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REVIEW

Severity of effect considerations regarding the use of mutation as a toxicological endpoint for risk assessment: A report from the 8th International Workshop on Genotoxicity Testing (IWGT)

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Abstract

Exposure levels without appreciable human health risk may be determined by dividing a point of departure on a dose–response curve (e.g., benchmark dose) by a composite adjustment factor (AF). An "effect severity" AF (ESAF) is employed in some regulatory contexts. An ESAF of 10 may be incorporated in the derivation of a health-based guidance value (HBGV) when a "severe" toxicological endpoint, such as teratogenicity, irreversible reproductive effects, neurotoxicity, or cancer was observed in the reference study. Although mutation data have been used historically for hazard identification, this

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endpoint is suitable for quantitative dose–response modeling and risk assessment. As part of the 8th International Workshops on Genotoxicity Testing, a sub-group of the Quantitative Analysis Work Group (WG) explored how the concept of effect severity could be applied to mutation. To approach this question, the WG reviewed the prevailing regulatory guidance on how an ESAF is incorporated into risk assessments, evaluated current knowledge of associations between germline or somatic mutation and severe disease risk, and mined available data on the fraction of human germline mutations expected to cause severe disease. Based on this review and given that mutations are irreversible and some cause severe human disease, in regulatory settings where an ESAF is used, a majority of the WG recommends applying an ESAF value between 2 and 10 when deriving a HBGV from mutation data. This recommendation may need to be revisited in the future if direct measurement of disease-causing mutations by error-corrected next generation sequencing clarifies selection of ESAF values.

KEYWORDS
genetic disease, germ-line mutation, mosaicism, mutation, risk assessment

1 | INTRODUCTION

Over the past decade, there has been increasing recognition that mutation is a bona fide toxicological endpoint, and that in vivo mutation data could be analyzed quantitatively to obtain a point of departure (PoD) that could then be used to establish a health-based guidance value (HBGV) for regulatory decision making (Heflich et al., 2020; Johnson et al., 2021; MacGregor et al., 2015; Menz et al., 2023; White et al., 2020). The Quantitative Analysis Work Group (WG) of the International Workshops on Genotoxicity Testing (IWGT) systematically evaluated genetic toxicology data analysis and dose–response modeling approaches, to define best practices for the quantitative interpretation of in vivo mutagenicity data. Past WGs evaluated the strengths and weaknesses of PoD metrics and reported the following order of preference: the statistical lower bound metric of the benchmark dose (BMD) > the no observed genotoxic effect level > threshold or breakpoint dose levels (MacGregor et al., 2015). Additional IWGT Quantitative Analysis WG efforts evaluated the critical effect size appropriate for BMD modeling of genotoxicity data (White et al., 2020) and enunciated the need for a better conceptual frame of reference regarding how adjustment/uncertainty factors, particularly the effect severity adjustment factor (ESAF), should be applied to interpretation of genotoxicity data (Heflich et al., 2020; White et al., 2020). Consequently, as part of the 8th IWGT, a Quantitative Analysis WG explored how the concept of effect severity can be applied to mutation as an endpoint, with recommendations resulting from this effort presented here. Recognizing that repairability and the potentially transient nature of some genetic toxicology endpoints would add complexity to the topic, the WG elected to focus on the application of an ESAF to gene mutation data rather than to genotoxicity data more broadly. IWGT recommendations regarding the application of adjustment factor (AF) values to genetic toxicity data that account for interspecies extrapolation, intraspecies variability and susceptibility, and study duration were addressed by a separate team.

This report considers the history, rationale, merits, and concerns regarding the use of an ESAF when determining a HBGV (i.e., a dose without appreciable risk). The HBGV could be a Reference Dose, Permitted Daily Exposure (PDE) or Tolerable Daily Intake (TDI) depending on the risk assessment jurisdiction. Additionally, the report describes the toxicological endpoints that are considered severe and summarizes available guidance regarding the use of an ESAF for deriving a HBGV. The report then addresses the nature of mutation as a toxicological endpoint, what is known regarding human diseases associated with germline and somatic mutation, the numbers of human mutations known to cause phenotypes recognized as severe, and considerations surrounding the use of an ESAF for mutagenic effects. When an ESAF is used in regulatory settings, the WG recommends a flexible approach that permits a range of values, with value selection based on the impact on germ cells, the nature of the mutational target, the dose at which a significant increase in mutation is observed in relation to human exposure levels, what is known about mechanism(s) of mutagenesis at that dose, and/or any additional information regarding the potential for severe adverse effects associated with mutation.

1.1 | Overview of the use of ESAF in setting a HBGV

A human HBGV can be calculated by dividing the dose that produces a defined effect (i.e., PoD) by a composite AF that accounts for uncertainties in extrapolating from the endpoint observed in the experimental system to human population risk (Dankovic et al., 2015). Uncertainties related to extrapolation include extrapolation from

animal to human, from an average to a sensitive human, from a lowest observed adverse effect level (LOAEL) to a no observed adverse effect level (NOAEL), and from short-term to long-term exposure. The PoDs employed are generally a NOAEL, LOAEL, or benchmark dose (BMD). Although the term AF is used here, alternative nomenclature includes assessment factor, uncertainty factor (UF), and safety factor; some have reserved AF for data-derived factors (WHO, 2020a). An ESAF of 1 has been ascribed when the endpoint defining the reference study PoD is a mild and reversible toxicological endpoint. According to review articles published in 2007 and 2016 (Ritter et al., 2007; Sussman et al., 2016), an ESAF value of 10 is generally incorporated into a composite AF when the substance under consideration induces genetic toxicity, carcinogenicity, developmental/teratogenic effects (e.g., malformations), or reproductive effects (failure to produce viable offspring), all of which are considered irreversible outcomes. Indeed, irreversibility has been a consistent feature of endpoints characterized as severe. Articulation of endpoints considered severe varies across regulatory guidance documents (specifics provided below), but the endpoints recognized as severe in multiple guidance documents are neurotoxicity, reproductive effects, teratogenicity, and cancer. In this context, it is important to differentiate "severity of effect" as it refers to toxicological endpoints of greater regulatory concern (addressed further in Section 1.2 below) from the use of the qualitative descriptor "severe," which can be used when grading the magnitude of a toxicological effect (i.e., mild, moderate, marked, or severe).

The ESAF is qualitatively different from other AFs, which are intended to compensate for uncertainty in aspects of human health risk assessment and regulatory decision-making. Although other AFs address uncertainties routinely evaluated in risk assessment (Dankovic et al., 2015; Sussman et al., 2016), the ESAF incorporates the scientific judgment that more conservatism is warranted when neurotoxic, irreversible reproductive, teratogenic, or carcinogenic effects are associated with the exposure being evaluated. Although quantitative dose–response analyses and PoD derivation are routinely used to assess other toxic endpoints (Johnson et al., 2014), mutation data have been used primarily for hazard identification rather than quantitative risk assessment (Menz et al., 2023; White et al., 2020). Consequently, the appropriate ESAF value to use when determining a HBGV from a mutation reference study is largely an open question, as well as a somewhat controversial issue.

1.2 | Regulatory guidance regarding the use of an ESAF in setting a HBGV

As described by Ritter et al. (2007), the first use of an ESAF was attributed to the 1987 International Programme on Chemical Safety (IPCS) document *Environmental Health Criteria 70*: *Food Additives and Contaminants in Food*, *Principles for the Safety Assessment of Food Additives and Contaminants in Food* (WHO, 1987). This document described the potential use of "judgemental factors" that may be incorporated into the regulation of food additives, with "irreversibility of the observed effect in embryotoxicity studies" (e.g., skeletal abnormalities, teratogenicity), "age-related effects in reproduction studies," and "finding of carcinogenicity" provided as circumstances justifying use of an additional factor.

The use of an ESAF is not applied consistently by different regulatory agencies, but when used, ESAF values between 1 and 10 are prescribed (Sussman et al., 2016). An ESAF is not mentioned in Environmental Protection Agency (EPA) or Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) guidelines (Sussman et al., 2016). The ESAF is designated as F4 in quality guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). The most explicit text regarding the use of an ESAF, particularly with regard to reproductive toxicology, is found in the following current guidance documents: *ICH Impurities*: *Guideline for Residual Solvents Q3C(R8)* (ICH, 2021); *Guideline for Elemental Impurities Q3D(R2)* (ICH, 2022); and *Impurities*: *Residual Solvents in New Veterinary Medicinal Products*, *Active Substances and Excipients (Revision) VICH GL18(R)* (VICH, 2011). The relevant text states the following:

> F4 = a factor that may be applied in cases of severe toxicity, for example, non-genotoxic carcinogenicity, neurotoxicity or teratogenicity. In studies of reproductive toxicity, the following factors are used:
> - F4 = 1 for fetal toxicity associated with maternal toxicity.
> - F4 = 5 for fetal toxicity without maternal toxicity.
> - F4 = 5 for a teratogenic effect with maternal toxicity.
> - F4 = 10 for a teratogenic effect without maternal toxicity.

Examples regarding ICH's selection of the ESAF values are provided in Table 1. Severity of effect is also mentioned in ICH Guidelines regarding AF "F5," which is defined as "a variable factor that may be applied if the no-effect level was not established" (ICH, 2021; ICH, 2022; VICH, 2011). The ICH Guidance includes the text "when only a LOEL is available, a factor of up to 10 could be used depending on the severity of the toxicity." An additional 10-fold AF based on F5 was applied when the reference study endpoint was carcinogenicity with no defined NOAEL—see cumene example in (ICH, 2011b). The rationale for this practice and the impact of applying two separate AFs of 10 (F4 and F5) to PoDs based on a carcinogenicity reference study are discussed below (see Section 1.12).

