Committee on MUTAGENICITY

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

Guidance on the genotoxicity testing strategies for germ cell mutagens

Background

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 views regarding the most appropriate strategy for genotoxicity testing are outlined in full in the COM (2011)[RB1] "*Guidance On A Strategy For Genotoxicity Testing Of Chemical Substances*".

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

3. 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.

~~1.~~4. The COM affirms 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, as most, if not all, germ cell mutagens are also genotoxic in somatic cells. It has been noted however, that there are some rare examples that are contrary to this statement (e.g. sodium orthovanadate, (Attia et al., 2005)[RB2][RB3][RB4].

5. There are also examples of germ cell mutagens which affect specific stages of gametogenesis in males (Adler, 2008) and where there are differences between male and female germ cell genotoxicity (Bishop, 2003). Currently, the focus for germ cell mutagenicity assays is on male germ cells due to the accessibility of sperm. However, the gap relating to female germ cell assays in regulatory testing is recognised. The male germ cell assays described in this discussion document differ in the specificity, sensitivity and the endpoint detected. It should be noted that all such assays must ensure that the most appropriate phases of spermatogenesis are being tested through specified sample collection timings (Yauk et al., 2015).

~~2.~~6. The development of testing strategies for germ cell mutagens is a rapidly evolving field. Therefore the COM considered it appropriate to prepare a supplementary document on the topic, to support the COM (2011) "*Guidance On A Strategy For Genotoxicity Testing Of Chemical Substances*" which can be updated at regular intervals as new information becomes available (COM, 2011). This discussion paper seeks to provide a brief summary of test methodologies that are currently used or under development and/or validation, to assess germ cell mutagenicity.

OECD Test Guidelines

~~3.~~7. Classification of a substance as a germ cell mutagen should be based on the findings from well conducted, scientifically validated tests in a weight of evidence approach. Where germ cell testing is indicated, there are a number of OECD test guidelines to assess germ cell mutations.

Heritable translocation (OECD TG 485) and specific locus tests

~~4.~~8. The mouse heritable translocation test (HTT; OECD TG 485) (OECD, 1986) was previously viewed as the gold standard assay for determining the transmission of germ cell mutations to the offspring of exposed parents. The mouse HTT is defined by the COM as detecting '*heritable structural chromosome changes (i.e. translocations) in mammalian germ cells as recovered in first-generation progeny*'. The mouse specific locus test (SLT) is described by the COM as '*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*'.

~~5.~~9. Following the development of molecular cytogenetics and genomics technologies, these assays are now viewed negatively as requiring large numbers of

animals (including the use of a mutant mouse strain in the SLT) and as being labour intensive. As a result, these assays are no longer performed in the UK and Europe.

Dominant lethal test (OECD TG 478)

~~6.~~10. The dominant lethal test (DLT, OECD TG 478) (OECD, 2016a) has been the most widely used of the germ cell mutagenicity assays with only minor changes being introduced since its development in 1984. The DLT is usually conducted in male rats or mice and provides information on unstable chromosome changes in gametes that lead to fetal death after fertilisation in non-exposed mated females; indications on the stage of gametogenesis affected can also be determined (COM, 2011). Pre- and post-implantation embryonic losses are considered to be due to severe structural or numerical chromosomal changes inherited from the father (Brewen et al., 1975; Marchetti et al., 2004). The limitations of the assay are that cytotoxicity cannot be excluded as a cause of embryonic death and that the endpoint is not truly heritable. However, the DLT has been well standardised and used to assess many chemicals; some of these have also been tested in the HTT assay with a good correlation of positive results being seen between the two assays (Yauk et al., 2015).

