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Inverse association of highly chlorinated dioxin congeners in maternal breast milk with dehydroepiandrosterone levels in three-year-old Vietnamese children

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#### Highlights

Dioxin levels in the breast milk were higher in the hotspot than the non-exposed region.

Salivary steroid hormones were analyzed from 3-year-old children of these mothers.

DHEA levels were significantly lower in the hotspot than in the non-exposed region.

DHEA levels were inversely correlated with highly chlorinated dioxin congeners.

**Abbreviations**

DHEA: Dehydroepiandrosterone

F: Cortisol

E: Cortisone

A-dione: Androstenedione

OCDD: Octachlorodibenzodioxin

TCDD: 2,3,7,8-Tetrachlorodibenzo-p-Dioxin

CYP17: Cytochrome P450C17

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

GC-MS: Gas chromatography-mass spectrometry

## **Abstract**

This study aims to evaluate the endocrine-disrupting effect of dioxin congeners on adrenal steroid hormones in mother–child pairs. In our previous study, we found that cortisol and cortisone levels were higher in the blood and the saliva of mothers living in a dioxin hotspot area than in mothers from a non-exposed region in Vietnam. In this follow-up study, we determined the salivary steroid hormone levels in 49 and 55 three-year-old children of these mothers in the hotspot and non-exposed region, respectively. Steroid hormones were determined by liquid chromatography-tandem mass spectrometry, and dioxin in the maternal breast milk was determined by gas chromatography-mass spectrometry. Dioxin levels in the breast milk of mothers from the hotspot (median total toxic equivalents polychlorinated dibenzodioxins / polychlorinated dibenzofurans; (TEQ PCDD/Fs) of 11 pg/g lipid) were three to four times higher than that of mothers in the non-exposed region (median TEQ PCDD/Fs of 3.07 pg/g lipid). Salivary dehydroepiandrosterone (DHEA) levels in children were found to be significantly lower in the hotspot than in the non-exposed region, while cortisol and cortisone levels were not different between the two regions. Highly chlorinated dioxin congeners, such as octachlorodibenzodioxin (OCDD), 1,2,3,4,6,7,8-heptachlorodibenzodioxin (HpCDD) and 1,2,3,4 (or 6), 7,8-hexachlorodibenzodioxin Hx(CDD), showed stronger inverse associations with the children's salivary DHEA than other lowly chlorinated dioxin congeners. Glucocorticoid levels in the mothers exhibited a significantly positive correlation with OCDD and HpCDD/F (polychlorinated dibenzofurans). In conclusion, highly chlorinated dioxin congeners are more strongly correlated with endocrine-disrupting effects on adrenal hormones, resulting in high cortisol levels in the mothers and low DHEA levels in their three-year-old children.

Key words: Dehydroepiandrosterone (DHEA), Cortisol, Endocrine-disruption, Dioxin, Vietnamese children

## 1. Introduction

Dioxin (polychlorinated dibenzodioxins, polychlorinated dibenzofurans) is one of the most toxic chemical substances known and is a persistent environmental contaminant. It can be released into the environment as a by-product of various chemical manufacturing and combustion processes.

Dioxin involves a number of isomers and congeners with a dibenzo-*p*-dioxin, dibenzofuran or biphenyl skeleton, and different numbers of chloride atoms, with the toxic potency differing markedly from one isomer to the next. As such, and to allow a simple evaluation of their hazards to health, the toxic equivalency factor (TEF) was established and has been widely used for some time (Berg et al., 2006). Although dioxin was suspected to cause endocrine disruption for a long time, very few epidemiological studies were carried out on its effects on the steroid hormone biosynthesis in humans (Nhu et al., 2011; Manh et al., 2013; Kido et al., 2014; Sun et al., 2014). In our previous research on women from a dioxin hotspot region in Vietnam, the salivary and serum levels of six steroid hormones, including sex hormones, were simultaneously determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Kido et al., 2014). The results of that study demonstrated that the levels of cortisol (F) and cortisone (E) were higher in the hotspot than in a non-exposed region, and these hormone levels were positively associated with dioxin concentrations in breast milk. Furthermore, we found saliva to be a useful matrix for hormone assays in epidemiological studies.

