, 2003), could help to identify pathways leading to secondary genotoxicity.

Clear negative results for genotoxicity were obtained with the NGTxC chemicals with both biomarkers for most test conditions. Inconclusive results were obtained for the highest concentrations of DEA and DHEP. However, for both compounds, all probability scores were below (or equal to 0.55) and the inconclusive result was due to a prediction score between 0.55 and 0.45 in one of the two GENOMARK prediction models. As all concentrations of both compounds were also classified as NDDI by TGx-DDI, they were concluded to be non-genotoxic. This is in agreement with the toxicological profiles of DEHP, TAA, DEA, PFOA, and TPA, as these compounds have been shown to be non-genotoxic, and to induce carcinogenicity through non-genotoxic modes of action. Indeed, they mainly exert their carcinogenic activity through epigenetic effects, oxidative stress, or receptor-mediated pathways, rather than by causing direct DNA damage (Veltman et al., 2023). It should be noted that the highest concentration of TPA tested in our study (20 µM) yielded a positive classification for genotoxicity with both biomarkers. This is probably due to cytotoxicity since considerable toxicity was observed in the ATP assay conducted in parallel with the principal study with 3D HepaRG cells. These findings highlight how GENOMARK and TGx-DDI can accurately identify true DNA damage-inducing agents from chemicals that cause cancer through non-genotoxic pathways (Audebert et al., 2023) in 3D systems.

Previously, 3D HepaRG spheroids were shown to generate promising results when combined with different NAM-based genotoxicity tests (Štampar & Žegura, 2024). For example, HepaRG spheroids have been used in the comet assay, high-throughput CometChip and the micronucleus assay (Mandon et al., 2019; Barranger and Le Hégarat, 2022; Seo et al., 2023). These methods demonstrated significant sensitivity in assessing primary DNA damage and chromosomal

instability in 3D HepaRG models. While TGx-DDI and GENOMARK have never been tested with 3D HepaRG spheroids, they demonstrated high classification accuracy when using 2D HepaRG cells. In a study conducted by Thienpont et al. (2024) the authors showed positive calls for AFB1, and CPA tested with 2D HepaRG cells. Moreover, iRowan-Carroll et al. (2025) reported that TGx-DDI classified AFB1, and CPA as positive genotoxic chemicals in 3D PHH, whereas PFOA was categorized as non-DDI after a short 4-hour treatment (Rowan-Carroll et al., 2025). These results are in line with our findings in a 3D HepaRG model, confirming the capability and applicability of both biomarkers on the gene expression profiles generated from 3D HepaRG spheroids treated with different GTx chemicals and NGTxC such as PFOA.

Despite the promising outcomes of our study, some limitations of this approach must be acknowledged. Although 3D HepaRG spheroids can be combined with high-throughput analysis/scoring technologies, the ULA culture system used in our study to conduct spheroid formation and chemical treatment suffers from low throughput, considering the number of spheroids required per treatment condition. In addition, accurate dilution and exposure of compounds with the spheroid culture systems are challenging, as the spheroids are non-adherent cells, and consequently, the media cannot be completely replaced. Future work should focus on finding solutions to overcome these issues. For example, as the TempO-Seq ^{TM} technology is highly sensitive even when using a small amount of extracted mRNA (minimum 0.5 cell/ml), fewer spheroids could be included per condition to increase the throughput of the ULA system. In addition, other cellular systems could be used to form spheroids, such as Microtissues (mold system), which can form 35 spheroids/well in multi-well plates (Svoronos et al., 2014). In addition, a future step could consist of performing a quantitative analysis of the dose response effect from chemicals to quantify the chemical potency and determine the point of departure (POD) through BMD analysis (Thienpont et al., 2024).

In summary, this study demonstrates the applicability of TGx-DDI and GENOMARK transcriptomic biomarkers for classifying GTx, non-GTx, and NGTxC compounds in 3D HepaRG spheroids using gene expression data generated by the TempO-Seq^{TM} sequencing technology. Both biomarkers displayed a high accuracy in the classification of the tested chemicals in a 3D HepaRG spheroid model, especially when evidence from both biomarkers was combined. In addition, this study highlights the potential of applying more physiologically relevant models, such as 3D HepaRG spheroids, to enhance genotoxicity assessment. Finally, combining the 3D HepaRG model with the transcriptomic biomarkers provides a powerful, multi-evidence approach that enhances the predictivity of *in vitro* genotoxicity testing.

References

Allemang, A., De Abrew, K. N., Shan, Y. K., Krailler, J. M., & Pfuhler, S. (2021). A comparison of classical and 21st century genotoxicity tools: A proof of concept study of 18 chemicals comparing in vitro micronucleus, ToxTracker and genomics-based methods (TGx-DDI, whole genome clustering and connectivity mapping). *Environmental and Molecular Mutagenesis*, *62*(2), 92–107. https://doi.org/10.1002/em.22418

Anderson, D., Bishop, J. B., Garner, R. C., Ostrosky-Wegman, P., & Selby, P. B. (1995). Cyclophosphamide: Review of its mutagenicity for an assessment of potential germ cell risks. *Mutation Research/Reviews in Genetic Toxicology*, *330*(1–2), 115–181. https://doi.org/10.1016/0027-5107(95)00039-L

Anthérieu, S., Chesné, C., Li, R., Guguen-Guillouzo, C., & Guillouzo, A. (2012). Optimization of the HepaRG cell model for drug metabolism and toxicity studies. *Toxicology in Vitro*, *26*(8), 1278–1285. https://doi.org/10.1016/j.tiv.2012.05.008

Ates, G., Doktorova, T. Y., Pauwels, M., & Rogiers, V. (2014). Retrospective analysis of the mutagenicity/genotoxicity data of the cosmetic ingredients present on the annexes of the cosmetic EU legislation (2000-12). *Mutagenesis*, *29*(2), 115–121. https://doi.org/10.1093/mutage/get068

Ates, G., Mertens, B., Heymans, A., Verschaeve, L., Rogiers, V., & Doktorova, T. Y. (2018). A novel genotoxin-specific qPCR array based on the metabolically competent human HepaRG™ cell line as a rapid and reliable tool for improved in vitro hazard assessment. *Archives of Toxicology*, *92*(4), 1593–1608. https://doi.org/10.1007/s00204-018-2172-5

Audebert, M., Assmann, A. S., Azqueta, A., Babica, P., Benfenati, E., Braeuning, A., ... & Žegura, B. (2023). New approach methodologies to facilitate and improve the hazard assessment of non-genotoxic carcinogens—a PARC project. *Frontiers in Toxicology*, *5*, Article 1220998. https://doi.org/10.3389/ftox.2023.1220998

Auletta, A. E., Dearfield, K. L., & Cimino, M. C. (1993). Mutagenicity test schemes and guidelines: U.S. EPA Office of Pollution Prevention and Toxics and Office of Pesticide Programs. *Environmental and Molecular Mutagenesis*, *21*(1), 38–45. https://doi.org/10.1002/em.2850210106

Barranger, A., & Le Hégarat, L. (2022). Towards better prediction of xenobiotic genotoxicity: CometChip technology coupled with a 3D model of HepaRG human liver cells. *Archives of Toxicology*, *96*(7), 2087–2095. https://doi.org/10.1007/s00204-022-03292-4

Barutcu, A. R. (2024). Assessment of TGx-DDI genes for genotoxicity in a comprehensive panel of chemicals. *Toxicology Mechanisms and Methods*, *34*(7), 761–767. https://doi.org/10.1080/15376516.2024.2335966

Bruin, A., Maiti, B., Jakoi, L., Timmers, C., Buerki, R., & Leone, G. (2003). Identification and characterization of E2F7, a novel mammalian E2F family member capable of blocking cellular proliferation. *The Journal of Biological Chemistry*, *278*(43), 42041–42049. https://doi.org/10.1074/jbc.M308105200

Buick, J. K., Rowan-Carroll, A., Gagné, R., Williams, A., Meier, M. J., Kroese, E. D., ... & Yauk, C. L. (2022). Integrated genotoxicity testing of three anti-infective drugs using the TGx-DDI transcriptomic biomarker and high-throughput CometChip® assay in TK6 cells. *Frontiers in Toxicology*, *4*, Article 991590. https://doi.org/10.3389/ftox.2022.991590

Buick, J. K., Williams, A., Gagné, R., Aubrey, S., Brandon, E. F., & Yauk, C. L. (2020). Flow cytometric micronucleus assay and TGx-DDI transcriptomic biomarker analysis of ten genotoxic and non-genotoxic chemicals in human HepaRG™ cells. *Genes and Environment*, *42*(1), Article 1. https://doi.org/10.1186/s41021-019-0139-2

Buick, J. K., Williams, A., Meier, M. J., Swartz, C. D., Recio, L., Li, H. H., ... & Yauk, C. L. (2021). A modern genotoxicity testing paradigm: Integration of the high-throughput CometChip® and the TGx-DDI transcriptomic biomarker in human HepaRG™ cell cultures. *Frontiers in Public Health*, *9*, Article 694834. https://doi.org/10.3389/fpubh.2021.694834

Cartus, A., & Schrenk, D. (2017). Current methods in risk assessment of genotoxic chemicals. *Food and Chemical Toxicology*, *106*(Part B), 574–582. https://doi.org/10.1016/j.fct.2016.09.012

Carvajal, L. A., Hamard, P. J., Tonnessen, C., & Manfredi, J. J. (2012). E2F7, a novel target, is up-regulated by p53 and mediates DNA damage-dependent transcriptional repression. *Genes & Development*, *26*(14), 1533–1545. https://doi.org/10.1101/gad.184911.111

Chen, R., Lin, Y. T., Fornace, A. J., Jr., & Li, H. H. (2022). A high-throughput and highly automated genotoxicity screening assay. *ALTEX - Alternatives to Animal Experimentation*, *39*(1), 77–86. https://doi.org/10.14573/altex.2102121

Guo, K., & van den Beucken, T. (2024). Advances in drug-induced liver injury research: In vitro models, mechanisms, omics and gene modulation techniques. *Cell & Bioscience*, *14*(1), Article 134. https://doi.org/10.1186/s13578-024-01317-2

Luijten, M., Wackers, P. F. K., Rorije, E., Pennings, J. L. A., & Heusinkveld, H. J. (2021). Relevance of in vitro transcriptomics for in vivo mode of action assessment. *Chemical Research in Toxicology*, *34*(2), 452–459. https://doi.org/10.1021/acs.chemrestox.0c00313

Menz, J., Götz, M. E., Gündel, U., Gürtler, R., Herrmann, K., Hessel-Pras, S., Kneuer, C., Kolrep, F., Nitzsche, D., Pabel, U., Sachse, B., Schmeisser, S., Schumacher, D. M.,

Schwerdtle, T., Tralau, T., Zellmer, S., & Schäfer, B. (2023). Genotoxicity assessment: Opportunities, challenges and perspectives for quantitative evaluations of dose-response data. *Archives of Toxicology*, *97*(9), 2303–2328. https://doi.org/10.1007/s00204-023-03553-w

Sachse, B., Schmeisser, S., van Benthem, J., Corvi, R., Dogliotti, E., Ertych, N., Frötschl, R., Gündel, U., Herrmann, K., Johnson, G., Kneuer, C., König, J., Martus, H. J., Pfuhler, S., Smith-Roe, S., Stopper, H., White, P., Schwerdtle, T., ... & Tralau, T. (2026). Quantitative evaluation of genotoxicity data for risk assessment and regulatory decision-making: Time for a paradigm shift. *Environment International*, *208*, Article 110070. https://doi.org/10.1016/j.envint.2026.110070

Chen, T., Dusinska, M., & Elespuru, R. (2022). Thymidine kinase+/- mammalian cell mutagenicity assays for assessment of nanomaterials. *Frontiers in Toxicology*, *4*, Article 864753. https://doi.org/10.3389/ftox.2022.864753

Chen, Y., & Liu, Y. (2019). Non-coplanar and coplanar polychlorinated biphenyls potentiate genotoxicity of aflatoxin B1 in a human hepatocyte line by enhancing CYP1A2 and CYP3A4 expression. *Environmental Pollution*, *246*, 945–954. https://doi.org/10.1016/j.envpol.2018.12.041

Cho, E., Buick, J. K., Williams, A., Chen, R., Li, H. H., Fornace, A. J., Jr., & Yauk, C. L. (2019). Assessment of the performance of the TGx-DDI biomarker to detect DNA damage-inducing agents using quantitative RT-PCR in TK6 cells. *Environmental and Molecular Mutagenesis*, *60*(2), 122–133. https://doi.org/10.1002/em.22257

Corcuera, L. A., Vettorazzi, A., Arbillaga, L., Pérez-Nebra, M. L., de Cerain, A. L., & Cerain, A. L. (2015). Genotoxicity of aflatoxin B1 and ochratoxin A after simultaneous application of the in vivo micronucleus and comet assay. *Food and Chemical Toxicology*, *76*, 116–124. https://doi.org/10.1016/j.fct.2014.12.003

Corton, J. C., Williams, A., & Yauk, C. L. (2018). Using a gene expression biomarker to identify DNA damage-inducing agents in microarray profiles. *Environmental and Molecular Mutagenesis*, *59*(9), 772–784. https://doi.org/10.1002/em.22243

Ebmeyer, J., Braeuning, A., Glatt, H., These, A., Hessel-Pras, S., & Lampen, A. (2019). Human CYP3A4-mediated toxification of the pyrrolizidine alkaloid lasiocarpine. *Food and Chemical Toxicology*, *130*, 79–88. https://doi.org/10.1016/j.fct.2019.05.019

Fortin, A. V., Long, A. S., Williams, A., Kuo, B., Luijten, M., & Yauk, C. L. (2023). Application of a new approach methodology (NAM)-based strategy for genotoxicity assessment of data-poor compounds. *Frontiers in Toxicology*, *5*, Article 1098432. https://doi.org/10.3389/ftox.2023.1098432

Fowler, P., Smith, K., Young, J., Jeffrey, L., Kirkland, D., Pfuhler, S., & Carmichael, P. (2012). Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. I. Choice of cell type. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *742*(1–2), 11–25. https://doi.org/10.1016/j.mrgentox.2011.10.014

Froetschl, R., Corton, J. C., Li, H., Buick, J. K., Yauk, C. L., ... & Dearfield, K. (in press). Consensus findings of an international workshops on genotoxicity testing workshop on using transcriptomic biomarkers to predict genotoxicity. *Environmental and Molecular Mutagenesis*. https://doi.org/10.1002/em.22645

Groopman, J. D., Fowler, K. W., Busby, W. F., & Wogan, G. N. (1981). Interaction of aflatoxin B2 with rat liver DNA and histones in vivo. *Carcinogenesis*, *2*(12), 1371–1373. https://doi.org/10.1093/carcin/2.12.1371

Guo, X., Xu, H., & Seo, J. E. (2024). Application of HepaRG cells for genotoxicity assessment: A review. *Journal of Environmental Science and Health, Part C*, *42*(3), 214–237. https://doi.org/10.1080/26896583.2024.2331956

Hendriks, G., Atallah, M., Morolli, B., Calléja, F., Vrieling, H., & van Steeg, H. (2012). The ToxTracker assay: Novel GFP reporter systems that provide mechanistic insight into the genotoxic properties of chemicals. *Toxicological Sciences*, *125*(1), 285–298. https://doi.org/10.1093/toxsci/kfr281

Jacociunas, L. V., de Andrade, H. H. R., Lehmann, M., & Dihl, R. R. (2012). Artichoke induces genetic toxicity and decreases ethyl methanesulfonate-related DNA damage in Chinese hamster ovary cells. *Journal of Medicinal Food*, *15*(10), 873–878. https://doi.org/10.1089/jmf.2012.0032

Kirkland, D., Aardema, M., Henderson, L., & Müller, L. (2005). Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens: I. Sensitivity, specificity and relative predictivity. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *584*(1–2), 1–256. https://doi.org/10.1016/j.mrgentox.2005.02.004

Kirkland, D., Pfuhler, S., Tweats, D., Czich, A., Lynch, A. M., Müller, L., ... & Speit, G. (2007). How to reduce false positive results when undertaking in vitro genotoxicity testing and thus avoid unnecessary follow-up animal tests: Report of an ECVAM workshop. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *628*(1), 31–55. https://doi.org/10.1016/j.mrgentox.2006.11.008

Kopp, B., Le Hégarat, L., & Audebert, M. (2020). Differential toxic effects of food contaminant mixtures in HepaRG cells after single or repeated treatments. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *850-851*, Article 503161. https://doi.org/10.1016/j.mrgentox.2020.503161

Krishna, G., Petrere, J., Anderson, J., & Theiss, J. (1995). Use of cyclophosphamide as a positive control in dominant lethal and micronucleus assays. *Mutation Research/Environmental Mutagenesis and Related Subjects*, *335*(3), 331–337. https://doi.org/10.1016/0165-1161(95)00038-0

Li, H. H., Aubrecht, J., Doktorova, T. Y., Froetschl, R., Yauk, C. L., ... & Fornace, A. J., Jr. (2025). Review of transcriptomic biomarkers that predict in vitro genotoxicity in human cell lines. *Environmental and Molecular Mutagenesis*. Advance online publication. https://doi.org/10.1002/em.70004

Li, H. H., Yauk, C. L., Chen, R., Hyduke, D. R., Williams, A., Froetschl, R., ... & Fornace, A. J., Jr. (2019). TGx-DDI, a transcriptomic biomarker for genotoxicity hazard assessment of pharmaceuticals and environmental chemicals. *Frontiers in Big Data*, *2*, Article 36. https://doi.org/10.3389/fdata.2019.00036

Li, X., He, X., Chen, S., Guo, X., & Mei, N. (2020). Evaluation of pyrrolizidine alkaloid-induced genotoxicity using metabolically competent TK6 cell lines. *Food and Chemical Toxicology*, *145*, Article 111662. https://doi.org/10.1016/j.fct.2020.111662

Lim, H. K., Hughes, C. O., Landry, T., Tan, C. W. J., Ayehunie, S., & Smith, B. P. C. (2025). Qualification of the human reconstructed intestine micronuclei cytome assay for site-of-contact genotoxic hazard identification. *NAM Journal*, *1*, Article 100015. https://doi.org/10.1016/j.namjnl.2025.100015

Long, A. S., Lemieux, C. L., Arlt, V. M., & White, P. A. (2016). Tissue-specific in vivo genetic toxicity of nine polycyclic aromatic hydrocarbons assessed using the Muta™Mouse transgenic rodent assay. *Toxicology and Applied Pharmacology*, *290*, 31–42. https://doi.org/10.1016/j.taap.2015.11.010

Luijten, M., van Benthem, J., Morita, T., ... & Yauk, C. L. (2024). Evaluation of the standard battery of in vitro genotoxicity tests to predict in vivo genotoxicity through mathematical modeling: A report from the 8th International Workshop on Genotoxicity Testing. *Environmental and Molecular Mutagenesis*. Advance online publication. https://doi.org/10.1002/em.22640

Mandon, M., Huet, S., Dubreil, E., Fessard, V., & Le Hégarat, L. (2019). Three-dimensional HepaRG spheroids as a liver model to study human genotoxicity in vitro with the single cell gel electrophoresis assay. *Scientific Reports*, *9*(1), Article 10548. https://doi.org/10.1038/s41598-019-47114-7

Marchese, S., Polo, A., Ariano, A., Velotto, S., Costantini, S., & Severino, L. (2018). Aflatoxin B1 and M1: Biological properties and their involvement in cancer development. *Toxins*, *10*(6), Article 214. https://doi.org/10.3390/toxins10060214

Nesslany, F. (2017). The current limitations of in vitro genotoxicity testing and their relevance to the in vivo situation. *Food and Chemical Toxicology*, *106*(Part B), 609–615. https://doi.org/10.1016/j.fct.2016.08.035

Phillips, D. H., & Arlt, V. M. (2009). Genotoxicity: Damage to DNA and its consequences. *EXS*, *99*, 87–110. https://doi.org/10.1007/978-3-7643-8336-7_4

Phillipson, C. E., & Ioannides, C. (1989). Metabolic activation of polycyclic aromatic hydrocarbons to mutagens in the Ames test by various animal species including man. *Mutation Research/Genetic Toxicology*, *211*(1), 147–151. https://doi.org/10.1016/0027-5107(89)90115-2

Pool, B. L., Bos, R. P., Niemeyer, U., Theuws, J. L., & Schmähl, D. (1988). In vitro/in vivo effects of mesna on the genotoxicity and toxicity of cyclophosphamide—a study aimed at clarifying the mechanism of mesna's anticarcinogenic activity. *Toxicology Letters*, *41*(1), 49–56. https://doi.org/10.1016/0378-4274(88)90007-0

Pridgeon, C. S., Schlott, C., Wong, M. W., Toyoda, Y., Goldring, C. E., Park, B. K., ... & Sidaway, J. E. (2018). Innovative organotypic in vitro models for safety assessment: Aligning with regulatory requirements and understanding models of the heart, skin, and liver as paradigms. *Archives of Toxicology*, *92*(2), 557–569. https://doi.org/10.1007/s00204-018-2152-9

Quintás, G., Martínez-Sena, T., Conde, I., Ibars, E. P., Kleinjans, J., & Castell, J. V. (2021). Metabolomic analysis to discriminate drug-induced liver injury (DILI) phenotypes. *Archives of Toxicology*, *95*(9), 3049–3062. https://doi.org/10.1007/s00204-021-03114-z

Rabiet, L., Jeger-Madiot, N., García, D. R., ... & Fessard, V. (2024). Improved functionality of hepatic spheroids cultured in acoustic levitation compared to existing 2D and 3D models. *Scientific Reports*, *14*(1), Article 21528. https://doi.org/10.1038/s41598-024-72059-x

Ramaiahgari, S. C., & Ferguson, S. S. (2019). Organotypic 3D HepaRG liver model for assessment of drug-induced cholestasis. *Methods in Molecular Biology*, *1981*, 313–323. https://doi.org/10.1007/978-1-4939-9420-5_20

Ranade, A. R., Wilson, M. S., McClanahan, A. M., & Ball, A. J. (2014). High content imaging and analysis enable quantitative in situ assessment of CYP3A4 using cryopreserved differentiated HepaRG cells. *Journal of Toxicology*, *2014*, Article 291054. https://doi.org/10.1155/2014/291054

Revet, I., Feeney, L., Bruguera, S., ... & Bonner, W. M. (2011). Functional relevance of the histone γH2Ax in the response to DNA damaging agents. *Proceedings of the National Academy of Sciences*, *108*(21), 8663–8667. https://doi.org/10.1073/pnas.1105866108

Roebuck, B. D., Siegel, W. G., & Wogan, G. N. (1978). In vitro metabolism of aflatoxin B2 by animal and human liver. *Cancer Research, 38*(4), 999–1002.

Rowan-Carroll, A., Meier, M. J., Yauk, C. L., ... & Atlas, E. (2025). Deciphering per- and polyfluoroalkyl substances mode of action: Comparative gene expression analysis in human liver spheroids. *Toxicological Sciences, 205*(1), 124–142. https://doi.org/10.1093/toxsci/kfaf023

Saleh, M., Hogeveen, K., Fessard, V., & Le Hégarat, L. (2025). Mechanistic toxicity profiling of chemicals: A comparative study in 2D and 3D HepaRG models using high-content analysis. *Toxicology, 154280*. https://doi.org/10.1016/j.tox.2025.154280

Seo, J. E., Li, X., Le, Y., Mei, N., Zhou, T., & Guo, X. (2023). High-throughput micronucleus assay using three-dimensional HepaRG spheroids for in vitro genotoxicity testing. *Archives of Toxicology, 97*(4), 1163–1175. https://doi.org/10.1007/s00204-023-03461-z

Štampar, M., & Žegura, B. (2024). In vitro hepatic 3D cell models and their application in genetic toxicology: A systematic review. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 900*, Article 503835. https://doi.org/10.1016/j.mrgentox.2024.503835

Svoronos, A. A., Tejavibulya, N., Schell, J. Y., Shenoy, V. B., & Morgan, J. R. (2014). Micro-mold design controls the 3D morphological evolution of self-assembling multicellular microtissues. *Tissue Engineering Part A, 20*(7–8), 1134–1144. https://doi.org/10.1089/ten.tea.2013.0297

Theumer, M. G., Henneb, Y., Khoury, L., Itria, S. D., Pfohl-Leszkowicz, A., & Cavret, S. (2018). Genotoxicity of aflatoxins and their precursors in human cells. *Toxicology Letters, 287*, 100–107. https://doi.org/10.1016/j.toxlet.2018.02.007

Thienpont, A., Cho, E., Williams, A., Yauk, C. L., ... & Mertens, B. (2024). Unlocking the power of transcriptomic biomarkers in qualitative and quantitative genotoxicity assessment of chemicals. *Chemical Research in Toxicology, 37*(3), 465–475. https://doi.org/10.1021/acs.chemrestox.3c00318

Thienpont, A., Verhulst, S., Van Grunsven, L. A., Rogiers, V., Vanhaecke, T., & Mertens, B. (2023). Novel prediction models for genotoxicity based on biomarker genes in human HepaRG™ cells. *ALTEX - Alternatives to Animal Experimentation, 40*(2), 271–286. https://doi.org/10.14573/altex.2206201

Turkez, H., Arslan, M. E., & Ozdemir, O. (2017). Genotoxicity testing: Progress and prospects for the next decade. *Expert Opinion on Drug Metabolism & Toxicology, 13*(10), 1089–1098. https://doi.org/10.1080/17425255.2017.1375097

Veltman, C. H. J., Pennings, J. L. A., van de Water, B., & Luijten, M. (2023). An adverse outcome pathway network for chemically induced oxidative stress leading to (non)genotoxic carcinogenesis. *Chemical Research in Toxicology*, *36*(6), 805–817. https://doi.org/10.1021/acs.chemrestox.2c00396

Wong, J. J., & Hsieh, D. P. (1976). Mutagenicity of aflatoxins related to their metabolism and carcinogenic potential. *Proceedings of the National Academy of Sciences*, *73*(7), 2241–2244. https://doi.org/10.1073/pnas.73.7.2241

Xia, Q., Chou, M. W., Edgar, J. A., Doerge, D. R., & Fu, P. P. (2006). Formation of DHP-derived DNA adducts from metabolic activation of the prototype heliotridine-type pyrrolizidine alkaloid, lasiocarpine. *Cancer Letters*, *231*(1), 138–145. https://doi.org/10.1016/j.canlet.2005.01.023

Yadav, V., Krishnan, A., Zahiruddin, S., Ahmad, S., & Vohora, D. (2023). Amelioration of cyclophosphamide-induced DNA damage, oxidative stress, and hepato- and neurotoxicity by Piper longum extract in rats: The role of γH2AX and 8-OHdG. *Frontiers in Pharmacology*, *14*, Article 1147823. https://doi.org/10.3389/fphar.2023.1147823

Yamanaka, H., Nagao, M., Sugimura, T., Furuya, T., Shirai, A., & Matsushima, T. (1979). Mutagenicity of pyrrolizidine alkaloids in the Salmonella/mammalian-microsome test. *Mutation Research/Genetic Toxicology*, *68*(3), 211–216. https://doi.org/10.1016/0165-1218(79)90152-6

Yeakley, J. M., Shepard, P. J., Goyena, D. E., VanSteenhouse, H. C., McComb, J. D., & Seligmann, B. E. (2017). A Trichostatin A expression signature identified by TempO-Seq targeted whole transcriptome profiling. *PLoS One*, *12*(5), Article e0178302. https://doi.org/10.1371/journal.pone.0178302

Zeller, A., Pfuhler, S., Albertini, S., ... & Fautz, R. (2018). A critical appraisal of the sensitivity of in vivo genotoxicity assays in detecting human carcinogens. *Mutagenesis*, *33*(2), 179–193. https://doi.org/10.1093/mutage/gey005

Zhu, X., Huo, J., Zeng, Z., ... & Tan, X. (2022). Determination of potential thresholds for N-ethyl-N-nitrosourea and ethyl methanesulfonate based on a multi-endpoint genotoxicity assessment platform in rats. *Environmental Science and Pollution Research*, *29*(56), 85128–85142. https://doi.org/10.1007/s11356-022-21605-z

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Transcriptomic–based analysis to discriminate Non-Genotoxic Carcinogens from genotoxic carcinogens and non-carcinogens in a human HepaRG spheroid model: Mechanistic insights and biomarker identification

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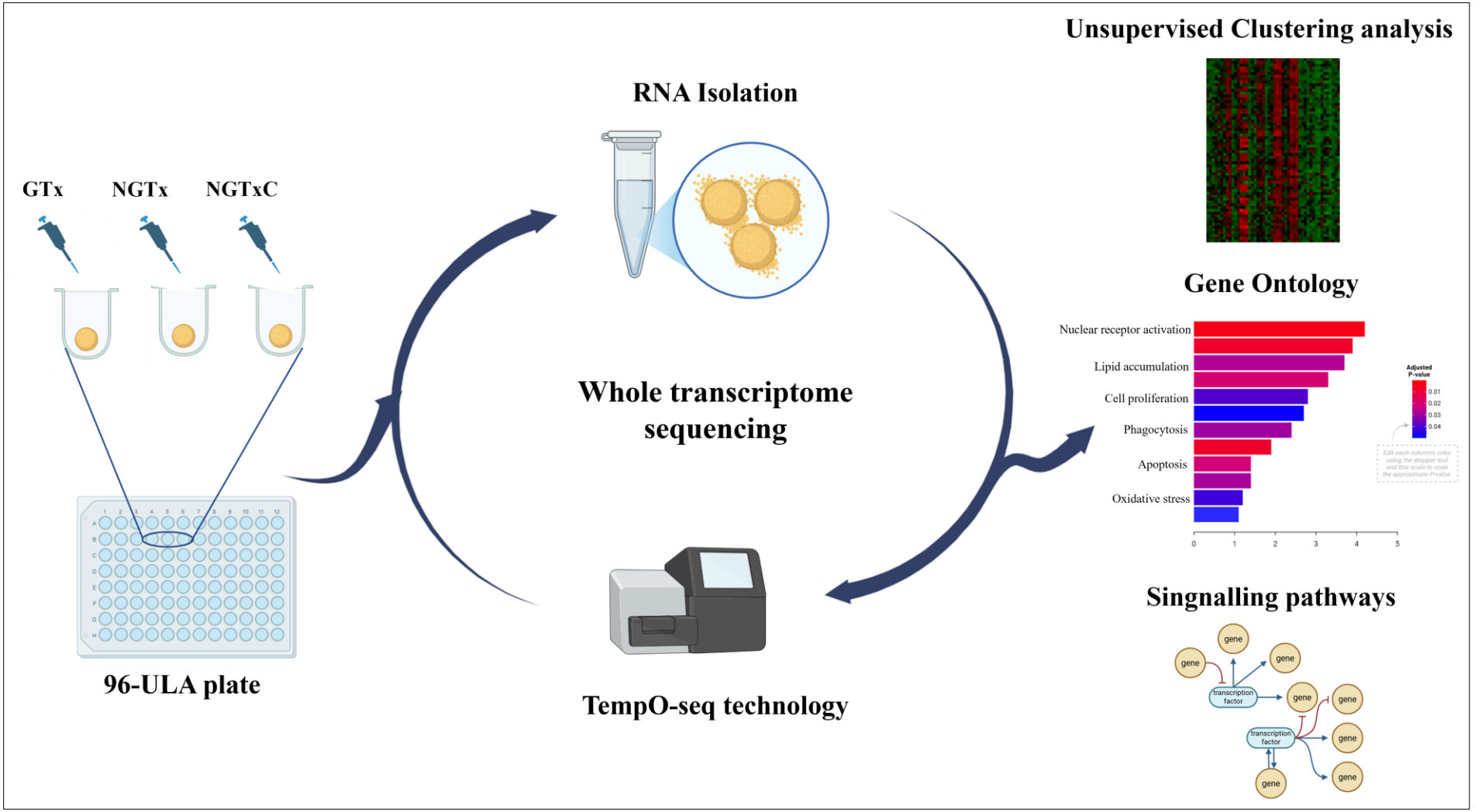


Abstract

Carcinogenic compounds are generally categorized into two classes, genotoxic carcinogens (GTxC) and non-genotoxic carcinogens (NGTxC). While GTxCs can be detected based on a battery of *in vitro* and *in vivo* genotoxicity assays, detection of NGTxCs remains based on 2-year *in vivo* rodent studies that are time-consuming, expensive, pose ethical concerns, and may not be directly extrapolated to humans due to species-specific responses. Thus, it is urgent to develop *in vitro* New Approach Methodologies (NAMs) to detect NGTxCs. In this context, and as part of the PARC project, we applied a NAMs-based *in vitro* transcriptomic approach using 3D human hepatic HepaRG spheroids to identify changes at the molecular level in relation to different NGTxC Modes of Action (MoA). 3D HepaRG spheroids were exposed for 72 h repetitive treatment with three different groups of chemicals: (A) five GTxCs, (B) five NGTxCs, and (C) five non-genotoxic non-carcinogens, at concentrations corresponding to the IC_{10} and $IC_{10/2}$. Clustering analysis and Gene Ontology (GO) enrichment analysis were applied to classify the different groups of chemicals based on their gene expression profiles and to identify the key molecular events associated with the NGTxCs. So far, the analysis of the results obtained shows clusters where some of the tested NGTxC chemicals clearly display similar gene profiles, which are enriched with overexpression of nuclear receptor genes and genes associated with lipid metabolism. In addition, down-expression of genes related to caspase activity was enriched within the gene expression profiles of the tested NGTxCs. However, some NGTxCs did not show these changes. Consequently, further analyses of this preliminary data are needed to more comprehensively investigate the clustering results in greater depth to characterize chemical-specific transcriptomic responses. Such analyses will help identify integrated and enriched response pathways associated with individual NGTxCs and improve our understanding of their potential MoAs. Moreover, additional investigation will determine whether this approach could support the classification of non-genotoxic carcinogens based on their transcriptomic profiles.

Highlights

- NGTxC alters lipid metabolism in a 3D HepaRG spheroid model.
- PPAR γ nuclear receptor shows a role in non-genotoxic MoA in 3D HepaRG.
- Apoptotic genes implicated in caspase activity are down regulated in 3D HepaRG spheroids treated with NGTxC.
- Unsupervised clustering and gene ontology offer useful insights for NGTxC profiling, but they remain insufficient for reliably classifying non-genotoxic carcinogens.

Key words

New Approach Methodologies (NAMs), High-throughput sequencing, TempO-Seq™ technology, unsupervised clustering, Gene Ontology (GO), Human-hepatic 3D cellular model, Non-Genotoxic Carcinogens (NGTxC).

1. Introduction

Cancer is a complex, heterogeneous disease characterized by uncontrolled cell proliferation, and the ability to invade surrounding tissues and metastasize to distant organs (Liu et al., 2021). The complexity of this disease is reflected in its multifaceted nature, defined by various functional capabilities acquired by cancer cells during malignant transformation. These functional capabilities, referred to as the Hallmarks of Cancer, include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan, 2022).

Carcinogens can induce cancer through a complex multistep process known as carcinogenesis (Stewart, 2019). Based on their mode of carcinogenicity, carcinogens are generally divided into genotoxic carcinogens (GTxC) and non-genotoxic carcinogens (NGTxC). GTxCs exert their effects through direct interaction with DNA, resulting in mutations, DNA adducts, and chromosomal aberrations that can initiate malignant transformation (Hoenerhoff et al., 2022). In contrast, NGTxCs do not directly damage DNA; instead, these compounds promote tumour development through diverse mechanisms such as sustained proliferation, nuclear receptor activation, epigenetic alterations, oxidative stress, and metabolic reprogramming (Delft et al., 2004; Louekari and Jacobs, 2024). These various mechanistic changes, or Modes of Action (MoA), that may be triggered following exposure to NGTxCs, present a challenge for their hazard identification and assessment, as a single NGTxC may act through multiple MoAs. By perturbing several MoAs simultaneously, NGTxCs can accelerate the transition from pre-neoplastic states to malignant tumours without initiating genetic mutations (Hernández et al., 2009).

The International Agency for Research on Cancer (IARC) estimated that 10%–20% of human carcinogens classified as Group 1 may act as NGTxCs (Audebert et al., 2023). In addition, long-term (2-year) rodent bioassays remain the gold standard for their identification (Heusinkveld et al., 2020). This reliance on time-consuming, costly, and ethically challenging animal-based studies, combined with the complex MoAs of NGTxCs, underscores the urgent need for an *in vitro*, mechanistic-based New Approach Methodologies (NAMs) strategy for NGTxC identification (Audebert et al., 2023).

The identification of NGTxCs is still considered a regulatory challenge; however, the Organization for Economic Co-operation and Development (OECD) has recognized the value of mechanistically based approaches and developed Integrated Approaches to Testing and

Assessment (IATA). IATA provides a structured framework for integrating diverse evidence streams, including human-relevant *in vitro* assays, *in silico* modeling, and high-throughput omics, for the assessment of NGTxCs (Jacobs et al., 2016). Within this framework, transcriptomic approaches play a key role by identifying gene expression changes linked to specific NGTxC MoAs (Oku et al., 2022). Pathway-level information can support the mapping of these changes onto Adverse Outcome Pathways (AOPs), which describe the progression from the molecular initiating event (MIE) through key events to the resulting adverse outcome, thereby enabling early mechanistic insight into NGTxC activity (Leist et al., 2017; Louekari and Jacobs, 2024). Thus, transcriptomic data can provide mechanistic anchors that link chemical exposures to adverse outcomes, enabling more predictive assessments (Smith, 2025).

The liver has been identified as a primary organ affected by many carcinogens. Heusinkveld et al. (2020) highlighted the liver as the main target organ for various NGTxCs with diverse MoAs (Heusinkveld et al., 2020). In recent years, the development of advanced *in vitro* hepatic models has significantly progressed (Kaur et al., 2022). Traditional two-dimensional (2D) cell cultures lack the complex architecture and cellular interactions of the liver, resulting in limited predictive power (Saleh et al., 2025). In contrast, three-dimensional (3D) liver models, such as HepaRG spheroids, provide a more physiologically relevant environment by maintaining cellular polarity, intercellular junctions, and liver-specific functional activities (Saleh et al., 2025).

As part of the European Partnership for the Assessment of Risk from Chemicals (PARC) project, which aims to develop *in vitro* strategies to identify conserved molecular signatures shared among NGTxCs (Audebert et al., 2023), we applied a high-throughput NAMs-based *in vitro* transcriptomic approach using human hepatic HepaRG 3D spheroids to identify molecular changes related to various NGTxC MoAs. 3D HepaRG spheroids were exposed for 72 hours to repetitive treatments with three different groups of chemicals: (A) five genotoxic chemicals; (B) five non-genotoxic, non-carcinogenic chemicals; and (C) five non-genotoxic carcinogens, at concentrations corresponding to the IC_{10} or $IC_{10/2}$. Unsupervised clustering and GO enrichment analysis were applied to classify the different groups of chemicals based on their gene expression profiles and to identify the molecular events associated with NGTxCs.

2. Materials and Methods

2.1. HepaRG cell culture and 3D spheroid formation

Human hepatic HepaRG cells were purchased from Biopredic International (Rennes, France) and cultured according to the methods described by Saleh et al., (2025). Briefly, cells were maintained in Williams E medium 1X + GlutamaxTM-I (Gibco, Noisy le Grand, France), 10% FBS FetalClone II (Cytiva, Saint Germain en Laye, France), Penicillin 5000 units/mL / Streptomycin 5000 µg/mL (Gibco, Noisy le Grand, France), 5 µg/mL insulin (Sigma, Saint Quentin Fallavier, France), and 18 µg/mL Hydrocortisone hemisuccinate (Upjohn, Paris, France). The cells were expanded and differentiated in DMSO (Sigma, Saint Quentin Fallavier, France) over a one-month period, as previously detailed (Saleh et al., 2025). For spheroid formation, differentiated cells were seeded without DMSO at a density of 2,000 cells/well into 96-well ultra-low attachment (ULA) plates (Sigma, Saint Quentin Fallavier, France). These spheroids were ready for chemical exposure one week after seeding.

2.2. Cell Viability assay

A pre-cytotoxicity assay was performed for all the selected chemicals to determine the concentrations for the transcriptomic experiments. For the GTx and NGTxC chemicals, six concentrations were selected for the pre-cytotoxicity assay based on a previously published toxicity range in 3D HepaRG spheroids with the same assay in literatures. For the NGTx chemicals, the six different concentrations were selected starting from the highest soluble concentrations (not more than 10mM) were selected (Figure APP. II 1) (Figure APP. II 2) (Figure APP. II 3). After 72h repeated dose treatment, cytotoxicity was assessed using the CellTiter-Glo 3D Cell Viability kit (Promega G9682), according to the manufacturer's instructions. One spheroid per condition was used for the cytotoxicity measurements. ATP luminescence was measured using a FLUOstar Optima microplate reader (BMG Labtech, Champigny-sur-Marne, France). Blank-corrected raw data were used to calculate the relative cell viability using the following formula:

Relative ATP level (%) = [Blank corrected raw data (sample) / Blank corrected raw data (control)] x 100

Next, IC_{10}, IC_{50}, and IC_{90} values of each chemical were calculated based on the relative ATP values using GraphPad prism (version 9.5.1). In addition, concurrent cytotoxicity assays were

conducted with the selected IC_{10} and $IC_{10/2}$, as described above, to assess cytotoxicity across all conditions tested in the transcriptomics study.

2.3. Chemical exposure

Fifteen different chemicals were selected in this study based on their known MoAs (Table C.II-B 1). With the exception of PA, which was obtained from Phytoplant Research S.L. (Córdoba, Spain), all chemicals were supplied from Sigma (Saint-Quentin-Fallavier, France). Spheroids were exposed to a concentrations equivalent to IC_{10} or $IC_{10/2}$ repetitively for 72h, with treatment media replaced every 24h. All chemicals were prepared in 1% DMSO, and FBS free media were applied throughout the exposure. For each condition, 35 spheroids were collected, centrifuged at 500g for 3 min, washed with PBS, then stored at dry pellets at -80 °C for no longer than one week prior to RNA lysate preparation. This experimental design was repeated independently 3 times using different HepaRG cell batches.

2.4. mRNA Lysate preparation and TempO-Seq™ assay

RNA lysate preparation and sequencing analysis with TempO-Seq™ were performed as described previously with Saleh, et al. (2025a). In brief, 35 spheroids were lysed using BioSpyder Enhanced 2X Lysis Buffer (P/N 201247), diluted to 1X with PBS, following the TempO-Seq™ Assay user guide. Spheroids were incubated with lysis buffer at 37 °C for 10 minutes, and the resulting lysate were shipped on dry ice to Bioclavis (Glasgow, UK), a spinout company of BioSpyder technologies. The whole-genome expression profiling was performed using TempO-Seq™ technology with the Human Whole Transcriptome (v2.1) probe set and reagent kit in a 96-well format. Negative (1X lysis buffer) and positive controls (Human Reference Total RNA, Brain Total RNA) were included in quadruplicate. RNA-lysates were hybridized to transcriptome probes, followed by nuclease digestion of unbound probes. Bound probes were amplified in single-plex PCR reactions to add sample-specific tags and sequencing adapters. Libraries were sequenced on an Illumina NextSeq 2000, with a median depth of six million reads per sample. Yeakley et al., (2017) have previously described the TempO-Seq™ protocol in detail.

Table C.II-B 1. Chemicals tested with 3D HepaRG spheroids in this study. The table presents the full name of the chemicals, the chemicals abbreviation, its CAS number, the chemical MoA, the concentrations corresponding to IC_{10} or $IC_{10/2}$ tested in this study, and previously published information concerning their hepatoxicity.

Chemicals	CAS	MoA	Concentrations (µM)	Reference
GTx				
Aflatoxin B1 (AFB1)	1162-65-8	Hepatotoxic DNA Adducts	0.1	(Moloi et al., 2024)
Benzo[a]pyrene (BaP)	50-32-8	Hepatotoxic DNA adducts	3.6	(Kim et al., 2024)
Cyclophosphamide (CPA)	6055-19-2	Hepatotoxic Alkylation	2000	(DeChiara et al., 2023)
Ethyl methanesulfonate (EMS)	62-50-0	Hepatotoxic Alkylation	100	(Siddique et al., 2024)
Lasiocarpine (PA)	303-34-4	Hepatotoxic Alkylation	0.5	(Dubreil et al., 2024)
NGTx				
2-Deoxy-D-glucose (2DDG)	154-17-6	Not hepatotoxic	10000	(Bischof et al., 2025)
Aflatoxin B2 (AFB2)	7220-81-7	Unknown Hepatotoxicity	15	(Gemede, 2025)
Ampicillin trihydrate (AMP)	7177-48-2	Not hepatotoxic	10000	(Ooi et al., 2023)
Caffeine (CAFF)	58-08-2	Not hepatotoxic	5000	(Abbas-Hashemi et al., 2024)
D-mannitol (D-man)	69-65-8	Not hepatotoxic	10000	(DeMaria et al., 2025)

NGTxC				
Perfluorooctanoic acid (PFOA)	3825-26-1	Hepatotoxic Oxidative stress Mitochondrial activity	40	(Feng et al., 2025)
Thioacetamide (TAA)	62-55-5	Hepatotoxic Liver fibrosis	7000	(Ezhilarasan; 2023)
12-O-Tetradecanoylphorbol-13-acetate (TPA)	16561-29-8	Hepatotoxic Promoting cell proliferation	10	(Aniqa et al. 2022)
Diethanolamine (DEA)	111-42-2	Hepatotoxic Phospholipid metabolism	100	(Lehman-McKeeman et al., 2002)
Di(2-ethylhexyl) phthalate (DEHP)	117-81-7	Hepatotoxic PPARα nuclear receptor activation	100	(Yasuda et al., 2024)

2.5. Clustering and gene ontology analysis

Gene expression profiles of 3D HepaRG cells treated with the selected chemicals were generated using TempO-Seq™ technology, which provides raw gene-level count data for each condition. For genes associated with multiple probe IDs, counts were summed prior to normalization using R (version 4.0.4). Raw counts were then normalized with the DESeq2 median-of-ratios method (R version 4.0.4), which corrects for library size and compositional biases. Subsequently, the likelihood ratio test (LRT) implemented in DESeq2 was applied to compute global p-values across all conditions, enabling the identification of genes exhibiting significant expression changes between groups. These normalized expression profiles and associated p-values were used as input for downstream unsupervised clustering analyses to explore patterns of gene expression across samples.

Unsupervised Bayesian clustering was performed using the AutoClass Web algorithm (version 2.2.1), grouping conditions based on the expression profiles of differentially expressed genes, focusing on genes with significant abundance changes (adjusted p-value < 0.05) obtained from DESeq2. Within each cluster, the sets of differentially expressed genes are visualized by colour intensity for each treatment condition: red indicates upregulated genes, green indicates downregulated genes, and black represents no significant change relative to the negative control (1% DMSO). To further characterize the biological relevance of each cluster, Gene Ontology (GO) enrichment analysis was performed on the gene sets defining each cluster. GO analysis was conducted using ClueGO, a Cytoscape plug-in (version 3.8.2). Significant GO terms were identified using an adjusted p-value threshold of 0.05 (Benjamini–Hochberg correction), with all quantified genes used as the reference set.

Differentially expressed genes (DEGs) represented with the MA plots (application of Bland-Altman plot), were created using the [TempO-SeqR] web-based platform by BioSpyder. Box plots of the differentially expressed genes observed within the clusters were done on MetaboAnalyst 6.0 web-based platform [MetaboAnalyst]. The normalized data with DESeq2 were used in this analysis.

