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Human urinary biomarkers of dioxin exposure: Analysis by metabolomics and biologically driven data dimensionality reduction

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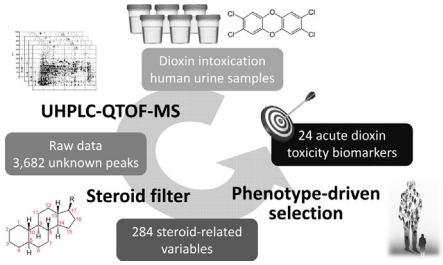
HIGHLIGHTS

- Highlight of biomarkers of dioxin exposure by metabolomics.
- Data dimensionality reduction done by chemical and biological knowledge.
- Steroid and bile acid pattern are modified by dioxin exposure.

GRAPHICAL ABSTRACT



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ABSTRACT

Untargeted metabolomic approaches offer new opportunities for a deeper understanding of the molecular events related to toxic exposure. This study proposes a metabolomic investigation of biochemical alterations occurring in urine as a result of dioxin toxicity. Urine samples were collected from Czech chemical workers submitted to severe dioxin occupational exposure in a herbicide production plant in the late 1960s. Experiments were carried out with ultra-high pressure liquid chromatography (UHPLC) coupled to high-resolution quadrupole time-of-flight (QTOF) mass spectrometry. A chemistry-driven feature selection was applied to focus on steroid-related metabolites. Supervised multivariate data analysis allowed biomarkers, mainly related to bile acids, to be highlighted. These results supported the hypothesis of liver damage and oxidative stress for long-term dioxin toxicity. As a second step of data analysis, the information gained from the urine analysis of Victor Yushchenko after his poisoning was examined. A subset of relevant urinary markers of acute dioxin toxicity from this extreme phenotype, including glucuro- and sulfo-conjugated endogenous steroid metabolites and bile acids, was assessed for its ability to detect long-term effects of exposure. The metabolomic strategy presented in this work allowed the determination of metabolic patterns related to dioxin effects in human and the discovery of highly predictive subsets of biologically meaningful and clinically relevant compounds. These results are expected

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to provide valuable information for a deeper understanding of the molecular events related to dioxin toxicity. Furthermore, it presents an original methodology of data dimensionality reduction by using extreme phenotype as a guide to select relevant features prior to data modeling (biologically driven data reduction).

1. Introduction

Dioxins are toxic and extremely persistent and as they degrade very slowly and accumulate through the food chain, many people may be repeatedly exposed to low amounts of dioxin-like compounds, making this a question of public health concern (Gies et al., 2007; Steenland et al., 2001). Their influence on human health at low levels remains poorly understood. The effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are mainly mediated by its binding to the aryl hydrocarbon receptor (AhR) (Bock and Kohle, 2009). Hepatic damages, neurological effects, diabetes, atherosclerosis and hypertension were associated with human acute dioxins exposure. TCDD has been classified as a human carcinogen by the World Health Organisation and the US National Toxicological Program (IARC, 1997). This fact is controversial, because all evidences for a relationship between TCDD and human carcinogenesis are indirect. TCDD has a very long biological half-life and is not mutagenic, and no study could demonstrate that TCDD is a carcinogen in humans (Boffetta et al., 2011).

The most severe human intoxications occurred after industrial accidents due to uncontrolled formation of TCDD, *e.g.* Seveso (Italy) in 1976 (Bertazzi et al., 2001), Neratovice (Central Bohemia in the former Czechoslovakia) in 1965–1968 (Pelclova et al., 2001) and Ludwigshafen (Germany) in 1953 (Zober et al., 1997). The massive spreading of Agent Orange, a TCDD-contaminated herbicide, during the Vietnam War also led to severe intoxications (Gochfeld, 2001). Additional TCDD exposure cases were reported, including two women exposed in Vienna (Austria) in a textile research institute in 1997–1998 (Geusau et al., 2001) and the deliberate poisoning of Ukrainian president Victor Yushchenko (VY) in September 2004 (Saurat et al., 2012; Sorg et al., 2009). All these cases of acute TCDD intoxications resulted in chloracne. This skin lesion plays the role of a sentinel sign for dioxin or dioxin-like compounds exposure, as there is currently no other reliable human biomarker of such intoxication. The confirmation is usually done by direct dioxin measurement in blood, a protocol which is not routinely implemented in most hospital laboratories.

Relevant and sensitive biomarkers are therefore highly desirable to detect the onset of dioxin toxicity before the appearance of clinical manifestations and metabolomics constitutes a promising approach for that purpose. Metabolomics is a blossoming research field which has been demonstrated as a valuable approach to monitor the chemical diversity of complex biological systems from a holistic angle (Goodacre et al., 2004). It aims at depicting the molecular events associated with biological processes characterizing living systems, at the tissue, the organ or the whole organism level. This description can be related to physiological conditions or to the reaction to pathological, environmental or developmental perturbations (Nicholson and Lindon, 2008). Thanks to its ability to monitor alterations of endogenous metabolites levels, but also xenobiotics and their biotransformation products, metabolomics was quickly promoted as a relevant tool for assessing biochemical phenotypes related to toxicity (Lindon et al., 2003).

Due to the ever-increasing sensitivity and resolution of modern analytical platforms commonly used in metabolomics, such as mass spectrometry (MS), a key challenge when achieving untargeted analyses is treating the very high number of recorded signals (Boccard et al., 2010). Biologically relevant hypotheses are harder to find when handling these massively multivariate data. Variable selection intends to reduce the size of the data by selecting subsets of relevant variables, *i.e.* biomarkers able to represent the salient characteristics of the phenomenon under study. Knowledge-based dimensionality reduction is very useful to reduce the size of the hypothesis space and provide more parsimonious solutions to biological problems (Mitchell, 1997).

To date only few metabolomic studies assessing TCDD toxicity based on cell lines or rodent models were reported. Most results highlighted changes related to amino acids and lipid metabolism including fatty acids or bile acids (Forgacs et al., 2012; Lin et al., 2011a,b; Ruiz-Aracama et al., 2011). A metabolomic investigation of serum samples from workers exposed to TCDD was achieved recently but no major alteration could be detected (Saberi Hosnijeh et al., 2013).

