Committee on Mutagenicity

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

GUIDANCE ON A STRATEGY FOR GENOTOXICITY TESTING OF CHEMICALS

Continued consideration and comments of the updated COM 'Guidance document on a strategy for genotoxicity testing of chemicals'.
This paper has been amended according to comments received from members of COM following the meeting in November 2020 and the 'sub-group' meeting in January 2021. This document incorporates all comments received, members are asked to:

1. Please provide advice where this is indicated in the text.
2. Following any changes that are discussed, this guidance will now be published via Chairs action - do the members agree?

Secretariat
February 2021

GUIDANCE ON A STRATEGY FOR GENOTOXICITY TESTING OF CHEMICALs

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GUIDANCE ON A STRATEGY FOR GENOTOXICITY TESTING OF CHEMICALS.

Executive Summary

1. The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) has a remit to provide UK Government Departments and Agencies with advice on the most suitable approaches to testing chemical substances for genotoxicity. The COM published guidance in 1981, 1989, 2000 and again in 2011. This document incorporates some significant changes and reports the COM views regarding the most appropriate strategy for genotoxicity testing (Figure 1) reached in 2020, bringing the guidance document up to date.

2. It should be noted that in this updated guidance, several key areas have been identified as potentially requiring frequent updating, due to their fast-moving nature. To facilitate such updates, standalone documents have been prepared outlining the currently available status of the use of Quantitative Structure Activity Relationship (QSAR) modelling (30 - 31) and testing strategies for germ cell mutagens (paras 88 - 91), both of which were included in the previous version of the guidance document. In addition, standalone documents have been prepared detailing the use of 3D tissue models for genotoxicity testing and test guidance strategies for manufactured nanomaterials. Both of these areas were not included in the previous version of the guidance document and are now briefly detailed in paras 28 and 35 respectively.

3. The COM recommends a staged approach to testing:

- **Stage 0** consists of preliminary considerations which include physico-chemical properties of the test chemical substance, Structure Activity Relationships (SAR), and information from screening tests[1]. However, data from SAR and screening tests should not overrule test data from adequately designed and conducted genotoxicity tests.

- **Stage 1** consists of *in vitro* genotoxicity tests. The COM recommends a core-test battery of the Ames test combined with the *in vitro* micronucleus test. This combination provides information on three types of genetic damage for which

[1] Note that the terms ‘test’ and ‘assay’ are used interchangeably throughout the document to reflect naming conventions.

data are required (namely, gene mutation, chromosomal damage and aneuploidy) and gives appropriate sensitivity to detect chemical genotoxins. As also supported by the OECD, the COM consider that there is no need to independently replicate adequately designed and conducted core *in vitro* tests which are either clearly negative or clearly positive. The strategy document also considers the contribution that can be made by a number of non-core *in vitro* tests.

- **Stage 2** consists of *in vivo* genotoxicity tests. A case-by-case strategy should be developed to answer one or more of the following specific queries;

    1) Investigation of genotoxic end point(s) identified in Stage 1,
    2) Investigation of genotoxicity in tumour target tissue(s),
    3) Investigation of potential for germ cell genotoxicity,
    4) Investigation of *in vivo* genotoxicity for chemicals which were negative in Stage 1 but where there is high or moderate and prolonged exposure,
    5) Investigation of genotoxicity in site of contact tissues.

4. The core tests in Stage 2 are the rodent micronucleus/chromosome aberration assays for aneuploidy and clastogenicity, the transgenic rodent gene mutation assay and the rodent alkaline comet assay for DNA damage.

5. Usually, negative results obtained in a carefully selected *in vivo* test (possibly studying more than one endpoint and tissue) will be sufficient to address positive results found *in vitro*. However, a further test(s) may be needed if some of the genotoxic effects seen in Stage 1 *in vitro* tests had not been adequately studied *in vivo* (e.g. the chemical affects multiple mutagenic endpoints), or other aspects of the genotoxic potential of the chemical had not been fully resolved (e.g. a human metabolite is identified that is not formed, or only in small amounts, in rodents, or in the case where an investigation of heritable effects was required). The strategy document also considers the contribution that can be made by a number of non-core *in vivo* tests. In most instances information from core *in vivo* tests is sufficient to evaluate the *in vivo* genotoxicity of chemical substances. A supplementary *in vivo* test strategy can provide additional information on a case-by-case basis, to investigate aspects such as further characterisation of germ cell genotoxicity, and DNA adduct data which can provide information to elucidate the mode of genotoxic action of carcinogenic chemicals.

I. Preface

6. The COM is an independent expert advisory committee whose members are appointed by the secretary of state for health and social care and the chair of the Food Standards Agency (FSA) following an appointments exercise involving public advertisement. Members serve in their own capacity as independent experts and observe a published code of practice including principles relating to the declaration of possible conflicting interests.

7. The remit of the COM is to advise any UK government departments and agencies with an interest in the safety of chemicals across various sectors on the human health aspects of the mutagenicity and genotoxicity of chemicals (these terms are defined for the purposes of this guidance document in paragraphs 9 - 11 below). The Secretariat is provided by Public Health England (PHE), who lead, and the FSA. Other government departments with an interest provide assessors to the COM; these are specifically from the Department of Health and Social Care (DHSC), the Department of Environment, Food and Rural Affairs (Defra), the Chemicals Regulation Division (CRD) of the Health and Safety Executive (HSE) (responsible for legislation regulating chemicals, pesticides, biocides and detergents), the Environment Agency (EA), the Veterinary Medicines Directorate (VMD; a Defra agency responsible for the licensing of veterinary drugs) and the Medicines and Healthcare products Regulatory Agency (MHRA; a DHSC agency responsible for the licensing of human medicines). In addition, there are assessors from the Scottish Government, the Welsh Assembly Government and the Northern Ireland Assembly.

8. The role of the COM is advisory. It has no regulatory status, although its advice may be provided to a body that does have such a role (e.g. HSE CRD for occupational aspects and for pesticides etc). Its remit is to advise on the human health aspects of the genotoxicity of chemicals, and this may involve advice on a specific chemical, and also on testing strategies and research. This guidance document focuses on testing strategies for chemicals for which there are no available genotoxicity data. Separate guidance on a strategy for the genotoxicity testing and mutagenic hazard assessment of chemicals with inadequate genotoxicity data was published in 2011 (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/315802/strategy_for_chemicals_with_inadequate_genotoxicity_data.pdf). Throughout this guidance the COM has referred to the genotoxicity testing of chemical(s) which refers to a specified chemical or material, including any additive necessary to preserve its stability and any impurity deriving from the process used.

The COM usually provides advice on a specific chemical which can be equated to a single chemical or compound. Provision of advice on radiation aspects is not within the scope of the COM.

9. The COM also has a general remit to advise on important general principles or new scientific discoveries in connection with potential mutagenic and genotoxic hazards (inherent properties of chemicals) or risk (the likelihood of mutagenic or genotoxic effects occurring after a given exposure to a chemical) and to present recommendations for genotoxicity testing. In practice the bulk of the work of the COM relates to assessing genotoxicity tests and providing advice on the genotoxic hazard of chemicals.

10. In the context of testing strategies, the COM first published guidelines for the testing of chemicals for mutagenicity in 1981, and these were revised in 1989 and 2000 (DOH, 2000). These provided guidance to the relevant government departments and agencies on best practice for testing at those times. The rationale developed by COM in 2000, particularly in relation to the testing of all potential mutagenic endpoints, was adopted by the International Workshops on Genotoxicity Testing (IWGT) (Muller et al., 2003). The need for guidance to be periodically updated, to reflect advances in development and validation of methods, was recognised and substantially revised guidance was published in 2011 (DOH, 2011). Testing strategies, the same or similar to those outlined in the 2011 COM guidance, have been adopted by some regulatory bodies, including the European Food Safety Authority (EFSA) (EFSA, 2017) and included in the notes on Guidance from the Scientific Committee on Consumer Safety (SCCS) (SCCS, 2016) and in the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation (ECHA, 2017).

11. A further revision of the guidance has been undertaken. This version (COM, 202x) of the guidance outlines the strategy that COM consider to be the most scientifically appropriate given available methods, and recognises the need to avoid the use of live animals where practical and validated alternative methods are available. The COM believes that the approach outlined presents an overview of the core principles of genotoxicity testing and will remain valid for several years. It is acknowledged that existing national or international testing strategies will be at different stages of review and hence inconsistencies are expected. The COM guidance is not intended to supersede or replace existing national or international sector-specific genotoxicity testing strategies (e.g. those recommended for pharmaceuticals by the International Conference on Harmonisation of Technical Requirements for Registration

of Pharmaceuticals for Human Use (ICH) (ICH, 2011), for chemicals assessed under REACH Regulations (EC1906/2006) (ECHA, 2017), or by EFSA (EFSA, 2011).

II. Introduction

12. The COM last published guidance on a strategy for the testing of chemicals for mutagenic potential in 2011 (DOH, 2011). The guidance outlined in 2011 was based on the development of new approaches to identifying genotoxic hazards *in vitro* including new approaches to identify misleading positive results and evaluate target organ genotoxicity *in vivo*. There is also a need for a testing strategy which can encompass chemicals such as cosmetics where no animal tests are permitted under European Union (EU) law. It is the objective of this paper to set out a scientifically valid testing strategy comprising those methods which the COM believe to be the most informative with regards to the detection of genotoxic hazard and (when possible) are well validated. There is no discussion of methods which experience has shown to be suboptimal in determining genotoxicity. Details of methodologies are not given since they are provided in the Organisation for Economic Cooperation and Development (OECD) test guidelines (TG), the EU Test Methods Regulation (EC 440/2008) and the IWGT guidance.

13. The genome can be damaged in a variety of ways either spontaneously or from exposure to genotoxic agents. The term “mutagenic” refers to the ability of a chemical to induce a permanent change in the amount or structure of the genetic material of an organism, which may result in an heritable change in the characteristics of the organism. Chemicals inducing mutations are referred to as mutagens (they are mutagenic). These alterations may involve individual genes, blocks of genes, or whole chromosomes. Mutations involving single genes may be a consequence of effects on single DNA bases (point mutations) or of larger changes, including deletions and rearrangements of DNA. The potential to induce mutation is measured in test systems that detect a broader range of genetic changes than simply mutation – they measure genotoxicity. Mutagenicity is accepted as a key event in carcinogenicity. Epigenetic changes, that could also be heritable, fall outside the scope of this guidance.

14. Genotoxicity refers to interaction with, or damage to, DNA and/or other cellular components which regulate the fidelity of the genome. It is a broad term that, as well as mutation, includes damage to DNA such as the production of DNA adducts, by the chemical itself or its metabolites. Cells have the capacity to protect themselves from such potentially lethal or mutagenic genotoxic effects by many repair processes and therefore many genotoxic events do not become evident as mutations. However, the

capacity to damage the genome (genotoxicity) is an indicator of potential mutagenicity. Thus, some methods that measure genotoxicity do not provide direct evidence of heritable mutation.

15. The objective of genotoxicity testing is to exclude or identify potential hazards to humans and, for those chemicals that are positive, to aid in the elucidation of the mode of action (MoA). This guidance therefore presents a strategy for genotoxicity testing since this term encompasses all the assays included in the strategy. Consequently, it is important to generate information on three types of genetic damage, namely gene mutation, changes to chromosome structure (i.e. clastogenicity) and number (i.e. aneuploidy), to provide comprehensive coverage of the mutagenic potential of a chemical.