3. Results and discussion

3.1. Cytotoxicity evaluation

Pre-cytotoxicity evaluation was performed for all selected chemicals and negative control (1% DMSO) to determine non-toxic concentrations corresponding to the IC_{10} and/or $IC_{10/2}$ (Figure APP. II 1) (Figure APP. II 2) (Figure APP. II 3). In addition, concurrent cytotoxicity assessments were conducted to ensure that no cytotoxicity-related effects were present that could potentially lead to misleading results during the analysis. The results of the cytotoxicity tests showed that all the selected treatment conditions and negative control tested with 3D HepaRG spheroids for a 72h repetitive treatment exhibited no more than 30% associated cytotoxicity (Figure C.II-B 1). This absence of cytotoxicity provides sufficient evidence that the observed changes in gene expression were attributable to chemical exposure rather than cytotoxic effects. The PFOA concentration selected for this study was based on the IC_{10} value established in preliminary cytotoxicity assays; however, the cytotoxicity observed in the concurrent assessment was marginally lower than this target threshold. This slight deviation likely reflects the inherent biological variability associated with low-toxic concentrations, which are frequently subject to minor fluctuations between independent experimental runs. Given the minimal inhibitory nature of an IC_{10} concentration, these small variations remain consistent with the preliminary findings and represent the typical response range for PFOA at this level (Figure C.II-B 1).

3.2. Differentially expressed genes

Qualitative evaluation of the gene expression profiles of the treated 3D HepaRG spheroids was done through the analysis of the significant DEGs based on the adjusted p-value (p-value-adj < 0.05) and presented with MA plots (Figure C.II-B 2) (Figure C.II-B 3) (Figure C.II-B 4). BaseMean represents the mean of normalized counts across all samples for each gene. Log2-transformed ratios represents expression levels between each condition vs. negative control (DMSO solvent control).

Within the GTx group of chemicals (AFB1, BaP, CPA, EMS, and PA), all the tested conditions within this group induced a significantly high number of over- or down-regulated genes in the gene profiles of the treated 3D HepaRG spheroids. However, the BaP group was associated with only one significant differentially expressed gene. This might be due to the low concentration used in this study, which might not have been sufficient to induce changes at the

gene expression level. These results could also explain the previous findings reported earlier in the previous publication of this chapter obtained with the application of the genotoxic transcriptomic biomarkers TGxDDI and GENOMARK, where B[a]P was negative at 3.6 µM (Figure C.II-B 2).

Within the NGTx group of chemicals (2DDG, AFB2, AMP, D-man), as expected, all the treatment conditions resulted in only a few changes at the transcriptomic level in the treated HepaRG spheroids. As discussed in the previous chapter, CAFF was an exception, and treatment of HepaRG spheroids resulted in a high number of genes with significantly modified expression levels (Saleh et al., 2025a) due to the very high concentration that was used (Figure C.II-B 3). In addition, this chemical at a concentration of 5000 µM was classified as a positive inducer of DNA damage with TGxDDI and GENOMARK applied in the previous study (Saleh et al., 2025a). With Figure CII-B 4, the NGTxC group of chemicals (PFOA, TAA, TPA, DEA, and DEHP) showed a significant change in the gene expression profiles of the treated HepaRG spheroids.

The qualitative analysis conducted after the cytotoxicity check was primarily to investigate the effect level of each treatment on the gene expression profiles of the 3D HepaRG spheroids. For instance, the NGTx (non-carcinogens) group of chemicals, with all the tested conditions except CAFF, showed a very low effect on the gene expression profiles. Thus, compared to the GTx and NGTxC groups, this group of chemicals could be used as an important negative control in our study. In addition, as these figures represent the BaseMean of each gene, the evaluation of the number of important genes with high mean values associated with each treatment could be visually analyzed. This would help in the qualitative analysis of the more effective treatments on the gene expression profiles of the 3D HepaRG spheroids. In addition, the preliminary qualitative analysis confirmed that the selected IC_{10} concentrations represent biologically active exposure levels capable of inducing molecular alterations. Using concentrations associated with comparable cytotoxicity ensured a balanced experimental design, allowing meaningful comparison between treatments while avoiding excessive cytotoxicity that could confound transcriptomic responses. This analysis therefore supports the interpretation of observed molecular changes as treatment-related rather than secondary to overt cytotoxic effects.

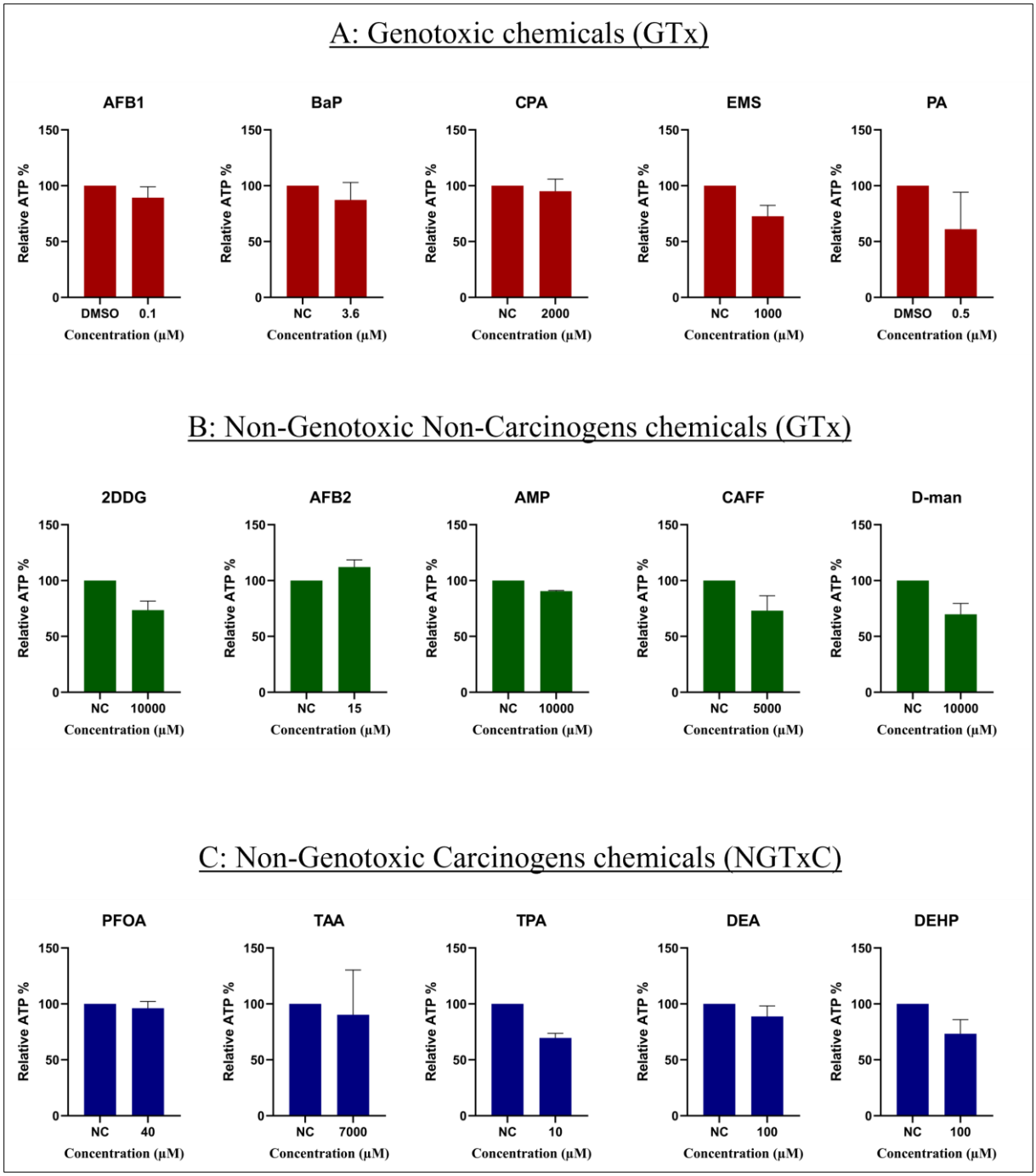


Figure C.II-B 1. Relative ATP levels following 72 h repetitive treatments with the selected chemicals in 3D HepaRG spheroids. (A): Genotoxic chemicals (GTx) selected AFB1, BaP, CPA, EMS, and PA. (B): Non-Genotoxic chemicals (NGTx) selected 2DDG, AFB2, AMP, CAFF, and D-man. (C): Non-Genotoxic Carcinogens (NGTxC) chemicals selected PFOA, TAA, TPA, DEA, and DEHP. Data are presented as means (3 biological replicates) ± SEM.

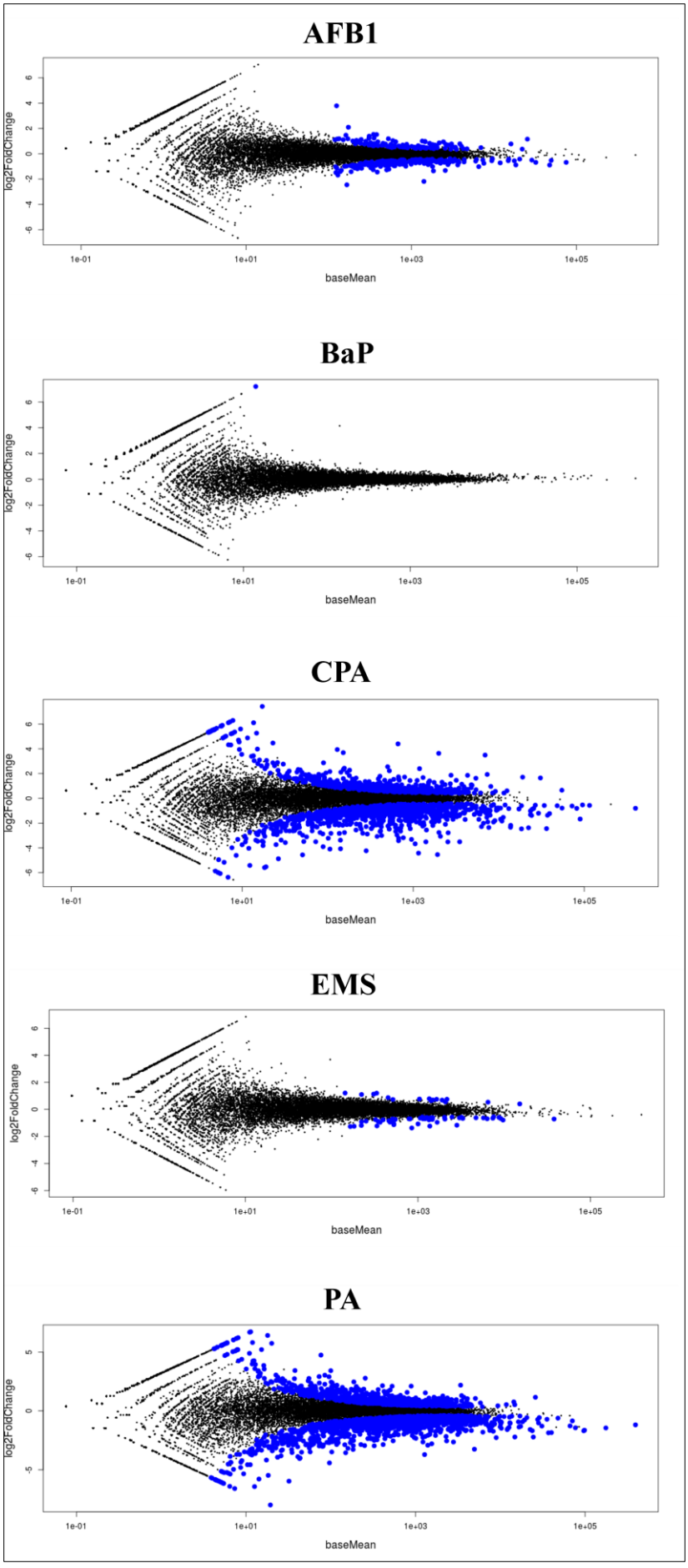


Figure C.II-B 2. MA plots of the differentially expressed genes (DEGs) with GTx group of chemicals (AFB1, BaP, CPA, EMS, PA). baseMean represents the mean of normalized counts across all samples for each gene (log scale). Log_2-transfromed ratio of expression levels between each condition vs. negative control (DMSO solvent control). Blue points highlight the genes of the significantly overexpressed genes represent the genes with Log2Foldchange > 0 (up), and the significantly down expressed genes represents the genes with Log2Foldchange < 0 for (down). Black points represent all other non-significant genes analysed.

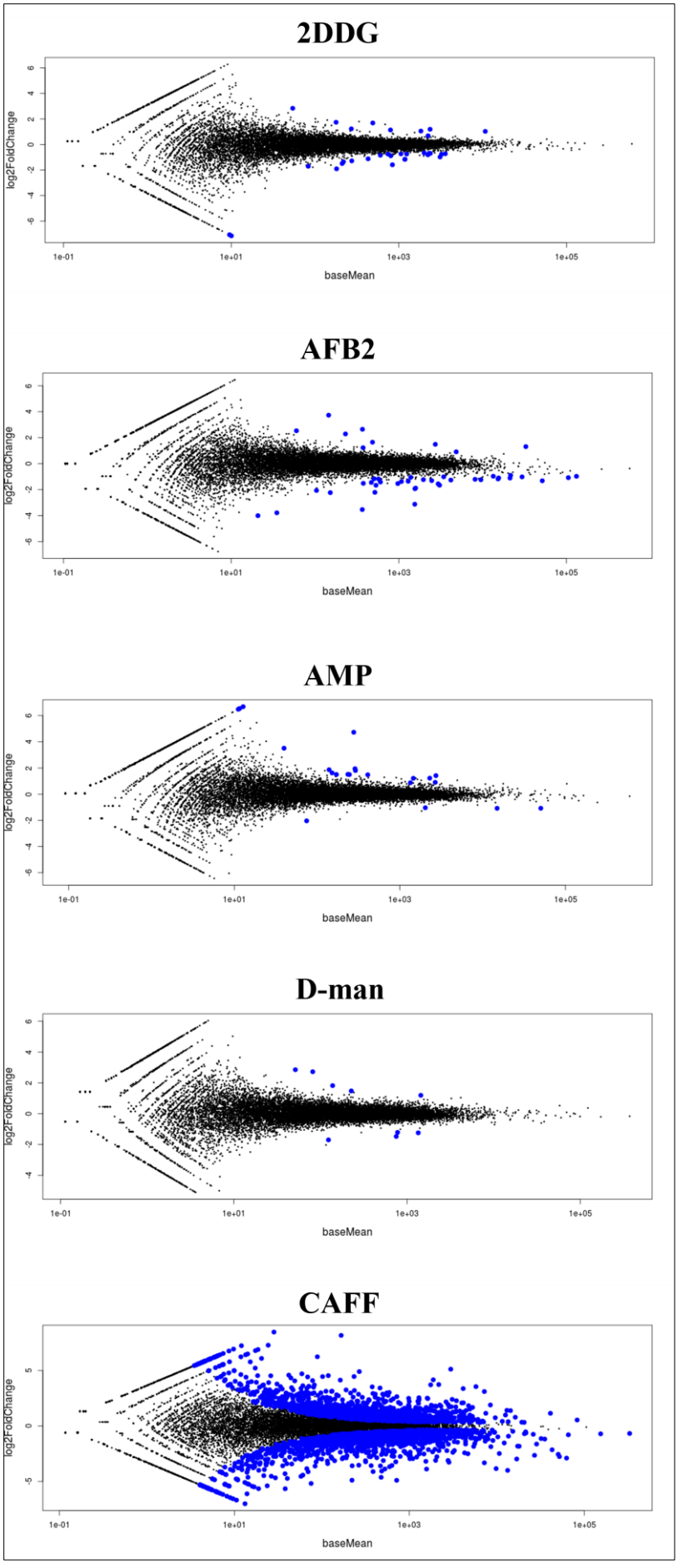


Figure C.II-B 3. MA plots of the differentially expressed genes (DEGs) with NGTx group of chemicals (2DDG, AFB2, AMP, D-man, CAFF). baseMean represents the mean of normalized counts across all samples for each gene (log scale). Log_2-transfromed ratio of expression levels between each condition vs. negative control (DMSO solvent control). Blue points highlight the genes of the significantly overexpressed genes represent the genes with Log2Foldchange > 0 (up), and the significantly down expressed genes represents the genes with Log2Foldchange < 0 for (down). Black points represent all other non-significant genes analysed.

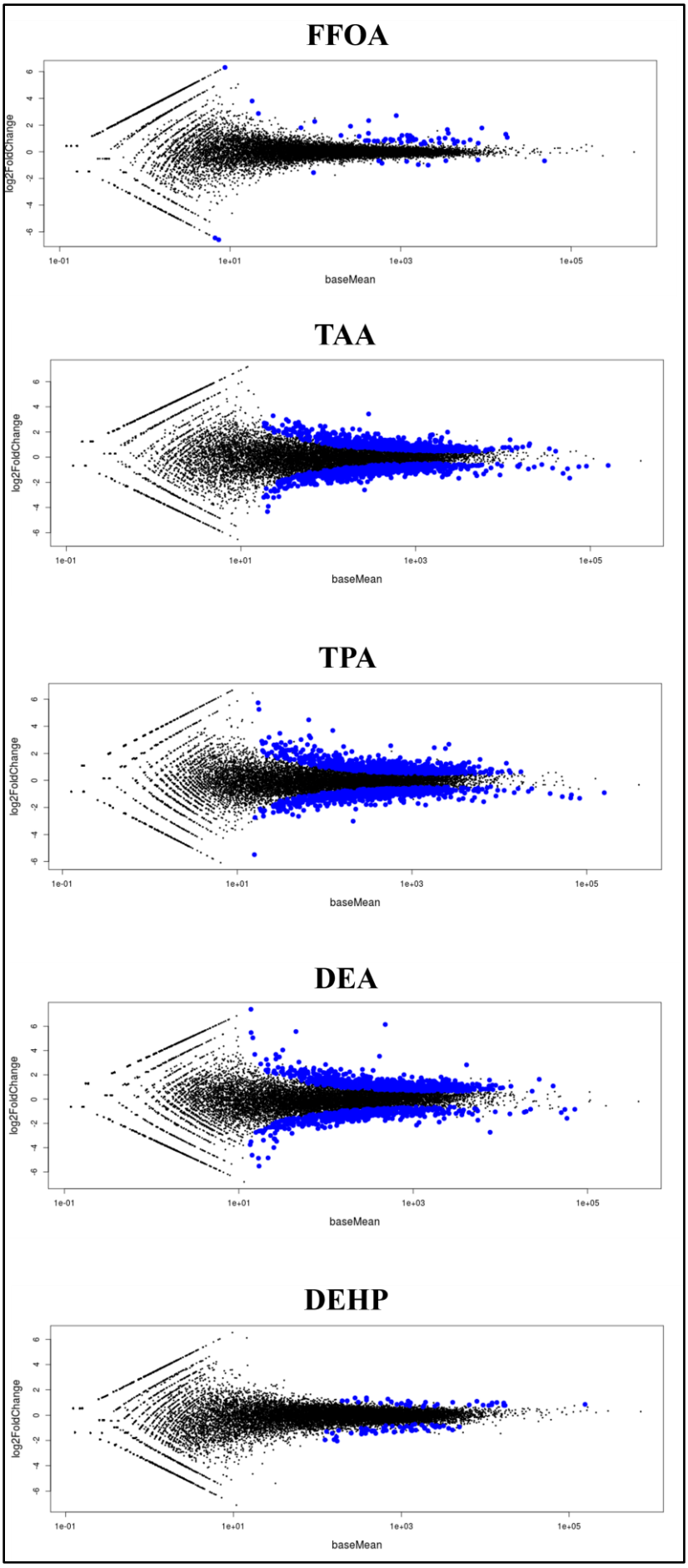


Figure C.II-B 4. MA plots of the differentially expressed genes (DEGs) with NGTxC group of chemicals (PFOA, TAA, TPA, DA, DEHP). baseMean represents the mean of normalized counts across all samples for each gene (log scale). Log_2-transfromed ratio of expression levels between each condition vs. negative control (DMSO solvent control). Blue points highlight the genes of the significantly overexpressed genes represent the genes with Log2Foldchange > 0 (up), and the significantly down expressed genes represents the genes with Log2Foldchange < 0 for (down). Black points represent all other non-significant genes analysed.

3.3. Clustering analysis and gene ontology

3.3.1. Cross-talk between nuclear receptor activators and lipid metabolism

The unsupervised clustering analysis resulted in distinct clusters. The first two clusters and their GO enrichment analysis with the term ‘p-Value corrected with Benjamini-Hochberg' along with the associated genes in each term represented in (Figure C.II-B 5 A-B). Cluster one (C1) (Figure C.II-B 5 A), contains a subset of highly upregulated genes (red) with the NGTxC group of chemicals, especially with the TAA, TPA, and DEA treatment conditions. This cluster was significantly enriched in the GO terms “*nuclear receptor activity (GO:0004879)”* and *“positive regulation of phagocytosis, engulfment (GO:0060100)”*. The GO term “*nuclear receptor activity” was* represented by the genes NR5A2, NR6A1, PPARG, and RORA. In addition, the set of genes that represented the GO term *“positive regulation of phagocytosis, engulfment”* were ABCA7, APPL2, and PPARγ. Similarly, in Cluster 2 (C2) (Figure C.II-B 5 B), the NGTxC group of chemicals including PFOA, TAA, TPA, DEA, and DEHP were associated with an upregulation of a set of genes associated with different GO terms related to lipid metabolism. This cluster was significantly enriched in “*fatty-acyl-CoA biosynthetic process (GO:0046949)”*, “*carboxylic ester hydrolase activity (GO:0052689)”*, and “*triglyceride lipase activity (GO:0004806)”* terms with their associated genes presented in (Figure C.II-B 5 B).

In the first cluster where TAA, TPA, and DEA are significantly enriched in GO terms related to nuclear receptor activation and positive regulation of phagocytosis, one of the differentially expressed genes associated with both GO terms is PPARG (PPARγ). Peroxisome proliferator-activated receptors (PPARs) constitute a small family of three nuclear receptors (PPARα, PPARβ/δ, and PPARγ) that act as lipid sensors, and thereby regulate the transcription of genes having key roles in lipid metabolism and energy homeostasis (Fougerat et al., 2024). It has been reported that PPARγ overexpression can induce a significant increase in fat accumulation in the liver (Lee et al., 2018). The activation of such nuclear receptors can influence the expression of genes involved in lipid metabolism, thereby affecting the lipid accumulation. For instance, it has been reported that PPARγ promotes the expression of downstream genes that are directly involved in fatty acid uptake and storage either directly, or indirectly through a cascade of activated genes (Lee et al., 2018). Similarly, the Retinoic acid-related orphan receptor α (RORα), associated with GO term “*nuclear receptor activity (GO:0004879)”* of Cluster one (C1), regulates various target genes related to lipid metabolism in hepatic cells (Han et al., 2021). In contrast, RORα regulates lipid homeostasis by inhibiting PPARγ to restore the metabolic homeostasis in liver thereby preventing excessive lipid accumulation (Kim et al.,

2017). However, Park et al. (2012) demonstrated that under certain conditions RORα may play a role in lipid accumulation (Park et al., 2012).

In addition, genes involved in phagocytosis, such as those which are significantly and differentially expressed in the conditions of TAA, TPA, and DEA shown in C1, can regulate the uptake of lipid particles, influencing lipid accumulation. For instance, ABCA7 gene involved in the GO term "*positive regulation of phagocytosis, engulfment (GO:0060100)"* associated with C1, was demonstrated to be a key gene involved in mediating cellular cholesterol and phospholipid release (Iwamoto et al., 2006). This gene encodes a human ATP-binding cassette (ABC) transporter that mediates lipid transport and plays a role in lipid metabolism (Fang et al., 2025).

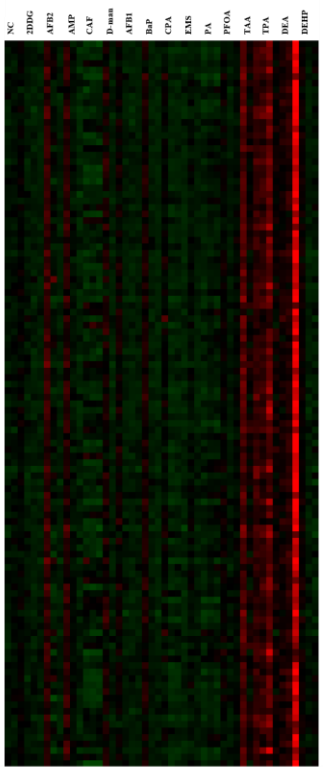
Interestingly, the clustering and the GO analysis within the NGTxC group of chemicals (FFOA, TAA, TPA, DEA, and DEHP) in cluster two (C2) showed different enriched GO terms related to lipid metabolism in the treated 3D HepaRG spheroids (Figure C.II-B 5 B). ACSL5, one of the genes associated with the GO term "*fatty-acyl-CoA biosynthetic process (GO:0046949)"* is known to be one of the main genes involved in fatty acid metabolism. Acyl-CoA synthetase long-chain family member 5 (ACSL5) is a key member of ACSL genes, widely expressed in mammalian liver that plays an important role in fatty acid metabolism (Luo et al., 2023). In addition, it has been reported that PPAR-γ agonists upregulated ACSL5 expression in rat liver (Way et al., 2001). Moreover, other genes associated with the GO term enriched in C2 such as CES1, ACOT1, APOA1, and ENPP2 play a significant role in lipid metabolism though the hydrolysis of the long-chain acyl-CoA to free fatty acid (Cavalli et al., 2021), cholesterol transport (Xiao et al., 2024), and by promoting lipogenesis (Lu et al., 2024) respectively. For instance, CES1 overexpression resulting from xenobiotic exposure is known to be strongly linked to hepatocellular carcinoma tumor progression in mice through lipid metabolism (Li et al., 2023). Li et al. (2023) reported that both CES1 and PPAR-γ or PPAR-α expression results in tumor progression and chemoresistance, and their inhibition or their down expression significantly slowed the growth of HCC tumors in mice. Similarly, Li et al (2024) demonstrated that cancer cells have altered metabolic patterns and regulate lipid metabolism to promote their own survival and progression, and that this metabolic reprogramming occurs through PPARs (Li et al., 2024).

These results suggests that the impact on lipid metabolism in the 3D HepaRG spheroids treated with the NGTxC group, and in particular with TAA, TPA, and DEA, might be due to the nuclear receptor activities such as PPARγ that could directly and or indirectly regulate the expression

of genes implicated in lipid metabolism and thus, result in altered lipid metabolism. Hence, changes in the expression of the nuclear receptor PPAR-γ and its link to lipid metabolism in 3D HepaRG spheroids treated with NGTxC could represent one of the key events in the adverse effects of such carcinogens.

Therefore, the interplay between nuclear receptors that regulate the expression of genes involved in lipid metabolism, and genes involved in phagocytosis and lipid metabolism provides evidence on how and why lipid metabolism is disrupted following treatment of 3D HepaRG spheroids with NGTxCs. Understanding these interactions can provide insights into the mechanisms underlying lipid-related changes in treated HepaRG spheroids, especially with NGTxC.

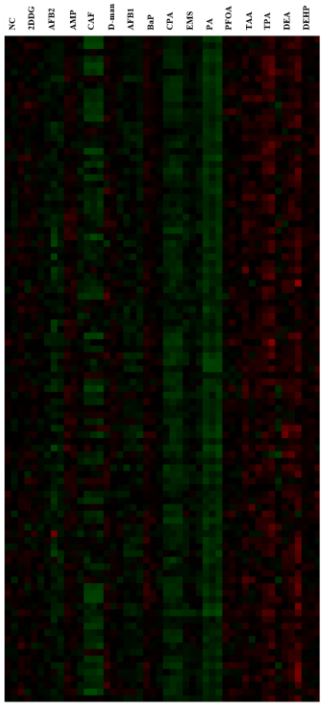
A



Cluster 1 (C1)

GOID	GO:0004879	GO:0060100
GOTerm	nuclear receptor activity	positive regulation of phagocytosis, engulfment
Term PValue Corrected with Benjamini-Hochberg	3.80E-04	1.36E-04
Associated Genes	[NR5A2, NR6A1, PPARG, RORA]	[ABCA7, APPL2, PPARG]

B



Cluster 2 (C2)

GOID	GO:0052689	GO:0004806	GO:0052689
GOTerm	fatty-acyl-CoA biosynthetic process	triglyceride lipase activity	carboxylic ester hydrolase activity
Term PValue Corrected with Benjamini-Hochberg	1.19E-03	2.50E-03	6.65E-05
Associated Genes	[ACSL4, ACSL5, HSD17B8]	[APOA1, CES1, PCSK6]	[ABHD6, ACOT1, APOA1, BPHL, CES1, ENPP2, PCSK6, PRDX6]

Figure C.II-B 5. Clustering analysis of the GTx, NGTx, and NGTxC tested groups of chemicals with 3D HepaRG spheroids, and Gene ontology (GO) enrichment analysis of each cluster. (A): Cluster one (C1) with its enriched GO terms, Term P-Value, and associated genes. (B): Cluster two (C2) with its enriched GO terms, Term P-Value, and associated genes. Within each cluster, set of the differentially expressed genes are represented by colour intensity with each treatment condition, where red indicates upregulated genes, green indicates downregulation of the genes in the cluster, and black represents no significant change relative to the negative control.

3.3.2. Interplay between cell proliferation and caspase activity: pathways of growth and apoptotic resistance

The second two clusters result from the unsupervised clustering and their GO enrichment analysis with the term p-Value corrected with Benjamini-Hochberg along with the associated genes in each term represented in (Figure C.II-B 6 A-B). Cluster three (C3) (Figure C.II-B 6 A), contains a subset of highly upregulated genes (red) with NGTxC group of chemicals especially with TAA, TPA, and DEA conditions. This cluster was significantly enriched in the GO terms "*regulation of double-strand break repair (GO:2000779)"* and *"double-strand break repair via homologous recombination (GO:0000724)".* The GO term "*regulation of double-strand break repair" was* represented by the genes CHEK1, FIGN, FIGNL1, RADX, and TWIST1. In addition, the set of genes represented the GO *"double-strand break repair via homologous recombination"* term was CHEK1, FIGN, FIGNL1, MCM3, RADX, RECQL, and REV3L. Similarly, in Cluster four (C4) (Figure C.II-B 6 B), all chemicals with NGTxC group shows downregulation of a set of genes that are significantly enriched in GO term "*positive regulation of cysteine-type endopeptidase activity involved in apoptotic signalling pathway (GO:2001269)",* with their associated genes BAX, FAS, and GSN represented in (Figure C.II-B 6 B).

The enriched biological processes especially with TAA, TPA, and DEA in C3 of "*regulation of double-strand break repair (GO:2000779)"* and *"double-strand break repair via homologous recombination (GO:0000724)"* represents a central process to maintain genome integrity and ensuring accurate DNA replication prior to cell proliferation (Valerie & Povirk, 2003; Mladenov et al., 2016).

As double strand breaks (DSBs) are among the most deleterious form of DNA damage, as these lesions attenuates the cellular proliferation and their repair mechanism is critical for the transformation of the genetic material to a daughter cell (Kumari et al., 2025). The regulation of the DSB repairs encompasses various mechanisms that modulates the frequency, efficiency, and fidelity of repair. However, homologous recombination (HR) represents a high-fidelity pathway that causes a homologous DNA sequence as a template to restore the broken DNA strands (Li & Heyer, 2008). Since cells respond to DNA damage by activating a network of pathways that enable damage recognition and initiation of apoptotic pathways or senescence, the DNA repair activation processes are intimately linked to cell-cycle progression (Mladenov et al., 2016). These repairs are particularly linked to the S phase, where DNA replication occurs,

and the G2/M phases, where cells prepare for and execute mitosis. Robust checkpoint mechanisms ensure that DSBs are adequately repaired before proceeding through these phases, preventing the propagation of genomic instability and potential apoptosis (Liu et al., 2023).

Several genes associated with the enriched GO terms within C3 and investigated in this study (Figure C.II-B 6) play central role in linking DNA repair machinery with cell-cycle regulation, demonstrating a strong functional connection to cell proliferation process. For instance, CHEK1 (Checkpoint Kinase 1) serves as a central checkpoint kinase, monitoring replication and response to DNA damage by arresting the cell cycle in S and G1 or G2 phases until the repair is complete (Patil et al., 2013; Baran & Mayer, 2023). Its activation is crucial for replication fork integrity and genomic stability, highlighting its essential role in enabling proliferation under DNA stress. Similarly, RADX stabilizes single-stranded DNA at replication forks by preventing the fork collapse, and directly supporting cell proliferation under conditions that would otherwise impede replication (Mohamed et al., 2022). In addition, MCM3 and RECQL represent core components of the replication machinery, as MCM3 ensures the DNA unwinding of the replication machinery, while the RECQL helicase facilitates fork stabilization and restart during replication stress, preventing the accumulation of DNA damage that could trigger cell-cycle arrest or apoptosis (Li et al., 2020; Benedict et al., 2020). Moreover, Li et al (2020) reported that MCM3 represents an essential protein implicated in tumor migration and invasion through the enhancement of the anchorage-independent cell growth, cell migration and invasion in HCC (Li et al., 2020). In addition, TWIST1is a transcriptional factor known for its role in epithelial to mesenchymal transition, which also influences proliferation through the modulating of the genes involved in cell cycle progression, indirectly supporting S-phase entry and G2/M transition (Cordani et al., 2023). Collectively, these genes, whose expression was modulated following treatment of HepaRG spheroids with NGTxC, illustrate a spectrum of mechanisms from checkpoint enforcement to replication fork management, which are strongly implicated in ensuring successful DNA synthesis and proliferation.

Importantly, the upregulation of the genes involved in the DNA repair machinery and sustained proliferation could be closely linked to a response to cellular oxidative stress (Molinaro et al., 2021). It was reported by Saleh et al. (2025) the cellular stress effect associated with 3D HepaRG spheroids treated with TAA for 72h through Reactive Oxygen Species (ROS) induction (Saleh et al., 2025). These results highlight more the oxidative stress effect associated with these treatments in 3D HepaRG spheroids.

In our results with clustering analysis and GO, we did not find direct evidence to support a role for the oxidative stress pathway in the tested conditions. However, ROS could accumulate through a disrupted metabolic pathway (Ghosh et al., 2018). One of the important disrupted metabolic pathways in our results is the lipid metabolism (Figure C.II-B 5). Enríquez-Cortina et al., (2017) reported that cholesterol accumulation in the liver induces redox imbalance and oxidative stress- mediated DNA damage and contributes to hepatocarcinogenesis. (Enríquez-Cortina et al., 2017). Thus, the genes involved in lipid metabolism identified in cluster C2 (ACOT1, APOA1, CES1, PCSK6, and PRDX6) could influence cellular redox balance through the regulation of membrane lipid composition. For instance (Shen et al., 2023).

This interplay establishes a functional axis wherein lipid metabolism influences oxidative stress levels, oxidative stress induces DNA damage, and DNA repair machinery responds to maintain replication fidelity and cell-cycle progression. By integrating checkpoint signalling, replication fork stabilization, and homologous recombination-mediated repair, cells can navigate S and G2/M phases while sustaining proliferation, even under oxidative challenges imposed by endogenous metabolism or xenobiotic compounds. The combination of DNA repair efficiency, oxidative stress mitigation, and cell-cycle regulation underscores the tightly coordinated network that allows cells to balance metabolic activity with genome integrity, ensuring survival and proliferation. In summary, the genes examined here demonstrate a multifaceted relationship with DNA repair machinery, lipid metabolism, oxidative stress, and cell proliferation. Thus, understanding these interconnections provides insights into the molecular networks that safeguard genomic integrity during proliferation under stress, and integrate metabolic and signalling pathways, which may have implications in cancer biology, toxicology, and metabolic disease.

Interestingly, transcriptomic profiles of 3D HepaRG spheroids treated with NGTxC (Figure C.II-B 6 B), were enriched in the GO term "*positive regulation of cysteine-type endopeptidase activity involved in apoptotic signalling pathway (GO:2001269)".* which was associated with regulation of the genes BAX, FAS, and GSN (Figure C.II-B 6 B) This significant downregulation (green) was observed with PFOA, TAA, TPA, and DEA. This pathway is directly linked to the regulation of caspase activity (programmed cell death), reflecting a fundamental safeguard against the survival of damaged or stressed cells. Within this GO term, three central regulators of apoptosis were identified; BAX, FAS and GSN (Figure C.II-B 6 B). BAX is a pro-apoptotic member of BLC62 family that is involved in various biological

processes such as caspase activation. FAS encodes a death receptor that activates the extrinsic apoptotic pathway via caspase-8 recruitment. GSN (gelsolin), in turn, modulates actin cytoskeleton dynamics and contributes to the amplification of apoptotic signalling. These genes were significantly downregulated in 3D HepaRG spheroids treated with these NGTxCs (shown in green in Figure C.II-B 6 B. The sown regulation of BAX, FAS, and GSN suggests a suppression of pro-apoptotic signalling, thereby diminishing the capacity of the cells to undergo programmed cell death in response to the treatment with NGTxC. Such downregulation implies a shift in the cellular balance toward apoptotic evasion, a hallmark of carcinogenic progression and represents one of the important NGTxC Key Characteristics (Smith et al., 2020). Within the context of the liver, the attenuation of apoptosis in hepatocyte-like cells can permit the survival of gnomically compromised cells, contributing to long-term risk of malignant transformation. These findings indicate that NGTxCs can induce transcriptomic alterations in 3D HepaRG spheroids, suppressing apoptosis-related gene expression and potentially creating conditions that favour hepatocarcinogenesis (Kulbay et al., 2022).

These findings suggest that 3D HepaRG spheroids treated with the NGTxC group of chemicals exhibit distinct molecular-level effects. They are consistent with published research describing the key molecular events associated with NGTxC MoAs. One of the major contributors is the activation of nuclear receptors, which promotes lipid accumulation and disrupts lipid metabolism. In addition, increased cell proliferation and inhibition of apoptotic pathways represent central molecular events commonly linked to NGTxC activity (Louekari & Jacobs, 2024).

Moreover, the gene sets implicated in the DNA repair mechanism that is upregulated in NGTxC, are not included in the gene list of the genotoxic transcriptomic biomarkers TGxDDI and GENOMARK (described in first part of this chapter). There are other direct genes implicated in the DNA repair mechanism used to analysis genotoxicity using transcriptomic biomarkers. Moreover, the upregulation of genes associated with the apoptotic pathway (associated with the GTxC group in our data) sometimes result in downregulation of genes associated with the DNA repair mechanism with GTxC (Foster et al., 2012). Finally, GTxC exert a wide range of effects on DNA which result in genotoxicity. While some compounds may induce direct DNA damage, others may have effects on destabilizing the DNA repair machinery (Giaginis et al., 2006).

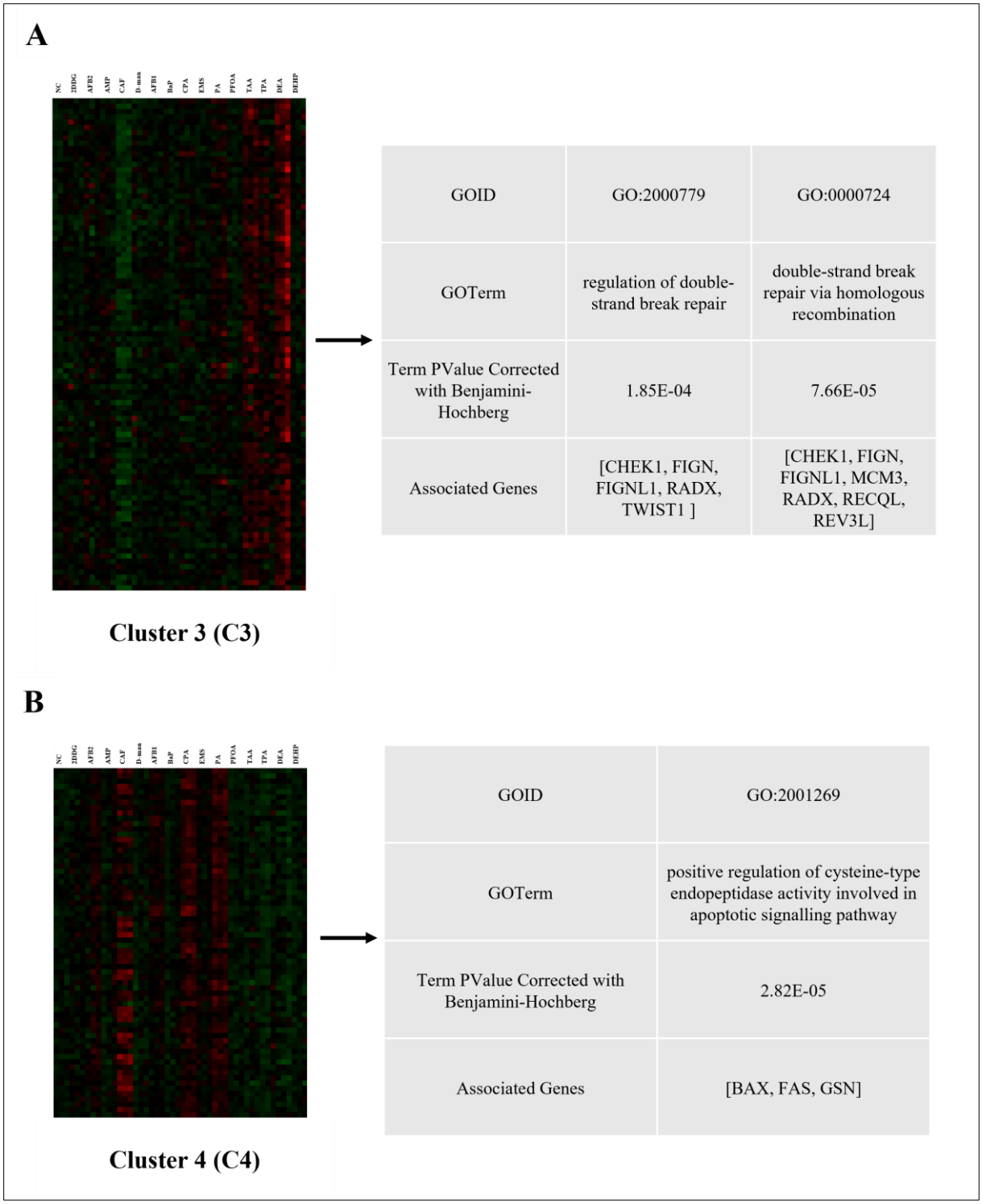


Figure C.II-B 6. Clustering analysis of the GTx, NGTx, and NGTxC tested groups of chemicals with 3D HepaRG spheroids, and Gene ontology (GO) enrichment analysis of each cluster. (A): Cluster three (C3) with its enriched GO terms, Term P-Value, and associated genes. (B): Cluster four (C4) with its enriched GO terms, Term P-Value, and associated genes. Within each cluster, set of the differentially expressed genes are represented by colour intensity with each treatment condition, where red indicates upregulated genes, green indicates downregulation of the genes in the cluster, and black represents no significant change relative to the negative control.

3.3.3. PPARγ as a key player among differentially expressed genes associated with NGTxC

The PPAR-γ upregulation gene was differentially expressed, in particular in HepaRG spheroids treated with a NGTxC chemicals and especially with TAA, TPA, and DEA (Table C.II-B 2) (Figure C.II-B 7).

PPARγ is involved in lipid accumulation in liver and is linked to carcinoma as an adverse outcome. PPARγ could have different effects depending on the chemical (Maloney & Waxman, 1999). For instance, TPA, a known tumor promoter, can activate protein kinase C pathways that may indirectly influence PPARγ (Ricote et al., 1998; Kim et al., 2006). This was in line with our results as shown in (Figure C.II-B 7) as the differential expression of this gene was significantly higher in 3D HepaRG spheroids treated with TPA compared to the negative control. In contrast, exposure to PFOA and DEHP does not lead to PPARγ upregulation. Despite its DEG profile presented before, DEHP has been found to activate PPARα rather than PPARγ (Ito & Nakajima, 2008). Similarly, PFOA has been shown to activate PPARα rather than PPARγ (Evans et al., 2022). These results suggest the importance of the chemical specific effects and the challenge in classifying NGTxC having different mode of actions.

Table C.II-B 2. PPARγ gene expression in the NGTxC group of chemicals (PFOA, TAA, TPA, DEA, and DEHP). Statistical analysis shows the log2fold change of PPARγ gene and its adjusted p-Value calculated with DESeq2 R-package.

Chemicals/statistics	log2FoldChange	Adjusted p-Value
PFOA	-0.0145008356993773	0.9992532
TAA	1.35750728783037	0.0001260
TPA	1.4031706125012	0.0060952
DEA	1.24736041681425	0.0162984
DEHP	0.195964570301452	0.9999876

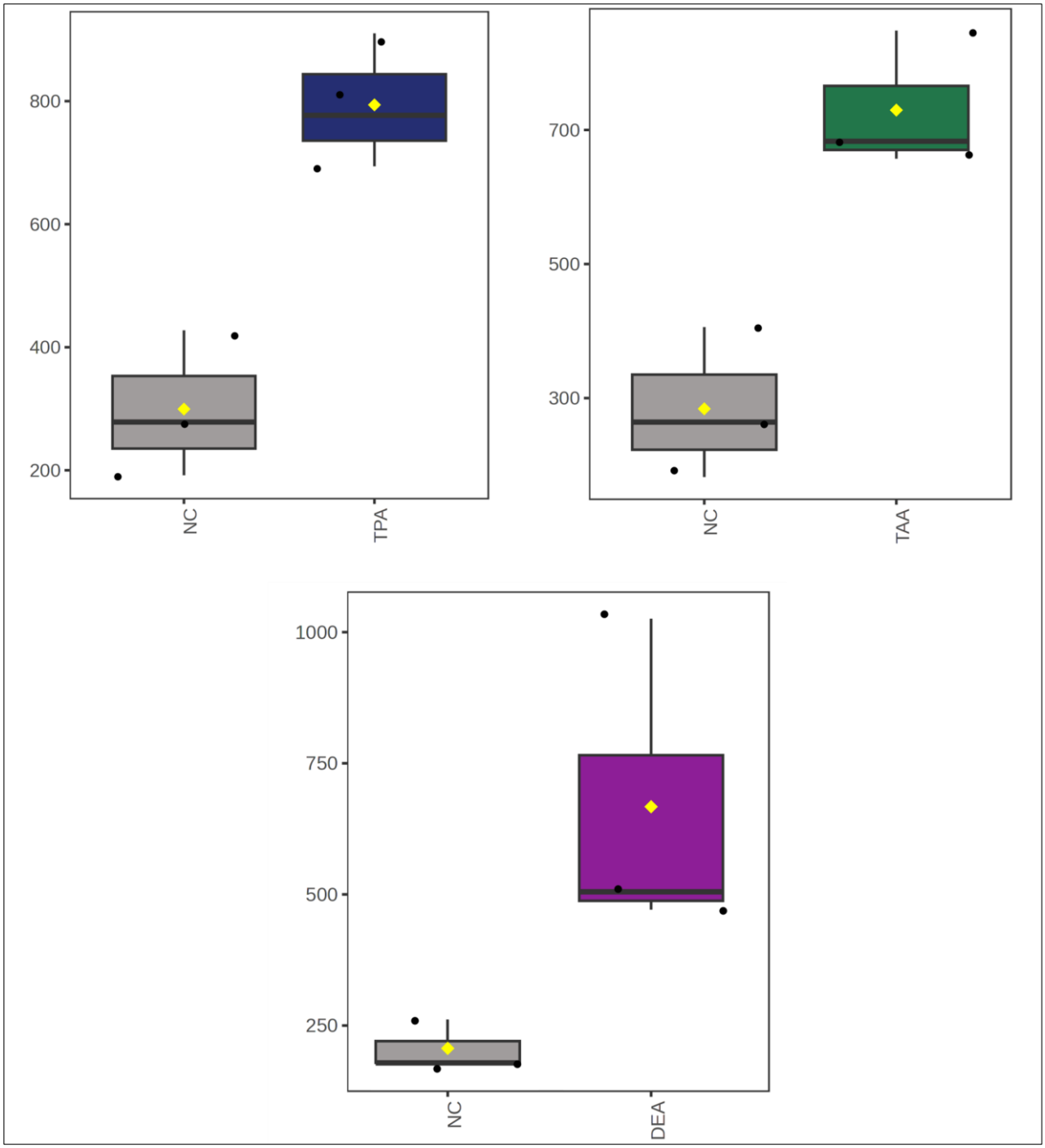


Figure C.II-B 7. Box plots of PPARγ gene expression in 3D HepaRG spheroids treated with NGTxC group of chemicals (TPA, TAA, and DEA) after 72h repetitive treatment.

