Later childhood effects of perinatal exposure to background levels of dioxins in the Netherlands

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4.1 PERINATAL DIOXIN EXPOSURE AND CYTOCHROME P-450 ACTIVITY, THYROID AND LIVER FUNCTIONS AT FOLLOW-UP AFTER 7 – 12 YEARS

EVIDENCE FOR TRANSIENT EFFECTS ON THYROID AND LIVER FUNCTIONS
Abstract

Objectives: Perinatal exposure to Dutch "background" dioxin levels is rather high. Health effects may span many years. Therefore we determined plasma TSH, FT4, ALAT and ASAT levels amongst our longitudinal cohort, as was done perinatally and at 2½ years. The children underwent a caffeine loading test to determine CYP1A2 activity.

Study design: The longitudinal cohort of 37 healthy children (7 - 12, mean 8.2 years), with documented perinatal dioxin exposure, ingested 3 mg caffeine/kg BW 6 hours prior to blood withdrawal. Paraxanthine/caffeine molar ratio, TSH, FT4, ALAT and ASAT were determined in venous blood.

Results: Linear regression of TSH and FT4 revealed no relation with prenatal and postnatal dioxin exposure. No relation was found between ASAT and ALAT and prenatal and postnatal exposure. No correlation was found between the paraxanthine/caffeine molar ratio and prenatal and postnatal dioxin exposure.

Conclusion: This follow-up has shown a normalisation of previously abnormal thyroid hormone homeostasis and ALAT levels, indicating a transient effect. CYP1A2 activity, measured by means of a caffeine-loading test, revealed no correlation with the pre- and postnatal exposures. This study provides new data on longterm follow-up after perinatal dioxin exposure to background levels of dioxins.

Introduction

Polychlorinated dibenzo-p-dioxins and dibenzofurans and some PCBs, such as PCB-77, -126 and -169 (henceforth jointly referred to as dioxins) belong to the group of most toxic substances known, and have been associated with malignancy, congenital malformations, immuno-suppression, enzyme induction and endocrine disruption (1-6). Dioxins are formed as waste products of combustion processes and municipal incinerators are amongst the primary sources of these compounds in The Netherlands. Heating of polychlorinated biphenyls (PCBs) is a notable source of polychlorinated dibenzofurans. Dioxins, being poorly
degradable in nature, persist in the environment, accumulating in the human food chain via fish-oils and animal fats (7). These chlorinated polyaromatic compounds are highly lipophilic and are in the human primarily stored in adipose tissues and liver. Their lipophilicity allows them to readily pass the placenta, whereupon they are stored in fetal adipose tissues (8;9). In 1986 relatively high background dioxin concentrations in the breast milk of Dutch mothers was first reported, followed by similar findings in other industrialised countries (10-12). As a result fetuses and breastfed children are exposed to relatively high “background” dioxin levels of averaging approximately 30 ng/kg fat, as measured in breastmilk (10-14). Yet, very little is known about the effects of this exposure on the (longterm) health of children.

Dioxins influence thyroid and liver determinants in humans (6;13;14), and induce cytochrome P-450 1A2 (CYP1A2) activity in animals (15). Pluim, in the same group of children as the current cohort, found a tendency toward higher thyroxine (T4) concentrations at birth in relation to increasing prenatal dioxin exposure, and increased mean T4 concentrations and increased T4/thyroxine-binding globulin ratio, at one and eleven weeks of age, related to dioxin exposure. TSH levels, while being similar in the higher and lower exposure groups at birth and one week post partum, were significantly higher in the higher exposure group at eleven weeks of age (13;16). Furthermore, plasma alanine (ALAT) and aspartate (ASAT) aminotransferase activity was found to be significantly increased at eleven weeks of age, in relation to increasing cumulative (postnatal) dioxin exposure (14). In contrast, Ilsen, investigating the same children at two and a half years of age, found no correlations between the perinatal dioxin exposures and thyroid hormones. Neither did she find a correlation between perinatal dioxin exposures and ASAT or ALAT concentration (17).

