



OPZ Geel
Openbaar Psychiatrisch Zorgcentrum

Luik III: follow-up study

Neurobehavioural and cognitive effects of prenatal exposure to persistent environmental toxicants in three year old children (2002-2007): Final results

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Introduction

Environmental exposures to a variety of neurotoxins remain a concern in western industrialized countries, as well as in developing countries, especially concerning health problems in vulnerable populations such as the unborn child. PCB's and dioxin-like compounds and heavy metals such as lead (Pb) and Cadmium (Cd) are regarded to belong to the most ubiquitous developmental neurotoxins (Guo et al., 2004; Chiodo et al., 2004) PCBs (polychlorinated biphenyls) have been widely produced and/or applied e.g. as flame retardants, lubricants for heavy machinery and dielectric fluid in transformers. They are belonging to the POPs (persistent organic pollutants) due to their very stable chemical structures and consequently very long half lives in the environment and living species, including humans. Therefore the handling of PCBs has been strictly regulated in many countries for quite some time. Nevertheless, they will persist in many surroundings for some more decades and will stay a potential health hazard, and today they can be measured in almost every human body. Human exposure to PCBs is largely due to ingestion of

contaminated foods, especially milk, fish and meat products. PCBs can cross the placenta causing prenatal exposure in the developing foetus and accumulates in breast milk.

Literature suggests that children are much more susceptible to the neurotoxic effects of PCB exposure especially prenatally and possibly also in the first years of life children compared to the effects of exposures later in life (Ribas-Fito, 2001).

Part of the literature is reporting deleterious effects on general IQ and/or cognitive development. Children born to mothers involved in the Yucheng disaster had a delay in growth, a delay in cognitive development and more behavioural problems than non-exposed children (Yu et al. 1991) These Yucheng children reached almost all the developmental milestones (32 of the 33 milestones measured) at a later stage (Yu et al., 1991). This resulted in the conclusion that foetus are especially vulnerable to the toxic effects of PCBs (Yueliang et al. 2004). With regard to PCB concentrations found in western industrialised countries, Patandin et al. (1999) and Vreugdenhil et al (2002) found an inverse relation between maternal PCB concentrations and cognitive performance at 42 months and 7.5 years of age respectively. Again prenatal exposure seemed to be more hazardous than postnatal exposures because even though breast fed babies had higher postnatal exposures to PCBs compared to formula fed babies, the breastfed children performed better at 42 months (Patandin et al., 1999) and on some cognitive aspects at 9 years of age. Therefore, the deleterious effects of PCB exposure may be more pronounced in formula-fed babies. There might even be a gender specific effect, as higher exposed boys did worse on constructive tasks in the Yucheng study (Guo et al. 1995). Gender specific learning defects in early developmental stages were also reported in rats exposed to PCBs. But not all authors could reproduce these findings. Daniels et al. (2003) didn't find any relation between prenatal PCB concentration levels and the cognitive or psychomotor development in young children. Differences in results might be caused by many factors, e.g. different tests or testing procedures, differences in methods measuring covariates and differences in biomarkers of prenatal PCB exposures (different congeners measured as biomarkers of exposure; exposure to other mixtures of congeners; prenatal PCB concentration measurements in maternal blood vs. umbilical blood).

In addition to the effects on general IQ and cognitive development, attention deficits, increased reaction times on P300 and psychomotor slowing have been reported in the Yucheng study (Chen et al. 1994, Lai et al. 2002). Also in the Dutch cohort, Vreugdenhil et al. (2004) reported increased reaction times and higher standard deviations on reaction time tests in 9 year old children related to higher prenatal PCB exposures. In addition, Shallice et al. (1982) found in children of the same age worse scores on the Tower of London test related to higher prenatal PCB concentrations, and a dose-dependent association between cord blood PCBs and errors of commission (response inhibition) was found (Stewart et al,

2003). Thus far, animal data have shown even stronger correlations between attention and psychomotor slowing and PCB exposure levels compared to the epidemiological data in children.

As PCBs and dioxin-like compounds have an endocrine disrupting, it is interesting to notice that play behaviour is reported to change in boys and girls (less masculine and more feminine play behaviour) according to the level of prenatal PCB exposure (Vreugdenhil et al, 2002). Some data in girls suggest that even the postnatal PCB exposure might influence play behaviour, although in the opposite direction (more masculine) (Vreugdenhil et al, 2002). Although these data are certainly not conclusive, as the questionnaire (PSAI) which was used was not appropriate for the age of the children and test-retest results were rather poor (0.6) (Kaufman, 2003.)

In addition to PCB exposures, prenatal as well as postnatal environmental exposures to heavy metals, especially Pb may result in behavioural changes and intellectual deficits in young children as well as in adolescents, and to a lesser extent in adults (Viaene et al., 2000, Vermeir et al. 2005). Co-exposure to other heavy metals is relatively common (e.g. Cd) and it could result in a more pronounced cognitive decline. Although the effects of very low environmental exposure to Pb (blood Pb <10 µg/dL) is most probably negligible in adults, the threshold below which there is an insignificant influence of Pb-exposure in children is still uncertain. Typical effects of Pb-exposure and possibly also Cd-exposure are concentration deficits, diminished visuomotor functioning, diminished planning and organisation and altered behaviour (Viaene et al., 2000, Vermeir et al. 2005).

Epidemiological studies have many possibilities of bias. In general, maternal IQ, socio-economic status (measured e.g. by yearly income or by the highest educational level of one of both parents), the home environment [observed by e.g. Home Measurement for Observation of the Environment, age of the mother, parity, gender, alcohol use, drugs use and smoking during pregnancy, complications at birth or during pregnancy, and breastfeeding, are regarded as possible influencing factors in the degree of neurobehavioral development of a young child. Even if the greatest care has been given to possible biases in some of these studies, not all influencing factors were or are known. For instance, Van den Bergh and co-workers (2005) have recently shown that the prenatal anxiety state and trait of the mother is a very important factor in the cognitive and behavioural development of children, expanding its effects even until teenage years, and which may have even more influence than other known factors. On the other hand the possible additive effects of co-exposure to chemicals which are obviously structural and biochemical totally different, but which also may have comparable negative influences on certain domains of the neurobehavioral development of children, has never been studied. Recently experts asked to review a FWO-research proposal on this topic have argued that significant co-exposures

never occur in the environment, which is untrue in Western industrialised countries like Belgium (Nawrot et al., 2002)

Interference with neurotransmitter levels, disruption of intracellular calcium homeostasis as well as changes in the thyroid axis and interference with oestrogen receptors and oestrogen levels are the major observed effects related to PCB-neurotoxicity. The knowledge that thyroid hormones are essential for normal brain development and that PCBs can disrupt the thyroid function, combined with the fact that PCBs influence neurobehavioral development (30, 32-36), has led to the hypotheses that PCBs disrupt brain development through the disruption of the prenatal thyroid function. But, in addition, they disrupt the activity of sex hormones due to the oestrogen or anti-oestrogen actions, which might be an explanation of the sex-linked differences as reported in epidemiological studies. Recent research has shown that during embryogenesis, and even in adulthood, oestrogens are essential in the functionality and neurogenesis of neurons which are important in learning processes and behaviour such as anxiety and gender-specific behaviour. Other neurotoxic compounds such as lead and mercury are known to disrupt the calcium homeostasis of the cell, but they also have an oestrogen disruptive effect. By this reason, some authors suggest that the neurotoxicity of mixtures of PCB-congeners and of mixtures of PCBs with heavy metals could be responsible for similar and/or synergistic effects, possibly through this or other overlaying mechanisms.