16. The COM reaffirms its view, published in 1989, 2000 and 2011, that there is currently no single validated assay that can provide comprehensive information on all three types of genetic damage and thus, it is necessary to subject a given test chemical to several different assays. The range of assays discussed in this document include those using prokaryotes (bacteria) and mammalian cells *in vitro*, and whole mammals, where effects in a wide range of target organs including germ cells can be measured. Assays may be classified on the basis of genetic endpoints (e.g. gene mutation, clastogenicity, aneugenicity and tests for DNA damage) or by consideration of the different phylogenetic levels (e.g. bacteria, and mammalian cells) represented and also in mammals by the tissues or target organs studied.

III Significance of Chemical-Induced Mutation for Human Health

17. A mutation in the germ cells of sexually reproducing organisms may be transmitted to the offspring, whereas a mutation that occurs in somatic cells may be transferred only to descendant daughter cells. Mutagenic chemicals may present a hazard to health since exposure to a mutagen carries the risk of inducing germ-line mutations, with the possibility of inherited disorders, and the risk of somatic mutations including those leading to cancer.

18. A separate statement discussing the significance of chemical-induced mutation to human health was published in 2012: (https://www.gov.uk/government/publications/the-significance-of-chemical-induced-mutation-for-human-health).

IV. General Principles of Testing Strategy

19. The COM recommends a two-stage testing strategy (Stages 1 and 2) for the detection of the genotoxic hazard of chemicals which can be supported by appropriate preliminary screening tests and/or *in silico* data (Stage 0).

20. Initial testing for genotoxic potential in Stage 1 is based upon two core *in vitro* tests that are chosen to provide information on gene mutation, clastogenicity and aneuploidy, with case-by-case additional testing and investigation depending on the results of these initial genotoxicity tests. All *in vitro* tests should be designed to provide the best chance of detecting potential activity, with respect to (a) the exogenous metabolic activation system (S9 - see glossary); (b) the ability of the compound or its metabolite(s) to reach the target DNA and/or targets such as the cell division apparatus, and; (c) the ability of the genetic test system to detect the given type of genotoxic event. Where international guidelines are available, the assays should be carried out to conform to those internationally recognised documents e.g. as published by the OECD, the IWGT and in the EU test methods Regulation (EC 440/2008). The same approach to testing can be used for chemicals where *in vivo* genotoxicity testing is not permitted (e.g. cosmetics). Investigations regarding MoA are important to derive conclusions on biological relevance of *in vitro* genotoxicity test results, to aid in overall risk assessment, and to inform on the strategy for *in vivo* tests. This is of particular importance for those chemicals where no *in vivo* genotoxicity testing is permitted.

21. For most chemicals, results from the two Stage 1 core tests should be sufficient to reach a conclusion on the presence or absence of mutagenic potential. However, in some instances, even when Stage 1 tests are negative, regulatory authorities may require consideration of the need for *in vivo* Stage 2 testing particularly where exposure is considered to be high, or moderate and prolonged (e.g. most human medicines), or where there is a chemical class precedent (i.e. structural relationship) of positive *in vivo* genotoxicity data. Guidance on the level of exposure which equates to high, moderate or prolonged is beyond the remit of the COM.

22. Stage 2 consists of a number of *in vivo* tests designed to investigate whether *in vitro* genotoxic activity including specific end points identified by *in vitro* tests can be expressed in the whole animal. This may also include assays for specific target organs (e.g. rodent tumours detected in carcinogenicity bioassays) or in germ cells. Few chemicals are active only *in vivo* and in such cases this may be due to a number of factors such as metabolic differences, the influence of gut flora, higher exposures *in*

vivo compared to *in vitro,* pharmacological (e.g. folate depletion or receptor kinase inhibition) and extreme physiological effects (Tweats et al, 2007b).

23. There is currently no single *in vivo* test which can assay all three types of genetic damage (Thybaud et al., 2007) and thus a strategy for Stage 2 has to be designed based on the nature of genotoxic effects identified in Stage 1 and the possibility that genotoxic activity will only be expressed *in vivo* as discussed above. However, consideration should be given to the possibility of evaluating different genotoxicity endpoints in a single set of test animals.

24. There should be a clear strategy for planning tests within each stage and for progressing from Stage 1 to Stage 2. Clear statements can be made regarding the initial *in vitro* tests to be used in Stage 1 as these methods have been well studied, whereas the strategy for Stage 2 is more complex and, if not a specific regulatory requirement, needs to be developed on a case-by-case basis.

25. Under the strategy recommended by COM, the use of animals in genotoxicity testing is primarily required when it is necessary to investigate whether genotoxic activity detected in Stage 1 *in vitro* is reproduced *in vivo,* to study target organ genotoxicity (for example involvement of genotoxicity in rodent tumours) and to evaluate the potential for heritable mutagenic effects. Genotoxicity testing using animals, when required by guidance, should be carried out when there is no suitable alternative, and the minimum number of animals should be used, consistent with obtaining valid results. If feasible, studies can be conducted as an adjunct to single or repeat dose toxicity studies. The COM supports current and future developments to replace, refine or reduce the need for animals, consistent with the principles of the 3Rs[2].

V Genotoxicity Testing Strategy

26. The COM guidance provides a strategy for testing chemicals where no genotoxicity data are available. Test chemicals may also contain impurities at varying levels which may exhibit genotoxic activity. Separate guidance on the genotoxicity assessment of impurities is available at https://www.gov.uk/government/publications/genotoxicity-assessment-of-impurities-in-chemical-substances. The assessment and control of genotoxic impurities is the subject of an ICH Guideline (M7) and ICH M7(R1) and a Question and Answer

[2] https://www.nc3rs.org.uk/the-3rs

document (https://www.ema.europa.eu/en/ich-m7-assessment-control-dna-reactive-mutagenic-impurities-pharmaceuticals-limit-potential).

27. The strategy recommended in the following sections is concerned with testing for genotoxic activity of chemicals and does not specifically address complex mixtures of chemicals.

Stage 0: Preliminary Considerations Prior to Genotoxicity Testing

28. The intrinsic chemical and toxicological properties of the test chemical must be considered before devising the genotoxicity testing programme. Manufactured nanomaterials present particular considerations with regards to genotoxicity testing and these are discussed in a separate document 'Test Guidance Strategies for Genotoxicity Testing of Manufactured nanomaterials' (COM, 202x).

Physico-chemical and Toxicological Properties

29. The physico-chemical properties of the test chemical (for example, acid dissociation constant (pKa), partition coefficient, solubility, volatility and stability in, and potential reactions with, solvents/vehicles) and its purity can affect the ease of conduct and results of *in vitro* tests. For example, the tolerance of cells to acidic chemicals can be enhanced by neutralisation but this may affect the inherent reactivity of chemicals with DNA (Hiramoto et al., 1997). Potential reactions of the test chemical with solvent /vehicle should also be considered (e.g. cisplatin reacts with dimethyl sulfoxide (DMSO)) (Fischer et al., 2008). Alternatively, low solubility may limit the feasibility of undertaking some or all of the *in vitro* mutagenicity tests recommended in this strategy. The potential for auto-oxidation of the test chemical in the culture medium can also affect the outcome of *in vitro* genotoxicity tests (Long et al., 2007). It is noteworthy that the toxic properties of test chemicals, such as target organ effects, or irritancy/corrosivity in contact with skin or mucous membranes and their toxicokinetics and metabolism will influence the choice of route of administration and the highest dose level achievable in Stage 2 *in vivo* mutagenicity tests.

Quantitative Structure Activity Relationships (QSAR)

30. The expected mutagenic potential of a chemical can be assessed from its chemical structure, which may provide structural alerts for mutagenicity. The COM has previously agreed that where no genotoxicity data are available, initial assessment of potential genotoxicity can be based on publicly available QSAR models. A range of QSARs have been developed to predict genotoxicity and COM considered updated information on these models in February 2018. The discussions formed the basis of the COM Statement 'Use of QSAR models to predict genotoxicity' (COM, 202x). It was

concluded that whilst it remained useful to evaluate data generated from QSAR models, in particular as a negative predictor for screening purposes, no changes to the previously recommended guidance (detailed more fully within the 2011 version of the COM Guidance document; COM, 2011) were warranted.

31. Overall, QSAR approaches for the prediction of genotoxic activity can be a valuable tool to aid in the high throughput screening of compounds, the provision of assessments for chemicals for which no genotoxicity test data are available and also prioritisation for genotoxicity testing. QSAR can also aid in the interpretation of genetic toxicology tests. Expert judgement is needed when reaching conclusions on mutagenic hazard on the basis of QSAR information alone, and such predictions cannot replace the need to undertake the *in vitro* and *in vivo* genotoxicity tests required to derive conclusions on mutagenic hazard and risk. In reaching conclusions, data from well conducted *in vitro* or *in vivo* genotoxicity tests should be attributed a much higher weight of evidence than QSAR predictions, although all information should be assessed on a case-by-case basis.

Screening Tests

32. With regard to this guidance, genotoxicity screening tests refers to high throughput or scaled-down tests which have been designed to be rapid, economical, reproducible, require only small amounts of test chemicals (typically below 50 mg) and have a high concordance with comparator genotoxicity end points in genotoxicity tests; these tests are also often referred to as pre-screening tests. None of the available genotoxicity screening tests have reached the stage of development where they could routinely be used to replace data generated from guideline-compliant *in vitro* genotoxicity testing. COM therefore does not recommend any particular test for screening purposes.

33. A number of *in vitro* systems for use as screening tests have been proposed and are described in full in the previous version of the COM Guidance (COM, 2011). COM is currently preparing a stand-alone document detailing recent advances in screening tests that will sit alongside this Guidance Document.

Stage 1: In Vitro Genotoxicity Testing

Overview of strategy

34. The COM concluded in 1989, 2000 and 2011 that it was appropriate to concentrate on a relatively small number of assays, using validated, sensitive methods particularly chosen to ***avoid misleading negative or positive results*** when compared to *in vivo* testing results (Kirkland et al., 2005a, 2007c; Fowler et al.,

2012a, b; Matthews et al., 2006; Pfuhler et al., 2011). A detailed justification of the strategy is given in the previous version of the COM Guidance (COM, 2011) and, as such, is not included here.

35. Misleading positive results are considered to be caused by a number of factors, including inappropriately high doses of chemical and the use of cell lines of rodent origin (e.g., V79, CHO, CHL) that partially lack normal cell cycle control,[RB1][RB2] have limited metabolic capacity (even with the addition of S9) and do not mimic site-specific metabolic capacity (Reus et al., 2013). The use of p53-competent human cells and careful control of cytotoxicity can help reduce the number of misleading positive results without compromising sensitivity (Fowler et al., 2012a, b). The development of 3D tissue models is also hoped to reduce the number of misleading positive findings and improve the accuracy of predictions due to their improved metabolic capacity and proximity to *in vivo* gene expression and protein functions (Andres et al., 2012; Barcham et al., 2018; Pfuhler et al. 2020a, b). The current state of the science for 3D model development and validation is discussed in '3D Tissue Models for Genotoxicity Testing' (COM, 2020).

36. As outlined above in paragraph 20 and shown in Figure 2, Stage 1 involves tests for genotoxic activity using *in vitro* methods and comprises a two test core system; namely an *in vitro* bacterial test for gene mutation (Ames test) and an *in vitro* micronucleus test (MNvit), with the objective of assessing genotoxic potential by investigating three different end points (gene mutation, structural chromosomal damage and changes in chromosome number). A detailed justification of the strategy is given in the previous version of the COM Guidance (COM, 2011).

37. **A clear positive result in either of these two core tests is sufficient to define the chemical as an *in vitro* genotoxin, although further *in vitro* and/or *in vivo* testing may be undertaken to understand the relevance of the positive results**. The Committee considers this strategy allows for efficient identification of all genotoxic endpoints and that, by reducing the number of mammalian cell tests and following the most current version of the methodologies, the risk of misleading positive results (i.e. when compared with *in vivo* genotoxicity data) is decreased.

