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Relative effect potency estimates of dioxin-like activity for dioxins, furans, and dioxin-like PCBs in adults based on cytochrome P450 *1A1* and *1B1* gene expression in blood

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ABSTRACT

Background: In the risk assessment of PCDDs, PCDFs, and dioxin-like (DL) PCBs, regulatory authorities support the use of the toxic equivalency factor (TEF)-scheme derived from a heterogeneous data set of the relative effect potency (REPs) estimates.
Objectives: We sought to determine REPs for dioxin-like compounds (DLCs) using expression of cytochrome P450 (*CYP*) *1A1* and *1B1* mRNA in human peripheral blood mononuclear cells representing two different pathways.
Methods: We used a sex and age adjusted regression-based approach comparing the strength of association between each DLC and the cytochrome P450 (*CYP*) *1A1* and *1B1* mRNA expression in 320 adults residing in an organochlorine-polluted area of eastern Slovakia.
Results: We calculated REPs based on *CYP1A1* expression for 4 PCDDs, 8 PCDFs, and 1 PCB congener, and based on *CYP1B1* expression for 5 PCDFs and 11 PCB congeners. REPs from *CYP1A1* correlated with REPs previously derived from thyroid volume ($\rho = 0.85$; $p < 0.001$) and serum FT4 ($\rho = 0.77$; $p = 0.009$). The 13 log REPs from *CYP1A1* correlated with log WHO-TEFs ($r = 0.63$; $p = 0.015$) and 11 log PCB REPs with PCB consensus toxicity factors (CTFs) for compounds with WHO-TEFs ($r = 0.80$; $p = 0.003$). The complete set of derived 56 log REPs correlated with the log CTFs ($r = 0.77$; $p = 0.001$) and log WHO-TEFs ($r = 0.81$; $p < 0.001$).
Conclusions: REPs calculated from thyroid and cytochrome P450 endpoints realistically reflect human exposure scenarios because they are based on human chronic and low-dose exposures. While the *CYP 1A1* seems more suitable for toxicity evaluation of PCDD/Fs, the *CYP 1B1* is more apt for PCDFs and PCBs and reflects different pathways.

1. Introduction

The name "dioxins" is often used for the family of structurally and chemically related polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). Certain dioxin-like polychlorinated biphenyls (DL-PCBs), with similar toxic properties, are also often included under the term "dioxins" (WHO, 2014). For risk assessment purposes, the World Health Organization (WHO) assigned 29 individual compounds with a toxic equivalency factor (TEF) (Van den Berg et al., 2006). This factor indicates a relative toxicity compared to the most toxic congener, TCDD, which is given a reference value of 1.

We addressed the issue of relative potencies (REPs) of dioxin-like compounds (DLCs) by examination of cross-sectional data on thyroid impairment among a population exposed to a mixture of organochlorines. We identified relationships between serum concentration of individual mixture components and thyroid volume or free thyroxine (FT_4) serum level (Trnovec et al., 2013). The aim of the present study is to derive REPs based on the systemic plasma concentration in combination with the cytochrome P450 (*CYP*) *1A1* and *1B1* gene expression in peripheral blood mononuclear cells (PBMCs) and to compare them with REPs based on thyroid data and with the WHO-TEFs (Van den Berg et al., 2006).

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2. Materials and methods

2.1. Subjects, exposure assessment, and determination of cytochrome P450 1A1 and 1B1 gene expression

Information on participants, chemical analyses, assessment of thyroid outcomes, statistical analysis, and approaches for estimation of REPs were described previously (Trnovec et al., 2013). In brief, our initial sample group of 2047 adults was drawn from a population living in the Michalovce, Svidnik, and Stropkov districts in eastern Slovakia, an area known to be contaminated by a mixture of organochlorines (Jursa et al., 2006; Langer et al., 2007; Petrik et al., 2006; Wimmerová et al., 2015). Of the 2047 adults, 320 were willing to provide 90 mL of blood for analysis of PCDDs, PCDFs, and PCBs. Quantification of *CYP1A1* and *CYP1B1* mRNA levels in human un-stimulated PBMCs was described previously (van Duursen et al., 2005b; Canton et al., 2003). White blood cells were collected from the buffy coat by osmotic hemolysis. RNA was obtained from these PBMCs for simultaneous quantification of *CYP1A1* and *CYP1B1* gene expression with quantitative real-time polymerase chain reaction (PCR) by TaqMan technology. The expression of *CYP1A1* and *1B1* genes from these 320 individual blood samples was calculated by using the ΔΔCt method, a relative quantification method, as described elsewhere (ABI PRISM, 2001). Briefly, in this method the amount of copies (target) is normalized to a constant amount of copies from an endogenous reference (β-actin) and relative to a standard. The Ct values for the endogenous gene expression levels (β-actin) were subtracted from the Ct values determined for *CYP1A1* and *1B1* (ΔCt) and then compared with the standard value (ΔΔCt). This test does not provide information on protein production or activity.

2.2. Statistical analysis

To estimate the relative potencies of individual components of the mixture, we used an approach based on comparing the magnitude of the regression coefficient (β) for *CYP1A1* or *1B1* mRNA levels regressed on the serum concentration of the individual congeners (Brown et al., 2001; Trnovec et al., 2013). We considered participants' sex, age at blood draw, and smoking status, as well as concentrations of PCDDs, PCDFs, and PCBs determined in the exposure mixture as potential confounders. We present results with adjustment only for age, sex, and smoking, because the addition of other organochlorines had negligible influence on estimates (data not shown), as in our previous study with thyroid outcomes (Trnovec et al., 2013). The REPs of the individual congeners were calculated as the ratio of the β coefficient obtained for the *i*th congener to β coefficient for TCDD: β_i/β_{TCDD}.

3. Results

3.1. Characteristics of the participants

Characteristics of the participants have been described previously (Trnovec et al., 2013). Briefly, the subgroup of 320 participants consisted of 197 males 44.9 ± 11.47 years of age (mean ± SD; median, 48 years) and 120 females 47.3 ± 9.24 years of age (median, 48 years), with an overall mean age of 45.8 ± 10.7 years (median, 48 years).

