Environmental and Molecular Mutagenesis 54:558–565 (2013)

Research Article

Serum Metabolomic Pertubations Among Workers Exposed to 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD)

Fatemeh Saberi Hosnijeh,[1,2] Alexandros Pechlivanis,[3] Hector C. Keun,[3] Lützen Portengen,[1] H. Bas Bueno-de-Mesquita,[4,5] Dick Heederik,[1] and Roel Vermeulen[1,6]*

[1]*Institute for Risk Assessment Sciences (IRAS), Division Environmental Epidemiology, Utrecht University, Utrecht, the Netherlands*
[2]*Zanjan University of Medical Science, School of Medicine, Biostatistics and Epidemiology department, Zanjan, Iran*
[3]*Department of Surgery and Cancer, Imperial College London, London, United Kingdom*
[4]*National Institute for Public Health and Environment (RIVM), Bilthoven, The Netherlands*
[5]*Department of Gastroenterology and Hepatology, University Medical Center, Utrecht, The Netherlands*
[6]*Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands*

Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been associated with multiple health effects. Mechanistic studies using metabolomics could provide supporting evidence for such associations by identifying relevant biological pathways. In this study, we investigated metabolic perturbations in a cohort of TCDD exposed workers to better understand TCDD related health effects. Eighty one workers who had been exposed to TCDD in the past and 63 nonexposed workers were included in the study. Serum metabolites were detected using ultra high pressure liquid chromatography coupled online to a Q-TOF Premier mass spectrometer with a scan range of 70–1,000 *m/z*. Current plasma levels of TCDD were determined by high-resolution gas chromatography/isotope dilution high resolution mass spectrometry. TCDD blood levels at the time of last exposure were estimated using a one-compartment first order kinetic model. Differentially expressed metabolites were identified using linear regression models, partial least squares regression (PLSr) and a regression-based Bayesian variable selection approach. Features that were present in all quality control samples and had a coefficient of variation <30% were included in the analyses ($n = 421$ features). Adjusted linear regression analysis showed several significant perturbations ($n = 27$; $P < 0.05$) but these observations did not survive multiple testing correction (q value > 0.05). PLSr analyses and Bayesian variable selection regression analyses revealed no obvious metabolic perturbations associated with TCDD levels. This is the first metabolomic analysis related to TCDD exposure in humans. No significant metabolic features were identified. It is concluded that TCDD exposure at levels present in this study does not lead to significant perturbations of the serum metabolome. Environ. Mol. Mutagen. 54:558-565, 2013. © 2013 Wiley Periodicals, Inc.

Key words: dioxin; TCDD; metabolomics; cross sectional study

INTRODUCTION

Over the past 30 years, several studies reported on the adverse health effects of exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a persistent environmental contaminant generated as an unwanted by-product of numerous chemical reactions involving chlorine compounds. It produces a broad spectrum of effects on human organs including the skin, liver, reproductive, nervous,

*Correspondence to: Roel Vermeulen, PhD, Institute for Risk Assessment Sciences, Division Environmental Epidemiology, Yalelaan 2, PO Box 80178, 3508 TD, Utrecht, The Netherlands. E-mail: R.C.H.Vermeulen@uu.nl

Received 30 May 2013; provisionally accepted 12 June 2013; and in final form 13 June 2013

DOI 10.1002/em.21802

Published online 1 August 2013 in Wiley Online Library (wileyonlinelibrary.com).

hematopoietic and immune system [IARC, 1997]. Moreover, based on animal and human epidemiology data, TCDD was classified by the WHO's International Agency for Research on Cancer (IARC) as a "known human carcinogen." This spectrum of toxicities is known to be mediated via its binding to the aryl hydrocarbon receptor, a specific intracellular protein expressed by major cell types of the immune system [Marshall and Kerkvliet, 2010].

Recently, comprehensive analysis of endogenous small molecules (metabolites) present in cells, tissues, organs, and biological fluids commonly referred to as metabolomics has found broad application in the identification of markers of exposure and disease. Such analyses may aid in our understanding of disease mechanisms and the effects of toxicants on the biological system as metabolic markers result from a complex interplay among gene expression, protein expression, and the environment [Kaddurah-Daouk and Krishnan, 2008]. Recently, animal and *in vitro* studies have shown metabolic changes in lipid accumulation, fatty acid beta-oxidation, inflammation and alteration of amino acids and phase II drug-like metabolism related to TCDD exposure [Lin et al., 2011; Ruiz-Aracama et al., 2011].

