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Dioxin-like exposures and effects on estrogenic and androgenic exposures and micronuclei frequency in mother–newborn pairs

Marie Pedersen [a,*], Thorhallur I. Halldorsson [b,c], Line Mathiesen [a], Tina Mose [a], Abraham Brouwer [d], Morten Hedegaard [e], Steffen Loft [a], Jos C.S. Kleinjans [f], Harrie Besselink [d], Lisbeth E. Knudsen [a]

[a] Section of Environmental Health, Department of Public Health, University of Copenhagen, Oester Farimagsgade 5A, 1353 Copenhagen K, Denmark
[b] Faculty of Food Science and Nutrition, School of Health Sciences, University of Iceland, Hjarðarhagi 2-6, 107 Reykjavík, Iceland
[c] Maternal Nutrition Group, Department of Epidemiology, Statens Serum Institut, Artillerivej 5, 2300 Copenhagen S, Denmark
[d] BioDetection Systems B.V., Science Park 406, 1098 XH Amsterdam, The Netherlands
[e] Department of Obstetrics, University Hospital of Copenhagen, Blegdamsvej 9, 2100 Copenhagen Oe, Denmark
[f] Department of Health Risk Analysis and Toxicology, University of Maastricht, Universiteitssingel 50, 6229 ER Maastricht, The Netherlands

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ABSTRACT

In utero exposure to environmental dioxin-like, estrogen and androgen compounds can cause adverse health effects. Little is known about potential interactions *in vivo* between dioxin-like compounds, estrogens and androgens during fetal development in humans. Therefore we explored the potential interactions *in vivo* between dioxin-like compounds, estrogens, androgens using chemical-activated luciferase expression (CALUX)® bioassays in maternal and umbilical cord blood plasma concurrently collected at the time of planned Caesarean section from 98 healthy pregnancies. The dioxin-like activity was also determined after placental transfer of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the *ex vivo* human placenta perfusion system. Similar dioxin-like activity in maternal and cord blood (37 versus 33 pg CALUX®-TEQ/g plasma lipids, $P>0.05$) was detected and it demonstrates transplacental transfer. Increased dioxin-like activity in the perfused placenta tissue after *ex vivo* TCDD perfusions (from 17 to 280 pg CALUX®-TEQ/g plasma lipids) suggest that accumulation in the placenta prevents immediate transplacental transfer of TCDD. Androgenic activity were also similar in the paired mother–newborns (0.10 versus 0.18 ng CALUX®-AEQ/mL plasma), whereas cord blood plasma estrogenic activity was higher than maternal levels (22.6 versus 18.5 ng CALUX®-EEQ/mL plasma). In cord blood plasma androgenic activity was strongly positively associated with maternal levels ($Rs=0.8$, $P<0.001$) whereas dioxin-like and estrogenic activities were modestly associated with maternal levels ($Rs\leq0.4$, $P<0.001$). The micronuclei frequency, an indicator of genetic instability was significantly associated with dioxin-like activity in cord blood, independently of other recorded factors ($Rs=0.4$, $P<0.003$). This study demonstrated interactions *in vivo* between dioxin-like, estrogenic and androgenic exposures during fetal development of humans.

1. Introduction

Dioxin-like compounds, e.g. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzo-furans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs) are a widespread and diverse group of persistent, lipophilic and hazardous environmental pollutants. In utero exposure to dioxin-like compounds at background levels have been associated with adverse health effects, e.g. reduced birth weight, adverse effects on endocrine function, cognitive development, immune-, and respiratory system observed in epidemiological studies (Lundqvist et al., 2006; ten Tusscher and Koppe, 2004). Health risks in relation to fetal exposure has been underlined by the fact that transplacental transfer of dioxin-like compounds has been demonstrated in the range of 25–80% by congener-specific analyses in human umbilical cord blood, placenta and fetal tissues in several studies (Covaci et al., 2002; Fukata et al., 2005; Suzuki et al., 2005; Sala et al., 2001; Jaraczewska et al., 2006; Schecter et al., 1998; Koppe et al., 1992; Wang et al., 2004).

Dioxin-like compounds have also been associated with human cancer risks. The carcinogenic mechanisms of action of TCDD and dioxin-like compounds are not yet fully understood, but the majority of the toxic effects induced by dioxin-like compounds are mediated

* Corresponding author. Tel.: +45 35327653; fax: +45 35327686.
E-mail addresses: marp@sund.ku.dk (M. Pedersen), Lur@ssi.dk (T.I. Halldorsson), lima@sund.ku.dk (L. Mathiesen), tina@sund.ku.dk (T. Mose), Bram.Brouwer@bds.nl (A. Brouwer), Morten.Hedegaard@rh.dk (M. Hedegaard), stl@sund.ku.dk (S. Loft), J.Kleinjans@grat.unimaas.nl (J.C.S. Kleinjans), Harrie.Besselink@bds.nl (H. Besselink), liek@sund.ku.dk (L.E. Knudsen).

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through activation of the aryl hydrocarbon receptor (AhR) resulting in changed gene expressions, altered metabolism, cell growth and differentiation and disruption of the steroid-hormone and growth-factors signal transduction pathways (Mandal, 2005). Although, dioxins are not direct genotoxins, activation of the AhR is clearly associated with cellular oxidative stress, mediated in part by the induction of cytochrome P450 enzymes with relatively loose coupling between oxygen and NADPH utilization, on the one hand, and substrate oxidation, on the other hand (Dalton et al., 2002). This can lead to genotoxicity through oxidative damage to DNA, which together with the well known promoter effect of dioxin can explain the specific induction of tumors in rodent livers (Knerr and Schrenk, 2006). In addition, it may be hypothesized that dioxin-mediated activation of the AhR-receptor and consequent up regulation of Cytochrome P450 leads to bioactivation of pre-carcinogens to which dioxin-exposed subjects are simultaneously exposed, for instance through dietary intake of polycyclic aromatic hydrocarbons e.g. benzo[a]pyrene and heterocyclic amines e.g. 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (DiMeIQx), and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP).

