Intended for

Denka Performance Elastomer LLC, Request for Correction

Exhibit A

Date

July 15, 2021

SUPPLEMENTAL MATERIALS G
RESPONSES TO PEER REVIEWER COMMENTS

Ramboll's Response to External Peer Review Tier 1 and Tier 2 Comments/ Recommendations[1]

Ramboll scientists have reviewed the Post-Meeting Peer Review Summary Report entitled "External Peer Review of a Report on Physiologically Based Pharmacokinetic (PBPK) Modeling for Chloroprene (Ramboll 2020) and a Supplemental Analysis of Metabolite Clearance (USEPA 2020)" and dated December 8, 2020. These Supplemental Materials list the Tier 1 Key Recommendations and Tier 2 Suggestions from the reviewers and provide Ramboll's responses, along with a description of the associated revisions to the original PBPK model documentation.

> Tier 1: Key Recommendations – Recommendations that are necessary for strengthening the scientific basis for the PBPK model, reducing model uncertainties (especially with respect to typical expectations for a PBPK model) or accurately evaluating such uncertainties before the model is applied for risk assessment.
>
> Tier 2: Suggestions – Recommendations that are encouraged in order to strengthen confidence before the PBPK model is potentially applied in risk assessment. It is understood that other factors (e.g. timeliness) may also be considered before deciding to conduct the suggested additional research or model revisions.

The responses are organized by general topic, with the question from the charge to the peer reviewers noted with each comment. All Tier 1 and Tier 2 comments have been addressed and resolved. We appreciate the reviewers' comments and believe that the additional analyses we have performed at their request have increased the strength of the revised PBPK model.

COMMENTS AND RESPONSES RELATED TO KGL

Tier 1

Annie Lumen (Question 1.b.): Specifically, to evaluate the degree of under-estimation of metabolic parameters from the current estimates, in other words what is the maximum dose-metric value will the lowest possible value of Kgl (0.11 L/hr as stated in Pg. 6 of Supplementary Materials B or whatever the appropriate equivalent is) would yield in each tissue.

Ramboll response*: Table 2 of the Ramboll chloroprene PBPK model report provides a comparison of the dose metrics obtained with the model assuming (1) that there was no diffusion limitation on transport in the metabolism studies, and (2) that there was a transport limitation with Kgl =0.22. The resulting dose metrics only differed by roughly 30%, and the differences were similar across concentrations and in mouse and human. Therefore, the impact of Kgl on risk estimates derived from the model would be negligible.*

In response to the reviewer's recommendation, we have conducted a new sensitivity analysis of the impact of Kgl values ranging from 0.055 to 1000 on the metabolism parameter estimates and the dose metrics obtained with the model. This new analysis, which has been added to the methods and results sections of the Model Documentation, demonstrates that (1) a value of Kgl = 0.22 provides the best fit to the data, (2) the impact of the choice of Kgl on the values estimated for Vmaxc is less than 10% and the effect on estimates of Km is less than a factor of

[1] The comments/recommendations are all direct quotes from the Draft "Post-meeting Peer Review Summary Report" December 8, 2020.

2, (3) the female mouse dose metrics are essentially unaffected by the value of Kgl assumed, while the human dose metric decreases about 30% as Kgl is increased from 0.175 (the lowest value for which the MCMC analysis could converge) to 1000.

Overall, the value of Kgl = 0.22 that was selected for use in the in vitro modeling (Supplemental Materials D) is both scientifically defensible and risk-conservative, based on (1) it was derived from a joint MCMC analysis for Kgl and Km in the male mouse, which was the most informative metabolic data (Supplemental Materials B), (2) it provides the best goodness of fit of the in vitro model to the experimental metabolism data in the human liver (Table 1 of the main report), and (3) lower risk estimates would be obtained using higher values of Kgl. While a value of Kgl=0.175 would provide a higher risk estimate, it did not provide as good a fit to the in vitro data as Kgl = 0.22; in fact, attempting to decrease Kgl any further than 0.175 made it impossible to fit the data at all.

Annie Lumen (Question 3): The authors of the report have cited the range of Km values for similar compounds as supportive reasoning for the choice of this value fixing for Chloroprene. I recommend that since this overall process is to estimate the respective metabolic parameters in each tissues/species including Km perhaps it would be useful to at least understand whether and by how much would this initial choice of fixed Km value impact the final metabolic parameter estimations – perhaps a range of values around the 1.0 µmol/L (below and above) be evaluated to see if the initialization of that value carries any considerable impact.

***Ramboll response**: A new sensitivity analysis of the Kgl value has been conducted in response to the comments from the reviewers (see response to previous comment). The analysis is documented in the methods and results sections of the revised documentation. The results of the new analysis provide support for the value of 0.22 L/h currently used in the model as the most scientifically defensible and conservative estimate.*

Annie Lumen (Question 6): Evaluate the worst-case scenario using Kgl value of 0.11L/h instead of 0.22 L/h given the high correlation to Km

***Ramboll response**: A new sensitivity analysis of the Kgl value has been conducted in response to the comments from the reviewers (see initial response in this section). The analysis, which includes a comparison of Kgl = 0.11 vs 0.22, is documented in the methods and results sections of the revised documentation. The result of the analysis provide support for the value of 0.22 L/h currently used in the model as the most scientifically defensible and conservative estimate. A value of Kgl=0.175 would provide a higher risk estimate, but it did not provide as good a fit to the in vitro data as Kgl = 0.22; attempting to decrease Kgl any further than 0.175 made it impossible to fit the data at all.*

Kan Shao (Question 1.b.): (1) Comparing the estimated results (including confidence intervals) of Vmax and Km before and after introducing the "Kgl" parameter, so that the impact of introduction "Kgl" can be evaluated.

***Ramboll response**: Table 2 of the Ramboll chloroprene PBPK model report provides a comparison of the dose metrics obtained with the model assuming (1) that there was no diffusion limitation on transport in the metabolism studies, and (2) that there was a transport limitation with Kgl =0.22. The resulting dose metrics only differed by roughly 30%, and the differences were similar across concentrations and in mouse and human. Therefore, the impact on risk estimates derived from the model would be negligible.*

A new sensitivity analysis of the Kgl value has been conducted in response to the comments from the reviewers. The analysis is documented in the methods and results sections of the revised documentation. The results of the analysis (see initial response in this section) provide support for the value of 0.22 L/h currently used in the model as the most scientifically defensible and conservative estimate.

(2) Investigating the impact of the specified prior distribution, i.e. log-uniform distribution, on the "Kgl" estimation. The estimated confidence interval shown on Figure B-5 is very narrow, I am wondering if this is related to the specified uninformative prior for these parameters. Additionally, it seems to me that the resulting posterior distribution of "Kgl" is not closely related to the specified lower bound "0.11" used in the prior distribution for "Kgl". Therefore, it is worth to investigate if the "Kgl" estimate is sensitive to its specified prior distribution.

Ramboll response*: A sensitivity analysis of the Kgl value has been conducted in response to this comment. The analysis is documented in methods and results sections of the revised documentation. The results of the analysis (see initial response in this section) provide support for the value of 0.22 L/h currently used in the model as the most scientifically defensible and conservative estimate.*

Kan Shao (Question 3): perform a more detailed analysis (as suggested in my response to Charge Question 1) to understand the possible value range of "Kgl" with uncertainty and sensitivity.

Ramboll response*: See initial response in this section.*

Kan Shao (Question 4): As mentioned earlier, additional analysis results should be presented to better evaluate if introducing the "Kgl" parameter is appropriate, i.e. how the estimates of Vmax and Km changed before and after including "Kgl".

Ramboll response*: See initial response in this section.*

Raymond Yang (Question 1.b.): (1) Dr. Clewell and Team at Ramboll provide more detailed descriptions, to be included in this Report, of the incubation system as well as explaining how 500 rpm stirring was achieved in such a system.

Ramboll response*: It has not been possible for Ramboll to obtain a more detailed description of the incubation system beyond what was provided in the publications of the work. The studies were performed more than 10 years ago, and the original investigators no longer have access to the raw data.*

(2) Dr. Schlosser and Team at the USEPA provide written description of how he and the USEPA colleagues examining the kinetic behavior of the above system and reached their conclusion that the high speed agitation at 500 rpm had not denatured the microsomal enzymes.

Ramboll response*: If the high-speed agitation had denatured the microsomal enzymes, it would be apparent in the time-course and dose-response relationships of the experimental data. In particular, the fact that the data in the liver tissues is well described by a Michaelis-Menten metabolic description is clear evidence that the microsomal enzymes are functioning normally. This response has been added to the documentation on page 10 in the section discussing re-estimation of in vitro metabolism parameters.*

Kenneth M. Portier (Question 3): Resolve the confusion between the bounds on km provided in Table B-1 and the suggested limits in Figure B-3.