The aim of the current study was to investigate pertubations in the serum metabolome possibly related to TCDD exposure in a retrospective cohort of Dutch workers, part of the IARC multinational study of workers exposed to chlorophenoxy herbicides, chlorophenols and dioxins [Bueno de Mesquita et al., 1993; Kogevinas et al., 1997; Hooiveld et al., 1998; Boers et al., 2010]. We previously found evidence for several health effects associated with TCDD exposure including mortality from all causes, ischemic heart disease, and non-Hodgkin's lymphoma (NHL) within this population [Boers et al., 2012].

MATERIAL AND METHODS

Study Population

The cohort study design and exposure assessment have been previously described in detail [Bueno de Mesquita et al., 1993; Hooiveld et al., 1998; Boers et al., 2010]. The cohort consists of workers from two chlorophenoxy herbicide producing factories. Current analyses utilized a subset of workers from factory A ($n = 92$) who were exposed to TCDD as a byproduct of production of 2,4,5-trichlorophenoxyacetic acid and 2,4,5-trichlorophenol during 1953 to 1969, and/or during an occupational accident in 1963 and a random sample of workers from factory B ($n = 78$) who were not exposed to TCDD. In factory B, the main products were 4-chloro-2-methylphenoxyacetic acid (MCPA), 4-chloro-2-methylphenoxy propanoic acid (MCPP), and 2,4-dichlorophenoxyacetic acid (2,4-D). These compounds are unlikely to be contaminated with TCDD, but can be contaminated with other chlorinated dioxins including dichlorodibenzo-*p*-dioxins and hexachlorodibenzo-*p*-dioxins. All study subjects were male. The study was conducted with the approval of the Medical Ethics Committee of the University Medical Center Utrecht, the Netherlands and written informed consent was obtained from each study subject after the study was explained. Participants were asked to complete a self-administered questionnaire, which included questions on occupational history, personal medical history, medication used in the weeks prior to the blood collection, anthropometric characteristics, smoking status and alcohol intake. Blood plasma samples were collected according to a standard protocol during home visits between May 2007 and September 2008 [Boers et al., 2012; Saberi Hosnijeh et al., 2012].

Exposure Measurements

Heparin plasma samples of all subjects were analyzed for TCDD, at the Centers for Diseases Control and Prevention (CDC; Atlanta, USA) using high-resolution gas chromatography/isotope-dilution high-resolution mass spectrometry. Results were lipid adjusted and reported as parts per trillion (ppt) [Patterson et al., 1991]. TCDD is highly persistent with a long half-life in blood and human tissues. As we measured current levels of TCDD ($TCDD_{current}$) ~35 years since last exposure (lag), a one-compartment first order kinetic model with a TCDD half-life ($t_{1/2}$) of 7.1 years was used to estimate TCDD blood levels at the time of last exposure ($TCDD_{max}$) [Saberi Hosnijeh et al., 2011; Boers et al., 2012; Saberi Hosnijeh et al., 2012]:

$$\mathrm{TCDD_{max}} = \mathrm{background} + (\mathrm{measured\,TCDD} - \mathrm{background}) \times \exp(\ln(2) \times \mathrm{lag}/t_{1/2})$$

Current TCDD levels and estimated maximum TCDD levels were subsequently used to investigate exposure-response relations between TCDD levels and serum metabolites.

Serum ion Metabolites

Sample Preparation

Nearly 60 μl of cold MeOH was added to 20 μl of each serum sample and after vortexing, incubated overnight in −20°C. The samples were centrifuged and 35 μl of the supernatant were mixed with 25 μl of the standard mix. The standard mix consisted of Methionine C13, Tryptamine D4, Phenylalanine C13, Hippurate D2 and Acetylcarnitine D3. Nearly 20 μl of each sample were pooled and mixed to make the quality control (QC) sample.

Chromatography

The samples (5 μL) were injected onto a 2.1 × 100 mm^2 (1.7 μm) HSS T3 Acquity column kept at 50°C (Waters, Milford, MA) and eluted using a 27 min gradient of 99.5% A to 99.5% B (A = water, 0.1% formic acid; B = acetonitrile, 0.1% formic acid), with the last 4 min as column re-equilibration. Samples were analyzed using an ultra high pressure liquid chromatography (UPLC) system (UPLC Acquity, Waters, Elstree, UK) coupled online to a Q-TOF Premier mass spectrometer (Waters MS Technologies, Manchester, UK).

Mass Spectrometry

The mass spectrometer was operating in positive electrospray mode (ESI) with a scan range of 70–1,000 *m/z*. ESI conditions were as follows: source temperature 120°C, desolvation temperature 350°C, desolvation gas flow 800 L h^{-1}, capillary voltage 3 kV, sample cone voltage 10 V. Mass spectrometric conditions were optimized through direct infusion of available BA standards. The Q-TOF Premier was operated in V optics mode with scan time of 0.5 s and an interscan delay of 0.1 s. A solution of 200 pg μL^{-1} (50:50 acetonitrile: water) leucine enkephalin (*m/z* 556.2771) was used as the lock mass. Data were collected in centroid mode. The paired samples were analyzed consecutively in two batches. The QC sample was injected regularly throughout the run (after every six samples) to assess the stability of the analytical platform.