38. Additional investigations of chemicals which give positive or repeated equivocal results in Stage 1 tests can include an assessment of mode(s) of *in vitro* genotoxic action. There are a number of reasons (discussed in paragraphs 43-45) why positive results in *in vitro* genotoxicity tests might occur by mode(s) of action not relevant to human health hazard assessment. Such MoA evaluation *in vitro* is particularly relevant

for those chemicals (e.g., cosmetics) where there is a regulatory constraint which precludes the use of *in vivo* genotoxicity assays in the testing strategy. The COM does not recommend the use of Stage 1 in *vitro* genotoxicity assays that have not been considered in detail in this guidance or for which OECD guidelines either do not exist or have been deleted. This includes assays for sister chromatid exchange, the *in vitro* Unscheduled DNA Synthesis (UDS) assay, the *in vitro* comet assay or tests using fungi or *Drosophila*, A table of genotoxic endpoints detected by each assay cited in Stage 1 of this strategy is given in Annex 1.

39. For chemicals which give equivocal results or repeated small positive effects, when considering biological relevance, it is important to consider evidence of reproducibility in the same assay or in different assays detecting similar effects, and the magnitude of the induced genotoxic effect in relation to historical negative control data, and then consider whether further *in vitro* genotoxicity testing is needed (Hayashi et al., 2011; Kirkland et al., 2007a). Further consideration of SAR data for these chemicals may also give valuable information (Dearfield et al., 2010).

40. If clear negative results are obtained in both core *in vitro* tests undertaken, it can generally be concluded that the chemical has no genotoxic activity. However, there are some occasions when additional *in vitro* and/or *in vivo* genotoxicity testing may be undertaken for chemicals giving a negative response in the two *in vitro* core genotoxicity tests. For example, in situations where tumours are found in rodents, where the *in vitro* metabolic activation systems are not optimal or where there are human-specific metabolites, there may be a need for further genotoxicity assessment. A further testing strategy would have to be designed on a case-by-case basis (Kirkland et al., 2007b; Muller et al., 2003). An IWGT working group has published guidance on this topic (Kasper et al., 2007). An important part of any additional *in vitro* strategy should be consideration of the appropriate exogenous metabolic activation system (including alternative sources of S9 or other metabolic systems including genetically engineered cell lines) (Ku et al., 2007b), or even the testing of specific, relevant metabolites. Further information on *in vivo* genotoxicity testing of such test chemicals is provided in Stage 2 of this strategy.

41. Information from other combinations of genotoxicity tests, which may include one or more non-core tests outlined below in paragraphs 66-71, may also give adequate data on all three endpoints on a case-by-case basis. *In vitro* genotoxicity tests (micronucleus scoring and comet) using human reconstructed skin may provide useful information on *in vitro* mutagenic hazard in circumstances where *in vivo* testing is not permitted, or when extensive dermal exposure is anticipated (e.g. cosmetic

ingredients) (Aardema et al., 2013; Chapman et al., 2014; Roy et al., 2016; Reisinger et al., 2018).

42. The full Stage 1 strategy should be performed, and the results of studies evaluated before a decision is made on whether to proceed to Stage 2 testing or whether a conclusion on mutagenic hazard can be derived for test chemicals where no *in vivo* genotoxicity testing is permitted. An outline of Stage 0 and Stage 1 (*in vitro* genotoxicity testing) is given in Figure 2 and a description of the assays recommended is provided in the following paragraphs.

Discussion of Stage 1 Tests- General Aspects

43. The conduct of genotoxicity assays has improved over time and the overall sensitivity of *in vitro* testing strategies regarding prediction of rodent carcinogens is very high (Pfuhler et al., 2011; Corvi and Madia, 2017).

44. Kirkland et al. assessed the sensitivity of a combination of the Ames test and MNvit test to detect rodent carcinogens and *in vivo* genotoxicants (Kirkland et al., 2011). The authors stated that it is difficult to draw precise conclusions from the available sensitivity and specificity data since the databases of chemicals used vary. However, these data do show that mammalian cell genotoxicity tests can have low specificity and that combinations of *in vitro* genotoxicity tests result in high sensitivity for rodent carcinogens and *in vivo* genotoxicants. High sensitivity has always been a priority of genotoxicity testing strategies recommended by the COM (DOH, 2000, 2011). COM evaluated the use of *in vitro* genotoxicity tests to predict rodent carcinogens and *in vivo* genotoxicants in June 2010 and concluded **that there is no convincing evidence that any [relevant or DNA reactive] rodent carcinogen or *in vivo* genotoxicant would fail to be detected by using an *in vitro* genotoxicity test battery consisting of Ames and MNvit tests** (http://webarchive.nationalarchives.gov.uk/20140506144308/http://www.iacom.org.uk/meetings/index.htm).

45. It is most likely that one of the few occasions where *in vitro* test strategies fail to detect mutagenic activity (i.e. misleading negative results) could be explained by the absence of appropriate metabolic activity *in vitro* (Brambilla and Martelli, 2004) or that the test chemical does not reach the cells. Approaches to resolving potential inadequacies in metabolic activation include structure based metabolism predictions, use of genetically modified target organisms (e.g. CYP2E1 in *Salmonella* YG7108pin3ERb$_5$) (Emmert et al., 2006), the use of exogenous metabolic activation

systems derived from human sources, or recombinant human cytochrome P450 systems as an external activation system (Ku et al., 2007b). Testing of isolated or synthesised metabolites may also be considered.

46. There are a number of MoAs by which a chemical may demonstrate an *in vitro* genotoxic effect that is either not relevant for humans (e.g., a rat specific metabolite) or has a threshold. The COM has reviewed the evidence for a number of threshold MoAs and published a general guidance statement in 2010 (https://www.gov.uk/government/publications/assessment-of-thresholds-for-in-vitro-mutagens)

47. Threshold MoAs can generally be attributable to non-DNA interactions or an overload of normal cellular physiology. In such cases a No Observed Effect Concentration (NOEC) can be determined and may be useful in evaluating risk. Investigations of a threshold-based MoA need to be designed on a case-by-case basis and can be complex to interpret (Kirkland et al., 2007a).

48. There has been considerable debate regarding the highest concentration that should be used routinely in mammalian cell assays. The ICH has stated the maximum concentration tested for human pharmaceuticals should be 1 mM (or 500 µg/mL; whichever is lower) in mammalian cell genotoxicity assays when not limited by solubility in solvent or culture medium or by cytotoxicity. (ICH, 2012) This would have the effect of reducing the number of misleading positive results by avoiding the excessive concentrations where the cellular defence mechanisms might be overwhelmed (ICH, 2012). However, a reduction to 1 mM is not consistent with the OECD recommendation for a top concentration of 10 mM (or 2000 µg/mL; whichever is lowest) in mammalian cell genotoxicity assays, when not limited by solubility in solvent or culture medium or by cytotoxicity (OECD, 2016a,c,d,e). Morita et al. (2014) showed that the reduction in the top concentration from 5000 to 2000 µg/mL for mammalian cell tests had no impact on sensitivity or specificity of *in vitro* chromosomal aberration tests. Another analysis of published data for the top concentration in mammalian cell genotoxicity tests identified a small number of carcinogens that (according to the publications) would not be detected in any part of a three test *in vitro* genotoxicity test battery (consisting of the Ames, mouse lymphoma and *in vitro* chromosomal aberration (CA) tests) if the testing concentration limit for mammalian cell assays were reduced from 10 mM to 1 mM (Parry et al., 2010). A further investigation of these carcinogens found that some positive results at concentrations above 1 mM were not reproducible (i.e. they were not genotoxic in mammalian cells under current OECD guideline protocols) and others were positive at concentrations

below 1 mM, particularly when continuous treatments in the absence of S-9 (not included in the original publications) were conducted. An upper limit for mammalian cells tests of 1 mM or 500 µg/ml (whichever is lower) has been proposed as sufficient to detect all genotoxic carcinogens that are negative in the Ames test (Kirkland and Fowler, 2010). Several international organisations have updated their guidance regarding upper limit selection (e.g. ICH, 2012, OECD, 2020a; Galloway et al., 2011) although currently no international consensus has been reached. Precipitation of the chemical in the medium can also be used to define a maximal concentration or upper limit for testing. On balance, COM agreed that care should be taken to follow the appropriate guidance, depending on the chemical of interest.

49. There has also been considerable investigation of the role of excessive cytotoxicity in mammalian cells and choice of cell type as possible causes of misleading positive results (Blakey et al., 2008; Fellows et al., 2008b; Pfuhler, 2009; Pfuhler et al., 2011). The method used to assess cytotoxicity may affect the selection of the highest concentration tested and potentially the results obtained using mammalian cell genotoxicity assays (Fowler et al., 2012b; Kirkland et al., 2007c; Kirkland, 2012a) and recommendations have been made to use cytotoxicity measures based on cell proliferation (Galloway, 2000). However, it is important to note that although excessive cytotoxicity may lead to misleading positive results, it may also result in misleading negative results when pronounced cell cycle delay occurs. A similar conclusion was reached at an international symposium on regulatory aspects of genotoxicity testing (Blakey et al., 2008).

50. Most cell lines used for genotoxicity testing lack appropriate metabolism leading to reliance on exogenous metabolic activation systems. These cell lines may often have impaired p53 function and altered DNA repair capacity (Kirkland et al., 2007c). There is some evidence that human lymphocytes are less susceptible to misleading positives than the rodent cell lines currently used (e.g. Chinese Hamster Ovary (CHO), V79, Chinese hamster lung (CHL)). The use of human cell lines HepG2, TK6 and MCL5 cells and the reconstructed human skin models and HepaRG have been evaluated (Fowler et al., 2012a; Kirkland et al., 2007c; Le Hegarat, 2010). A brief summary of 3D models currently used for genotoxicity testing and those under development and/or validation has been prepared by COM (COM, 202x).

51. The COM agrees that it is not necessary to undertake independent confirmatory *in vitro* tests when clear negative or positive results have been obtained provided the following criteria are satisfied:

- there is no doubt as to the quality of the study design and the conduct of the test;
- the spacing and range of test chemical concentrations rule out missing a positive response; and
- sufficient treatment conditions and sampling times have been used.

52. It is recognised that it can be difficult to provide convincing evidence for absence of genotoxic effects. The investigator should consider the power of the study design and the past performance of the test system when formulating a protocol in order to optimise the chances of obtaining an unequivocal result from a single experiment and to ensure that any potential genotoxic effect is not missed.

53. There is a need to undertake further *in vitro* genotoxicity testing [DK3][RB4][OS5][RB6]when an equivocal result is obtained (i.e., neither clearly negative nor clearly positive by appropriate biological or statistical criteria). In the case of the MNvit and CAvit assays an equivocal result may be resolved by scoring more cells from the existing study (paragraph 86) and this should be assessed in the first instance. Additional genotoxicity tests need to be planned on a case-by-case basis and need not necessarily be undertaken in an identical fashion to the initial experiment(s). Indeed, it may be preferable to alter certain aspects of the study (e.g. concentration levels investigated, treatment and sampling times, concentration of metabolic activation mix) to obtain supplementary data. It may also be appropriate to use a different genotoxicity test system, e.g. a chromosomal aberration (CA) test, if there is equivocal evidence of clastogenicity from an *in vitro* micronucleus test, or an *in vitro* cell mutation assay (e.g. TK or HPRT mutation assays) if there is equivocal evidence of gene mutations from an Ames test.

