

**Chlorine Chemistry Division of the
American Chemistry Council**

**Technical Comments on the Derivation of Cancer and
Non-Cancer Toxicity Criteria in**

*EPA's Reanalysis of Key Issues Related to Dioxin Toxicity
and Response to NAS Comments*

Comments to the EPA Science Advisory Board Dioxin
Review Panel

July 9, 2010

Table of Contents

Executive Summary

Comments on Reference Dose

Attachment A	Comments On The U.S. EPA Draft Proposed Reference Dose (RfD) For 2,3,7,8-Tetrachlorodibenzo-p-Dioxin	Lesa L. Aylward, Ph.D.
Attachment B	Setting of a RfD Based on Results of the Baccarelli et al., 2008, and Mocarelli et al., 2008, Studies: A Point of View	Dr. Warren Foster
Attachment C	Specific Comments Regarding the Animal Bioassays Used in EPA's Derivation of a Chronic RfD Value	Amy Lavin Williams, PhD, DABT Michael Garry, PhD John M DeSesso, PhD, DABFM, Fellow ATS

Comments on Cancer Risk Assessment

Attachment D	Dioxin's MOA and EPA's Decision to Reject Non-Linearity and Thresholds for Cancer Risk Characterization	Robert A. Budinsky, Ph.D.
Attachment E	Comments On The U.S. EPA Draft Cancer Slope Factor Derivation For 2,3,7,8-Tetrachlorodibenzo-p-Dioxin	Lesa L. Aylward, Ph.D.

Executive Summary

The Chlorine Chemistry Division of the American Chemistry Council (ACC) offers the following technical comments directed towards correcting errors in EPA's non-cancer and cancer risk characterization. It is the intent of these comments to assist the SAB Dioxin Review Panel in examining the scientific validity of EPA's approach and conclusions and to illustrate the necessary scientific modifications to the RfD and the cancer potency estimates to bring EPA into alignment with the National Academy of Sciences (2006) recommendations. The ACC comments, which include numerous attachments, provide information on the: a) modeling of the Seveso TSH and sperm parameter data in deriving a reference dose (RfD), b) Mode-of-Action (MOA) basis for a threshold approach to modeling the human and rodent carcinogenicity data, and c) the dose-response modeling of the NIOSH cohort leading to the linear cancer slope factor. The key conclusions of ACC's comments are presented below with specific cross-references to the SAB Charge Questions.

Comments Related to Reference Dose (RfD) Derivation (SAB Charge Questions, Section 4)

We present comments addressing the selection of datasets and the relevance of the endpoints, as well as on the technical details of the derivation of the proposed RfD.

Attachment A presents detailed technical comments on the derivation of the proposed RfD by Lesa L. Aylward, Ph.D., of Summit Toxicology. Dr. Aylward examines the use of the studies by Baccarelli et al. and Mocarelli et al. in RfD derivation. She provides detailed comments and proposed modifications of the EPA's use of these studies based on weight of the evidence evaluation that draws in numerous supporting studies. These additional studies inform the selection of the point of departure, the application of pharmacokinetic modeling to estimate intake doses associated with internal dose measures of interest in children, and the selection of uncertainty factors. Key conclusions from Dr. Aylward's comments include:

- (Charge Question 4.1) Contrary to EPA's assertion, the study by Baccarelli et al. (2008) provides a clear basis for estimating a no-observed-adverse-effect-level (NOAEL) for impacts on neonatal thyroid stimulating hormone (TSH) levels:

“All positive associations were dependent on the presence in the analyses of participants with very high plasma TCDD level (>50 ppt, n=5). When the analysis was restricted to individuals with TCDD \leq 50 ppt, *none of the correlations... was statistically significant.*” (Baccarelli et al. 2008, p. 1136, emphasis added).

This NOAEL is supported by the dose-response relationships observed in several other large studies, encompassing nearly 600 maternal-infant pairs. The identification of this robust NOAEL, with substantial support from the weight of the evidence from numerous other studies, provides the basis for reduced uncertainty factors in the derivation of the RfD.

- (Charge Question 4.1) The pharmacokinetic modeling conducted for the analysis of Mocarelli et al. (2008) did not account for the known increased elimination rate of TCDD in children from Seveso and elsewhere (Milbrath et al. 2010; Kerger et al. 2006) because the PBPK model used by EPA omits the clearance of TCDD through passive intestinal lipid clearance, which is much more rapid than in adults. This omission underestimates the dose rate of TCDD required to attain the tissue concentrations identified by EPA at the point of departure (POD).
- (Charge Question 4.2) The EPA did not identify a preferred dose metric (average vs. peak) related to the observed effects on sperm parameters in Mocarelli et al. (2008). However, the large body of data from rodent studies indicates that although effects on sperm counts and quality are not consistently found in such studies, when they do occur, they are associated with acute bolus dosing regimens rather than with environmentally relevant dosing through diet (data reviewed in Bell et al., 2010). This supports the selection of peak concentrations as the basis for risk assessment for this endpoint.
- (Charge Question 4.6) EPA concludes that it is not necessary to consider non-TCDD TEQ exposure because these exposures are “minimal” and “background,” however, in both of the Seveso studies, non-TCDD TEQ was present at substantial levels at the point of departure selected: approximately 80 ppt non-TCDD TEQ in Mocarelli et al. (2008) (in addition to 68 ppt TEQ due to TCDD); and 25 to 50 ppt non-TCDD TEQ at the POD of 39 or 50 ppt TCDD in Baccarelli et al. (2008). These additional TEQ exposures must be considered in the assessment of POD and uncertainty factors.

Based on these considerations, modifications to the RfD derivation are presented and result in a proposed RfD of >2 to 10 pg/kg-d, depending on the selection of endpoint and assumptions.

Attachment B presents comments from Warren Foster, PhD., Professor of Obstetrics and Gynecology, McMaster University. Dr. Foster authored a recent review on the mechanism of action of TCDD impacts on sperm quality based on animal and human studies (Foster et al. 2010). Dr. Foster addresses considerations related to the applicability, robustness, and clinical significance of the observed outcomes in Baccarelli et al. (2008) and Mocarelli et al. (2008). Professor Foster's observations include the following:

- (Charge Question 4.1) Both papers describe outcome measures that are useful clinical markers that guide further investigation but are not stand alone indicators of adverse health status.
- (Charge Question 4.1) Natural variability and potential confounders for both endpoints evaluated by EPA were only partially addressed or accounted for in the studies.
- (Charge Question 4.2) The use of these data to set an RfD is questionable because the exposure scenario experienced in Seveso is markedly different from exposures common to the general population. It is uncertain if the adverse effects documented in these two studies would occur with sustained low level exposure in the absence of an initial high exposure pulse.

Dr. Foster's detailed comments discuss numerous additional clinical issues that should be considered as these studies and endpoints are evaluated and used in RfD derivation.

Attachment C presents comments by Amy Williams, Ph.D., Michael Garry, Ph.D., and John DeSesso, Ph.D., on the numerous animal studies used by EPA to develop "candidate" RfDs. These comments address several concerns regarding a number of the studies included by EPA in this exercise:

- (Charge Question 4.1) Some of the animal studies used to support derivation of a chronic RfD evaluate endpoints that are not indicative of adverse events, have not been specifically linked to adverse events, or are of questionable toxicological relevance.

- (Charge Question 4.1) Some of the studies cited in support of EPA's derivation of an RfD report findings that conflict with those of other studies, thus indicating that the associated responses to TCDD treatment have not been well-elucidated.
- (Charge Question 4.1) Many of the animal studies used dosing regimens that cannot be properly extrapolated to chronic exposures, and thus, are inappropriate for derivation of a chronic RfD.
- (Charge Question 4.1) Some of the findings that are addressed in derivation of an RfD are actually precancerous lesions, and as such, are more appropriate to consider for use in cancer risk assessment.
- (Charge Question 4.1) In developmental studies, the appropriate unit for statistical analysis is the litter; however, many of the developmental studies considered by EPA incorrectly used the individual pup as the statistical unit for analysis.
- (Charge Question 4.1) Some of the data are derived from guinea pigs, which are known to be substantially more susceptible to the effects of TCDD treatment than are humans.

The comments include detailed discussions and examples related to these issues and present a thorough evaluation of many of the studies included by EPA in this effort.

Comments Related to Cancer Risk Assessment (EPA Charge Questions, Section 5)

Two sets of technical comments related to the cancer risk assessment chapter of the EPA document are presented. The National Academy of Sciences (NAS) 2006 review of the EPA Dioxin Reassessment was unequivocal in recommending a non-linear, mode of action-based risk assessment approach for cancer risk assessment for TCDD. NAS emphasizes the scientific justification for a non-linear approach in numerous places in the report. NAS notes that, rather than being a scientifically justified approach, the linear approach is a policy default and the choice to rely upon this approach should be part of risk management rather than risk assessment.

Attachment D presents comments from Robert A. Budinsky, Ph.D., addressing the voluminous data available related to the mode of action for TCDD-induced carcinogenesis. These data are reviewed in the context of the mode of action human relevance framework and include the following points:

- (Charge Question 5.2) The NAS panel was firm in its recommendation that EPA model dioxin's cancer risk using a non-linear threshold model for both the animal and human data sets. Their recommendation was based on the Mode-of-Action (MOA) understanding of how dioxins promote tumors in laboratory animals.
- (Charge Question 5.2) EPA rejected the NAS recommendation claiming that no MOA was known and yet at the same time, EPA stated an established MOA for dioxin's carcinogenicity supported TCDD's classification as a known human carcinogen and the justification for modeling the epidemiological cancer risk on all cancer mortality.
- (Charge Question 5.2) While EPA identified a number of key events related to dioxin's MOA, all of which are threshold phenomenon, they failed to conduct a thorough weight-of-the-evidence MOA assessment using the Human Relevance Framework process as specified in their 2005 Cancer Guidelines.
- (Charge Question 5.2) In opposition to the EPA's rejection of a threshold MOA, the scientific and regulatory community, e.g., the Joint Exposure Committee on Food Additives from the World Health Organization, recognize the threshold nature of dioxin's effects, including cancer. Because dioxin is a tumor promoter that works via activation of a nuclear receptor (the arylhydrocarbon receptor or AHR), it is widely understood to act in a threshold manner.
- (Charge Question 5.2) Dioxin is one of the most studied chemicals and has an extensive published literature that provides critical data for evaluating key events that support the tumor promotion MOA. EPA did not adequately address this literature.
- (Charge Question 5.4) Our MOA comments provide an outline of dioxin's tumor promotion, the key events, and the underlying published literature to support the MOA conclusions. This information clearly illustrates the data that the EPA should have subjected to a formal Human Relevance Framework Process in arriving at their conclusions regarding the non-linearity of dioxin's tumor promotion properties.
- (Charge Question 5.4) The weight-of-evidence on how dioxin promotes tumors, in the context of the biology of tumor promoters and the Human Relevance Framework, clearly supports a non-linear, threshold basis for dioxin's cancer risk characterization. This conclusion arises from tissue accumulation of dioxin, zonal AHR activation leading to reversible adaptive effects, stress responses that can be either reversible or irreversible,

inhibition of apoptosis in initiated hepatocytes, hepatopathy, and regenerative repair-induced cell turnover stimulating clonal growth of spontaneously initiated altered hepatic foci into adenomas and carcinomas.

Attachment E presents comments from Dr. Lesa Aylward regarding the use of results from Cheng et al. (2006) (on which she is a coauthor) in deriving linear cancer slope factors. As noted above, the NAS (2006) report noted that the most scientifically justified approach to cancer risk assessment for TCDD would employ a non-linear, mode of action approach, and that use of a linear approach is a policy decision. However, if a linear approach is used, Dr. Aylward's comments are offered in the interest of ensuring that the derivation is appropriate and consistent with the underlying data sets and models.

The EPA derivation results in a range of estimated cancer slope factors that span an order of magnitude, from approximately 100,000 to 1,300,000 (mg/kg-d)⁻¹, and EPA emphasizes the upper end of the range, recommending a slope factor of 1,000,000 (mg/kg-d)⁻¹. Dr. Aylward presents comments on two major aspects of the EPA's derivation:

- The selection and interpretation of regression coefficients from the Cheng et al. (2006) study, and
- The application of PBPK modeling to derive risk-specific doses and cancer slope factors.

Both of these steps necessarily entail several choices and decisions, but the choices and decisions made by USEPA (2010) are inconsistent in critical aspects with the underlying data and PBPK model. In summary, the key comments are as follows:

- (Charge Question 5.5) The selection of the Cheng et al. (2006) regression coefficient from the analysis, which omits the 5% of cases with the highest estimated exposures, results in a slope factor nearly 200 times greater than that obtained from the untrimmed data set. Thus, this regression coefficient is already an upper bound on the regression coefficient that is consistent with this data set. The best estimate of this regression coefficient, not a statistical upper bound on this coefficient, should be used as the basis for cancer risk estimates from Cheng et al. (2006).

- (Charge Question 5.5) The Emond et al. (2005) PBPK model should be used to estimate incremental intake doses resulting in incremental fat concentrations at body concentrations consistent with current background tissue concentrations. Use of the model to estimate intake doses associated with vanishingly small absolute TCDD concentrations is a) inconsistent with practical accepted application in risk assessment (which seeks to assess risks associated with potential incremental doses of dioxins from a given source); and b) requires use of the model in a concentration range that results in increasingly extreme predicted elimination half lives and which is well outside the range of validation.

When these modifications are adopted, cancer slope factors in the range of 80,000 to 290,000 (mg/kg-d)⁻¹ are derived. This narrower range of cancer slope factors is appropriate for recommendation for use by risk managers for assessing incremental risks associated with potential exposures to dioxins in a variety of contexts.

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Mocarelli, P., Gerthoux, P. M., Patterson, D. G., Jr., Milani, S., Limonta, G., Bertona, M., Signorini, S., Tramacere, P., Colombo, L., Crespi, C., Brambilla, P., Sarto, C., Carreri, V., Sampson, E. J., Turner, W. E., Needham, L. L., 2008. Dioxin exposure, from infancy through puberty, produces endocrine disruption and affects human semen quality. *Environ Health Perspect.* 116, 70-7.

Attachment A:

Comments On The U.S. EPA Draft Proposed Reference Dose (RfD) For 2,3,7,8-Tetrachlorodibenzo-p-Dioxin

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Introduction

The USEPA has proposed a non-cancer reference dose of 0.7 pg/kg-d derived based on two studies of the population in Seveso exposed during a trichlorophenol reactor accident in 1976. The key outcomes identified by USEPA were increased neonatal thyroid stimulating hormone (TSH) levels associated with maternal serum TCDD in infants born to mothers exposed during the Seveso accident¹ and reduced sperm number and motility in young men exposed as children during the Seveso accident². For both data sets, USEPA a) identified a serum lipid concentration associated with an estimated point of departure (POD); b) modeled the human intake associated with that POD using a physiologically-based pharmacokinetic (PBPK) model³; and c) applied uncertainty factors to derive the estimated RfD. These studies and endpoints appear to present an appropriate basis for RfD derivation, and the general approach used by USEPA, which integrates human epidemiologic data and sophisticated pharmacokinetic modeling, is a scientifically justifiable and valuable approach.

However, several of the choices made in the examination and analysis of the key datasets by EPA can be refined by consideration of the weight of evidence available from the many other human and animal datasets available for TCDD. By considering the other available data, uncertainties can be reduced in the selection of points of departure and relevant dose metrics and in the modeling of corresponding external doses. Consideration of the full weight of evidence provides context for and increased confidence in the quantitative choices required for derivation of an RfD value.

Specifically, these comments propose modifications to several of the EPA decisions based on a weight of evidence evaluation of the available data from human studies of TCDD including data on pharmacokinetics in children⁴, data on the role of peak vs. average exposure levels in alterations of sperm parameters in animals⁵, and a weight of evidence evaluation of numerous studies of associations between maternal or cord blood dioxin levels and alterations in neonatal thyroid hormone levels.⁶ The EPA decisions and proposed modifications based on a consideration of the full weight of evidence are summarized in Table 1; details are discussed in the following text.

Table 1: Summary of EPA draft derivation of proposed reference dose (RfD) and alternative assumptions and choices informed by consideration of the weight of evidence on several aspects of the derivations.

Derivation Step	Draft EPA Determination	Comment	Alternative Approach	Comment
Endpoint: Neonatal TSH (Baccarelli et al. 2008)				
1. POD Selection	LOAEL: 39 ppt maternal serum lipid TCDD (Baccarelli et al. 2008)	Selected from categorical analysis reporting geometric mean maternal serum TCDD for infants with TSH>5 µU/ml. See Figure 1.	NOAEL: 50 ppt maternal serum lipid TCDD	<p>Authors note that the relationship between TSH and maternal TCDD was not significant when 5 individuals with highest TCDD levels (>50 ppt TCDD;; >75 ppt TEQ) were omitted; see Figure 1.</p> <p>This NOAEL supported by dose-response relationships from 4 other large studies of maternal infant pairs (total of 570 infants); reviewed in Goodman et al. 2010 (see Figure 2).</p>
2. Estimation of Intake Dose	24 pg/kg-d	Appears to be dose associated with maternal serum concentrations of 270 ppt, corresponding to regression prediction for TSH=5 µU/ml (see Appendix D of USEPA 2010), rather than intake dose associated with serum levels of 39 ppt.	9.5 pg/kg-d	Estimated intake dose under gestational model associated with maternal serum lipid TCDD = 50 ppt (see Appendix C.4.3 of USEPA 2010, assuming serum lipid concentration equals fat concentration).
3. Identification of Uncertainty Factors	UF = 30	10 for LOAEL to NOAEL; 3 for interindividual variability	UF = 1	<p>Robust NOAEL identified from consideration of detailed data in Baccarelli et al. (2008) and supported by four other large studies (see Figure 2).</p> <p>Sensitive subpopulation studied in each of the five studies, and overall sample size is 570 maternal-infant pairs.</p> <p>Quantitation of intake ignores additional non-TCDD serum TEQ of 25 ppt present in addition to the TCDD NOAEL of 50 ppt, and so is a conservative estimate.</p> <p>Based on these factors, no additional UF for interindividual variability required.</p>
4. Resulting RfD	0.8 pg/kg-d		9.5 pg/kg-d	As discussed above, the value is conservative due to neglect of substantial non-TCDD TEQ.

Derivation Step	Draft EPA Determination	Comment	Alternative Approach	Comment
Endpoint: Effects on Sperm Parameters (Mocarelli et al. 2008)				
1. POD Selection	LOAEL: 68 ppt serum lipid TCDD Extrapolated to estimated peak of 248 ppt or average of 57.7 ppt (3.8 yrs after accident)	Median TCDD concentration, first quartile, measured in children ages <10 during the first year following the Seveso accident. Lower sperm counts and motility observed in all quartiles compared to comparison group, with no obvious dose-response.	No alternative proposed.	Selection is generally appropriate. Note that non-TCDD TEQ in serum in children in the Seveso area in 1976 averaged 80 ppt TEQ: “If TCDD acts in concert with other dioxin-like chemicals in affecting sperm quality, the total dioxin toxic equivalency (TEQ) should be considered. In nine serum pools from females residing in the uncontaminated area in 1976, Eskenazi et al. (2004) found an average TEQ of 100 ppt.” (Mocarelli et al. 2008). So the quantitative estimate of the LOAEL (68 ppt) accounts for less than half of the TEQ present.
2. Estimation of Intake Dose	32 pg/kg-d or 8 pg/kg-d average: 20 pg/kg-d	Intake dose rates required to achieve peak or average serum TCDD concentrations during childhood estimated. Due to uncertainties regarding appropriate dose metric (peak vs. average), these two intake rates were averaged.	>60 pg/kg-d	Animal data from numerous studies in rodents show inconsistent effects on sperm parameters, but effects are only seen in studies employing bolus or repeated bolus, rather than environmentally relevant dietary, dosing regimens. ⁵ These studies indicate that peak is the appropriate dose metric. Emond et al. (2005) PBPK model has not been validated for children against available datasets for Seveso children ⁴ and does not incorporate demonstrated TCDD elimination via intestinal lipid clearance. Failure to include this mechanism leads to a substantial underestimate of the intake rate required to achieve target serum concentrations under environmental exposure conditions. Estimate of >60 pg/kg-d presented here is rate required to achieve a peak concentration of 248 ppt TCDD by age 10 based on a simple first-order model employing the observed serum TCDD elimination half-life of 1.5 years in Seveso children. ⁴
3. Identification of Uncertainty Factors	UF = 30	10 for LOAEL to NOAEL; 3 for inter-individual variability	No alternative proposed.	These uncertainty factors appear to be reasonable.
4. Resulting RfD	0.7 pg/kg-d		>2 pg/kg-d	As discussed above, the value is conservative due to neglect of substantial non-TCDD TEQ (average of 80 ppt TEQ in this population in 1976).

Detailed Comments

Several aspects of the derivation of the RfD were assessed for each of the key data sets selected by USEPA in light of consideration of the weight of evidence from the available published data:

- Identification of the POD and quantification of the serum concentration associated with the POD;
- Quantification of the intake dose associated with the POD; and
- Identification and application of uncertainty factors

For each aspect, the choices and decisions made by USEPA are presented, and alternative choices and recommendations are presented based on the full range of available data.

Neonatal TSH

Selection of POD. Baccarelli et al. (2008) present several analyses indicating that neonatal TSH is positively associated with maternal exposure to TCDD. As noted by USEPA, the most useful analyses for derivation of an RfD are those based on the dataset of 51 maternal-infant pairs that include measurement of levels of maternal serum TCDD and other contributors to dioxin toxic equivalency (TEQ).

The draft USEPA (2010) assessment presents conflicting assessments of the appropriate point of departure. In section 4.3.4.1, the geometric mean of the maternal serum TCDD concentration in the group with TSH > 5 μ U/ml (39 ppt) is identified as a lowest observed adverse effect level (LOAEL). However, in Appendix D, the maternal serum concentration corresponding to TSH of 5 μ U/ml based on the statistical regression as presented in Figure 2A of Baccarelli et al. (2008) was identified as the LOAEL. In each case, USEPA asserts that a no-observed adverse effect level (NOAEL) could *not* be identified based on the data set.

Figure 1 presents the TSH vs. maternal TCDD data from Baccarelli et al. (2008). EPA's selected LOAEL is derived from the geometric mean of maternal TCDD for 7 infants with TSH level in

excess of 5 $\mu\text{U}/\text{mL}$. However, the WHO screening criteria for TSH of 5 $\mu\text{U}/\text{mL}$ anticipates that approximately 3% of normal infants will exceed this level. It is important to note that a neonatal TSH concentration in excess of 5 $\mu\text{U}/\text{mL}$ is not, in itself, indicative of an adverse effect. Rather, this concentration reflects a screening value used to identify potential hypothyroidism, triggering testing for T4 and T3 hormone levels in order to detect hypothyroidism and avert the possibility of adverse developmental impacts. In a group of 51 normal infants, exceedence of the 5 $\mu\text{U}/\text{mL}$ screening value would be expected in 1 or 2 infants. Selection of a LOAEL based on the geometric mean of maternal TCDD for all infants in excess of this criterion ignores this normal variation and ignores the much more explicit dose-response data available in the regression analyses presented by the authors.

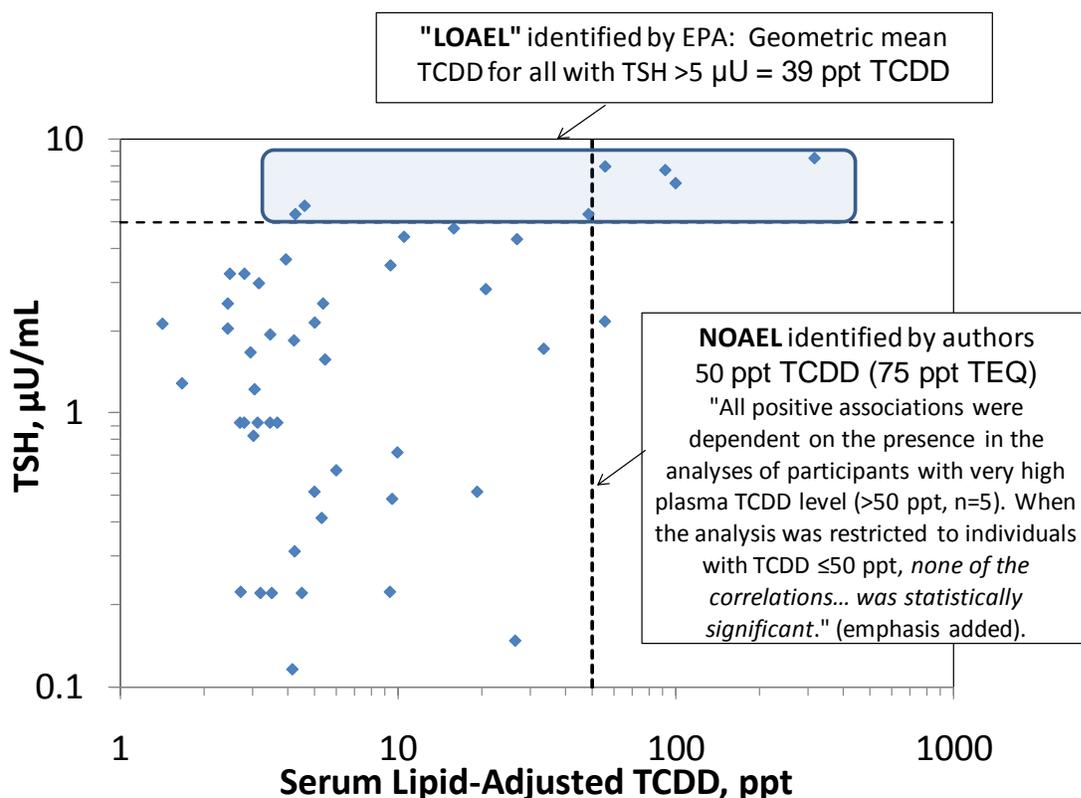


Figure 1: Data from Baccarelli et al. (2008), Figure 2A, presenting neonatal TSH concentrations versus maternal serum lipid-adjusted TCDD concentration. The WHO screening criterion for infant TSH of 5 $\mu\text{U}/\text{mL}$ is presented as a horizontal dashed line. Approximately 3% of infants in a normal population are expected to exceed this criterion (corresponding to 1 to 2 infants in a population with $n=51$). The vertical dashed line indicates the exclusion of the five individuals with TCDD levels in excess of 50 ppt. No statistically significant relationship between TSH and maternal TCDD levels was present when the analysis was restricted to individuals with measured serum TCDD below this level.

The authors present a clear assessment of a statistically-based NOAEL in the study:

“All positive associations were dependent on the presence in the analyses of participants with very high plasma TCDD level (>50 ppt, n=5). When the analysis was restricted to individuals with TCDD \leq 50 ppt, *none of the correlations... was statistically significant.*”¹ (Baccarelli et al., 2008, p. 1136, emphasis added).

The selection of this cutpoint, 50 ppt TCDD in maternal serum (corresponding to 75 ppt TEQ), as a NOAEL for this dataset is supported by visual inspection of the data from Baccarelli et al. (2008) (Figure 1).

More importantly, selection of this maternal serum level as a NOAEL is also consistent with numerous other, larger studies examining the relationship between maternal TEQ concentrations and infant thyroid hormone levels. In a recent comprehensive review, Goodman et al. (2010) identified more than two dozen studies examining this endpoint as a function of various measures of maternal or infant exposure to dioxin-like compounds. Statistically significant associations between maternal TEQ concentrations and neonatal TSH levels were only reported in studies with very high exposure levels relative to current general population levels, particularly in the Dutch cohorts studied during the 1980s and the Baccarelli et al. (2008) analyses (see Goodman et al. 2010 for details).

Figure 2 presents the exposure ranges for several of the largest studies examining the relationship between maternal TEQ and infant neonatal TSH, including both positive and negative studies. These studies bolster selection of 50 ppt TCDD (~75 ppt TEQ) from the Baccarelli et al. (2008) dataset as a NOAEL for impacts on TSH. The exposure ranges from three of the largest studies⁷⁻⁹, which report no association with TSH, are shown in Figure 2 along with the Baccarelli et al. (2008) exposure range and that from Koopman-Esseboom et al. (1994), which reported a positive correlation coefficient (but no values outside the normal range) for TSH versus TEQ. The weight of evidence is strong and consistent across many studies including 570 maternal-infant pairs: maternal serum concentrations below approximately 75 ppt TEQ are not associated with any statistically significant impact on neonatal TSH levels.

