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Stochastic Pharmacokinetic-Pharmacodynamic Modeling for Assessing the Systemic Health Risk of Perfluorooctanoate (PFOA)

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The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

ABSTRACT

A phase 1 dose-escalation trial assessed the chemotherapeutic potential of ammonium perfluorooctanoate (APFO). Forty-nine primarily solid-tumor cancer patients who failed standard therapy received weekly APFO doses (50–1200 mg) for 6 weeks. Clinical chemistries and plasma PFOA (anionic APFO) were measured predose and weekly thereafter. Several clinical measures including total cholesterol, high-density lipoproteins (HDLs), thyroid stimulating hormone (TSH), and free thyroxine (fT4), relative to PFOA concentrations were examined by: Standard statistical analyses using generalized estimating equations (GEE) and a probabilistic analysis using probability distribution functions (pdf) at various PFOA concentrations; and a 2-compartment pharmacokinetic/pharmacodynamic (PK/PD) model to directly estimate mean changes. Based on the GEE, the average rates of change in total cholesterol and fT4 associated with increasing PFOA were approximately -1.2×10^{-3} mmol/l/μM and 2.8×10^{-3} pmol/l/μM, respectively. The PK/PD model predicted more closely the trends observed in the data as well as the pdfs of biomarkers. A decline in total cholesterol was observed, with a clear transition in shape and range of the pdfs, manifested by the maximum value of the Kullback-Leibler (KL) divergence, that occurred at plasma PFOA between 420 and 565 μM (175 000–230 000 ng/ml). High-density lipoprotein was unchanged. An increase in fT4 was observed at a higher PFOA transition point, albeit TSH was unchanged. Our findings are consistent with some animal models and may motivate re-examination of the epidemiologic studies to PFOA at levels several orders of magnitude lower than this study. These observational studies have reported contrary associations, but currently understood biology does not support the existence of such conflicting effects.

Key words: APFO; cholesterol; PFOA; phase 1 trial; PK/PD modeling; thyroid.

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The ammonium salt of perfluorooctanoate (APFO, NH_4^+ $C_7F_{15}COO^-$) was used for decades in industrial applications as an emulsifier in fluoropolymer manufacturing, including the polymerization of tetrafluoroethylene (PTFE; Buck *et al.*, 2011). Due to the environmental and toxicologic concerns, the industrial use of APFO has been phased-out in the United States (US EPA, 2006).

Ammonium perfluorooctanoate is absorbed orally (Kennedy *et al.*, 2004), readily dissociates to perfluorooctanoate (herein also referred to as PFOA) in the blood, and binds to serum protein. PFOA does not metabolize and is excreted in urine and feces. Urinary elimination rates vary among the perfluorocarboxylate homologs and renal tubular secretion and reabsorption depends on sex, species, and chain-length. For PFOA, serum elimination half-life values range from hours/days (rats), weeks (mice), or months (monkeys) (Butenhoff *et al.*, 2004; Han *et al.*, 2012; Lou *et al.*, 2009), whereas in humans it ranges between 2.3 and 3.5 years (Bartell *et al.*, 2009; Olsen *et al.*, 2007a; Russell *et al.*, 2015). Serum PFOA concentrations have declined in the United States general population between 2000 and 2015 (Olsen *et al.*, 2017).

Although APFO is not genotoxic (Butenhoff *et al.*, 2014), chronic bioassays in Sprague Dawley rats with dietary administration of APFO (up to 300 ppm) resulted in hepatic and pancreatic acinar cell adenomas (Biegel *et al.*, 2001) or a proliferative response in the acinar pancreas (Butenhoff *et al.*, 2012a; Caverly Rae *et al.*, 2014). Both studies also reported an increase in Leydig cell adenomas. Various levels of confidence regarding the mode of action for these 3 tumors have been attributed to activation of the xenosensor nuclear receptor peroxisome proliferator-activated receptor α (PPARα) (Klaunig *et al.*, 2012). Weight of evidence indicates that the mode of action steps for liver tumors is not likely relevant to humans (Gonzalez *et al.*, 1998; Rosen *et al.*, 2009; US EPA, 2003); whereas a similar PPARα-mediated mode of action was considered plausible for pancreatic tumors (Klaunig *et al.*, 2012). Although the prevalence of Leydig cell tumors is low in humans (0.00004%) compared with the occurrence in Sprague Dawley rats (5.3%) (Cook *et al.*, 1999), the increased incidence of such tumor type in rats with exposure to APFO and its relevance to humans remains recondite.

Occupational epidemiology studies have reported inconsistent associations between PFOA and kidney cancer that may have been confounded by other workplace exposures (Consonni *et al.*, 2013; Raleigh *et al.*, 2014; Steenland and Woskie, 2012). A study of a mid-Ohio river valley community and worker population ($n = 32\ 254$), exposed to drinking water containing PFOA from an industrial source, reported a significant trend for testicular cancer (17 cases) with a nonsignificant trend observed for kidney cancer (105 cases) (Barry *et al.*, 2013). The International Agency for Research on Cancer (IARC) categorized PFOA as a possible human carcinogen for testicular and kidney cancer (group 2B) (Benbrahim-Tallaa *et al.*, 2014).

In light of this IARC qualitative hazard index listing and the presence of PFOA in the general population, it is highly unusual that an environmental toxicant such as PFOA would ever be considered for its chemotherapeutic properties. Interestingly, PFOA has been shown to cause endoplasmic reticulum stress in tumor cells, activity against PIM kinases, and activity in 5 xenograft models of solid tumors (Barnett *et al.*, 2010). Because PIM kinases can be overexpressed in many cancers that involve cell survival, cell cycle progression, and cell migration, inhibitors of PIM kinases have become a focus for drug discovery research, including APFO (Blanco-Aparicio and Carnero, 2013). Based on APFO's antitumorgenicity profile, a phase 1 trial was sponsored by CXR Biosciences, Ltd. (2 James Lindsay Pl, Dundee DD1 5JJ, UK) to determine the safety, dose limiting toxicity, and maximum tolerated dose (MTD) of APFO.

This phase 1 trial also enabled detailed evaluation of various clinical chemistries. Observational epidemiologic research has reported positive associations between measured or modeled serum PFOA concentrations and higher serum cholesterol (Nelson *et al.*, 2010; Steenland *et al.*, 2009; Winquist and Steenland 2014a). These findings, however, are contrary to the PPARα-mediated lowering of serum lipids that occur in rodents treated with APFO (Kennedy *et al.*, 2004) as well as the pharmacodynamics (PD) reduction of serum lipids in humans mediated by the PPARα agonist fibrate drugs (Roy and Pahan, 2009).

The purpose of this paper is to describe this APFO phase 1 trial and the time-dependent relationships that were observed over the course of this study between administered doses of APFO, plasma concentrations of PFOA, and several clinical markers, including cholesterol, in the participating subjects.

MATERIALS AND METHODS

Study Design for Phase 1 Trial of APFO

This open-label, nonrandomized, phase 1 study used a dose-escalation design (Le Tourneau *et al.*, 2009). The study was conducted in 2008–2011 at 2 centers in Scotland: Beatson West of Scotland Cancer Centre (Glasgow) and the Aberdeen Royal Infirmary. CXR Biosciences (Dundee, Scotland) was the study sponsor. The study was conducted in accordance with the International Conference on Harmonization of Good Clinical Practice and approved by the Glasgow West Research Ethics Committee. All subjects provided written informed consent prior to undergoing any study-related procedure.