54. The use of historical negative control data to aid in the interpretation of genotoxicity test results has been considered particularly in relation to equivocal and small magnitude genotoxic effects (Hayashi et al., 2011). Advice has been published on approaches to collecting historical control data. Ideally data should be reported in terms of means and confidence intervals for the distribution of baseline genotoxic effects rather than observed ranges where outliers can have a disproportionate effect. The dataset should be updated regularly and should be as large as possible. In addition, it is important that negative historical control data should have been generated using consistent methodology unless it can be demonstrated that changes in protocol do not impact on the range of values reported in studies (Hayashi et al., 2011). In their most recent guidance OECD places an increased emphasis on the use

of historical concurrent negative control data in the assessment of genotoxicity test results, including recommendations on how to build an historical control database (OECD, 2020a).

55. If a chemical is considered on the basis of Stage 1 genotoxicity test results to have *in vitro* genotoxic potential but has not been tested *in vivo,* the COM considers it prudent to assume that the chemical may have *in vivo* genotoxic potential.

Discussion of Stage 1 Strategy: Specific Core Tests

In Vitro Bacterial Tests for Gene Mutations

56. The most widely used *in vitro* mutagenicity test is the bacterial reverse mutation assay for gene mutations developed by Ames and his colleagues using *Salmonella typhimurium* (Gatehouse et al., 1994) which forms the basis of OECD TG471 (Bacterial Reverse Mutation Test). The very extensive database available for this assay justifies its inclusion in any initial genotoxicity testing for mutagenic hazard. Several strains of bacteria capable of detecting both base-pair and frame-shift mutations must be included, the validated strains being TA1535, TA1537 (or TA97 or TA97a), TA98 and TA100. These strains detect effects at G-C-rich sites. To detect certain oxidising mutagens or hydrazines, that produce effects at A-T-rich sites, an additional strain such as TA102 or a repair-deficient *Escherichia coli* strain (WP2*uvrA* or WP2*uvrA* (pKM101)) should be included. To detect cross-linking agents, it may be preferable to include TA102 or to add a repair proficient *Escherichia coli* strain (WP2 or WP2 (pKM101)). Testing should be carried out both in the presence and absence of an appropriate exogenous metabolic activation system such as induced rat liver S-9. Both plate-incorporation and pre-incubation methods are widely used, and either is acceptable in all test guidelines. There is ongoing consideration of the bacterial strains used. For example, the sensitivity and selectivity of the bacterial strains specified in OECD TG471 have been assessed (Williams et al., 2019) and the current criteria for a valid Ames test and interpretation of test results have been evaluated (Levy et al., 2019).

57. Developments to the Ames test have been suggested to automate and minimise the amount of test chemical required; for example the Spiral *Salmonella* mutagenicity assay (Claxton et al., 2001)[RB7], Ames II™ test (Fluckigetr-Isler et al., 2004) and Ames MPF (Fluckigetr-Isler and Kamber, 2012; Spiliotopoulos and Koelbert, 2020). [RB8] Whilst discussions at the OECD around assay developments are ongoing[RB9], the Committee considers that these methods have not currently been developed to a point where they can be routinely used for regulatory submissions.

In Vitro Mammalian Cell Micronucleus Assay (MNvit) for Clastogenicity and Aneuploidy

58. The COM recommends that equivalent information on clastogenicity could be obtained from the MNvit compared with CA testing in mammalian cells (metaphase analysis) but that aneuploidy could be more easily detected by MNvit. There have been extensive and authoritative investigations of the utility of the MNvit which have concluded that the MNvit is reliable and can be used as an alternative to the *in vitro* CA for the assessment of clastogenicity and has the benefit of more easily detecting aneuploidy (Corvi et al., 2008). The MNvit is available as OECD TG 487 (In Vitro Mammalian Cell Micronucleus Test) (OECD, 2016a).

59. The MNvit can be carried out in the absence or presence of cytochalasin B, which is used to block cytoplasmic division and generate binucleate cells (cytokinesis block methodology (CBMN)). The advantage of using cytochalasin B is that it allows clear identification that treated and control cells have divided *in vitro* during or after treatment and provides a simple assessment of cell proliferation. Moreover, a defined population of binucleate cells is available for scoring. In general, the use of cytochalasin B has no impact on the sensitivity of the test results (Garriott et al., 2002; Lorge et al., 2006; Oliver et al., 2006; Wakata et al., 2006), however this is not the case for nanoparticles (COM, 202x). In the absence of cytochalasin B, where all cells will be mononucleate, it is essential to have evidence that cells have divided.

60. MNvit protocol development and assay performance have been previously described (COM, 2011; Fowler et al., 2012a, b). A flow cytometric approach to the micronucleus assay has also been developed (Bryce et al., 2013). MNvit assay can be performed using most mammalian cell lines used in genotoxicity testing however there is evidence that rodent cell lines with compromised p53 activity such as V79, CHO and CHL cells can give more misleading positive results than cell lines proficient for p53 activity such as TK6 and human lymphocytes (Fowler et al., 2012a). Overall, the COM's preference is for human lymphocytes which have a number of advantages over cell lines (e.g. normal diploid primary human cells with some protection against oxidative damage when whole blood cultures are used). If cell lines are used, it is important that the cells have defined provenance (Lorge et al., 2016) and that the impact of potential genetic drift of the cells cultured is understood (Kirkland et al., 2007c). One particular area of protocol development that has been under considerable investigation is the most appropriate method(s) for estimating cytotoxicity. It has been suggested that using relative cell counts (RCC) may underestimate cytotoxicity, as proliferation is not measured, and lead to potentially misleading positive results (Fowler

et al., 2012b). In addition, it should also be recognised that cytotoxicity may be underestimated when using vital stains as these also do not measure proliferation. [DK10][RB11][OS12][RB13]In the absence of cytokinesis block, the relative increase in cell count (RICC) or relative population doubling (RPD) are comparable with replication index (RI) used with the cytokinesis block assay and are the most appropriate methods of cytotoxicity estimation. Consensus recommendations embedded in the OECD guideline 487 indicate that the target range for cytotoxicity in the MNvit is 55 ± 5%.

61. The MNvit assay in combination with the CB methodology and with pancentromeric or chromosome specific centromeric probes fluorescence *in situ* hybridisation (FISH) provides a sensitive assessment of cell proliferation and allows discrimination between chromosome breaks, chromosome loss (using pan-centromeric or anti-kinetochore antibodies) and chromosome non-disjunction and polyploidy (using chromosome-specific centromere probes) (Kirsch-Volders et al., 2002). It is therefore a useful model for assessing mode of action (Parry, 2006).

62. Binucleate cells obtained with the CBMN will usually be needed for determination of non-disjunction of chromosomes between daughter nuclei. Fenech has proposed that the CBMN assay can be further modified to provide comprehensive information on nucleoplasmic bridges (NPBs). This may provide information on chromosome rearrangements or telomere end fusions, and nuclear buds (NBUDs) which may provide information on gene amplification (Fenech, 2006, 2007). Fenech proposed that the comprehensive CBMN assay should be considered as a 'cytome' method for measuring chromosomal instability and altered cellular viability (Fenech, 2006). The 'cytome' method is complex and requires large amounts of blood and considerable technical skill. It is currently not suitable for routine testing of chemicals for genotoxicity but may provide useful information on MoA.

63. The flow-cytometry-based micronucleus assay (FCMMN) was developed to increase reproducibility and decrease turnaround time for the micronucleus test (Laingam et al., 2008; Avlasevich et al., 2011). However, the modified assay did not overcome the potential issue of misleading positive results. A number of approaches were undertaken to overcome this and have been previously described (COM, 2011). A separate approach to automation of the CBMN assay involves automated image analysis (Decordier et al., 2009; Avlasevich et al., 2011; Seager et al, 2014, Chapman et al, 2014, Lyulko et al 2014; Thougaard et al., 2014; Buick et al., 2020). This does provide some advantages over the FCMMN assay as the cells are not destroyed in the analysis and it can be applied to the cytokinesis blocked micronucleus assay. Thus, micronuclei can be scored in binucleated cells, cells containing multiple micronuclei

can be easily identified and scored as a single event, and the image galleries and slides can be stored, allowing the experiment to be re-visited at a later date.

64. An interlaboratory evaluation of the MultiFlow DNA Damage kit— p53, gamma H2AX, Phospho-Histone H3 and polyploidy has been described by Bryce et al. (2017). This is a multiplexed *in vitro* genotoxicity assay based on flow cytometric analysis in which detergent-liberated nuclei are simultaneously stained with propidium iodide and labelled with fluorescent antibodies against p53, gH2AX, and phospho-histone H3. Polyploidy can be quantified as the proportion of 8n-positive events relative to the number of total events with 2n and greater DNA content.

65. From seven laboratories assessing chemicals representing clastogens, aneugens and non-genotoxicants, with analysis based on global evaluation factors and using a multinomial logistic regression, assay sensitivity, specificity and concordance in relation to a priori MoA grouping were 92%. The authors suggest that the two distinct analysis strategies utilised can be used to rapidly and reliably predict a genotoxic MoA for new chemicals.

Discussion Stage 1: Non-Core Tests

In Vitro Chromosomal Aberration Assay in Mammalian Cells (Metaphase Analysis) for Clastogenicity and Aneuploidy

66. The *in vitro* CA assay in mammalian cells has been widely used in genotoxicity testing for many decades and provides information on chromatid and chromosome breaks, deletions and re-arrangements that are indicative of damage associated with adverse health outcomes. Only limited information can be obtained on potential aneugenicity by recording the incidence of polyploidy and/or modification of mitotic index (Aardema et al., 1998). The COM notes that polyploidy may not be a reliable indicator for aneugenicity and may result from a number of different genetic changes (Galloway, 2000; Mitchell et al., 1995). It is possible to adapt the chromosome aberration assay to include the use of chromosome specific centromeric probes with fluorescence *in situ* hybridisation (FISH) to assess the potential for aneuploidy (Maierhofer et al., 2002). An IWGT report (Galloway , 2000) concluded that the preferred measure of cytotoxicity in the CA test should be one based on cell proliferation (e.g. relative population doubling or relative increase in cell counts) compared to negative control cultures rather than simple cell counts. On balance it is considered preferable to use the *in vitro* micronucleus test for the initial assessment of clastogenic and aneugenic potential. The latest revision of the OECD test guideline

(OECD, 2016c) utilises a maximum test concentration corresponding to 10 mM (or 2 mg/mL) which is in-line with the revised MNvit assay (OECD, 2016a).

In Vitro Mouse Lymphoma Assay for Gene Mutation and Clastogenicity

67. The COM reaffirms the view stated in the 1989, 2000 and 2011 guidance, that the most appropriate *in vitro* mammalian cell gene mutation test is the mouse lymphoma assay. Protocol development and test data interpretation strategies were discussed previously (COM, 2011).

68. A re-evaluation of published studies, many of which were undertaken by the US NTP, showed that a large number of these were uninterpretable or the outcomes equivocal (Schisler et al., 2018). This assay is now described in a separate OECD TG (Test 490: In Vitro Mammalian Cell Gene Mutation Test Using the Thymidine Kinase Gene) which was published in 2016 (OECD, 2016d). Some authors have reported that the mouse lymphoma assay can detect, in addition to gene mutations and clastogenicity, information on recombination, deletion and aneuploidy (Ogawa et al., 2009; Sofuni, 1996; Wang et al., 2009). However, this has been contested from results showing that none of 7 reference aneugens were reliably detected at acceptable levels of cytotoxicity (Fellows et al., 2011b). It is possible that aneuploidy in these cells could be a secondary effect of chromosomal rearrangement. However, the COM considers that this assay does not reliably detect aneugens.