The present study is a part of the Environmental Health Action Program (EHAP) (2002-2006) in Flanders, investigating environmental exposure to e.g. Pb, Cd, PCBs, compounds with 'dioxin-like', chlorinated pesticides [DDE and hexachlorobenzene (HCB)], and organic solvents in eight selected regions of Flanders. In this part of the EHAP, concentration abilities, language development, visuomotor abilities, mental development and general intelligence will be examined at the age of 36 months. Social and sexual behavioural patterns will also be measured at the same age. The relation between these neuro-developmental parameters and the prenatal exposure markers of PCB's and dioxin-like compounds, Pb, Cd and chlorinated pesticides [DDE and hexachlorobenzene (HCB)] (umbilical cord blood concentrations) will be studied. In addition, the possibility of defining subgroups at risk (boys, low premorbid IQ, no breastfeeding) will be investigated. Measuring development in the different domains of human brain function is a lengthy and difficult task. Therefore an additional aim is to investigate if behaviour (questionnaires, play behaviour observations) is a useful bio-effect parameter in follow-up studies in populations exposed to toxic agents and if computerized testing of attention is feasible in toddlers. As no funding was available for analysis of biomarkers of postnatal exposure, it will be tried to calculate qualitative exposure estimates from repetitive food intake questionnaires.

Materials and methods

Study population (Participants)

Participants were recruited as part of an Environmental Health Action Program (2002-2006) in Flanders. Between September 2002 and February 2004, mother-child pairs from the general population were recruited through 26 maternities which were selected by stratified sampling in eight study areas. The study areas included two urban areas (Antwerp and Ghent), an area characterized by fruit orchards, a rural area and four types of industrial areas (harbour, non ferrous smelters, chemical industry and household combustion incinerators). The selected areas were representative for 20% of the inhabitants of Flanders. Pregnant mothers could be included if they lived for at least five years in the area of interest if they gave informed consent and if they were able to fill out Dutch questionnaires. This part of the study recruited in a rural area and the areas around the harbours, the waste incinerators, and the areas near the non ferrous smelters.

The study group was selected based on following inclusion criteria by the co-workers of the Provincial Institute of Hygiene -Antwerp: pregnancy without major complications, born at term, no major congenital abnormalities or diseases, no twins, no abnormal or asymmetrical reflexes during standard neurological screening during the first days and that the children would be raised with Flemish as their mother-tongue to prevent differences in language development. Informed consent was asked to participants whom were eligible in the sampled areas. The inclusion was planned to stop after 1 year. But due to a lower birth rate as expected in some areas, the sampling period had to be extended with half a year. At the end, 206 baby-mother pairs had given informed consent.

Blood sampling and measurements

PCBs, Calux TEQ, DDE, HCB

Cord blood was aliquoted and plasma was separated by centrifugation within one day in either the maternity or blood bank laboratories. The aliquoted samples were kept in the refrigerator for maximal one week. Afterwards they were put at -20°C until analysis. In cord plasma, marker polychlorinated biphenyls (PCB 138, 153 and 180), PCB 118 and 170 which may have more neurotoxic potentials, chlorinated pesticides [DDE and hexachlorobenzene (HCB)], and dioxin-like compounds (CALUX® assay) were analysed. The PCBs and

chlorinated pesticides were analyzed by GC/ECD using the method of Gomara et al. (Gomara, Ramos et al. 2002). The analyses were performed by two labs. Both laboratories participated in the AMAP proficiency testing scheme (Institute National de Santé Publique, Quebec, Canada). The measurement uncertainty (sum of systematic error and two times the reproducibility) was estimated from the results of the ClinChek and AMAP samples, and ranged between 21% and 34% for all the compounds except for HCB (64%). The limit of detection for all chlorinated compounds was 0.02 µg/L. Routinely measured cholesterol and triglycerides were used to express the results on a lipid weight basis (Covaci, Voorspoels et al. 2006). Exposure to dioxin-like compounds was assessed via the CALUX® assay, based on in vitro activation of the aryl hydrocarbon receptor (AhR) of cultured H4IIE rat hepatoma cells by the dioxin-like compounds present in 5 mL cord plasma (BioDetection Systems BV, Amsterdam, The Netherlands). The extraction and clean-up procedures were performed as described in Koppen et al. 2001. The limit of detection was calculated as the light signal measured from the dimethyl sulfoxide-control plus 3 times its standard deviation on each well plate (= 16 pg CALUX-TEQ/g cord plasma fat).

In cord plasma, lead and cadmium were analyzed using the method of HR-ICP-MS. The limits of detection (LD) and quantification (LQ) were calculated in such a way that the whole procedure of analysis was accounted for (digestion, calibration, measurement). The limit of detection was calculated as 3.3 multiplied by the standard deviation of the procedure blanco multiplied by the dilution-factor. The limit of quantification was calculated as 10 times the standard deviation of the procedure blanco multiplied by the dilution-factor. The final concentration in blood was made as followed. If the measured value was below the LD, it was set at LD/2. If the measured value was above the LQ, the measured concentration of the heavy metals was multiplied by the dilution-factor (DF) of the blood (with DF=1 if the blood wasn't diluted). If calculating the DF wasn't possible due to a lack of data, no final concentration was calculated.

Analysis of Cortisol in saliva

Saliva samples were taken by means of a Salivette®. Samples were analysed with a LUMINESCENCE-ENHANCED ENZYME IMMUNOASSAY (LEIA) as developed and described by Westermann, Demir, & Herbst (2004).

General history, medical file data and general health questionnaires

Shortly after birth/delivery, data regarding the child, data on life style of the parents and information about the current and former pregnancies were collected through the general

self-assessment questionnaire of the Environmental Health Action Program (2002-2006). These data and questionnaires were collected by trained staff members of the Provincial Institute for Hygiene and Epidemiology (V. Nelen). Women were asked e.g. whether they or their partner had been treated for fertility disorders, whether they had had any infections, hypertension, diabetes or other diseases and/or complications during pregnancy, whether they had used medication (0/1), alcohol (0/1), or had been smoking (0/1) during pregnancy. Almost all mothers proved to have taken some vitamin supplementation throughout their pregnancy. Data regarding mother and child were also collected in the medical files of the hospital: weight, length, Apgar scores, standard neurological, medical history of the mother, weeks pregnant, complications during birth, age of the mother, congenital diseases and breastfeeding (0/1). After screening the files one baby was going to be raised in a foreign language and excluded. The neurologic examination at birth was normal in all children except two children with a symmetrical babinski sign, which were excluded. Four children had minor birth defects (a supplementary finger, a small skin-patch, minor dysplasia of the outer ears, one foot with moderate valgus malformation), which were considered as without significance. One child proved to have an hydronefrose and has been excluded. All the other clinical signs were normal in all babies. In total, 202 mother-children pairs met all the inclusion criteria. If the Apgarscore at the beginning (1 min) was 6 or less and/or other signs of fetal asphyxia were present (meconium stained amniotic fluid, aspiration, abnormal fetal heart rate, neonatal resuscitation) the baby was considered to have had perinatal asphyxia (0/1).

During the first three years of the study, several questionnaires prepared by the Neurotoxicology Expertise Centre of The Governmental Psychiatric Hospital (Geel) were send to the mothers and returned by post, fax or e-mail. After the first month (4-6 weeks after delivery) all mothers received a supplementary questionnaire that was send only once. This questionnaire repeated and extended the questions used in the general questionnaire at birth. Semi-quantitative ethanol consumption (g ethanol/week) was calculated. Also, the Edinburgh Postnatal Depression Scale was included.

In the first year the mothers fulfilled a second questionnaire every month. After the first year this was repeated every three months. The second questionnaire questioned nutritional and developmental data of the child. Additionally, important life events and stress factors were questioned with the STAI (State-Trait Anxiety Inventory) (Handleiding bij de Zelf-beoordelings vragenlijst ZBV, Swets Test Publishers, 2000).

Neurobehavioral test methods and neurobehavioral questionnaires

An overview of the tests is summarised in Table 1.

At the time the child reached the age of 12, 24 and 36 months, the mother and pre-school playgroup/teacher completed questionnaires regarding possible emotional and behavioural problems [Infant Behaviour Questionnaire (IBQ) (Rothbart, 1981) at 12 and 24 months, Child Behaviour Checklist (CBCL) & Caregiver-Teacher's Report Form (C-TRF) (Achenbach and Rescola, 2000) at 24 and 36 months].

At the time the child reached the age of 36 months, an appointment was made to carry out the neurobehavioral evaluation of mother and child.