3.2. Exposure to dioxin-like compounds (DLCs)

The descriptive data on serum concentration of DLCs for these participants are shown in Table 1. Subjects were simultaneously exposed to several DLC congeners and, moreover, the serum concentrations of individual congeners were interrelated. Specifically, the serum PCDDs correlated with PCDFs ($\rho = 0.41$; $p < 0.001$), less strongly with PCBs ($\rho = 0.22$; $p < 0.001$) and most strongly between serum PCBs and PCDFs ($\rho = 0.63$; $p < 0.001$) (Table 2). Therefore, due to the correlated nature of these compounds, it is difficult to distinguish between their independent effects (Directorate-General for Health and Consumers, 2011).

Table 1
Descriptive statistic data on serum concentration of DLCs for participants of the study (pg WHO TEQ/g lipid).

	Males	Females	Total
N	197	120	317
Mean	32.9	31.6	32.4
Standard deviation	38.9	31.6	36.3
Minimum	4.8	9.7	4.8
Median	23.3	23.8	23.3
Maximum	359.0	253.5	359.0
Geometric mean	24.5	25.2	24.7
p-Value (Mann-Whitney test)	0.6		

3.3. REPs for PCDD, PCDF, and DL-PCB congeners calculated from CYP 1A1 and 1B1 gene expression

We present the REPs for PCDD, PCDF, and DL-PCB congeners calculated from *CYP 1A1* and *1B1* gene expression in Table 3. For comparison we also show the REPs for thyroid outcomes which we published previously (Trnovec et al., 2013). It can be seen that the slopes of the regression (β) of levels of *CYP1A1* against DLC congener concentrations were negative for all members of the exposure mixture except for 1,2,3,7,8,9-HxCDD and for PCB congeners 126, 169, 105, 114, 118, 123, 156, 157, 167 and 189. This means that for this group of congeners, TCDD inclusive, increased exposure was associated with lower *CYP1A1* expression.

For *CYP1B1* when compared with *CYP1A1* expression, we observed a completely different pattern. None of the regressions of *CYP1B1* for PCDD congeners concentrations had a negative sign (<0), except for the index compound, TCDD. In contrast, regressions for PCDF and PCB congener concentrations were negative except for 1,2,3,7,8-PeCDF and OCDF. Discrepancy in the direction of association between the index TCDD and tested chemical suggests a different mode of action. Furthermore, the basic assumption of the TEF methodology, that the effect of individual aryl hydrocarbon receptor (AHR) agonist act *via* the same AHR-mediated mechanism, does not seem valid. On the other hand, for all PCBs examined and the 5 PCDFs, with regard to identical, negative sign of regression coefficients, we assume dose additivity with TCDD and the derivation of REPs as justified.

3.4. Comparison of REPs calculated from various outcomes between themselves

We compare REPs originating from thyroid and *CYP1A1* and *CYP1B1* outcomes pairwise as indicated in Fig. 1, and show the respective plots in Fig. 2. We present the corresponding Spearman's correlations in Table 4. It can be seen that a stepwise addition of REP data on members of DLCs mixture increased the statistical significance of correlations of REPs derived from *CYP1A1* mRNA level in PBMCs and thyroid volume (Fig. 3A), from *CYP1A1* mRNA level in PBMCs smoking adjusted and thyroid volume (Fig. 3B), from *CYP1A1* mRNA level in PBMCs and serum FT4 (Fig. 3C) and from *CYP 1A1* mRNA level in PBMCs smoking adjusted

Table 2
Spearman rank-order correlations between serum concentration of groups of DLCs.

Spearman's correlations		PCDDs	PCDFs	PCBs
PCDDs	ρ	1	0.411	0.219
	p		<0.001	<0.001
PCDFs	ρ		1	0.625
	p			<0.001
PCBs	ρ			1
	p			

Table 3
The calculated relative potencies (REPs) of PCDD, PCDF and DL-PCB congeners. We included the minimum, maximum, and median values published for *in vivo* REPs in the REP2004 database (see Table 8 of Haws et al., 2006). Abbreviations: n: number of cases; β: regression coefficient; FT4: free thyroxin; TEF: toxic equivalency factor; CTF: consensus toxicity factor.

		CYP 1A1		*CYP 1B1*		TV		FT4		WHO-TEF	REP_{2004} database			CTF human	CTF rat
	n	β	REP as β_i/β_{TCDD}	β	REP as β_i/β_{TCDD}	β	REP as β_i/β_{TCDD}	β	REP as β_i/β_{TCDD}		Minimum	Median	Maximum		
2378-TCDD	70	−347.16	1	−0.350	1	−1.101	1	−0.508	1	1				1	
12378-PeCDD	132	−140.15	0.40371	2.642		−0.45	0.432	−0.24	0.471	1	0.044	0.4	1.5	1	
123478-HxCDD	81	−181.76	0.52356	1.762		−0.283	0.257	−0.409	0.805	0.1	0.0076	0.059	0.35	0.03	
123678-HxCDD	286	−4.81	0.01386	0.344		−0.091	0.082	−0.064	0.126	0.1	_	_	_	0.06	
123789-HxCDD	76	34.51		1.541		0.146		−0.245	0.482	0.1	0.029	0.029	0.029	0.002	
1234678-HpCDD	316	−6.75	0.01943	0.225		−0.009	0.008	−0.015	0.029	0.01	0.001	0.01	0.035	0.2	
OCDD	319	0.33		0.041		−0.003	0.003	0.002		0.0003	0.00025	0.00025	0.00025	0.005	
2378-TCDF	43	−142.51	0.41050	−0.715	2.0429	−0.912	0.828	−0.051	0.1	0.1	_	_	_	0.1	
12378-PeCDF	13	−213.67	0.61548	0.386		−0.382	0.347	0.657		0.03	0.0027	0.022	0.95	0.6	
23478-PeCDF	314	−3.09	0.00891	−0.058	0.1657	−0.019	0.016	−0.01	0.02	0.3	0.0065	0.2	3.7	1	
123478-HxCDF	311	−18.17	0.05234	−0.259	0.7400	0.023		0.043		0.1	0.014	0.05	0.16	1	
123678-HxCDF	312	−83.59	0.24078	0.413		−0.161	0.146	0.012		0.1	0.0031	0.081	0.16	0.04	
234678-HxCDF	51	−206.34	0.59437	−0.415	1.1857	−0.86	0.78	1.084		0.1	0.015	0.018	0.1	0.06	
1234678-HpCDF	314	−73.21	0.21088	−0.390	1.1143	−0.059	0.054	−0.027	0.053	0.01	_	_	_	0.01	
OCDF	80	−4.31	0.01243	1.026		0.127		−0.19	0.373	0.0003	0.000004	0.000077	0.0016	0.2	
PCB 81	234	−0.3220	0.00093	−0.072	0.2057	−0.0111	0.0101	−0.009	0.017	0.0003	_	_	_		0.0002
PCB 126	319	0.881		−0.006	0.0171	0.0009		0.00004		0.1	0.000067	0.1	0.86	0.003	0.09
PCB 169	320	0.369		−0.013	0.0371	0.0034		0.0022		0.03	0.0000018	0.019	0.74		0.002
PCB 105	276	0.00738		−0.000052	0.00015	0.0000006		−0.0000009	0.0000018	0.00003	0.00000047	0.000042	0.0022		0.00001
PCB 114	315	0.012474		−0.000236	0.00067	0.000063		0.0000213		0.00003	0.0002	0.00034	0.00048		0.00006
PCB 118	301	0.001576		−0.000012	0.000034	0.0000032		0.0000005		0.00003	0.00000042	0.00002	0.0023		0.000009
PCB 123	276	0.027125		−0.000038	0.00011	0.000033		0.000022		0.00003	0.000034	0.000044	0.000055		0.000009
PCB 156	315	0.002117		−0.000027	0.000077	0.0000075		0.0000022		0.00003	0.0000021	0.000055	0.42		0.00008
PCB 157	315	0.016712		−0.000174	0.000497	0.0000291		0.0000087		0.00003	0.000420	0.0011	0.0017		0.00003
PCB 167	315	0.007217		−0.000059	0.00017	0.0000192		0.0000027		0.00003	_	_	_		0.000007
PCB 189	315	0.002919		−0.000096	0.00027	0.0000265		0.0000117		0.00003	0.000037	0.000055	0.00018		0.000007