Ramboll response*: Figure 3 shows the relationship between Kgl and Km in the range of* $log_{10}(Kgl) = 0.85 - 1$ *(Kgl = 0.14 – 0.10), to demonstrate that above a Kgl value of approximately 0.11, a lower estimate of Kgl is associated with a lower estimate of Km, whereas further reduction in Kgl below a value of 0.11 no longer effects the estimate of Km (because Kgl becomes rate-limiting). Table B-1 displays values of Km that have been estimated for compounds that, like chloroprene, are substrates for CYP2E1, demonstrating that the range of likely values of the Km for chloroprene is in the range from 1 – 7 µM. This range of Kms is well within the region in which Km and Kgl are highly correlated (above a Km of about 0.05, Figure 3). This point has been clarified in Supplemental Materials B.*

Yiliang Zhu (Question 3): (1) Given that the three kinetic parameters Vmax, Km, and Kgl are biologically and statistically dependent, the MCMC analysis must sample data from the joint posterior distribution. This requires specification of the likelihood for the parameters, a prior for each parameter, the joint posterior, and MCMC implementation strategies.

(2) Supp B failed to describe the model log(u) in the likelihood (Supp B, Eq 1) and the joint posterior distribution. The first step to implement MCMC is to specify the likelihood function where log(u) must be explicit with respect to u=(Vmax, Km, and Kgl), and the kinetic model underlying log(u) should be also specified. A log-normal likelihood is reasonable. Re-parameterization of the kinetic parameters may be useful or even necessary to utilize the fact that a normal likelihood in conjunction with appropriate prior (e.g. non-informative) implies normal posterior for the kinetic parameters.

(3) A non-informative prior for each kinetic parameter can be specified if an informative prior is not plausible. However, use of the log-normal distribution as a prior for SD is highly unusual, justifications are needed. Common priors for SD include uniform and inverse gamma (ref: Gelman A. Prior distributions for variance parameters in hierarchical models. Bayesian Analysis (2006) 1, Number 3, pp. 515–533).

(4) MCMC can be implemented most effectively in an iterative fashion as illustrated below:

a) draw posterior k samples from P(σ|data);

b) for fixed σ (e.g. the kth sample), draw k samples from the posterior distribution p (Kgl |σ;data)

c) for fixed σ and Kgl, draw k samples from posterior p(Km | Kgl, σ; data)

d) for fixed σ, Kgl, and Km, draw k samples from posterior p(Vmax | Km, Kgl, σ; data)

e) iterate between steps a)-d)

(5) The iterative approach above ensures a multivariate posterior distribution resembling what Figure B-3 depicts. Consider presentation of the MCMC results in a joint fashion when feasible.

(6) Describe the MCMC sampling process and report results in greater details to ensure transparency and reproducibility.

(7) Describe convergence criteria adopted, including graphic tools such as trace plot.

Ramboll response: *As a point of clarification, Kgl was a fixed parameter in the Ramboll analysis of in vitro Vmax and Km. A value of Kgl=0.22 was derived in two separate ways: (1) from scaling of the mixing rate in the experimental determination of Kgl to the mixing rate in the*

metabolism studies, and (2) from simultaneous estimation of Kgl. Km and Vmax using the data for the male mouse liver, which had the highest rates of metabolism. The Kgl estimated from the male mouse liver was used for the estimation of Vmax and Km in all of the tissue data, on the assumption that the mixing conditions in the vials were the same throughout the studies. Due to the collinearity between Km and Kgl, we conducted an analysis of the data on metabolism in the male mouse liver to simultaneously estimate Vmax, Km and Kgl, using uninformative priors except that (1) the prior for Kgl was bounded from below at 0.11 L/hr, the minimum value that we had previously determined could support the observed rate of metabolism, and (2) the prior for Km was bounded from below at a value of 0.5 µmol/L, a factor of 2 below the lowest value for substrates of CYP2E1 from our review of the literature. Importantly, there was no evidence that the posterior distributions from this analysis were clipped by the use of these lower bounds on the priors. Kgl would be expected to have minimal deviation from vial to vial in the robotic system used in the experiments.

While we did investigate the interaction between Kgl and Vmax (see Supplemental Material B), this was not the basis of the final analysis presented in Ramboll (2020). We will expand on the description of the kinetic model below as it was used to estimate posterior distributions of Vmax and Km in the "Re-estimation of In Vitro Metabolism Parameters" (Supplemental Material B).

The kinetic model is a series of differential equations:

$$d(Aa)/dt = Kgl/PC * Am/Vm - Kgl * Aa/Va - Rloss * Aa/Va$$

$$d(Am)/dt = Kgl * Aa/Va - Kgl/PC * Am/Vm - (Vmax * Am/Vm)/(Km + Am/Vm)$$

where: Aa is the amount of CP in the vial headspace, Va is the volume of the vial headspace, Kgl is the mass transfer rate between air and media, PC is the air:media partition coefficient, Am is the amount of CP in media, Vm is the volume of media, Vmax is the maximal rate of metabolism, Km is the affinity constant for CP metabolism and Rloss is the background loss from the vial headspace. The observed headspace concentrations (obs) are modeled as:

$$log(obs_{t,i}) \sim Normal\ (log(u_t), \sigma^2)$$

where u = Aa/Va which is the predicted headspace concentration at sampling time (t). The differential equations are solved numerically in acslX using a stiff system algorithm. The MCMC routine within the software package implements an adaptive random walk Metropolis Hasting algorithm to draw samples from the joint posterior distribution. Parameters are sampled individually and updated with the exception that all parameters except the one being updated are fixed at their last value (acslX MC Modeler User's Guide, Version 3.1). For the analysis, observed data, Vmax and Km were log transformed. The prior for the model parameters (Vmax and Km) were given broad uniform distributions (-10, 5) and the residue standard deviation σ was given a truncated normal distribution (u=1, sd=1, lb=0.01, ub=100). Convergence was assessed using Gelman-Rubin potential scale reduction factor (PSRF) conditional on an upper threshold value of 1.1 for each parameter and σ^2. Plots for the posterior chains, density and correlation between posterior Vmax and Km for the female mouse liver and lung and the human liver are shown in Figures B-6 through B-10.

Tier 2

Annie Lumen (Question 1): I've not conducted such assays to speak from experience about the validity of treating the air and liquid phases to be always at equilibrium for these class of chemicals. If this has not been confirmed experimentally it might be good to evaluate by making this assumption that the two phases are in equilibrium if and by how much the metabolic parameters would be under-estimated by. Especially, since under-estimation of metabolic capacities could contribute to lesser health-conservative dose metrics.

***Ramboll response**: As noted above for the Tier 1 comment, the sensitivity of the model predictions to Kgl was evaluated during the development of the model and the results were shown in Table 2 of the Ramboll PBPK report, which provides a comparison of the dose metrics obtained with the model assuming (1) that there was no diffusion limitation on transport in the metabolism studies, and (2) that there was a transport limitation with Kgl =0.22. The resulting dose metrics only differed by roughly 30%, and the differences were similar across concentrations and in mouse and human. Therefore, the impact on risk estimates derived from the model would be negligible. In addition, a new sensitivity analysis of the impact of the Kgl value has been conducted in response to the comments from the reviewers. The results are presented in the methods and results sections of the revised documentation. The results of the analysis (see initial response in this section) provide evidence that the value of 0.22 L/h currently used in the model is the most scientifically defensible and conservative estimate from a risk perspective.*

Annie Lumen (Question 3): I wanted to note that for the second approach, I was a little confused when the Kgl value of 0.22 L/h estimated for male mouse liver was stated to be used in the re-analysis of metabolism data for all tissues (Pg. 8 of Supplementary Materials B) but elsewhere in the same document Kgl was said to be fixed at 0.45 L/hr in the MCMC analysis (Figure legends for Figures B-6, B-7,B-8 in Pgs. 11,12,13). During the meeting, it was clarified that 0.45 L/hr was not used in the final metabolic parameter estimations. The implications of this as it relates to Figure B-6, B-7, B-8 and estimated parameters needs to be verified.

***Ramboll response**: The Kgl value used in the final metabolic parameter estimations was 0.22. Supplemental Material B was corrected accordingly.*

Kan Shao (Question 1): I comment on this question only from a perspective of quantitative analysis. Using this simplified assumption may ignore the potential uncertainty and variability in the rate of air:liquid transfer, which should be reasonably characterized. A sensitivity analysis is suggested to justify the validity of the assumption.

***Ramboll response**: As noted above (Annie Lumen comments on Question 1): The sensitivity of the model predictions to Kgl was evaluated during the development of the model and the results were provided in Table 2 of the Ramboll PBPK report, which provides a comparison of the dose metrics obtained with the model assuming (1) that there was no diffusion limitation on transport in the metabolism studies, and (2) that there was a transport limitation with Kgl =0.22. The resulting dose metrics only differed by roughly 30%, and the differences were similar across concentrations and in mouse and human. Therefore, the impact on risk estimates derived from the model would be negligible. In addition, a new sensitivity analysis of the Kgl value has been conducted in response to the comments from the reviewers (see above). The analysis is documented in the methods and results sections of the revised documentation. The results of*

the analysis (see initial response in this section) provide support for the value of 0.22 L/h currently used in the model as the most scientifically defensible and conservative estimate.