Finding a clinically relevant metabolic pattern of dioxin toxicity in humans constitutes a key issue in the perspective of diagnostic and prognostic procedures and long-term exposure is probably the most relevant situation in the context of human healthcare. The objective of this work was to investigate long-term effects of dioxin toxicity related to occupational exposure to TCDD. Urine samples from a cohort of Czech workers were analyzed on a platform hyphenating ultra-high pressure liquid chromatography (UHPLC) to quadrupole time-of-flight (QTOF) mass spectrometry. Since dioxins have been regularly described as perturbing the steroid profile, a validated method dedicated to the analysis of glucuro- and sulfo-conjugated forms of some androgens was applied (Badoud et al., 2011). As the QTOF technology allows the untargeted screening of metabolites, it could help finding new biomarkers, as reported recently for testosterone intake in the context of anti-doping (Boccard et al., 2011). An original data treatment strategy using biological knowledge as well as statistical tools was implemented to obtain a small subset of biomarkers able to characterize cases of TCDD intoxication. For this purpose, an extreme phenotype case was used as a reference. Phenotypic extremes are expected to provide characteristic patterns of toxicity for the detection of reliable biomarkers. The most contributing features detected during the investigation of a case of acute intoxication (extreme phenotype) were selected for further analysis of the long-term effects of dioxin exposure. Therefore, the data analysis strategy integrated chemical and phenotypic information for the detection of relevant toxicity biomarkers.

2. Experimental

2.1. Chemicals and reagents

5α-Androstan-3α-ol-17-one sulfate sodium salt (androsterone sulfate), 4-androsten-17β-ol-3-one sulfate sodium salt (testosterone sulfate), 4-androsten-17α-ol-3-one sulfate sodium salt (epitestosterone sulfate), 5β-androstan-3α-ol-17-one sulfate sodium salt (etiocholanolone sulfate), 5-androsten-3β-ol-17-one sulfate sodium salt (dehydroepiandrosterone sulfate), 4-androsten-17β-ol-3-one glucosiduronate (testosterone glucuronide), 4-androsten-17α-ol-3-one glucosiduronate (epitestosterone glucuronide), 5β-androstan-3α-ol-17-one glucosiduronate (etiocholanolone glucuronide), 5-androsten-3β-ol-17-one glucosiduronate (dehydroepiandrosterone glucuronide) and 5α-androstan-17β-ol-3-one glucosiduronate (dihydrotestosterone glucuronide) were purchased from Steraloids (Newport, RI, USA). 4-Androsten-17α-ol-3-one sulfate (epitestosterone sulfate), 5α-androstan-3α-ol-17-one-3α-D-glucuronide (androsterone glucuronide), [16,16,17α-$^{2}H_3$]androst-4-en-17α-ol-3-one sulfate triethylammonium salt (TS-d3), [16,16,17β-$^{2}H_3$] androst-4-en-17β-ol-3-one sulfate (ES-d3), [2,2,3β,4,4-$^{2}H_5$]5β-androstan-3α-ol-17-one sulfate (EtioS-d5), [2,2,4,4-$^{2}H_4$]5α-androstan-3α-ol-17-one sulfate (AS-d4), [16,16,17α-$^{2}H_3$]androst-4-en-17β-ol-3-one glucuronide (TG-d3), [16,16,17α-$^{2}H_3$]androst-4-en-17α-ol-3-one glucuronide (EG-d3), and

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[2,2,4,4-$^2H_4$]5α-androstan-3α-ol-17-one glucuronide (AG-d4) were purchased from LGC Standard GmbH (Wesel, Germany). Water, acetonitrile (ACN) and methanol (MeOH) were ULC–MS quality and were obtained from Biosolve (Valkenswaald, The Netherlands). Formic acid (FA) and ammonium hydroxide were purchased, respectively, from Merck (Darmstadt, Germany) and from Sigma–Aldrich (Buchs, Switzerland).

2.2. Urine samples

2.2.1. Czech cohort

An uncontrolled decomposition reaction occurred during the production of the herbicide trichlorophenol acetic acid in a Czech plant during the years between 1965 and 1968. It leaded to the formation of TCDD. The TCDD blood levels of 80 workers were estimated to about 5000 pg/g blood lipid at the time of exposure (Pelclova et al., 2001). Among these patients, 11 are still medically followed by the Charles University of Prague in Czech Republic (Pelclova et al., 2002, 2006, 2007, 2009, 2011; Urban et al., 2007). 24 h urine samples were collected in 2011 and kept at −80 °C. These 11 workers were born between 1940 and 1946 and were exposed to dioxin for periods from 10 days to 23 months (Pelclova et al., 2009). Samples from healthy volunteers matched for age (65–70 years old) were also collected to constitute the control group (CTRL65 group, $n = 11$). These samples were provided by the Geneva University Hospital (anonymous urines with non-pathological results, protocol number CE 11-111 approved by the ethical commission).

2.2.2. Victor Yushchenko

VY was poisoned the 6th September 2004. His TCDD blood level was about 108,000 pg/g fat four months after intoxication (Sorg et al., 2009). Various urine samples were collected in Ukraine or in Switzerland the months and years following the poisoning (Saurat et al., 2012). These samples were then kept at −80 °C in Geneva. Eleven samples were available for analysis: the first samples were collected about 15 months after the poisoning and the last sample about 3 years later. The samples were labeled with the abbreviation VY followed by the number of months after intoxication. The day of intoxication (06.09.2004) was set to time 0. Samples: VY-15.5 (22.12.2005), VY-21.5 (23.06.2006), VY-22.9 (05.08.2006), VY-23 (08.08.2006), VY-28.5a (20.01.2007), VY-28.5b (21.01.2007), VY-28.5c (22.01.2007), VY-32.2 (14.05.2007), VY-34.1 (11.07.2007), VY-39.3 (14.12.2007) and VY-46.1 (10.07.2008). Samples VY-28.5a to VY-28.5c corresponded to three collects of 24 h at three successive days. Samples from healthy volunteers matched for age (50–55 years old) were also collected to constitute the control group (CTRL50 group, $n = 12$). These samples were provided by the Geneva University Hospital (anonymous urines with non-pathological results, protocol number CE 11-111 approved by the ethical commission).