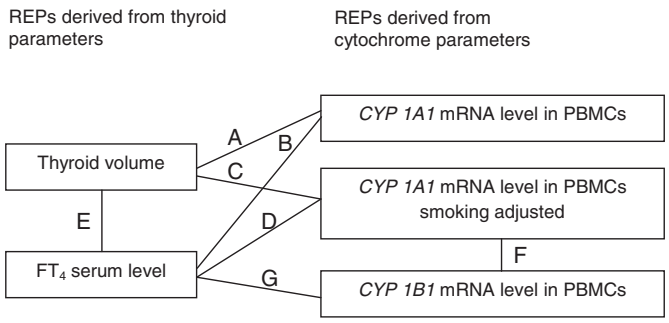

Fig. 1. Schedule indicating pairwise linear regressions of REPs originating from thyroid and cytochrome P450 outcomes.

and serum FT4 (Fig. 3D). However, we observed a different outcome when correlating REPs derived from thyroid volume and serum FT4 (Fig. 3E). Here the addition of the REP for PCB 81 decreased the level of statistical significance. Finally, we compared the seven REP values derived from *CYP1B1* with REPs derived from *CYP1A1* (for 2,3,7,8-TCDD, 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF, 1,2,3,4,7,8-HxCDF, 2,3,4,6,7,8-HxCDF, 1,2,3,4,6,7,8-HpCDF and PCB 81) (Fig. 1, connection F) and six REP values derived from *CYP1B1* with REPs derived from FT4 (for 2,3,7,8-TCDD, 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF, 1,2,3,4,6,7,8-HpCDF, PCB 81 and PCB 105) (Fig. 1, connection G). For the former comparison we obtained $\rho = 0.71$ and $p = 0.071$ and for the latter one $\rho = 0.77$ and $p = 0.072$ which shows that the REPs derived from *CYP1A1* and *CYP1B1* are associated at the marginal level of significance and similarly those from *CYP 1B1* with FT4.