There are two main contaminated regions in the world as a result of dioxin exposures with one in Southern Vietnam and the other at Seveso in Italy (Stellman et al., 2003; Warmer et al., 2011). Although many Vietnamese were exposed to herbicide/dioxin to a greater extent, most studies concerning adverse health effects have been carried out on American veterans (Giri et al., 2004). Large numbers of residents in Southern Vietnam have been known to suffer from adverse health effects as a result of herbicide/dioxin exposure. Similarly, dioxin levels in human milk were found to be higher than 950 pg/g lipid at the end of the war in 1970 (Schechter et al., 1995). Current levels in the sprayed region of Vietnam are much lower (0.2–0.5%) due to the wash-off by tropical rain and chemical breakdown over the 40 years since spraying ceased (Schechter et al., 1991; Manh et al., 2014). However, levels are still three to five times higher in breast milk and serum from residents in and around the three former US air bases (Bien Hoa, Da Nang and Phu Cat) than in non-exposed regions (Manh et al., 2014; Hue et al., 2014; Thuong et al., 2014; Pham et al., 2015). In addition to direct exposure from soil, indirect exposure is known to occur as a result of apparent food-chain transfer of dioxins to humans. This is a particularly important source of exposure for the health of babies fed with maternal milk on a daily basis. Like other endocrine-disrupting chemicals, dioxin is suspected to have an effect on human hormones at low doses (Vandenberg et al., 2012). Indeed, the adverse effects such as

cancer, diabetes, immunosuppression and neurotoxicity associated with dioxin exposure may be considerably mediated by alterations to endocrine function (Huisman et al., 1995; Diamanti-Kandarakis et al., 2009; Miyashita et al., 2011).

Recent human studies have shown that high circulating levels of maternal cortisol during pregnancy correlate negatively with birth weight, thereby suggesting that excess glucocorticoids can cross the placental barrier (Braun et al., 2013; Reynolds, 2013). Similarly, an increase in the frequency of low birth weights was found to be associated with high dioxin concentrations in the milk and blood of mothers from Japan (Tawara et al., 2009; Konishi et al., 2009). It is also very important to monitor the development from child to adult as intrauterine growth retardation or a low birth weight have been linked to a late onset of diseases such as cardiovascular disease and type 2 diabetes in adulthood (Pinney et al., 2011). These concepts have led to the developmental origin of health and disease (DOHaD) hypothesis (Pinney et al., 2009). As such, endocrine-disrupting chemicals may affect both exposed individuals and their children and subsequent generations.

In this study, we focused on the adrenal hormone levels of mother-child pairs and elucidated the dioxin effects on the steroid biosynthesis pathway. As it is difficult to obtain blood samples from infants in epidemiological studies, we have developed a simple technique for collecting saliva from children and determining the hormone levels by LC-MS/MS.

The first aim of this study was to determine the adrenal hormone levels in three-year-old children and to compare the results for their mothers in the previous report (Kido et al., 2014). Then, any hormone relations among these mother-child pairs will be identified.

The second aim was to identify which dioxin congeners were associated with adrenal hormone variations in the mothers and their children. In the previous report, we only reported the total TEQ of PCDD/Fs; therefore in this report, we further describe the relation of each dioxin congener to the hormone levels. Cytochrome P450C17 (CYP17) has two catalytic actions, 17 $\alpha$ -hydroxylase and 17,20-lyase, on the steroid (pregnane) and plays a role in the turning point into androgen and corticoid biosynthesis (Li and Wang, 2005). We therefore note that the ratio of androgen (C19 steroid) / corticoid (C21 steroid) can reflect the two enzymatic activities.

## 2. Subjects and Methods

### 2.1. Study region

**Agent Orange/dioxin hot-spot:** The dioxin hot-spot selected was Phu Cat air base, where chemical herbicides were stored during the Vietnam War and the aircraft used to spray Agent Orange/dioxin were washed (Manh et al., 2014). The Phu Cat district is located in Binh Dinh province and is one of the three representative dioxin hotspots in South Vietnam (Manh et al., 2014; Hue et al., 2014; Pham et al., 2015). Records show that approximately 17,000 drums of Agent Orange, 9000 drums of Agent White and 2900 drums of Agent Blue were stored at Phu Cat (Young, 2008).

**Control region:** The non-exposed region selected as the control region was the Kim Bang district in Ha Nam province in the north of Vietnam, which was not exposed to chemical herbicides during the war and has not been affected by industrial pollution (Manh et al., 2014).