Cytogenetic analysis of spermatogonia or embryos (OECD TG 483)

~~7.~~11. The cytogenetic analysis of spermatogonial metaphases (OECD TG 483) (OECD, 2016b) is a standardised method to detect chromosomal aberrations in male germ cells of mice and rats (Yauk et al., 2015). Chromosome painting techniques have been applied to the method which allows stable balanced aberrations (e.g. reciprocal translocations) to be distinguished from unstable aberrations (e.g. acentric fragments, dicentric chromosomes) (Marchetti and Wyrobek, 2003). Technically the method is challenging and so not widely used. The main limitation however, is that transmission of mutagenic effects to mature gametes and offspring is not demonstrated, as any possible mutagenicity is observed at the beginning of germ cell differentiation (Marchetti and Wyrobek, 2005).

Transgenic rodent somatic and germ cell mutation assay (OECD TG 488)

~~8.~~12. The transgenic rodent mutation assays (TGR; OECD TG 488) (OECD, 2013a)[1] are based on the detection of a mutation in a transgenic sequence that can be isolated from most rodent tissues and expressed in a bacterial system (Yauk et al., 2015). The assays can be used to assess gene mutations in a wide range of rodent tissues (including germ cells) using all routes of administration (Lambert et al., 2005; Kirkland et al., 2019a) and is particularly valuable when investigating gene mutation as the genotoxic endpoint. Determination of the mutation spectrum (base substitutions, insertions/deletions, frameshifts) following chemical exposure of testicular cells and epididymal sperm has been described (Lambert et al., 2005). However, at the current

[1] A draft update of TG 488 was published by OECD in 2019 – at the current time this has not been adopted.

time the chemical database for this test in germ cells is limited, and its ability to detect changes during different spermatogenic phases is still unknown.

~~9.~~13. Limitations of TG 488 include the use of some mutation reporter genes that are limited in use and availability. There are sufficient data to assess the performance of the Muta™mouse, BigBlue® mouse and rat (including use of λ cII transgene), *LacZ* plasmid mouse, and the *gpt* delta mouse models, although it is noted that the *gpt* models are not widely used and are less well validated (COM, 2011). In addition, the TGR assay only infers potential inheritance of mutations, however good correlations are found with the detection of positive chemicals with the SLT, which does directly assess heritability (Singer et al., 2006). It has also been reported that the assays may not detect some types of mutations, including large deletions/insertions for some TGR loci, and rearrangements or copy number variants (CNVs) (Yauk et al., 2015).

14. The ability to use the standard somatic cell TGR assay with testicular tissue would allow significant reductions in animal usage, in line with the 3Rs principles[2], cost, and time. Further, it would allow a quantitative comparison of the same mutagenic endpoints between somatic and germ cell tissues (Yauk et al., 2015). Further development of the assay is underway to try and achieve this, discussed further in paragraphs 22 - 23.

~~10.~~15. Development of the TGR assay for detecting female germ cell mutations is not considered possible due to the low numbers of oocytes available per female for analysis, and is not considered further here (Yauk et al., 2015).

Detection of genotoxic and mutational changes in sperm

~~11.~~16. Genotoxicity tests in sperm can be applied in the same way to humans and animals, providing a direct comparison between biomonitoring and experimental data. There are a number of assay systems that detect different types of pre-mutational and mutational changes in sperm; these are outlined below. Importantly, these could offer quick, higher throughput pre-screening tools for detecting germ cell mutagens, even though they do not assess heritable effects. Many of these have not currently undergone standardisation and harmonisation processes and are discussed more fully in paragraphs 28 - 37.

- Comet - detects DNA strand breaks and abasic sites;[RB5][RB6][RB7]
- TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labelling detects DNA fragmentation;
- SCSA – sperm chromatin structure assay detects chromatin packing alterations;
- FISH – fluorescent in situ hybridisation detects numerical and structural chromosome changes.

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

Other toxicity assays providing evidence of potential germ cell genotoxicity

~~12.~~17. Standard repeat dose and reproductive toxicity studies are a potential source of information that may indicate germ cell genotoxicity. In both types of study, cytotoxic and reproductive endpoints can indicate that a substance has been delivered to particular organs, including gonadal tissue and associated male and female germ cells. These are described further in paragraphs 18 - 23. It should be noted that these studies do not assess mutagenicity specifically and further studies would need to be carried out to confirm this as a mechanism of action.

