ORIGINAL ARTICLE

Increased oxidative/nitrosative stress markers measured non- invasively in patients with high 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin plasma level

Daniela Pelclova [1], Tomas Navratil [2], Zdenka Fenclova [1], Stepanka Vlckova [1], Karel Kupka [3], Pavel Urban [1], Petr Ridzon [1], Vit Zikan [4], Ludmila Landova [5], Kamila Syslova [6], Marek Kuzma [7], Petr Kacer [6]

1 Department of Occupational Medicine, 1st Medical Faculty, Charles University and General Teaching Hospital, Prague, Czech Republic
2 J. Heyrovský Institute of Physical Chemistry of AS CR, v. v. i., Prague, Czech Republic
3 Institute of Nuclear Medicine, 1st Medical Faculty, Charles University and General Teaching Hospital, Prague, Czech Republic
4 3rd Medical Department, 1st Medical Faculty, Charles University and General Teaching Hospital, Prague, Czech Republic
5 Institute of Clinical Chemistry and Laboratory Diagnostics Faculty, Charles University and General Teaching Hospital, Prague, Czech Republic
6 Institute of Chemical Technology, Prague, Czech Republic
7 Institute of Microbiology, AS CR, v.v. i., Prague, Czech Republic

Correspondence to: Prof. Daniela Pelclová
Department of Occupational Medicine,
1st Medical Faculty, Charles University and General Teaching Hospital
Na Bojisti 1, 120 00 Prague, Czech Republic.
TEL: +420 224 964 532; FAX: +420 224 964 629; E-MAIL: daniela.pelclova@LF1.cuni.cz

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Abstract

OBJECTIVES: 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) is a highly toxic persistent environmental contaminant, classified as a human carcinogen affecting any target organ. The mechanism of carcinogenesis by TCDD is unclear as TCDD shows a lack of direct genotoxicity. Experimental studies also support the role of oxidative stress in TCDD neurotoxicity and vascular dysfunction. The aim was to investigate markers of oxidative/nitrosative stress and inflammation using non-invasive methods in subjects who got ill due to severe occupational exposure to TCDD in the years 1965–1968.

METHODS: In 11 TCDD-exposed patients, and 16 controls, the analysis of following oxidative products of lipids, proteins and nucleic acids in plasma, urine and exhaled breath condensate (EBC) was performed: 8-*iso*-prostaglandin $F_{2\alpha}$ (8-isoprostane), 4-hydroxy-*trans*-2-nonenale (HNE), malondialdehyde (MDA), *o*-tyrosine (*o*-Tyr), 8-hydroxyguanosine (8-OHG), 8-hydroxy-2´-deoxyguanosine (8-OHdG), 5-hydroxymethyluracil (5-OHMeU). In addition, nitric-oxide-tyrosine (NO-Tyr) and leukotriene (LT) B4, C4, D4, and E4 were detected by liquid chromatography-mass spectrometry/mass spectrometry (LC-ESI-MS/MS). TCDD was measured by HRGC/HRMS, body lipid content by densitometry. Single-photon emission spectrometry (SPECT) of the brain was performed and compared with the findings of the patients in 2008.

RESULTS: Mean TCDD plasma level in 2010 was 175 ± 162 pg/g lipids (population level about 2 pg/g), total TCDD content in the body 5.16 ± 4.62 mg. Reduction of cerebral blood flow in SPECT progressed in 8 patients, finding was stable in 2 subjects, and improvement occurred in 1 patient.
In the EBC, 10 from 12 markers (all except LT D4 and LT E4), were significantly increased in the patients (p<0.05). In the urine, 7 markers were significantly higher than in the controls (p<0.05): 8-isoprostane, MDA, HNE, LT C4, LT E4, *o*-Tyr and NO-Tyr. In plasma, only NO-Tyr and 8-OHG were elevated (p<0.05).
CONCLUSION: NO-Tyr was increased in all matrices in dioxin-exposed patients. EBC is not limited to lung disorders as the markers of oxidative stress and inflammation were elevated in EBC of patients with normal lung functions. TCDD-induced oxidative stress and inflammation markers can be detected non-invasively in the EBC and urine in the follow-up of the highly-exposed patients. Their prognostic value, however, needs to be elucidated.