Increased CYP1A2 activity in humans has been associated with an increased risk of developing colorectal neoplasms (18), and dioxin exposure has been linked to sigmoid cancer in an 11-year-old boy (19). Perinatal exposure to dioxins and related compounds may have consequences spanning many years, due to their long residence time in the body. Based on the reported half lives, it may take up to a few
decades to attain steady state levels. In practice this means that accumulation will continue through childhood and puberty. Our working hypothesis is that perinatal exposure to background levels of dioxins in The Netherlands could:

1) have a life-long effect on thyroid hormone homeostasis (13;16);
2) cause persistent liver damage (seen in abnormal plasma aminotransferase levels) and thereby negatively affecting various metabolic reactions (14);
3) alter CYP1A2 activity resulting in a life-long reduced activity by a lowering of the setpoint, in accordance with Csaba’s study in laboratory animals (20;21).

In our ongoing study of the development of children with documented perinatal dioxin exposure, we therefore assessed the thyroid and liver determinants and the CYP1A2 activity in the study participants, using routine blood investigations and a caffeine-loading test, respectively. The children had previously been studied during the perinatal period and at the age of two and a half years.

Methods and materials

The institutional Medical Ethics Committees of the De Heel Zaans Medical Center (Zaandam, The Netherlands) and of the Academic Medical Center (Amsterdam, The Netherlands) approved the study. Written informed consent was obtained from parents/guardians and children.

Subjects
Eight years after Pluim’s Zaandam study (13;14), the cohort was again contacted, as was the group from his first study (up to twelve years ago) (22). The perinatal dioxin exposure is documented for all these study participants. Out of the original cohort of 61 subjects, those born prematurely, born out of suboptimal pregnancies necessitating medical intervention, or who were twins, were not included in this follow-up study (9 children). Two children had moved abroad, 5 children chose not
to participate in the follow-up study and 5 children were untraceable. The prenatal and postnatal dioxin exposures of the excluded subjects were varied and in no way did their exclusion introduce a population bias. The elder sister (2 years older) of a subject requested to take part in the study and was included in the cohort for this reason. Her breastfeeding period was known and she was assigned the same prenatal dioxin exposure (25.4 ng/kg fat) as her younger sister. [In reality, being the firstborn and the first child to be breastfed, her prenatal exposure would have been higher than that of her younger sister, caused by the fact that dioxins are so readily excreted in breastmilk].

The 37 study subjects were 7 to 12 years old (mean 8.0 years, SD 1.5 years). The children underwent the test and blood withdrawal on one of three Saturday afternoons in the spring of 1998, at De Heel Zaans Medical Centre, Zaandam. The dioxin toxic equivalency and cumulative toxic equivalency values as determined by Pluim in the breastmilk of their mothers, were used as such within this follow-up study. Briefly, the concentration of dioxins, using the I-TEQ method, in the mother’s breastmilk, shortly after having given birth, was taken as the prenatal dioxin exposure level of the child (teqdiox), a reliable approximation of the prenatal exposure (23). The postnatal cumulative dioxin exposure (teqcum) was calculated as: teqdiox multiplied by the amount of breastmilk ingested during the breastfeeding period of the child. A 2.5% fat concentration in the milk, 700 g daily milk intake during the exclusively breastfeeding period and 350 g daily intake during the transition period to formula feed was assumed (22). Formula feed has undetectable levels of dioxins, with the animal fats having been removed and replaced with plant fats (24). The prenatal exposure ranged from 8.74 to 88.80 (mean 34.6) ng TEQ dioxin/kg milk fat. The postnatal exposure ranged from 4.34 to 384.51 (mean 75.4) ng TEQ dioxin. These exposures are indicative of the background exposures in The Netherlands, and similar studies in other parts of the country have found similar background concentrations (11;12;25;26). Subsequent (childhood) dioxin exposures are about 25 times less than during the fetal and breastfeeding period (27), and it can safely be assumed that all the subjects had similar childhood exposures, seeing as they all had similar diets.
**Thyroid function**

Thyroid stimulating hormone (TSH) and free thyroxine (FT4) were measured in plasma immediately after venapuncture, on a routinely used Beckman Access® HYPERsensitive hTSH and FREE T4 immunoassay system respectively. TSH was quantified using a standard paramagnetic particle, chemiluminescent immunoassay, with a functional sensitivity of 0.01-0.02 mIU/L (product no. 33820). Similarly, FT4 was quantified using a standard paramagnetic particle, chemiluminescent immunoassay, using a two-step enzyme immunoassay and monoclonal anti-thyroxine antibodies (product no.33880).