### 2.2. Subjects and sampling

The study subjects comprised of 49 lactating women from the dioxin hotspot and 55 from the control region. The characteristics of these women were described previously (Kido et al., 2014). Breast milk (20 mL) was collected from the lactating mothers in September 2008, and serum samples were collected from the same subjects one year later (August 2009), as described in detail previously (Kido et al., 2014).

The 104 children, who were nursed by mother milk in the two regions described above, were followed-up at the age of three years. Body height, weight and circumference of these mother-child pairs were also measured. Saliva samples were collected from these children in August 2011 using hormone-free cotton swabs, which were previously washed three times with hot ethanol and dried at 60 °C for 3 days. These cotton swabs (approximately 250–300 mg) were then stored in individual conical tubes, and the tubes were weighed. For sample collection, the cotton swab was inserted into the child's mouth using tweezers and allowed to soak up saliva for 1 min. It was then placed into the tube (and this process was repeated). Blood and saliva samples were collected from 8:00–10:30 am. They were then stored in a cooling box and frozen in dry ice for two days. All samples were transported to Japan for analysis. The volume of saliva obtained from each child was calculated by weighing. The cotton swabs and serum samples were stored at –70°C until analysis. The Medical Ethics Committee of Kanazawa University approved this study. Participating mothers gave their consent to this plan for collecting saliva samples from their children.

### 2.3. Instruments

The LC-MS/MS system used was an API-4000 triple-stage quadrupole mass spectrometer (Applied Biosystems, MDS Sciex, Tronto ON, Canada) with an ESI ion source, equipped with an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) and a PTC auto-sampler. An Xterra-C18 column was used (Waters Co). The gas chromatography-mass spectrometry (GC-MS) system used was a high-resolution mass spectrometer (HRMS; JEOL MS station-JMS700) equipped with a GC (HP-6980, Hewlett-Packard, Palo Alto, CA, USA). The ENV-5MS column used was 30 m x 0.25 mm ID with a 0.25  $\mu\text{m}$  film thickness (Kanto Chemical Co., Inc., Tokyo, Japan).

### 2.4. Measurement of dioxin congeners by GC-MS

Dioxins in the breast milk were extracted and purified using a previously reported method, and 17 PCDD/Fs dioxin congeners were estimated by GC-MS (Tai et al., 2011; Kido et al., 2014). Dioxin detection limits were determined at a signal to noise ratio of 3 on a lipid basis, and congener concentrations below the detection limits were set to half the detection limits.

The estimated values were shown as concentrations (pg/g lipid) or were converted to toxic equivalents (TEQs) using the World Health Organization toxic equivalency factor (Van den Berg et al., 2006).

### 2.5. Serum hormone estimation by LC-MS/MS

Serum steroid analysis was carried out using the procedure described previously (Kido et al., 2014). Here, serum (200  $\mu\text{L}$ ) was diluted with purified water to a volume of 1.0 mL, and then cortisol- $^2\text{H}_4$  (1 ng), DHEA- $^2\text{H}_4$  (100 pg), progesterone- $^{13}\text{C}_3$  (100 pg), estrone- $^{13}\text{C}_4$  (100 pg) and estradiol- $^{13}\text{C}_4$  (100 pg) were added as internal standards. After extraction with ethyl acetate, derivatization with picolinic acid was carried out according to the procedure described by Yamashita et al. (Yamashita et al., 2009). Six types of hormones were simultaneously determined by the LC-MS/MS method. The lowest estimation levels for cortisol, cortisone, DHEA, A-dione, estrone and estradiol were 50, 50, 5, 10, 1.0 and 0.5 pg/assay, respectively. Both the accuracy and precision in inter- and intra-day assays were within  $\pm 20\%$  of the lowest levels, and both were within  $\pm 15\%$  for all concentrations other than the lowest concentration. Quality control for salivary DHEA estimation involved 6 samples at 3 different levels, namely 20, 100 and 500 pg.