The European Food Safety Authority (EFSA) document that addresses the ESAF is entitled *Guidance on Selected Default Values to be Used by the EFSA Scientific Committee, Scientific Panels and Units in the Absence of Actual Measured Data* (EFSA, 2012). Regarding "Severity and nature of the observed effect," the guidance states "The Scientific Committee considers that the need for an extra UF to allow for the severity of an effect is exceptional, and therefore recommends considering its use on a case-by-case basis." The guidance cites examples where an ESAF was considered necessary, which include fetal malformation, possible carcinogenicity with a mode of action that has a threshold, as well as developmental, neurotoxic, or immunotoxic effects (EFSA, 2012). It may be useful to note that "threshold" or "non-genotoxic carcinogens" are specified in some AF guidance,

TABLE 1 Examples of ESAF values employed by the ICH to calculate a dose without appreciable risk.

Guideline	Chemical (route of exposure)	Reference study design and point of departure	ESAF, and rationale for ESAF selection	Reference study
ICH Q3C(R8) and VICH GL18 (R)	Acetonitrile (inhalation)	Mice exposed to 50, 100, 200, and 400 ppm by inhalation 6.5 h/day, 5 days/week for 13 weeks. 100 ppm caused slightly increased liver weight in females. At higher levels changes in liver is seen, and RBC and WBC reduced.	F4 = 1, because no severe toxicity was encountered.	European Directorate for the Quality of Medicines & HealthCare (1997)
ICH Q3D(R1)	Cobalt (oral)	Cobalt dietary supplement was given orally. Hearing, vision, cardiac, and neurologic functions were assessed. NOAEL for polycythemia is 1 mg/day.	F4 = 1, because no severe toxicity was encountered.	Tvermoes et al. (2014)
ICH Q3D(R1)	Inorganic Mercury (oral)	6-month rat study with treatment by gavage. A $BMDL_{10}$ of 0.06 mg Hg/kg/day (adjusted for 5 days/week) was derived based on adverse renal effects (weight increase).	F4 = 1, because findings in the 6-month and 2-year studies were not considered significant at the lowest dose.	NTP (1993)
Q3D(R1)	Cadmium (parenteral)	Rats exposed to 0.6 mg/kg cadmium s.c. had renal damage at weeks 6–12. LOAEL of 0.6 mg/kg based on decreased body weight, increased urine volume, and urinary biomarkers.	F4 = 5, because cadmium is carcinogenic by the inhalation route and granulomas were observed by the subcutaneous route.	Prozialeck et al. (2009); Waalkes et al. (1999)
Q3D(R1)	Nickel (inhalation)	Groups of 65 male and 65 female F344/N rats were exposed to 0, 0.62, 1.25, or 2.5 mg nickel oxide/m^3 (equivalent to 0, 0.5, 1.0, or 2.0 mg nickel/m^3) by inhalation for 6 h/day, 5 days/wk for 104 weeks. 0.62 mg nickel oxide/m^3 (equivalent to 0.5 mg nickel/m^3) by inhalation produced chronic inflammation of the lung in most exposed rats by 7 months. Alveolar adenomas and carcinomas observed.	F4 = 10, because of the potential of relatively insoluble forms of Ni to accumulate in the lungs and inflammation was observed in the lungs upon histopathology after inhalation of all forms of Ni.	NTP (2006)
Q3D(R1)	Selenium (oral)	In a rat carcinogenicity study of selenium sulfide, the NOAEL for hepatocellular carcinoma was 3 mg/kg/day (1.7 mg Se/kg/day).	F4 = 10, because of the risk of selenosis (neurotoxicity).	NTP (1980)
ICH M7(R2) Addendum	Aniline and Aniline HCl (oral)	Aniline hydrochloride was administered to rats in the diet at 200, 600, and 2000 ppm (7.2, 22, and 72 mg/kg/day). Stromal sarcomas were observed in the mid and high dose groups.	F4 = 10, severe toxicity—non-genotoxic carcinogen.	CIIT (1982) ICH (2023b)

acknowledging that a risk assessment approach that employs AFs has traditionally not been used for mutagenic carcinogens. For genotoxic and carcinogenic substances, the Scientific Committee of EFSA recommends the use of a margin of exposure (MOE) approach, where an MOE is defined as the ratio of the dose needed to observe a small but measurable adverse effect relative to the human intake level. EFSA considers an MOE of 10,000 (based on the Benchmark Dose Lower Confidence Limit for an effect 10% above control, i.e., $BMDL_{10}$) to be of low concern for compounds that are genotoxic or carcinogenic (EFSA, 2012). The guidance also cites a European Union regulation that states "When the critical effect is judged of particular significance, such as developmental neurotoxic or immunotoxic effects, an increased margin of safety shall be considered, and applied if necessary" (Parliment of the European Union, 2009).

The Pest Management Regulatory Agency (PMRA) of Health Canada has published a guidance document entitled *A Framework for*

Risk Assessment and Risk Management of Pest Control Products (Health Canada Pest Management Regulatory Agency, 2021). According to this document, in addition to two 10-fold factors to account for interspecies and intraspecies variability applied to the dose that caused no adverse effect in animal studies, "additional factors may be applied to the reference dose to address the severity of an effect or any concerns of uncertainties about the toxicity information."

The World Health Organization (WHO) published *Guidelines for Drinking-water Quality* (WHO, 2017). In the derivation of chemical guideline values, this document indicates that UFs should be applied to the response considered most biologically significant, whether it is the NOAEL, LOAEL, or the BMD/BMDL. The Guidelines state "extra uncertainty factors may be incorporated to allow for database deficiencies and for the severity or irreversibility of effects." Also, "Situations in which the nature of severity of effect might warrant an additional uncertainty factor include studies in which the end-point is malformation of a fetus or in which the end-point determining the NOAEL is directly related to possible carcinogenicity."

A WHO document entitled *Assessing human Health Risks of Chemicals*: *Derivation of Guidance Values for Health-based Exposure Limits* (WHO, 1994) notes 'a number of bodies, including the WHO and Food and Agriculture Organization of the United Nations Joint Expert Committee on Food additives (JECFA) and the Joint Meeting on Pesticide Residues (JMPR) have incorporated an additional "safety factor" of up to 10 in cases where the NOAEL is derived for a critical effect that is a severe and irreversible phenomenon, such as teratogenicity or non-genotoxic carcinogenicity, especially if associated with a shallow dose-response relationship.'

In a 2020 WHO document entitled *Chapter 5*: *Dose–Response Assessment and Derivation of Health-Based Guidance Values* (WHO, 2020a), the use of the term "severity" appears to connote a high degree of (or more potent effect of) a toxicological endpoint, which is clarified by a description of the ordinal categorical responses that reflect severity categories.

In a European Chemicals Agency (ECHA) document entitled "*Guidance on information requirements and chemical safety assessment*, *Chapter R.8: Characterisation of dose [concentration]-response for human health*," the nature and severity of an effect is considered in the context of a Derived No Effect Level (DNEL) or Derived Minimal Effect Level (ECHA, 2012). The text states "The size of an assessment factor should take into account the dose spacing in the experiment (in recent study designs generally spacing of 2-4 fold), the shape and slope of the dose–response curve, and the extent and severity of the effect seen at the LOAEL." This is accompanied by the guidance that "When the starting point for the DNEL calculation is a NOAEL, the default assessment factor, as a standard procedure, is 1. However, a larger assessment factor may be applied in specific cases such as the following: exceptional cases of serious effects (e.g., severe irreversible effects, major malformations, foetal or offspring lethality) at dose levels slightly higher than the NOAEL (i.e., at the LOAEL)—this corresponds to a very steep dose–response curve" (ECHA, 2012). According to the ECHA guidance (ECHA, 2012), "for some endpoints, especially mutagenicity and carcinogenicity, the available information may not enable a threshold, and therefore a DNEL, to be established." Instead, for carcinogens and/or mutagens, the guidance recommends that a qualitative description of severity and potency of the endpoint be included in the chemical classification and labeling. Further, the guidance states that a DNEL value cannot be derived for reproductive and developmental toxicity data when genotoxicity is known to be an underlying mechanism.

1.3 | The nature of mutation

Before concluding how mutagenicity data can be interpreted with respect to the ESAF, the potential biological impact of mutation must be understood. Mutations are considered irreversible, permanent changes to a DNA sequence, even though a second mutation at the same genomic locus could theoretically reverse the result of a given mutation (Honma, 2020). Mutations are generally considered irreversible because, although theoretically possible, the probability of a rare mutagenic event being reversed by another rare mutagenic event is expected to be too low to be practically meaningful. A proportion of mutations have marked potential for causing adverse health effects (see Section 1.9 below), and are therefore considered adverse toxicological outcomes (Cho et al., 2022; Heflich et al., 2020). Mutations can cause either a gain or loss of function, or more commonly, have no discernable impact on function. Mutations can drive selection at the population, individual, cellular, and molecular levels (Lovell, 1995). Mutation can cause molecular variation that is a substrate for selection (Savino et al., 2022).

Mutations are categorized as non-synonymous or synonymous based on whether they do or do not cause an amino acid substitution in a protein-coding sequence, respectively. Non-synonymous mutations are more likely to alter protein structure and confer an altered phenotype to a mutant cell than synonymous mutations. Insertions and deletions in a protein coding sequence that cause reading frame shifts (i.e., frameshift mutations) can significantly alter protein structure thereby altering phenotype (Savino et al., 2022). Mutations that alter the function of proteins involved in normal DNA repair and metabolism have the potential to cause large numbers of secondary mutations and, consequently, are described as conferring a mutator phenotype (Kennedy et al., 2015). Mutations in genes that disrupt DNA polymerase proofreading (e.g., *Pol* ϵ or *Pol* δ) or DNA mismatch repair (e.g., *MSH2* or *MSH3*) confer strong mutator phenotypes (Loeb, 2001). In yeast, such mutations were shown to increase mutation rates 1000- to 10,000-fold above background (Kennedy et al., 2015). Somatic and germline mutations that confer a mutator phenotype increase the risk of certain cancers (Kennedy et al., 2015; Loeb, 2016). For example, pathogenic variants with a role in the repair of DNA double-stranded breaks by homologous recombination, particularly *BRCA1* and *BRCA2*, contribute significantly to the etiology of hereditary breast, ovarian, prostate, and pancreatic cancers (Sekine et al., 2021; Yamamoto & Hirasawa, 2022). Clearly, mutations capable of conferring a mutator phenotype have an associated human health risk.