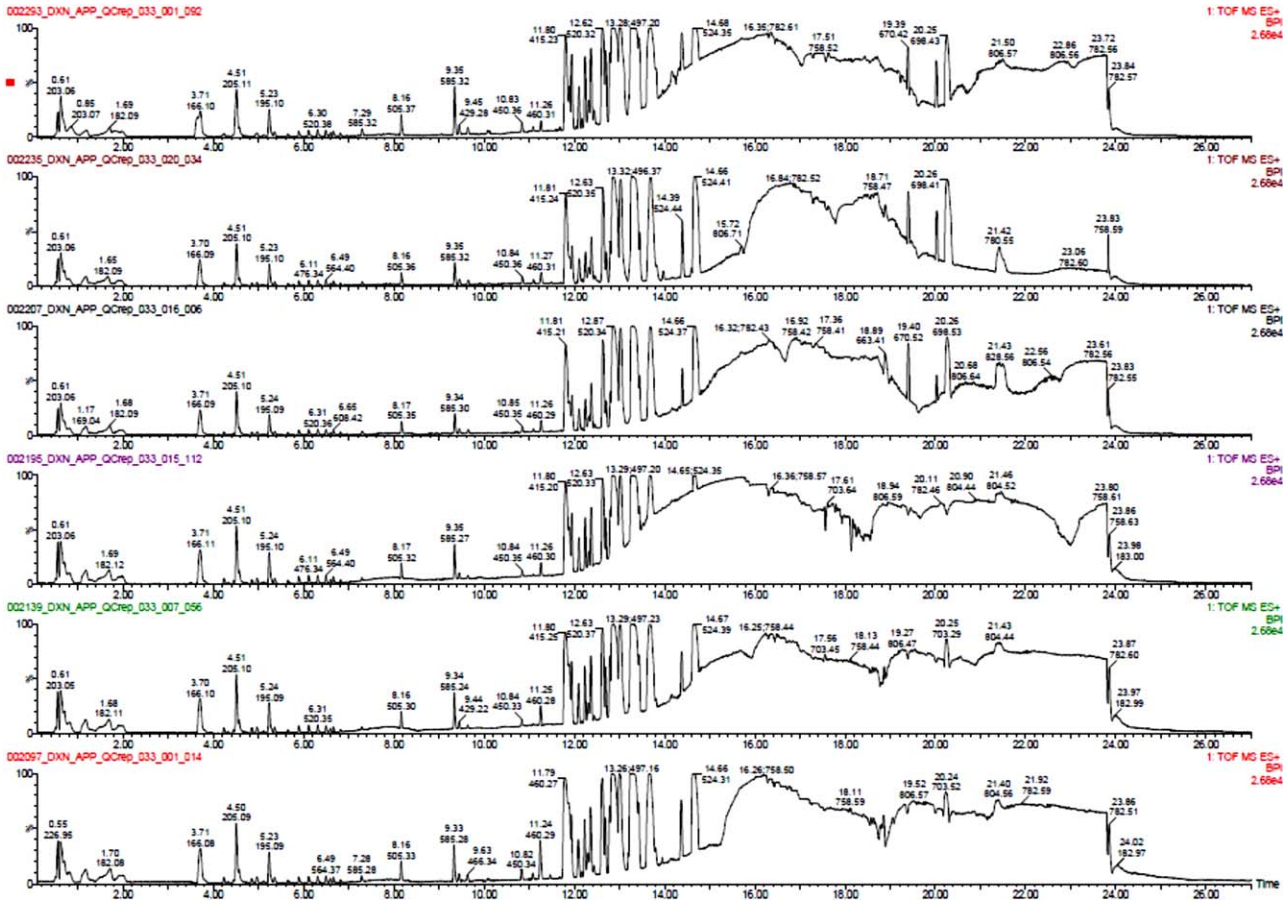

Fig. 1. Base peak ion chromatograms of quality control samples of both batches, showing good retention time and intensity reproducibility. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Data Extraction

Visual inspection of the chromatograms showed good retention time and intensity reproducibility up to 13 min where the lipids that could not be washed of the column were accumulated (Fig. 1).