4. Conclusions and perspectives

Transcriptomic analysis performed in human-relevant 3D hepatic models offer a promising and predictive NAMs-based approach for the hazard identification of carcinogens. In this study, we applied transcriptomic profiling using high-throughput TempO-Seq™ technology to characterize gene-expression responses in 3D HepaRG spheroids exposed to a panel of chemicals, with the aim of leveraging mechanistic signatures to classify carcinogenic compounds. Three groups of chemicals were selected: (1) GTx, (2) NGTx, and (3) five NGTxC. Spheroids were exposed to non-cytotoxic concentrations corresponding to the IC_{10} or $IC_{10/2}$ for 72 hours using a repeated-dose treatment.

Unsupervised global clustering analyses, followed by GO enrichment analysis, were performed on the transcriptomic profiles obtained from treated spheroids. We examined clustering patterns based on gene-set–level responses and evaluated the biological pathways enriched within each cluster, with particular focus on the NGTxC group. This approach enabled the identification of mechanistic signatures that may support the classification of NGTxC within a human-relevant *in vitro* framework. In the first phase of this study, the applied clustering analysis resulted in distinct clusters of which the NGTxC group of chemicals were clearly grouped and showed different gene expression profiles separated from both GTx and NGTx groups. This grouping highlights the potential of clustering approaches to distinguish NGTxC as a specific chemical class. However, certain compounds within the NGTxC group, such as PFOA and DEHP, did not cluster within the NGTxC group in some clusters, highlighting the challenges associated with the chemical-specific effects. These findings indicate that while global clustering strategies can be valuable tools to identifying and grouping NGTxC, challenges remain due to the heterogeneity of responses among individual NGTxC especially when having different MoA.

In the second phase of this study, we focused on identifying significant changes in gene expression at the molecular level that were specifically associated with the NGTxC group of chemicals. The upregulation of the expression of the PPARγ nuclear receptor which plays an important role in lipid metabolism and accumulation in liver cells was highly differentially expressed in NGTxC. In addition, lipid metabolism and accumulation can occur through different metabolic process, and interestingly, genes associated with such metabolism were overexpressed in the clusters grouping NGTxC.

Moreover, overexpression of genes related to cell proliferation and inhibition of apoptotic pathways were associated with the NGTxC group. The identification of such pathways, which

are related to hallmarks of cancer and associated with different tested NGTxC, may therefore play important roles in their carcinogenic MoA.

In summary, the analysis of gene expression profiles associated with NGTxC which were different than GTx and NGTx groups highlights several important points:

1. Lipid metabolism could be a major pathway targeted in hepatic models exposed to NGTxC, in particular through the activation of PPAR nuclear receptors.
2. Cell proliferation and inhibition of the apoptotic pathway could represent one of the important key events associated with NGTxC activity *in vitro*.
3. Chemical specific effect is challenging in analysing NGTxC MoA and identifying key events associated with such treatments.
4. Further statistical applications and analyses should be conducted with our data to confirm the outcomes and compare gene profiles of NGTxC with GTx group and NGTx.

While global clustering and GO analyses applied in this chapter have yielded significant insights, a comprehensive characterization of the dataset requires a more granular investigation. Future efforts will prioritize the interrogation of GTx GO terms and the identification of DEGs unique to individual compounds. Furthermore, a rigorous assessment of chemical-specific effects is necessary, with a particular focus on the NGTxC cohort to delineate their distinct toxicological signatures.

Moreover, although transcriptomic analysis were recommended to analyse different NGTxC MoA, study conducted by Schaap et al., (2012), demonstrated that gene expression profiles in primary hepatocytes could be able to detect some NGTxC MoA but not all (Schaap et al., 2012). This study outcomes are in line of what we have demonstrated so far with the data analysis in this study so far.

References

Abbas-Hashemi, S. A., Yari, Z., Hatami, B., Moradi-Moghaddam, O., Zali, M. R., & Hekmatdoost, A. (2024). Caffeine supplement, inflammation, and hepatic function in cirrhotic patients: A randomized, placebo-controlled, clinical trial. *Heliyon*, *11*(1), e41138. https://doi.org/10.1016/j.heliyon.2024.e41138

Aniqa, A., Kaur, S., Negi, A., Sadwal, S., & Bharati, S. (2022). Phytomodulatory effects of Murraya koenigii in DMBA/TPA induced angiogenesis, hepatotoxicity and renal toxicity during skin carcinogenesis in mice. *Journal of Advanced Scientific Research*, *13*(02), 153–165. https://doi.org/10.55218/JASR.202213221

Audebert, M., Assmann, A.-S., Azqueta, A., Babica, P., Benfenati, E., Braeuning, A., Braakhuis, H., Burgdorf, T., Corvi, R., Ebner, R., Fessard, V., Jacobs, M. N., Jennings, P., Luijten, M., Oberemm, A., O'Brien, J., Rousselle, C., Schmeisser, S., Stamou, M., ... & Žegura, B. (2023). New approach methodologies to facilitate and improve the hazard assessment of non-genotoxic carcinogens—a PARC project. *Frontiers in Toxicology*, *5*. https://doi.org/10.3389/ftox.2023.1220998

Baran, V., & Mayer, A. (2023). Checkpoint kinase 1 is a key signal transducer of DNA damage in the early mammalian cleavage embryo. *International Journal of Molecular Sciences, 24*(7), 6778. https://doi.org/10.3390/ijms24076778

Benedict, B., van Bueren, M. A., van Gemert, F. P., van Meel, E., van der Linden, R., & Knipscheer, P. (2020). The RECQL helicase prevents replication fork collapse during replication stress. *Life Science Alliance, 3*(10), e202000668. https://doi.org/10.26508/lsa.202000668

Bischof, T., Gsoellpointner, M., Kovacevic Miljevic, K. D., Marjanovic, J., & Zimmer, A. (2025). Safety, tolerability, and pharmacokinetics of intranasal 2-deoxy-D-glucose in normal healthy volunteers: A randomized, double-blind, placebo-controlled, single and multiple ascending dose phase 1 study. *European Journal of Pharmaceutical Sciences, 209*, 107069. https://doi.org/10.1016/j.ejps.2025.107069

Cavalli, M., Diamanti, K., Dang, Y., Tang, W., Ho-Fung, V., & Wadelius, C. (2021). The thioesterase ACOT1 as a regulator of lipid metabolism in type 2 diabetes detected in a multi-omics study of human liver. *OMICS: A Journal of Integrative Biology*, *25*(10), 652–659. https://doi.org/10.1089/omi.2021.0093

Cordani, N., Mologni, L., Piazza, R., & Redaelli, S. (2023). TWIST1 upregulation is a potential target for reversing resistance to the CDK4/6 inhibitor in metastatic luminal breast cancer cells. *International Journal of Molecular Sciences, 24*(22), 16294. https://doi.org/10.3390/ijms242216294

DeChiara, J. R., Birch, E. M., & Harper, H. (2023). Low-dose cyclophosphamide associated with hyponatremia and hepatotoxicity. *Cureus, 15*(9), e45375. https://doi.org/10.7759/cureus.45375

Delft, J. H. M. van, van Agen, E., van Breda, S. G. J., Herwijnen, M. H., Staal, Y. C. M., & Kleinjans, J. C. S. (2004). Discrimination of genotoxic from non-genotoxic carcinogens by gene expression profiling. *Carcinogenesis, 25*(7), 1265–1276. https://doi.org/10.1093/carcin/bgh108

DeMaria, S., Bachner, E. M., Mroz, V., & Miller, R. (2025). The efficacy of mannitol in attenuating postreperfusion syndrome in orthotopic liver transplantation: A retrospective cohort study. *Journal of Clinical Medicine, 14*(6), 1897. https://doi.org/10.3390/jcm14061897

Dubreil, E., Darney, K., Delignette-Muller, M.-L., & Verre, L. (2024). Modeling HepaRG metabolome responses to pyrrolizidine alkaloid exposure for insight into points of departure and modes of action. *Journal of Hazardous Materials, 474*, 134721. https://doi.org/10.1016/j.jhazmat.2024.134721

Enríquez-Cortina, C., Bello-Monroy, O., Rosales-Cruz, P., & Bucio, L. (2017). Cholesterol overload in the liver aggravates oxidative stress-mediated DNA damage and accelerates hepatocarcinogenesis. *Oncotarget, 8*(61), 104136–104148. https://doi.org/10.18632/oncotarget.22024

Evans, N., Conley, M. J., Cardon, M., Hartig, P., Medlock-Kakaley, E., & Gray, L. E. (2022). In vitro activity of a panel of per- and polyfluoroalkyl substances (PFAS), fatty acids, and pharmaceuticals in peroxisome proliferator-activated receptor (PPAR) alpha, PPAR gamma, and estrogen receptor assays. *Toxicology and Applied Pharmacology, 449*, 116136. https://doi.org/10.1016/j.taap.2022.116136

Ezhilarasan, D. (2023). Molecular mechanisms in thioacetamide-induced acute and chronic liver injury models. *Environmental Toxicology and Pharmacology, 99*, 104093. https://doi.org/10.1016/j.etap.2023.104093

Fang, S.-C., Wang, L., Cheng, M.-T., & Sun, L. (2025). Structural insights into human ABCA7-mediated lipid transport. *Structure, 33*(3), 583-593.e5. https://doi.org/10.1016/j.str.2024.12.015

Feng, Y., Wang, H., Lu, B., & Zhang, Y. (2025). Perfluorooctanoic acid (PFOA) induces adipose-derived ADMSC senescence via oxidative stress pathway. *Environmental Chemistry and Ecotoxicology, 7*, 2067–2079. https://doi.org/10.1016/j.enceco.2025.08.020

Foster, S. S., De, S., Johnson, L. K., Petrini, J. H. J., & Stracker, T. H. (2012). Cell cycle- and DNA repair pathway-specific effects of apoptosis on tumor suppression. *Proceedings of*

the National Academy of Sciences of the United States of America, 109(25), 9953–9958. https://doi.org/10.1073/pnas.1120476109

Fougerat, A., Bruse, J., Polizzi, A., Montagner, A., Guillou, H., & Wahli, W. (2024). Lipid sensing by PPARα: Role in controlling hepatocyte gene regulatory networks and the metabolic response to fasting. *Progress in Lipid Research, 96*, 101303. https://doi.org/10.1016/j.plipres.2024.101303

Gemede, H. F. (2025). Toxicity, mitigation, and chemical analysis of aflatoxins and other toxic metabolites produced by Aspergillus: A comprehensive review. *Toxins, 17*(7), 331. https://doi.org/10.3390/toxins17070331

Ghosh, N., Das, A., Chaffee, S., Roy, S., & Sen, C. K. (2018). Chapter 4 - Reactive oxygen species, oxidative damage and cell death. In S. Chatterjee, W. Jungraithmayr, & D. Bagchi (Eds.), *Immunity and inflammation in health and disease*. Academic Press. https://doi.org/10.1016/B978-0-12-805417-8.00004-4

Giaginis, C., Gatzidou, E., & Theocharis, S. (2006). DNA repair systems as targets of cadmium toxicity. *Toxicology and Applied Pharmacology, 213*(3), 282–290. https://doi.org/10.1016/j.taap.2006.03.008

Han, Y.-H., Kim, H.-J., & Lee, M.-O. (2021). RORα regulates hepatic lipolysis by inducing transcriptional expression of PNPLA3 in mice. *Molecular and Cellular Endocrinology, 522*, 111122. https://doi.org/10.1016/j.mce.2020.111122

Hanahan, D. (2022). Hallmarks of cancer: New dimensions. *Cancer Discovery, 12*(1), 31–46. https://doi.org/10.1158/2159-8290.CD-21-1059

Hernández, L. G., van Steeg, H., Luijten, M., & van Benthem, J. (2009). Mechanisms of non-genotoxic carcinogens and importance of a weight of evidence approach. *Mutation Research, 682*(2–3), 94–109. https://doi.org/10.1016/j.mrrev.2009.07.002

Heusinkveld, H., Braakhuis, H., Gommans, R., Havinga, J., van der Ven, L., van Steeg, H., & Luijten, M. (2020). Towards a mechanism-based approach for the prediction of nongenotoxic carcinogenic potential of agrochemicals. *Critical Reviews in Toxicology, 50*(9), 725–739. https://doi.org/10.1080/10408444.2020.1841732

Hoenerhoff, M. J., Boyle, M., Minocherhomji, S., & Pandiri, A. R. (2022). Chapter 8 - Carcinogenesis: Mechanisms and evaluation. In W. M. Haschek, C. G. Rousseaux, M. A. Wallig, & B. Bolon (Eds.), *Haschek and Rousseaux's handbook of toxicologic pathology* (4th ed.). Academic Press. https://doi.org/10.1016/B978-0-12-821044-4.00013-3

Ito, Y., & Nakajima, T. (2008). PPARα- and DEHP-induced cancers. *PPAR Research, 2008*, 759716. https://doi.org/10.1155/2008/759716

Iwamoto, N., Abe-Dohmae, S., Sato, R., & Yokoyama, S. (2006). ABCA7 expression is regulated by cellular cholesterol through the SREBP2 pathway and associated with phagocytosis. *Journal of Lipid Research, 47*(9), 1915–1927. https://doi.org/10.1194/jlr.M600127-JLR200

Jacobs, M. N., Colacci, A., Louekari, K., Luijten, M., Hakkert, B. C., Paparella, M., & Vasseur, P. (2016). International regulatory needs for development of an IATA for non-genotoxic carcinogenic chemical substances. *ALTEX, 33*(4), 359–392. https://doi.org/10.14573/altex.1601201

Kaur, S., Kidambi, S., Ortega-Ribera, M., van Grunsven, L. A., Karandikar, K., & Hebbar, S. (2022). In vitro models for the study of liver biology and diseases: Advances and limitations. *Cellular and Molecular Gastroenterology and Hepatology, 15*(3), 559–571. https://doi.org/10.1016/j.jcmgh.2022.11.008

Kim, H. J., Woo, I. S., Kang, E. S., Kim, H. J., Nam, J. S., Ahn, C. W., Cha, B. S., Lim, S. K., Kim, K. R., & Lee, H. C. (2006). Phorbol ester potentiates the growth inhibitory effects of troglitazone via up-regulation of PPARgamma in A549 cells. *Biochemical and Biophysical Research Communications, 349*(2), 660–667. https://doi.org/10.1016/j.bbrc.2006.08.085

Kim, K., Boo, K., Yu, Y. S., Oh, S. K., Kim, H., Jeon, Y., Bhin, J., Hwang, D., Kim, K. I., & Baek, S. H. (2017). RORα controls hepatic lipid homeostasis via negative regulation of PPARγ transcriptional network. *Nature Communications, 8*(1), 162. https://doi.org/10.1038/s41467-017-00215-1

Kim, M., Jee, S.-C., & Sung, J.-S. (2024). Hepatoprotective effects of flavonoids against benzo[a]pyrene-induced oxidative liver damage along its metabolic pathways. *Antioxidants, 13*(2), 180. https://doi.org/10.3390/antiox13020180

Kim, Y., Kim, H., & Kim, Y. (2025). Advancing hepatotoxicity assessment: Current advances and future directions. *Toxicological Research, 41*(4), 303–323. https://doi.org/10.1007/s43188-025-00289-w

Kulbay, M., Paimboeuf, A., Ozdemir, D., & Bernier, J. (2022). Review of cancer cell resistance mechanisms to apoptosis and actual targeted therapies. *Journal of Cellular Biochemistry, 123*(11), 1736–1761. https://doi.org/10.1002/jcb.30173

Kumari, N., Kaur, E., Raghavan, S. C., & Sengupta, S. (2025). Regulation of pathway choice in DNA repair after double-strand breaks. *Current Opinion in Pharmacology, 80*, 102496. https://doi.org/10.1016/j.coph.2024.102496

Lee, Y. K., Park, J. E., Lee, M., & Hardwick, J. P. (2018). Hepatic lipid homeostasis by peroxisome proliferator-activated receptor gamma 2. *Liver Research, 2*(4), 209–215. https://doi.org/10.1016/j.livres.2018.12.001

Lehman-McKeeman, L. D., Gamsky, E. A., Hicks, S. M., Vassallo, J. D., Mar, M.-H., & Zeisel, S. H. (2002). Diethanolamine induces hepatic choline deficiency in mice. *Toxicological Sciences, 67*(1), 38–45. https://doi.org/10.1093/toxsci/67.1.38

Leist, M., Ghallab, A., Graepel, R., Marchan, R., Hassan, R., Bennekou, S. H., Limonciel, A., Vinken, M., Schildknecht, S., Waldmann, T., Danen, E., van Ravenzwaay, B., Kamp, H., Gardner, I., Godoy, P., Bois, F. Y., Braeuning, A., Reif, R., & Hengstler, J. G. (2017). Adverse outcome pathways: Opportunities, limitations and open questions. *Archives of Toxicology, 91*(11), 3477–3505. https://doi.org/10.1007/s00204-017-2045-3

Li, G., Li, X., Mahmud, I., Ding, S., Chen, Y., Roper, J., & Guo, H. (2023). Interfering with lipid metabolism through targeting CES1 sensitizes hepatocellular carcinoma for chemotherapy. *JCI Insight, 8*(2), e163624. https://doi.org/10.1172/jci.insight.163624

Li, H.-T., Wei, B., Li, Z.-Q., & Jiang, H. (2020). Diagnostic and prognostic value of MCM3 and its interacting proteins in hepatocellular carcinoma. *Oncology Letters, 20*(6), 308. https://doi.org/10.3892/ol.2020.12171

Li, X., & Heyer, W.-D. (2008). Homologous recombination in DNA repair and DNA damage tolerance. *Cell Research, 18*(1), 99–113. https://doi.org/10.1038/cr.2008.1

Li, Y., Pan, Y., Zhao, X., & He, J. (2024). Peroxisome proliferator-activated receptors: A key link between lipid metabolism and cancer progression. *Clinical Nutrition, 43*(2), 332–345. https://doi.org/10.1016/j.clnu.2023.12.005

Liu, M., Yang, J., Xu, B., & Zhang, X. (2021). Tumor metastasis: Mechanistic insights and therapeutic interventions. *MedComm, 2*(4), 587–617. https://doi.org/10.1002/mco2.100

Liu, S., Byrne, B. M., Byrne, T. N., & Oakley, G. G. (2023). Role of RPA phosphorylation in the ATR-dependent G2 cell cycle checkpoint. *Genes, 14*(12), 2205. https://doi.org/10.3390/genes14122205

Louekari, K., & Jacobs, M. N. (2024). A modular strategy for the testing and assessment of non-genotoxic carcinogens. *Archives of Toxicology, 98*(8), 2463–2485. https://doi.org/10.1007/s00204-024-03753-y

Lu, L., Hu, X., Han, Y., & Zhang, Q. (2024). ENPP2 promotes progression and lipid accumulation via AMPK/SREBP1/FAS pathway in chronic lymphocytic leukemia. *Cellular & Molecular Biology Letters, 29*, 159. https://doi.org/10.1186/s11658-024-00675-6

Luo, Q., Das, A., Oldoni, F., & Sahoo, D. (2023). Role of ACSL5 in fatty acid metabolism. *Heliyon, 9*(2), e13316. https://doi.org/10.1016/j.heliyon.2023.e13316

Maloney, E. K., & Waxman, D. J. (1999). Trans-activation of PPARalpha and PPARgamma by structurally diverse environmental chemicals. *Toxicology and Applied Pharmacology, 161*(2), 209–218. https://doi.org/10.1006/taap.1999.8809

Mladenov, E., Magin, S., Soni, A., & Iliakis, G. (2016). DNA double-strand-break repair in higher eukaryotes and its role in genomic instability and cancer: Cell cycle and proliferation-dependent regulation. *Seminars in Cancer Biology, 37–38*, 51–64. https://doi.org/10.1016/j.semcancer.2016.03.003

Mohamed, T., Adolph, M. B., & Cortez, D. (2022). Oligomerization of DNA replication regulatory protein RADX is essential to maintain replication fork stability. *The Journal of Biological Chemistry, 298*(3), 101672. https://doi.org/10.1016/j.jbc.2022.101672

Molinaro, C., Martoriati, A., & Cailliau, K. (2021). Proteins from the DNA damage response: Regulation, dysfunction, and anticancer strategies. *Cancers, 13*(15), 3819. https://doi.org/10.3390/cancers13153819

Moloi, T. P., Ziqubu, K., Mazibuko-Mbeje, S. E., Mabaso, N. H., & Ndlovu, Z. (2024). Aflatoxin B1-induced hepatotoxicity through mitochondrial dysfunction, oxidative stress, and inflammation as central pathological mechanisms: A review of experimental evidence. *Toxicology, 509*, 153983. https://doi.org/10.1016/j.tox.2024.153983

Oku, Y., Madia, F., Lau, P., & Corvi, R. (2022). Analyses of transcriptomics cell signalling for pre-screening applications in the integrated approach for testing and assessment of non-genotoxic carcinogens. *International Journal of Molecular Sciences, 23*(21), 12718. https://doi.org/10.3390/ijms232112718

Ooi, H., Asai, Y., & Sato, Y. (2023). Risk evaluation of ampicillin/sulbactam-induced liver injury based on albumin-bilirubin score. *Journal of Infection and Chemotherapy, 29*(9), 900–904. https://doi.org/10.1016/j.jiac.2023.06.003

Park, U.-H., Jeong, J.-C., Jang, J.-S., & Kim, E.-J. (2012). Negative regulation of adipogenesis by kaempferol, a component of Rhizoma Polygonati Falcatum in 3T3-L1 cells. *Biological & Pharmaceutical Bulletin, 35*(9), 1525–1533. https://doi.org/10.1248/bpb.b12-00254

Patil, M., Pabla, N., & Dong, Z. (2013). Checkpoint kinase 1 in DNA damage response and cell cycle regulation. *Cellular and Molecular Life Sciences, 70*(21), 4009–4021. https://doi.org/10.1007/s00018-013-1307-3

Ricote, M., Huang, J., Fajas, L., & Glass, C. K. (1998). Expression of the peroxisome proliferator-activated receptor gamma (PPARgamma) in human atherosclerosis and regulation in macrophages by colony stimulating factors and oxidized low density lipoprotein. *Proceedings of the National Academy of Sciences of the United States of America, 95*(13), 7614–7619. https://doi.org/10.1073/pnas.95.13.7614

Saleh, M., Hogeveen, K., Fessard, V., & Le Hégarat, L. (2025). Mechanistic toxicity profiling of chemicals: A comparative study in 2D and 3D HepaRG models using high-content analysis. *Toxicology, 510*, 154280. https://doi.org/10.1016/j.tox.2025.154280

Saleh, M., Hogeveen, K., Thienpont, A., Demuynck, E., Fessard, V., Mertens, B., & Le Hégarat, L. (in preparation). Advancing genotoxicity assessments with transcriptomic biomarkers: From 2D to 3D HepaRG spheroid model.

Schaap, M. M., Zwart, E. P., Wackers, P. F. K., van Herwijnen, M. H., Thas, O., Kleinjans, J. C., & Luijten, M. (2012). Dissecting modes of action of non-genotoxic carcinogens in primary mouse hepatocytes. *Archives of Toxicology, 86*(11), 1717–1727. https://doi.org/10.1007/s00204-012-0883-6

Shen, W., Yang, L., Yang, Y., Zhang, Q., & Wang, J. (2023). PRDX6 promotes fatty acid oxidation via PLA2-dependent PPARα activation in rats fed high-fat diet. *Antioxidants & Redox Signaling, 38*(16–18), 1184–1200. https://doi.org/10.1089/ars.2022.0065

Siddique, Y. H., Varshney, H., Subhan, I., Gaur, K., Fatima, J., & Jyoti, S. (2024). Evaluation of the toxic potential of ethyl methanesulphonate (EMS) on Hydra vulgaris. *Toxicology Reports, 13*, 101839. https://doi.org/10.1016/j.toxrep.2024.101839

Smith, M. T. (2025). The key characteristics concept. *Current Opinion in Toxicology, 41*, 100515. https://doi.org/10.1016/j.cotox.2024.100515

Smith, M. T., Guyton, K. Z., Kleinstreuer, N., Borrel, A., Cardenas, A., Chiu, W. A., Felsher, D. W., Gibbons, C. F., Goodson, W. H., Houck, K. A., Kane, A. B., La Merrill, M. A., Lebrec, H., Lowe, L., McHale, C. M., Naylor, S., O'Brien, A. J., Pollak, M. N., Rieswijk, L., ... & Thomas, R. S. (2020). The key characteristics of carcinogens: Relationship to the hallmarks of cancer, relevant biomarkers, and assays to measure them. *Cancer Epidemiology, Biomarkers & Prevention, 29*(10), 1887–1903. https://doi.org/10.1158/1055-9965.EPI-19-1346

Stewart, B. W. (2019). Mechanisms of carcinogenesis: From initiation and promotion to the hallmarks. In R. A. Baan, B. W. Stewart, & K. Straif (Eds.), *Tumour site concordance and mechanisms of carcinogenesis*. International Agency for Research on Cancer. http://www.ncbi.nlm.nih.gov/books/NBK570326/

Valerie, K., & Povirk, L. F. (2003). Regulation and mechanisms of mammalian double-strand break repair. *Oncogene, 22*(37), 5792–5812. https://doi.org/10.1038/sj.onc.1206679

Way, J. M., Harrington, W. W., Brown, K. K., Gottschalk, W. K., Sundseth, S. S., Mansfield, T. A., Ramachandran, R. K., Willson, T. M., & Kliewer, S. A. (2001). Comprehensive messenger ribonucleic acid profiling reveals that peroxisome proliferator-activated receptor gamma activation has coordinate effects on gene expression in multiple insulin-

sensitive tissues. *Endocrinology, 142*(3), 1269–1277. https://doi.org/10.1210/endo.142.3.8037

Xiao, Q., Wang, J., Wang, L., & Ding, H. (2024). APOA1/C3/A4/A5 gene cluster at 11q23.3 and lipid metabolism disorders: From epigenetic mechanisms to clinical practices. *Biomedicines, 12*(6), 1224. https://doi.org/10.3390/biomedicines12061224

Yasuda, A., Murase, W., Kubota, A., Kamata, S., & Shioda, T. (2024). Effects of di-(2-ethylhexyl) phthalate and its metabolites on transcriptional activity via human nuclear receptors and gene expression in HepaRG cells. *Toxicology in Vitro, 101*, 105943. https://doi.org/10.1016/j.tiv.2024.105943

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Mariam Saleh: Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – Review & Editing, **Kevin Hogeveen**: Conceptualization, Supervision, Writing – Review & Editing, **Thibaut Leger**: Data curation, **Valérie Fessard:** Writing – Review & Editing, **Ludovic Le Hégarat:** Conceptualization, Supervision, Writing – Review & Editing.

Chapter III

"Phenotypic footprints of chemical carcinogens"

Revealing carcinogenic potential of chemicals: an *in vitro* evaluation using the Bhas42 cell transformation assay

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Preamble

The final objective of this study is to investigate phenotypic changes at the apical level induced by selected non-genotoxic carcinogens (NGTxC) using the Bhas42 Cell Transformation Assay (CTA). This includes assessing the assay's predictive capacity for identifying human carcinogens and comparing its outcomes with those obtained from other NAMs-based approaches implemented in chapter one of this project.

While Chapters One and Two primarily focused on elucidating NGTxC mode of action (MoAs) at cellular and molecular levels, Chapter Three shifts toward evaluating chemicals in a functional assay that more directly reflects the terminal phase of carcinogenicity. This chapter plays a pivotal role in the overall workflow, bridging early mechanistic insights with late-stage adverse outcomes such as tumor formation.

Given its sensitivity to both initiators (genotoxic carcinogens, GTxC) and promoters (NGTxC), the Bhas42 CTA was applied to a curated set of non-genotoxic chemicals selected based on toxicity profiles identified in Chapter One. The assay's core principle lies in detecting morphological and behavioral alterations in cultured cells with neoplastic transformation potential, specifically using Bhas42 cells. These changes, including enhanced cell proliferation, loss of contact inhibition, and acquisition of anchorage-independent growth, represent key events (KEs) in the multistage carcinogenesis process. Unlike mechanistic assays that target specific molecular or cellular pathways, the CTA offers an apical endpoint that integrates multiple underlying mechanisms of carcinogenicity.

Although the assay is not yet fully standardized, ongoing international validation efforts particularly those focused on the promotion assay, highlight its growing relevance for NGTxC detection. Incorporating the Bhas42 CTA into this project enables the evaluation of apical responses to a diverse set of NGTxC with varying toxicity profiles, while also allowing assessment of the assay's predictive performance, assay's applicability, and its potential utility for chemical classification. Given that only about 100 chemicals have been tested with the Bhas42 CTA to date, this study also contributes to expanding the existing dataset by including previously untested compounds, thereby helping to address critical data gaps in NGTxC evaluation.

Préambule

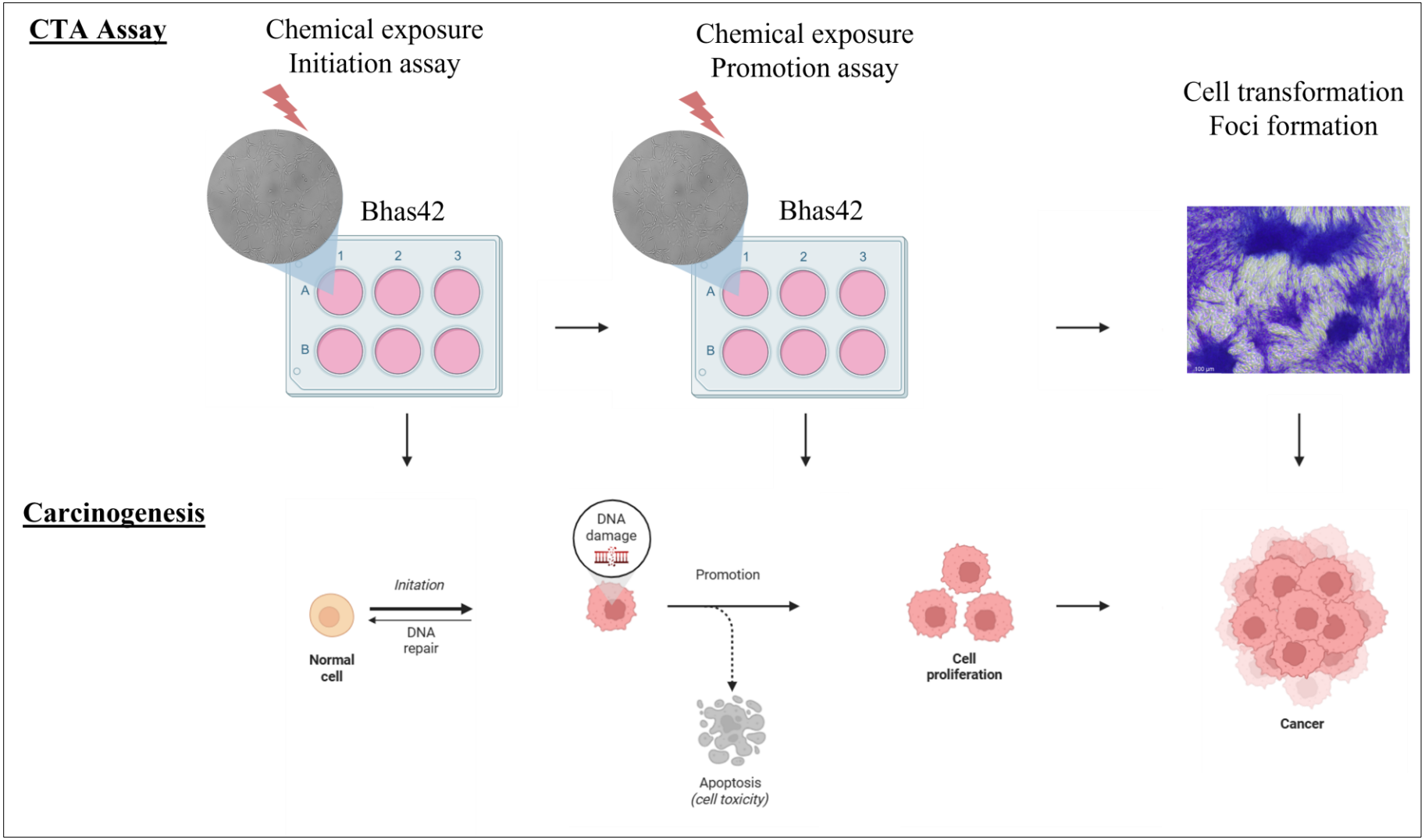
L'objectif final de cette étude est d'examiner les modifications phénotypiques au niveau apical induites par une sélection de cancérogènes non génotoxiques (NGTxC) à l'aide du test de transformation cellulaire Bhas42 (Bhas42 Cell Transformation Assay, CTA). Il s'agit également d'évaluer la capacité prédictive de ce test pour l'identification des cancérogènes humains et de comparer ses résultats à ceux obtenus avec d'autres approches fondées sur les NAMs, appliquées dans le premier chapitre de ce projet.

Alors que les chapitres 1 et 2 étaient principalement consacrés à l'élucidation des modes d'action (MoA) des NGTxC aux niveaux cellulaire et moléculaire, le chapitre 3 se concentre sur l'évaluation de substances chimiques dans un test fonctionnel reflétant plus directement la phase terminale du processus cancérogène. Ce chapitre occupe une place essentielle dans le déroulement global du projet, en établissant un lien entre les premiers événements mécanistiques et les effets indésirables tardifs, tels que la formation de tumeurs.

Compte tenu de sa sensibilité à la fois aux initiateurs (cancérogènes génotoxiques, GTxC) et aux promoteurs (NGTxC), le Bhas42 CTA a été appliqué à un ensemble ciblé de substances non génotoxiques sélectionnées sur la base des profils de toxicité identifiés au chapitre 1. Le principe fondamental de ce test repose sur la détection d'altérations morphologiques et comportementales dans des cellules en culture présentant un potentiel de transformation néoplasique, en particulier les cellules Bhas42. Ces modifications constituent des événements clés (KE) du processus multistade de cancérogenèse. Contrairement aux tests mécanistiques ciblant des voies moléculaires ou cellulaires spécifiques, le CTA fournit une mesure apicale intégrant plusieurs mécanismes sous-jacents de cancérogénicité.

Bien que ce test ne soit pas encore entièrement standardisé, les efforts internationaux de validation, notamment ceux portant sur l'essai de promotion, soulignent sa pertinence croissante pour la détection des NGTxC. L'intégration du Bhas42 CTA dans ce projet permet d'évaluer les réponses apicales à un ensemble diversifié de NGTxC présentant différents profils de toxicité, tout en examinant la performance prédictive du test, son applicabilité et son potentiel pour la classification des substances chimiques. Étant donné qu'environ 100 substances seulement ont été testées à ce jour avec le Bhas42 CTA, cette étude contribue également à enrichir la base de données existante en incluant des composés encore jamais évalués, comblant ainsi des lacunes critiques dans l'analyse des NGTxC.

Graphical abstract



Abstract

Carcinogens are broadly classified into Genotoxic Carcinogens (GTxC) and Non-Genotoxic Carcinogens (NGTxC). While a variety of *in vitro* genotoxicity assays are well established and available for testing genotoxic potential of chemicals, the identification of NGTxCs still relies heavily on long-term *in vivo* rodent bioassays, which are costly, time-consuming, and raise ethical concerns regarding animal use. Consequently, the development of alternative *in vitro* models to evaluate the carcinogenic potential of chemicals has become a key focus in regulatory toxicology. The Bhas42 Cell Transformation assay (CTA) has been recognized and used to assess both GTxC and NGTxC. This study applies the Bhas42 CTA to assess six chemicals (Chlorothalonil, Tebuconazole, Thioacetamide, Diethanolamine, Diethyl maleate,and Clofibrate) with diverse toxicity profiles, and aims to fill existing data gaps, to support the integration of Bhas42 CTA within New Approach Methodologies (NAMs) for chemical carcinogenicity assessment. Among the six tested chemicals, three produced reproducible positive responses in the promotion assay. The positive responses are consistent with previously reported evidence regarding the promotion potential of these chemicals, supporting the relevance of the assay for detecting tumor-promoting activity. In contrast, two negative compounds in promotion assay may reflect limitations related to metabolic competence in the *in vitro* system. In the initiation assay, the responses were not reproducible between experiments, preventing a clear classification of the tested compounds as initiators. This variability underscores the difficulty of achieving consistent initiation outcomes and indicates that additional experimental work, methodological refinement, and broader chemical testing are needed to strengthen understanding of this assay and improve its overall applicability.

Highlights

- Bhas42 assay demonstrated highly reproducible results in promotion assay.
- The Bhas42 CTA was able to predict 3 three out of the six tested chemicals in promotion assay.
- The Bhas 42 CTA is capable of assessing carcinogens with different MoA.
- Further advancement is needed to validate CTA Bhas42 assay.

Key words

New Approach Methodologies (NAMs), Carcinogens, Initiation potential, promotion potential, transformed foci.

1. Introduction

Cancer represents one of the major diseases causing death worldwide, with incidence and mortality rates continuing to rise significantly (Park et al., 2025). Evaluating the cancer-causing potential of both chemicals currently being used as well as the continually growing number of new chemicals and contaminants represents a significant global concern (Boobis et al., 2017). This concern underlines the importance of distinguishing between the different types of carcinogens and understanding the appropriate methods to detect their carcinogenic potential.

Carcinogens are broadly classified into Genotoxic Carcinogens (GTxC) and Non-Genotoxic Carcinogens (NGTxC) (Nohmi, 2018). GTxC directly damage DNA causing mutations, chromosomal breaks or alterations which can be detected via a standardized battery of *in vitro* assays (Baldrick, 2021). However, positive results from this *in vitro* genotoxicity battery often requires further *in vivo* follow up validation (Leopardi et al., 2010). In contrast, NGTxCs induce cancer via various mechanisms, other than genotoxic, for example through the disruption of cellular and molecular functions, or alterations of cell proliferation rates. The only validated method to assess carcinogenic potential is the long-term 2-year rodent assay *in vivo* bioassays (Jacobs et al., 2020; Suarez-Torres et al., 2021).

Rodent bioassays are still considered the gold standard for the assessment of the carcinogenic potential of chemicals (Suarez-Torres et al., 2020). Nevertheless, *in vivo* tests require a high number of animals resulting in extensive cost, prolonged experimental durations, and the extrapolation of the results from animals to humans can be challenging (Norman, 2019). Hence, there is an urgent need in regulatory toxicology to develop alternative *in vitro* approaches to evaluate carcinogenic potential of chemicals (Lilienblum et al., 2008; Barguilla et al., 2023).

In vitro Cell Transformation Assays (CTAs) represent a possible alternative to detect carcinogenic effects of chemicals (Chatterjee & Alfaro-Moreno, 2023). OECD (Organisation for Economic Co-operation and Development) test guidance already exists for the Syrian Hamster Embryo (SHE) and the Bhas42 CTAs (Steinberg, 2016). Based on these CTAs, the malignant transformation of the cells is associated with cellular alterations based on: 1) changes in cell morphology, 2) changes in growth patterns, 3) anchorage-independent growth (Steinberg 2016).

The Bhas 42 cell line was derived as a clone formed by the stable transfection of the v-Ha-*ras* oncogene into the BALB/3T3 A31-1-1 (Sasaki et al., 1988). Compared to the SHE and BALB/c 3T3 CTAs, the Bhas42 CTA demonstrate enhanced sensitivity and greater robustness. It

provides a shorter assay duration, with the ability to capture both initiation and/or promotion activities of the tested chemicals covering different steps in the carcinogenesis process (Sasaki et al., 2014; Pu et al., 2016). The assay is also amongst the *in vitro* tests that are under consideration for inclusion in the Integrated Approach to Testing and Assessment (IATA) for NGTxC (Colacci et al., 2023). Hence, the Bhas42 CTA could represent a promising alternative to animal testing for the evaluation of chemical carcinogenicity (Guichard et al., 2023).

Despite their advantages, Bhas42 CTAs also present several limitations, including challenges in inter-laboratory transferability, reliance on manual foci counting, the absence of metabolic competence and intercellular communication, and the lack of organ-specific carcinogenicity. In addition, the current limited chemical coverage generates notable data gaps, ultimately constraining its potential for full regulatory recognition and acceptance (Sakai et al., 2010; Colacci et al., 2023).

In this study, we applied the Bhas42 CTA to evaluate a panel of six chemicals (Chlorothalonil, Tebuconazole, Thioacetamide, Diethanolamine, Diethyl maleate, and Clofibrate) with distinct and well-defined toxicity profiles. By examining the assay's applicability across mechanistically diverse chemicals, this work contributes to filling existing data gaps and reinforcing the evidence base needed to support CTA performance. In doing so, it aligns with ongoing international initiatives aimed at validating CTAs and advancing their integration into *in vitro* carcinogenicity assessment frameworks within NAMs. Through the generation of new experimental data and the characterization of assay behaviour across different chemical profiles, this study seeks to strengthen the positioning of the Bhas42 CTA as an important tool that requires further validation for its integration into NGTxC assessment.

2. Materials and methods

2.1. Cell culture

Bhas42 cells, derived from the mouse fibroblastic BALB/c 3T3 clone A31-1-1 transfected with the v-Ha-ras gene (Sasaki et al., 1988), were obtained from the Japanese Collection of research Bioresources (Ibaraki City Osaka Prefecture, Japan) Cell Bank No.: JCRB0149. Bhas42 cell culture, master cell bank, and working cell banks were prepared following the OECD Guidance Document (GD) "GD No. 231" (OECD, 2016). Briefly, for initial expansion Bhas42 cells at low passage were maintained in Minimum Essential Medium (MEM, Merck, Darmstadt, Germany) supplemented with 10% FBS (Gibco, Thornton, Australia), and antibiotics (10.000 U/ml penicillin and 10.000 µg/ml streptomycin (Sigma, St. Louis, MO, USA). Cells were cultured in 75 cm2 culture flasks (Corning, Durham, USA) and seeded at a density of 5×10^5 cells/mL in a humidified 5% CO2 incubator at 37 °C. When cells reached about 70% confluence, they were sub-cultured with 1X Trypsin-EDTA (Paisley, UK), seeded for the Cell Growth Assay (CGA) in 96-well plates (Corning, Durham, USA) and for CTA in 6-well plates Corning, Durham, USA) and maintained in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12, Paisley, UK) supplemented with 5% FBS (Gibco, Thornton, Australia) and antibiotics. Schematic diagrams of cell seeding strategies are shown in Figure C.III 1 and Figure C.III 2.

2.2. Chemical selection and exposure

Six chemicals were selected for this study (Table C.III 1). Diethanolamine, Diethyl Maleate, Clofibrate, and Thioacetamide were supplied from Sigma (Saint-Quentin-Fallavier, France); Chlorothalonil and Tebuconazole were supplied from LGC (Molsheim, France). All chemicals were dissolved in DMSO (Sigma, Saint-Quentin-Fallavier, France) at a 0.1% final DMSO concentration. Concentrations for the Cell Growth Assay (CGA), Initiation (I) and Promotion (P) assays were prepared as serial dilutions in DF5F complete medium. All chemicals were prepared at a final concentration of 0.1% DMSO, except 3MCA which was used at a final concentration of 0.3% DMSO.

Table C.III 1. Chemicals selected for this study with information related to their CAS number, IARC classification, *in vitro* genotoxicity, available data on the mode of action (MoA), *in vivo* carcinogenicity, previous CTA data. "MLTK" *in vitro* Mouse lymphoma TK assay, "ND" No Data, "I" Initiation, "P" Promotion.

Chemical	Chlorothalonil (CT)	Tebuconazole (Tebu)	Thioacetamide (TAA)	Diethanolamine (DEA)	Diethyl Maleate (DEM)	Clofibrate (CF)
CAS	1897-45-6	107534-96-3	62-55-5	111-42-2	141-05-9	637-07-0
IARC classification	Group 2B	Not evaluated	Group 2B	Group 2B	Not evaluated	Group 3
In vitro genotoxicity (Vasseur &Lasne, 2012)	(-) Ames (+) MLTK	(ND) Ames (ND) MLTK	(-) Ames (-) MLTK	(-) Ames (-) MLTK	(ND) Ames (ND) MLTK	(-) Ames (ND) MLTK
MoA	Oxidative stress (Wang et al., 2024)	Lipid accumulation, Oxidative stress (Lee et al., 2024)	Mitochondrial dysfunction, Oxidative stress (Ezhilarasan, 2023)	Choline homeostasis (Niculescu et al., 2007)	Oxidative stress (Stevens & Anders, 1981)	Mitochondrial dysfunction, Lipid metabolism disruption, Oxidative stress (Zhao et al., 2023)
In vivo carcinogenicity	Paraneoplastic and neoplastic lesions for kidneys and forestomach in rat (EFSA, 2018)	No evidence in rodents and humans (EFSA, 2013)	Hepatocellular carcinoma and tumors of the bile duct in rodents (IARC, 1994)	Hepatocellular adenoma, and renal tubule adenoma in rodent (IARC, 2013)	ND	Hepatocellular carcinoma (IARC, 1996)
CTA (Vasseur &Lasne, 2012)	(+) 7.1-7.35 pH SHE (ND) Balb/c 3T3 (ND) C3H10T1/2	ND	(+) 7.0 pH SHE (-) C3H/10/T1/ 2	(+) 6.7 pH SHE (-) C3H10T1/2 (-) BALB/c 3T3	ND	(+) 6.7 pH SHE (+) 7.0 pH SHE (-) BALB/c 3T3 (+ P) C3H/ 10/T1/2

2.3. Pre-Cell Growth Assay

For concentration selection, a pre-CGA assay for I and P were conducted following the OECD GD No. 231. A panel of different concentrations was tested based on the previously reported toxicity and solubility in the literature (Table C.III 2).

Bhas42 cells were sub-cultured and seeded in 96-well plates (Corning life sciences, Durham, USA) at a density of 200 cells / well for pre-CGA (I) and a density of 400 cells / well for pre-CGA (P) (Figure C.III 1) (Figure C.III 2). Cells were treated with the chemical concentrations at day 1 post-seeding for (I), and day 4 for (P). In both pre-CGA assays, cells were washed twice with PBS at day 7 and incubated for two hours with 10% Alamar blue (Eugene, Oregon, USA) at 370 C and 5% CO2. Following incubation, 40µl from each well was transferred to a flat bottom 96-well polystyrene microplate (Thermo Fisher Scientific, Roskilde, Denmark). Alamar Blue fluorescence was measured at 530 nm excitation, and 590 nm emission using a FLUOstar Optima Microplate reader (BMG Labtech, Champigny-sur-Marne, France).

The percentage of cell viability was calculated according to the following equation:

Cell viability (%) = [Average of 8 replicates of Blank corrected raw data (sample) / Average of 8 replicates of Blank corrected raw data (control)] x 100

This experiment was repeated independently at least 2 times with 8 technical replicates for each tested concentration using the same batch of Bhas42 cells at passage 3. Cell viability percentage was analysed relative to the negative control (solvent) in each tested concentration, and IC_{10}, IC_{50}, and IC_{90} values were calculated using GraphPad prism (version 9.5.1) (Figure APP. III 1) (Figure APP. III 2)

2.4. Concentration selection

Based on the concentration selection criteria mentioned in OECD GD No 231, at least five concentrations per chemical were selected for the CTA (I & P): for the I assay, one non-toxic concentration, two concentrations near IC_{50}, and two concentrations near IC_{90}. For the P assay, two non-toxic concentrations, two concentrations near the IC_{50}, and one concertation near the IC_{90} for chemicals that induce cell inhibition (Figure C.III 1) (Table C.III 3).