Even though they may not cause an amino acid substitution, synonymous mutations can potentially alter cell phenotype. For example, some synonymous mutations can alter protein translational efficiency (Hunt et al., 2014; Robert & Pelletier, 2018). Synonymous mutations have the potential to alter gene expression levels by creating or altering promoter sequences, which can in turn impact cell phenotype (Lebeuf-Taylor et al., 2019). Mutations in exon–intron boundaries and regulatory sequences can result in aberrant transcripts and expressed proteins with altered structure and function (Anna & Monika, 2018). Increasingly, specific mutations in a variety of non-coding RNAs are being linked to disease (de Almeida et al., 2016). Given the continued advances in our understanding of gene regulation, mutations currently considered silent may have yet undiscovered functional impacts. A pan-cancer analysis concluded synonymous mutations account for 6%–8% of all mutations conferring a selective advantage (Sharma et al., 2019). According to the Human Gene Mutation Database (HGMD), ~11.7% of cancer-causing mutations were identified as splicing or regulatory in nature (Stenson et al., 2017).

Mutation frequency increases with age similarly in post-mitotic neurons and polyclonal smooth muscle, independent of cell division (Abascal et al., 2021). This phenomenon is observed across a wide range of species, with varying mutation rates, and seems to be correlated with life span. Rodents (mice and rats) with short life spans, have a high mutation rate, whereas humans, with longer life spans, have lower mutation rates (Cagan et al., 2022). Somatic mutations can cause cancer or non-neoplastic mosaicism. Interestingly, germline variants can impact the rate of accumulation of somatic variants (Olafsson & Anderson, 2021). Consequently, a disease phenotype may create a selective environment that favors the expansion of mutant clones, complicating the interpretation of mutation-disease associations.

Disease-causing somatic mutations that confer a positive selective advantage have been referred to as advantageous mutations or driver mutations, and cells carrying such mutations can spread within the tissue where they arise (i.e., via clonal expansion), thereby increasing a tissue's mutation burden (Brunner et al., 2019; Fiala & Diamandis, 2020; Gomes, 2022; Olafsson & Anderson, 2021). The same mutation may not confer a selective advantage in a different context. Accumulation of "advantageous" somatic mutations can increase the probability of developing a disease due to somatic mosaicism or neoplasia. For example, congenital overgrowth syndromes can predispose affected individuals to hypoglycemia, embryonal tumors, seizures, developmental delay, intellectual disability, and musculoskeletal complications (Manor & Lalani, 2020). Interestingly, some of the same mutations are involved in both somatic mosaicism (as overgrowth syndromes) and cancer (e.g., *PIK3CA* mutations) (Iriarte Fuster et al., 2021; Madsen et al., 2018; Wasilewska et al., 2022). In some instances, somatic mosaicism is considered a pre-neoplastic condition (e.g., clonal hematopoiesis of indeterminant potential [CHIP]) (Marnell et al., 2021).

A subset of advantageous mutations are cancer driver mutations (CDMs), which can be defined as changes in DNA sequence that confer a growth advantage on the cells carrying them and have been selected positively during the evolution of a cancer (Stratton et al., 2009). Cancer genomes contain large numbers of mutations, yet only a small fraction of these is responsible for the cell transformation(s) that lead to cancer (Dietlein et al., 2020). Many CDMs have been identified based on their prevalence in neoplastic and pre-neoplastic tissues, with the impact of functional mutations confirmed in experimental systems (Korenjak & Zavadil, 2019).

It is important to recognize that not all driver mutations in normal tissues will lead to cancer, and not every somatic cell carrying a disease-associated mutation will manifest as an individual with the mutation-associated disease. Mutations may occur in genes of cells where that gene's function is not relevant and, if a mutant cell's altered function is detrimental, a cell may be removed by immune surveillance or apoptosis (Campbell et al., 2015). Interestingly, although NOTCH1 has been implicated in several forms of cancer (Aster et al., 2017), clonal expansion of *NOTCH1* mutants in esophageal epithelia has been associated with decreased cancer risk (Colom et al., 2020, 2021), again exemplifying that alteration of cell fitness by mutation will be context-dependent. Some mutations that accumulate with age may be responsible for age-related diseases and functional declines (Cagan et al., 2022; Choudhury et al., 2022; Colom et al., 2020, 2021; Evans & Walsh, 2023; Haring et al., 2022; Martincorena et al., 2018; Moore et al., 2021; Yokoyama et al., 2019), but mutations also may have no phenotype (i.e., silent mutations). Thus, linkages between somatic mutation and disease should be considered probabilistic rather than deterministic because disease penetrance may depend on the probability of additional biological conditions having been met. Clearly the nature of mutation as a toxicological endpoint is heterogeneous and complex. Nevertheless, because of its role in the etiology of human diseases, mutation has considerable potential as an endpoint for quantitative risk assessment.

1.4 | Mutation as a toxicological endpoint for risk assessment and regulatory decision-making

Studies conducted to support product development and regulatory decisions generally assess mutation by one of several in vitro and in vivo tests that measure mutant frequency (MF) in a reporter gene (Lambert et al., 2005). According to the ICH *Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use* [S2(R1)], "Fixation of damage to DNA in the form of gene mutations, larger scale chromosomal damage or recombination is generally considered to be essential for heritable effects and in the multi-step process of malignancy, a complex process in which genetic changes might possibly play only a part" (ICH, 2011a). Currently available assays are based on detecting mutations in genes that have easily selectable phenotypes (e.g., *HPRT and xprt* in in vitro mammalian cell tests, *Tk* in the mouse lymphoma assay and human TK6 cells, along with *gpt*, *lacI*, and *lacZ* in transgenic rodent (TGR) somatic and germ cell assays, and *Pig-a* in the mammalian erythrocyte assay) (Salk & Kennedy, 2020). Methods to conduct these standard approaches for the assessment of MF and to appropriately interpret their results are described in regulatory guidelines (e.g., ICH, ECHA, and OECD)

(ECHA, 2017; ICH, 2011a; OECD, 2016a, 2016b, 2022a, 2022b). These mutation assays detect the "background" level of mutation in vehicle-treated or untreated control samples, which may be distinguished from test article-induced mutation via statistical analysis combined with the application of scientific judgment. This analysis may demonstrate a significant increase in MF over the concurrent control, a significant trend of increasing MF with increasing dose, and/or a MF in treated cultures/animals increased above historical control levels (OECD, 2016b, 2022a, 2022b). There is considerable precedent regarding the use of these assays, as well as extensive chemical databases that provide context when interpreting results, for example the *Pig-a* in vivo gene mutation assay database (Shemansky et al., 2019) (https://www.pharmacy.umaryland.edu/centers/cersi-files/) or the Transgenic Rodent Assay Information Database (Lambert et al., 2005). Information regarding the spectra of observed mutations can be derived using some currently available assays (e.g., *gpt*, *lacI*, and *lacZ* TGR assays). Analysis of mutational spectra can, in some cases, discern whether mutations were caused by endogenous or exogenous exposure, and/or elucidate mutagenic mechanisms (OECD, 2022a; Phillips, 2018). An induced mutation spectra observed in a toxicological assessment may be compared with established human mutational signatures (https://cancer.sanger.ac.uk/signatures) (Alexandrov et al., 2020), as an approach to explore etiology, understand mechanism, and further inform risk assessment (MacGregor et al., 2015).

Given the importance of mutation in human disease manifestation, the argument has been made that increases in MF induced by an etiological agent of concern could be considered, independent of any disease that may result from them, as a toxicological endpoint per se for dose–response assessment, PoD determination, and regulatory decision-making (Heflich et al., 2020). Indeed, genetic toxicologists have been advocating for the use of mutagenicity test results in a quantitative manner, as is done for other toxicological endpoints (Clayson et al., 1993; Heflich et al., 2020; Menz et al., 2023; White et al., 2020). Use of quantitative mutation dose–response data represents an advance in the state of the art for genetic toxicology when compared with the common practice of using mutation data for hazard identification to classify test articles as either mutagenic or non-mutagenic. Since mutation can be regarded as a bona fide toxicological endpoint that is irreversible in nature, with a wealth of information linking mutation to human disease etiology (discussed below), quantitative analyses of mutation per se, such as by dose–response modeling of mutation data, and extrapolation below a PoD to determine a HBGV, are expected to be useful in risk assessment (Heflich et al., 2020; Johnson et al., 2014; MacGregor et al., 2015; Menz et al., 2023; White et al., 2020). To use mutation response data (MF) for setting HBGVs, it is important to consider what ESAF value will be applied to mutagenicity-derived PoD values (White et al., 2020).

Three publications provide examples of the application of an ESAF in derivation of an HBGV, based on measurement of gene mutations in reference studies. MutaMouse mutation data, collected to evaluate the risk associated with Viracept contamination with ethylmethane sulfate (EMS), established 25 mg/kg/day as the NOAEL for mutation in the gastrointestinal tract, which represented an apparent practical threshold below which mutation induction was not observable (Bercu et al., 2009). A PDE for EMS of 0.104 mg/day (70-fold greater than that recommended by the threshold of toxicological concern, 1.5 μg/day) was calculated based on this NOAEL, using what were described as the most conservative uncertainty factors (including an ESAF of 10). Gollapudi et al. (2020) calculated an ethylene oxide PDE based on increased MF (i.e., induced mutations) measured in several gene loci (*cII*, *Kras*, *lacI*, and *Hprt*). When calculating the PDE for ethylene oxide based on mutagenicity dose–response data, a conservative default ESAF of 10 was applied to the $BMDL_{50}$ estimates "to account for the potential severity of the effect induced by genotoxicity/mutagenicity," (note: the appropriate critical effect size for mutation will be addressed in a separate Quantitative Analysis WG report). Similarly, Johnson et al. (2021) calculated PDEs for *N*-nitrosodimethylamine and *N*-nitrosodiethylamine based on previously published mutagenicity dose–response data from the *lacI* gene of Big Blue rats and the *gpt* gene of gpt delta rats, respectively (Akagi et al., 2015; Gollapudi et al., 1998). The authors noted that mutation is the most relevant key event in the adverse outcome pathway for cancer induced by alkylating agents. In the calculation of PDEs for these two chemicals, an ESAF of 10 was used because "both mutation and cancer are considered irreversible severe effects." However, Johnson et al. also noted that the ESAF was "open for modification based on increased understanding of biology and translation" (Johnson et al., 2021). Providing biological context for mutation as a toxicological endpoint and elaborating considerations in its translation to quantitative risk assessment, was a goal of the IWGT Quantitative Analysis WG.