2.3. Analysis of urine samples

Urine samples were analyzed in triplicate by using a solid phase extraction protocol prior to UHPLC–MS. A thorough description can be found in Badoud et al., 2011. Briefly, 1 mL of urine was diluted with 1 mL of 2% FA spiked with 10 μL of the internal standard solution (ES-d3, EtioS-d5, AS-d4, TG-d3, EG-d3 and AG-d4). Oasis HLB cartridges of 30 mg (96-well plate) were conditioned with 500 μL of MeOH and equilibrated with 1 mL of 2% FA. The diluted urine samples were added to the cartridges. Washing was done by 1 mL of 2% FA, followed by 1 mL of a 5% ammonium hydroxide/MeOH (90/10, v/v) solution. Metabolites were eluted with 500 μL of MeOH/water (40/60, v/v) and then evaporated to dryness under azote stream. The dry residues were resuspended in 100 μL of ACN/water (30/70, v/v).

2.4. Instrumental conditions

An Acquity UHPLC-QTOF-MS (Xevo™, Waters) system was used for the analysis of steroid and other metabolites in urine. Compounds were separated on an Acquity UPLC column (BEH C18 150 mm × 2.1 mm, 1.7 μm), preceded by a Van Guard precolumn (BEH C18, 5 × 2.1 mm, 1.7 μm). A gradient of mobile phase A (0.1% FA in water) and mobile phase B (0.1% FA in ACN) was used linearly from 5% to 37% B over 25 min, followed by a washing step of 95% B for 3 min and a re-equilibration of 8 min. The sample volume injected was 10 μL in the full loop mode and the samples were maintained at 4 °C in the autosampler.

The QTOF Xevo was equipped with an electrospray ionization (ESI) source operated in the negative mode. Following operating conditions were used: the desolvation gas flow was 800 L/h with a temperature of 360 °C, the capillary voltage was defined at 2.4 kV, the cone voltage was kept constant at 50 V, the source temperature was 120 °C, the cone gas flow and the collision gas flow were set to 10 L/h and 0.25 mL/min, respectively. Two functions were used for the acquisitions: a wide-pass quadrupole mode with low collision energy (5 eV) for the first function (range m/z 95–1000) and a collision energy ramp of 5–70 eV for the second function (MS^E, range m/z 50–1000). Data were collected in centroid mode with a scan time of 0.2 s and an interscan delay of 0.02 s. Dynamic range enhancement (DRE) was used and recalibration of the data were made with the infusion of a solution of 2 ng/mL of Leucine-enkephalin (Sigma–Aldrich, Buchs, Switzerland) through the Lock Spray probe at 20 μL/min.

2.5. Data analysis

Raw data pre-treatment, including baseline correction, peak detection, chromatogram alignment, peak integration and normalization were achieved with MarkerLynx XS™ (SCN 854, Waters). The parameters were set as follows: peak detection from 1 to 29.5 min in the m/z 95–1000 mass range, the mass tolerance set to 0.05 amu, chromatogram alignment performed using EG-d3 internal standard, the peak width at 5% height set to 25 s, the peak-to-peak baseline set to 15, the mass window set to 0.05 amu, retention time window set to 0.8 min, noise elimination level set to 15, the marker intensity threshold set to 500 counts and the normalization performed according to the sum of the total reconstituted spectra. A first filtering procedure was achieved with the MarkerLynx XS™ *Replicate* function to select only reliable peaks. Individual samples were then considered as biological replicates and peaks were kept for further analysis when they were present at least in 50% of a defined group (VY samples, Czech workers, *etc.*). This strategy allowed the removal of noisy and irrelevant variables from the data matrix and a reduced data table of 3682 ions was obtained.

Chemistry-driven dimensionality reduction was performed with in-house routines under the MATLAB® 7 environment (The MathWorks, Natick, USA). Orthogonal partial least squares discriminant analysis (OPLS-DA) models were computed with the SIMCA-P software (version 12, Umetrics, Umeå, Sweden). A leave-one-out cross-validation was achieved to assess the predictive ability of the models. CV-ANOVA (Eriksson et al., 2008) and permutation tests were performed to ensure the validity of the models.

3. Results and discussion

3.1. Exploratory analysis of the Czech cohort: long-term effect of dioxin toxicity following an acute occupational exposure

Between 1965 and 1968, a group of Czech chemical workers (approximately 80 people) were subjected to intoxication with dioxins in a herbicide production plant in Central Bohemia, a region of former Czechoslovakia. Early examinations were done in the 1970s and all men suffered from chloracne. This symptom was still reported for two patients, 30 years after exposure with extremely high plasma levels of TCDD. Plasma lipid alterations were observed during the follow-up period and several patients manifested neuropsychological, and neurological abnormalities (Pelclova et al., 2001). TCDD levels between 25 and about 400 times the normal population and chronic effects including hyperlipidaemia, hypertension and diabetes were further described 40 years after exposure (Pelclova et al., 2009). These previous studies underlined therefore the elevated persistence of TCDD in the body associated with long-term effects on human health. It has to be noted that these effects can be observed several decades after severe intoxication and metabolic disorders are expected to subsist. Of the 80 intoxicated workers, urine samples of 11 workers collected in 2011 were available for the present study. These samples were analyzed to evaluate the long-term effect of dioxin toxicity (about 40 years after exposure).

Several advantages are derived from the use of urine as a reference biofluid to monitor toxicity, such as easy and non-invasive sampling and reduced sample preparation compared to blood as no protein precipitation is required (Ryan et al., 2011). However, the complexity of urine samples remains high when performing untargeted experiments, even after ensuring the proper selection of consistent data from the raw signal. Additionally, their content can be greatly modified by several physiological and environmental factors, such as age, diet, diurnal variation and medication. Measuring metabolites in urine in an untargeted manner remains therefore a challenging task due to the numerous confounding factors. In clinical practice, a knowledge-based dimensionality reduction may therefore be highly desirable to avoid idiosyncratic responses related to a therapeutic intervention during untargeted analyses. Chemical data may constitute a valuable source of information to define criteria for selecting variables and simplifying multivariate models.