Abbreviations:

5-OHMeU	- 5-hydroxymethyluracil
8-isoprostane	- 8-*iso*-prostaglandin $F_{2\alpha}$
8-OHdG	- 8-hydroxy-2′-deoxy-guanosine
8-OHG	- 8-hydroxyguanosine
5-OHMeU	- 5-hydroxymethyluracil
AhR	- aryl hydrocarbon receptor
BMI	- body mass index
CDT	- carbohydrate-deficient transferin
DNA	- deoxyribonucleic acid
EBC	- exhaled breath condensate
HNE	- 4-hydroxy-*trans*-2-nonenale
HRGC	- high resolution gas chromatography
HRMS	- high resolution mass spectrometry
ICAM-1	- intercellular adhesion molecule 1
LC-ESI-MS/MS	- liquid chromatography-electrospray ionization-tandem mass spectrometry
LDL-cholesterol	- low-density lipoprotein cholesterol
LT	- leukotriene
MDA	- malondialdehyde
NO-Tyr	- nitric-oxide-tyrosine
o-Tyr	- *o*-tyrosine
PAI-1	- tissue plasminogen activator
ROS	- reactive oxygen species
RNS	- reactive nitrogen species
SPECT	- single-photon emission computer tomography
TCDD	- 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin
TEQ	- toxic equivalent (to TCDD concentration)
VCAM-1	- vascular cell adhesion molecule 1

INTRODUCTION

2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) is considered to be a human carcinogen (IARC 1997), however the relationship between carcinogenesis by TCDD and the biochemical effects in humans exposed to TCDD is not fully understood. In addition, there are no markers available that can be used for cancer risk assessment in people exposed to dioxins. In spite of many studies showing a lack of direct genotoxicity, oxidative DNA damage was detected in experimental studies *in vivo* and *in vitro* (Reichard *et al.* 2006, Yoshida & Ogawa 2007).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can lead to multi-organ dysfunction failure, aging, mutations, and cancer. If uncontrolled, they can harm biomacromolecules constituting cells, such as phospholipids, proteins, enzymes, DNA and thus affect physiological processes and/or cause cell death. To quantify oxidative stress level in humans, following specific biomarkers, products of lipids, proteins and nucleic acids can be measured in plasma, urine and exhaled breath condensate (EBC): 8-*iso*-prostaglandin $F_{2\alpha}$ (8-isoprostane), 4-hydroxy-*trans*-2-nonenale (HNE), malondialdehyde (MDA), *o*-tyrosine (*o*-Tyr), 8-hydroxyguanosine (8-OHG), 8-hydroxy-2′-deoxy-guanosine (8-OHdG), 5-hydroxymethyluracil (OHMeU) (Ichinose *et al.* 2004, Chapman *et al.* 2010). In parallel, nitric-oxide-tyrosine (NO-Tyr) (Radi 2004) and inflammation markers leukotriene (LT) B4, C4, D4, and E4 can be analyzed in these matrices. LT B4 is best known for its role in initiating the inflammatory response, produced by leukocytes residing in the tissue in response to stimuli like infection or stress. Recently, roles of LTs′ have been discovered in different kinds of cancers, atherosclerosis and other disorders (Funk 2005).

Oxidative stress was suggested as the crucial pathogenic mechanism inducing impairment of vascular function (Nedeljkovic *et al.* 2003, Radi 2004), nitric-oxide-derived oxidants as inflammatory mediators in coronary artery disease (Shishehbor *et al.* 2011) and endothelial dysfunction was found in TCDD-exposed subjects (Pelclova 2007). However data concerning the effect of RNS in TCDD exposure are very spare.

In humans, the levels of oxidative stress markers in plasma and urine, especially of 8-isoprostane, HNE and MDA, reportedly reflect the aging processes and systemic production (Deravaj 2001). The measurement of the concentration of 8-isoprostane in the exhaled breath condensate (EBC) appears valuable in non-invasive assessing oxidative stress in lung diseases (Loukides *et al.* 2011, Pelclova *et al.* 2007, Pelclova *et al.* 2008). The usefulness of EBC for systemic disorders has not been studied yet.

MATERIAL AND METHODS

Subjects

Eleven former chemical workers (66±1.5 years, BMI 31.2±4.1), exposed to TCDD in the years 1965–1968 during herbicide production and 16 health care workers (7 men, 9 women, 57±9.7 years, BMI 28.4±3.9) were examined. Their smoking and drinking habits did not significantly differ.