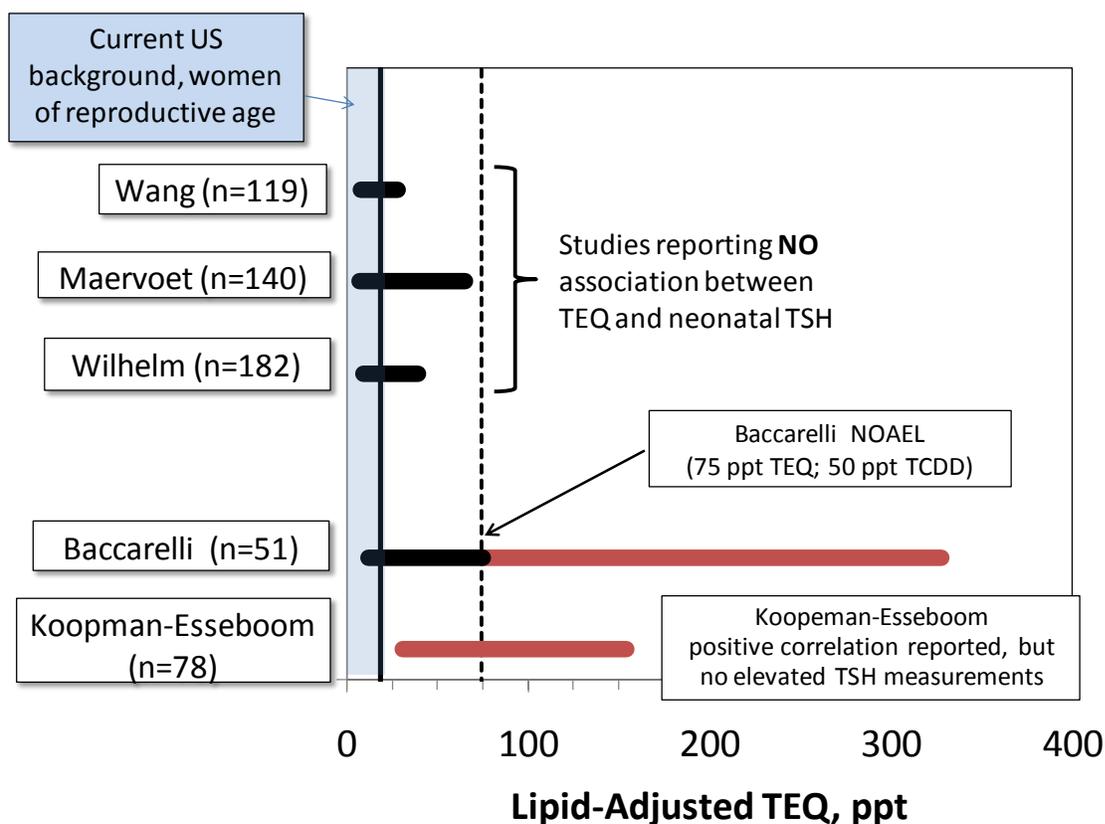


Figure 2: Exposure ranges from several other, larger, studies of neonatal TSH levels⁷⁻¹⁰ as well as the range of background US serum TEQ for women of reproductive age¹¹. Dark bars illustrate the exposure ranges over which no statistically significant relationship was found, while red bars illustrate the exposure ranges where statistically significant correlations are apparent. Note that the study of Koopman-Esseboom, while reporting a positive correlation coefficient, did not report a regression slope, nor did they find any abnormal TSH measures in the studied population. The data from these studies are entirely consistent with the NOAEL from Baccarelli et al. (2008). Altogether, these studies encompass 570 maternal infant pairs from five countries, providing confidence in the identified NOAEL of 50 ppt TCDD or 75 ppt TEQ from Baccarelli et al. (2008).

Quantification of human intake dose associated with POD. USEPA identified a daily intake rate of 24 pg/kg-d associated with the LOAEL calculated from the regression model presented in Baccarelli et al. (2008). Using the alternative POD selected above (50 ppt TCDD) and the pharmacokinetic modelling results presented in Appendix C of USEPA (2010) for the human gestational PBPK model, a daily intake rate of approximately 9.5 pg/kg-d would be associated with a maternal serum lipid TCDD concentration of 50 ppt (assuming that serum lipid concentration would be equivalent to fat concentration).

This intake rate is conservative, in that it does not incorporate any contribution from intake of other TEQ contributors, which accounted for a 50% increase in the total maternal TEQ compared to the TCDD concentration at this NOAEL (75 ppt TEQ vs. 50 ppt TCDD). However, the PBPK models available do not provide a reliable basis for estimation of the intake rates of the complex mixture accounting for this additional 25 ppt TEQ, so the POD is estimated only for TCDD.

Identification and application of uncertainty factors. USEPA selected an uncertainty factor of 10 for extrapolation from a LOAEL to a NOAEL, as well as a factor of 3 for potential inter-individual variation. The POD of 50 ppt TCDD identified above, which is based not only on Baccarelli et al. (2008) but also on consideration of the weight of evidence from many studies including 570 maternal-infant pairs, is a clear NOAEL and does not require application of any uncertainty factor for LOAEL to NOAEL extrapolation, so this UF is set to 1. In addition, the large number of studies and maternal-infant pairs providing consistent results indicates that sensitive subpopulations have been well-studied and accounted for, indicating that no uncertainty factor for inter-individual variation in sensitivity is required, so this UF is set to 1 as well. Thus, the estimated RfD based on this dataset is 9.5 pg/kg-d. This RfD is necessarily conservative because it does not take into account the substantial non-TCDD contributors to TEQ at the selected POD.

Effects on Sperm Parameters

Selection of POD. The USEPA identified boys in the lowest quartile of those exposed at Seveso in the Mocarelli et al. (2008) study as a LOAEL group, with a median serum concentration of 68 ppt TCDD measured on average one half year after the Seveso accident. This LOAEL was based on lower sperm concentrations and motility compared to a comparison group not exposed during the Seveso accident. This is a reasonable POD. However, it is important to note that the general population levels of non-TCDD TEQ were substantial in that area in that time period (similar to the US and other Western European countries):

“If TCDD acts in concert with other dioxin-like chemicals in affecting sperm quality, the total dioxin toxic equivalency (TEQ) should be considered. In nine

serum pools from females residing in the uncontaminated area in 1976, Eskenazi et al. (2004) found an average TEQ of 100 ppt.” (Mocarelli et al. 2008).

The analyses by Eskenazi et al. (2004)¹² of pooled blood samples found that in children, average non-TCDD TEQ was approximately 80 ppt TEQ in serum lipid, or more than twice the POD of 68 ppt TCDD identified by USEPA. This level of non-TCDD TEQ was typical in Western Europe and the United States during the 1970s.¹³

Quantification of human intake dose associated with POD. USEPA estimated two dose metrics for the boys in the lowest quartile from Mocarelli et al. (2008): peak serum lipid TCDD concentration immediately following the Seveso accident, and average serum lipid concentration over ages 1-10. USEPA uses the PBPK model of Emond et al. (2005) to estimate the chronic daily intake rates required to either a) reach that peak concentration, or b) to attain that average concentration during that age range (32 and 8 pg/kg-d, respectively). There are two issues associated with this analysis.

First, the Emond et al. (2005) PBPK model has not been validated against data sets for elimination of TCDD in infants and children. In particular, the highly relevant data on elimination rates in children from Seveso (Kerger et al. 2006) provides data that indicate that the model of Emond et al. (2005) substantially underestimates the intake rate needed under environmental exposure conditions to achieve the estimated peak or average concentrations identified as the PODs of interest. Kerger et al. (2006) found that over a period of 16 years following the accident, elimination half-lives in children under age 12 averaged 1.5 yrs with a relatively narrow degree of variation. The Emond et al. (2005) PBPK model does not include the demonstrated elimination pathway for TCDD through intestinal lipid clearance. This mechanism has a relatively small effect in adults, but intestinal clearance of lipids in infants and children occurs at many times the adult rate.¹⁴ Because intestinal lipid clearance and concomitant elimination of TCDD is much faster in infants and children than in adults, the Emond et al. (2005) model does not accurately reflect the accelerated clearance of TCDD in children compared to adults, and therefore likely underpredicts the intake rate associated with the target serum lipid concentrations by a factor of 2 or more.

A second issue related to the selection of the appropriate intake rate that can be informed by consideration of the full weight of evidence is the consideration of peak vs. average concentrations. Bell et al. (2010) provides a comprehensive review of the numerous studies examining the effect of *in utero* and developmental exposure to TCDD on male rat reproductive system parameters. Two conclusions are clear from consideration of the full weight of evidence regarding these endpoints: a) effects on sperm are not a consistent finding of such studies, particularly when the data sets are restricted to those with a robust sample size; and b) when such effects are observed, they are associated with bolus or repeated bolus dosing, rather than with environmentally relevant administration in feed. Thus, the weight of evidence suggests that peak rather than average exposure level is most relevant to assessing this endpoint.

Assuming simple first order kinetics with a half-life of 1.5 years⁴ and an average bodyweight of 20 kg with 20% body fat, a calculation of the daily intake rate required to obtain the peak serum concentration of 248 ppt TCDD identified by EPA by the age of 10 can be made. Under these assumptions, the daily intake rate would be at least 62 pg TCDD/kg-d. This calculation likely substantially underestimates the true dose needed to reach the relevant peak concentration for several reasons. First, the simple first-order kinetic model ignores sequestration in the liver, which will increase the required daily dose needed to reach the target serum lipid concentration. Second, the calculation allows for attainment of the peak concentration at age 10, rather than earlier in childhood (the average age at peak exposure in Mocarelli et al. 2008 was 6 years) – greater daily intake rates would be required to reach the target at a younger age. Note that this daily intake rate of 62 pg/kg-d is more than *130-fold* lower than the bolus dose of 8,100 pg/kg-d estimated by USEPA to have been received by the children studied in Mocarelli et al. (2008). Finally, the calculation again ignores exposure to non-TCDD TEQ, which in these individuals was significant, with serum concentrations averaging approximately 80 ppt TEQ, more than *double* the median TEQ concentration due to TCDD in the lowest quartile.²

Identification and application of uncertainty factors. USEPA selected an uncertainty factor of 10 for extrapolation from a LOAEL to a NOAEL, as well as a factor of 3 for potential inter-individual variation. These factors appear to be appropriate, even in light of the modifications suggested above. Based on application of a composite uncertainty factor of 30 to the POD of 62

pg/kg-d identified above, an RfD would be approximately 2 pg/kg-d. However, as discussed in the previous paragraph, the estimated intake rate at the POD is likely to underestimate the actual intake required to achieve the target serum lipid concentration and ignores non-TCDD TEQ (present at greater concentrations than TCDD in these individuals). Thus, the estimated RfD of at least 2 pg/kg-d is conservative.

Conclusions

The USEPA analysis makes use of recent data sets that include internal measures of exposure and examination of endpoints considered to be of high interest based on the animal data. The choices entailed in using these data sets for derivation of an RfD can be informed by considering these data sets in the context of the weight of evidence from the full range of data sets available on the key responses (reviewed by Bell et al. and Goodman et al.) and on the pharmacokinetics of TCDD in children from Seveso.⁴ Based on the full weight of evidence and available body of data, the estimated RfD is >2 to approximately 10 pg/kg-d, depending on the data set and endpoint used. This value is in the range of previous international assessments.¹⁵

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Attachment B:

Setting of a RfD Based on Results of the Baccarelli et al., 2008, and Mocarrelli et al., 2008, Studies: A Point of View

July 9, 2010

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Dr. Foster received his doctorate from McMaster University in 1991 in Reproductive Biology. He joined the staff of the Environmental Health Directorate at Health Canada as a reproductive toxicologist in 1990 and in 1992 assumed the position of Head, Reproductive Toxicology Section. While at Health Canada Dr. Foster established the Departmental Endocrine Disrupter Committee and served for several years as the Canadian representative to the Organization for Economic Cooperation and Development (OECD) Test Guideline Programme. In 1999, he joined the Department of Obstetrics and Gynecology at Cedars Sinai Medical Center in Los Angeles as the Assistant Director of Women's Health and Director of Research. In 2001 he joined the faculty in the Department of Obstetrics and Gynecology at McMaster University as an Associate Professor rising to the rank of Professor in 2005. Between 2002 and 2010 Dr. Foster served as the Director of the Reproductive Biology Division and received a career award from the Canadian Institutes of Health Research and the Ontario Women's Health Council in recognition for his research. Between 2005 and 2008 Dr. Foster also served as the Medical Director for the Centre for Reproductive Care. Dr. Foster has served on several study section panels including the NIH Integrative and Clinical Endocrinology and Reproduction Study Section (2009-2010), US-EPA-Star Program and Canadian Institutes of Health Research Clinical Investigation A panel (2007-2010). Dr. Foster has been an invited member of several expert panels including: NIH/NIEHS-NTP, Reproductive & developmental effects of soy products and genistein (2009-present) and member of Council of Canadian Academies expert panel for the Integrated Testing of Pesticides (2009-present). He is a member of the Society of Toxicology and a member of the Reproductive and Developmental Toxicology Specialty Section (1999-present) where he was recently elected to the position of secretary treasurer. He is also a member of several other professional societies and has recently served as President of the Canadian Fertility and Andrology Society (2007-2008). He is currently the Chair of the Society for the Study of Reproduction Committee on Reproduction and the Environment. His research interests are in dysregulation of estrogen regulated proliferation/apoptosis and metastasis, and in particular the role of environmental estrogens in breast cancer progression and the pathophysiology of endometriosis. He has published in excess of 125 peer reviewed scientific articles and has given more than 60 invited lectures. He teaches in several graduate courses at the Medical School in toxicology and reproductive endocrinology. He also lectures in undergraduate courses in toxicology and reproductive medicine.

General comments: I have reviewed the EPA charge to the panel and the two papers (Baccarelli et al., 2008; Mocarelli et al., 2008) used in setting the lowest observable adverse effect level (LOAEL) and candidate point of departure (POD) for derivation of a candidate reference fixed dose (RfD). The papers demonstrate an association between exposure to TCDD and: (a) elevated TSH concentrations in neonates (Baccarelli et al., 2008); and (b) decreased sperm concentration and motile sperm count in adult males exposed to TCDD when they were between 1 and 9 years of age. While both papers describe well designed statistically robust

epidemiological studies, they also have a number of limitations that deserve comment. Specifically, both papers describe outcome measures that are useful clinical markers that guide further investigation but are not stand alone indicators of adverse health status. Results of these two studies demonstrate that accidental exposure to very high concentrations of TCDD can increase circulating TSH concentrations in neonates. Moreover, adult men who were accidentally exposed to these high concentrations of TCDD when they were between the ages of 1 and 9 years of age have lower sperm concentrations and decrease motile sperm counts compared to a reference population. These data are important because they demonstrate that accidental exposure to high levels of TCDD can adversely affect thyroid and testicular function. However, the relevance of these findings for the general population with low level background exposure to TCDD is unclear. The use of these data to set an RfD is questionable because the exposure scenario is markedly different from exposures common to the general population. It is uncertain if the adverse effects documented in these two studies would occur with sustained low level exposure in the absence of an initial high exposure pulse. High concentration exposure to TCDD over several days followed by continued exposure over years describes a very different exposure scenario from one of sustained background exposure over a lifetime. Indeed, it is more probable that the adverse effect documented in these studies is the consequence of very high concentration exposure to TCDD over a period of time during a susceptible developmental stage. The most significant results of these two studies is the demonstration of an adverse effect on thyroid function and thus the need for continued follow-up in these study subjects. However, suggestion that the findings of these studies points to the potential for adverse effects in the general population with exposures representative of background is not warranted. Detailed comments on the interpretation of the two studies are as follows:

Baccarelli et al., 2008 study and TCDD effects on thyroid function: The association between maternal exposure to TCDD and neonatal TSH concentrations greater than 5 $\mu\text{U/ml}$ was described by Baccarelli and co-workers (Baccarelli *et al.*, 2008) and used by EPA to establish a maternal intake of 0.024 ng/kg-day as the lowest observable adverse effect level (LOAEL). This LOAEL was also used by EPA as a candidate point of departure (POD).

As described above the relevance of the exposure is a concern when trying to translate results to the general population. In addition, there are some limitations to this study in terms of control for potential confounders as well as the limited number of subjects in this study from zone A. Finally, use of a single point measure of TSH is raised as an indicator of adverse of outcome.

Exposure – The population studies included women resident in Seveso, Italy at the time of the accident and a reference population giving birth between the years 1994 and 2005. While there does not appear to be any difference in the age of mothers in the different groups included in this study it is unclear when they delivered and if there was a disproportionate number of women in any study group that came from the later years of the study vs. the earlier years of the inclusion period. Specifically, was there an equal proportion of mother's who delivered and were recruited

to the study for each group and year of study. It can be expected that the body burden of TCDD will be lower as the time from the initial exposure increases and thus if a disproportionate number of women were recruited to any group either closer to the time of exposure to more distant from the exposure then results could easily be skewed.

Confounders – Circulating TSH is known to be affected by a number of factors such as gestational age and neonatal age at the time of sample collection. Indeed, circulating TSH levels can change markedly during the first year of life including the first days of life (Hubner *et al.*, 2002). In general circulating TSH concentrations are highest in the first few days of life and then begin to decline. Thus it is important to know if the neonate was delivered prematurely. Furthermore, although the authors state that blood was collected within the first 72 hours of life it would also be helpful although not critical to evaluate when the blood was collected and to document that the distribution of sample collection time was equivalent across the three study groups assessed in this study. Other potentially important confounders that were not mentioned in this study are method of conception (natural conception vs. assisted reproduction techniques), maternal body mass index, and history of maternal and paternal cigarette smoking.

Relevance of TSH – Blood TSH concentration is used as a screening tool to identify neonatal hypothyroidism (Rose *et al.*, 2006) owing primarily to the importance of thyroid function in neurodevelopment. Circulating levels of TSH change profoundly over the course of the first couple of years of life and even the first few days of life (Hubner *et al.*, 2002; Zurakowski *et al.*, 1999). It is worth noting that although the number of neonates with TSH concentrations above the WHO level of 5 μ U/ml, the reported reference range for newborns between 1 and 3 days of age (0.13-9.23 mIU/L), is very broad (Hubner *et al.*, 2002) and thus the majority of neonates described in the Baccarelli *et al.*, (2008) study would fall within the normal range. The most relevant observation is the distribution of the blood TSH concentrations was shifted to the right and the percentage of neonates whose values were outside of the normal range was increased. A single measure of elevated TSH concentration on its own would not typically result in a diagnosis of hypothyroidism. Rather an abnormal TSH result would mark a neonate for continued clinical follow-up. Specifically, the TSH test should be repeated and accompanied by a measure of circulating free thyroxine (fT4) concentration to determine if the hypothalamic-pituitary- thyroid axis is functioning normally. Patients with elevated TSH but normal circulating concentrations of fT4 are defined as subclinical hypothyroid. Of those with subclinical hypothyroidism approximately 88% will have normal thyroid function (Wasniewska *et al.*, 2009). Regardless, in the first two years of life, it is generally accepted that when in doubt it is appropriate to treat (Zadik, 2009).

Summary and conclusions – This is a very interesting and well designed study that despite its limitations suggests that there is an association between historical exposure to high concentrations of TCDD and blood TSH concentrations in the newborn. However, owing to limitations in control for confounding variables, difficulty in translating exposure scenario to the

general population, relevance of the main outcome measure, the results of this study are suitable for hypothesis generation but are not strong enough on their own for generation of a RfD.

Link between TCDD exposure and decreased sperm concentration (Mocarelli et al., 2008):

Exposure of young boys between the ages of 1 and 9 years TCDD was associated with a 20% decrease in sperm concentration and a 11% decrease in motile sperm in adulthood (Mocarelli *et al.*, 2008). Of note follicle stimulating hormone (FSH) was increased whereas estradiol (E2) was decreased. In contrast there were no differences in circulating concentrations of luteinizing hormone (LH), inhibin B (INH-B), and testosterone (T).

This is a particularly strong report in that the study population is very well described and the data is provided for potential confounding factors such as cigarette smoking, number of cigarettes smoked, history of maternal smoking, and alcohol use. The exclusion criteria were well described and include common disorders known to affect semen quality. However, information was not provided on the use of medications known to affect semen quality such as anti-inflammatory agents such as sulfasalazine, prednisone, and the cyclooxygenase inhibitors. While there is no reason to anticipate that study subjects from zones A and B would be different from the reference population in their use of such medications this information would have been easy to collect and should have been considered in a study of this design. Although a very strong study there are a number of limitations that deserve comment as follows:

Dependence on a single measure of sperm concentration - Sperm concentration is notorious for its high variability between samples from the same study subject. While study subject participation in such studies is often low and request for more than one sample could lower recruitment success even further, the requirement for a second sample cannot be overlooked. Furthermore, the relationship between traditional markers of semen quality and exposure to environmental contaminants remains unclear. For example, there are numerous reports in the literature describing a decline in sperm concentration (Carlsen *et al.*, 1992; Auger *et al.*, 1995; Bendvold *et al.*, 1991; Bendvold, 1989; Almagor *et al.*, 2003; De Celis *et al.*, 2000) or poor semen quality in men occupationally exposed to chemical contaminants (Abell *et al.*, 2000; Alexander *et al.*, 1996) whereas others have reported no change (Andolz *et al.*, 1999; Larsen *et al.*, 1998). Still others have reported regional differences in semen quality that could be tied to environmental contaminant exposure (Fisch and Goluboff, 1996; Jorgensen *et al.*, 2006; Jorgensen *et al.*, 2001; Younclai *et al.*, 1998). Thus the debate continues concerning the effect of environmental exposures on semen quality. The report of Mocarelli and coworkers (Mocarelli *et al.*, 2008) expands the literature but is not in any way definitive but presents an interesting hypothesis that deserves testing. Specifically, is there a critical window between birth and nine years of age that is critical to adult spermatogenesis and semen quality?

Relevance of the outcome measures – The biological relevance of the outcome measures used are not necessarily adverse. Specifically, the values for semen quality reported in this study are

above clinical thresholds that would suggest any adverse effect on fertility. Although statistically different from the comparison group it is unclear if these differences would translate to differences in time-to pregnancy for those attempting to achieve a pregnancy. Indeed, it is generally acknowledged that there is poor agreement between semen quality measures above clinical thresholds and fertility. The absence of any detectable differences in circulating concentrations of LH and T is troubling in the face of differences in semen quality. These data could suggest that exposure to TCDD has no effect on Leydig cell function or the process of spermatogenesis. However, evidence of differences in circulating levels of FSH could indicate an effect of exposure on Sertoli cell number or function. Sertoli cell numbers are regulated during fetal development and for a short period after birth and thus it is conceivable that TCDD exposure could affect the number of Sertoli cells and thus the number of cells that could support sperm production which would affect overall sperm count. However, this hypothesis is not supported by any of the existing animal literature (Foster *et al.*, 2010). An effect of TCDD exposure on epididymal function cannot be excluded; however, this hypothesis has yet to be evaluated even in the animal literature.

Summary and conclusions – This is a well designed study that provides evidence that a sustained pulse of high concentration TCDD exposure between the ages of 1 and 9 years of age is associated with a decrease in sperm concentration, the percentage of motile sperm, and the number of progressively motile sperm. Moreover, results suggest that this exposure to TCDD is linked with increased circulating FSH and decreased E2 concentrations. Exposure at later ages is associated with an increase in semen quality. The Mocarelli and co-worker study (Mocarelli *et al.*, 2008) is important because it suggests that there is a critical period of development during which semen quality later in life may be affected. However, the poor clinical relevance of the outcome measures employed and the absence of a mechanistic explanation for the observations limits the value of this study for establishing a critical POD value but highlights important questions raised by this study for future research.

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Attachment C:

Specific Comments Regarding the Animal Bioassays Used in EPA's Derivation of a Chronic RfD Value

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Specific Comments Regarding the Animal Bioassays Used in EPA's Derivation of a Chronic RfD Value

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The animal studies that the U.S. Environmental Protection Agency (EPA) has cited in support of its derivation of an oral reference dose (RfD) for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), as listed in Figure 4-4 of the *EPA's Reanalysis of Key Issues Related to Dioxin Toxicity and Response to NAS Comments* (EPA, 2010), were critically reviewed. The results of this study-by-study evaluation are provided in Appendix 1. Based on this critical examination, a number of deficiencies were identified regarding this body of studies, which limit its utility to support derivation of a chronic RfD value for TCDD. These deficiencies are as follows:

- 1. Some of the animal studies used to support derivation of a chronic RfD evaluate endpoints that are not indicative of adverse events, have not been specifically linked to adverse events, or are of questionable toxicological relevance.**

A number of studies cited by the EPA assessed endpoints that are not considered adverse. Examples include:

- Amin et al. (2000), which examined the effects of gestational TCDD exposure on saccharin preference;
- Fatorre et al. (2000), which evaluated effects of TCDD treatment on hepatic vitamin A concentrations;
- Cantoni et al. (1981), which found effects of treatment on urinary porphyrins, but did not demonstrate liver toxicity;
- Hojo et al. (2002), which assessed the effects of gestational TCDD exposure on behavior using a complex testing paradigm, showing sex-dependent responses (no effects were observed using more simplistic behavioral testing) ;
- Li et al. (1997), which found transient hormonal changes following single dose exposure; these effects were only observed in immature female rats, and not in males or in adult females; and
- Van Birgelen et al. (1995b), which assessed liver retinol concentrations.

Further, a number of studies examined the effects of TCDD exposure on serum thyroid hormone concentrations (Crofton et al., 2005; Seo et al., 1995; Sewall et al., 1995). However, few of these studies evaluated whether these changes were transient or permanent. Further, none determined whether the findings were associated with alterations in thyroid organ weights or histopathology.

Finally, Kattainen et al. (2001), Keller et al. (2007, 2008a,b), and Miettinen et al. (2006) evaluated dental caries and molar development in rats and mice. These species only develop one set of teeth in their lifetime, have enamel on the lingual surface of the incisors only, and consume a diet that is substantially different from that of humans. Further, humans generally practice dental care, which rats and mice do not. Thus, not only is assessment of these “developmental” endpoints highly unusual, they are of questionable relevance for assessing potential effects on human embryonic development.

As EPA notes in their document (pg 4-6), “(s)ome endpoints/effects may be sensitive, but lack general toxicological significance due to not being clearly adverse, being an adaptive response or not being clearly linked to downstream function or pathological alterations.” Most of the above noted effects are primary examples of toxicologically irrelevant findings that EPA has failed to exclude from its analysis.

2. Some of the studies cited in support of EPA’s derivation of an RfD report findings that conflict with those of other studies, thus indicating that the associated responses to TCDD treatment have not been well-elucidated.

In Amin et al. (2000), gestational TCDD treatment reduced the saccharin preference of female, but not male, rats. The authors suggested that these findings may be due to an anti-estrogenic effect of TCDD, resulting in masculinization of the female response. This conclusion, however, runs counter to their own interpretation of effects on male reproductive behavior (*i.e.*, that TCDD causes feminization of male reproductive behavior), as reported in other studies (Bjerke et al., 1994; Gray et al., 1995; Mably et al., 1992). In another example, Markowski et al. (2001) reported that female rats exposed to TCDD during gestation exhibited a dose-related reduction in wheel-running activity/responses. These findings, however, contrast with those of Hojo et al. (2002), which showed an increased response of females following TCDD treatment on GD8 using a complex behavioral testing paradigm. They also conflict with the findings of other researchers (Gray and Ostby, 1995) that showed no effect of GD15 TCDD treatment on wheel running activity. The lack of agreement among studies regarding the evaluated responses following TCDD treatment suggests that these endpoints likely are not sensitive indicators of TCDD-mediated effects. Thus, they should not be used to support the derivation of an RfD for TCDD.

3. Many of the animal studies used dosing regimens that cannot be properly extrapolated to chronic exposures, and thus, are inappropriate for derivation of a chronic RfD.

In particular, a number of developmental studies were cited that administered an acute dose of TCDD to animals on a single day during gestation (Hojo et al., 2002; Kattainen et al., 2001; Keller et al., 2007, 2008a,b; Markowski et al., 2001; Miettinen et al., 2006); another study administered a single dose of TCDD to immature female rats (Li et al., 1997). Additionally, some of the cited studies used short-term exposures of two weeks or less (Amin et al., 2000; Li et al., 2006; White et al., 1986), while others reported administration of weekly or biweekly doses of TCDD (Cantoni et al., 1981; NTP, 1982; Sewall et al., 1995; Shi et al., 2005; Toth et al., 1979; Vos et al., 1973). None of these dosing regimens is appropriate for modeling the effects associated with chronic daily TCDD exposure.

TCDD is well-absorbed and tends to distribute to most tissues quickly; however, TCDD is ultimately stored in the adipose tissue and its diffusion into this tissue occurs much more slowly than that into other body compartments. Further, TCDD is slowly metabolized and eliminated from the body. Thus, because of these unique characteristics, the kinetics following a single or short-term exposure will be substantially different from that observed during chronic TCDD exposure. Similarly, weekly exposures will not properly model the kinetics associated with daily administration of TCDD over the long-term. Because the kinetics associated with these different dosing regimens are likely to be substantially different from those associated with chronic daily TCDD exposure, they do not accurately predict the chronic daily doses at which adverse findings may be observed.

4. Some of the findings that are addressed in derivation of an RfD are actually precancerous lesions, and as such, are more appropriate to consider for derivation of a cancer slope factor for TCDD.

In EPA's treatment of the 2006 NTP report on TCDD, the Agency focuses on findings of liver hypertrophy and hyperplasia. Using interim sacrifices, these effects of TCDD were shown to be related to subsequent development of hepatocellular adenomas at the end of two years. Thus, the hypertrophy and hyperplasia noted in this study are part of the progression towards cancer, and should be used in derivation of a cancer slope factor, but not for development of a chronic RfD value.

5. In developmental studies, the appropriate unit for statistical analysis is the litter; however, many of the developmental studies considered by EPA incorrectly used the individual pup as the statistical unit for analysis.

The best example of this is Shi et al. (2007), which reported statistically significant findings based on 10 pups per group derived from three dams only. Other studies by Keller et al. (2007, 2008a,b) and Miettinen et al. (2006) do not specifically report whether they used the litter or pup

for the basis of their statistical analyses; however, the data are presented based on individual animals and appear to have been statistically analyzed as such. Data from developmental studies that have been incorrectly evaluated using the individual pup should not be used as the basis for derivation of an RfD. Alternatively, the original study data may be reanalyzed using the litter as the statistical unit of analysis.