The ratios of C-19 steroid to C21-steroid in the serum were calculated from individual levels using the formulas below:

$$\text{Ratio} = (\text{C19 steroid levels}) / (\text{Non-exposed: C21 steroid levels})$$

$$\text{Ratio} = (\text{DHEA} + \text{A-dione} + \text{Estrone} + \text{Estradiol}) / (\text{Non-exposed: Cortisol} + \text{Cortisone})$$



## 2.6. Estimation of child salivary hormones by LC-MS/MS

After extracting the saliva-soaked cotton swabs three times with ethanol (1.5 mL), the solution obtained was evaporated on a centrifugal evaporator at 40 °C. Cortisol-2H4 (1 ng) and DHEA-2H4 (100 pg) in methanol (100 µL) were added to the tubes as internal standards, and then the solution was diluted with water. After the mixture was extracted with ethyl acetate, the extract was applied to a C-18 cartridge column. The obtained sample was derivatized with anhydrous picolinic acid and then purified as described above. The steroid hormones in saliva were simultaneously estimated by LC-MS/MS according to the previous method (Kido et al., 2014).

The lowest analytical limits for cortisol, cortisone, DHEA were 50, 50, 5 pg/assay, respectively. The ratios of C-19 steroid to C21-steroid in saliva were calculated from individual levels using the formulas below:

$$\text{Ratio} = (\text{C19 steroid level}) / (\text{Non-exposed: C21 steroid level})$$

$$\text{Ratio} = (\text{DHEA}) / (\text{Non-exposed: Cortisol + Cortisone})$$

## 2.7. Statistical analyses

Data are shown as the mean  $\pm$  SD or the median and the interquartile range. The mean difference in each indicator between the two regions was calculated using Student's t-test in the case of a normal distribution or the Mann-Whitney U-test in the case of a non-normal distribution, as determined by the Shapiro-Wilk test. Pearson's correlation coefficients were calculated between each dioxin congener and the steroid hormones. Finally, multiple linear regressions were used to evaluate the relation between dioxin congeners and DHEA levels after adjusting for the child's gender, maternal age and parity. The significance level was set to  $p < 0.05$ . All statistical analyses were performed using the SPSS 12.0 Software and the JMP@ 9 Software package (SAS institute, Cary, NC, USA).

### 3. Results

#### 3.1. Comparison of study subjects from the dioxin hot-spot and non-exposed regions

In the previous report (Kido et al., 2014), there was a total of 109 mothers at the beginning of the study. However, due to not following up at the time when their children become 3 years old, 5 children were lost to follow-up, 104 mother-child pairs remained for this study. For the mothers (N=104), characteristics such as age, weight, height, BMI, residence and income did not differ significantly between the hotspot and non-exposed regions; therefore, we did not show these mothers' data again in this report.

Similarly, the height, weight and head circumference (mean  $\pm$  SD) for children (N=104) in the hot spot and non-exposure regions were  $92.29 \pm 3.88$  and  $91.47 \pm 3.77$  cm,  $12.88 \pm 1.74$  and  $12.77 \pm 1.56$  kg,  $48.92 \pm 1.62$  and  $48.53 \pm 1.36$  cm, respectively. These estimated values did not significantly differ between the two regions ( $p > 0.05$ ).

#### 3.2. Comparison of hormone levels in mother-child pairs

Table 1 shows the serum levels of six hormones for mothers from the dioxin hotspot and non-exposed regions. Because the distributions of hormones are not normal distributions, we presented the data as median values and inter-quartile ranges. Only cortisol and cortisone levels were significantly higher in the hotspot than in the non-exposed region ( $p < 0.004$ ). No statistically significant differences were found for the other hormones nor the ratio of C19-steroid (DHEA + Adione + Estrone + Estradiol) to C21-steroid (cortisol and cortisone) between the hotspot and non-exposed regions.

In contrast, the salivary DHEA levels for children from the hotspot were significantly lower than those from the non-exposed region, and this decrease was found to be higher in females than in males (see Table 1). However, cortisol and cortisone levels of these children did not differ significantly between the two regions.

The ratio of C19-steroid (DHEA) to C21-steroid (cortisol and cortisone) was significantly lower in the hotspot than in the non-exposed region ( $p < 0.01$ ).

#### 3.3. Comparison of dioxin congener levels in mothers from the dioxin hot-spot and the non-exposed region

The dioxin congener levels are shown as median values and interquartile ranges in Table 2. Most of the dioxin congener levels were higher in the hotspot than that in the non-exposed region. The total TEQ PCDD/F concentrations in breast milk from lactating mothers from the hot-spot were over three times higher than those in mothers from the non-exposed region.