Subject Eligibility

Patients with histologically or cytologically confirmed advanced solid tumors, refractory to standard anticancer therapy or for which no standard therapy existed, were recruited in this study. Other inclusion criteria for study subjects were: ≥18 years, physician-assessed life expectancy greater than 3 months, adequate hematologic function, able to comply with study procedures, and written consent. Exclusion criteria included any anticancer therapy within the last 4 weeks (including chemotherapy, radiotherapy, endocrine therapy, immunotherapy, or use of other investigational agents), HIV infection, hepatitis B or hepatitis C positivity, inadequate renal function, abnormal liver function tests, lack of physical integrity of the GI tract that might lead to impaired administration and absorption of the oral therapy, and uncontrolled cardiac disease. Ammonium perfluorooctanoate is a potent inhibitor of CYP2C (Elcombe *et al.*, 2011); therefore, patients taking warfarin, phenytoin, or tolbutamide were excluded from the trial.

Pretreatment Evaluation

For each subject, pretreatment evaluations included a full medical history, tumor evaluation, chest X-ray and 12-lead ECG, full blood count and coagulation screen, biochemical profile including urea, electrolytes, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase, bilirubin, total

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cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, albumin, calcium, urea, uric acid, thyroid stimulating hormone (TSH), free thyroid hormone (fT4), blood glucose, urinalysis, and a physical examination.

Trial Treatment

Based on known APFO pharmacokinetics (PKs) in monkeys (Butenhoff *et al.*, 2004), treatment could be administered orally via gelatin capsules that would result in the desired plasma PFOA concentrations. Therefore, the study drug was administered in powder-filled hard gelatin capsules containing 50 mg of APFO. The bulk active pharmaceutical ingredient was manufactured under Good Manufacturing Practices conditions by Chimete Srl (Tortona, Italy). The capsules were manufactured according to GMP by Penn Pharmaceutical Services, LTD (Tredegar, UK).

The pharmacy at each institution controlled the dispensing of APFO. The administration of APFO to the subject occurred by authorized staff to ensure treatment compliance. Prophylactic antiemetics were not administered, and subjects were requested to fast for 1 h after ingestion of APFO. If a subject vomited after ingestion of the oral capsule, then the time of the event was recorded but another capsule was not administered to replace this administration of APFO. Blood samples for PK analysis as well as for clinical chemistries were taken over the following 6-week period. Following this 6-week trial period, the subjects could continue the treatment with weekly administration of drug should they so desire in consultation with their physician. For the purpose of this paper, analyses focus on the 6-week trial period.

Treatment Cohorts

Initial dose cohort. Ammonium perfluorooctanoate was administered orally as a single dose of 50 mg in the morning after an overnight fast in the first cohort of 3 subjects and PK and PD (PK/PD) parameters were measured over a 6-week period. No further dosing was given to this cohort during the 6-week period.

Dose-escalation cohorts. In a 3 + 3 dose-escalation trial design for a phase 1 clinical trial (Le Tourneau *et al.*, 2009), 3 subjects are enrolled into a given dose cohort. If there is no dose-limiting toxicity (DLT) observed among these subjects, the trial proceeds to enroll additional subjects into the next higher dose cohort. If DLT occurs in 1 of the 3 subjects within an individual dose cohort, then up to 3 more subjects can be treated at this dose level and dose escalation is only allowed if no further DLT is observed. If DLT occurs in 2 of the 3 subjects within an individual dose cohort, then no further dose escalation is allowed. The dose level immediately below the maximum administered dose is then defined as the MTD. An additional cohort of subjects could be treated at this MTD up to a total of 12 subjects.

In the dose cohorts that followed the initial dose cohort, subjects were treated with weekly administration of APFO from the start of dosing. Dose escalation was performed only after the subjects at the preceding dose level had completed a 3-week repeat dosing period. If a subject had not completed 3 weeks of repeat dosing with APFO for reasons other than toxicity (eg, withdrawal of consent), the subject was replaced for the purpose of toxicity evaluation. All subjects who were dosed were included in this analysis of the data.

In the present study, the dose of APFO was initially doubled in successive cohorts until a ≥grade 2 drug-related toxicity was observed. The dose assigned to a specific dose cohort was reviewed and determined by the investigators and study sponsor before any subjects were treated at a new dose level. No intrasubject dose escalation was allowed.

Subjects were allowed to receive supportive care therapies. All medications (prescription and over-the-counter) taken during the course of the trial were documented. No other chemotherapy, immunotherapy, hormonal therapy, radiation therapy, or other experimental therapies were permitted during the trial. However, a subject's clinical needs were paramount, and if a restricted concomitant medication or therapy was required whereas the subject was enrolled in the trial then this may have necessitated withdrawal of the subject.

Treatment Discontinuation

Treatment discontinuation was considered in the event of a subject decision to withdraw consent to further treatment, treatment-related serious adverse events, recurrent DLT despite appropriate dose-modifications, progression of the underlying malignant disease, and if further treatment with APFO was contraindicated in the opinion of the investigator.

PK Assessment

For the initial dose cohort who received a single dose (50 mg) of APFO, PFOA in plasma was measured at predose, and then 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 24, 48, and 72 h after administration and then once weekly at weeks 2 through 6. For those subjects with repeat dose administration, blood samples were taken at predose and then 2, 3, 4, and 24 h after each weekly administration for 6 weeks. A complete PK assessment is reported elsewhere (Campbell *et al.*, in preparation).

Analytical Measurement of PFOA

Details are presented in the Supplementary Data. All analytical measurements of PFOA were conducted by CXR Biosciences (Dundee, Scotland). Plasma PFOA was reported in μM (μM PFOA = 413 ng/ml). The method used 40 μL plasma, solvent extraction followed by LC/MS/MS, and provided sufficient detectability (ie, lower limit of quantitation [LLOQ] of 5 ng/ml) to fully define the PKs following the initial dose. This LLOQ is inappropriate for the levels found in general human populations. However, calibration and quality control standards were adjusted as appropriate to cover the range required with increasing dose. Accuracy was within 15% of the actual value and precision did not exceed 15% of the coefficient of variation at all concentrations above the LLOQ. The LLOQ was established as the lowest point on the calibration with accuracy and precision within 20% limits.

PD Assessment

For those subjects with repeat dose-administration, blood samples for clinical measures were taken after an overnight fast: Predose, 2 and 24 h postdose for 6 weeks and analyzed in the hospital clinical laboratories. Units of reported measurement were the following serum lipids: total cholesterol, LDL, and HDL (mmol/l = 38.6 × mg/dL); serum triglycerides (mmol/l = 88.5×mg/dl); thyroid: TSH (mIU/l) and fT4 (pmol/l = 0.08×ng/dl); glucose (mmol/l = 18×mg/dl); liver and pancreatic enzymes and bilirubin: ALT, AST, gamma glutamyl transaminase (GGT), alkaline phosphatase, amylase (IU/l), and total bilirubin (mg/l); liver function blood clotting tests: Fibrinogen (g/l), prothrombin time (PTT) (s), activated partial thromboplastin time (aPTT) (s); serum proteins: total albumin and globulin (mg/dl); and renal clinical chemistries: serum creatinine (μmol/l = 0.0113 ×mg/dl); urea

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(mmol/l = BUN [mg/dl] × 0.3571); and uric acid (μmol/l = 0.0168 × mg/dl).

Tumor Response

Tumor response was assessed clinically every three weeks and involved radiologic evaluation of disease at the end of the six-weeks, as well as for those who requested continued treatment. Response to APFO treatments was recorded using the RECIST criteria (Therasse *et al.*, 2000) in subjects with measureable disease. These tumor response findings are not reported herein.

Statistical Analyses

Internal dosimetry of PFOA, not the dose group, was considered the most appropriate metric for the evaluation of the clinical chemistries. The baseline (before first dose) and subsequent 24-h postdose plasma PFOA and clinical chemistry results (through the 6 treatments) were considered for statistical analyses and characterized by estimating the univariate and bivariate distributions, and examining for outliers and other anomalies. Simple graphic displays were used to characterize these distributions, including box plots and scatter plots. Autocorrelations and intraclass correlations (ICC) of the time series were estimated and Box-Cox transformations to normalize raw data were evaluated. To accommodate correlation of values within subject, generalized estimating equation (GEE) models were fitted to estimate the trends in clinical chemistries over concentrations of PFOA.