Finally, it should be mentioned that both Hojo et al. (2002) and Markowski et al. (2001) used only one pup per litter (or one pup per sex per litter) in their analyses. Using a single animal from each litter allows for the data to be evaluated on both a litter and individual pup basis. This is an appropriate statistical treatment of the data. However, by using a single animal per litter, informative data that could be derived from using all animals in a litter are lost. Therefore, although the use of such procedures is considered “correct”, it is not the most suitable method to determine the true effects associated with developmental exposures.

6. Some of the data are derived from guinea pigs, which are known to be substantially more susceptible to the effects of TCDD treatment than are humans.

Both Decaprio et al. (1986) and Vos et al. (2003) investigated the effects of subchronic TCDD exposure in guinea pigs, which are much more sensitive to the toxic effects of TCDD than are other species. Vos et al. (2003) also examined the effects of TCDD treatment in rats and mice; however, the EPA focuses only on the findings in guinea pigs to support their derivation of an RfD. The exquisite sensitivity of guinea pigs to TCDD toxicity is demonstrated in Vos et al. (2003) by the 100% mortality at 1,000 ng TCDD/kg per week versus the complete absence of death in rats at doses up to 5,000 ng/kg per week and the loss of only one mouse at doses of up to 25,000 ng/kg per week. Because of the extreme sensitivity of guinea pigs, a 3x uncertainty factor for animal-to-human extrapolation is unfounded for these studies. Further, the doses from the guinea pig studies were likely biased low, as noted by EPA (on page 4-18) because they were extrapolated using first-order body burden HED estimates (EPA, 2010).

Summary

As a whole, the body of animal studies evaluated by the EPA exhibit a number of deficiencies that limit their utility to support EPA’s derivation of a chronic RfD value for TCDD. Some of the studies evaluate endpoints that are not linked with adverse events, and therefore of questionable toxicological relevance. Additionally, a few of the cited studies report findings that conflict with those of others. Many of the studies use dosing regimens that are not appropriate for extrapolation to chronic exposures, including acute dosing, short-term treatment, and weekly/biweekly exposures. The 2006 NTP study appears to have been well-conducted; however, it specifically assesses the development of precancerous lesions, which should not be considered in the derivation of a non-cancer RfD value. A few of the developmental toxicity studies cited by EPA improperly use the individual pup, rather than the litter, as their statistical unit for analysis. Further, EPA inappropriately applies a 3x uncertainty factor to studies in the

guinea pig for extrapolation of the results to humans, although the guinea pig is known to be the most susceptible species to TCDD toxicity.

Finally, the study inclusion criteria used by the EPA for consideration of animal bioassays does not address certain critical parameters that should be considered in determining what studies are appropriate for derivation of an RfD. These include:

- Toxicological relevance of the endpoint – This parameter is supposedly considered in EPA's process to select/identify candidate points of departure, but should be considered in advance of this step.
- Duration of exposure – Studies that use a single dose or a short-term exposure are not appropriate for derivation of a chronic toxicity reference value and should have been dropped from inclusion altogether, as discussed in Point 3 above.
- Number of animals per group, number of doses, the spread of the doses – These criteria all address whether the study is appropriately designed; it is not enough that the study clearly explains what was done, it needs to have been done appropriately to be considered a quality study.

Further, EPA does not adequately explain the rationale for using a cut-off of ≤ 30 ng/kg/d as one of the inclusion criteria.

Appendix 1

Critical Review of the Animal Bioassays Relied on for Derivation of TCDD RfD

Amin et al. (2000)

Amin et al. (2000) examined the effects of gestational exposure to TCDD, PCB77 or PCB 126 on the development of a non-reproductive sexually dimorphic behavior (specifically, saccharin preference) in male and female Sprague-Dawley rats. For the purposes of this review, only findings related to TCDD exposure will be discussed. Pregnant rats were received from the supplier in four separate shipments spaced 8 weeks apart and equal numbers of rats from each shipment were assigned to each test group. Dams (exact number per group not reported) were gavaged with 0, 25, or 100 ng/kg/day of TCDD in a corn oil vehicle on gestational days (GDs) 10-16. Following delivery, litters were culled to 8 pups each (equally divided between males and females on postnatal day (PND) 2; this is a little earlier than normal (PND 4). Litters that had fewer than 5 pups per litter were excluded from evaluations, resulting in 10-11 litters per group for evaluation. TCDD treatment was reported to have no effect on reproductive outcomes, including gestational body weight gain, litter size, live birth percentage, or pup birth and weaning weights; however, statistical analyses on these endpoints were not presented. Further, it should be noted that gestational body weight gain in the TCDD low dose group was substantially less than that of the control group (139.5 ± 8.7 g versus 154.8 ± 9.4 grams, respectively); mean body weight gain in the TCDD high dose group, however, was comparable to that of control. Pups were weaned on PND21 and one male and one female from each litter were retained for later testing.

Saccharin preference testing was conducted at 189-234 days of age. The reason for the large range in ages at the time of testing was not reported; further, it is not known if the mean age at testing differed among groups or if all animals were tested at the same time. During the first 4 days of testing, animals were provided with two bottles of tap water and daily water consumption was determined. On days 5 & 6, one of the tap water bottles was replaced with a 0.25% saccharin solution. On days 7 & 8, the saccharin solution was increased to 0.50%. The placement of the bottles was changed daily to control for any positional preferences. TCDD treatment had no significant effect on the saccharin preference of males. However, females of dams treated with either the low or high dose of TCDD consumed significantly less saccharin solution (expressed as either the amount of solution consumed per 100 g body weight or the amount of total fluid consumption that consisted of saccharin solution) than controls (exception: using the 0.25% solution, the effect at the low dose was not statistically significant when

measured relative to body weight). The response using a 0.50% solution was greater than that observed using a 0.25% solution. Further, the reduction exhibited a dose-response relationship. However, there is no indication that the authors controlled for multiple comparisons to maintain alpha at 0.05 and limit Type I errors in this study.

BRIEF SUMMARY: The authors suggest that the findings of this study (altered saccharin preference in female rats) may be due to an anti-estrogenic effect of TCDD, resulting in masculinization of the female response. This conclusion runs counter to their interpretation of effects on male reproductive behavior resulting from developmental TCDD exposure, as reported in other studies (Bjerke et al., 1994; Gray et al., 1995; Mably et al., 1992). Specifically, they suggest that TCDD exposure causes feminization of male reproductive behavior. Further, their results conflict with those of Ikeda et al. (2005), which found no effect of gestational TCDD exposure on female saccharin preference and an increased preference in males. Regardless, the toxicological relevance of a change in saccharin preference is unclear because such a finding should not be interpreted as an adverse effect. Thus, the use of this study, which evaluates a toxicologically irrelevant endpoint, to derive a chronic reference dose is inappropriate.

Bell et al. (2007)

Bell et al. (2007) investigated the reproductive effects of TCDD exposure on male offspring in a GLP-compliant study conducted at Covance. Groups of 65 female Wistar rats (5-6 weeks of age; n=75 in the control group) were fed diets containing 0, 28, 93 or 530 ng TCDD/kg diet (TCDD dissolved in acetone) through a 12-week pre-mating acclimation period, mating, and gestation. Diets were discontinued once animals gave birth. A total of 45 animals in the control group and 35 animals in each of the treated groups were sacrificed during the course of the study prior to delivery for other analyses not discussed in the study report. The remaining 30 animals per group were allowed to deliver. Litters were culled to 8 pups each on PND4 and to 5 males per litter (when possible) at weaning on PND21. One male per litter was sacrificed in postnatal week 10 (designated PND70; total pups per group = 25); the rest of the animals were sacrificed in postnatal week 17 (designated PND120). At necropsy, the weights of the liver, kidneys, spleen, thymus, and male reproductive and sex accessory organs were recorded. Histopathological evaluation of the testes, epididymides, liver, thymus, and prostate was conducted for the control and high dose group males only. Sperm from the cauda epididymis were counted and 500 sperm per animal evaluated for motility, viability and morphology.

In other examinations, 20 animals per group were evaluated in weeks 12 and 13 using the swim maze, motor activity tests, and a functional observation battery (week 13 only). In postnatal week 16, 20 males per group were mated with virgin females; these females were sacrificed on GD16 for evaluation of developmental endpoints. All statistical evaluations were conducted using the litter as the unit of analysis.

TCDD intake based on feed consumption was 0, 2.4, 8 and 46 ng TCDD/kg/d in the control, low, medium and high dose groups, respectively. Dams in the high dose group gained slightly more weight than controls during the first 5 weeks of the pre-mating period, which corresponded with a greater food intake during pre-mating than that of controls. No statistically significant differences from control were noted in fertility and mating indices, gestational duration, the number of pups per litter, pup sex ratio, or pup survival. Pup weights showed a dose-related decrease from PND1-PND21; however, it is not known if these differences were considered statistically significant. Balano-preputial separation was significantly delayed in all TCDD-treated groups in a dose-related manner (1.8, 1.9, and 4.4 days in the low, medium and high dose groups, respectively); further, differences in body weights among the treatment groups did not appear to account for the delays. Body weights were not significantly different among groups in postnatal week 10; however, a dose-related decrease in body weights was observed in postnatal week 17, with the difference between high dose and control animals being statistically significant. Various organ weight changes from control were noted at necropsy; however, none of these (with the exception of relative brain weights in postnatal week 17, which are likely related to reduced body weights) exhibited a dose-related trend. In postnatal week 10, the proportion of abnormal sperm was higher and the number of spermatids was lower in the high dose group compared to control; however, no significant differences were observed at postnatal week 17, suggesting that the findings were either transient or due to chance. No major histopathological findings were noted.

No significant differences were noted among treatment groups in swim maze performance or in the functional observational battery. Animals in the high-dose group were reported to be significantly less active than control in the motor activity test; however, the data were not shown. Following mating of the male offspring to virgin females, no differences from control were noted in reproductive or developmental endpoints.

BRIEF SUMMARY: Bell et al. (2007) appears to be a well-conducted, GLP-compliant reproductive toxicity study. It assessed an adequate number of animals per group and evaluated a large number of endpoints, including developmental outcomes in F₁ male and F₂ offspring; reproductive performance of F₁ males; bodyweights; organ weights; histopathology; sperm viability, motility, and morphology; and various neurobehavioral parameters. The majority of these endpoints were unaffected by treatment. In a few cases, statistically significant differences from control were noted in TCDD-treated groups; however, these findings often did not demonstrate a dose-response relationship or were not consistent across time points. Gestational TCDD treatment appears to be associated with a significant, dose-related delay in balano-preputial separation, reduced body weights in postnatal week 17, and possibly decreased motor activity in male offspring.

Cantoni et al. (1981)

Cantoni et al. investigated the porphyrinogenic effects of TCDD in rats. The authors note that rats are less sensitive than mice to porphyria induced by TCDD and other polyhalogenated aromatic hydrocarbons, but female rats are more sensitive than males. Female CD-COBS rats were administered 0, 0.01, 0.1, and 1 µg/kg/week TCDD in corn oil:acetone (6:1) by oral gavage for 10 months (N = 4, 4, 3, and 3, respectively). Porphyrins were measured in 24-hour urine collected at 2, 3, 4, 6, 8, and 10 months, and in liver, kidney, and spleen at study termination.

At 10 months, total urinary porphyrins were slightly, but significantly increased in the 0.01 and 0.1 µg/kg dose groups (2.4- and 3.4-fold, respectively; $P < 0.05$) and increased more than 80-fold in the 1 µg/kg group ($P < 0.01$). The pattern of porphyrin speciation in the 1 µg/kg group also shifted over the time course of the study from 70% coproporphyrin (as a percentage of total urinary porphyrins) at the beginning of the study, to 80% uroporphyrin at 10 months. Coproporphyrins remained at 50% to 70% throughout the study in the control group (data not presented for the 0.01 and 0.1 µg/kg dose groups). Although coproporphyrin was significantly increased for all dose groups, beginning at 2 months in the 1 µg/kg group and at 3 months in the 0.01 and 0.1 µg/kg dose groups, the ultimate porphyrin species, uroporphyrin, was significantly increased only in the 0.1 µg/kg ($P < 0.05$) and 1 µg/kg ($P < 0.01$) dose groups. Thus, the small increase in total porphyrins in the low dose group was mostly the result of increased coproporphyrins.

Signs of porphyria were only present in the 1 µg/kg group, in which livers were pigmented, 38% larger (data not shown), and liver porphyrins were increased 450-fold ($P < 0.01$). In addition, kidney and spleen porphyrins were elevated 15-fold ($P < 0.01$) and 7-fold ($P < 0.05$), respectively.

EPA concluded that the LOAEL for this study was 0.01 µg/kg/week (1.43 ng/kg/day) based on a 2- to 3-fold increase in urinary coporphyrins, and that a NOAEL could not be determined. It is difficult to determine the toxicological significance of this small difference given the absence of frank effects, and more broadly the 2.4 to 3.4-fold difference in total porphyrins in the 0.01 and 0.1 µg/kg groups, without knowing the normal range for rats of this strain. In humans, the normal range for a 24-hour urine sample is 50-300 mg total porphyrins, a 6-fold difference.¹ In this regard, it is noteworthy that even in the control group there was a considerable amount of variability in mean urinary coporphyrins over the course of the study, ranging from approximately 0.5 (at 4 months) to 1.3 (at 0 months) µg/24-hour urine, a 2.6-fold difference.

BRIEF SUMMARY: Porphyrin levels were measured in female rats administered TCDD once weekly by oral gavage for 10 months. Total urinary porphyrin levels were increased slightly in the 0.01 and 0.1 µg/kg/week dose groups (2.4- and 3.4-fold, respectively) and more than 80-fold

¹ MedlinePlus, U.S. National Library of Medicine and National Institutes of Health, <http://www.nlm.nih.gov/medlineplus/ency/article/003614.htm>

in the 1 µg/kg/week group. Signs of porphyria (liver pigmentation, increased liver porphyrins, and increased liver size) were observed only in the 1 µg/kg/week group. EPA concluded that the LOAEL was 0.01 µg/kg/week (1.43 ng/kg/day) and that there was no NOAEL. However the toxicological significance of the small changes in the low and medium dose groups is unclear in the context of normal variability for urinary porphyrins and the lack of frank effects.

Chu et al. (2001)

Chu et al. (2001) investigated the effects on rats of TCDD exposure alone, and in combination with PCBs. Consistent with EPA's review, only the results from TCDD exposure alone are summarized here. Female Sprague Dawley rats (5/dose group) were administered 0, 2.5, 25, 250, or 1,000 ng TCDD in corn oil daily by oral gavage for 28 days. Body weights were recorded three times per week and clinical observations made daily. At study termination blood was collected for measurement of serum biochemical and hematological parameters, and selected organs removed, weighed, and prepared for analysis of enzyme activity and other parameters.

In the 1,000 ng/day dose group body weight and relative thymus weight were reduced and liver weight was increased ($P<0.05$). Several biochemical parameters were also significantly altered in the 1,000 ng/day dose group, including increases in EROD, MROD, BROD, UDPGT, serum cholesterol, vitamins A and C, and serum albumin ($P<0.05$). EROD, MROD, UDPGT, and serum cholesterol were significantly increased in the 250 ng/day group as well ($P<0.05$). Most hematological parameters were unaffected, although there were small (7%) decreases in mean corpuscular volume and mean corpuscular hemoglobin in the 1,000 ng/day group ($P<0.05$).

Although there were significant changes in some biochemical measurements in the 250 ng/day dose group, the toxicological significance of these changes in the absence of frank effects on body or organ weights is questionable. Therefore, the LOAEL for this study is 1,000 ng/day based on reduced body and thymus weight, increased liver weight, and associated increases in several biochemical parameters. The NOAEL is 250 ng/day. EPA concurred with this conclusion in their review.

BRIEF SUMMARY: The effects of TCDD exposure on body and organ weights as well as on biochemical and hematological parameters were investigated in female rats administered TCDD daily by oral gavage for 28-days. A LOAEL of 1,000 ng/day was determined based on reduced body and thymus weights, increased liver weight, and associated increases in several biochemical parameters. Some biochemical parameters were also affected in the 250 ng/day group, but there were no associated effects on body or organ weight. Therefore, the NOAEL for the study is 250 ng/day.

Crofton et al. (2005)

Individual dose-response curves were developed for the compounds that would comprise an environmentally modeled mixture. Female Long-Evans rats were exposure to TCDD via gavage

for four days at 0, 0.1, 3, 10, 30, 100, 300, 1,000, 3,000, or 10,000 ng/kg-day TCDD (purity >99%). Four to 12 animals were in the treatment groups and 14 animals were in the control group. Serum T4 levels were determined the day after the last dose. No statistics were reported and the T4 concentrations and standard deviations are reported as a % of the control. It does not appear that any differences in T4 levels exist at doses ≤ 30 ng/kg-day TCDD. However, at 100 ng/kg-day TCDD T4 levels were reduced to 70.94% and continue to decrease in a dose-related manner up to 10,000 ng/kg-day TCDD. EPA selected 100 ng/kg-day TCDD as the LOAEL and 30 ng/kg-day TCDD as the NOAEL.

BRIEF SUMMARY: Crofton et al. (2005) developed dose-response curves for TCDD-induced reductions in serum T4 concentration. Substantial reductions in T4 levels were observed at 100 ng/kg-day TCDD, which EPA selected as the LOAEL. Use of thyroid hormone changes as a point of departure for setting regulatory threshold is inappropriate.

DeCaprio et al. (1986)

Guinea pigs are the most sensitive experimental animal species to the toxic effects of 2,3,7,8-TCDD. This was the first subchronic study to investigate the effects of daily oral administration of 2,3,7,8-TCDD to guinea pigs. Guinea pigs were administered 2,3,7,8-TCDD in the diet for 90 days at 0, 0.12, 0.61, 4.9, and 26 ng/kg-day (males) and 0, 0.12, 0.68, 4.86, and 31 ng/kg-day (females). Significant mortality was observed at the highest dose therefore, remaining animals were sacrificed at Day 46 (males) or Day 60 (females). Body weights were statistically significantly reduced at the two highest dose levels. In males, absolute kidney, absolute thymus, and relative thymus weights were statistically decreased and relative brain and liver weights were increased at 4.9 ng/kg/day. In females, only relative liver weight was increased at 4.86 ng/kg/day (Note: female liver weight changes not mentioned in EPA's review). At the 4.9 ng/kg/day dose level, elevated triglycerides were seen in male guinea pigs and decreased ALT was observed in female guinea pigs. In a companion study, the reversibility of the effects at the highest dose level were also investigated following administration of 2,3,7,8-TCDD for 11, 21, or 35 days. Body weight changes were observed within the first week in all regimens and no recovery was seen in the two groups exposed for the longer durations, but the 11-day group recovered and gained weight at the same rate as the controls, although they never regained the initial weight loss. Mortality was 10% and 70% in the 21- and 35-day exposure and recovery groups, respectively. However, no mortality was observed in the 11-day exposure recovery group (Note: EPA's summary reports a 10% mortality for this group).

The NOAEL identified by the authors, 0.61 ng/kg-day (males) and 0.68 ng/kg-day (females) is the same as that selected by EPA and generally based on the same results. This NOAEL is based on the statistically significant decreases in body weight in both genders, decreased relative thymus weights and increased triglycerides in males. Although an increase in relative liver weight was seen in both genders at approximately 4.9 ng/kg/day, the authors highlight the lack of

other signs of liver toxicity and question the toxicological significance of these findings in the guinea pig.

BRIEF SUMMARY: DeCaprio et al. (1986) conducted the first subchronic, 90-day study with guinea pigs, the most sensitive species to TCDD, decreases in body weight and organ weight changes were reported. Although significant increases in relative liver weights were seen, no other signs of hepatic toxicity were observed. The NOAEL was established on decreased body weight and relative thymus weights, and increase triglycerides in males.

Fattore et al. (2000)

Fattore et al. (2000) investigated the effect of TCDD and other dioxin and furan congeners on hepatic vitamin A levels in Sprague Dawley (Iva:SIV 50) rats. Consistent with EPA's review of the study, only the TCDD results are summarized here. In the first experiment, male and female rats (N = 6/sex/dose group) were maintained on diets containing 0, 0.2, 2, and 20 µg/kg TCDD for 13-weeks. The authors estimated the respective intake levels at 0, 0.3, 3, and 32 µg/day for females and 0, 0.5, 5, and 43 µg/day for males. The diets were also supplemented with 3 µg-vitamin A/kg-diet. At study termination, animals were sacrificed and livers prepared for vitamin A analysis. Experiments 2 and 3 used similar experimental designs, but Experiment 2 had a single TCDD dose group (2 µg/diet) and Experiment 3 had dose groups of 2 and 10 µg/kg-diet. Experiment 4 included three sub-experiments, using female rats only, with one TCDD dose group each of 1 µg/kg-diet for 13, 26, and 39 weeks, respectively.

In Experiment 1, there was a dose-dependent decrease in hepatic vitamin A of 26%, 72%, and 99% from controls in females in the 0.2, 2, and 20 µg/kg dose groups, respectively, and a similar decrease in males. However, vitamin A levels were significantly decreased only in the 2 µg/kg ($P < 0.05$ for males and females) and 20 µg/kg ($P < 0.01$ for males, $P < 0.001$ for females) dose groups. Food consumption and dietary vitamin A intake of were similar across all dose groups. No significant effects were reported in the low dose group. As reported in previous studies and summarized by Fattore et al. (2000), liver and body weight were significantly affected in 2 and 20 µg/kg dose groups, and there was an increased incidence of liver lesions in the 20 µg/kg group, and to a lesser extent in the 2 µg/kg group. Therefore, the LOAEL for Experiment 1 should be interpreted as 2 µg/kg (3 and 5 µg/day for females and males, respectively), and the NOAEL as 0.2 µg/kg (0.3 and 0.5 µg/day for females and males, respectively). In contrast, EPA identified the low dose group (0.2 µg/kg), which they estimated as a daily dose of 20 ng/kg/day by assuming food consumption to be 10% of body weight, as the LOAEL for decreased hepatic vitamin A. EPA did not identify a NOAEL for the study.

Experiments 2 and 3 also resulted in significant reductions in hepatic vitamin A, but because they used dose levels at or above the LOAEL for Experiment 1 (i.e., 2 µg/kg) they do not provide additional information. Hepatic vitamin A levels decreased in a time-dependent manner in the female rats administered a diet with 1 µg/kg TCDD in Experiment 4 to 30%, 19%, and 5%

of controls at 13, 26, and 39 weeks ($P < 0.05$). Based on these results, the LOAEL for Experiment 4 (and the study as a whole) can be interpreted as 1 $\mu\text{g}/\text{kg}$ (2, 3, and 5 $\mu\text{g}/\text{day}$ for 13, 26, and 39 week old rats, respectively). The study NOAEL is 0.2 $\mu\text{g}/\text{kg}$, from Experiment 1.

BRIEF SUMMARY: Fattore et al. (2000) investigated the effect of TCDD administered in the diet to rats for 13 weeks on hepatic vitamin A levels. There was a dose-dependent reduction in hepatic vitamin A, with significant reductions at TCDD diet concentrations of 1, 2 and 20 $\mu\text{g}/\text{kg}$ but not at 0.2 $\mu\text{g}/\text{kg}$. Although EPA identified the lowest dose as the LOAEL, the LOAEL for the study should be interpreted as 1 $\mu\text{g}/\text{kg}$ -diet (2 $\mu\text{g}/\text{day}$ for 13-week old female rats) with a NOAEL of 0.2 $\mu\text{g}/\text{kg}$ (0.3 $\mu\text{g}/\text{day}$ for 13-week old female rats).

Franc et al. (2001)

Franc et al. (2001) evaluated the effect of TCDD exposure on AhR regulation, and of AHR levels on sensitivity to TCDD-induced toxicity. Female Long-Evans (L-E), Sprague Dawley (SD), and Han/Wistar (H/W) rats ($n=8/\text{strain}/\text{dose}$ group) were administered doses of 0, 140, 420, or 1,400 ng/kg TCDD (>99% purity) in corn oil by oral gavage every 2 weeks for 22 weeks, corresponding to dose equivalents of 0, 10, 30, or 100 $\text{ng}/\text{kg}/\text{day}$. The three rat strains exhibit varying sensitivity to acute toxicity from TCDD: L-E ($\text{LD}_{50} \sim 10 \mu\text{g}/\text{kg}$) > SD ($\text{LD}_{50} \sim 50 \mu\text{g}/\text{kg}$) > H/W ($\text{LD}_{50} > 9,600 \mu\text{g}/\text{kg}$).

L-E rats had decreased body weight gain over the course of the study relative to the other two strains ($P < 0.001$). Although the effect of TCDD on body weight appeared significant in the L-E rats, the authors did not report results in this way, focusing instead on the strain effect and the time \times strain \times TCDD interaction. There was little apparent effect of TCDD on body weight in the SD and H/W rats. TCDD-induced liver hypertrophy occurred in all strains, with statistically significant increases in relative liver weight for all three rat strains at all doses ($P < 0.05$), but particularly for SD rats in the 100 $\text{ng}/\text{kg}/\text{day}$ group (~60% increase from controls). Although statistically significant, the relative liver weight was increased <10% from controls for all strains in the 10 $\text{ng}/\text{kg}/\text{day}$ group and for H/W rats in the 30 $\text{ng}/\text{kg}/\text{day}$ group. These small changes are not considered toxicologically relevant. EPA concurred with this view. Relative liver weight increased ~20% from controls in 30 $\text{ng}/\text{kg}/\text{day}$ group for SD and L-E rats, and in the 100 $\text{ng}/\text{kg}/\text{day}$ group for L-E and H/W rats. Relative thymus weight was significantly reduced in the 30 and 100 $\text{ng}/\text{kg}/\text{day}$ dose groups for all strains to ~25 to 75% of controls ($P < 0.05$). The SD and L-E rats responded similarly, and the H/W appeared slightly less sensitive.

AhR mRNA was significantly increased in a similar fashion in all three rat strains at the 10 and 30 $\text{ng}/\text{kg}/\text{day}$ doses, but only in L-E rats at the 100 $\text{ng}/\text{kg}/\text{day}$ dose level. Hepatic AhR protein levels were also increased in the SD and L-E rats in the 30 $\text{ng}/\text{kg}/\text{day}$ dose groups, but only in the L-E rats at the 10 $\text{ng}/\text{kg}/\text{day}$ dose level ($P < 0.05$). SD rats exhibited the greatest induction, with a greater than two-fold induction in the 30 and 100 $\text{ng}/\text{kg}/\text{day}$ groups when measured by radioligand binding assay. AhR protein levels were not increased in H/W rats in any dose group.

Contrary to the study authors' conclusion, there was some degree of correlation between AhR regulation and TCDD-induced liver toxicity. The H/W rats experienced no upregulation of AhR protein and were also the least sensitive to relative liver weight increases. The SD rats had, by a large margin, the greatest amount of AhR protein upregulation and the largest increase in relative liver weight in the 100 ng/kg/day dose group.

The LOAEL for this study is 30 ng/kg/day, based on increased relative liver weight in SD and L-E rats and decreased relative thymus weight in all three strains. Although relative liver weight was also increased in the 10 ng/kg/day group, the increase was small and not considered biologically relevant. Thus, the NOAEL for this study was 10 ng/kg/day. This conclusion is consistent with EPA's review of the study. In this particular study, the NOAEL is the same regardless of strain. But the variability in susceptibility raises potential questions for interpretation of other studies using one rat strain. The study used relatively few animals, further limiting evaluation of strain differences.

BRIEF SUMMARY: Susceptibility to TCDD exposure was evaluated in three rat strains with known variability in susceptibility to TCDD acute toxicity. Although the results yielded the same NOAEL (10 ng/kg/day) and LOAEL (30 ng/kg/day) for all strains based on liver and thymus weight changes, there were differences in the magnitude of response. The liver weight changes correlated, to some degree, with upregulation of Ah receptor protein levels.

