MUT/2022/11

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

First draft document for COM Guidance Statement (G0X): The Use of Biomarkers in Genotoxicity Risk Assessment

1. At the February 2022 meeting, COM considered the revised COC Guidance Statement G04 'The Use of Biomarkers in Carcinogenic Risk assessment' (MUT/22/03). Following discussions, it was considered that it would be helpful for COM to produce its own, more comprehensive, guidance on biomarkers of genotoxicity, relevant to its area of expertise. This document could then be referred to by the other Committees when needed and as appropriate.

2. A draft scoping document was prepared to provide an overview of the proposed content of the new COM guidance, for discussion and agreement by members at the meeting in June 2022 (MUT/22/06).

3. The document presented here at Annex A is a first draft document which has been amended according to Committee discussions and recommendations. Revised text is presented in red.

Questions for the Committee

1. Members are asked to consider the amended guidance document outline, and, in particular, to:

   i. Comment on the suggested sections and whether additional themes need to be included.

   ii. Comment on the key themes that need to be covered under each section.

   iii. Address questions included in the text.

   iv. Consider next steps in progressing the document

IEH Consulting under contract supporting the UK HSA COC and COM Secretariat
October 2022

MUT/2022/11 – Annex A

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

COM Guidance Statement (G0X): The Use of Biomarkers in Genotoxicity Risk Assessment

First draft document

Secretariat

October 2022

Proposed guidance document outline

Introduction

- The focus of the guidance statement is biomarkers of genotoxic relevance for risk assessment purposes.[RB1]

The specific focus of this guidance statement are biomarkers of relevance for genotoxicity. Readers are also referred to COC Guidance Statement G04 'The Use of Biomarkers in Carcinogenic Risk Assessment' (COC, XX) which outlines biomarkers specific to the carcinogenic process and those of more general toxicological relevance. A biomarker is defined as "any substance, structure or process that can be measured in an organism or tissue, related to a specific exposure or effect or which can influence the incidence of the effect" (Choi et al., 2015). For the purposes of this guidance statement, the specific effect would be genotoxicity, i.e., the induction of DNA damage, mutation, or both (Smith et al., 2016). Biomarkers can provide valuable information to aid chemical risk assessment processes and are used as investigative tools in both animal and human studies which aim to evaluate carcinogenic hazards and risk (COC, XX).

The COM 'Guidance on a strategy for genotoxicity testing of chemicals' defines genotoxicity as the 'interaction with, or damage to, DNA and/or other cellular components which regulate the fidelity of the genome'. Further, '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 (COM, 2021).

- Distinction between genotoxic and non-genotoxic carcinogens, to include consideration of mechanistic information - this is particularly relevant for risk assessment, with the assumption of the existence of no-effect concentrations (threshold levels) in case of the latter group. In contrast, genotoxic carcinogens, their metabolic precursors and DNA reactive metabolites are considered to represent risk factors at all concentrations since even one or a few DNA lesions may, in principle, result in mutations and, thus, increase tumour risk.

Carcinogens can thus be divided into two broad classes based on their mechanism of chemical carcinogenicity: genotoxic and nongenotoxic (epigenetic) carcinogens. Genotoxic carcinogens initiate the process of chemical carcinogenesis by damaging DNA and acting as mutagens. Nongenotoxic carcinogens promote carcinogenesis without binding, damaging or interacting with DNA. They act via many modes of action (MoA): causing cytotoxicity, binding to receptors such as oestrogen, androgen, aryl hydrocarbon, peroxisome or constitutive active receptors, suppressing immune system, increasing oxidative stress, or inhibiting DNA damage repair; i.e.,

they do not act as a ‘traditional’ initiator in the development of carcinogenesis. MoA data is therefore key in establishing the genotoxic or nongenotoxic nature of a carcinogen.

For risk assessment purposes, genotoxic carcinogens, their metabolic precursors and DNA reactive metabolites have generally been considered to represent risk factors at all concentrations since even one or a few DNA lesions may, in principle, result in mutations and, thus, increase tumour risk, i.e., there is no threshold of toxicity. However, the COM has published guidance on possible threshold modes of genotoxicity which can include: i) involvement of non-DNA targets (for example, aneugen inhibition of microtubules); ii) the contribution of protective mechanisms (for example, repair of DNA adducts formed from many low molecular weight alkylating agents); iii) overload of detoxication pathways (for example, paracetamol) (COM, 2010).