Segmented reproductive toxicity tests

~~13.~~18. Segmented studies assess adverse effects following exposure at particular time periods of development rather than the entire life cycle. There are a number of segmented designs within guidance from The International Conference on Harmonization (ICH) and the OECD:

- ICH guideline S5(R2) describes three segmented phases for the testing of pharmaceuticals; a fertility and early embryonic development study with exposure of males for 4 weeks prior to mating and of females for 2 weeks prior to mating, through to implantation; exposure of the pregnant dam from implantation through fetal development (assessing organogenesis); pre- and post-natal developmental (PPND) with exposure of the dam from implantation, through lactation until pup weaning.
- OECD TG 421 Reproduction/Developmental Toxicity Screening Test (OECD, 2016c) has a similar pre-mating exposure in males and females, with continuous exposure of females to PND 4. TG 421 is designed to provide limited information regarding the effects of exposure of the test chemical on fertility (male and female reproductive performance such as gonadal function, mating behaviour, conception) and development of the conceptus and parturition. Although this test provides an assessment of transferred effects from exposed males (which may include mutagenic effects), any effects may also be due to exposure *in utero*. No specific assessment of mutagenicity is carried out.
- OECD TG 414 Prenatal Developmental Toxicity Study (OECD, 2018a) assesses the effects of *in utero* exposure to a test chemical from the implantation phase through to parturition. TG 414 is designed to provide general information on the effects of prenatal exposure to a test chemical on the developing organism. The parameters assessed in TG 414 include maternal effects (including death), structural abnormalities and/or altered growth in the fetus. Although it is possible that some effects seen will be due to mutagenicity, no specific assessment of this is carried out.

~~14.~~19. Within these studies, adverse effects on fertility and litter size are determined from the pregnant female, and developmental outcomes can be assessed in fetal

tissue which can be examined to assess morphologic changes and through functional tests in pups, including reproductive performance testing (Yauk et al., 2015).

Continuous cycle reproductive toxicity tests

~~15.~~20. Continuous cycle study designs assess all the different stages of the reproductive life cycle from germ cell through fetal development to adulthood and are often multigenerational. There are two main approaches for continuous study designs:

- The National Toxicology Program's (NTP) reproductive assessment by continuous breeding (RACB) (Gulati et al., 1991);
- The OECD Two-Generation Reproduction Toxicity Study (OECD TG 416) (OECD, 2001).

~~16.~~21. As described for the segmented reproductive toxicity test (paragraphs 18 – 19), the continuous cycle reproductive studies indirectly assess potential effects of germ cell mutagenicity through histopathological analysis of the reproductive and endocrine systems; however, mutagenicity *per se* is not assessed. Effects on fertility and fecundity are assessed through mating of the F0 animals. However, limitations arise in that for any effects arising in the F1 generation, it is not possible to distinguish between those passed on from the F0 generation or those due to *in utero* exposure.

One generation reproduction toxicity study

~~17.~~22. The extended one-generation study design (enhanced pre and postnatal study) (OECD TG 443) (OECD, 2018b) has been developed from the one generation reproduction toxicity study (OECD TG 415) (no longer an active Test Guideline) and [RB8]multigenerational reproductive studies. In the extended study rodents are dosed before mating through gestation with exposure being stopped at various times, with either necroscopy or mating to produce an F1/F2 generation.

Repeat dose toxicity studies

~~18.~~23. Short-term and long-term repeat dose toxicity studies (e.g. 90 day studies) can be combined with reproduction/development toxicity screening tests (e.g. OECD TG 408 Repeated Dose 90-day Oral Toxicity Study and 422 Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test) (OECD, 2018c; 2016c). As previously discussed (paragraphs 17 – 23), the assessment of ovarian and testicular histopathology, sperm count, motility and morphology, might be used to indicate potential germ cell effects.