A chemistry-driven dimensionality reduction was recently proposed to filter untargeted metabolomic data and specifically

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assess urinary steroid metabolites in the context of doping control (Boccard et al., 2011). As dioxin is known to alter steroid metabolism, a similar procedure was applied in this study to focus on the steroid subset of urinary compounds (Egeland et al., 1994; Grochowalski et al., 2001; Kleeman et al., 1990; Manh et al., 2013; Sechman et al., 2011). In practical terms, raw data were processed with MarkerLynx XS^{TM} to generate a list of 3682 peaks per sample (see Section 2 for more details). Each of the 3682 unidentified peaks was then compared with a series of reference m/z values extracted from the LipidMaps database, related to endogenous glucuronidated and sulfated steroid conjugates from the *sterol lipids* class (Fahy et al., 2009). Peaks matching a reference entry within a tolerance interval of ±0.025 amu were considered as putative steroid-related metabolites and retained for further analysis, while retention times were not considered in the selection process. A series of 284 steroid-related compounds was obtained after this second selection step corresponding to approximately 7.7% of the detected features. The steroid nature of the selected compounds could be checked by the analysis of fragmentation patterns obtained in the MS^{E} mode.

Data were Pareto-scaled and an OPLS-DA model was computed to distinguish exposed patients ($n = 11$) from a group of healthy volunteers ($n = 11$) on the basis of the 284 variables (model#1). This modeling approach allows a straightforward interpretation of the results by separating the predictive variation from orthogonal sources of systematic variability (Trygg and Wold, 2002). The predictive scores and loading vectors are less prone to undesirable variations and relevant patterns can be more easily related to a meaningful biological context. The control group was composed of healthy men matched for age (65–70 years old) with non-pathological results for urine dipstick chemical analyses. A model with 2 latent variables (one predictive and one orthogonal) was obtained and a clear separation of the two classes was observed on the score plot (Fig. 1A). The model validity was estimated by LOOCV and satisfactory statistical indices were reported, *i.e.* global accuracy of 95.5%, specificity of 90.9%, sensitivity of 100.0% and CV-ANOVA p-value $p < 0.05$. Due to the relatively limited number of samples available for building the model ($n = 22$) and as cross-validation may be overly optimistic when assessing predictive ability, the risk of overfitting must be considered. Despite this limitation, the contribution of the 284 variables was assessed by the investigation of the S-plot related to the predictive component (Fig. 1B). This representation allows both the amplitude of variation and the reliability of each variable to be summarized in a practical way by combining covariance and correlation information, respectively (Wiklund et al., 2008). By these means, the most discriminant variables could be highlighted (black circles, ●). Some of them, such as etiocholanolone glucuronide and androsterone glucuronide, were unambiguously identified by comparing their mass spectra and retention times with authentic standards.

Additionally, other steroid metabolites were partially identified on the basis of accurate mass measurements and several unknown features were tentatively associated to bile acids. Dioxin-induced increased levels of glycocholic acid and its glucuronide metabolite (m/z 464.299 at 13 min and m/z 640.333 at 6.5 min), glycodeoxycholic acid (m/z 448.306 at 20.3 min), chenodeoxycholic acid sulfate or ursodeoxycholic acid sulfate (m/z 471.24 at 23.2 min) and both glucuro-and sulfoconjugates of glycochenodeoxycholic acid (m/z 624.338 at 22 min and m/z 528.262 at 20.3 min). Decreased concentrations due to dioxin exposure were associated with glucuroconjugated forms of the following steroids: hydroxyandrosterone or hydroxyetiocholanolone (m/z 481.241 at 10.1 min), hydroxy DHEA or hydroxytestosterone (m/z 479.228 at 9.6 min), etiocholanolone (m/z 465.246 at 20.6 min) and estrone (m/z 445.19 at 6.3 min). Glycoursodeoxycholic acid glucuronide (m/z 624.336 at 14 min) and sulfate (m/z 528.261 at 11.8 min) metabolites were also decreased. Steroids and bile acids have numerous structural isomers or diastereoisomers that cannot be resolved by mass spectrometry fragmentation. Therefore the mentioned identified metabolites could also correspond to an isomer or a diastereoisomer. The identification of these markers can only be made with authentic standards, but modified levels of this class of compounds were detected.

These results were in accordance with previous studies, as altered cholesterol metabolism and bile acid biosynthesis was associated to TCDD toxicity in several *in vitro* and *in vivo* models. Transcriptomics highlighted altered mRNA levels of genes related to cholesterol and fatty acids biosynthesis and glucose metabolism in the liver of TCDD daily-exposed mice (Sato et al., 2008). Expression changes in genes involved in cholesterol metabolism and bile acids synthesis were similarly reported in rats after exposure to high-dose of TCDD (Fletcher et al., 2005; Moffat et al., 2010). TCDD exposure was also associated with the disruption of lipid metabolism in guinea pigs by altering genes involved in adipogenesis and lipogenesis (Nishiumi et al., 2008). As a whole, these results support the hypothesis of cholesterol homeostasis dysregulation following TCDD exposure mediated by AhR.

As already observed by several authors in various metabolomic studies, the number of variables in the current model (284) is too high for an exhaustive identification of all putative biomarkers and for a direct interpretation. Another filtering step was thus performed by taking advantage of the data originating from an identified dioxin acute intoxication, *i.e.* urines of Victor Yushchenko.

3.2. Exploratory analysis of Victor Yushchenko urine samples: extreme phenotype due to an acute poisoning with TCDD

Victor Yushchenko (VY), candidate for the presidency in Ukraine was poisoned with a single high dose of dioxin during a dinner in September 2004. He became severely ill, suffering of gastritis, colitis, hepatitis, neuropathy and pancreatitis. Chloracne appeared a few weeks later. The symptoms were clearly described and prior analyses suggested an acute intoxication with pure TCDD (Saurat et al., 2012; Sorg et al., 2009).

A significant approach in epidemiology consists in the identification and the specific study of patients with clinically relevant phenotypes, rather than unselected patients, to progress more efficiently in the understanding of a disease (Perez-Gracia et al., 2010). As multifactorial effects can produce intricate phenomena hindering proper interpretation in human studies, these *phenotypic extremes* are expected to facilitate detection of the characteristic symptoms of a disease. In the context of toxicology, a collection of various phenotypes can range from chronic low dose to acute toxic exposure. More particularly, the characterization of extreme phenotypes such as acute intoxication episodes may provide valuable information for a deeper understanding of the cellular and molecular events occurring after toxic exposure. In other words, the discovery of biomarkers of an acute intoxication could provide information for a better understanding of TCDD exposure.

