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Influence of the uncertainty in the validation of PBPK models: A case-study for PFOS and PFOA

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ABSTRACT

Physiologically-based pharmacokinetic (PBPK) models are mathematical representations of the human body aimed at describing the time course distribution of chemicals in human tissues. Since parameterization of PBPK models is based on empirical estimation and experimental data, simulation results may have high degree of uncertainty. As a consequence, the reliability of model validation is highly affected. In this study, the parametric uncertainty associated with PBPK models developed for perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) were analyzed and the different validation approaches were discussed for a case-study in Tarragona County (NE of Spain). Physicochemical parameters and dietary intake of PFOS and PFOA were estimated from previous investigations performed in Tarragona County. A sensitivity analysis (SA) was performed to understand the degree of influence of input parameters on the final outcomes. The uncertainty of the PBPK models' outcome was assessed by propagating the parametric uncertainty using the Latin Hypercube Sampling (LHS) technique. The elimination constants (*Tm* and *Kt*) as well as the *Free fraction* and the *Intake*, were the most influential parameters according to the SA results, being up to 83% for PFOS and 99.9% for PFOA. The validation of the PBPK model, which was performed using different approaches, showed clear discrepancies in the visual validation when compared with the statistical analysis.

KEY WORDS: Physiologically based pharmacokinetic (PBPK) model, perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA), uncertainty analysis, model validation

1. INTRODUCTION

Physiologically-based pharmacokinetic (PBPK) modeling is extensively used nowadays in drug development and human health risk assessment (Chiu et al., 2007; Rowland et al., 2011). PBPK models are aimed at simulating the time course concentration of chemical compounds in the human body (Nestorov, 2007). In PBPK modeling, tissues are considered compartments linked by the blood flow, where the chemical concentrations are described by mathematical equations that can be solved computationally (Thompson and Beard, 2011; Thompson and Beard, 2012). The complexity of the PBPK models depends on the administration, distribution, metabolism and elimination (ADME) processes of the chemicals in the human body. Although the first PBPK model was developed by Teorell (1937), the mathematical resolution of the equations was not achieved until the 1970s (Bischoff et al., 1970; Jones and Rowland-Yeo, 2013). However, the scarcity of pharmacokinetic data as well as the implementation of *in vivo* and *in silico* tools to estimate critical model parameters, have delayed an extensive use of PBPK models (Mumtaz et al., 2012; Rowland et al., 2011).

PBPK modeling involves the use of input parameters generated by experimental assays. Therefore, it owes a certain degree of variability and uncertainty associated with the measurements. Variability is the inter-individual differences in the anatomical and physiological characteristics among individuals (Holzkaemper et al., 2009; Linares et al., 2010), whereas uncertainty give a value of the precision and accuracy of the parametric estimates. Although variability cannot be reduced, a better understanding can be provided by increasing the set of experimental values. This also allows a reduction of the uncertainty (Bois et al., 2010). In the past, a number of approaches have been used to estimate the

variability and uncertainty of PBPK models, such as Monte-Carlo simulation, Fuzzy simulations, and Bayesian Markov chain Monte Carlo (MCMC) (Gueorguieva et al., 2005; Hack, 2006; Sweeney et al., 2001; Woodruff et al., 1992). Moreover, a clear differentiation of uncertainty and variability in modelling has been also conducted (Chiu et al., 2009; Holzkaemper et al., 2009; Huizer et al., 2012; Mörk et al., 2009). The sensitivity of the parameters has been identified as a key aspect in the evaluation of the variability and uncertainty of input parameters. Sensitivity analysis (SA) provides a quantitative assessment of the degree of influence of input parameters on the model results (Loizou and McNally, 2010; McNally et al., 2011).

Validation is the process of evaluation of a model with the reliability and relevance of a particular approach, which is established within its domain of applicability (Chiu et al., 2007; WHO, 2010). Although PBPK models can be validated by using different methods, a single approach has not been yet accepted by regulatory agencies. Uncertainty plays a key role in the validation of the PBPK models. Validity relates to the correctness of the basic structure of a model as well as parameter values. A model can be completely invalid if it is incorrectly structured, even if it happens to give an answer that is close to correct with some set of inputs. Although, in general closeness is the most important criteria and simulations within a factor of 2 of experimental results may be considered as valid (WHO, 2010). The most common approach is the visual comparison of the experimental data with the simulated results (Chiu et al., 2007; Loccisano et al., 2011). However, this approach is highly affected by the structural and parametric uncertainties of the PBPK models. Normally, high parametric uncertainty can increase the range of the model outputs and most of the experimental values (validation data) may fall within the range of simulation output and inferred as valid. As a result the model may be erroneously considered as a

valid. Since the uncertainty associated to PBPK model parameterization is very high (Chiu et al., 2007; Farrar et al., 1989; Nestorov, 2001), the validation of PBPK models without a proper consideration of uncertainty might be invalid.

Taking this into account, this study was aimed at estimating the uncertainty associated to PBPK modeling parameterization, prior to its validation. Parametric uncertainty in this study represents both variability and uncertainty and "uncertainty" has been used as a generic term to whole range uncertainty estimated from existing information. The model for this study comes from a previous study, where PBPK models for perfluorooctane sulfonate acid (PFOS) and perfluorooctanoic acid (PFOA) in humans were developed. and tested for a case-study in Tarragona County (NE of Spain) (Fàbrega et al., 2014). PFOS and PFOA are the two most well-known perfluoroalkyl substances (PFASs), a group of chemical pollutants currently of great environmental concern. Although they are easily absorbed via oral route, they are not metabolized in human body, being poorly eliminated. The mechanism of elimination of PFASs is mainly through urine elimination in the majority of the congeners. The excretion in the feces and the amount that undergo to enterohepatic circulation is negligible in comparison with the urine elimination for most of the congeners (Zhang et al., 2013). However, there is a resorption mechanism from urine to plasma (Andersen et al., 2006; Loccisano et al., 2011; Tan et al., 2008). Moreover, PFASs strongly bind to serum albumin (Chen and Guo, 2009), being only a small fraction (unbound free fraction) available to move to tissues and susceptible to elimination (Bischel et al., 2010; Chen and Guo, 2009; Han et al., 2003).

The objective of the present paper is to assess the uncertainty of a previous PBPK model developed for PFOS and PFOA and to compare the process of visual validation (commonly used in development of many published PBPK models) with the statistical

validation included in this work. In a first step, a SA was done and the most sensitive parameters were identified. Secondly, the uncertainty of the PBPK model was assessed by propagating the parametric uncertainty, using the Latin Hypercube Sampling (LHS). Finally, the uncertainty of PBPK models' simulation was statistically validated with experimental values collected from the population living in the case study area under evaluation.