1.5 | Sources of human genetic variation

In considering how mutation should be viewed in terms of *effect severity*, the WG reviewed the current knowledge of associations between human genetic variation and disease. Although this issue has been addressed for decades (e.g., Berg et al., 1986), the WG found it to be of foundational importance. An individual's disease risk is shaped by inherited variation, environmentally induced variation, and de novo variation that is a consequence of normal DNA replication/cell division. Of these, mutation arising from normal DNA replication likely has a major role, although this may not be the case for all diseases and affected tissues (Tomasetti et al., 2017). Based on sequencing of parent and offspring trios, the spontaneous mutation rate within human germ cells was estimated as $1–3 \times 10^{-8}$ de novo mutations per base-pair per generation; the mutation rate was shown to increase with paternal age (Abecasis et al., 2010; Conrad et al., 2011; Kong et al., 2012). From such observations, it was estimated that 30–100 de novo mutations will occur during gametogenesis (Acuna-Hidalgo et al., 2015; Morris, 2015). If 100 mutations occur at each generation, and each generation inherits half of the mutations that occurred in previous generations, it is not surprising that human genomes carry large amounts of genetic variation (Morris, 2015).

1.6 | The magnitude of human genetic variation

By analyzing 2504 individuals from 26 different populations, the 1000 Genomes Project identified 84.7 million single nucleotide polymorphisms (SNPs or population-level variants defined as having allele frequencies of ≥1%), 3.6 million short insertions or deletions (indels), and over 60,000 larger structural variants (Auton et al., 2015). The Consortium reported that >99.9% of variants are SNPs and small indels, but that structural variants affect a greater percentage of the genome. Structural variants include variation in numbers of short tandem repeats, deletions, duplications, copy number variants (CNVs), insertions, inversions, and translocations (Feuk et al., 2006). In a Sri Lankan cohort, 1.7% of individuals undergoing cytogenetic testing carried a translocation (Paththinige et al., 2019). CNVs have been observed in sequences that correspond to 12% of the genome (Redon et al., 2006). According to the 1000 Genomes Project, the average individual differs from the reference human genome at 4.1–5 million sites, including 2100–2500 structural variants affecting ~20 million bases (~1000 large deletions, ~160 CNVs, ~1100 insertions, ~4 nuclear-embedded mitochondrial DNA variants, and 10 inversions) (Auton et al., 2015).

1.7 | Functional impacts of human genetic variation

A variety of methods and prediction algorithms have been used to estimate the fraction of genetic variation that is deleterious. According to the 1000 Genomes Project (Auton et al., 2015) a typical genome contains 149–182 protein truncating variants, 10,000–12,000 peptide sequence-altering variants, and 459,000–565,000 variants within known gene regulatory regions. It has been estimated that, on average, each individual carries 250–300 loss-of-function variants in annotated genes and 50–100 variants implicated in an inherited disorder (Abecasis et al., 2010). Based on impacts to 3-dimensional protein structure, Sunyaev et al. (2001) estimated the average human genotype carries 1000 damaging non-synonymous SNPs. Using informatic tools to predict the functional impact of non-synonymous mutation (PolyPhen2, SIFT, a likelihood ratio test, and MutationTaster) and synonymous mutations (GERP, PhyloP, and SFS-Del), Tennessen et al. estimated 47% of non-synonymous and 6% of synonymous variants are deleterious (Tennessen et al., 2012). Using PolyPhen2, Subramanian estimated that 48% of non-synonymous SNPs and 53% of non-synonymous mutations with allele fractions of <0.002 are deleterious (Subramanian, 2012a; Subramanian, 2012b). A proteome-wide missense variant effect prediction tool, AlphaMissense predicted 32% of all missense variants are likely pathogenic and 57% are likely benign (Cheng et al., 2023).

1.8 | Associations between human genetic variation and disease

Several factors make it challenging to associate disease-causation with specific genetic variants. Inheritance pattern of variants (autosomal/X-linked/Y-linked, dominant/recessive), and their degree of penetrance (complete or incomplete), may obscure genotype–phenotype associations (Jackson et al., 2018). A recent study that analyzed 37,780 clinical variants involved in 197 diseases by whole exome sequencing of 72,434 individuals reported 6.9% penetrance of known pathogenic variants (Forrest et al., 2022). Generally, recessive mutations may not confer a phenotype on an individual early in life. However, recessive mutations may manifest early in the offspring of consanguineous marriage, may be manifested later in life, and can be passed to future generations (Hanany et al., 2020; Lovell, 1995). Consequently, the recessive carrier frequency in a population is a public health concern.

A few human health beneficial germline mutations (i.e., polymorphisms) have been reported, such as those protective against developing type 2 diabetes, HIV infection, or bubonic plague (Flannick et al., 2014; Klunk et al., 2022; Unutmaz, 2022). Other mutations that increase a cell's fitness and cause cells to acquire a selective/proliferative advantage can lead to clonal expansion (Martincorena, 2019; Martincorena et al., 2018). Clonal expansions of mutant cells may be pathologically benign and indistinguishable from normal cells, but some clones may result in cancer initiation, thereby increasing the risk of cancer. Mutations also can be neutral or decrease cell fitness, resulting in either no impact on cell status or an increase in the potential for senescence/cell death, respectively (Tenaillon & Matic, 2020). For example, in *HPRT* mutant heterozygous females, where random X-inactivation should render 50% of cells mutant, only 10% of T and B cells are *HPRT* mutant (Hakoda et al., 1995). Although this has been interpreted as evidence of selection against *HPRT*-negative blood cells, the same sequence changes appear to be neutral when they occur as rare somatic cell mutations (Hakoda et al., 1995). There are examples of mutations associated with disease in one context that are protective in another context. Mutation in the ß-globin gene causes the sickle cell trait, which can have detrimental effects (exercise-related injury, renal complications, and venous thromboembolism) in affected carriers, although the mutation is a largely protective in the context of malaria (Naik & Haywood, 2015), potentially explaining why the mutation persists in the gene pool. Inherited mutations in the *Bruton tyrosine kinase* (*BTK*) gene block B-cell development giving rise to X-linked (Bruton's) agammaglobulinemia, however these mutations also make carriers impervious to infection by Epstein–Barr virus (Faulkner et al., 1999).

Mutations that contribute to multigenic disease causation will be more difficult to discover than those that exhibit Mendelian patterns of inheritance. Polygenic obesity, for example, is believed to be due to hundreds of polymorphisms, each having a small effect (Loos & Yeo, 2022). Regarding osteoporosis, 501 loci and 1103 independent associations explain only ~20% of bone mineral density (Abood & Farber, 2021). Multigenic causation is being addressed by efforts to associate polygenic risk scores with disease phenotypes, so more information regarding disease-conferring combinations of mutations may be available in the future (Dehestani et al., 2021; He et al., 2022; Liu et al., 2021; Torkamani et al., 2018). Diseases with causation that involves non-genetic determinants, in addition to genetic determinants, may be difficult to identify. Non-genetic determinants of

disease causation may include environmental, physical, immune, epigenetic, or other biological triggers. For example, diet and exercise may modify the penetrance of a SNP associated with diabetes, and smoking might modify the penetrance of a mutation that drives lung cancer (Jackson et al., 2018). In many cases, the reason one individual with a genetic variant develops disease and another individual with the same variant does not is unknown (e.g., *BRCA1/2* variants and breast/ovarian cancer) (Jackson et al., 2018).

Despite these obstacles, genome-wide association studies (GWAS) have identified thousands of genetic variants linked to the risk of human disease (Sun et al., 2022). The Online Mendelian Inheritance in Man (OMIM) database contains 7378 phenotypes for which the molecular basis is known, including 4804 phenotype-causing mutations (OMIM, 2023). The HGMD (release 2023.1) contains 410,743 unique disease-associated mutations of which 286,571 are categorized as disease-causing (Stenson et al., 2017). Human genetic variation is shaped by selective pressures operating at the population level. The majority of polymorphisms (population frequency ≥1%) are believed to be neutral; whereas, deleterious mutations are selected against and, consequently, rarer (Morris, 2015). There are estimated to be 5000–8000 monogenic diseases (i.e., single-gene disorders) (Jackson et al., 2018; Prakash et al., 2016). There are reported to be over 6000 inherited disorders and it has been estimated that 65% of people have a health problem resulting, at least in part, from congenital mutations (Acuna-Hidalgo et al., 2016). In Europe, chromosome abnormalities account for ~15% of the major congenital anomalies diagnosed before age 1, and chromosome abnormalities are associated with 25% of perinatal deaths due to congenital anomalies (Wellesley et al., 2012). In the United States, it has been reported that chromosomal disorders account for 5%–7% of still births (Lovell, 1995).