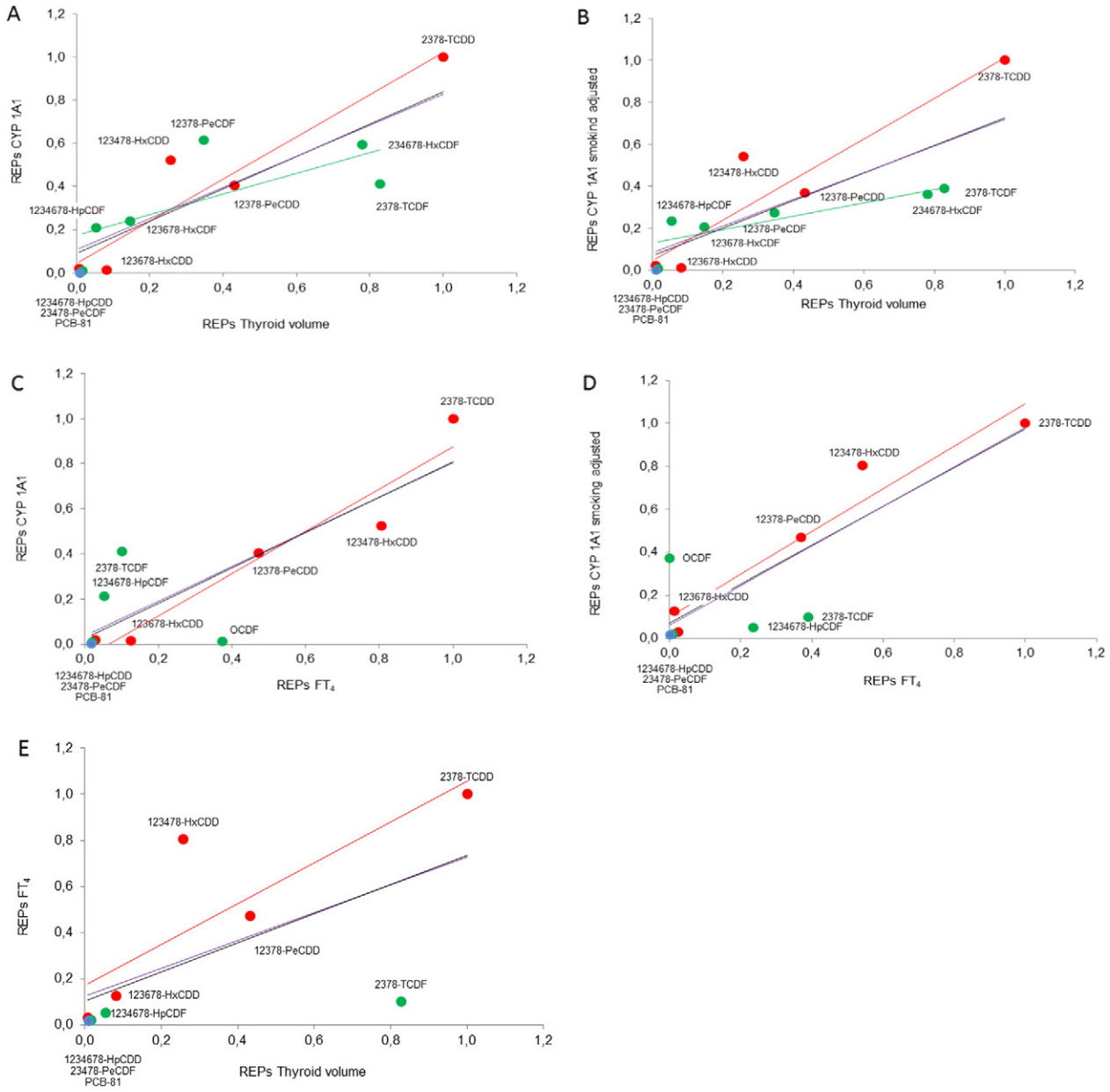

Fig. 2. Correlations between REPs derived from 4 various health outcomes. A. Plot of REPs derived from *CYP1A1* gene expression against REPs derived from thyroid volume data. B. Plot of REPs derived from smoking adjusted *CYP1A1* gene expression against REPs derived from thyroid volume data. C. Plot of REPs derived from *CYP1A1* gene expression against REPs derived from serum FT4 data. D. Plot of REPs derived from smoking adjusted *CYP1A1* gene expression against REPs derived from serum FT4 data. E. Plot of REPs derived from thyroid volume data against REPs derived from serum FT4 data. All data were sex and age adjusted and alternately to smoking and not smoking. PCDDs points and lines are red, PCDFs are green, combined PCDDs and PCDFs are violet and combined PCDDs, PCDFs and PCB 81 are black. The corresponding parameters of the Spearman rank correlations are in Table 4. Schedule indicating pairwise linear regressions of REPs originating from thyroid and cytochrome P450 outcomes is in Fig. 1.

Table 4
Parameters of Spearman's correlations linking REPs derived from *CYP 1A1* mRNA level in PBMCs, thyroid volume and FT4 serum level. The relationships are depicted in Fig. 2. The correlated variables are shown in Fig. 1.

	Group of dioxin-like compounds or their combinations	Spearman's correlation coefficient ρ	p
Correlation of REPs derived from *CYP 1A1* mRNA level in PBMCs and thyroid volume (Fig. 3A). Connection A, Fig. 1	PCDDs	0.8	0.104
	PCDFs	0.771	0.072
	PCDDs and PCDFs	0.836	0.001
	PCDDs, PCDFs and PCB 81	0.853	0.001
Correlation of REPs derived from *CYP 1A1* mRNA level in PBMCs smoking adjusted and thyroid volume (Fig. 3B). Connection B, Fig. 1	PCDDs	0.8	0.104
	PCDFs	0.943	0.005
	PCDDs and PCDFs	0.836	0.001
	PCDDs, PCDFs and PCB 81	0.853	0.001
Correlation of REPs derived from *CYP 1A1* mRNA level in PBMCs and serum FT4 (Fig. 3C). Connection C, Fig. 1	PCDDs	0.9	0.037
	PCDFs	0.4	0.6
	PCDDs and PCDFs	0.683	0.042
	PCDDs, PCDFs and PCB 81	0.77	0.009
Correlation of REPs derived from *CYP 1A1* mRNA level in PBMCs smoking adjusted and serum FT4 (Fig. 3D). Connection D, Fig. 1	PCDDs	0.9	0.037
	PCDFs	−0.2	0.8
	PCDDs and PCDFs	0.6	0.088
	PCDDs, PCDFs and PCB 81	0.636	0.048
Correlation of REPs derived from thyroid volume and serum FT4 (Fig. 3E). Connection E, Fig. 1	PCDDs	0.9	0.037
	PCDFs		
	PCDDs and PCDFs	0.81	0.015
	PCDDs, PCDFs and PCB 81	0.833	0.05

In summary, it can be stated that presently derived REPs from expression of *CYP1A1* and partly of *CYP1B1* correlate well with previously (Trnovec et al., 2013) derived REPs both from thyroid volume and FT4 serum level.

3.5. Comparison of our human REPs with the WHO-TEFs, CTFs, and values in the REP_{2004} database

We compared our human REPs with WHO-TEF values (Van den Berg et al., 2006), with consensus toxicity factors (CTF) for compounds, World Health Organization toxic equivalency factors (Larsson et al., 2015), and with published data on REPs for DLCs (Haws et al., 2006; van Ede et al., 2016).

Comparing REPs derived from *CYP1A1* expression in PBMCs with the published WHO-TEFs (Van den Berg et al., 2006) (Fig. 3), we observed that the group of 14 REPs fits well with the respective WHO-TEF values (Pearson, $r = 0.633$; $p = 0.015$). To make comparisons easier, in Fig. 4 we have plotted our REP values against the corresponding WHO-TEFs. In addition to our REPs, we plotted the consensus toxicity factors for compounds with World Health Organization toxic equivalency factors (CTFs) (Larsson et al., 2015). Note that the PCDD/F and PCB congeners in Fig. 4 were ranked in descending order of WHO-TEF values. Fig. 5 illustrates the REP values in relation to WHO-TEFs, normalized to the respective WHO-TEFs. Note that in Fig. 5 the congeners were grouped as PCDDs, PCDFs, and PCBs, and within each group with regard to number of chlorine substituents. Such presentation makes a comparison between the three DLCs groups easier.

Visual inspection of our REPs and CTFs derived for PCB in Fig. 4 indicates that there might be a certain parallelism between the two groups. The strong association observed in Fig. 6 between our PCB REPs and the PCB CTFs (Pearson $r = 0.798$; $p = 0.003$) provides evidence for this hypothesis. For PCB 126, we used the human CTF value (Larsson et al., 2015).

In sum we have derived 56 REP values. Of them, 14 originate from *CYP 1A1*, 17 from *CYP 1B1*, 13 from thyroid volume, and 12 from FT4 level. The 4 REPs = 1 for each outcome are for TCDD as the index chemical. In Fig. 7 we relate the complete set of 56 REPs with the consensus toxicity factors for compounds and with World Health Organization toxic equivalency factors (Larsson et al., 2015). For PCBs, except PCB 126, the rat CTF data were used, and for PCB 126 human data were available. We observed a strong correlation between our REP and the CTFs (Pearson $r = 0.774$; $p = 0.001$).