All psychological tests and questionnaires used are validated in Flanders for clinical use and suitable for use in a research context. The tests were administered by especially trained test leaders with substantial experience in testing children and adults. The tests were divided over two test sessions. Saliva samples were taken in the beginning and at the end of the session from mother and child, and the mother was instructed to take the same samples the day before the second test (see supra). Questionnaires for mother and teacher were given to the mother and it was asked to complete them before the second test. The different tests were trained in group with all test leaders and co-workers. Afterwards, test sessions and procedures were tried out in six volunteer mother-child pairs from May till July 2006. Subsequently tests and scoring procedures were refined to further minimize differences between different persons.

Table 1 Overview of questionnaires and tests concerning the children

Questionnaires	Measurement type	Time of assessment
Infant Behaviour Questionnaire (IBQ)	Temperament as observed by the mother	12 months and 24 months
Child Behaviour Checklist (CBCL) & Caregiver-Teacher's Report Form (C-TRF)	Functioning of the children as perceived by the mother and the teacher/kindergarten. Includes questions about sports, hobby's, school and a series of behavioural and emotional problems of the last 6 months.	24 and 36 months
<i>Child Sexual Behaviour Inventory (CSBI)</i>	<i>Sexual behaviour as observed by the mother</i>	<i>36 months</i>
<i>PSAI (PreSchool Activity Inventory)</i>	<i>Play behaviour (masculine/feminine)</i>	<i>36 months</i>
Milestones	Developmental abilities	0-36 months
Tests		
Bayley Scales of Infant Development (BSID-II-NL)	cognitive development: mental and motor developmental indices	36 months
Reynell Language Developmental Scale (RTOS)	receptive language development level measured by oral instructions to carry out small tasks while playing with toys	36 months
<i>NES 3 (neurobehavioral evaluation system) Continuous Performance Test</i>	<i>Computerised vigilance task (adapted for children by our research group)</i>	<i>36 months</i>
Snijders-Oomen non-verbal intelligence test (SON-R 2.5-7)	Intelligence: reasoning and visuospatial abilities	36 months
Observation of toy preference	7 min. lasting observation of masculine/feminine play behaviour	36 months

The questionnaires and tests in italic proofed to be ineffective for the purpose they were selected in our study context. The results of these items were not further analysed.

Questionnaires:

Infant Behaviour Questionnaire (IBQ) :

This instrument assesses 6 domains of infant temperament (activity level, soothability, distress and latency to approach sudden or novel stimuli, distress to limitations, smiling and laughter, and duration of orienting).

Child Behaviour Checklist (CBCL1½-5) & Caregiver-Teacher's Report Form (C-TRF1½-5):

The CBCL/1½-5 obtains parents' ratings of 99 items.

The C-RTF/1½-5 obtains teacher's (or caregiver) ratings of the same items.

Following cross-informant syndromes from both forms: Emotionally Reactive, Anxious/Depressed, Somatic Complaints, Withdrawn, Attention Problems, and Aggressive Behaviour. Also a Sleep Problems syndrome is derived from the CBCL/1½-5.

Additionally, the ratings result in a profile of DSM (Diagnostic and Statistical Manual)-oriented scales. The DSM-oriented scales are: Affective Problems, Anxiety Problems, Pervasive Developmental Problems, Attention Deficit/Hyperactivity Problems, & Oppositional Defiant Problems.

Child Sexual Behaviour Inventory (CSBI):

The CSBI is a parent report (mother or primary female caregiver) measure of sexual behaviour in children ages 2-12 years. In this study the 47 item-version (adapted by Schoentjes et al., 1999) was used, assessing a wide range of sexual behaviours that cover nine major content domains: Boundary Issues, Sexual Interest, Exhibitionism, Sexual Intrusiveness, Gender Role Behaviour, Sexual Knowledge, Self-Stimulation, Voyeuristic Behaviour, and Sexual Anxiety.

PSAI (PreSchool Activity Inventory):

In order to distinguish behaviours of boys and girls in relation to their degree of masculinity and femininity, the Pre-School Activities Inventory (PSAI) was developed. The PSAI has been standardised on more than 2000 subjects in the UK.

Milestones

In children, there is some variation in stages of development. The milestones included in this study are the most 'basic' abilities and skills that most children learn somewhere before reaching the age of 36 months. The milestones included in this study are: Holding Head Upright, Holding Back Upright, Smiling, Clapping One's Hands, Gripping, Rolling, Sitting strait Up, Eating Cookie Alone, Talking (first words), Crawling, First Steps Alone, Pulling Oneself Up, Potty Trained, and Clean (day and night).

Tests

Bayley Scales of Infant Development (BSID-II-NL):

The Bayley Scales measure the mental and motor development and test behaviour of infants from one to forty-two months of age. The scales have been used extensively worldwide to assess the development of infants. The examiner presents a series of test materials to the child and observes the child's responses and behaviours. The test contains items designed to identify young children at risk for developmental delay. In this study, mental and motor scales were assessed. In the Mental scale, several types of abilities are evaluated: sensory/perceptual acuities, discriminations, and response; acquisition of object constancy; memory learning and problem solving; vocalization and beginning of verbal communication; basis of abstract thinking; habituation; mental mapping; complex language; and mathematical concept formation. In the Motor scale, the degree of body control, large muscle coordination, fine motor skills of the hands and fingers, dynamic movement, postural imitation, and the ability to recognize objects by sense of touch (stereo gnosis) were assessed.

Reynell Language Developmental Scale (RTOS)

This instrument measures the language perception as well as the language production. The total score gives an indication of the overall language development.

NES 3 (neurobehavioral evaluation system) Continuous Performance Test

The frame of the continuous performance test of the NES3 was used to develop a test feasible for 3 year old children. The test was first tried out in three year old school children before it was used in this study.

They had to touch the mouse pad hit the spacebar of the computer every time a kitten appeared on the screen because mother cat had lost all her kittens and they had to help her find them back between all the other little animals (little rabbit, piglet). The construct of this test was not fully validated in children of this age but the mean latency of the correct answers, the mean latencies on the whole trial, the percentage of correct answers, and the last completed section can be regarded as measurements of concentration abilities. False positives can be regarded as measurement of impulse control.

Snijders-Oomen non-verbal intelligence test (SON-R 2.5-7)

The SON-R 2.5-7 is an individual nonverbal intelligence test which is suitable for children aged from two and a half to seven years. The test consists of six subtests of about 15 items each. The subtests Mosaics, Puzzles and Patterns involve spatial insight. The subtests Categories, Analogies and Situations call upon reasoning abilities. Separate total scores are presented for the performance scale (SON-PS) and the reasoning scale (SON-RS). The

intelligence score (SON-IQ) is derived from the six subtests. For both scales: mean is 100, standard deviation is 15.

Observed toy preference

“Toy preference” is assessed by measuring the time a child is playing with toys which are generally preferred either by girls (feminine) or by boys (masculine), or which is liked by both equally (neutral) (Connor & Serbin, 1977; Liss, 1981). The toys consisted of four feminine toys, two neutral toys, and four masculine toys. The toys are presented in a standardised way. A 7 minutes play was videotaped. The total time playing with feminine, masculine and neutral toys respectively and the time spent without playing was calculated.

This task was reported to have a high test-retest reliability (Henderson and Berenbaum, 1997), which was our own experience (5 videotapes were scored by two different persons and the difference was maximum some seconds per toy and consequently less than ten seconds per category, not changing anything to the percentage of preference).

In this study, an additional dependent variable was measured: the number of switches the child makes between the toys during the play session.