#### 3.4. Correlation between maternal serum cortisol or child DHEA levels and dioxin congener

*concentrations in maternal breast milk*

Table 3 shows the Pearson correlation between salivary DHEA in the children, serum cortisol in the mothers and 17 dioxin congeners in breast milk. OCDD, 1,2,3,4,6,7,8-HpCDD and 1,2,3,4(6),7,8-HxCDD were found to be highly negatively correlated with salivary DHEA ( $p < 0.01$ ), whereas TCDD was weakly correlated with this hormone. Furthermore, this correlation was generally stronger in females than in males. Fig 1 shows the correlation between salivary DHEA levels in male, female children and some highly chlorinated dioxin congeners.

Table 4 shows the relation between dioxin congeners and children salivary DHEA levels by using multiple regressions to adjust for the child's gender, maternal age and parity. The results showed a negative correlation between dioxin levels and DHEA levels and remained even after adjusting for other confounders. In particular, OCDD and 1,2,3,4,6,7,8-HpCDD were strongly correlated with salivary DHEA in children.

#### 4. Discussion

To our best knowledge, this is the first report of adrenal endocrine disruption by dioxins in mother and child pairs. Our epidemiological study showed an alteration to adrenal hormone levels, namely high cortisol levels in the mothers and low DHEA levels in their three-year-old children, from a dioxin-exposed region of Vietnam.

The purpose of this study was to elucidate whether the mother's dioxin burden was associated with steroid hormone levels in their children from a herbicide-exposed region in Vietnam after 40 years when spraying occurred. We already found that dioxin influenced adrenal steroid hormone levels in women from the dioxin-exposed region (Kido et al., 2014). Thus, we focused on adrenal hormones in children from previously characterized mothers to elucidate the effect of dioxins on subsequent generations (in this case 104 mother-child pairs).

It was difficult to obtain blood samples from infants in epidemiological studies. Therefore, in this study with children, we used only saliva as a matrix for hormone analysis as this can be taken non-invasively from even one-year-old children. Good correlations were found between the levels of six steroid hormones in saliva and those in serum (Kido et al., 2014). In the children's saliva, we focused on 3 adrenal hormones, including cortisol, cortisone and DHEA because other hormones were present in only trace quantities.

Salivary DHEA levels in children were approximately 30–50% lower in the hot-spot region than in the non-exposed region, whereas cortisol and cortisone levels did not differ significantly between the two regions (Table 1). Cortisone in saliva is well known to be predominant over cortisol due to 11 $\beta$ -hydroxydehydrogenase (type II) in the salivary membrane (Kido et al., 2014).

We analyzed 6 types of steroid hormones in the serum of the mothers as shown Table 1.

The cortisol and cortisone levels in the serum of the mothers from the hot-spot region were significantly higher (30% and 20%, respectively) than those from the non-exposed region, whereas DHEA levels did not differ significantly between the two regions.

The correlation between maternal serum cortisol and the child salivary DHEA levels were not significant ( $p > 0.44$ ). We speculate that the child adrenal hormone levels are not associated with the reactivity of the maternal adrenal gland.

DHEA and cortisol are both well-known adrenal hormones that are regulated by the adrenocorticotrophic hormone (ACTH) in humans (Rege et al., 2013). If dioxin acts on the pituitary or hypothalamus, DHEA and cortisol may change simultaneously in the mother or the children. However, we observed only a change of DHEA in the children and cortisol in the mothers. This result allows us to conclude that dioxin may act directly upon the steroid biosynthetic pathway in the adrenal cortex rather than on ACTH secretion through the pituitary.

In light of the above, we decided to elucidate whether dioxin affects the pathway leading to

the biosynthesis of DHEA and cortisol by using the ratio of C19 steroid to C21 steroid hormone levels in the serum or the saliva. Cytochrome P450C17 (CYP17) plays a key role in corticoid and androgen biosyntheses (Rege et al., 2010) as a result of catalytic actions of 17 $\alpha$ -hydroxylase and 17, 20-lyase (Miller, 2009; Kinoshita et al., 2014). In mothers, CYP17 17 $\alpha$ -hydroxylase might be promoted as a result of the serum cortisol and cortisone levels shown in Table 1. We also evaluated lyase and hydroxylase activities of CYP17 from the ratio of the DHEA and 2 types of corticoid levels, respectively, in saliva from children. As shown in Table 1, the DHEA/corticoids ratio decreased by approximately 50% for children in the hotspot. These findings showed that dioxin significantly inhibited the lyase activity of CYP17 in children from the hot-spot.