GWAS initially focused on discovering the genetic bases for common diseases (Zuk et al., 2014), consequently much remains to be discovered regarding the genetics underlying rare diseases. Rare diseases are defined differently in different countries. Rare diseases are defined as those effecting <200,000 people in the United States or <1 in 2000 people in the European Union. Genetic disorders represent 80% of rare disorders (Jackson et al., 2018). It has been reported there are 5000–8000 rare genetic diseases that affect 30 million people in the United States (1 in every 10 individuals) and 300–400 million people world-wide (Haendel et al., 2020; Lee et al., 2020; Marwaha et al., 2022). Only 3654 unique genes have been associated with 3551 rare diseases (Boycott et al., 2017). Variants represented at low frequency in populations may contribute to rare diseases. According to various reports, 76%–95% of single nucleotide variants (SNVs) have a minor allele fraction of <0.5% and, therefore, are appropriately identified as mutations (population frequency <1%) (Auton et al., 2015; Nelson et al., 2012; Tennessen et al., 2012). Nelson et al. (2012), state "because of rapid population growth and weak purifying selection, human populations harbor an abundance of rare variants, many of which are deleterious and have relevance to understanding disease risk." Future progress in associating mutations with rare diseases will likely require the use of larger sample sizes, technologies to detect non-coding genetic changes (e.g., transcriptomics, epigenetic analyses), and novel strategies for case matching (Kierczak et al., 2022; Marwaha et al., 2022).

1.9 | Population risk of severe disease associated with germ cell and somatic cell mutation

The WG collected information on the relative contributions of germline and somatic mutations to human morbidity/mortality correlates of toxicological endpoints that are considered severe (neurotoxic, irreversible reproductive, teratogenic, or carcinogenic effects) and believed to have a genetic etiology. The documentation provided in Table 2 is stratified in terms of the genetic impact on germ cells or somatic cells, but does not impute endogenous or exogenous etiology to the causal events.

The extent to which de novo and inherited mutations in germ cells contribute to human disease remains uncertain; new mutation phenotype associations are still being discovered. When genomes of more than 13,500 UK and Irish families having a child with a severe undiagnosed developmental disorder were sequenced, genetic diagnoses were derived for 5500 children that involved more than 800 different genes and 60 new conditions were identified (Wright et al., 2023). Of the 3599 family trios analyzed, 2750 (76%) had a pathogenic de novo variant (Wright et al., 2023). According to the information collected in Table 2, infertility is the most frequent health effect related to germ cell mutation, even though only ~50% of infertility may be due to genetic defects (Zorrilla & Yatsenko, 2013). Although difficult to quantify, some infertility is a consequence of germline mutations that are incompatible with life (i.e., mutations known to cause obligatory mosaic diseases) (Youssoufian & Pyeritz, 2002). Other severe disease consequences of germline mutations are birth defects, neurological diseases, inherited cancer syndromes, and germ cell tumors.

Worldwide, 6% of births (accounting for 7.9 million children per year) manifest a serious birth defect of genetic or partially genetic origin (Zarocostas, 2006). A third of all infant deaths are due to a genetically influenced condition or serious birth defect (Lovell, 1995). In the United States, major structural or genetic birth defects occur in 3% of births and are a major contributor to infant mortality, as well as long-term disability (Centers for Disease Control and Prevention, 2008). Mutation in germ cells is an underlying cause of many birth defects, with a varying impact of gene mutation (versus chromosomal effects) for different types of birth defects.

The genetic etiology of neurodevelopmental disorders is heterogeneous (Brunet et al., 2021). Yet, de novo mutations may be important factors for patients with neurological disorder (Brunet et al., 2021; Erickson, 2016; Karam et al., 2015; Pekeles et al., 2019). The majority of neurodevelopmental disorder-causing variants identified by Brunet et al. (2021) were de novo rather than inherited variants, and 746 gene mutations have been associated with intellectual disability (Kochinke et al., 2016). Single detrimental de novo mutations that occur predominantly in egg or sperm have been reported to

TABLE 2 Population impact of severe human diseases with genetic causation.

Type of mutation	Nature of the risk		Estimated fraction of population effected (rate per 100,000)	Description of the reported statistic and association with mutation	References
Germ cell	Individual and Multigenerational	Infertility[a]	12,500/10,000 (~6250/~5000)	Lifetime risk of experiencing infertility (and that attributed to genetic defects) among women/men[b]	Zorrilla & Yatsenko (2013); Datta et al. (2016)
		Birth defects[a]	2534	All anomalies reported in Surveillance of Congenital Anomalies in Europe (EUROCAT), includes all fetal deaths, still births, live births, and termination of pregnancy for congenital abnormalities reported in 2020.	European Commission (2022)
			3000	Major structural or genetic birth defects affect ~3% of births in the United States.	Centers for Disease Control and Prevention (2008)
		Neurological disease	90.9	Neurologic conditions known to be caused by highly penetrant mutations in monogenic disorders in the North of England based on literature published between 1966 and 2015	Bargiela et al. (2015)
		Cancer	9–19	The age-standardized rate for all cancers (excluding non-melanoma skin cancer) for men and women combined was 190 per 100,000 in 2020. 5%–10% of cancer cases are due to genetic changes in germ cells (de novo or inherited).	Tsaousis et al. (2019); WCRF International (2023)
		Germ cell tumors	1.2–6.7	Testicular germ cell tumors reported in US men between 1998 and 2011, with variation in the rate related to race/ethnicity	Ghazarian et al. (2015)
			<1	Worldwide incidence of ovarian germ cell tumors reported between 2008 and 2012	Hubbard & Poynter (2019)
Somatic cell	Individual	Cancer	130	Worldwide deaths in 2019	Global Burden of Disease Cancer Collaboration (2022)
			3240	Worldwide disability-adjusted life years in 2019	
			144	US Deaths in 2020	Centers for Disease Control and Prevention (2022)
		Non-cancer mosaicism	Not yet available	Topographic, local diseases and overgrowth syndromes, hematologic diseases, autoimmune and other immunological diseases, autism and neurologic diseases, cardiovascular, and liver disease	Mustjoki & Young (2021); Ng et al. (2021); Choudhury et al. (2022); Wasilewska et al. (2022)

[a]To avoid redundancy, the human disease corresponding to reproductive toxicity was captured as infertility and teratogenicity captured as birth defects.
[b]Infertility is defined as failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse.

TABLE 3 Search terms applied to the Human Gene Mutation Database (HGMD).

Severe endpoints	Toxicological definition	Terms used to search the HGMD for pathologies/diseases related to a severe endpoint
Cancer	Malignant growth or tumor resulting from division of abnormal cells, which may be created or promoted by toxic substances	Cancer, tumor, neoplasia, hyperplasia, malignant neoplasm
Neurotoxicity	ICH Q3C/D: The ability of a substance to cause adverse effects on the nervous system Damage to the brain or peripheral nervous system caused by exposure to natural or man-made toxic substances	Intellectual disability, nervous system disorder, neurodegenerative, central nervous system, cognition, neurological, Huntington, Parkinson, Alzheimer, dementia, encephalopathy, microcephaly, peripheral neuropathy, convulsions, ataxia, myoclonus, optic neuritis, mental retardation, hearing, vision, blindness, visual, nystagmus, speech, psychomotor, seizure, learning, hyperexcitability (selected based on Moser et al. (2013))
Reproductive toxicity	"The antagonistic effects of a substance on any characteristics of the male or female sexual reproductive cycle, together with an impairment of reproductive function, and the induction of adverse effects in the embryo, such as growth retardation, malformations, and death which would interfere with the production and development of normal offspring that could be reared to sexual maturity, capable in turn of reproducing the species" [Bremer et al. (2005) PMID: 16194149]	Stillbirth, preterm, infertility, premature ovarian insufficiency, recurrent spontaneous abortion, miscarriage, developmental, overgrowth (selected based on Toragall et al. (2022))
Teratogenicity	ICH Q3C/D: The occurrence of structural malformations in a developing fetus when a substance is administered during pregnancy The property or capability of producing congenital malformations	Malformation, congenital heart, neural tube, encephalocele, polydactyly, syndactyly, thumb, myelomeningocele, club foot, talipes equinovarus, Fryns, Miller, acrofacial, Robin, craniofacial, cleft, dysostosis, cerebrocostomandibular, Guion-Almeida, choanal atresia, micrognathia, microtia, aural atresia, coloboma, microcephaly, limb deficiency, spina bifida, skeletal, short stature, omphalocele, gastroschisis, urogenital, duodenal atresia, esophageal atresia, intestinal malrotation, hypospadias (selected based on Cassina et al. (2017), Holmes (2010), Toragall et al. (2022))

cause rare developmental disorders, including Schinzel–Giedion syndrome, Baraitser–Winter syndrome, Kabuki syndrome, intellectual disability, and autism (Acuna-Hidalgo et al., 2015).

Hereditary cancer predisposition syndromes represent 5%–10% of all cancers (Tsaousis et al., 2019). Pathogenic germline variants were identified in 7.8% of 6009 cancer patients, and ~5% of the variants were characterized as highly-penetrant (Srinivasan et al., 2021). Analysis of 746 individuals with a family history of cancer identified known pathogenic variants in 22%, of which ~90% of the variants were frameshifts, nonsense, missense, or splicing mutations (Tsaousis et al., 2019). Pathogenic SNPs have been observed in germ cell tumors, particularly in the neoplasms of adolescents (Fonseca et al., 2019). Single gene mutations are observed, but uncommon in testicular germ cell tumors (Sheikine et al., 2012).

The health impact of germline mutations has been evaluated in terms of years of lost and impaired life (Czeizel et al., 1988). Specifically, the impact of heritable genetic variation in terms of disability-adjusted life years (DALY) has been described for over 80 diseases (Jukarainen et al., 2022). Disease manifestation due to germ cell mutations can be exacerbated by consanguinity and it has been estimated that 10% of marriages worldwide are between first and second cousins (Bittles & Black, 2010; Lovell, 1995). Given this information, a judgment can be made that birth defects are the most impactful societal risk due to germ cell mutation, based on the frequency with which they occur and their potential to cause mortality or life-long disability.