Observed Mother-child interaction

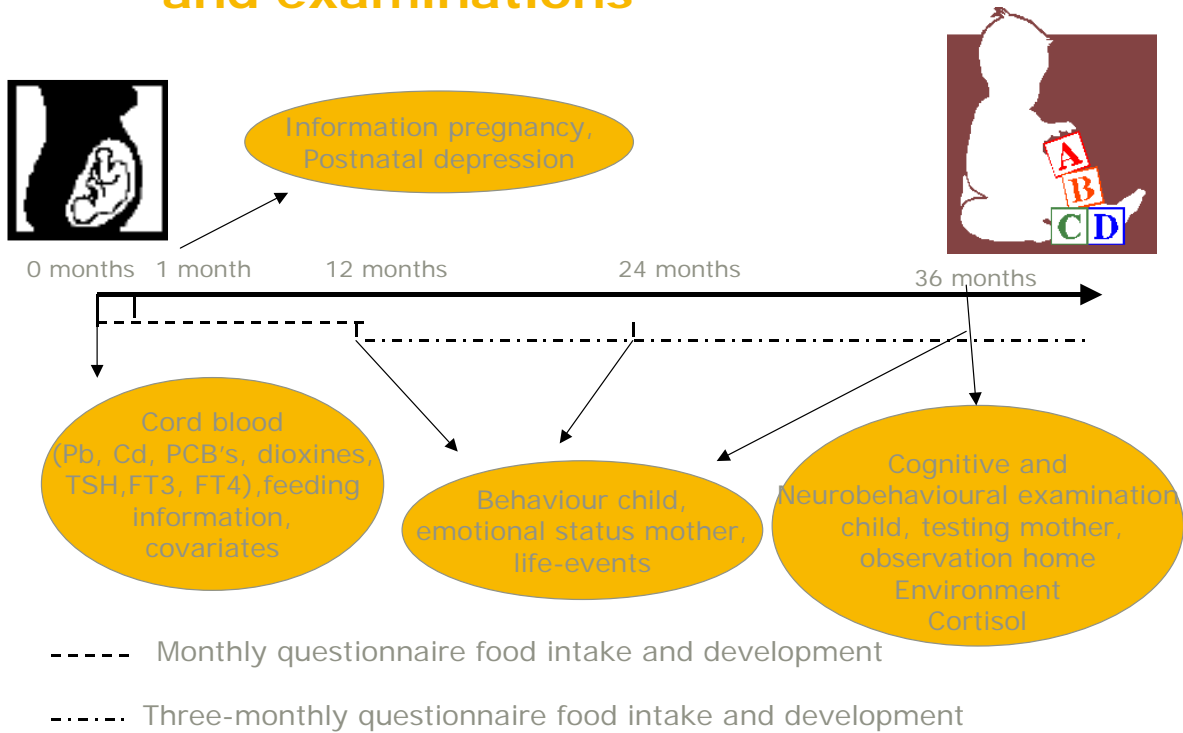
Three standardized play moments which require a mutual interaction between mother and child were videotaped and will be scored. There are two possible ways of scoring: from the quality of interactional behaviour from the mother and the quality of interactional behaviour of the child. Although these are not independent because in both scoring systems both mother and child factors are crucial (Erikson sensitivity scales).

Maternal factors and home environment

Maternal intelligence was assessed by the WASI (Wechsler Abbreviated Scale of Intelligence) (Wechsler, 2000) and an observation of the home environment was done by the HOME (Home Measurement for Observation of the Environment) (Caldwell, 1988). The State Trait Anxiety Inventory (STAI) (Ploeg, 2000) was assessed as a measure of state and trait anxiety, the Perceived Stress Scale (PSS) (Cohen et al., 2004) as a measure of self perceived stress. A life-events questionnaire was filled in to control for the impact of important life-events and the NEO-PI-R was assessed as a general measure of personality (Hoekstra et al, 1996).

Summary of the study design

Overview measurements and examinations



Statistical analysis

The statistical analysis was done on a group of 189 subjects. Data on co-variables are taken from the general questionnaire of the Environmental Health Action Program. Also, as anxiety of the mother seems to be a very important factor in the development of the child (Van den Bergh, 2005), different correction factors (cortisol values) regarding this possible confounder must be taken into account.

Database management and statistical analyses were performed with the SPSS program, version 12.0.

With ANCOVA variance the possible significant difference in testing methods and scoring between the different test-leaders was checked. The same covariates as for the stepwise regression analysis were used in the analysis (see below).

As asked for by the contractor, data will be compared between regions with an ANCOVA, using the same covariates as were used in the stepwise regression (see below).

Stepwise regression was performed in the total group using the sum of the marker polychlorinated biphenyls (PCB 138, 153 and 180) (sumPCB), Calux TEQ, Pb-umbilical cord blood, Cd-umbilical cord blood, DDE-umbilical blood and HCB-umbilical cord blood as biomarkers of exposure. Total IQ of the mother (WAIS), gender, highest educational level of the parents, parity, mother's age at birth, smoking during pregnancy, alcohol use during pregnancy, neonatal asphyxia, medication use during pregnancy, infections or other interfering diseases during pregnancy, birth season, breastfeeding, STAI score as a marker of prenatal stress, and total score of the HOME were used as covariates for the neurobehavioral test data.

After correlations analysis of the covariates in our study group, two variables were deleted from the covariates list. Highest Education Level Of The Parents correlated very high with the Total IQ Of The Mother ($r=.52$; $p<.001$) and Parity was highly correlated with the HOME score ($r=.34$; $p<.05$). Therefore Highest Education Level and Parity were excluded from the regression analyses.

A nominal STAI score was computed as a marker of prenatal stress. The trait score of the STAI scale has proven to be stable across years, whereas the state score is more vulnerable to acute mood changes. Therefore a 0/1 variable was created, based on the P90 of STAI trait score (measured at 12 months) ($n=142$). For the participants that had no score at 12

months, the 24 (n=8) or 36 months (n=16) score were used. This was allowed because of the stability of the STAI trait score over time. For 3 participants, the NEO-PI-R score (\geq P90) on Neuroticism was used, because this scale is highly correlated with the STAI trait ($r=.736$; $p<.001$) (n=3).

In addition, stepwise regression will be done including interactions between the different exposures. Stepwise regression analysis concerning gender-specific behaviour was done in boys and girls separately and in the mother-child interaction, the STAI was not incorporated in the model, as it is part of the scoring system, and thus of the results.

ANCOVA will be used to study the effects of prenatal PCB-exposure on cognitive and behavioural development to detect more vulnerable groups: in the formula-fed versus breastfed children, boys versus girls, low IQ ($<P50$) versus high IQ ($\geq P50$) and low neuroprotective metal concentrations (Zn, Fe) versus high concentration ($<P50$, $\geq P50$).

Results

General information

The results of the nutritional questionnaires proved not to be useful (too many uncertainties, the mother's compliance to fill in this questionnaire over a period of three years was rather poor), also the adapted computerised vigilance task proved to be too difficult for most children, making the data often not reliable (too much help of the test leader needed or too much interference by the mother). The CSBI was also frequently not filled out properly (lacking data, difficulties in interpretation of the questions by the mothers). At last, in coding the PSAI, we encountered difficulties in the interpretation of the scores and consequently also in the statistical analysis, probably the same as Vreugdenhil et al. (2002). After contact with the authors of the test, an alternative scoring system, as applied by Vreugdenhil et al. (2002), was prohibited. Therefore, the results of these three questionnaires were not further analysed.

Group characteristics

The group characteristics of the total group are summarized in Tabel 2a and 2b.

When starting the first test sessions in September 2006, 4/5 mother-child pairs still participated in the cohort and had sent back the questionnaires at (semi-)regular basis. Collecting the questionnaires over the three years was rather difficult as many participants proved to be in a busy part of their life. Drop-out was because of loss of interest (60%), or due to contact problems (e.g. families moved away and didn't gave us their new address) (40%).

