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MUT/2020/18

**COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER
PRODUCTS AND THE ENVIRONMENT (COM)**

Guidance on the use of 3D Tissue Models for genotoxicity testing.

Continued consideration and comments of the updated COM Guidance document on the use of 3D Tissue Models for genotoxicity testing.

Members are asked to complete review of this latest draft as attached and consider the following questions:

1. Are members happy with the changes made to the section 'Application of 3D models for genotoxicity testing' (paragraphs 4 – 8) to better reflect that uses of these assays are wider than just reducing false positives.
2. Do members agree with the IWGT statement provided in paragraph 14 ('initial data show that the comet assay can be applied to the 3D airway models and the WG encourages further development of this assay').
3. Are members aware of any other regulatory challenges that are not listed that should be included in the document.
4. Do members agree with the statement made in paragraph 28.
5. Are members happy with the other changes made to the document following the meeting in June 2020.
6. This statement will now be published - do the members agree

Secretariat

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26 Background

27 1. The Committee on Mutagenicity of Chemicals in Food, Consumer Products and
28 the Environment (COM) has a remit to provide UK Government Departments and
29 Agencies with advice on the most suitable approaches to testing chemical substances
30 for genotoxicity. The COM views regarding the most appropriate strategy for
31 genotoxicity testing are outlined in full in the COM (202x) “*Guidance On A Strategy
32 For Genotoxicity Testing Of Chemical Substances*”.

33 2. In brief, the COM recommend a staged approach to genotoxicity testing. **Stage**
34 **0**, in the absence of test data from adequately designed and conducted genotoxicity
35 tests, consists of preliminary considerations of the test chemical substance, including,
36 physicochemical properties, Structure Activity Relationships (SAR), and information
37 from screening tests. **Stage 1** consists of *in vitro* genotoxicity tests that provide
38 information on three types of genetic damage (namely, gene mutation, chromosomal
39 damage and aneuploidy) and gives appropriate sensitivity to detect chemical
40 genotoxins. **Stage 2** consists of *in vivo* genotoxicity tests which are chosen on a case-
41 by-case basis to address any genotoxic endpoints identified in Stage1; investigate
42 genotoxicity in tumour target tissue(s) and/or site of contact tissues; investigate
43 potential for germ cell genotoxicity; and investigate potential genotoxicity for chemicals
44 where high/moderate and prolonged exposure is anticipated, even if negative in Stage
45 1.

46 3. The use of 3D models for genotoxicity testing has not previously been
47 discussed in the full COM guidance document (COM, 202x). However, as the
48 development of 3D models is a rapidly evolving field, members considered it
49 appropriate to prepare guidance in this area, that can be updated at regular intervals.
50 As such, a brief summary of this area is provided in the full guidance document, while
51 this document outlines in more detail the 3D models currently used for genotoxicity
52 testing and those under development and/or validation.

53

54 Application of 3D models for genotoxicity testing

55 4. The main drivers for the development/use of 3D models were the Cosmetics
56 Directive, which prevented the use of *in vivo* testing for cosmetics, and the 3Rs
57 principle that requires the reduction, replacement and refinement of the use of animals
58 in toxicity testing. 3D models have also been developed to undertake testing for which
59 there is no robust in vivo system, such as site of contact studies, and have shown a
60 utility that is now being assessed for genotoxicity testing.

61

62 4.5. Although currently used *in vitro* genotoxicity testing batteries can reliably
63 identify *in vivo* genotoxicants, there are a number of positives which, when tested *in*

vivo, are non-genotoxic i.e. these are misleading positive findings, commonly referred to as 'false positives'. As a consequence, animal usage, testing time and costs can be unnecessarily increased which go against current initiatives which attempt to reduce the number of misleading positives from in vitro testing.

5-6. Such misleading positive findings are considered to be due to a number of reasons, including the use of cell lines of rodent origin (V79, CHO or CL) that partially lack normal cell cycle control, have limited metabolic capacity (even with the addition of rat liver S9) and do not mimic site-specific metabolic capacity (Reus et al., 2013). However, the impact of these factors has become increasingly recognised and has led to the development of models which more closely reflect tissue structure and tissue metabolic activity.