Data Normalization

DataBridge tool, implemented in MassLynx4.1 software (Waters), was used to export the raw data in netCDF format. Data processing was performed using XCMS software. As the peak-picking algorithm "Centwave" was chosen, and the peak width for the peak picking was set to values 3–20 sec [Veselkov et al., 2011]. Different data pretreatment methods (i.e., Normalization of the data to the total area, median fold change normalization, pareto scaling, log transformation, and feature-wise normalization) were tested on the data set. Finally, a feature-wise normalization was performed in which the average intensity for every feature on the QCs of each batch was calculated and the corresponding intensities of each sample in that batch were divided with this value. The two batches were analyzed separately using the exact same parameters and then the results "stitched" in a single table. For further analysis of the data we used the features that were present in all the QC samples and had a coefficient of variation (CV) $<30\%$ ($n = 421$ features).

Statistical Analysis

Individual TCDD levels which were below the limit of detection (exposed workers $n = 30$, nonexposed workers $n = 45$) were imputed using a maximum likelihood estimation method previously described [Boers et al., 2012]. TCDD measures were log-transformed as measured levels appeared to follow a log-normal distribution. Differences in continuous and categorical parameters between exposed and nonexposed subjects were tested using a two sample t test and chi-square test, respectively.

Differentially expressed metabolites were identified using linear regression analysis of the relation between log-transformed TCDD (as the independent variable) and individual metabolites (as dependent variable), adjusted for potential confounders body mass index (in kg m^{-2}; continuous variable); alcohol intake (unit/week; continuous variable), smoking status (categorical variable), medication (categorical variable), and chronic and acute medical conditions (categorical variables). P values were calculated using a Wald test and corrected for multiple testing using the positive false discovery rate (pFDR) approach [Storey, 2003]. The pFDR has been defined as the conditional FDR given that at least one hypothesis is rejected. Calculated q values are a measure of significance related to the proportion of false positive findings.

Partial least squares (PLS) regression [Mevik and Wehrens, 2007], a dimension reduction technique, was used to find the latent variables formed by linear combination of predictor variables that maximize the covariance between these latent variables and the response variable(s). We used leave-one-out cross-validation to estimate the optimal number of latent variables (components) to create. Predictor variables (metabolites) were mean-centered and scaled before PLS regression analyses. In addition a regression-based Bayesian variable selection with spike-and-slab priors of (nonlinear) generalized additive models [Scheipl et al.,

TABLE I. General Characteristics of Exposed and Nonexposed Workers

	Exposed (n = 81)	Nonexposed (n = 63)	P value[a]
Age (years)[b]	69.03 (7.65)	59.54 (9.30)	<0.0001
Body mass index (kg m^{-2})[b]	26.94 (3.00)	26.95 (3.60)	0.99
Alcohol intake (units/week)[b]	13.58 (13.43)	14.37 (15.32)	0.74
Smoking status, N (%)			0.92
Current smoker	18 (22.2%)	15 (23.8%)	
Former smoker	49 (60.5%)	36 (57.1%)	
Never smoker	14 (17.3%)	12 (19.0%)	
Skin cancer, N (%)	6 (7.4%)	2 (3.2%)	0.27
Infectious disease in the past 4 weeks, N (%)	7 (8.6%)	5 (7.9%)	0.88
Chronic disease, N (%)[c]	43 (53.1%)	27 (42.9%)	0.22
Chronic inflammatory disease, N (%)[d]	20 (24.7%)	15 (23.8%)	0.90
Medication, N (%)			0.27
Immunosuppressant	7 (8.6%)	2 (3.2%)	
NSAIDs	20 (24.7%)	12 (19.0%)	
Antibiotics	0	1 (1.6%)	
$TCDD_{current}$ (ppt)[e]	2.09 (6.74)	0.44 (5.13)	<0.0001
$TCDD_{max}$ (ppt)[f]	29.91 (34.22)	0.44 (5.13)	<0.0001

[a]P values from t tests for continuous variables and X^2 tests for categorical variables.
[b]Mean (standard deviation); NSAIDs: nonsteroidal anti-inflammatory drugs.
[c]Chronic diseases included: diabetes, coronary heart disease, and hypertension.
[d]Chronic inflammatory diseases: chronic obstructive pulmonary disease, psoriasis, sarcoidosis, asthmatic bronchitis, rheumatoid arthritis, liver failure, Crohn's disease, fibromyalgia and allergy.
[e]Current levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin ($TCDD_{current}$) parts per trillion, geometric mean (geometric standard deviation).
[f]Estimated maximum levels of TCDD ($TCDD_{max}$) parts per trillion, geometric mean (geometric standard deviation).

2012] was used to (1) choose an appropriate subset of potential covariates and their interactions, (2) to determine whether linear or more flexible functional forms are required to model the effects of the respective covariates, and (3) to estimate their shapes.