Hojo et al. (2002)

Hojo et al. (2002) examined the effects of gestational TCDD exposure on the operant behavioral response of male and female Sprague Dawley rats using various fixed-ratio (FR) and differential reinforcement schedules. Pregnant dams were gavaged with 0, 20, 60, or 180 ng/kg TCDD in a corn oil vehicle on GD8. A total of 36 pregnant dams were treated; however, only 5-6 litters per group were ultimately tested. The reason certain litters were excluded from evaluation is not clear. It is also unclear why the control dams lost weight during gestation days 4-8, which is atypical of pregnant Sprague-Dawley dams. Furthermore, the authors only presented body weight data through gestation day 16, at which time the high-dose dams had gained approximately 11% less weight than control dams (GD 0-16). Gestational duration, gestational weight, the number of live pups, sex ratios and pup weights were recorded and found not to be significantly affected by treatment. Litters were culled to 10 pups each on PND4, with equal distribution of sexes in each litter. In the retained litters, the high-dose group had 1.1 fewer pups/litter than the control group. At weaning, pups were housed in same sex litter pairs until PND60, then individually thereafter. On PND80, one pup per sex per litter was randomly selected for behavioral testing. Testing was conducted starting on PND90 in operant chambers containing two levers each, only one of which was used in the experiments. Rats were first trained to lever-press using a variable-time FR schedule, which dispensed a food pellet after a single lever press (FR1) or after 30.5 seconds had elapsed, then switched to a FR1-only schedule

for 3-4 sessions. Next, the rats were subjected to an incremental FR schedule, which incrementally increased the number of lever pushes required to dispense a food pellet every four sessions in the following sequence: 1, 6, 11, 21, 31, 41, 51, 61 and 71. Finally, rats were retrained, then tested on a multiple FR11, differential reinforcement of low rate (DRL) 10-second schedule. On this schedule, rats were required to press the lever 11 times to obtain a food pellet during one component of the session or to respond with 10-second delay between lever-pushes to obtain a food pellet during the other session component. The test animals showed no treatment-related differences in responding during the FR1 schedule or the incremental FR schedule. During the multiple FR11, DRL 10-second reinforcement schedule, control males exhibited a higher response rate than control females during both components of the session. Also, TCDD-treated males exhibited a lower rate of response than control males, while TCDD-treated females exhibited a higher rate of response than control females. However, no clear dose-response relationships were observed for either sex during either component of the session despite the 9-fold increase in TCDD dose between to low and high dose groups. Further, the increased rate of response in females conflicts with the reduced rates of response in females observed after TCDD treatment on GD18, as noted in another study by the same group of researchers (Markowski et al., 2001). Many of the endpoints examined in this study were highly correlated. Furthermore, the authors conducted some mathematical manipulations of the data, subtracting the mean female response rate from that of her paired male littermate for each session block of each component (FR11 or DRL 10-sec) of the operant schedule, then took the mean of these values for benchmark modeling and calculate of ED₁₀ and ED₀₁ values. The validity of conducting such calculations for determining effective doses (i.e., comparing male versus female behavior rates instead of treated versus control rates), however, is not clear. Finally, the toxicological relevance of the findings, if any, is not known.

BRIEF SUMMARY: Hojo et al. (2002) is a single dose study using a small number of animals per group. An effect on behavior was observed using a complex testing paradigm; however, this effect was not seen using more simplified operant testing schedules. Further, the direction of the change appears to depend on the sex of the animal, which is highly unusual. These complexities contribute to the further weakening of any toxicological relevance of these findings. Due to the questionable relevance of the findings and the fact that the single doses used on this study cannot be appropriately adjusted to chronic exposure concentrations, Hojo et al. (2002) is considered unsuitable for derivation of a chronic reference dose for TCDD.

Hutt et al. (2008)

Sensitivity to disruption of morphogenesis was evaluated in pre-implantation embryos of Sprague Dawley rats harvested from pregnant dams that had been exposed to TCDD in corn oil by oral gavage at various life stages. In the first experiment, 0 or 50 ng/kg TCDD (purity >99%) was administered to pregnant dams (N = 3/dose group) on GD 14, GD 21, PND 7, and PND 14. On PND 21 female pups (N = 3/dose group) were weaned and began receiving weekly doses of 0

or 50 ng/kg TCDD. At 3 months of age, the F1 females were mated and pre-implantation embryos were collected on day 4.5 post coitum. In a second experiment, 50 day old female rats (N = 3-6/dose group) were administered a single dose of 0, 50, 1,000 or ng/kg TCDD in corn oil by oral gavage on the evening of proestrus. Pre-implantation embryos were collected on day 4.5 or 5.5 post coitum. Embryos from both experiments were analyzed using immunofluorescent confocal microscopy and classified for abnormalities.

In Experiment 1, the number of embryos per animal present at compaction stage (8-16 cell) was not affected by 50 ng/kg/week TCDD, however, the percent of morphologically normal embryos decreased from 80% in controls to 37% in the TCDD treatment group ($P < 0.05$). Abnormalities included irregularly sized and shaped blastomeres, alterations in nuclear shape, disruption of cytokinesis, spindle defects, and aberrant f-actin caps.

The single TCDD exposure in experiment 2 induced similar results: the percent of morphologically normal embryos was significantly reduced in both TCDD exposure groups ($P < 0.05$) from 80% in controls to 52 and 46% in the 50 and 1,000 ng/kg dose groups, respectively. Despite the high incidence of abnormalities at the compaction stage, however, both survival to blastocyst stage and the average number of cells/blastocyst were similar in all groups. In the 1,000 ng/kg dose group the percentage of normal blastocysts was significantly reduced to 46% compared to 76% in controls and 52% in the 50 ng/kg group ($P < 0.05$), however the abnormalities were considered less severe than those observed in the compaction stage and typically occurred in isolated blastomeres within otherwise normal blastocysts (including normal blastocoels). This study did not investigate whether abnormalities persisted beyond the blastocyst stage.

EPA identified a LOAEL for the study of 50 ng/kg/week (7.1 ng/kg/day) based on an increased incidence of pre-implantation embryos with morphological abnormalities at the compaction stage. A NOAEL was not identified by EPA. However, for several reasons this study is not suitable for deriving a toxicity factor for risk assessment. First, results were analyzed and reported on a per animal basis, not a per litter basis, which is the appropriate denominator for reproductive toxicity studies. Second, there were relatively few blastocysts analyzed in the study and they were collected from only a three to five dams per group (only 3 dams/group in the multiple exposure study). Third, there are no comparative historical data or other quantified experience with these kinds of observations. Fourth, given the lack of impact on survival to blastocyst stage and the apparent reduced incidence and severity of abnormalities in blastocysts (compared to compaction stage embryos), it is unclear what long-term effects if any the pre-implantation effects observed would ultimately have on fetal development.

BRIEF SUMMARY: Sensitivity to disruption of morphogenesis was evaluated in pre-implantation embryos of rats exposed to TCDD by oral gavage. Both repeated exposures (beginning *in utero* and continuing postnatally through 12 weeks of age) and a single exposure at

conception resulted in an increased incidence of abnormal pre-implantation embryos at a dose level of 50 ng/kg/week. A single TCDD exposure of 50 or 1,000 ng/kg did not affect survival of embryos to blastocyst stage. Although there were a greater number of blastocyst stage embryos with abnormalities in the 1,000 ng/kg dose group, both the incidence and severity were less than at the compaction stage. Although EPA identified a LOAEL for the study, for several reasons the results from this study are not suitable for risk assessment.

Kattainen et al. (2001)

The effects of *in utero* and lactational TCDD exposure on tooth development were evaluated in rat Lines A, B, and C, derived from a selective cross of Han/Wistar rats, which are resistant to TCDD-induced acute toxicity and other effects, and TCDD-sensitive Long-Evans rats (F10 generation). The resistance of the Han/Wistar strain is associated with mutations in an aryl hydrocarbon receptor (AHR) allele (*Ahr^{hw}*) and allele *B^{hw}* from another unidentified gene. Line A is homozygous for the *Ahr^{hw}* mutation, Line B for the *B^{hw}* mutation, and Line C has neither mutation. The three lines represent a gradient of sensitivity to TCDD-induced acute toxicity, with LD50s for lines A, B, and C of >10,000, 830, and ≤40 µg/kg, respectively. A single dose of 0, 0.03, 0.1, 0.3 or 1 µg/kg TCDD (>99% purity) was administered by oral gavage in corn oil to 4-8 pregnant dams on gestational day 15 (GD 15). On post-natal day 1 (PND 1) offspring were counted and litters adjusted to 3 males and 3 females (if possible). The pups were weaned on PND 28 and subsequently housed with same-sex littermates. On PNDs 35 and 70 female and male pups, respectively, were sacrificed and lower jaws (and a subset of upper jaws) were collected and examined for tooth development.

No effects on body weight or clinical signs of toxicity were observed in the dams. Pup body weight was decreased in the 1 µg/kg group for line B during PNDs 1-7 and for Line C during PNDs 1-70 (data not shown). Line C was more sensitive to effects of TCDD on third molar development than Lines A or B ($P<0.05$). Specifically, lower third molars were completely absent in 11 of 20 Line C pups in the 1 µg/kg group (with no gender difference), but only one each in Lines A and B (both females). Only one female pup from each line lacked a third upper molar in the 1 µg/kg dose group. All molars were present in all animals in all other dose groups. Even when present, third molar eruption was significantly reduced in females from Lines B and C even in the absence of TCDD exposure. Third molars were partially or fully erupted in less than 30% Line B and C control females, but 94% of Line A control females. In Line A females, third molar eruption was significantly reduced in the 0.3 and 1 µg/kg dose groups ($P<0.05$). The effect of TCDD on molar eruption was not evaluated in Lines B and C females because of the low prevalence of eruption in the control group. In males, all third molars that developed were completely erupted by PND 70. When lower third molars were present, the mesio-distal length was significantly reduced in Lines A and C females from all treatment groups, and in Line B females from all but the 0.03 µg/kg group ($P<0.05$). Lower second molar size was also significantly reduced in females pups in the 0.3 µg/kg dose group for Line B, and in the 1 µg/kg

dose group for all lines ($P < 0.05$). Although males were less sensitive than females to TCDD-induced reductions in molar size, they still experienced significant reductions in mesio-distal length of lower second and third molars, albeit at higher dose levels than females. Incisor pulpal perforation was also evaluated, but was not present in any of the pups evaluated.

The NOAEL for maternal toxicity in this study was the highest dose, based on evaluation of body weight and clinical signs. The LOAEL for developmental toxicity in this study was the lowest dose tested, a maternal dose of 0.03 $\mu\text{g}/\text{kg}$ (30 ng/kg), based on impaired molar development in females from Lines A and C. A developmental NOAEL was not identified. This is consistent with EPA's review of the study.

Relative to lactational exposure, placental transfer of TCDD is limited (Li et al. 1995). Given the dosing regimen employed and lacking body burden measurements, it is difficult to place in context the dose-response information from this study. In addition, TCDD exposure itself alters Ah receptor levels and consequently, can alter the dose response relationship. Thus, a single large dose may have different effects than repeated low-dose exposures. The rat strains used in this study may not be best suited for evaluating TCDD-induced impairment of tooth development. Females from two of the lines (B and C) had significantly reduced molar eruption even in the control group. Line A, on the other hand, was selected for a mutation that confers resistance to TCDD-induced acute toxicity, but with unknown relative sensitivity to effects on tooth development.

BRIEF SUMMARY: Tooth development was evaluated in rat pups following *in utero* and lactational exposure to TCDD after a single maternal exposure during gestation. Molar tooth development in offspring was significantly impaired with TCDD exposure, with a LOAEL established for reduction of molar growth at the maternal dose of 30 ng/kg . A developmental NOAEL was not established. Maternal body weight was not affected and no clinical signs of maternal toxicity were observed at any dose group.

Keller et al. (2007)

Keller et al. (2007) evaluated the effect of TCDD on molar development in mice and differential susceptibility between six inbred mouse strains (C57BL/6J, BALB/cByJ, A/J, CBA/J, C3H/HeJ, and C57BL/10J). Pregnant females (number not specified) were administered 0, 0.01, 0.1, and 1 $\mu\text{g}/\text{kg}$ TCDD (purity not specified) in corn oil by oral gavage on GD 13. Although morphological signs of tooth development appear at GD 11, the first visible signs of molar development appear on GD 13-14 and continues past GD 15 (Cobourne and Sharpe 2003). F1 offspring were weaned and housed by sex at PND 28, sacrificed at PND 70. Pups were evaluated for the presence of upper and lower molars, and for cuspal development of lower first molars (M_1).

All four third molars were present in all treatment groups for the C57BL/6J, BALB/cByJ, and C57BL/10J strains, and for nearly all A/J strain mice (only 3 of 51 mice were missing third molars). Only the CBA/J and C3H/HeJ strains demonstrated susceptibility to TCDD effect on molar development. All mice in the 1 µg/kg group were missing at least one third molar. None of the CBA/J mice and only 1 of C3H/HeJ mice in the control group were missing a third molar. However, the 3% background frequency of missing third molars in the CBA/J strain has been reported (Murai 1975). The authors note that even low background frequencies suggests the likelihood of a genetic predisposition towards impaired molar development that may be triggered by less than optimal prenatal conditions. The specific insult (i.e., TCDD) may not be as important as the timing. In addition, previous studies have shown lower third molars more susceptible to loss than upper third molars following TCDD exposure. But Keller and colleagues (2007) report a loss of upper and lower third molars to be highly correlated.

Five of the six mouse strains tested had low susceptibility to variants in first molar development following TCDD exposure (prevalence of 0-8%). Variants were only prevalent (55%) in the C57BL/10J strain. In fact, first molar variants were observed in 33% of pups in the control group, in addition to 68, 59, and 58% of pups in the 0.01, 0.1, and 1 µg/kg dose groups, respectively. The differences between dose groups did not reach statistical significance using logistic regression analysis ($P=0.078$), and there was not a dose-response relationship. The authors did report that the frequency of variants was significantly higher in all three of the dose groups than in the control group (p-values not provided). Response in the three treatment groups did not differ significantly ($P=0.71$) and was, if anything, slightly lower in the middle and high dose groups than the low dose group. The high frequency of spontaneous variants (i.e., in the control group) limits interpretation of the results and suggests this strain may not be an appropriate model for evaluating this effect. Nevertheless, the consistently increased response in all treatment groups compared to controls strengthens the conclusion of an association between TCDD exposure and increased frequency of first molar variants.

The LOAEL for this study is the maternal dose of 0.01 µg/kg (10 ng/kg/day) on GD 13, based on an increased frequency of variants in cuspal development of the third molar. In their review of this study, EPA also identified first molar variants as the critical effect with a LOAEL of 0.01 µg/kg. A NOAEL was not established in this study.

All of the mouse strains tested possess the susceptible *b* allele coding for the AhR protein, yet demonstrated a wide variability in susceptibility to effects on molar development following *in utero* TCDD exposure. It is likely that variability in other genes, in addition to *Ahr*, modulate susceptibility to the effects of TCDD on tooth development. Clearly choice of the mouse strain will affect the results and ultimately, interpretation of study data for human health risk assessment.

BRIEF SUMMARY: This study evaluated the effect of *in utero* TCDD exposure on molar development in six mouse strains. Four of six strains were not susceptible to effects on third molar development and five of six were not susceptible to effects on first molar development. In the mouse strain susceptible to variants in the first molar, there was a relatively high frequency of variants in the control group (33%) but a significantly higher frequency of variants in all of the TCDD treatment groups. Therefore, the LOAEL for the study was based on the lowest tested maternal dose of 10 ng/kg/day to the most responsive mouse strain, and a NOAEL was not established.

Keller et al. (2008a)

Keller et al. (2008a) investigated differences in susceptibility to alterations in jaw structure induced by TCDD exposure in five inbred mouse strains (C57BL/6J, BALB/cByJ, CBA/J, C3H/HeJ, and C57BL/10J). The study began with a sixth strain (A/J) that was eventually dropped because of non-treatment related difficulties in rearing the animals. Pregnant females (number not specified) were administered 0, 0.01, 0.1, and 1 µg/kg TCDD (purity not specified) in corn oil by oral gavage on GD 13. F1 offspring (4-8 litters/treatment group (EPA identified this as 4 or 5 offspring/treatment group) were sacrificed at PND 70. Mandibles were removed and analyzed for size and shape.

Offspring survival and ten-week body weight were not affected in any dose group for any of the mouse strains. Mandible size was significantly different for both males and females between strains ($P < 0.0001$), but not between treatment groups for most of the strains. Two-way ANOVA did not identify a significant difference between treatments for C3H/HeJ males or any other strain/sex. Using logistic regression, however, C3H/HeJ males exhibited a significant, negative effect of TCDD on mandible size ($P = 0.003$). Post-test results for individual comparisons within strain were not reported, but examination of the results shown in Figure 1A of the article show that the 0.01 and 0.1 µg/kg dose groups could not have been significantly different than the control group judging by the overlap in standard error bars. The mean mandible size of the 1 µg/kg group was ~1 mm smaller than the control group mean and appears responsible for the significant, negative trend.

Using a digital morphometric analysis program to quantitate and analyze differences in mandible shape, treatment related differences were identified in three of the five mouse strains. Compared to their sex and strain-specific controls, males in the 0.01 (C3H/HeJ only), 0.1 (C57BL/6J), and 1 µg/kg (C3H/HeJ, C57BL/6J, and C57BL/10J) dose groups differed significantly in mandible shape ($P < 0.01$). TCDD did not significantly affect mandible shape female mice.

The LOAEL for this study is the maternal dose of 0.01 µg/kg (10 ng/kg) on GD 13, based on alterations in mandible shape in male C3H/HeJ mice. A NOAEL was not established. This conclusion is consistent with EPA review of the study, although EPA also considered the low dose to be a LOAEL for effects on mandible shape in male C3H/HeJ mice. However, as

discussed, mandible shape was not likely significantly reduced in either the 0.01 or 0.1 µg/kg dose groups, but may be significantly reduced in the 1 µg/kg dose group.

BRIEF SUMMARY: Differential susceptibility to alterations in lower jaw structure induced by *in utero* TCDD exposure was investigated in five mouse strains. A significant reduction in lower jaw size was observed only in male C3H/HeJ mice. Jaw shape was affected in male mice of three strains, but the C3H/HeJ was again more sensitive. The LOAEL for the study was the lowest tested maternal dose of 10 ng/kg, and a NOAEL was not established.

Keller et al. (2008b)

In a study similar to Keller et al. (2008a), Keller et al. (2008b) investigated differences in susceptibility to alterations in molar size, shape, and symmetry induced by *in utero* TCDD exposure in five inbred mouse strains (C57BL/6J, BALB/cByJ, CBA/J, C3H/HeJ, and C57BL/10J). Pregnant females (number not specified) were administered 0, 0.01, 0.1, and 1 µg/kg TCDD (purity not specified) in corn oil by oral gavage on GD 13. F1 offspring (107-110 per strain) were sacrificed at PND 70. Mandibles were removed and analyzed for size, shape, and right/left symmetry. Although some details of the two Keller studies differ (e.g., the total number of pups included), it is unclear if the two Keller studies represent independent experiments or reanalysis of the same dataset. Both studies began with a sixth strain (A/J) that was eventually dropped because of non-treatment related difficulties in rearing the animals. Both studies also began with a 10 µg/kg TCDD group that was discontinued because of extensive cannibalization.

Although three-way ANOVA identified significant differences in molar shape among strains, sexes, and litters, there was not a statistically significant treatment related effect ($P=0.06$). The sex \times treatment ($P=0.2$), treatment \times strain ($P=0.55$), and the sex \times strain \times treatment ($P=0.94$) interactions were not significant. The authors (and EPA) discuss a hormesis-like trend, with possible increased molar size at 0.1 µg/kg TCDD and decreased size at 1 µg/kg. But such a pattern is not readily discernable from the data presented. The authors identify a significant treatment effect when they exclude the BALB/cByJ strain ($P=0.0045$), but this appears to be a *postpriori* analysis that seems to preferentially exclude data that is not consistent with a specific outcome.

A significant treatment related effect on molar shape was observed ($P<0.0001$), and this effect differed by strain ($P<0.0001$). The C3H/HeJ and CBA/J mice in the 1 µg/kg group differed significantly from controls for both males and females. C57BL/6J females in the 0.1 µg/kg group (but not the 1 µg/kg group) also differed significantly from controls, but this finding does not appear treatment related since it was not dose-dependent. Symmetry in molar size and shape did not differ significantly by TCDD treatment. However, there was a significant trend towards increased molar shape asymmetry ($P<0.03$) and the authors hypothesize that higher doses of TCDD may lead to significant differences.

EPA identified the lowest dose level from this study (0.01 µg/kg) as the LOAEL based on significant differences in molar shape in male C3H/HeJ mice, and they concluded that a NOAEL could not be identified. However, Keller et al. (2008b) only reported a significant difference in molar shape in male C3H/HeJ mice at the 1 µg/kg. Molar shape was also significantly altered at this dose level for female C3H/HeJ mice and CBA/J mice of both sexes. Therefore, contrary to EPA's conclusion, the true LOAEL for this study appears to be the maternal dose of 1 µg/kg (1,000 ng/kg) at GD 13, and the NOAEL is 0.1 µg/kg (100 ng/kg). It is unclear whether the molar shape differences identified should be considered an adverse effect and if they are relevant to humans.

BRIEF SUMMARY: Differential susceptibility to alterations in molar size, shape, and symmetry induced by *in utero* TCDD exposure was investigated in five mouse strains. Molar size and left/right symmetry was not affected. A significant difference in molar shape occurred in the two most sensitive mouse strains. The LOAEL for this effect was 1,000 ng/kg and the NOAEL was 100 ng/kg. This differs from EPA's interpretation of the study.

Kociba et al. (1978)

Kociba et al. (1978) conducted a chronic toxicity and carcinogenicity study of TCDD in Sprague Dawley rats. The rats (50/sex/TCDD dose group, 86/sex in the control group) were administered 0, 1, 10, or 100 ng/kg/day TCDD (>99% purity) in the diet for 2 years. Average body burden at study termination was measured at 540, 1,700, and 8,100 ppt in fat and 540, 5,100, and 24,000 ppt in liver for the 1, 10, and 100 ng/kg/day dose groups, respectively.

There were no toxicologically significant observations in the 1 ng/kg/day dose group. Several hematological and clinical chemistry parameters were affected in the 100 ng/kg/day group ($P<0.05$). Coproporphyrin, uroporphyrin, δ -aminolevulinic acid, and serum SGPT and AP were all significantly increased for females in the high dose group. Coproporphyrin was also increased for females in the 10 ng/kg/day dose group. Hemoglobin was reduced in the high dose group for both males and females.

Mortality was significantly increased for females only in the 100 ng/kg/day group, and for males only in the 10 ng/kg/day group. The male mortality was not considered toxicologically significant because it was isolated to the middle dose. Both males and females had decreased body weight in the 100 ng/kg/day group and, to a lesser extent, body weight was decreased for females in the 10 ng/kg/day group.

Multiple degenerative, inflammatory, and necrotic changes in the liver were observed in both the 10 and 100 ng/kg/day dose groups, with females more affected than males ($P<0.05$). Other significant pathology observations included various non-neoplastic lesions in the 10 (females only) and 100 ng/kg/day (males and females) dose groups, and uterine effects in the 100 ng/kg/day group ($P<0.05$). Significant changes in organ weight were observed in the liver and

thymus. Absolute liver weight was increased in the 100 (males and females) and 10 ng/kg/day dose groups (males only). Relative liver weight increased for females in the 10 and 100 ng/kg/day groups. Thymus weight decreased for females in the 100 ng/kg/day group.

Although not the focus of this review, a number of tumors were statistically increased in the 100 ng/kg/day group (e.g., hepatocellular carcinomas in females only, squamous cell carcinoma of the hard palate/nasal turbinates, lung, tongue), and the incidence of several others were reduced (e.g., mammary, pituitary, pancreas, adrenal).

The LOAEL for this study is 10 ng/kg/day based on increased absolute and relative liver weight along with increased liver histopathological changes. Coproporphyrin was also increased in females at this dose level. The NOAEL is 1 ng/kg/day. This conclusion is consistent with EPA's review of the study.

BRIEF SUMMARY: Chronic dietary exposure to TCDD was evaluated in Sprague Dawley rats. Significant and consistent effects in the liver were observed at dose levels as low as 10 ng/kg/day, including increased liver weight, histopathological changes, and coproporphrin excretion. This dose level was associated with a body burden of 1,700 ppt in fat and 5,400 ppt in liver. The NOAEL for the study is 1 ng/kg/day.

Latchoumycandane and Mathur (2002)

Latchoumycandane and Mathur (2002) evaluated the effects of TCDD (purity not reported) on the testis of male Wistar rats (n=6 per group) that were orally administered doses of 0, 1, 10, or 100 ng/kg/d in an olive oil:acetone (19:1) vehicle for 45 days. Additional groups of animals were administered the same doses of TCDD in combination with 20 mg vitamin E/kg/d for 45 days. The volume of the dosing solution was not indicated. The study report states that, for dosing, "the pipette tip was placed gently just inside the mouth and the dosing solution was slowly expelled from the tip, allowing the animal to lick the compound." This is an unusual administration method and may have resulted in incomplete dosing of some animals. The day after the last dose, animals were weighed, sacrificed, and the reproductive organs excised and weighed. Daily sperm production was determined using the left testis; the right testis was evaluated for markers of oxidative stress. Body weights were not affected by TCDD treatment; however, the absolute weights of the testis, epididymis, seminal vesicles, and ventral prostate were statistically reduced at all doses. Relative organ weights were not reported. Although these findings were dose-dependent, the weights at 100 ng TCDD/kg/d were all $\geq 90\%$ those of control animals. Daily sperm production was statistically reduced in a dose-dependent manner at all TCDD doses. Activity of superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase were all statistically decreased while hydrogen peroxide generation and lipid peroxidation were both statistically increased in the testes at all TCDD doses. All findings in animals co-administered vitamin E and TCDD were equivalent to those observed in vehicle-treated animals, suggesting that vitamin E protected against the adverse effects of TCDD in the

testis. It should be noted that the reproductive organ weight data were also reported in a companion report (Latchoumycandane et al., 2002).

BRIEF SUMMARY: The study of Latchoumycandane and Mathur (2002) suffers from a number of weaknesses. The number of animals per group (n=6) is relatively small and the method of dosing is unusual. Nevertheless, the findings show a dose-dependent reduction in male reproductive organ weights and sperm number following subchronic oral exposure to TCDD. The reduced sperm numbers, however, conflict with the data of Bell et al. (2007), which found no effect of 12 weeks treatment with up to 46 ng/kg/d TCDD on fertility or reproductive indices in rats, suggesting that the finding in Latchoumycandane and Mathur (2002) is either an anomaly or of no biological consequence.

Li et al. (1997)

Li et al. (1997) investigated the effects of a single oral dose of 0.03-30 µg TCDD/kg on lutenizing hormone (LH) and follicle stimulating hormone (FSH) serum concentrations in immature female Sprague Dawley rats (22 days of age; 10 animals/group). The authors do not indicate the number of litters from which these weanlings originated, nor do they state that the effect of litter was controlled in either their pup assignments or their statistical analyses, which makes data interpretation problematic. A dose-dependent increase in both FSH and LH serum concentrations was observed in response to TCDD treatment at 24-hours post-exposure. LH concentrations were statistically increased at doses $\geq 3,000$ ng/kg (NOT 300 ng/kg, as reported in EPA's reanalysis report [EPA, 2010]), while FSH concentrations were elevated at doses ≥ 30 ng/kg. This effect was transient, with hormones returning to control levels within 48 hours of dosing. Based on these data, the authors calculated an ED₅₀ of 5,000 ng/kg (NOT 500 ng/kg, as reported in EPA's reanalysis report [EPA, 2010]). They also conducted *in vitro* studies to further characterize the mechanisms involved in this response.

BRIEF SUMMARY: Li et al. (1997) is an acute, single administration study that characterized a transient change in hormone levels in response to TCDD treatment. The doses cannot be appropriately adjusted to chronic exposure concentrations. Further, the transient nature of the characterized response indicates that the endpoint is likely not to be toxicologically relevant with respect to chronic exposure conditions. Therefore, Li et al. (1997) is considered unsuitable for derivation of a chronic reference dose for TCDD. Further, as the authors note in their report, this increase in LH and FSH serum concentrations is not observed in similarly dosed adult female rats or male rats of either age.

Li et al. (2006)

Li et al. (2006) evaluated the effects of oral TCDD exposure on both pregnant and pseudo-pregnant NIH mice (pseudo-pregnant mice were produced by mating females with vasectomized males). Doses of 0, 2, 50 and 100 ng/kg/day TCDD in sesame oil were administered to groups

of 10 animals each on GD1-8, GD1-3, or GD4-8. Mice were sacrificed on GD9. Pregnant mice were evaluated for the number of uterine implantation sites (methods not described); pseudo-pregnant mice were examined for relative uterine weights. Serum concentrations of estradiol and progesterone were measured by radioimmunoassay; however, it is not clear from the study report if these measurements were made in the pregnant females, pseudo-pregnant females, or both. Finally, liver, kidney, brain, fat and uterine tissues were collected for indirect measurement of TCDD concentrations. Tissues (0.2 grams per sample) were homogenized in acetone, centrifuged, the supernatant collected, and dried down. The resulting residue was reconstituted in DMSO and measured for activity in a lacZ yeast aryl hydrocarbon (Ah) receptor assay. The response obtained from tissues of control animals was subtracted from that of TCDD-treated animals. TCDD tissue concentrations were then extrapolated from a TCDD standard dose-response curve.

In the pregnant females, doses of 50 and 100 ng/kg/d TCDD were associated with a statistically significant dose-dependent reduction in implantations; the effect at GD1-8 was similar to that on GD1-3 and greater than that of GD4-8. Relative uterine weights in the pseudo-pregnant mice were statistically decreased at 50 and 100 ng/kg/d on both GD1-8 and GD1-3, but only at 100 ng/kg/d on GD4-8. Absolute weights and body weights were not presented; thus, it is not known if this finding relates to a dose-related reduction in body weights rather than a direct effect on the uterus. No dose-related differences in serum estradiol concentrations were noted. Progesterone concentrations were statistically reduced at all doses for all three dosing regimens, with dose-response relationships evident for GD1-8 and GD1-3; it should be noted, however, that the group of animals from which these data are derived (pregnant, pseudo-pregnant, or both) is not identified. Finally, TCDD tissue concentrations were extrapolated using a reporter gene assay. No TCDD was measured in the tissues of animals treated with 2 ng/kg/d TCDD. Kidney tissues exhibited substantially lower TCDD concentrations than liver, fat and uterine tissues; TCDD concentrations were not measured in the brain. Due to confounding from the presence of endogenous Ah receptor agonists and antagonists and issues with extraction, it is unlikely that this method provides an accurate measurement of TCDD concentrations. It should be further noted that the amount of TCDD measured in the uterus did not correspond with the reduced implantations response observed after different exposure regimens. More specifically, uterine TCDD concentrations were similar after treatment on GD1-8 and GD4-8, but substantially lower on GD1-3; however, the effect on implantations was less after treatment on GD4-8 than after treatment on GD1-8 and GD1-3.