In Fig. 8 we show the relationship between the set of our REPs and the WHO-TEFs (Van den Berg et al., 2006). Similar to previous comparison (Fig. 7), we observed a strong correlation (Pearson $r = 0.808$; $p < 0.001$).

Finally, we compared our REP data with values in the REP_{2004} database (Haws et al., 2006) and the newly derived REPs published recently (van Ede et al., 2016). From the REP_{2004} database we included into Table 3 the minimum, maximum, and median values published for *in vivo* data (see Table 8 of Haws et al., 2006). It can be seen that our REPs are predominantly within the published extremes.

4. Discussion

We found that REPs derived from four independent biomarkers in adults environmentally exposed to DLCs are internally strongly

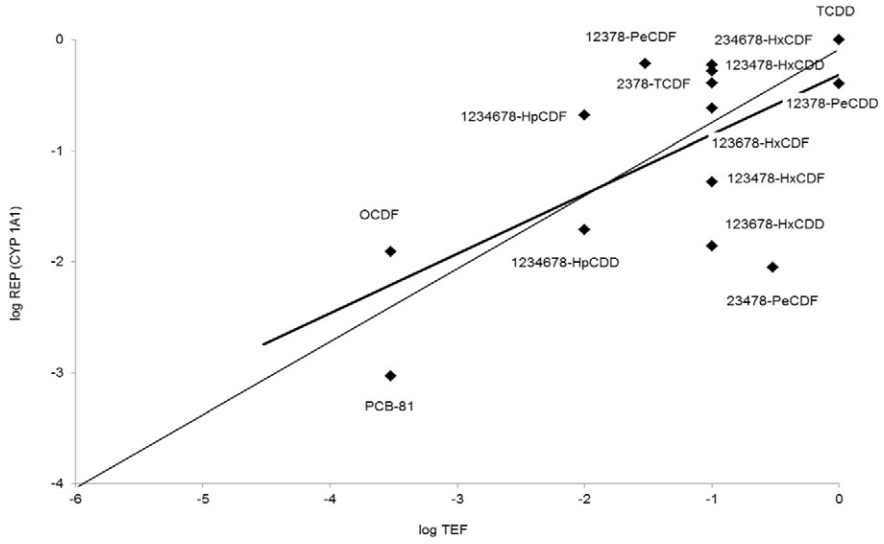

Fig. 3. Relationship between derived REPs for individual mixture components and the published WHO-TEFs (Van den Berg et al., 2006) as measured by *CYP1A1* expression in PBMCs (Pearson, $r = 0.633$; $p = 0.015$).

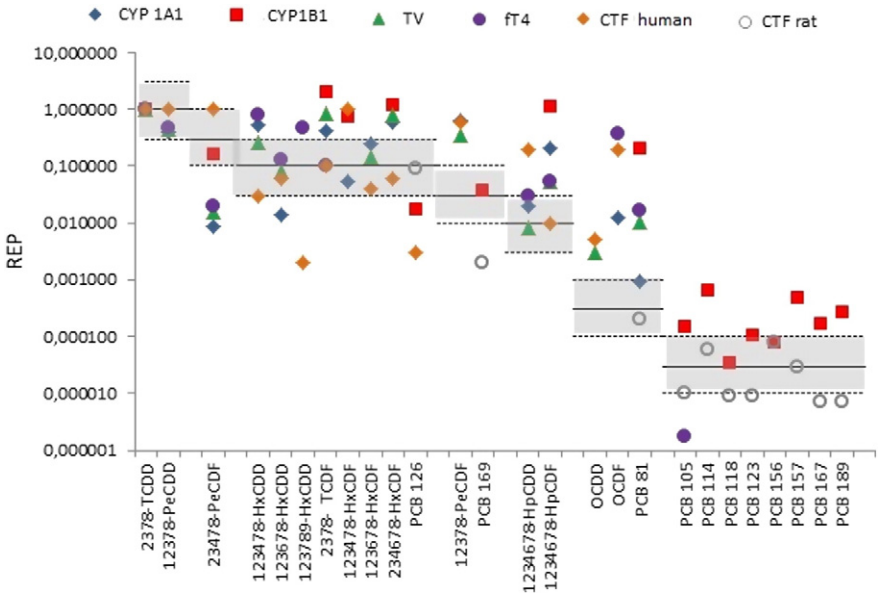

Fig. 4. Relative effect potencies (REPs) determined for individual PCDD/F and PCB congeners for *CYP1A1*, *CYP1B1*, thyroid volume and FT4 serum level outcomes plotted against the corresponding World Health Organization toxic equivalency factors (WHO-TEFs). The gray shaded area represents the half log uncertainty range around the WHO-TEF. Values for *CYP1A1* are marked by blue diamonds, *CYP1B1* by red squares, thyroid volume by green triangles and FT4 by violet circles. The consensus toxicity factors for compounds with World Health Organization toxic equivalency factors (Larsson et al., 2015) for human data are marked by orange diamonds and rat data by gray circles.

interrelated between themselves and, moreover, they are associated with WHO-TEFs (Van den Berg et al., 2006) and the newly described consensus toxicity factors for compounds with World Health Organization toxic equivalency factors (Larsson et al., 2015). The so far published REPs (Haws et al., 2006; van Ede et al., 2016) for PCDD, PCDF, and DL PCB congeners are based exclusively on *in vitro* data or on *in vivo* animal experiments. In contrast, we have estimated REPs by examining two human thyroid endpoints (Trnovec et al., 2013) and in the current work, expression of *CYP1A1* and *CYP1B1* in PBMCs in adult humans exposed to DLCs. The REPs estimated herein are highly relevant for human risk assessment as the underlying studies were *in vivo* on human subjects, with serum concentrations reflecting body burden and very probably targeting a AHR-mediated outcome.

Unlike *in vitro* or *in vivo* animal studies, the investigator cannot manipulate the magnitude of exposure in human epidemiological studies. Until recently it has remained unclear whether real-life background exposure to dioxin related compounds is associated with altered thyroid and some other functions (Arisawa et al., 2005). We compared the exposure level of our participants (Table 1) with the relatively recent review data on blood levels of dioxins, furans, and DL-PCBs in other populations (Consonni et al., 2012). The authors reported from 161 studies for blood levels of sum of DLCs median and mean values of 10.2 and 13.2 lipid-adjusted TEQs, respectively. Analogous concentrations for our adults were more than two times higher, *i.e.* 23.3 and 32.4 TEQs, respectively, which increases the probability of a dioxin related adverse response in our study.