Cross-talk between AhR agonists such as dioxin-like compounds and the estrogen receptor alpha (ERα) and androgen receptor (AR) *in vitro* have been observed (Ohtake et al., 2008). In human breast carcinoma cells and in human umbilical vascular endothelial cells, some dioxin-like compounds act as agonists or antagonist of the ERα (Pliskova et al., 2005; Tavolari et al., 2006), but little is known about potential interactions *in vivo* between dioxin-like compounds, estrogens and androgens during fetal development in humans. Increased estradiol secretion measured in explants of human placental tissue after exposure to mixtures of dioxin-like compounds whereas decreased estradiol secretion after TCDD exposures have been reported (Augustowska et al., 2003). Disruption of the balance between estrogen and androgen in utero has, among other adverse effects, been proposed to influence the maternal–placental–fetal blood circulating and thereby the risks of intrauterine growth restriction (Carlsen et al., 2006), hormone-related cancer e.g. breast and testicular later in life (Grotmol et al., 2006; Ekbom, 1998) and reproductive disorders e.g. cryptorchidism, hypospadias, and decreased sperm count (Sharpe, 2003).

In view of this spectrum of dioxin-related health risks, already at early life, it is important to learn about actual fetal exposure levels. The collection of umbilical cord blood from the placenta is more directly linked to fetal exposure and less invasive than venipuncture of pregnant women, although the amount of available blood and thus the diversity of analysis of dioxin-like compounds are limited. Most commonly, the exposure assessment therefore rest on a narrow range of congeners determined in maternal/cord plasma. In some cases it can, however, be difficult to predict in utero exposures to dioxin-like compounds accurately from maternal plasma concentrations due to distinct differences between pregnant women and fetus related to the exposure windows, the route of exposures, body composition, plasma protein and lipid composition, metabolism and excretion (Morgan, 1997). Furthermore, the essential formation and transformation of steroid hormones (Pasqualini, 2005), which are present at elevated and differential concentrations in the mother, the placenta and fetus during pregnancy may be of importance in the assessment of this critical period of exposure and vulnerability.

Exposure misclassification may be reduced by measurement of specific receptor activations through *in vitro* reporter gene bioassays as variation in uptake and affinity to the receptor are integrated in the toxic potency measurements (Murk et al., 1997). Multiple interactions between co-exposures, their metabolites as well as endogenous compounds of similar mode of actions are also taken into account.

In view of the high sensitivity of the chemical-activated luciferase expression (CALUX)® bioassays, only small amounts of blood plasma are required for these measurements.

In the present study, we used a cross-sectional sample of paired mother–newborn plasma collected concurrently at the time of delivery in Copenhagen, Denmark for studying the exposures and effect related to dioxin-like, androgenic and estrogen activities during fetal life. We explored the associations between maternal and cord blood plasma level. The CALUX® bioassays were used to study transplacental transfer and potential impact of child exposures of maternal origin. In parallel, the transfer of TCDD, measured as dioxin-like activity by the CALUX® bioassay was studied in the *ex vivo* human placenta perfusion system. Potential effects in terms of genotoxicity, which could be related to oxidative stress, were assessed by the micronuclei (MN) frequencies in cord blood lymphocytes and maternal peripheral blood lymphocytes. The MN frequency is a well established biomarker of chromosomal breakage and/or whole chromosome loss that are unrepaired, misrepaired or malsegregated due to chromosome, cellular and nuclear dysfunction (Fenech, 2007). In prospective studies with adults elevated MN frequencies above the spontaneous baseline levels have been associated with increased cancer incidence (Bonassi et al., 2007) whereas later health outcomes associated with MN frequencies in umbilical cord blood have yet to be investigated.

The present study serves as a feasibility study in the context of the EU NewGeneris (FP6) project, in which analyses of biomarkers of exposure, effects and susceptibility in blood from several European mother–newborns cohorts are developed and applied in the context of children cancer risks (Merlo et al., 2009).

2. Materials and methods

2.1. Study population

We sent written invitations to participate to selected healthy pregnant women giving singletons births by planned Caesarean section at the University Hospital of Copenhagen, Rigshospitalet, 1–5 weeks prior to delivery (December 2006 to December 2007). Potential study participants were identified from the pregnancy medical records and the inclusion criteria were: (i) no chronic health complications e.g. diabetes; (ii) older than 18 years; (iii) no private commercial banking of cord blood; (iv) signed written informed consent; (v) capacity to understand the informed consent and (vi) willingness to complete the questionnaires in Danish. During a meeting at the hospital the day before delivery additional recruitment of participants for the placenta perfusion studies took place (May 2007 to May 2008). We limited our sampling to Caesarean sections in order to avoid potential variation in the biomarkers due to the labor stress, although this is much less likely to affect CALUX measurements and MN, which are more long term biomarkers. However, our sample was limited in size and reduction of potential variation not related to exposure or outcome was highly desirable.

Information on maternal potential confounders e.g. age, parity, education, weight, height, current and ex-smoking and alcohol consumption during pregnancy was obtained from self-administered maternal questionnaires (90 min) checked at the participant's home or at the hospital at the time of delivery. Birth outcomes e.g. gestational age, gender of the newborns, birth weight, birth length, birth head circumference, birth abdominal circumference and placenta weight were collected from the medical birth records. Gestational age estimation was done by the midwives and based on the first day of the last menstrual period corrected after ultrasound examination.

Written informed consent was obtained from all participants upon collection of blood, placenta, birth outcomes and questionnaires for the purpose of in utero exposure studies through the use of biomarkers and *ex vivo* placenta perfusion studies. The Ethics Committee of the Capital Region of Denmark (J. Nr. H-KF-01-327603; J. Nr. KF-11-260063) and the Danish Data Protection Agency (J. Nr. 2007-41-0415; J. Nr. 2005-41-4961) reviewed and approved the studies prior to initiation.