BRIEF SUMMARY: Li et al. (2006) involved administration of three different doses of TCDD over a limited duration (from 3-8 days) to groups of 10 mice each. Although exposure was associated with a dose-dependent reduction in uterine implantations, reporting deficiencies make interpretation of the data difficult. Further, the methods used for evaluation of tissue TCDD

concentrations are inadequate. Finally, the results of these latter analyses do not correlate with the implantations data.

Markowski et al. (2001)

Markowski et al. (2001) investigated the effects of a single gavage exposure to TCDD during gestation on wheel-running and lever-pressing to earn wheel-running opportunities in female Holtzman rats. Dams were gavaged with 0, 20, 60, or 180 ng/kg TCDD in olive oil on G18; the numbers of litters per group were 7, 4, 6, and 7, respectively. Litter size, pup weight, and sex ratio of pups were recorded and found to be generally unaffected by TCDD treatment, although the high-dose group had approximately 1.6 fewer pups/litter. The exception is that pups from the 60 ng/kg group were significantly heavier than control pups. Litters were culled to 8 pups each on PND4, with equal distribution of sexes in each litter. At weaning, pups were housed in same-sex litter pairs until PND60, then individually thereafter. Around PND77, one female pup per litter was randomly selected for behavioral testing using a wheel apparatus. Pressing a lever would disengage the wheel's brake, allowing the animal an opportunity to use the exercise wheel for 20 or 30 seconds. After training on a fixed-ratio (FR) 1 schedule that required one lever-press to disengage the brake, the FR ratio was increased every 5 test intervals to 2, 5, 10, 20 and 30. After each test session, animals were subjected to vaginal lavage to determine stage of estrous cyclicity. A dose-related decrease in the number of earned opportunities to run per session and on the mean number of wheel revolutions per session were observed. The lever response rate was also reportedly decreased with treatment (data not shown). It is unclear whether the authors counterbalanced the dose groups at the time of testing and whether diurnal variations in response were considered. Furthermore, it is unclear why the authors chose to analyze the final 3 FR 1 sessions and the first 3 sessions under the other FR levels instead of analyzing the full five sessions collected at each FR level. With these multiple comparisons (prenatal exposure x six FR values x 18 sessions), control for multiple comparisons to maintain alpha at 0.05 and limit Type I errors is important and it is unclear whether the authors made these statistical adjustments. Within each FR session, dose-response relationships were not well maintained. These findings contrast with those of Hojo et al. (2002), which showed an increased response of females following TCDD treatment on GD8, as well as with those of other group of researchers (Gray and Ostby, 1995), which reportedly showed no effect of GD15 TCDD treatment on wheel running activity. The effects on activity did not appear to be related to the stage of estrous cyclicity. The authors conducted benchmark dose modeling of the study results to determine ED₁₀ and ED₀₁ values for both earned opportunities to run and total wheel revolutions. Again, as with the work of Hojo et al. (2002), the toxicological relevance of the findings is not clear.

BRIEF SUMMARY: Markowski et al. (2001) is a single exposure study showing an effect on behavior using an operant testing regimen. The findings of this study conflict with those of others. Due to the questionable relevance of the findings and the fact that the single doses used

on this study cannot be appropriately adjusted to chronic exposure concentrations, Markowski et al. (2001) is considered unsuitable for derivation of a chronic reference dose for TCDD.

Miettinen et al. (2006)

This study evaluated the effect of *in utero* and lactational TCDD exposure on susceptibility to dental caries in Line C rats (described in review of Kattainen et al. 2001). Pregnant dams received a single dose of 0.03, 0.1, 0.3, or 1 µg/kg TCDD in corn oil by oral gavage on GD 15. The pups from the TCDD exposure groups were inoculated in the mouth with *Streptococcus mutans* and received a cariogenic, sugar rich diet. In addition, there were three control groups: C-1 received corn oil only, a normal diet, and no *Streptococcus mutans*; C-2 received corn oil only, the *S. mutans* inoculation, and a sugar rich diet; C-3 received the same amount of TCDD as the high dose group (1 µg/kg), but the pups were not inoculated with *S. mutans* and received the same normal diet as C-1.

Maternal weight gain during pregnancy and pup weight on PND 0 were not affected by TCDD exposure. Body weight at 11 weeks was significantly elevated for both male and female pups in the 0.03 and 0.3 µg/kg dose groups, and for females only in the C-2 and 0.1 µg/kg group compared to C-1 ($P<0.05$). Food consumption was decreased for the males on the sugar rich diet but not for the females. Compared to C-2, only males in the 0.3 µg/kg group and females in the C-3 and 1 µg/kg groups had significantly elevated body weight ($P<0.05$). The sporadic differences in offspring body weight are not likely treatment related, but rather attributable to the sugar rich diet. However, treatment related mortality did occur: pup survival to termination (at PND 77) decreased in both groups administered 1 µg/kg TCDD dose (i.e., the high dose treatment group and C-3). Survival was 83% 50%, and 58% in the C-1, C-3 and high dose groups, respectively.

As in Kattainen et al. (2001), TCDD exposure affected molar tooth development. 100% of pups in the 1 µg/kg dose group had missing third molars, whereas in the C-1, C-2, 0.03 µg/kg, and 0.3 µg/kg groups and 8% in the 0.1 µg/kg group ($P<0.05$ compared to C-2). This effect was not the result of an additive or synergistic effect with the cariogenic diet, because 100% of pups in the C-3 group were also missing third molars. The number of caries lesions was significantly increased in all groups on the sugar rich diet relative to C-2 ($P<0.05$), the effect was exacerbated by TCDD exposure. There was a trend toward more caries with greater TCDD exposure, with caries lesions present in 79, 76, 83, and 91% of pups in the 0.03, 0.1, 0.3, and 1 µg/kg dose groups, respectively. All but the 0.1 µg/kg dose group differed significantly compared to the 60% rate in C-2 pups ($P<0.05$). However, co-exposure to the cariogenic diet and/or *S. mutans* is also required for the high rate of caries lesions observed, as demonstrated by the 8% caries lesion rate in the C-3 group, although the rate was still significantly elevated in the C-3 pups relative to the 0% rate in C-1 pups ($P<0.05$). Enamel and dentin mineral composition of the lower third

molars was also evaluated but there were no consistent differences associated with the observed caries lesions rates.

The NOAEL for maternal toxicity in this study was the highest dose, based on maternal body weight. The LOAEL for developmental toxicity in this study was the lowest dose tested, a maternal dose of 0.03 µg/kg (30 ng/kg), based on an increased rate of caries lesions. A developmental NOAEL was not identified. This is consistent with EPA's review of the study; however, it should be noted that the endpoint of dental caries (which develop long after birth) in a monophyodont species that has enamel only on the lingual surface of the incisors is not a traditional endpoint in developmental toxicology. The relevance of this finding to human embryonic development is questionable.

As with the Kattainen et al. (2001) study, the dosing regimen and lack of body burden data limit interpretation of the dose-response information from this study.

BRIEF SUMMARY: Susceptibility to dental caries was evaluated in rat pups following *in utero* and lactational exposure to TCDD after a single maternal exposure during gestation, along with a sugar rich diet and exposure to cariogenic bacteria. The proportion of offspring with dental caries lesions was significantly increased with TCDD exposure, with a LOAEL established for increased dental caries at the maternal dose of 30 ng/kg. A developmental NOAEL was not established. Maternal body weight gain was not affected in any dose group.

Murray et al. (1979)

Murray et al. (1979) conducted a three-generation reproduction study of Sprague Dawley rats administered 0, 0.001, 0.01, and 0.1 µg/kg/day TCDD (purity >99%) in their diet beginning at 7 weeks of age in the F0 generation. After 90 days, F0 rats were mated to produce an F1a generation. At weaning, the pups were placed on the diet of their parents. Thirty-three days after weaning of the F1a pups, F0 rats were mated again to produce an F1b generation, which also received the parental diet after weaning. F1b were mated at 130 days of age to produce an F2 generation, and F2 rats similarly mated to produce the F3 generation.

The Fertility Index (FI) was significantly reduced in the 0.01 µg/kg/day dose group for both the F1 and F2 generations ($P < 0.05$). At 0.1 µg/kg/day fertility was so substantially reduced in the F0 generation (3 and 10% for the two matings) that the dose was discontinued for subsequent generations. The fertility rate was unusually low for controls in the first mating of the F0 generation (44%), and was similarly low for the 0.001 and 0.01 µg/kg/day groups (50 and 60%, respectively). The mating procedure (one male placed with two females for 15 days) was subsequently altered such that one female was placed with one male for 6 days, then placed with another male for 6 days after a 6 day resting period in between. Control F0 fertility in the second mating period was improved to 66%, but was still unusually low compared to F1 (85%) and F2 (88%) controls.

The average number of days from beginning of cohabitation to delivery was significantly increased at the 0.01 µg/kg/day dose level in the F1 and F2 generations ($P<0.05$). The average number of pups per litter was significantly decreased in the 0.01 µg/kg/day dose group from 11 in controls to 8 and 9 in the F2 and F3 generations, respectively ($P<0.05$). The number of pups per litter was affected at the 0.1 µg/kg/day dose level in only the F1a and F1b generations, but the number of pups at birth was unusually low in the F1a litters (mean = 4 pups/litter). The Gestational Survival Index (GSI) was also slightly, but significantly reduced at the 0.01 µg/kg/day dose level in the F1 and F2 generations ($P<0.05$). GSI was also reduced at the 0.001 µg/kg/day dose level, but only in the F2 generation and was likely not treatment related.

Both postnatal survival to PND 21 and postnatal body weight were significantly reduced in a few instances, but there was not a consistent effect or dose-response pattern. For example, postnatal survival was reduced in the F1b pups at 0.001 µg/kg/day, but not at 0.01 or in the few 0.1 µg/kg/day dose group pups. F2 pups in the 0.01 µg/kg/day group had reduced body weight at PND 1, but not at PNDs 7, 14, or 21. Body weight was, however, reduced in the 0.01 µg/kg/day dose group at both 14 days and 21 days (for females) in the F3 generation ($P<0.05$).

No treatment related gross abnormalities were identified at necropsy and 21 day. Thymus weight was significantly reduced (males and females) and liver weight increased (males only) for the F3 generation only in the 0.01 µg/kg/day dose group. No other organ weight differences were observed.

In a supplemental cross-mating study to determine if male or female mediated effects might play a larger role, a subset of F0 dams from the 0.1 µg/kg/day dose group were mated a third time with untreated younger animals. There was no effect on pregnancy incidence or implants/dam for either TCDD treated F0 males or females cross-mated with untreated rats. The percentage of resorptions was also unaffected in litters sired by F0 males, but significantly increased for litters from TCDD treated F0 females. The authors noted that the rate of pregnancy was very low for F0 females in both the control (5%) and TCDD group (15%), likely because of their advanced age. Consequently, the results from this cross-mating study should be interpreted cautiously.

Based on effects observed in several reproductive parameters (days from cohabitation to deliver, GSI, reduced body and thymus weight, increased liver weight), the LOAEL for this study is 0.01 µg/kg/day and the NOAEL is 0.001 µg/kg/day. This is consistent with EPA's review of the study.

BRIEF SUMMARY: The effects of TCDD exposure on rats were evaluated in a three-generation reproductive toxicity study. TCDD administered in the diet affected several reproductive parameters, including the number of days from cohabitation to delivery, the number of live pups at birth, and body, thymus, and liver weight in pups. The LOAEL for the study was 0.01 µg/kg/day and the NOAEL was 0.001 µg/kg/day.

Ohsako et al. (2001)

Ohsako et al. (2001) investigated the effects of TCDD exposure on the male reproductive system of Holtzman rats, a strain that is highly responsive to CYP1A1 induction by TCDD. Twelve week old pregnant dams (N = 6/dose group) were administered a single dose of 0, 12.5, 50, 200, or 800 ng/kg TCDD (purity >99.5%) in corn oil by oral gavage on GD 15. Litters were randomly culled to 5 males and 3 females at PND 2, and then housed by sex after weaning. Two males from each litter were sacrificed on PNDs 49 and 120 and male reproductive parameters evaluated, as described below. In addition, 5 α -reductase type 2 and androgen receptor mRNA levels were evaluated in the caput epididymis and ventral prostate, but only three animals per dose group were analyzed and it is unclear whether they were selected from separate litters. Thus, the mRNA data are not directly relevant for risk assessment and not reviewed here.

There was no effect on either maternal weight gain during pregnancy or pup body weight in any of the dose groups. TCDD also did not affect the number of live pups. There were no treatment related effects on paired testicular weight, epididymal weight (total paired or cauda epididymis), daily sperm production, cauda epididymal sperm reserve, or testicular histopathology. Testicular weight was significantly lower in the 50 ng/kg dose group ($P < 0.01$) and cauda epididymal weight increased in the 200 ng/kg group at PND 120 ($P < 0.05$). In both cases, however, there were no differences in other dose groups. Therefore the findings were not considered toxicologically significant or TCDD related. Serum luteinizing hormone, follicle stimulating hormone, and testosterone levels were not affected.

Several other parameters did appear to be sensitive to TCDD exposure. Both urogenital complex weight ($P < 0.05$) and ventral prostate weight ($P < 0.01$) were significantly reduced at PND 120 in both the 200 and 800 ng/kg dose groups, and ventral prostate weight was also reduced at PND 49, but only in the 800 ng/kg group ($P < 0.05$). Anogenital distance was slightly, but significantly reduced at PND 120 in the 50, 200, and 800 ng/kg dose groups, but unaffected at PND 49.

A critical point regarding interpretation of this study is that data were reported on a per animal rather than per litter basis. Reporting and analysis on a per litter basis is the standard of practice for reproductive toxicology studies. Although these results bear consideration for interpretation of other studies examining similar endpoints, they are not suitable for deriving toxicity factors for use in risk assessment. Nevertheless, EPA concluded the LOAEL for developmental effects from the study is the maternal dose of 50 ng/kg administered on GD 15, based on reduced anogenital distance in male offspring, and the NOAEL is 12.5 ng/kg.

BRIEF SUMMARY: The effects of a single *in utero* TCDD exposure at GD 15 on the male reproductive system were investigated in a rat strain that is highly responsive to TCDD. The study reported reduced urogenital complex and ventral prostate weights at TCDD doses as low as 200 ng/kg, and reduced anogenital distance at doses as low as 50 ng/kg. However, the data were

analyzed and reported on a per animal basis, not a per litter basis, and are not suitable for deriving toxicity factors for risk assessment.

NTP (1982)

The National Toxicology Program (NTP) conducted a chronic carcinogenesis assay on TCDD in Osborne-Mendel rats and B6C3F1 mice (n=50/sex/TCDD dose group; n=75/sex for controls). The rats and male mice were administered TCDD (purity not specified) by oral gavage in corn oil:acetone (9:1) twice weekly for 104 weeks, for a dose equivalent to 0, 0.01, 0.05, or 0.5 µg/kg/wk. Female mice received a dose equivalent of 0, 0.04, 0.2, or 2 µg/kg/wk. The study focuses primarily on carcinogenic endpoints but also provides some data on noncancer effects.

The authors report that body weight was reduced in the high dose group for male rats after week 55 and female rats after 45 weeks, but statistics were not provided. The differences appear significant from the data figure, but body weight differences may also have disappeared for males by study termination. Body weight did not differ for mice between dose groups. No differences in mortality or other adverse clinical signs were observed for rats or mice. Non-neoplastic histopathological observations included an increased incidence of liver pathology, identified as “toxic hepatitis”, in the high dose group for rats (14/50 males and 32/49 females vs. 0/74 males and 0/75 females). In mice, the incidence of toxic hepatitis was similarly increased in the high dose group (44/50 males and 34/47 females vs. 1/73 males and 0/73 females for controls in mice). However, for male mice there also appeared to be an increased incidence in the low (5/49) and medium (3/49) dose groups. Statistics were not provided.

Assuming the increased incidence of toxic hepatitis is significant, the LOAEL for the mouse study is the lowest dose, 0.01 µg/kg/wk (1.4 ng/kg/day), and there is no NOAEL identified. This conclusion is consistent with EPA’s review of the study. Although the EPA review indicates that toxic hepatitis occurred only in the high dose group for both rats and mice, they identified the low dose for male mice as the LOAEL with no NOAEL. NTP notes in the study summary: “Increased incidences of toxic hepatitis related to the administration of the test chemical were detected among high-dose rats and high-dose mice of each sex”, raising the possibility that the low incidence rates in the low and medium dose groups were not significant. While the LOAEL for the rat study appears to be 0.5 µg/kg/wk (71 ng/kg/day) based on liver pathology, and the NOAEL is 0.05 µg/kg/wk (7 ng/kg/day), EPA did not identify a separate LOAEL and NOAEL for rats.

BRIEF SUMMARY: This National Toxicology Program sponsored study focuses on the potential carcinogenicity of TCDD in rats and mice. However, it provides some data on noncancer effects. The study identified an increased incidence of liver pathology in both rats and mice. The LOAEL for mice was 1.4 ng/kg/day and a NOAEL was not established. The LOAEL for rats was 71 ng/kg/day, with a NOAEL of 7 ng/kg/day, although EPA did not differentiate between species in identifying their LOAEL.

NTP (2006)

NTP (2006) conducted a GLP-compliant two-year carcinogenicity study of TCDD in female Sprague-Dawley rats (n=81-82/group; 3-5 per cage). Male rats, although not part of the study, were kept in the colony to ensure proper estrous cycling of the females. Doses of 0, 3, 10, 22, 46, or 100 ng/kg/d TCDD in a corn oil:acetone (99:1) vehicle were administered by gavage (5 days per week) for 105 weeks. Another group of 50 rats was administered 100 ng/kg/d TCDD (5 days per week) for 30 weeks, then switched to the dosing vehicle alone for the remainder of the study (referred to as the stop-exposure group). Animals were observed daily, body weights measured weekly for the first 13 weeks, then monthly thereafter. Interim sacrifices of ≤ 10 animals per group (except the stop-exposure group) were conducted at 14, 31, and 53 weeks, at which point supplemental analyses were conducted. As part of these supplemental analyses, blood was collected for measurement of thyroid stimulating hormone (TSH), triiodothyronine (T_3) and free thyroxine (T_4) serum concentrations and cell turnover rates of the liver were measured in animals that received 40 mg/ml BrdU in drinking water for 5 days. Liver and lung tissue samples were collected for determination of microsomal cytochrome P450 (cyp1a1, cyp1a2, and cyp2b) activities. Complete necropsies were conducted on all rats on study. For the interim analyses, the left kidney, liver, lung, left ovary, spleen and thyroid were weighed; the thymus was also weighed at 14 weeks. Pathologic examinations were conducted on a limited suite of tissues at the interim sacrifices (adrenal gland, liver, lung, mammary gland, ovary pancreas, pituitary, spleen, stomach, thymus, thyroid, uterus and vagina); the full panel of organs was examined at the end of 2 years in all groups, including the stop exposure group. Results of the pathologic evaluation were evaluated by the Pathology Working Group. The study report notes that, "...there were hepatocellular proliferative lesions for which the criteria used for common diagnoses did not appear to fit. Furthermore, classification was sometimes confounded by significant liver damage (toxic hepatopathy) that was present in many animals from these studies."

No clinical findings related to TCDD treatment were noted and survival was similar in all treatment groups. By the end of study, mean body weights were reduced at 22, 46, and 100 ng/kg/d and in the stop-exposure group, although whether these findings were statistically significant was not reported. TSH levels were statistically increased at ≥ 46 ng/kg/d in week 14, but unaffected in weeks 31 and 53. Serum T_3 concentrations were increased at ≥ 46 ng/kg/d in weeks 14 and 31, and at ≥ 10 ng/kg/d in week 53. Total T_4 serum concentrations were reduced at ≥ 22 ng/kg/d in weeks 14 and 31 and at ≥ 46 ng/kg/d in week 53; free T_4 concentrations were similarly affected, except in week 53, when no statistically significant changes were noted. Hepatic cell proliferation was statistically increased at 22 ng/kg/d in week 14; however, the lack of a dose-response suggests that this is a chance finding. In week 31, hepatic cell proliferation was statistically increased at all TCDD doses. By week 53, however, proliferation was statistically increased at 46 and 100 ng/kg/d only. Cytochrome P450 activity levels were statistically increased in both the liver and lung at all TCDD doses and at all time points of

analysis (14, 31 and 53 weeks). TCDD concentrations were highest in the liver, then the adipose tissue and generally exhibited a dose-response relationship. TCDD was not detected in lung tissue and blood.

Both absolute and relative liver weights were statistically increased at all doses at 14 and 31 weeks and relative weights were statistically increased at ≥ 10 ng/kg/d in week 53. Absolute and relative thymus weights were statistically reduced at ≥ 46 ng/kg/d in week 14; this endpoint was not evaluated at 31 and 53 weeks. Relative lung weights were statistically increased at all doses in week 31. Other absolute and/or relative organ weights were noted, but were generally observed in the absence of a dose-response or with a lack of consistency across interim sacrifices.

In week 14, liver hypertrophy was noted at doses ≥ 10 ng/kg/d. By week 31, hypertrophy was seen at all doses; increased pigmentation was noted at ≥ 10 ng/kg/d and a dose-related increase in multinucleated hepatocytes was seen at ≥ 46 ng/kg/d. At week 53, liver hypertrophy was again noted in all dose groups, along with an increased incidence/severity of multinucleated cells at 100 ng/kg/d and pigmentation at doses of ≥ 10 ng/kg/d. At the end of 2 years, liver hypertrophy and inflammation were increased at all doses; multinucleated cells, pigmentation, diffuse fatty change and toxic hepatopathy were more prevalent at ≥ 10 ng/kg/d than in controls. Hepatocyte hyperplasia and cellular necrosis were also observed at doses of ≥ 22 ng/kg/d. The incidences of hepatocellular adenomas and cholangiocarcinoma were statistically increased at 100 ng/kg/d.

Pathological changes were noted in other tissues as well at the end of 2 years. These changes include increased incidences of bronchiolar metaplasia and hyperplasia of the alveolar epithelium at all doses and cystic keratinizing epitheliomas of the lung at 100 ng/kg/d; squamous hyperplasia of the gingival tissues at all doses and an increased incidence of squamous cell carcinoma of the mouth at 100 ng/kg/d. As well, various noncarcinogenic changes were noted in the uterus, pancreas, adrenal cortex, cardiac tissue, clitoral gland, ovary, kidneys and stomach.

BRIEF SUMMARY: The NTP (2006) study appears to be a well-conducted two-year oncogenicity study in female rats. It followed GLP and involved peer-review of the pathological findings to ensure agreement on diagnoses. An adequate number of animals were included in all dose groups and interim sacrifices were conducted to characterize the development/progression of pathological changes over time. Of particular note is the liver hypertrophy, which was observed as early as 14 weeks into the study, and occurred at all doses by week 31. Possible limitations of the study are that only female animals were evaluated and dosing was only done 5 days per week. Thus, in order to derive chronic exposure values, EPA had to adjust these values to account for continuous dosing.

Seo et al. (1995)

TCDD was administered to time-mated rats on gestational days 10-16 via gavage at 25 and 100 ng/kg/day. Thyroid hormone levels, EROD activity, and UDP-GT activity were measured in these animals and reproductive/developmental outcomes and necropsy of pups performed. No reproductive/developmental effects were reported. EROD and UDP-GT activity was statistically significantly increased at the high dose, although EPA considers the EROD response to be adaptive and not adverse. Serum T4 concentrations were statistically decreased in female rats only. No differences in T3 or TSH were observed and thyroid weights were not affected. The authors note “ the decrease in plasma T4 ranged from about 15-20%. This small degree of T4 depression is unlikely to be of any clinical significance to the animal, particularly given the normal plasma T3 and TSH concentrations.” Decreased pup thymus weights and thymus atrophy were seen at 100 ng/kg/day. EPA established the LOAEL on the decreased thymus weights and decreased T4 levels at the high dose, 100 ng/kg/day. Given the lack of a clear toxic effect on the thyroid, the reliance on changes in T4 hormone levels is inappropriate. However, this does not impact the LOAEL in this study because statistically significant effects on the thymus were seen at this same dose.

BRIEF SUMMARY: Seo et al. (1995) exposed pregnant rats to TCDD during development and reported decreased serum T4 levels and decreased thymus weights with atrophy in the 21-day old pups at . The authors did not consider the decreased T4 levels to be clinically significant; however, based on the thymus effects the LOAEL is 100 ng/kg/day.

Sewall et al. (1995)

Diethylnitrosamine was administered to one-half of the animals as an initiator for evaluating hepatic pre-neoplastic foci and the remaining animals were administered saline. EPA only relied on the saline-treated animals in establishing NOAEL/LOAEL values. TCDD was administered by gavage once every two weeks for 30 weeks at doses equivalent to 0, 0.1, 0.35, 1.0, 3.5, 10.7, 35.7, or 125 ng/kg-day (n = 9 per group). Serum thyroid hormone levels (T3, T4, and TSH) were presumably measured at the end of treatment.

A dose-related decrease in T4 concentrations were observed (Fig 1). A statistically significant decrease was seen at 35.7 ng/kg-day in saline non-initiated animals (in the DEN-initiated animals a significant decrease was seen at the next lower dose, 10.7 ng/kg-day). These results were based on serum concentration for only 6 to 9 animals. Although T4 concentrations within each dose group did not correlate to liver TCDD concentrations, overall, a positive correlation (0.66) was observed between liver TCDD concentrations and decreasing T4 levels. T3 concentrations were not affected by TCDD administration at even the highest dose, 125 ng/kg-day. Dose-related TSH concentrations were only provided for the DEN-initiated animals because it was the “most complete set of samples for analysis” based on data from 7-9 animals per group. The TSH in these animals were slightly decreases at the low doses and statistically significantly increased

at 3.5 and 125 ng/kg-day. No changes in thyroid weights were observed and no thyroid histopathological changes were seen that correlated with changes in thyroid hormone levels.

Additionally, liver RNA was extracted reverse transcription-polymerase chain reaction (RT-PCR) used to measure UGT1 and CYP1A1 mRNA levels in animals initiated with DEN. Appropriately, these data were not relied on by EPA in their derivation of an RfD.

BRIEF SUMMARY: Sewall et al. (1995) reported a dose-related decrease in T4, but not T3 or TSH in the saline non-initiated rats; these findings are limited by the analysis in a small number of animals. Additionally, the change in T4 hormone levels was not associated with changes in thyroid weight or histopathology. Thus, the NOAEL/LOAEL selected by EPA may not reflect a permanent toxic effect, rather an adaptive response.

Shi et al. (2007)

Three dams per dose group were administered TCDD via gavage on a weekly basis during gestation and through lactation. Pups, 10 per group, were administered TCDD until reproductive senescence defined as two extended (6-9 days) estrus cycles. The doses administered were 0, 1, 5, 50, or 200 ng/kg/week or 0, 0.14, 0.71, 7.41, and 28.6 ng/kg/day.

Significant effects included delayed vaginal opening at the highest dose, 28.6 ng/kg/day. EPA commented that a delay, albeit not statistically significant was also reported at the lowest dose (0.14 ng/kg/day) and the second highest dose (7.14 mg/kg/day) – the difference in the mean delay is less than one day. In addition, a statistically significant decrease in estradiol was seen in all dose groups except the lowest dose and was dose-dependent. Statistics of the dose-trend are not provided.

The primary limitation of these data is the evaluation of 10 pups that were the offspring of only 3 dams, which is a very small number on which to conduct statistical analysis. Specifically, the pups cannot be considered individual data points in the statistical analysis because the pups from each dam shared gestation and lactational exposure. This is why in GLP-compliant reproductive/development studies the litter is the statistical unit of analysis.

BRIEF SUMMARY: Shi et al. (2007) conducted a reproductive/developmental study which is limited by the very small numbers of animals in each dose group. The 10 pups/dose group were offspring of only 3 dams and therefore represent only 3 unique data points for statistical analysis. The determination of a NOAEL and LOAEL based on the analyses as provided by the authors is not appropriate for deriving a regulatory threshold value.

Smialowicz et al. (2008)

Smialowicz et al. (2008) examined the effects of subchronic treatment with TCDD or mixtures containing TCDD and various polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated

dibenzofurans (PCDFs) or polychlorinated biphenyls (PCBs) on the immune response of female B6C3F1 mice (8 weeks of age). The purpose of this study was to determine if the toxic equivalency (TEQ) assigned to each of the mixtures based on their chemical make-ups would accurately predict their immunosuppressive effects. For the purposes of this review, only data derived from the administration of TCDD alone are discussed. Female mice (8-15/group) were gavaged with 0, 1.5, 15, 150, or 450 ng/kg/d TCDD in a corn oil vehicle 5 days/week for 13 weeks. Treatment was done in, “two blocks of 7-8 animals/dose group.” It is not known if differences in response were observed between the two treatment blocks. Three days following the last treatment, the mice were immunized with sheep red blood cells (SRBCs) injected via the tail vein. Four days later, the mice were sacrificed, spleen, thymus and liver weights recorded, and the plaque-forming colony (PFC) assay conducted, the methods for which were not well reported. Briefly, spleen cells were mixed with SRBCs and guinea pig complement in an agar/medium mixture, then added to petri dishes, which were incubated at 37°C for 3 hours. Anti-SRBC antibodies secreted by the spleen cells in culture will bind to the SRBCs; the addition of complement will activate lysis of the SRBCs, resulting in formation of a plaque. Both the number of plaques formed per 10⁶ spleen cells and the number of plaques formed per spleen were counted. TCDD treatment reduced relative spleen weights at doses ≥15 ng/kg/d, increased relative liver weights at doses ≥1.5 ng/kg/d, and reduced the antibody response to SRBCs at doses ≥1.5 ng/kg/d. In all cases, these effects exhibited dose-response relationships. At the highest dose tested (450 ng/kg/d), antibody responses were decreased ~90% from control.