6-7. A number of types of 3D model, ranging from single cell microtissues to multi cell types grown within scaffolds have been developed. It is hoped the use of such models will improve the accuracy of predictions due to their improved metabolic capacity and the proximity to *in vivo* gene expression and protein function (Andres et al., 2012; Barcham et al., 2018).

7-8. The International Workshop on Genotoxicity Testing (IWGT) concluded that '3D tissue models offer a more 'in-vivo-like' behaviour for key parameters like cell viability, proliferation, differentiation, morphology, gene and protein expression, and function and therefore provide a valuable complement to the classical '2D' cell culture-based assays' (Pfuhler et al., 2020a).

3D models of skin

8-9. 3D models have, to date, mainly been developed for the skin. These models mimic the architectural features and behaviour of normal human skin and the changes that occur during early skin cancer progression and wound re-epithelialisation. Reconstructed 3D human epidermal skin models are used in OECD TG 431 (*in vitro* skin corrosion: Reconstituted human epidermis (RHE) test) (Kandárová et al., 2006; Kidd et al., 2007; OECD, 2016a) , which can be used in addition to the acute dermal irritation/corrosion test in rats (OECD TG 404). OECD TG 439 (*in vitro* skin irritation: Reconstituted human epidermis test) also utilises reconstructed 3D epidermal skin models (Alépée et al., 2010; Kandarova et al., 2009; OECD, 2015). Assessment of phototoxic properties (Jírová et al., 2005; Lelièvre et al., 2007) and sensitisation potential (dos Santos et al., 2011; Teunis et al., 2013) are also being explored using reconstructed 3D skin models and are considered to have a high potential to be accepted as OECD TGs (Reus et al., 2013).

9-10. For genotoxicity testing purposes, 3D skin models have been linked to the standard genotoxicity endpoints of the micronucleus test and Comet assay. Two endpoints are utilised to reflect different types of genetic damage, namely

clastogenicity and aneugenicity and DNA strand breaks, incomplete repair sites and alkali labile sites, respectively. The 3D Skin Comet assay and Reconstituted Skin Micronucleus (RSMN) test are described in paragraphs 10 to 15 below. These assays allow the *in vitro* assessment of DNA damage following dermal exposure, which has only previously been possible using *in vivo* assays; this is despite dermal exposure being a common route for a number of compounds found in household products, cosmetics, and industrial chemicals (Reisinger et al., 2018).

3D Comet assay

~~10-11.~~ The Comet assay has been adapted for use with two reconstructed full thickness human skin models: the EpiDerm™- and Phenion® FullThickness Skin Models. Both skin models are comprised of primary and p53 competent cells of human origin. These models have a number of advantages over current monolayer-type assays including: species specificity, with a phenotype close to native human skin; normal cell cycle control; DNA-repair competence; similar gene and protein expression patterns; and the mimicking of conditions of use for dermally applied substances/products (Reisinger et al., 2018).

~~11-12.~~ As the Comet assay does not rely on proliferating cells and can be used with a wide range of cell types, it is particularly suitable for application to skin tissue models. The assay also detects a wide range of DNA damage including double-stranded and single-strand breaks from direct interaction of the test chemical or related to incomplete excision repair as well as alkali labile sites (OECD, 2016b). This ensures that both clastogenic DNA damage and lesions that may give rise to gene mutation are detected.

~~12-13.~~ The 3D Skin Comet assay has undergone inter-laboratory validation using the Phenion® Full-Thickness Skin Model to assess its potential use as a new *in vitro* tool for following up positive findings from the standard *in vitro* genotoxicity test battery for dermally applied chemicals. The authors reported that the skin model has similar metabolic competency to natural human skin. Further, for the 32 substances tested, there was a high predictive capacity with a sensitivity of 77%, a specificity of 88% and an overall accuracy of 83% when compared to *in vivo* animal genotoxicity test outcomes. Improved ‘positive predictability’ of the assay was also seen when combined with the RSMN assay in a testing battery (Reisinger et al., 2018; Pfuhler et al., 2020b).