2. MATERIALS AND METHODS

2.1. PBPK model for PFOS and PFOA

In a recent study, we developed a PBPK model to estimate the distribution of PFOS and PFOA within the human body (Fàbrega et al., 2014). The previous model structure, equation and data set of the PBPK model were maintained in the present work. However, the range of parametric uncertainty derived from previous study has been included in order to perform sensitivity analysis and assess the uncertainty of the model. The PBPK model, whose structure is depicted in Fig.1, is based on 10 differentiated compartments: plasma, gut, liver, kidney, filtrate, storage, fat, brain, lungs and rest of the body. The PBPK model was tested for a case-study in Tarragona County (NE of Spain). The intake of PFOS and PFOA was assumed to exclusively occur by the consumption of food and water (Domingo et al., 2012a; Domingo et al., 2012b), while other potential exposure pathways, such as air inhalation or dermal exposure, were considered as negligible. Experimental data regarding PFOS and PFOA in human tissues other than plasma and breast milk are very scarce (Karrman et al., 2010; Maestri et al., 2006; Pérez et al., 2013). For validation purposes, concentrations of PFOS and PFOA in human tissues were obtained in the framework of

previous biological monitoring studies in Tarragona County (Ericson et al., 2007; Pérez et al., 2013). Levels of PFOS and PFOA were analyzed in samples of plasma from 48 donors, with an average age of 40 years old (aged 20-60) (Ericson et al., 2007). Complementarily, the concentrations of the same PFASs were previously assessed in autopsy samples of liver, brain, lung, bone marrow and kidney from 20 individuals, who at the time of their death had been living in Tarragona County for at least 10 years (Pérez et al., 2013). At the time of death, the average age of the individuals was 57 years old, ranging between 28 and 86 years old.The partition coefficients (Pks), defined as the concentration of a chemical in a tissue over its concentration in plasma, were identified as the key parameters of the PBPK model. *Pks* values were estimated by using available data of PFOS and PFOA in plasma and human tissues from the previous biological monitoring studies (Ericson et al., 2007; Pérez et al., 2013). The elimination was based on a resorption mechanism in kidney. Similarly to *Pks*, the elimination constants, maximum resorption (Tm) and affinity constant (Kt), were also calculated according to experimental values in human autopsy tissues (Fàbrega et al., 2014). Tm is the maximum amount of PFOS or PFOA that can be resorbed from urine to plasma, while Kt is obtained when V (Kt) is equal to Vmax/2, in the trend of the reaction, analogous to the Michaelis-Menten curve. Tm and Kt are analogous parameters to the maximum velocity (Vmax) and Michaelis-Menten constant (Km), respectively, in the Michaelis-Menten kinetics. While PFOS and PFOA are strongly bound to plasma albumin, the remaining unbound fraction was called Free fraction, being one of the key parameters in the distribution of PFOS and PFOA in PBPK modelling.

2.2. Case-study

The mean dietary intake of PFOS in adults living in Tarragona County (Spain) was previously estimated in 158.2 ng/day, with minimum and maximum values of 109.9 and 316 ng/day, respectively (Domingo et al., 2012b). With respect to PFOA, the average intake was 327.6 ng/day, ranging between 58.1 and 1330 ng/day. In addition to food intake, the contribution of water consumption, as another potentially important exposure pathway of PFOS and PFOA, was also studied. The concentrations of PFOS and PFOA in samples of drinking water were also determined, being 1.8 and 2.4 ng/L, respectively (Domingo et al., 2012a). Considering a daily consumption of water of 1.23 L day (US EPA, 2011), the mean intake of PFOS for adults living in Tarragona County was estimated in 0.17µg/day (range: 0.11-0.32 µg /day), while that of PFOA was calculated in 0.33 µg/day (range: 0.06-1.33 µg/day) (Domingo et al., 2012a; Domingo et al., 2012b). Due to the lack of lifetime intake data, the constant rate of intake with different uncertainty scenarios was considered along the lifetime of the individual. .

2.3. Sensitivity Analysis (SA)

SA of the PBPK model was performed to identify the degree of influence of input parameters on the model outputs (Evans and Eklund, 2001). SA was carried out by using the method of sensitivity index (SI), defined as the absolute value of the difference between the maximum and minimum output values over the maximum output value of the model output (Hamby, 1994):

$$SI = \left| \frac{D\max - D\min}{D\max} \right| \quad (1)$$

The whole range of PBPK model parameters was evaluated in the SA, including body weight (BW), volume of each human tissue, cardiac output to liver, brain, lungs, and kidney, elimination constants (Tm and Kt), Free fraction, and Intake. Moreover, the Pk

value of PFOS and PFOA in gut, liver, kidney, fat, brain, lungs and the rest of the body, were also taken into account. These were previously assessed using experimental data obtained in human tissues (Fàbrega et al., 2014; Maestri et al., 2006). In addition, physiological data of tissue volumes and cardiac output were obtained from Huizer et al. (2012) and Brown et al. (1997). The mean and uncertainty range (minimum – maximum) for the parametric data was assessed using the setof data provided by Fàbrega et al., 2014; Maestri et al., 2006; Huizer., 2012 and Brown et al,. 1997. The values were summarized in Table 1. Data of elimination constants (Tm and Kt) and Free fraction were taken from our previous study (Fàbrega et al., 2014) and a coefficient of variation (CV) of 0.3 (30%) was used for these parameters, according with the CV used in previous studies (Allen et al., 1996; Brochot et al., 2007; Sweeney et al., 2001). The duration of the simulation exposure was the maximum age (90 years) where all the parameters had achieved the convergence. The contribution of each parameter to the total sensitivity was assessed as percentage of individual sensitivity (SI) of the total sensitivity (sum of SIs) of all the parameters. PFOS and PFOA concentration in plasma/blood is used as output variable of interest for the sensitivity analysis.