Combining Toxicologic and Epidemiologic Information Via an Information Theoretic PK/PD Model

In order to replicate the observed PK/PD, we tested different metamodels (ie, different models with different parameters or values of parameters) for which the goal was to reproduce selected observed patterns with the highest accuracy. These patterns were (1) the variation of PFOA over time; (2) the probability distribution functions (pdf) of PFOA and clinical chemistries over time; and (3) the patterns of clinical chemistries (eg, total cholesterol, HDL, triglycerides, TSH, and fT4) conditional on different levels of PFOA. Note that these 3 patterns encompass the classical PK and PD relationships, better defined as the concentration-time, effect-time, and effect-concentration relationships, respectively. The model architecture used for the PK/PD modeling was the one proposed by Lindhardt and Gennemark (2014). This model has been implemented in the "PopED lite" computational platform (http://www.bluetree.me/PopED_lite.html). PopED lite is a computational technology for clinical PK and PD studies (software and documentation is found at http://arxiv.org/abs/1505.06658v1). PopED lite focuses on optimization of the dosage and PK/PD sampling (or observation) times to improve the accuracy of the parameter estimates of fixed-effect PK/PD models. Thus, model design ("metamodeling"; Saltelli *et al.*, 2008) via global sensitivity and uncertainty analyses (GSUA) in this context refers to the selection of the optimal probability distribution functions and value ranges that maximize the accuracy of predicting the three patterns considered. Prediction accuracy is assessed by three accuracy indicators: The root mean square error (RMSE), the Kullback-Leibler divergence over time (KL), and the Akaike Information Criterion (AIC). These three indicators are deterministic, probabilistic and model validation criteria, respectively, and are calculated for all three patterns considered. An RMSE is a classical indicator of model performance that depends on average values of variables; KL is a probabilistic indicator that considers the whole probability distribution of variables; and AIC is a computational complexity indicator that considers the number of parameters in the model. The selection of these evaluation indicators allowed for global assessment of model performance and determination of its reliability. One advantage was examination of the sensitivity of the estimated effect of interest to the distributions underlying the other parameters in the model. From this there can be an assessment to what extent the findings can be influenced by the model form and parameterization. For a more complete development of the GSUA model (Sobol, 1993; Ludtke et al., 2008) and pertinent implementation details, see Supplementary Data.

The PK of the population cohort was modeled by a linear two-compartment model as (Figure 1)

$$C'_{pu} = \frac{D}{V} \times k_a \times e^{-k_a \times t} - k_{12} \times C_{pu}(t) + k_{21} \times C_2(t) - k_{10} \times C_{pu}(t) \quad (1)$$

$$C'_2(t) = k_{12} \times C_{pu}(t) - k_{21} \times C_2(t) \quad (2)$$

where C_{pu} and C_2 represent unbound concentration in the plasma compartment and in a second tissue compartment, respectively. D is the weekly dose given (zero for the baseline value) and the parameters V (volume of distribution), k_a, k_{12}, k_{21}, and k_{10} are calibrated simultaneously in a fitted model that considers the data and aims to maximize multi-pattern prediction accuracy (see Supplementary Data for details). The PD of the population cohort of the drug was modeled as follows. A first-order distribution delay of compound to and from the biophase (compartment) was assumed to be the rate-limiting step, and thus requires no assumptions about the timing of response, and an effect-compartment model was used, where the concentration in the effect compartment was linked to the plasma compartment by a first-order equilibrium rate constant k_e. The rate of change of compound concentration in the effect compartment could then be expressed by

$$C'_{eu}(t) = k_e \times [C_{pu}(t) - C_{eu}(t)] \quad (3)$$

where C'_{eu} denotes the unbound effect compartment concentration (Figure 1). The observed PD effect was modeled by a power function as

$$E(t) = a \times C_{eu}(t)^b \quad (4)$$

where E denotes the effect variable (such as total cholesterol and fT4 but in general could be any clinical chemistry), and a and b are calibrated input factors whose specific value depends on the effect compartment but whose probability distribution does not (see Supplementary Data for details). In this way, we keep the power exponent b and the scaling factor a as effect-dependent factors but the structure of the observed PD effect is invariant, as should be expected. The effect compartment represents the target site concentration, and is used to infer concentration-effect relationships (or PD relationships versus the concentration-time PK relationships) without ambiguity from variability of delays in effect. The model was calibrated for 1 week and validated for the next 5 weeks of the 6-week phase 1 trial in which the population cohort was studied.

A sensitivity analysis was conducted to determine the influence of the study subjects' treatment with cholesterol lowering drugs, thyroid medications, and corticosteroids.

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Table 1. Treatment Data for the 49 Subjects in the Phase 1 Clinical Trial

	APFO Dose (mg)	Number of Subjects	6 Weeks (wks) of Treatment (Yes/No)
Initial single dose cohort	50	3	(Single dose week 1 only)
Repeat weekly dose cohorts	50	1	Yes = 1; No = 0
	100	3	Yes = 2; No = 1 (missed wks 5–6)
	200	3	Yes = 3; No = 0
	300	4	Yes = 2; No = 2 (1 missed wk 6; 1 missed wks 2–6
	450	3	Yes = 2; No = 1 (missed wk 6)
	600	7	Yes = 4; No = 3 (1 missed wk 5; 2 missed wks 4–6)
	750	3	Yes = 2; No = 1 (missed wk 6)
	950	4	Yes = 2; No = 2 (1 missed wk 6; 1 missed wks 2–6)
	1000	12	Yes = 7; No = 5 (1 missed wk 4; 1 missed wk 5; 1 missed wk 3 and wk 6; 2 missed wks 4–6)
	1200	6	Yes = 2; No = 4 (1 missed wk 4; 1 missed wks 5–6; 2 missed wk 6)

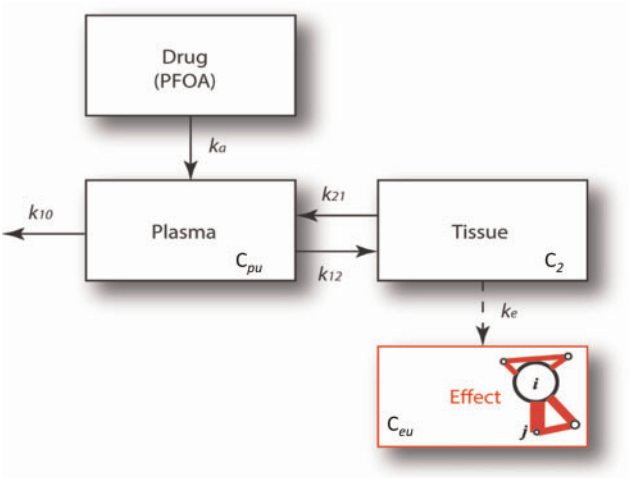


Figure 1. Conceptual diagram of the PK/PD compartmental model. The black and red boxes refer to the stochastic pharmacokinetic (PK) and pharmacodynamics (PDs) model, respectively. Dose of the drug, plasma concentration, unbound concentration in the tissue and effect in the biologic compartments are the quantities that are calculated by the model. Cpu and C2 represent unbound concentration in the plasma compartment and in a second tissue compartment. The parameters V, k_a, k_{12}, k_{21}, and k_{10} are calibrated simultaneously in a fitting model that considers the data and aim to maximize prediction accuracy. The concentration in the effect compartment was linked to the plasma compartment by a first-order equilibrium rate constant k_e. C_{eu} denotes the unbound effect compartment concentration. *E* denotes the effect variable, and *a* and *b* (see equation 4) are calibrated input factors whose specific value depends on the effect compartment (or clinical biomarker) but their probability distribution does not. i and *j* refer to any clinical biomarker that are mutually dependent.