Assays under development and/or validation

~~19.~~24. A number of new assays to assess germ cell mutagens are currently being developed and validated. In addition, modifications to current OCED TGs are being explored.

Transgenic rodent somatic and germ cell mutation (TGR) assay

~~20.~~25. The TGR assay (OECD TG 488) (OECD, 2013a) is generally considered to enable efficient and effective screening of chemical germ cell mutagens. However, as discussed in paragraphs 10 – 12, there are some limitations of the assay which are currently being addressed.

26. One of the major efforts underway is to develop an optimal protocol for assessing mutagenic effects in germ cells whilst integrating germ cell and somatic cell testing. Currently under TG 488, sampling of mice for somatic cell mutation and germ cells from the seminiferous tubules is at 28 +3 days, and sampling of mature sperm from the cauda epididymis is at 28 +49 days; this has been shown to be the minimum period for sperm maturation in mice and is used as standard in male germline stem cell testing (Marchetti and Wyrobek, 2005). To combine these timepoints would double the number of mice used and the costs, and so there is reluctance to do that currently.

27. Investigations are being carried out to assess whether cells from the seminiferous tubule at the 28 +3 day time point, which are a mixed population, can be used to represent various germ cell stages. Early findings showed that although an acceptable estimate of stem cell mutation frequency can be made at this time point, the effects in dividing spermatogonia may be considerably underestimated (Yauk et al., 2015). Marchetti et al. (2018a) reported use of an equilibrium population model to define exposure of the cell population from seminiferous tubules in mice during the standard 28 +3 day time point. The authors reported that during the proliferating phase, the germ cells~~,~~ were only exposed to 42 % of the total exposure. A protocol of 28 +28 days resulted in 99 % exposure and 100 % exposure was reached using a 28 +30 day protocol. They concluded that false negative and/or conflicting results may have resulted from the standard protocol for germ cells.

28. In a further study, Marchetti et al. (Marchetti et al., 2018b) assessed the impact on the sampling time of tubule germ cells, using published data performed using TG 488. They concluded that evaluating mutant frequencies in sperm from the cauda epididymis using the standard 28 +3 days sampling time did not provide meaningful mutagenicity data. Although more reliable mutagenicity data were obtained from tubule germ cells at the same time point, this was only considered reliable for positive findings. A 28 +28 day protocol produced reliable, positive and negative findings in both mice and rats. The authors concluded that the amended regimen could provide an approach that assesses somatic and germ cell mutagenicity simultaneously in the same animal. However, they also stressed that work was needed to confirm the new protocol for tissues other than slowly proliferating ones, as currently included in TG 488.

Expanded simple tandem repeat (ESTR) assays

~~21.~~29. ESTRs are long homogenous arrays of relatively short repeats (4–9bp) which have a very high spontaneous mutation rate of length changes both in germline and somatic cells (Bois et al., 1998). ESTR loci are considered to be a class of expanded microsatellites, with the spontaneous mutation being replication-driven (Hardwick et al., 2009; Shanks et al., 2008). The analysis of length change mutations occurring at ESTR loci has been utilised for assessing male germ cell mutagenicity in mice (Barber et al., 2009; Dubrova et al., 2000; Vilariño-Güell et al., 2003; Marchetti et al., 2011).

~~22.~~30. The sensitivity of the assay has been increased through the use of single-molecule PCR to detect ESTR mutations, which has also decreased the numbers of animals required and assay duration. In addition, this approach is applicable to human studies; for example, mutation induction has been measured in mice using human clinically-relevant doses of anticancer drugs (Glen et al., 2008).

~~23.~~31. There are some limitations currently reported for the ESTR assay. The mutations detected occur in a very specific genomic context of tandem repeats. In addition, the mechanism underlying ESTR mutation induction is not fully defined. One hypothesis is that non-targeted events cause mutagen-related DNA damage elsewhere in the genome, which leads to an increased mutation rate at the ESTR loci.