Table C.III 2. The concentrations tested with the pre-Cell Growth Assay (Pre-CGA) for both initiation and promotion Cell Transformation Assays (I & P CTA).

Chemicals	**Pre-CGA (I) Tested concentrations (µM)**
CT	0.012, 0.024, 0.048, 0.097, 0.78, 1.56, 3.123, 6.25, 12.5, 25
Tebu	1.56, 3.123, 6.25, 12.5, 25, 50, 100
TAA	156.25, 321.5, 625, 1250, 2500, 5000, 10000
DEA	3.123, 6.25, 12.5, 25, 50, 100, 200, 250, 500, 1000
DEM	0.78, 1.56, 3.123, 6.25, 12.5, 25, 50, 100
CF	3.123, 6.25, 12.5, 25, 50, 100, 200, 250, 500, 1000
Chemicals	**Pre-CGA (P) Tested concentrations (µM)**
CT	0.048, 0.097, 0.195, 0.39, 0.78, 0.56, 1.56, 3.123, 6.25, 12.5, 25
Tebu	1.56, 3.123, 6.25, 12.5, 25, 50, 100, 150
TAA	321.5, 625, 1250, 2500, 5000, 10000, 20000
DEA	3.123, 6.25, 12.5, 25, 50, 100, 200, 250, 500, 1000
DEM	3.123, 6.25, 12.5, 25, 50, 100
CF	3.123, 6.25, 12.5, 25, 50, 100, 200, 250, 500, 1000

Table C.III 3. The concentrations selected for each chemical for the Cell Transformation Assay (CTA) experiment and its corresponding Cell Growth Inhibition Assay (CGA). In addition, the table summarizes the controls used in the study.

Chemicals	CAS	Solvent	Concentrations (µM)	
			Initiation	Promotion
CT	1897-45-6	DMSO 0.1%	10, 15, 20, 25, 27 Or 0.025, 0.05, 0.1, 0.2, 0.4	0.4, 0.80, 1, 1.2, 1.4
Tebu	107534-96-3	DMSO 0.1%	3.125, 6.25, 50, 100, 150	3.125, 6.25, 50, 100, 150
TAA	62-55-5	DMSO 0.1%	625, 1250, 1500, 2000, 2500	625, 1250, 1500, 2000, 2500
DEA	111-42-2	DMSO 0.1%	12.5, 25, 50, 100, 200	12.5, 25, 50, 100, 200
DEM	141-05-9	DMSO 0.1%	12.5, 25, 40, 55, 85	12.5, 25, 40, 55, 85
CF	637-07-0	DMSO 0.1%	12.5, 25, 50, 100, 200	12.5, 25, 50, 100, 200
Positive and negative controls				
3MCA	56-49-5	DMSO 0.3%	11 µM (3 µg/Ml)	/
TPA	16561-29-8	DMSO 0.1%	/	0.08 µM (0.05 µg/mL)
CAFF	58-08-2	H2O 5%	0.52 µM (0,1 µg/ml)	0.52 µM (0,1 µg/ml)

2.5. Cell Transformation Assay

In the CTA (I), Bhas 42 cells were seeded in 6-well plates (Corning, Durham, USA) at a density of 4000 cells/well and maintained with DF5F complete medium at 37 °C. One day 1, media was replaced and cells were treated with the chemicals as shown in Figure C.III 1 On day 4, media was replaced with fresh media. The duration of the assay was 21 days and media was changed every 2 days. Foci were stained at day 21 (Figure C.III 1).

In the CTA (P), Bhas 42 cells were seeded in 6-well plates (Corning, Durham, USA) at a density of 40000 cells / well and maintained with DF5F complete medium at 37 °C. Medium containing test chemicals was added at day 4 as shown in Figure C.III 2. Cells were treated for 10 days. Every second day during the treatment, the medium was replaced with fresh medium containing chemicals. At day 14, media was replaced with fresh media without treatments. Fresh medium was replaced every 2 days until day 21 when foci were stained (Figure C.III 2). For each concentration, one 6-well plate (6 technical replicates) was tested in both initiation and promotion CTAs, and two independent experiments (biological replicates) were conducted using the same Bhas42 cells at passage 3. Each assay included appropriate controls including positive controls: 3MCA for the initiation assay (Sigma, St Louis, USA) and TPA for the promotion assay (Sigma, Buchs, Switzerland). Caffeine (Sigma, St Louis, USA) was used as a negative control, and solvent and medium controls were also used, in accordance with OECD GD No. 231 (Table C.III 3). In addition, a concurrent CGA test was conducted with both CTAs (I and P) as described earlier.

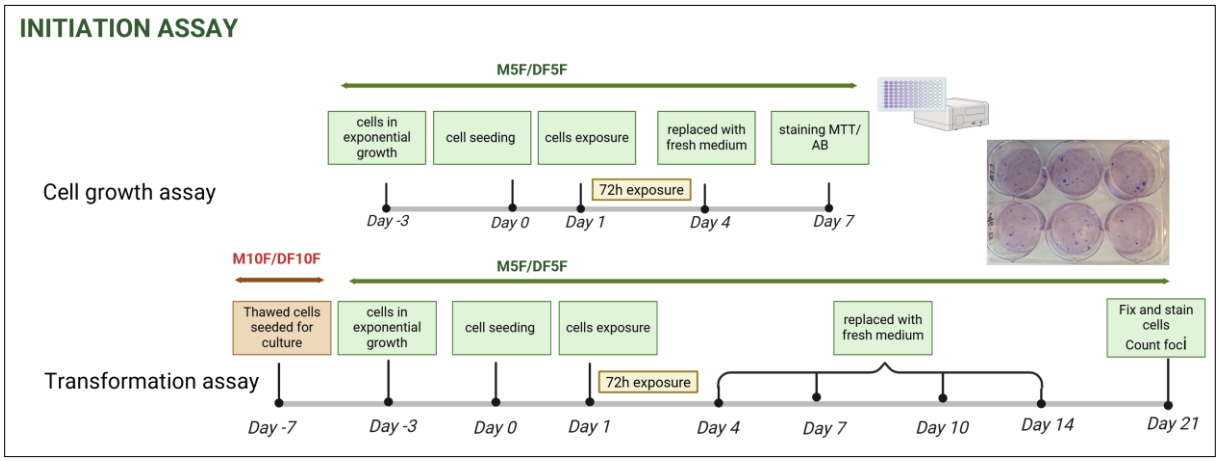


Figure C.III 1. Illustration representing the CTA initiation protocol and the concurrent CGA test.

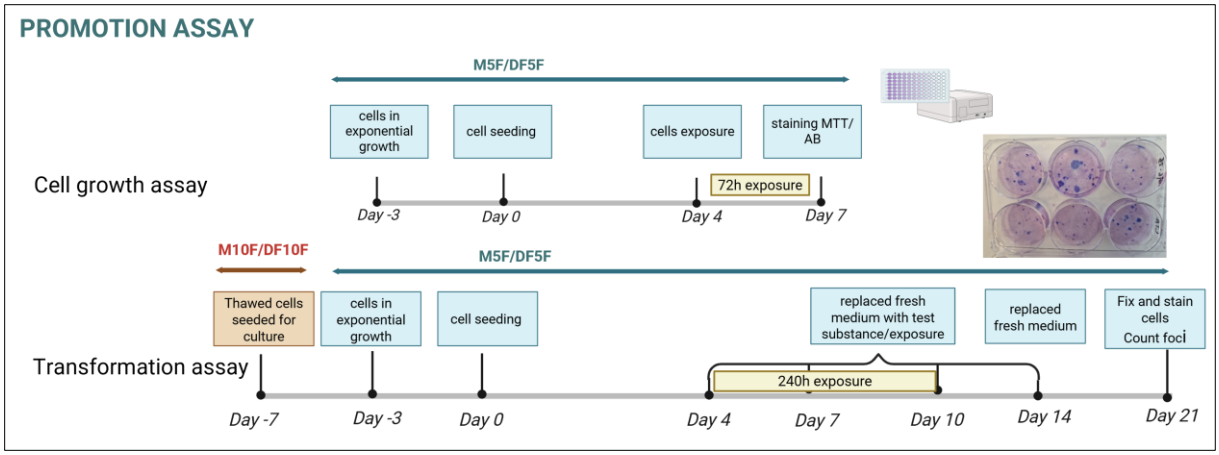


Figure C.III 2. Illustration representing the CTA promotion protocol and the concurrent CGA test.

2.6. Foci fixation, staining and evaluation

At day 21 in both CTAs (I and P), cells in 6-well plates were washed twice with PBS and fixed with 100% Ethanol (Merck, Darmstadt, Germany) at room temperature for 10 minutes. After fixation, cells were stained for 30 min at room temperature with 2 mL/well 5% Giemsa (Merck, Darmstadt, Germany) diluted in milliQ-water with gentle shaking. Cells were gently washed with tap water to remove the excess of Giemsa staining solution and plates were stored before evaluation and foci counting. Two different operators counted transformed foci visually under an inverted transmitted light microscope. The determination of the malignant transformed 'positive' foci were evaluated based on the previously published foci catalogue by (Sasaki et al., 2012) and OECD GD No. 231. As reported, the positive foci were assessed based on their morphological appearance including: 1) deep basophilic staining, 2) multilayer cell growth, 3) spindle shaped cells with random orientations, 4) invasive cells. The criteria of the foci evaluation used in this study are shown in Table APP. III 1.

2.7. Data and statistical analysis

Foci counting in each CTA experiment (CTA1 and CTA2) were analysed relative to the negative control (six technical replicates of the solvent control) using one-way ANOVA, followed by a Dunnett's post-hoc test using GraphPad prism (version 9.5.1). Data are presented as means (six replicates) ± SD. p-values<0.05 were considered statistically significant *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

The transformation frequency (TF) was calculated from the average of the six technical replicates / concentration from the two independent biological experiments, versus the negative

control taken from each biological experiment. This gave rise to a mean value presented as Transformation Frequency (TF).

2.8. Judgment

The results in the Bhas 42 CTAs (I or P) were considered positive when a statistically significant increase in the number of transformed foci compared to the negative for at least two sequential concentrations was observed. Results were considered negative when there was no OECDse showing a statistically significant increase in the number of foci in the two independent biological experiments. When the statistically significant increase was at only one dose, the chemical was evaluated to be positive if, in the second assay, a statistically significant increase in the number of transformed foci resulted at same first significant concentration or more. When differences in significance were obtained between the two independent biological experiments, the results were evaluated as equivocal, and more experiments are needed to evaluate the results.

3. Results

3.1. Evaluation of the carcinogenic initiation activity of the tested chemicals

The results of foci counting from the initiation assays performed with all test compounds (CT, Tebu, TAA, DEA, DEM, CF), together with the assay controls, are presented in Table C.III 4. In the first biological replicate (CTA1), the negative control (1% & 3% solvent control) did not meet the OECD acceptance criteria because the background level of transformed foci in the exceeded 10 foci. In addition, the fold-change between the mean amount of foci/plate in the positive control (3-MCA) and its corresponding solvent control (3% DMSO) was low, with only about 12-foci difference per plate. In the second biological replicate (CTA2), the assay again did not fully meet the OECD acceptance criteria, as the background level of transformed foci in the DMSO control was slightly above the threshold (12.33 ± 2.06), although very close to the acceptable limit. However, the fold-change between DMSO and 3-MCA in CTA2 was higher than in CTA1, indicating a clearer separation between negative and positive controls. Across both replicates, CF and TAA can be considered negative in the initiation assay, as no statistically significant increase in foci frequency was observed compared with the negative control (1% DMSO). For CT, Tebu, DEM, and DEA, a significant increase in foci frequency was detected at their highest tested concentrations in CTA2 but not in CTA1.

3.2. Evaluation of the carcinogenic initiation activity of the tested chemicals

Results from foci counting in the promotion assay for all tested chemicals (CT, Tebu, TAA, DEA, DEM, CF), together with the assay controls, are presented in Table C.III 5. In both biological replicates (CTA1 and CTA2), the negative control (0.1% DMSO) met the OECD acceptance criteria, as the number of background foci did not exceed 12 foci and are equal to 12.33 ± 1.21. A clear and statistically significant increase in transformed foci was observed with the positive control TPA compared with the solvent control in both replicates, confirming the responsiveness of the assay. A statistically significant concentration-dependent increase in transformed foci was observed for Tebu in both CTA1 and CTA2, up to 6.25 µM, demonstrating consistent promoter activity across experiments. In addition, DEM at 85 µM and CF at 200 µM induced significant increases in foci frequency compared with the negative control in both biological replicates. In contrast, CT, TAA, and DEA did not show significant promoter activity in either replicate. Overall, the promotion assay demonstrated high reproducibility between CTA1 and CTA2, with the same chemicals identified as positive or negative and with comparable response across both experiments.

Table C.III 4. Foci formation in the Initiation assay (I) for the tested chemicals and positive and negative controls in both independent biological experiments (CTA1 and CTA2). Data are presented as relative cell growth (%), mean of the foci counted from each plate (mean of 6 technical replicates), and the evaluation based on the within-run statistical analysis (6 technical replicates / condition vs 6 technical replicate of solvent negative control) performing using a one-way ANOVA, followed by a Dunnett's post-hoc test. Data are presented as means (six replicates) ± SD. p-values <0.05 were considered statistically significant *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. (N/A) data not available.

Chemical	Concentration (µM)	Initiation (I)					
		CTA1			CTA2		
		Relative cell growth (%)	Mean Foci/plate	Judgment	Relative cell growth (%)	Mean Foci/plate	Judgment
Controls							
DMSO	0.1 %	99,73	20 ±2	-	94.15	12.33 ±2.06	-
DMSO	0.3 %	83,20	19,33 ±1,03	-	86,04	8,66 ±1,7512	-
CAFF	0.52	88,35	21,5 ±2,25	-	94.11	14.66 ±2.94	-
H2O	5%	86,53	21,33 ±1,86	-	95.43	11.8 ±1.64	-
3MCA	11	66,36	30,33 ±2,58	+ ****	82.28	21.66 ±1.63	+ ****
Treatments							
CT	10(CTA1) 0.025(CTA2)	90,13	18,66 ±3,55	-	81,50	15.5 ±3.44	-
	15 (CTA1) 0.05(CTA2)	93,21	18,33 ±2,73	-	84,92	18.00 ±1.78	+ ***
	20(CTA1) 0.1(CTA2)	96,56	20,16 ±4,02	-	90,35	17.66 ±1.21	+ ***
	25(CTA1) 0.2(CTA2)	94,60	20 ±3,46	-	86,65	13.66 ±1.86	-

	27(CTA1) 0.4(CTA2)	96,79	18,66 ± 2,50	-	96,00	15.50 ±2.07	-
Tebu	3.125	96,90	13,83 ±2,56	-	87.72	11.16 ±2.71	-
	6.25	88,08	13 ±1,67	-	92.86	7.5 ±2.42	-
	50	75,59	16,33 ±1,96	-	100.2	11 ±2.28	-
	100	51,94	19,33 ±5,50	-	75.02	17 ±2.09	+ ***
	120	6,10	22,5 ±1,37	-	12.11	21 ±0.89	+ ****
TAA	625	113,14	11 ±1,26	-	115.78	12.83 ±2.48	-
	1250	111,98	11,33 ±1,21	-	122.28	14.83 ±1.47	-
	1500	110,43	11,5 ±1,04	-	116.59	13.66 ±1.50	-
	2000	113,88	12,66 ±2,06	-	110.69	14 ±2.19	-
	2500	101,12	16,83 ±1,94	-	116.19	14.16 ±2.13	-
DEA	12.5	104,91	16,16 ±1,72	-	88.65	17 ±2.09	+ **
	25	102,68	15,66 ±1,63	-	90.34	18.83 ±2.22	+ ***
	50	98,60	16,66 ±1,03	-	88.97	15.83 ±4.11	-
	100	92,93	16,5 ±2,42	-	94.33	15.16 ±3.81	-

	200	106,56	15,5 ±1,76	-	101.96	17.66 ±2.80	+ **
DEM	12.5	97,56	21 ±3,34	-	91.27	12.66 ±2.80	-
	25	99,51	21 ±1,41	-	94.09	16.83 ±1.60	+ **
	40	81,98	20,66 ±1,96	-	97.11	18.16 ±2.71	+ ***
	55	62,95	14,66 ±2,80	-	74.66	17 ±2.60	+ **
	85	42,57	16,16 ±4,07	-	56.88	15.66 ±2.50	-
CF	12.5	91,24	14,66 ±1,86	-	78.29	12.66 ±1.21	-
	25	88,56	17,66 ±2,25	-	81.59	12.16 ±2.48	-
	50	90,93	19,83 ±1,60	-	85.20	13 ±1.54	-
	100	87,27	17,16 ±2,13	-	84.54	11.83 ±1.16	-
	200	71,70	17,66 ±2,42	-	90.13	11 ±1.26	-

Table C.III 5. Data Foci formation in the Promotion assay (P) with the tested chemical and the assay controls in both biological independent experiments (CTA1 and CTA2). Data are presented as relative cell growth (%), mean of the foci counted from each plate (mean of 6 technical replicates), and the evaluation based on the within-run statistical analysis (6 technical replicates / condition vs 6 technical replicates of solvent negative control) using a one-way ANOVA, followed by a Dunnett's post-hoc test using GraphPad prism (version 9.5.1). Data are presented as means (six replicates) ± SD. p-values <0.05 were considered statistically significant: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. (N/A) data not available.

Chemical	Concentration (µM)	Promotion (P)					
		CTA1			CTA2		
		Relative cell growth (%)	Mean Foci/plate	Judgment	Relative cell growth (%)	Mean Foci/plate	Judgment
Controls							
DMSO	0.1 %	101,23	12,33 ±1,21	-	88,84	12,16 ±2,04	-
CAFF	0.52	88,09	7 ±1,41	-	91,80	9 ±1,78	-
H2O	5%	93,20	5,5 ±1,64	-	79,17	8,33 ±1,21	-
TPA	0.08	112,18	29 ±3,68	+ ****	113,28	33 ±3,68	+ ****
Treatments							
CT	0.4	90,56	10,66 ±1,36	-	63,74	11,5 ±0,83	-
	0.80.	69,77	17,66 ±2,50	-	65,31	11,5 ±0,54	-
	1	30,95	12 ±2,91	-	59,54	11,66 ±1,36	-
	1.2	1,76	6,83 ±1,72	-	32,95	9,16 ±0,75	-
	1.4	0	0,5 ±0,54	-	36,79	10,83 ±1,32	-

Tebu	3.125	95,08	15,33 ±1,21	-	80,59	11,33 ±1,03	-
	6.25	93,74	20 ±1,89	+ ****	75,44	18,33 ±1,86	+ ****
	50	80,54	23,16 ±1,94	+ ****	69,36	22,66 ±1,75	+ ****
	100	64,02	9,16 ±1,47	-	58,27	19 ±0,89	+ ****
	120	28,93	3,83 ±1,16	-	29,08	7,83 ±2,31	-
TAA	652	118,25	8,5 ±0,83	-	107,48	8,66 ±1,21	-
	1250	121,16	13,5 ±3,78	-	102,502	8,83 ±1,16	-
	1500	133,43	9 ±1	-	103,31	8,66 ±0,81	-
	2000	133,23	9,33 ±2,08	-	117,84	7,83 ±1,47	-
	2500	127,21	N/A	-	113,54	8,66 ±1,21	-
DEA	12.5	105,67	9,8 ±1,09	-	78,51	11,5 ±1,37	-
	25	108,37	11,33 ±1,21	-	76,87	11,83 ±0,98	-
	50	109,61	10,83 ±0,75	-	81,57	11,33 ±1,03	-
	100	112,49	11,8 ±0,83	-	86,71	12,33 ±1,63	-
	200	113,15	12,2 ±1,09	-	96,33	13,5 ±1,22	-

DEM	12.5	119,56	12,4 ±1,51	-	91,24	11,5 ±1,048	-
	25	126,43	10,8 ±0,83	-	94,94	12,33 ±1,63	-
	40	120,18	15,33 ±2,06	-	93,67	15 ±2,09	-
	55	130,69	16,16 ±1,60	-	97,67	14 ±2,19	-
	85	112,54	25,16 ±3,12	+ ****	87,81	26,66 ±2,42	+ ****
CF	12.5	96,76	14,83 ±1,72	-	65,99	13 ±1,78	-
	25	98,80	14 ±3,89	-	66,26	12,33 ±1,50	-
	50	95,27	13,83 ±3,60	-	71,11	13,16 ±1,60	-
	100	100,84	12,16 ±3,48	-	73,74	12,16 ±1,94	-
	200	101,56	20,66 ±1,75	+ ****	76,31	18,66 ±1,21	+ ****

3.3. Overall carcinogenic potential

The TF percentage was calculated for all chemicals at all tested concentrations for each independent Initiation and Promotion CTA experiments in Figure C.III 3 and Figure C.III 4.. In the initiation assay, all the tested compounds showed no trend or increase in the TF except highest concentrations tested with Tebu (Figure C.III 3). However, due to the equivocal results obtained in the initiation assay the two independent experiments with Tebu, along with DEA, and DEM, the overall evaluation was for these chemicals inconclusive (Table C.III 6).

The results for CT were not considered from the initiation assays as two different sets of concentrations were tested in the two independent biological replicates. The rest of chemicals such as CT, TAA, and CF were evaluated as negative for initiation (Table C.III 6). In the Promotion assay, Tebu, DEM, and CF, were attributed an overall positive evaluation (Figure C.III 4) (Table C.III 6).

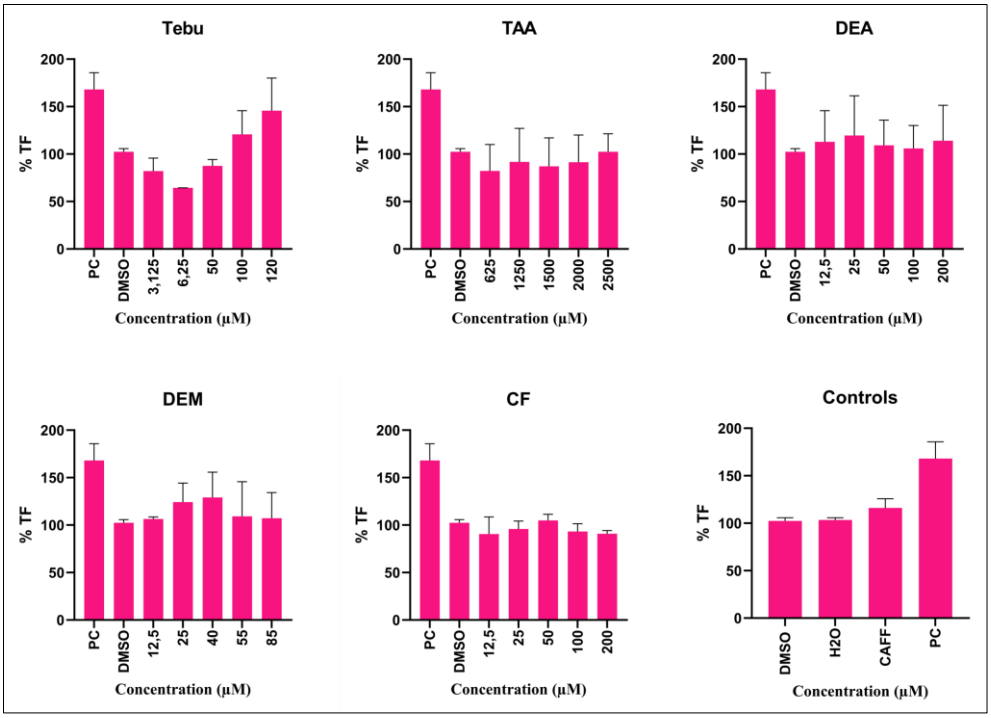


Figure C.III 3. Transformation Frequency (TF) across 2 independent initiation experiments. The mean of the six technical replicates from each biological independent experiment across all the tested concentrations such as CT, Tebu, TAA, DEA, DEM, and CF and the assay controls such as solvent (DMSO 0.1%), H2O (5%), CAFF (0.1µg/ml), and 3MCA (1µg/ml). Data are presented as mean ± SD.

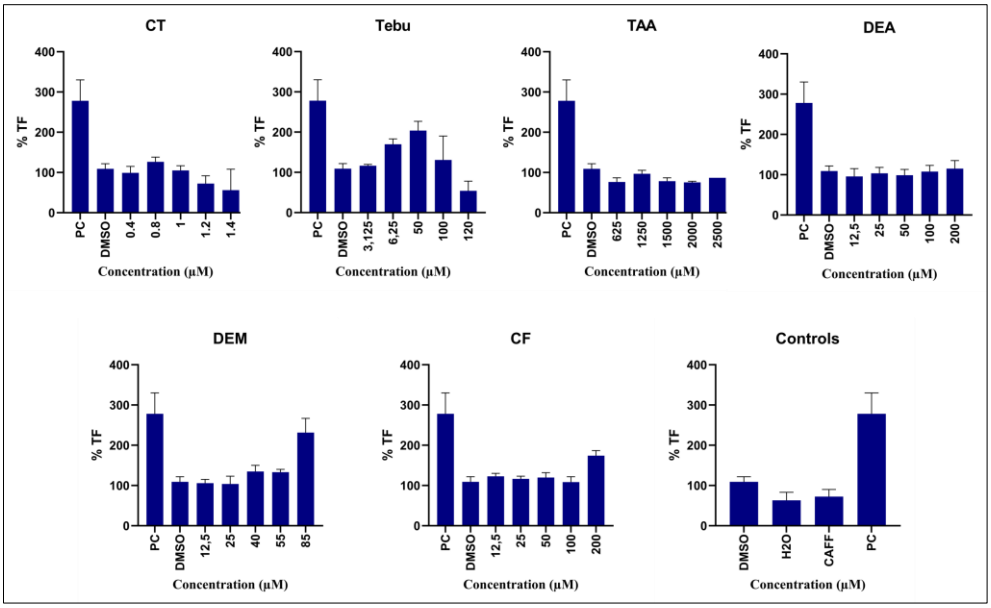


Figure C.III 4. Transformation Frequency (TF) across 2 independent promotion experiments. The mean of the six technical replicates from each biological independent experiment across all the tested conditions such as CT, Tebu, TAA, DEA, DEM, and CF and the assay controls such as solvent (DMSO 0.1%), H2O (5%), CAFF (0.1 µg/ml), and TPA (0,05 µg/ml). Data are presented as mean ± SD.

Table C.III 6. Overall assessment across the two independent experiments (CTA1 and CTA2).

Chemicals	Initiation		Overall evaluation	Promotion		Overall evaluation
	CTA1	CTA2		CTA1	CTA2	
DMSO	-	-	-	-	-	-
H2O	-	-	-	-	-	-
CAFF	-	-	-	-	-	-
3MCA	+	+	+	/	/	/
TPA	/	/	/	+	+	+
CT	-	+	**Inconclusive**	-	-	-
Tebu	-	+	**Inconclusive**	+	+	+
TAA	-	-	-	-	-	-
DEA	-	+	**Inconclusive**	-	-	-
DEM	-	+	**Inconclusive**	+	+	+
CF	-	-	-	+	+	+

4. Discussion

Assessing NGTxC activity remains particularly challenging both *in vivo* and *in vitro*. *In vivo* assessment relies heavily on long-term animal bioassays, which are resource-intensive and time-consuming. *In vitro* evaluation is also complex because NGTxCs act through diverse MoAs, including inflammation, immune modulation, sustained proliferative signalling, epigenetic alterations, and metabolic disturbances rather than direct DNA damage. Consequently, no single *in vitro* assay can capture the full spectrum of biological events involved in NGTxC processes. To address these limitations, integrated testing strategies have been proposed, most notably the IATA for NGTxCs developed by the OECD, which integrates mechanistic information from multiple sources within a weight-of-evidence (WoE) framework.

Within this context, the CTA has gained attention as an *in vitro* model capable of predicting the carcinogenic potential of chemicals, including both initiation and promotion events. Among available models, the Bhas42 CTA is currently under consideration for inclusion within the NGTxC IATA. This assay enables the modeling of chronic exposure conditions through extended treatment periods of up to 21 days for both initiation and promotion activities. However, despite these advantages, its broader regulatory acceptance remains limited due to the relatively small number of chemicals tested to date, existing data gaps, and challenges related to inter-laboratory transferability and protocol standardisation.

In this context, the Bhas42 CTA was applied to evaluate six chemicals with different MoAs that had not previously been assessed using this assay. This study aimed to contribute to filling existing data gaps and to further evaluate the applicability and potential challenges associated with the Bhas42 CTA. Both initiation and promotion assays were conducted, each accompanied by a concurrent CGA to assess cytotoxicity and support the interpretation of transformation responses.

TAA and CF were negative in both CTA experiment initiation assay. While CT, Tebu, DEA, and DEM were negative in the first biological experiment of the Bhas42 initiation assay (CTA1), a statistically significant concentration-dependent increase in transformation frequency was observed with CT, Tebu, DEA, and DEM in the second biological experiment (CTA2). These overall equivocal responses resulted from variability between independent tests, which represents a commonly observed issue associated with the initiation assay and can arise from several biological and technical factors, such as high background TF levels (Sakai et al., 2010). As a result, when the effects of test chemicals are weak, elevated background TF levels

can influence the relative increases observed between independent experiments, leading to equivocal outcomes. Importantly, despite the lack of statistical significance in CTA1, the overall patterns for Tebu were similar across both experiments, where a clear upward trend was already visible in CTA1 and the magnitude of the response closely matched that observed in CTA2. The apparent "negative" outcome in CTA1 therefore likely reflects assay performance constraints, including the elevated background in the DMSO control and the low fold-change difference between the negative and positive controls. These relatively small differences between DMSO and 3-MCA in CTA1 may have reduced the assay's ability to resolve treatment-related increases. While 3-MCA is the recommended initiator, such variability suggests that its performance may not always provide optimal contrast under all experimental conditions. Taken together, the lack of statistical significance in CTA1 is more likely attributable to technical limitations than to true differences in initiation activity.

Studies *in vitro* have reported that Tebuconazole can induce effects resembling genotoxicity under certain conditions, although this does not necessarily indicate a primary genotoxic mechanism. In human HCT116 cells, Tebuconazole exposure caused cytotoxicity associated with increased reactive oxygen species (ROS) production, lipid peroxidation, mitochondrial dysfunction, and DNA fragmentation, suggesting stress-mediated damage rather than direct DNA interaction (Gutiérrez-Praena et al., 2021). Similarly, sub-cytotoxic concentrations of Tebuconazole in HEp-2 cells induced DNA damage as measured by micronucleus and comet assays (Martins et al., 2023). *In vivo*, Ben Othmène et al. (2020) reported that Tebuconazole induced DNA damage and apoptosis in the kidneys of rats. Together, these findings suggest that Tebuconazole has the potential to trigger DNA damage markers both *in vitro* and *in vivo*, often linked to oxidative stress and cytotoxicity, but they do not necessarily demonstrate that Tebuconazole acts as a classical genotoxic agent through direct interaction with DNA. This in line with our finding, Tebu was positive in the second initiation assay (CTA2), and these findings suggest that Tebu could result in DNA damage.

Building on this, and considering that there is little *in vitro* or *in vivo* evidence indicating that chemicals such as CT, DEA, and DEM induce genotoxic effects and are therefore less likely to generate positive results in the initiation assay, these chemicals are more likely to be classified as non-genotoxic compounds. Indeed, DEA has been classified as a non-genotoxic carcinogen by Kirkland et al. (2016) (Kirkland et al., 2016). CT, Tebu, and DEM are also act through different mechanisms rather the direct DNA effect and are known inducers of oxidative stress (da Silva Barreto et al., 2020; Vilas Bôas Correia et al., 2025; Li et al., 2025). Consequently,

the variability observed with CT, Tebu, DEA, and DEM in the initiation assay should be interpreted as biologically inconclusive, pending confirmation in additional experiments. Furthermore, the negative results observed in the initiation assay with TAA and CF at the concentrations tested in this study suggest that these compounds do not exert direct DNA-damaging or genotoxic initiation effects, which is consistent with their known negative genotoxicity profiles *in vitro* and *in vivo* (Boerrigter, 2004; Sui et al., 2015).

Bhas42 cells are pre-initiated due to their transfection with an activated ras oncogene (v-Ha-ras), which increases sensitivity for detecting chemicals with non-genotoxic tumor-promoting mechanisms (Sakai et al., 2010). The clear positive dose-response effect observed in the promotion assay strengthens our confidence that Tebu could represent a potential carcinogen acting via a promotion mechanism, capable of promoting cell transformation in pre-initiated cells. The results obtained were highly reproducible, demonstrating the robustness of the assay. DEM and CF showed positive responses in both replicates but did not exhibit a clear dose-response relationship. Despite the absence of a dose-response effect, both chemicals were considered positive because transformation was reproducibly observed in two independent experiments, supporting that these compounds exert genuine promotion activity even without a clear dose-response. These findings provide additional evidence that these compounds may act as carcinogens through a non-genotoxic (NGTx) mechanism. For CF, the results align with *in vivo* evidence indicating its potential to induce hepatocellular carcinoma in humans (IARC, 1996), suggesting that CF is likely a carcinogen acting as a promoter since it was negative in initiation and positive in promotion. There is no *in vivo* evidence of human carcinogenicity for Tebu and DEM, and no human studies have evaluated cancer incidence or long-term outcomes following Tebu and DEM exposure.

Our promotion assay results further support the regulatory validation of the Bhas42 promotion CTA, emphasizing reproducibility across biological replicates for positive chemical classification in a CTA (Sakai et al., 2011) and reinforcing the assay's utility for identifying potential tumor promoters. DEM and CF were both positive in the promotion assay, and literature evidence indicates that these compounds do not exert genotoxic effects *in vivo* or *in vitro*, leaving their carcinogenic classification uncertain. Nevertheless, DEM and CF display mechanisms of toxicity *in vitro* that could contribute to non-genotoxic carcinogenesis. For example, in the HepaRG 3D liver model, CF induces neutral lipid accumulation and affects mitochondrial membrane hyperpolarization (Saleh et al., 2025). Since these compounds were

positive in promotion and negative in initiation assays, this suggests that CF may act as a carcinogen by promoting malignant cell transformation. These findings are consistent with Vasseur and Lasne (2012), where CF was positive in the C3H/10T1/2 CTA. Similarly, the positive promotion results obtained with DEM could be explained by its ability to induce oxidative stress (Saleh et al., 2025). Moreover, DEM's positive result in the second initiation assay replicate (CTA2) could also be attributed to high oxidative stress induction, similar to Tebu. In contrast, CT, which also induces oxidative stress, did not elicit a promotion response in our assay, although it showed a positive initiation result in CTA2 similar to Tebu and DEM. These findings hypothesis align with the broader IATA framework for non-genotoxic carcinogens (NGTxCs), in which multiple mechanistic events contribute to tumor development, including receptor-mediated signalling alterations, sustained stimulation of cell proliferation, oxidative stress, epigenetic modifications, disruption of gap junction intercellular communication, immune modulation, and resistance to apoptosis. Persistent activation of mitogenic pathways promotes continuous cell division, increasing the likelihood of spontaneous mutations and clonal expansion, while oxidative stress activates inflammatory pathways and cellular stress responses that facilitate tumor promotion. Additionally, epigenetic dysregulation, such as alterations in DNA methylation, histone modifications, and non-coding RNA expression, can modify gene expression to activate oncogenic pathways or silence tumor suppressor genes without direct DNA damage. Collectively, these mechanistic events correspond to the hallmarks of cancer, which define the key biological capabilities acquired during tumorigenesis (Jacobs et al., 2024).

Surprisingly, TAA yielded negative results in both the initiation and promotion CTA experiments. TAA is a well-known hepatotoxicant with demonstrated hepatocarcinogenic activity in rodents and potential carcinogenicity in humans, consistently inducing hepatocellular carcinoma *in vivo*. Its activity has also been reported as positive under (+) 7.0 pH SHE conditions. The carcinogenic and toxic effects of TAA depend on extensive CYP2E1-mediated conversion to its S-oxide metabolites. Without this metabolic activation, TAA's activity cannot be detected *in vitro* (Kang et al., 2008; Hajovsky et al., 2012). Therefore, the negative results observed in our study likely reflect the limited metabolic capacity of Bhas42 cells, which may be insufficient to generate the active metabolites required for TAA-mediated toxicity and transformation.

Similarly, the negative results observed with DEA in the promotion assay were unexpected, given that it was reported as positive in a previous CTA study using SHE cells. This lack of response may reflect species-specific differences in cellular and metabolic properties necessary to trigger cell proliferation or transformation pathways. DEA acts as a disruptor of choline homeostasis, and species-specific variations in choline metabolism have been proposed to explain why DEA is carcinogenic in mice but not in rats (Niculescu et al., 2007; Li et al., 2025). These differences may account for the discrepancy between its positive effect in SHE cells and the negative outcome observed in our Bhas42 promotion assay.

The compounds that we tested in the Bhas42 CTA are known to act through various MoAs, some of which, such as oxidative stress, are common between chemicals. These compounds are known to act through multiple MoAs, targeting diverse cellular pathways such as lipid accumulation, mitochondrial dysfunction, and oxidative stress. Despite these differences in the underlying mechanisms, the CTA is able to detect their potential to act as promoters, demonstrating its ability to detect promoters with multiple biological responses. However, CTA might be insensitive to some promoter's activity, and in particular to compounds that require metabolic activation.

Our results support the utility of the Bhas42 assay for predicting NGTxC potential and distinguishing promoter-type activity from direct genotoxic initiation. However, several important improvements could enhance the assay's performance. For example, according to OECD guidance, the pre-CGA test for the promotion assay is recommended to last 3 days; however, the cytotoxicity results from this pre-CGA test were markedly different from those obtained during the concurrent 10-day cytotoxicity assessment in the CTA. To achieve more accurate concentration settings that reflect the toxicity observed throughout the CTA, the pre-cytotoxicity test should be extended in duration. These observations are consistent with findings reported by Arai et al. (2010), who documented similar discrepancies between pre-test dose-setting results and concurrent cytotoxicity measurements in the Bhas42 CTA promotion assay. In contrast, no such issues were observed for the initiation assay, as the pre-cytotoxicity assessment and the treatment phase occur under the same conditions, ensuring consistency.

Additional mechanistic information could be generated in the context of the CTA through the integration a more mechanistic-based method such as transcriptomics to evaluate the carcinogenic potential of chemicals at the molecular level (Ohmori et al., 2022). This may be particularly important in the case of weak positive compounds where the suspected carcinogen

might induce critical changes at the molecular level but not entirely sufficient for the induction of foci formation in 21 days. In addition to difficulties associated with manual foci counting which can be subjective, the transformics assays (coupling CTA assay with transcriptomic analysis) could be promising for the evaluation of morphological and molecular modifications accompanying the carcinogenic process. This could allow for more predictive assessments of carcinogenic potential by taking into account the diverse underlying molecular mechanisms. In parallel to the current study, we have conducted a transformics study with the effective concentrations of compounds showing a positive response in the Bhas CTA both initation and promotion assays.

Overall, the Bhas42 CTA represents a promising approach for the evaluation of chemical carcinogenicity, particularly for promoter-type agents. With the assay now progressing through the OECD validation program, there is strong momentum toward establishing an OECD Test Guideline in the near future. This study provides further support for the reliability of the Bhas42 CTA and represents an important step forward in the adoption of mechanistically informed non-genotoxic carcinogenicity testing. Nevertheless, additional work is required to fully validate this *in vitro* method for regulatory use.

In the present study, we applied the Bhas42 CTA to, with the overarching aim of assessing the assay's predictivity and sensitivity across diverse NGTxC-related MoAs, and determining its ability to detect and discriminate non-genotoxic carcinogens. Beyond evaluating overall performance, this work also explored how consistently the assay captures key phenotypic changes associated with neoplastic transformation across chemicals with differing toxicity profiles. By testing compounds spanning multiple mechanistic domains, we were able to better define the strengths and limitations of the assay, identify potential gaps in sensitivity for specific pathways, and evaluate its potential contribution to chemical classification within a NAMs-based carcinogenicity framework.

Given the historically limited number of chemicals tested with the Bhas42 CTA, inclusion of additional substances expands the existing dataset and supports ongoing efforts to refine and validate the assay at the international level. In this context, the present study not only benchmarks the assay's performance but also strengthens the evidence base required for its broader regulatory and scientific applicability.

References

Andrioli, N. B., Nieves, M., Poltronieri, M., Bonzon, C., & Chaufan, G. (2023). Genotoxic effects induced for sub-cytotoxic concentrations of tebuconazole fungicide in HEp-2 cell line. *Chemico-Biological Interactions*, *373*, 110385. https://doi.org/10.1016/j.cbi.2023.110385

Arai, S., Tanaka, N., Sasaki, K., & Sakai, A. (2010). A study on the dose setting of test chemicals for the promotion assay in Bhas 42 cell transformation assay. *Alternatives to Animal Testing and Experimentation*, *15*(1), 6–13. https://doi.org/10.11232/aatex.15.6

Audebert, M., Assmann, A.-S., Azqueta, A., et al. (2023). New approach methodologies to facilitate and improve the hazard assessment of non-genotoxic carcinogens—a PARC project. *Frontiers in Toxicology*, *5*. https://doi.org/10.3389/ftox.2023.1220998

Baldrick, P. (2021). Genotoxicity test battery – An assessment of its utility in early drug development. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *868–869*, 503388. https://doi.org/10.1016/j.mrgentox.2021.503388

Barguilla, I., Maguer-Satta, V., Guyot, B., Pastor, S., Marcos, R., & Hernández, A. (2023). In vitro approaches to determine the potential carcinogenic risk of environmental pollutants. *International Journal of Molecular Sciences*, *24*(9), 7851. https://doi.org/10.3390/ijms24097851

Boerrigter, M. E. T. I. (2004). Mutagenicity of the peroxisome proliferators clofibrate, Wyeth 14,643 and di-2-ethylhexyl phthalate in the lacZ plasmid-based transgenic mouse mutation assay. *Journal of Carcinogenesis*, *3*(1), 7. https://doi.org/10.1186/1477-3163-3-7

Boobis, A., Brown, P., Cronin, M. T. D., et al. (2017). Origin of the TTC values for compounds that are genotoxic and/or carcinogenic and an approach for their re-evaluation. *Critical Reviews in Toxicology*, *47*(8), 705–727. https://doi.org/10.1080/10408444.2017.1318822

Chatterjee, N., & Alfaro-Moreno, E. (2023). In vitro cell transformation assays: A valuable approach for carcinogenic potentiality assessment of nanomaterials. *International Journal of Molecular Sciences*, *24*(9), 8219. https://doi.org/10.3390/ijms24098219

Colacci, A., Corvi, R., Ohmori, K., et al. (2023). The cell transformation assay: A historical assessment of current knowledge of applications in an integrated approach to testing and assessment for non-genotoxic carcinogens. *International Journal of Molecular Sciences*, *24*(6), 5659. https://doi.org/10.3390/ijms24065659

da Silva Barreto, J., de Melo Tarouco, F., & da Rosa, C. E. (2020). Chlorothalonil causes redox state change leading to oxidative stress generation in *Danio rerio*. *Aquatic Toxicology*, *225*, 105527. https://doi.org/10.1016/j.aquatox.2020.105527

European Food Safety Authority (EFSA). (2013). Conclusion on the peer review of the pesticide risk assessment of the active substance S-abscisic acid. *EFSA Journal*, *11*(8), 3341. https://doi.org/10.2903/j.efsa.2013.3341

European Food Safety Authority, Arena, M., Auteri, D., et al. (2018). Peer review of the pesticide risk assessment of the active substance chlorothalonil. *EFSA Journal*, *16*(1), e05126. https://doi.org/10.2903/j.efsa.2018.5126

Ezhilarasan, D. (2023). Molecular mechanisms in thioacetamide-induced acute and chronic liver injury models. *Environmental Toxicology and Pharmacology*, *99*, 104093. https://doi.org/10.1016/j.etap.2023.104093

Guichard, Y., Savoy, C., & Gaté, L. (2023). Can a 12-gene expression signature predict the cell transforming potential of tumor promoting agents in Bhas 42 cells? *Toxicology Letters*, *389*, 11–18. https://doi.org/10.1016/j.toxlet.2023.10.006

Hajovsky, H., Hu, G., Koen, Y., et al. (2012). Metabolism and toxicity of thioacetamide and thioacetamide S-oxide in rat hepatocytes. *Chemical Research in Toxicology*, *25*(9), 1955–1963. https://doi.org/10.1021/tx3002719

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. (1994). *Some industrial chemicals*. International Agency for Research on Cancer. https://www.ncbi.nlm.nih.gov/books/NBK507472/

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. (1996). *Printing processes and printing inks, carbon black and some nitro compounds*. International Agency for Research on Cancer. https://www.ncbi.nlm.nih.gov/books/NBK424318/

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. (2013). *Some chemicals present in industrial and consumer products, food and drinking-water*. International Agency for Research on Cancer.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. (2013). *Some chemicals present in industrial and consumer products, food and drinking-water* (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 101). International Agency for Research on Cancer. https://publications.iarc.who.int/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Some-Chemicals-Present-In-Industrial-And-Consumer-Products-Food-And-Drinking-water-2012

Jacobs, M. N., Colacci, A., Corvi, R., et al. (2020). 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. *Archives of Toxicology*, *94*(8), 2899–2923. https://doi.org/10.1007/s00204-020-02784-5

Kang, J. S., Wanibuchi, H., Morimura, K., et al. (2008). Role of CYP2E1 in thioacetamide-induced mouse hepatotoxicity. *Toxicology and Applied Pharmacology*, *228*(3), 295–300. https://doi.org/10.1016/j.taap.2007.11.010

Kirkland, D., Kasper, P., Martus, H.-J., et al. (2016). Updated recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *795*, 7–30. https://doi.org/10.1016/j.mrgentox.2015.10.006

Leopardi, P., Cordelli, E., Villani, P., et al. (2010). Assessment of in vivo genotoxicity of the rodent carcinogen furan: Evaluation of DNA damage and induction of micronuclei in mouse splenocytes. *Mutagenesis*, *25*(1), 57–62. https://doi.org/10.1093/mutage/gep043

Li, C. Y., Zhao, W. J., Qu, Y. N., & Zhang, Y. B. (2025). Proteomic insights into DEM-induced oxidative stress: Glycolytic and antioxidant synergy in beef colour stability. *Food Chemistry*, *494*, 146202. https://doi.org/10.1016/j.foodchem.2025.146202

Lilienblum, W., Dekant, W., Foth, H., et al. (2008). Alternative methods to safety studies in experimental animals: Role in the risk assessment of chemicals under the new European chemicals legislation (REACH). *Archives of Toxicology*, *82*(4), 211–236. https://doi.org/10.1007/s00204-008-0279-9

Louekari, K., & Jacobs, M. N. (2024). A modular strategy for the testing and assessment of non-genotoxic carcinogens. *Archives of Toxicology*, *98*(8), 2463–2485. https://doi.org/10.1007/s00204-024-03753-y

Niculescu, M. D., Wu, R., Guo, Z., da Costa, K. A., & Zeisel, S. H. (2007). Diethanolamine alters proliferation and choline metabolism in mouse neural precursor cells. *Toxicological Sciences*, *96*(2), 321–326. https://doi.org/10.1093/toxsci/kfl200

Nohmi, T. (2018). Thresholds of genotoxic and non-genotoxic carcinogens. *Toxicological Research*, *34*(4), 281–290. https://doi.org/10.5487/TR.2018.34.4.281

Othmène, Y. B., Hamdi, H., Ben Salem, I., Annabi, E., Amara, I., Neffati, F., Najjar, M. F., & Abid-Essefi, S. (2020). Oxidative stress, DNA damage and apoptosis induced by tebuconazole in the kidney of male Wistar rat. *Chemico-Biological Interactions*, *330*, 109114. https://doi.org/10.1016/j.cbi.2020.109114

Othmène, Y. B., Hamdi, H., Ben Salem, I., et al. (2020). Oxidative stress, DNA damage and apoptosis induced by tebuconazole in the kidney of male Wistar rat. *Chemico-Biological Interactions*, *330*, 109114. https://doi.org/10.1016/j.cbi.2020.109114

Othmène, Y. B., Salem, I. B., Hamdi, H., Annabi, E., & Abid-Essefi, S. (2021). Tebuconazole induced cytotoxic and genotoxic effects in HCT116 cells through ROS generation.

