PO婴儿 AND CHILD COHORT STUDY — DEFINING THE PROBLEM, THE AIM OF THE STUDY AND METHODOLOGICAL ASSUMPTIONS

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Abstract
Objectives: Exposures during prenatal period have implications for pregnancy outcome as well as for children’s health, morbidity and mortality. Prospective cohort study design allows for the identification of exposures that may influence pregnancy outcome and children’s health, verification of such exposures by biomarker measurements and notification of any changes in exposure level. Materials and methods: Polish Mother and Child Cohort Study (REPRO_PL) is multicenter prospective cohort study conducted in 8 different regions of Poland. The final cohort is intended to comprise 1300 mother-child pairs to be recruited within 4-year period (2007–2011). The recruitment and all scheduled visits are conducted in maternity units or clinics in the districts included in the study. The women are followed-up 3 times in pregnancy (once in each trimester) and after delivery for the notification of pregnancy outcome. During each visit, detailed questionnaire and biological samples are collected including saliva, urine, hair, maternal blood and cord blood. About 6 weeks postpartum, breast milk from part of the women is collected. The study concentrates on the identification and evaluation of the effects of prenatal environmental exposure on pregnancy outcome and children’s health. Specific research hypotheses refer to the role of heavy metals, exposure to polycyclic aromatic hydrocarbons (PAHs) and environmental tobacco smoke (ETS) in the aetiology of small-for-gestational-age (SGA) and preterm delivery (PD). The role of oxidative stress putative mechanism and pregnant women nutritional status will be investigated. Based on questionnaire data, the impact of occupational exposures and stressful situations will be evaluated. Results: The results of the study will become available within the next few years and will help to determine levels of child prenatal exposure in several areas of Poland and its impact on course and outcome of pregnancy.

Key words: Birth cohort, Pregnancy, Prenatal exposure, Biological sample
INTRODUCTION

Epidemiological data indicate that disease etiology has to be evaluated with life-course perspective, starting from prenatal period or, if possible, close to the time of conception and followed until birth and later even until adult life. Exposures during prenatal period have implications for pregnancy outcome as well as for children’s health, morbidity and mortality occurring later in life, including asthma, allergy, delayed neurodevelopment, diabetes, cardiovascular diseases, cancer and many other. The assessment of factors influencing birth outcome and children’s health should concentrate on genetic predisposition, environmental exposure and social context.

Birth cohort studies that address prenatal outcomes have been conducted all over the world for at least 20 years. It is important to conduct such studies as new medicines and preventive measures are developed on one hand and new occupational and environmental hazards emerge on the other, which may affect pregnancy outcome. Additionally, new tools for detection of infections, environmental exposures, and the role of genetic factors offer major new research approaches and opportunities to explain the etiology of many reproductive failures.

Prospective cohort study design enables identification of exposures that may influence pregnancy outcome and children’s health, verification of such exposures by biomarker measurements and notification of any changes in exposure levels. In the studies in which exposure status is evaluated from questionnaire data, recall bias can significantly influence the results, especially if evaluated retrospectively. It is also important to note that for some exposures, such as smoking or alcohol consumption, for which detrimental health impacts are well established, the exposure levels may be underestimated.

Several birth cohorts have been established in Europe in the last decades [1–4]. Some of these cohorts have addressed identification of exposures for which the consequences become manifest before or shortly after birth, while other have followed the children until adolescence or even adulthood. For example, one of the biggest cohort study — Norwegian Mother and Child Cohort Study (MoBa) has comprised a cohort of 100 000 pregnant women [1]. In the study the biological samples have been collected from mother, father and child. The children are intended to be followed until the age of 6. The study has covered different factors, such as medication, nutrition, infection and work exposure. Genetic factors and the interaction between genes and the environment has been also studied. About 100 000 pregnant women are examined early in pregnancy in the Danish National Birth Cohort (DNBC) with the long-term follow-up of the offspring [2]. In that study, exposure assessment is done by interviewing the women twice during pregnancy and when their children are six and 18 months old. The biological bank has been set up with blood taken from mother two times in pregnancy and blood from the umbilical cord taken shortly after birth. In the French PELAGIE cohort study, the association between fish and shellfish intake and length of gestation, birthweight and the risk of preterm delivery (PD), low birthweight (LBW) or small-for-gestational-age (SGA) babies is analysed [4].