**ALAT and ASAT**

Standard aspartate aminotransferase (ASAT) (product no. 07 3737 2) and alanine aminotransferase (ALAT) (product no. 07 3734 8) measurements in plasma, using a Roche Cobas Integra 700 system®, were performed immediately after venapuncture. The standard pyridoxal phosphate activated test (product no. 07 3902 2) was used for both ASAT and ALAT.

**Caffeine loading test**

The subjects refrained from caffeine intake for 48 hours prior to the ingestion of caffeine, in a single dosage of 3 mg/kg body weight, dissolved in ‘AA’® soda drink, which contains no caffeine, but to which the caffeine was added. Six hours later blood was drawn and later analysed in the laboratory of the AMC Clinical Pharmacy Department (Amsterdam, The Netherlands).

The venapuncture was performed by an experienced clinical laboratory worker. The blood was centrifuged within two hours and thereafter cooled at 4° C until the following Monday, whereupon it was analysed. In these blood samples paraxanthine and caffeine concentrations were determined, and the paraxanthine/caffeine molar ratio was calculated. Methods of analysis for simultaneous determination of paraxanthine and caffeine have been described (28;29), however, to our knowledge, only in a single, recent publication the necessary validation of the assay is described (29).
We therefore developed a HPLC assay for simultaneous determination of paraxanthine and caffeine and validated this assay before the above mentioned study was performed (30).

Caffeine and paraxanthine in plasma was quantitated with a ‘reversed-phase’-HPLC using UV-detection. Only analytical grade chemicals were used, and included paraxanthine (Sigma, Zwijndrecht, batch no. 117H4066), caffeine (OPG, Utrecht, batch no. 92K10AL-WP04732), sulfadinoxine, theophylline, methanol (Merck, Amsterdam), trichloracetic acid, tri-ethylamine (Merck, Amsterdam, batch no. 40613917), and sodium dihydrogenphosphate monohydrate (Merck, Amsterdam, batch no. A858546).

The analysis was performed using a HPLC-system consisting of a Waters M45 pump, Applied Biosystems 757 UV-detection system, set to a wavelength detection of 273 nm, Shimadzu-CR3A integrator, Supelco LC-18-DB-087311AC column, Rheodyne 7125 injector. The pH of the mobile phase was adjusted using a Radiometer Copenhagen PHM83 Autocal and filtrated over a Millipore GVHP04700 filter (poresize 0,22 μm). During sample preparation a Hettich Rotanta/P centrifuge was used.

A stock solution of 15 mg caffeine and 15 mg paraxanthine in eluent was used for the preparation of standard solutions of caffeine and paraxanthine. All standard solutions were prepared by diluting this stock solution. The validation and calibration samples were prepared by spiking human blank serum with the above-mentioned stock solution. Serum proteins were precipitated before the sample was injected onto the chromatographic system. Protein precipitation was achieved using 50 μL serum + 20 μL internal standard (sulfadinoxine 5 mg/L) + 50 μL methanol + 50 μL 10% trichloracetic acid, mixed on a Vortex for 1 minute and centrifuged at 4000 rpm for 10 minutes. 15 μL of supernatant was injected onto the HPLC system. Quantification of caffeine and paraxanthine was achieved by comparing peak areas, using sulfadinoxine as an internal standard.
Statistics
Linear regression, which is a dose-response analysis and two-sided test, was performed using SPSS 10.0®. P-values were calculated and P-values less than 0.05 were considered statistically significant. Smoking (mothers and children) was considered a confounder as smoking may induce CYP1A2 activity. Gender was also considered as a confounder, as metabolism and hormone effects may be different between males and females.

Results

Subjects
Of the 42 children taking part in the study, 4 declined to allow venapuncture, and 1 was not available on the planned Saturdays due to his residing outside the country. Therefore 37 blood samples were obtained for analysis. None of the children smoked at the time of the study. Statistical correction for mothers who smoked at the time of the study, or who smoked during pregnancy, had no effect on the results. Smoking was therefore not considered to be a confounder in this study.