Jeffrey Heys (Question 1.b.): Retain the current two compartment model with separate air and liquid phases. If the experiments recommended in response to question 2 demonstrate that mass transfer is nearly instantaneous, then the model can be modified to use a single compartment and an equilibrium assumption.

***Ramboll response**: The current two-compartment model was retained.*

Kenneth M. Portier (Question 3): (1) Perform a literature search to better justify that the mass-transfer coefficient for volatile compounds is likely to be proportional to mixing speeds.

***Ramboll response**: The dependence of mass-transfer on mixing rate is a well-established principle, and the value of Kgl=0.22 was confirmed by simultaneous estimation of Kgl and Km using MCMC analysis with the data for the male mouse, which had the highest rates of metabolism. Further, the sensitivity analysis conducted in response to peer reviewer comments (see above) provides support for the value of 0.22 L/h currently used in the model as the most conservative estimate.*

(2) Perform a sensitivity analysis on the impact of placing bounds on the range of prior distributions as well as modifying the form of the prior distribution. The current assumptions are given in Table B-2. Table B-1 suggests that an informed upper bound for ln(km) is closer to -7 than to 5. What is the impact of assuming a priori that Km ~ Log-Uniform (-10, -7) instead of Log-Uniform (-10,5)? What is the impact of assuming a priori that Kgl ~ Log-Uniform (-4,0) instead of Log-Uniform (-3,0)? It is plausible that the lower bound for Kgl is below exp(-3)=0.05.

***Ramboll response:** In the new analysis conducted to estimate Kgl, the prior for Kgl was bounded from below at 0.11 L/hr, the minimum value that we had previously determined could support the observed rate of metabolism, and the prior for Km was bounded from below at a value of 0.05 µmol/L, a factor of 2 below the lowest value for substrates of CYP2E1 from our review of the literature. Importantly, there was no evidence that the posterior distributions from this analysis were clipped by the use of these bounds on the priors.*

(3) Following the approach by Lampert et al. (2005), perform a sensitivity analysis to determine how specification of the prior distribution of the standard deviation impacts the estimates of Vmax, Km and Kgl in the re-analysis.

***Ramboll response:** As noted previously, Table 2 of the Ramboll chloroprene PBPK model report provides a comparison of the dose metrics obtained with the model assuming (1) that there was no diffusion limitation on transport in the metabolism studies, and (2) that there was a transport limitation with Kgl =0.22. The resulting dose metrics only differed by roughly 30%, and the differences were similar across concentrations and in mouse and human. Therefore, the impact on risk estimates derived from the model would be negligible. However, a new sensitivity analysis of the Kgl value has been conducted in response to reviewer comments. The analysis is documented in the methods and results sections of the revised documentation. The results of the analysis (see initial response in this section) provide support for the value of 0.22 L/h currently used in the model as the most scientifically defensible and conservative estimate.*

Jordan Smith (Question 6): I suggest a sensitivity analysis should be conducted to better identify sensitive parameters (referencing Kgl specifically).

Ramboll response: *See responses above.*

COMMENTS AND RESPONSES RELATED TO A1

Tier 1

Annie Lumen (Question 6): Consider the range values the parameter A1 can take and evaluate its impact as a part of supplemental uncertainty analysis if seen fit. For example, from Table 2 of Lorenz et al. 1984 a range of value for A1 can be derived. Of which the highest bound of A1 value is 0.0083 (0.0013/0.156) which is approximately 6-fold higher than the average value currently proposed to be used.

***Ramboll response**: The value of A1 used in the chloroprene PBPK model is the same value used by USEPA in their IRIS risk assessments for methylene chloride. A multi-faceted analysis of the uncertainty in the estimate of A1 was conducted in response to the peer reviewers' comments, including estimating a 95% confidence interval for the Lorenz et al. (1984) value and conducting a literature search to support estimation of an A1 value for chloroprene based on CYP expression. These additional analyses are documented in the methods and results sections of the revised model documentation and support the current approach for A1 used in the PBPK model.*

Annie Lumen (Question 7): (1) If CYP2E1 is shown to be the only enzyme metabolizing Chloroprene (please gather sufficient evidence from the literature or other sources to verify this), and if 7-ethoxycoumarin is a CYP2E1 specific substrate (please gather sufficient evidence from the literature or other sources to verify this), then assuming that Km and the enzyme catalytic rate are the same (please gather sufficient evidence from the literature or other sources to verify this assumption), and that the in vitro activity translates well in vivo (please gather sufficient evidence from the literature or other sources to verify this assumption), then the ratio of Vmax in human liver and lung for 7-ethoxycoumarin can be used to estimate the proportional enzyme expression levels between the two tissues.

***Ramboll response**: The value of A1 used in the chloroprene PBPK model is the same value used by USEPA in their IRIS risk assessments for methylene chloride. Prior to using this value, we reviewed the literature to determine whether there were more recent data that could be used as an alternative to, or in support of, the A1 value used previously. The only alternative we were able to find at that time was a study on tissue mRNA expression of CYPs (Nishimura et al. 2003), where the ratio of total CYP2E1 plus CYP2F1 mRNA expression in human lung and liver was consistent with the value of A1 used by USEPA for methylene chloride. In further support of the applicability to chloroprene of the human value of A1 based on Lorenz et al. (1984), the value of A1 in the male mouse (0.414) based on data from Lorenz et al. (1984) is very close to the ratio of the in vitro Vmax estimates in the lung and liver of the male mouse in our new analysis (0.56, see Table S-3). In addition, in response to comments from the reviewers, additional analyses of the uncertainty in A1 have been conducted that provide additional support for the current value used in the PBPK model (see description of A1 uncertainty analysis in the methods and results sections of the revised model documentation).*

(2) Each of the verification criteria stated above are of equal ranking in my opinion to confidently use the 7-ethoxycoumarin relative activity to predict chloroprene metabolism in human lung. Appropriate uncertainty analysis can be undertaken if some of the verification criteria are not met. This ratio can then perhaps be used to scale the Vmax for chloroprene oxidative metabolism from human liver to human lung.

***Ramboll response**: See response to previous recommendation. We believe the A1 approach used by USEPA in the IRIS assessment for methylene chloride remains the best approach for estimating human lung metabolism. As explained in our report, the* in vitro *metabolism studies conducted with chloroprene were unable to detect any metabolism in the human lung, as evidenced by the fact that the rate of change in chloroprene concentrations in the human lung metabolism vials was similar to, and in some cases less than, the rate of change of chloroprene concentrations in the control vials.*

Annie Lumen (Question 8): For rats it would be good to confirm that other enzymes such as CYP2F does not contribute to chloroprene metabolism (please gather sufficient evidence from the literature or other sources to verify this) and if found to be the case please evaluate if CYP2E1 substrate is a good choice to estimate relative activity between tissues in that specific species. My only additional comment is that if the parallel approach is agreed to be appropriate for use in estimating human lung metabolism from liver values then it seems reasonable that the same approach will be applied to other metabolically relevant tissues as well. For CYP2E1 the mRNA expression correlates very poorly to its protein expression levels and that protein expression levels are better correlated to CYP2E1 activity than mRNA expression levels (Ohtsuki et al. 2012; Sadler et al. 2016).

***Ramboll response**: CYP2F4 does contribute to the metabolism of chlorinated alkenes in the rat. In general, CYP2E1 and CYP2F have similar substrates. In deriving values of A1 from mRNA expression, we added the expression of CYP2E1 and CYP2F1. Because the CYP enzymes are subject to induction by substrate stabilization (Parkinson 1996), an individual's protein expression varies with exposure to substrates, meaning that the activity in a particular donor tissue would reflect effects from recent exposures to food and drugs. CYP mRNA expression, on the other hand, provides an assessment of the baseline enzyme activity in the individual. Therefore, mRNA expression provides a more reliable value for estimating an average value for a human population from a small number of subjects.*

Parkinson A. 1996. An overview of current cytochrome P450 technology for assessing the safety and efficacy of new materials. Toxicologic Pathology, 24(1): 48-57.

Jochem Louisse (Question 8): Perform a literature study to obtain data on the in vitro conversion of CYP2E1 substrates in human liver and human lung tissue fractions (see as example above butadiene), also including other organs of interest. These data may provide insight into whether the derivation of a factor to scale the Vmax obtained with liver microsomes to a Vmax for lung microsomes (and other tissue fractions) may be feasible. If these analyses provide values for A1 that largely differ, this would indicate that this approach is not valid. If these data are limited to do such a comparative assessment, one should be cautious using the approach. In that case, the recommendation below would become a Tier 1 recommendation. In any case, in order to obtain the most reliable estimation of chloroprene oxidation in the lung, I would highly recommend to perform in vitro biotransformation studies with microsomes and determine the time- and concentration-dependent formation of metabolites (epoxide metabolites), instead of using a substrate depletion approach, to derive kinetic constants for chloroprene oxidation.