Biomarker types and their use in risk assessment

- Description of biomarkers of exposure, effect and susceptibility (as *per* COC current guidance).

For the purposes of this document and for consistency with the terminology used in COC G04 (COC, XX), biomarkers will be broadly characterised as those of *exposure* and those of *effect*, although the distinction between these two is not always clear-cut. Biomarkers in the context of carcinogenicity can mean proof of exposure to a carcinogen, detection of a reaction product or an indication that a preliminary genotoxic event or actual DNA damage has occurred. Other types of biomarkers exist, for example biomarkers of susceptibility, which were initially introduced as interpretative aids to epidemiological investigations of chemically-induced carcinogenesis.

Biomarkers of exposure - “an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism” (Laideira and Viegas, 2016). Biomarkers of exposure are further divided into those reflecting ‘internal dose’ and those reflecting ‘effective dose’. The concentration of a chemical (or metabolite) in blood following exposure is a basic measure of the internal dose, indicating the likely level of chemical (or metabolite) at the target site. The effective dose is a more accurate measurement of the exposure levels associated with the target molecule, structure or cell itself (Laideira and Viegas, 2016) (refer to paragraphs XX-XX).

Biomarkers of effect - “a measurable biochemical, physiological, behavioural or other alteration within an organism that, depending upon the magnitude, can be recognised as associated with an established or possible health impairment or disease” (IPCS, 1993; Jeddi et al., 2021), for example measures of chromosome damage, related to carcinogenicity (refer to paragraphs XX - XX).

Biomarkers of susceptibility - A biomarker of susceptibility may be defined as an indicator of an inherent or acquired ability of an organism to respond to the

challenge of exposure to a chemical (Manno et al., 2010) (refer to paragraphs XX-XX).

- How are biomarkers used in the risk assessment of genotoxicity – to include an overview of the current COM guidance on a strategy for genotoxicity testing of chemicals and how biomarkers of genotoxicity are utilised within the individual components of the risk assessment process.[RB2]

- Validation and characterisation of biomarkers (as *per* COC). In addition, it is important to highlight the importance of confirming biological significance e.g. by measuring DNA adducts and mutation in parallel. DNA adducts have an important role in the risk assessment process and in establishing a mode of carcinogenic action, although the association of an adduct with a disease does not automatically indicate causality.

COM has a role in evaluating the entire spectrum of biomarkers including the development, validation and assessment of practicality of new techniques and the applicability and interpretation of well-established methods.

Biomarkers must be appropriately characterised and validated before conclusions are drawn from their use. Particular emphasis may be placed on the early events (both mutagenic and non-mutagenic) in the carcinogenic process. There are a number of criteria that should be considered when selecting and validating suitable biomarkers for use in human biomonitoring [RB3]studies (Vorkamp et al., 2021). For example, the general criteria used for the evaluation of the most suitable exposure biomarkers (EB) and matrix (M) for the current European initiative, HBM4EU for carcinogens and non-carcinogens, include:

- Specificity – concentration of the exposure biomarker in the selected matrix should exclusively reflect exogenous exposure and is a consequence of environmental and/or occupational exposure. It is noted that for chemicals with very limited data, exclusivity may not be achievable, however concentrations should be a correct indication of exposure.
- Biological sensitivity - the measured concentration of the EB/M correlates strongly with the substance intake dose. Variations of EB/M concentration reflect the variation of exposure to the substance of interest. For chemicals with very limited data, the measured concentration of the EB/M is an acceptable indication of the substance intake dose.
- Half-life - the EB/M should preferably have a half-life sufficiently long to avoid an excessive intra-individual variability in EB/M concentration measurement.
- Stability after sample collection - cryo-preservability of EB/M is sufficient to guarantee a high stability during storage in the biobank or analyse the sample as soon as possible.
- Matrix availability and sample collection - the sample collection of the relevant matrix is not considered too invasive. Easy collection and transportation of the required amount of sample with a validated

sampling protocol is beneficial. It is advantageous if it is possible to determine more than one EB in the same matrix where it is relatively easy to obtain a sufficient sample volume for a required number of samples.