2.4. Uncertainty analysis

The uncertainty of the PBPK model was assessed for the most contributive parameters, according to the SA results. Uncertainty analysis was performed using Latin Hypercube Sampling (LHS) method. LHS is a stratified sampling methodology used to reduce the number of runs necessary for a Monte Carlo (MC) simulation, thus achieving a distribution with an acceptable number of calculation.(McKay et al., 1979). In the LHS, the range of each variable is divided into N intervals, where N is the number of iterations of the

MC simulation. Then, a random value is obtained for each segment following uniform distributions which are randomly grouped for each MC simulation. The 1000 parametric samples of Tm, Kt, Free fraction and Intake were sampled using LHS method assuming lognormal distribution (Table 2) and used to simulate the concentrations of PFOS and PFOA in human tissues using respective PBPK model. For the given set of parameters, we assumed that there ware no correlation between the values of different uncertain parameters used in the model simulation. The input parameters set from stratified sampling exercise were used to simulate the minimum and maximum concentrations of PFOS and PFOA in the selected human tissues (plasma, liver, brain, lung and kidney). In case of non-convergence simulation, Tm, Kt and Free were recalibrated to get a stable solution. The minimum and maximum simulation bands were used to validate the experimental results by comparing the experimental data with the simulation results obtained through the PBPK model (Chiu et al., 2007).

2.5. Statistical analysis

To assess the statistical validity of the PBPK model for the given case study data, a Student's *t*-test was performed between the simulated results obtained by the PBPK model and the experimental data. This test is used to determine if the means of two populations are equal (Snedecor and Cochran, 1989), according to the following expression:

$$t = \frac{\bar{x} + \bar{y}}{\sqrt{\frac{s_x^{\,2}}{n} + \frac{s_y^{\,2}}{m}}} \qquad (2)$$

where x and y are the means, s_x and s_y are the standard deviations, and "n" and "m" are the size of the samples. When data variances are different, the statistical test under the

null hypothesis has a Student's distribution. Therefore, the sample standard deviation is replaced by the pooled standard deviation, following this equation:

$$s = \sqrt{\frac{(n-1)s_x^{\ 2} + (m-1)s_y^{\ 2}}{n+m-2}} \quad (3)$$

To test the validity of the statistical hypothesis (in this case the model validity for a given case study data), test output as p-value is compared with α-value. The p-value is a probability function derived from the observed sample results that the observed statistic occurred by chance alone. A level of significance, which is called alpha (α), need to be set. Alpha explains how the observed extreme results must be in order to reject the null hypothesis of the *t*-test. Alpha is associated to the confidence level of the *t*-test. Commonly, alpha has a value of 0.05 or 0.01, indicating a level of confidence of 95% or 99%, respectively. . To test the significance, α is compared with the p-value to accept or reject the null hypothesis.

3. RESULTS AND DISCUSSION

3.1 Sensitivity analysis

SA was performed to identify the degree of influence of input parameters on the model outputs. SA outcomes are summarized in Table 1, in which the parameters are ranked according to their sensitivity. For PFOS, the highest values of SA were obtained for cardiac output to kidney (Q. kidney), Free Fraction, affinity constant (Kt), followed by maximum resorption (Tm), Partition Coefficient (Pk) for the rest of the body, and oral intake. For PFOA, the parameter with the highest contribution to the SA was Free fraction,

and Kt followed by oral intake, Tm, Q. kidney, and Pk for the rest of the body. In general terms, cardiac output and volumes of tissues showed the smallest contribution, with the only exception of Q. kidney. The reason of the high sensitivity of Q. kidney might be due to the fact that kidney is the elimination tissue, and it has been reported that Q. kidney is a physiological parameter with a low uncertainty (Huizer et al., 2012). Similarly, Pk of all the individual tissues showed a relatively low sensitivity, with the only exception of the rest of the body. This could be due to the fact that this compartment is a lumped tissue, which receives all the uncertainty of all other tissues (Thompson and Beard, 2012). There is no plausible kinetic mechanism can be found for higher sensitivity of Free fraction and Kt in PFOA than in PFOS. We believe that this may be due to different uncertainty range of data for these four parameters for PFOS and PFOA. Intake was also identified as a sensitive parameter for PFOS and PFOA. The study of the uncertainty was focused on the most sensitive parameters (or the parameters with the highest contribution to the model output), namely elimination constants (Tm and Kt), Free fraction, as well as Intake. Though Q.kidney is one of the most sensitive parameter for PFOS and PFOA, it most was not considered for general uncertainty analysis as it is linked to particular organ and may influence the results only for particular organ. For PFOS, around 83% of the uncertainty comes from these four parameters. Regarding PFOA, the percentage of SA contribution of these four parameters was 99.9%.

3.2. Uncertainty assessment of parametric data

The mean, minimum and maximum concentrations of PFOS and PFOA in plasma, liver, brain, lung and kidney were simulated and compared with experimental data (Fig. 2-6). The parameters under study were selected from the SA outcomes with higher SI score

and representing the influential input parameters with more than 80% contribution to output uncertainty. These parameters were elimination constants (*Tm* and *Kt*), Free fraction and Intake. In many cases, the result was a set of non-converging simulations. For those set of unconverged simulation, recalibrations of Tm, Kt, Free Fraction and intake values were performed to get a stable solution. These multi-parameters calibrations were performed in the reported uncertainty range of these parameters. This process of calibration may underestimate the final value for the true range of uncertainty. However, it allows obtaining simulations that converges (more stable) with close approximation to the true range of uncertainty.

The behaviour of the elimination parameters, Tm and Kt, are analogous to the maximum velocity of reaction (Vmax) and the Michaelis-Menten constant (Km), respectively, in the Michaelis-Menten reactions (Kou et al., 2005). Tm is the maximum amount of PFOS or PFOA that can be resorbed from urine to plasma, while Kt is obtained when V (Kt) is equal to Vmax/2, in the trend of the reaction, analogous to the Michaelis-Menten curve. When Tm increases, the final concentration values in tissues also increase. In turn, the concentration in tissues decreases when Kt increases. Free Fraction (or unbound chemical) is available for passive diffusion to extravascular or tissue sites where the toxicological effects of the chemical take place. Therefore, the *Free Fraction* typically determines chemical concentration at the active site and, thus, efficacy. On the other hand, when the intake is high, the amount of bound chemical approaches an upper limit determined by the number of available binding sites. For a highly tissue-bound chemical, a higher intake results in a higher concentration in human.