***Ramboll response**: We performed the literature search suggested by the reviewer during the development of the chloroprene PBPK model. The only data we were able to find was on tissue mRNA expression of CYPs in the human (Nishimura et al. 2003), where the ratio of total CYP2E1 plus CYP2F1 mRNA expression in human lung and liver was consistent with the value of A1 used*

by USEPA for methylene chloride. Additional literature searching conducted in response to the peer review of the model resulted in the identification of a second publication (Bieche et al. 2007) publication that provided additional data (see discussion of A1 uncertainty analysis in the methods and results sections of the revised model documentation).

In further support of the applicability to chloroprene of the A1 based on Lorenz et al. (1984), the value of A1 in the male mouse (0.414) from Lorenz et al. (1984) is very close to the ratio of the in vitro Vmax estimates in the lung and liver of the male mouse in our new analysis (0.56, see Table S-3).

Tier 2

Jochem Louisse (Question 4): Assess whether information on metabolic conversion of model substrates are available for the microsomal badges that have been used for the in vitro kinetic studies. These data should then be compared with metabolic conversion data of well-characterized batches of human microsomes (e.g. available by suppliers of these materials). This may provide some insight into whether the microsomal badges that have been used in the original studies can be considered representative for the 'average' human.

Ramboll response*: Due to the age of the Lorenz et al. (1984) study, and the inability to access the original data, it is not possible to obtain such information.*

Kenneth M. Portier (Question 4): Provide an estimate of the standard deviation of A1, compute an approximate confidence interval and use this to discuss the likelihood that A1 is close to 1.

Ramboll response*: The requested analysis has been performed and is documented in the methods and results sections of the revised model documentation.*

Kan Shao (Question 4): Regarding the pool sizes for the human microsomes, the estimate results presented in Supplemental Materials A and B demonstrate that the pool sizes are reasonably sufficient to generate adequate parameter estimates with confidence intervals. However, sensitivity analysis on the prior distribution is highly recommended which will be very useful to determine whether the relatively small confidence intervals are resulted from narrow priors or sufficient sample sizes.

Ramboll response*: To be clear, the human liver microsome data are based on a single purchased pool, and are not data that were pooled after incubations with microsomes from different subjects. The pool size for the human liver microsomes was presumably based on the available pool from the vendor at the time of the study. The pooled microsomes would have undergone multiple quality control tests conducted by the vendor to ensure the pool provided reasonable levels of metabolism based on a battery of standard compounds (i.e. EROD and PROD substrates). It is unclear why the reviewer appears to believe the prior distributions may have been narrow. The prior distributions for Vmax and Km in the in vitro analysis covered 15 orders of magnitude on the log of the parameter (-10, 5). This is a highly uninformative prior chosen to allow the data to predominate in the analysis. The narrowness of the posterior confidence intervals is due to the consistency of the pooled human liver incubation data across concentrations and times. We agree that the prior, if too narrow, can influence the posterior distribution; however, that is not the case in this analysis. The reviewer could be noting the prior distribution of Kg in Table B-2 (-2.996-0). The prior for Kg was only used in initial examination of the transport limitation in the male mouse liver and a semi-informative prior for Kg was*

necessary to allow the simulation to converge, as there is a very strong correlation between Kg and Km. For the MCMC analysis of the in vitro incubation data presented in Supp Mat D, Kg was fixed at 0.22 L/hr.

Annie Lumen (Question 7): The validity of using mRNA expression ratio to support enzyme activity ratio was discussed in detail as part of the meeting. Panel members who have expertise in this area provided supporting references that suggests that this could be true for some enzymes but for CYP2E1 the mRNA expression correlates very poorly to its protein expression levels and that protein expression levels are better correlated to CYP2E1 activity than mRNA expression levels (Ohtsuki et al. 2012; Sadler et al. 2016). Based on this information my suggestion is perhaps to not rely on the mRNA expression ratios to support the choice of A1 value. And if possible, other approaches be sought or the associated uncertainties in this value be appropriately evaluated.

Ramboll response*: Because the CYP enzymes are subject to induction by substrate stabilization (Parkinson 1996), an individual's protein expression varies with exposure to substrates, meaning that the activity in a particular donor tissue would reflect effects from recent exposures to food and drugs. CYP mRNA expression, on the other hand, provides an assessment of the baseline enzyme activity in the individual. Therefore, mRNA expression provides a more reliable value for estimating an average value for a human population from a small number of subjects.*

Parkinson A. 1996. An overview of current cytochrome P450 technology for assessing the safety and efficacy of new materials. Toxicologic Pathology, 24(1): 48-57.

Annie Lumen (Question 8): In my preliminary comments I had indicated that a consensus be reached on what is the lowest limit of metabolism below which we accept that no metabolism needs to be described for that particular tissue. During the meeting this was clarified that if by using MCMC analysis Vmax and Km values were found to be identifiable from the low metabolism data then metabolism parameters were derived from that data.

Ramboll response*: No response required.*

COMMENTS AND RESPONSES RELATED TO MODELING OF THE REACTIVE METABOLITES OF CHLOROPRENE

Raymond S.H. Yang ("Post-meeting Thoughts"):

(1) Ramboll should use the kinetic information in Cottrell et al. (2001) and Munter et al. (2003) in their chloroprene PBPK modeling. For instance, capturing as many data points as needed, digitally, from the curves in Figure 9 by software such as Getdata-Graph-Digitizer. After obtaining Vmax and Km from double reciprocal plots for chloroprene for this reaction, PBPK model simulations could be done without the issue of using surrogates. Since Cottrell et al. (2001) and Munter et al. (2003) also did comparisons of microsomal enzyme assays with or without epoxide hydrolase (EH) inhibitor, quantitative information on the detoxication of EH was buried in there somewhere to be uncovered.

(2) Ramboll scientists should effectively use the 1,3-butadiene pharmacokinetic and metabolism data as additional supporting information in the building of a robust and complete PBPK model for chloroprene. (This last sentence should be considered as a Tier 1 recommendation).

(3) Another important note is that the Cottrell et al. (2001) and Munter et al. (2003) studies are microsomal enzyme studies. They did not investigate the role of glutathione S-transferase (GST) which is a cytosolic enzyme in chloroprene metabolism. GST, being a high capacity low affinity enzyme (Andersen et al. 1987), would serve as an important detoxifying enzyme for chloroprene epoxides as indicated by similar data in 1,3-butadiene metabolism studies (Csanady et al. 1992; Kohn and Melnick 2000, 2001). Ramboll scientists should incorporate kinetic information of reactive metabolites detoxication into their PBPK modeling.)

(4) The Cottrell et al. (2001) and Munter et al. (2003) studies also reported the GSH detoxication of reactive metabolites of chloroprene as a body's chemical defense system as outlined in Scheme 2 in Cottrell et al. (2001) and Scheme 1 in Munter et al. (2003). These chemical detoxication processes, as other detoxifying enzymatic processes, would certainly impact on the "dose metric" of the Ramboll/Denka PBPK modeling approach. Ramboll scientists should incorporate such detoxication processes into their PBPK modeling.

(5) Ramboll scientists should compare the simulation results of three PBPK modeling approaches: (i) their present approach modified and refined with consideration of downstream detoxication processes; (ii) PBPK modeling using kinetic constants estimated using data from Cottrell et al. (2001) and Munter et al. (2003) with consideration of downstream detoxication processes; and (iii) PBPK modeling using kinetic constants from 1,3-butadiene data from Csanady et al. (1992) and Kohn and Melnick (2000, 2001) with consideration of downstream detoxication processes.

***Ramboll response**: The five comments from Dr. Yang raise his concerns about the adequacy of the chloroprene PBPK model for assessing appropriate dose measures for carcinogenicity of chloroprene in the mouse lung and extrapolating the dose metric from the mouse to the human. Concerns voiced were (1) the need to make more quantitative use of the Cottrell et al. (2001) and Munter et al. (2003) data on specific chloroprene metabolites to establish kinetic parameters for EH hydrolysis and GSH-conjugation of the epoxides formed by microsomal oxidation; (2) the need to account for knowledge available from butadiene modeling for parametrizing aspects of the chloroprene model; (3) the need to ensure appropriate consideration of the role of GSH conjugation, both enzymatically and non-enzymatically, in the detoxification of reactive intermediates (a concern that covers both comments 3 and 4 from Dr. Yang). Dr. Yang*

suggested comparing the results from the present model with (1) a model using quantitative reaction rates for chloroprene metabolism extracted from Cottrell et al. and Munter et al. and (2) a model using kinetic constants from the PBPK models for butadiene. In response to the concerns raised by Dr. Yang we have added a description of the downstream metabolism of chloroprene to epoxides and other reactive products. The development of the extended PBPK model, and comparison of dose-metric predictions with the current parent chemical model, is described in Supplemental Materials F. The extended model incorporates information derived from the Cottrell and Munter studies and includes a description of the role of glutathione in detoxification, including the impact of glutathione depletion and regeneration. Before summarizing the results of the reactive metabolite modeling, we will directly address two of the key concerns that were raised.