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Methods

EBC collection was performed with EcoScreen, Jaeger. All subjects breathed tidally through a mouthpiece connected to the condenser (–20 °C). A constant volume of exhaled air of 120l was maintained. Samples were immediately frozen to –80 °C. Lung functions in patients and controls were measured.

Analysis of oxidative products of lipids, proteins and nucleic acids in EBC, plasma and urine were performed by a combination of a pre-treatment method - lyophilization (conditions: temperature –47 °C; pressure 9 kPa). Detection method – liquid chromatography with mass spectrometry/mass spectrometry analysis (LC-ESI-MS/MS) operated in the selective reaction monitoring mode (SRM) under the stable-isotope-dilution assay conditions was used. Details of the methods are given elsewhere (Syslova *et al.* 2009, 2010 and 2011).

Following markers were detected: 8-isoprostane, HNE, MDA, *o*-Tyr, NO-Tyr, 8-OHG, 8-OHdG, 5-OHMeU, and leukotriene (LT) B4, C4, D4, and E4. Blood and spot urine sample was taken between 8 and 12 a.m. for above mentioned markers of oxidative stress and inflammation, blood count, erythrocyte sedimentation, cholesterol, triacylglyceroles, glycohemoglobin, urea, creatinine, liver enzymes, carbohydrate-deficient transferin (CDT), a marker of chronic ethanol intake, and for urinalysis. Fibrinolysis was characterized by plasma concentrations of tissue plasminogen activator (t-PA), and inhibitor of tissue plasminogen activator (PAI-1); both were determined by ELISA method using Elisatest commercial kits (Hyphen BioMed, France). Serum E-selectin, P-selectin, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) were used as biochemical markers of endothelial activity and were assessed by commercial ELISA kits manufactured by RD System Europe, Ltd (Abingdon, UK).

TCDD was measured by high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS). Body composition measurements were determined using Dual Energy X-ray absorptiometry (DXA) with the Hologic QDR 4500A densitometer (Waltham, USA). Single-photon emission spectrometry (SPECT) of the brain was performed, as previously described (Pelclova *et al.* 2009).

Statistical analysis

Evaluation used Statistical Analysis Software (S.A.S.) 8.2. ANOVA, Student's t-test or Wilcoxon's test, Mann-Whitney and Kolmogorov-Smirnov tests were selected for comparing data between groups. Pearson's and Spearman's correlations were used for analysis of relationships between measured parameters.

RESULTS

TCDD plasma level in 2010 was 175±162 pg/g lipids (population level 2 pg/g), total TCDD content in the body fat 5.16±4.62 mg. Reduction of cerebral blood flow in SPECT progressed comparing with the examination in 2008 (Pelclova *et al.* 2009) in 8 patients (73%), was stable in 2 subjects (18%), and improved in 1 patient (9%).

Blood lipids and glucose in the patients were not significantly elevated. Cholesterol in the patients and controls was 4.83±0.47 and 5.33±0.53 mmol/l, respectively (reference value 3.83–5.8 mmol/l), triacylglycerol 1.7±0.44 mmol/l and 1.16±0.25 mmol/l (reference value 0.68–1.69 mmol/l) and glucose 6.1±1.2 mmol/l and 5.13±0.41 mmol/l (reference value 3.9–5.6 mmol/l). Glycohemoglobin in the patients was 4.97±0.81% (reference value 2.8–4%).

Mean CDT was 1.31±0.24%, and no subject achieved the upper limit of 2.60%.