A special website (www.birthcohorts.net) has been created to facilitate exchange of knowledge and collaboration between researchers on already founded, established and ongoing cohorts and research in the initial phase. The existing birth cohorts are heterogeneous in design and focus but, for specific purposes, data from multiple cohorts could be successfully pooled together. Advantages of this approach include feasibility and low costs.

In Poland, the Kraków centre has continued cohort studies concentrating mostly on the assessment of impact of exposure to air pollution and mercury on birth outcome and children’s health [5,6]. The Polish Mother and Child Cohort is multicentre study on different exposures. The aim of the study is to evaluate the impact of exposure to different environmental factors during pregnancy on pregnancy outcome and children’s health. Specific research hypotheses refer to the role of heavy metals, exposure to polycyclic aromatic hydrocarbons (PAHs) and environmental tobacco smoke (ETS) in the etiology of SGA and PD. It is also intended to explain the role of oxidative stress and nutritional status of the pregnant women. The impact of occupational exposures
and stressful situations will be evaluated from questionnaire data. The results of the study will become available within the next few years and will help to determine levels of child prenatal exposure in several areas of Poland and their impact on course and outcome of pregnancy.

**MATERIALS AND METHODS**

**Study design and population**

Polish Mother and Child Cohort Study (REPRO_PL) is the prospective cohort study conducted in 8 different regions of Poland. The study concentrates on the identification and evaluation of the effects of prenatal environmental exposures on pregnancy outcome and children’s health. For the reliable verification of such exposures, extensive assessments are carried out in pregnant women included in the study. The final cohort is intended to comprise 1300 mother-child pairs to be recruited within 4-year period (2007–2011). The recruitment and all scheduled visits are conducted in maternity units or clinics in the districts participating in the study. The women are followed-up 3 times in pregnancy (once in each trimester) and after delivery for the notification of pregnancy outcome. In future studies it is planned to follow the child till the age of 2 years to determine long term effects of pre- and postnatal environmental exposures.

The study was approved by the Ethical Committee of the Nofer Institute of Occupational Medicine, Łódź, Poland (Decision No. 7/2007). All study participants are informed about the aims and procedures of the study and are asked to sign an informed consent form. Data collected during the study are confidential. All staff involved in the collection, processing, and analysis of study data are aware of the important responsibility to safeguard the rights of study participants. Respondents are assured that all identifying data, such as their name and address, are not available to anyone outside the project team and are not associated with their responses. All answers are used only for research purposes and are not combined with those of other participants. Appropriate technical and organizational measures are implemented to protect personal data against any unlawful form of processing. All biological material stored and used for the work is identified by a code number only. Participants have the right to have their data removed from the cohort at any time and the right not to be subject to more interviews or sampling. So far, only 1 out of about 700 women has asked to be deleted from the cohort.

**Power calculation /Sample size**

We conducted power analysis to calculate the minimum effect size that is likely to be detected in a study using a given sample size. We have calculated power function for assumed sample size (1300 pregnancies). As incidences for LBW and PD are at a similar level (approximately 6% in Poland) we have performed the calculations only once. We have distinguished between dichotomous and continuous exposure variables (Fig. 1). We have used function bpower from Hmisc R package to calculate power for binary exposure [7]. For each combination of OR and exposure prevalence we have constrained fraction of cases to be equal to assumed in population. For continuous variables we have assumed normal distribution of exposure. We have conducted 1000 simulations for each effect size from 1 to 1.8 (with 0.01 step size) to calculate power. Then we have fitted logistic curve to data points from simulations. The power of our study is equal to 80% for risk factors with prevalence 0.05, 0.1, 0.3 when its effect sizes are, respectively, 3, 2.5 and 2. In any case of exposure prevalence we have low power for effect size lower than 1.25.
In case of continuous exposures we have power above 80% threshold for effect size equal to 40%. Non-differential misclassification will reduce some of the power, and this needs to be taken into account. In any case, the size provides new research opportunities for rare exposures.

**Inclusion criteria**
We include into the study women between 8–12 weeks of single pregnancy, not assisted with reproductive technology, and not expected to be finished as spontaneous abortion. All women with the serious chronic diseases specified in study protocol such as diabetes, hypertension, nephropathy, epilepsy and cancer are excluded from the study. The same refers to suspicion of serious child malformations.