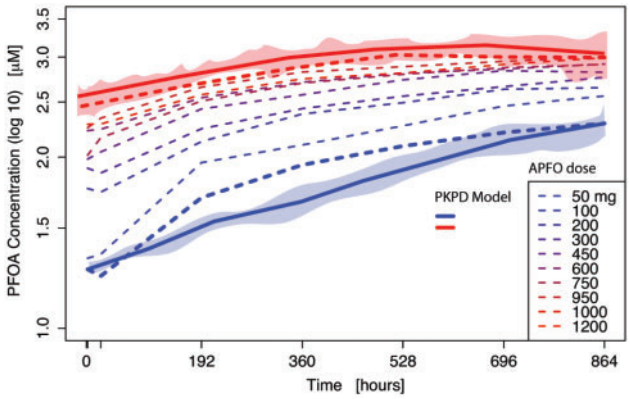


Figure 2. Observed and predicted plasma concentration of PFOA over time conditional on the assigned dose. Predictions and data are solid and dashed lines as a function of dose category. The PK/PD model predictions are shown only for the lowest and highest dose category. Variability around PK curves is a function of the pdf assigned to the input factors and numerical Monte Carlo variability related to the Sobol sampling scheme.

RESULTS

Systemic Response

A total of 50 subjects (22 females, 28 males) were initially enrolled and 49 underwent the treatment in this phase 1 trial of APFO (see Table 1). These 49 subjects were on average 61 years of age, were 1.7 m in height, and weighed 75 kg (Supplementary Table 1). Estimates of autocorrelation and ICC were small enough to ignore, thus for the purposes of modeling, all measurements were treated as independent. Supplementary Table 2 provides the distribution of tumor sites and a breakdown of the colorectal and pancreatic cancers by stage.

No more than one subject showed DLT at any dose so therefore the protocol-defined MTD was not reached. The recommended phase 2 dose (RP2D) of 1000 mg weekly was based on tolerability of common cumulative drug-related toxicities which consisted primarily of fatigue, nausea, vomiting, and diarrhea. Stable disease for >12 weeks was observed in 8 subjects including subjects with anaplastic thyroid (40 weeks), pancreatic (35 weeks), and colon (34 weeks) cancer (MacPherson *et al.*, 2011).

Plasma PFOA Concentrations

The trajectory of both observed and modeled plasma concentrations of PFOA over time for different dose groups are provided in Figure 2. The plot shows that, although cumulative dose goes up linearly, plasma concentration begins to plateau with increasing dose categories. The highest dose category showed a plateau at around 600 h into the dosing period.

PK/PD Modeling

The two clinical chemistry measures that showed clearly observable associations with PFOA concentrations were total cholesterol and fT4.

Based on the GEE analysis for total cholesterol (Figure 3), the average rate of change in total cholesterol with increased PFOA concentration was approximately -1.2×10^{-3} mmol/l/μM (Figure 3).

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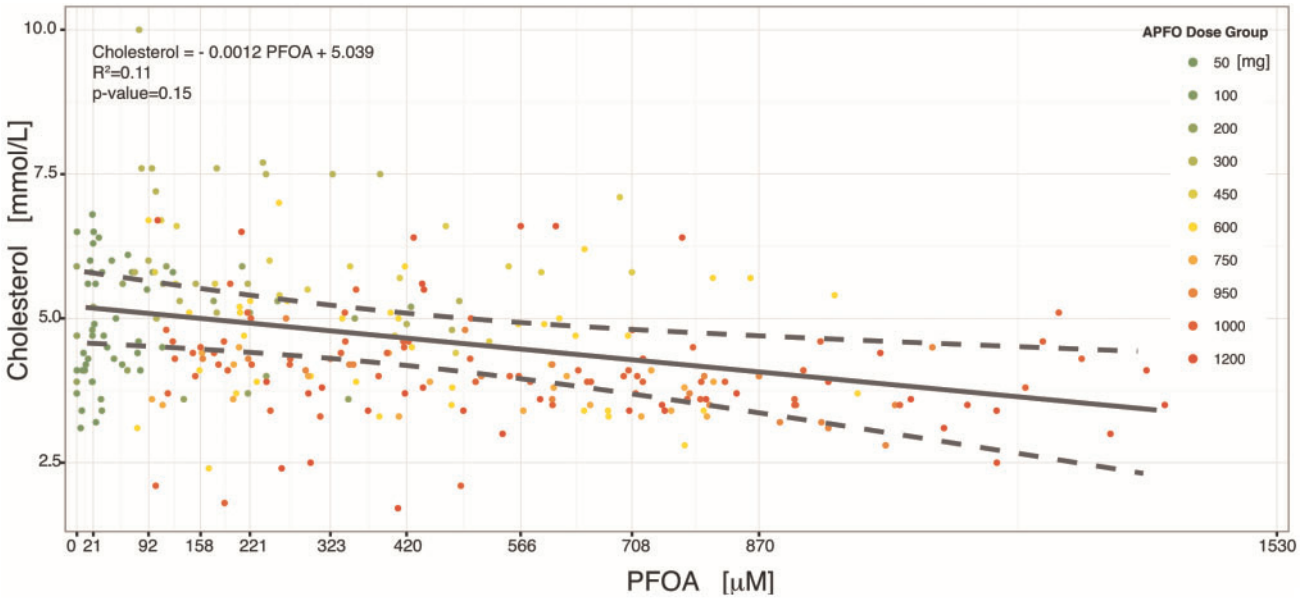


Figure 3. Generalized estimating equations analysis of cholesterol and PFOA. Slope and 95% CI presented. Dots of different colors are for different dose groups.

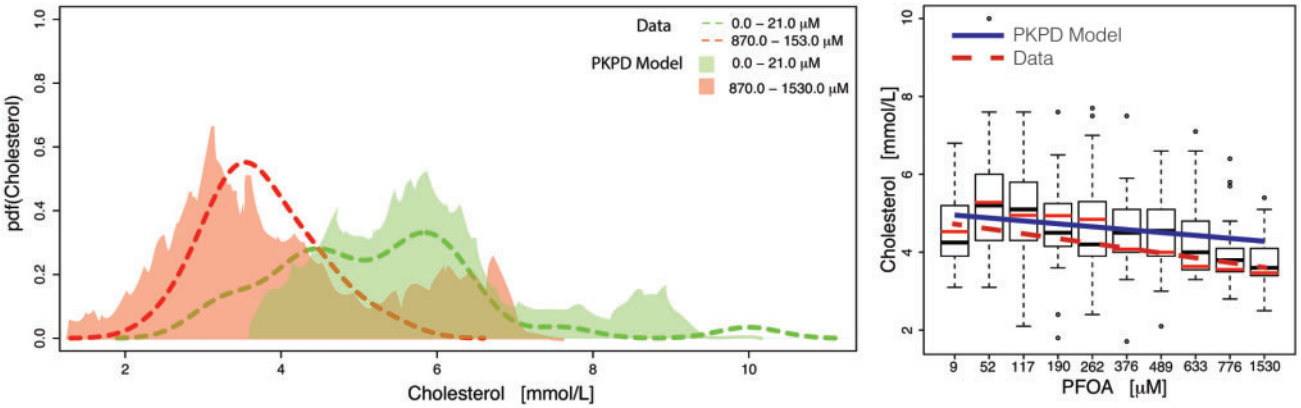


Figure 4. Observed and predicted probability distribution function of cholesterol and dependent on PFOA concentration. (Left) The solid pdfs are cholesterol levels for the lowest and highest PFOA concentration categories predicted by repeated runs of the PK/PD model whereas the dashed pdf are from smoothed observations. (Right) Box plots and slope of the observed and slope of the model-predicted average value of cholesterol plotted as a function of each of the 10 PFOA serum concentration categories. The black and red bars in the boxes represent the median and the mean value respectively. Dots above boxes are outliers (upper dots are more than 3/2 times of upper quartile, whereas lower dots are less than 3/2 times of lower quartile). The extremes of the whiskers are the maximum and minimum values for each category excluding outliers. The extremes of the boxes are the third and first quartiles.