2.2. Biological sample collection

Maternal peripheral blood samples (~50 mL) were drawn from 98 pregnant women by venipuncture 1–2 h before planned section in the morning hours, between 7 and 11 a.m. The women had been fasting in approximately 6 h. Umbilical cord blood (0.5–80 mL) was drawn by umbilical puncture from 96 placentas at the hospital immediately after delivery. All blood samples were collected into heparinized tubes (Vacutainer, Becton Dickinson, Oxford, UK) and paired mother–newborn samples were processed within <1–5 h from each other. Sufficient cord blood was available for separation (650 g, 10 min) of >3 mL plasma from approximately two-thirds of the participants. The placentas used for the *ex vivo* placenta perfusion studies were obtained immediately after the deliveries by section. Upon collection of umbilical cord blood (~20 mL), Krebs–Ringer buffer with heparin (25 IU/mL) was slowly injected into chorionic vessels in the placenta. All biological samples were stored at −20 °C until shipment on dry ice.

2.3. Cultures, slide preparation and scoring of micronuclei

The MN frequencies per 1000 (once-divided) binucleated cells (MNBN‰) were analysed using the cytokinesis-block micronucleus assay (Fenech and Morley, 1985). Paired mother–newborn whole blood cultures, in duplex, were initiated simultaneously on average within 6 h from collection. At 72 h following phytohaemagglutinin stimulation, cells were harvested and fixed (Pedersen et al., 2009). The cell suspensions were dropped onto clean labeled slides, air-dried and after 48 h half of the slides were stained in freshly filtered 5% Giemsa (Sigma-Aldrich, Denmark) for 20 min (pH 6.8). All slides were coded and randomized at the end of recruitment. The scoring of ~2000 binucleated cells per donor was carried out by the same person using a light microscope (Olympus BX 41) at 400× magnification following the HUMN criteria for scoring MN (Fenech et al., 2003).

2.4. Ex vivo placenta perfusions with TCDD

We used a dual recirculating perfusion system with separate maternal and fetal circulations to study the transfer of TCDD, as previously described in details (Mathiesen et al., 2009). The fetal vein and artery in an isolated villous tree was perfused by cannulation within 1 h after delivery. In four out of ten perfusions, human serum albumin (HSA) at 30 mg/mL was added to the perfusate (RPMI 1640 medium supplemented with 2% L-glutamine, 1% penicillin and streptomycin) while dextran at 2 mg/mL was added to increase the viscosity of the fluids in the experiments without HSA. A final concentration of 6.0 pg/mL of the test substance, TCDD [CAS No 1746-01-6] (Cambridge Isotope Laboratories, Inc., Andover, USA) diluted in 0.5 mL dimethyl sulfoxide (DMSO) and 100 µg/mL of antipyrine, the positive control of transplacental transfer, were added to the maternal perfusate of 100 mL. Gassing of 95% O_2 and 5% CO_2 and 95% N_2 and 5% CO_2 in maternal and fetal perfusate, respectively, was done throughout the perfusion. The flow rate during the perfusions was 9 mL/min in the maternal circulation and 3 mL/min in the fetal circulation. After preperfusion with perfusate without added test substance and positive control for 30 min the perfusion was initiated. A perfusion time of 6 h at 37 °C was used for the perfusions with TCDD.

Maternal and fetal perfusion fluid samples were collected before and during the perfusion (at 0, 30, 60, 120, 240 and 360 min). The pellets of red blood cells (4000 g, 5 min) were discarded and the supernatants (~5 mL) were stored for dioxin-like activity determination and for antipyrine (200 µL) detection by high performance liquid chromatography.

We obtained tissue samples (10 g) from the placenta used for perfusion prior to cannulation for assessment of background levels of dioxin-like activity. After the end of perfusion, tissue samples were collected from the perfused cotyledon and its surrounding tissue for assessment of potential accumulation of TCDD.

2.5. AhR-mediated activation

Dioxin-like activity, expressed as AhR mediated activation of the extractable lipid from plasma, placenta and placenta perfusion fluids, was determined through the DR CALUX® bioassay developed by BioDetection System (Aarts et al., 1996; Brouwer et al., 2004). This reporter gene bioassay is based on a genetically modified H4IIE rat hepatoma cell line containing the luciferase gene under transcriptional control of AhR. Proportional to the strength of the AhR binding, luciferase as well as proteins and enzymes associated with AhR activation are produced *in vitro* after exposure of the cells. With addition of the substrate luciferine for the luciferase enzyme, it is converted and then light is emitted. The luminance is calibrated with respect to TCDD in units of toxic equivalency quantity (TEQ) and sample results are expressed as pg CALUX®-TEQ per gram of extractable lipid. The DR CALUX® bioassay has previously been validated and used in human biomonitoring, using 0.1–3 mL adult blood plasma (Brouwer et al., 2004; Koppen et al., 2002; Pauwels et al., 2000) and 5–10 mL cord blood plasma (Koppen et al., 2009).