**Follow-up of the women**
All women who have agreed to participate in the study are interviewed by obstetrician and/or midwife using detailed questionnaire. During that visit, saliva and blood sample is collected (Fig. 2). The second visit scheduled between 20 and 24 weeks of pregnancy includes all elements from the first visit and, additionally, collection of urine sample. The third examination between 30–34 weeks of pregnancy contains: third questionnaire and collection of saliva, urine, blood and hair samples. At the time of delivery, blood from the mother and cord blood is sampled. After delivery, pregnancy outcome is recorded and about 6 weeks postpartum breast milk is collected from some of the women.

**Questionnaire**
All women participating in the study are interviewed three times during pregnancy. Socio-demographic information, including place of residence, age, marital status and level of education is collected. Detailed information relating to previous and current pregnancies (including the medication and vitamin intake, complications if any, ultrasound measurements) is noted. The midwives also collect the information about active and passive smoking at different stages of pregnancy. Assessment of various occupational exposures is based on detailed questions, updated three times during pregnancy. The questionnaire is concerned with the time and type of work performed, way of performing it and specific exposures, if any. Food frequency questionnaire conducted at 20–24 weeks of pregnancy allows for maternal diet assessment. At the same time additional questionnaires are conducted including questionnaire for subjective perception of work and family environment and Cohen questionnaire.

**Assessment of pregnant women exposure**
All biological samples (saliva, urine, hair, blood, cord blood and breast milk) are collected, processed and stored in each study centres according to specified protocol. The biological samples are transported to the coordinating centre at Nofer Institute of Occupational Medicine, Łódź, Poland where they are analysed. Details of exposure assessment are presented in Table 1.

**The air pollution exposure**
There is a growing body of literature reporting associations between ETS and atmospheric pollutants and reproductive outcomes, in particular birth weight and gestational duration [8–11]. The main limitations of the existing literature relating to the impact of such exposure on pregnancy outcome include exposure misclassification and confounding related to socio-economic status. Even less information is
Geographic Information System (GIS) techniques are used to link the information about place of residence to the data from local stations measuring the air pollution levels. All available data about air quality are analysed (including PM$_{10}$, SO$_2$, NOx, PM$_{2.5}$).

**Assessment of nutrition status, oxidative stress**

Trace elements (Se, Zn, Cu) and bioactive substances, including vitamins A, C, E, carotenoids, polyphenols, flavonoids etc.), are necessary nutrients that should be delivered to the body with diet. The dietary intakes of the microelements vary considerably in various geographical regions due to varied amounts present in the soil and, consequently, in food products (meat, dairy, cereals, fruits and vegetables) and varied eating habits [12]. There is statistically significant correlation between intake of microelements and their blood concentration in healthy humans [13].
Microelements as well as bioactive nutrients are involved in cellular metabolism as an integral part of many enzymes or activators of metabolic pathways. Beneficial health effects of bioactive nutrients are in part attributable to reduction of oxidative stress and improved antioxidant status. Prolonged process of exposure to airborne xenobiotics, even to low environmental levels of these chemicals, may contribute to development of pathological processes [14]. Insufficient antioxidative protection (insufficient dietary supply of antioxidants, depletion of low-molecular antioxidants, damage of important cell molecules — proteins, membrane lipids and nucleic acids — or insufficient repair processes) can intensify oxidative processes indicative of chronic oxidative stress [15,16].

Removal of these reactive oxygen species (ROS) depends on dynamic interaction between a wide spectrum of antioxidative defense components: glutathione, vitamins C, E, carotenoids, antioxidant enzymes: ZnCu-superoxide dismutase, Se-dependent family of glutathione peroxidases, ceruloplasmin, catalase.

In pregnancy, ROS production is intensified, probably due to intensified cell metabolism and increased oxygen demand [17]. Wąsowicz et al. [18] found elevated levels of markers of oxidative stress in pregnant women. Chronic maternal oxidative stress, accompanying insufficient defenses of antioxidants could impair fetal development and growth; thus, antioxidative potential of fetus is affected by the course of pregnancy. Delivery and period directly after birth are periods during which neonate is subjected to considerably intensified attack of ROS produced by rapid exposure to oxygen at atmospheric pressure. Despite insufficient protection which is provided by placenta and maternal organism during pregnancy, the biomarkers of oxidative stress in fetal plasma at birth had a lower level compared to the levels recorded in their mothers at delivery [19,20].