There was a clear transition in shape and range of the pdf for a decrease in total cholesterol (Figs. 4 and 5) that occurred at roughly PFOA levels between 420 and 565 μM (approximately 175 000–230 000 ng/ml). This transition is manifested by the maximum value of the KL divergence quantified between pdfs of all PFOA categories. These PFOA concentrations are several orders of magnitude higher than reported in workers or the general population. Figure 6 displays pdf results for total cholesterol, HDL, and LDL demonstrating that the impact of PFOA was on decreased LDL but not HDL. In addition, the variability of total cholesterol was higher at lower PFOA levels. The higher dosed subjects experienced a relatively narrower range of total cholesterol values but much higher variability in PFOA plasma concentration. Supplementary Figure 1 demonstrates the same results for subjects grouped in 3 different treatment bins used to calculate the average value of total cholesterol, verifying the robustness of these analyses.

Based on the GEE analysis for fT4 (Figure 7), the average rate of change in fT4 was 2.8 $\times 10^{-3}$ pmol/l/μM with a transition in shape and range of the pdf at higher concentrations of PFOA (Figure 8) than seen with cholesterol. This transition is manifested by the maximum value of the KL divergence quantified between pdfs of all PFOA categories. In Figure 9, which graphs both TSH and fT4 pdf as a function of PFOA plasma concentration, it is clear that only fT4 was influenced (increased) by PFOA, not TSH. Compared with cholesterol, the distribution of fT4 appeared not to narrow with increasing PFOA level. Supplementary Figure 1 also presents the fT4 results with subjects grouped by the three treatment bins.

Based on the analysis of the PK/PD model, the average rate of decrease in total cholesterol and increase of free T4 associated with increasing PFOA were approximately -0.30×10^{-2} mmol/l/μM and 2.66×10^{-3} pmol/l/μM, respectively;

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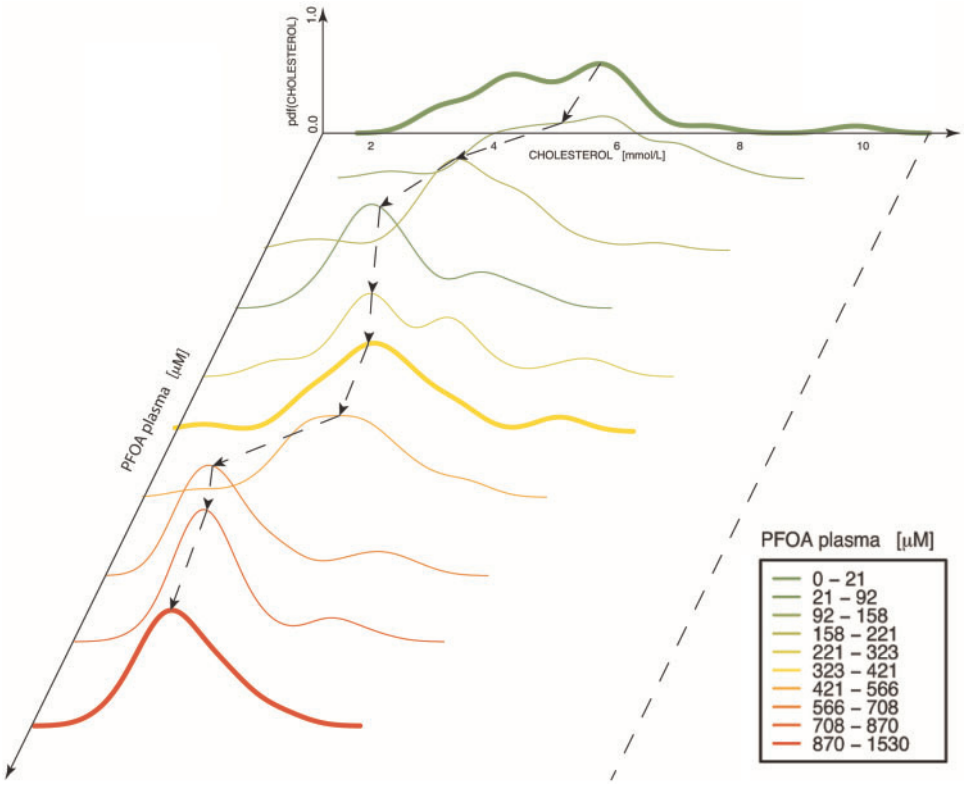


Figure 5. Probability distribution functions of total cholesterol over increasing PFOA concentrations for all subjects in the cohort. The colors of the pdf correspond to the PFOA concentrations groups as in the legend.

thus, the GEE underestimates the change of cholesterol, but predicts relatively correctly the change in fT4. Note that the change in fT4 was not as nonlinear as the change in cholesterol considering the pdfs of these biomarkers. Despite the largest change is observed for cholesterol (probabilistically speaking), the change in the average value (explained linearly) is small, and smaller than the average change of fT4, despite the latter is changing less than cholesterol probabilistically. This is clearly related to the nonlinear dynamics where seemingly small changes in average values actually produce large effects.

Figures 10 and 11 present pdfs for ALT and serum creatinine, respectively, by plasma PFOA group. Both clinical chemistries did not show appreciable changes as a function of PFOA levels. It is possible that serum creatinine was higher for a few individuals for highest values of PFOA, but there is little evidence of any impact on ALT.

The dynamics of other clinical measures—triglycerides, urea, glucose, AST, GGT, alkaline phosphatase, total bilirubin, fibrinogen, PTT, and aPTT—show almost no observable differences with measured plasma PFOA concentrations (Supplementary Figs. 2–6).

Sensitivity analyses displays the results for cholesterol and fT4 robust to subject treatment with statins ($n = 6$ subjects), thyroxine ($n = 3$ subjects), and corticosteroids ($n = 18$ subjects). (Supplementary Figs. 7–10).

The most important result of GSUA performed for the stochastic PK/PD model showed the relative importance between compartment effects factors and dose-plasma factors (Supplementary Figs. 11–13). Details of these analyses are given in the Supplementary Data. Viewed from the perspective of the plasma concentration, the factor D is the most important driver of the whole model. The second most important factors are a, b, and ke, which determine the PD response, our main interest here. For each of these factors, the dependence on other factors is balanced by their independent impact. Although somewhat dependent on other factors, these estimates are generally robust. Non-negligible, particularly in terms of model factor interactions are the plasma-compartment factors k12, k21, and k10. In other words, the way in which the drug is assimilated by each model compartment is important to the concentration. The volume V and the absorption rate k_a did not seem to determine significantly the PK/PD dynamics.

DISCUSSION

PFOA and Lipid Dynamics

The plasma PFOA concentrations measured in this phase 1 study are the highest ever reported in humans. At plasma PFOA concentrations between 420 and 565 µM (approximately 175 000–230 000 ng/ml), there was a marked decrease in serum total cholesterol but not in HDL. This reduction in non-HDL cholesterol is consistent with the known PDs of PPARα agonists used to treat hyperlipidemia in humans. For example, the fibrate drugs (eg, clofibrate), work through the regulation of genes involved in lipid metabolism (Roy and Pahan, 2009). PFOA activates human PPARα both in vitro in primary hepatocytes (Bjork and Wallace, 2009) and in vivo in humanized PPARα mice (Albrecht *et al.*, 2013).