Approximately, 1 g of maternal plasma, 3 g of cord blood plasma, 5 g of perfusion fluid and 10 g of placenta tissue were extracted in 97% n-hexane 3% diethyl ether solution by means of shake-solvent extraction. The acid-labile matrix components of extractable fat were removed by passage through two acid silica (first 20% and then 33% wet/wet) columns topped with sodium sulfate. The oxidized fat and chemically instable AhR ligands such as polycyclic aromatic hydrocarbons (PAHs), other combustion products, flavonoids, indole-3-carbinol or endogenous compounds were hereby removed, and then the cleaned extracts were evaporated under nitrogen and re-dissolved in 8 µL DMSO. The AhR activity of the extracts was measured after 24 h of incubation of cells at 95% confluency exposed to the extracts in 0.8% DMSO in triplicate on 96-well microtiter plates containing the standard TCDD calibration range, a DMSO control, a procedure blank, and an internal reference material. The luciferase activity was measured using a luminometer equipped with two dispensers (Lucy2; Anthos Labtec Instruments, Wals, Austria). The limit of detection (LOD) was calculated as the average background signal measured from the DMSO solvent control on each plate plus three times its standard deviation. LOD of the DR CALUX® assay varied between maternal and cord blood plasma samples dependent on the plasma lipid content. It ranged from 0.15 to 11.0 and from 0.09 to 34.1 pg CALUX®-TEQ/g lipid, respectively. The LOD of perfusion fluid varied between perfusions, ranging from 0.01 to 0.05 while the LOD of the placental tissue was 1.57 pg CALUX®-TEQ/g lipid. Limit of quantification was determined as the average background plus 10-times the SD. Lipid content of the plasma, placenta tissues and perfusion fluids was determined gravimetrically (Rylander et al., 2006).

2.6. ERα- and AR-mediated activity

Similarly, we determined the internal dose of compounds having estrogenic and androgenic activity of the extractable lipid fraction of the plasma samples using the ERα and the AR CALUX® bioassays, respectively (Sonneveld et al., 2005). These validated bioassays are derived from the same human U2-OS osteosarcoma cell line and used in human biomonitoring (Besselink et al., 2007; Pliskova et al., 2005). Extractable lipid from 0.5 mL of plasma using methyl tert-butyl ether was evaporated under nitrogen and re-dissolved in DMSO. The ERα and AR activities of the extracts were measured after 24 h of incubation in cells at 95% confluency exposed to the extracts in 0.1% DMSO in triplicate on 96-well microtiter plates containing a concentration series of 17 ß-estradiol or dihydrotestosterone, DMSO, procedure blank and an internal reference material. The luminescence read outs were then calibrated with respect to E2 and DHT and sample results

were expressed as ng estrogenic equivalents (EEQ) per mL or ng androgenic equivalents quantity (AEQ) per mL of plasma, respectively. The LOD of ERα ranged 0.011–0.022 ng CALUX®-EEQ/mL while the LOD of AR was 0.03 ng CALUX®-AEQ/mL.

2.7. Statistical analyses

Nonparametric Wilcoxon Signed Ranks tests (two-tailed) were used for the comparison between maternal and cord blood levels and between boys and girls. We reported results as median and range. Spearman Rank correlations coefficient (Rs) with level of significance set to $P<0.05$ were used to explore the relations between potential predictors of cord blood activity levels and partial function was used to take away effects of potential confounders such as gestational age (*results not shown*). In the negative binomial regression models for investigation of associations between MN frequencies and the CALUX® activities the rounded MN frequencies were used. A priori potential confounders, which included maternal current smoking, pre-pregnancy BMI, and parity, were included in the final model. Samples below the LOD did not differ in lipid content (*data not shown*) from those above LOD and were assigned a value equal to 0.5 × LOD prior to analyses for associations. We performed the statistical analyses in SAS version 9.1 (SAS Institute Inc. Cary NC. USA).

3. Results

3.1. Study population characteristics

Healthy pregnant women ($N=98$) with planned singleton delivery, 94% Caucasians, 3% Asian, 2% Mestizo and 1% Aryan ethnicity, in the early-30s participated (Table 1). Maternal age at delivery, maternal education, pre-pregnancy body-mass index (BMI), parity (except for estrogenic activity), previous breast feeding, current smoking and alcohol intake during pregnancy were not significantly associated with dioxin-like, estrogenic or androgenic activity levels in maternal or cord blood plasma (*results not shown*). Estrogenic activity in maternal plasma (EEQ/mL) was positively associated with parity (0/1+) ($Rs=0.2$, $P=0.05$) and total lipids (g/L) ($Rs=0.3$, $P=0.02$). Inverse associations between maternal androgenic activity (AEQ/mL) and total lipids ($Rs=-0.3$, $P<0.001$) were observed. Higher androgenic activity levels among ex-smoking as compared with never-smoking mothers (0.19 versus 0.06 CALUX®-AEQ/mL, $P=0.002$) and their newborns (0.20 versus 0.06 CALUX®-AEQ/mL, $P=0.09$) were observed.

3.2. Transplacental transfer of dioxin-like compounds in human plasma and after ex vivo placenta perfusions with TCDD

Dioxin-like activity was determined as AhR activation in the majority of samples (Table 2). Maternal plasma lipid concentrations were more than 3 times higher than those measured in cord blood. This means that dioxin-like activity expressed per g plasma lipid the values were similar (37 versus 33 pg CALUX®-TEQ/g lipid, $P>0.05$) for mothers and children respectively. When comparing dioxin-like activities expressed per volume of plasma, maternal levels were significantly higher than those measured in cord blood (0.33 versus 0.11 pg CALUX®-TEQ/mL, $P<0.001$).

The background dioxin-like activity in the five placentas used for perfusions was comparable to the corresponding cord blood samples (17 (9–20) versus 18 (15–68) pg CALUX®-TEQ/g lipid). After successful perfusion of the placentas with TCDD, the activity levels increased to 54 and 146 pg CALUX®-TEQ/g lipid in the surrounding tissue and the perfused cotyledon, respectively (Fig. 1). Perfused cotyledon levels increased even more when HSA was added to the perfusate (280 (240–690) pg CALUX®-TEQ/g lipid) and also the activity level in surrounding placenta tissue was higher (215 (63–390) pg CALUX®-TEQ/g lipid) (*data not shown*). In four of ten performed placenta perfusions, HSA was added in the perfusion medium. Five perfusions were successful e.g. the leakage from the fetal reservoir was less than 3 mL/h, and the fetal/maternal ratio of antipyrine was greater than 0.75 during the perfusion and in two of these HSA was included in the medium. As illustrated in Fig. 1, the initial high dioxin-like activity in maternal perfusate decreased during the perfusion time as a result of TCDD transfer from the maternal circulation to the placenta tissue where it was accumulated. We found that the dioxin-like activity in the fetal circulation remained low throughout the perfusion indicating no transfer of TCDD across the perfused tissue under the experimental conditions used in these experiments with two types of perfusion media and 6 h perfusion. A similar transport from maternal circulation to placenta tissue and no increased dioxin-like activity in the fetal circulation was also observed in the five perfused placentas which are not presented in Fig. 1 due to the increased leaking from the fetal circulation during the last hour of the perfusion.