As more than 50% of high and low exposed workers had a chronic disease, analyses were also run using subjects free of chronic disease at the time of blood draw as a sensitivity analysis. Moreover, analyses were repeated for the exposed factory only (factory A).

Statistical analyses were performed using the R 2.13.2 language and environment (The R Foundation for Statistical Computing, Auckland University, Auckland, New Zealand) and SAS 9.2 (SAS Institute, Cary, NC).

RESULTS

Characteristics of Participants

Out of 170 subjects, TCDD was measured successfully in 165 workers. We excluded 16 subjects (seven workers from factory A and nine from factory B) with a previous cancer diagnosis (other than skin cancer) from the analyses to remove the possibility that the metabolite profile may have been changed due to malignant disease or medications used. Five subjects with high amount of polyethylene glycol that can cause ion suppression were excluded as well. This resulted in 144 subjects available for analysis: 81 exposed workers from factory A and 63 nonexposed workers from factory B. Subject characteristics are shown in Table I. Exposed workers were significantly older than nonexposed workers. Distribution of other covariates among exposed and nonexposed workers was similar. More than 50% of exposed and nonexposed workers suffered from chronic diseases such as diabetes, cardiovascular diseases and hypertension. Geometric mean (GM) and geometric standard deviation (GSD) levels of $TCDD_{current}$ and historical maximum exposure ($TCDD_{max}$) were significantly higher in exposed workers ($TCDD_{current}$: 2.09 ± 6.74 ppt; $TCDD_{max}$: 29.91 ± 34.22 ppt) compared to presumed nonexposed workers ($TCDD_{current}$ and $TCDD_{max}$: 0.44 ± 5.13 ppt).

Metabolite Data

Pairwise (Pearson) correlations between the 421 different metabolite levels were generally modest (Fig. 2), with over 80% of the correlations below 0.2 and only 5% exceeding 0.4.

Dimension Reduction and Variable Selection Results

Linear regression models adjusted for covariates did not reveal any obvious candidates as illustrated by the flat P value distribution (Fig. 3). Although, 27 features were identified to be associated with current TCDD level using an adjusted linear regression model, these features were not significant after pFDR adjustment (Table II). Cross-validation of the PLS regression model resulted in a single-component model that explained 24% of the variation in log-transformed TCDD levels (Fig. 4). However, the loading plot (Fig. 5) indicates that the PLS regression did not result in a model with clear single metabolite effects, but rather indicates general higher metabolite levels

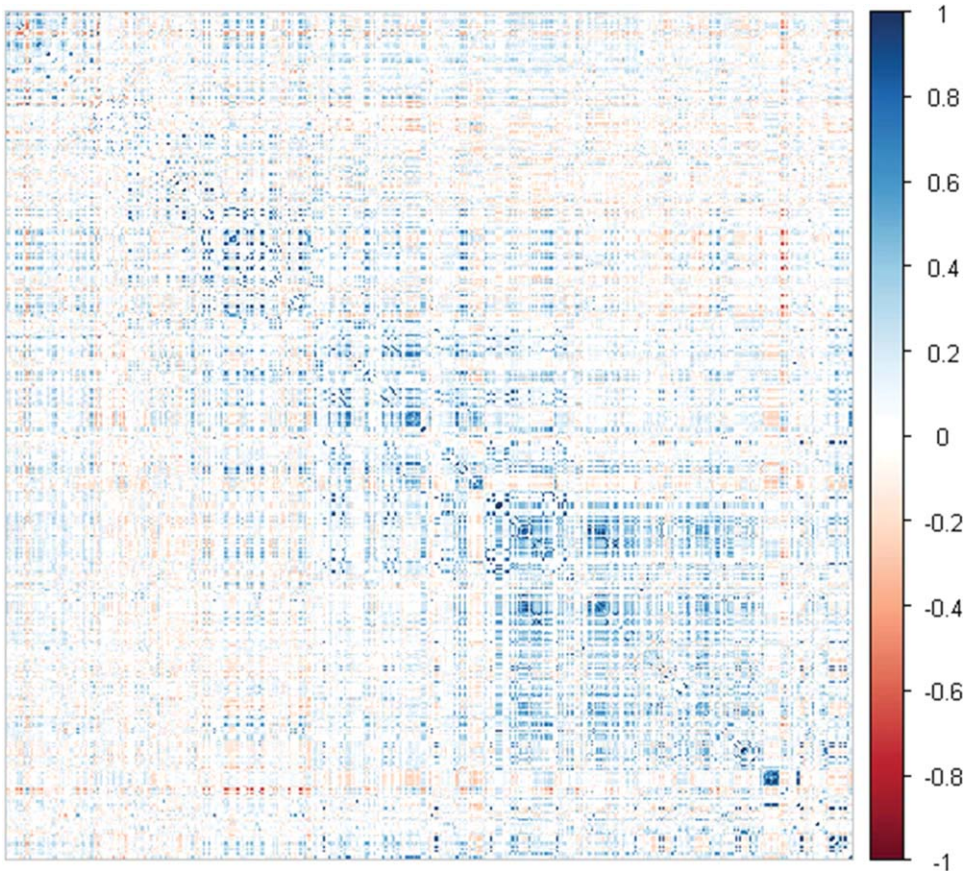

Fig. 2. Heatmap showing the pairwise Pearson correlations between different metabolites.