The phase 1 results are generally consistent with the toxicologic evidence, where PFOA has been shown to activate PPARα and regulate the transcription of genes involved with lipid metabolism in rats and mice (Albrecht *et al.*, 2013; Elcombe *et al.*, 2010). Rodents treated with APFO over 14 days showed

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decreased serum lipids at PFOA levels between 20 000 and 51 000 ng/ml in rats and 10 000–14 000 ng/ml in mice (Loveless *et al.*, 2006). A study of C57BL/6 and BALB/c mice fed a high cholesterol and fat diet containing PFOA, found elevated hypercholesterolemia (primarily HDL) (Rebholz *et al.*, 2016) but this was not supported by a study of humanized ApoE*3.Leiden.CETP mice (Westerterp *et al.*, 2006), in which a Western diet containing APFO resulted in decreased plasma non-HDL cholesterol and triglycerides (Princen *et al.*, 2016). Hypolipidemia was not observed in cynomolgus monkeys with repeated oral administration of APFO over six months (Butenhoff *et al.*, 2002). Human PPARα receptors have comparable affinity to PPARα agonists (Corton *et al.*, 2014), but less than 10% the level of PPARα receptors compared with rats and mice (Gonzalez *et al.*, 1998). It is, therefore, reasonable to conclude that the hypolipidemic response observed in the phase 1 trial subjects may be consistent with a PPARα-mediated mode of action.

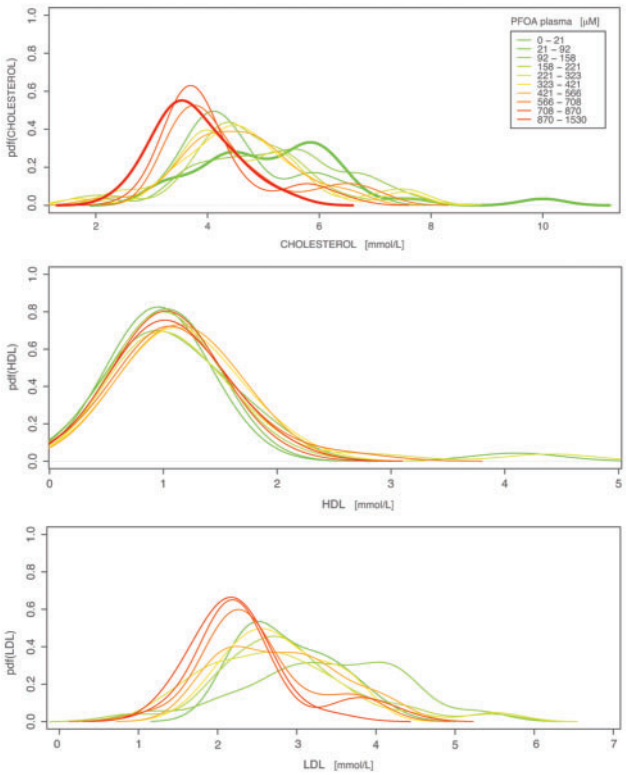


Figure 6. Observed probability distribution function of cholesterol. Total cholesterol, HDL, and LDL are considered. HDL is invariant for any PFOA class, and by LDL that is decreasing for increasing values of PFOA. The pdfs of cholesterol for the lowest and highest PFOA plasma classes are shown with a thick line to emphasize the change in their probabilistic structure. Pdfs of different colors correspond to the PFOA concentration groups as in the legend.

Several observational epidemiologic cross-sectional studies of general populations have reported positive associations between serum cholesterol (primarily LDL) and PFOA (Eriksen *et al.*, 2013; Geiger *et al.*, 2014; Nelson *et al.*, 2010; Starling *et al.*, 2014) at approximately 4 orders of magnitude lower concentrations than in this phase 1 study. Furthermore, the modest association observed in studies of general populations is inconsistent with the weaker associations reported in more highly exposed workers (Costa *et al.*, 2009; Olsen and Zobel, 2007; Sakr *et al.*, 2007a,b; Steenland *et al.*, 2010a, 2015). An association between high cholesterol and measured PFOA (Fitz-Simon *et al.*, 2013; Frisbee *et al.*, 2010; Steenland *et al.*, 2009) and model-derived cumulative PFOA (Winquist and Steenland 2014a) has been reported in a mid-Ohio river valley community whose drinking water contained PFOA from a nearby fluoropolymer plant. However, there was no increased risk for coronary artery disease related to PFOA exposure in this population. The C8 Science Panel (2012a) considered the observed increased risk for high cholesterol with exposure to PFOA small in magnitude and would therefore not necessarily result in increased heart disease given its other attributable risk factors. Studies of highly exposed PFOA occupational cohorts have also not reported increased risks with either coronary artery disease incidence (Steenland *et al.*, 2015) or mortality (Raleigh *et al.*, 2014; Steenland and Woskie, 2012) when using internal referent comparisons to minimize confounding by nonrepresentative general referent populations.

Because of the consistency from this phase 1 trial and toxicologic studies demonstrating lower cholesterol with high concentrations of PFOA, on the one hand, and the inconsistency with the observational epidemiologic associations showing higher cholesterol with markedly lower PFOA concentrations on the other, future research should address noncausal biologic explanations for the latter. Several possibilities include:

1. Inherent variability in the glomerular filtration rate (GFR), which confounds other associations reported with PFOA, including lower birthweight (Verner *et al.*, 2015) and chronic

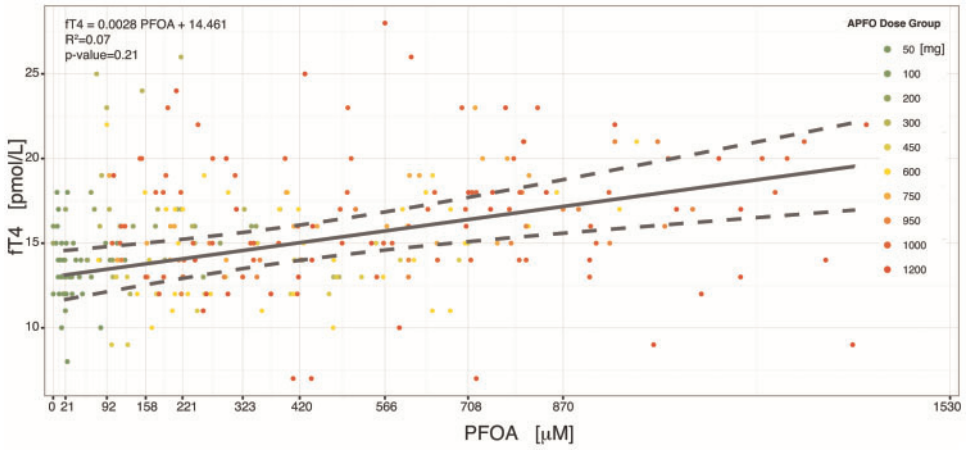


Figure 7. GEE analysis of fT4 and PFOA. Slope and 95% CI presented. Dots of different colors are for different dose groups.