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Tabel 2a: group characteristics

Total group N=214 ¹					
		Minimum	Maximum	Mean	Std. Deviation
Parity	214	1,00	4,00	1,59	,79
Duration of Pregnancy		36,00	42,00	39,4059	1,19313
Total weeks of breastfeeding	190	0	104	14,05	17,51
Number of weeks only breastfeeding (n=53)	186	0	52	7,60	9,34
Neurotism scale Mother	104	92	197	137,93	21,47
Total IQ mother (WASI)	129	75	136	103,93	12,06
SumPCB/total lipid conc*	198	2,81	305,56	87,93	59,57
PCB 118/total lipid conc	198	0	71,05	14,86	11,29
PCB 170/total lipid conc	197	0	55,56	8,93	7,42
CaluxTEQ/total lipid conc	148	5,05	130,15	30,10	20,50
DDE-conc/total lipid conc	206	8,17	1274,51	198,18	193,48
HCB-conc/total lipid conc	197	2,35	132,08	27,79	20,41
Pb-conc Umbilical Cord Blood(µg/L)	201	1,00	87,27	19,20	15,75
Cd-conc Umbilical Cord Blood(µg/L)	201	,05	13,87	,56	1,12
Fe-conc Umbilical Cord Blood	201	120868,55	899797,80	557206,17	129326,07
Cu-conc Umbilical Cord Blood	201	282,92	2264,19	597,81	213,13
Zn-conc Umbilical Cord Blood	201	285,42	30000,00	1868,70	2377,73
FT3 Umbilical Cord Blood#	200	,60	7,45	1,57	,85
FT4 Umbilical Cord Blood#	201	,85	4,30	1,32	,48
TSH (heel prick)		1,92	39,79	8,37443	6,235403
Total lipid conc Umbilical Cord Blood	211	52,00	614,0	211,38	73,85

¹ CaluxTEQ-values were available only in n=148 and questionnaires of the results NEO-PI-R were available in n=104 and of the STAI in n=166.

*SumPCB= PCB 138+ PCB 153+ PCB 180

Thyroid function

Table 2b: group characteristics

0/1 %	Frequency	Missing data
Boys/Girls	107/107	0
Working during pregnancy	10/90 (9,8% / 88,2%)	2 (2,0%)
Smoking during pregnancy	25/187 (11,7% / 87,4%)	2 (0,9%)
Alcohol during pregnancy	15/197 (92,1% / 7,0%)	2 (0,9%)
Spontaneous labour	160/52 (74,8% / 24,3%)	2 (0,9%)
Disease of the mother before pregnancy	100/2* (98,0% / 2,0%)	0
Regions 2/4/6/7 [#]	84/68/10/52 (39,3% / 31,8% / 4,7% / 24,3%)	0
IVF	211/1 (98,6% / 0,5%)	2 (0,9)

*fibromyalgia, colitis ulcerosa

Influence of different test leaders

Coincidentally, certain test leaders tested significantly more boys while other test leaders tested more girls. When corrected for gender of the child, there was no significant difference in any of the outcome variables regarding the different test-leaders ($p > 0,050$) (multiple linear regression).

Influence of Regions

There was no significant difference in any of the outcome variables regarding the different regions ($p > 0,050$) (multiple linear regression). No correlation was found between Pb and sumPCB concentrations in the total group, nor in the regions. No difference in Pb concentrations between boys and girls were found.

Cord blood - Relationship pollutants & Thyroid Hormones

(Preliminary results, in collaboration with Johan Maervoet, UIA)

Pollutant ($\mu\text{g/L}$)	Log ft_4 (ng/dl)		Log ft_3 (pg/ml)		Log TSH (mIU/L)	
	β	p	β	p	β	p
PCB 118	-0.318	< 0.0001	-0.192	0.010	-0.087	0.261
PCB 138	-0.392	< 0.00001	-0.235	0.004	-0.158	0.063
PCB 153	-0.176	0.041	-0.083	0.327	-0.050	0.567
PCB 170	-0.346	< 0.0001	-0.192	0.022	-0.101	0.242
PCB 180	-0.332	< 0.001	-0.248	0.004	-0.058	0.522
Calux TEQ	-0.165	0.038	-0.154	0.043	-0.018	0.830
HCB	-0.270	< 0.001	-0.151	0.052	-0.108	0.179
p,p'-DDE	-0.169	0.018	-0.104	0.137	-0.042	0.569
Zn	+0.277	0.0001	+0.185	0.008	+0.005	0.940

$n \geq 182$. Multiple linear regression - Adjusted for total lipids, age mother, sex, gestational age, and alcohol consumption

There is a negative relation between FT_4 and FT_3 and all PCB-congeners, dioxin-like compounds and chlorinated pesticides biomarkers. There is no relation with TS

The relation between neurobehavioral development and prenatal Pb-exposure

Questionnaires:

INFANT BEHAVIOUR QUESTIONNAIRE

IBQ (12 months) (n=93)	PbB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-
Activity level	0,012	0,005	0,221	0,029	gender (M>F)	-0,269 0,008

By analysing at the gender groups separately, only a significant relation is found in the boys group:

IBQ (12 months) (n=47; boys group)	PbB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-
Activity level	0,022	0,008	0,203	0,007	-	

No significant relation was found with the other category scores.

CHILD BEHAVIOUR CHECKLIST/TEACHER REPORT FORM FOR CHILDREN

CBCL (24 months) (n=58)	PbB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-
Withdrawn	0,027	0,009	0,351	0,004	neonatal asphyxia IQ mother	0,392 0,001 -0,233 0,050

CBCL (36 months) (n=74)	PbB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value
Withdrawn	0,019	0,009	0,214	0,048	STAI trait mother	0,305 0,005

Anxious/Depressed	-0,028	0,012	0,260	0,022	Prenatal alcohol	0,722	0,000
					BMI child at birth	0,206	0,041

For the CBCL 36 months questionnaire, borderline significant relations were found between PbB and the Internal Scale (Beta=0,204; p=.064).

For the TRF 24 months questionnaire, borderline significant relations were found between PbB and the Attention Deficit/Hyperactivity Problem Scale (Beta= -0,0315; p=.056).

For the TRF 36 months questionnaire, borderline significant relations were found between PbB and the Anxiety Problem Scale (Beta= -0,2370; p=.056)71.

No significant relation was found with the other category scores.

MILESTONES QUESTIONNAIRE

milestones (0-36 months) (n=91)	PbB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-
rolling over	0,029	0,012	0,255	0,015	-	

milestones (0-36 months) (n=85)	PbB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-
<i>Pulling oneself up</i>			0,185	0,062	<i>Age of mother</i>	0,419 0,000

For the milestones, borderline significant relations were found between PbB and Pull Oneself Up (Beta= -0,2370; p=.056).

No significant relation was found with the other category scores.

Tests and observations

SNIJDERS-OOMEN NONVERBAL INTELLIGENCE TEST: REASONING AND PERFORMANCE IQ SCORES

SON (n=111)	PbB				Other significant covariates	
	B	SE (B)	Beta	p-	Beta	p-value
SON total IQ			-0.139	0.096	Gender (F>M)	0.406 0,000
					HOME	0.247 0,004
					STAI trait	-0.203 0,014
SON Reasoning IQ	-0,165	0,068	-0,194	0,018	Gender (F>M)	0,419 0,000
					BMI child	0,221 0,008
					HOME	0,220 0,009
SON Performance IQ				n.s.		

By analysing at the gender groups separately, no significant relations were found with PbB.

BAYLEY SCALES OF INFANT DEVELOPMENT: MENTAL AND MOTOR SCALE INDICES

BSID (n=100)	PbB				Other significant covariates	
	B	SE (B)	Beta	p-	Beta	p-value
Mental Scale Index				n.s.	Gender (F>M)	0,231 0,021

No significant relation was found with the Motor Scale Index.

By analysing at the gender groups separately, only a significant relation is found in the girls group:

BSID (n=49; girls group)	PbB				Other significant covariates	
	B	SE (B)	Beta	p-	Beta	p-value
Mental Scale	-0,274	0,123	-0,273	0,031	Total IQ	0,476 0,001
					mother	
					Breastfeeding	-0,363 0,008

No significant relation was found with the Motor Scale Index.

GENDER RELATED PLAY BEHAVIOUR OBSERVATION

Observation Toy Preference (Girls n=50)	PbB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value
% Masculine play behaviour	0,563	0,208	0,364	0,009	-	-
% Feminine play behaviour	-0,743	0,228	-0,426	0,002	-	-

No relation was found between PbB and the RTOS.