Pesticide Biochemistry and Physiology, *174*, 104797. https://doi.org/10.1016/j.pestbp.2021.104797

Park, J. Y., Georges, D., Alberts, C. J., Bray, F., Clifford, G., & Baussano, I. (2025). Global lifetime estimates of expected and preventable gastric cancers across 185 countries. *Nature Medicine*. Advance online publication. https://doi.org/10.1038/s41591-025-03793-6

Pu, J., Wang, Y., Qi, N., et al. (2016). A high-throughput Bhas 42 cell transformation assay for the determination of the carcinogenicity of three herbal extracts. *Fundamental Toxicological Sciences*, *3*(4), 157–166. https://doi.org/10.2131/fts.3.157

Sakai, A., Sasaki, K., Hayashi, K., et al. (2011). An international validation study of a Bhas 42 cell transformation assay for the prediction of chemical carcinogenicity. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *725*(1), 57–77. https://doi.org/10.1016/j.mrgentox.2011.07.006

Sakai, A., Sasaki, K., Muramatsu, D., et al. (2010). A Bhas 42 cell transformation assay on 98 chemicals: The characteristics and performance for the prediction of chemical carcinogenicity. *Mutation Research*, *702*(1), 100–122. https://doi.org/10.1016/j.mrgentox.2010.07.007

Saleh, M., Hogeveen, K., Fessard, V., & Le Hégarat, L. (2025). Mechanistic toxicity profiling of chemicals: A comparative study in 2D and 3D HepaRG models using high-content analysis. *Toxicology*, 154280. https://doi.org/10.1016/j.tox.2025.154280

Sasaki, K., Bohnenberger, S., Hayashi, K., et al. (2012). Photo catalogue for the classification of foci in the BALB/c 3T3 cell transformation assay. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *744*(1), 42–53. https://doi.org/10.1016/j.mrgentox.2012.01.009

Sasaki, K., Huk, A., El Yamani, N., Tanaka, N., & Dusinska, M. (2014). Bhas 42 cell transformation assay for genotoxic and non-genotoxic carcinogens. In *Genotoxicity and DNA repair*. Humana Press. https://doi.org/10.1007/978-1-4939-1068-7_20

Sasaki, K., Mizusawa, H., & Ishidate, M. (1988). Isolation and characterization of ras-transfected BALB/3T3 clone showing morphological transformation by 12-O-tetradecanoyl-phorbol-13-acetate. *Japanese Journal of Cancer Research*, *79*(8), 921–930. https://doi.org/10.1111/j.1349-7006.1988.tb00056.x

Steinberg, P. (2016). In vitro–in vivo carcinogenicity. In *In vitro environmental toxicology – Concepts, application and assessment*. Springer. https://doi.org/10.1007/10_2015_5013

Stevens, J. L., & M. W. Anders. (1981). Effect of cysteine, diethyl maleate, and phenobarbital treatments on the hepatotoxicity of [1H]- and [2H]chloroform. *Chemico-Biological Interactions*, *37*(1), 207–217. https://doi.org/10.1016/0009-2797(81)90178-2

Suarez-Torres, J. D., Jimenez-Orozco, F. A., & Ciangherotti, C. E. (2020). The 2-year rodent bioassay in drug and chemical carcinogenesis testing: Sensitivity, according to the framework of carcinogenic action. *Toxicology Mechanisms and Methods*, *30*(6), 462–475. https://doi.org/10.1080/15376516.2020.1760986

Suarez-Torres, J. D., Orozco, C. A., & Ciangherotti, C. E. (2021). The 2-year rodent bioassay in drug and chemical carcinogenicity testing: Performance, utility, and configuration for cancer hazard identification. *Journal of Pharmacological and Toxicological Methods*, *110*, 107070. https://doi.org/10.1016/j.vascn.2021.107070

Sui, H., Matsumoto, H., Wako, Y., & Kawasako, K. (2015). Evaluation of in vivo genotoxicity by thioacetamide in a 28-day repeated-dose liver micronucleus assay using male young adult rats. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *780–781*, 81–84. https://doi.org/10.1016/j.mrgentox.2014.10.001

Sui, H., Matsumoto, H., Wako, Y., & Kawasako, K. (2015). Evaluation of in vivo genotoxicity by thioacetamide in a 28-day repeated-dose liver micronucleus assay using male young adult rats. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *780–781*, 81–84. https://doi.org/10.1016/j.mrgentox.2014.10.001

Van Norman, G. A. (2019). Limitations of animal studies for predicting toxicity in clinical trials. *JACC: Basic to Translational Science*, *4*(7), 845–854. https://doi.org/10.1016/j.jacbts.2019.10.008

Vasseur, P., & Lasne, C. (2012). OECD detailed review paper (DRP) number 31 on "cell transformation assays for detection of chemical carcinogens": Main results and conclusions. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *744*(1), 8–11. https://doi.org/10.1016/j.mrgentox.2011.11.007

Vilas Bôas Correia, L., Carvalhal de Aguiar, G., Dos Santos Pereira, A. M. R., de Santana Cardoso Thomaz, L., Correia Dos Santos de Oliveira, I. C., de Britto Mari, R., Perobelli, J. E., Araki Ribeiro, D., & Barbosa da Silva, R. C. (2025). Triazole fungicides induce genotoxicity via oxidative stress in mammals in vivo: A comprehensive review. *Reviews on Environmental Health*, *41*(1), 142–151. https://doi.org/10.1515/reveh-2025-0051

Wang, Y.-S., Yang, S.-J., Wan, Z.-X., et al. (2024). Chlorothalonil exposure compromised mouse oocyte in vitro maturation through inducing oxidative stress and activating MAPK pathway. *Ecotoxicology and Environmental Safety*, *273*, 116100. https://doi.org/10.1016/j.ecoenv.2024.116100

Zhao, J., Pike, B., Huang, J., Feng, Z., Odle, J., & Lin, X. (2023). Effects of medium chain triglycerides on hepatic fatty acid oxidation in clofibrate-fed newborn piglets. *Animal Nutrition*, *12*, 334–344. https://doi.org/10.1016/j.aninu.2022.12.001

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Conclusion and perspectives

In support of NAM-based CSA, particularly for chemical hazard identification, and as part of the PARC project, the present thesis addresses various challenges in terms of improving and proposing in vitro-based testing strategies for identifying NGTxCs. The approaches applied in this thesis provide insights into the modes of how NGTxCs act at different levels (cellular, molecular, and apical), while also addressing the applicability and critical limitations of the different *in vitro* methods applied in this project.

In the first chapter, we investigated the predictive power of high-throughput screening approaches using a human hepatic cell line to provide a rapid and human-relevant method for evaluating chemical toxicity at the cellular level, generating a comprehensive mechanistic toxicity profile for the selected hepatotoxic chemicals. Through the application of rapid high-content imaging and the use of various statistical tools, toxicity profiles of the tested chemicals obtained from 3D HepaRG spheroids showed higher sensitivity than those from 2D HepaRG cells, indicating that more complex cellular models may offer an improved *in vitro* predictivity of chemical-associated toxicity. The high-throughput nature of these screening methods also enables the simultaneous assessment of multiple endpoints, supporting the rapid evaluation and classification of chemical lists for further follow-up testing.

While rapid methods are valuable for assessing multiple cellular endpoints, they often fail to provide detailed insights into the screened effects and their underlying molecular mechanisms. To address this limitation within a battery of tests, in Chapter Two we applied a complementary *in vitro* approach using the sensitive 3D HepaRG models. A panel of chemicals was selected, including genotoxic, non-genotoxic, and NGTxC chemicals (based on the observations of Chapter One, two chemicals were selected). A high-throughput sequencing approach using TemO-seq™ technology was employed to generate the gene expression profiles of the treated HepaRG spheroids.

First, the gene expression profiles were evaluated using genotoxic transcriptomics biomarkers, which proved highly sensitive in identifying genotoxic chemicals and distinguishing them from non-genotoxic chemicals. Together with the results obtained in Chapter One, these findings provide further evidence of the sensitivity of the 3D HepaRG spheroid model not only in toxicity profiling but also in distinguishing genotoxic from non-genotoxic chemicals through the application of transcriptomics biomarkers at the molecular level. Moreover, the sensitivity and applicability of the transcriptomics biomarkers tested in this thesis project in 3D HepaRG

support this method as a NAM-based complementary test that could be integrated into a battery of assays to provide more mechanistic insights when assessing chemical toxicity.

Second, unsupervised clustering and gene ontology (GO) analysis were applied to explore the molecular insights of chemical exposures and to attempt classification of NGTxC chemicals. Different activated genes related to similar intact pathways were analyzed in the tested NGTxC chemicals. One of the most consistently intact biological pathways in the analyzed gene sets was the lipid metabolism process. However, due to chemical-specific effects, the intact genes implicated in the same general biological process (GO term) differed among the treated 3D spheroids. Furthermore, the altered mechanisms associated with NGTxC, particularly the lipid accumulation process, could be influenced by tissue specificity. Since the HepaRG cell line represents a human-liver model, it might serve as a sensitive indicator of lipid homeostasis that could be affected by chemicals, as lipid metabolism is one of the major metabolic processes in the liver. Here we can raise an important question regarding the tissue-specific effects of NGTxC and the implications for *in vitro* hazard assessment. NGTxC are inherently more challenging to detect and interpret than genotoxic carcinogens because they do not interact directly with DNA and instead act through a diverse set of MoAs such as epigenetic modulation, altered cell signaling, receptor-mediated effects, and tissue-specific toxicity processes, without a unifying MoAs. This diversity underscores the tissue specificity of NGTxC effects, where mechanisms relevant in one cell type (e.g., hepatic lipid homeostasis) may be less evident or absent in others, highlighting why a single generic *in vitro* model often fails to capture all relevant NGTxC MoAs.

This inherent variability in NGTxC mechanisms is also reflected in our transcriptomic analyses. The clustering method was not able to completely group the selected NGTxC chemicals based on their transcriptomic signatures. Although transcriptomic analysis can provide valuable mechanistic insights at the molecular level, identifying NGTxC through a global analysis of gene expression data remains highly challenging. Because NGTxC act via different MoAs, they generate highly variable gene expression profiles, making it difficult to detect common signals across the global dataset of tested chemicals. Furthermore, analyzing these profiles requires substantial time and the use of sophisticated statistical tools to identify the key events (KEs) and mechanisms for each chemical. Therefore, focusing on individual gene expression and GO profiles for each chemical may offer more meaningful and specific mechanistic insights, while also acknowledging the need for an *in vitro* model capable of capturing the full diversity of NGTxC MoAs at the molecular level.

Although the MoAs of NGTxCs can be detected at both cellular and molecular levels, apical endpoints represent an important outcome in evaluating NGTxCs. The apical effects of different carcinogens represent the final step in the carcinogenicity of chemicals after the upstream molecular and cellular perturbations, thereby offering a more integrated and biologically relevant perspective on NGTxC toxicity. In this regard, in the third chapter of this thesis project, the third in vitro-based testing strategy applied was the Cell Transformation Assay (CTA).

Since CTA is the only *in vitro* method used to identify NGTxC that is under OECD consideration and validation, it was applied in this thesis project to test the apical changes induced by a list of chemicals selected based on their evaluation at the cellular level in Chapter One, and which had not been tested before with this assay. The application of this assay was first intended to fill data gaps regarding the number of chemicals previously tested with Bhas42 CTA and to understand its functionality for different chemicals showing different non-genotoxic activity at the cellular level. The application of the Bhas42 CTA assays shows high reproducibility in the promotion assay which is known to detect tumor promoters (NGTxC); however, different challenges were associated with this assay, as discussed in the chapter, the CTA lacks mechanistic insights and does not explain how chemicals drive the transformation *in vitro*. For that, and as mechanistic insight could be more promising in understanding the signalling pathways associated with foci formation caused by NGTxC, we applied a transcriptomic analysis to the treated Bhas42 cells in both initiation and promotion assays. Different concentrations were tested at different time points of the assays to validate the results obtained with the Bhas42 CTA. However, the data analysis is still ongoing. This analysis will provide a future perspective to analyse the gene sets implicated at different time points of the CTA assays and to investigate the gene sets involved in early stages prior to complete foci transformation, in addition to the genes implicated in the late transformation event. That could be use in a future perspective to predict carcinogenicity using transcriptomic technology.

To state it differently, each applied approach discussed in this thesis project has shown a specific limitation. For example, high-content screening and CTA methods provide limited molecular insights, while focusing solely on transcriptomics may miss the upstream effects of the chemicals being tested. The analysis of the gene expression profiles alone helps in identifying certain insights at the molecular level; however, it remains limited in determining whether these molecular or even cellular changes associated with the chemicals can drive malignant transformation. Moreover, the *in vitro* cell-based CTAs used to study the apical effects of different carcinogens lack mechanistic information.

Consequently, the challenges observed and assessed with each *in vitro* strategy applied in this project, highlights the importance of applying various complementary approaches to identify multiple line of evidences when identifying NGTxC at different levels. Thus, the workflow included in this project emphasizes the need for employing a comprehensive battery of NAMs-based strategy identify the adverse effects associated with different NGTxC and try to investigate a KE associated with their carcinogenicity. Therefore, based on the findings presented throughout this research work, this project proposed (Figure C.P 1):

1. As the selection of the cellular model represent one of the essential steps in conducting research work related to hazard identification especially with the NGTxC. The first step in the proposed framework is to select an appropriate cell model that is known to be sensitive, human-relevant, and easy to be manipulated. Hence, from our results, 3D HepaRG spheroids showed to be suitable for different high-throughput mechanistic approaches applied in this project.

2. The chemical classification and prioritization based on their toxicity profiles constitute a key step in organizing chemicals and determining a priority list for further studies. This could be achieved using a rapid screening method.

3. The identification of KE at the molecular level provides more mechanistic-based information related to the chemical hazardous effect. This approach enables a more advanced prediction of the toxic effects of such chemicals at higher biological levels. However, relying solely on such analysis might not be enough and combining evidences from the cellular and apical levels is important.

4. The last step in the suggested framework represents the identification of the apical changes induced by NGTxC. This step could be applied using the Bhas 42 CTA assay. However, mechanistic insights through the transcriptomic analysis are recommended to validate such results.

5. In addition, the application of appropriate statistical tools also represents an important step to be applied with the different steps of this framework.

6. For more targeted screening analysis at the cellular level, transcriptomic analysis could be informative in mapping the targeted pathways by chemicals.

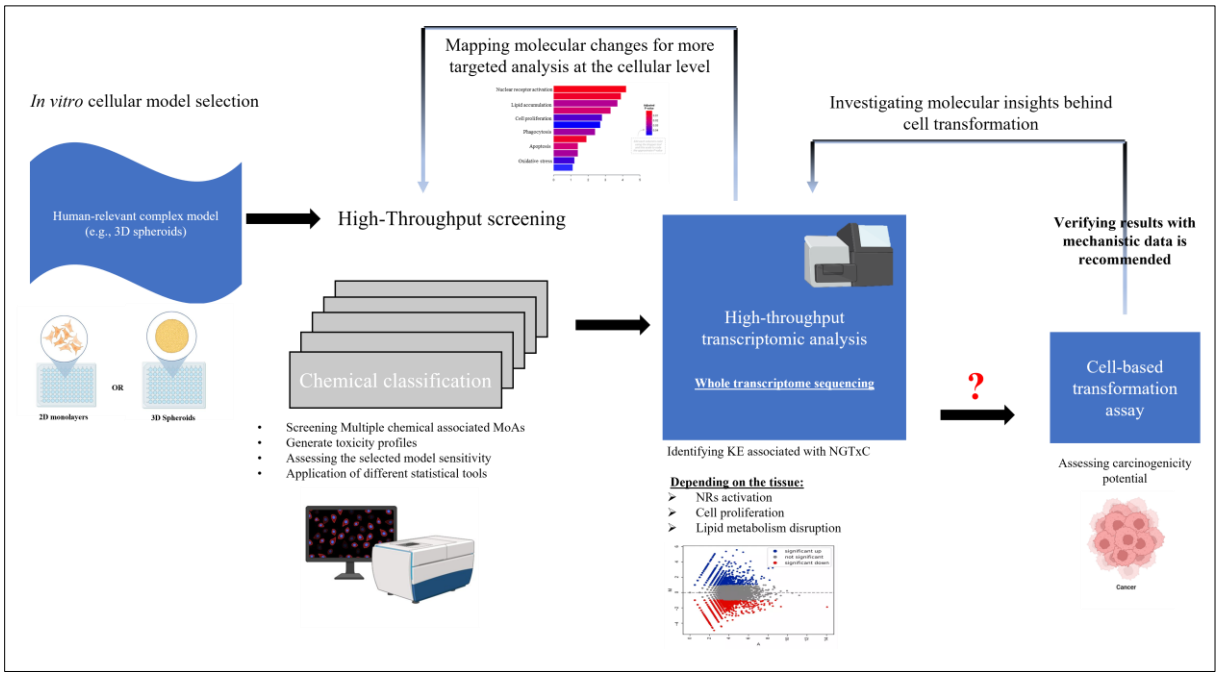

Figure C.P 1. A NAMS-based *In Vitro* framework for investigating NGTxC MoAs: insights from our findings.

Across the three chapters of this thesis, this work investigated three complementary experimental approaches addressing different levels of biological organization: high-content phenotypic screening, transcriptomic profiling, and CTA. When combined, these methodologies provide a multi-layered view of chemical-induced toxicity at cellular, molecular, and phenotypic levels *in vitro*, enabling more comprehensive mechanistic insights. Furthermore, integrating these functional assays can enhance the predictive capacity of NAMs for chemical hazard evaluation. This multi-method strategy aligns with the principles of IATA, as promoted by the OECD, which advocate the integration of mechanistic evidence across multiple lines of data rather than relying on single-test outcomes.

Moreover, this project contributed to generating data that helps fill existing data gaps regarding different NGTxCs and how they may act at different levels. Beyond methodological evaluation, this thesis also generated a substantial body of experimental data describing the cellular, phenotypic, and transcriptional responses to chemicals exhibiting diverse carcinogenic MoAs. The resulting dataset represents a valuable resource for future computational toxicology applications, including the development of predictive models based on Quantitative Structure–Activity Relationship (QSAR) approaches or other machine-learning strategies. By linking chemical exposure to mechanistically anchored biological responses, these data may contribute

to addressing current gaps in NGTxC hazard characterization and may support the development of data-driven tools for hazard prediction.

Importantly, the emphasis of this work was on hazard identification and screening rather than quantitative risk assessment. The assays and datasets developed here are therefore intended to support early-stage hazard detection and the prioritization of chemicals, providing mechanistic evidence that can inform regulatory decision-making within integrated testing strategies. The research conducted in this thesis also contributes to identifying areas can be further improved within NAMs-based approaches to enhance their robustness and regulatory applicability. By systematically evaluating different *in vitro* approaches and comparing their strengths and limitations, this work highlights critical methodological aspects that influence assay performance, including model complexity, endpoint selection, and data-interpretation strategies.

Moreover, the integration of phenotypic screening, transcriptomics, and transformation assays provided complementary mechanistic evidence, strengthening the overall confidence in the generated data. These findings help clarify how NAMs can be refined, standardized, and combined to support chemical hazard evaluation. Consequently, the methodological advancements and datasets generated in this thesis contribute to building scientific confidence in NAM-based strategies and represent an important step toward their broader integration into future chemical safety assessment and regulatory decision-making frameworks

A key contribution of this thesis lies in the incorporation of more physiologically relevant systems, particularly the use of 3D spheroid cultures of HepaRG cells, which have not previously been applied or are not commonly used in the assays employed in this work. This supports the value of advanced human-based in vitro systems for hazard screening and highlights the advancement of human-relevant in vitro models through the implementation of 3D HepaRG spheroids. By incorporating greater structural and functional complexity into the experimental design, this work demonstrates how advanced liver models can improve both the sensitivity and biological relevance of in vitro toxicological testing.

Such models are particularly valuable for the study of NGTxCs, whose mechanisms often involve metabolic activation, chronic cellular adaptation, human-relevance, processes that are difficult to capture in simplified *in vitro* systems.

Consequently, the application of a more complex multi-cellular models represents a promising direction, as these systems mimics more the *in vivo* complexity through the interaction between different types within human tissue. In addition, the influence of the microenvironment on toxicity outcomes, providing a more physiologically relevant platform compared to traditional monocultures. For instance, 3D HepaRG co-cultured with other cell types found in the liver such as LX2 cells (represents the stellates cells), and macrophages (represents the Kupffer cells). Currently, these models are under development in our research unit, with a validation protocol that will be published in the near future (Appendix IV). Furthermore, application of different tissue cellular models such as breast, intestine and others that could help in generating more information regarding the tissue specificity of NGTxC. This work is being done within different Labs and partners of PARC project. However, the implication of the multicellular complex model with the different tissue cellular models could be promising as well and represents an important future perspective to this work.

Together, this thesis contributes to the ongoing transformation of toxicological testing by demonstrating how advanced human-relevant in vitro systems, combined with high-content screening and transcriptomics, can collectively improve the detection and mechanistic understanding of non-genotoxic carcinogens. By generating new experimental evidence and structured datasets aligned with IATA/OECD principles, this work supports the development of next-generation predictive toxicology tools and provides a foundation for future computational modeling and integrated hazard assessment strategies.

Additionally, expanding the range of the tested chemicals will improve the robustness and applicability of the proposed framework. By incorporating chemicals that act through multiple different pathways than those selected for this study. In this step in the future could help in assessing and covering broader NGTxC effects, which in terns could act as data base for a better understanding, and better characterization of different MoAs.

Moreover, mapping the findings within the Adverse Outcome Pathway (AOP) framework can enhance the understanding of the mechanistic effects associated with different NGTxCs. By connecting molecular initiating events (MIE) with significant cellular events and adverse outcomes, AOPs aid in identifying predictive biomarkers and promote the creation of more precise, mechanism-focused testing approaches, helping to understand how the perturbation of one event can lead to another and how these events are interconnected. In this context, this thesis opens several perspectives for future research. One important direction will be the

integration of the datasets generated across the three experimental chapters into a unified mechanistic framework, potentially structured around the AOP concept. Mapping the observed molecular and cellular perturbations onto established or emerging AOPs for non-genotoxic carcinogenesis could facilitate the identification of key events and improve the interpretability of *in vitro* findings.

In conclusion, the research conducted in this thesis advances the development and application of human-relevant NAMs for assessing non-genotoxic carcinogens, providing mechanistic insights and structured datasets that strengthen hazard identification and regulatory decision-making. In the short term, this work contributes to the identification of early biomarkers associated with NGTxC, the development of improved screening tools for chemical and pharmaceutical safety, and the generation of mechanistic data that support weight-of-evidence approaches. Medium-term perspectives include the integration of 3D multicellular *in vitro* models into regulatory testing strategies, contributing to ongoing OECD and European discussions on alternative carcinogenicity assessment, and fostering industrial partnerships for the validation of predictive NAM platforms. In the long term, this thesis represents a critical step toward reducing and ultimately replacing traditional long-term rodent carcinogenicity studies by providing robust, human-relevant, mechanism-based *in vitro* platforms capable of reliably predicting chemical hazards.

By establishing data-driven approaches and enhancing confidence in NAMs, this work lays the foundation for future regulatory frameworks in which animal testing can be minimized or replaced, supporting safer chemical design, sustainable innovation, and ethical research practices. Collectively, these advances contribute to public health protection, reduce the socio-economic costs associated with toxicity testing, and reinforce the transition toward responsible, sustainable, and human-relevant toxicology.

Appendix

Appendix Int.

Advancing NGTxC hazard assessment: Integration of PARC Project

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Introduction “State of the Art” supplementary information

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New approach methodologies to facilitate and improve the hazard assessment of non-genotoxic carcinogens—a PARC project

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Carcinogenic chemicals, or their metabolites, can be classified as genotoxic or non-genotoxic carcinogens (NGTxCs). Genotoxic compounds induce DNA damage, which can be detected by an established *in vitro* and *in vivo* battery of genotoxicity assays. For NGTxCs, DNA is not the primary target, and the possible modes of action (MoA) of NGTxCs are much more diverse than those of genotoxic compounds, and there is no specific *in vitro* assay for detecting NGTxCs. Therefore, the evaluation of the carcinogenic potential is still dependent on long-term studies in rodents. This 2-year bioassay, mainly applied for testing agrochemicals and pharmaceuticals, is time-consuming, costly and requires very high numbers of animals. More importantly, its relevance for human risk assessment is questionable due to the limited predictivity for human cancer risk, especially with regard to NGTxCs. Thus, there is an urgent need for a transition to new approach methodologies (NAMs), integrating human-relevant *in vitro* assays and *in silico* tools that better exploit the current knowledge of the multiple processes involved in carcinogenesis into a modern safety assessment

toolbox. Here, we describe an integrative project that aims to use a variety of novel approaches to detect the carcinogenic potential of NGTxCs based on different mechanisms and pathways involved in carcinogenesis. The aim of this project is to contribute suitable assays for the safety assessment toolbox for an efficient and improved, internationally recognized hazard assessment of NGTxCs, and ultimately to contribute to reliable mechanism-based next-generation risk assessment for chemical carcinogens.

KEYWORDS

non-genotoxic carcinogens, NGTxC, new approach methodologies, NAM, PARC

Introduction

Cancer is one of the leading causes of death in Europe, with nearly 3 million new cases and 1.3 million deaths per year (www.ecris.jrc.ec.europa.eu) (Dyba et al., 2021). The importance of cancer prevention is recognized in the EU's Chemicals Strategy for Sustainability Towards a Toxic-Free Environment, as well as in Europe's Beating Cancer Plan (EC, 2023). These ambitious programs are challenged by the fact that cancer is a highly diverse and complex disease that affects various organs and involves multiple steps and mechanisms of progression that vary with cancer type and tissue affected. This complexity is also reflected in the different functional capabilities that cancer cells must acquire during malignant transformation, as described by the concept of "hallmarks of cancer" (Hanahan, 2022). Moreover, the vast majority of cancer-related deaths occurs at the late stages of carcinogenesis (i.e., invasive growth and metastasis) (Lyden et al., 2022).

The multiple causes of cancer can be divided into endogenous risk factors, comprising genetic predisposition, aging, hormone levels, stem cell division and DNA replication infidelity, and exogenous risk factors like (natural) radiation, lifestyle, infections and exposure to environmental chemicals. Environmental chemicals causing cancer can be grouped into genotoxic and non-genotoxic substances (NGTxCs). In a recent report by the European Environment Agency, it is estimated that such exogenous risk factors are responsible for a not negligible part of cancer cases (EEA, 2022). Interestingly, the International Agency for Research on Cancer (IARC) estimates that 10%–20% of human carcinogens classified as Class 1 may act as NGTxCs (Hernandez et al., 2009). A comprehensive knowledge of the carcinogenic potential of substances is urgently needed to minimize exposure and thus the risk of cancer. Genotoxic chemicals interact directly with DNA or interfere with the cellular machinery that maintains genomic integrity, leading to mutations and/or chromosomal alterations. In contrast, NGTxCs promote tumor formation through a variety of mechanisms and pathways without evidence of genotoxicity (Jacobs et al., 2020). To classify carcinogenic chemicals, the concepts of key characteristics (Smith et al., 2016; Guyton et al., 2018; Smith et al., 2020; Madia et al., 2021) or the hallmarks of environmental insults (Peters et al., 2021) have been proposed to link carcinogenic modes of action and the hallmarks of cancer concept.

In the different regulations, carcinogenicity is evaluated in long-term (2-year) exposure studies with rodents, in combination with *in vitro* and *in vivo* genotoxicity testing (OECD, 2016a; OECD, 2016b; OECD, 2018a; OECD, 2018b; Madia et al., 2019; OECD, 2022a; OECD, 2022b). However, long-term exposure rodent bioassays are time-consuming, costly, ethically questionable, and considered of limited relevance for humans. Indeed, some mechanisms induced by NGTxCs are potentially confounding biological and physiological species differences (Heusinkveld et al., 2020a). Since less than 5% of chemicals registered worldwide have been tested in long-term carcinogenicity rodent studies (Doe et al., 2019), there is an urgent need for a transition to new approach methodologies (NAMs). This allows for the inclusion of human-relevant *in vitro* and *in silico* methods that enable using recent advances in our understanding of diseases and employ the latest non-animal methods available in a modern safety assessment toolbox.

NGTxCs may have multiple modes of action, including epigenetic alterations, endocrine modulation, immune suppression, or continuous stimulation of inflammatory responses, sustained proliferation and migration (Hernandez et al., 2009). However, various carcinogenic processes are not exclusively cancer related, since they are also involved in non-cancer processes. In addition, tissue-specific effects must also be considered (Bianchi et al., 2020; Madia et al., 2021; Arner and Rathmell, 2022; Doe et al., 2022).

In 2020, the European Commission adopted its Chemicals Strategy for Sustainability (EC, 2020) with the main objective of promoting innovation for safe and sustainable chemicals, and improving the protection of human health and the environment from hazardous chemicals. This led to the establishment of the EU-funded Partnership for the Assessment of Risk from Chemicals (PARC) (Marx-Stoelting et al., 2023). This partnership consists of several sub-projects and sub-tasks. In the current sub-task project, we aim to provide appropriate assays for efficient and improved hazard assessment of NGTxCs, in order to support a mechanism-based next-generation risk assessment (NGRA) of chemical carcinogens.

To achieve this goal, a consortium of 13 partners across Europe has been established within the PARC project. Each partner contributes with its own expertise and knowledge in assay development that are compiled into a comprehensive strategy to identify and predict the carcinogenic potential of NGTxCs. The selected assays address a range of endpoints relevant to human carcinogenesis, and cover a broad spectrum of MoA that fit into the concept of hallmarks of cancer.

The objective: *improve and develop NAMs for the identification and the characterization of NGTxCs and the establishment of a reliable and efficient testing strategy for development of a safety assessment toolbox for NGRA.*

TABLE 1 Overview of the different cell types, complexity levels, exposure scenarios and endpoints addressed by the various partners of the NGTxC project. A = acute, R = repeat.

Partner	Cell/Tissue models	2D/3D	Treatment	Endpoints								
				Genomic instability	Proliferation	Cytotoxicity	Oxidative stress	NR-activation	Migration	Phenotypic changes	Inflammation	Epigenetic changes
ANSES	Liver	2D/3D	A/R		•	•	•	•		•	•	•
BfR	Breast, Liver, Colon	2D	A/R		•	•		•	•	•		
INRAE	Multicellular liver	3D	A/R	•	•	•	•			•	•	
INSERM	Breast, Adipocytes	2D/co-culture	A		•	•			•	•		
IRFMN	NA	NA	NA	•				•				
NIB	Liver	3D	R	•	•	•	•	•		•		•
NILU	Breast	2D/3D	A		•	•	•				•	
RECETOX	Liver	2D	A		•	•						
RIVM	Zebrafish	NA	A/R		•		•					
RPTU	Colon	2D	A/R	•	•	•	•		•			
UL-LACDR	Breast, Liver	2D	A		•	•	•	•	•	•	•	
UNAV	Liver	2D/3D	A/R	•		•	•					

ANSES: french agency for food, Environmental and Occupational Health and Safety, BfR: german federal institute for risk assessment, INRAE: national research institute for agriculture, Food and the Environment, France INSERM: national institute for health and medical research, France, IRFMN: istituto di ricerche farmacologiche mario negri, Italy, NIB: national institute of biology, Slovenia, NILU, the climate and environmental research institute, Health Effects Laboratory, RECETOX: research centre for toxic compounds in the environment czech republic, RIVM: national institute for public health and the environment, the Netherlands, RPTU: Rheinland-Pfälzische Technische Universität, Germany, UL-LACDR: leiden academic centre for drug research, the Netherlands, UNAV: University of Navarra, Spain.

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In light of the limitations of the 2-year rodent bioassay, which under some regulations is the required test for the identification and characterization of carcinogens, there is an urgent need for an efficient and reliable *in silico* and *in vitro* testing battery. Several past and ongoing European research initiatives, not exclusively dedicated to NGTxCs detection, aim to develop new *in vitro* toxicity test methods. These include the EU-ToxRisk and RISK-HUNT3R projects as well as the ASPIS (https://aspis-cluster.eu/) and EURION (https://eurion-cluster.eu/) project clusters. Over the last years, a number of new and improved methods have become available that might play a pivotal role in the development of a regulatory acceptable integrated approaches for testing and assessment (IATA) for NGTxCs. In 2020, an OECD expert group developed an IATA for NGTxCs and published a consensus paper describing the overarching IATA, with the molecular initiating events (MIE) of cellular metabolism and receptor interactions, followed by the early key events (KEs) of inflammation and immune dysfunction, mitotic signaling, and cell injury, leading to (sustained) proliferation, morphological transformation and tumor formation (Jacobs et al., 2020). Several assay evaluations and reviews have been conducted and published in relation to epigenetics (Desaulniers et al., 2021), cell transformation (Colacci et al., 2023), gap junction (Sovadinova et al., 2021), gene signaling (Oku et al., 2022), cell proliferation and oxidative stress (Veltman et al., 2023). These publications provide details on the next steps needed to facilitate developments that will have regulatory relevance for the safety assessment of NGTxCs.

Our project aims to provide a comparison of the different selected methods to check how they could be applied and how they may complement in the context of an IATA. In this context, the project can also help to improve knowledge on the relationships between specific toxicity mechanisms and adverse outcome pathways (AOPs) leading to cancer. AOPs correspond to chains of events starting from a MIE, then describing all the steps involving KEs at different biological levels (cellular, tissue, organism) leading to an adverse outcome (toxic impact or pathology). For the *in vitro* identification of NGTxCs, it is mandatory that the developed methods cover MIEs and/or KEs associated with hallmarks of cancer. In this context, AOPs can be a useful framework for mapping aspects of these complex relationships of carcinogenesis.

In this project, the partners provide a wide variety of methods using model systems of increasing complexity, from 2D human cell lines to advanced 3D human systems and zebrafish embryos models. These innovative human-relevant NAMs focus on organs (liver, breast and colon) commonly affected by chemical carcinogens, and include state-of-the-art transcriptomic approaches, cell painting, high-content image analysis and high-throughput-compatible reporter systems. The assays address well established and relevant mechanisms involved in carcinogenesis, (Table 1). The challenge here is to sufficiently capture the high complexity of NGTxCs triggered carcinogenesis with a manageable testing battery covering key features of human cancer (Table 1) (Hanahan, 2022). Moreover, factors supporting tumor initiation and progression as oxidative stress, activation of nuclear receptors and (tissue-specific) cytotoxicity leading to continuous regenerative proliferation are important mechanisms, but do not count to the hallmarks of cancer themselves. Excessive oxidative stress is known to play an important role in tumorigenesis by causing oxidative DNA damage that can directly lead to mutations. On the other hand, oxidative stress can also stimulate proliferation and epithelial-mesenchymal transition (EMT), leading to cancer progression through metastatic processes, and can be associated with inflammatory responses and cytotoxicity (Heusinkveld et al., 2020a; Hayes et al., 2020).

Interestingly, methods based on Nrf2 gene reporter cell lines, a key mediator of oxidative stress response, as well as a direct assay for measuring reactive oxygen species (ROS) content have already been accepted by the OECD as test guideline (TG) TG 442D and TG 495, with a focus on skin sensitization and phototoxicity, respectively. Estrogen receptor activation is a common therapeutic target, closely linked to the proliferation of endometrial, colorectal, prostate and breast cancer, but also associated with metastasis, EMT and epigenetic changes (Garcia-Martinez et al., 2021). Similarly, the role of AhR-mediated functions in cancer development is not limited to the generation of carcinogenic metabolites, but also feeds into various other cancer-related processes, including cell migration, cell transformation, inflammation, and epigenetic processes (Larigot et al., 2018; Larigot et al., 2022). Finally, sustained cytotoxicity has been shown to be an important mechanism of action for carcinogenic chemicals, particularly inducing regenerative proliferation (Hernandez et al., 2009). Thus, the endpoints addressed by the consortium partners are related to several hallmarks of cancer. As the same endpoint is addressed by different methods, their respective applicability will be established by comparing the responses of appropriate reference substances. Moreover, the comparison will also investigate the relevance of cell models and type of exposure methods in effects of carcinogenic chemicals. The variety of models and methods in this project should facilitate the development of an efficient and reliable test battery to identify NGTxCs and analyze their responses. Experimental approaches will be complemented by the optimization of *in silico* tools for the identification of NGTxCs.

A large set of reference compounds (positive or negative for carcinogenicity), including some prioritized PARC compounds (toxins and bisphenol analogues), will be tested, allowing chemical specific comparison of the different methods. The number and chemical classes of reference chemicals to be tested for the various mode of actions will depend upon the availability of reliable human-relevant data, including rodent bioassay data. The sensitivity and specificity of the various systems as well as their complementarity in detecting the different mechanisms involved in non-genotoxic carcinogenicity will be supplied.

Next, we will describe in more detail the methods applied in the project by the individual consortium partners.

ANSES: High content analysis of oxidative stress, nuclear receptor translocation, epigenetic markers, inflammatory responses, and mitochondrial metabolism in liver cell models

High-content analysis (HCA) is the application of automated high-resolution microscopy combined with quantitative image analysis for cell-based or organoid-based assays. This multiparametric image-based approach is capable of quantifying

a large number of features at single cell and population levels. In addition, the high throughput nature of the approach enables a large number of chemicals or endpoints to be tested in a single experiment. Furthermore, HCA can be used for the analysis of live and fixed cells, which makes this approach an important *in vitro* method for toxicological and mechanistic studies needed to predict NGTxCs (Li and Xia, 2019). Through the use of antibodies or fluorescent probes specific for cellular processes, this system is well suited for the detection of different hallmarks of cancer, including oxidative stress, nuclear receptor translocation, epigenetic markers, inflammatory responses, and mitochondrial metabolism. HCA therefore represents a promising *in vitro* approach to detect NGTxCs. In this context, a HCA approach to assess potential mode of action of NGTxC compounds using the human bi-potent progenitor HepaRG cell line in 2D and more complex 3D spheroid models will be applied (Marion et al., 2010).

BfR: Identification of NGTxCs by phenotypic screening for carcinogenic effects on cell adhesion, migration, proliferation, and mitochondrial dysfunction

The BfR will apply phenotypic screening methods in order to investigate and group compounds according to their modes of action. The first model, called E-morph assay, is based on ER-alpha expressing MCF7 breast cancer cells and evaluates the changes in adhesion junction (AJ) organization as an indicator for anti-estrogenic or estrogenic effects (Kornhuber et al., 2021). The Estrogen-dependent changes in the AJ directly influence cell stiffness and motility, two functions that are highly relevant for cancer progression, in particular in respect to progression from benign to malignant tumors or metastasis and address the relevant endpoints of phenotypic changes-migration and nuclear receptor activation at the same time By applying a cytoplasmic stain on the parental cells or by using an E-Cadherin-EGFP-expressing reporter cell line (de Beco et al., 2009; Klutzny et al., 2022), the appearance of the plasma membrane can be quantified by high content imaging. Depending on the assay set-up specific competitive compounds can be included allowing to screen for (anti-) estrogenic or (anti-) glucocorticoid compounds.

In our second model, cell-painting techniques (Bray et al., 2016) will be applied in order to generate compound-specific morphological profiles. In this assay, the breast cancer cell line MCF7 and the non-tumor cell line hTERT-HME1 are labeled with 6 different stains, followed by high content imaging and computational image analysis. The extraction of up to 50 features (e.g., size, shape, intensity, texture) allows the generation of compound-specific data. The direct comparison of a rather normal, immortalized cell line with a cancer cell line might also facilitate the detection of specific effects on the process of tumor progression, also in respect to proliferation, oxidative stress and cytotoxicity. Based on the assumption that compounds with similar profiles share common modes of action (Fetz et al., 2016), the applicability to identify carcinogenic compounds will be evaluated. Within this project, it will also be tested if the classical cell painting set up or another dye-set allowing the detection of different cellular structures can contribute best in the discrimination of such compounds. In parallel, the effects on the composition of the extracellular matrix (ECM) will be analyzed. Changes in the composition of the extracellular matrix have long been known to play an important role in the activation of "silent" neoplasia (Bissell and Hines, 2011) and, thus, a thorough analysis of chemically induced changes in ECM composition will also provide a better mechanistic insight into the effects of potential carcinogens on cellular migration and metastasis.

INRAE: Distinguishing genotoxic carcinogens from NGTxCs

In the EU, carcinogenicity studies are required for new active substances in the pesticides (1107/2009) and biocide (528/2012) regulations, whereas they are only required for high tonnage industrial chemicals under REACH (1907/2006). They can be waived if no evidence for hyperplasia or pre-neoplastic lesions are detected in repeated-dose studies and the substance is found non-mutagenic in genetic toxicity tests. Cancer has long been known to be induced by mutations, a molecular process that led to the development of the classical battery of genotoxicity tests available to detect chemicals that induce gene mutations (OECD TG 471, 476, 490) and chromosomal aberrations (OECD TG 473, 484). Although the sensitivity of this battery of the current regular genotoxicity assays is high, the specificity is rather low leading to a high false-positive rate compared with *in vivo* data, especially with the mammalian cell-based assays.

The methodology proposed by the INRAE team allows an evaluation of genotoxicity using two biomarkers, phosphorylated histones H2AX (γH2AX) and H3 (pH3), specific for clastogenic and aneugenic chemicals, respectively. The γH2AX biomarker is also related to genomic instability, is detected in cancerous cells and clinically used in cancer biology (Palla et al., 2017). The γH2AX/pH3 method was developed and pre-validated on 2D cell models and demonstrated a predictivity higher than 90% (Kopp et al., 2019). Moreover, some NGTxCs chemicals were detected with this method, indicating that this assay may permit to detect genotoxicity of some NGTxCs not revealed previously through the regular OECD genotoxicity assays (Kopp et al., 2019). In the PARC project, the assay will be further developed with a 3D multicellular liver model. The spheroids will include different cell types: the HepaRG hepatoma cell line, Kupffer cells and hepatic stellate cells (Yan et al., 2021). Repeated chemical treatment over numerous days will be used to take into account the real human exposure more efficiently. Additional specific biomarkers of cytotoxicity, oxidative stress, proliferation and inflammation will be included to discern some specific chemical toxic MoA. In the same idea, cell painting experiments will be performed on this 3D model to detect phenotypic changes induced by the compounds.

INSERM: Analysis of cell migration

The methodology proposed by the METATOX team (Inserm T3S) allows the real-time monitoring of proliferative, morphologicaly and migratory capacities of breast cancer cells in a context that mimics the tumor microenvironment. Additionally, a change in the cellular

morphology provides a qualitative indication of potential cytotoxicity (to be then confirmed using other specific tests). The system is based on the co-culture of MCF7 human breast cancer cells and hMADS human pre-adipocytes in multi-well plates equipped with a permeable membrane insert that allows communication between the two cell types. We already demonstrated that by exposing this model to Seveso dioxin or a mixture extracted from cigarette smoke, the phenotype of the cancer cells was modified, with the setting up of a process suggesting an epithelial-mesenchymal transition (EMT), the first step in the formation of metastasis (Koual et al., 2021; Benoit et al., 2023). Changes in cell phenotype (morphology and migration) can be followed in real-time using the xCELLigence system. In case of modification upon exposure to a chemical, morphological changes can be further characterized using microscopy and expression profile analysis for biomarkers of tumor progression taking proliferation and cytotoxicity into account. This model has the great advantage of mimicking the early stages of metastatic cell formation and appears relevant to study the influence of NGTxCs that may promote metastasis, which is involved in up to 90% of cancer mortality.

IRFMN: In silico identification of NGTxCs

At the basis of the *in silico* models there is the possibility of anticipating the activity of a compound starting from its chemical structure. It is known that, in fact, certain physico-chemical properties, such as the melting point, are related to the chemical structure. Similarly, certain types of toxicities are predicted with QSAR (Quantitative Structure-Activity Relationship) models, too. As initial steps, we collect information on the feasible *in silico* models related to carcinogenicity (Benigni et al., 2008; Fjodorova et al., 2010; Benfenati and Gini, 2013; Benigni et al., 2013; Golbamaki et al., 2016; Toma et al., 2020) and associated endpoints, such as genotoxicity (Roncaglioni et al., 2008; Baderna et al., 2020; Van Bossuyt et al., 2020) and nuclear receptor activity. Indeed, there are many QSAR models freely available, for instance within the platform of *in silico* tools, VEGAHUB (https://www.vegahub.eu/). These tools can be used on the selected compounds, to evaluate the carcinogenicity potential and the binding to specific nuclear receptors (https://www.vegahub.eu/portfolio-item/vega-nrmea/), for instance. The analysis of the predictivity of the *in silico* approach towards this kind of biological activity will be examined in depth developing new *ad hoc* models starting from big sets of data (e.g., ICE–Integrated Chemical Environmental of NTP) related to the key characteristics of carcinogens. An additional activity is using the tools for the so called read-across approach (https://www.vegahub.eu/portfolio-item/vera/) (Vigano et al., 2022). In this case, the model explores the similarity between the target substance and related compounds with experimental data.

NIB: High Content analysis of genotoxicity, epigenetic changes, AhR activation, stress response and cell proliferation in liver spheroids

A multi-labeling approach using specific antibodies and stains and the use of flow cytometry to simultaneously detect multiple endpoints in the same cell and cell population represents a HCA tool. The effects of chemicals, including NGTxCs, on cell proliferation will be studied by detecting fluorochrome signals for proliferation markers (e.g., Ki67, PCNA) and by analyzing the distribution of cells in the cell cycle. In addition, other hallmarks of carcinogenesis will be investigated simultaneously, including genomic instability by detecting DNA damage (γH2AX marker) reflecting clastogenic activity, and mitotic cells (histone H3-positive cells) reflecting aneugenic activity, oxidative stress, nuclear receptor activation (AhR), and epigenetic modifications of histones (e.g., H3K27me3, H3K9ac, and H3K9me2) that serve as predictors of carcinogenicity (Stampar et al., 2022). The effects on cell growth and proliferation will be assessed using the MTS assay.

Toxicogenomics, the application of gene expression analysis techniques in toxicological studies, identifies global changes in gene expression associated with a toxicological outcome and contributes to the refinement of toxicity and carcinogenicity testing. It provides supporting mechanistically based data on the molecular pathways underlying the apical effects (Ellinger-Ziegelbauer et al., 2005). Recently, gene expression profiling has been proposed as a useful tool for hazard identification and human health risk assessment, providing not only qualitative, but also quantitative information related to relevant mechanisms of action induced by the stressors (Corvi and Madia, 2017). It is well known that stressors with similar biological activities (e.g., oxidative stress inducers, genotoxic carcinogens/NGTxCs) can deregulate the expression of specific genes, which can therefore be used to distinguish different molecular mechanisms of action of the stressors under study (biomarkers of exposure or effect). Using a combination of multi-labeling and toxicogenomics approaches, the mechanisms of action of NGTxCs will be investigated in a hepatic *in vitro* 3D cell model (spheroids) developed from human hepatocellular carcinoma (HepG2) cells. The advantage of spheroids over traditional monolayer cultures is that they more closely resemble tissue cell organization and therefore better mimic the *in vivo* microenvironment and provide more predictive data for human exposure (Stampar et al., 2020).