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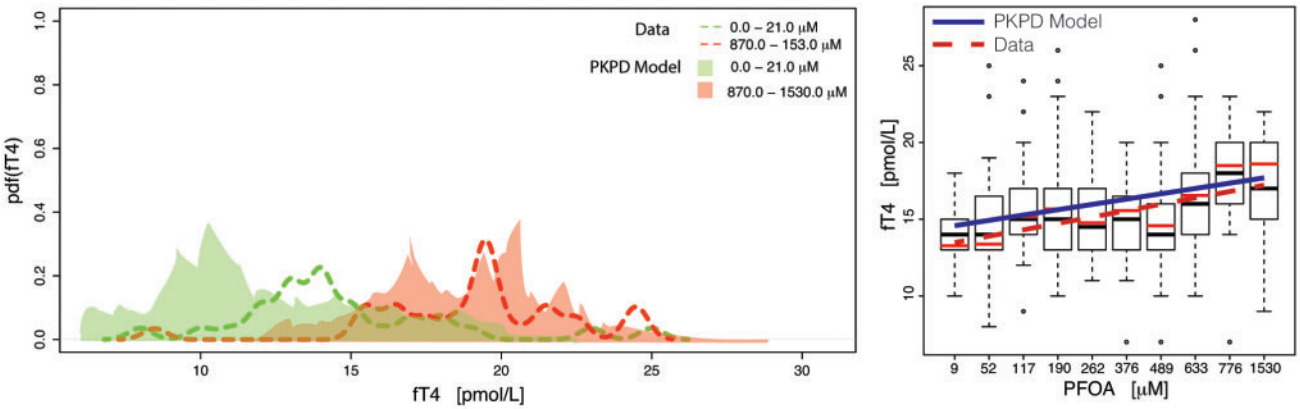


Figure 8. Observed and predicted probability distribution function of fT4 and dependent on PFOA concentration. (Left) The solid pdfs are fT4 levels for the lowest and highest PFOA concentration categories predicted by repeated runs of the PK/PD model whereas the dashed pdf are from smoothed observations. (Right) Box plots and slope of the observed and slope of the model-predicted average value of fT4 plotted as a function of each of the 10 PFOA serum concentration categories. The black and red bars in the boxes represent the median and the mean value respectively. Dots above boxes are outliers (upper dots are more than 3/2 times of upper quartile, whereas lower dots are less than 3/2 times of lower quartile). The extremes of the whiskers are the maximum and minimum values for each category excluding outliers. The extremes of the boxes are the third and first quartiles.

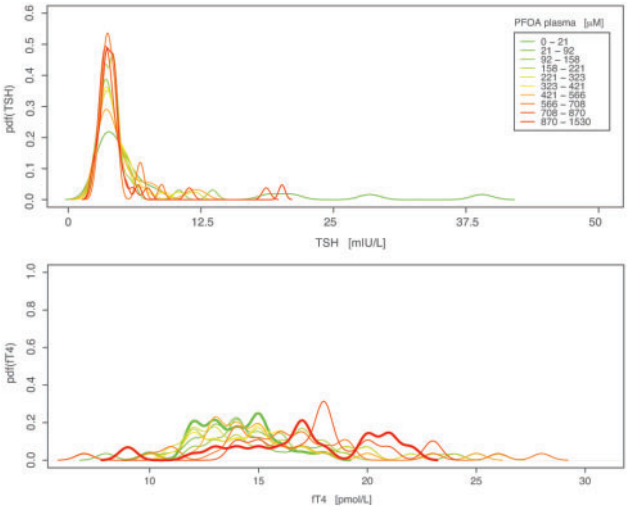


Figure 9. Observed probability distribution function of thyroid function. TSH is invariant and fT4 is higher for higher values of PFOA. The pdfs of fT4 for the lowest and highest PFOA plasma classes are shown with a thick line to emphasize the change in their probabilistic structure. Pdfs of different colors correspond to the PFOA concentration groups as in the legend.

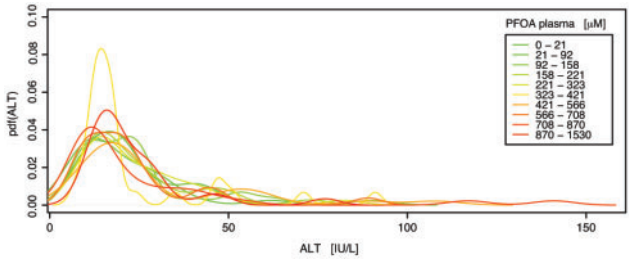


Figure 10. Observed probability distribution function of ALT. ALT is invariant for any PFOA categorization. Pdfs of different colors correspond to the PFOA concentration groups as in the legend.

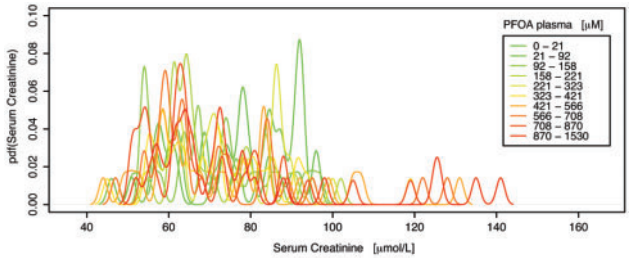


Figure 11. Observed probability distribution function of serum creatinine. Serum creatinine appears to be slightly increasing for increasing values of PFOA but this was based on one individual. Pdfs of different colors correspond to the PFOA concentration groups as in the legend.

kidney disease (Watkins *et al.*, 2013). Studies have shown that individuals with dyslipidemia, as well as those taking a much less aggressive atorvastatin treatment for their dyslipidemia, have lower GFR (Lin *et al.*, 2015; Shepherd *et al.*, 2007). These studies may suggest that elevated cholesterol may affect GFR which may then result in increased retention of PFOA.

2. Organic anion transporters in the gastrointestinal tract and liver that may share binding affinity with lipids and PFOA, analogous to the URATE transporter in the proximal tubule and uric acid and PFOA (Han *et al.*, 2012). Organic anion transporting polypeptides in human and rodent hepatocytes and enterocytes have been shown to transport PFOA, thus contributing to its enterohepatic circulation (Zhao *et al.*, 2017). Thus, persons with increased enterohepatic circulation transport of lipids may also have increased retention of PFOA.
3. Saturation in an underlying physiologic mechanism given the nonlinear association between PFOA and cholesterol, as was suggested by Steenland *et al.* (2009) and Frisbee *et al.* (2010). This could involve reabsorption at the proximal tubule and/or enterohepatic reuptake.
4. Lipoproteins that may bind with PFOA, but this is less plausible since 99% of the PFOA in one human donor sample was distributed in lipoprotein-depleted plasma (Butenhoff *et al.*, 2012b). Additional study subjects would be reassuring to assess this result.

Plausible biologic modes of action that support the hypercholesterolemia positive association reported at low ng/ml PFOA concentrations, whereas explaining negative association

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at high concentrations, also need to be investigated. Serum PFOA concentrations as low as 50 ng/ml have not resulted in higher cholesterol levels in humanized ApoE*3.Leiden.CETP mice given the reduction in cholesterol at higher PFOA concentrations (personal communication H Princen, TNO Biosciences). Therefore, there needs to be a concerted effort to focus on modes of action to address the existence of a PFOA-mediated effect with the positive cholesterol associations reported in observational epidemiologic studies.

PFOA and Thyroid Function

The observed increase in fT4 seen in this phase 1 trial, with no apparent effect on TSH, suggested that the increase in fT4 was not clinically significant but may be due to displacement of the thyroid bound hormone by PFOA. These findings appear similar to the toxicologic evidence in laboratory studies with perfluorooctanesulfonate (PFOS), which displaced thyroxine from binding proteins in rats, transiently increasing free thyroxine without altering overall thyroid hormone homeostasis (Chang *et al.*, 2007, 2008; Weiss *et al.*, 2009). PFOA is structurally similar to PFOS in that both compounds resemble a fatty acid in their amphiphilic nature and compete for binding with free fatty acids on albumin and liver fatty-acid binding protein (Luebker *et al.*, 2002), and therefore, PFOA can also similarly displace thyroxine (Butenhoff *et al.*, 2012c). Such displacement has been shown with aspirin, heparin, and free fatty acids (Koulouri *et al.*, 2013).