The relation between neurobehavioral development and prenatal Cd-exposure

Only a few significant relations were found between the prenatal Cd-umbilical cord concentration and the outcome variables. This was expected. Cd concentrations are very low as Cd is not crossing the placenta. The possible deleterious effects of the Cd-body burden are probably due to postnatal environmental exposure. As already mentioned, no funds were available to measure Cd in blood or urine at 36 months.]

Questionnaires

INFANT BEHAVIOUR QUESTIONNAIRE

IBQ (12 months) (n=95)	CdB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-
Distress and Latency to Approach Sudden or Novel Stimuli	0,412	0,152	0,272	0,008	age of mother at birth	-0,231 0,036

IBQ (24 months) (n=60)	CdB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-
Smiling and Laughter	-0,320	0,131	-0,288	0,018	Smoking during pregnancy	0,349 0,005

No significant relation was found with the other category scores.

CHILD BEHAVIOUR CHECKLIST/TEACHER REPORT FORM FOR CHILDREN

TRF-C (24 months) (n=33)	CdB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value
Anxious/Depressed	0,757	0,368	0,347	0,0048	-	

TRF-C (36 months) (n=57)	CdB				Other significant covariates		
	B	SE (B)	Beta	p-value	Beta	p-value	
Anxiety Problems	-1,224	0,564	-0,270	0,034	alcohol during pregnancy	0,340	0,008

MILESTONES QUESTIONNAIRE

milestones (0-36 months) (n=105)	CdB				Other significant covariates		
	B	SE (B)	Beta	p-value	Beta	p-	
Grasping	0,514	0,231	0,214	0,029	-		

No significant relation was found with the other milestones.

No significant relations were found with CdB, SON, BSID and RTOS.

GENDER RELATED PLAY BEHAVIOUR OBSERVATION

Observation Toy Preference (Girls n=50)	CdB				Other significant covariates		
	B	SE (B)	Beta	p-value	Beta	p-value	
% Feminine play behaviour	-14,796	7,300	-0,281	0,048	-		

The relation between neurobehavioral development and the prenatal exposure levels of PCB and dioxin-like compounds.

Questionnaires

INFANT BEHAVIOUR QUESTIONNAIRE

IBQ (12 months) (n=68)	CALUX TEQ				Other significant covariates		
	B	SE (B)	Beta	p-value	Beta	p-	

Distress and Latency to Approach Sudden or Novel Stimuli	-0,011	0,005	-0,247	0,038	age of mother at birth	-0,266	0,026
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IBQ (24 months) (n=61)	PCB118				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-
Distress to limitations	-0,021	0,010	-0,259	0,044	-	-

No significant relation was found with the other category scores.

CHILD BEHAVIOUR CHECKLIST/TEACHER REPORT FORM FOR CHILDREN

CBCL (36 months) N=49	Calux TEQ				Other significant covariates		
	B	SE (B)	Beta	p-value		Beta	p-value
Withdrawn	-0,024	0,010	-0,290	0,016	STAI	0,450	0,000
					IQ child	-0,324	0,007
Emotionally Reactive	-0,044	0,016	-0,329	0,009	STAI	0,344	0,005
					breastfeeding	0,257	0,038
Internal Scale	-0,102	0,035	-0,314	0,006	STAI	0,499	0,000
					IQ child	-0,321	0,004
Pervasive Developmental Problems	-0,032	0,014	-0,259	0,030	STAI	0,502	0,000
					Gender (M>F)	-0,331	0,006

CBCL (36 months) N=71	sumPCB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value
Somatic Complaints	-0,009	0,004	-0,257	0,031	-	-

TRF (24 months) N=24		Calux TEQ			Other significant covariates		
	B	SE (B)	Beta	p-value		Beta	p-value
Pervasive developmental problems Internal Scale	-0,067	0,029	-0,440	0,031	-		
	-0,122	0,041	-0,526	0,007	STAI	-0,455	0,017

TRF (36 months) N=60		Sum PCB (138+153+180)			Other significant covariates		
	B	SE (B)	Beta	p-value		Beta	p-value
Other Problems	-0,012	0,005	-0,264	0,035	HOME	-0,293	0,020

TRF (36 months) N=58		PCB118			Other significant covariates		
	B	SE (B)	Beta	p-value		Beta	p-value
Emotionally Reactive	-0,031	0,015	-0,259	0,045	-		
Other Problems	-0,050	0,025	-0,241	0,050	HOME	-0,283	0,022
					Prenatal alcohol	0,245	0,046

For the CBCL 36 months questionnaire, borderline significant relations were found between CALUX TEQ, the Total Problems scale (Beta=-0,179; p=0,087) and the Other Problems Scale (Beta=-0,169;p=.099) and between sumPCB, the Emotionally Reactive Scale (Beta= -0,231, p=0,076) and the Anxiety Problems Scale (Beta=-0,291, p=0,082).

For the TRF 36 months questionnaire, borderline significant relations were found between PCB118 and the Anxiety Problems scale (Beta= -0,228; p=0,067).

MILESTONES QUESTIONNAIRE

milestones (0-36 months) (n=47)		Sum PCB (138+153+180)			Other significant covariates		
	B	SE (B)	Beta	p-value		Beta	p-

first steps alone	0,015	0,006	0,338	0,020	-
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milestones (0-36 months) (n=47)	PCB 118				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-
first steps alone	0,095	0,032	0,409	0,004	-	

No relation was found with the results of the IBQ_12 months, 24 months and CBCL_24 months and sum PCBs, PCB118, PCB170 or Calux-TEQ.

Tests and observations

SNIJDERS-OOMEN NONVERBAL INTELLIGENCE TEST: REASONING AND PERFORMANCE IQ SCORES

SON (Boys: n=57)*	PCB 118				Other significant covariates		
	B	SE (B)	Beta	p-		Beta	p-value
SON total IQ	-0,446	0,155	-0,347	0,006	STAI	-0,348	0,006
SON Reasoning IQ				n. s.			
SON Performance IQ	-0,482	0,189	-0,308	0,014	STAI	-0,391	0,002

*On the total group, no significant relations were found with PCB 118 (only with gender: F>M).

REYNELL LANGUAGE DEVELOPMENTAL SCALES: LANGUAGE COMPREHENSION AND LANGUAGE PRODUCTION

RTOS (n=60)	CALUX_VET				Other significant covariates		
	B	SE (B)	Beta	p-value		Beta	p-value
Vocabulary	-0,055	0,026	-0,252	0,041	Age of testing	0,323	0,010

RTOS (n=99)	sumPCB				Other significant covariates		
	B	SE (B)	Beta	p-value		Beta	p-value
Language	-0,125	0,062	-0,200	0,047	-		

Comprehension		
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	RTOS (n=98)				PCB 170		Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value		
Language	-1,377	0,561	-0,243	0,016			-	
Comprehension								

GENDER RELATED PLAY BEHAVIOUR OBSERVATION

	Observation Toy Preference (total group: n=84)				SumPCB		Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value		
Switching toys (number)	-0,017	0,008	-0,236	0,030			-	

A borderline significant relation was found between sumPCB and % masculine play behaviour (Beta= -0,174, p=0,057).

	Observation Toy Preference (total group: n=84)				PCB 170		Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value		
% non-gender specific	0,628	0,274	0,246	0,024			-	

A borderline significant relation was found between PCB 170 and % masculine play behaviour (Beta= -0,157, p=0,086).

	Observation Toy Preference (total group: n=84)				PCB 118		Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value		
% masculine play	-0,641	0,236	-0,242	0,008			Gender (M>F)	-0,569 0,000

Observation Toy Preference (girls: n=47)	PCB 170				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value
% non-gender specific	0,686	0,309	0,314	0,032	-	

Observation Toy Preference (girls: n=45)	PCB 118				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value
% masculine play behaviour	-0,593	0,263	-0,318	0,029	-	

In the boys' group, a borderline significant relations was found between sumPCB and % masculine play behaviour (Beta=-0,262, p=0,094).