As a complement to the above literature review on associations between germ cell mutation and severe disease, an analysis was conducted to extract information from the HGMD on the human germline mutations known to produce diseases corresponding with rodent endpoints recognized as severe in guidance documents. Search terms were collected from reference materials that could be used to identify human phenotypes corresponding to severe toxicological endpoints (cancer, neurotoxicity, irreversible reproductive toxicity, and teratogenicity) (see Table 3). Using the search terms identified in Table 2, disease/phenotype searches were conducted within the HGMD, which is comprised of two separate databases for single base substitutions (SBSs) and micro-lesions (defined as insertions/deletions ≤21 base pairs) (QIAGEN HGMD® Professional 2022.1; Stenson et al., 2017). Information was collected on the numbers and genomic positions of mutations considered causal for the identified phenotypes (see Table 4). The HGMD is comprised of germline variants within coding, splicing, and regulatory regions of human nuclear genes. Somatic mutations and mutations in the mitochondrial genome are not included. Each mutation is entered into the database only once, to avoid confusion between recurrent and identical-by-descent mutations. HGMD mutations are identified by a unique mutation

TABLE 4 Numbers of HGMD mutations and mutable sites underlying human phenotypes corresponding to "severe" toxicological endpoints.

HGMD mutation database searched	Human search terms selected based on a severe toxicological endpoint	Number of unique HGMD mutations recovered	Number of unique HGMD mutations based on GrCh38 position	Number of unique HGMD mutations combined for all search terms	Number of HGMD mutated GrCH38 positions combined for all search terms
Disease-causing Single base substitutions	Cancer	5218	4560	26,644	25,195
	Neurotoxicity	16,401	15,797		
	Reproductive toxicity	4309	4234		
	Teratogenicity	4231	4148		
Disease-causing micro-lesions (insertions/deletions ≤21 base pairs)	Cancer	6085	5960	13,247	13,089
	Neurotoxicity	5306	5285		
	Reproductive toxicity	1573	1566		
	Teratogenicity	1625	1623		
Single base substitutions and micro-lesions combined	Cancer	11,303	10,520	39,891	37,967
	Neurotoxicity	21,707	21,082		
	Reproductive toxicity	5882	5800		
	Teratogenicity	5856	5771		

identification number and their location in the human reference genome (GrCh38), allowing mutations recovered in different searches to be deduplicated. Only mutations in the HGMD categorized as "disease-causing" were collected (i.e., those derived from publications where the authors indicated the alteration described conferred the specified clinical phenotype) (Stenson et al., 2017).

Several conclusions were drawn from this analysis. Of the 226,502 disease-causing mutations included in the HGMD release 2022.1, ~40,000 (17.6%, considering SBSs and micro-lesions) have the potential to cause diseases/phenotypes corresponding to the toxicological endpoints identified as severe. SBSs capable of causing the diseases/phenotypes that mirror severe toxicological endpoints occurred at ~38,000 positions within the genome. Given that a haploid human genome contains ~3.117 billion bases (human genome version, T2T CHM13v2.0/hs1) (Nassar et al., 2023; T2T Consortium, 2023) and ~25,000 disease-causing SBSs have been documented in the HGMD, it can be estimated that 1 of every ~125,000 bases may cause a disease equivalent to a severe toxicological endpoint when mutated.

There are many caveats related to the above-mentioned estimates. The number of disease-causing mutations could be underestimated for several reasons. It is likely only a subset of all severe disease-causing SBSs, small insertions/deletions, and frameshift mutations are represented in the HGMD. Many mutations contributing to multigenic or multifactorial causation of severe disease may be undiscovered. Copy number variation, loss of heterozygosity, chromothripsis, and chromosome rearrangements are not represented in the HGMD, and such events are also expected to contribute to severe disease. Given that mutations with the highest level of evidence were evaluated, mutations that cause severe disease, but have not been studied sufficiently, may be omitted. Also, given that the HGMD curates inherited mutations, germline mutations that result in loss of viability (i.e., lethal mutations) are not represented in the HGMD. A different approach, like mouse embryo viability screening, is needed to identify lethal, homozygous, loss of function mutations (Cacheiro et al., 2022). In addition, some mutations may have been incorrectly identified as disease-causing. Finally, it is important to acknowledge that mutagenesis varies across target sequences, so any estimate of mutations per number of nucleotides calculated for the entire genome may not be accurate for specific regions of the genome. Despite these caveats, the analysis presented in Table 3, along with the information above, provides a snapshot of current understanding regarding human mutation burden, germline mutation–phenotype associations, and an estimate of the minimum number of positions that when mutated in the human genome could cause a severe disease.

Diseases associated with somatic cells can be categorized as those related to cancer and those related to non-cancer somatic mosaicism. There is a wealth of information describing the occurrence of CDMs detected in cancers (Bailey et al., 2018; Martínez-Jiménez et al., 2020; Poulos & Wong, 2019). The tissue-specific impact of different driver mutations, and how that relates to the selective advantage conferred on a cell, has been reviewed (Bianchi et al., 2020; Harris et al., 2020). Databases have been developed that contain astounding numbers of mutations observed in tumors. The Catalog of Somatic Mutations in Cancer (COSMIC, https://cancer.sanger.ac.uk/cosmic) curates reports of ~23 million mutations and COSMIC's cancer gene census includes mutations in 579 genes identified as having the highest level of evidence of functional impact (Tier 1). The International Cancer Genome Consortium (ICGC, https://dcc.icgc.org/) curates ~81 million tumor mutations, with ~760,000 defined as having high functional impact. The Cancer Genome Atlas Project (TCGA, https://portal.gdc.cancer.gov/) curates almost 3 million tumor mutations. The strength of known associations between cancer etiology and driver mutations is highlighted by the ever-expanding number of resources available for selecting therapy based on the genetic profile of a patient's cancer (Berger & Mardis, 2018; Damodaran et al., 2015).

A precision oncology knowledge base (Oncokb, https://www.oncokb.org/) annotates mutations in terms of their oncogenic effect(s), level of evidence, prognostic implications, and their predictive value in terms of clinical benefit or resistance associated with specific therapies (Chakravarty et al., 2017). Clearly, the disease impacts of CDMs are well-studied compared to mutations associated with other diseases.

The disease consequences of somatic mosaicism and obligatory mosaic diseases have been reviewed (Campbell et al., 2015; Erickson, 2003; Erickson, 2010; Olafsson & Anderson, 2021; Wasilewska et al., 2022; Youssoufian & Pyeritz, 2002). Significant progress is being made toward identifying and studying the human health impacts of non-cancer somatic mosaicism (Mustjoki & Young, 2021; Thorpe et al., 2020), but clarity regarding the portion of non-cancer diseases attributable to genetic mosaicism lags far behind the cancer field. Somatic mosaicism is implicated in a number of disease phenotypes, such as paroxysmal nocturnal hemoglobinuria and neurofibromatosis I (Erickson, 2003), Duchenne Muscular Dystrophy (Erickson, 2010), inflammatory bowel disease (Olafsson & Anderson, 2021), and cardiovascular disease (Choudhury et al., 2022; Evans & Walsh, 2023; Haring et al., 2022; Heimlich & Bick, 2022).

It has been recognized that for disease-causing somatic mutations to have a significant effect on phenotype they must reach a minimal prevalence within a tissue (Olafsson & Anderson, 2021). The accumulation and spread of mutant cells through a tissue have the potential to override normal physiological function, which can lead to increased disease risk (Eng et al., 2020; Mustjoki & Young, 2021; Youssoufian & Pyeritz, 2002). Consequently, the degree of disease manifestation due to specific mutations driving somatic mosaicism is impacted by the developmental timing of mutation, with mutations occurring early during development having the greatest impact on phenotype due to the greater potential for exponential cellular expansion (Campbell et al., 2015; Freed et al., 2014). The rate of accumulation of mutation can be rapid in early development, that is, before birth (Manders et al., 2021) and, consequently, the most severe non-cancer outcomes due to mosaicism have been linked to pre-natal or peri-natal development. This is manifest in cellular overgrowth syndromes, a diverse set of conditions defined by excessive proliferation of organs or tissues in association with vascular anomalies, where a specific set of causal mutations is associated with each condition (e.g., Klippel–Trenaunay Syndrome, Parkes Weber Syndrome, or Proteus Syndrome) (Eng et al., 2020).

It has been reported that 6.5% of presumed germline mutations are in fact post-zygotic mosaic mutations (Acuna-Hidalgo et al., 2015). Therefore, every individual likely carries some amount of somatic mosaicism. An analysis of embryonic mosaic mutations, conducted by sequencing RNA from 49 normal tissues of 570 individuals, concluded newborns carry 0.5–1 exon mutations affecting multiple organs (Muyas et al., 2020). The predominant mutational specificity observed was consistent with spontaneous deamination of methylated cytosines (Muyas et al., 2020). Somatic mosaicism has been implicated in more than 30 monogenic disorders (Youssoufian & Pyeritz, 2002).

A precise understanding of the extent of human disease related to somatic mosaicism in adults remains uncertain. Broadly, somatic mosaicism has been described as causing benign disease (Mustjoki & Young, 2021), because it most often contributes to declining health during aging rather than outright lethality. Somatic mosaicism has been reported in genes that impact immune disorders (Solis-Moruno et al., 2021). Autism and liver disease have been associated with high burdens of somatic mosaicism (Brunner et al., 2019; Rodin et al., 2021). Somatic mutations increase in differentiated liver cells with age, where they may causally contribute to age-related functional decline (Brazhnik et al., 2020). Also, age-related increases in somatic mutation have been reported for human neurons, esophageal epithelial cells, and cardiomyocytes, with relatively high levels of mutation being observed in middle-aged individuals with apparently "normal tissues" (Choudhury et al., 2022; Lodato et al., 2018; Martincorena et al., 2018). This suggests expression of disease state may involve additional factors.