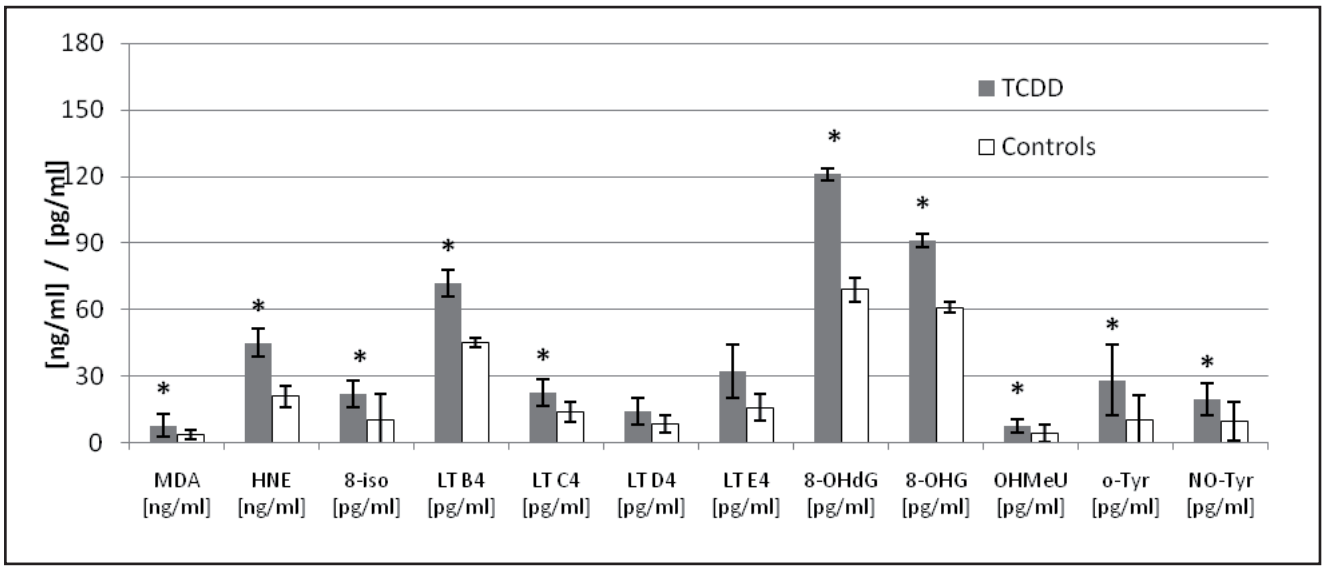

Fig. 1. Oxidative stress markers and leukotrienes of TCDD-exposed patients and controls in the exhaled breath condensate (EBC). MDA and HNE are expressed in ng/ml, all other markers in pg/ml. * $p<0.05$

Serum concentration of ICAM-1 and PAI-1 was significantly higher ($p<0.05$) in exposed subjects (303±42 ng/ml vs. 187±20 ng/ml, and 89±16 ng/ml vs. 48±14 ng/ml, respectively). Other markers of endothelial dysfunction, except P-Selectin, were mildly elevated in the patients but did not reach statistical significance.

Lung function in the patients and controls did not significantly differ. In the EBC, 10 from 12 markers (except LT D4 and LT E4), were significantly increased in the patients, as can be seen in Figure 1. In the urine, 7 markers were significantly higher than in the controls, as shown in Figure 2. In plasma, only NO-Tyr (175±25 vs. 96±19 pg/ml, respectively) and 8-OHG (190±36 vs. 115.1±9.2 pg/ml, respectively) were elevated ($p<0.05$).

NO-Tyr in urine of the patients correlated with the TCDD level, as shown in Table 1. The concentration of NO-Tyr in blood, urine and EBC is presented in Figure 3, where the patients are listed according to the descending level of TCDD.

TCDD in plasma total body TCDD, and I-TEQ correlated with several markers of oxidative/nitrosative stress and inflammation measured in urine, as can be seen in Table 1. On the other hand, no correlations of EBC and blood markers with TCDD parameters have been observed.

Importantly, no impact of sex, body mass index (BMI), high blood glucose, blood lipids, or alcohol consumption (expressed as CDT) on the markers of oxidative/nitrosative stress and inflammation was observed.

Other positive correlations were found, such as I-TEQ with glycaemia (0.633), TCDD body depot with glycaemia (0.620), glycohemoglobin with LDL-cholesterol (0.671), BMI with cholesterol (0.682), and ICAM-1 with SPECT impairment (0.656). On the other hand, SPECT impairment nor ICAM-1 correlated with the TCDD level.

Tab. 1. Correlation of TCDD/I-TEQ with markers of oxidative/nitrosative stress and inflammation measured in urine.

TCDD in plasma	MDA	0.655	$p<0.05$
TCDD in plasma	HNE	0.755	$p<0.01$
TCDD in plasma	LT C4	0.713	$p<0.01$
TCDD in plasma	LT D4	0.738	$p<0.01$
TCDD in plasma	OHMeU	0.703	$p<0.01$
TCDD in plasma	NO-Tyr	0.614	$p<0.05$
TCDD body depot	HNE	0.664	$p<0.05$
TCDD body depot	LT C4	0.624	$p<0.05$
TCDD body depot	LT D4	0.636	$p<0.05$
TCDD body depot	OHMeU	0.630	$p<0.05$
I-TEQ in plasma	HNE	0.690	$p<0.05$
I-TEQ in plasma	LT C4	0.635	$p<0.05$
I-TEQ in plasma	LT D4	0.661	$p<0.05$
I-TEQ in plasma	OHMeU	0.603	$p<0.05$