~~13-14.~~ A number of 3D human airway models (also called lung models) have been established that closely resemble the lining of the human airway. As such, their utility for the genotoxicity testing of inhaled chemicals is being evaluated. The IWGT reported that ‘initial data show that the comet assay can be applied to the 3D airway models and the WG encourages further development of this assay’. It was emphasised that ‘the lack of 3D airway assays that can detect aneugenicity is considered a gap and the development of such an assay is strongly encouraged’. Use of the MN assay with the current 3D airway models was thought to be restricted by the limited proliferation rate of the cells in the models (Pfuhler et al., 2020a).

3D human reconstituted skin micronucleus assay

~~44-15.~~ The RSMN assay has been developed to assess the genotoxicity of dermally applied compounds [incorporated into cosmetics](#) and utilises a highly differentiated *in vitro* model of the human epidermis (Episkin LM™) with automated micronucleus detection using the standard cytokinesis block micronucleus assay (Barcham et al., 2018). The RSMN offers a close approximation of natural human skin due to the origin of the cells used and its physiological properties [for cosmetic testing](#). The model also allows topical administration which ensures that all parts of the model are exposed, regardless of the lipophilic nature of the test substance. In addition, the Episkin LM™ model has been shown to have a similar metabolic capacity to that of native human skin (Eilstein et al., 2014) allowing the assessment of genotoxic potential by metabolic activation as an intrinsic feature.

~~45-16.~~ A global validation of the assay has been carried out with the blinded testing of over 40 coded chemicals. Findings showed an overall accuracy of 84%, a sensitivity of 80% and specificity of 87% when compared to *in vivo* genotoxicity outcomes (Pfuhler et al., 2020). IWGT noted that the sensitivity of the 72 h protocol was superior to that of the 48 h and that the assay was now suitable for OECD TG development. [A submission has now been made to the OECD to include this assay into the Test Guideline programme](#). Further the WG concluded that the 'RSMN assay was an acceptable alternative to the *in vivo* test [for cosmetic testing](#) and that the high predictivity also demonstrates that the test complies with all requirements to be accepted as a 2nd tier test (Pfuhler et al., 2020a).

Other 3D tissue models

3D liver microtissue model

~~46-17.~~ Conventional *in vitro* monolayer assays using hepatic cell lines may not be the most relevant assays to carry out functional and metabolic studies as the cells lose key liver specific functions, in particular cytochrome P450 activity (Godoy et al., 2013; Kim et al., 2011; Mingoia et al., 2007; Pfuhler et al., 2020a). In addition, non-parenchymal cells are absent which play an important role in clearance and in the initiation of an immune response. Due to the limited lifespan of the conventional assays, repeated exposures are not possible (Kermanizadeh et al., 2014).

~~47-18.~~ A 3D liver microtissue model has been described (Messner et al., 2013; Kermanizadeh et al., 2014; Kratschmar DV, 2013) which has a number of advantages over conventional hepatic assays. These include: the use of primary human hepatic cells; viability of cells for long periods which allows multiple exposures to be assessed; maintenance of a high level of metabolic activity across the lifespan of cells.

~~48-19.~~ A 3D liver model utilising HepG2 cells grown using a 'hanging-drop' technique has been assessed for genotoxicity testing, with micronucleus detection in the 3D spheroid models. Micronucleus induction was seen to be greater in the 3D structures than in the 2D format (Shah et al., 2018). The IWGT concluded that for 3D liver

spheroids ‘initial data show that the MN assay can be applied to 3D liver spheroids and the WG encourages further development of this assay’. It is also recognised by the WG that this technique is being investigated within the EU Horizon 2020 project PATROLS which includes characterisation of their metabolic competence ([Llewellyn et al., 2020](#); Pfuhler et al., 2020a).