Our epidemiological studies showed that dioxin influenced the production of adrenal hormones such as corticoid and androgen. Dioxin influences the adrenal cortex in two different ways, namely by promoting CYP17 17 $\alpha$ -hydroxylase activity in the mother's adrenal gland, and by inhibiting the lyase activity of CYP17 in the zona reticulatae (ZR) layer of the adrenal gland in children (Suzuki et al., 2000; Rege et al., 2014). Li and Wang reported that dioxin suppressed 17, 20-lyase activity and activated 17- and 18-hydroxylase, followed by an increase in cortisol and aldosterone in human adrenal cancer cells (Li and Wang, 2005). It is clear that dioxin can influence adrenal hormone levels, although its mechanism is unknown. The discrimination between 17 $\alpha$ -hydroxylase and 17, 20-lyase activities is regulated by the allosteric action of cytochrome b5 (Kok et al., 2010; Rege et al., 2014). From these findings, we suggest the possibility that dioxin may affect the action of cytochrome b5 on CYP17 due to its allosteric effect.

The second aim of this study was to identify which dioxin congeners were associated with the variations of adrenal hormones in the mothers and their children. Some dioxin congeners exhibited a positive correlation with the mother's serum cortisol. These congeners were OCDD ( $p < 0.003$ ,  $r = 0.29$ ) and 1,2,3,4,7,8,9-HpCDF ( $p < 0.004$ ,  $r = 0.28$ ) in mothers. In contrast, only a weak correlation was found between TCDD in breast milk and cortisol in serum ( $p < 0.05$ ). In children, some dioxin congeners in breast milk were negatively correlated with salivary DHEA levels ( $p < 0.01$ ), with the strongest correlations observed for 1,2,3,4(6),7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD and OCDD. We continue to follow up subjects for a total of up to 7 years children in both areas at present. The sex differences in dioxin effects on the steroid biosynthesis will be clear. There was no strong correlation between TCDD and DHEA levels in children (Table 3, Table 4). The effect of these dioxin congeners on adrenal hormone levels agrees with the recent findings of Kishi et al. who reported that 1,2,3,4,6,7,8-HpCDD and 1,2,3,7,8,9-HxCDD were negatively associated with the mental and psychomotor developmental indices in BSID-II (Bayley Scales Infant Development, version II) for

six-month-old infants (Kishi et al., 2013). Moreover, Tsukimori et al. also reported that 1,2,3,6,7,8-HxCDD is the most important causative congener for the development of fetal Yusho disease (Tsukimori et al., 2013). In addition, it has also been reported that low birth weights are caused by 2,3,7,8-TCDD and 2,3,4,7,8-PeCDF exposures (Konishi et al., 2009; Pinney et al., 2011). We have recently observed that the frequency of low birth weights (<2500 g) linked to maternal dioxin and cortisol levels (Kido et al., 2015 in submission). In this context, it also seems uncertain whether the various toxicities of dioxin and dioxin-like compounds are in agreement with the magnitudes of the toxic equivalent factor (TEF) defined by WHO. Indeed, these findings suggest that some dioxin congeners, such as HpCDD/F and OCDD, are more toxic in humans than would be indicated by the WHO-TEF value, depending on the binding assay to aryl hydrocarbon receptors (Berg et al., 2006).

It is still not known whether an increase in glucocorticoid levels in breast milk causes any adverse health effects in the woman or her child. In our research, the dioxin level (PCDDs + PCDFs) in breast milk was three- to four-times higher in samples from the hotspot region than in those from the control region (see Table 2). As such, we suppose that the daily dioxin intake (DDI) in babies is also three- to four-times higher in the hotspot. This result agrees well with some previous reports (Tai et al., 2011; Rege et al., 2013). In Vietnam, dioxin levels in sprayed regions (hotspots) are currently much lower due to the effects of tropical rain, erosion and chemical breakdown over the past 40 years. However, it was recently noted that even low doses of dioxin may cause adverse health effects in humans (Vandenberg et al., 2012). The fetal adrenal layer differentiates into three parts known as the zona glomerulosa, zona fasciculata and ZR within 2–3 years, and these three layers are responsible for hormone production (Voutilainen et al., 2015). Moreover, fetal tissue changes to form the ZR after birth. As such, dioxin may influence the differentiation process in the adrenal zone. DHEA is produced in relatively large quantities in the fetal adrenal gland. After delivery, DHEA levels decrease rapidly. DHEA levels change markedly during the first five years of the child's development. Thus, the level is the highest immediately after birth, reaching a minimum at the age of two to three years (Miller et al., 2009), and subsequently increases again to a maximum at approximately 15 years of age, then decreases again with age (Parker et al., 1997; Voutilainen et al., 2015;). As such, we speculate that the low DHEA levels found in the children studied herein reflected a delay in the increase of DHEA production.