Thyroid hormones
Four of the 37 children (11%) had a TSH value above the upper limit of the normal values (4.4 mU/L) with the highest value being 4.64 mU/L. One value fell below the lower limit of normal (0.7 mU/L), being 0.62 mU/L. The average TSH value amongst the 37 samples was 2.14 (SD 1.20) mU/L. All the FT4 results fell within the normal range (13 - 27 pmol/L for 2 - 7 year olds, and 10 - 25 pmol/L for 8 - 20 year olds), with the exception of one 7 year old child with a FT4 value of 12.6 pmol/L. The lowest FT4 value was 12.5 pmol/L and the highest was 23.1 pmol/L. The average FT4 value was 17.4 (SD 2.69) pmol/L. It must be stressed that the normal values used are based on American children, as European normal values are not available (31). None of the children exhibited clearly pathological thyroid hormone levels.
Linear regression revealed no correlation between TSH and prenatal dioxin exposure (p=0.74), TSH and postnatal dioxin exposure (p=0.17),
FT4 and prenatal exposure (p=0.65), nor with FT4 and postnatal exposure (p=0.84). Finally there was no correlation between either TSH (p=0.90) or FT4 (p=0.29) levels and CYP1A2 activity, although the scatter diagram of FT4 against CYP1A2 suggests an inverse gradient (figure 1).

![Free Thyroxine versus CYP1A2 Activity](image)

Figure 1: Free thyroxine (FT4) versus cytochrome P-450 1A2 activity, measured by the ratio of paraxanthine to caffeine.

**ASAT/ALAT**
The ASAT measurements ranged from 16 to 33 U/L with a mean of 20.4 (SD 3.28) U/L. The normal ASAT range for children from 3 to 15 years is 0 to 40 U/L, which means that all the children had normal aspartate aminotransferase levels. Linear regression showed no correlation between ASAT and prenatal dioxin exposure (p=0.22), nor with ASAT and postnatal exposure (p=0.49).
The ALAT measurements ranged from 8 to 24 U/L with a mean of 12.6 (SD 3.65) U/L. The normal ALAT range for children from 1 to 15 years is 0 to 45 U/L, which means that all the children had normal alanine aminotransferase levels. Linear regression showed no correlation between ALAT and prenatal dioxin exposure (p=0.19), nor with ALAT and postnatal dioxin exposure (p=0.30).

**Histogram of Paraxanthine/Caffeine ratio**

![Histogram of Paraxanthine/Caffeine ratio](attachment:image.png)

Figure 2: Histogram of the paraxanthine/caffeine ratio; the ratio is an indication of the cytochrome P-450 1A2 activity.

**Caffeine loading test**

The distribution of the paraxanthine/caffeine molar ratio within the group of children is displayed in a histogram (figure 2). The ratio ranged from 0.41 to 2.48, with a mean of 1.35 (SD 0.48). Linear regression of the paraxanthine/caffeine molar ratio, indicative of the CYP1A2 activity, versus prenatal dioxin exposure revealed no significant linear correlation
In the same manner there was no correlation between the molar ratio and postnatal dioxin exposure (p=1.00).

Discussion

Thyroid hormones
During the neonatal period of our cohort, Pluim detected a trend towards a higher T4 and T4/TBG ratio (as indicator of the FT4 concentration) in the cord blood of the higher dioxin exposure group. This trend attained statistical significance at one and eleven weeks post partum. The TSH level was also increased at eleven weeks post partum (13;16). The thyroid parameters of the mothers of the higher and lower exposure group were comparable and all the infants were breastfed. The hypothesis was that dioxins ultimately decrease the nuclear occupancy of T3 receptors, leading to a stimulation of TSH secretion, and a higher thyroxine synthesis. In this manner the FT4 setpoint, or optimal FT4 level, is increased. From a physiological point of view, for optimal metabolism in children a certain level of occupation of T3 receptors is needed. Peripheral thyroid hormone problems, such as thyroid hormone hypo-responsiveness (an hereditary illness), lead to supraphysiological levels of FT4 with only slightly increased TSH levels. In this case the setpoint for the thyroid hormone is set higher in order to compensate. The setpoint is the optimal thyroid hormone concentration, being a characteristic of the whole body, with the hypophysis as custodian. While the setpoint certainly has a genetic basis, it is also susceptible to (transient) external influences, such as is seen in non-thyroidal illnesses, or shortly after birth when there is an increased need.