In Vitro HPRT assays for Gene Mutation

69. An *in vitro* cell mutation assay which uses forward mutation in the hypoxanthine guanine phosphoribosyl transferase (HPRT) gene to assess mutations has been developed in several cell lines, principally CHO cells and is described in the revised OECD 476 guideline (OECD, 2016e). TG476 recommends that the minimum number of cells required for the assay should allow for at least 10 spontaneous mutants being present in all phases of the test. The COM have previously considered the power of this assay and it was concluded that 10^7 surviving cells are required for a valid test, (http://webarchive.nationalarchives.gov.uk/20140506144831/http://www.iacom.org.uk/meetings/02.10.2003.htm), providing sufficient numbers of cells to maintain between 10 and 100 spontaneous mutations.

70. As discussed in para 43, a number of research groups have developed genotoxicity assays based on MN measurement using commercial sources of human reconstructed skin (such as Episkin® and EpiDerm™) (Chapman et al., 2014; Curren et al., 2006; Flamand et al., 2006; Hu et al., 2009; Mun et al., 2009; Roy et al., 2016; Pfuhler 2020a,b) or a co-culture technique involving reconstructed skin and mouse

lymphoma L5178Y cells (Flamand et al., 2006). Measurement of DNA damage using the comet assay in reconstructed skin has also been reported (Pfuhler et al., 2011; Reisinger et al., 2018; Pfuhler et al., 2020b) and is considered to be sufficiently validated to start the OECD Test Guideline development process (Pfuhler et al., 2020b). The primary purpose in developing genotoxicity tests using reconstructed skin has been to supplement genotoxicity data-packages for cosmetic chemicals where no *in vivo* genotoxicity tests are permitted.

In Vitro Alkaline Comet Assay for DNA Damage

71. The *in vitro* alkaline comet assay for DNA damage has been proposed as an alternative to clastogenicity assessment in mammalian cells since cell proliferation is not needed, therefore any cell type can be used (Hartmann et al., 2001; Witt et al., 2007). The alkaline comet assay detects a wide range of genetic damage including single and double strand breaks, repair induced breaks, alkali labile lesions and abasic sites. There is evidence that the *in vitro* comet assay can be modified to detect DNA cross-linking agents (Spanswick et al., 2010). The comet-FISH assay has been developed to provide information on site specific DNA strand breaks (Glei et al., 2009; Rapp et al., 2000; Santos et al., 1997). There is evidence that the *in vivo* comet assay can detect chemicals that induce gene mutations *in vitro* and *in vivo* (Dertinger et al., 2010; Kirkland and Speit, 2008; Kirkland et al. 2019a, b). Extrapolation from this suggests that the *in vitro* comet assay can also detect chemicals that induce gene mutations and this capability has been demonstrated (Dertinger et al., 2010). However, it is not recommended as a routine replacement for gene mutation tests *in vitro*. Thus, the comet assay measures DNA damage irrespective of genotoxic endpoint, with the exception of aneuploidy. A positive comet assay result may be due to repairable DNA damage or lesions which lead to cell death and not necessarily lead to mutations or MN. Negative results from an Ames test and MNvit would reduce the level of concern associated with positive results from an *in vitro* comet assay. Thus, the *in vitro* comet assay can serve as a useful adjunct to the recommended core-tests, especially in instances where *in vivo* testing is not permitted such as in cosmetics testing. Pfuhler et al. (2020b) has reviewed the status of the development of the 3D organ-based models for genotoxicity testing. The authors concluded that the 3D skin comet assay was sufficiently validated to start the process of OECD Test Guideline development.

Summary Stage 1 (*In Vitro* Genotoxicity Testing)

72. The COM recommendations for Stage 1 testing remain the same as in the 2011 guidelines, namely that the three key endpoints of gene mutation, clastogenicity and

aneuploidy can be detected by using two core *in vitro* tests. These should be undertaken according to the best international guidance available to avoid misleading positive or negative results. Data should be interpreted using appropriate statistical analysis and use of historical negative control data. It is important to note that the *in vitro* tests should be undertaken prior to any *in vivo* testing.

73. The COM confirms the need to understand MoA in order to derive conclusions regarding the biological importance of results. Data on MoA are important in elucidating whether genotoxicity tests give misleading negative or positive results, and also understanding of the MoA can help decisions with regard to devising a strategy for Stage 2 *in vivo* genotoxicity testing. There is a particular need to understand MoA for chemicals which cannot be subjected to *in vivo* genotoxicity tests (e.g. cosmetics). In this particular instance, some useful additional information on genotoxicity may be provided by undertaking further testing, for example using *in vitro* mammalian cell gene mutation assays or *in vitro* MN and comet tests using reconstructed human skin.

74. The recommended two core genotoxicity tests in Stage 1 are the Ames test and MNvit. These recommended assays, when combined, provide sufficient information for the genotoxicity assessment of most chemicals and provide high sensitivity for the identification of rodent carcinogens and *in vivo* genotoxicants, and reduce the risk of misleading positive results when compared with a battery containing more than one mammalian cell test.

75. Results from non-core tests described in this document may provide useful additional information on *in vitro* mutagenic hazards on a case-by-case basis. In most instances misleading negative *in vitro* results are due to inadequate exogenous metabolic activation (Ku et al., 2007b).

76. Some regulatory authorities may require an *in vivo* genotoxicity test where high, or moderate and prolonged, levels of exposure are expected (e.g. most human medicines) in order to provide additional reassurance even when Stage 1 tests have given negative results. If a chemical is considered on the basis of Stage 1 test results to have *in vitro* mutagenic potential but has not been tested *in vivo,* the COM considers it prudent to assume that the chemical may have *in vivo* mutagenic potential.

Stage 2: *In Vivo* Genotoxicity Tests

Overview of Strategy

Stage 2 of the testing strategy involves an assessment of genotoxic activity *in vivo* in somatic tissues and in germ cells (when there is a need for the assessment of heritable effects and/or information on hazard classification of mutagens) (see Figure 3). The *in*

vivo genotoxicity testing strategy has to be designed on a case-by-case basis and can be used to investigate aspects of *in vivo* mutagenicity, for example;

1. key end point(s) identified in Stage 1,
2. genotoxicity in tumour target tissue(s),
3. potential for germ cell genotoxicity,
4. *in vivo* genotoxic potential for chemicals which were negative in Stage 1 but where there is high or moderate and prolonged exposure.
5. genotoxicity [RB14]in site of contact tissues.

77. It is thus possible for there to be one or more separate Stage 2 strategies designed to assess the above objectives for a particular test chemical. A revised *in vivo* Stage 2 strategy was presented in the previous COM guidance document (COM, 2011) based on the selection of tests to provide information on one or more specific aspects such as species and/or tissue genotoxicity combined with investigation of particular genotoxic end points and modes of genotoxic action. This approach does not necessarily lead to the selection of the rodent BMMN test as the first assay. Furthermore, the rat liver UDS assay is no longer recommended as a second assay (EFSA, 2017 - discussed in para 104). A table of *in vivo* genotoxicity tests and endpoints is provided in Annex 1.

78. Other factors that should be considered when determining an *in vivo* genotoxicity testing strategy include whether the testing strategy can be integrated into other regulatory toxicity tests (such as subacute or subchronic toxicity studies). Consideration needs to be given to the nature of the chemical (including physico-chemical properties), the results obtained from *in vitro* genotoxicity tests and the available information on the toxicokinetic and metabolic profile of the chemical (for example when selecting most appropriate species, tissue and end point). The routes of exposure in animal studies should be appropriate to ensure that the chemical reaches the target tissue. Routes unlikely to give rise to significant absorption in the test animal should therefore be avoided. Unless systemic exposure can be confirmed from other toxicological studies, or evident toxicity in the target organ is seen, or the intravenous route is used, confirmatory toxicokinetic studies to measure blood or tissue exposure as appropriate should be undertaken to accompany all *in vivo* genotoxicity studies to assess the adequacy of any negative results obtained (Hardy et al., 2017).

79. The design of *in vivo* genotoxicity tests should incorporate appropriate approaches to reduce the number of animals used in tests, such as the integration of

genotoxicity endpoints into repeat-dose studies. Options for reduction in animal usage include:

- use of one sex only (if supported by metabolism data or other data indicating equivalence),
- reduced numbers of sampling times for micronucleus and CA assays when repeat dosing is performed,
- combining micronucleus and comet assays into a single acute test employing repeated administrations of test chemical; integration of micronucleus and comet end points into repeat-dose toxicity (including transgenic mutation) studies, although it should be noted that the comet assay is difficult to integrate without using satellite groups (Bowen et al., 2010; Bowen and Beevers, 2011; Pfuhler et al., 2009; Vasquez, 2010).

80. It should also be possible to omit the concurrent positive control administrations in micronucleus, CA and transgenic rodent mutation assays (but not for the comet assay) where the test facility has appropriate historical positive control data (Pfuhler et al., 2009) as long as positive control slides or tissues from positive control treated rodents "banked" from previous treatments and coded in with the experimental samples, are included to demonstrate technical proficiency.

81. The toxic properties of test chemicals (such as acute toxicity, subchronic toxicity (including target organ effects), irritancy/corrosivity in contact with skin or mucous membranes), toxicokinetic and metabolism data will influence the choice of route of administration and the highest dose level achievable in *in vivo* mutagenicity tests. Dose selection for *in vivo* genotoxicity testing [DK15][RB16][OS17][RB18]requires estimation of the limit dose (LD), maximum tolerated dose (MTD), consideration of tissue-specific effects and in some instances (as discussed in paragraph 78), appropriate toxicokinetic data or toxicity data in the target tissue from other studies, to support tissue exposure to the chemicals and/or metabolites (EFSA, 2017). OECD recommend the use of the LD in circumstances where "toxicity and solubility are not limiting factors, and if genetic toxicity is not expected based on data from structurally related substances". A LD of 2000 mg/kg bw/day for a treatment period of < 14 days and 1000 mg/kg bw/day for a treatment period > 14 days are stated. In circumstances where toxicity is the limiting factor, OECD recommend use of the which is defined by OECD as "the highest dose that will be tolerated without evidence of study-limiting toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality or evidence of pain, suffering or distress necessitating

humane euthanasia" (OECD, 2000). It is possible that for some chemicals, the maximum dose may not be achievable (e.g., due to solubility issues) and, in this case, the maximum feasible dose (MFD) may be applied.

82. The approach outlined for Stage 2 in Figure 3 takes account of evidence to suggest that *in vivo* comet and rodent transgenic mutation assays have better sensitivity and specificity for the identification of rodent carcinogens compared with the rat liver UDS test, particularly for carcinogens that are negative in the *in vivo* micronucleus test (Kirkland and Speit, 2008). The initial *in vivo* genotoxicity testing strategy should therefore involve selection of one or more of the core Stage 2 tests in rodents; namely, micronucleus tests (accompanied by specific modifications for aneuploidy if necessary), the transgenic gene mutation tests, or comet DNA damage assays in rodents. It is acceptable to undertake one *in vivo* genotoxicity test to investigate a specific end point identified from Stage 1 *in vitro* genotoxicity tests. In some instances, there may be a need to investigate more than one end point before reaching a full conclusion on *in vivo* genotoxic potential.

83. Stage 2 *in vivo* genotoxicity tests should be undertaken for test chemicals that are positive in any of the *in vitro* Stage 1 genotoxicity tests where there is a need to ascertain whether genotoxic activity can be expressed *in vivo*. There are many reasons why activity shown *in vitro* may not be observed *in vivo* (for example, lack of absorption, inability of the active metabolite to reach DNA, rapid detoxication and elimination). Data from *in vivo* genotoxicity tests are, therefore, essential before any definite conclusions can be drawn regarding the potential mutagenic or genotoxic hazard to humans from test chemicals which have given positive results in one or more *in vitro* genotoxicity tests. However, conclusions on mutagenic or genotoxic hazard and MoA may have to be derived from *in vitro* genotoxicity data for test chemicals when no *in vivo* genotoxicity testing is permitted.