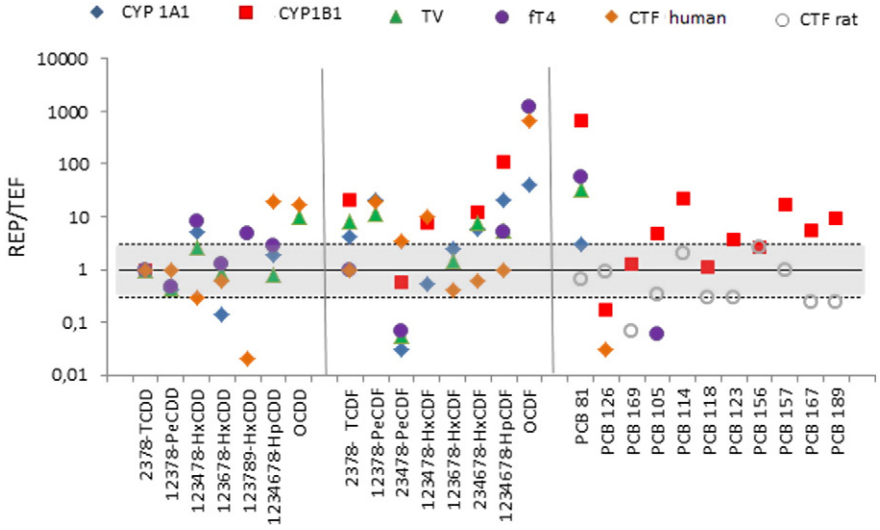

Fig. 5. Ratios of relative effect potencies (REP) determined in 4 different bioassays to the corresponding World Health Organization toxic equivalency factors (WHO-TEFs) (REP/TEF). Ratios were determined for individual PCDD/F and PCB congeners in *CYP1A1*, *CYP1B1*, thyroid volume and FT4 serum level models. Values for *CYP1A1* are marked by blue diamonds, *CYP1B1* by red squares, thyroid volume by green triangles and FT4 by violet circles. Included are ratios of the values of the consensus toxicity factors (CTF) for compounds with World Health Organization toxic equivalency factors (Larsson et al., 2015) to the corresponding WHO-TEFs. Human CTF data are marked by orange diamonds and rat CTF data by open gray circles.

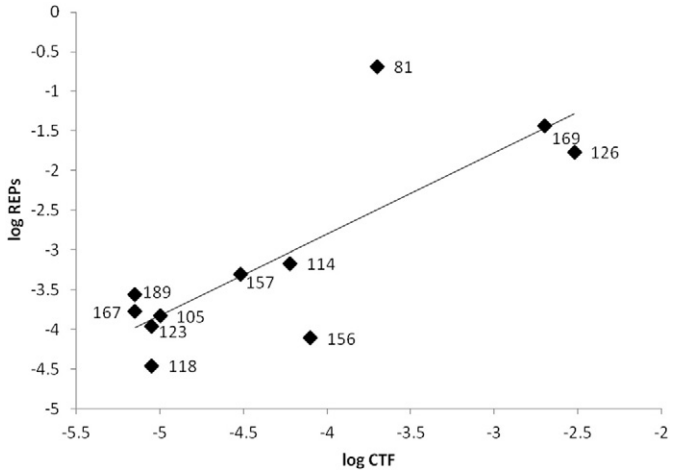

Fig. 6. Relationship between derived REPs for PCBs and the consensus toxicity factors for compounds with World Health Organization toxic equivalency factors for PCB congeners (Larsson et al., 2015) (Pearson $r = 0.798$; $p = 0.003$). For PCB 126 the human CTF value was used. The numbers denote PCB congeners.

Sources of DLC exposure besides that from PCBs is not well documented in our study population from eastern Slovakia (Kocan et al., 2001). There are no data on dioxin or furan impurities in the PCBs produced locally in eastern Slovakia. Nevertheless, interrelations between serum concentrations of PCDDs, PCDFs and DL-PCBs (Table 2) may help to trace the source of exposure of our subjects. We found a strong association between PCDFs and DL-PCBs, while dioxins did not correlate as strongly with PCBs.

A prerequisite for derivation of the REPs in the present study was responsiveness of the outcomes, *i.e.*, the expression of *CYP 1A1* and *CYP1B1* in PBMCs, to concentrations of DLCs in serum. Induction of *CYP1A1* has been suggested as an extremely sensitive marker for exposure and/or tissue responsiveness to TCDD (Vanden Heuvel et al., 1994). Both *CYP1A1* (Vanden Heuvel et al., 1994) and *CYP1B1* (Spencer et al., 1999) mRNA levels in peripheral lymphocytes have been proposed as biomarkers of TCDD biological effective dose in humans. These conclusions were however drawn from single dose administration of TCDD to mice or *in vitro* exposure, respectively. On the other hand, the results of our study and of a few comparable human studies (Landi et al., 2003; Toide et al., 2003; van Duursen et al., 2005a) are based on long-term and low-dose exposures. In the Seveso accident, expression of *CYP1A1* could not be detected and expression of *CYP1B1* was not significantly associated with TCDD or TEQ plasma level in contrast to short-term exposures (Landi et al., 2003). In lymphocytes from subjects occupationally exposed to dioxins at waste incinerators, expression of *CYP1A1* mRNA was also not observed (Toide et al., 2003). Too, based on their findings, the authors of the *in vitro* study with human lymphocytes exposed to TCDD and PCB 126 concluded that *CYP1A1* and *CYP1B1* expression in human lymphocytes might not be applicable as biomarkers of exposure to dioxin and dioxin-like compounds (van Duursen et al., 2005b). In addition, data on mRNA expression of *CYP1B1* in lymphocytes indicate that environmental exposure to PCBs had no significant effect on *CYP1B1* expression (van Duursen et al., 2005a). *CYP1A1* and AHR could not be detected in uncultured bovine lymphocytes while the *CYP1B1* expression was higher in lymphocytes collected from animals reared in the DLCs contaminated area compared with controls (Girolami et al., 2013). In contrast to these data, we observed associations between *CYP* expression, positive and negative (see the sign of β in Table 3), and exposure to DLCs. The decreased expression after exposure to TCDD is in agreement with the hypothesis that long-term presence of dioxin in the human body does not result in an increase in AHR pathway responsiveness or that responsiveness is eventually lost or reduced decades after the initial exposure (Baccarelli et al., 2004). An alternative explanation may be that the internal dose may be high enough for *CYP 1A2* to be induced which means that much of the TCDD and other DLCs are sequestered in the liver (Aylward et al., 2005; Staskal et al., 2005; van Ede et al., 2016) instead of circulating peripherally exerting toxic activity. With 2,3,4,7,8-PeCDF, known for hepatic sequestration (Diliberto et al., 1997), we have observed much lower REPs (0.0089; 0.166; 0.0160; 0.02, see Table 3) than the 0.3 TEF (Figs. 4 and 5), which possibly is related to the higher liver sequestration of this congener compared with TCDD (van Ede et al., 2016).