DISCUSSION

Pathogenesis of TCDD-caused damage is consistent with the hypothesis that TCDD produces most of its toxic effects by binding to a gene regulatory protein, the aryl hydrocarbon receptor (AhR). TCDD, through interaction with AhR, is also known to regulate the expression of a wide range of additional drug-metabolizing enzymes, genes that participate in cell cycle regulation, and inflammatory mediators. While TCDD did not induce DNA damage in most genotoxicity tests, it did induce oxidative DNA damage or increase oxidative stress in several situations, which proves that TCDD is able to induce reactive oxygen species (ROS), even if indirectly. Evidence obtained in animal experiments shows that oxidative stress may be associated with carcinogenesis by TCDD, which also induces complex changes in enzymes of oxidative stress in both adipocytes and liver (Kern *et al.* 2002, Aly 2009).

Subchronic exposure to low doses of TCDD induced oxidative tissue damage in brain tissues which may play a role in the effects of TCDD on the central nervous system (Hassoun *et al.* 1998). TCDD caused dose-dependent increases in superoxide anion and lipid peroxidation in cerebral cortex and hippocampus. Hassoun *et al.* (2003) also demonstrated that subchronic TCDD exposure can stimulate or suppress different antioxidant enzymes in select regions of rat brain. Regional selectivity may be relevant to a consideration of neuropsychological and neurological disorders in humans associated with long-term exposure to TCDD, including our patients (Pelclova *et al.* 2001, Pelclova *et al.* 2002, Urban *et al.* 2007, Preiss *et al.* 2010).

Oxidative/nitrosative stress markers and ICAM-1 and PAI-1, as endothelial dysfunction and fibrinolysis markers were increased in the patients. This confirms our earlier findings (Pelclova 2007), and is in agreement with experimental data. Kopf *et al.* (2010) described recently that TCDD increases reactive oxygen species production in human endothelial cells via induction of cytochrome P4501A1.

Circulating levels of NO-Tyr may serve as a biomarker to assess atherosclerosis risk and appears to be modulated by statin therapy (Shishehbor *et al.* 2003). However, limited data was published concerning TCDD and RNS.

The potential role of LTs in the pathogenesis of TCDD-induced cancers and atherosclerosis damage is not elucidated, however it has been recently suggested that these markers might be used during the treatment of these diseases (Klingerberg 2009).

Highest concentrations of markers of oxidative/nitrosative stress were measured in the blood, however only two markers were significantly elevated comparing to the controls – NO-Tyr and 8-OHG. Most significant differences from the controls were seen in the EBC, and urine. The spectrum of markers was the largest in the EBC, and included markers of nucleic acids oxidation. As lung functions in the patients were not significantly