The degree of influence of *Pks* on the tissue concentrations was very high. A number of mathematical algorithms were used to provide a good estimation of *Pks* for

different chemicals. Unfortunately, the amphiphilic structure of PFOS and PFOA makes it difficult to predict the Pks, because the algorithms are not optimized for amphiphilic substances(Peyret et al., 2010). This left us with the use of experimental data as the only way to estimate the *Pks* for PFOS and PFOA. The *Pks* value used in this study were previously calculated by dividing the concentrations in each tissue by the level in plasma (Fàbrega et al., 2014; Maestri et al., 2006). Simulated concentrations found in some tissues were far from experimental values. For instance, mean PFOS concentration estimated in lung and kidney tissue was much lower than that found experimentally. It demonstrated that these *Pks* should be better estimated by an improved mathematical algorithm and more experimental data. Considering the high sensitivity of *Pks*, and the relatively good fitting between simulation and experimental results in plasma, it can be safely assumed that one of the main factors responsible for the relatively poor results obtained in tissues, other than plasma, were the *Pks*, being added to the small contribution of other parametric uncertainties.

3.3. Model validation

3.3.1 Visual validation

The most common process of model validation in the PBPK models is the visual comparison of the simulation results with experimental values (Chiu et al., 2007). The simulation of the mean, minimum and maximum values was conducted in plasma, liver, brain, lung and kidney for PFOS and PFOA, and compared with the experimental values found in Tarragona Country (Ericson et al., 2007; Pérez et al., 2013). The comparison of experimental and simulation data are depicted in Fig. 2-4. The simulations were run for 90

335 years, being this the maximum age among all the evaluated subjects. All the simulation
336 trends followed a hyperbolic profile, similar to the Michaelis-Menten saturation curve (Kou
337 et al., 2005). It reaches convergence when the age of the population is between 10 and 60
338 years old. Simulation for the minimum concentration range converges earlier (close to 10
339 years old), whereas the maximum concentration range convergence is at an older age (close
340 to 60). Most modelled results were within the range of experimental data. The differences
341 between the minimum and maximum values of the experimental results were statistically
342 significant ($p<0.05$). The minimum and maximum concentrations levels in the steady state
343 for PFOS ranged 0.03-150, 0.002-14.32, 0.001-8.7 and 0.01-84.57 ng/g w.w. and for PFOA
344 ranged 0.0005-47, 0.0001-7.74, 0.0006-57.84 and 0.0005-64 ng/g w.w. in liver, brain, lung
345 and kidney, respectively. A visual validation was established considering that the
346 experimental values should be between the minimum and the maximum values found in the
347 simulations. In plasma, all the experimental results were between the maximum and
348 minimum estimated levels of PFOS and PFOA (Fig. 2), and most of them being close to the
349 mean. With respect to PFOA, the distribution of the experimental plasma concentrations
350 was more aligned to the mean than PFOS, for which a higher dispersion was noted. Results
351 for the other tissue compartments are shown in Fig. 3-4. In case of PFOS, results are varied
352 with liver, brain and kidney are showing good agreement with experimental values,
353 whereas in lung, most experimental samples are out of the simulated range (Fig. 3). In case
354 of PFOA, the experimental concentrations for the whole set of samples were in the
355 uncertainty range of simulated levels except lung (Fig. 4). In both cases, lung has shown
356 poor agreement with experimental data. Overall, visual inspections confirm that simulations
357 results are in accordance with experimental data, validating the PBPK model. Moreover,

PFOA results were slightly better than the estimations for PFOS, because most of the experimental results are within the range of the PBPK simulations.

A second visual validation was performed by depicting the results in box plots (Fig. 5-6). A box plot is a tool to graphically depict a set of numerical data using their quartiles. Box plots in Fig.5-6 are constructed using a box where the lower and upper ends of the box represents the first and third quartile (Q1 and Q3), and the vertical line within the box represent the median. Moreover, two horizontal lines are constructed in the ends of the box that represent the minimum and maximum values. In plasma, experimental data of both PFASs were grouped in two clusters, according to the age of the subjects: 1) population from 20 to 30 years old, with an average age of 25 years, and 2) population from 50 to 60 years old, aging 54 years old as a mean. An important number of outliers were found in the simulation box plots of plasma. However, the simulated and experimental box plots were very coincident in the ranges. In the other tissues, the box plots showed, in general terms, a larger dispersion of the results when compared to simulation results. This spread higher in lungs for both compounds, as well as in kidney for PFOS. The analysis of the box plots were in agreement with the above-shown simulation trends, however it reveals some more statistical facts than previous visual validation.

3.3.2. Statistical validation

To assure the statistical validity of the final results, the Student's *t*-test was performed between steady state simulated results and experimental data. Results for plasma are summarized in Table 3, while those corresponding to other human tissues are shown in Table 4. Two levels of statistical significance (α=1% and α=5%) were established. In

plasma, two subpopulation groups were differentiated according to the mean age, either 25 or 54 years old. The *p* values were 0.69 and 0.86 for PFOS and PFOA, respectively, assuming an equal variance. Therefore, there were no significant differences between the experimental and the simulated results. Similarly, if an unequal variance is assumed, *p* values ranged from 0.53 to 0.73 for PFOS and PFOA, respectively, showing a lack of significant differences between the experimental data and the simulation results. In other tissues, the validity of the model was dependant on the level of significance. For α=1%, significant differences for PFOS were found in lung and kidney, as well as in brain and lung for PFOA. For α=5%, significant differences were observed in liver, lung and kidney for PFOS, and in liver, brain and lung, for PFOA. In contrast with the visual validation, it seems that the PBPK model was only validated for the simulation of PFASs in plasma, while varied degrees of disagreement were registered for data in other tissues. The results of the model validation, either visual or statistical, differed notably. It must be highlighted that, in a visual validation, most of the simulation results are considered valid when most experimental values fall in the range of simulation results. In contrast, in the statistical analysis that avoids human biases in judgment, the results may significantly differ in many tissue compartments and put the PBPK model validation in question. Although visual analysis is the predominant method in PBPK modelling, statistical analysis should be incorporated to avoid biases in the visual validation.

4. CONCLUSIONS

The parametric uncertainty and the statistical validation of a specific PBPK model were studied and applied to a case-study, where the body burdens of PFOS and PFOA in

the adult population of Tarragona County (Spain) were estimated. The elimination constants (*Tm* and *Kt*) as well as the *Free fraction* and the *Intake,* were the most influential parameters according to the SA results, being up to 83% for PFOS and 99.9% for PFOA. The validation of the PBPK model, which was performed using different approaches, showed clear discrepancies in the visual validation when compared with the statistical analysis. According to the visual validation, the concentrations of both, PFOS and PFOA, fell within the range of the model simulations, resulting in the PBPK model being considered valid. In turn, according to the Student's *t*-test, the model was not validated for PFOS in lungs and kidney, as well as for PFOA in brain and lungs. It clearly indicates that the statistical analysis is an important step in the validation process of PBPK modelling to avoid potential biases. The study of the uncertainty depends on the parametric data and the structure of the model, becoming then model-dependent. Therefore, the conclusions cannot be extrapolated to all the PBPK models. However, our findings may help PBPK modelers to know the most uncertain parameters and the influence of the uncertainty in their models. Further, greater confidence in uncertainty and variability analyses will result if the parameter distributions, correlations among parameters, etc., are based on the best available biological understanding of the systems. Therefore, the real uncertainty associated to the PBPK parameters may be underestimated. There are enormous possibilities of improvement. However, the present study has presented discrepancies in our scientific reporting of model validation, putting forward a strong argument to consider the uncertainty in the PBPK modelling and need of statistical analysis to be incorporated or substituted for the predominant visual validation in the process of PBPK model validation.