Due to the pathologies VY suffered and his political schedule, no structured experimental setup could be defined for sample collection: sampling and collection times of the urinary samples were noted but irregular. Urines samples of VY were collected at different time points after the intoxication (about 1 year to 4 years after the poisoning, see Section 2).

Preliminary investigations were done to evaluate the urines of VY as a homogeneous group and to select the most relevant features able to distinguish VY from a control group of the same age. Furthermore, this preliminary study was mandatory to exclude the presence of undesirable trend or temporal bias in this particular urine set. For the latter, no temporal drift after the poisoning could be clearly established using various statistical tools. To decrease the

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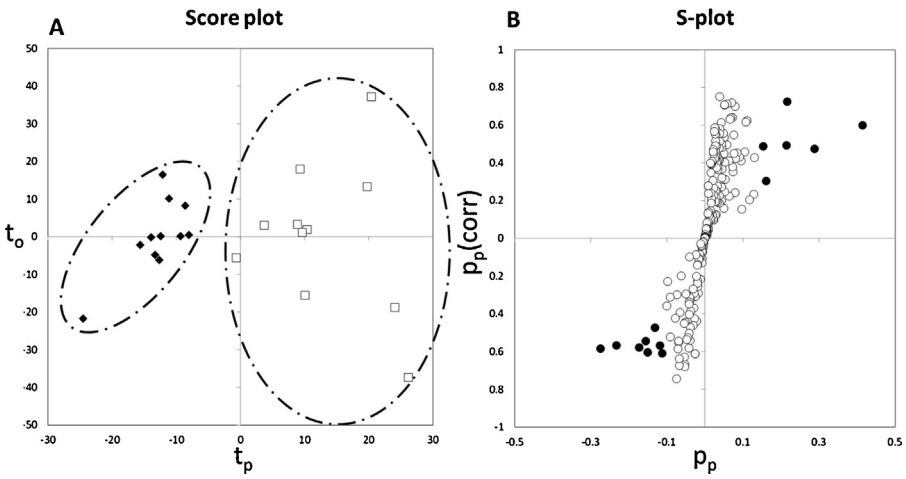

Fig. 1. OPLS-DA model#1 based on 284 steroid-related variables. (A) Score plot: urine samples from Czech chemical workers exposed to dioxin are symbolized with black diamonds (♦) and samples taken from healthy volunteers by white squares (□). (B) S-plot: discriminating variables are symbolized by black circles (●).

number of exogenous markers originating from therapy at a particular sampling time, features detected in at least 50% of VY samples were retained (see Section 2). This procedure allowed metabolites related to specific drug delivery to be removed from the data and to decrease intra-individual variability.

Multivariate data analysis was performed to differentiate urine samples from a control group of volunteers matched for age and the samples collected during Yushchenko's medical care. Pareto scaling was applied to the data table and an OPLS-DA model was computed. A satisfactory model with one predictive and one orthogonal latent variable was obtained (model#2) and a clear separation of the two classes of observations was observed on the score plot (data not shown). Cross-validation was carried out indicating a significant CV-ANOVA p-value $p < 0.001$. No false negative or false positive prediction was reported. A S-plot was then built to highlight the most relevant markers. Identifications were investigated in multiples open sources databases such as the Human Metabolome Database, the METLIN Metabolite Database or Chemspider for exogenous compounds. Despite the removal of unreliable MS signals during data pre-treatment, first observations indicated putative structures mainly matching exogenous compounds. These results were closely related to the various drug therapies VY received during the years following poisoning.

The two first top-ranked elevated markers were assigned to propofol glucuronide (m/z 353.150 at 16 min) and 4-hydroxy-propofol hydrogensulfate (m/z 273.072 at 7.2 min). Propofol is a widespread sedative-hypnotic agent used for the induction and maintenance of general anesthesia. The metabolism of propofol is well studied and various urinary metabolites were described (Favetta et al., 2000). The variable corresponding to the propofol glucuronide was found to be very intense in some urine samples (*e.g.* VY-28.5a) and its MS^E spectra corresponded to the expected fragmentation pattern. As a complementary indication for this identification, the medication report confirmed that VY was in surgery at the corresponding sampling time points. Other anesthetic agents, such as sevoflurane (m/z 166.992 at 6.4 min) or its metabolite hexafluoroisopropanol (HFIP, same chemical formula: $C_3H_2F_6O$) were also highlighted. According to the literature, sevoflurane, HFIP and HFIP glucuronide could be retrieved in urine (Accorsi et al., 2005). Some non-steroidal anti-inflammatory drugs were also detected, such as ibuprofen metabolite (m/z 381.151 at 21.6 min). Overall, several of the most characteristically elevated biomarkers of VY samples were due to medication. Nevertheless, poisoning with a very high dose of TCDD could lead to expressed biomarkers that should be detected whatever the drug therapy.

It has to be noted that previous results of blood and urine VY samples highlighted some abnormal levels of steroids (Sorg and Saurat, unpublished results). Therefore, the steroid-based filtering strategy used for the analysis of samples from the Czech Cohort was implemented. Pareto scaling was applied to the data subset of 284 steroid-related features and an OPLS-DA model was assessed. A satisfactory model with one predictive and one orthogonal latent variable was obtained (model#3) and a clear separation of the two classes of observations was observed on the score plot (Fig. 2). Cross-validation was carried out indicating a high accuracy of prediction (90.9%) and a highly significant CV-ANOVA p-value $p < 0.001$. No false negative (sensitivity of 100%) and only a few false positive cases (specificity of 81.8%) were reported.

An S-plot was investigated to highlight relevant biomarkers of acute TCDD toxicity. The urinary levels of a series of 12 compounds were increased in VY samples, while 12 others were decreased when compared to controls. These variables and their putative identification based on reference entries of the steroid filter are displayed in Table 1. Among them, some steroids such as DHEA sulfate, androsterone sulfate and androsterone glucuronide were identified with authentic standards, while others were evaluated by MS^E fragmentation and search in the literature (see Supplementary Material for examples of putative identification).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2013.10.031.