NILU: *In vitro* cytotoxicity, oxidative stress (ROS), proliferation and inflammatory markers

Breast cancer (BC) is one of the most common cancer type in the world and the second leading cause of cancer deaths among women (Bray et al., 2018). Exposure to environmental chemicals and pollutants have been linked to BC incidence (Kortenkamp, 2006; Bonefeld-Jorgensen et al., 2011). BC mortality is associated mainly with the development of metastases (Ruscitto et al., 2022). Therefore, identification of the mechanisms involved in metastasis formation is of high importance. The signaling pathways involved in metastatic tumor cells emergence and progression are increasingly linked to exposure to environmental chemicals and pollutants (Koual et al., 2020; Gabet et al., 2021; Kay et al., 2022). Oxidative stress is one of the best documented mechanisms for carcinogenesis, together with impacts on cell cycle progression (Martínez-Reyes and Chandel, 2021; Dharshini et al., 2023). Environmental pollutants may induce cell damage by

induction of ROS and oxidative stress, which can trigger an inflammatory response which may also evolve into a chronic inflammation (Dharshini et al., 2023). Inflammation has been linked to various steps in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis. NILU´s contribution to this project will focus on breast cancer and breast cancer cell models. We will study oxidative stress and ROS production as an early KE in carcinogenesis, as well as induction of relevant pro-inflammatory cytokines (ELISA) (Esquivel-Velazquez et al., 2015). Further, we will study cell proliferation. One of the promising advanced *in vitro* cellular models which mimic more closely the *in vivo* situation, is the 3D spheroids models. Different cell lines with features representing different BC subtypes, JIMT-1, MDA-MB-231 and T-47D have been used to establish advanced 3D multicellular aggregates. NILU will focus on one of these cell lines and apply the above mentioned endpoints. Both 2D and 3D models of JIMT-1 cells will be used, and their sensitivity compared. In addition to the use of cancer cell lines, the use of normal cell lines should also be considered, e.g., the epithelial MCF-10A cell line. These cells are subline of spontaneously immortalized human breast epithelial cells derived from human fibrocystic mammary tissue with characteristics of normal breast epithelium, which make the MCF-10A a valuable *in vitro* model to study normal breast cell function and determine the potential of environmental chemicals, including NGTxCs, to induce tumor transformation.

RIVM: Analysis of oxidative stress responses in zebrafish

RIVM's contribution to this project is focused on oxidative stress. Oxidative stress, resulting from an imbalance between the generation of oxidants and their scavenging by antioxidants, may lead to sustained cytotoxicity and regenerative proliferation and is therefore a MoA relevant for carcinogenesis (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Leuthold et al., 2019; Hanahan, 2022). Oxidative stress can be induced through endogenous processes, but also upon exposure to chemical substances (Heusinkveld et al., 2020b; Veltman et al., 2023). This is known to promote carcinogenesis through DNA damage and impaired repair, as well as through indirect actions influencing homeostasis and signaling (Klaunig, 2018). Additionally, ROS play a role in numerous stages of the multistep carcinogenic process (Weinberg et al., 2019).

RIVM will use zebrafish embryos as whole-organism model for measuring oxidative stress for a diverse set of substances. The added value of this model for toxicity testing of both single substances and chemical mixtures, as well as MoA identification has been shown by us and others (Leuthold et al., 2019; Heusinkveld et al., 2020b; Bauer et al., 2021; Van Der Ven et al., 2022). Various parameters for oxidative stress will be measured, including ROS levels, lipid peroxidation and changes in gene expression. For this purpose, both fluorophore-based techniques in wild-type zebrafish and transgenic reporter lines are available, responsive to activation of the Nrf2 pathway allowing for microscopic determination of oxidative stress on a multi-organ level in live embryos. The rationale for using zebrafish embryos is to generate data in a more complex test system, complementary to other models available to the project. In particular, the zebrafish can provide data for higher-tier key events in AOP approaches (organ-organism levels) that are not covered by (complex) cellular models.

RECETOX: Analysis of intercellular communication

The RECETOX team will explore the applicability of the scrape loading-dye transfer (SL-DT) assay (Dydowiczova et al., 2020) assessing gap junction intercellular communication (GJIC) *in vitro* using rodent and human liver cells to distinguish between known NGTxCs and non-carcinogens. GJIC is a vital process in multicellular animals, facilitating the exchange of ions and molecules between cells and coordinating tissue actions (Nielsen et al., 2012; Zefferino et al., 2019). Disruption of GJIC has been a well-recognized but overlooked hallmark of NGTxCs for a long time (Trosko et al., 2004; Aasen et al., 2016; Jacobs et al., 2016; Zefferino et al., 2019; Jacobs et al., 2020). In the recently proposed IATA for identification of NGTxCs, GJIC has been linked to pivotal key events of mitogenic signaling and cell injury (Jacobs et al., 2020), thus related to the endpoints of cell proliferation and cytotoxicity. However, there are no validated methods for GJIC assessment. While several methods can be used to evaluate the dysregulation of GJIC, many have limited throughput and require specialized equipment (Sovadinova et al., 2021). The SL-DT assay is the most commonly used *in vitro* method for evaluating the impact of toxicants or carcinogens on GJIC with a proven potential for high-content analysis and screening (HCA/HCS) (Dydowiczova et al., 2020; Sovadinova et al., 2021). The SL-DT assay has been widely employed to study alterations in GJIC caused by hundreds of chemicals, including NGTxC. Overall, the sensitivity of the SL-DT assay to predict IARC carcinogens (Group 1, 2A, or 2B) is 77% (Sovadinova et al., 2021). However, most of the published data were generated using a rat liver epithelial cell line (WB-F344), and negative compounds (non-carcinogens) have not been widely assessed. These issues will be addressed in this project by testing a common set of reference compounds using the multiparametric SL-DT assay allowing assessment of GJIC along with cell proliferation and cytotoxicity (Dydowiczova et al., 2020). The results obtained in WB-F344 cells will be validated the results using human cells. This will help evaluate the potential of the multiparametric SL-DT assay as a screening/testing tool for NGTxCs within integrated testing approaches.

RPTU: Identification of NGTxCs using human colon cell models

Non-malignant human colonic epithelial cells (HCEC) (Roig et al., 2010) and HCT116 human colorectal cancer (CRC) cells (Ahmed et al., 2013) have already being used to study dietary chemical carcinogens, bacterial toxins as well as drug candidates in the context of CRC (Mimmler et al., 2016; Seiwert et al., 2017; Dorsam et al., 2018; Seiwert et al., 2020; Arnold et al., 2022). To identify NGTxCs, a test battery covering the following endpoints will be applied: cell proliferation, formation of ROS, EMT, cell

migration and genotoxicity. The latter endpoint is based upon the sensitive detection of γH2AX as established DNA damage marker (Kinner et al., 2008) using InCell Western or immunofluorescence methodology, which will help to distinguish genotoxic from non-genotoxic compounds. Increased cell proliferation is a well-described hallmark of cancer (Hanahan, 2022) and known to be triggered by various NGTxCs. Effects on cell growth and proliferation will be first assessed using the Alamar Blue cell viability assay (Vieira-da-Silva and Castanho, 2023), allowing for extensive concentration-range finding studies in a high-throughput manner. This will be complemented by the 5-ethynyl-2′-deoxyuridine (EdU) assay, which measures the incorporation of EdU into nascent DNA during replication (Salic and Mitchison, 2008) and thus correlates with cell proliferation. EdU incorporation is visualized via Click chemistry using fluorescent azides, such as 6-fluorescein azide, followed by fluorescence microscopy or flow cytometry in low-to-medium throughput.

The induction of oxidative stress will be analyzed as a further endpoint using the cell-permeable probe CM-H_2DCFDA, which is converted intracellularly by ROS into a fluorescent dye (Kalyanaraman et al., 2012). This can be measured in a multi-well plate reader using an additional Hoechst staining for normalization to cell counts. Depending on its level, spatiotemporal formation and chemical nature, oxidative stress contributes to carcinogenesis via both non-genotoxic and genotoxic mechanisms (Sies and Jones, 2020). Another important endpoint in the context of metastasis is EMT. This process involves the activation of specific transcription factors, leading to the reorganization of the actin cytoskeleton and degradation of extracellular matrix as a prerequisite for migration and invasion (Kalluri and Weinberg, 2009). Here, the F-actin will be stained using a fluorescent phalloidin probe, which will be combined with the detection of E-cadherin as a marker of epithelial morphology that decreases upon EMT. Both markers are then visualized by fluorescence microscopy, but may alternatively be measured using a multi-well plate reader to adjust to more samples.

Finally, cell migration as pivotal step during the metastasis of cancer cells will be studied using the so-called wound healing assay that is also termed scratch assay (Vang Mouritzen and Jenssen, 2018). To this end, confluent cells with a scratch/gap are exposed to the compound of interest and cell migration is then monitored as gap closure via microscopy (endpoint measurement), thus somewhat limiting the number of samples to be analyzed.

UKHSA: Augmentation of the CYP induction test method for use in the NGTxC IATA and validation management for the cell transformation Assay using transcriptomics: the transformics assay

UKHSA will provide input on the further development of the HepaRG CYP induction test method (Bernasconi et al., 2019) (Jacobs et al., 2022), to support the NGTxC IATA (Jacobs et al., 2020) and will be implicated for the (pre)validation of promising test methods that address the outstanding needs as identified in the work of the OECD NGTxC IATA expert group, specifically here in relation to cell transformation (Colacci et al., 2023) and gap junction (Sovadinova et al., 2021) in the first instance. A regulatory framework for the IATA for NGTxC developed with ECHA will also be provided (Louekari et al. paper in prep), this will assist in supporting the regulatory targeted assay development.

UL-LACDR: High-Throughput screening for oxidative stress as well as mitogenic and inflammatory signaling

UL-LACDR focuses on quantitative, mechanism-based, and human-relevant hazard characterization, employing an AOP/AON conceptual thinking in direct relation to the hallmarks of cancer concepts and taking advantage of high-throughput modular assays using automated confocal microscopy and high throughput transcriptomics. This allows for testing redundancy and validation of methodologies whilst permitting constant improvement and innovation of integrated testing strategies. The approach will follow a tiered testing strategy that includes high-content 2D and 3D cell-based imaging platforms for next-generation risk assessment of NGTxCs. In the context of this project, the focus will be on mammary gland and liver, two of the most critical target organs for chemically induced carcinogenesis in 2-year rodent cancer bioassays (Sistare et al., 2011). Pivotal key events in the proposed integrated approach to testing and assessment (IATA) of NGTxCs (Jacobs et al., 2020) will be addressed, in particular oxidative stress, inflammatory signaling and mitogenic signaling. Since several known human NGTxCs target estrogen receptor signaling, fluorescent BAC reporters for ERα-induced proliferation and cell cycle progression in human ERα-positive MCF7 breast cancer cells will be included (Duijndam et al., 2021; Duijndam et al., 2022), as well as a panel of CRISPR-based endogenously tagged fluorescent human induced pluripotent stem cell reporter lines that can be differentiated to relevant lineages including progenitor cells of mammary epithelial cells and hepatocytes. These reporters cover diverse critical cellular signaling responses that are essential in cancer development. Using validation sets of chemical compounds with known toxic effects, the reporter lines will be evaluated with high content live cell confocal imaging for sensitivity and specificity in the detection of specific types of stress-response in different target organ lineages, in 2D or 3D. This includes the analysis of general markers of cytotoxicity and effects on proliferation, migration and other cellular phenotypes. The imaging-based approaches will be complemented by high throughput targeted mRNA sequencing as well as high throughput single-cell sequencing using TempO-Seq (https://www.biospyder.com) or BART-seq (Uzbas et al., 2019), to get broader insight in AOP activation and thresholds of carcinogenic events.

UNAV: Detection of repairable DNA lesions induced by NGTxCs

Genotoxic carcinogens are compounds that give a positive response in classical batteries of genotoxicity testing, which includes *in vitro* and *in vivo* assays that mainly detect point mutations or chromosomal aberrations [e.g., ICH (ICH, 2008)

and EFSA (EFSA, 2011)], and in the *in vivo* 2-years carcinogenicity studies. Compounds that induce DNA lesions that are completely repaired may not be detected by the genotoxicity battery.

On the other hand, NGTxCs are compounds that induce tumors in the *in vivo* carcinogenicity studies but are negative in the aforementioned genotoxicity testing batteries. However, the current regulatory genotoxicity testing strategies are mainly focused on point mutations or chromosomal aberrations. A key question is whether the presence of repairable DNA lesions could be a marker for some NGTxCs. To explore this option the detection of DNA lesions, also called pre-mutagenic lesions, that cause genomic instability, will be carried out *in vitro* using different versions of the alkaline comet assay.

The standard version of the alkaline comet assay detects DNA strand breaks and alkali labile sites (ALS), such as abasic sites, at a single cell level (Collins et al., 2023). The assay is used in genotoxicity testing, in both *in vitro* and *in vivo* models. The *in vivo* version is included in the ICH and EFSA genotoxicity testing strategies (ICH, 2008; EFSA, 2011); its OECD test guideline was published in 2014 and reviewed in 2016 (OECD, 2016c). Indeed, this is the only assay included in the standard testing strategies that does not detect point mutations or chromosomal aberrations, but DNA strand breaks and ALS.

On the other hand, the comet assay has been modified to detect specific DNA lesions (Collins et al., 2023) such as altered bases and it is widely used for the detection of oxidized bases, a marker of oxidative stress. Oxidized DNA bases can be induced directly by a compound or indirectly through a physiological toxic response. In PARC the alkaline comet assay will be used in its standard version but also combined with the enzyme Fpg for the detection of oxidized purines and with the enzyme hAAG for the detection of alkylated DNA bases (Muruzabal et al., 2020; Muruzabal et al., 2021). These three versions of the assay will be applied in 2D and 3D HepG2 human liver cells after acute and repeated exposure. Cytotoxicity will be also measured in the same exposure conditions.

Concluding remarks

In the course of this project, a wide variety of already established methods will be optimized to test the effects of NGTxCs on different KEs known to be involved in carcinogenesis. In addition, new methods and NAMs will be developed and tested to complement existing methods with respect to the various modes of action of NGTxCs. Other work packages from PARC focus on the development of IATAs and AOPs for genotoxic and NGTxCs. It will be important to align this work with the efforts of the OECD that has established an expert group to evaluate the currently available NAMs suitable for the published NGTxC IATA (Jacobs et al., 2020). Most importantly, several PARC projects are addressing toxicological endpoints that are directly related to (non-) genotoxic carcinogenesis, in particular immunotoxicity, inflammation and metabolic endocrine disruption. In the future, NAMs developed in these specific PARC projects as well as new methods currently being developed in EU Horizon 2020 projects, in particular in the EURION and ASPIS clusters, but also methods that are already established and are undergoing validation, will be taken into account. It is important to ensure that there is sufficient overlap in the selection of compounds tested to allow, at a later stage, a comprehensive analysis of various combinations of these methods in testing strategies. In combination with the assays developed in additional PARC and Horizon 2020 projects, test methods for several of the defined hallmarks of cancer will be (further) developed. Comprehensive coverage of all the hallmarks of cancer will not be possible in the PARC project alone or even in cooperation with ongoing EU projects, including processes such as de- or trans-differentiation or the influence of the microbiome.

This project provides a unique opportunity to directly compare a wide variety of methods and approaches, and the results will feed into other projects and work packages to support the development of AOPs and IATAs, in close cooperation with parallel activities in Europe and the OECD. This work will facilitate the development of more efficient and human-relevant assessment of chemicals for potential non-genotoxic carcinogenic activities by developing NAMs for identification of NGTxCs, mapping KEs for further developing AOPs and IATA for carcinogenicity, and contributing to the safety assessment toolbox to support NGRA of carcinogens (Bischoff et al., 2020).

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Aasen, T., Mesnil, M., Naus, C. C., Lampe, P. D., and Laird, D. W. (2016). Gap junctions and cancer: Communicating for 50 years. *Nat. Rev. Cancer* 16 (12), 775–788. doi:10.1038/nrc.2016.105

Ahmed, D., Eide, P. W., Eilertsen, I. A., Danielsen, S. A., Eknæs, M., Hektoen, M., et al. (2013). Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis* 2 (9), e71. doi:10.1038/oncsis.2013.35

Arner, E. N., and Rathmell, W. K. (2022). Mutation and tissue lineage lead to organ-specific cancer. *Nature* 606 (7916), 871–872. doi:10.1038/d41586-022-01535-z

Arnold, C., Demuth, P., Seiwert, N., Wittmann, S., Boengler, K., Rasenberger, B., et al. (2022). The mitochondrial disruptor devimistat (CPI-613) synergizes with genotoxic anticancer drugs in colorectal cancer therapy in a bim-dependent manner. *Mol. Cancer Ther.* 21 (1), 100–112. doi:10.1158/1535-7163.MCT-21-0393

Baderna, D., Gadaleta, D., Lostaglio, E., Selvestrel, G., Raitano, G., Golbamaki, A., et al. (2020). New in silico models to predict *in vitro* micronucleus induction as marker of genotoxicity. *J. Hazard Mater* 5 (385), 121638. doi:10.1016/j.jhazmat.2019.121638

Bauer, B., Mally, A., and Liedtke, D. (2021). Zebrafish embryos and larvae as alternative animal models for toxicity testing. *Int. J. Mol. Sci.* 22 (24), 13417. doi:10.3390/ijms222413417

Benfenati, E. M. A., and Gini, G. (2013). "VEGA-QSAR: AI inside a platform for predictive toxicology in CEUR workshop proceedings," in Proceedings of the workshop "Popularize Artificial Intelligence 2013 Turin, Italy: December 5, 2013.

Benigni, R., Bossa, C., Jeliazkova, N., Netzeva, T., and Worth, A. (2008). *The Benigni/Bossa rulebase for mutagenicity and carcinogenicity - a module of Toxtree*. EUR 23241 EN - 2008 Brussels, Belgium: European Commission.

Benigni, R., Bossa, C., and Tcheremenskaia, O. (2013). Nongenotoxic carcinogenicity of chemicals: Mechanisms of action and early recognition through a new set of structural alerts. *Chem. Rev.* 113 (5), 2940–2957. doi:10.1021/cr300206t

Benoit, L., Tomkiewicz, C., Delit, M., Khider, H., Audouze, K., Kowandy, F., et al. (2023). Cigarette smoke and tumor microenvironment copromote aggressiveness of human breast cancer cells. *Toxicol. Sci.* 192 (1), 30–42. doi:10.1093/toxsci/kfad013

Bernasconi, C., Pelkonen, O., Andersson, T. B., Strickland, J., Wilk-Zasadna, I., Asturiol, D., et al. (2019). Validation of *in vitro* methods for human cytochrome P450 enzyme induction: Outcome of a multi-laboratory study. *Toxicol Vitro* 60, 212–228. doi:10.1016/j.tiv.2019.05.019

Bianchi, J. J., Zhao, X., Mays, J. C., and Davoli, T. (2020). Not all cancers are created equal: Tissue specificity in cancer genes and pathways. *Curr. Opin. Cell. Biol.* 63, 135–143. doi:10.1016/j.ceb.2020.01.005

Bissell, M. J., and Hines, W. C. (2011). Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nat. Med.* 17 (3), 320–329. doi:10.1038/nm.2328

Bonefeld-Jorgensen, E. C., Long, M., Bossi, R., Ayotte, P., Asmund, G., Krüger, T., et al. (2011). Perfluorinated compounds are related to breast cancer risk in Greenlandic inuit: A case control study. *Environ. Health* 10, 88. doi:10.1186/1476-069X-10-88

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 68 (6), 394–424. doi:10.3322/caac.21492

Bray, M. A., Singh, S., Han, H., Davis, C. T., Borgeson, B., Hartland, C., et al. (2016). Cell Painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes. *Nat. Protoc.* 11 (9), 1757–1774. doi:10.1038/nprot.2016.105

Colacci, A., Corvi, R., Ohmori, K., Paparella, M., Serra, S., Da Rocha Carrico, I., et al. (2023). The cell transformation assay: A historical assessment of current knowledge of applications in an integrated approach to testing and assessment for non-genotoxic carcinogens. *Int. J. Mol. Sci.* 24 (6), 5659. doi:10.3390/ijms24065659

Collins, A., Møller, P., Gajski, G., Vodenková, S., Abdulwahed, A., Anderson, D., et al. (2023). Measuring DNA modifications with the comet assay: A compendium of protocols. *Nat. Protoc.* 18 (3), 929–989. doi:10.1038/s41596-022-00754-y

Corvi, R., and Madia, F. (2017). *In vitro* genotoxicity testing-Can the performance be enhanced? *Food Chem. Toxicol.* 106 (Pt B), 600–608. doi:10.1016/j.fct.2016.08.024

de Beco, S., Gueudry, C., Amblard, F., and Coscoy, S. (2009). Endocytosis is required for E-cadherin redistribution at mature adherens junctions. *Proc. Natl. Acad. Sci. U. S. A.* 106 (17), 7010–7015. doi:10.1073/pnas.0811253106

Desaulniers, D., Vasseur, P., Jacobs, A., Aguila, M. C., Ertych, N., and Jacobs, M. N. (2021). Integration of epigenetic mechanisms into non-genotoxic carcinogenicity hazard assessment: Focus on DNA methylation and histone modifications. *Int. J. Mol. Sci.* 22 (20), 10969. doi:10.3390/ijms222010969

Dharshini, L. C. P., Rasmi, R. R., Kathirvelan, C., Kumar, K. M., Saradhadevi, K. M., and Sakthivel, K. M. (2023). Regulatory components of oxidative stress and inflammation and their complex interplay in carcinogenesis. *Appl. Biochem. Biotechnol.* 195 (5), 2893–2916. doi:10.1007/s12010-022-04266-z

Doe, J. E., Boobis, A. R., Cohen, S. M., Dellarco, V. L., Fenner-Crisp, P. A., Moretto, A., et al. (2022). A new approach to the classification of carcinogenicity. *Arch. Toxicol.* 96 (9), 2419–2428. doi:10.1007/s00204-022-03324-z

Doe, J. E., Boobis, A. R., Dellarco, V., Fenner-Crisp, P. A., Moretto, A., Pastoor, T. P., et al. (2019). Chemical carcinogenicity revisited 2: Current knowledge of carcinogenesis shows that categorization as a carcinogen or non-carcinogen is not scientifically credible. *Regul. Toxicol. Pharmacol.* 103, 124–129. doi:10.1016/j.yrtph.2019.01.024

Dorsam, B., Seiwert, N., Foersch, S., Stroh, S., Nagel, G., Begaliew, D., et al. (2018). PARP-1 protects against colorectal tumor induction, but promotes inflammation-driven colorectal tumor progression. *Proc. Natl. Acad. Sci. U. S. A.* 115 (17), E4061–E4070. doi:10.1073/pnas.1712345115

Duijndam, B., Goudriaan, A., van den Hoorn, T., van der Stel, W., Le Dévédec, S., Bouwman, P., et al. (2021). Physiologically relevant estrogen receptor alpha pathway reporters for single-cell imaging-based carcinogenic hazard assessment of estrogenic compounds. *Toxicol. Sci.* 181 (2), 187–198. doi:10.1093/toxsci/kfab037

Duijndam, B., Tedeschi, M., van der Stel, W., van den Hoorn, T., van der Burg, B., Bouwman, P., et al. (2022). Evaluation of an imaging-based *in vitro* screening platform for estrogenic activity with OECD reference chemicals. *Toxicol Vitro* 81, 105348. doi:10.1016/j.tiv.2022.105348

Dyba, T., Randi, G., Bray, F., Martos, C., Giusti, F., Nicholson, N., et al. (2021). The European cancer burden in 2020: Incidence and mortality estimates for 40 countries and 25 major cancers. *Eur. J. Cancer* 157, 308–347. doi:10.1016/j.ejca.2021.07.039

Dydowiczova, A., Brózman, O., Babica, P., and Sovadinová, I. (2020). Improved multiparametric scrape loading-dye transfer assay for a simultaneous high-throughput analysis of gap junctional intercellular communication, cell density and viability. *Sci. Rep.* 10 (1), 730. doi:10.1038/s41598-020-57536-3

EC (2020). *Chemicals strategy for sustainability*. Brussels, Belgium: European Commission.

EC (2023). URLs. Available at: https://commission.europa.eu/resources-partners/europa-web-guide/planning-your-web-presence/define-and-plan/urls-and-redirects/url-structure_en.

EEA (2022). *Beating cancer — The role of Europe's environment*. Norway, Europe: EEA.

EFSA (2011). Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. *EFSA J.* 9, 1–68. doi:10.2903/j.efsa.2011.2379

Ellinger-Ziegelbauer, H., Stuart, B., Wahle, B., Bomann, W., and Ahr, H. J. (2005). Comparison of the expression profiles induced by genotoxic and nongenotoxic carcinogens in rat liver. *Mutat. Res.* 575 (1-2), 61–84. doi:10.1016/j.mrfmmm.2005.02.004

Esquivel-Velazquez, M., Ostoa-Saloma, P., Palacios-Arreola, M. I., Nava-Castro, K. E., Castro, J. I., and Morales-Montor, J. (2015). The role of cytokines in breast cancer development and progression. *J. Interferon Cytokine Res.* 35 (1), 1–16. doi:10.1089/jir.2014.0026

Fetz, V., Prochnow, H., Brönstrup, M., and Sasse, F. (2016). Target identification by image analysis. *Nat. Prod. Rep.* 33 (5), 655–667. doi:10.1039/c5np00113g

Fjodorova, N., Vracko, M., Novic, M., Roncaglioni, A., and Benfenati, E. (2010). New public QSAR model for carcinogenicity. *Chem. Cent. J.* 4 (1), S3. doi:10.1186/1752-153X-4-S1-S3

Gabet, S., Lemarchand, C., Guénel, P., and Slama, R. (2021). Breast cancer risk in association with atmospheric pollution exposure: A meta-analysis of effect estimates followed by a health impact assessment. *Environ. Health Perspect.* 129 (5), 57012. doi:10.1289/EHP8419

Garcia-Martinez, L., Zhang, Y., Nakata, Y., Chan, H. L., and Morey, L. (2021). Epigenetic mechanisms in breast cancer therapy and resistance. *Nat. Commun.* 12 (1), 1786. doi:10.1038/s41467-021-22024-3

Golbamaki, A., Benfenati, E., Golbamaki, N., Manganaro, A., Merdivan, E., Roncaglioni, A., et al. (2016). New clues on carcinogenicity-related substructures derived from mining two large datasets of chemical compounds. *J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev.* 34 (2), 97–113. doi:10.1080/10590501.2016.1166879

Guyton, K. Z., Rieswijk, L., Wang, A., Chiu, W. A., and Smith, M. T. (2018). Key characteristics approach to carcinogenic hazard identification. *Chem. Res. Toxicol.* 31 (12), 1290–1292. doi:10.1021/acs.chemrestox.8b00321

Hanahan, D. (2022). Hallmarks of cancer: New dimensions. *Cancer Discov.* 12 (1), 31–46. doi:10.1158/2159-8290.CD-21-1059

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell.* 144 (5), 646–674. doi:10.1016/j.cell.2011.02.013

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell.* 100 (1), 57–70. doi:10.1016/s0092-8674(00)81683-9

Hayes, J. D., Dinkova-Kostova, A. T., and Tew, K. D. (2020). Oxidative stress in cancer. *Cancer Cell.* 38 (2), 167–197. doi:10.1016/j.ccell.2020.06.001

Hernandez, L. G., van Steeg, H., Luijten, M., and van Benthem, J. (2009). Mechanisms of non-genotoxic carcinogens and importance of a weight of evidence approach. *Mutat. Res.* 682 (2-3), 94–109. doi:10.1016/j.mrrev.2009.07.002

Heusinkveld, H., Braakhuis, H., Gommans, R., Botham, P., Corvaro, M., van der Laan, J. W., et al. (2020a). Towards a mechanism-based approach for the prediction of nongenotoxic carcinogenic potential of agrochemicals. *Crit. Rev. Toxicol.* 50 (9), 725–739. doi:10.1080/10408444.2020.1841732

Heusinkveld, H. J., Schoonen, W. G., Hodemaekers, H. M., Nugraha, A., Sirks, J. J., Veenma, V., et al. (2020b). Distinguishing mode of action of compounds inducing craniofacial malformations in zebrafish embryos to support dose-response modeling in combined exposures. *Reprod. Toxicol.* 96, 114–127. doi:10.1016/j.reprotox.2020.06.002

ICH (2008). Guideline S2 (R1) on genotoxicity testing and data interpretation for pharmaceuticals intended for human use, https://www.ema.europa.eu/en/ich-s2-r1-genotoxicity-testing-data-interpretation-pharmaceuticals-intended-human-use-scientific.

Jacobs, M. N., Colacci, A., Corvi, R., Vaccari, M., Aguila, M. C., Corvaro, M., et al. (2020). 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.* 94 (8), 2899–2923. doi:10.1007/s00204-020-02784-5

Jacobs, M. N., Colacci, A., Louekari, K., Luijten, M., Hakkert, B. C., Paparella, M., et al. (2016). International regulatory needs for development of an IATA for non-genotoxic carcinogenic chemical substances. *ALTEX* 33 (4), 359–392. doi:10.14573/altex.1601201

Jacobs, M. N., Kubickova, B., and Boshoff, E. (2022). Candidate proficiency test chemicals to address industrial chemical applicability domains for *in vitro* human cytochrome P450 enzyme induction. *Front. Toxicol.* 4, 880818. doi:10.3389/ftox.2022.880818

Kalluri, R., and Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. *J. Clin. Invest.* 119 (6), 1420–1428. doi:10.1172/JCI39104

Kalyanaraman, B., Darley-Usmar, V., Davies, K. J. A., Dennery, P. A., Forman, H. J., Grisham, M. B., et al. (2012). Measuring reactive oxygen and nitrogen species with fluorescent probes: Challenges and limitations. *Free Radic. Biol. Med.* 52 (1), 1–6. doi:10.1016/j.freeradbiomed.2011.09.030

Kay, J. E., Cardona, B., Rudel, R. A., Vandenberg, L. N., Soto, A. M., Christiansen, S., et al. (2022). Chemical effects on breast development, function, and cancer risk: Existing knowledge and new opportunities. *Curr. Environ. Health Rep.* 9 (4), 535–562. doi:10.1007/s40572-022-00376-2

Kinner, A., Wu, W., Staudt, C., and Iliakis, G. (2008). Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Res.* 36 (17), 5678–5694. doi:10.1093/nar/gkn550

Klaunig, J. E. (2018). Oxidative stress and cancer. *Curr. Pharm. Des.* 24 (40), 4771–4778. doi:10.2174/1381612825666190215121712

Klutzny, S., Kornhuber, M., Morger, A., Schönfelder, G., Volkamer, A., Oelgeschläger, M., et al. (2022). Quantitative high-throughput phenotypic screening for environmental estrogens using the E-Morph Screening Assay in combination with in silico predictions. *Environ. Int.* 158, 106947. doi:10.1016/j.envint.2021.106947

Kopp, B., Khoury, L., and Audebert, M. (2019). Validation of the γH2AX biomarker for genotoxicity assessment: A review. *Arch. Toxicol.* 93 (8), 2103–2114. doi:10.1007/s00204-019-02511-9

Kornhuber, M., Dunst, S., Schönfelder, G., and Oelgeschläger, M. (2021). The E-Morph Assay: Identification and characterization of environmental chemicals with estrogenic activity based on quantitative changes in cell-cell contact organization of breast cancer cells. *Environ. Int.* 149, 106411. doi:10.1016/j.envint.2021.106411

Kortenkamp, A. (2006). Breast cancer, oestrogens and environmental pollutants: A re-evaluation from a mixture perspective. *Int. J. Androl.* 29 (1), 193–198. doi:10.1111/j.1365-2605.2005.00613.x

Koual, M., Tomkiewicz, C., Cano-Sancho, G., Antignac, J. P., Bats, A. S., and Coumoul, X. (2020). Environmental chemicals, breast cancer progression and drug resistance. *Environ. Health* 19 (1), 117. doi:10.1186/s12940-020-00670-2

Koual, M., Tomkiewicz, C., Guerrera, I. C., Sherr, D., Barouki, R., and Coumoul, X. (2021). Aggressiveness and metastatic potential of breast cancer cells Co-cultured with preadipocytes and exposed to an environmental pollutant dioxin: An *in vitro* and *in vivo* zebrafish study. *Environ. Health Perspect.* 129 (3), 37002. doi:10.1289/EHP7102

Larigot, L., Benoit, L., Koual, M., Tomkiewicz, C., Barouki, R., and Coumoul, X. (2022). Aryl hydrocarbon receptor and its diverse ligands and functions: An exposome receptor. *Annu. Rev. Pharmacol. Toxicol.* 62, 383–404. doi:10.1146/annurev-pharmtox-052220-115707

Larigot, L., Juricek, L., Dairou, J., and Coumoul, X. (2018). AhR signaling pathways and regulatory functions. *Biochim. Open* 7, 1–9. doi:10.1016/j.biopen.2018.05.001

Leuthold, D., Klüver, N., Altenburger, R., and Busch, W. (2019). Can environmentally relevant neuroactive chemicals specifically Be detected with the locomotor response test in zebrafish embryos? *Environ. Sci. Technol.* 53 (1), 482–493. doi:10.1021/acs.est.8b04327

Li, S., and Xia, M. (2019). Review of high-content screening applications in toxicology. *Arch. Toxicol.* 93 (12), 3387–3396. doi:10.1007/s00204-019-02593-5

Lyden, D., Ghajar, C. M., Correia, A. L., Aguirre-Ghiso, J. A., Cai, S., Rescigno, M., et al. (2022). Metastasis. *Cancer Cell.* 40 (8), 787–791. doi:10.1016/j.ccell.2022.07.010

Madia, F., Pillo, G., Worth, A., Corvi, R., and Prieto, P. (2021). Integration of data across toxicity endpoints for improved safety assessment of chemicals: The example of carcinogenicity assessment. *Arch. Toxicol.* 95 (6), 1971–1993. doi:10.1007/s00204-021-03035-x

Madia, F., Worth, A., Whelan, M., and Corvi, R. (2019). Carcinogenicity assessment: Addressing the challenges of cancer and chemicals in the environment. *Environ. Int.* 128, 417–429. doi:10.1016/j.envint.2019.04.067

Marion, M. J., Hantz, O., and Durantel, D. (2010). The HepaRG cell line: Biological properties and relevance as a tool for cell biology, drug metabolism, and virology studies. *Methods Mol. Biol.* 640, 261–272. doi:10.1007/978-1-60761-688-7_13

Martínez-Reyes, I., and Chandel, N. S. (2021). Cancer metabolism: Looking forward. *Nat. Rev. Cancer* 21 (10), 669–680. doi:10.1038/s41568-021-00378-6

Marx-Stoelting, P., Rivière, G., Luijten, M., Aiello-Holden, K., Bandow, N., Baken, K., et al. (2023). A walk in the PARC: Developing and implementing 21st century chemical risk assessment in Europe. *Arch. Toxicol.* 97 (3), 893–908. doi:10.1007/s00204-022-03435-7

Mimmler, M., Peter, S., Kraus, A., Stroh, S., Nikolova, T., Seiwert, N., et al. (2016). DNA damage response curtails detrimental replication stress and chromosomal instability induced by the dietary carcinogen PhIP. *Nucleic Acids Res.* 44 (21), 10259–10276. doi:10.1093/nar/gkw791

Muruzabal, D., Sanz-Serrano, J., Sauvaigo, S., Gützkow, K. B., López de Cerain, A., Vettorazzi, A., et al. (2020). Novel approach for the detection of alkylated bases using the enzyme-modified comet assay. *Toxicol. Lett.* 330, 108–117. doi:10.1016/j.toxlet.2020.04.021

Muruzabal, D., Sanz-Serrano, J., Sauvaigo, S., Treillard, B., Olsen, A. K., López de Cerain, A., et al. (2021). Validation of the *in vitro* comet assay for DNA cross-links and altered bases detection. *Arch. Toxicol.* 95 (8), 2825–2838. doi:10.1007/s00204-021-03102-3

Nielsen, M. S., Axelsen, L. N., Sorgen, P. L., Verma, V., Delmar, M., and Holstein-Rathlou, N. H. (2012). Gap junctions. *Compr. Physiol.* 2 (3), 1981–2035. doi:10.1002/cphy.c110051

OECD (2018a). *TG451 Carcinogenicity studies*. Paris, France: OECD.

OECD (2018b). *TG453 combined chronic toxicity/carcinogenicity studies*. Paris, France: OECD.

OECD (2022a). *TG470 mammalian erythrocyte pig-a gene mutation assay*. Paris, France: OECD.

OECD (2016b). *TG474 mammalian erythrocyte micronucleus test*. Paris, France: OECD.

OECD (2016a). *TG475 mammalian bone marrow chromosomal aberration test*. Paris, France: OECD.

OECD (2022b). *TG488 transgenic rodent somatic and germ cell gene mutation assays*. Paris, France: OECD.

OECD (2016c). *TG489* in vivo *mammalian alkaline comet assay*. Paris, France: OECD.

Oku, Y., Madia, F., Lau, P., Paparella, M., McGovern, T., Luijten, M., et al. (2022). Analyses of transcriptomics cell signalling for pre-screening applications in the integrated approach for testing and assessment of non-genotoxic carcinogens. *Int. J. Mol. Sci.* 23 (21), 12718. doi:10.3390/ijms232112718

Palla, V. V., Karaolanis, G., Katafigiotis, I., Anastasiou, I., Patapis, P., Dimitroulis, D., et al. (2017). gamma-H2AX: Can it be established as a classical cancer prognostic factor? *Tumour Biol.* 39 (3), 1010428317695931. doi:10.1177/1010428317695931

Peters, A., Nawrot, T. S., and Baccarelli, A. A. (2021). Hallmarks of environmental insults. *Cell.* 184 (6), 1455–1468. doi:10.1016/j.cell.2021.01.043

Roig, A. I., Eskiocak, U., Hight, S. K., Kim, S. B., Delgado, O., Souza, R. F., et al. (2010). Immortalized epithelial cells derived from human colon biopsies express stem cell markers and differentiate *in vitro*. *Gastroenterology* 138 (3), 1012–1021.e1-5. doi:10.1053/j.gastro.2009.11.052

Roncaglioni, A., Piclin, N., Pintore, M., and Benfenati, E. (2008). Binary classification models for endocrine disrupter effects mediated through the estrogen receptor. *Sar. QSAR Environ. Res.* 19 (7-8), 697–733. doi:10.1080/10629360802550606

Ruscitto, F., Roda, N., Priami, C., Migliaccio, E., and Pelicci, P. G. (2022). Beyond genetics: Metastasis as an adaptive response in breast cancer. *Int. J. Mol. Sci.* 23 (11), 6271. doi:10.3390/ijms23116271

Salic, A., and Mitchison, T. J. (2008). A chemical method for fast and sensitive detection of DNA synthesis *in vivo*. *Proc. Natl. Acad. Sci. U. S. A.* 105 (7), 2415–2420. doi:10.1073/pnas.0712168105

Seiwert, N., Neitzel, C., Stroh, S., Frisan, T., Audebert, M., Toulany, M., et al. (2017). AKT2 suppresses pro-survival autophagy triggered by DNA double-strand breaks in colorectal cancer cells. *Cell. Death Dis.* 8 (8), e3019. doi:10.1038/cddis.2017.418

Seiwert, N., Wecklein, S., Demuth, P., Hasselwander, S., Kemper, T. A., Schwerdtle, T., et al. (2020). Heme oxygenase 1 protects human colonocytes against ROS formation,

oxidative DNA damage and cytotoxicity induced by heme iron, but not inorganic iron. *Cell. Death Dis.* 11 (9), 787. doi:10.1038/s41419-020-02950-8

Sies, H., and Jones, D. P. (2020). Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. *Nat. Rev. Mol. Cell. Biol.* 21 (7), 363–383. doi:10.1038/s41580-020-0230-3

Sistare, F. D., Morton, D., Alden, C., Christensen, J., Keller, D., Jonghe, S. D., et al. (2011). An analysis of pharmaceutical experience with decades of rat carcinogenicity testing: Support for a proposal to modify current regulatory guidelines. *Toxicol. Pathol.* 39 (4), 716–744. doi:10.1177/0192623311406935

Smith, M. T., Guyton, K. Z., Gibbons, C. F., Fritz, J. M., Portier, C. J., Rusyn, I., et al. (2016). Key characteristics of carcinogens as a basis for organizing data on mechanisms of carcinogenesis. *Environ. Health Perspect.* 124 (6), 713–721. doi:10.1289/ehp.1509912

Smith, M. T., Guyton, K. Z., Kleinstreuer, N., Borrel, A., Cardenas, A., Chiu, W. A., et al. (2020). The key characteristics of carcinogens: Relationship to the hallmarks of cancer, relevant biomarkers, and assays to measure them. *Cancer Epidemiol. Biomarkers Prev.* 29 (10), 1887–1903. doi:10.1158/1055-9965.EPI-19-1346

Sovadinova, I., Upham, B. L., Trosko, J. E., and Babica, P. (2021). Applicability of scrape loading-dye transfer assay for non-genotoxic carcinogen testing. *Int. J. Mol. Sci.* 22 (16), 8977. doi:10.3390/ijms22168977

Stampar, M., Breznik, B., Filipič, M., and Žegura, B. (2020). Characterization of *in vitro* 3D cell model developed from human hepatocellular carcinoma (HepG2) cell line. *Cells* 9 (12), 2557. doi:10.3390/cells9122557

Stampar, M., Žabkar, S., Filipič, M., and Žegura, B. (2022). HepG2 spheroids as a biosensor-like cell-based system for (geno)toxicity assessment. *Chemosphere* 291 (Pt 1), 132805. doi:10.1016/j.chemosphere.2021.132805

Toma, C., Manganaro, A., Raitano, G., Marzo, M., Gadaleta, D., Baderna, D., et al. (2020). QSAR models for human carcinogenicity: An assessment based on oral and inhalation slope factors. *Molecules* 26 (1), 127. doi:10.3390/molecules26010127

Trosko, J. E., Chang, C. C., Upham, B. L., and Tai, M. H. (2004). Ignored hallmarks of carcinogenesis: Stem cells and cell-cell communication. *Ann. N. Y. Acad. Sci.* 1028, 192–201. doi:10.1196/annals.1322.023

Uzbas, F., Opperer, F., Sönmezer, C., Shaposhnikov, D., Sass, S., Krendl, C., et al. (2019). BART-seq: Cost-effective massively parallelized targeted sequencing for genomics, transcriptomics, and single-cell analysis. *Genome Biol.* 20 (1), 155. doi:10.1186/s13059-019-1748-6

Van Bossuyt, M., Raitano, G., Honma, M., Van Hoeck, E., Vanhaecke, T., Rogiers, V., et al. (2020). New QSAR models to predict chromosome damaging potential based on the *in vivo* micronucleus test. *Toxicol. Lett.* 329, 80–84. doi:10.1016/j.toxlet.2020.04.016

Van Der Ven, L. T. M., Van Ommeren, P., Zwart, E. P., Gremmer, E. R., Hodemaekers, H. M., Heusinkveld, H. J., et al. (2022). Dose addition in the induction of craniofacial malformations in zebrafish embryos exposed to a complex mixture of food-relevant chemicals with dissimilar modes of action. *Environ. Health Perspect.* 130 (4), 47003. doi:10.1289/EHP9888

Vang Mouritzen, M., and Jenssen, H. (2018). Optimized scratch assay for *in vitro* testing of cell migration with an automated optical camera. *J. Vis. Exp.* 138, 57691. doi:10.3791/57691

Veltman, C. H. J., Pennings, J. L. A., van de Water, B., and Luijten, M. (2023). An adverse outcome pathway network for chemically induced oxidative stress leading to (Non)genotoxic carcinogenesis. *Chem. Res. Toxicol.* 36, 805–817. doi:10.1021/acs.chemrestox.2c00396

Vieira-da-Silva, B., and Castanho, M. (2023). Resazurin reduction-based assays revisited: Guidelines for accurate reporting of relative differences on metabolic status. *Molecules* 28 (5), 2283. doi:10.3390/molecules28052283

Vigano, E. L., Colombo, E., Raitano, G., Manganaro, A., Sommovigo, A., Dorne, J. L. C., et al. (2022). Virtual extensive read-across: A new open-access software for chemical read-across and its application to the carcinogenicity assessment of botanicals. *Molecules* 27 (19), 6605. doi:10.3390/molecules27196605

Weinberg, F., Ramnath, N., and Nagrath, D. (2019). Reactive oxygen species in the tumor microenvironment: An Overview. *Cancers (Basel)* 11 (8), 1191. doi:10.3390/cancers11081191

Yan, L., Messner, C. J., Zhang, X., and Suter-Dick, L. (2021). Assessment of fibrotic pathways induced by environmental chemicals using 3D-human liver microtissue model. *Environ. Res.* 194, 110679. doi:10.1016/j.envres.2020.110679

Zefferino, R., Piccoli, C., Gioia, S. D., Capitanio, N., and Conese, M. (2019). Gap junction intercellular communication in the carcinogenesis hallmarks: Is this a phenomenon or epiphenomenon? *Cells* 8 (8), 896. doi:10.3390/cells8080896

Appendix I

Mechanistic toxicity profiling of chemicals: A comparative study in 2D and 3D HepaRG models using high-content analysis

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Chapter I supplementary information

Table APP. I 1. Results summary of chemicals tested with both models. Summary table of results obtained with the tested chemicals in both HepaRG cell models. Red: statistically significant changes, Green: no change. ↑: increase, ↓: decrease.

Assay	Cytotoxicity		Mitochondrial ΔΨm changes		Oxidative stress		Lipid accumulation	
Compound	2D	3D	2D	3D	2D	3D	2D	3D
CT								↑
Tebu							↑	↑
TAA								↑
BPO								↓
DEM								↑
CF								↑
IP								
3MCA								↑

Table APP. I 2. Results summary of chemicals tested with 3D models. Summary table of results obtained with the chemicals tested only in the 3D HepaRG cell model. Red: statistically significant changes, Green: no change. ↑: increase, ↓: decrease.