~~24.~~32. The ESTR assay can be integrated with standard genetic toxicology tests in mice, however it is currently not known whether ESTR mutations can be assayed in testicular cells sampled under these protocols. Further, integration may require additional animals to be used specifically for the ESTR assay, with an additional appropriate sampling time. From a methodological perspective, the assay is also technically challenging which can lead to variable inter-laboratory results.

Spermatid micronucleus (MN) assay

~~25.~~33. OECD TGs currently exist for the analysis of MN formed as a consequence of chromosome damage and/or spindle malfunction, in *in vitro* (OECD TG 487 In Vitro Mammalian Cell Micronucleus Test) (OECD, 2016d) and *in vivo* (OECD TG 474 Mammalian Erythrocyte Micronucleus Test) (OECD, 2016e) somatic cells. These are widely used and predominant assays for somatic cell testing, with high sensitivity facilitated by flow cytometric analysis. Attempts are being made to develop an equivalent germ cell assay.

~~26.~~34. The spermatid MN assay detects MN originating during meiosis. It was originally developed in rats and subsequently adapted for mouse spermatids. The assay is able to be combined with other genotoxicity tests, including the transgenic rodent assay, and potentially analysis in erythrocytes (Yauk et al., 2015).

~~27.~~35. Some current limitations of the spermatid MN assay include its labour-intensive nature, which limits the number of cells that can be scored and hence the sensitivity. This is being addressed through development of automated detection of MN by flow cytometry, as exists for somatic cells. In addition, although it is not known what the fate of a sperm cell carrying MN is, it is considered unlikely that the micronuclei would be inherited (Yauk et al., 2015).

Sperm Comet assay

~~28.~~36. OECD TG 489 (In Vivo Mammalian Alkaline Comet Assay) (OECD, 2016f) describes the *in vivo* alkaline Comet assay for the measurement of DNA strand breaks in single cells. The Comet assay has been used to assess genotoxic hazard for a large number of chemical and physical genotoxicants both *in vivo* and *in vitro*. Although the main use of the assay has been for the assessment of somatic cells, the assay has been conducted both on mature sperm and on germ cells isolated from the seminiferous tubules (Speit et al., 2009; Haines et al., 2001; Haines et al., 2002). When applied to germ cells, the assay does not show heritability but does indicate genotoxicity.

~~29.~~37. There are, however, a number of limitations that need to be addressed before the assay could potentially be applied to assess germ cell DNA damage for regulatory purposes (Kirkland et al., 2019b). The exposure protocol outlined in TG 489 would result in only fully mature sperm being exposed, which have a high resistance to DNA damage. Although the analysis of germ cells collected from the seminiferous tubules is not fully validated, it is known that two different germ cell populations (spermatocytes and elongating spermatids) are present. For both cell populations, DNA double strand breaks are part of the normal process of development (meiotic recombination in spermatocytes and chromatin compaction in elongating spermatids) which may lead to false positive findings. Mature sperm also require a pre-digestion step before analysis in the Comet assay, which can lead to poorly reproducible results (Yauk et al., 2015).

~~30.~~38. There are currently initiatives underway to standardise the Comet assay, with 10 laboratories worldwide developing fully validated protocols to ensure data [RB9]reproducibility (Yauk et al., 2015).

Sperm chromatin quality assays

~~31.~~39. Two other commonly used assays to assess the integrity of sperm DNA include the sperm chromatin structure assay (SCSA) and the terminal deoxynucleotidyl transferase-mediated (TdT) deoxyuridine triphosphate (dUTP) nick end labeling assay (TUNEL). Both assays were developed around 30 years ago and validation is more advanced in humans than in animals.

~~32.~~40. SCSA uses flow cytometry methods to assess the susceptibility of sperm DNA to acid-induced denaturation, as denaturation is linked to the presence of single stranded DNA, an indicator of potential genotoxicity (Sills et al., 2004). The TUNEL assay measures DNA breaks *in situ* as assessed by the incorporation of dUTP at the sites of breaks (Gorczyca et al., 1993). Both assays, therefore, measure different aspects of DNA integrity.