Four different thyroxine receptors have been identified (alpha 1 & 2 and beta 1 & 2). This heterogenicity could explain why dioxins seem to have varied effects in different tissues. An example of this may be the enhanced maturation of the neuromotor system (in relation to perinatal dioxin exposure), as documented by Ilsen (17), which she explained as an effect of FT4 agonism.

Until such time as it is clear precisely how dioxins influence the thyroidal system, it is probably safest to assume that the different effects seen are a
result of receptor differences and binding differences. In other tissues, with a (slightly) different receptor for T4, dioxins might not bind as well, and this imperfect binding may result in a blockade of the function, with a decrease in nuclear thyroxine occupancy in the hypophysis and hypothalamus. In other words, in such a setting the regulatory system is affected.

Taking this into consideration, it is interesting to note that Koopman-Esseboom et al. noted a lower T3 and T4 level in relation to total dioxin and PCB exposure, in their study of mothers before and after delivery (32). This may be ominous, bearing in mind that women with low normal T4 levels have been shown to produce children with impaired psychomotor development (33;34). Furthermore, Koopman-Esseboom, in a group of babies comparable to our cohort, found a significantly lower T4 and FT4, and an increased TSH level, in the second week after birth. In umbilical cord plasma and at the age of three months the TSH level was slightly increased (32). The contradictory findings between her lower T4 and that of Pluim, with his higher T4, might be explained by a population bias or methodological error in the determinations. The latter is unlikely, seeing as the determination of thyroidal system hormone concentrations in plasma are standard determinations.

The fact that both the Amsterdam and Rotterdam groups, working independently of each other, detected increased TSH levels strengthens this finding, and makes it unlikely that the finding is accidental. The supposedly contradictory results from both studies in the neonatal period can only be explained by an influence of dioxins on both the thyroid hormone regulating systems and peripheral tissues. In our cohort, both at two and a half years (17) and now at the age of eight years, no abnormalities were detected in thyroid hormone regulation. The external influence of dioxins on the setpoint then seems transient. Very young children have a relatively greater thyroxine need than older children. This is especially evident shortly after birth when the neonate’s energy supply comes from free fatty acids, with the glucose transport from the mother having ceased, and gluconeogenesis not yet operational. Furthermore, non-shivering thermogenesis is necessary for temperature control, and a rapid breakdown of triglycerides in the brown adipose tissue is used for this. In 1984, Rozman already hypothesised that brown adipose tissue is a
main target for dioxin exposure (35;36). The mobilisation of fat after birth is dependent on thyroxine, and this probably explains why the abnormalities in T4 and TSH levels attain significance, in relation to dioxin exposure, during the first few weeks after birth. After the neonatal period, the T4 need diminishes, from 10 micrograms/kg in the neonate to 2 micrograms/kg in the adult. This is a result of a physiological lowering of the setpoint, as a result of the reduced T4 need, and probably also as a result of the relative decrease of dioxin exposure, including a greater dilutional effect. This would result in the normalisation of the setpoint of T4 seen at the age of two and eight years. Further follow-up is necessary in order to establish that the apparent transient influence has no later effects on the thyroid and its regulatory system.

The FT4 concentration and CYP1A2 activity seem to show an inverse correlation in the scatter diagram (figure 1), yet statistically show no significant relation. The latter might be due to the limited size of the group. Cytochrome P450 1A2 possibly plays a role in FT4 metabolism.

**ALAT and ASAT**

In contrast to ALAT, a literature search failed to uncover an effect of dioxin exposure on ASAT. Acute dioxin exposure is known to increase plasma ALAT levels in laboratory animals, such as mice (37) and rhesus monkeys (38), and in children(14;39), yet these changes seem to be transient. Seefeld’s study of adult rhesus monkeys, exposed to various concentrations of dioxin, revealed increased ALAT levels during the course of intoxication, indicating liver damage. The maximum effect seen after two to three weeks was again normalised at about 6 weeks, indicating a transient effect (38). Mocarelli’s six year follow-up study, of the Seveso children exposed to dioxins following a chemical plant explosion, revealed a similar increase in ALAT amongst the highest exposed boys, which was decreased to levels comparable to the control population after about five years (39). This again indicates a transient effect of dioxin on ALAT levels, even at high exposure concentrations. Follow-up of industrially exposed workers in the U.S. showed no increase in ALAT activity (40). In the light of these studies, it is then not surprising that our current study has no longer found the relation between
the perinatal dioxin concentration and the ALAT levels, seen by Pluim in this same cohort shortly after birth (14). It is during the prenatal (in utero) and breastfeeding period that children get exposed to the highest dioxin concentrations (11;12;27). The later exposure, being predominantly determined by dietary exposure, is much less (27). It is then to be expected that the acute rise in ALAT levels, the reaction to the acute high dioxin exposure, will again normalise as was seen in our two follow-up studies of the same children, namely this study and that of Ilsen (17). We, too, are then inclined to interpret these findings as indicating that dioxin causes reversible damage to the liver. Whether the explanation for this lies in the rapid distribution of dioxin to adipose tissue, to the liver’s regenerating capacity, to an (as yet) unknown mechanism, or to a combination of factors remains to be elucidated.