3.3. Estrogenic and androgenic plasma activities

Estrogenic activities were detected at levels, above LOD, in all the samples, irrespectively of the gender of the newborns. Maternal estrogenic activity levels were significantly lower than cord blood levels (18.5 versus 22.6 ng CALUX®-EEQ/mL, $P<0.03$). Androgenic activities were above LOD in the majority of samples analysed. No significant difference between maternal and cord blood samples were detected (0.10 versus 0.18 ng CALUX®-AEQ/mL plasma, $P>0.05$). The ratio of androgenic/estrogenic activity was 0.005 in mothers and 0.008 in newborns. Fig. 2 illustrates the activity levels in the mother–newborn pairs.

3.4. Dioxin-like activity and relations with estrogenic and androgenic plasma activities

We found a strong positive association between fetal and maternal androgenic activity ($Rs=0.8$, $P<0.001$) (Table 3) (Fig. 2c). Dioxin-like and estrogenic activities in cord blood plasma were also positively associated with the corresponding maternal activities ($Rs=0.3$, $P=0.003$; $Rs=0.4$, $P<0.01$, respectively). Dioxin-like and androgenic activities were strongly inversely associated in cord blood plasma ($Rs=-0.7$, $P<0.001$), in maternal plasma ($Rs=-0.4$, $P<0.001$), as well as between cord blood plasma dioxin-like activity and maternal plasma androgenic activity ($Rs=-0.3$, $P=0.04$). In contrast, dioxin-like and estrogenic activities were positively associated at modest and borderline levels in cord blood plasma, in maternal plasma and between cord blood and maternal plasma, respectively. Estrogenic and androgenic activities were inversely associated in cord blood plasma ($Rs=-0.4$, $P=0.005$) and in maternal plasma ($Rs=-0.3$, $P=0.01$). Associations remained significant after inclusion of parity and total lipids in relation to maternal estrogenic activity and after inclusion of ex-smoking and total lipids in relation to maternal androgenic activity in partial Spearman Rank correlation analysis (*results not shown*).

Dioxin-like activity was inversely associated with gestational age (days) in cord blood plasma ($Rs=-0.4$, $P=0.002$) while estrogenic activity was not associated with gestational age and androgenic activity was positively associated with gestational age ($Rs=<0.3$, $P=0.02$). Fetal dioxin-like activity was positively associated with placenta thickness. Androgenic activity in maternal and fetal samples associated positively with placenta weight, at borderline levels too. These associations were similar in boys and girls and before and after adjustment for maternal current smoking (no/yes), prepregnancy BMI, gestational age, and parity (0/1+). No other differences between boys and girls were observed (data not shown). Androgenic activity in cord blood samples was positively associated with the length of the newborn at birth. At borderline level androgenic activity in cord blood samples negatively associated with head circumference.

Table 1
Characteristics of the study population of 98 mother–newborn pairs.

	Median	(Range)
Maternal age at delivery (years)	33	(21–43)
Pre-pregnancy BMI (kg/m^2)	22.2	(17.3–38.7)
Parity (0/1+)	1	(0–4) 17/75
Maternal education (high/low)[a]	71/24	
Current smoking (no/yes)	92/6	
Ex-smoking (no/yes)	41/54	
Pregnancy alcohol (no/yes)	46/47	
Maternal micronuclei frequency (MNBN ‰)[b]	7.2	(3–17)
Newborn micronuclei frequency (MNBN ‰)	3.2***	(0–9)
Gender (girls/boys)	49/49	
Birth length (cm)	51	(47–55)
Birth weight (g)[c]	3460	(2400–4320)
Gestational age (days)[d]	270	(245–281)
Birth head circumference (cm)[e]	36	(31–39)
Birth abdominal circumference (cm)	32	(27–36)
Placenta weight (g)[f]	744	(358–1256)

[a] Education for 4 years above secondary school (~ bachelor levels or more) was classified as high.
[b] Micronuclei frequencies per 1000 binucleated cells were used as indicator of chromosomal breakage and/or whole chromosome loss expressed in white blood cells after cell division.
[c] Considering birth weight at term (37 completed weeks of gestation) below 2500 g as low, then 2% had low birth weight.
[d] Considering preterm birth as before 37 completed weeks of gestation, then 3% of the newborns were born preterm.
[e] Boys were born with bigger heads than the girls (36.5 versus 35.0 cm, $P=0.002$).
[f] Placenta weight included the umbilical cord and membranes.
*** Significant difference between maternal and cord blood levels (Wilcoxon Signed Ranks Test (Two-tailed) ($P<0.001$).

Table 2
Dioxin-like, estrogenic and androgenic activities in plasma from mother–newborn pairs measured by the CALUX® bioassays.

	Mothers			Newborns		
	Median (range)	*N*	*N*>LOD	Median (range)	*N*	*N*>LOD
DR CALUX® (pg TEQ/g lipid)	37 (6–118)	98	97	33 (15–141)	71	52
DR CALUX® (pg TEQ/mL)	0.33 (0.03–1.18)***	98	97	0.08 (0.01–0.38)	71	52
ERα CALUX® (ng EEQ/mL)	18.5 (1.3–57.0)	85	85	22.6 (1.8–62.0)*	56	56
AR CALUX® (ng AEQ/mL)	0.10 (0.02–0.91)	85	66	0.18 (0.02–0.29)[a]	54	45
Total lipids (g/L)	9.0 (5.5–23.3)***	98	98	2.5 (0.3–4.1)	71	52

[a] Androgenic activities were higher at borderline levels in boys than in plasma from girls (0.21 versus 0.06 ng AEQ/mL plasma, $P=0.09$) Dioxin-like and estrogenic activity did not differ between girls and boys.
* Significant difference between maternal and cord blood levels (Wilcoxon Signed Ranks Test (Two-tailed) ($P<0.05$)).
*** Significant difference between maternal and cord blood levels (Wilcoxon Signed Ranks Test (Two-tailed) ($P<0.001$)).