3D tissue models of the airway epithelium

[19-20](#). In conventional monolayer (2D) cultures of basal cells, only maintenance and expansion of cells is possible. However, in 3D airway tissue models, basal cells can differentiate into a mucociliary pseudostratified epithelium containing ciliated, goblet and basal cells. Other properties similar to the native human airway epithelium include beating cilia, mucus secretion, barrier properties and remodelling and restoration properties (Rock et al., 2009).

[20-21](#). The two most widely used models of the airway epithelium are 3D microtissue models and co-cultures of multiple cell types, both of which can be grown at the air-liquid interface (ALI) (Evans et al., 2017; Evans et al., 2019; Barosova et al., 2020; Pfuhler et al., 2020a).

[21-22](#). ALI cultures reflect physiological conditions *in vivo*, with the respiratory epithelium being exposed to the air. These cultures are currently used to study cell biology and infection, culture patient-derived cells to model diseases, and test the effects of aerosolised particles (including drug formulations and cigarette smoke) on the respiratory epithelium (for example (Azzopardi et al., 2015)). IWGT considered that these models may enable a more realistic (geno)toxicity assessment of inhaled compounds. In addition, as the models can be kept in culture for months, this presented the possibility of assessing subchronic exposures (Pfuhler et al., 2020a).

Regulatory challenges

[23](#). There is a requirement within OECD TGs to show proliferative index and viability when undertaking *in vitro* assays for genotoxicity. This is challenging to do with 3D models and requires further consideration.

[24](#). The cosmetics industry is more accepting of the findings of 3D models as no *in vivo* testing can be carried out. However, the application and acceptance of data to other areas of chemical genotoxicity testing is currently not known. In both cases though, data from such models would be considered as part of an overall weight of evidence.

Conclusion

[25](#). 3D human tissue models may offer an alternative testing strategy to *in vivo* assays for substances that are found to be positive using the traditional *in vitro* genotoxicity battery of tests. Extensive progress has been made on the development

and validation of 3D genotoxicity models and models are available for the major routes of exposure in humans.

26. The most advanced of such models, the 3D Skin models, have undergone inter-laboratory validation and been shown to comply with all requirements to be accepted as a 2nd tier test for cosmetic ingredients testing.

27. The 3D RSMN assay is currently moving into OECD TG development. For the 3D airway model, measurement of clastogenicity and gene mutation are possible, but detection of aneuploidy is not developed at the present time. The 3D liver model a test for gene mutation is required; both models also need to undergo validation.

22-28. Using historic data, chemicals that are positive for genotoxic activity *in vivo* have been shown to be positive in either the 3D-micronucleus or 3D-Comet assay. In the main, chemicals that are negative for genotoxic activity *in vivo* are also negative in the two 3D models (Kirkland et al., 2014).

References

- Aardema, M. J., Barnett, B. B., Mun, G. C., et al. 2013. Evaluation of chemicals requiring metabolic activation in the EpiDerm 3D human reconstructed skin micronucleus (RSMN) assay. *Mutat Res*, 750, 40-9.
- Alépée, N., Tornier, C., Robert, C., et al. 2010. A catch-up validation study on reconstructed human epidermis (SkinEthic™ RHE) for full replacement of the Draize skin irritation test. *Toxicology in Vitro*, 24, 257-266.
- Andres, E., Molinari, J., Remoue, N., et al. 2012. Successful micronucleus testing with the EPI/001 3D reconstructed epidermis model: preliminary findings. *Mutat Res*, 743, 36-41.
- Azzopardi, D., Haswell, L. E., Foss-Smith, G., et al. 2015. Evaluation of an air-liquid interface cell culture model for studies on the inflammatory and cytotoxic responses to tobacco smoke aerosols. *Toxicology in Vitro*, 29, 1720-1728.
- Barcham, R., Orsini, N., Andres, E., et al. 2018. Successful proof of concept of a micronucleus genotoxicity assay on reconstructed epidermis exhibiting intrinsic metabolic activity. *Mutat Res*, 829-830, 75-86.
- Barosova, H., Drasler, B., Petri-Fink, A., et al. [Multicellular Human Alveolar Model Composed of Epithelial Cells and Primary Immune Cells for Hazard Assessment. J. Vis. Exp. \(159\), e61090.](#)
- COM. 2011. *Guidance On A Strategy For Genotoxicity Testing Of Chemical Substances* [Online]. https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/315793/testing_chemicals_for_genotoxicity.pdf. [Accessed].
- Dos Santos, G. G., Spiekstra, S. W., Sampat-Sardjoepersad, S. C., et al. 2011. A potential in vitro epidermal equivalent assay to determine sensitizer potency. *Toxicology in Vitro*, 25, 347-357.
- Eilstein, J., Lereaux, G., Budimir, N., et al. 2014. Comparison of xenobiotic metabolizing enzyme activities in ex vivo human skin and reconstructed human skin models from SkinEthic. *Arch Toxicol*, 88, 1681-1694.