84. In addition, an *in vivo* genotoxicity test may give positive results for chemicals which only act *in vivo*; experience though, has shown that such chemicals are rare (Tweats, 2007a, b). Such agents include some kinase inhibitors, glucocorticoid receptor antagonists (Hayes et al., 2013) and long-acting beta-2-agonists (Ponten et al., 2013). In some instances positive results might be obtained from *in vitro* genotoxicity tests that are adapted to evaluate specific characteristics of the test chemical; for example, by the use of modified or non-standard exogenous metabolising fractions (Muller et al., 2003).

85. Positive results in any Stage 2 genotoxicity test should be assessed for an indication of a MoA and for evidence which may suggest a threshold of effect or irrelevant positive responses. The COM has previously discussed the relevance of high-dose only positives and recognises that these results may be secondary to non-genotoxic effects rather than being a genotoxic effect of the compound (http://webarchive.nationalarchives.gov.uk/20140506144902/http://www.iacom.org.uk/statements/COM03S5.htm).

86. Examples of MoAs that may lead to irrelevant positive responses in micronucleus tests, include hypothermia or hyperthermia in rodents and compound induced increases in cell division of bone marrow erythroblasts (Blakey et al., 2008; Shuey et al., 2007; Tweats et al., 2007a). If the conclusion is reached that a[RB19] relevant MoA occurs, then the chemical should be considered as an *in vivo* mutagen. MoA data will be important in considering whether a threshold or non-threshold approach to risk assessment can be used. The COM has published guidance on possible threshold modes of genotoxicity which can include; i) involvement of non-DNA targets, (e.g. aneugen inhibition of microtubules), ii) the contribution of protective mechanisms (e.g. repair of DNA adducts formed from many low molecular weight alkylating agents) and, iii) overload of detoxication pathways (e.g. paracetamol) (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/315698/assessment_of_threshold_for_in_vivo_mutagens.pdf).

87. Equivocal results may be resolved in some assays such as MNvit or CAvit by scoring more cells. . In the absence of equivocal results or if there is a need to investigate specific mutagenic endpoints, tumour target organs, or the potential for heritable effects, supplementary *in vivo* genotoxicity tests should be undertaken [DK20][RB21][OS22][RB23] (Figure 3) or. This may involve repeating all or aspects of the initial Stage 2 testing strategy, or performing supplementary investigations (e.g. mode of action investigations, such as DNA adducts or more specific germ cell testing) to investigate aspects of the genotoxicity of the test chemical which have not been resolved. There is a need to select the most appropriate test(s) on a case-by-case basis. All relevant factors, such as results from previous tests, and available information on toxicokinetics, toxicological effects and metabolism of the chemical, should be considered.

88. The development of testing strategies for germ cell mutagens is a rapidly evolving field. A summary of test methodologies that are currently under development and/or validation are outlined in the COM document ‘Test Strategies for Germ Cell Mutagens’ (COM, 202x).

89. One aspect of the approach to testing outlined in Figure 3 is that hazard characterisation of germ cell genotoxicity can be included in the initial *in vivo* genotoxicity testing strategy if considered necessary. This is because there are multi tissue *in vivo* genotoxicity assays (e.g. transgenic rodent mutation assays and comet assay, though it should be noted that the standard comet assay has not been validated using mature sperm) which can also be used if a need to evaluate germ cell genotoxicity has been established (COM, 202x). Additionally, germ cell mutation assays might be valuable on a case-by-case basis to provide information on heritable mutagenic effects, but these would form part of a supplementary *in vivo* genotoxicity testing strategy, if considered appropriate.

90. The COM reaffirms that a chemical considered a positive *in vivo* somatic cell mutagen should also be considered as a possible germ cell mutagen unless data can be provided to the contrary. The position held previously, that most if not all germ cell mutagens are also genotoxic in somatic cells, still holds true. It has been noted that some rare examples (e.g. sodium orthovanadate, (Attia et al., 2005) where the mouse bone marrow micronucleus assay does not predict germ cell genotoxicity have been reported. However, the data on such compounds are conflicting and it is not known, for example, whether somatic mutations or DNA strand breaks would have been identified if other test systems (e.g. transgenic assays and the comet assay) had been used and other tissues sampled (Attia et al., 2005; Ciranni et al., 1995; Witt et al., 2003).

91. It is plausible that other targets during the process of meiotic cell division may be unique to germ cells but not necessarily identical in both sexes (Pacchierotti et al., 2007). The COM evaluated advances in germ cell mutagenicity testing and some theories and hypotheses regarding human germ cell mutagenesis. It was concluded that it is not known whether unique germ cell mutagens exist (i.e., chemicals that are germ cell mutagens but not somatic cell mutagens), but that this is partially because of the underutilisation of the currently accepted tests for assessing germ cell mutagenicity and a lack of investigations examining this. Recommended regimes for the analysis of mutations in germ cells are discussed fully in the COM document 'Test Strategies for Germ Cell Mutagens' (COM, 202x).

Discussion of Stage 2 Initial Testing Strategy - General Aspects

92. There are many publications debating *in vivo* genotoxicity testing strategies. These include those developed by the GUM (German speaking section of the European Environmental Mutagen Society) which recommended a single study using

a combined analysis for MN and comet induction in selected tissues (Pfuhler et al., 2007), and those from the World Health [OS24][RB25]Organization/International Programme on Chemical Safety (WHO/IPCS) which recommended cytogenetics (bone marrow) or gene mutation or alternative tests as defined by genotoxic endpoint, chemical class and reactivity (with consideration of factors such as bioavailability and metabolism) (Eastmond et al., 2009). ICH (2011), EFSA (2017) and ECHA (2017) have also proposed similar strategies to these. The *in vivo* genotoxicity testing strategy recommended by the COM acknowledges there can be a variety of reasons for undertaking *in vivo* genotoxicity tests and it is important to identify clearly the objective of the study and the critical aspects of *in vivo* genotoxicity to be addressed (as set out in the Overview of Stage 2 strategy) in order to develop a strategy accordingly, rather than simply specify preferred first and second tests. There is less data on the performance of *in vivo* genotoxicity assays for prediction of rodent carcinogenicity compared with data on the performance of *in vitro* genotoxicity tests. Transgenic rodent mutation assays and the *in vivo* micronucleus assay have been shown to exhibit complementarity regarding prediction of rodent carcinogenicity, consistent with the assessment of different mutagenic endpoints by these two assays (Morita et al., 2016). The IWGT has reported that an evaluation of 91 chemicals showed that TGR and *in vivo* comet assays have a similar ability to detect *in vivo* genotoxicity when tested with bacterial mutagens and Ames-positive carcinogens (Kirkland et al., 2019b). Thus, genotoxic endpoint and MoA analysis of *in vitro* mutagenic activity is of considerable importance in helping to develop an initial *in vivo* genotoxicity testing strategy. The COM recommends that the initial *in vivo* genotoxicity testing strategy should be based on one or more tests selected from a relatively limited number of *in vivo* genotoxicity tests that have been specifically designed to provide the optimum amount of information on *in vivo* mutagenic potential of the test chemical. Where possible, consideration should be given to integrating in vivo genotoxicity testing into repeat-dose toxicity studies.

Discussion of Stage 2 - Recommended *In Vivo* Genotoxicity Tests

93. Three recommended *in vivo* genotoxicity tests are outlined below and in Figure 2. Information from one or more of these recommended core tests should provide sufficient *in vivo* genotoxicity data for most chemicals.

Rodent Bone Marrow and Peripheral Blood MN Assay for Clastogenicity and Aneuploidy
OR Rodent Bone Marrow CA Assay for Clastogenicity

1004 94. The *in vivo* bone marrow or blood micronucleus (MNviv) assay is still the most
1005 widely used *in vivo* genotoxicity test (OECD TG 474: Mammalian Erythrocyte
1006 Micronucleus Test) (OECD, 2016f). Most of the available *in vivo* data on the
1007 mutagenicity of chemicals have been obtained from studies using the MNviv test in
1008 bone marrow of mice. The bone marrow is readily accessible to chemicals that are
1009 present in the blood and a wide range of structurally diverse clastogens and aneugens
1010 has been detected using these methods. The use of peripheral blood is an alternative
1011 approach for both mice and rats (when the youngest fraction of reticulocytes are
1012 sampled) which provides equivalent data to the bone marrow assay and is technically
1013 less demanding. High throughput approaches to the peripheral blood MNviv utilising
1014 flow cytometry have been published (De Boeck et al., 2005; Torous et al., 2000;
1015 Dertinger et al., 2011a) and the assay is well validated. The MNviv assay detects
1016 clastogenicity by measuring MN formed from acentric chromosome fragments in young
1017 (polychromatic) erythrocytes in the bone marrow or in reticulocytes of peripheral blood.
1018 It may also be used to identify the induction of chromosome loss. MN containing whole
1019 chromosomes (as opposed to fragments) can be identified with molecular kinetochore
1020 or centromeric labelling techniques. It should be noted that only aneuploidy produced
1021 by chromosome loss can be measured in the MNviv assay. The MNviv can be used in
1022 the initial *in vivo* genotoxicity strategy for generic testing for *in vivo* genotoxic potential
1023 and for assessment of clastogenicity and aneuploidy. Clastogenicity may be measured
1024 by metaphase analysis of CA in bone marrow of rodents as an alternative approach to
1025 the use of the micronucleus assay.

1026 95. Proposals have been published to incorporate micronucleus assays into routine
1027 rodent 28 day subacute toxicity studies following demonstration of the feasibility of
1028 such an approach (Hamada et al., 2001; Krishna et al., 1998; Madrigal-Bujaidar et al.,
1029 2008). The evidence from one evaluation of micronucleus tests conducted on samples
1030 from short-term, subchronic and from a few chronic studies in mice has been published
1031 (Witt et al., 2000). In mice, MN in polychromatic erythrocytes represent DNA damage
1032 occurring in the last 72h, whilst MN in normochromatic erythrocytes represent average
1033 damage during the 30 day period prior to sampling (Witt et al., 2000).

1034 96. The development of a simultaneous liver and peripheral blood micronucleus
1035 assay in adult rats has also been reported (Suzuki et al., 2005). A correlation between
1036 micronucleus induction in hepatocytes and hepatocarcinogenicity was shown and the
1037 authors proposed that the assay could detect micronucleus-inducing chemicals that
1038 require metabolic activation. Takasawa et al. (2007), Suzuki et al., (2009) and Hamada
1039 et al. (2015) have also reported developments of an *in vivo* liver micronucleus assay,

1040 which has been discussed by IWGT (Uno et al., 2015b; Kirkland et al., 2019b), and it
1041 has been recommended that an OECD guideline should be developed.
1042 97. *Transgenic Rodent (TGR) Mutation Assay for Gene Mutations*
1043 The transgenic rodent somatic and germ cell gene mutation assays (OECD TG 488,
1044 OECD, 2020b) can be used to assess gene mutations in a wide range of rodent tissues
1045 (including germ cells) using all routes of administration and is particularly valuable
1046 when investigating gene mutation as the genotoxic endpoint (Kirkland et al., 2019a, b).
1047 There are sufficient data to support the use of the Muta™mouse, BigBlue® mouse and
1048 rat (including use of λ cII transgene), *LacZ* plasmid mouse, and the *gpt* delta models
1049 in TG 488.
1050 98. Molecular sequencing of induced mutations in transgenic targets can aid in
1051 interpretation of study results (particularly equivocal responses) and also provide
1052 mechanistic information. The OECD published a Detailed Review Paper (DRP) on
1053 Transgenic Rodent Gene Mutation Assays which led to the development of an OECD
1054 guideline that was adopted in July 2011, with revision in 2013 (OECD, 2013) and in
1055 2020 (OECD, 2020b). The latest version focuses on updating recommended regimes
1056 for the analysis of mutations in germ cells (discussed fully in the COM document 'Test
1057 Strategies for Germ Cell Mutagens' (COM, 202x)). TG488 states that "when both
1058 somatic and germ cells need to be collected and/or tested, based on regulatory
1059 requirements, or toxicological information, a 28+28d regimen [i.e., 28 days treatment
1060 with sampling 28 days following administration of the final dose] permits the testing of
1061 mutations in somatic tissues and tubule germ cells from the same animals" (Marchetti
1062 et al., 2018; Marchetti et al., 2019).