3.5. Associations between dioxin-like, estrogens and androgens activities and MN frequency

We found a positive association between chromosomal damage expressed as MN frequency and dioxin-like activity in cord blood (Table 4). Cord blood estrogenic and androgenic activities were also associated with MN frequency, though at borderline significance. The association between dioxin-like activity and MN frequency remained unchanged in multivariate models adjusting for estrogenic and androgenic activity as well as when adjusting for maternal smoking, pre-pregnancy BMI and parity.

We also found that maternal MN frequency was not associated with dioxin-like (Rs<0.1, $P=0.5$), estrogenic (Rs<0.1, $P=0.3$) or androgenic activity (Rs<0.1, $P=0.6$).

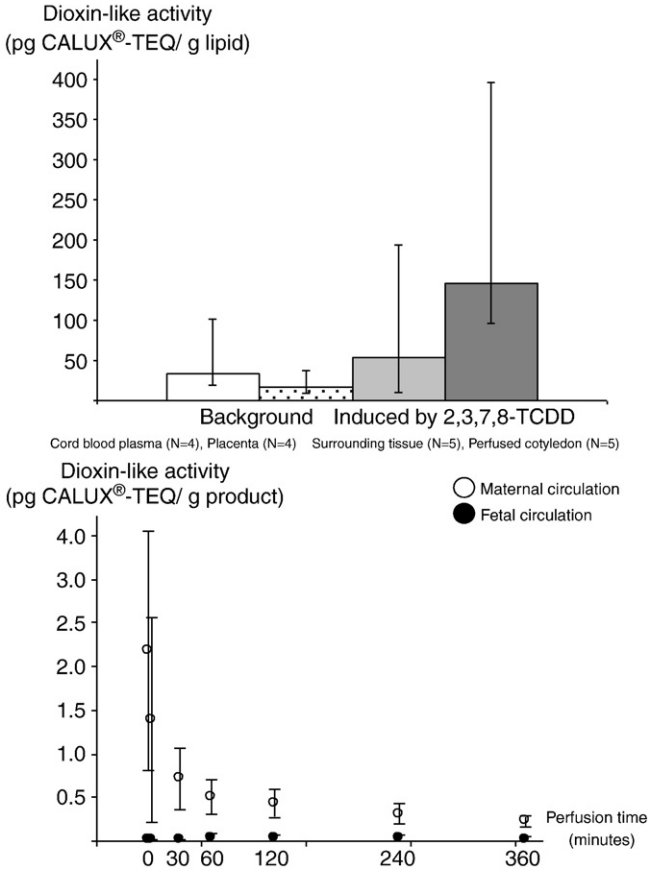

Fig. 1. Dioxin-like activity in maternal and fetal circulation in the *ex vivo* human placenta perfusion system before and after perfusions with 2,3,7,8-tetrachlorodibenzo-p-dioxin.

A significant association between maternal MN frequency ($\beta=0.035$, 95% C.I. (0.011–0.060) and age while no association with dioxin-like activity ($\beta=0.004$, 95% C.I. (−0.003–0.012) was found in a multivariate model.

4. Discussion

This study shows, for the first time to our knowledge, that the dioxin-like, estrogenic and androgenic activity levels are significantly correlated between plasma samples concurrently collected from mother–newborn pairs and that the MN frequency is significantly associated with the dioxin-like activity in cord blood.

Background dioxin-like activity levels reflect interactions of a related group of stable, lipophilic and exogenous compounds with AhR. The median maternal plasma dioxin-like activity in the present study were taken 2 h before delivery was similar to the level measured by the same laboratory in blood collected predelivery during gestation week 8–25 in 1998–2002 from a subset of 100 pregnant women from the Danish National Birth Cohort (*median (range);* 38 (4–214) pg CALUX®-TEQ/g lipid, 88% of the samples above LOD) (Halldorsson et al., 2009). The present cord blood levels were higher than the dioxin-like activity levels in cord plasma samples from 2002–2004 in Belgium (*median (range)*; 23 (4–158) pg CALUX®-TEQ/g lipid), although a higher fraction (89%) of the Belgian cord samples were above LOD (Koppen et al., 2009). The background dioxin-like activity measured in placenta tissue in the present study (17 (9–20) CALUX®-TEQ/g lipid*)* was comparable to the sum of dioxins measured by chemical-analyses in 50 placentas from Taiwan (*mean (95% CI);* 12.8 (11.5–14.1) pg WHO-TEQ/g lipid) (Wang et al., 2006).

Our findings of similar mother-cord blood dioxin-like activity levels and the positive association between the paired samples indicate transplacental transport of dioxin-like compounds. In our experimental model, TCDD accumulated in the placenta indicated by the increased (up till 38 times higher) dioxin-like activity in the tissue after *ex vivo* perfusions with TCDD preventing immediate transplacental transfer within 6 h in the term placenta. Elevated levels of TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF have previously been detected in the placentas as compared with corresponding maternal and cord blood samples (Suzuki et al., 2005; Wang et al., 2006). The latter suggesting that congeners with high AhR affinity accumulate in placenta, a tissue with high density of AhR (Manchester et al., 1987). However, short term effect observations in *ex vivo* models under experimental conditions with media different from whole blood are not directly comparable to long term observations of the environmental transplacental transfer *in vivo* which are mediated by whole blood and initiated earlier and throughout pregnancy.