Observation Toy Preference (boys: n=37)	SumPCB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value
Switching toys (number)	-0,027	0,012	-0,367	0,025	-	

In the boys' group, a borderline significant relations was found with PCB 118 (Beta=-0,271, p=0,090).

The relation between neurobehavioral development and the prenatal exposure levels of DDE and HCB.

Questionnaires

INFANT BEHAVIOUR QUESTIONNAIRE

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On the IBQ 12 months, a borderline significant relation was found between HCB and the Smiling and Laughter scale (Beta= 0,180, p=0,065).

+

IBQ (24 months) (n=61)	HCB				Other significant covariates		
	B	SE (B)	Beta	p-value	Beta p-		
Distress to limitations	-0,011	0,005	-0,271	0,034	-		
Smiling and Laughter	0,011	0,004	0,316	0,010	Prenatal smoking	0,355	0,004

No significant relation was found with the other category scores.

CHILD BEHAVIOUR CHECKLIST/TEACHER REPORT FORM FOR CHILDREN

CBCL (36 months) N=70	HCB				Other significant covariates		
	B	SE (B)	Beta	p-value	Beta		
Internal scale	-0,054	0,026	-0,229	0,043	STAI	0,333	0,004
Total Problems	-0,172	0,082	-0,221	0,041	STAI	0,435	0,000
Other Problems	-0,056	0,024	-0,233	0,023	STAI	0,508	0,000

TRF (24 months) N=35	HCB				Other significant covariates		
	B	SE (B)	Beta	p-value	Beta		
Sleep Problems	-0,235	0,007	-0,356	0,020	Gender (F>M)	0,378	0,013
					STAI	-0,322	0,033
Other Problems	-0,004	0,001	-0,399	0,005	Gender (F>M)	0,474	0,001
					STAI	-0,270	0,050

TRF (24 months) N=35	DDE				Other significant covariates		
	B	SE (B)	Beta	p-value	Beta		

Total Problems	-0,017	0,007	-0,356	0,020	Gender (F>M)	0,378	0,013
Other Problems	-0,004	0,001	-0,399	0,005	STAI	-0,322	0,033
					Gender (F>M)	0,474	0,001
					STAI	-0,270	0,050

A borderline significant relation was found between DDE, the External Scale (Beta= -0,294, $p=0,061$), Oppositional Defiant Problems (Beta= -0,304, $p=0,062$) and Anxiety Problems (Beta= -0,323, $p=0,068$).

TRF (36 months) N=35	HCB				Other significant covariates		
	B	SE (B)	Beta	p-value	Beta		
Emotionally Reactive	-0,021	0,009	-0,293	0,023	-	-	
Somatic complaints	-0,004	0,002	-0,276	0,033	-		
Aggressive behaviour	-0,074	0,035	-0,268	0,038	-		
Other Problems	-0,034	0,015	-0,271	0,030	HOME	-0,281	0,025
Total Problems	-0,163	0,080	-0,258	0,047	-		

A borderline significant relation was found between DDE and the Other Problems Scale (Beta= -0,216, $p=0,087$) and between and the Anxiety Problems Scale (Beta= -0,221, $p=0,079$).

On the CBCL 24 months, a borderline significant relation was found between HCB and the Sleep Problems scale (Beta=-0,235; $p=0,060$).

MILESTONES QUESTIONNAIRE

milestones (0-36 months) (n=47)	HCB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-

First steps	0,045	0,019	0,330	0,023	-
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A borderline significant relation was found between HCB and the milestone Keeping Head Up (Beta= 0,162, p=0,096).

Tests and observations

SNIJDERS-OOMEN NONVERBAL INTELLIGENCE TEST: REASONING AND PERFORMANCE IQ SCORES

A borderline significant relation was found between HCB and the SON Performance Scale (Beta= -0,154, p=0,086).

REYNELL LANGUAGE DEVELOPMENTAL SCALES: LANGUAGE COMPREHENSION AND LANGUAGE PRODUCTION

RTOS (n=98)	HCB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value
Language Comprehension	-0,487	0,184	-0,262	0,010	-	

GENDER RELATED PLAY BEHAVIOUR OBSERVATION

Observation Toy Preference (boys: n=39)	DDE				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value
% masculine play behaviour	-0,056	0,026	-0,326	0,037	HOME	-0,326 0,037

In the total group, a borderline significant relation was found between HCB and % masculine play behaviour (Beta=-0,162, p=0,086).

The relation between neurobehavioral development and the interaction between the prenatal exposure levels of sum-PCB and Pb.

In order to look at possible interaction and/or addition effects, the test and scale scores were transformed into standardised (z) scores. All potential factors were brought into the model (stepwise Multiple Linear Regression).

Pb and PCBs

None of the interaction or addition factors had a surplus effect (no changes in Beta) on the cognitive endpoints.

PCBs and DDE/HCB

RTOS (n=97)	sumPCB*HCB				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-value
Language comprehension	-7,884	2,341	-0,327	0,001	-	0,323 0,010

The interaction factor of the standardised PCB and HCB values has a surplus effect (beta > -0,252 and > -0,262).

milestones (0-36 months) (n=47)	SumPCB*DDE				Other significant covariates	
	B	SE (B)	Beta	p-value	Beta	p-
first steps alone	0,665	0,271	0,343	0,018	-	

The interaction factor of the standardised PCB and DDE values has a surplus effect (beta > -0,338).

Overall interaction/addition.

No surplus effect was found.

Summary results

The higher the prenatal Pb concentrations the lower the non-verbal intelligence (SON-IQ: Reasoning IQ) in the total group, and the lower The Mental Scale Index (Bayley) in the girls group. Higher prenatal Pb exposure is associated with prolonged motor development, more specific in acquiring skills needed to roll over (milestones 0-36 months). A higher activity level is reported in relation to higher prenatal Pb concentrations at 12 months (IBQ), especially in the boys group. Higher prenatal Pb concentrations are related to a lower ability to focus on tasks and more behavioural problems (Attention Problems, more Withdrawn, Total Problems, and Other Problems) at 24 and at 36 months (CBCL/TRF). Girls with higher prenatal Pb concentrations show more masculine and less feminine play behaviour (gender related play observation).

At 36 months, boys with higher prenatal exposure to PCBs and dioxin-like compounds have lowered nonverbal IQ-scores, with more specific decreased performance (visuomotor) skills (PCB118) and they tend to be less sensitive to novel stimuli (more passive behaviour)(sumPCB).

Prenatal exposure to PCBs is reported to be associated with a prolonged language development (RTOS), more specific the comprehension of language (CALUX-TEQ, sumPCB, PCB170). Children with higher prenatal PCB or dioxin-like compounds concentrations are reported to show less affective mood swings, show less emotional reactivity (Calux-TEQ) and express less somatic complaints (sumPCB) (CBCL_36 months). These children react less alert at new stimuli (CALUX-TEQ)(IBQ_12 months) and are to a lesser extend distressed to limitations (PCB118)(IBQ_24 months). Children with high prenatal PCB concentrations start to walk significantly later (sumPCB, PCB118)(milestones). These children also show less masculine and more non-gender specific play behaviour(PCB118, PCB170), with less switching between toys (less sensitive to novel stimuli)(sumPCB).

Prenatal exposure to pesticides (DDE and HCB) is associated with impaired language comprehension (HCB). These children start to walk later (milestones), indicating a prolonged motor development (HCB). These children show less distress to limitations, are more easy going (IBQ_24 months)(HCB), express less affective problems, have less sleep problems, show less emotional reactivity and less aggressive (acting-out) behaviour (CBCL_36 months, TRF_24months, TRF_36 months)(DDE, HCB).

Boys show less masculine play behaviour at 36 months (DDE).

An interaction effect between sumPCB, HCB and DDE was found on language comprehension (RTOS) and motor development (starting to walk, milestones).