<u>Regarding the extraction of kinetic parameters/information from Munter et al. and Cottrell et al.:</u> There are two questions to consider about parameterizing the CP model to explicitly include oxidation, EH hydrolysis and glutathione conjugation based on these papers. The first question is whether the Cottrell and Munter studies were designed in a manner to allow confident estimation of rate constants for hydrolysis and GSH conjugation, and the second is whether this information, if available, would enhance the ability of the CP model to estimate dose metrics for highly reactive metabolites in the lung.

On the first point, we agree that the two papers provide important qualitative information about pathways of metabolism and the metabolite formed, and we took this information into account in designing our PBPK model for CP. However, for several reasons, these papers were not well-designed to extract quantitative information as suggested by Dr. Yang. First, CP was introduced into the vials by injection in 5 μL acetonitrile (described explicitly in Munter et al. but only inferred by a careful reading of Cottrell et al.) with resultant CP concentrations ranging from 0.01 to 10 mM in Munter et al. and from 1 to 40 mM in Cottrell et al. The estimated end of exposure CP concentration in blood at 80 ppm is only 0.015 mM, well below most of the concentrations examined in these two papers. A second confounder is the use of another potential low-molecular weight CYP2E1 substrate – acetonitrile – as diluent, raising concerns for competitive inhibition of CP metabolism between CP and acetonitrile. Thirdly, when GSH was added, the achieved levels were much below those that occur in tissues, which are from 2 to 6 mM.

Despite these concerns for extracting specific kinetic constants, the papers provided solid information on the pathways of metabolism to the epoxides and expected reactive metabolites – especially the reactive aldehydes and ketones. The Cottrell paper discussed incubations with and without an EH inhibitor and incubations with GSH. In incubations with active EH, neither 1-CEO or 2-CEO were found in the incubations, leading to respectively to diol from 1-CEO and various reactive aldehyde and ketone metabolites from 2-CEO. Using the EH inhibitor, it was found that the 2-CEO epoxide was still very unstable, producing metabolites that quickly reacted with GSH either in the presence or absence of cytosol, i.e. the conjugation reaction does not require soluble glutathione transferases. Cottrell also noted that the reaction of the 2-CEO breakdown product, 1-hydroxy-but-3-ene-2-one with GSH is rapid and likely an effective detoxication pathway. The takeaway from these results was that the dominant pathway – formation of 2-CEO – leads to rapid, nearly quantitative production of GSH-reactive metabolites, observations that supported a dose metric of amount metabolized per unit tissue for this arm of the pathway, which accounts for about 90% of total metabolism (i.e. α in Figure 1 is 0.9) as reported in Himmelstein et al. (2004a).

The remainder of the total oxidation (1-α) is through 1-CEO. The half-life of 1-CEO must be longer than 2-CEO since 1-CEO is found in the airspace over the microsomal incubations (Himmelstein et al. 2004a); however, 1-CEO formation is a relatively minor pathway compared to 2-CEO production. With respect to GSH conjugation of 1-CEO, Munter et al. also report that 1-CEO either does not react with GSH or the reaction is very slow (Munter et al. pages 1294-1295). Thus, there would be no need to consider glutathione transferase reactions in the overall metabolic scheme with either 2-CEO or 1-CEO.

With regard to exposure of the lung tissue to a slightly longer-lived 1-CEO, one interspecies difference could arise based on EH activities. Humans have higher EH activity than does the mouse (Himmelstein et al. 2004a). If a quantitative correction were to be included in the reactive metabolite tissue dose metric in the CP model for longer tissue exposure to 1-CEO, the expected tissue dose would decrease for human compared to mouse, i.e. the present configuration of our risk-assessment estimate of tissue dose is conservative and would produce even lower risk estimates if we included interspecies EH hydrolysis rates to reduce tissue exposure to 1-CEO.

<u>Regarding the use of kinetic constants from the PBPK model for butadiene to describe the kinetics of the chloroprene epoxides:</u> Chloroprene differs substantially from non-chlorinated dienes, such as BD or isoprene, due to the presence of the electron withdrawing chloride atom at the 2-position. As pointed out in Cottrell et al. the chorine atom leads the formation of chloro-aldehydes and ketones in addition to epoxides. These metabolites themselves rapidly react with glutathione and, when formed in sufficient amounts, could lead to GSH depletion and attendant tissue toxicity. These differences in epoxide reactivity have been well documented for ethenes with the studies on ethylene metabolism to ethylene oxide versus kinetic models for vinyl chloride or 1,1-dichloroethylene with metabolism to halogenated epoxides that are unstable and whose reaction products readily react with GSH. The glutathione depletion with these halogenated ethene epoxides plays important roles in their toxicity and similarly are important in the tissue effects of chloroprene noted in in older studies in rats.

The purposes in developing models for CP and BD were quite different: the CP model was designed to understand dose of locally produced reactive metabolites in lung and the BD model was intended to examine the production of epoxides in tissues, primarily liver, and the expected steady-state circulating concentration of the mutagenic mono- and diepoxide arising from subsequent distribution of the epoxides to other tissues. The Kohn and Melnick work, called a privileged-access model and referenced by Dr. Yang, was essentially an extension of an earlier model by Johanson and Filser (1993) that included first-pass clearance of the CYP produced epoxide by EH in a microsomal compartment in the liver. In both cases (privileged access and intrahepatic first-pass), the model structure was designed to enhance epoxide clearance before its appearance in the tissue and its transfer to tissue blood and the general circulation (shown by the inclusion of both EH and CYP2E1 in the reaction scheme from BD to butadiene monoepoxide (BMO) and from BMO to butadiene diepoxide (BDO). These BD epoxides are much more stable than those produced by CP and due to longer half-lives are available for downstream reactions and diffusion into blood. In addition, there was a significantly greater data base on which to parametrize the model – especially the gas uptake studies from Kreiling et al. (1987) and concentrations of circulating epoxides. The CP model depends primarily on the detailed in vitro examinations of CP metabolism from Cottrell et al., Munter et al. and Himmelstein et al.

<u>Key differences in modeling with BD and CP:</u> Modeling systemic exposures to stable metabolites (BMO and BDO for BD) is very different from modeling for transient tissue exposures to reactive

intermediates that do not leave tissues in significant amounts. The metabolism pathways are similar, but the consequences of forming stable versus highly reactive metabolites lead to very different demands on the PBPK model for tissue of formation dose measures –proportion escaping the liver as BMO and BDO for BD - versus total amount metabolized for CP. With BD, the goal was to understand the systemic delivery of epoxides to tissue throughout the body, including blood time course for the epoxides; with CP the goal is more restricted to understand tissue dose in a specific tissue containing CYP2E1 and how possible species differences in CYP2E1 and EH in this tissue might affect local exposures. The Himmelstein et al. paper provided the key data for parameterizing the model and the papers by Munter and Cottrell provided key data for understanding the reactive metabolites from 1-CEO and especially 2-CEO.

<u>Results of reactive metabolite modeling:</u> The extended model of chloroprene metabolism described above was exercised to evaluate three potential dose metrics for the lung toxicity and carcinogenicity of chloroprene: (1) total lung metabolism per gram lung (TMet), the dose metric used in the published PBPK models and previously submitted to the USEPA; (2) average concentration of reactive products of metabolism in the lung (PReact), and (3) average concentration of 1-CEO in the lung (1-CEO).

The first comparison performed was an evaluation of the consistency of the alternative dose metrics with the gene expression dose-response data reported in Thomas et al. (2013). In this study, female mice and rats were exposed to chloroprene by inhalation 6 hours per day, for 5 or 15 days. Mice were exposed at the bioassay concentrations, but the concentration range was extended in the rat to provide similar tissue doses based on predicted total amount of chloroprene metabolized per gram of lung tissue per day from a preliminary version of the PBPK model of Yang et al. (2012). For this comparison, two genomic responses were used: the lowest Benchmark Dose (BMD) for any gene expression change and the lowest BMD for any gene expression change related to regulation of glutathione homeostasis. A successful dose-metric for cross-species extrapolation should predict that cellular responses in the lung begin to occur at similar values of the dose metric.

Consistent with the expectations that drove the experimental design in Thomas et al. (2013), the inhaled concentrations at which there was genomic evidence of cellular stress in the lungs of the rat were much higher than in the mouse. The predicted dose metric values for 1-CEO concentration associated with similar genomic biomarkers of cellular effects were also nearly an order of magnitude higher in the rat than in the mouse. In contrast, the model predicts similar dose metric values for both TMet and PReact in the rat and mouse, consistent with the expectation that cellular responses to chloroprene in the lung would begin to occur at similar levels of cellular stress. The consistency of these two dose metrics with the observed genomic dose-response in the female mouse and female rat, and the inconsistency of the 1-CEO or inhaled CP dose metrics, support the dominant role of reactive product formation in the mode of action for chloroprene.

The second comparison performed was an evaluation of the consistency of the alternative dose metrics with the tumor incidence in the bioassays for the female mouse and rat. While both the TMet and Preact metrics provided a reasonable dose-response relationship with tumor incidence, the 1-CEO metric did not. In fact, using the 1-CEO concentration as the dose metric would predict that the female rat should have had a higher tumor incidence than the female mouse. The inconsistency of the 1-CEO dose metric with the relationships for both toxicity and carcinogenicity between the female mouse and female rat is likely due to the small proportion of total chloroprene metabolism that it represents. At the bioassay concentrations, the predicted concentrations of 1-

CEO are less than 0.4% of the concentrations of reactive products in the female mouse and less than 5% in the rat.