Analogous to the cholesterol studies mentioned above, studies of thyroid hormone measurements and thyroid diseases can be categorized into: (1) general populations for which many have been cross-sectional (eg, Melzer *et al.*, 2010; Wang *et al.*, 2013; Webster *et al.*, 2016; Wen *et al.*, 2013); (2) the mid-Ohio river valley community (Knox *et al.*, 2011; Lopez-Espinosa *et al.*, 2012; Winquist and Steenland 2014b), and (3) occupational studies (Costa *et al.*, 2009; Olsen and Zobel, 2007; Sakr *et al.*, 2007a,b; Steenland *et al.*, 2015). The studies of general populations did not yield consistent results with measured thyroid hormones and could be subject to reverse causation (Webster *et al.*, 2016) as GFR will decrease in the untreated or subclinical hypothyroid state and increase in the untreated hyperthyroid or subclinical state, both of which can be normalized upon treatment (Dousdampanis *et al.*, 2014; Koulouri *et al.*, 2013; Woodward *et al.*, 2008). Such an effect on GFR may retain or eliminate unbound PFOA, respectively. In the mid-Ohio river valley studies, PFOA exposure was measured in cross-sectional studies (Knox *et al.*, 2011; Lopez-Espinosa *et al.*, 2012) and estimated via exposure models in a longitudinal study of a community with diagnosed functional thyroid disease (Winquist and Steenland 2014b). The latter study reported associations for women diagnosed mainly with hyperthyroidism and for men with hypothyroidism. Occupational studies that have measured thyroid hormones did not report consistent associations with thyroid hormones nor did they provide workers' thyroid disease histories, except for a prospective study (Steenland *et al.*, 2015) that reported an exposure trend with PFOA for hypothyroidism in male workers.

PFOA and Liver Enzymes and Function

During this 6-week phase 1 trial, ALT and all other liver enzymes appeared unaffected by plasma concentrations of PFOA up to 1530 μM. Nor did it appear hepatic functional changes were altered as analyzed by fibrinogen, PTT, and aPTT.

In rats and mice treated with APFO, liver effects such as increased serum ALT and AST values (Butenhoff *et al.*, 2012a; Minata *et al.*, 2010; Son *et al.*, 2008); peroxisome proliferation with subsequent hepatocellular hypertrophy (Elcombe *et al.*, 2010; Haughom and Spydevold, 1992; Loveless *et al.*, 2006;); and increased incidence of hepatocellular adenoma in rats (Biegel *et al.*, 2001; Butenhoff *et al.*, 2012a) were reported, but these changes were attributed to PPARα activation, a mode of action unlikely to result in human liver tumors (Corton *et al.*, 2014; Das *et al.*, 2017). An increased liver weight reported in an APFO feeding study of monkeys was due, in part, to adaptive hepatocellular hypertrophy possibly as a result of mitochondrial proliferation (Butenhoff *et al.*, 2002); no corroborative changes in either serum ALT or alkaline phosphatase occurred. Studies of the mid-Ohio river valley community reported small shifts in liver enzymes with increasing PFOA (Darrow *et al.*, 2016; Gallo *et al.*, 2012). The C8 Science Panel (2012b) considered this within normal physiologic ranges and found other study population results inconsistent; thus, they were uncertain whether PFOA was the cause of these modest liver enzyme associations but this did not reflect in any increased risk in liver disease, including medically validated enlarged liver, fatty liver, or cirrhosis.

PFOA and Kidney Function

Serum creatinine, urea, and uric acid were not associated with the PFOA concentrations in this phase 1 trial. Two-year bioassays in rats (Biegel *et al.*, 2001; Butenhoff *et al.*, 2012a) and a 6-month study in monkeys (Butenhoff *et al.*, 2002) did not find treatment-related renal effects with APFO. Epidemiologic cross-sectional studies have associated chronic kidney disease in children and adults with PFOA (Kataria *et al.*, 2015; Shankar *et al.*, 2011a) or with hyperuricemia (Geiger *et al.*, 2013; Shankar 2011b; Steenland *et al.*, 2010b); however, these findings were likely confounded by the GFR (Watkins *et al.*, 2013; Dhingra *et al.*, 2017). Dhingra *et al.* (2016) reported the mid-Ohio river valley population was not at an increased risk from chronic kidney disease based on their community worker longitudinal study of modeled cumulative serum PFOA concentrations.

PK/PD Modeling

Individual response is the result of a complex interaction of exposure and biology and it is very difficult to untangle the causal effects (via dependent clinical measures) of an exposure unless they are considered altogether systemically. The novelty of the PK/PD model is the information theoretic GSUA-driven model design and evaluation approach. This approach, incorporating stochastic parameters and state variables, optimizes the trade-off between model complexity, uncertainty, and relevance. Nonlinearity is assessed by considering all pdfs of model input factors and their interdependencies for multiple predicted patterns, interdependencies that are usually neglected. Optimization is achieved by minimizing three independent evaluation criteria (RMSE, KL, and AIC). This modeling approach addresses the uncertainty of data and extends the observed and discrete biologic dynamics to a continuous domain. Hence, the model itself also evaluates the consistency and reliability of the data. Additionally, it helps to identify transition ranges and predict unobserved exposure dynamics.

Study Limitations

The main limitation of the study is that it used as subjects late-stage cancer patients whose metabolic activity may differ considerably from healthy individuals. Although there is no evidence that any of the cancers involved or treatments received prior to the study had systematic effects on the metabolic functions studied, it cannot be asserted with absolute confidence that no such systematic effects existed.

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The sample size and study length is limited, but the observation of impacts on two clinical chemistries (total cholesterol and fT4) suggests that this limitation is not absolute. It may be that other chemistries were affected, but the power of the study design was inadequate to detect the effects. Although subjects were not selected randomly for inclusion in dose groups, but were taken serially over time, this limitation leading to systematic bias by group would depend upon mechanisms not apparent to the investigators. In addition, changes over time within individuals are consistent with changes seen in the population averages and pdfs.

Another limitation as it applies to the epidemiologic generalization of the results is that much lower serum PFOA concentrations, as previously reported in workers, communities affected through PFOA-containing drinking water, and general populations, were not studied. Very low doses having opposite effects on cholesterol, such that increases in cholesterol are seen for PFOA concentrations that are orders of magnitude smaller than those doses administered to these cancer patients, is perhaps plausible. However, we have not uncovered research identifying such a mechanism in humans.

CONCLUSIONS

Health concerns about the low levels of PFOA in the environment have been raised by observational studies. Using data from this phase 1 study of PFOA, we addressed the effects of exposure to a very wide range of PFOA, including extremely high PFOA concentrations. For levels of PFOA more than four orders of magnitude higher than the levels observed in general populations, there was no evidence of any major effects other than a decrease in total cholesterol (but not HDL) and an increase in fT4 (but not TSH) for increasing levels of PFOA plasma concentration. These nonlinear effects that show changes into the probability distribution of cholesterol and fT4 are evidence for the importance of probabilistic versus linear models. Our findings are consistent with animal models and may contribute to focusing the evaluation of human health risks of PFOA in the environment, by motivating re-examination of the implications of population studies exposed to much lower levels of PFOA. These observational studies have reported contrary associations, but currently understood biology does not support the existence of such conflicting effects.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

FUNDING

This work was supported by the sponsor of this phase 1 clinical trial, CXR Biosciences (Dundee, UK). 3M Company (St Paul, Minnesota) licensed a part of the database from CXR Biosciences for the purpose of the analyses presented herein.

CONFLICT OF INTEREST

Matteo Convertino and Timothy Church, from the University of Minnesota, are recipients of research grants from the 3M Company. Geary Olsen is an employee of 3M Company, a former manufacturer of PFOA. Eddie Doyle and Clifford Elcombe were employees of CXR Biosciences. Drs Leslie Samuel, Iain MacPherson, and Thomas Jeffrey Evans are physicians who conducted this phase 1 clinical trial that was sponsored by CXR Biosciences. Anna Barnett was the study director for CXR Biosciences for this phase 1 clinical trial study. Yang Liu has no competing interest.

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