BRIEF SUMMARY: Smialowicz et al. (2008) illustrates the immunosuppressive effects of TCDD in groups of 8-15 mice each using four different doses of TCDD and administration 5 days/week over a period of 13 weeks. Only a single endpoint related to immune function—the plaque-forming colony assay response—was evaluated; organ weights were also assessed. A further limitation is that treatment was done in two blocks of animals; however, the response between blocks was not reported.

Toth et al. (1979)

Toth et al. (1979) investigated tumor incidence and the occurrence of amyloidosis in Swiss mice exposed to TCDD alone, 2,4,5-trichlorophenoxyethanol (TCPE) alone, and TCDD/TCPE combined. The carcinogenicity data are not included in this review. In the amyloidosis portion of the study, 45 10-week old male mice per dose group were administered 0, 0.007, 0.7, or 7 µg/kg TCDD (purity not specified) in sunflower oil by oral gavage once per week for one year. After the one-year exposure period, animals were followed until spontaneous death or until they became moribund and were sacrificed.

TCDD negatively affected survival in the 7 µg/kg/wk dose group, in which average lifespan was decreased (424 days vs. 588 days for controls). Statistics were not provided, but it is unclear whether the difference was not statistically significant or if the parameter was not analyzed

statistically. Statistical methods were only described for the tumor incidence. Given the magnitude of difference in survival (28% reduction in lifespan), the result would appear significant. The EPA review mistakenly identifies this as a 72% decrease in lifespan rather than a lifespan 72% of controls. Survival was increased by 10% and 7.6% in the 0.007 and 0.7 $\mu\text{g}/\text{kg}/\text{wk}$ dose groups, respectively, but again, it is unclear whether these differences are statistically significant. Skin lesions occurred in 0, 11, 30, and 58% of mice in the 0, 0.007, 0.7, or 7 $\mu\text{g}/\text{kg}/\text{wk}$ TCDD dose groups, respectively. In most, but not all of these animals the lesions progressed to generalized lethal amyloidosis, which occurred in 0, 11, 23, and 40% of mice in the 0, 0.007, 0.7, or 7 $\mu\text{g}/\text{kg}/\text{wk}$ TCDD dose groups, respectively. As with survival, statistics were not reported for skin lesions or amyloidosis. However, the increased incidence is likely significant for all dose groups considering the 0% incidence rate in the control group.

Assuming the increased incidence of reported effects are statistically significant, the LOAEL for this study is 0.007 $\mu\text{g}/\text{kg}/\text{wk}$ (1 $\text{ng}/\text{kg}/\text{day}$), based on skin lesions and lethal amyloidosis. A NOAEL was not identified. This conclusion is consistent with EPA's review of the study.

BRIEF SUMMARY: The incidence of skin lesions and amyloidosis was investigated in mice chronically exposed to TCDD. Animals were administered weekly doses of TCDD by oral gavage for one year, then followed for the remainder of their lives. The incidence of skin lesions and amyloidosis increased in all treatment groups. The LOAEL for the study was 1 $\text{ng}/\text{kg}/\text{day}$ and a NOAEL was not established.

Van Birgelen et al. (1995a, 1995b)

In a 13-week study, 0, 200, 400, 700, 5,000, or 20,000 ng/kg TCDD in the diet (purity >99%) was administered to Sprague-Dawley rats ($n=8$); these dietary concentrations were estimated to approximately equal 0, 14, 26, 47, 320, or 1,024 $\text{ng}/\text{kg}\text{-day}$.

Van Birgelen et al (1995a) report the findings for CYP1A1, UGT1A1, and T4UGT activities and plasma levels of free and total T4 measurements in the animals at the end of the 13 weeks. CYP1A1, UGT1A1, and T4UGT activities were significantly induced at all dose levels with the exception of T4UGT at the lowest dose. Free T4 was significantly reduced at 320, or 1,024 $\text{ng}/\text{kg}\text{-day}$ and total T4 was reduced at ≥ 47 $\text{ng}/\text{kg}/\text{day}$; the highest dose was associated with a 38% reduction in total T4. A significant correlation ($p<0.001$) between total T4 concentrations and T4UGT activity was reported, although it is important to recognize the wide variability of individual activity/thyroid hormone levels. The authors reported the following NOAELs: UGT1A1 activity <14 $\text{ng}/\text{kg}/\text{day}$, T4UGT activity 14 $\text{ng}/\text{kg}/\text{day}$, plasma total T4 26 $\text{ng}/\text{kg}/\text{day}$, and plasma free T4 47 $\text{ng}/\text{kg}/\text{day}$.

Van Birgelen (1995b) report on the other endpoints examined in the subchronic toxicity study. A dose-dependent decrease in body weights were seen, with statistically significant decreases observed at ≥ 47 $\text{ng}/\text{kg}/\text{day}$. Absolute and relative liver weight increases were reported at 320

and 1,024 ng/kg/day, respectively. Absolute and relative thymus weight were significantly decreased in a dose-related manner beginning at the lowest dose, 14 ng/kg/day; the weights were comparable at the three lowest dose levels: 14, 26, and 47 ng/kg/day. Liver enzyme activity (EROD and 40OH-AA) was significantly increased at all dose levels. Plasma retinol concentrations were measured at 4 weeks and 13 weeks; a statistically significant increase was seen at 47 ng/kg/day at 13 weeks. The data from 4 weeks is not presented in a tabular manner, but in the discussion it is noted that at the highest dose (1,024 ng/kg/day) a 3-fold increase was seen at 4 weeks and at 13 weeks the increase was approximately 2-fold, suggesting an adaptive response. Liver retinoid levels at 13 weeks were significantly decreased at all dose levels.

EPA identifies 14 ng/kg/day as the LOAEL based on significantly decreased absolute and relative thymus weights and significantly decreased liver retinoid levels. Although the liver retinoid levels were significantly decreased at 13 weeks, based on the plasma retinol concentration that decreased between 4 and 13 weeks, this may be an adaptive response and cannot be considered a toxic effect. Therefore, the LOAEL should only be based on the changes in thymus weight.

BRIEF SUMMARY: Van Birgelen et al. (1995a, 1995b) conducted a subchronic, 13 week dietary study in rats and measured a number of biological markers and (e.g., EROD activity, T4 concentrations) cannot be considered direct toxic effects. Body weights, liver weights and thymus weight changes were observed and are considered to be indicators of toxicity. Significant thymus weight decreases were seen at the lowest dose, 14 ng/kg/day, and is considered the LOAEL.

Vos et al. (1973)

Vos et al. (1973) examined the immune system effects of TCDD (purity not reported) administered to laboratory animals in a series of four separate experiments. In the first experiment, female Hartley guinea pigs (n=10 per group) were administered weekly oral doses of 0, 8, 40, 200 or 1,000 ng TCDD/kg in an acetone-corn oil vehicle (exact formulation of vehicle unknown) over a period of 8 weeks (56 days). Body weights were recorded weekly, various immune system organs weighed and differential leukocyte counts conducted at sacrifice. To evaluate antibody mediated immunity, 0.1 mL tetanus toxoid was injected subcutaneously into the right foot pad on days 28 and 42. It should be noted that the toxoid was aluminum phosphate-adsorbed in one case and unadsorbed in the other. Blood was collected on days 35, 49 and at study termination; however, the day of study termination was not reported. Serum concentrations of tetanus antibodies were determined using radial immunodiffusion. This method involves the reaction of serum antibodies with antigen in an agar gel; using equal aliquots of serum, the size of the antibody diffusion ring is generally proportional to the concentration of antibodies present in the serum. However, the authors did not report whether

they used known concentrations of antibodies to develop a standard curve for quantitation. Although relatively imprecise compared with currently available methods, radial immunodiffusion is considered an adequate method for the time of the study.

In a second set of experiments, groups of 10 female Hartley guinea pigs were treated as described for experiment one. Again, body weights were recorded weekly, and immune organs weighed and differential leukocyte counts conducted at sacrifice. To evaluate cell-mediated immunity, 0.05 mL of an oil suspension of inactivated tuberculin (in complete H₃₇Ra adjuvant) was injected into the hind foot pad on day 35. Intradermal injections of tuberculin (site of injections not reported) were then administered on days 47 and 54, and the diameter and thickness (minus that of normal skin) of the subsequent skin reactions determined at 24 and 48 hours post-injection. Serum samples were also evaluated for cortisol and corticosterone concentrations; however, when the serum samples were collected was not reported.

In these experiments, all guinea pigs administered 1,000 ng TCDD/kg per week died between 24 and 32 days and those administered 200 ng TCDD/kg per week experienced a statistically significant reduction in body weights. Absolute thymus weights were statistically reduced at 200 ng/kg per week in both experiments and at 40 ng/kg per week in experiment one; relative thymus weights were reduced at 200 ng/kg per week (statistically so in the first experiment). Relative adrenal weights were statistically increased at 200 ng/kg per week in both experiments as well. Methods for microscopic analysis were not described in the study report; however, “severe atrophy of the thymic cortex,” was reported. Total leukocyte counts were statistically reduced at 40 ng/kg per week in experiment one and at 200 ng/kg per week in experiment two. Lymphocyte counts were statistically reduced at 8, 40 and 200 ng/kg per week in experiment one; however, a dose-response was not evident and the finding was not repeated in experiment two. In experiment one, the secondary antibody response to tetanus toxoid was statistically reduced at 200 ng/kg per week on both days 49 and 56; further, a dose-response trend was noted. In experiment two, the diameter of the skin reaction was statistically reduced in a dose-dependent manner at 40 and 200 ng/kg per week at 48 hours after injection of tuberculin on day 47 and 24 and 48 hours after injection on day 54. The thickness of the reaction was also reduced at these same doses on day 54 (statistically so at 200 ng/kg per week). Serum corticosteroid concentrations were unaffected by TCDD treatment.

In a third experiment, cell-mediated responses were evaluated in groups of 10 CD rats (gender not reported) administered weekly oral doses of 0, 200, 1,000 or 5,000 ng TCDD/kg for 6 weeks. An oil suspension of inactivated tuberculin (0.05 mL) was injected into the right hind foot pad on day 28. An intradermal injection of tuberculin was then administered at the shaved flank on day 42. The diameter and thickness (minus that of normal skin) of the subsequent skin reactions determined at 24 and 48 hours post-injection. Five animals per group were sacrificed on day 45 for evaluation of immune organ weights and differential leukocyte counts. Again, the results of

microscopic evaluations were discussed, although the methods for these analyses were not reported.

All rats were reported to have survived treatment, although body weights were statistically reduced in those animals administered 5,000 ng TCDD/kg per week. Absolute and relative thymus weights were statistically reduced and relative spleen weights were increased at 5,000 ng TCDD/kg per week; as well, absolute adrenal weights were decreased at both 1,000 and 5,000 ng TCDD/kg per week. Although not statistically significant, total leukocyte and lymphocyte counts were increased at 5,000 ng TCDD/kg per week. These findings conflict with those of the guinea pig in that spleen weights were unaffected and leukocyte counts were reduced, rather than increased, in the guinea pig. Also in contrast to the guinea pig, TCDD treatment had no effect on the cell-mediated response to tuberculin at either 24 or 48 hours post-injection in the rat.

In the fourth experiment, the effect of TCDD treatment on graft-versus-host response was measured in B₆D₂F₁ mice (the progeny of a mating between C57Bl/6 and DBA-2 mice) that had been injected with spleen cells derived from TCDD-treated C57Bl/6 mice. Groups of 5-7 C57Bl/6 mice (gender not reported) were orally administered 0, 200, 1,000, 5,000 or 25,000 ng TCDD/kg per week for four weeks, then sacrificed. Thymuses were weighed at this time. Spleen cells, “from the pooled spleens of each donor group,” were then injected into B₆D₂F₁ mice (10⁷ cells/mouse; the number of recipient mice per group not reported). Although the donor spleen cells are tolerated by recipient mice, they react against the DBA-2 antigens of the host animal, causing enlargement of injected lymph nodes. This graft-versus-host response can be measured as the ratio of the injected lymph node weight to that of the uninjected lymph node.

One B₆D₂F₁ mouse in the high dose group died after 24 days; however, body weights were generally unaffected by TCDD treatment. Absolute and relative thymus weights were statistically reduced in the animals receiving 25,000 ng TCDD/kg per week. Although the data were not shown, spleens of the high dose group were reported to be too small to allow for preparation of sufficient numbers of donor cells; thus, graft-versus-host data for this dose group are not available. Regardless, the graft-versus-host activity of donor spleen cells was statistically reduced at the next lower dose of 5,000 ng TCDD/kg per week.

BRIEF SUMMARY: Vos et al. (1973) illustrates the immune-suppressive effects of TCDD in three species of animals using at least four doses and 10 animals per group. In EPA’s treatment of this study, they fail to discuss data from the rat and mouse studies; rather, they present the guinea pig data only. Guinea pigs are known to be the most sensitive species to the toxic effects of TCDD. Their exquisite sensitivity is demonstrated by their 100% mortality at 1,000 ng TCDD/kg per week versus the complete absence of deaths in rats at doses up to 5,000 ng/kg per week and the loss of only one mouse at doses of up to 25,000 ng/kg per week. It should be also noted that humans are not extremely sensitive to TCDD toxicity, as demonstrated by follow-up with victims of the Seveso accident and other acute poisonings. Consequently, the use of a 3x

uncertainty factor for animal-to-human extrapolation is unfounded in this case. Further, EPA adjusted the weekly doses used in this study to continuous daily exposures by dividing by 7. Daily exposure to 1/7th of the weekly dose likely will not result in the same toxicokinetics (and thus, not the same toxicity patterns) as weekly exposure to TCDD.

White et al. (1986)

White et al. (1986) examined the effects of TCDD and HCDD treatment on complement activity in female B6C3F1 mice (8 per group; 10 in the vehicle-control). For the purposes of this review, data derived from HCDD-treated animals are not discussed. Doses of 0, 10, 50, 100, 500, 1,000 and 2,000 ng TCDD/kg/d (purity not reported) were administered by gavage in a corn oil vehicle for 14 days, after which serum was collected. Serum complement activity was measured using the microtiter hemolytic assay; complement C3 was evaluated using anti-mouse C3 serum and a spectrometric assay that measured precipitation of antigen-antibody complexes. Complement activity was statistically reduced at all doses of TCDD; however, the response was not dose related below 100 ng/kg/d. Complement C3 was similarly statistically reduced at TCDD doses of 500-2,000 ng/kg/d. Further experiments were conducted to look at the reversibility of effects on the complement system following 14-day gavage 1,000 or 10,000 ng TCDD/kg/d. Serum complement activity began to recover 14 days post-exposure and were similar to control by 50 days post-exposure. C3 showed a more rapid recovery, returning to vehicle control levels by 14 days post-exposure.

In additional experiments, the effect of 14 days gavage treatment with 1,000 ng TCDD/kg/d on host resistance was measured in groups of 8 mice each that were inoculated ip with *Streptococcus pneumoniae*. Mortality was substantially increased with TCDD treatment following inoculation with 2.4×10^7 or 3.4×10^7 colony-forming units (CFU); the effect at 7.2×10^7 CFU, in contrast, was limited because mortality at this level of inoculation was greater than 90% in the vehicle-treated controls. Finally, studies were conducted on the complement activation system following acute TCDD treatment. These studies showed that both complement activity and C3 were statistically reduced for up to 12 days following a single 14,000 ng TCDD/kg dose. The results of these later studies, however, are not useful for risk assessment as they were conducted using only one dose of TCDD.

BRIEF SUMMARY: The study of White et al. (1986) illustrates the immunosuppressive effects of TCDD in groups of 8 mice each using six different doses of TCDD and administration over a period of 14 days. Endpoints evaluated include serum complement activity, concentrations of complement C3, and resistance to infection. The effects on complement were shown to be reversible following cessation of treatment.

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Attachment D:

Dioxin's MOA and EPA's Decision to Reject Non-Linearity and Thresholds for Cancer Risk Characterization

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I. Comment Outline

1. Introduction
2. NRC Recommendations (for deriving a non-linear cancer potency factor)
3. EPA's Position and Basis for Rejecting MOA and Thresholds (Chapter 5. Cancer Assessment)
4. The Human Relevance Framework (e.g., EPA's 2005 Cancer Guidelines)
5. Scientific Precedent ("Weight-of-Evidence Basis") Behind Tumor Promotion Biology: Baseline Information Necessary For Examining An MOA
6. Proposed Dioxin MOA for Rodent Liver Tumors
 - a. Key Events
 - b. Dose Concordance
 - c. Temporality Concordance
 - d. Hill Criteria Elements of the Human Relevance Framework
 - e. Alternate MOA(s)

[**Note:** A bibliography of publications is included after certain sections of the comments in order to facilitate a quick review of the supporting studies. The reader should be aware that these references may not be the conclusion of the comments and that additional sections may follow.]

II. Introduction

EPA has concluded no Mode-of-Action (MOA) exists for explaining how dioxin promote tumors in laboratory animals or in the modeling of the NIOSH all cancer mortality data. EPA reviewed limited MOA information in support of their conclusion. However, an extensive list of publications is available and the EPA should have reviewed these studies for understanding aryl hydrocarbon receptor (AHR) activation and the subsequent events leading to tumor promotion. Because the EPA failed to provide a detailed examination of the MOA behind dioxin tumor promotion, the SAB will not have this information available to them unless it is provided or the panel members take it upon themselves to pull together and analyze all of this information. We are providing these comments to ensure there is full consideration of the robust data base in place to explain the mode of action for dioxin's carcinogenicity.

The following comments address TCDD's tumor promotion MOA for development of female rat liver tumors. These tumors have historically been used to derive dioxin's cancer slope factor. The central MOA key event can be generically explained as excessive nuclear receptor activation and gene transcription culminating in dysregulation of cell function. A nuclear receptor MOA and key event explanation for tumor promotion is sufficient to establish a non-linear, threshold basis for TCDD's cancer potency.

The AHR MOA and key event comments have been streamlined in these comments in consideration of the SAB's enormous task of reviewing 1800 pages of the EPA's document. Citations are provided under the various MOA and key event subheadings in the event the SAB members wish to further explore supporting data. This outline constitutes a preliminary assessment of dioxin's MOA and may be updated in the near term as the SAB deliberates over its charge questions or if the SAB would like additional information in support of this analysis.

III. NRC Recommendations

The EPA released its latest partial response to the NAS panel's recommendations on May 21, 2010 almost four years after the National Academy of Sciences' expert panel rendered its set of recommendations for correcting EPA's Draft Dioxin Reassessment. The NAS panel unequivocally recommended EPA develop a non-linear cancer potency value, based on TCDD's receptor-based mode of action (MOA):

“The committee unanimously agrees that the current weight of evidence on TCDD, other dioxins, and DLCs carcinogenicity favors the use of nonlinear methods for extrapolation below the point of departure (POD) of mathematically modeled human or animal data. However, the committee recognizes that it is not scientifically possible to exclude totally a linear response at doses below the POD, so it recommends that EPA provide risk estimates using both approaches and describing their scientific strengths and weaknesses to inform risk managers of the importance of choosing a linear vs. nonlinear method of extrapolation. To the extent that EPA favors using default assumptions for regulating dioxin as though it were a linear carcinogen, such a conclusion should be made as part of risk management. EPA should strictly adhere to the distinction between risk assessment, which is a scientific activity, and risk management, which takes into account other factors.” [pg. 190]

EPA has decided to reject the NRC recommendations for development of both (emphasis added) a linear and non-linear cancer potency estimate by only endorsing a linear cancer slope factor of $1,000,000 \text{ mg/kg/day}^{-1}$. The basis for this rejection is EPA's argument that no MOA exists for explaining how dioxins promote tumors in laboratory animals. This MOA issue also extends to efforts to model the NIOSH epidemiological results – a threshold should be established for the human evidence provided the epidemiological data rises to the level of quantitative certainty for conducting dose-response modeling.

IV. EPA’s Position and Basis for Rejecting MOA and Thresholds **(Chapter 5. Cancer Assessment)**

EPA correctly identified the rodent target organ cancers reported in the cancer bioassays published over the last 30 years, i.e. , hepatic adenoma, hepatocellular and cholangiolar carcinoma, lung, oropharyngeal tumors (palate/mucosa), thyroid, pancreas, and adrenal cortex tumors.

- Of these, the liver and lung are consistent target organs for tumor development with liver tumors providing the historical basis for risk characterization.
- EPA has concluded that no single MOA can be clearly identified for these tumors, even for rodent liver tumors “Furthermore, no single definitive mode of action of TCDD-mediated carcinogenicity has been identified” (page 5-16, lines 30-31); and “The sequence of key events following binding of TCDD to the AHR and that ultimately leads to the development of cancers is unknown. Therefore, in the **strictest sense**¹ (emphasis added), TCDD’s interaction with the AHR does not constitute a mode of action as defined by the 2005 Cancer Guidelines because information about the progression of necessary events is **lacking** (emphasis added) (pages 5-10 to 5-11, lines 31 and lines 1-4, respectively)”. Remarkably, EPA then further states: “...but there are compelling carcinogenicity data in animals and mechanistic information in animals and humans demonstrating similar modes of carcinogenic action” (pg 5-2, lines 26-27). EPA asserts a known MOA to support their use of all cancer mortality for modeling a linear cancer slope factor and in support of EPA’s argument that the similarity of AHR biology between humans and rodents establishes TCDD’s classification as a known human carcinogen. EPA then offers “Overall, the data demonstrate that TCDD is a tumor promoter and potentially harbors only weak initiating activity” (pg 5-15, lines 30-31)...”One hypothesized mode of carcinogenic action of TCDD in the liver is mediated through hepatotoxicity” (page 5-17, lines 3-4).
- In reviewing potential MOA and key events information, EPA describes a very limited and selected number of possible key events for lung and liver cancer by citing papers on PPAR-alpha (Woods et al., 2007) with an occasional relevant AHR study or review (e.g., Haarman-Stemmann et al., 2009).
 - EPA’s MOA discussion for rodent liver tumors includes: a) AHR-mediated gene expression, b) hepatotoxicity, c) oxidative stress, and d) hepatocellular proliferation (pg 5-122, Figure 5-9). These key events are threshold-dependent biochemical effects widely recognized as such in the scientific literature.

¹ EPA does not define “Strictest Sense” but this terminology suggests EPA is replacing mechanism of action for MOA in arguing for a higher standard of biological understanding for how dioxins act as promoters. EPA should have provided criteria for defining “Strictest Sense” from their own 2005 Cancer Guidelines to support such a statement.

- EPA's proposed key event discussion for rodent lung tumors included: a) AHR-mediated gene expression, b) toxicity, c) retinoid depletion, d) induction of CYPs and COX2 enzymes, and e) cell proliferation (**pg 5-123, Figure 5-10**) All of these EPA proposed key events occur at threshold doses and can be modeled with non-linear approaches as part of the overall MOA for dioxin's tumor promotion of rodent liver tumors. However, in doing so, EPA should be cautioned against the choice of a 1% POD as described in the NAS critique of EPA's draft dioxin reassessment.
- EPA cites their 2003 Draft Dioxin Reassessment for further information supporting their decision but it should be noted that this document heavily relied on CYP1A1 induction and changes in epidermal growth factor (EGF) and the EGF receptor as justification for the linear cancer slope factor. Furthermore, a thorough weight-of-evidence examination of MOA was not provided in the 2003 Draft Dioxin Reassessment.

V. The Human Relevance Framework (EPA’s 2005 Cancer Guidelines)

- The Human Relevance Framework is a weight-of-evidence process for examining the MOA, the MOA’s relevance to humans, and its dose-response implications. The development of the Human Relevance Framework is a collaborative result of regulatory (IPCS), academic and ILSI efforts that the EPA adopted in its 2005 Cancer Guidelines. The “Key Events Dose-Response Framework” is a follow up to the Human Relevance Framework in order to further define dose-response approaches for evaluating the MOA and key events.
- EPA’s rejection of a dioxin MOA does not follow the Human Relevance Framework process. The EPA’s conclusion is based on a limited discussion of selected studies of key events. EPA’s effort falls far short of the effort needed to fully explore the numerous critical studies that show how AHR activation can culminate in tumors, especially rodent liver tumors that have been historically relied upon to derive the cancer slope factor.
- Other relevant EPA documents include EPA’s Risk Principles and Practices or the Risk Characterization Handbook which provides information on how the Agency could or should have examined this important issue in greater detail.

Example of Human Relevance Framework References

Boobis, A.R., Cohen, .M, Dellarco, V., McGregor, D., Meek, M.E., Vickers, C., Willcocks, D. and Farland, W. 2006 IPCS Framework for Analyzing the Relevance of a Cancer Mode of Action for Humans. *Crit Rev Toxicol* 36: 781-792

Meek, M.E., Bucher, J.R., Cohen, S.M., Dellarco, V., Hill, R.N., Lehman-McKeeman, L.D., Longfellow, D.G., Pastoor, T., Seed, J and Patton, D.E. 2003 A framework for human relevance analysis of information on carcinogenic modes of action. *Crit. Rev. Toxicol* 33: 591-653

U.S. EPA 2005 Guidelines for Carcinogen Risk Assessment. EPA/630/P-03/001F

U.S. EPA 2004 An Examination of EPA Risk Assessment Principles and Practices. EPA/100-B-04/001

U.S. EPA 2000 Risk Characterization Handbook EPA 100-B-00-002

VI. Scientific Precedent (“Weight-of-Evidence Basis”) Behind Tumor Promotion Biology: Baseline Information Necessary for Examining an MOA

Even though EPA proposed plausible but partial MOAs and key events in discussing this topic, the agency asserts that no MOA can be clearly identified. The agency position is not a true representation of the body of scientific evidence that supports the well-recognized tumor promotion MOA of nuclear receptor activation, changes in gene transcription and a number of key events involved in tumor promotion. As previously noted, EPA’s rejection of an MOA is contrary to the findings of the NRC report that concluded a threshold-dependent MOA for dioxin-induced rodent tumors does exist.

The scientific community consistently accepts the fact that tumor promoters are threshold carcinogens in nature, e.g., “...the kinetics of human risk assessment is not like genotoxic carcinogens where low-dose linearity with a linearized multistage model is used, but rather a non-linear approach (threshold) is applied.”², or, “...nongenotoxic carcinogens and non-DNA-reactive carcinogens (for these compounds a true [perfect] threshold is associated with a clearly founded no-observed adverse-effect level.”³ Internationally, regulatory organizations, such as the WHO’s Joint Exposure Committee on Food Additives (JECFA) that concluded dioxin’s MOA is threshold in nature in developing their tolerable daily food intake of 2.3 pg/kg/day, view dioxin’s risks as threshold in nature.

Some of the baseline information behind dioxin’s nuclear receptor MOA and key events and an approach for examining this information includes the following:

- TCDD (and other dioxins/furans, e.g., Waern et al., 1991) are not mutagenic nor genotoxic, but rather act as tumor promoters in rodent liver. There are many dioxin initiation-promotion studies that demonstrate tumor promotion (e.g., Teeguarden et al., 1999). Published studies have examined the tumor promotion MOA and key events of dioxin, e.g., Simon et al., 2009. A number of review articles on dioxin tumor biology and MOA similarly describe key events that underpin TCDD’s threshold MOA for rodent tumors (e.g., Kohle et al., 2008; Bock and Kohle, 2005)
- The proposed MOA for TCDD-induced liver tumors and the underlying key events are more clearly illuminated when organized using the elements of the human relevance framework set forth in EPA’s 2005 Cancer Guidelines - a Weight-of-Evidence approach for examining this issue. More information on these points follows below.
- There are numerous scientific papers on liver tumor promotion biology not reviewed by EPA that have important relevance to the issues under discussion (See below). A

² Hernandez et al., 2009 Mechanisms of non-genotoxic carcinogens and importance of a weight of evidence approach. *Mutat Res* 682: 94-109

³ Bolt and Degen 2004 Human carcinogenic risk evaluation. Part II. Contributions of the EUROTOX Specialty Section for Carcinogenesis *Tox Sci* 81: 3-6

careful review of the tumor promoter literature and MOA is the first step in developing a MOA examination for dioxin. Against the fundamental knowledge of how chemicals promote rodent liver tumors, the Agency should have ruled-in or ruled-out possible MOAs consistent with what is known. EPA did not do this. There are a number of studies and reviews that provide information on tumor promotion and the MOA for the SAB's consideration. Information from this published literature was relied upon for supporting the following proposed MOA and key event analyses involved in dioxin tumor promotion of rodent liver tumors.

Therefore, given the scientific community consensus regarding the dioxin cancer MOA, one can only conclude that the EPA position is based on policy rather than science.