These results were consistent with the scarce clinical data reporting the effects of acute dioxin toxicity with altered steroid metabolism and liver damage. The strong induction of the cytochrome P450 1A1 (CYP1A1) coding gene was reported in a study combining transcriptomics analyses and immunostaining of Yushchenko's skin lesions (Saurat et al., 2012). Cytochrome P450 enzymes form a multigene family metabolizing thousands of endogenous compounds such as steroids as well as exogenous chemicals such as drugs or toxic chemicals. The observed activation of CYP1A1 was associated with dioxin metabolization and the reduction of the systemic toxicity. Altered cholesterol metabolism and bile acid biosynthesis was also associated to TCDD toxicity and cytochrome P450 modulation in several *in vitro* and *in vivo* models (Fletcher et al., 2005; Moffat et al., 2010; Rifkind, 2006). These results support the hypothesis of the dioxin-induced development of oxidative stress by alterations of cytochrome P450s expression and bile acids homeostasis dysregulation (Pelclova et al., 2011; Reichard et al., 2005). Furthermore, other transcripts implicated in the regulation of bile acid synthesis such as CYP7A1, CYP46A1 and

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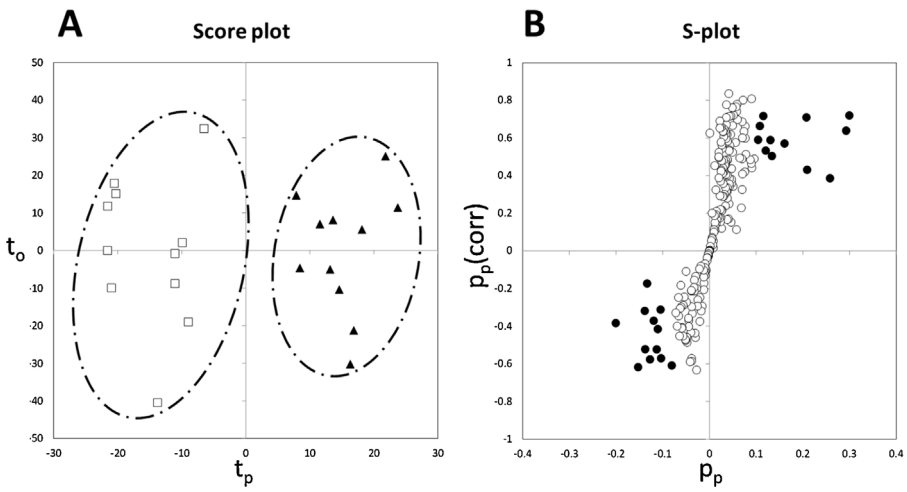

Fig. 2. VY OPLS-DA based on 284 steroid-related variables (model#3). (A) Score plot: urine samples from VY are symbolized with black triangles (▲) and samples taken from healthy volunteers by white squares (□). (B) S-plot: discriminating variables are symbolized by black circles (●).

CYP27A1 were expressed in the skin biopsy of VY (Sorg and Saurat, unpublished results). The 24 variables highlighted to be the most important features to discriminate VY samples were proposed as a metabolic pattern for the detection of acute dioxin toxicity as well as a basis for the selection of features in the Czech dataset.

3.3. *Application of the phenotype-based dimensionality reduction to the Czech cohort*

Based on the biomarkers obtained from the analysis of VY samples, an OPLS-DA model was computed for the Czech dataset on the basis of the 24 selected variables (model#4) as a phenotypic selection filter. A scheme of the knowledge-based variable selection procedure is presented in Fig. 3.

A model with 2 latent variables (model#4, one predictive and one orthogonal) was obtained with a satisfactory prediction accuracy of 90.9% estimated by LOOCV and a significant CV-ANOVA p-value $p < 0.05$. It has to be noted that the two classes were not perfectly separated on the score plot (Fig. 4A), but similarly to model#1, no false negatives (sensitivity of 100%) and a small number of false positive cases (specificity of 81.8%) were reported during cross-validation. These results are particularly relevant in the context of chronic toxicity detection, as no real case of intoxication was wrongly predicted. This further confirms the reliability of the selected biomarkers and their ability to characterize both acute and long-term dioxin intoxication phenotypes.

Interestingly, several compounds highlighted in model#1 were also reported as relevant biomarkers in the restricted subset. According to the S-plot (Fig. 4B), a small number of compounds could be related to the class separation. The upper right region corresponding to decreased concentrations after dioxin exposure included glycoursodeoxycholic sulfate (m/z 528.261 at 11.8 min), hydroxy DHEA or hydroxytestosterone glucuronide (m/z 479.228 at 9.6 min), and hydroxyandrosterone or hydroxyetiocholanolone glucuronide (m/z 481.241 at 10.1 min). The lower left area related to increased levels included glucuronide metabolites of

Table 1
24 Acute dioxin toxicity biomarkers.

Markers	m/z	RT (min)	Putative identifications (or isomeric structure)	Proposed formula
1	367.156	15.4	***DHEAS***	$C_{19}H_{28}O_5S$
2	369.172	10.8	Dihydrotestosterone-S or isomer	$C_{19}H_{30}O_5S$
3	369.172	17.3	Isomeric structure	$C_{19}H_{30}O_5S$
4	369.172	18.6	***Androsterone-S***	$C_{19}H_{30}O_5S$
5	383.151	7.8	Dihydroxyandrostenone-S	$C_{19}H_{28}O_6S$
6	445.190	8.8	Estrone-G or isomer	$C_{24}H_{30}O_8$
7	463.232	17.2	Isomer of epitestosterone-G	$C_{25}H_{36}O_8$
8	464.299	13.0	Glycocholic acid	$C_{26}H_{43}NO_6$
9	465.245	21.2	***Androsterone-G***	$C_{25}H_{38}O_8$
10	465.248	14.3	Isomeric structure (like isomer of dihydrotestosterone-G)	$C_{25}H_{38}O_8$
11	471.246	26.3	Chenodeoxycholic acid-S or ursodeoxycholic acid-S	$C_{24}H_{40}O_7S$
12	479.225	12.0	Hydroxy DHEA-G or hydroxyTestosterone-G or oxo-androsterone-G	$C_{25}H_{36}O_9$
13	479.228	7.7	Hydroxy DHEA-G or hydroxyTestosterone-G or oxo-androsterone-G	$C_{25}H_{36}O_9$
14	481.241	10.1	Hydroxyandrosterone-G or hydroxyetiocholanolone-G	$C_{25}H_{38}O_9$
15	483.259	9.3	Hydroxyandrostane-G	$C_{25}H_{40}O_9$
16	495.294	25.0	Pregnanediol-3-G	$C_{27}H_{44}O_8$
17	528.261	11.8	Glycoursodeoxycholic acid-S	$C_{26}H_{43}NO_8S$
18	567.316	20.1	Deoxycholic acid-G or chenodeoxycholic acid-G	$C_{30}H_{48}O_{10}$
19	583.310	17.2	Cholic acid-G or isomer	$C_{30}H_{48}O_{11}$
20	583.312	9.7	Cholic acid-G or isomer	$C_{30}H_{48}O_{11}$
21	624.338	21.1	Glycochenodeoxycholic acid-G or isomer	$C_{32}H_{51}NO_{11}$
22	624.338	22.0	Glycochenodeoxycholic acid-G or isomer	$C_{32}H_{51}NO_{11}$
23	640.333	6.5	Glycocholic acid 3-G or isomer	$C_{32}H_{51}NO_{12}$
24	640.333	13.4	Glycocholic acid 3-G or isomer	$C_{32}H_{51}NO_{12}$