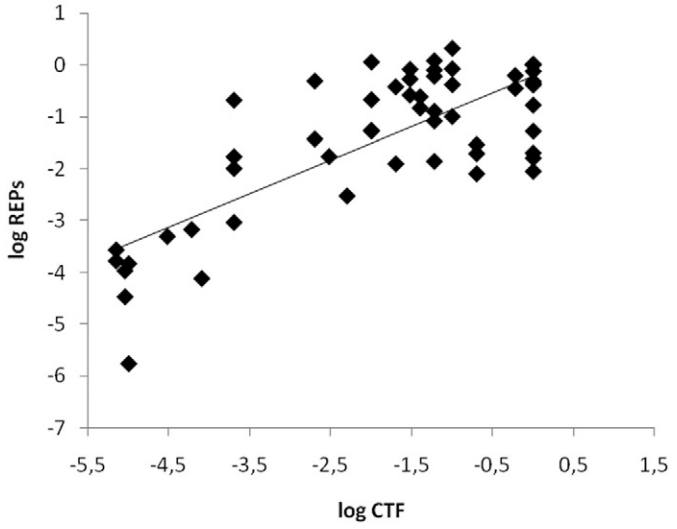

Fig. 7. Relationship between derived REPs for individual mixture components and the consensus toxicity factors for compounds with World Health Organization toxic equivalency factors (Larsson et al., 2015) (Pearson $r = 0.774$; $p = 0.001$). For PCBs the rat CTF data were used, for PCB 126 the human data were available. The points are labeled with congeners in Fig. 7S in the supplement.

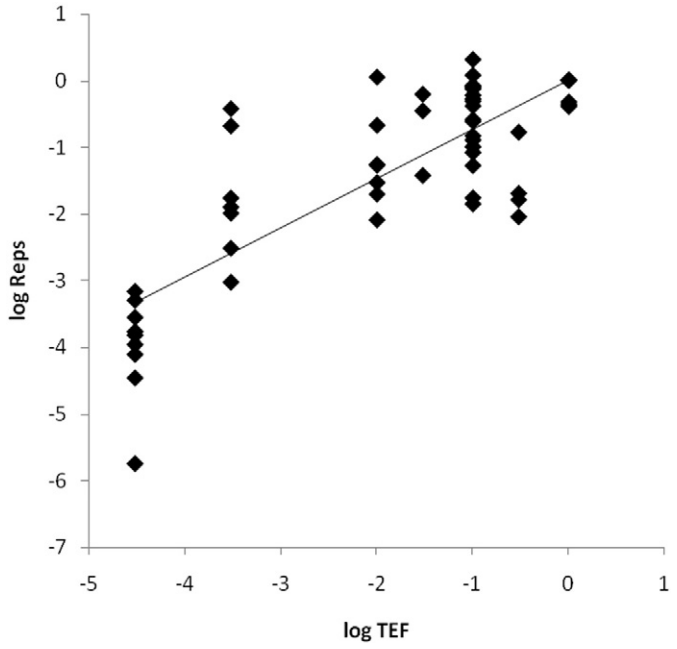

Fig. 8. Relationship between derived REPs for individual mixture components and the published WHO-TEFs (Van den Berg et al., 2006) (Pearson $r = 0.808$; $p < 0.001$). The points are labeled with congeners in Fig. 8S in the supplement.

It has been shown that the relative potencies of DLCs are depending on both, the interaction with the AHR and the pharmacokinetics of the substance (DeVito et al., 1997; Devito et al., 1998; van Ede et al., 2013). Observed marked differences in elimination half-lives among DLCs (Ogura, 2004; Milbrath et al., 2009) may contribute to the

differences between REP estimates. For the two congeners with short half-lives, 2,3,7,8-TCDF and PCB 81 (Ogura, 2004), we have determined an REP for each outcome. It can be seen from Table 3 and Fig. 5 that all are much smaller than the corresponding TEFs, reflecting fast metabolism of these congeners.

Compared with laboratory exposures the environmental exposure scenario is more complex. The observed endpoints result from exposures to mixtures of DLCs and in addition to AHR agonists of non DLC type, not to mention all the dietary and pharmacological compounds that activate the AHR. The exposures are different, oral, dermal, by inhalation, *etc.*, and of variable magnitude which taking into account the non-linear behavior of some of the agents, contributes to the variability of the outcomes. Furthermore, in contrast to laboratory studies of developmental TCDD exposure and immune function (Vorderstrasse et al., 2004; Hogaboam et al., 2008), the present study was limited to outcomes in adults, with exposure assessed during middle age. Since PCB production began in 1959 in this area of Slovakia, it is likely that a proportion of our participants were also exposed to dioxin-like compounds prenatally (83 of our 317 adults were born after 1959 (see Supplement), *i.e.* after PCB production commenced in this region). We have previously documented adverse immune associations with early life PCB exposure in this population (Jusko et al., 2012; Jusko et al., 2016). Whether these early-life associations are transient, limited to certain periods of the life course, or the result of developmental programming, is the subject of future research.

In spite of this, the resulting framework of the 56 assessed REPs appears very robust. When deriving this set of REPs we strictly observed the basic requirement of the TEF approach, the same mechanism of action of the index chemical (TCDD) and the congener studied, formally represented by the same sign of the regression coefficients linking the outcome with serum concentration.