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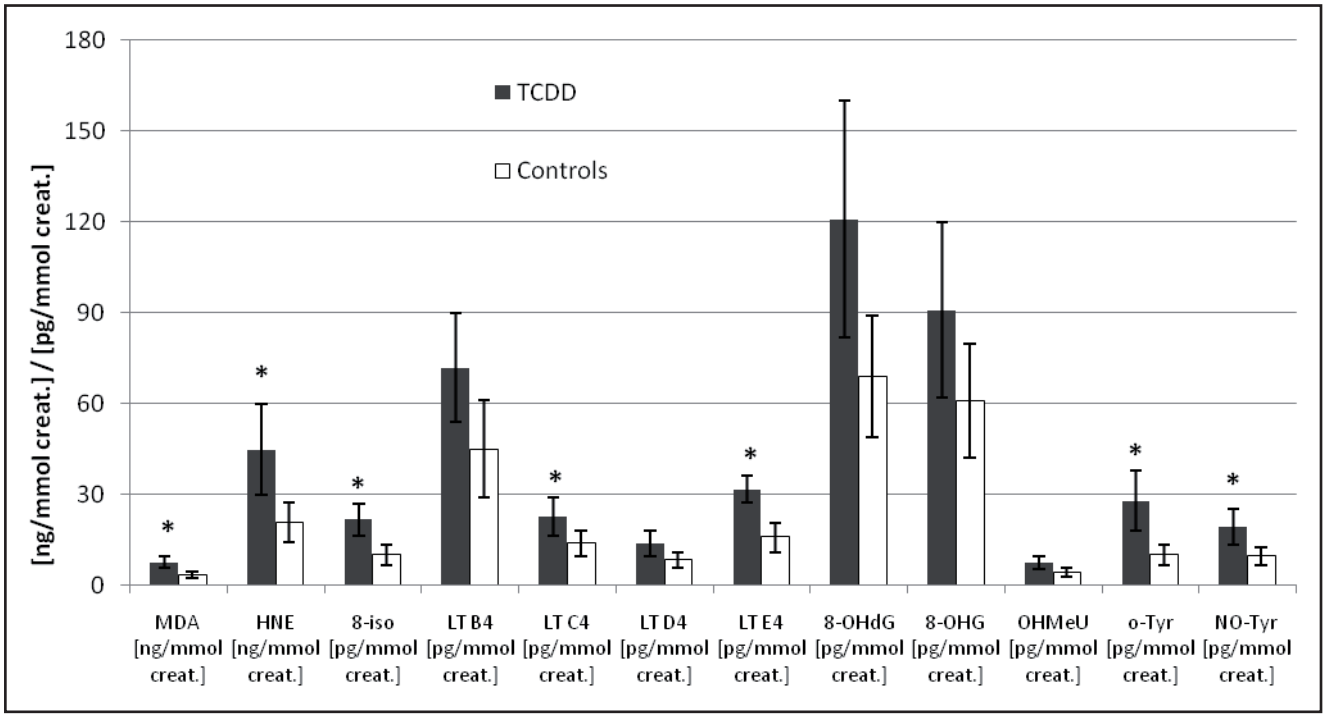

Fig. 2. Oxidative stress markers and leukotrienes of TCDD-exposed patients and controls in the urine. MDA and HNE are expressed in ng/mmol creatinine (creat.), all other markers in pg/mmol creat. * $p<0.05$.

impaired comparing to the controls, the EBC findings cannot be attributed to a respiratory disorder.

Plasma lipids in the TCDD-exposed patients in 2010 were not significantly different from the control group, due to hypolipidemic treatment with statins (6 patients), and fibrates (5 patients); only two (18%) patients had no hypolipidemic treatment. In 1996, before the treatment was started in the patients, plasma cholesterol and triacylglyceroles levels correlated with plasma TCDD level. In the recent study, this correlation was not found. Hypolipidemic therapy is crucial (Laufs 2003), as there is no method available, eliminating TCDD from the body.

CONCLUSION

Induction of oxidative/nitrosative stress by TCDD plays an important role in cardiovascular, neurotoxic and cancer development, as was shown experimentally. In this study, we investigated for the first time, the markers of oxidative/nitrosative stress and leukotrienes in vivo in highly dioxin-exposed patients in EBC, urine and plasma. EBC is not limited to lung disorders as the markers of oxidative stress and inflammation were elevated in TCDD-exposed patients without respiratory disorders. The largest spectrum of TCDD-induced stress or inflammation markers has been detected in the EBC (10 markers) and in urine (7 markers). These non-invasive examinations might be utilized in the follow-up of the patients. Their prognostic value needs to be elucidated.

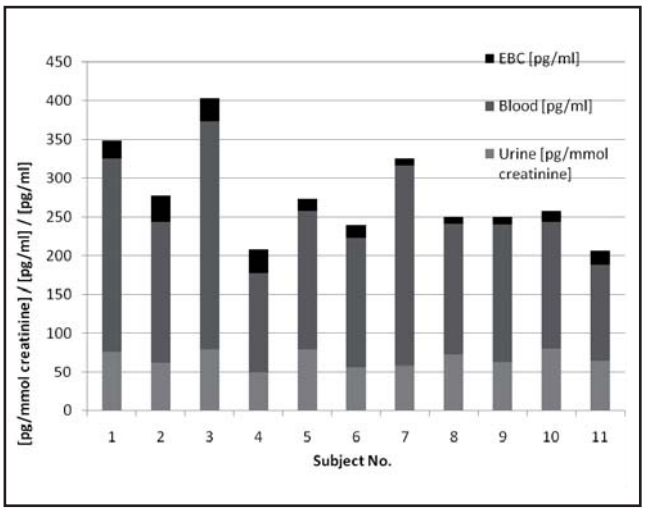

Fig. 3. NO-Tyr in patients in descending level of TCDD in blood (pg/ml), urine (pg/mmol creatinine) and exhaled breath condensate (pg/ml).

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