We clearly demonstrated steroid hormone disruptions caused by dioxin in humans using LC-MS/MS analysis capable of tracing steroid hormones. This is the first study that showed the adverse effects of highly chlorinated dioxin congeners on adrenocortical steroid hormones in the children and their mothers after nearly 40-45 years of exposure. These results have provided more scientific evidence of adverse dioxin effects on a child's development in the 3rd-4th

generation in exposed regions.

Several limitations should be considered in this study. Our study evaluated the correlation between dioxin concentrations in maternal breast milk with steroid hormones in their 3-year-old children. Because we do not have accurate data during the breast-feeding period, dioxin concentrations in the breast milk may not reflect the burden of dioxins in the bodies of the children. However, both study areas are rural areas, where breast milk is the main source of nutrition for infants; we assume that most of our infants drank breast milk as their main nutrition.

In summary, our epidemiological study showed an alteration to adrenal hormone levels, namely, high cortisol levels in the mothers and low DHEA levels in their three-year-old children, in a dioxin-exposed region of Vietnam. However, it remains unclear whether the DHEA decrease will result in any adverse health effects. To gain a better understanding of the developmental process in children, it is important to continuously monitor the levels of DHEA and other hormones in bodily fluids and to further evaluate the influence of low dose dioxin exposure on fetal and postnatal development. This will help to reduce the risk of endocrine-disrupting chemicals affecting subsequent generations.

## **5. Conclusions**

Higher cortisol levels in the mothers and lower DHEA levels in their three-year-old children were found in an epidemiological study in a dioxin-exposed region in Vietnam. The alteration of steroid hormones was more intensely correlated with higher chlorinated dioxin congeners, such as hexa-, hepta- and octa-CDDs, than with their lesser chlorinated counterparts, such as TCDD.

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## Legends

Table 1

Serum or saliva hormone levels in mother-child pairs from the dioxin hot-spot and non-exposed regions

- 1) Because data are not normal distributions, we present the median (interquartile) and test by the Mann-Whitney test
- 2) C19/C21(%):  $(\text{DHEA} + \text{A-dione} + \text{Estrone} + \text{Estradiol}) / (\text{Non-exposed: Cortisol} + \text{Cortisone}) \times 100$
- 3) C19/C21(%):  $\text{DHEA} / (\text{Non-exposed: Cortisol} + \text{Cortisone}) \times 100$

Table 2

Dioxin concentrations in the breast milk of lactating mothers in the dioxin hot-spot and non-exposed regions

Note: Because data are not normal distribution, we present the median (interquartile) and test by the Mann-Whitney test

Table 3:

Correlation between the child's salivary DHEA and the mother's serum cortisol levels and dioxin congener concentrations in maternal breast milk from the dioxin hot-spot and non-exposed regions

- 1) Mother: Serum cortisol
- 2) Child: Salivary DHEA
- 3) r: Correlation coefficient

Table 4:

Correlation of salivary DHEA in the child and dioxin congeners adjusted for the child's sex, parity and maternal age

$\beta$ : Standardized coefficients

Fig 1

Correlation between pairs of child's salivary DHEA level and dioxin congener concentrations in the breast milk from the dioxin hot-spot and non-exposed regions:

- 1) A1–A3: Male
- 2) B1–B3: Female

Table 1

Subjects	Matrix	Hormone	Hotspot region			Non-exposed region			p value
			N	Median	Interquartile range	N	Median	Interquartile range	
Mother	Serum	Cortisol (ng/ml)	49	94.2	71.8 - 133.8	55	66.8	53.3 - 103.6	0.001
		Cortisone (ng/ml)	49	25.7	21.8 - 30.8	55	21.9	17.2 - 27.6	0.004
		DHEA (ng/ml)	49	4.52	3.40 - 6.51	55	4.54	3.35 - 6.72	0.987
		A-dione (ng/ml)	49	1.48	1.11 - 2.12	55	1.65	1.22 - 2.07	0.237
		Estrone (ng/ml)	49	22.7	13.6 - 38.5	55	26.2	19.4 - 45.1	0.163
		Estradiol (ng/ml)	49	21.3	11.2 - 42.4	55	22.1	12.1 - 38.1	0.855
		C19/C21 (%)	49	4.86	3.82 - 7.57	55	6.57	4.77 - 8.20	0.822
Male child	Saliva	Cortisol (ng/ml)	28	0.47	0.22 - 0.91	26	0.33	0.19 - 0.55	0.194
		Cortisone (ng/ml)	28	3.09	1.79 - 5.19	26	2.86	1.99 - 4.20	0.377
		DHEA (pg/ml)	28	39	29 - 59	26	72	34 - 105	0.013
		C19/C21 (%)	28	1.06	0.66 - 2.07	26	2.26	1.44 - 3.32	0.001
Female child	Saliva	Cortisol (ng/ml)	21	0.39	0.21 - 0.74	29	0.39	0.25 - 0.68	0.437
		Cortisone (ng/ml)	21	2.53	1.22 - 5.51	29	3.14	2.05 - 4.22	0.366
		DHEA (pg/ml)	21	31.0	21.5 - 56.5	29	77.0	57.0 - 112.0	0.000
		C19/C21 (%)	21	1.25	0.76 - 1.79	29	2.31	1.40 - 3.00	0.012

Table 2

Dioxin congeners  
(pg/g lipid)

s	49	4.42	3.07 - 5.60	55	1.32	1.09 - 1.78	< 0.001
TEQ Total PCDDs + PCDFs							

Table 3

	hers		DHEA			
	(n=104)		Male child (n=54)			
	r	p	r	p	r	p
Dioxin congeners						
2,3,7,8-TeCDD	0.197	0.050	-0.286	0.038	-0.256	0.079
1,2,3,7,8-PeCDD	0.218	0.026	-0.251	0.070	-0.399	0.005
1,2,3,4,7,8-HxCDD	0.152	0.213	-0.333	0.015	-0.391	0.006
1,2,3,6,7,8-HxCDD	0.175	0.076	-0.289	0.036	-0.419	0.003
1,2,3,7,8,9-HxCDD	0.146	0.130	-0.275	0.047	-0.350	0.015
1,2,3,4,6,7,8-HpCDD	0.213	0.030	-0.343	0.040	-0.394	0.006
OCDD	0.288	0.003	-0.351	0.010	-0.411	0.004
2,3,7,8-TeCDF	0.046	0.643	-0.167	0.232	0.115	0.434
1,2,3,7,8-PeCDF	0.218	0.026	-0.297	0.031	-0.263	0.071
2,3,4,7,8-PeCDF	0.165	0.093	-0.340	0.013	-0.195	0.185
1,2,3,4,7,8-HxCDF	0.214	0.029	-0.325	0.018	-0.313	0.030
1,2,3,6,7,8-HxCDF	0.208	0.034	-0.318	0.021	-0.291	0.045
1,2,3,7,8,9-HxCDF	0.142	0.151	-0.231	0.096	-0.309	0.033
2,3,4,6,7,8-HxCDF	0.159	0.108	-0.268	0.052	-0.290	0.046
1,2,3,4,6,7,8-HpCDF	0.236	0.016	-0.289	0.036	-0.298	0.040
1,2,3,4,7,8,9-HpCDF	0.278	0.004	-0.310	0.024	-0.351	0.014
OCDF	0.171	0.082	-0.174	0.212	-0.288	0.047
TEQ Total PCDDs	0.270	0.006	-0.278	0.044	-0.395	0.006
TEQ Total PCDFs	0.228	0.020	-0.342	0.012	-0.298	0.040
TEQ Total PCDDs + PCDFs	0.269	0.006	-0.315	0.022	-0.376	0.008



Table 4

Dioxin congeners	p value	DHEA $\beta$	$R^2$
			7
1,2,3,4,7,8,9-HpCDF	0.001	-0.338	0.136
OCDF	0.027	-0.224	0.078
TEQ Total PCDDs	0.0015	-0.343	0.127
TEQ Total PCDFs	0.0011	-0.343	0.133
TEQ Total PCDDs + PCDFs	0.0008	-0.360	0.138

Fig 1