- Evans SJ, Clift MJ, Singh N, et al. 2017. Critical review of the current and future challenges associated with advanced in vitro systems towards the study of nanoparticle (secondary) genotoxicity. *Mutagenesis*. 32(1), 233-241.
- Evans SJ, Clift MJD, Singh N, et al. 2019. In vitro detection of in vitro secondary mechanisms of genotoxicity induced by engineered nanomaterials. *Part Fibre Toxicol*. 16(1), 8.
- Godoy, P., Hewitt, N. J., Albrecht, U., et al. 2013. Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Arch Toxicol*, 87, 1315-530.
- JíRová, D., Kejlová, K., Bendová, H., et al. 2005. Phototoxicity of bituminous tars—correspondence between results of 3T3 NRU PT, 3D skin model and experimental human data. *Toxicology in Vitro*, 19, 931-934.
- Kandarova, H., Hayden, P., Klausner, M., et al. 2009. In vitro skin irritation testing: Improving the sensitivity of the EpiDerm skin irritation test protocol. *Altern Lab Anim*, 37, 671-89.
- Kandárová, H., Liebsch, M., Spielmann, H., et al. 2006. Assessment of the human epidermis model SkinEthic RHE for in vitro skin corrosion testing of chemicals according to new OECD TG 431. *Toxicology in Vitro*, 20, 547-559.
- Kermanizadeh, A., Lohr, M., Roursgaard, M., et al. 2014. Hepatic toxicology following single and multiple exposure of engineered nanomaterials utilising a novel primary human 3D liver microtissue model. *Part Fibre Toxicol*, 11, 56.
- Kidd, D. A., Johnson, M. & Clements, J. 2007. Development of an in vitro corrosion/irritation prediction assay using the EpiDerm™ skin model. *Toxicology in Vitro*, 21, 1292-1297.
- Kim, B.-S., Park, I.-K., Hoshiba, T., et al. 2011. Design of artificial extracellular matrices for tissue engineering. *Progress in Polymer Science*, 36, 238-268.
- Kirkland, D., Aardema, M., Henderson, L., et al. 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. *Mutat Res*, 584, 1-256.
- Kirkland, D., Aardema, M., Muller, L., et al. 2006. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens II. Further analysis of mammalian cell results, relative predictivity and tumour profiles. *Mutat Res*, 608, 29-42.
- Kirkland, D., Pfuhler, S., Tweats, D., et al. 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. *Mutat Res*, 628, 31-55.
- Kirkland, D., Zeiger, E., Madia, F., et al. 2014. Can in vitro mammalian cell genotoxicity test results be used to complement positive results in the Ames test and help predict carcinogenic or in vivo genotoxic activity? I. Reports of individual databases presented at an EURL ECVAM Workshop. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 775-776, 55-68.
- Kratschmar Dv, M. S., Moritz W, Et Al. 2013. Characterization of a Rat Multi-Cell Type 3D-Liver Microtissue System. *J Tissue Sci Eng*, 4, 130 - 136.
- Lelièvre, D., Justine, P., Christiaens, F., et al. 2007. The episkin phototoxicity assay (EPA): Development of an in vitro tiered strategy using 17 reference chemicals to predict phototoxic potency. *Toxicology in Vitro*, 21, 977-995.