Assay	Cytotoxicity	Mitochondrial ΔΨm changes	Oxidative stress	Lipid accumulation
Compound	3D	3D	3D	3D
DCMU				↑
FICZ				↓
TPA				↑
Azox				↑

Appendix II

Advancing genotoxicity assessment with transcriptomic biomarkers: From 2D to 3D HepaRG spheroid model

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Transcriptomic–based analysis to discriminate Non-Genotoxic Carcinogens from genotoxic carcinogens and non-carcinogens in a human HepaRG spheroid model: Mechanistic insights and biomarker identification

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Chapter II supplementary information

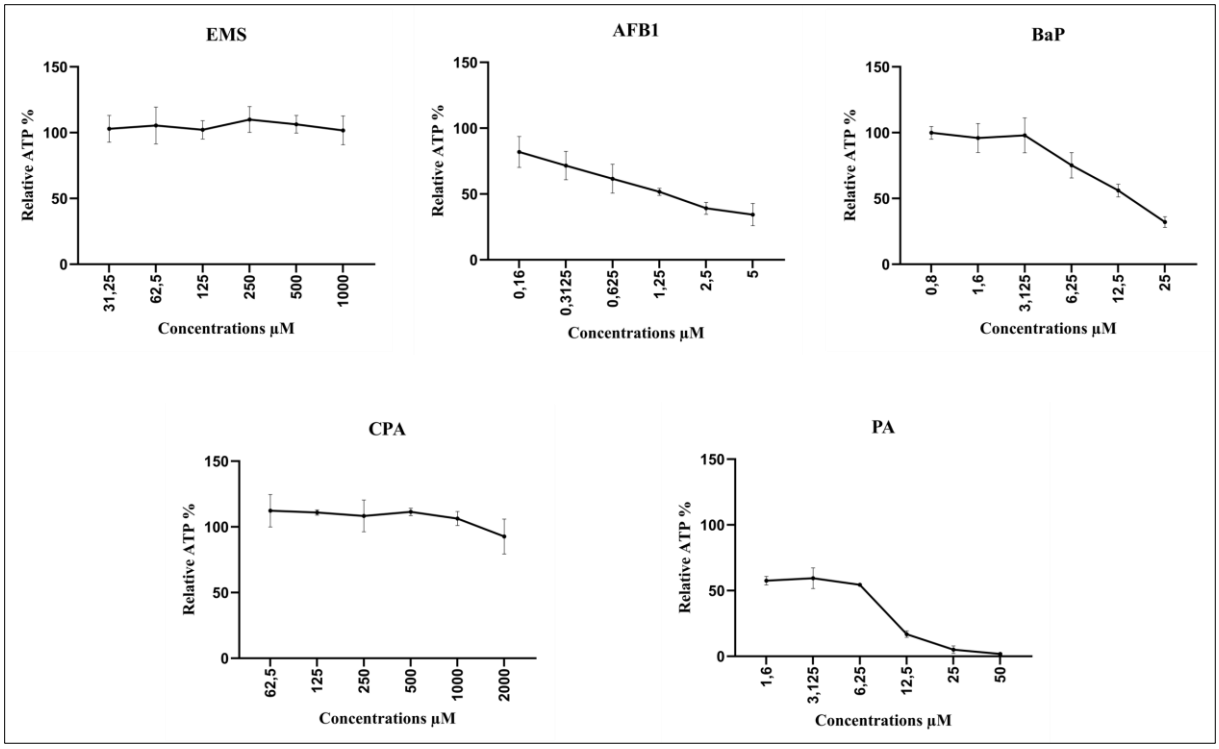

Figure APP. II 1. Pre-Cytotoxicity of the Genotoxic (GTx) selected chemical list (EMS, AFB1, BaP, CPA, and PA). The linear line represents the relative ATP percentage of the concentrations tested of each chemical with 3D HepaRG spheroids. Data presented as mean (three independent biological replicates) ± SEM.

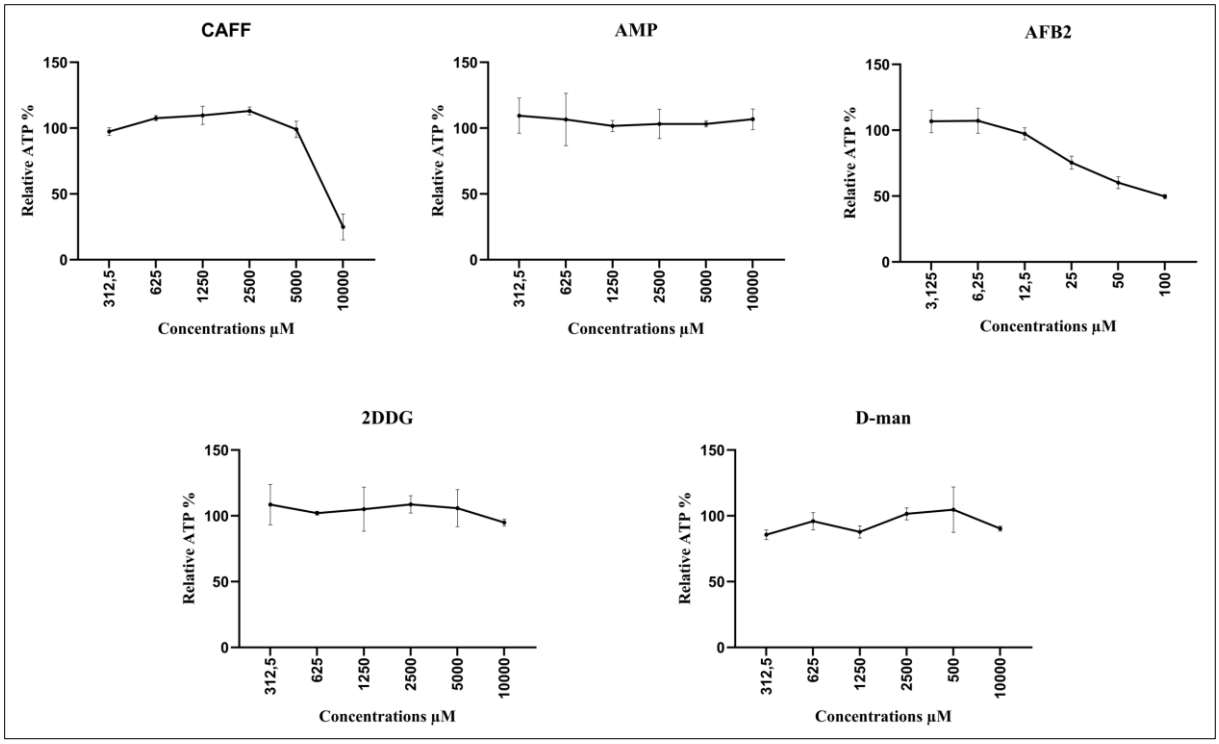

Figure APP. II 2. Pre-Cytotoxicity of the Non-genotoxic Non-carcinogens (NGTx) selected chemical list (CAFF, AMP, AFB2, 2DDG, and D-man). The linear line represents the relative ATP percentage of the concentrations tested of each chemical with 3D HepaRG spheroids. Data presented as mean (three independent biological replicates) ± SEM.

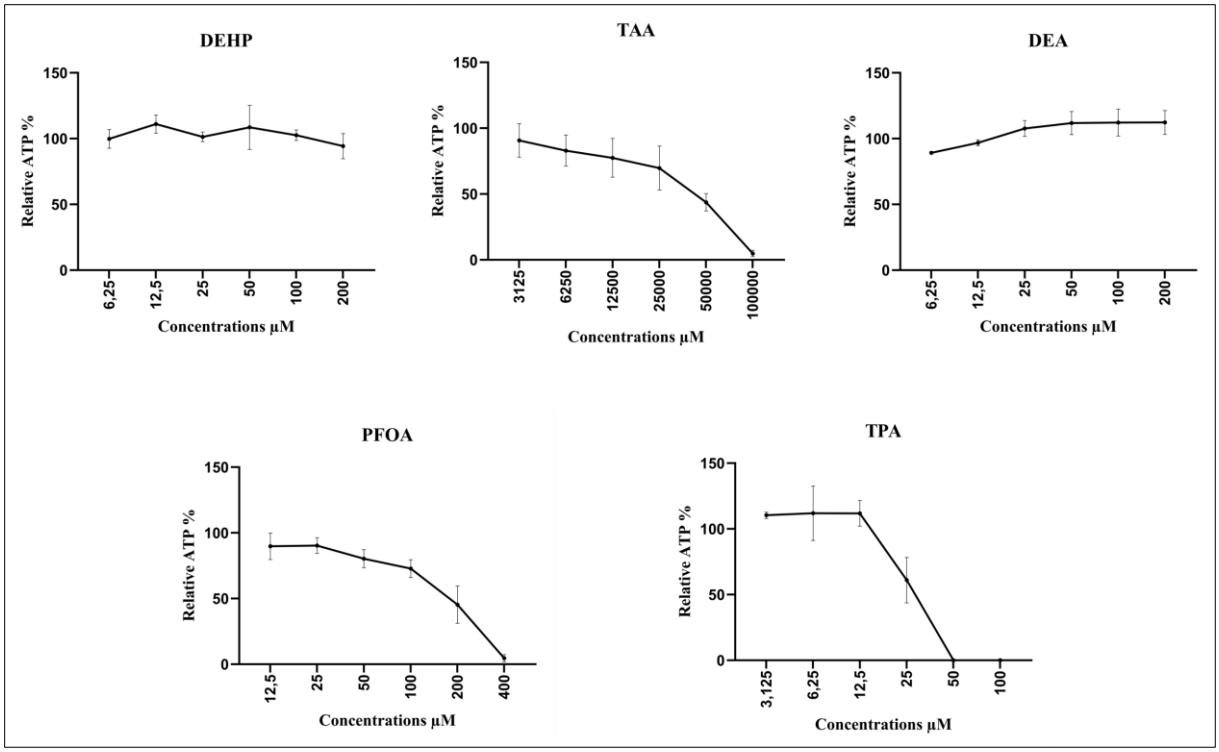

Figure APP. II 3. Pre-Cytotoxicity of the Non-genotoxic carcinogens (NGTxC) selected chemical list (DEHP, TAA, DEA, PFOA, and TPA). The linear line represents the relative ATP percentage of the concentrations tested of each chemical with 3D HepaRG spheroids. Data presented as mean (three independent biological replicates) ± SEM.

Appendix III

Advancing genotoxicity assessment with transcriptomic biomarkers: From 2D cell systems to 3D HepaRG spheroid model

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Chapter III supplementary informations

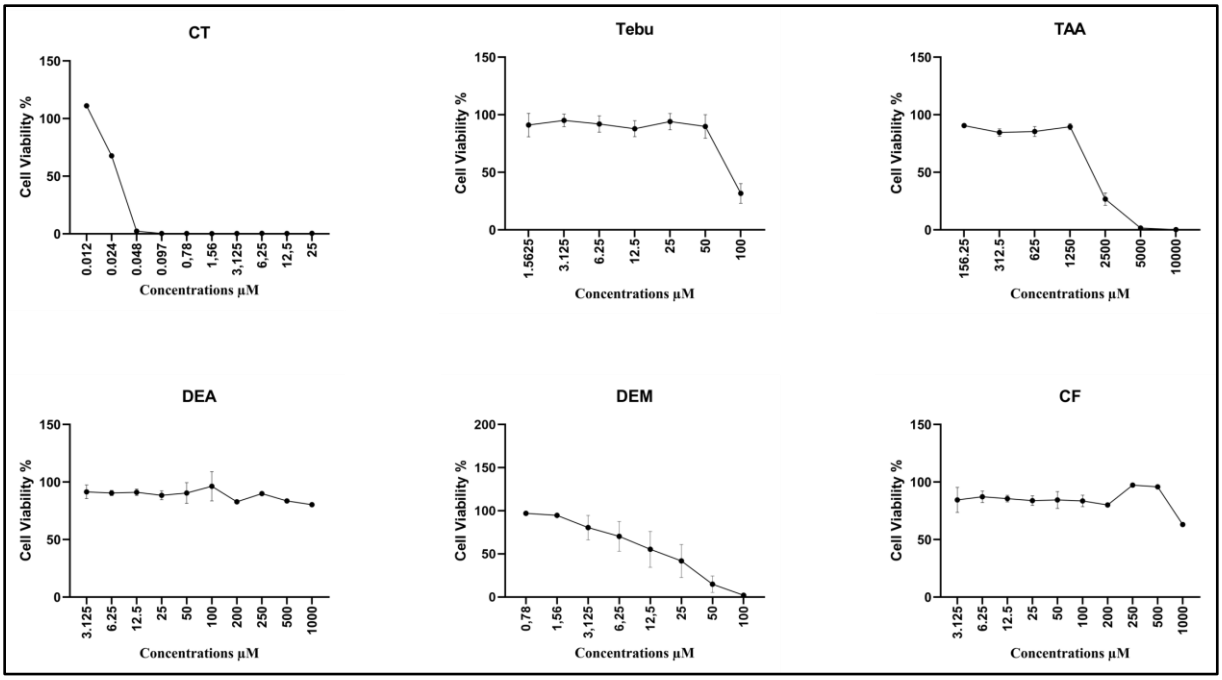


Figure APP. III 1. Pre-CGA (I) of the selected chemicals. The linear line represents the relative cell growth percentage in relative to the negative control (solvent).

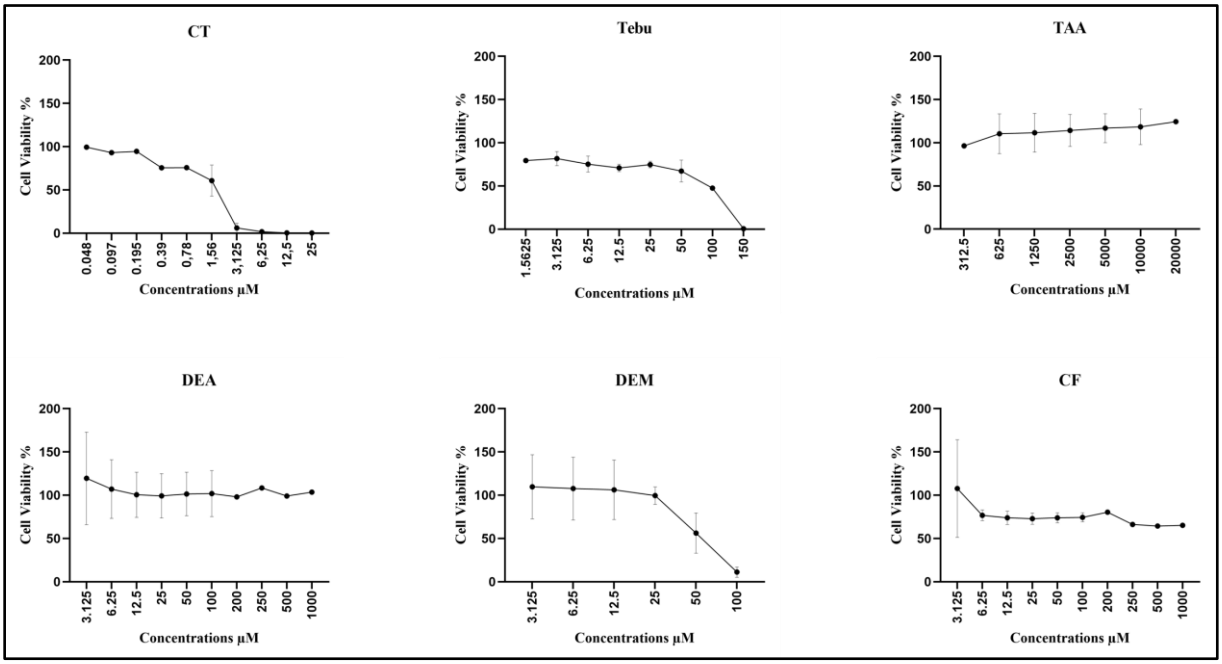
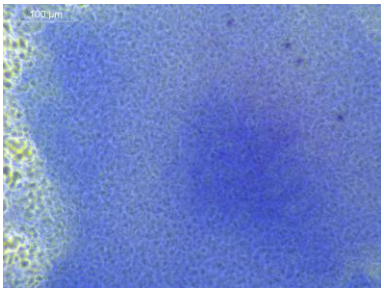
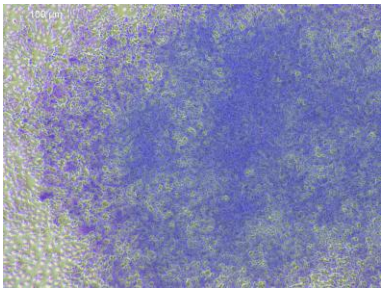
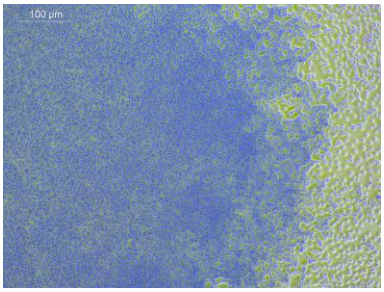
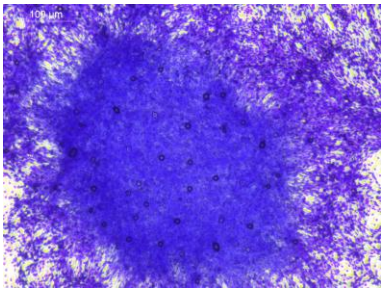
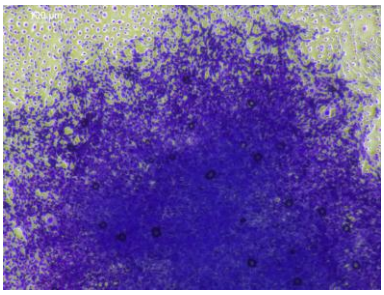
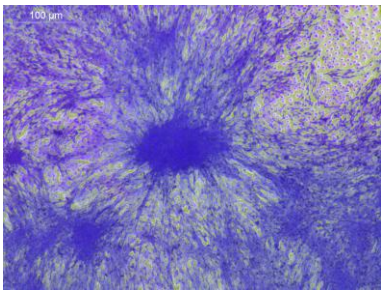
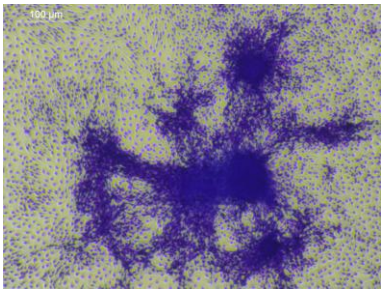
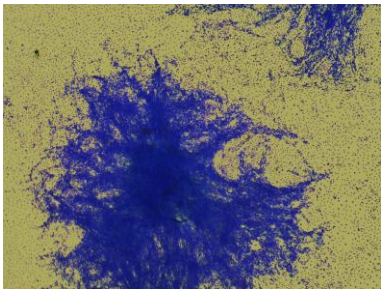


Figure APP. III 2. Pre-CGA (P) of the selected chemicals. The linear line represents the relative cell growth percentage in relative to the negative control (solvent).

Table APP. III 1. Transformed foci criteria. The applied criteria in the Bhas42 foci evaluations (positive and negative) obtained from our results.

Foci	Characteristics	Judgment
	Basophilic Cell aggregation	Negative foci
	Cell aggregation	Negative foci
	Basophilic Cell aggregation	Negative foci
	Highly Basophilic Multi-layered	Positive Foci

	Highly Basophilic Multi-layered Spindle shape extremities Random orientation	Positive Foci
	Highly Basophilic Multi-layered Spindle shape extremities Invasive	Positive Foci
	Highly Basophilic Multi-layered Spindle shape extremities Random orientation Invasive	Positive Foci
	Highly Basophilic Multi-layered Spindle shape extremities Random orientation Invasive	Positive Foci

Appendix IV

Multi-Cellular Human Liver Organoids for Modeling Metabolic Dysfunction–Associated Steatotic Liver Disease and Drug-Induced Liver Injury

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Publicatoin “Protocol”

Published – *Current Protocols* (Toxicology Section) - *Wiley*

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Template for a Protocol Article

Multi-Cellular Human Liver Organoids for Modeling Metabolic Dysfunction–Associated Steatotic Liver Disease and Drug-Induced Liver Injury

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Abstract

In vitro modeling of human liver function is essential for assessing drug-induced toxicity, particularly in the context of metabolic dysfunction associated liver disease (MASLD). However, standard liver organoid systems often lack xenobiotic-metabolizing enzyme activity and fail to replicate the pathological features of (MASLD). Here, we present a method for generating functional, multi-cell-type human liver organoid model (HML organoids) from HepaRG cells, primary human macrophages, and LX-2 hepatic stellate cells. These 3D organoids are cultured under defined conditions that support key hepatic functions. To mimic MASLD, we expose HML organoids to a mixture of stearic and oleic acids for 9 days, inducing steatosis and fibrogenic responses characteristic of the disease. These MASLD-HML organoids retain liver-specific functions and express key fibrosis markers. We further demonstrate the usefulness of this method to produce standardized organoids useful for the evaluation of drug-induced liver injury through IC_{50} and benchmark dose (BMD) calculations, particularly in MASLD context. Together, HML and MASLD-HML organoids provide a robust model for studying *in vitro* drug metabolism, toxicity, and adverse drug reactions in healthy and diseased liver states.

Basic Protocol: Modeling drug-induced liver injury and MASLD using multi-cell-type human liver organoids

Keywords: NAMs, Organoids, NAMS, MASLD, 3D hepatic model

1. Introduction

3D multi-cell-type liver organoids provide a powerful *in vitro* model to study liver disease and drug-induced toxicity. Metabolic dysfunction associated liver disease (MASLD) is now recognized as a major risk factor for drug-induced liver injury (DILI) (Perumpail et al., 2017), yet human-relevant *in vitro* models that recapitulate both steatosis and fibrogenesis remain limited. Primary human hepatocytes (pHHs) are often considered the gold standard for hepatotoxicity testing but face significant challenges, including limited availability and loss of function during prolonged culture. Moreover, hepatocytes like cells in two-dimensional monocultures usually fail to capture the complex multicellular interactions involved in MASLD progression and DILI (Kammerer, 2021).

Liver organoids are explored as a support model for the development of new approach methodologies (NAMs) for toxicological assessment. Commonly used models often rely on poorly matured cell systems such as HepG2 or THP-1 cells, which limits their ability to fully mimic the *in vivo* hepatic physiology (Yokoyama et al., 2018). In addition, the use of DMSO, high serum concentrations, and incompatibility with high-throughput assessment represents major limitations to such cellular models. Moreover, long-term stability and scalability, along with the good integration of non-parenchymal cells restrict their capacity to model complex responses such as inflammation and fibrosis. These limitations underscore the need for robust and easily deployable liver organoids that can be broadly implemented as reliable new approach methodologies (NAMs).

Human-hepatic HepaRG cell line provides a relevant *in vitro* model for studying liver function and toxicology (Aninat et al., 2006; Ashraf et al., 2019). As a human-derived hepatic cell line, HepaRG cells retain key metabolic and functional characteristics of primary hepatocytes, including phase I and phase II drug-metabolizing enzymes and transporter activity (Hart et al., 2010). These properties make them particularly valuable for mimicking *in vivo* liver complexity compared to other hepatic cell lines. Owing to their stability, reproducibility, and functional relevance, HepaRG cells are increasingly recognized as a promising cell model used in (NAM) for toxicological assessments mainly with liver toxicity.

Here, we describe a detailed protocol to generate three-dimensional (3D) multi-cellular liver organoids composed of differentiated human hepatic cell line HepaRG cells, primary human

unstimulated M1 macrophages, and human hepatic stellate cell-derived LX-2 cell line. These self-assemble organoids are called HML (**H**epaRG **M**acrophages **L**X-2).

This organoid model is produced under scaffold-free conditions while reproducing key features of the liver microenvironment through the production of major extracellular matrix proteins by stellate cells (Arteel, 2024). Thus, it maintains metabolic competence, including the expression of phase I and II enzymes, without the need for DMSO or complex differentiation conditions. in addition, it allows for chronic exposure of up to 3 weeks to different conditions, including mixtures of stearic and oleic acids, leading to lipid accumulation, basal inflammation, and fibrogenic responses characteristic of steatohepatitis (Bronsard et al., 2023). Moreover, this model provides a reproducible, human-relevant alternative to animal models, mimicking *in vivo* liver complexity and enabling the evaluation of DILI risk in the context of metabolic liver diseases.

The described protocol represents two different culture methods, ultra-low attachment (ULA) plates and agarose casts, to produce HML organoids. In ULA plates, cells spontaneously aggregate into spheroids, providing a simple and reproducible setup that enables the assessment of multiple conditions within the same plate. In contrast, agarose casts allow the generation of 35 organoids per condition, which is advantageous for downstream analyses requiring larger amounts of biological material such as DNA or protein. However, agarose casting is more labor-intensive and less easily adaptable to high-throughput formats compared with ULA plates.

The protocol below describes the culture and co-seeding of differentiated HepaRG cells, primary human unstimulated M1 macrophages, and LX-2 cells, organoid formation using low-attachment conditions, induction of steatosis with fatty acids, and subsequent analyses including viability assays, histological staining, gene expression, and dose–response modeling. With this protocol, researchers can generate robust liver organoid model to study the mechanistic basis of MASLD and drug-induced liver injury.

Caution: All appropriate institutional guidelines for human-derived cells must be followed, and standard BSL II practices should be employed for all procedures involving primary human macrophages and blood-derived materials.

2. Strategic planning

2.1. Independent culture protocols for HepaRG cells, primary human M1 macrophages, and LX-2 cells

Strict adherence to culture protocols is essential to ensure the preservation of the phenotypic characteristics of each cell type used in the model. Here, we describe for each cell type the required materials and reagents for their culture, along with a detailed step-by-step protocol for cell culture procedures.

2.2. Organoid formation in agarose molds and ULA plates

Tri-cellular organoids composed of differentiated HepaRG cells, unstimulated M1 macrophages, and LX-2 cells can be generated in two different systems. Here, we describe how to prepare agarose molds and how to seed the three cell types in ULA plates and in agarose molds. The protocols also include the necessary steps to maintain these cultures over time up to 3 weeks.

2.3. Harvesting organoids from agarose molds and processing for histological or transcriptomic analyses

Producing organoids in agarose molds allows the generation of up to 35 organoids per condition, thereby increasing the amount of biological material that can be collected for multi-omics analysis (histology, transcriptomic, proteomic and metabolomic). Here, we describe how to harvest organoids and process the required number for these downstream analyses.

2.4. Using organoids in ULA plates to perform cytotoxicity assays

To perform cytotoxicity assays, we prefer producing organoids in 96-well Ultra-Low Attachment plates to obtain a single organoid per well. Here, we describe how to treat these organoids with compounds to carry out cytotoxicity tests.

3. Cell Culture Protocols

Warm up steps for cell culture

To ensure good laboratory practices, a clean and sterile working environment is essential. This includes wearing gloves, properly disposing of all types of waste (sharps, liquids, and solids), and cleaning the biosafety hood before and after use according to laboratory protocols. Additionally, when working with cell lines, careful documentation is required on all flasks and seeding plates, including the cell line name, passage number, and treatment conditions. Working with human cells, particularly human primary cells and blood samples, requires a biosafety level 2 environment. We strongly recommend having an up-to-date hepatitis B vaccine when handling blood samples.

3.1. HepaRG cell culture

3.1.1. Introductory paragraph

The HepaRG cell line was originally derived from a hepatocellular carcinoma in a patient co-infected with chronic hepatitis C virus (Gripon et al., 2002). HepaRG cells have the remarkable ability to undergo both differentiation and transdifferentiation processes (Cerec et al., 2007). When cultured at low density, they display a strong self-renewal capacity, maintaining an elongated, undifferentiated morphology and expressing markers characteristic of bipotent hepatic progenitor cells. As they proliferate, their morphology progressively evolves toward that of granular epithelial cells, surrounded by more flattened and clear epithelial cells. To promote full differentiation, the addition of 1.7% DMSO 15 days after seeding at confluence induces their maturation into two distinct cell populations: hepatocyte-like cells, closely resembling primary human hepatocytes, and biliary-like cells. Typically, around 50% to 55% of the resulting population consists of hepatocyte-like cells.

3.1.2. Materials

Undifferentiated HepaRG cells (Biopredic, Ref: BPHPR101)

William’s E Medium, Glutamax supplement (Gibco, Ref: 32551020)

Fetal Bovine Serum (FBS) Fetalclone 2 (Cytiva, Ref: SH30066.33)

Penicillin-Streptomycin (PS) 100X (Gibco, Ref 15070-063)

Insulin (Sigma, Ref: PHR8925)

Hydorcortisone hemisuccinate (Upjohn Pharmaceuticals, Ref: SERB 100 mg)

PBS 1X (Gibco, Ref: 14190-094)

Trypsin-EDTA (0.05%) (Gibco, Ref: 25300-054)

Tube 50 mL (Corning, Ref: 430290)

Flask T75 (Corning, Ref: 430720U)

Cell counter

Centrifuge

37°C incubator

3.1.3. Cell medium preparation

1. Pre warm a William's E medium, Glutamax supplement (500 ml bottle) at 37°C (for 1 hour maximum to preserve media components from degradation)
2. Retrieve 56 mL of Willam's E medium as equivalent of total volume of complements
3. Add 5 mL of stock penicillin-streptomycin at 100X for a final concentration of 1X
4. Add 500 µL of Insulin stock solution 5 mg/mL to 500 mL of medium for a final concentration at 5 µg/mL
5. Add 500 µL of Hydrocortisone stock solution at 50 mM in 500 mL of medium for a final concentration at 50 µM
6. Add 50 mL of FBS in 500 mL of medium for a final concentration at 10% FBS.
7. Gently mix the media components while avoiding foam formation.

3.1.4. Cell defrost

1. Defrost the vial containing 1.10^6 cells rapidly (in about 2 minutes) in a water bath at 37 °C.
2. Transfer the cells in a 50 mL tube containing 9 mL of fresh media and centrifuge the cells 2 minutes at 500G in order to wash out DMSO from cryopreservation media.
3. Aspirate the media and transfer the cells in a 75 cm² flask containing 15 ml of full culture medium (with 10 % FBS).

3.1.5. Subculture protocol

1. Remove the culture medium and rinse the cells once with approximately 10 ml of PBS.
2. Add 1 ml of Trypsin-EDTA (0.05%), incubate for about 5 minutes in the incubator. Monitor cell detachment under the microscope and gently tap the flask to complete cell detachment.
3. Resuspend the cells in 5 ml of complete medium and pipette up and down several times while avoiding foaming.
4. Add 5 ml of complete medium (final volume: 10 ml) and transfer the cell suspension into a 15 ml tube.
5. Count the cell in the suspension (using Mallassez for example)
6. Centrifuge at 136 g (800 rpm) for 5 minutes at room temperature.
7. Discard the supernatant and resuspend the cell pellet in 10 ml of complete medium.
8. Seed into 75 cm^2 flasks at approximately 2 x 10^6 cells per flask.

3.1.6. Differentiation protocol

1. After 14 days of culture according to subculture protocol, cells are incubated in the presence of complete medium supplemented with 1% DMSO.
2. After 16 days of culture, cells are incubated in the presence of complete medium supplemented with 1.5% DMSO.
3. After 18 days of culture, cells are incubated in the presence of complete medium supplemented with 1.7% DMSO, with a renewal of complete medium with 1.7% DMSO 2 days until cell usage for one week until D28 (from Day 18 to Day 28).

3.2. LX2 cell culture

3.2.1. Introductory paragraph

LX-2 is a human hepatic stellate cell line derived from primary stellate cells and immortalized to maintain a stable, activated phenotype suitable for *in vitro* fibrosis research. LX-2 cells express key markers such as α-SMA, GFAP, and type I collagen, reflecting their fibrogenic capacity. They respond reliably to cytokines and growth factors involved in liver fibrosis, making them a robust and reproducible model for studying stellate cell activation, extracellular matrix deposition, and the effects of anti-fibrotic treatments. Due to their stable phenotype and ease of culture, LX-2 cells are widely used for mechanistic studies and high-throughput screening of compounds targeting fibrogenesis.

3.2.2. Materials

LX-2 cells (SCC064, Sigma-Aldrich)

Dulbecco's Modified Eagle Medium supplemented with GlutaMAX (Gibco, Ref: 15440544)

Fetal Bovine Serum FBS (Gibco, Ref 10270-106)

Penicillin-Streptomycin (PS) 100X (Gibco, Ref 15070-063)

PBS 1X (Gibco, Ref: 14190-094)

Trypsin-EDTA (0.05%) (Gibco, Ref: 25300-054)

Tube 50 mL (Corning, Ref: 430290)

Flask T75 (Corning, Ref: 430720U)

Cell counter

Centrifuge

37°C incubator

3.2.3. Cell medium preparation

1. Pre warm a Dulbecco's Modified Eagle Medium supplemented with GlutaMAX (500 ml bottle) at 37°C (for 1 hour maximum to preserve media components from degradation)
2. Add 1 % of stock penicillin-streptomycin (100X) (i.e 5 ml/500 ml of in the medium)
3. Add 10 mL of FBS in 500 mL of medium for a final concentration at 2% FBS.

3.2.4. Cell defrost

1. Defrost the vial containing 1.10^6 cells rapidly (in about 2 minutes) in a water bath at 37 °C.
2. Transfer the cells in a 50 mL tube containing 9 mL of fresh media and centrifuge the cells 2 minutes at 500G in order to wash out DMSO from cryopreservation media.
3. Aspirate the media and transfer the cells in a 75 cm^2 flask containing 15 ml of full culture medium (with 2 % FBS).

3.2.5. Subculture protocol

1. Remove the culture medium and rinse the cells once with approximately 10 ml of PBS.
2. Add 1 ml of Trypsin-EDTA (0.05%), incubate for about 5 minutes in the incubator. Monitor cell detachment under the microscope; tap the flask to complete cell detachment.
3. Resuspend the cells in approximately 4 ml of complete medium; pipette up and down several times, avoiding foaming.
4. Add approximately 5 ml of complete medium (final volume: approximately 10 ml) and transfer the cell suspension into a 15 ml tube.
5. Centrifuge at 136 g (800 rpm) for 5 minutes at 20°C.
6. Discard the supernatant and resuspend the cell pellet in 7 ml of complete medium.
7. Seed into 75 cm^2 flasks at approximately 2 x 10^6 cells per flask.

Optional activation step

The LX-2 cell line does not require differentiation steps. However, the cells can be activated under specific culture conditions. LX-2 cells display a quiescent phenotype when cultured with low serum concentrations (≤ 2%) and get activated in high serum concentrations (10%). Quiescent cells proliferate very slowly and store lipid droplets, whereas activated cells are characterized by an increase proliferation rate, loss of lipid droplets volume, cell migration and secretion of ECM components (Schinagl et al., 2021).

3.3. Primary human unstimulated M1 macrophages culture

3.3.1. Introductory paragraph

Primary human unstimulated M1 macrophages are derived from peripheral blood mononuclear cells (PBMCs) isolated from donor blood. Blood contains various cell types suspended in plasma, including monocytes — a population of white blood cells that can migrate into tissues and differentiate into macrophages or dendritic cells. To obtain monocytes, PBMCs are typically isolated using a Ficoll density gradient from a buffy coat. The purified monocytes are then cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), which promotes their differentiation into macrophages.

Under these conditions, monocytes mature into unstimulated M1 macrophages, an inflammatory subset specialized in pathogen clearance and immune activation. M1 macrophages are characterized by their production of pro-inflammatory cytokines and enzymes

that degrade microbial components and cellular debris. This protocol ensures a reliable and reproducible generation of primary human M1 macrophages, providing a relevant cell type for *in vitro* models investigating immune responses, inflammation, and interactions with other hepatic cells in the context of liver disease. Additional steps for M1 macrophage activation can be performed using LPS or IFN-γ to polarize them toward a pro-inflammatory profile, but these preliminary steps are not included in our protocol.

3.3.2. Materials

RPMI Culture Medium (Gibco, Ref: 61870-010)

Decomplemented Fetal Bovine Serum FBS (Gibco, Ref 10270-106)

Penicillin-Streptomycin (PS) 100X (Gibco, Ref 15070-063)

PBS 1X (Gibco, Ref: 14190-094)

HEPES 1M (Fisher Bioreagents BP299-1)

GM-CSF (Miltenyi, Ref: 130-095-372)

EDTA (Gibco, Ref: 03690)

Trypsin-EDTA (0.05%) (Gibco, Ref: 25300-054)Unisep Maxi (Eurobio, Ref: U10)

Pre separation filter (Miltenyi, Ref 130-101-812)

LS Column (Miltenyi, Ref 130-0420-401)

CD14 Microbeads (Miltenyi, Ref: 130-050-201)

Erythrocyte lysis buffer (Eurobio, CMSLYS00-07)

MACS Multistand (Myltenyi, Ref: 130-042-303)

Tube 50 mL (Corning, Ref: 430290)

Flask T75 (Corning, Ref: 430720U)

Cell counter

Centrifuge

37°C incubator

3.3.3. Cell medium preparation

1. Prepare 500 mL of PBS 1X containing 1 mM EDTA
2. Prepare 50 mL of Miltenyi buffer containing PBS 1X with 0,45% FBS
3. Prepare monocytes culture medium containing RPMI with 1% L-Glutamine, Hepes 1 mM, 0.1% P/S and 10% FBS

3.3.4. PBMC isolation from buffy coat

4. Fresh buffy coat (leukocytes platelet concentrates) is obtained from the French Blood Establishment (EFS).
5. Pour an equivalent volume of PBS-EDTA into a T75 flask to match the blood volume in the bag (~45 mL).
6. Cut one of the bag's tubes and pour the blood into the PBS (1:1 dilution of blood in PBS).
7. After mixing, carefully transfer the mixture into UNI-SEP tubes (maximum 30 mL per tube, 20 mL recommended).
8. Centrifuge for 20 minutes at 1000g at 20°C, with minimal acceleration and braking (set to level 1).

3.3.5. PBMC Washing

1. Prepare four 50 mL Falcon tubes with 10 mL of PBS-EDTA.
2. Carefully collect the PBMC ring using a 10 mL pipette and transfer it into the prepared Falcons.
3. Centrifuge for 10 minutes at 500g at 20°C.
4. Discard the supernatant.
5. Resuspend the pellet in PBS-EDTA and adjust the volume to 45 mL.
6. Centrifuge for 15 minutes at 160g at 20°C.
7. Discard the supernatant gently with aspiration.
8. (optional) Erythrocyte lysis: resuspend the pellet in 1mL of PBS+EDTA.
9. Add 9mL of Erythrocyte lysis buffer.
10. Incubate 10 min at 4°C.
11. Adjust the volume to 45 mL with PBS+EDTA.
12. Centrifuge for 10 min at 500g at 20°C.

3.3.6. CD14+ Cell Isolation and Elution

1. Resuspend all pellets in 5–10 mL of PBS-EDTA.
2. Filter cells through the pre-separation filter
3. Transfer into a 50 mL tube and adjust the volume to 45 mL.
4. Perform a cell count.
5. Centrifuge for 10 minutes at 500g at 20°C.
6. Discard the supernatant.
7. Resuspend the pellet in Miltenyi Buffer (80 µL for every 10 million PBMCs).
8. Add CD14+ MicroBeads to the suspension (10 µL for every 10 million cells, maximum 600 µL).
9. Mix and incubate for 15 minutes at 4°C.
10. Wash the suspension with 15 mL of Miltenyi Buffer.
11. Centrifuge for 10 minutes at 500g.
12. Discard the supernatant.
13. Resuspend the pellet in 500 µL of Miltenyi Buffer (500 µL for every 100 million PBMCs).
14. Place the LS Column on the magnetic stand, ensuring it is vertical and seated properly.
15. Position a 15 mL Falcon tube under the column to collect the CD14- fraction.
16. Rinse the column with 3 mL of Miltenyi Buffer.
17. Apply the cell suspension to the column and collect the CD14- fraction in the tube.
18. Perform three washes with 3 mL of Miltenyi Buffer (3 × 3 mL).
19. Remove the column from the magnetic stand and place it on a new 15 mL tube.
20. Add 5–7 mL of Miltenyi Buffer and use the plunger to release the CD14+ cells.
21. Perform a cell count to prepare for cell seeding.

3.3.7. Monocytes culture and Differentiation into M1 Macrophages

1. Monocytes can be cultured at 37°C), typically for no more than 24 hours in complete, in monocytes complete medium. To differentiate into Macrophages M1 for HML organoids production, monocytes are seed in T75 following the seeding density table below:

Type	Surface area (cm2)	Monocytes seeding density	Growth medium (mL)
6-well	9.6	5.12E+05	1 to 3
12-well	3.5	1.87E+05	1 to 2
24-well	1.9	1.01E+05	0.5 to 1.0
48-well	1.1	5.86E+04	0.2 to 0.4
96-well	0.32	1.71E+04	0.1 to 0.2
T-25	25	1.33E+06	3 to 5
T-75	75	4.00E+06	8 to 15
T-175	175	9.33E+06	35 to 53

1. Prepare M1 Macrophages differentiation medium with monocyte culture medium supplemented with 50 ng/mL of GM-CSF.
2. Resuspend the pellet in the right amount of differentiation medium.
3. Incubate à 37°C and 5% CO2.
4. Renew the medium every two days during 7 days.

Note: Alternatively, cryopreserved human PBMCs can be commercially obtained and subsequently differentiated into macrophages *in vitro*. Some vendors also provide cryopreserved, pre-differentiated human macrophages, which may be used directly for organoids production.

4. Adapting organoids production to your needs

The choice between small-scale and large-scale organoid production should be guided by the specific experimental needs. Small-scale production using 96-well ULA plates enables the generation of individual organoids per well, allowing for high-throughput applications such as cytotoxicity screening, where multiple treatment conditions and replicates are required in parallel. In contrast, large-scale production using agarose-cast 3D Petri Dishes® allows the formation of up to 35 uniform organoids per condition. This format is particularly well suited for experiments requiring bulk collection of organoids, such as transcriptomic, proteomic, or metabolomic analyses. Users should therefore select the appropriate culture format depending on whether their priority is high-throughput screening or the acquisition of large quantities of biological material for downstream multi-omics assays.

Note: Although both agarose-cast 3D Petri Dishes® and ULA plates can be seeded with equivalent cell numbers and identical cell compositions, the physical format of the culture systems differs significantly. The size, geometry, and surface properties of the wells influence nutrient diffusion, oxygenation, and potentially organoid morphology. As a result, direct comparison of biological readouts between organoids generated in these two systems should be interpreted with caution. Wherever possible, experiments should be internally controlled within the same culture format, and any cross-format comparison should be validated to account for potential system-specific effects.

4.1. Organoid seeding procedure 1 in Agarose Molds

4.1.1. Introductory paragraph

The MicroTissues® 3D Petri Dish® is a user-friendly and efficient system designed to produce precision micro-molds that enable the culture of organoids in a scaffold-free 3D environment that maximizes cell–cell interactions. Each micro-mold is autoclavable and reusable. Once the agarose gel is prepared and cast into the micro-molds, it is allowed to solidify and then carefully transferred to a standard 24-well plate. This approach ensures the reproducible generation of 35 organoids per molds in a format easy harvestable (Figure APP. IV 1).

4.1.2 Materials

William's E Medium, Glutamax supplement (Gibco, Ref: 32551020

Fetal Bovine Serum (FBS) Fetalclone 2 (Cytiva, Ref: SH30066.33)

Penicillin-Streptomycin (PS) 100X (Gibco, Ref 15070-063)

Insulin (Sigma, Ref: PHR8925)

Hydorcortisone hemisuccinate (Upjohn Pharmaceuticals, Ref: SERB 100 mg)

MicroTissues® 3D Petri Dish®(5x7 array) (Sigma, Ref: Z764051)

Ultrapure® Agarose (Invitrogen, Ref: 16500-100)

PBS 1X (Gibco, Ref: 14190-094)

Autoclave

Incubator

4.1.3. Cells seeding medium preparation

1. Pre warm a William's E medium, Glutamax supplement (500 ml bottle) at 37°C
2. Retrieve 31 mL of Willam's E medium as equivalent of total volume of complements
3. Add 5 mL of stock penicillin-streptomycin at 100X for a final concentration of 1X
4. Add 500 µL of Insulin stock solution 500 mg/mL to 500 mL of medium for a final concentration at 5 mg/mL
5. Add 500 µL of Hydrocortisone stock solution at 50 mM in 500 mL of medium for a final concentration at 50 µM
6. Add 50 mL of FBS in 500 mL of medium for a final concentration at 10% FBS.

4.1.4. Organoids medium preparation

1. Pre warm a William's E medium, Glutamax supplement (500 ml bottle) at 37°C
2. Retrieve 11 mL of Willam's E medium as equivalent of total volume of complements
3. Add 5 mL of stock penicillin-streptomycin at 100X for a final concentration of 1X
4. Add 500 µL of Insulin stock solution 500 mg/mL to 500 mL of medium for a final concentration at 5 mg/mL
5. Add 500 µL of Hydrocortisone stock solution at 50 mM in 500 mL of medium for a final concentration at 50 µM
6. Add 5 mL of FBS in 500 mL of medium for a final concentration at 1% FBS.

4.1.5. Prepare agarose solution and sterilize micro-molds

1. Rinse the micro-molds thoroughly with distilled water (dH_2O).
1. Place the rinsed micro-molds in a container that is suitable for autoclave sterilization.
2. Weigh out 2 g of agarose powder and transfer it into a dry, 100 mL autoclave-safe glass bottle, making sure that both the bottle and the agarose powder are completely dry and free of any moisture. Screw the bottle cap on so that it is secure but still loose to allow pressure equalization during sterilization.
3. Autoclave both the micro-molds and the bottle containing the agarose powder for 30 minutes using a dry cycle to ensure sterility.
4. Under sterile condition, add 50 mL of PBS 1X to the bottle containing the sterilized agarose powder. Secure the lid loosely again and gently swirl the bottle to disperse the agarose powder evenly in the solution.

5. Use a microwave oven to heat the mixture until the agarose is fully melted and completely dissolved, pausing the microwave every 10 seconds to swirl the bottle gently. This helps ensure uniform heating and prevents localized overheating. Carefully check that no small bits of translucent undissolved agarose remain.

Caution: Molten agarose is extremely hot, can boil over when the bottle is swirled, and may cause severe burns. Always wear appropriate personal protective equipment, including heat-resistant gloves, eye protection, and a lab coat, when working with molten agarose.

4.1.6. Prepare the agarose cast for Organoid culture

1. Allow the molten agarose to cool to approximately 60–70 °C. Pipette 350 µL of molten agarose into each well of a 24 series micro-mold. Take care to avoid introducing bubbles while pipetting.
2. Remove any small bubbles that may be trapped in the micro-mold features by gently pipetting or scraping before the agarose solidifies.
3. After the agarose has completely gelled, carefully flex the micro-mold to release the agarose cast. Avoid over-flexing, as this may crack the agarose dish; some practice may be required to perform this step without damaging the mold. Transfer the released agarose cast to a standard 24-well tissue culture plate.
4. To equilibrate the agarose cast, add 500 µL of cell culture medium around the cast.
5. Incubate for at least 15 minutes to 24 hours, then remove the medium prior to cell seeding procedure.

4.1.7. Cell Culture Preparation for Mixed Organoid Formation

1. The cell mixture consists of 80% differentiated HepaRG, 10% LX-2, and 10% Macrophages M1. Refer to the cell culture protocol to ensure that all cell types are properly prepared and differentiated in advance, so they are ready to be seeded together on the same day to form the organoids. We recommend to begin with Macrophages M1 as they took more time to be trypsinized.
2. After confirming that the cells are well dissociated (single-cell suspension, no clumps), centrifuge all three cell types for 5 minutes at 500 x g.
3. For each cell type, remove the supernatant and resuspend in cell seeding medium the cell pellet in a volume that results in a final concentration of 1 million cells/ml.

4. Calculate the required volume for each cell type according to the table below:

	1 Organoid	1 mold (35 orgs)		24 + 3 Molds (1 plate + 3 molds)	
HepaRG 80%	1,600 cells	56 000 cells	56 µL	1 512 000 cells	1 512 µL
LX-2 10%	200 cells	7 000 cells	7 µL	189 000 cells	189 µL
Macrophage 10%	200 cells	7 000 cells	7 µL	189 000 cells	189 µL
Resuspension Volume	100 µl	70 µl		1 890 µL	

By preparing all cell suspensions at 1 million cells/mL, the mixed cell solution is ready to be seeded into the cast with a seeding volume of 70 µL, since each mold contains 35 organoids with a total of 2,000 cells per organoid. We recommend using an additional 10% dead volume to avoid pipetting issues during seeding.

5. Mix the pool cell suspension
6. Dispense 70 µL of the final cell suspension into each cast.
7. Incubate for 20 minutes at room temperature to allow the cells to settle at the bottom of the microwells.
8. Gently add 500 µl of HML medium with 10% FBS to each well by letting it flow down the wall of the well.
9. Place the plate in a 37°C incubator with 5% CO2.
10. Caution: Once the organoids are formed, it is important to avoid shaking the plate or slamming the incubator door, as this could cause the organoids to dislodge from the microwells.