- Measurement validity - the EB/M concentration in the sample is not likely to be altered by contamination with a ubiquitous parent substance from the environment preceding and during the analysis. Variations in matrix composition can be easily corrected for (e.g. creatinine in urine, lipids in serum). Sample contamination by a ubiquitous parent substance might occur, but the level of contamination is low compared to expected levels and special precautions can be applied to minimize the amount of contamination.

Biomarkers used in animal studies must also be suitably characterised and validated and this should be based on the principles detailed for human biomarkers. In relation to biomarkers, the STrengthening the Reporting of OBservational studies in Epidemiology – Molecular Epidemiology (STROBE-ME) initiative (Gallo et al., 2012) provides standardised guidelines and a 'checklist' for the reporting of biomarker and molecular epidemiology studies (see http://www.equator-network.org/reporting-guidelines/strobe-me/, accessed August 2022). An extension to STROBE is the STrengthening the REporting of Genetic Association studies (STREGA) which includes additions to 12 of the 22 items on the STROBE checklist that are important to consider in genetic association studies (Little et al., 2009).

The appropriate interpretation and application of DNA adduct data to inform risk assessment decisions have been debated for the past several decades. The intensity of this debate has increased more recently with the advent of new, highly specific, and exquisitely sensitive, analytical methods. It should be appreciated that DNA adduct data cannot be used in isolation in the risk assessment process but must be used in an integrated fashion with other information that establishes links between DNA adducts (e.g., type of adduct, frequency, persistence, type of repair process) and accepted outcome measures (e.g., dosimetry, toxicity, mutagenicity, genotoxicity, and tumor incidence) to inform characterisation of the mode of action. DNA adducts are considered biomarkers of exposure, whereas gene mutations and chromosomal alterations are often biomarkers of early biological effects and also can be bioindicators of the carcinogenic process (Jarabek et al., 2009).[LL4][RB5][RB6]

Strategic uses of human biomonitoring

- Use of HBM has been developed in occupational settings where exposures to an identified chemical of particular concern might be relatively high. Subsequent application to population exposure has made progress but genotoxicity biomarkers are not applied extensively in large population studies[RB7].

Probably the most well-developed use of HBM has been in occupational settings where exposures to a chemical of particular concern might be relatively high. Here, routine HBM might be more informative about risk than air monitoring, particularly where skin uptake is an important contributing exposure pathway, and various types

of reference values used for risk management might exist for the chemical of concern. In the general population, HBM is often used to inform on exposure to chemical of particular concern and also, for changes over time (increase or decrease) for substances of interest related to industrial or consumer usage to existing or newly introduced substances (Bevan et al., 2017).

Ongoing national and international surveillance programmes such as the US National Health and Nutrition Examination Survey (NHANES), the Canada Health Measures Survey (CHMS) and the German Environmental Survey (GerES) typically use well-established biomonitoring techniques (e.g. biomarkers which are known to reflect exposure to the chemical of interest, standardised sampling methods and verified analytical techniques) to collect information on population exposures to environmental hazards that are known to be significant to public health. As biomonitoring does not generally determine exposure sources and routes of exposure, environmental monitoring remains crucial (Ladeira and Viegas, 2016).

A Biomonitoring Equivalent (BE) is an estimated concentration or range of concentrations of an environmental chemical in humans that is consistent with existing health-based guidance values such as the Tolerable Daily Intake (TDI) or reference dose or concentration (RfD, RfC). It provides a way of interpreting biomonitoring data in the context of these values (Hays et al., 2008; LaKind et al., 2008). It is envisaged that they will be useful for understanding and prioritising risk management practices and will enable the available biomonitoring data to be utilised more fully. However, to date, there is limited information on the use of BEs for estimating chemical exposure in the context of carcinogenesis (Faure et al., 2020).

Human biomonitoring guidance values (HBM-GVs) are being derived by the European Human Biomonitoring Initiative referred to as HBM4EU. There is currently a diversity in the derivation of health-based guidance values for both the general population and for occupational exposure. The HBM4EU initiative aims to increase confidence in HBM-GVs derived using a harmonised, systematic and generally accepted strategy for the derivation of HBM-GVs at the European level (Vorkamp et al., 2021).