~~33.~~41. There are currently initiatives underway to standardise the SCSA and TUNEL assays, with 10 laboratories worldwide developing fully validated protocols to ensure data reproducibility. It is hoped that validation of the assays in humans will allow rapid transfer to animal models (Yauk et al., 2015). [RB10]

~~34.~~42. The main limitation with using sperm DNA integrity as an endpoint for genotoxicity testing is that currently we do not understand the mechanisms and consequences of sperm chromatin damage. The integrity of sperm chromatin has been identified as a contributing factor to a healthy pregnancy and offspring (Aitken et al., 2013; Aitken et al., 2009; Lewis and Simon, 2010; Robinson et al., 2012) however, clinically relevant parameters that would allow chromatin integrity to be assessed have not currently been defined.

Whole genome sequencing

~~35.~~43. Advancements in genome sequencing technologies allow detection of the effects of mutagens on heritable germ cells. These technologies have been applied to the full genomic sequencing of 78 individuals and findings suggested that the father's age is a dominant factor in determining the number of *de novo* mutations in their offspring (Kong, 2012). If genome-wide mutation spectra and frequencies in rodent models are shown to be comparable to humans, this technology has the potential to determine phenotypic consequences to an organism as a whole (Yauk et al., 2015).

~~36.~~44. However, as the methodology is still in development it has not been applied from a toxicological basis, and extensive validation will be needed. Other limitations include the high costs and long analysis time, which are expected to be reduced with improved data handling (Yauk et al., 2015).

Copy number variants (CNVs)

~~37.~~45. CNVs comprise a structural variation of DNA ranging in size from 50 base pairs to megabases, which alters or rearranges the number of copies of specific DNA segments. CNVs account for around 12 % of genetic variation in humans and are considered to be related to a broad range of human genetic disorders (Stankiewicz and Lupski, 2010; Campbell, 2013; Girirajan and Eichler, 2010; Sebat et al., 2004; Lupski, 2007). CNVs are not detected using currently available genotoxicity testing assays and require high- resolution array comparative genomic hybridization (or

aCGH) and SNP (single nucleotide polymorphism) microarray technologies (Yauk et al., 2015).

~~38.~~46. Both technologies have been applied in the clinic to identify the sources of idiopathic diseases (Dittwald et al., 2013; Wiszniewska et al., 2014; Cheung et al., 2005; Boone et al., 2013; Pham et al., 2014) but only limited assessments of the effects of mutagens on CNV formation have been reported (Arlt et al., 2009; Arlt, 2011; Arlt et al., 2014). It has been shown *in vitro* that replication stress (for example through exposure to hyroxyurea or low doses of ionising radiation) can lead to the formation of CNVs. Increases in *de novo* CNVs is also associated with increasing paternal age (Sun et al., 2012).

~~39.~~47. The major current limitation of this technology is the lack of evidence to show its application *in vivo* and extensive development and validation is therefore required (Yauk et al., 2015).

High-throughput analysis of egg aneuploidy in nematode C. elegans

~~40.~~48. High throughput screening (HTS) tools for chemical testing is a rapidly developing field, which is aimed at increasing chemical testing capacity whilst reducing animal use. A major gap exists in HTS assays for the detection of mutagens and aneugens (Knight et al., 2009). Existing assays focused on the initiation of a DNA damage response have low sensitivity and do not consider effects on germ cells. A new screening tool, which is currently under development, utilises the nematode C. elegans to measure chromosome segregation errors occurring in eggs and has been proposed as an HTS assay for Tier 1 screening of female germ cells (Yauk et al., 2015). Preliminary validation using 50 chemicals showed an accuracy of 69 % (average of sensitivity and specificity) in predicting the ability of chemicals that cause reproductive toxicity in rodents (Allard et al., 2013).