In summary, the results of the reactive product modeling support the use of total metabolism as the most appropriate dose metric for the carcinogenicity of chloroprene and demonstrate that the use of inhaled concentration or epoxide area under the curve is inconsistent with the cross-species relationship of the toxicity and carcinogenicity of chloroprene.

COMMENTS AND RESPONSES RELATED TO OTHER TOPICS

Tier 1

Jochem Louisse (Question 5): (1) Make a clear overview of the kinetic data in a Table that has been used to derive the kinetic constants for chloroprene conversion. This allows a better assessment as to whether the data, with regard to number of replicates, independent experiments, etc., can be considered as being adequate to provide robust data.

Ramboll response*: The section of the documentation entitled "Model Parameters" outlines the sources for the kinetic data used in the modelling. Further, it points the reader to Supplemental Materials A which provides tables comparable to those suggested by the peer reviewer. Additional information is also provided in Supplemental Materials D. In addition to these data, all of the underlying data that have been relied upon and provided as part of the modeling scripts are included with citations along with the model code in Supplemental Materials E. The raw data underlying these modeling scripts are not currently available. The studies were performed more than 10 years ago, and the original investigators no longer have access to the raw data.*

(2) It is not clear to me whether all data have been obtained from one experiment or whether independent studies have been performed. It would have been of help for the analysis to present an overview of the data points that have been obtained in the Himmelstein et al. (2004) and Yang et al. (2012) papers, e.g. in a Table like this:

		Himmelstein et al. (2004), experiment 1	Himmelstein et al. (2004), experiment 2	Yang et al. (2012), experiment 1	Yang et al. (2012), experiment 2
Female mouse	Liver	concentrations technical replicates per concentration time points per replicate			
	Lung				
	Kidney				
Human	Liver				
	Lung				
	Kidney				

Ramboll response*: As discussed in the text, all of the metabolic parameters in both the Yang et al. (2012) paper and the current analysis are based on the in vitro chloroprene metabolism time-course data reported by Himmelstein et al. (2004a) and IISRP-17520-1288 (2009). The key differences between the new analysis and the original Yang et al. (2012) analysis were: (1) the incorporation of an additional parameter in the analysis of the* in vitro *metabolism data (Kgl) to describe the rate of transfer of chloroprene from the headspace to the media in the metabolism studies, (2) the use of updated tissue microsomal protein concentrations for scaling the* in vitro *results to* in vivo *values appropriate for the PBPK model, and (3) the adoption of a previously published approach for estimating the metabolism parameters in the human lung (Andersen et*

al. 1987). The differences in these parameters in the Yang et al. (2012) paper and the current analysis are documented in Table S-3 of Supplemental Materials A.

Jochem Louisse (Question 6): (1) To facilitate assessment of the kinetic data, it would be of help to not only present the data points in graphs but to include these in accompanying tables, allowing better assessment by the reviewers.

Ramboll response*: For Figures 2, 3 and 7, as noted in the text, the data are provided in Supplemental Materials A. In addition, as noted previously, the underlying data relied upon for the model are provided with citations along with the model code in Supplemental Materials E.*

(2) Assess whether in vitro kinetic data of optimization studies are available and include these in the table presenting all in vitro kinetic data. This allows reviewers to assess whether kinetic studies have indeed been performed at optimal conditions.

Ramboll response*: All of the in vitro kinetic data are now listed in Supplemental Materials E, as they were received from the original investigators.*

Annie Lumen (Question 6): Based on the description in Pg. 17 of Ramboll report I calculated the metabolic clearance in the lung to be 0.24 L/h/g of protein (0.052*0.00143*1000/ 0.316) but the report indicates an estimate of metabolic clearance of 0.16 L/h/g of protein. I doubt this glitch is real and could be something that I've missed but I thought I'll bring it up since it caught my eye and I'm fine with being proved wrong.

Ramboll response*: The reviewer is correct. The number in the report must not have been updated with the final MCMC results. This has been corrected in the current documentation. The calculated metabolic clearance should be 0.24 L/hr/kg, which is close to the upper bound estimate of metabolism from Yang et al. (2012) of 0.32 L/hr/g.*

Annie Lumen (Question 10): Approximately, 36% of mouse body weight and 24% of human body weight doesn't seem to be contributing to chemical disposition. This seems to be a rather large fraction of body weight to remain unperfused. Please verify.

Ramboll response*: The bones contribute about 10% to body weight and the gut lumen, hair and integuments contribute another 10% to 15%. The fraction of non-perfused body weight in the chloroprene PBPK model results from the use of the tissue data in Brown et al. (1997).*

Annie Lumen (Question 14): (1) Since blood flow to the liver is identified as the primary determinant of the overall elimination of Chloroprene and no influence of tissue-specific metabolism is noted to affect blood concentrations, the predictive evaluation using blood concentration data should not be weighted to validate the extrapolative performance of IVIVE, its related PBPK parameterization, and subsequent overall predictive performance of tissue-dose metrics (parameters that influence blood concentrations have normalized sensitivity coefficients < 0.5 in influencing lung dosimetric).

Ramboll response*: We agree with the reviewer that the in vivo study does not serve as validation of model predictions for tissue-specific metabolism. The sole purpose of the* in vivo *validation study, which was performed in response to an USEPA concern, was to demonstrate the ability of the model to predict the* in vivo *pharmacokinetics of chloroprene inhalation, since that is the exposure route of concern. The validity of the model predictions of for target tissue (e.g. lung) metabolism rests on the correctness of the IVIVE methodology. This dichotomy of the*

validation process was also the case with the PBPK model for methylene chloride that was used by the USEPA in their IRIS assessments. In that case, the lung metabolism parameters were also based on in vitro *data and the available* in vivo *data was insensitive to their values.*

(2) The average measured blood concentrations between 13ppm and 90ppm are only 3.8-fold and 5.1-fold apart at 3h and 6h respectively (Table S-6). That said, from a health-protective standpoint, an overprediction of blood concentration I suppose in better than an under-prediction. Nevertheless, this (the observed discrepancy in model predictions and observations) is a model uncertainty and needs to be evaluated as such.

Ramboll response*: On the contrary, the model predictions for blood concentrations during and after the 6-hr chloroprene exposures are in good agreement with the data collected in the study. Consistent with the WHO/IPCS (2010) guidance on the use of PBPK modeling in risk assessment, model predictions are generally within roughly a factor of two of the means of the experimental data. It was not necessary to adjust any of the model parameters to provide agreement with the new data.*

I recommend caution be exercised when making predictions of tissue-dosimetric (rate of metabolite production) in other metabolically relevant tissues with only blood concentrations.

Ramboll response*: It's unclear what the reviewer is trying to say, but caution is always a good thing in risk assessment. It is not possible to obtain* in vivo *validation data on the production of a reactive metabolite in a tissue. As in the case of methylene chloride and vinyl chloride, the validity of such model predictions must be based on biological plausibility and correct methodology.*

Kan Shao (Question 6): The comparison results presented in Table S-3 in the Supplemental Material A confuse me. First of all, without showing the confidence interval estimates for these parameters, it is very difficult to judge the magnitude of uncertainties in the estimated results obtained using Yang et al. (2012) approach.

Ramboll response*: The confidence intervals for the Yang et al. analysis are available in the publication. Only the means were included in this appendix, have been added to Table S-3 in Supplemental Materials A as requested.*

Jordan Smith (Question 13): I suggest that male and female physiological parameters are implemented independently to ensure that physiologies of both sexes are adequately considered. ICRP (2002) could serve as a reference for male and female physiologies across various life-stages.

Ramboll response*: Concerns regarding potential sensitive human populations, including the effect of gender, is part of the application of the model for a specific risk assessment application, which USEPA will undertake if they accept the model. The physiological and metabolic structure of the PBPK model provides the necessary framework for conducting such investigations, and appropriate parameters are available in the literature (Clewell et al. 2004, Mallick et al. 2020).*

Clewell HJ, Gentry PR, Covington TR, Sarangapani R, Teeguarden JG. 2004. Evaluation of the potential impact of age- and gender-specific pharmacokinetic differences on tissue dosimetry. Toxicol. Sci. 79:381-393.

Mallick P, Moreau M, Song G, Efremenko AY, Pendse SN, Creek MR, Osimitz TG, Hines RN, Hinderliter P, Clewell HJ, Lake BG, Yoon M. 2020. Development and Application of a Life-Stage

Physiologically-Based Pharmacokinetic (PBPK) Model to the Assessment of Internal Dose of Pyrethroids in Humans. Toxicol Sci. 173(1): 86–99

Tier 2

Annie Lumen (Question 10): I had suggested a proof of concept modeling evaluation for epoxide hydrolase activity using available literature data as an added component to the current model. In the public meeting, when discussing this comment, it was mentioned that 1-CEO might only constitute 4-5% of the total metabolites produced and that other metabolites might be more reactive. Therefore, I'll leave this suggestion here as a lower tiered one for consideration if potential concern for downstream metabolite accumulation is seen to be likely.