The estrogenic activities observed in pregnant women and newborns most likely reflect high levels due to pregnancy buildup of estrogens and to a minor extend to some environmental exposure at low levels. The high estrogenic plasma activities especially in cord blood in this study probably reflect the elevated estrogen production in the placenta, reaching 100–120 mg/24 h at term in uncomplicated pregnancies (Pasqualini, 2005). Estradiol and testosterone in pregnant women at higher concentrations than in cord blood have been reported (Troisi et al., 2003), but considering the sum of estrogens as well as the sum of androgens concentrations were higher in the cord blood samples. Among these hormones modest associations between maternal and cord blood levels were observed. Strong estrogenic activities of endogenous estrogens (parent compounds and metabolites), xenoestrogens as well as a weak estrogenic activity of benzo[a]pyrene have been detected using the ERα CALUX® bioassay (Legler et al., 2002). Some PCBs have shown estrogenic and antiestrogenic potencies (Pliskova et al., 2005) and estrogenic activity as well as anti-androgenic activity in the ERα - and AR-CALUX® bioassays have been reported from several pesticides (Sonneveld et al., 2005). In serum collected from third trimester from pregnant women, inverse and positive associations between dioxin-like compounds and estrogen metabolites have been

found (Wang et al., 2006). Antagonistic *in vitro* effects at the AR have been reported for a variety of environmental compounds such as plasticizers (Kruger et al., 2008).

The present study shows positive associations between dioxin-like activity and estrogenic activities as well as inverse associations between dioxin-like activity and androgenic activities in both maternal and fetal plasma. These interactions are puzzling and similar studies in other study population may further elucidate these findings. The strong association between maternal and cord blood plasma androgenic activity in the present study suggest that the sources of the maternal and fetal exposure are shared.

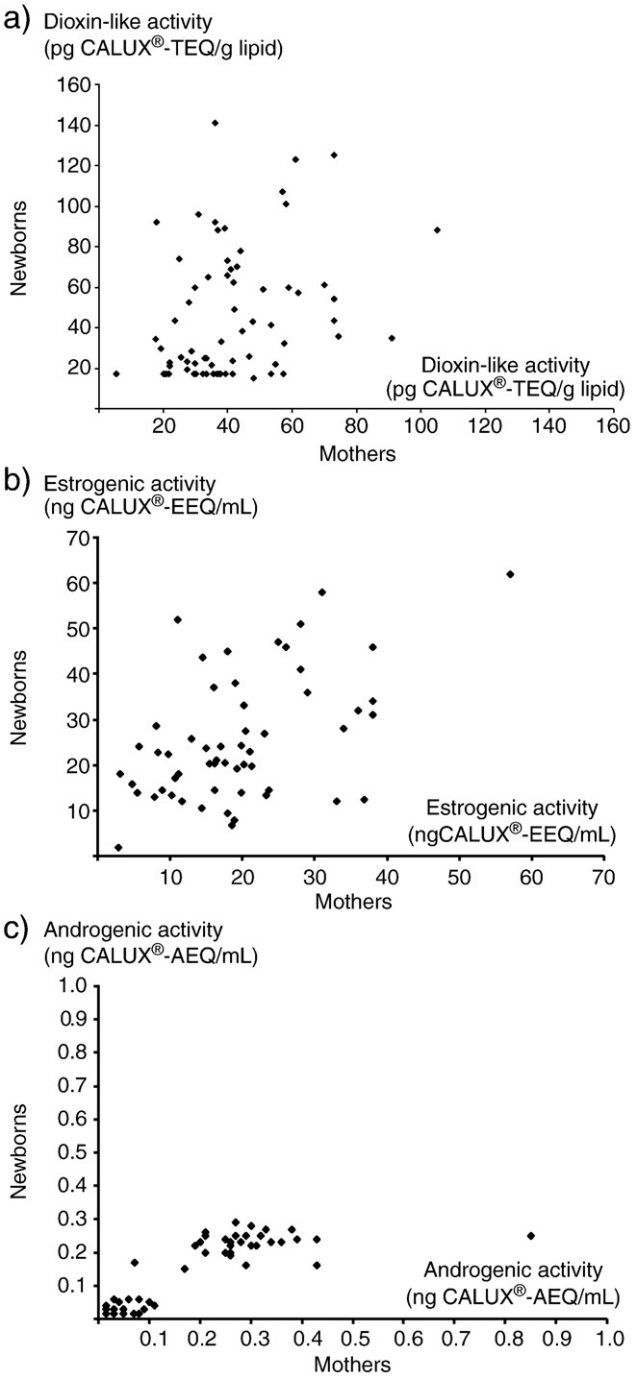

Fig. 2. Dioxin-like (a), estrogenic (b) and androgenic (c) activities in maternal and human umbilical cord blood.

We found a positive association between internal dose to persistence compounds having dioxin-like activity and MN frequency in the cord blood, but not in maternal blood. Similarly, we have reported associations between maternal exposure to air pollution and MN frequency in cord blood and not in maternal blood (Pedersen et al., 2009). In mothers, an age-related increase in MN frequency was found. Our results support that elevated MN frequencies above the low spontaneous baseline level of MN found in umbilical cord blood reflect genotoxic exposure during fetal life (Neri et al., 2005). In general, environmental exposure to clastogens or aneugens, genetic defects in genome maintenance e.g. cell-cycle checkpoint, DNA repair genes as well as aging and micronutrient deficiency or excess can induce MN in PBLs (Mateuca et al., 2006).