- To focus on, with relevant examples, of exposure to genotoxic carcinogens and their application to risk assessment. [RB8]

Biomarkers of exposure

- Discussion of biomarkers of DNA damage – to include single-stand breaks, double-strand breaks, covalently bound chemical DNA adducts, oxidative-induced lesions and DNA–DNA or DNA–protein cross-links.

Examples of DNA damage include DNA adducts (a molecule bound covalently to DNA), DNA strand breaks (breaks in the phosphodiester bonds), DNA crosslinks, and DNA alkylation. DNA damage by itself is not a mutation and generally does not alter the linear sequence of nucleotides (or bases) in the DNA, whereas a mutation is a change in the DNA sequence and usually arises as the cell attempts to repair the DNA damage (Shaughnessy and DeMarini 2009).

- Temporality considerations of biomarkers of DNA damage related to other critical events.

Biomarkers of internal dose may indicate, depending on their nature, a recent or very recent exposure as well as a long-term exposure (Gallo et al 2011).

- Equivalent endogenous biomarkers of DNA damage and their effect on the dose-response curve of the exogenous biomarker.

DNA damage can be spontaneous in origin through errors of nucleic acid metabolism or can be induced by endogenous or exogenous agents. In some cases, the exogenous agents may also be generated endogenously, such as formaldehyde and acetaldehyde, producing a background level of DNA damage.

Biomarkers of effect[RB9]

- Discussion of biomarkers of genotoxic effect – to include cytogenetic endpoints such as micronuclei (MN) and chromosome aberrations (CA), which are considered to be biomarkers of early carcinogenic effects and are thought to be predictive for cancer risk in humans.

Mutations can be classified into three groups based on their location or involvement in the genome. Gene or point mutations are changes in nucleotide sequence within a gene (e.g., base substitutions, frameshifts, and small deletions/duplications). Chromosomal mutations are changes in nucleotide sequence that extend over multiple genes (e.g., chromosome aberrations, translocations, large deletions, duplications, insertions, inversions, or micronuclei due to chromosome breakage). Genomic mutations involve the duplication or deletion of nucleotide sequences of an entire chromosome, an example of which is aneuploidy or formation of micronuclei that contain a centromere. A large proportion of Group 1 carcinogens ("The agent (mixture) is carcinogenic to humans") are genotoxic, as documented in IARC Monographs Volume 100 A–F.

In terms of human biomarkers of genotoxic effect, there are a number of genotoxicity and mutational endpoints that have been studied in humans. These include; lymphocyte micronuclei, PigA mutation in erythrocytes, DNA strand breaks in lymphocytes with the Comet assay and H2AX.

The Micronucleus (MN) assay

The human lymphocyte micronucleus assay has been used to study genotoxic events in humans for several decades. The Human Micronucleus (HuMN) project was launched in 1997 (https://pubmed.ncbi.nlm.nih.gov/10517999/) and in 2007, a key paper showing the link between baseline MN levels and future cancer risk suggested a link between this genotoxic biomarker and 31dr5carcinogenesis [RB10](Bonassi 2007). Importantly, the MN levels in circulating lymphocytes were a predictor for internal solid cancers. Over the years, lymphocytes MN levels have been shown to be linked to exposures to a range of genotoxic compounds and links between MN levels and cancer risks have been explored. In 2021, a special issue of

Mutation Research/Review in Mutation Research was dedicated to the human MN studies (https://www.sciencedirect.com/journal/mutation-research-reviews-in-mutation-research/special-issue/10NQ1MFQNFK) .

PigA

The PigA mutation assay is well established in rodents (Lemieux 2011 , Cao 2014) and measures mutations through loss of glycophosphatidylinositol (GPI) linked cell surface proteins. Recent developments have been made to optimise a human erythrocyte *PIG-A* assay for biomonitoring purposes, with a limited number of studies identifying a similar low background mutation frequency in the erythrocytes of the 'healthy subjects' studied (Cao 2016, Dobrovolsky 2011, Dertinger 2016).

The minimally invasive nature of *PIG-A* testing (pin-prick sampling optional) offers the potential of the assay to be used as a biomonitoring tool, assessing the risk of environmental, occupational and pharmaceutical exposures. The paucity of mutant red blood cells in healthy volunteers enhances the ability to identify mutagenic exposures above a very low background level and repeat sampling may permit the analysis of long term, chronic exposure.