- [Llewellyn, S. V., Conway, G. E., Shah, U. K., et al. 2020. Advanced 3D Liver Models for In vitro Genotoxicity Testing Following Long-Term Nanomaterial Exposure. *J. Vis. Exp.* \(160\), e61141.](#)
- Matthews, E. J., Kruhlak, N. L., Cimino, M. C., et al. 2006. An analysis of genetic toxicity, reproductive and developmental toxicity, and carcinogenicity data: I. Identification of carcinogens using surrogate endpoints. *Regulatory Toxicology and Pharmacology*, 44, 83-96.
- Messner, S., Agarkova, I., Moritz, W., et al. 2013. Multi-cell type human liver microtissues for hepatotoxicity testing. *Arch Toxicol*, 87, 209-13.
- Mingoia, R. T., Nabb, D. L., Yang, C. H., et al. 2007. Primary culture of rat hepatocytes in 96-well plates: effects of extracellular matrix configuration on cytochrome P450 enzyme activity and inducibility, and its application in in vitro cytotoxicity screening. *Toxicol In Vitro*, 21, 165-73.
- OECD 2015. *Test No. 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method*, Paris, France, OECD Publishing.
- OECD 2016a. Test No. 431: In vitro skin corrosion: reconstructed human epidermis (RHE) test method. *OECD Guidelines for the Testing of Chemicals, Section 4*. Paris: OECD Publishing.
- OECD 2016b. *Test No. 489: In Vivo Mammalian Alkaline Comet Assay*, Paris, France, OECD Publishing.
- Pfuhler, S., Van Benthem, J., Curren, R., et al. 2020a. Use of in vitro 3D tissue models in genotoxicity testing: Strategic fit, validation status and way forward. Report of the working group from the 7(th) International Workshop on Genotoxicity Testing (IWGT). *Mutat Res*, 850-851, 503135.
- Pfuhler, S., Pirow, R., Downs, T. R., et al. 2020b. Validation of the 3D reconstructed human skin Comet assay, an animal-free alternative for following-up positive results from standard in vitro genotoxicity assays. *Mutagenesis*.
- Reisinger, K., Blatz, V., Brinkmann, J., et al. 2018. Validation of the 3D Skin Comet assay using full thickness skin models: Transferability and reproducibility. *Mutat Res*, 827, 27-41.
- Reus, A. A., Reisinger, K., Downs, T. R., et al. 2013. Comet assay in reconstructed 3D human epidermal skin models--investigation of intra- and inter-laboratory reproducibility with coded chemicals. *Mutagenesis*, 28, 709-20.
- Rock, J. R., Onaitis, M. W., Rawlins, E. L., et al. 2009. Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proc Natl Acad Sci U S A*, 106, 12771-5.
- Shah, U. K., Mallia, J. O., Singh, N., et al. 2018. Reprint of: A three-dimensional in vitro HepG2 cells liver spheroid model for genotoxicity studies. *Mutat Res*, 834, 35-41.
- Teunis, M., Corsini, E., Smits, M., et al. 2013. Transfer of a two-tiered keratinocyte assay: IL-18 production by NCTC2544 to determine the skin sensitizing capacity and epidermal equivalent assay to determine sensitizer potency. *Toxicol In Vitro*, 27, 1135-50.

Abbreviations used in the document

3D Tissue Model	Artificially created environment in which biological cells are permitted to grow or interact with their surroundings in all three dimensions.
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2D	Two dimensional
RSMN	Reconstituted Skin Micronucleus
ECVAM	European Center for Validation of Alternative Methods
HepG2 cells	Immortalised cell line consisting of human liver carcinoma cells

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376 **NCET at WRc/IEH-C under contract supporting the PHE COT Secretariat**

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