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539 **Table 1.** Mean, range and sensitivity of the parameters used in the PBPK model

PFOS				PFOA			
Parameter	Mean	Range	Sensitivity	Parameter	Mean	Range	Sensitivity
Free	0.03	9.45E-7-7.0E-2	0.999	Free	0.03	9.46E-7-7.0E-2	0.999
Kt	0.018	3.30e-7-5.0E-5	0.999	Kt	0.116	1.12E-4-3.0E-2	0.998
Q. kidney	0.17	0.118-0.265	0.999	Intake	0.331	0.061-1.33	0.952
Tm	3.50	0.617-17.2	0.957	Tm	6	1.46-20.9	0.929
Pk Rest body	0.2	0.0007-17.9	0.781	Q. kidney	0.177	0.118-0.265	0.801
Intake	0.161	0.109-0.316	0.624	Pk Rest body	0.12	0.0002-40.2	0.664
Pk fat	0.033	0.004-15.0	0.438	BW	71.4	64.7-79.1	0.139
BW	71.4	64.7-79.1	0.136	Pk fat	0.467	0.008-16.2	0.055
Pk gut	0.57	0.013-17.7	0.073	Pk gut	0.05	9.97E-6-33.7	0.025
Pk brain	0.255	0.002-16.8	0.017	Pk liver	1.03	0.077-14.1	0.002
Pk lung	0.155	0.0003-24.8	0.017	Pk brain	0.17	0.0005-20.8	0.0008
Pk liver	2.67	0.494-13.6	0.007	Pk kidney	1.17	0.096-11.5	0.0006
Pk kidney	1.26	0.105-14.7	0.003	Volume plasma	0.04	0.026-0.059	0.0004
Volume brain	0.021	0.014-0.032	0.001	Volume lung	0.014	0.009-0.021	0.0003
Q. brain	0.117	0.078-0.176	0.0008	Volume liver	0.023	0.015-0.034	0.0003
Volume liver	0.023	0.015-0.034	0.0007	Pk lung	1.27	0.105-13.723	0.0002
Q. liver	0.189	0.126-0.283	0.0005	Q. liver	0.189	0.126-0.283	0.0002
Q. lung	0.034	0.023-0.051	0.0002	Volume kidney	0.004	0.003-0.006	0.0002
Volume plasma	0.04	0.026-0.059	0.0002	Q. brain	0.117	0.078-0.176	0.0002
Volume kidney	0.004	0.003-0.006	0.0001	Q. lung	0.034	0.023-0.051	0.0002
Volume lung	0.014	0.009-0.021	0.0001	Volume brain	0.021	0.014-0.032	0.0001

540 BW: Body weight (kg); Tm: Resorption maximum (µg/h); Kt: Affinity constant (µg/L); Free: free fraction (unitless); Pk: partition coefficient (unitless); Intake
541 (µg/day), Q: cardiac output. Cardiac output to tissues and tissue volumes are given according to the fraction of total cardiac output and total volume.

542 **Table 2.** Parametric Distribution data for PFOS and PFOA used in the PBPK model for uncertainty
543 assessment.

Parameters	Distribution	PFOS		PFOA	
		Mean	Std.	Mean	Std.
Tm[a]	Lognormal	3.5	1.03	6	1.34
Kt[a]	Lognormal	0.02	0.08	0.12	0.20
Free[a]	Lognormal	0.03	0.09	0.03	0.09
Intake[b]	Lognormal	0.16	0.11	0.33	0.67

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545 Tm: Maximum Resorption (µg/h); Kt: Affinity constant (µg/L); Free: Unbound Free Fraction (unitless);
546 Intake (µg/day); Std: Standard Deviation; [a] based on assumption of CV=0.3 from previous studies (Allen et
547 al., 1996; Brochot et al., 2007; Sweeney et al., 2001); [b] Intake of PFOS and PFOA was based on previous
548 study of Domingo et al., (2012a) & Domingo et al., (2012b)
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572 **Table 3**: Two sample *t*- test of simulated and experimental concentrations of PFOS and PFOA in
573 plasma for different age subpopulation groups, assuming equal and unequal variances.

		Equal variances			Unequal variances		
	Mean age	$H_{\alpha = 1\%}$	$H_{\alpha = 5\%}$	P	$H_{\alpha = 1\%}$	$H_{\alpha = 5\%}$	P
PFOS	25 years	0	0	0.69	0	0	0.61
	54 years	0	0	0.69	0	0	0.53
PFOA	25 years	0	0	0.86	0	0	0.72
	54 years	0	0	0.85	0	0	0.73

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598 **Table 4**: Two sample *t*- test of simulated and experimental concentration of PFOS and PFOA.

	PFOS			PFOA		
Tissue	$H_{\alpha = 1\%}$	$H_{\alpha = 5\%}$	P	$H_{\alpha = 1\%}$	$H_{\alpha = 5\%}$	P
Liver	0	1	0.027	0	1	0.05
Brain	0	0	0.34	1	1	1.56E-15
Lung	1	1	5.42E-07	1	1	0.003
Kidney	1	1	5.23E-04	0	0	0.162