The potential use of this mutation test as a biomarker for disease and a biomonitoring tool has recently been assessed in a limited number of studies. It has been demonstrated that oesophageal cancer patients had elevated levels of GPI-deficient erythrocytes compared to non-neoplastic controls (Haboubi, 2019) suggesting a link between circulating mutations in blood cells and internal cancer risk. One study has shown patients with inflammatory bowel disease (IBD) treated with azathioprine over prolonged periods had increased levels of *PIG-A* mutant erythrocytes (Cao 2019). In terms of studying human mutation levels with a view to human biomonitoring, the human PigA assay has also revealed mutational links to diet, medication and age (Lawrence, 2020) and to exposure to heavy metals such as lead (Cao 2020).

Comet assay

The Comet assay measures DNA strand breaks and is its use in human lymphocytes (hCOMET) is often regarded as a measure of exposure rather than effect, as the DNA can be repaired and/or the damaged cells may die. The dynamic changes in Comet tail lengths has also be used to imply DNA repair levels and this repairability has been linked to human cancer risk (https://www.sciencedirect.com/science/article/pii/S1383574218300899) . The use of the Comet assay in lymphocytes has been well studied and there are many papers showing Comet assay levels mirroring exposures to a range of genotoxic compounds (https://www.sciencedirect.com/science/article/pii/S1383574219300456) (https://www.sciencedirect.com/science/article/pii/S1383574221000089) . Links between Comet tail length and current cancer risk have also been suggested. (https://www.nature.com/articles/s41598-021-95976-7).

H2AX gamma?[RB11]

Ionizing radiation (IR) exposure is inevitable in our modern society and can lead to a variety of deleterious effects including cancer and birth defects. A reliable, reproducible and sensitive assessment of exposure to IR and the individual response to that exposure would provide much needed information for the optimal treatment of each donor examined. a diagnostic test for IR exposure based on detection of the phosphorylated form of variant histone H2AX (γ-H2AX), which occurs specifically at sites of DNA double-strand breaks (DSBs) has been developed The cell responds to a nascent DSB through the phosphorylation of thousands of H2AX molecules flanking the damaged site. This highly amplified response can be visualized as a γ-H2AX focus in the chromatin that can be detected *in situ* with the appropriate antibody (Redon et al., 2009).

- Temporality considerations of biomarkers of genotoxic effect related to other critical biological events.[RB12]

- Equivalent endogenous biomarkers of genotoxic effect and their influence on the dose-response curve of the exogenous biomarker.[RB13][RB14]

Biomarkers of susceptibility

- Evidence suggests that genetic susceptibility plays a role in an individual's response to exogenous and environmental exposures. Provide relevant examples.

Evidence suggests that genetic susceptibility plays a role in an individual's response to exogenous and environmental exposures. Consequently, a number of studies have explored the interactions between genetics and exposures in the aetiology of disease (for example, Kelly and Vineis, 2014).

Additionally, the use of detailed PBPK models for interpreting biomonitoring data further allows for the modelling of different sources of interindividual variability of the absorption, distribution, metabolism and excretion processes, such as body weight, age, genetic polymorphisms in xenobiotic metabolic pathways, excretion and elimination rates and others. The previously so-called confounders or uncertainty factors can be treated as analysable variables which reflect variations in the susceptibility within a population that is exposed to environmental pollutants (Ladeira and Viegas, 2016).[RB15]

Biomarkers of acquired susceptibility - such as biomarkers of previous diseases or biomarkers of previous exposures such as epigenetic changes (Gallo et al., 2011).[RB16]

- Application of biomarkers of susceptibility to risk assessment, challenges for interpretation [RB17]

Measurement of biomarkers of genotoxic exposure and effect[RB18]

- Established quantitative *in vitro* and *in vivo* methods – to include omics (genomics, proteomics, metabolomics) and, potentially, transcriptomics and next generation sequencing.

- Developing methods - to include DNA adducts and mutational signatures (adductome analysis), epigenetic changes (DNA methylation, histone modifications, and miRNAs) and gene expression biomarkers.

Summary

To be finalised once the document is complete.

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