Discussion

The SON-R is a non-verbal intelligence test and is as a consequence a visual test. The subtests are almost completely in black-and-white and not very attractive to three-year-olds. As a consequence, next to the different aspects of intelligence, sustained attention is really necessary to fulfil this test with success. The SON-IQ decreases with 1 IQ-point when Pb increases with 0,606 µg/L in our study. The range of Pb concentrations in umbilical cord blood in the studied regions in Flanders is between 0,1,00 and 8,727µg/dL, meaning that between the lowest and the highest exposed 14,234 non-verbal IQ points are lost. Generally a lowering of a mean IQ of 100 with 15 points or more will shift a child from a normal IQ to a borderline normal IQ, which has grave consequences for schooling and professional careers, especially in a region like Flanders, which prospers on a 'knowledge economy'.

In contrast to the SON, is the BSID a test measuring developmental milestones. It consists of very attractive and very familiar little toys with which the child has to execute short and varying tasks, alike the daily life of the child. This test is much less demanding on concentration than the SON, which may explain why no prominent adverse effects of Pb are found on this test (only in girls).

In addition, at 12 months (IBQ) prenatal Pb concentrations are related to a lower ability to focus on tasks and thus a lesser duration of task-specific orientation. The CBCL/TRF reported more behavioural problems. Prenatal Pb exposure is associated with a higher activity level, especially in boys. This subgroup seems to be at risk here. Earlier findings have shown diminished intelligence scores, poor visuomotor integration and difficulties with planning and organisation in children exposed to Pb (Canfield et al, 2003; Bellinger & Dietrich, 1994; Lamphaer et al, 2000; Chiodo et al, 2004), sustaining our findings.

A relative new effect is the relation between higher prenatal Pb concentrations and gender related play behaviour in girls. An increase in masculine play behaviour is observed to the detriment of feminine play behaviour (gender related play observation).

Contrary to the findings regarding prenatal Pb exposure levels, prenatal PCB levels seem to produce more passive behaviour. They are easier to handle, are reported to show less affective mood swings, and less emotional reactivity (Calux-TEQ). This indicates possibly the existence of a more 'smoothed' affect and a certain passivity in contrast to lower exposed children. Prenatal exposure to PCBs has an effect on language development (RTOS), more

specific on language comprehension. The range between the lowest and the highest exposed child in our group was associated with impaired language comprehension, up to 38% lower scores.

Also, in the toy preference task, higher sumPCB exposed children alternate less between toys and are playing more with a puzzle and a book compared to children with lower prenatal sumPCB-levels. An increase of 58,82 points (range 2,81 till 305,56) of the sumPCB (corrected for serum lipids) gives one time less switching during 7 minutes. Meaning that the highest exposed child switches toys almost once per minute less compared to the lowest exposed child, plays almost 64% less with his boys-specific toys and 62% more with non-gender specific (neutral) toys. Switching in this case means also crawling and turning, as toys were displayed in a semi-circle. The changes in switching behaviour are most pronounced in boys (1 switch less with an increase of 37,04 points of sumPCB levels). Higher prenatal sumPCB-exposed children are attaining some motor skills at a later age. The milestone First Steps is a marker of motor development, requires balance and coordination, but also requires active behaviour (in contrast to passive waiting) and an urge to achieve new goals.

Jacobson et al. (1990) also found a relationship between PCB exposure and activity level, they found reduced activity with 4-year old children with higher PCB levels. Similar effects were found in the Dutch cohort (Patandin, 1999). In both studies, this finding was most associated with postnatal exposure. Unfortunately, we don't have data on the postnatal PCB exposure levels in this study.

That prenatal exposure to PCBs was related to less masculine play behaviour and more neutral play behaviour in boy was also reported by Vreugdenhil et al (2002). These authors used the PSAI, but were criticized for a nonconforming scoring of the test (Kaufman, 2003). After consulting the authors (personal communication with Golombok, 2006), we scored the test conform to the initial instructions. But in analysing the results of the PSAI in our population, we probably encountered the same problems as Vreugdenhil et al. (2002). The significance of an increasing or decreasing score is depending on the gender of the child and the level of the score itself. This made the results of the PSAI difficult to analyse and interpret. In contrast, the Observation of Toy Preference is a much easier, parent-independent measurement of gender-specific behaviour, with a highly inter-observer reliability. These observations confirm the previous impressions of influences on gender-specific behaviour by PCB exposures in fetuses and infants.

Prenatal exposure to the pesticides DDE and HCB is associated with retarded language comprehension, they start to walk at a later age and show also signs of a 'smoothed' affect.

They express less emotions, are very easy to handle and show somewhat passive behaviour. DDE-exposed boys show less masculine play behaviour.

The interaction between high prenatal sumPCB and prenatal HCB gave a more pronounced effect on RTOS language development test, more specific on language comprehension. The interaction between sumPCB and DDE has a surplus (significant, although very small) effect on the developmental milestone First Steps Alone.

PCBs, DDE and HCB are all organochlorine compounds and might have similar effects, as was observed in this study. By co-exposure on these chemicals, it seems an interaction occurs, which enlarges the separate effects of the individual compounds.

Our results show that the deleterious effects of environmental pollutants may be different (play behaviour) or more extensive and more pronounced (Bayleys) in boys and girls separately. This may be explained by the different impact that endocrine disruptors may have on the development of the male and female brain. Moreover, our results show that the normal development of neurobehavioral abilities has a small but significantly different course in boys and girls (SON, Bayleys, RTOS, Toy preference, ...), a finding which is known from literature. Cognitive development is sex-dependent, boys and girls show differences in cognitive style, as a result of the actions of sex hormones and steroids on the brain during early development (Willams et al. 1990). Therefore, it may be very well possible that the neurobehavioral bio-effect markers of neurotoxic exposure differ between the sexes at the same age. In this study, in contrast to the former adolescent study (Vermeir et al, 2005), the differences between boys and girls can not be explained by different exposure levels in boys or girls (all $p > 0,100$, except for the PCB 118/total lipid concentration which was slightly higher in girls, t-test, $p = 0,030$).

In addition, the results have shown that PCBs have an impact on the thyroid function of the newborn child. This finding was not consistent in literature. We suggested that this may be caused by the frequent use of TSH as marker for thyroid function. In contrast to FT_3 and FT_4 concentrations, TSH concentration can change more rapidly, especially under stressful conditions. Therefore we hypothesised that TSH was most likely a less reliable marker to study the impact of other factors on thyroid function under the circumstances of birth.

Recently has been found further evidence to support the hypothesis that organochlorine compounds can alter the thyroid system, even at background levels of exposure, particularly total T3 levels (Alvarez-Prederol et al, 2008). Our results sustain this hypothesis. This finding supports the hypothesis that environmental exposure parameters such as organochlorine compounds can interfere with the thyroid system and therefore have an effect on brain development.

Summary:

As was known from literature, our data support the assumption that the neurobehavioral development of young children is negatively influenced by environmental concentrations of PCBs as they exist at present in Flanders. The effect of prenatal PCB exposure can be described as more passive, more quite children, with a slower mental development and a more 'smoothed' affect. In addition, higher exposed children seem to show more neutral (non-gender) specific behaviour.

As was also reported in literature before, the present environmental Pb concentrations, which were thought to be safe (all <10 microgram/dL) make children less able to sustain attention for a longer period of time, is associated with more behavioural problems, lowers non-verbal intelligence, increases activity level in boys and more masculinised and less feminized play in girls.

Our results sustain the hypothesis that PCBs have an impact on the thyroid function of newborns, which may be one mode of action of their developmental neurotoxicity, although other modes of action are still possible. More human and animal studies are needed to clarify this issue.

In addition, our study results seem to confirm that the neurotoxic effects of PCBs are different in boys and girls, which was suspected before. Moreover, prenatal exposure to a combination of PCBs and DDE or HCB, at concentrations like they exist in large parts of Flanders, may increase the deleterious effects on mental development and on some aspects of motor development in young children, which has not yet been reported before.

At last, non-verbal IQ testing and short observations of play are very reliable, easy to perform and sensitive tests to detect neurotoxic effects of chemicals with impact on essential cell functions and/or hormones in very young children. And thus, they can be easily used as screening tools for this purpose, especially if new potentially neurotoxic environmental pollutants are emerging (e.g. perfluoro derivates, polybromated compounds, phtalates, cobalt, ...).

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