-S is for sulfate and -G is for glucuronide compound. Steroid confirmed with authentic standards are written in bold italic.
Markers elevated by VY in comparison to the controls are highlighted in gray.

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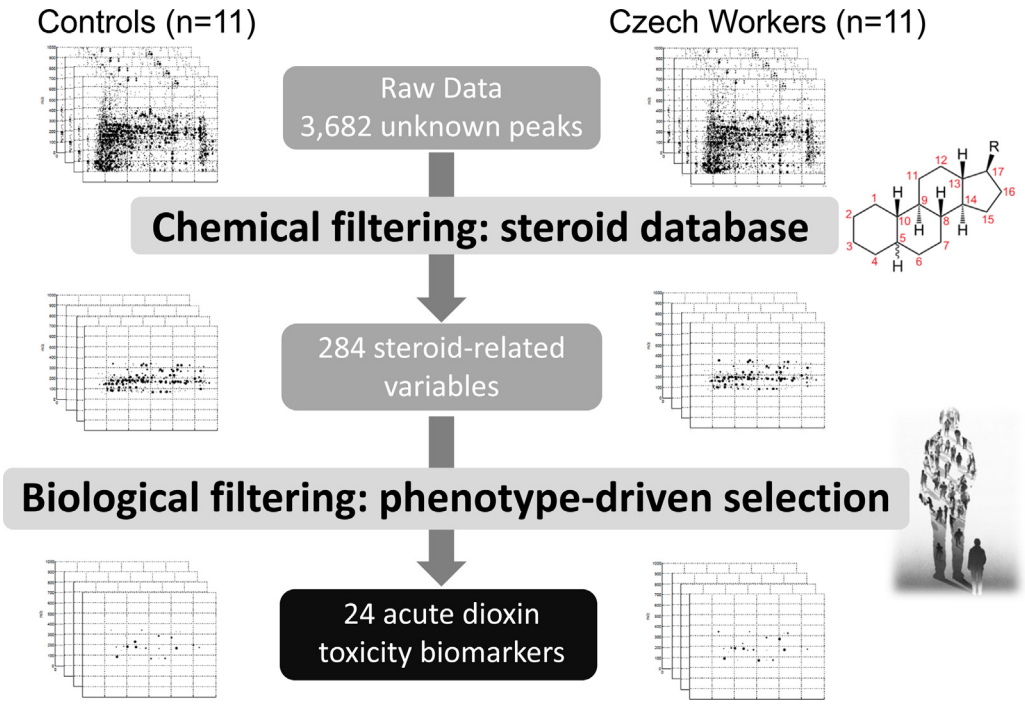

Fig. 3. Knowledge-based variable selection procedure.

chenodeoxycholic acid (*m*/*z* 567.316 at 20.1 min), glycochenodeoxycholic acid (*m*/*z* 624.338 at 22 min) and estrone (*m*/*z* 445.19 at 8.8 min). Furthermore, glycocholic acid and its glucuronide metabolite (*m*/*z* 464.299 at 13 min and *m*/*z* 640.333 at 6.5 min) and dihydrotestosterone sulfate (*m*/*z* 369.172 at 10.8 min) were also tentatively identified as increased biomarkers.

However, it has to be pointed out that the role of each variable is not necessarily similar in both cases and can even be opposite. Actually, the combination of the biomarkers is more relevant than the evaluation of a single parameter taken individually. VY and the Czech cohort are cases of acute exposure. Urine sampling was carried out about 40 years after the exposure of the Czech workers and about 1–5 years after the poisoning of VY. This difference in sampling could lead to different biomarkers of acute exposure, some related to long-term toxicity and some for short/medium term toxicity. The respective role of the biomarkers were investigated by examining their normalized loadings (p(corr)) to the predictive latent variable of both model#3 and model#4 (Fig. 5). Interestingly, some markers had a similar contribution to both dioxin exposure cases. On the other hand, biomarkers characterized by increased levels in VY samples were mainly related to steroids, while samples from the Czech cohort followed a different trend.

Common biomarkers may be due to similar toxic pathway and dissimilarities to different mechanisms. The aryl hydrocarbon receptor does not only act as a transcription factor binding the dioxin responsive element (DRE) and activating metabolizing enzymes such as CYP1A1 and CYP1B1. AhR is also involved in the regulation of other pathways in a DRE-independent manner. As an example, AhR regulates cholesterol biosynthesis by interacting with the sterol element-binding protein 2 (SREPB2) transcription factor (Tanos et al., 2012). Furthermore, cross-talks between AhR and some nuclear receptors such as the estrogen receptor (ER) and the androgen receptor (AR) are well described (Ohtake et al., 2011; Patel et al., 2007). AR itself influences the cholesterol homeostasis