4.1.8. Maintenance of organoids

1. For organoid maintenance, the medium should be changed at the same frequency as for other cells: Monday / Wednesday / Friday
2. Aspirate the medium surrounding the molds carefully, without touching the mold itself.
3. Add fresh organoids medium with 5% FBS, allowing the medium to flow gently along the wall of the well.
4. Return the plates to the 37°C incubator with 5% CO_2.
5. After 5 days of culture, switch to HML medium with 1% FBS.

4.2. Organoid seeding procedure 2 in ULA 96 well plate

4.2.1. Introductory paragraph

This protocol describes low scale method different spheroid culture system or different spheroid formation system for seeding the same mixed cell populations used in agarose micro-molds, but instead cultured in a different System: ultra-low attachment (ULA) 96-well plates. In this setup, each well functions as an individual microenvironment, allowing the formation of one organoid per well. This approach facilitates downstream analyses at the single-organoid level. This protocol should be followed as an alternative to the standard agarose mold protocol when a single-organoid-per-well format is required, for example for high-content imaging, drug screening, or individual organoid tracking.

4.2.2. Materials

William's E Medium, Glutamax supplement (Gibco, Ref: 32551020

Fetal Bovine Serum (FBS) Fetalclone 2 (Cytiva, Ref: SH30066.33)

Penicillin-Streptomycin (PS) 100X (Gibco, Ref 15070-063)

Insulin (Sigma, Ref: PHR8925)

Hydrocortisone hemisuccinate (Upjohn Pharmaceuticals, Ref: SERB 100 mg)

96-well Black/Clear Round Bottom Ultra-Low Attachment Surface Spheroid Microplate (Corning, Ref: 4520)
Cell counter

Centrifuge

Incubator

4.2.3. Cell Culture Preparation for Mixed Organoid Formation

1. The cell mixture consists of 80% differentiated HepaRG, 10% LX-2, and 10% Macrophages M1. Refer to the cell culture protocol to ensure that all cell types are properly prepared and differentiated in advance, so they are ready to be seeded together on the same day to form the organoids. We recommend to begin with Macrophages M1 as they took more time to be trypsinized.

2. After confirming that the cells are well dissociated (single-cell suspension, no clumps), centrifuge all three cell types for 5 minutes at 500 x g.
3. For each cell type, remove the supernatant and resuspend in cell seeding medium the cell pellet in a volume that results in a final concentration of 1 million cells/ml.
4. Calculate the required volume for each cell type according to the table below:

	1 Organoid	1 plate (96 orgs)	
HepaRG 80%	1,600 cells	160 000 cells	160µL
LX-2 10%	200 cells	20 000 cells	20 µL
Macrophage 10%	200 cells	20 000 cells	20 µL
Resuspension Volume	100 µL	10 mL	

Note: By preparing all cell suspensions at 1 million cells/mL, the mixed cell solution is ready to be seeded into the ULA plate with a seeding volume of 100 µL, withand a total of 2,000 cells per organoid. We recommend using an additional 10% dead volume to avoid pipetting issues during seeding.

5. Mix the pool cell suspension
6. Dispense 100 µL of the final cell suspension into each well.
7. Place the plate in a 37°C incubator with 5% CO2.

4.2.4. Maintenance of organoids

8. For organoid maintenance, the medium should be changed at the same frequency as for other cells: Monday / Wednesday / Friday
9. Aspirate 50 µL of the medium in each well carefully, without touching the bottom of the well. Try to sense the cone-marker inside each well to help you place correctly the cone and not aspirate your organoid.
10. Add 50 µL of fresh organoids medium with 5% FBS, allowing the medium to flow gently along the wall of the well.
11. Return the plates to the 37°C incubator with 5% CO_2.
12. After 5 days of culture, switch to HML maintenance medium.

4.3. Alternative protocol "Organoid seeding procedure from cryopreserved cells"

4.3.1. Introductory paragraph

Instead of using freshly cultured cells to generate organoids, it is possible to use cryopreserved cells for each cell type. This protocol will describe the cell culture preparation for organoid formation using cryovials of differentiated HepaRG cells supplied by Biopredic, activated LX-2 cells and differentiated macrophages isolated from human monocytes. Once thawed, these cells can be seeded in agarose molds or in ULA 96-wells plate. The protocol does not detail the preparation of agarose solution and casts, as well as organoid maintenance, as it is identical to the procedure described above. The use of cryopreserved cells allows to decrease the procedure duration and enhance the reproducibility between experiments, as cells of the same culture batch can be used in different experiments.

4.3.2. Materials

Cryopreserved differentiated HepaRG® cells (HPR116-TA08, Biopredic)

Cryopreserved activated LX-2 cells

Cryopreserved primary human macrophages differentiated from peripheral blood mononuclear cells (7 days of culture with GM-CSF)

William's E Medium, Glutamax supplement (Gibco, Ref: 32551020)

HepaRG® Serum-free Induction Supplement with antibiotics (ADD650C, Biopredic) without DMSO

Fetal bovine serum (FBS) (Biopredic)

Trypsin-EDTA (0.05%) (Gibco, Ref: 25300-054)

Incubator

Centrifuge

4.3.3. Cryopreservation of LX-2 and macrophages cells

1. Harvest cells at the desired confluency by trypsinization and resuspend them in fresh complete medium.
2. Count the cells and adjust the cell density to approximately 0.5 10^6 cells/mL.

3. Prepare freezing medium consisting of complete culture medium supplemented with 10% (v/v) sterile DMSO.
4. Resuspend the cell pellet gently in the freezing medium at the appropriate density.
5. Aliquot 1 mL of the cell suspension into sterile cryovials.
6. Place the cryovials into a controlled-rate freezing container (e.g., Mr. Frosty®) prefilled with isopropanol to ensure gradual cooling at approximately –1 °C per minute.
7. Transfer the container to a –80 °C freezer and leave for at least 24 hours.
8. After 24 hours, transfer the cryovials to liquid nitrogen (–196 °C) for long term storage

4.3.4. Cells thawing medium preparation

1. Thaw Biopredic FBS by placing the tube in a 37°C water bath until completely thawed.
2. Thaw HepaRG® Serum-free Induction Supplement with antibiotics (ADD650C) without DMSO by placing the tube in a 37°C water bath until completely thawed.
3. In sterile conditions, reconstitute the thawing medium by adding 1.6 mL of supplement (ADD650C without DMSO) and 10 mL of FBS per 100 mL of Williams' E Medium with GlutaMAX™.
4. The reconstituted medium is ready for use and can be stored at 4°C for up to one month.

5. Cell culture preparation for mixed organoid formation

5.1. Cell thawing

<u>One cryovial per cell type is thawed: differentiated HepaRG® cells (HPR116), LX-2 cells, and primary human macrophages.</u>

1. Retrieve the 24 well plates with the agarose micro-molds and remove the media from the wells using an aspiration pipette with a 200 µl micropipette cone.
2. Carefully remove the media in the seeding chamber of the agarose micro-molds using an aspiration pipette with a 200 µl micropipette cone. Be gentle to avoid damage to the agarose micro-molds.
3. Pre-warm the Thawing medium 10% FBS in a 37°C water bath.
4. Pipet 9 mL (per cryovial to be used) of pre-warmed Thawing medium 10% FBS into a sterile 15 mL polystyrene round-bottom tube or similar container.
5. Prepare an absorbent paper with 70% absolute ethanol.

6. Remove the cryovial from the liquid nitrogen.
7. Under the laminar flow hood, briefly twist the cap a quarter-turn (do not open the cryovial completely) to release the internal pressure, and then close it again.
8. Quickly transfer the cryovial to the water bath at 37°C. Do not submerge it completely, being careful not to allow water to enter the cap. While holding the tip of the cryovial, gently agitate the vial for about 2 minutes. A small ice crystal should still be present in the cryovial when removed from the water bath.
9. Wipe the outside of the cryovial with 70% absolute ethanol on an absorbent paper and place the cryovial under the laminar flow hood.
10. Aseptically transfer the "semi"-thawed cell suspension into the tube containing 9 mL of the pre-warmed Thawing medium 10% FBS (resulting in a 1:10 ratio of cell suspension to total volume). Careful pipetting (at low speed) reduces shear forces on freshly thawed, fragile cells, and increases recovery of viable cells.
11. Rinse out the cryovial once with approximately 1 mL of the Thawing medium 10% FBS and return the resulting suspension to the 15 mL tube.
12. Centrifuge the cell suspension for 3 min at 500 x g at room temperature.
13. Aspirate and discard the supernatant. To avoid aspiration of cells, let a little volume of medium on the pellet.
14. Gently resuspend the cell pellet with Thawing medium 10% FBS in 5 mL by pipetting. In the case of differentiated HepaRG® cells, don't try to dissociate the bigger clusters.

5.2. Cell seeding

1. After cell counting, centrifuge cell suspension for 3 min at 500g.
2. For each cell type, remove the supernatant and resuspend in cell thawing medium the cell pellet in a volume that results in a final concentration of 1 million cells/ml.

5.3. In agarose molds

1. Calculate the required volume for each cell type according to the table below

	1 Organoid	1 mold (35 orgs)		24 + 3 Molds (1 plate + 3 molds)	
HepaRG 80%	1,600 cells	56 000 cells	56 µL	1 512 000 cells	1 512 µL
LX-2 10%	200 cells	7 000 cells	7 µL	189 000 cells	189 µL
Macrophage 10%	200 cells	7 000 cells	7 µL	189 000 cells	189 µL
Resuspension Volume	x	70 µl		1 890 µL	

2. By preparing all cell suspensions at 1 million cells/mL, the mixed cell solution is ready to be seeded into the cast with a seeding volume of 70 µL, since each mold contains 35 organoids with a total of 2,000 cells per organoid. We recommend using an additional 10% dead volume to avoid pipetting issues during seeding.
3. Mix the pool cell suspension
4. Dispense 70 µL of the final cell suspension into each cast.
5. Incubate for 20 minutes at room temperature to allow the cells to settle at the bottom of the microwells.
6. Gently add 500 µl of HML medium with 10% FBS to each well by letting it flow down the wall of the well.
7. Place the plate in a 37°C incubator with 5% CO2.

Caution: Once the organoids are formed, it is important to avoid shaking the plate or slamming the incubator door, as this could cause the organoids to dislodge from the microwells.

5.4. In ULA 96-well plate

1. Calculate the required volume for each cell type according to the table below

	1 Organoid	1 plate (96 orgs)	
HepaRG 80%	1,600 cells	160 000 cells	160µL
LX-2 10%	200 cells	20 000 cells	20 µL
Macrophage 10%	200 cells	20 000 cells	20 µL
Resuspension Volume	100 µL	10 mL	

Note: By preparing all cell suspensions at 1 million cells/mL, the mixed cell solution is ready to be seeded into the ULA plate with a seeding volume of 100 µL, withand a total of 2,000 cells per organoid. We recommend using an additional 10% dead volume to avoid pipetting issues during seeding.

2. Mix the pool cell suspension
3. Dispense 100 µL of the final cell suspension into each well.
4. Place the plate in a 37°C incubator with 5% CO2.

6. Preparation of the fatty acid mixture

6.1. Introductory paragraph

To model hepatic steatosis *in vitro*, free fatty acids (FFAs) such as stearic acid and oleic acid are commonly used to induce lipid accumulation within hepatocytes. This mimics the pathophysiological features of metabolic dysfunction associated liver disease (MASLD). In order to make these fatty acids bioavailable for cultured cells, they must be complexed with bovine serum albumin (BSA), which acts as a carrier molecule to increase solubility and facilitate cellular uptake. A saponification step is required prior to complexing the fatty acids with BSA. This converts the fatty acids into their saponified form, which improves their dispersion in the aqueous culture medium. The resulting fatty acid–BSA stock solutions are then stored at –20 °C until use.

6.2. Materials

Stearic acid (Sigma, Ref: S4751)

Oleic acid (Sigma, Ref: 01008)

Free fatty acid BSA (Sigma, Ref: A9418)

NaOH (Fischer, Ref: S4920)

Ethanol (Supelco, Ref: 81760)

6.3. Preparation of compounds solution

1. Prepare BSA solution 1 day before fatty acid preparation
2. To adjust the volume, first dissolve the required amount of BSA in 80% of the final volume and incubate overnight at 4°C with agitation
3. After complete solubilization, adjust the solution to the final volume.
4. Prepare NaOH solution 0.1 M by dissolving 0.2 g of NaOH in 50 mL of water
5. Prepare Ethanol 50% by dilution 25 mL of Ethanol in in 25 mL of water

6.3. Preparation of fatty acids solution

6. To prepare a stearic acid solution at 5 mM final concentration, we need to prepare a working solution at 8X concentration as follows:

Compound	Molecular Weight:	C stock [µM]	V EtOH [mL]	Required weight [mg]
Stearic acid (C18:0)	284.47	5000	2	22.8

7. Incubate the working solution at 70°C.
8. To prepare oleic acid solution at 10 mM final concentration, we need to prepare a working solution at 8X concentration as follows:

Compound	Molecular Weight:	C stock [µM]	V EtOH [mL]	Pesée théorique [mg]
Oleic acid (C18:1)	282.47	10000	2	45.2

9. Incubate the working solution at 70°C.

6.4. Saponification of fatty acids

10. Preheat the fatty acid solutions prepared in a 50% ethanol solution in a tube heater at 70°C.
11. Mix: 150 µL of fatty acid solutions + 150 µL of 0.1 M NaOH.
12. Incubate for 10 minutes at 70°C.
13. Preheat tubes containing 750 µL of BSA in a tube heater at 56°C.

14. Quickly mix: 250 µL of the FA-NaOH solution + 750 µL of BSA.
15. Immediately vortex and store at -20°C.

Note: The saponified solutions of stearic and oleic acids should appear translucent. In particular, stearic acid solutions tend to be more prone to incomplete saponification. If the aliquot appears turbid, this indicates that the fatty acid has not been properly saponified and complexed with BSA. We strongly recommend discarding such preparations and repeating the procedure.

6.5. Preparation of control BSA

To prepare the negative control for lipid exposure experiments, a solution of bovine serum albumin (BSA) is processed following the same thermal conditions used for fatty acid–BSA complexation, but without the addition of fatty acids. This BSA-only control is essential to distinguish the specific cellular effects of stearic and oleic acid treatment from those potentially associated with BSA exposure or thermal processing alone.

16. Mix 150 µL 50% with 150 µL of .01M NaOH
17. Incubate for 10 minutes à 70°C
18. Preheat tubes containing 750 µL of BSA in a tube heater at 56°C.
19. Quickly mix: 250 µL of the EtOH-NaOH solution + 750 µL of BSA.
20. Immediately vortex and store at -20°C

7. MASLD Induction

7.1. Introductory paragraph

To mimic the pathophysiological features of metabolic dysfunction associated liver disease (MASLD), organoids are cultured for 9 days in the presence of stearic and oleic acids mixture. This chronic lipid overload led to the progressive accumulation of intracellular lipids within the organoids, reproducing the characteristic steatotic phenotype of MASLD including lipid accumulation, expression of fibrotic markers, phase 1 and 2 dysfunction and basal inflammation. This simple and reproducible *in vitro* induction protocol allows the generation of MASLD-like organoids in agarose cast or ULA 96 well plate.

7.2. Materials

Fatty acids mixture stock solution

Organoids medium

7.3. Fatty acids medium preparation

To ensure the stability of the medium components, we recommend preparing the full volume of fatty acid–supplemented culture medium required for the entire 9-day treatment period in advance. Avoid storing the medium for extended periods, as prolonged storage may lead to degradation of its components and reduced reproducibility of experimental outcomes.

1. Preheat both the organoid culture medium and the fatty acid stock solution aliquots to 37 °C.
2. Vortex the fatty acid aliquots thoroughly to ensure homogeneity.
3. Dilute the 5 mM stearic acid stock solution into the prewarmed organoid medium to achieve a final concentration of 150 µM.
4. Dilute the 10 mM oleic acid stock solution into the same medium to achieve a final concentration of 300 µM.
5. Store the prepared fatty acid–supplemented medium at 4 °C and use within a short time frame to avoid degradation.

7.4. Organoids treatments with fatty acids

After 5 days of culture, the organoid culture medium is replaced with fatty acid–supplemented medium and maintained until day 14 (Figure APP. IV 2).

7.5. Xenobiotic treatment of organoids

Xenobiotic exposure can be initiated from day 5 of organoid culture, as HML organoids have reached a functionally mature state at this time point (Bronsard et al., 2023). However, if modeling drug responses in a steatotic context is required, it is important to note that theMASLD phenotype becomes fully established only after 14 days of lipid treatment. Users should therefore define the treatment window according to the specific objectives of their study—whether the aim is to assess toxicity in healthy-like organoids, in early-stage steatosis, or in a well-established NAFLD context. The choice of treatment starting day, duration of exposure, and xenobiotic concentration must be optimized.

8. Harvesting organoids in agarose casts

To harvest the organoids formed in the agarose cast, we recommend following the procedure available at https://www.microtissues.com/protocols. In summary, the cast is inverted into a well to allow organoids to be released from the microwells and collected at the bottom of the plate by centrifugation. This method enables efficient recovery of all organoids within the cast but requires a centrifuge compatible with SBS-format plates.

8.1. Procedure

1. Carefully remove the culture medium surrounding the agarose cast and store it for further use if necessary.
2. Add 500 µL of fresh medium to the well.
3. Using a sterile pipette tip or tweezers, gently invert the agarose cast into the well, avoiding the formation of air bubbles.
4. Centrifuge the plate in a swinging-bucket rotor at **500 rpm for 5 minutes** to facilitate organoid release.
5. Observe under a microscope to confirm that the organoids have detached from the microwells.
6. Remove the empty agarose cast with tweezers, then collect the organoid-containing medium using a P1000 pipette and transfer it to a sterile tube.
7. Allow the organoids to settle by gravity, or gently centrifuge if needed. Wash them with **1X PBS**, and proceed with downstream applications.

9. Harvesting organoids in ULA plate

Organoid harvesting from ULA (Ultra-Low Attachment) plates is simpler than from agarose molds but must be done well-by-well. Using a 200 µL pipette, each organoid is individually collected and pooled into a tube as needed.

1. Carefully remove 50 µL of culture medium from each well and store it for further use if necessary.
2. Using a P200 pipette, gently collect each organoid individually and pool them into separate tubes if needed.

3. Observe under a microscope to ensure that the organoids have been fully detached from the well.
4. Let the organoids settle by gravity or centrifuge gently if required. Wash them with 1X PBS, then proceed with downstream applications.

10. Troubleshooting

This table recapitulates the most common problems encountered in this protocol, as well as their probable cause and solution.

Problem	Possible Cause	Solution
Fused organoids	Mechanical disruption of the plate	Discard the concerned wells
Contamination	Contamination of one of the components or solution used	Discard contaminated cells and make fresh solutions

10. Commentary:

Comparison ULA and agarose cast

A direct comparison between organoids produced in ULA plates and those generated in agarose casts is difficult because of differences in exposure conditions that directly affect toxicological outcomes. In ULA plates, only half of the medium is renewed at each change, leading to a partial dilution of compounds and metabolites, whereas in agarose casts, two-thirds of the surrounding medium is replaced. In addition, the total medium volume per organoid differs between the two systems, resulting in distinct compound-to-biomass ratios. These factors alter the actual dosimetry experienced by the organoids, influencing both the intensity and kinetics of toxic responses. For their use as new approach methodologies (NAMs), these discrepancies highlight the importance of carefully considering culture format when interpreting toxicity data and underscore the need for standardization of medium renewal and dosimetry to ensure reproducibility and comparability across platforms.

11. Critical Parameters

In order to obtain reproducible organoids, cell culture of the different cell types before organoid seeding must be done thoroughly, with a particular attention to cell morphology, indicative of cell differentiation or phenotype.

Multiple sources of contamination are possible, from classical media components to agarose or fatty acids mixture.

Organoids cultured in agarose micro-molds are very sensitive to mechanical disruption, which could dislodge them of the micro-wells, thereby facilitating their contact with one another.

Primary human macrophages are the major source of biological variability in this model, and no complete study can be done using macrophages from only one donor, so this must be considered before conceiving experiments.

12. Time Considerations

The overall timeline of the experimental workflow reflects the different requirements of each cell type and different preparation step listed in the protocol. Differentiation of HepaRG cells requires approximately 30 days, while LX-2 cells can be maintained on a weekly passaging schedule. Primary monocytes are isolated within 4 hours and differentiation into macrophages is complete after 7 days of culture. Preparation of agarose casts is relatively short, ranging from 30 minutes to 2 hours depending on the number of wells needed. The setup of co-cultures for mixed organoid formation takes about 3 to 4 hours. Once established, organoids are maintained and subjected to MASLD induction for 14 days. Prior to fatty treatment, the preparation of the mixture requires around 2 hours, with a previously prepared BSA solution.

Graphical illustrations:

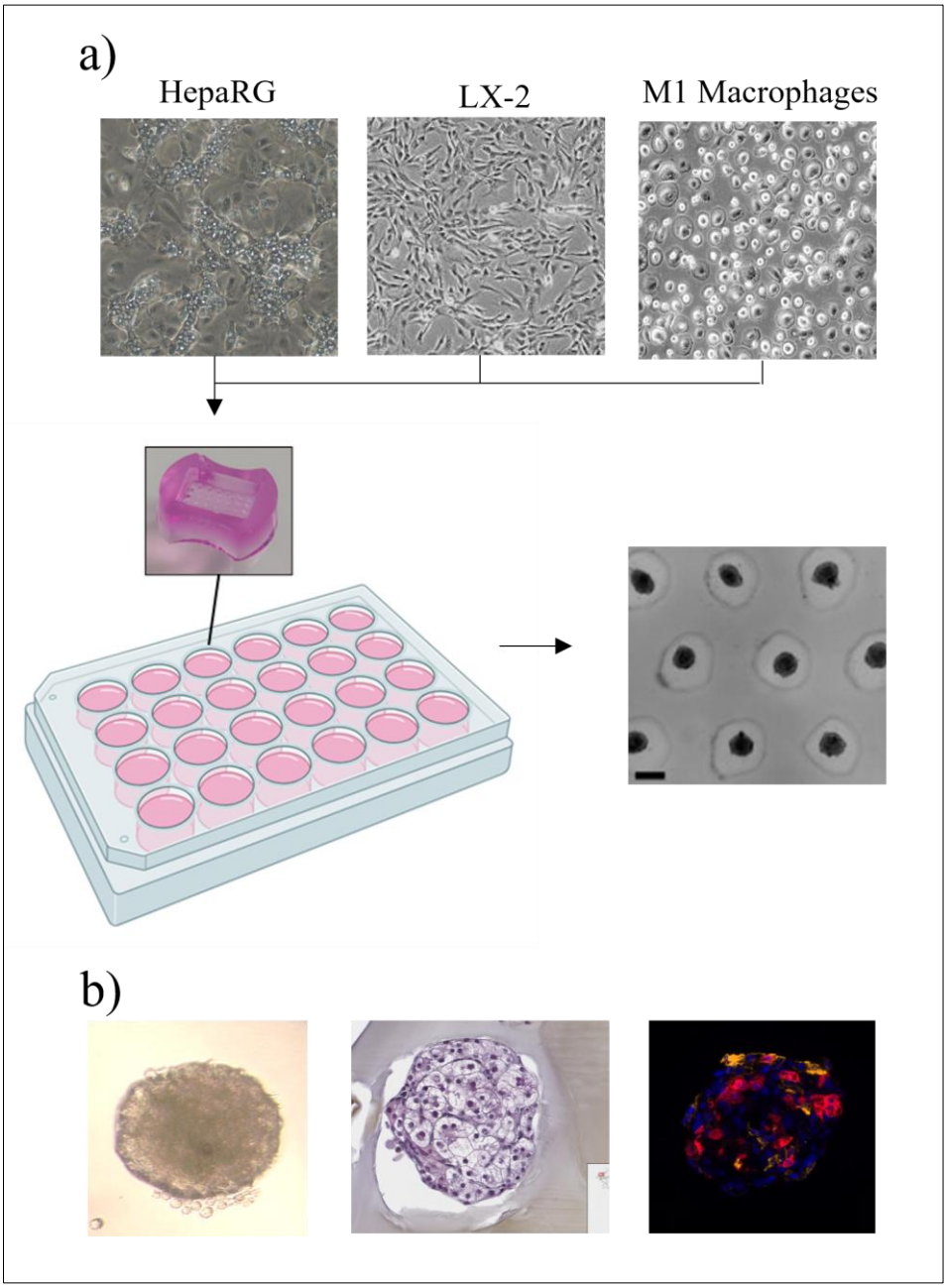

Figure APP. IV 1. Cell seeding to form organoids in agarose cast. a) Differentiated HepaRG, activated LX-2, and unstimulated M1 macrophages are cultured separately before being co-seeded in agarose cast at 2000 cells per organoid. The proportions are 80% HepaRG, 10% LX-2, and 10% M1 macrophages. b) After 14 days of culture, organoids can be harvested for various analyses, including histological and immunostaining imaging.

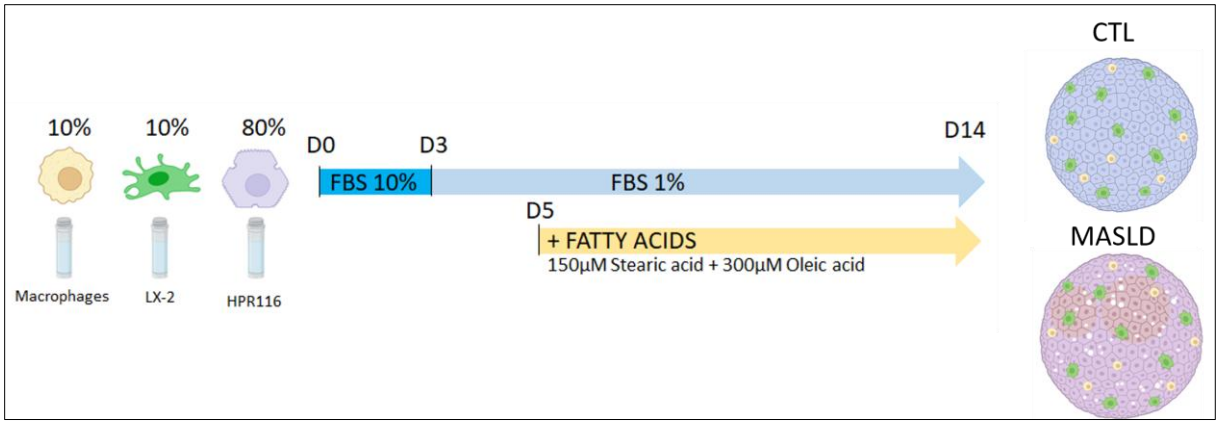

Figure APP. IV 2. Organoid exposure to fatty acids. After 3 days of culture, the FBS concentration is reduced from 10% to 1%. Then, at day 5, organoids are exposed to fatty acids through supplementation of the culture medium with 150 µM stearic acid and 300 µM oleic acid. After 14 days of culture with the fatty acid cocktail, HML organoids display a MASLD-like phenotype and can be harvested.

References

Aninat, C., Piton, A., Glaise, D., Charpentier, T. L., Langouët, S., Morel, F., Guguen-Guillouzo, C., & Guillouzo, A. (2006). Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metabolism and Disposition*, *34*(1), 75–83. https://doi.org/10.1124/dmd.105.006759

Arteel, G. E. (2024). Hepatic extracellular matrix and its role in the regulation of liver phenotype. *Seminars in Liver Disease*, *44*(3), 343–355. https://doi.org/10.1055/a-2404-7973

Ashraf, M. N., Asghar, M. W., Rong, Y., Doschak, M. R., & Kiang, T. K. L. (2019). Advanced in vitro HepaRG culture systems for xenobiotic metabolism and toxicity characterization. *European Journal of Drug Metabolism and Pharmacokinetics*, *44*(4), 437–458. https://doi.org/10.1007/s13318-018-0533-3

Bronsard, J., Savary, C., Massart, J., Viel, R., Moutaux, L., Catheline, D., Rioux, V., Clement, B., Corlu, A., Fromenty, B., & Ferron, P. J. (2023). 3D multi-cell-type liver organoids: A new model of non-alcoholic fatty liver disease for drug safety assessments. *Toxicology in Vitro*, *93*, 105728. https://doi.org/10.1016/j.tiv.2023.105728

Cerec, V., Glaise, D., Garnier, D., Morosan, S., Turlin, B., Drenou, B., Gripon, P., Kremsdorf, D., Guguen-Guillouzo, C., & Corlu, A. (2007). Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. *Hepatology*, *45*(4), 957–967. https://doi.org/10.1002/hep.21536

Gripon, P., Rumin, S., Urban, S., Le Seyec, J., Glaise, D., Cannie, I., Guyomard, C., Lucas, J., Trepo, C., & Guguen-Guillouzo, C. (2002). Infection of a human hepatoma cell line by hepatitis B virus. *Proceedings of the National Academy of Sciences*, *99*(24), 15655–15660. https://doi.org/10.1073/pnas.232137699

Hart, S. N., Li, Y., Nakamoto, K., Subileau, E., Steen, D., & Zhong, X. (2010). A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. *Drug Metabolism and Disposition*, *38*(6), 988–994. https://doi.org/10.1124/dmd.109.031831

Kammerer, S. (2021). Three-dimensional liver culture systems to maintain primary hepatic properties for toxicological analysis in vitro. *International Journal of Molecular Sciences*, *22*(19), 10214. https://doi.org/10.3390/ijms221910214

Perumpail, B. J., Khan, M. A., Yoo, E. R., Cholankeril, G., Kim, D., & Ahmed, A. (2017). Clinical epidemiology and disease burden of nonalcoholic fatty liver disease. *World Journal of Gastroenterology*, *23*(47), 8263–8276. https://doi.org/10.3748/wjg.v23.i47.8263

Yokoyama, Y., Sasaki, Y., Terasaki, N., Kawataki, T., Takekawa, K., Iwase, Y., Shimizu, T., Sanoh, S., & Ohta, S. (2018). Comparison of drug metabolism and its related hepatotoxic effects in HepaRG, cryopreserved human hepatocytes, and HepG2 cell cultures. *Biological and Pharmaceutical Bulletin*, *41*(5), 722–732. https://doi.org/10.1248/bpb.b17-00913

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Authorship contribution

Duclos M: Conceptualization, methodology, visualization, writing–original draft, writing–review and editing; **Marzougui Z**: Conceptualization, methodology writing–review and editing; **Saleh M**: Conceptualization, methodology, writing–review and editing; **Corlu A**: writing–review and editing; **Massart J**: writing–review and editing; **Ferron PJ**: Conceptualization, methodology, visualization, writing–original draft, writing–review and editing.

Appendix V

Thesis communication

Poster presentations

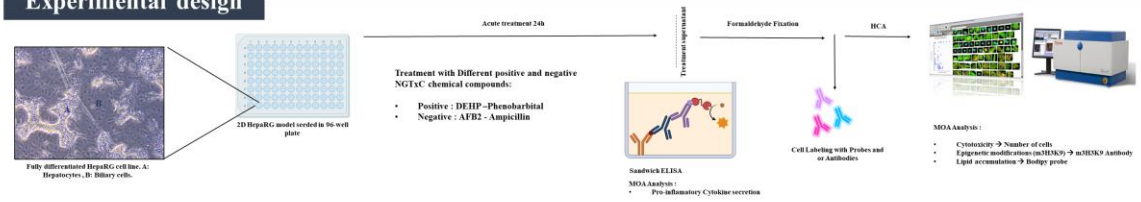
In vitro based testing strategy for the identification of non-genotoxic carcinogens (NGTxC)

SALEH Mariam[1], PORCQ Chloe[1], HOGEVEEN Kevin[1], LE HEGARAT Ludovic[1]
[1]ANSES: French Agency for Food, Environmental and Occupational Health & Safety, Fougeres Laboratory, Toxicology of Contaminants Unit, Fougeres, France

Context

NGTxCs promote carcinogenesis via a series of diverse **Modes of Action (MOA).** Unlike Genotoxic carcinogens, there is no in vitro-based testing strategy to accurately detect NGTxC, Therefore, their identification requires 2-year in vivo rodent studies that are time consuming, expensive, pose ethical concerns, and may not be directly extrapolated to humans due to species-specific responses. Thus, the transition into in vitro **New Approach Methodologies (NAMs)** could be an **alternative method** for human-relevant chemical safety assessment. In this context, and as a part of the **PARC project**, we aim to develop an **in vitro testing strategy** using different models of the **human hepatic HepaRG cell line** in combination with **Transcriptomics** and **High Content Analysis (HCA)** to assess different NGTxC MOAs.

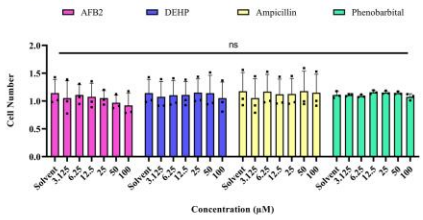
Experimental design



Results

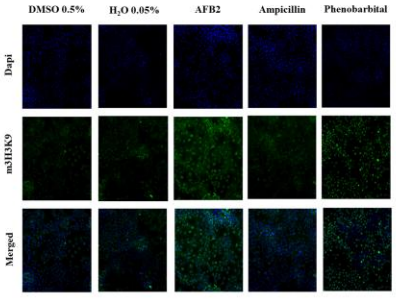
Results obtained with HCA:

A. Cytotoxic effect:

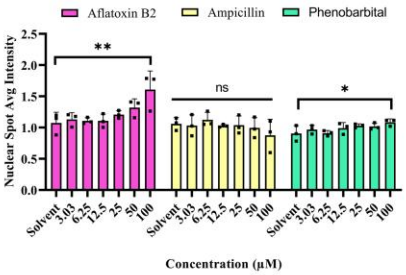


Cytotoxic effect on the Number of cells of AFB2, DEHP, Ampicillin and Phenobarbital. DMSO solvent used for AFB2 and DEHP, H2O used for Ampicillin and Phenobarbital.

B. Epigenetic modifications – Histone H3K9 trimethylation :

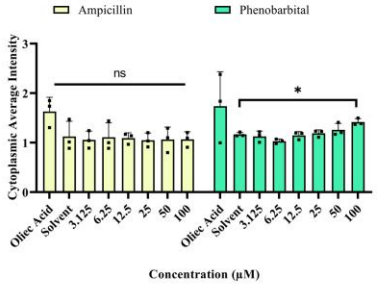


Representative images of the Histone H3K9 trimethylation labeling changes after 24h treatment with 100µM of AFB2, Ampicillin and Phenobarbital. DMSO represents negative solvent control for AFB2, and H_2O for Ampicillin and Phenobarbital.



Quantitative graphs of the Epigenetic H3K9 modification effect of AFB2, DEHP, and Phenobarbital. DMSO solvent used for AFB2 and DEHP, H_2O solvent used for Phenobarbital. P<0.05. No effect on m3H3K9 associated with DEHP treatment.

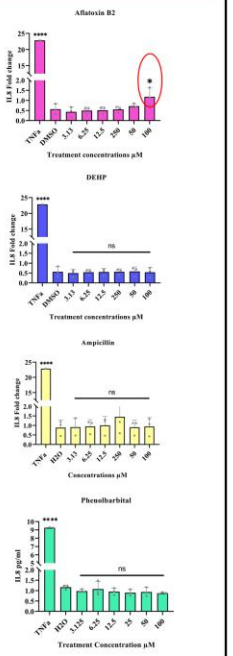
C. Neutral Lipid accumulation:



Lipid accumulation effect of Ampicillin and Phenobarbital. H_2O solvent used as negative control and Olieic Acid used as positive control. P<0.05.AFB2 and DEHP treatments shows no change in the lipid accumulation MOA.

Results obtained with ELISA:

A. Pro-inflammatory cytokines (IL8):



Pro-inflamatory cytokines secretion (IL8) effect of AFB2, DEHP, Ampicillin and Phenobarbital. DMSO solvent used for AFB2 and DEHP, H_2O solvent used for Ampillin and Phenobarbital. TNFa used as positiv control. P<0.05

Perspectives

- **Repetitive treatments (3 or 5 days) are likely to be more informative concerning NGTxC MOA.**
- **Through the application of the 3D co-culture model we will be able to test the contribution of other liver cells such as Stellate and immune cells on our treatment assessments.**
- **The application of transcriptomic analysis (RNA Sequencing) will allow the investigation of the response pathways involved in the MOAs of compounds with NGTxC potential.**

In vitro based testing strategy for the identification of non-genotoxic carcinogens

SALEH Mariam[1*], HOGEVEEN Kevin[1], LE HEGARAT Ludovic[1]

[1]Toxicology of Contaminants Unit, French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Fougeres, France

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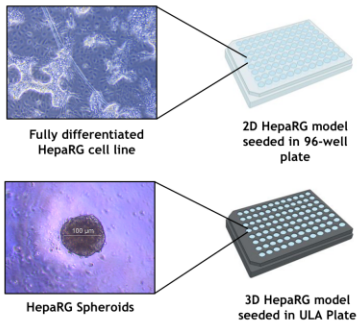
Context

Since there is no in vitro-based testing strategy to precisely detect NGTxC, their identification requires 2-year in vivo rodent studies that are time consuming, expensive, pose ethical concerns, and may not be directly extrapolated to humans due to species-specific responses. Genotoxic carcinogens exert carcinogenicity via direct effects on DNA, in contrast, NGTxCs promote carcinogenesis via a series of diverse Mode of Action (MOA) such as cytotoxicity, oxidative stress, dysregulated cellular metabolism, epigenetics, and many others. Therefore, the transition into in vitro New Approach Methodologies (NAMs) could be an alternative method to enhance the prediction of their carcinogenic potential for a human-relevant chemical safety assessment. In this context, and as a part of the PARC project, we aim to develop an in vitro testing strategy using different models of the human hepatic HepaRG cell line in combination with Transcriptomics and High Content Analysis (HCA) to assess different NGTxC MOAs.

in vitro Methodology

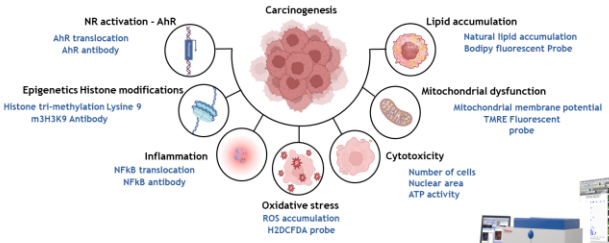
1. Different models of human HepaRG cell line used in our analysis



2. Treatment with different NGTxC chemical library and or chemicals have NGTxC activity



3. Assessment of different NGTxC MOAs using different probes and antibodies



4. Microscopic analysis using High Content Analysis (HCA)



High Content Analysis results

A. Cytotoxic effect on 2D HepaRG model

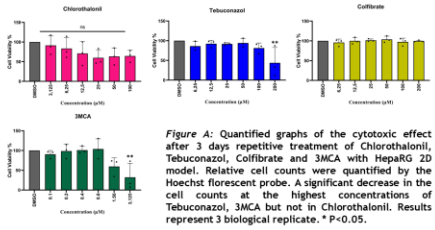


Figure A: Quantified graphs of the cytotoxic effect after 3 days repetitive treatment of Chlorothalonil, Tebuconazol, Colfibrate and 3MCA with HepaRG 2D model. Relative cell counts were quantified by the Hoechst florescent probe. A significant decrease in the cell counts at the highest concentrations of Tebuconazol, 3MCA but not in Chlorothalonil. Results represent 3 biological replicate. * P<0.05.

B. Mitochondrial dysfunction effect on 2D HepaRG model

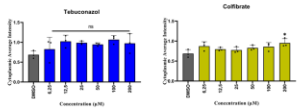


Figure B: Quantified graphs of the mitochondrial dysfunction after 3 days repetitive treatment of Tebuconazol and Colfibrate on HepaRG 2D model. Mitochondrial membrane potential changes were quantified by the TMRE fluorescent probe. A significant increase in the HepaRG membrane potential was observed at the highest concentrations of Colfibrate. Results represent 3 biological replicate. * P<0.05.

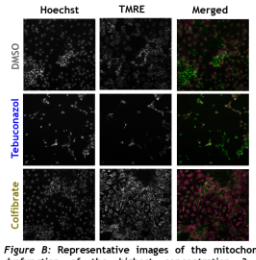


Figure B: Representative images of the mitochondrial dysfunction of the highest concentration 3 days repetitive treatment of Tebuconazol and Colfibrate on HepaRG 2D model. quantified by TMRE fluorescent probe.

A. Cytotoxic effect on 3D HepaRG Spheroids

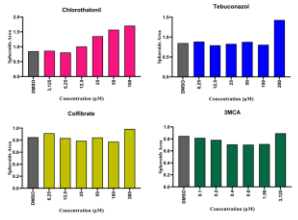


Figure A: Quantified graphs of the cytotoxic effect after 3 days repetitive treatment of Chlorothalonil, Tebuconazol, Colfibrate and 3MCA with HepaRG 3D model present by spheroids Area quantified by the Hoechst fluorescent probe. An increase in the HepaRG 3D spheroid Areas at highest concentrations of Chlorothalonil and Tebuconazol. Results represent one biological replicate.

B. Oxidative stress effect on 3D HepaRG Spheroids

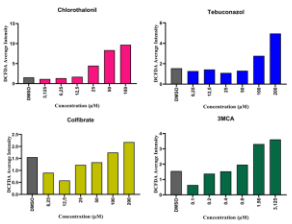


Figure B: Quantified graphs of the oxidative stress effect after 3 days repetitive treatment of Chlorothalonil, Tebuconazol, Colfibrate and 3MCA with HepaRG 3D spheroids. Oxidative stress was quantified with the fluorescent H_2DCFDA probe. Results represent one biological replicate.

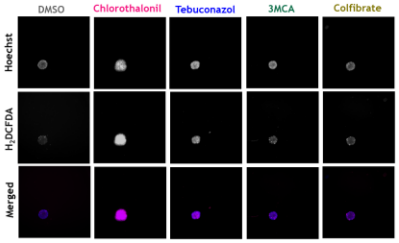


Figure B: Representative images of the oxidative stress effect in a 3D HepaRG model following a 3 days repetitive treatment with the second highest concentration of Chlorothalonil, Tebuconazol, Colfibrate and 3MCA quantified with the fluorescent H_2DCFDA probe.

Perspectives

- Through the application of the 3D co-culture model we will be able to test the contribution of other liver cells such as Stellate and immune cells on our treatment assessments.
- The transcriptomic analysis will allow the investigation of the response pathways involved in the MOAs of compounds with NGTxC potential.

Acknowledgments

EPAA are acknowledged to award Mariam Saleh the 3R's full travel grands to participate in the ESTIV 2024 congress

Titre: **Approche intégrée à haut débit pour la caractérisation des agents cancérigènes non génotoxiques (NGTxC)**

L'augmentation massive du nombre de substances chimiques interroge sur leur impact sur la santé humaine et environnementale. Cependant, les méthodes réglementaires actuelles de détection et de caractérisation des agents cancérogènes reposent principalement sur des études à long terme sur des animaux de laboratoire, qui ne sont pas suffisantes pour évaluer la toxicité d'un si grand nombre de produits chimiques dont ils pourraient être susceptibles de causer le cancer chez l'humain. De plus, ces méthodes ont des limites scientifiques, notamment en ce qui concerne les différences de sensibilité entre les humains et les animaux, le coût élevé, le faible débit et les considérations éthiques liées à la souffrance animale qui ne peuvent plus être ignorées.
Les cancérogènes sont généralement classés en cancérogènes génotoxiques (GTxC) et en cancérogènes non génotoxiques (NGTxC). Alors que l'identification des cancérogènes génotoxiques repose sur une batterie relativement robuste de tests de *génotoxicité in vitro* et *in vivo*, les essais biologiques sur 2 ans sur les rongeurs représentent l'étalon-or pour l'identification du NGTxC.
Par conséquent, il apparaît urgent d'élaborer une stratégie basée sur des méthodologies de nouvelle approche (NAM) pour l'identification et la caractérisation des substances chimiques potentiellement cancérogènes pour l'homme. Pour cela, ce travail, qui s'inscrit dans le cadre du projet européen PARC (Partnership for the Assessment of Risks from Chemicals), vise à étudier l'applicabilité de différentes approches basées sur les NAM pour identifier et caractériser les NGTxC avec différents modes d'action (MoA).
Les objectifs de ce travail de recherche ont été abordés à travers trois axes complémentaires : Dans un premier temps, un criblage à haut contenu couplé à différents critères de toxicité a été appliqué à des modèles HepaRG 2D et 3D. Cette application consistait à générer des profils de toxicité de produits chimiques ayant divers modes d'action et à comparer la sensibilité des deux modèles cellulaires afin de sélectionner le modèle cellulaire le plus approprié pour les objectifs ultérieurs. Deuxièmement, nous avons effectué le séquençage du transcriptome des cellules HepaRG 3D exposées à différents ensembles de produits chimiques. Une analyse génotoxique ciblée à l'aide de biomarqueurs transcriptomiques GENOMARK et TGx-DDI a été appliquée pour déterminer les réponses du modèle 3D HepaRG pour détecter les composés génotoxiques. Ensuite, grâce à une analyse non ciblée, nous avons analysé les profils transcriptomiques générés pour chaque produit chimique et étudié les principaux événements moléculaires sous-jacents aux mécanismes NGTxC. Enfin, nous avons étudié la réponse des NGTxC dans le test de transformation cellulaire (CTA) Bhas42 afin de déterminer la sensibilité et la spécificité de ce test dans la détection des cancérogènes humains. En résumé, ce travail de thèse contribue à proposer un cadre multi-NAM pour l'identification et la caractérisation du NGTxC.

Mots clés : NAMs, NGTxC, MoAs, PARC, Criblage à haut débit, Transcriptomq

Title: **Integrated high-throughput approach for the characterization of non-genotoxic carcinogens**

The massive increase in the number of chemical substances raises questions about their impact on human and environmental health. However, current regulatory methods for detecting and characterizing carcinogens relies mainly on long-term laboratory animal studies, which are not sufficient for assessing the toxicity of such a large number of chemicals of which they could have a potential of causing cancer to human. Furthermore, these methods have scientific limitations, particularly concerning differences in sensitivity between humans and animals, high cost, low throughput, and the ethical considerations related to animal suffering that can no longer be ignored.

Carcinogens are generally classified into genotoxic carcinogens (GTxC), and non-genotoxic carcinogens (NGTxC). While the identification of genotoxic carcinogens relies on a relatively robust battery of *in vitro* and *in vivo* genotoxicity tests, the 2-years rodent bioassays represent the gold standard for the identification of NGTxC.
Therefore, it appears urgent to develop a strategy based on New Approach Methodologies (NAMs) for the identification and characterization of chemical substances that are potentially carcinogenic to humans. For that, this work, which is part of the European project PARC (Partnership for the Assessment of Risks from Chemicals), aims to study the applicability of different NAMs-based approach to identify and characterize NGTxCs with different Mode of Actions (MoA).
The objectives of this research work were addressed through three complementary axes: First, high-content screening coupled with different toxicity endpoints was applied to HepaRG 2D and 3D models. This application was to generate toxicity profiles of chemicals with various MoAs, and to compare the sensitivity of both cellular models to select the most appropriate cellular model for the subsequent objectives. Second, we performed transcriptome sequencing of the 3D HepaRG cells exposed to different sets of chemicals. A targeted genotoxic analysis through GENOMARK and TGx-DDI transcriptomic biomarkers were applied to determine the responses of HepaRG 3D model to detect genotoxic compounds. Then, through an untargeted analysis, we analysed the generated transcriptomic profiles for each chemical and studied the main molecular events underlying NGTxC mechanisms. Finally, we studied the response of NGTxCs in the Bhas42 Cell Transformation Assay (CTA) to determine the sensitivity and specificity of this test in detecting human carcinogens. In summary, this thesis work contributes in proposing a multi-NAMs framework for the identification and characterization of the NGTxC.

Key words : NAMs, NGTxC, MoAs, High-throughput Approaches, Transcriptomic