in exposed workers. The Bayesian variable selection approach (unadjusted) suggested that linear component of one feature (*m/z* feature of 381.28) was related to TCDD exposure [marginal posterior inclusion probabilities *P* (gamma = 1) = 0.263, term importance (pi) = 0.067, level of significance > 0.25] (data not shown). However, the model adjusted for all covariates showed no significant (non)-linear effects in measured metabolites either.

Sensitivity analyses among exposed workers from factory A only, among subjects without chronic disease only, and models with estimated TCDD blood levels at the time of last exposure ($TCDD_{max}$) showed overall similar results.

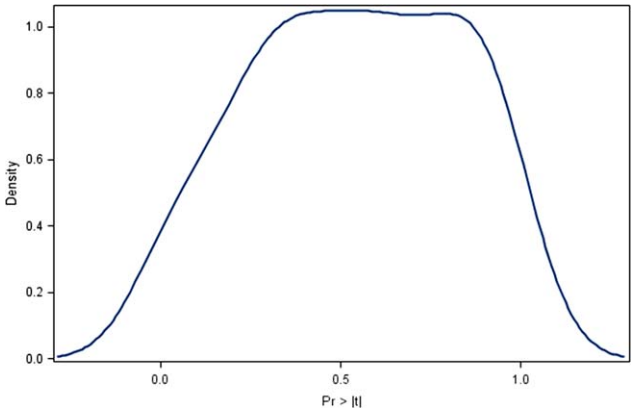

Fig. 3. *P* value distribution of adjusted generalized linear regression models for TCDD blood level in relation to *m/z* features. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

TCDD is the most toxic form of dioxins which has been comprehensively examined in multiple acute, subchronic, and chronic animal and human studies. It can cause reproductive and developmental problems, damage the immune system, and interfere with hormones [IARC, 1997]. Moreover, TCDD is carcinogenic in experimental animals, but has not been conclusively proven to cause cancer in humans. As such, evidence for a carcinogenic effect in humans has remained controversial [Boffetta et al., 2011].

In this study we looked at perturbations in metabolic profiles of TCDD exposed workers to provide more insight in the disease and exposure processes. The exposed subjects in our study are among the highest historically exposed occupational individuals, based on back-extrapolated blood TCDD concentrations, and

TABLE II. Mean and Standard Deviation of Log-Transformed TCDD Levels for Exposed and Nonexposed Workers and Adjusted Linear Regression Slope Estimates for the Relation Between Individual *m/z* Features and Log-Transformed TCDD Blood Levels and Corrected *P* Values

		Mean (standard deviation)				
m/z feature	Retention time	Exposed	Nonexposed	Estimate[a]	*P* value	pFDR; *Q* value
426.3317	642.709	0.839 (1.551)	0.798 (1.795)	−0.364	0.0004	0.4657
373.2862	642.7964	0.936 (1.653)	0.792 (1.601)	−0.32	0.0023	0.4657
511.336	464.4749	1.158 (0.492)	0.984 (0.228)	1.097	0.0038	0.4657
445.2994	446.8566	1.046 (0.340)	0.962 (0.244)	1.418	0.0054	0.4657
525.3186	378.8389	0.899 (0.157)	0.886 (0.106)	3.142	0.0057	0.4657
489.3256	458.9924	1.106 (0.413)	0.980 (0.228)	1.174	0.0080	0.4657
467.3123	453.1441	1.082 (0.377)	0.963 (0.233)	1.232	0.0095	0.4657
209.9357	1432.553	0.985 (0.119)	0.996 (0.101)	3.439	0.0108	0.4657
302.2439	510.5203	1.205 (0.868)	0.867 (0.668)	−0.488	0.0111	0.4657
401.2803	433.2547	1.050 (0.313)	0.951 (0.208)	1.428	0.0111	0.4657
520.3628	378.8389	0.928 (0.150)	0.905 (0.086)	3.057	0.0131	0.4657
696.4804	417.6791	1.005 (0.255)	0.939 (0.168)	1.707	0.0135	0.4657
564.3918	389.7549	0.945 (0.171)	0.918 (0.107)	2.553	0.0148	0.4657
653.4544	409.1598	0.980 (0.222)	0.946 (0.158)	1.895	0.0154	0.4657
565.396	389.7549	0.944 (0.168)	0.911 (0.110)	2.466	0.0189	0.4805
609.4255	399.7146	0.956 (0.201)	0.920 (0.135)	2.04	0.0197	0.4805
608.4216	399.714	0.969 (0.199)	0.919 (0.129)	2.071	0.0201	0.4805
652.4506	409.1605	0.987 (0.230)	0.924 (0.156)	1.774	0.0207	0.4805
569.3483	389.7549	0.944 (0.177)	0.888 (0.115)	2.347	0.0230	0.4805
740.5136	425.5416	1.003 (0.283)	0.954 (0.228)	1.339	0.0236	0.4805
327.2289	361.8583	0.036 (0.092)	1.109 (5.819)	−0.093	0.0239	0.4805
521.3669	378.8389	0.917 (0.153)	0.900 (0.088)	2.657	0.0277	0.5271
357.2933	749.3755	0.949 (0.925)	1.023 (1.078)	−0.351	0.0288	0.5271
461.2917	708.3252	0.854 (0.122)	0.911 (0.141)	−2.508	0.0318	0.5582
309.0766	1431.932	0.951 (0.306)	0.958 (0.136)	1.316	0.0358	0.6036
488.2906	900.2296	1.114 (0.270)	0.596 (0.387)	−0.978	0.0384	0.6226
586.2607	1105.999	0.921 (0.517)	0.837 (0.306)	−0.716	0.0480	0.7492