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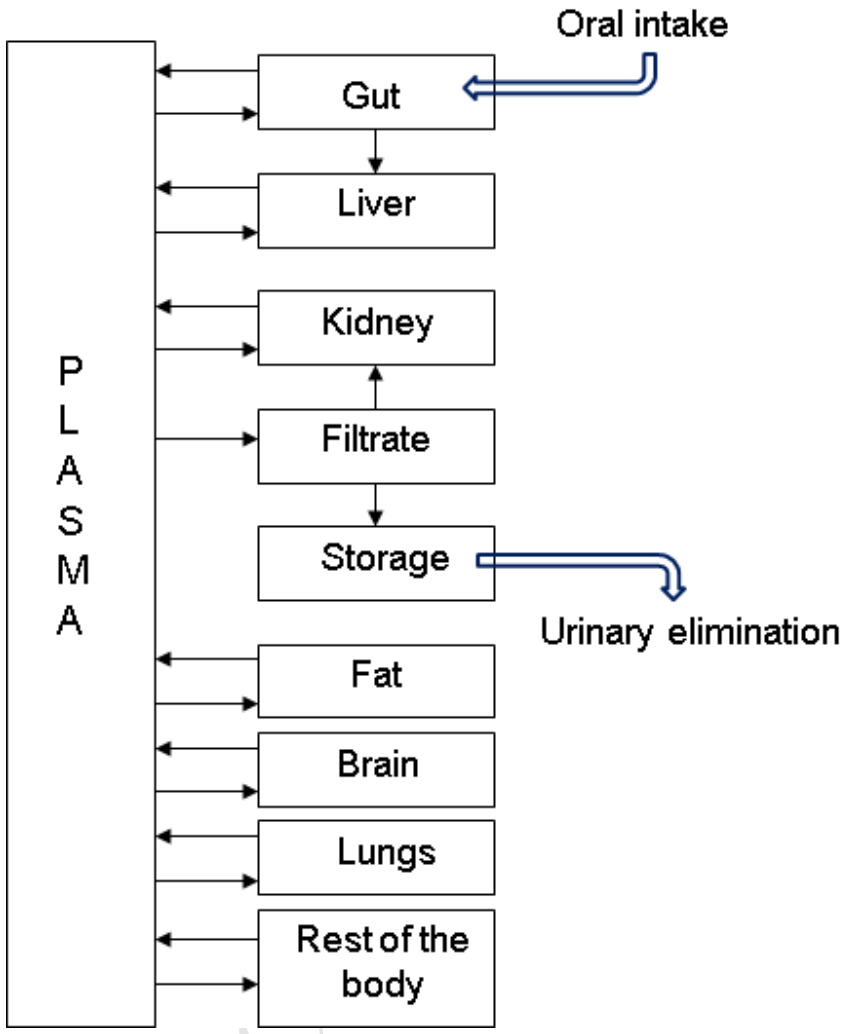
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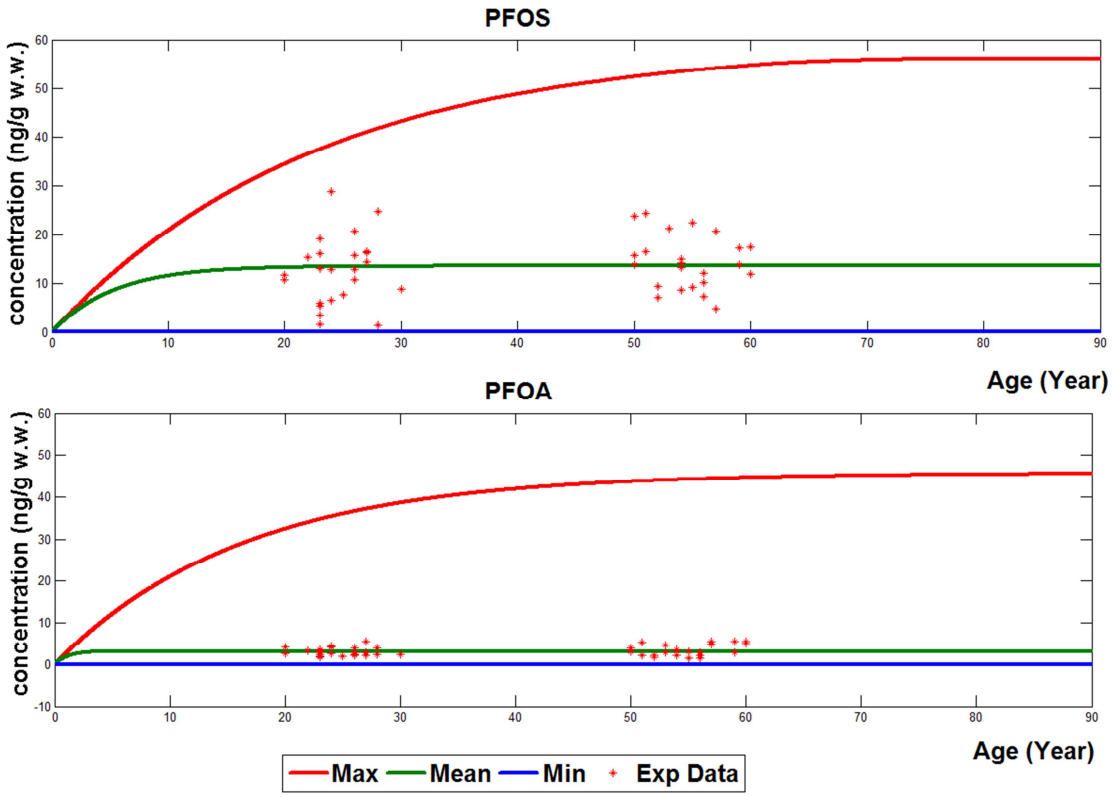