~~41.~~49. C. elegans is an established model system in genetics as there is a good degree of conservation with humans in key meiotic pathways. Limitations concerning the applicability of the relationship of aneuploidy in C. elegans to the same potential outcome in humans, has been raised (Yauk et al., 2015).

Summary of assays under development or undergoing validation

Table 1 - Advantages and disadvantages of assays under development or undergoing validation

Endpoint	Advantages	Disadvantages
Transgenic rodent mutation	Can be performed on a wide range of tissues; Allows a comparison of somatic and germ cell sensitivity/specificity; Neutral gene; Scores gene mutation; OECD guideline; Can be integrated into multiple test strategies.	Transgenic rodent model used; Scores mutations in a non-transcribed exogenous gene; Performed on germ cells so inheritance is assumed; May miss some types of mutations.
Tandem repeat assays	Endogenous loci; High spontaneous mutation rate; Adaption to any species possible; Links shown between some markers and disease; Sensitive at low doses; Integration into multiple test strategies possible (requires validation).	Indirect mechanism of mutation with unknown mode; Non-coding markers; Relevance of tandem repeat mutation to gene mutations is unclear; Small dynamic range; Technically challenging.
Spermatid micronucleus (MN)	Can be integrated into transgene mutation reporter assay and other toxicity tests; Can be performed in any species; Directly comparable to somatic MN to assess germ cell specificity/sensitivity.	Methodology is laborious (but potential for flow cytometry modifications); Small database; Performed on germ cells so inheritance is assumed.
Sperm comet assays	Can be performed in any species; Technically simple; Directly comparable to most somatic cell types; Detects a variety of DNA damage.	Difficult to integrate with other tests; High inter-laboratory and inter-study variability; Biological relevance of endpoint unclear; Technically challenging; Pre-mutational damage only detected.
Sperm chromatin structure	Rapid technique (flow cytometry approach); Can be performed in any species including humans; Major validation exercises are underway.	Performed on germ cells so inheritance is assumed; Pre-mutagenic lesion detected; Mechanisms causing changes in chromatin are not known; Technically challenging giving high inter-laboratory and inter-study variability.

Source: adapted from (Yauk et al., 2015)

Do the available assays reflect human relevant endpoints?

~~42.~~50. There is a spectrum of mutational events occurring *in vivo* that have the potential to impact on human health, and new genomics tools allow for the quantitation of genome-wide mutation rates. Single nucleotide variants (SNVs) and CNVs may affect coding and non-coding DNA sequences; for example, it has been reported that 76 % of SNVs originate in the paternal lineage (Campbell et al., 2012; Conrad et al., 2011; Roach et al., 2010).

~~43.~~51. The mutation rate (per locus and per total nucleotide number affected) is higher for CNVs than SNVs. It has been estimated that one large *de novo* CNV (>100 kbp) occurs per 42 births in humans, compared to an average of 61 new SNVs per birth; however, the average number of base pairs affected by large CNVs is 8–25 kbp per gamete versus 30.5 bp per gamete for SNVs (Yauk et al., 2015). CNVs are caused by chromothripis events whereby multiple *de novo* rearrangements in a single event (Stankiewicz and Lupski, 2010).

~~44.~~52. A number of additional functional genomic changes also arise, including:

- small insertions and deletions;
- mobile element insertions;
- tandem repeat mutations;
- translocations; and
- aneuploidies

~~45.~~53. Proportionally higher *de novo* mutation rates are reported for microsatellites than for SNVs, which is considered an important source of genetic variation (Sun et al., 2012). An inverse relationship has been reported between mutation size and frequency, meaning that although more rare, the number of nucleotides affected by large genomic changes, including CNVs and aneuploidies, is orders of magnitude greater (Yauk et al., 2015).