Features with *P* value <0.05 have been shown.

[a]Linear regression models adjusted for factory, age, body mass index, chronic disease, chronic inflammatory disease, infectious disease, medication, smoking status, and alcohol intake; statistical significance of the slope estimates (*P* value) was determined using a Wald test and was used to calculate corrected *P* values (*Q* values) according to the positive false discovery rate (pFDR) approach.

certainly higher than levels that have been found in environmental settings. This is the first study in humans, to our knowledge, that evaluated the patterns or fingerprints of metabolites in response to TCDD exposure. Screening with linear regression analysis did show some significant perturbations ($P < 0.05$) but these did not survive multiple testing correction (q values > 0.05). Application of two complementary statistical methods namely PLSr and regression-based Bayesian variable selection did not reveal any obvious targets as well, except an indication of higher metabolite levels in exposed workers in PLSr analyses. However, it should be note that these analyses were unadjusted for confounder variables which cannot be directly done in PLSr analyses. Although our study did not show significant changes in measured features in relation to TCDD blood levels, several animal and *in vitro* studies have shown metabolic changes of TCDD toxicity. A recent animal study that evaluated the toxic effects of TCDD using metabolite profiling of blood samples of AhR sensitive mice showed a variety of metabolic shifts in pathways

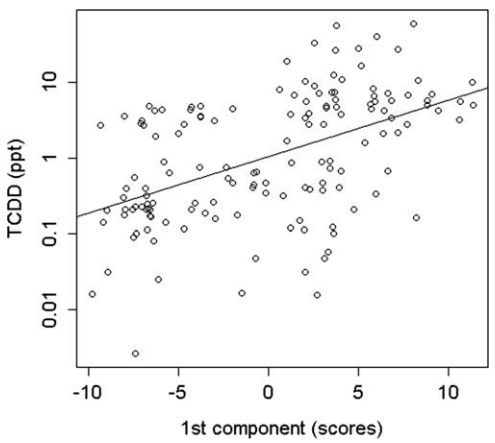

Fig. 4. Regression of log-transformed TCDD levels on the estimated latent variable from a single-component PLS model that explains 24.6% of the variation in log-transformed TCDD levels between individuals.

of lipid accumulation, fatty acid beta-oxidation, inflammation and alteration of amino acids and phase II drug-like metabolism [Lin et al., 2011]. Another *in vitro*