1063 *Rodent Alkaline Comet Assay for DNA Damage*[RB26][RB27]
1064 99. The *in vivo* comet assay (OECD TG 489: In Vivo Mammalian Alkaline Comet
1065 Assay) (OECD, 2016b) detects a wide spectrum of DNA damage including repairable
1066 DNA damage. A report of an international validation of the *in vivo* alkaline comet assay
1067 has been published (Uno et al., 2015a) and formed the basis for the OECD guideline.
1068 An overview of the types of genetic lesions detected is given above in paragraph 71.
1069 The *in vivo* comet assay can detect chemicals that induce gene mutations and has
1070 produced positive results for nearly 90% of rodent carcinogens not detected by the
1071 rodent BMMN assay (Kirkland and Speit, 2008). It also shows high sensitivity when
1072 compared with TGR results in liver and the GI tract, and high sensitivity at detecting
1073 bacterial mutagens and mutagenic carcinogens (Kirkland et al., 2019a, b).
1074 Developments regarding the conduct of the *in vivo* alkaline comet assay were detailed
1075 in the previous COM guidance (COM, 2011). This assay can be used for elucidating

positive *in vitro* genotoxicity findings and to evaluate genotoxicity in target organs of toxicity (Hartmann et al., 2004), however, it would not be an appropriate follow-up for a chemical causing aneuploidy *in vitro*. The comet assay can be applied to a wide range of species and in many tissues including site-of-contact tissues. In the absence of data indicating particular tissues of interest (e.g. toxic findings or tissue accumulation seen in other studies), the IWGT concluded that comet analysis of the liver combined with the bone marrow or peripheral blood micronucleus assay will be sufficient in most cases. However, if systemic exposure is expected, or found, to be low then site-of-contact-effects in GI tract are effective (Kirkland, 2019b). Validation of a protocol for a germ cell comet assay is ongoing.

100. The Committee considers that the *in vivo* comet assay has appropriate sensitivity to detect chemicals which induce both gene mutations and/or clastogenicity. Thus the *in vivo* comet assay is recommended as a core test in the initial *in vivo* genotoxicity testing strategy to assess DNA damage in multiple somatic tissues in a single study. It is possible to include the comet assay within other *in vivo* genotoxicity tests (Vasquez, 2010) or within standard subacute or subchronic regulatory toxicity tests (Rothfuss et al., 2010), although the logistics of achieving the correct sampling time in relation to the final doses must be carefully considered (Speit et al., 2015).

Non-Core In Vivo Test: Rat Liver UDS Assay for DNA Damage

101. The rodent liver UDS assay is an established approach for investigating genotoxic activity in the liver with the endpoint measured being indicative of DNA damage and subsequent repair in liver cells. The COM consideration of this assay and published evaluations now suggest it is less sensitive than the *in vivo* comet assay with regard to identification of genotoxicity in the liver. An analysis of the prediction of rodent carcinogens not identified by the micronucleus tests indicated that the comet assay was considerably better than the rat liver UDS assay at identifying rodent carcinogens (Kirkland and Speit, 2008; Speit et al., 2015). Based on these analyses, EFSA concluded that the UDS assay was of limited usefulness in genotoxicity testing strategies, being only suitable for the detection of chemicals causing damage in the liver, and with a lower predictive value than the TGR and comet assays in detecting chemicals which cause gene mutations. For existing datasets, where the UDS assay has been used as a follow up to positive *in vitro* gene mutation findings, a UDS study is considered adequate only for positive results (Hardy et al., 2017). The COM agree with this opinion and recommend use of the comet assay rather than rodent liver UDS in order to assess potential for DNA damage *in vivo*.

102. Another non-core test which is receiving increasing attention involves the detection of gene mutations at the endogenous phosphatidylinositol glycan complementation group A gene (*Pig-A*), a reporter gene in which mutations are currently detected in peripheral red blood cells of mammals (Bryce et al., 2008b; Dertinger et al., 2011; Miura et al., 2009). This assay has the potential advantage of being integrated into regulatory toxicity tests (Dertinger et al., 2010; Khanal et al., 2018) and it is noted that *Pig-A* mutations increase with duration of dosing (Miura et al., 2009). The development of the assay was discussed by the IGWT (Gollapudi et al., 2015) and it has since undergone validation in support of the development of an OECD TG (Dertinger et al., 2020; OECD 2020c; OECD 2020d).

Discussion of Stage 2-Supplementary Tests.

103. Supplementary *in vivo* genotoxicity tests need to be considered on a case-by-case basis taking into account all relevant information. It is considered that for most chemicals, supplementary *in vivo* genotoxicity data should be unnecessary but on a case-by-case basis, specific aspects of MoA (e.g. nature of DNA adducts) and further characterisation of germ cell genotoxicity (e.g. characterisation of male and/or female germ cell clastogenicity including use of FISH, and the evaluation of heritable effects) may be required. DNA adduct studies can provide valuable information on potential genotoxicity as a follow up for *in vitro* mutagens which have yielded negative results in *in vivo* genotoxicity assays (Phillips et al., 2000). DNA adduct data (including type of adduct, frequency, persistence, repair process) can be used to inform on MoA and its relationship to carcinogenesis, and should be considered in conjunction with other relevant data such as dosimetry, toxicity, genotoxicity and tumour data (Jarabek et al., 2009).

104. A brief outline of these additional Stage 2 methods is given in Table 1 below. Reference is also made in Table 1 to a number of tests for heritable genotoxic effects but it is noted that these tests, which involve the use of many animals and demand a high level of expertise, are comparatively rarely used. The COM is aware that there is the possibility that gender differences in germ cell mutagenesis may exist and this aspect may need to be considered on a case-by-case basis (Eichenlaub-Ritter et al., 2007). The conclusions of COM's evaluation of germ cell testing methods are provided in a separate document (COM, 202x).

1144 **Table 1 Supplementary in vivo genotoxicity tests**

Assay	Endpoint	Guidance	Main Attributes	Comments
Investigations of DNA Adducts				
^{32}P-postlabelling	DNA adducts	IWGT	Can be highly sensitive particularly with bulky adducts and if appropriate enrichment technique used.	Interpretation of results can be complex. Involves handling high-activity ^{32}P. (Phillips et al., 2020)
Covalent binding to DNA A variety of methods can be used such as those involving radioactive decay measurements (eg. ^{14}C-) or isotope measurements (eg Accelerator Mass Spectrometry AMS)	DNA Adducts	IWGT	Some methods (AMS) are potentially very sensitive and can provide data on DNA binding at levels of exposure similar to low level environmental exposures	Uses radiolabelled compound (very small amounts (e.g. nanograms) in the case of AMS). Interpretation of results can be complicated (e.g. by non-specific binding). (Himmelstein et al., 2009)
Supplementary investigations of germ cell mutagenicity				
Analysis for clastogenicity/ aneuploidy	Structural and numerical changes in spermatogonia, spermatocytes or oocytes	OECD	Can provide information on nature of effects in spermatogonia, spermatocytes and/or oocytes of mice or rats	Can provide useful information on MoA. (Russo, 2000)
Spermatid micronucleus assay	Chromosomal aberrations and or lagging chromosomes	None available	Provides information of clastogenic and/or aneugenic effects in spermatocytes.	(Allen et al., 2000)
Dominant lethal assay	Chromosomal/gene mutations	OECD	Provides information on unstable chromosomal changes in gametes that lead to fetal death after fertilization and can determine stage(s) of gametogenesis affected	Little used. needs relatively large numbers of animals (Adler et al., 1994)
Mouse specific locus test	Gene mutations	EPA	Provides information on genetic changes transmitted to the first generation progeny as basis for estimation of induced mutation frequency in humans	Very rarely used. Needs large numbers of animals (Adler, 2008)
Mouse heritable translocation test	Chromosomal changes	EPA	Provides information on chromosomal changes transmitted to the first generation progeny as basis for estimation of induced translocation frequency in humans	Very rarely used. Needs large numbers of animals (Adler, 2008)
Sperm Comet assay	Double strand breaks and/or apurinic sites in sperm head DNA	None available	Provides information on genetic instability in sperm	(Trivedi et al., 2010)
Spermatid UDS assay	Repair DNA synthesis in spermatocytes	EPA	Provides information on induction of DNA lesions	(Sotomajor and Sega, 2000)

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Summary Stage 2 (*In Vivo* Genotoxicity Testing)

105. The *in vivo* genotoxicity testing strategy has to be designed on a case-by-case basis and can be used to investigate aspects of *in vivo* mutagenicity, for example;

- key end point(s) identified in Stage 1,
- genotoxicity in tumour target tissue(s),
- potential for germ cell genotoxicity,
- *in vivo* genotoxic potential for chemicals which were negative in Stage 1 but where there is high or moderate and prolonged exposure.
- genotoxicity in site of contact tissues.

106. The recommended *in vivo* genotoxicity test(s) include micronucleus assay, bone marrow cytogenetics, alkaline comet assay in rodents and transgenic rodent mutation assay. In some instances there may be a need to undertake more than one *in vivo* test to perform an initial assessment of *in vivo* genotoxic potential (e.g. where endpoints cannot be assessed in one study and there is a need to investigate multiple endpoints before reaching conclusions on *in vivo* mutagenic potential). Multiple endpoints may be combined in a single study. If positive results are obtained it is important to consider the evidence for MoA and check the data for evidence of irrelevant positive results. Usually negative results obtained in a carefully selected *in vivo* test (possibly studying more than one endpoint and tissue) will be sufficient to address positive results found *in vitro,* provided that target tissue exposure is sufficient. However, a further test(s) may be needed if some of the genotoxic effects seen in Stage 1 *in vitro* tests have not been adequately studied *in vivo* (e.g., the chemical affects multiple mutagenic endpoints), or other aspects of the genotoxic potential of the chemical had not been fully resolved (e.g. in the case where an investigation of heritable effects was required). If equivocal results are obtained, then supplementary testing (including scoring of additional cells in the case of the comet and MN assays) may be needed. This may involve repeating some aspects of the recommended *in vitro* and/or *in vivo* genotoxicity tests or performing additional investigations (e.g. MoA investigations, such as DNA adducts and/or more detailed consideration of heritable effects). The supplementary *in vivo* genotoxicity testing strategy and selection of the most appropriate assays should be undertaken on a case-by-case basis. [RB28]All relevant factors such as results from previous tests, structural alerts and available information on toxicokinetics, tissue toxicity and metabolism of the chemical, should be considered. In the absence of appropriate germ cell genotoxicity data, the COM

considers it is reasonable to assume that all somatic cell mutagens have the potential to be germ cell mutagens.