Tumor Promotion Biology

Examples of General MOA Papers Relevant to Tumor Promotion

Allen, D.G., Pearse, G., Haseman, J.K. and Maronpot, R.R. 2004 Prediction of rodent carcinogenesis: An evaluation of prechronic liver lesions as forecasters of liver tumors in NTP carcinogenicity studies. *Toxicol Pathol* 32: 393-401

Bannasch, P., Haertel, T. and Qin, S. 2003 Significance of hepatic preneoplasia in risk identification and early detection of neoplasia. *Toxicol Pathol* 31: 134-139

Christensen, J.G., Gonzales, A.J., Cattley, R.C. and Goldsworthy, T.L. 1998 Regulation of apoptosis in mouse hepatocytes and alteration of apoptosis by nongenotoxic carcinogens. *Cell Growth and Differentiation* 9: 815-825

Elcombe, C.R., Odum, J., Foster, J.R., Stone, S., Hasmall, S., Soames, A.R., Kimber, I and Ashby, J. 2002 Prediction of rodent nongenotoxic carcinogenesis: Evaluation of biochemical and tissue changes in rodents following exposure to nine nongenotoxic NTP carcinogens. *Environ Health Perspect.* 110: 363-375

Enomoto, K. and Farber. E. 1982 Kinetics of phenotypic maturation of remodeling of hyperplastic nodules during liver carcinogenesis. *Can Res* 42: 2330-2335

Evarts, R.P., Hu, Z., Omori, N., Omori, N., Marsden, E.R. and Thorgeirsson, S.S. 1995 Effect of vitamin A deficiency on the integrity of hepatocytes after partial hepatectomy. *Am. J. Pathol.* 147: 699-706

Fabregat, I., Roncero, C. and Fernandez, M. 2007 Survival and apoptosis: a dysregulated balance in liver cancer. *Liver Int.* 155-162

Fielden, M.R. et al., 2008 Interlaboratory evaluation of genomic signatures for predicting carcinogenicity in the rat. *Tox Sci* 103: 28-34

Fielden, M.R., Brennan, R. and Gollub, J. 2007 A gene expression biomarker provides early prediction and mechanistic assessment of hepatic tumor induction by nongenotoxic chemicals. *Toxicol Sci* 99: 90-100

Goetz, M.E. and Luch, A. 2007 Reactive species: a cell damaging route assisting to chemical carcinogens. *Can Lett* 266: 73-83

Glauert, H.P., Schwarz, M., and Pitot, H.C. 1986 The phenotypic stability of altered hepatic foci: effect of the short-term withdrawal of phenobarbital and of the long-term feeding of purified diets after the withdrawal of phenobarbital. *Carcinogenesis* 7: 117-121

Goldsworthy, T.L., Conolly, R.B., and Fransson-Steen-R. 1996 Apoptosis and cancer risk assessment. *Mut Res* 365: 71-90

Greaves, P., Irisarri, E. and Monro, A.M. 1985 Hepatic foci of cellular and enzymatic alteration and nodules in rats treated with clofibrate or diethylnitrosamine followed by phenobarbital: Their rat of onset and their reversibility. *J. Natl Can Inst* 76: 475-484

Hasmall, S.C. and Roberts, R.A. 1999 The perturbation of apoptosis and mitosis by drugs and xenobiotics. *Pharmacol Ther* 82: 63-70

Holsapple, M.P., Pitot, H.C., Cohen, S.H., Boobis, A.R., Klaunig, J.E., Pastoor, T., Dellarco, V.L. and Dragan, Y.P. 2006 Mode of action in relevance of rodent liver tumors to human cancer risk. *Tox Sci* 89: 51-56

Kohle, C., Schwarz, M and Bock, K.W. 2008 Promotion of hepatocarcinogenesis in humans and animal models. *Arch Toxicol* (epub)

Mantovani, A., Allavena, P., Sica, A. and F. Balkwill 2008 Cancer-related inflammation. *Nature* 454: 436-444

Maronpot, R.R., Yoshizawa, K., Nyska, A., Harada, T., Flake, G., Mueller, G., Singh and Ward, J.M. 2010 Hepatic enzyme induction: Histopathology *Toxicol Path* 000: 1-20

Marsman, D.S. and Barrett, J.C. 1994 Apoptosis and chemical carcinogenesis. *Risk Analysis* 14: 321-326

Roberts, R.A., Nebert, D.W., Hickman, J.A., Richburg, J.H. and Goldsworthy, T.L. 1997 Perturbation of the mitosis/apoptosis balance: A fundamental mechanisms in toxicology. *Fund Appl Toxicol* 38: 107-115

Schulte-Hermann, R., Bursch, W., Kraupp-Grasl, B., Oberhammer, F., Wagner, A. Ad Jirtle, R. 1993 Cell proliferation and apoptosis in normal liver and preneoplastic foci. *Environ Health Perspect.* 101 (Suppl. 5): 87-90

Schulte-Herman, R., Timmermann-Trosiener, I., Barthel, G and Bursch, W. 1990 DNA synthesis, apoptosis, and pheotypic expression as determinants of growth of altered foci in rat liver during phenobarbital promotion. *Can. Res.* 50: 5127-5135

Schwarz, M., Buchmann, A., Bock, K.W. 1995 Role of cell proliferation at early stages of hepatocarcinogenesis. *Toxicol Lett* 82/83: 27-32

Tamm, I., Schriever and B. Dorken 2001 Apoptosis: Implications of basic research for clinical oncology. *Lancet Oncol* 2: 33-42

Tsuda, H., Fukushima, S., Wanibuchi, H., Morimura, K., Nakae, D., Imaida, K., Tatematsu, M., Hiro, M., Wakabayashi, K and Moore, M.A. 2003 Value of GST-P positive studies of hepatocarcinogenesis: Evidence for practical thresholds with both genotoxic and nongenotoxic carcinogens. A review of recent work. *Toxicol Pathol* 31: 80-86

Waern, F., Flodstrom, S., Busk, L., Kronevi, T., Nordgren, I and Ahlborg, .G. 1991 Relative liver tumour promoting activity and toxicity of some polychlorinated dibenzo-p-dioxin- and dibenzofuran-congeners in female Sprague-Dawley rats. *Pharmacol. Toxicol* 69: 450-458

Whysner, J and Williams, G.M. 1996 2,3,7,8-Tetrachlorodibenzo-p-dioxin mechanistic data and risk assessment: Gene regulation, cytotoxicity, enhanced cell proliferation and tumor promotion. *Pharmacol Ther* 71: 193-223

Williams, G.M. 1997 Chemicals with carcinogenic activity in the rodent liver; mechanistic evaluation of human risk. *Can Lett* 117: 175-188

Worner, W. and Schrenk, D. 1996 Influence of liver tumor promoters on apoptosis in rat hepatocytes induced by 2-acetylaminofluorene, ultraviolet light, or transforming growth factor B1. *Can Res* 56: 1272-1278

Examples of Dioxin-Specific Tumor Promotion Studies and Cancer Bioassays

Dragan, Y.P., Xu, X., Goldsworthy, T.L., Campbell, H.A., Maronpote, R.R. and Pitot, H.C. 1992 Characterization of the promotion of altered hepatic foci by 2,3,7,8-tetrachlorodibenzo-p-dioxin in the female rat. *Carcinogenesis* 13: 1389-1395

Dragan, Y.P. and Schrenk, D. 2000 Animal studies addressing the carcinogenicity of TCDD (or related compounds) with an emphasis on tumour promotion. *Food Add Contam* 17: 289-302

Graham, M.J., Lucier, G.W., Linko., P., Maronpot, R.R. and Goldstein, J.A. 1988 Increases in cytochrome P-450 mediated 17 β -estradiol 2-hydroxylase activity in rat liver microsomes after both acute administration and subchronic administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin in a two-stage hepatocarcinogenesis model. *Carcinogenesis* 9: 1935-1941

Kim, J.K., Han, B.S., Ahn, B., Hasegawa, R., Shirai, T, Ito, N. and Tsuda, H. 1997 Enhancement by indole-3-carbinol of liver and thyroid gland neoplastic development in a rat medium-term multiorgan carcinogenesis model. *Carcinogenesis* 18: 377-381

Kim, A.H., Kohn, M.C., Nyska, A. and Walker, N.J. 2003 Area under the curve as a dose metric for promotional responses following 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure. *Toxicol Appl Pharmacol* 191: 12-21

Lucier, G.W., Tritscher, A., Goldsworthy, T., Foley, J., Clark, G., Goldstein, J. and Maronpot, R. 1991 Ovarian hormones enhance 2,3,7,8-tetrachlorodibenzo-p-dioxin-mediated increases in cell proliferation and preneoplastic foci in a two-stage model for rat hepatocarcinogenesis. *Cancer Res* 51: 1391-1397

Maronpot, R.R., Foley, J.F., Takahashi, K., Goldsworthy, T., Clark, G., Tritscher, A., Portier, C. and Lucier, G. 1993 Dose-response for TCDD promotion of hepatocarcinogenesis in rats initiated with DEN: Histologic, biochemical, and cell proliferation endpoints. *Environ Health Perspect* 101: 634-642

NTP 1980 Bioassay of a mixture of 1,2,3,6,7,8-hexachlorodibenzo-p-dioxin and 1,2,3,7,8-hexachlorodibenzo-p-dioxin for possible carcinogenicity. NTP TR 198

NTP 1982 Carcinogenesis bioassays of 2,3,7,8-tetrachlorodibenzo-p-dioxin in Osborne-Mendel rats and B6C3F1 Mice (Gavage Study). NTP TR 209

NTP 2006a Toxicology and carcinogenesis studies of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in female Harlan Sprague-Dawley Rats, NTP TR 521

NTP 2006b Toxicology and carcinogenesis studies of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) in female Harlan Sprague-Dawley Rats, NTP TR 525

NTP 2006c Toxicology and carcinogenesis studies of a mixture of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 3,3',4,4',5-pentachlorobiphenyl (PCB 126) in female Harlan Sprague-Dawley Rats. NTP TR 526

Pitot, H.C., T. Goldworthy, H.A. Campbell and A. Poland 1980 Quantitative evaluation of the promotion by 2,3,7,8-tetrachlorodibenzo-p-dioxin of hepatocarcinogenesis from diethylnitrosamine. *Cancer Res* 40: 3616-3620

Pitot, H.C., Goldsworthy, T.L., S. Moran, W. Kennan, H.P. Glauert, R. R. Maronpot and H.A. Campbell 1987 A method to quantitate the relative initiating and promoting potencies of hepatocarcinogenic agents in their dose-response relationships to altered hepatic foci. *Carcinogenesis* 8: 1491-1499

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VII. Proposed Dioxin MOA for Rodent Liver Tumors

The following figure depicts a general schematic for a MOA and key event model (Figure 1). This figure was taken from a recent NRC report entitled “*Toxicity Pathways-Based Risk Assessment: Preparing For Paradigm Shift: A Symposium Summary*”

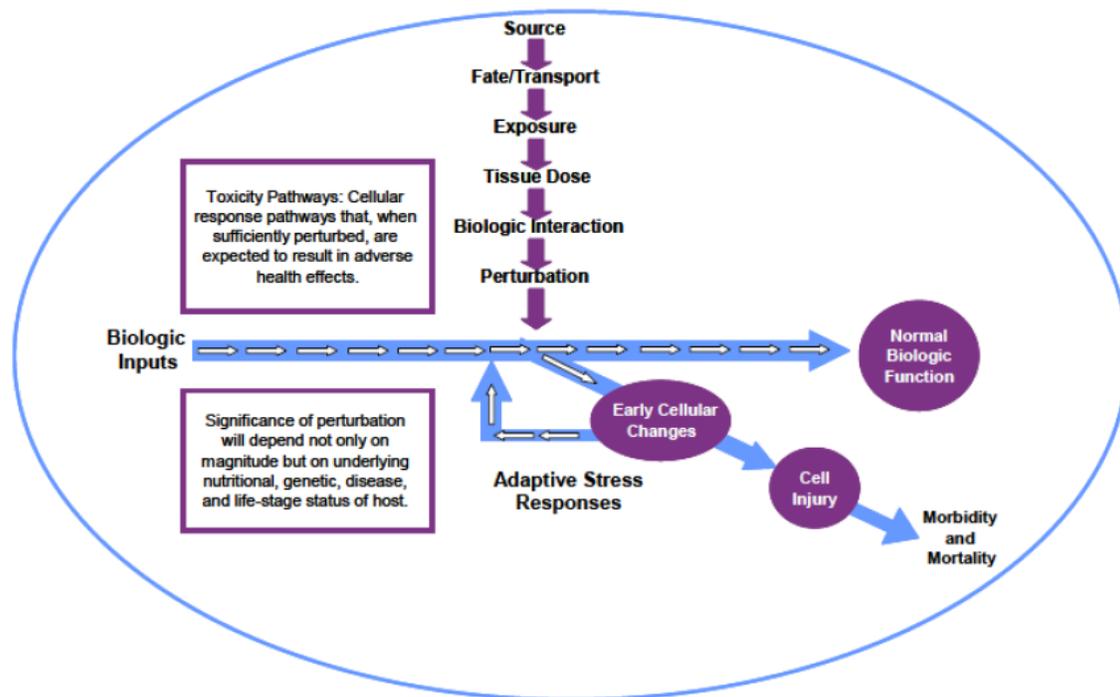


Figure 1. A model for dysregulation of cellular response pathways resulting in toxicity presented in the recent 2007 NRC report “*Toxicity Pathways-Based Risk Assessment: Preparing For Paradigm Shift: A Symposium Summary*.”

The schematic shown in Figure 1 was adapted for developing a dioxin-specific model of the proposed MOA and Key Events (next figure - Figure 2). All of these MOA and Key Event components can be evaluated as to their dose-response nature and then combined into an overall dose-response model for deriving cancer potency estimates.

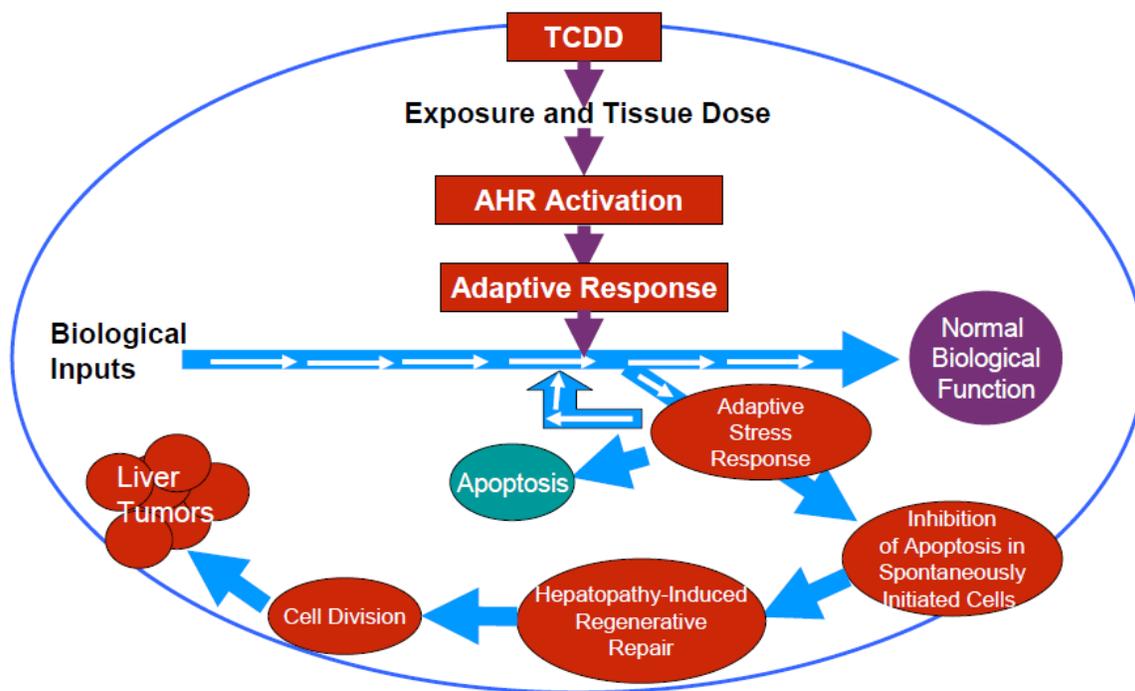


Figure 2. Insertion of MOA and Key Events Information from the Dioxin Literature into the 2007 NRC model.

The key events shown in this figure include:

a) Exposure and Tissue Dose

Hepatic dioxin concentrations increase in a dose-dependent, but non-linear fashion as a function of CYP1A2 induction and sequestration

b) AHR activation

TCDD-induced changes in altering gene transcription and cell signaling involving a complex number of steps:

- shedding of chaperone proteins
- translocation into the nucleus with a transporter system,
- binding with ARNT,
- competing with the dioxin response element with the AHRR,
- recruitment of a complex array of co-regulatory proteins,
- mRNA transcription, and
- protein translation and modification

Additional aspects of the AHR for consideration include QSAR-dependency of ligands and potency: The more avidly binding ligands are more potent promoters (NTP 2006). AHR polymorphisms and deletions directly affect dioxin-mediated tumor promotion, e.g., the DBA2 mouse, the constitutively active AHR mouse model, the Hans Kuptio resistant rat model. The AHR

Knockout mouse has shown to be very resistant to dioxin toxicity establishing the central role of the AHR in the overall MOA and Key Event model.

An important feature of AHR activation, central to MOA and key events, is the role of AHR zonal-activation (centrilobular to mid zonal to periportal in nature). Recent papers by Ong et al, (2010), Simon et al., (2008 and 2009) and Sheik-Bahaei et al. 2010) provide examples of quantitative approaches for evaluating these biological features of dioxin's liver tumor MOA and key events – more information on this dose-response factor related to the MOA is provided below

c) Adaptive Responses

The AHR has a normal biological role in development and homeostasis. Normal AHR activation is presumably modulated by naturally occurring AHR activity measured in human blood that is actually orders of magnitude greater in terms of AHR activity than background AHR activity attributable to dioxin and dioxin-like chemicals (Schechter et al., 1999; Connor et al., 2007). These naturally occurring ligands from food and from endogenous sources would modulate AHR activation, e.g., competitive inhibition, from exogenous AHR ligands such as dioxin. AHR activation, therefore, depending on the ligand, tissues and species, can result in normal biological responses necessary and beneficial.

d) Adaptive Stress Response

The adaptive stress response(s) consist of cell changes, such as CYP1A induction, increases in reactive oxygen defense, e.g., Nrf2 induction, DNA repair, e.g., TiPARP induction, and cell cycle slow down allowing DNA repair, e.g., evidence of G1/S cell cycle lock., in preventing cell damage and tumor promotion. However, excessive stress response stimulation can eventually lead to reversible and/or irreversible injury if normal biology is redirected towards sustaining these pathways. These normal and/or adaptive responses act counter to tumor promotion and must be factored into any low dose quantitative dose-response model for dioxin-promoted liver tumors.

e) Inhibition of apoptosis in spontaneously initiated liver cells and foci –

A common tumor promotion, key event for many promoters, especially those acting via nuclear receptors is inhibition of apoptosis in initiated hepatocytes. In initiated cells, AHR activation may act differently than in normal hepatocytes. AHR activation in initiated cells results in inhibition of apoptosis (Stinchcombe et al., 1995; Schrenk et al., 2005). This provides a growth advantage to initiated cells that might otherwise be directed towards apoptosis. AHR activation within altered hepatocytes/foci gives rise to an increase in the number and volume of altered hepatic foci. Absent nitrosamine and/or partial hepatectomy, the process of spontaneously

initiated hepatocyte development takes much longer, e.g., the late-stage development of liver tumors observed in dioxin cancer bioassays.

f) Hepatopathy-induced regenerative repair

Sustained AHR activation, culminating in hepatopathy provides for a critical mitogenic stimulus for further expansion of altered hepatocytes/foci and eventual development of liver tumors as evidenced by increased DNA synthesis (e.g., Brdu labeling (Hailey et al., 2006)). A number of toxicities may contribute to hepatopathy including: a) steatosis [e.g., non alcoholic fatty liver disease], b) Inflammation, c) Retinoid depletion, d) reactive oxygen generation, e) mitochondrial injury, and, e) estradiol promotion and/or metabolism.

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Increased DNA Synthesis/Cell Division

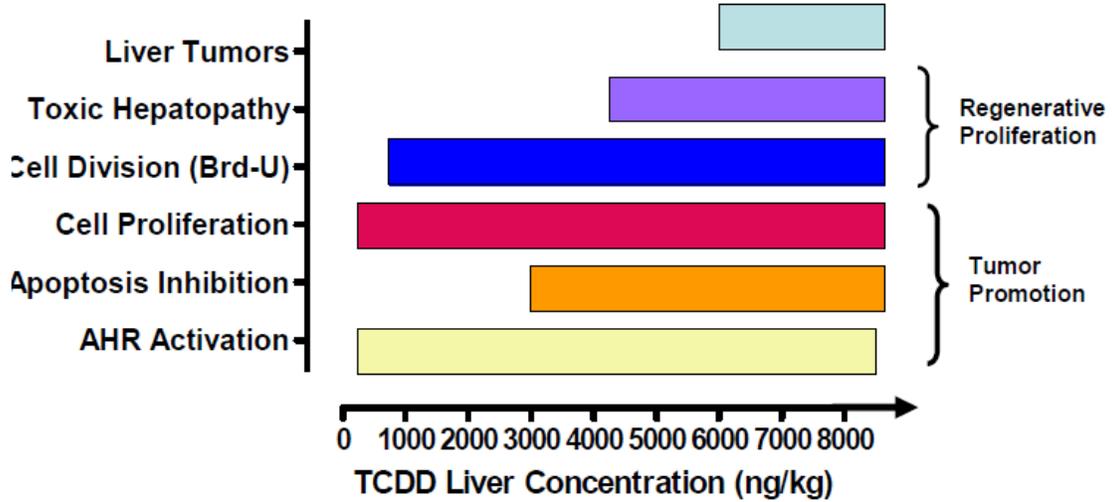
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All of the Key events and their underlying supporting data should be evaluated singly, and combined, in order to establish the best cancer risk characterization. The Human Relevance Framework specifically requires an assessment of dose-concordance between key events and the apical outcome (e.g., liver tumors).

Dose- and temporality-concordance between key events and the apical endpoints (liver tumors) can be established for dioxin, something that EPA did not adequately address (see following figures 3, 4 and 5 for examples of dose- and temporality-concordance – these figures were presented at the 2008 Winter ToxForum meeting: Budinsky, B and Rowlands, C. Dioxin (TCDD) Case Study Implications of the Mode of Action for Dose-Response in Risk Assessment)

Consistency of Dose-Response Findings With MOA, Key Events and Liver Tumors

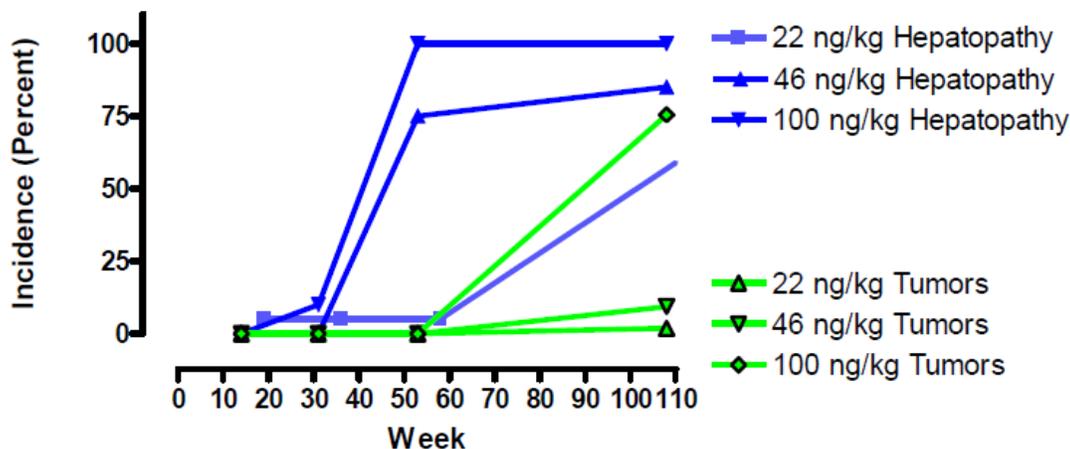


Liver conc. data estimated with Carrier and Aylward toxicokinetic model, BMD5 or BMD10 Estimates or liver concentrations estimated

19

Figure 3. Key Event tissue concentration range in relationship to the liver tumor apical outcome.

Example of Dose- and Temporality-Concordance (NTP 2006)



Liver tumors: Hepatomas and Cholangiocarcinomas

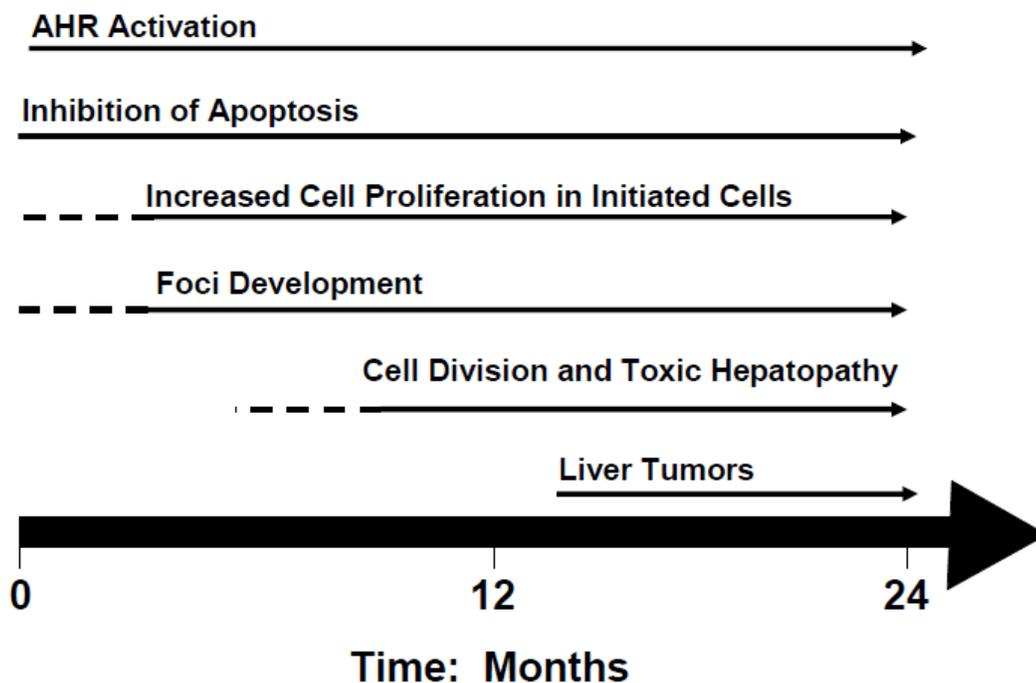
Toxic hepatopathy: characterized by increased incidences of numerous nonneoplastic liver lesions including hepatocyte hypertrophy, multinucleated hepatocytes, altered hepatocellular foci, inflammation, pigmentation, diffuse fatty change, necrosis, portal fibrosis, oval cell hyperplasia, bile duct hyperplasia, bile duct cysts, ²³ cholangiofibrosis, and nodular hyperplasia

Figure 4. Data taken from the 2006 NTP cancer bioassay on TCDD showing the temporal and dose-response relationships between hepatopathy that drives regenerative repair and the development of liver tumors.

The important points from the dose-response concordance examples are that dose-response relationships for the key events occur at dosages that are lower than those required to cause the apical tumor outcome thereby establishing a logical precedent for their role in the tumor promotion MOA. For example, hepatopathy occurs at lower dosages than liver tumors. If a greater dosage was required for hepatopathy than tumors one could argue that the two events were not closely linked, if linked at all. A published example of a dose-transition continuum for the MOA and key events is Simon et al., (2009) - see below.

Figure 5 is another illustration of the temporality-concordance between these key events and liver tumor development.

Temporal Progression of MOA and Key Events



21

Figure 5. An illustration of the timing between the onset of key events and the eventual development of liver tumors.

The key events occur in a temporal sequence consistent with the late stage development of hepatic tumors in rodents. AHR activation, as measured by CYP1A induction occurs within hours. AHR activation resulting in inhibition of apoptosis within damaged hepatocytes can also be measured within hours to days. However, clonal expansion of altered hepatocytes requires weeks to months. In rats hepatopathy requires months and this is associated with evidence of increased DNA synthesis. Finally, liver tumors occur late in the rat cancer bioassays consistent with the prior development of these key events.