Ramboll response*: At the request of the peer reviewers, we have conducted a more extensive analysis of the downstream metabolism of chloroprene (see previous section on COMMENTS AND RESPONSES RELATED TO MODELING OF THE REACTIVE METABOLITES OF CHLOROPRENE). The results of the reactive product modeling support the use of total metabolism as the most appropriate dose metric for the carcinogenicity of chloroprene and demonstrate that the use of inhaled concentration or epoxide area under the curve is inconsistent with the cross-species relationship of the toxicity and carcinogenicity of chloroprene.*

Annie Lumen (Question 11): Based on the current model structure, to understand the plausible mechanistic reasoning, perhaps it might be useful to run a time-dependent sensitivity analysis (particularly between 0-3hrs) to see if any unsuspected change in parameter sensitivities are observed at early timepoints across exposure levels. This could only be useful to generate hypothesis challenging the current assumptions of chloroprene uptake (e.g. need for saturable uptake kinetics at higher exposures for example) but might not provide a solution to the discrepancy directly.

Ramboll response*: We have performed the time-dependent sensitivity analysis suggested by the reviewer on PBPK models of similar chemicals and found that the early times are driven by ventilation and blood flow (Clewell et al. 1994).*

Clewell HJ III, Lee T, Carpenter RL. 1994. Sensitivity of physiologically based pharmacokinetic models to variation in model parameters: methylene chloride. Risk Analysis 14: 521-531.

Annie Lumen (Question 12): The logic of reducing any large discrepancy between ventilation rate and cardiac output and to have them match is reasonable. One recommendation is that since cardiac output value in a mouse model would most likely be a well-studied parameter, a secondary check from a source external to Brown et al. 1997 or the current Ramboll estimates/references could be useful as a confirmation to validate if the selected QCC is reflective of an average cardiac output for mouse models.

Ramboll response*: The value of QCC for the mouse in the chloroprene model (QCC=30), is similar to the mouse value (QCC=28) in the PBPK model of Andersen et al. (1987) that was used by EPA in the IRIS assessment for methylene chloride, and is consistent with the physiology of ventilation and perfusion.* As discussed in *Brown et al. 1997, while the value of cardiac output used in the PBPK model of Andersen et al. (1987) for the rat is in agreement with the experimental measurements reported in Table 22, the value for the mouse is about double the reported values. The higher value of QCC in the mouse was determined by comparisons of PBPK modeling with closed chamber exposure data for a number of chemicals (Gargas et al. 1986).*

Gargas ML, Andersen ME, Clewell HJ. 1986. A physiologically based simulation approach for determining metabolic constants from gas uptake data. Toxicol Appl Pharmacol 86:341 352.

Annie Lumen (Question 13): I did want to note that the blood-to-air partition coefficient, a sensitive parameter determining of chloroprene blood concentration, is determined in humans based on a sample size of 3 healthy male adult volunteers (Himmelstein et al. 2004 PartII). However, the values do seem to be tight and less variable so I'm not sure if there is any room for uncertainties here but changing this parameter considerably changes the model predictions of blood concentrations (as shown by the sensitivity analysis in Pg. 19 of the Ramboll report). It might be worth verifying that there is no room for uncertainties in the blood-to-air partition coefficient.

***Ramboll response**: The relationship of the experimental chloroprene blood-air partitions across species is consistent with results for similar volatile organic compounds, such as methylene chloride, vinyl chloride and trichloroethylene (Gargas et al. 1989).*

Gargas ML, Burgess RJ, Voisard DE, Cason GH, Andersen ME. Partition coefficients of low-molecular-weight volatile chemicals in various liquids and tissues. Toxicol Appl Pharmacol. 1989 Mar 15;98(1):87-99.

Jordan Smith (Question 11): I suggest an uncertainty analysis, such as a Monte Carlo type approach, to quantify model uncertainty. This would allow quantitative evidence to better assess if the model overpredictions are explained by data variability.

***Ramboll response**: An uncertainty analysis has now been conducted using the revised Ramboll PBPK Model, and the results are described in the PBPK Model documentation.*

Clewell HJ III, Campbell JL, Van Landingham C, Franzen A, Yoon M, Dodd DE, Andersen ME, Gentry PR. 2019. Incorporation of in vitro metabolism data and physiologically based pharmacokinetic modeling in a risk assessment for chloroprene. Inhalation Toxicology, 31(13-14): 468-483.

Yiliang Zhu (Question 11): the over-estimation of blood concentrations during and following 6-h exposure of B6C3F1 mice to Chloroprene raises questions regarding its reliability of model prediction and robustness toward key kinetic parameters. A systematic approach to sensitivity analysis involving the parameters of this high dimension would be useful. Sensitivity/robustness should be one criterion for selecting the kinetic parameters for prediction purposes.

***Ramboll response**: Such a sensitivity analysis was performed during the development of the PBPK model and the results are provided in the main report (Figures 8-10).*

Kan Shao (Question 14): A few technical issues undermine the overall quality of the PBPK model, including how to justify the necessity and validity of introducing a new parameter "Kgl" to quantify the air and liquid mass-transfer, the lack of detailed analytic results prevent better evaluating the statistical approaches' ability to characterize uncertainty and variability, etc. Therefore, better addressing and more clearly explaining these issues will certainly improve the quality of the report.

***Ramboll response**: As noted previously, Table 2 of the Ramboll chloroprene PBPK model report provides a comparison of the dose metrics obtained with the model assuming (1) that there was no diffusion limitation on transport in the metabolism studies, and (2) that there was a transport*

limitation with Kgl =0.22. The resulting dose metrics only differed by roughly 30%, and the differences were similar across concentrations and in mouse and human. Therefore, the impact on risk estimates derived from the model would be negligible. However, a new sensitivity analysis of the Kgl value has been conducted in response to this and other comments. The analyses are documented in the methods and results sections of the revised documentation. The results of the analysis (see initial response in the section on Kgl) provide support for the value of 0.22 L/h currently used in the model as the most scientifically defensible and conservative estimate.

COMMENTS AND RESPONSES RELATED TO THE RAMBOLL MCMC ANALYSES

Tier 2

Kan Shao (Question 5): I also would like to point out that the performance of MCMC simulation and consequently the estimation results are closely related to the modeling implementation: (1) using a single level MCMC analysis instead of a hierarchical structure essentially treated the samples from various incubation vials equally and increased the sample size, which may reduce the uncertainty/variability in the posterior sample and facilitate the convergence of posterior sample; (2) the selected prior distributions for the parameters may also have important impact on the resulting estimates. So, my suggestion is to employ additional sensitivity analysis to investigate how the various settings in the MCMC method may impact the results (**Tier 2**).

Ramboll response*: We do not believe this sensitivity analysis would provide significant additional value, since the USEPA is conducting their own MCMC analysis.*

Kan Shao (Question 6): it is likely that the uncertainty quantified in the present analysis may be underestimated. Additionally, it is not clear why the estimated value of Km_liver and the estimated value of Km_lung of the present analysis presented in Table S-3 in the Supplemental Material A are different given they were assumed to be the same (**Tier 2**).

Ramboll response*: The values of Km in liver and lung are for the mouse and rat. The assumption that the Km in the liver and lung were the same was made for the human. As explained in the main report, both CYP2E1 and CYP2F contribute to the metabolism of chloroprene in the mouse and rat, resulting in different estimated Km values in the two tissues. However, in the human, the activity of CYP2F1 is extremely low, so that the metabolic clearance in both liver and lung is dominated by CYP2E1, and the Km of CYP2E1 is the same.*

COMMENTS AND RESPONSES RELATED TO CONDUCTING ADDITIONAL EXPERIMENTAL STUDIES

Tier 1

Jordan Smith (Question 7): If the A1 approach is going to be used to extrapolate extrahepatic metabolism, I recommend that Ramboll experimentally determine which enzymes are responsible for chloroprene metabolism.

***Ramboll response**: As explained in the Ramboll PBPK report, both CYP2E1 and CYP2F contribute to the metabolism of chloroprene in the mouse and rat. However, in the human, the activity of CYP2F1 is extremely low, so that the metabolic clearance in both liver and lung is dominated by CYP2E1. Therefore, further experimentation is unnecessary.*

I also recommend that a substrate marker activity is then selected based on which enzymes are identified. For example, an alternative substrate marker for CYP2E1 may be chlorzoxazone activity, which is commonly used by vendors to assess CYP2E1 activity in commercially available samples.

***Ramboll response**: Although chlorzoxazone has been used to assess CYP2E1 activity in drug evaluations, it is also metabolized by CYP3A4, CYP1A2, CYP2A6, CYP2B6, and CYP2D6 (Shimada et al. 1999), so it would not provide a specific marker for CYP2E1.*

Shimada T, Tsumura F, Yamazaki H. 1999. Prediction of human liver microsomal oxidations of 7-ethoxycoumarin and chlorzoxazone with kinetic parameters of recombinant cytochrome P-450 enzymes. Drug Metabolism and Disposition, 27(11): 1274-1280.