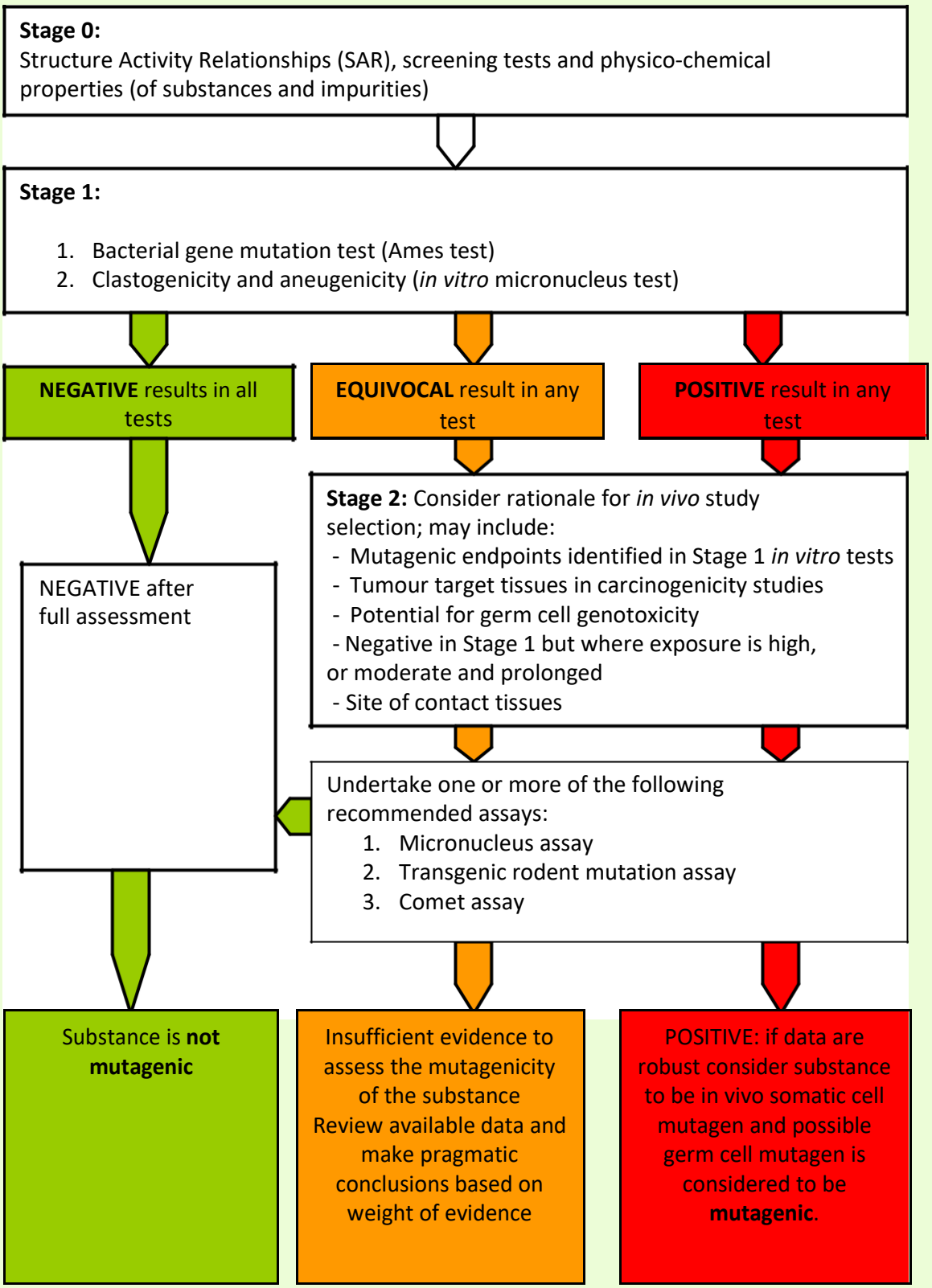
Possible Future Developments

107. The COM is aware that new assays and toxicogenomic approaches are under development which might be of value within genotoxicity testing. The ToxTracker assay uses a series of reporter cell lines expressing biomarker genes selected to detect chemically induced DNA damage and oxidative stress (Hendriks et al., 2012; Hendriks et al., 2011; Brandsma et al., 2020). Whilst the assay presents an interesting approach to identifying MoA, it is not currently considered to be a reliable genotoxicity test and is more suitable as a biomarker assay or in MoA investigations.

108. Other potential tests include investigation of instability in expanded simple tandem repeats in male gametes and offspring to evaluate heritable mutations (Singer et al., 2006). The development of new high throughput assays for the assessment of germ line mutations and the quantification of risk from such data may provide opportunities to protect future generations from mutated DNA sequences. Developments within the field of toxicogenomics are also likely to provide new methods for investigating genotoxic mechanisms and informing on MoA. The COM have reviewed data generated in this field several times [RB29]up to the drafting of this guidance statement but currently conclude that the evidence does not support the routine use of toxicogenomic approaches as an adjunct to genotoxicity testing.

109. HESI-GTTC has considered 'next generation' testing strategies for genotoxicity including the use of QSAR modelling, MoA assessments and their human relevance. The concept of quantitative assessment of genotoxicity data was also discussed (Gollapudi et al., 2013, 2014; Johnson et al., 2014; Dearfield et al., 2017). Quantitative approaches to the assessment of genotoxicity data was considered by COM in 2017-2018. Their conclusions were published in a statement (https://www.gov.uk/government/publications/quantitative-approaches-to-the-assessment-of-genotoxicity-data). IWGT have also published guidance on quantitative approaches to genotoxicity risk assessment (MacGregor et al., 2015a, b).

Figure 1: Overview of Strategy for testing chemical substances for genotoxicity



[DK30][RB31][RB32][RB33]

Figure 2: Screening (Stage 0) and *in vitro* tests (Stage 1)

Figure 3: *In vivo* tests (Stage 2)[RB34]

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[RB36]**Specific locus test:** A technique used to detect recessive induced mutations in diploid organisms; a strain that carries several known recessive mutants in a homozygous condition is crossed with a non mutant strain that has been treated to induce mutations in its germ cells; induced recessive mutations allelic with those of the test strain will be expressed in the progeny.

Spindle apparatus: In cell biology, the spindle apparatus is the structure that separates the chromosomes into the daughter cells during cell division. It is part of the cytoskeleton in eukaryotic cells. It is also referred to as the mitotic spindle during mitosis and the meiotic spindle during meiosis.

Structure Activity Relationships: the relationship between chemical structure and genotoxic effect based on predictions using computerised models (also Quantitative Structure Activity Relationships)

Test chemical: A chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the chemical or changing its composition.

Threshold: Dose or exposure concentration below which an effect is not expected[DPL37].

Topoisomerases: Enzymes which catalyze and guide the unknotting[DPL38][DK39] of DNA by creating transient breaks in the DNA using a conserved tyrosine as the catalytic residue. In so-called circular DNA, in which double helical DNA is bent around and joined in a circle, the two strands are topologically linked, or knotted. Topoisomerase I solves the problem caused by tension generated by winding/unwinding of DNA. It wraps around DNA and makes a cut permitting the helix to spin. Once DNA is relaxed, topoisomerase reconnects broken strands

Toxicogenomics: A new scientific subdiscipline that combines the emerging technologies of genomics and bioinformatics to identify and characterise mechanisms of action of known and suspected toxicants. Currently, the premier toxicogenomic tools are the DNA microarray and the DNA chip, which are used for the simultaneous monitoring of expression levels of hundreds to thousands of genes.

Toxicokinetics: The description of the fate of chemicals in the body, including a mathematical account of their absorption, distribution, metabolism and excretion. (see pharmacokinetics)

Transgenic: Genetically modified to contain genetic material from another species (see also genetically modified organism).

Transgenic rodent gene mutation models: Animals which have extra (exogenous) fragments of DNA incorporated into their genomes. This includes transgenic mice containing reporter genes[DPL40] to assess *in-vivo* mutagenicity in recoverable bacterial gene (lacZ or *lac I*). DNA can be isolated from a wide range of tissues following exposure to a test chemical and the genes assessed for induced mutations.

Translation: In molecular biology, the process during which the information in mRNA molecules is used to construct proteins[DPL41].

Tumour (Synonym - neoplasm): A mass of abnormal, disorganised cells, arising from pre-existing tissue, which are characterised by excessive and uncoordinated proliferation and by abnormal differentiation. **Benign** tumours show a close morphological resemblance to their tissue of origin; grow in a slow expansile fashion; and form circumscribed and (usually) encapsulated masses. They may stop growing and they may regress. Benign tumours do not infiltrate through local tissues and they do not metastasise. They are rarely fatal. **Malignant tumours** (synonym - cancer) resemble their parent tissues less closely and are composed of increasingly abnormal cells in terms of their form and function. Well differentiated examples still retain recognisable features of their tissue of origin but these characteristics are progressively lost in moderately and poorly differentiated malignancies: undifferentiated or anaplastic tumours are composed of cells which resemble no known normal tissue. Most malignant tumours grow rapidly, spread progressively through adjacent tissues and metastasise to distant sites. Tumours are conventionally classified according to the anatomical site of the primary tumour and its microscopical appearance, rather than by cause.

Unscheduled DNA Synthesis (UDS): DNA synthesis that occurs at some stage in the cell cycle other than the S period (the normal or 'scheduled' DNA synthesis period), in response to DNA damage. It is usually associated with DNA repair.

Weight of Evidence A quantitative ranking of evidence, or the qualitative appraisal of many different forms of evidence (e.g toxicological or genotoxicity data) to arrive at a conclusion regarding potential hazard (such as mutagenicity).

Annex 1

Tabulation of Genotoxicity Tests (in Stages 1 and 2) and Mutagenic/Genotoxicity Endpoints Detected.

Genotoxicity test	Mutagenic/genotoxicity endpoint detected	Comments
In vitro assays		
Ames	Gene mutation	Responds to wide range of DNA reactive mutagens when full set of *S. typhimurium* tester strains and *E. coli* with appropriate exogenous metabolic activation used.
Micronucleus test	Clastogenicity, aneuploidy	Centromere or kinetochore stains, with pancentromeric or chromosome specific centromeric probes using fluorescence in situ hybridisation (FISH) are required to distinguish between aneuploidy and clastogenicity
Chromosomal aberrations	Clastogenicity, aneuploidy	Indications of aneuploidy from induction of polyploidy or increased mitotic index, but the use of chromosome specific centromeric probes fluorescence in situ hybridisation (FISH) required to assess the potential for aneuploidy. Very similar assay performance compared with micronucleus test
Mouse Lymphoma Assay	Gene mutation, clastogenicity	Distribution of large and small colony mutants can give information on induction of gene mutations versus clastogenicity. No convincing evidence that MLA can detect aneuploidy consistently.
Comet assay	DNA strand breaks and alkali labile sites	Can respond to a wide range of gene mutagens and clastogens but gives no information about modes of mutagenic action.
In vivo assays		
Rodent Bone Marrow/peripheral blood micronucleus assay	Clastogenicity, aneuploidy	A wide range of structurally diverse clastogens and aneugens have been detected. Distingishing between clastogenic and aneugenic MoAs can be investigated by use of centromere or kinetochore probes. .
Rodent transgenic mutation assay	Gene mutations	Valuable for the investigation of gene mutation in a wide range of tissues including germ cells and particularly to confirm gene mutation as a mode of action.
Rodent Comet assay	DNA strand breaks, alkali labile sites	Can respond to a wide range of gene mutagens and clastogens but gives no information about modes of mutagenic action. Does

		not detect aneugens. Valuable for detection of DNA damage in a wide range of tissues, but the standard alkaline assay not validated for mature sperm.
Rodent Liver UDS	Unscheduled DNA synthesis	Endpoint measured is indicative of DNA damage and subsequent repair in liver cells, but now considered not as sensitive as other *in vivo* assays.
Pig-a gene mutation assay	Gene mutations	Endpoint measured is a reporter of gene mutation in rodents, but currently only extensively validated in blood cells.

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