1.10 | Evidence exogenous exposure to mutagens can cause severe disease

There is evidence exogenous human exposures induce somatic mutations that contribute to severe disease, most notably cancer. While many mutations that accumulate in tissue during normal aging are induced by endogenous processes, exogenously induced CDMs may cooperate with endogenously induced CDMs to initiate or progress cancer development (Parsons, 2018; Rosendahl Huber et al., 2021). Many somatic mutagens that cause human cancer have been identified (International Agency for Research on Cancer, 2019; Rosendahl Huber et al., 2021; Yoshida et al., 2020). Mutational signatures have been associated with known carcinogenic human exposures, including exposure to ultraviolet radiation, tobacco smoking/chewing, aristolochic acid, aflatoxin B1, platinum compound chemotherapy, azathioprine therapy, temozolomide, benzene, and occupational haloalkane exposure (Alexandrov et al., 2020; Poon et al., 2014). The percentages of human cancers that are due to intrinsic versus extrinsic causes have been estimated. Tomasetti and Vogelstein suggested that most cancers may be a consequence of replicative errors related to the extent of organ-specific cell division, with only onethird of cancers due to environmental factors and inherited variation (Tomasetti & Vogelstein, 2015). Parkin et al. (2011) asserted that less than optimal "exposure" to 14 modifiable factors accounts for 42.7% of tumors in the United Kingdom, with tobacco smoking, overweight/obesity, and alcohol consumption identified as the most important factors contributing to tumor incidence. It has been estimated that 80%–90% of lung cancers and 86% of melanomas involve exogenous mutagenic exposures (smoking and ultraviolet radiation) (Parkin et al., 2011; Peto et al., 2000).

Compared to the number of known human somatic cell mutagens, there are relatively few recognized germ cell mutagens. Tobacco smoke has been identified as a germ cell mutagen (Marchetti et al., 2011) for the spermatogonial stem cells of smokers and their offspring (Omolaoye et al., 2022). Maternal and paternal smoking has

also been associated with teratogenicity and neurotoxicity, respectively (Beal et al., 2017; Hackshaw et al., 2011). Ionizing radiation, chemotherapy, and air pollution have been suggested as possible human germ cell mutagens (Demarini, 2012). Exposure to heavy metals, like lead and cadmium, can be mutagenic, carcinogenic, neurotoxic, and cause reproductive effects (Ariza & Williams, 1999; Pugsley et al., 2022; Tchounwou et al., 2012).

The ability to draw causal relationships between exogenous exposures, mutation, and disease manifestation (including severe disease manifestation) is limited in critical ways. For any specific mutation, it is not possible to establish with certainty whether the mutation occurred spontaneously or was induced by an exogenous exposure (Lovell, 1995). Consequently, molecular epidemiological studies rely upon exposure assessment and statistical probability to draw conclusions at the population level (Ogino et al., 2016). Although information on the source of mutation can be gleaned through analysis of trios, it is often unclear whether the mutational event(s) leading to disease occurred de novo in affected individuals or occurred in the past and were inherited with additional factors needed for disease manifestation in the affected individual (Lovell, 1995). According to the data collected in Table 2, cancer is the most impactful manifestation of somatic mutation on human health, with 23.6 million new cases and 10 million deaths attributed to cancer worldwide in 2019 (Global Burden of Disease Cancer Collaboration, 2022). However, the WG recognized that germ cell mutations, generally, evince greater disease penetrance than somatic mutations (Godschalk et al., 2020) and germ cell mutagenicity health risks attach to multiple generations, rather than to an individual. Consequently, there was consensus among the WG that possible disease induction in unexposed offspring due to exogenously induced germ cell mutagenesis in exposed parents represents the most significant risk to human populations.

1.11 | Direct measurement of mutations with human health impact by error corrected NGS

The use of reporter gene assays to quantify mutation induction is sensitive and efficient but, as discussed above, has many caveats associated with interpretation in terms of disease risk. Error corrected NGS (ecNGS) technologies, like Duplex Sequencing, Hawk-Seq, NanoSeq, SMM-Seq, CODEC, PECC-Seq, and PacBio HiFi sequencing are quantitative approaches that can identify both germline variants and somatic mutations (Bae et al., 2023; Maslov et al., 2022; Matsumura et al., 2019; Miranda et al., 2022; Salk & Kennedy, 2020; You et al., 2023). The strength of these technologies in regulatory genetic toxicology is that, theoretically, induction of rare somatic mutations in any segment of the genome can be quantified, using DNA isolated from any tissue of any species (Marchetti et al. 2023b). This means ecNGS can be used to quantify mutations with known health impacts in human samples, as well as the homologs of human mutations in model systems. Thus, there is considerable enthusiasm regarding the potential of ecNGS to advance the testing needed for mutagenicity and carcinogenicity safety assessments and managing human health risks due to chemical exposures (Marchetti et al. 2023a; 2023b).

Moving forward, it will be important to identify the areas where ecNGS analyses will be most useful. ecNGS can be used to study mutagenesis in the same DNA targets currently used in phenotypic selection-based mutation assays (Marchetti et al. 2023a; 2023b; Valentine et al., 2020). However, in terms of understanding relationships between exposures, mutations, and disease, an advantage of ecNGS is the ability to examine DNA targets known to cause disease when mutated. Specifically, ecNGS has the potential to detect mutagen-induced low frequency somatic mutations equivalent to known disease-causing variants/single nucleotide polymorphisms. Using ecNGS for this purpose will add complexity to genetic toxicology assessments because the targets need to be identified in advance. Yet this approach may produce evidence and knowledge that was previously inaccessible.

Mutation analysis of generic targets by ecNGS could be used for dose–response modeling and PoD determination, as has been done for other mutational endpoints, with the application of appropriate AFs to derive a HBGV. In addition, the ability of ecNGS to analyze disease-associated mutational targets could be used to address specific risk assessment questions. For example, analysis of mutagenesis in human-relevant, disease- or tissue-specific in vitro models with human metabolic activation could be performed to better understand the relevance of potential human exposures. In vivo analyses of mutation using the model and panel of targets most relevant to potential toxicity in humans could be performed. An intellectual disability-associated gene panel could be used to investigate dose–response mutational data of a chemical linked to autism. A leukemia-associated gene panel could be used to investigate a chemical suspected of causing myeloproliferative disease. Specific panels could be dispensed with if a whole genome ecNGS method were used, like PacBio HiFi sequencing (Miranda et al., 2022). Potentially, disease-relevant DNA sequence targets in exposed or potentially exposed human populations could be analyzed (i.e., human biomonitoring), allowing those results to be correlated with results obtained using in vitro and in vivo models. Thus, analysis of disease-associated mutations by ecNGS has the potential to clarify the interpretation of mutation data with respect to effect severity.

1.12 | Considerations regarding the application of an ESAF in the derivation of an HBGV from mutation dose–response data

When selecting an ESAF for mutation, the WG recommends considering the totality of evidence regarding how the experimentally observed induced mutational response relates to the likelihood that exposure to the test article will induce a severe phenotype in humans. Factors that should be considered include the type of cell mutated (e.g., germline or somatic), the nature of the DNA sequences where mutation was observed, the dose at which a significant increase in mutation was observed in relation to human exposure levels, and

what is known about the mechanism(s) of mutagenesis at that dose. How these factors should influence selection of an ESAF is discussed briefly.

- Given the conclusion that germ cell mutations can confer multigenerational and potentially long-term population risks, evidence of germ cell mutagenicity should trigger consideration of an ESAF of 10, at least for regulatory scenarios where an increased population risk of germline effects cannot be ruled out.
- Not all mutational targets are expected to have the same susceptibility for mutagenesis. Notably, microsatellite sequences, ribosomal RNA gene clusters, segmental duplications, and mtDNA have high mutation rates, with higher mutation rates also observed in intergenic versus genic sequences and non-transcribed versus transcribed DNA strands (Campbell & Eichler, 2013; LeBlanc et al., 2022; Valentine et al., 2020). Thus, extrapolation from the reporter gene targets in transgenic models to the genome sequences in humans with the potential to cause disease is inexact, potentially due to differences in the sequence context (Oman et al., 2022), transcription-coupled repair (Vrieling et al., 1998), and the fraction of the target sequence that matches the mutational specificity of the mutagen. Despite these caveats, the transgenes of TGR models, the *Pig-a* reporter gene, and the *Hprt* gene are viewed as sensitive and useful sentinels of the in vivo mutagenesis occurring throughout the genome. In the future, uncertainty regarding the target for mutagenesis may be reduced by direct analysis of disease-relevant mutational targets (as per the above discussion of ecNGS). If mutation induction was observed in a target known to cause a severe phenotype, then an ESAF of 10 should be considered.
- In some circumstances, dose level(s) at which an adverse effect is detected may be a factor in selecting an ESAF. If it is established that significant induction of gene mutation occurs only at a dose that alters the chemical's mode of action from that observed at lower dose (e.g., the mutagenic effect is a consequence of increased cell proliferation resulting from frank toxicity that does not occur at lower doses), then use of an ESAF value <10 may be justified.

Some practical issues that should be considered for the proper application of the ESAF involve the endpoint to which the ESAF is applied and potential redundancy in AFs. In some cases, studies documenting a severe endpoint have been deemed inappropriate for PoD determination and the ESAF was applied to a different, non-severe endpoint (Renwick, 1995). Examples of this relative to cadmium and selenium are provided in Table 1. When a deficiency in a mutation study precludes its use for PoD determination, the WG does not recommend that an ESAF based on mutation data be applied to the PoD for a different toxicological endpoint (i.e., a PoD for a non-mutation endpoint). In such situations, if an extra AF is deemed necessary, an AF for database deficiency might be more appropriate, thereby communicating that the deficiency in the mutation data prohibits its use as a reference study for risk assessment.