Deficiencies of some microelements (e.g. Se) are of key significance for pregnancy outcome and fetus delivery. Monitoring of maternal oxidant-antioxidant status during pregnancy and at time of delivery may help ensure good pregnancy outcome, fetal growth and development. There are some publications in which authors showed significantly reduced risk of low birth weight in well nourished pregnant women [21]. Some authors implicated the important role of oxidative stress and insufficient antioxidant level/activity in etiology of premature delivery, eclampsia, intrauterine growth retardation, congenital malformations and developmental abnormalities in surviving offspring [22,23].

Imbalance in antioxidant status during the prenatal and early postnatal periods may affect individual’s future life. Oxidative stress has been suggested to be involved in the pathogenesis of several chronic diseases, including atherosclerosis, inflammatory diseases, cancer, and certain neurological disorders [22,24]. There is a lot of evidences that cellular bioavailability of bioactive compounds depends on individual susceptibility, which may be attributable to inherited genetic predispositions related to genetic polymorphisms of many genes [25].

Polymorphisms of genes have been shown to be associated with susceptibility to environmental xenobiotic exposure, oxidative stress generation, etc., but precise role of genetic, and thus enzyme functional polymorphism is still unclear. Enzymes involved in various metabolic pathways remain in gene-gene and gene-nutrient interactions with varying degree of complexity. Therefore, it is important to remember that analysis of only one enzyme coding gene is not sufficient to explain interindividual differences in activation of metabolic pathways, susceptibility to environmental xenobiotic exposure, and in consequence pregnancy outcome, fetal and neonatal development and child’s health.

Concentrations of Se, Zn, and Cu as well as the levels of oxidative stress markers and dietary antioxidants (Vit. A, E) are to be measured prospectively (1st, 2nd and 3rd trimester of pregnancy and cord blood). The level of Mg has been determined in blood collected in 1st and 3rd trimester of pregnancy and cord blood. Conventionally, trace element concentrations as well antioxidative vitamin levels are determined in plasma, antioxidative enzyme activity: GSH-Px, SOD and Cp — in plasma and/or in red blood cells. Plasma Se concentration is analysed using graphite furnace atomic absorption spectrometry (GFAAS), Zn, Cu and Mg — using atomic absorption spectrometry (AAS). Vitamins A and E are measured by HPLC.
enzyme activities are measured by spectrophotometry and marker of prooxidative processes — tiobarbituric acid reactive substances (TBARS) — by spectrofluorometry. Individual genetic susceptibility, expressed as polymorphic variants of genes, is determined in DNA samples isolated from blood lymphocytes. Genotype analysis is performed using real-time polymerase chain reaction (RT-PCR). Oligonucleotide sequences for PCR primers are designed using Molecular Beacon Software (Premier Biosoft International).

**Pregnant women exposure to mercury, lead, cadmium**

Although epidemiological data indicate that there is the association between heavy metals exposure and adverse pregnancy outcome and impaired child neurodevelopment, it is crucial to recognize the levels of the exposures in Polish population and the impact of the exposures at low levels.

In Repro_PL study, hair samples (clipped close to the scalp from the back of head) are collected from pregnant women (30–34 week of pregnancy). Total mercury is analysed using cold vapour atomic absorption spectrophotometry (CVAAS). The blood sample for determinations of lead and cadmium concentrations is obtained in 20–24 week of pregnancy and in cord blood. The concentrations of both metals are determined by GFAAS.

**PCDD/PCDF/PCB**

The high level of dioxins in breast milk is considered to be reflecting a high emission of PCDD/Fs and PCBs [26,27]. As it has been shown in WHO reports since 1987, the corrective measures can largely improve respective situation [28]. Reliable data on human exposure to PCDD/Fs and PCBs in Poland are not accessible. The project is intended to give some information on the content of seven congeners of PCDD, 10 of PCDF and 12 of PCB in breast milk samples.

Human breast milk samples collected during third to eight weeks of lactation were collected into 20 ml EPA washed and certified vials and stored at −20°C until analysis. The pool of aliquots will be analysed according to the USEPA 1613 and 1668 methods with HRGC-HRMS.


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