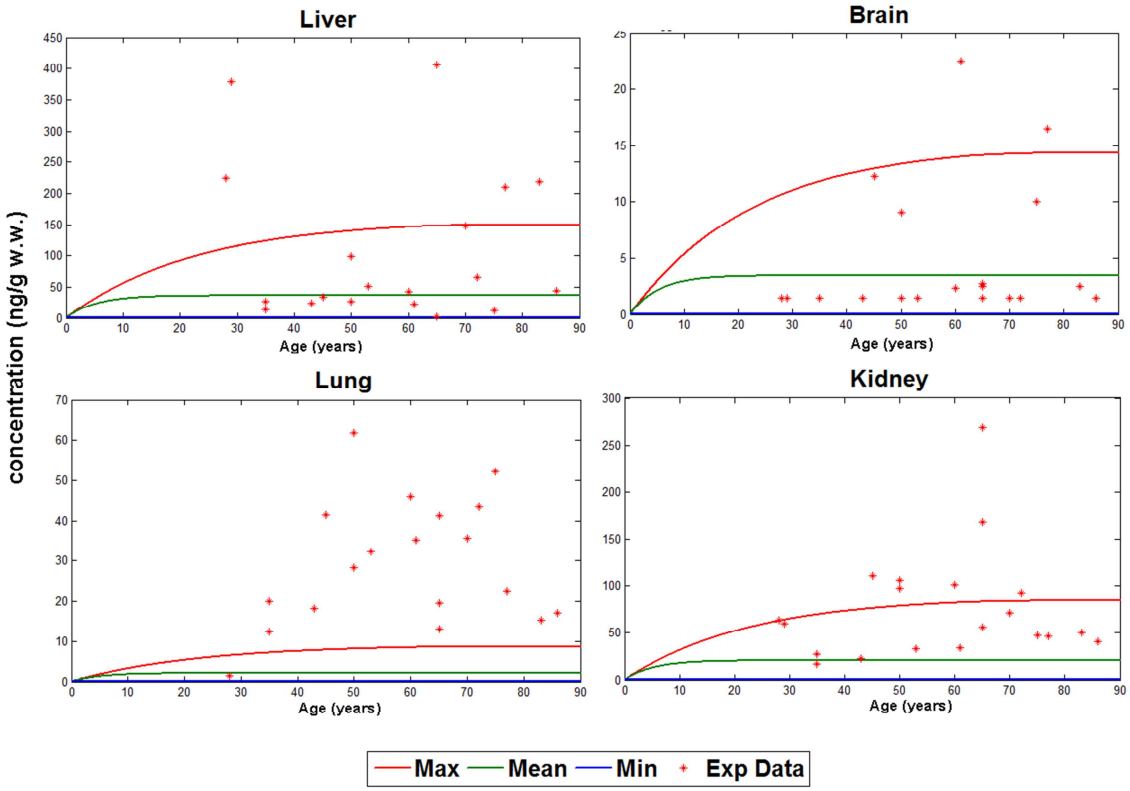

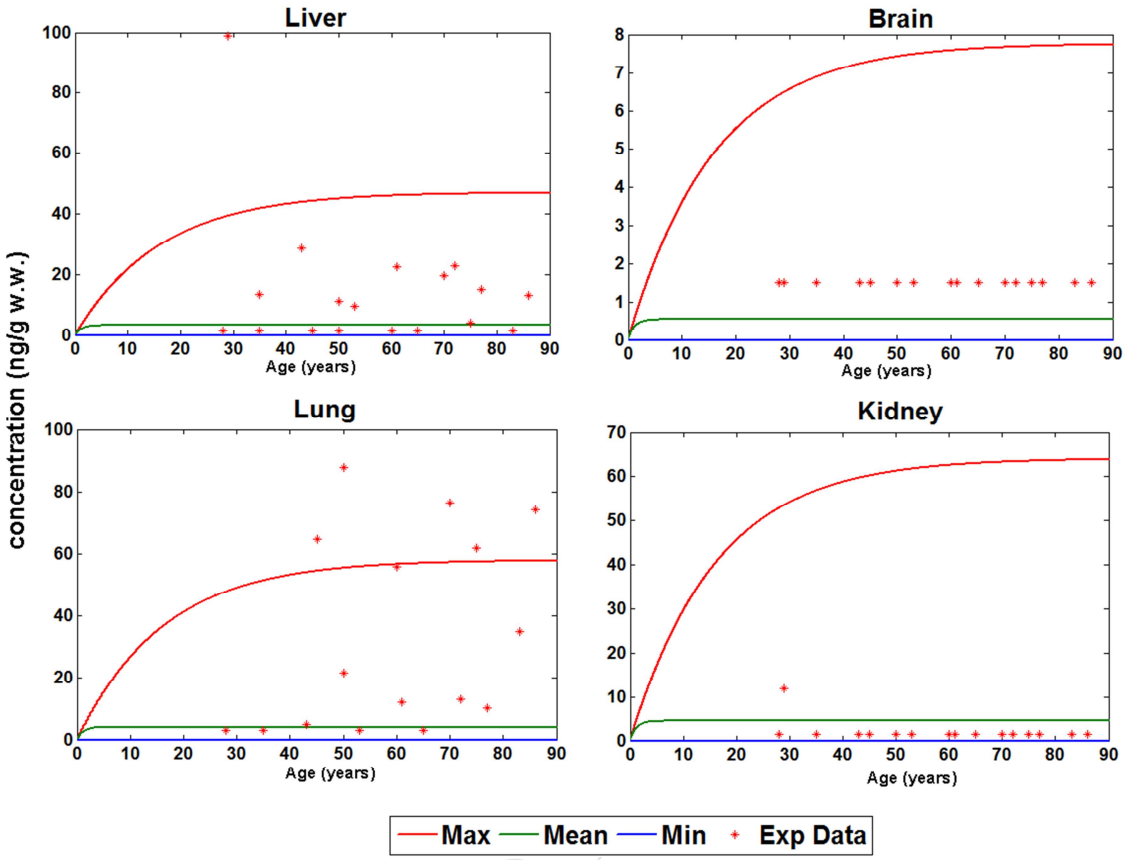

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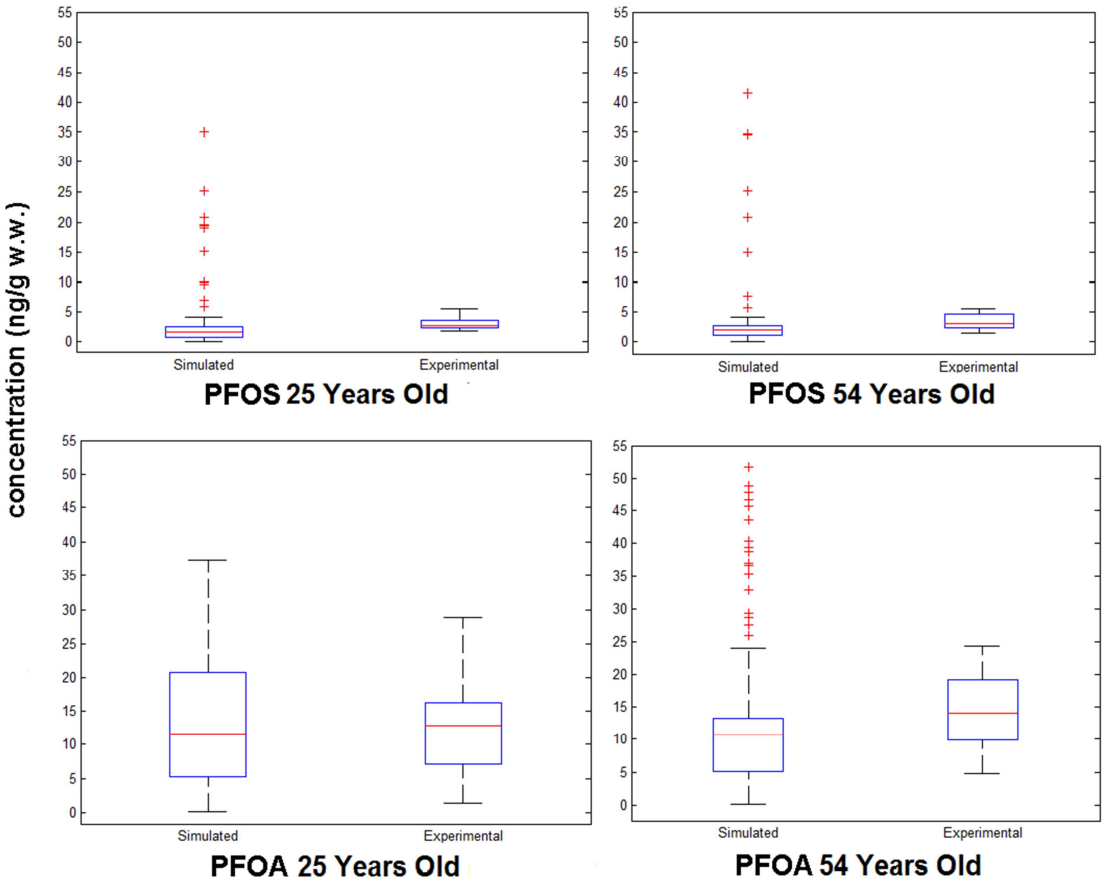