~~46.~~54. In humans, epidemiological studies look to measure the phenotypic effects of induced dominant mutations occurring in the descendants of exposed parents. Importantly, such studies have shown that as many mutations occurring in humans are recessive, phenotypic changes are not apparent for several generations until conception occurs with a complementary mutation or the mutation occurs in a somatic cell.

~~47.~~55. Some of these potentially important genomic changes may therefore not be effectively captured by both the existing battery of genotoxicity testing assays nor by those under development.

What is the current status of regulatory requirements for germ cell testing?

~~48.~~56. The testing of chemicals for germ cell mutagenicity is a regulatory requirement for many organisations worldwide, including the World Health Organisation / International Programme on Chemical Safety (WHO/IPCS), Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and the regulatory agencies in the US, Canada, Japan, UK and the EU. Although many other countries follow the approach taken by the US, India and Australia do not require germ cell mutation tests for regulatory purposes. Genetic toxicity tests used across organisations comprise three tiers, with Tier 1 containing *in vitro* and somatic *in vivo* tests and Tiers 2 and 3 the supporting germ cell studies that can be requested by regulatory bodies under certain conditions. For example, Tier 2 contains DNA damage assays in the testes or spermatogonia and Tier 3 the gene cell mutation tests.

~~49.~~57. The testing of pharmaceuticals for registration under the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) does not stipulate that germ cell assays should be carried out, rather it is assumed that *in vivo* somatic tests and carcinogenicity data will provide sufficient predictivity/protection for germ cell effects.

~~50.~~58. The WHO/IPCS Harmonised Scheme states that a positive *in vivo* somatic cell mutagen can trigger testing for germ cell mutagenicity, but this is not required. Optional recommended tests include transgenic mouse models, the ESTR assay, the spermatogonial chromosome aberration assay, chromosome aberration analysis by FISH, the Comet assay, and assays for DNA adducts. The WHO/IPCS tests in offspring include the ESTR assay, the DLT, the HTT, and the SLT (Yauk et al., 2015).

~~51.~~59. The GHS, together with OECD, ECHA and many other countries categorise mutagens according to three criteria:

- Category 1A – chemicals known to induce heritable mutations in germ cells of humans (based largely on human evidence);
- Category 1B – chemicals that should be regarded as if they induce heritable mutations in germ cells of humans (based largely on experimental animal data);
- Category 2 – chemicals that cause concern for induction of heritable mutations in germ cells of humans.

~~52.~~60. In the EU, for example, under the REACH regulations, any genotoxic agent in somatic cells is evaluated for germ cell mutagenicity using bioavailability and *in vivo* data. Where no data are available, the chemical can be further tested using relevant germ cell assays. Issues around the types of studies able to provide data suitable for distinguishing between mutagen categories 2 and 1B were discussed at a joint workshop between the Member State Committee (MSC) and Committee for Risk Assessment (RAC). Workshop participants agreed that refinement of the current MSC

approach was possible with regards to follow-up testing of positive somatic cell mutagens, including testing for mutagenic potential in both somatic and germ cells in the same study (ECHA, 2019).

~~53.~~61. Germ cell mutagenicity is an established regulatory endpoint and existing assays have identified >50 substances as germ cell mutagens in rodents. It has been noted by Yauk and colleagues that no agent has currently been regulated solely as a germ cell mutagen or evaluated to be a human germ cell mutagen (Yauk et al., 2015)~~.~~ i.e. there are no known Cat 1A substances.

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[RB11]

Abbreviations used in the document

HTT	Heritable translocation test
SLT	Specific locus test
DLT	Dominant lethal test
TGR	Transgenic rodent mutation assay
CNV	Copy number variant
ESTR	Expanded simple tandem repeat
MN	Micronucleus
SCSA	Sperm chromatin structure assay
TUNEL	Terminal deoxynucleotidyl transferase-mediated (TdT) deoxyuridine triphosphate (dUTP) nick end labeling assay
HTS	High throughput screening
SNV	Single nucleotide variants
WHO/IPCS	World Health Organisation / International Programme on Chemical Safety
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use