One of the main findings of our study is that REPs derived from *CYP1A1* strongly correlate with REPs derived from thyroid outcomes (Fig. 2 and Table 4) and marginally with REPs from *CYP1B1*. The discordant behavior of REPs derived from *CYP 1A1* and *CYP1B1* is in agreement with distinct properties of the *CYP1B1* gene family that clearly separate this P450 from the other well-established members of the CYP1 family (Murray et al., 2001; Ma and Lu, 2007). Though the regression slopes are relatively very small when relating *CYP 1B1* expression to serum PCBs, the REPs calculated from them are consistent between themselves and match well either to WHO-TEFs (Van den Berg et al., 2006) (Spearman $\rho = 0.74$, $p = 0.009$) or to CTFs (Pearson $r = 0.798$; $p = 0.003$) (Fig. 6). The *CYP 1B1* pathway proved a better predictor of toxic properties of PCBs compared with other outcomes as *CYP 1A1*, thyroid volume or FT4. The different toxicity of PCDDs as compared with PCDFs and PCBs, the first activating *CYP 1A1* and the latter ones *CYP 1B1* pathway, has already been demonstrated with vitamin K1 deficiency in breastfed children (Pluim et al., 1992; Pluim et al., 1994).

Even under strict observation of the principle of dose additivity, we were able to derive altogether 56 REPs and as much as 73.3% REPs for PCBs were derived from the *CYP 1B1* endpoint. Correlation between REPs derived from thyroid and CYP data is not surprising as links were described between thyroid hormone homeostasis and the cytochrome P450 system (Brtko and Dvorak, 2011). This holds mainly for *CYP 1A1*, however for *CYP 1B1*, no apparent interactions with thyroid hormones were reported except evidence that genetic variants in *CYP1B1* can be associated with serum T4, FT4 and FT3 levels in polycystic ovary syndrome patients (Zou et al., 2013) and that thyroid hormone affected testicular *CYP1B1* expression (Leung et al., 2009).

We had two options when analyzing computed REP data in relation to dioxin WHO-TEFs, CTFs, or REPs listed in the respective databases (Haws et al., 2006; van Ede et al., 2016): a. To focus on the data jointly and to identify a toxicological trend in the data set or b. To analyze each REP individually. The advantage of treatment of data as a set over individual approach has been recently stressed (Larsson et al., 2015). The set of REPs calculated in this study is generally within the range (minimum and maximum) of REPs listed in the REP_{2004} database (Table 8 Comparison of the range of *in vivo* REPs in the REP1997 and REP2004 databases) (Haws et al., 2006) (Table 3). It correlates both with the WHO-TEFs (Van den Berg et al., 2006) and consensus toxicity factors (CTFs) for compounds with World Health Organization toxic equivalency factors developed as a novel approach to establish toxicity factors for risk assessment of DLCs (Larsson et al., 2015). We analyzed the REPs and WHO-TEFs associations using several configurations:

First we examined the association of REPs derived from the *CYP 1A1* outcome with the WHO-TEFs. We obtained a statistically significant relationship between REP values and WHO-TEFs (Fig. 3). Next for easier comparison of our REPs with the newly suggested CTF values (Larsson et al., 2015) and WHO-TEFs, we present a graphical display of logarithms of REP and CTF values (Fig. 4). It can be seen that almost all of our REPs are within or above one order of magnitude of the WHO-TEFs (shaded area). The three REPs for 2,3,4,7,8-PeCDF and the REP for PCB 126 had however lower values compared with the TEF benchmark. The three REP values for 2,3,4,7,8-PeCDF, derived independently from *CYP 1A1* (0.0089), thyroid volume (0.016) and serum FT4 (0.020) data, are close, and support each other. The fourth value derived for this congener (0.1666) from *CYP 1B1* data is closer to the current TEF (0.3) and CTF_{human} (1.0) value. Interpretation of this divergence is difficult at the present state of knowledge.

For PCB 126, several authors describe species differences in response between rodents and humans, with human *in vitro* REPs being up to two orders of magnitude lower compared with the WHO-TEF (Carlson et al., 2009; Larsson et al., 2015; Silkworth et al., 2005; Sutter et al., 2010; van Duursen et al., 2005b; van Ede et al., 2014; van Ede et al., 2016). We derived a REP value (0.017) for PCB 126 from *CYP 1B1* expression. This value is almost one order of magnitude lower than the current 0.1 TEF. The PCB 126 TEF issue has been discussed also in light of the post 2006 studies (van Ede et al., 2016). With regard to validity of our REP value for PCB 126 regarding PCB 126 TEF it has to be taken into account that our REP is based on one observation and a single outcome and needs further confirmation by human studies.

Next we display in a logarithmic scale the REP/TEF and CTF/TEF values (Fig. 5). In this scheme the values ≥1 are greater than respective TEFs and vice versa. In Fig. 5 we grouped REP/TEF values for the PCDDs, PCDFs and PCBs. Such grouping made apparent the parallelism between *CYP 1B1* REPs and the CTFs for mono-ortho-substituted PCB congeners and PCB 169. This parallelism appeared as strong correlations ($r = 0.798$; $p = 0.003$) between PCB REPs and PCB CTFs (Fig. 6). For PCB 126 we used the CTF_{human} value of 0.003. Using the CTF_{rat} value of 0.09 (a value close to the current TEF value of 0.1) decreased the strength of the relationship ($r = 0.761$; $p = 0.007$). The peaking position of PCB 114 REP (0.00067) among REPs for mono-ortho-substituted PCB congeners, is noteworthy. An association of this congener with pathogenesis of endometriosis has been reported (Jirsova et al., 2005; Roy et al., 2012; Gennings et al., 2010). Finally we related the complete set of REPs derived presently and in our previous study (Trnovec et al., 2013) to the CTFs (Larsson et al., 2015) (Fig. 7) or the WHO-TEFs (Van den Berg et al., 2006) (Fig. 8). Both relationships were highly statistically significant sustaining validity of dioxin TEFs and CTFs regarding human risk assessment.

5. Conclusions

We demonstrate that REPs for several DLCs can be derived from four health outcomes, thyroid volume, FT4 serum level, *CYP 1A1* and *CYP 1B1* expression, examined in adult humans environmentally exposed to organochlorines and that these estimated REPs are consistent across endpoints. Furthermore, these REP values are based on human studies with "real-world" exposure scenarios, where chronic, low-dose exposures are typical, in contrast to much of the present literature on REPs, which often rely on *in vitro* models with high concentrations. Thus,

our results may be particularly useful in the context of human risk assessment.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2016.08.016.

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