**Cytochrome P-450 1A2**

Measuring paraxanthine/caffeine molar ratios in plasma, after a caffeine-loading test, has been shown to be an accurate method of estimating CYP1A2 activity (28;29;41).

The mean (1.35, range 0.41-2.48) of the paraxanthine/caffeine molar ratio in the cohort differs (though not statistically significantly) from the adult mean of 0.80 (range 0.16-1.7) [28,41]. However, it has long been assumed (although never demonstrated) that children show a higher CYP1A2 activity than do adults.

It is commonly known that CYP activity is altered by dioxin exposure [e.g. (42;43)] and the supportive literature is exhaustive. Yet, long term effects of perinatal exposure are still not clearly known, and this is especially so in humans. The degree of enzyme induction is seldom, if ever, in correlation to the dosage. In other words, should dioxins indeed cause induction, this induction need not be correlated to the level of exposure perinatally. We can therefore only conclude that we have found no visible decreased nor increased CYP1A2 activity, relative to perinatal dioxin exposure, seven to twelve years post exposure. It is possible that the activity was increased in the perinatal period (yet without permanently influencing the setpoint) due to the relatively far higher toxicity, and that the activity has normalised with the relative decrease in the toxin. Halperin also failed to show an association between industrial
exposure to TCDD and CYP1A2 induction in a larger adult group (44). Like CYP1A2, CYP1A1 also metabolises caffeine. However, CYP1A1 is expressed at very low levels in the liver, and caffeine metabolism is exclusively hepatic. It is therefore very unlikely that CYP1A1 colours the CYP1A2 results seen. There are no other human enzymes known to metabolise caffeine with high affinity and a similar pattern of metabolites as CYP1A2 (29). The results of the loading test then accurately represent the CYP1A2 activity in the children. We therefore could not confirm our hypothesis that dioxins decrease CYP1A2 activity in later childhood. The activity was not determined in the previous studies of the cohort.

While dioxins are widely known to be endocrine disrupters, it has been difficult to confirm their influence on humans. The cohort in this study is a group that was recruited antenatally, and in which the prenatal and postnatal dioxin exposures were determined perinatally. The children have since been studied at regular intervals and therefore form a usable longitudinal cohort, whereby the dioxin influence on the various developmental windows can be accurately followed over time. However, a longitudinal study often means losing subjects during the follow-up periods. Such is the case in this study, where the original group of 61 has been reduced to the current 37 children participating in this pre-pubertal follow-up. Yet, the grouping of values, without a directional tendency, seen in the scatter diagrams shows that the size of the group probably in no way limits the outcome. It is unlikely that a larger group would have produced a different outcome. The absence of statistically significant results at this age might be the effect of (partial) reversibility and/or recovery of the abnormalities seen in the perinatal period.

Concluding, follow-up of 7 to 12 year old children with documented prenatal and postnatal dioxin exposure has shown a normalisation of previously increased setpoint of thyroid hormone homeostasis. Similarly the relation between perinatal dioxin exposure and ALAT levels, seen shortly after birth, is no longer evident. These results point to a transient effect of the toxic influences of background levels of dioxins in The Netherlands on these determinants. This may be a result of the combination of body composition (less adipose tissue) in the perinatal
period and decreasing exposure from the relatively high perinatal to the much lower later childhood background exposure, and from a dilutional effect (more adipose tissue). The cytochrome P-450 1A2 activity, measured by means of a caffeine-loading test revealed no correlation with the pre- and postnatal dioxin exposures. Knowledge about the influence of perinatal background dioxin exposure on children is limited. This study provides new data.

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