It is acknowledged that the AFs are intended to account for different uncertainties or factors related to risk and care should be taken to ensure the applied AFs are not redundant (Sussman et al., 2016). According to ICH guidelines (ICH, 2021; ICH, 2022; VICH, 2011), an AF up to 10 could be used "depending on the severity of the toxicity" when a LOAEL is used as a PoD. Derivation of a PDE, therefore, may involve the application of two AFs of 10 based on the use of a carcinogenicity reference study, one factor of 10 for effect severity and one for the use of a LOAEL. The PDE derived for cumene provides a relevant example (ICH, 2021). Given high background rates of some tumors, the limited number of doses tested, and the difficulty in selecting doses for a carcinogenicity bioassay, it is common that a NOAEL for carcinogenicity is not established. Consequently, a carcinogenicity reference study often engenders two AFs of 10, decreasing the PDE by a factor of 100, which may be perceived as excessive. To reduce redundancy in the use of AFs, one may evaluate the dose–response to determine whether an AF between 1 and 10 for extrapolation from LOAEL to NOAEL is justified or explore whether BMD modeling, to obtain a BMDL, is a better option (e.g., to remove the need for an additional AF based on use of a LOAEL). In the long-term, more precise and expedient carcinogenicity testing and cancer risk assessment may be achieved using biomarkers of carcinogenic mechanisms in shorter-term studies that employ more dose groups with fewer animals per group, and a BMDL as a PoD (Long et al., 2022).

1.13 | WG views on appropriate ESAF values

A point of central interest and intense discussion among the WG was the range of values appropriate for mutation as an endpoint. A majority of the WG concluded that it is not appropriate to use an ESAF of 1 when deriving a HBGV from mutation data. Reasons supporting this conclusion are as follows. An ESAF of 1 (i.e., no adjustment to a HBGV) has been reserved for endpoints that are considered mild and reversible. Mutation is not considered a mild toxicological endpoint based on (1) areas of toxicology (e.g., food additives) for which guidance indicates a safe level of mutagen intake cannot be established (WHO, 2020b), and (2) evidence presented above indicating that mutations (irrespective of their etiology) have potential for inducing disease. For all practical purposes, mutation is considered an irreversible endpoint that can cause severe pathological conditions. As noted earlier, in some regulatory jurisdictions, the use of an ESAF of 10 is recommended when there is evidence an exposure is associated with neurotoxicity, teratogenicity, an irreversible reproductive effect, or cancer. With respect to mutation, only a subset of induced mutations are believed to be capable of causing these effects. Thus, it could be asserted that an ESAF <10 might be appropriate for mutation because mutation induction connotes a likelihood of inducing a severe effect rather than definitive evidence that a severe effect will occur. At the same time, the large number of mutagens that are also carcinogens, along with evidence that mutagens can cause teratogenicity, irreversible reproductive effects, and neurotoxicity, may justify applying an ESAF of 10 for mutation endpoints. Importantly, it was the opinion of

the WG that selection of an ESAF should be flexible enough to incorporate the expert scientific judgment of a risk assessor. Based on these factors, in regulatory contexts where the application of an ESAF is described by guidance (e.g., ICH, elemental and solvent impurities; PMRA, pesticides; WHO, drinking water; JEFCA, food additives; and JMPR, pesticide residues in food), the majority of the WG recommends an ESAF between 2 and 10 be incorporated into a composite AF when gene mutation is the endpoint of a reference study used to establish a HBGV.

A minority view was that ESAF values of 1 could be appropriate under certain situations when deriving a HBGV from a reference study that documented an increase in gene mutation. Specifically, it was the minority view that in situations where robust dose–response and biological data exist (e.g., data showing rapid detoxification before coming into contact with DNA, or by effective repair of induced damage, as mentioned in ICH (2023a) that support a threshold mechanism for mutagenesis), the use of an ESAF value of 1 is reasonable. In such situations the use of an ESAF of 1 is seen as reasonable because the general practice of extrapolation from a $BMDL_{10}$ and application of multiple AFs is sufficiently conservative.

For two reasons this minority view was not shared by the majority of the WG members. First, it is contrary to previous WG conclusions regarding the utility of experimentally established thresholds as a generalized approach for setting HBGVs. Specifically, according to the output of 2013 IWGT meeting (MacGregor et al., 2015), "The WG recognizes that scientific evidence suggests that thresholds below which genotoxic effects do not occur likely exist for both DNA–reactive and DNA–nonreactive substances, but notes that small increments of the spontaneous level cannot be unequivocally excluded either by experimental measurement or by mathematical modeling. Therefore, rather than debating the theoretical possibility of such low-dose effects, emphasis should be placed on determination of PoDs from which acceptable exposure levels can be determined by extrapolation using available mechanistic information and appropriate uncertainty factors." Second, the majority recognizes: (1) the historical and reasonable risk assessment practice of employing an ESAF of 1 for mild and reversible endpoints, and (2) that the decision to use an ESAF should be independent of issues like detoxification, repair, or threshold mechanisms of mutagenesis. Compensatory processes like detoxification and repair may increase BMD or BMD(L) values, but they do not change the nature of mutation as an endpoint or the judgment that mutation has greater effect severity than mild and reversible endpoints.

This literature review and report focused primarily on available data in deriving recommendations regarding the application of an ESAF to mutation data for the purpose of setting an HBGV from a critical reference study. These recommendations do not necessarily apply to other types of genetic toxicity endpoints (e.g., micronucleus or comet). Aneugens may induce abnormal chromosome segregations due to interactions with structural (non-DNA) targets of the mitotic/meiotic cell machinery (EFSA, 2021) and threshold-based mechanisms have been established for some aneugens (Elhajouji et al., 1995; Parry et al., 1994). Therefore, the application of an ESAF to genetic toxicity endpoints other than gene mutation may warrant a separate review, which should consider disease-causing potential, irreversibility, and dose–response, as well as cell viability and potential for expansion.

Finally, the preceding recommendations regarding the application of the ESAF to mutation data are based on the current state of the science. At present, a major source of uncertainty in applying an ESAF is that some mutations may have no phenotype, some may contribute to disease in ways that are not yet known, and some confer phenotypes that past regulatory judgments have identified as severe. As greater understanding develops regarding the relationships between mutation and disease, these recommendations may require reconsideration and/or refinement.

In summary, the WG supports the use of ESAFs of up to 10 for neutral reporter gene endpoints but recommends using an ESAF of 10 when there is evidence of germ cell mutagenesis or test article induction of mutations known to be associated with severe disease. While the majority view supports a minimum ESAF of 2, a minority view within the WG is that under certain circumstances a factor of 1 can also be appropriate.

2 | CONCLUSIONS AND CONSENSUS STATEMENTS

A subgroup of the members of the 8th IWGT Quantitative Analysis WG investigated the rationale and available regulatory guidance regarding the use of an ESAF, identified toxicological endpoints recognized as severe, and summarized available knowledge regarding the human risk of disease phenotypes associated with mutations. Application of an ESAF to mutation data is relatively unexplored. Consequently, the recommendations provided in the following consensus statements reflect the current state of knowledge; they may need to be revisited in the future when direct measurements of chemically induced disease-causing mutations may be available. With this background in mind, and considering the detailed information provided herein, the WG recommendations regarding how the ESAF should be applied to mutation data were captured in the following consensus statements.

Consensus Statement 1: Quantitative analysis of mutant frequency dose–response data has utility in setting health-based guidance values. Quantitative analysis of mutant frequency could be of value in mitigating the negative health outcomes of exposures to environmental mutagens.

Consensus Statement 2: From a practical standpoint, mutation can be considered irreversible in nature, and some chemically induced mutations could induce and/or contribute to diseases recognized as severe in present and future generations.

Consensus Statement 3: Despite known associations between mutation and severe disease, and the breadth of research relating mutation to disease, the number of sites in the human genome that when mutated would be neutral, disease-causing, or severe disease-causing has not been completely characterized.

Consensus Statement 4: In situations where an ESAF is deemed appropriate, the WG recommends that:

- The judgment used when selecting the ESAF should reflect the totality of evidence linking induced mutation to the likelihood of causing disease: including the occurrence in germ cells and/or somatic cells, the nature of the mutational target, and other factors (e.g., spectra of induced mutation, dose, and mechanism of mutagenesis) determined on a case-by-case basis.
- Guidance should be developed regarding how to apply the concept of severity to induced mutation.

Consensus Statement 5: The WG supports the development and evaluation of technologies for precise measurement of disease-causing mutations, which may help clarify the use of AFs, and improve risk assessment in the future.

AUTHOR CONTRIBUTIONS

The first draft of the manuscript was prepared by Barbara L. Parsons, based on extensive group discussion. Paul A. White and George E. Johnson served as co-Chairs and Andreas Zeller as Rapporteur of the 8th IWGT Quantitative Analysis WG. Co-authors provided substantial input during revision and preparation of the final version submitted for publication. All authors approved the final version before submission.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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APPENDIX A

A.1 | LIST OF ABBREVIATIONS

AF	adjustment factor
BMD	benchmark dose
BMDL	benchmark dose lower confidence limit
CDMs	cancer driver mutations
CHIP	clonal hematopoiesis of indeterminant potential
DALY	disability-adjusted life years
DNEL	derived no effect level
ECHA	European Chemicals Agency
ecNGS	error corrected next generation sequencing
EFSA	European Food Safety Authority
ESAF	effect severity adjustment factor
GTTC	Genetic Toxicology Technical Committee
HBGV	Health-Based Guidance Values
HGMD	human gene mutation database
Hprt	hypoxanthine-guanine phosphoribosyl transferase
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IWGT	International Workshops on Genotoxicity Testing
JECFA	Joint Expert Committee on Food additives
JMPR	Joint Meeting on Pesticide Residues
LOAEL	lowest observed adverse effect level
MF	mutation frequency
MLA	mouse lymphoma assay
MOE	margin of exposure
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
OMD(s)	obligatory mosaic disease(s)
PDE(s)	permitted daily exposure(s)
Pig-a	phosphatidylinositol glycan class A
PoD	point of departure
PRMA	Pest Management Regulatory Agency
REACH	Registration, Evaluation, Authorisation, and Restriction of Chemicals
RTBs	rodent tumor bioassays
SBSs	single base substitutions
SNVs	single nucleotide variants
TGR	transgenic rodent assay
WG	Working Group
WHO	World Health Organization