Tier 1/2

Jochem Louisse (Question 5): Perform in vitro studies with well-characterized batches of microsomes for which data on metabolic conversion of model substrates are available (provided by vendor). Include one or more of these substrates in the studies (reference chemicals) to assess whether the system works (quality control). I am actually of the opinion that such quality controls should always be included if one generates data to be used in a regulatory setting. From that perspective, this can also be seen as a Tier 1 recommendation. [I think it is important to have at least data of two independent experiments per type of microsome (so for a certain tissue/species/sex) in order to assess the robustness of the method (the experimental setup)].

***Ramboll response**: The laboratory at which the chloroprene metabolism studies were performed is no longer active and we were unable to find any commercial or academic laboratories that could perform such studies with chloroprene. Metabolism studies with volatile compounds are difficult and time-consuming, and require strong experience, such as the experience of the investigator, Dr. Matthew Himmelstein, who performed the studies used in the Ramboll PBPK model development. Further, as noted by Dr. Paul Schlosser during the peer review, the USEPA is not looking to conduct additional experimentation related to development of this model or its parameters. However, in response to the reviewers' concerns, we have conducted a new sensitivity analysis of the impact of Kgl values ranging from 0.055 to 1000 on the metabolism parameter estimates and the dose metrics obtained with the model. This new analysis, which has been added to the methods and results sections of the Model Documentation, demonstrates that (1) a value of Kgl = 0.22 provides the best fit to the data, (2) the impact of*

the choice of Kgl on the values estimated for Vmaxc is less than 10% and the effect on estimates of Km is less than a factor of 2, (3) the female mouse dose metrics are essentially unaffected by the value of Kgl assumed, while the human dose metric decreases about 30% as Kgl is increased from 0.175 (the lowest value for which the MCMC analysis could converge) to 1000. Overall, the value of Kgl = 0.22 that was selected for use in the in vitro modeling (Supplemental Materials D) is both scientifically defensible and risk-conservative, based on (1) it was derived from a joint MCMC analysis for Kgl and Km in the male mouse, which was the most informative metabolic data (Supplemental Materials B), (2) it provides the best goodness of fit of the in vitro model to the experimental metabolism data in the human liver (Table 1 of the main report), and (3) lower risk estimates would be obtained using higher values of Kgl. While a value of Kgl=0.175 would provide a higher risk estimate, it did not provide as good a fit to the in vitro data as Kgl = 0.22; in fact, attempting to decrease Kgl any further than 0.175 made it impossible to fit the data at all.

Tier 2

Jeffrey Heys (Question 2): (1) In Csanady, Guengerich et al. (1992), they state that equilibrium experiments employed "heat-inactivated microsomes or phosphate buffer." I think that conducting air-liquid equilibrium studies with heat-inactivated microsomes would provide some insight into the magnitude of non-specific binding (it would not provide insight into specific binding).

(2): If the first recommendation is not feasible, it also seems relatively straightforward to conduct equilibrium experiments identical to those shown in supplement B at various mixing rates up to at least 500 rpms to determine the impact of mixing on mass transport.

Ramboll response*: As noted previously, the laboratory at which the chloroprene metabolism studies were performed is no longer active and we were unable to find any commercial or academic laboratories that could perform such studies with chloroprene. Even if such a study could be performed, it would be difficult to assure that the experimental conditions in the new Kgl study were sufficiently similar to those in the original metabolism studies, so the actual value of Kgl in the metabolism studies would still be uncertain. However, in response to the reviewers' concerns, we have conducted a new sensitivity analysis of the impact of Kgl values ranging from 0.055 to 1000 on the metabolism parameter estimates and the dose metrics obtained with the model. This new analysis, which has been added to the methods and results sections of the Model Documentation, demonstrates that (1) a value of Kgl = 0.22 provides the best fit to the data, (2) the impact of the choice of Kgl on the values estimated for Vmaxc is less than 10% and the effect on estimates of Km is less than a factor of 2, (3) the female mouse dose metrics are essentially unaffected by the value of Kgl assumed, while the human dose metric decreases about 30% as Kgl is increased from 0.175 (the lowest value for which the MCMC analysis could converge) to 1000. Overall, the value of Kgl = 0.22 that was selected for use in the in vitro modeling (Supplemental Materials D) is both scientifically defensible and risk-conservative, based on (1) it was derived from a joint MCMC analysis for Kgl and Km in the male mouse, which was the most informative metabolic data (Supplemental Materials B), (2) it provides the best goodness of fit of the in vitro model to the experimental metabolism data in the human liver (Table 1 of the main report), and (3) lower risk estimates would be obtained using higher values of Kgl. While a value of Kgl=0.175 would provide a higher risk estimate, it did not provide as good a fit to the in vitro data as Kgl = 0.22; in fact, attempting to decrease Kgl any further than 0.175 made it impossible to fit the data at all.*

Jordan Smith (Question 2): I recommend that these hypotheses are tested using the *in vitro* metabolism experiment. Phase transfer experiments with inactivated microsomes at various concentrations could be used to measure Kgl at different RPMs. Properly designed experiments could address these hypotheses specifically and definitively.

Ramboll response*: Table 2 of the Ramboll chloroprene PBPK model report provides a comparison of the dose metrics obtained with the model assuming (1) that there was no diffusion limitation on transport in the metabolism studies, and (2) that there was a transport limitation with Kgl =0.22. The resulting dose metrics only differed by roughly 30%, and the differences were similar across concentrations and in mouse and human. Therefore, the impact on risk estimates derived from the model would be negligible.*

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Jordan Smith (Question 3): As recommended in question 2, measuring Kgl with an experiment specifically designed to assess mixing speed and microsome concentration with inactivated microsomes would be a preferred approach.

Ramboll response*: As noted previously, the laboratory at which the chloroprene metabolism studies were performed is no longer active and we were unable to find any commercial or academic laboratories that could perform such studies with chloroprene. Even if such a study could be performed, it would be difficult to assure that the experimental conditions in the new Kgl study were sufficiently similar to those in the original metabolism studies, so the actual value of Kgl in the metabolism studies would still be uncertain. However, in response to the reviewers' concerns, we have conducted a new sensitivity analysis of the impact of Kgl values ranging from 0.055 to 1000 on the metabolism parameter estimates and the dose metrics obtained with the model. This new analysis, which has been added to the methods and results sections of the*

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Kan Shao (Question 3): Therefore, my suggestions to the Ramboll/USEPA team are either (1) conducting a lab experiment to verify the "Kgl" value…

Ramboll response*: As noted previously, the laboratory at which the chloroprene metabolism studies were performed is no longer active and we were unable to find any commercial or academic laboratories that could perform such studies with chloroprene. Even if such a study could be performed, it would be difficult to assure that the experimental conditions in the new Kgl study were sufficiently similar to those in the original metabolism studies, so the actual value of Kgl in the metabolism studies would still be uncertain. However, in response to the reviewers' concerns, we have conducted a new sensitivity analysis of the impact of Kgl values ranging from 0.055 to 1000 on the metabolism parameter estimates and the dose metrics obtained with the model. This new analysis, which has been added to the methods and results sections of the Model Documentation, demonstrates that (1) a value of Kgl = 0.22 provides the best fit to the data, (2) the impact of the choice of Kgl on the values estimated for Vmaxc is less than 10% and the effect on estimates of Km is less than a factor of 2, (3) the female mouse dose metrics are essentially unaffected by the value of Kgl assumed, while the human dose metric decreases about 30% as Kgl is increased from 0.175 (the lowest value for which the MCMC analysis could converge) to 1000, and (4) the highest risk estimate is obtained for Kgl = 0.22. These results support the appropriateness of using a value of 0.22 for Kgl.*

Jordan Smith (Question 7): I recommend that CYP2E1 induction be evaluated in lung tissue, if it is determined that this enzyme is primarily responsible for chloroprene metabolism.

Ramboll response*: The recommended study would be extremely difficult due to the volatility of chloroprene and the difficulty of maintaining lung cell cultures over a sufficient period of time to see induction. Moreover, as discussed in the main report, there was no evidence of metabolic induction in liver microsomes from mice exposed to 90 ppm chloroprene for several weeks.*

Jochem Louisse (Question 10): Perform plasma binding studies to provide insight into whether chloroprene plasma protein binding is limited and whether description of plasma protein binding is indeed not needed in the PBPK model, or whether description of plasma protein binding should be included in the PBPK model.

***Ramboll response**: It is well known that plasma protein binding does not impact the kinetics of volatile organic compounds like chloroprene (Yoon et al. 2012), and it was not considered in the PBPK models used by EPA for similar compounds such as methylene chloride, vinyl chloride and trichloroethylene.*

Yoon, M, Campbell, JL, Andersen, ME, and HJ Clewell. 2012. Quantitative in vitro to in vivo extrapolation of cell-based toxicity assay results. Crit Rev Toxicol. 42(8):633-652.