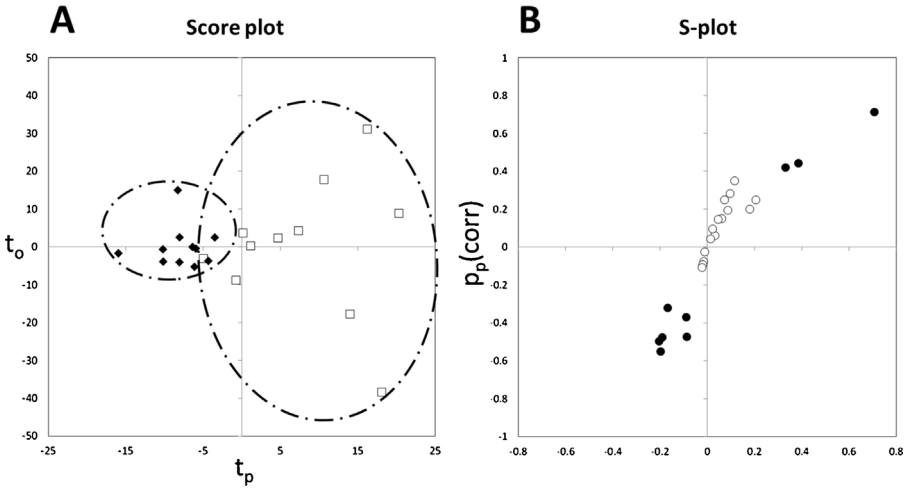

Fig. 4. OPLS model#4 based on 24 acute dioxin toxicity biomarkers. (A) Score plot: urine samples from Czech chemical workers exposed to dioxin are symbolized with black diamonds (♦) and samples taken from healthy volunteers by white squares (□). (B) S-plot: discriminating variables are symbolized by black circles (●).

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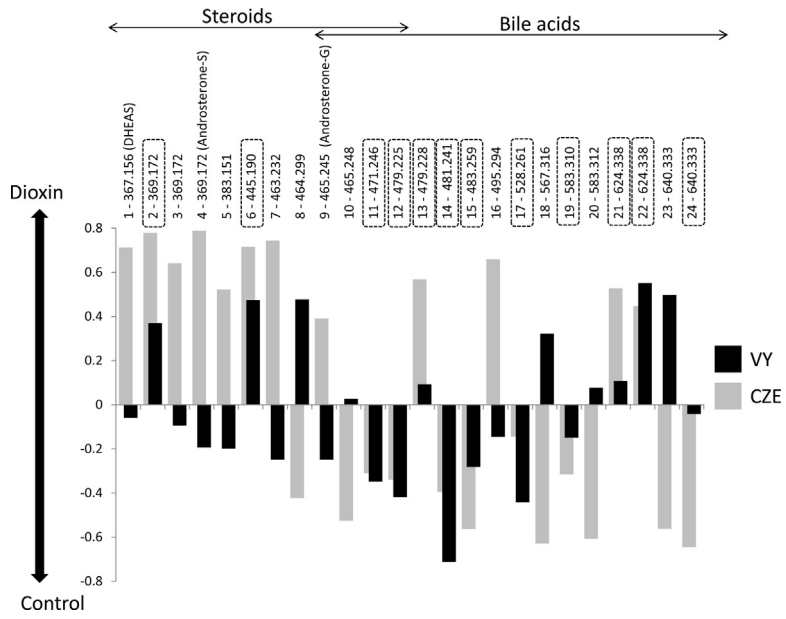

Fig. 5. Biomarkers' normalized loadings from model#3 and model#4. Common contributions are highlighted with dotted lines. For putative biomarkers candidates, see Table 1.

by interacting with the liver X receptor (LXR), which can regulate sulfotransferases and thereby androgens activity (Krycer and Brown, 2011). In mice, it was reported that TCDD deregulates genes of phase I and II metabolism such as genes implicated in cholesterol metabolism (CYP7A1), as well as two nuclear receptors, *i.e.* the short heterodimer partner (SHP) and farnesoid X receptor (FXR) (Fletcher et al., 2005). FXR, similarly to LXR, interacts with sulfotransferases and reduces androgens activity (Runge-Morris et al., 2013). The numerous cross-talks and interactions between nuclear receptors involved in steroids and bile acids regulations (FXR, LXR) indicate two-ways regulations between bile acids metabolism and steroid signaling. This two-way regulation and the description of dioxins effects on both pathways confirmed the perturbations of steroids and bile acids described in animals such as rodents or chickens (Fletcher et al., 2005; Forgacs et al., 2012; Sechman et al., 2011). Disturbances of steroids and bile acids reported in this study could be supported by inferring similar mechanisms between humans and animals exposed to dioxins, taking into account the great variation of doses between the species to observe a toxic effect (Mukerjee, 1998). Some nuclear receptors could be implicated in this process, such as AR and LXR or FXR, but the mechanisms still need to be elucidated. These results provided promising opportunities in the perspective of a better understanding of the effects of dioxin exposure on human health as a reduced list of potential biomarkers of dioxin intoxication was obtained. At this stage, the classical way of biomarkers validation involves their proper identification thanks to comparisons with authentic standard. The biological relevance and the clinical validity of the identified markers are then assessed by *in vivo* studies (animal in case of dioxin) and/or *in vitro* systems. An alternative validation is the analysis of these markers in an independent cohort. In the future, the biomarkers subset should be evaluated and validated in other human cohorts exposed to dioxin.

4. Conclusion

A metabolomic approach was proposed to assess the effects of acute dioxin exposure. Appropriate data pre-treatment procedures and a chemistry-driven filter were implemented to reduce the number of variables to consider and focus on the steroid metabolite content of urine samples. A series of 284 steroid-related variables was investigated and a predictive OPLS-DA model was obtained from the Czech cohort suffering from occupational dioxin exposure. The variables' contributions highlighted relevant biomarkers mainly related to bile acids. As a second step of data mining, prior knowledge collected during the treatment of VY after TCDD acute toxic exposure allowed biologically meaningful compounds to be selected. The 24 most relevant biomarkers were then used to investigate the long-term effects of exposure to dioxin. These results showed altered levels of endogenous steroid metabolites and modified urinary bile acids profiles. Taken together, these findings are compatible with an increased expression of cytochrome P450s, persistent hepatotoxicity, bile acid homeostasis dysregulation and oxidative stress. A reliable model could be evaluated; however, due to the small number of observations, additional targeted investigations of the biomarkers need to be performed for confirmation purpose. The selection of characteristic phenotypes was demonstrated as a very effective approach to detect toxicity biomarkers more easily. The integration of other types of knowledge-based filters such as metabolic pathways is expected to offer valuable alternatives to develop dedicated strategies for the selection of meaningful variables according to the biological context. The long-term follow-up of patients suffering from severe dioxin intoxication over decades is expected to provide new avenues of research to have a better understanding of the effects of these chemicals on human health.

Conflict of interest

The authors declare no conflict of interest.

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