The following figure from Simon et al. (2009) depicts dose- and temporality-concordance of some of the important key events relative to tumor development (LALC: Lifetime Average Liver Conc.) (Figure 6). This approach was built on the concept of dose-transitions in explaining toxicological effects (Slikker et al., 2003)

Example of Dose-Response Modeling and Concordance For Key Events; Simon et al., 2009

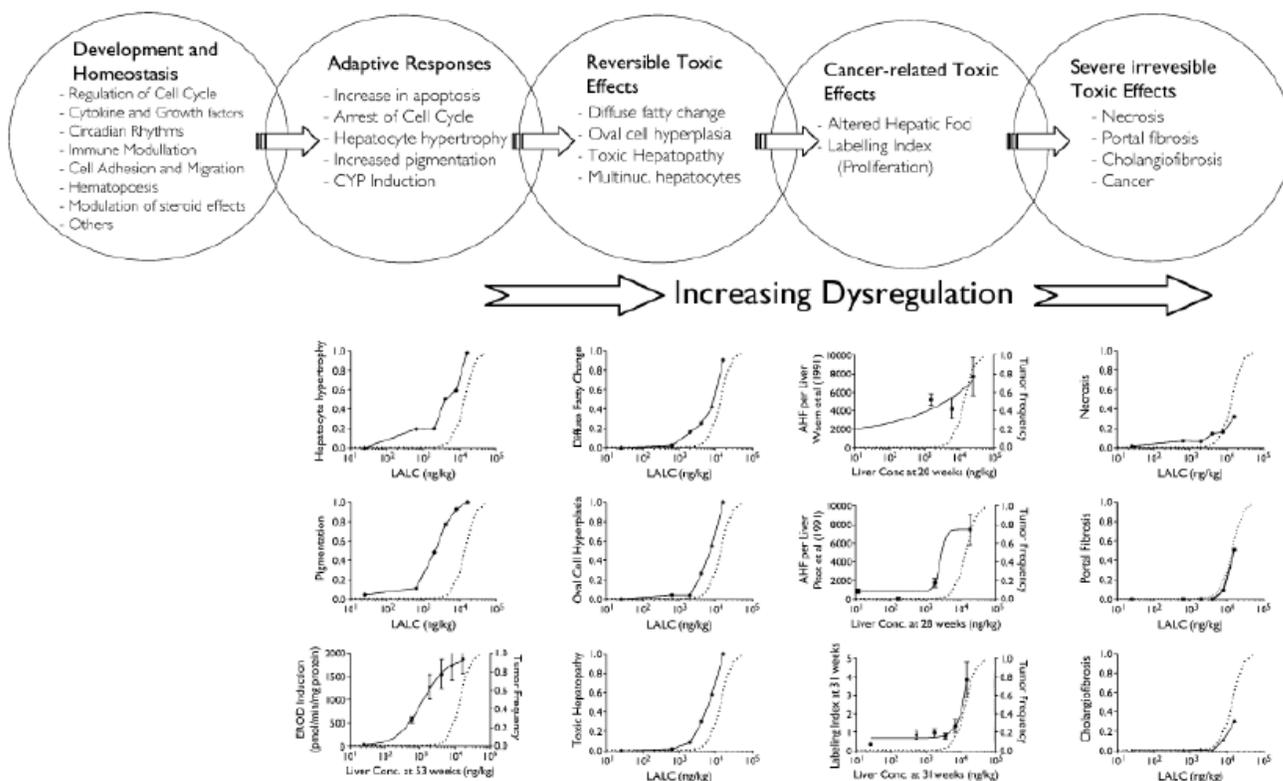


Figure 6: Qualitative and quantitative modeling information were carefully considered in deriving a non-linear cancer potency value for TCDD as published in Simon et al., (2009). This figure demonstrates the relationship between lifetime average liver concentrations (LALC) and/or liver concentrations in the 2006 NTP TCDD cancer bioassay and a number of key event and related endpoints.

Another important dose-response factor for modeling the AHR activation is zonal activation of AHR in the liver. The most sensitive AHR response (CYP1A induction) first occurs in centrilobular region where older polyploid hepatocytes are first activated (Andersen et al., 1997). These older centrilobular hepatocytes are distal from the more mid-zonal and peri-portal locations of stem cells, believed to be the pool of cells more susceptible to carcinogenesis (Roskams, 2006). These older centrilobular hepatocytes that are activated at lower dioxin dosages may not be as susceptible to the longer-lived stem cells and newly formed hepatocytes and bile duct cells for acquiring the cumulative DNA “hits” necessary for tumor development. If it is the stem cells or nascent hepatocytes/bile duct cells that give rise to dioxin-promoted tumors, then higher dosages of TCDD are necessary to zonally reach these cell types. Alternatively, as stated by Roskams (2006): “Large cells “dysplastic” foci, on the other hand, consist of mature senescent hepatocytes as a result of continuous proliferation in chronic liver disease and

not due to true precursor lesions of HCC.” If the latter is true for dioxin, i.e., adenomas and bile duct tumors arise from more mature liver cell types, the threshold-dependent development of chronic liver injury and its accompanying inflammation becomes even more important, i.e., an important “Time x Concentration” factor for hepatopathy necessary for final tumor promotion. Therefore, in addition to the dose-response aspects of the key events, there is the additional spatial feature of zonal AHR activation that adds to the non-linearity of AHR activation-dependent MOA and key events.

With respect to the Hill Criteria components of the Human Relevance Framework, the MOA and key events are consistent and coherent with, as well as biologically plausible with, both the scientific understanding of the possible MOA and key events involved in nuclear receptor-based tumor promotion and the published data on dioxin.

Finally, the Human Relevance Framework requires that alternative MOA possibilities be examined and either ruled-in or ruled-out. In this instance, the most important alternative MOA possibility is genotoxicity since this MOA results in a default linear outcome according to EPA’s 2005 Cancer Guidelines. Since TCDD is non-genotoxic it does not require, by default, a linear approach as specified in the 2005 U.S. EPA Cancer Guidelines. While oxidative stress and DNA oxidation have been reported in the literature, these key event-contributing effects do not appear to be mutagenic initiating events but rather are more appropriately linked with mitogenic stimuli and hepatopathy that contributes to the tumor promotion.

Examples of Dose-Response Literature

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Other Dose-Response Relevant Studies

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Guzelian, P., Quattrochi, I., Karch, N., Aylward, and Kaley, R. 2006 Does dioxin exert toxic effects in humans at or near current background body levels?: An evidence-based conclusion. *Human Exp Toxicol* 25: 99-105

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Attachment E:

**Comments On The U.S. EPA Draft Cancer Slope Factor
Derivation For 2,3,7,8-Tetrachlorodibenzo-p-Dioxin**

July 9, 2010

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Introduction

The USEPA (2010)¹ decided to estimate cancer risk by assuming a linear cancer dose-response function and derived cancer slope factors based on the analyses by Cheng et al. (2006)² of the National Institutes of Occupational Safety and Health (NIOSH) cohort previously studied and analyzed by Fingerhut et al. (1989)³ and Steenland et al. (1999, 2001).^{4,5} As noted in the American Chemistry Council's mode-of-action (MOA) comments included in this package and as explicitly recommended by the National Academy of Sciences expert panel, a non-linear assessment based on mode of action considerations is the scientifically justified approach to cancer risk assessment for dioxin; reliance on a linear approach for derivation of a cancer slope factor is a policy decision that ignores a large body of evidence regarding mode of action of TCDD. However, if USEPA decides to pursue derivation of a cancer slope factor based on the results of the Cheng et al. (2006) study (of which I am a coauthor), a number of technical issues in the analysis presented in the draft document need to be addressed.

Based on the results presented in Cheng et al. (2006) and application of the Emond et al. (2005)⁶ physiologically-based pharmacokinetic (PBPK) model, USEPA identified a range of potential cancer slope factors varying over more than an order of magnitude. Furthermore, USEPA focused on the upper end of the identified range to conclude that the appropriate cancer slope factor for use in cancer risk assessment for TCDD is 1,000,000 per mg/kg-d. The USEPA (2010) derivation of a cancer slope factor based on the Cheng et al. (2006) analysis is predicated on a significant number of assumptions, including (but not limited to):

- Assumption that a linear dose-response function for TCDD is scientifically justified, despite significant comment from the NAS (2006) panel to the contrary;
- Assumption that only studies showing a positive dose-response should be considered and used to quantify the slope factor.
- Assumption that "all cancers" is a biologically plausible and scientifically sound endpoint for cancer risk assessment;
- Assumption that all observed excess cancers in the cohort are due to TCDD exposure which included mesotheliomas, and significant excesses in bladder cancer in a subcohort exposed to the known bladder carcinogen, 4-aminobiphenyl; and

- Assumption that accurate dose reconstruction for an occupational cohort can be made based on limited industrial hygiene data which only included measurement of serum TCDD at a single time point decades following last exposure from a very small, non-representative sample of the cohort; and application of a pharmacokinetic model over decades.

Many of these assumptions will be addressed by other commenters. As a coauthor of the Cheng et al. (2006) study, my comments focus here on the quantitative derivation of the cancer slope factor based on this study. I present comments on two major aspects of the USEPA (2010) derivation:

- The selection and interpretation of regression coefficients from the Cheng et al. (2006) study, and
- The application of PBPK modeling to derive risk-specific doses and cancer slope factors.

Both of these steps entail several choices and decisions, and the choices and decisions made by USEPA (2010) are not consistent in critical aspects with the underlying data and PBPK model. I present information that more appropriately informs the decisions in a risk assessment context for each step. Based on these considerations, I present the most scientifically supportable and appropriate derivation of a cancer slope factor based on the Cheng et al. (2006) analyses and results.

Selection of Regression Coefficient

The Cheng et al. (2006) analysis of the NIOSH cohort mortality data reconstructed the dose using a concentration-dependent pharmacokinetic model and included several evaluations of the dose-response patterns in the data. These dose-response assessments assessed the relationships between increases in relative risk of all-cancer mortality and estimated area-under-the-curve (AUC) of TCDD in serum. The analyses included consideration of lagging (omission of the most recent period of contribution to AUC) and trimming of the data set. The trimming involved omitting the upper and lower 5% of estimated exposures from the dose-response assessment. This approach option was included because (1) the extremes of the estimated exposure ranges were considered to be the most uncertain, and (2) the upper end of the exposure-response range

exerts a substantial effect on the estimation of the exposure-response relationship in a linear analysis.

The coefficients relating relative risk of cancer mortality (RR) to AUC of serum TCDD from Cheng et al. (2006) were reproduced by USEPA (2010) in Table 5-2. This table is reproduced below. The USEPA (2010) table is inaccurate in one important respect. The regression coefficient for the lagged, linear analysis relying on the full data set (not trimmed) reported by Cheng et al. (2006) is *NOT* statistically significantly different from zero, contrary to the indication in the USEPA table.

The effect on the estimated regression coefficients of trimming the upper 5% of the exposure records is notable: the exposure-response relationship becomes statistically significant, and the estimated regression coefficient is nearly **200-fold higher** than the non-significant regression coefficient obtained from the untrimmed data set. The regression coefficient resulting from this trimmed analysis can and should be regarded as an upper bound of the exposure-response relationship that is consistent with the NIOSH data set.

The USEPA (2010) analysis goes further, however, and relies upon the statistical upper bound of the regression coefficient for the lagged, trimmed analysis. This increases the estimated regression coefficient by a further factor of almost 2, from 3.3×10^{-6} to 6.04×10^{-6} per ppt-yr. Reliance on the statistical upper bound of this regression coefficient, rather than on the best estimate from the lagged, trimmed analysis, represents an inappropriate additional source of conservatism in the risk assessment process.

The best estimate of the regression coefficient from the lagged, trimmed analysis is already an upper bound estimate of an exposure-response relationship that is consistent with the NIOSH data set, and is clearly appropriate and justified for use in deriving a linear cancer slope factor, if that is the policy decision pursued by USEPA. Thus, the incremental AUC and average fat concentrations presented in Table 5-4 (based on the central estimate of the regression coefficient) rather than those presented in Table 5-3 (based on the upper 95th percent confidence limit on the regression coefficient) are the more appropriate and scientifically sound set of risk-specific tissue concentration estimates. The extrapolation of those tissue concentrations to risk-specific dose

estimates and slope factors through use of the Emond et al. (2005) PBPK model is addressed in the next section.

Table 1: Table 5-2 reproduced from USEPA (2010). Note that the footnote “c” is incorrectly applied to the linear, full data regression coefficient; the coefficient is *not* statistically significantly different from zero, and should be annotated as such (footnote “d” according to this presentation). Footnote a is also incorrect.

1 **Table 5-2. Cox regression coefficients and incremental cancer-mortality risk**
 2 **for NIOSH cohort data**
 3

Model	Cox regression coefficient estimate (ppt-year) ⁻¹	Incremental risk ^a
Steenland et al. (2001, 197433) (unlagged exposures)		
Piecewise linear	1.5×10^{-5}	7.0×10^{-4}
Cheng et al. (Cheng et al., 2006, 523122) (exposures lagged 15 years)		
Linear, lower 95% of observations	3.3×10^{-6} ^b	1.2×10^{-4}
Linear, full data	1.7×10^{-8} ^c	6.3×10^{-7}

4
 5 ^aCompared to internal reference population (lowest exposure group), with a cancer mortality rate of 0.214; assumes
 6 background exposure of 5 ppt per year serum-lipid TCDD concentration.
 7 ^b $p \leq 0.05$.
 8 ^c $p \leq 0.05$.
 9 ^dNot statistically significant ($p > 0.05$).
 10
 11 Source: Cheng et al. (2006, [523122](#); Table IV).

Footnote should be “d”, not “c”. The result is not statistically different from zero.

Value should be 0.124. See Cheng et al. (2006).

Application of the PBPK model

One of the major changes in the USEPA (2010) risk assessment compared to previous drafts is the application of a PBPK model that reflects the concentration-dependent elimination behavior of TCDD. The underlying mechanism of the concentration-dependent model must be considered in the application of the model in the context of estimation of risk-specific doses and cancer slope factors. This section describes the model structure and basis for the concentration dependency, and then discusses the implications of these considerations for the quantitative estimates of risk-specific doses and cancer slope factors by USEPA.

Background on PBPK model. Concentration-dependent models such as the Emond et al. (2005) PBPK model (used by USEPA) and the Aylward et al. (2005)⁷ model (used in the Cheng et al.

Background on PBPK model. Concentration-dependent models such as the Emond et al. (2005) PBPK model (used by USEPA) and the Aylward et al. (2005)⁷ model (used in the Cheng et al. 2006 analyses) capture the dose dependence of hepatic sequestration of TCDD and the impact of that sequestration on the elimination rate for TCDD. The mechanism for sequestration is the induction of CYP1A2 protein in the liver by TCDD. CYP1A2 protein serves as a binding protein for TCDD. As exposure levels increase, CYP1A2 is induced in the liver, TCDD binds to the protein, and the proportion of TCDD in the body that is located in the liver increases. TCDD is eliminated (via metabolism or elimination of parent compound) from the liver more efficiently than from other depots, such as fat, and so the overall rate of elimination goes up (and half-lives decrease) at increasing exposure levels.

Thus, the key physiological factor influencing the elimination rate for TCDD is the level of induced CYP1A2. This level, in turn, is a function of the level of exposure to TCDD and the concentration of TCDD in the liver. The non-linear behavior of the elimination rate is particularly apparent at the lowest absolute exposure levels, with increasingly long half-lives predicted at the lowest liver tissue concentrations and exposure levels.

The dose-response curve describing the relationship between exposure to TCDD and induction of CYP1A2 is obviously very important in predicting the elimination behavior of TCDD. Substantial data are available from rodent species describing this process, although the data describing the shape of the dose-response curve at the tissue concentrations in the range of current background exposures are extremely limited. In the case of humans, *in vivo* data directly describing the dose-response curve for CYP1A2 induction are essentially non-existent. The human dose-response curve for the exposure-response relationship for CYP1A2 is either extrapolated from observed TCDD elimination behavior in highly exposed humans using a Michaelis-Menten function (Aylward et al. 2005) or extrapolated from rodent data sets (Emond et al. 2005), depending on the model. In both cases, the exposure-response behavior for CYP1A2 induction and resulting impact on elimination rates at the lowest exposure levels is uncertain.

Exposure to other TEQ contributors can also induce CYP1A2 protein, affecting the elimination rate for TCDD. Therefore, for humans in the general environment, where trace level exposure to a mixture of dioxin-like compounds occurs and is reflected in measured serum and tissue levels,

the contribution of non-TCDD congeners to CYP induction, and thus to enhancement of the TCDD elimination rate, must be taken into account.

Application of PBPK model to Cheng et al. (2006) results. As discussed above, Table 5-4 of USEPA (2010) presents results derived based on the best estimate of the regression coefficient from the linear, lagged, and trimmed analysis of the NIOSH cohort dose-response data in Cheng et al. (2006). Table 5-4 is reproduced below for convenience.

Table 2: Table 5-4 from USEPA (2010), presenting the assessment of AUC and fat concentrations associated with specified risk levels. The table also presents estimated risk-specific doses derived using the PBPK model of Emond et al. (2005) and corresponding cancer slope factors attributable to the absolute levels in fat in column 3.

1 **Table 5-4. Comparison of fat concentrations, risk specific dose estimates and**
 2 **associated central tendency slope estimates based on best estimate of**
 3 **regression coefficient^a of all fatal cancers reported by Cheng et al. (2006,**
 4 **[523122](#)) for selected risk levels**
 5

Risk level (RL)	AUC _{RL} , (ppt-yr)	FAT _{RL} (ng/kg)	Risk specific dose (D _{RL}) (ng/kg-day)	Central tendency slope estimates (mg/kg-day) ⁻¹
1 × 10 ⁻²	2.312 × 10 ⁴	3.303 × 10 ²	2.21 × 10 ⁻¹	4.5 × 10 ⁴
1 × 10 ⁻³	2.393 × 10 ³	3.419 × 10 ¹	6.97 × 10 ⁻³	1.4 × 10 ⁵
1 × 10 ⁻⁴	2.402 × 10 ²	3.431 × 10 ⁰	2.74 × 10 ⁻⁴	3.7 × 10 ⁵
1 × 10 ⁻⁵	2.403 × 10 ¹	3.432 × 10 ⁻¹	1.74 × 10 ⁻⁵	5.7 × 10 ⁵
1 × 10 ⁻⁶	2.403 × 10 ⁰	3.432 × 10 ⁻²	1.50 × 10 ⁻⁶	6.7 × 10 ⁵
1 × 10 ⁻⁷	2.403 × 10 ⁻¹	3.432 × 10 ⁻³	1.46 × 10 ⁻⁷	7.0 × 10 ⁵

6
 7 ^aBased on regression coefficient of Cheng et al (2006, [523122](#); Table III) excluding observations in the upper 5%
 8 range (≥252,950 ppt-year lipid adjusted serum TCDD) of the exposures; where reported β = 3.3 × 10⁻⁶ ppt-years;
 9 background cancer mortality risk is assumed to be 0.112 as reported by Cheng et al. (2006, [523122](#)).

The columns headed “AUC” and “FAT” present estimated risk-specific increments in cumulative area-under-the-curve of TCDD in serum lipid and the corresponding lifetime average fat (or lipid) concentrations (AUC in ppt-yrs divided by 70 years lifetime). These increments in the internal dose measures correspond to the incremental risk levels in column 1. The AUC and FAT concentrations presented here are calculated from the relative risk estimates in Cheng et al.

(2006) using the central tendency of the regression coefficient. It should be emphasized that the regression coefficient from Cheng et al. (2006) relates increases in relative risk (over a background risk) to increments in AUC of TCDD in serum lipid; this supports application of the derived exposure-response relationship in the context of incremental, rather than only absolute, exposures.

The column headed “Risk specific dose” presents the estimated lifetime intake rate required to obtain the absolute average fat concentration at a given risk level. These risk-specific doses are derived from the application of the Emond et al. (2005) PBPK model to estimate the daily intake rate of TCDD resulting in the identified risk-specific lifetime fat concentrations. The slope factor (SF) estimates presented in the final column are derived by dividing the risk level (RL) by the estimated risk-specific dose (RSD):

$$SF = RL/RSD \quad (1)$$

Note that the increments in fat concentration and AUC are essentially linearly related to risk up to risk levels of about 1×10^{-3} . However, the corresponding risk-specific doses are not linear. This is due to the use of the concentration-dependent PBPK model. Because the PBPK model is being applied by USEPA to the *absolute* fat concentrations identified in this table, elimination rates are being estimated at vanishingly small concentrations of TCDD in the body. As the levels of interest in fat get smaller and smaller, the model predicts decreasing elimination rates, and therefore, proportionally smaller risk-specific doses required to obtain the fat level of interest. Smaller risk-specific doses lead to higher cancer slope factors. Most importantly, these low TCDD concentrations are far below levels for which any *in vivo* data exist on CYP induction or TCDD elimination behavior (in rodents or humans), and thus are beyond the range of validation of the model.

This application of the PBPK model to vanishingly small concentrations of TCDD, and the resulting estimated RSDs and SFs runs counter to accepted risk assessment practice, which assesses cancer risk as an incremental risk. For risk managers who need to assess the incremental risk of potential exposures to dioxins in the environment or in foods, the question is: what slope factor should be used to assess the risk from an incremental exposure estimated for a given situation? The appropriate and accepted application of the PBPK model is to estimate the

doses required to produce *incremental* changes in fat concentrations corresponding to the risk levels of interest from Table 5-4 in the real-world general population.

Assessment of slope factors accounting for current background. Current background concentrations of TCDD in the general population range from less than 1 ppt in serum lipid in younger members of the population to approximately 5 to 10 ppt in older individuals.⁸ Furthermore, when other contributors to serum lipid TEQ are included, serum lipid TEQ concentrations in the general US population range up to approximately 20 ppt for young adults and approximately 60 ppt for older adults.⁹ Non-TCDD TEQ contributes to the induction of CYP1A2, which will influence the elimination rate for TCDD.^{10, 11}

These levels of current background tissue concentrations dictate the appropriate way to apply the PBPK model for estimating risk-specific doses and corresponding cancer slope factors. Given the current background body concentrations of TCDD and other TEQ contributors, the appropriate application of the PBPK model is to start from current background concentrations (including some accounting for non-TCDD TEQ) and estimate the incremental doses required to produce the incremental increases in lifetime fat concentrations identified in Table 5-4.

Tables 3 and 4 present the incremental risk levels and corresponding incremental fat concentrations from USEPA (2010) Table 5-4. These increments are added to two different estimates of baseline TCDD-equivalent tissue concentrations (3 ppt in Table 3, 30 ppt in Table 4, accounting for total TEQ) that are representative of the lower and mid-to-upper range of background TEQ concentrations, respectively. The following procedure was followed:

- The baseline intake dose resulting in the background fat level (3 ppt TCDD in Table 3; 30 ppt in Table 4) was estimated using the PBPK model results presented in USEPA (2010) Appendix C.4.
- Total fat concentrations corresponding to background plus incremental fat concentrations associated with varying levels of cancer risk (taken from USEPA 2010, Table 5-4, which is reproduced above as Table 2) were estimated.
- The lifetime intake rate required to attain each of the summed fat concentrations (background plus incremental risk-specific fat concentrations) is estimated, again using the PBPK model results presented in USEPA (2010) Appendix C.4.

- The intake rate corresponding to background fat level is subtracted from the intake rate corresponding to background + incremental fat concentration to estimate the risk-specific incremental intake at each risk level.
- The corresponding cancer slope factor is calculated according to equation 1 above.

This approach allows estimation of the relevant risk-specific doses using the PBPK model, but applies the model in the exposure range relevant to real-world exposures. In this way, the model reproduces the elimination behavior of TCDD relevant to risk assessment and risk management, taking into account current background body burdens of TCDD and non-TCDD contributors to TEQ and their impact on TCDD elimination behavior. This approach corresponds to the needs of risk managers in the risk assessment of dioxins in the environment.

When the PBPK model is used to estimate incremental risk-specific doses in the concentration ranges relevant to risk assessment for dioxins (background plus incremental fat concentrations), the estimated slope factors vary much less than when the PBPK model is applied to vanishingly small concentrations associated with absolute fat concentrations at the low risk level range (see Table 5-4 from USEPA 2010, reproduced as Table 2 above). This is because the increments in fat concentration associated with risk levels below 1×10^{-4} are generally small compared to background concentrations. Thus, the elimination behavior of TCDD predicted by the PBPK model is most influenced by the background concentration, not by the small risk-specific increments in fat concentration.

The slope factors presented in Tables 3 and 4, derived based on the evaluation of incremental fat concentrations over background concentrations in the ranges found in the US, are in the range of 80,000 to 290,000 $(\text{mg/kg-d})^{-1}$. This range is far narrower than the 30-fold range of slope factors (ranging up to values in excess of 1,000,000 per mg/kg-d) initially derived by USEPA based on the Cheng et al. (2006) study and is based on appropriate application of the PBPK model in the exposure ranges relevant to real-world exposures. This is an appropriate range of slope factors for recommendation to risk managers. In accordance with NAS recommendations (and emphasized in the OMB interagency comments), this range of cancer slope factors should be provided along with quantitative criteria based on a non-linear, mode-of-action assessment to risk managers. The decision of which approach is more appropriate and the impact of the two

approaches on risk management decisions can then be made in the context of the specific risk management challenge.

Table 3: Estimation of incremental risk-specific doses and corresponding cancer slope factors assuming a background TCDD concentration of 3 ppt in serum lipid and fat (accounts for TCDD and a contribution to CYP induction from non-TCDD TEQ). Incremental fat concentrations in the second column are those estimated by USEPA using the central tendency estimate of the regression coefficient for the lagged, trimmed analysis from Cheng et al. (2006).

Incremental risk level	Incremental fat concentration (from Table 5-4 of USEPA 2010), ng/kg lipid	Background + incremental fat concentration, ng/kg lipid	Intake dose from Appendix C.4 of USEPA 2010, lifetime model, ng/kg-d	Incremental intake risk-specific dose, ^b ng/kg-d	Slope factor, ^c (mg/kg-d) ⁻¹
0	0	3 ^a	2.31E-04	NA	NA
1.00E-07	0.0034	3.0034	2.31E-04	3.40E-07	2.94E+05
1.00E-06	0.034	3.034	2.34E-04	3.40E-06	2.94E+05
1.00E-05	0.34	3.34	2.67E-04	3.55E-05	2.82E+05
1.00E-04	3.4	6.4	6.27E-04	3.96E-04	2.53E+05
1.00E-03	34	37	8.09E-03	7.86E-03	1.27E+05

^a Effective lower bound of background serum lipid concentration including impact of non-TCDD TEQ on CYP1A2 induction and TCDD elimination.

^b Difference between intake at background+incremental fat concentration and intake associated with background only.

^c Calculated as SF=RL/RSD

Table 4: Estimation of incremental risk-specific doses and corresponding cancer slope factors assuming a background TCDD concentration of 30 ppt in serum lipid and fat (accounts for TCDD and a contribution to CYP induction from non-TCDD TEQ). Incremental fat concentrations in the second column are those estimated by USEPA using the central tendency estimate of the regression coefficient for the lagged, trimmed analysis from Cheng et al. (2006).

Incremental risk level	Incremental fat concentration (from Table 5-4 of USEPA 2010), ng/kg lipid	Background + incremental fat concentration, ng/kg lipid	Intake dose from Appendix C.4 of USEPA 2010, lifetime model, ng/kg-d	Incremental Intake risk-specific dose, ^b ng/kg-d	Slope factor, ^c (mg/kg-d) ⁻¹
0	0	30 ^a	5.75E-03	NA	NA
1.00E-07	0.0034	30.0034	5.75E-03	9.00E-07	1.11E+05
1.00E-06	0.034	30.034	5.76E-03	9.00E-06	1.11E+05
1.00E-05	0.34	30.34	5.84E-03	9.00E-05	1.11E+05
1.00E-04	3.4	33.4	6.75E-03	1.00E-03	1.00E+05
1.00E-03	34	64	1.82E-02	1.25E-02	8.03E+04

^a Effective upper bound of background serum lipid concentration including impact of non-TCDD TEQ on CYP1A2 induction and TCDD elimination.

^b Difference between intake at background+incremental fat concentration and intake associated with background only.

^c Calculated as SF=RL/RSD

Conclusions

As discussed above and in comments from other authors in this package, the National Academy of Sciences expert panel clearly recommended a non-linear assessment of cancer dose-response for TCDD based on mode of action considerations as the scientifically justified approach to cancer risk assessment for dioxin. Reliance on a linear approach for derivation of a cancer slope factor is a policy decision that ignores a large body of evidence regarding mode of action of TCDD. However, if USEPA decides to pursue derivation of a cancer slope factor based on the results of the Cheng et al. (2006) study, the following key issues should be addressed:

- The selection of the Cheng et al. (2006) regression coefficient from the trimmed analysis, which omits the 5% of exposure records with the highest estimated exposures, results in a slope factor nearly 200 times greater than that obtained from the untrimmed data set. Thus, this regression coefficient is already an upper bound on the exposure-response relationship that is consistent with this data set. The best estimate of this regression coefficient, not a statistical upper bound on this coefficient, should be used as the basis for cancer risk estimates from Cheng et al. (2006).
- The Emond et al. (2005) PBPK model should be used to estimate incremental intake doses resulting in incremental fat concentrations at body concentrations consistent with current background tissue concentrations, and this assessment should include accounting for the impact of non-TCDD TEQ concentrations present at background on TCDD elimination behavior. Use of the model to estimate intake doses associated with vanishingly small absolute TCDD concentrations is a) inconsistent with practical application in risk assessment (which seeks to assess risks associated with potential incremental doses of dioxins from a given source); and b) requires use of the model in a concentration range that results in increasingly extreme predicted elimination half lives and which is well outside the range of validation of that model.

When these issues are addressed and the approach outlined here is used, cancer slope factors in the range of 80,000 to 290,000 (mg/kg-d)⁻¹ are derived. This range of cancer slope factors is appropriate for recommendation for use by risk managers (in conjunction with quantitative criteria derived from a non-linear, mode-of-action analysis, as recommended by the NAS) for

assessing incremental risks associated with potential exposures to dioxins in environmental media, foods, and in other real-world contexts.

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