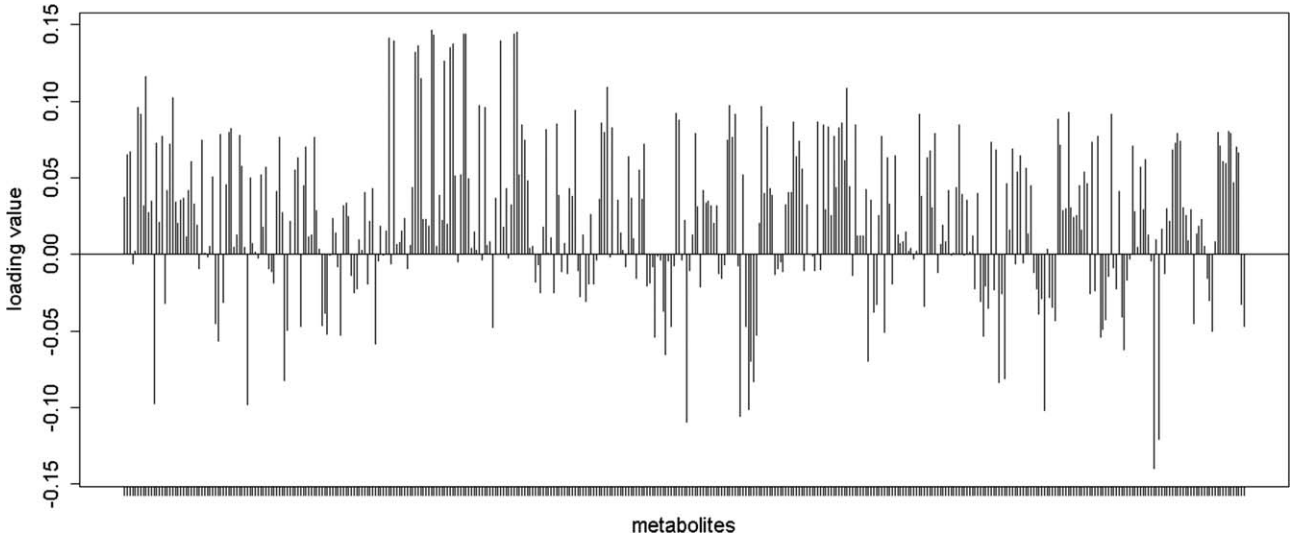

Fig. 5. Loading plot of the PLS regression model for log-transformed TCDD levels and metabolite levels. Ticks along the horizontal axis indicate individual metabolites.

study on liver cell line HepG2 exposed to TCDD also showed changes in the general metabolism of these cells, which involved fatty acids, amino acids and nucleotides [Ruiz-Aracama et al., 2011]. We did not confirm such perturbations in humans exposed to TCDD.

Typically samples show high variability in metabolite concentration, and the derived metabolic profiles by UPLC/MS method have a heteroscedastic noise structure characterized by increasing variance as a function of increased signal intensity. These sources of experimental and instrumental noise substantially complicate information recovery when statistical tools are used [Veselkov et al., 2011]. Our metabolite data had a good reproducibility up to 13 min in the chromatograms. Moreover, we used different normalization approaches to reduce the systematic variation and only features with a coefficient of variation <30% were used in the final analyses. As such data quality does not seem to be the reason for an absence of any obvious metabolic pertubations.

Our study had some limitations. First, workers with relatively high exposures may have died or been unable to participate, which might have led to selective survival bias in our results. Second, we assumed a single one-compartment first-order kinetic model for all workers. However several publications have reported that elimination of TCDD is more consistent with a two-compartment model, with a rapid elimination for high body concentrations in the first 3 years followed by a slower elimination [Michalek et al., 2002; Aylward et al., 2005]. We had no data available in our cohort to investigate elimination speed. Therefore, for simplicity and because of its widespread use, we used a first order kinetic model. Moreover, exposed workers were not closely matched to the non-exposed workers with regard to age. Although analyses were adjusted for age, some residual confounding might have remained due to changes in the levels of exposure, changes in life-style and differences in work setting. However, internal analyses among exposed only (Factory A), where age differences were minimal, did not reveal any effect either hinting that the age difference between exposed and unexposed subjects did not cloud the analyses. We allowed metabolic features with a coefficient of variance <30% into the analyses. This choice was based on an attempt to strike a balance between avoiding inclusion of metabolic features that are too noisy and thereby possibly weakening the ability to detect significant (other) findings and being overly conservative. Finally, as changes in metabolite concentrations are minimal and the levels within an individual vary over time, sufficient biological samples and repeated experiments are essential for reliability (in terms of statistical analysis) and to detecting relatively small differences in analyte concentrations. We can therefore not exclude that we missed minor biological perturbations due to the relatively small study size.

In conclusion, no significant TCDD related pertubations were identified in this study. However, future studies should extend on the current database and to potentially replicate some of the suggestive metabolic findings of this study.

ACKNOWLEDGMENTS

The authors thank Wayman E. Turner for blood plasma analyses of PCDDs, PCDFs, and PCBs (Division of Environmental Health Laboratory Sciences, Centers for Disease Control and Prevention, Atlanta GA, USA) and all workers for participation in the study.

AUTHOR CONTRIBUTIONS

RV, DH and HB designed the study and applied for Research Ethics Board approval, recruited the patients and collected the data and designed the experiments. AP and HK performed the experiments. FSH analyzed the data with important intellectual input from LP and prepared the manuscript. All authors approved the final manuscript.

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