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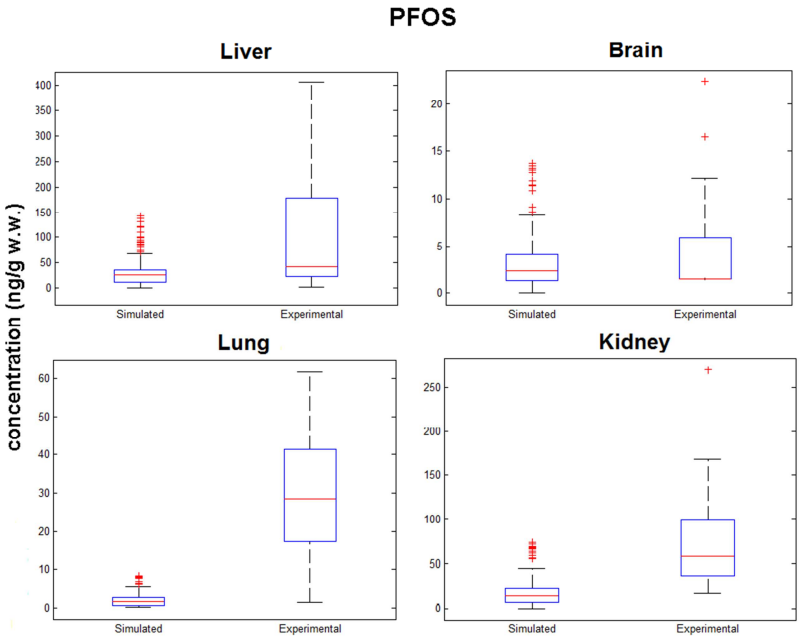
PFOS
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Brain
Lung
Kidney
concentration (ng/g w.w.)
Simulated
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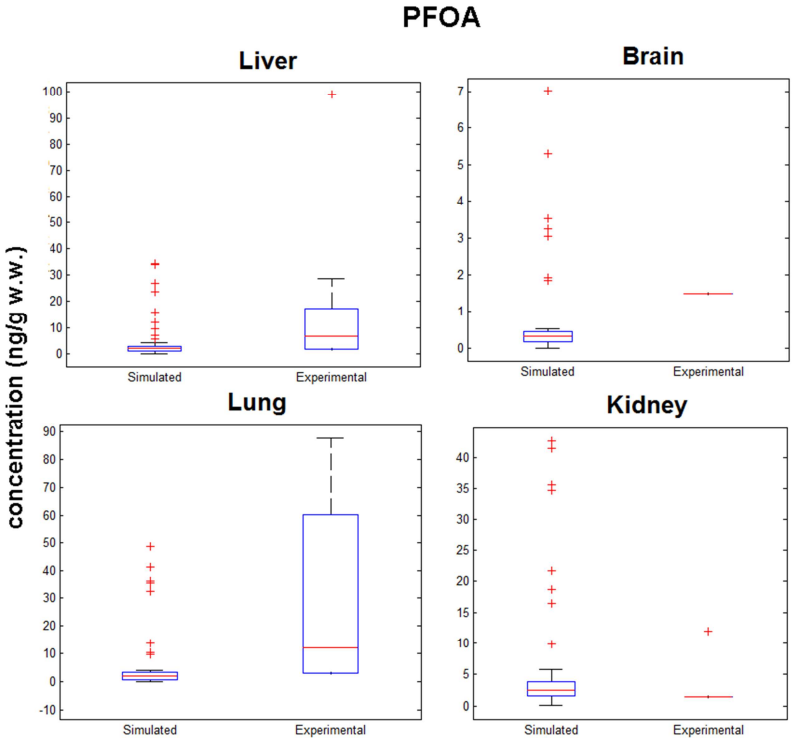
PFOA
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concentration (ng/g w.w.)
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Fig. 1. Compartmental Physiologically-based pharmacokinetic (PBPK) model structure.

Fig. 2. Time course simulated concentrations (mean, minimum and maximum) vs. measured concentrations of PFOS and PFOA in plasma.

Fig. 3. Time course Simulated concentrations (mean, minimum and maximum) vs. measured concentrations of PFOS in liver, brain, lung and kidney.

Fig. 4. Time course simulated concentrations (mean, minimum and maximum) vs. measured concentrations of PFOA in liver, brain, lung and kidney.

Fig. 5. Box plots showing the Simulated vs. experimental concentrations of PFOS and PFOA in plasma in two groups of data samples with age range of 20- 30 and 50-60 years old.

Fig. 6. Box plots showing the Simulated vs. experimental concentrations of PFOS and PFOA in liver, brain, lung and kidney in two groups of data samples with age range of 20- 30 and 50-60 years old.

Highlights

- **PBPK models for PFOA and PFOS were validated.**
- **Different validation techniques were compared.**
- **Visual validations of models are often erroneous.**
- **Validation of PBPK models are highly influenced by parametric uncertainty.**
- **Model validation should increase transparency and reduce discrepancies in scientific reporting.**