The finding of a positive association between dioxin-like activity and MN frequency in cord blood, is in accordance with a similar finding of high MN frequencies in human cord blood associated with exposure to environmental pollution with PCBs and heavy metals in Serbia (Milosevic-Djordjevic et al., 2005). A positive correlation between serum PCB 118, a dioxin-like PCB, and MN frequency among environmental exposed Belgian adults has also been reported (De Coster et al., 2008). Following extremely high internal doses of pure TCDD, a delayed and transient effect of increased MN frequency has been observed in two individuals (Valic et al., 2004). Enhanced induction of MN in human whole-blood cultures after exposures to mixture of organochlorine compounds similar to the dioxin-like congener-profile detected in the general population has been reported (Nagayama et al., 1997). TCDD induced changes in growth factor receptor signaling, other cytosolic signaling proteins, tumor suppressor proteins, and cell cycle proteins have been observed in endocervical cells (Enan et al., 1998). Activation of the AhR results in oxidative stress with potentially resulting damage to DNA as shown in several experimental models (Dalton et al., 2002; Knerr and Schrenk, 2006; Lin et al., 2007; Wyde et al., 2001). Moreover, associations between exposure to dioxin-like compounds and urinary excretion of 8-oxo-7,8-dihydrodexyguanosine, a product from oxidation of guanine in the nucleotide pool or DNA, have been found in occupationally exposed men (Wen et al., 2008), although some effects may be counterbalanced by up-regulation of defense systems (Yoshida et al., 2006). Increased strand breaks in white blood cells have also been found after combined dioxin and PAH exposure (Kim et al., 2004). In cell cultures homologous recombination appears to be affected by dioxins by mechanisms unrelated to oxidative stress (Chan et al., 2004a, b) and this may also cause genomic instability resulting in MN formation. These observations of multiple cancer predictive responses induced by exposure to dioxin-like compounds highlight the importance of AhR which may be involved in biotransformation, growth and differentiation.

Table 3
Dioxin-like, estrogens and androgens plasma CALUX® activities in cord blood and Spearman rank associations with potential predictors.

	Fetal DR (pg TEQ/g lipid)	Fetal ERα (ng EEQ/mL)	Fetal AR (ng AEQ/mL)
Maternal DR (pg TEQ/g lipid)	**0.3** $P=0.003$, $N=71$	0.2 $P=0.07$, $N=56$	**−0.3** $P=0.04$, $N=54$
Maternal ERα (ng EEQ/mL)	**0.4** $P<0.01$, $N=62$	**0.4** $P<0.01$, $N=56$	**−0.3** $P=0.01$, $N=54$
Maternal AR (ng AEQ/mL)	**0.7** $P<0.001$, $N=62$	**−0.5** $P<0.001$, $N=56$	**0.8** $P<0.001$, $N=54$
Fetal DR (pg TEQ/g lipid)	1	**0.4** $P=0.002$, $N=56$	**−0.7** $P<0.001$, $N=51$
Fetal ERα (ng EEQ/mL)	.	1	**−0.4** $P=0.005$, $N=54$
Placenta weight (g)	−<0.1 $P=0.3$, $N=67$	−<0.2 $P=0.1$, $N=52$	−<0.3 $P=0.08$, $N=50$
Birth length (cm)	−<0.1 $P=0.7$, $N=64$	−<0.0 $P=0.8$, $N=51$	**0.3** $P=0.03$, $N=49$
Birth head circumference (cm)	−0.2 $P=0.3$, $N=54$	−<0.2 $P=0.2$, $N=45$	0.3 $P=0.07$, $N=43$

Table 4
Dioxin-like, estrogenic and androgenic plasma CALUX activities and associations with micronuclei frequency (MNBN ‰) in cord blood.

	Univariate models					Multivariate models			
	Rs[a]	*P*	*N*	β[b] (95% C.I.)	*P*	β[c] (95% C.I.)	*P*	β[d] (95% C.I.)	*P*
DR (pg TEQ/g lipid)	0.4	0.02	43	0.006 (0.002–0.011)	0.008	0.005 (0.104–1.714)	0.027	0.006 (0.002–0.011)	0.010
ERα (ng EEQ/mL)	0.3	0.08	38	0.008 (−0.004–0.020)	0.186	0.004 (−0.003–0.001)	0.206	.	
AR (ng AEQ/mL)	−0.3	0.07	36	−1.526 (−3.326–0.274)	0.097	−0.166 (−2.836–2.504)	0.556	.	
Current smoking (no/yes)								0.243 (−0.514–1.001)	0.529
Pre-pregnancy BMI (kg/m^2)								0.243 (−0.046–0.042)	0.940
Parity (0/1/2/3/4)								0.127 (−0.185–0.438)	0.426

[a] Spearman rank correlations.
[b] Negative binomial regression model with MN frequencies per 1000 BN cells as dependent variable and dioxin-like, estrogenic and androgenic plasma activity, respectively, as independent variables.
[c] Negative binomial regression model with MN frequencies per 1000 BN cells as dependent variable including dioxin-like, estrogenic and androgenic plasma activity in the model.
[d] Negative binomial regression model with MN frequencies per 1000 BN cells as dependent variable and dioxin-like activity plus maternal smoking, pre-pregnancy BMI and parity.

Further studies of specific intrauterine exposures in combination with the use of reporter gene bioassays as well as transcriptomic and proteomic approaches in the present mother-cord blood samples are planned within the NewGeneris (FP6) framework. In summary comparable levels of dioxin-like activity and androgenic activity were demonstrated in paired mother–newborn samples, whereas cord blood plasma estrogenic activity was higher than maternal levels. Dioxin-like and estrogenic activities showed moderately strong associations between cord blood and maternal plasma, whereas a stronger association between fetal and maternal androgenic activity were observed, indicating some placental transfer of the responsible endogenous and exogenous compounds. The findings of significant associations between only cord blood levels and MN frequency and birth characteristics as well as the stronger associations between dioxin-like, estrogenic and androgenic activities in cord blood plasma as compared with those in maternal plasma emphasize that intrauterine exposure to dioxin-like compounds may interfere with the essential estrogen–androgen balance and lead to MN formation. The findings of the present study provide valuable information of potential interactions between multiple environmental transplacental exposures to mixtures and endogenous steroids during this critical period of development.

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Disclosure statement

No conflicts of interests.

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