Study Protocol

In utero exposure to endocrine disrupting chemicals, micro-RNA profiles, and fetal growth: a pilot study protocol

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Significance for public health

This research focuses on the developmental origin of disease with particular emphasis on maternal exposure to endocrine disrupting chemicals during pregnancy and fetal growth by examining microRNA profiles in maternal serum, placenta tissue, and cord blood. Pregnant mothers and offspring are the most vulnerable populations affected by environmental exposures including exposure to pesticides, metals, and contaminants in food. Results from our pilot study will inform a larger project proposal that will look not only at epigenetic modifications and fetal development, but also the epigenetic effects on longer term neurodevelopmental and metabolic outcomes in childhood and early adulthood.

Abstract

Background: The developing fetus is particularly vulnerable to the effects of endocrine disrupting chemicals (EDCs). Molecular fingerprints of EDCs can be identified via microRNA (miRNA) expression profiles and may be etiologically implicated in the developmental origin of disease (DOHaD).

Methods/design: This pilot study includes pregnant women at high risk (smoking at conception), and low risk (non-smoking at conception) for SGA birth (birthweight<10th percentile for gestational age). We have randomly selected 12 mothers (3 high-risk SGA birth, 3 low-risk SGA birth, 3 high-risk non-SGA birth, 3 low-risk non-SGA birth), with EDC measurements from gestational week 17. All offspring are female. We aim to test the stability of our samples (maternal serum, cord blood, placenta tissue), observe the differential expression of miRNA profiles over time (gestational weeks 17, 25, 33, 37, birth), and study the consistency between maternal EDC measures and miRNA expression profiles across our repeated measures.

Expected impact of the study for Public Health: Results from this pilot study will inform the development of a larger cohort wide analysis, and will impact the current state of knowledge in the fields of public health, epigenetics, and the DOHaD.

Introduction

The exposome refers to a cumulative measure of environmental exposures and associated biological responses from the prenatal period onward.[1,2] Cumulative environmental exposures are challenging to measure due in part to the short half-life of many chemicals, as well as transient exposure levels.[3] However, certain environmental exposures such as persistent organic pollutants (POPs) and tobacco smoke leave behind molecular fingerprints in the form of epigenetically modified microRNA (miRNA) expression profiles.[3] miRNAs are involved in many important biological mechanisms including cell differentiation,[4] cell apoptosis,[5] and development of individual tissues and organs;[6] the latter being particularly relevant to *in utero* fetal programming and epigenetic predisposition of disease.[7,8] Increasing evidence suggests that circulating miRNAs can be used as clinical biomarkers for diagnosis. For example, in women with preeclampsia, distinct miRNA expression profiles have been found in maternal serum and placental tissue,[9] whereas environmental exposures such as organochlorine pesticides[10] and tobacco smoke[11] have been associated with altered miRNA expression in cord blood. At the same time, miRNA expression in the placenta of growth restricted fetuses does not always correspond with maternal serum miRNA expression.[12] Taken together, these results suggest that miRNAs found in placenta, maternal serum, and cord blood may be novel responders to *in utero* environmental exposures. However, the biological mechanisms by which *in utero* exposures effect fetal growth indices, impact childhood outcomes, and influence the developmental origin of adult disease remains unclear.[9] An increasing body of evidence suggests that *in utero* exposure to endocrine disrupting chemicals (EDCs) may have consequential effects on the

epigenome of the developing fetus through to childhood.[13,14] Ours is the first study to consider the miRNA expression profiles across five time-points in pregnancy (17, 25, 33, 37 weeks, birth) and across bio-samples (maternal serum, cord blood, placenta tissue).

Materials and Methods

Cohort description

The current pilot study utilizes Norwegian data and bio-samples from the Scandinavian Successive Small for Gestational Age Births Study (the SGA Study) originally funded by the US National Institute of Child Health and Human Development.[15] Participant recruitment occurred over a 27-month period from 1986 to 1988. The SGA Study is a prospective birth cohort of Norwegian and Swedish mother-child pairs with comprehensive data on various maternal factors (*e.g.* tobacco smoking, diet, stress, environmental pollutants) and fetal growth, size at birth, as well as later bio-psycho-social development of offspring. A total of n=1009 Norwegian mother-child pairs are included in the cohort. Of these births, n=194 were term born SGA (cases), and n=815 were term born non-SGA (controls). SGA birth is defined as infants with birth weight below the 10th percentile for gestational age. The follow-up protocol included four comprehensive prenatal visits including collection and storage (-80°C) of maternal serum samples, a detailed neonatal offspring examination, collection and storage (-80°C) of cord blood samples, and complete examination of the placenta. Placenta tissue samples were collected, formalin fixed, and embedded in paraffin for later examination. The current pilot study includes 12 mother-child pairs from the Norwegian arm of the SGA Study.

Participant selection for pilot study

Twelve Norwegian mother-child pairs have been randomly selected within risk strata for SGA birth. Criteria for inclusion were: full range of endocrine disrupting chemicals previously measured in maternal serum at gestational week 17; serum samples available for miRNA analysis from gestational weeks 17, 25, 33, and 37; and cord blood samples available for miRNA analysis. Placental tissue samples from one of the twelve selected mother-child pairs are also included for miRNA analysis. All offspring were female to reduce possible confounding and/or environmental interaction by offspring sex.[13] Participants were further selected for SGA birth and non-SGA birth, as well as high risk for SGA birth (reported current smoking at conception), and low risk for SGA birth (reported non-smoking at conception) (Figure 1).

Placenta sample selection

As per the original SGA Study design, the full placenta was collected at term birth for histopathological examination. Tissue samples from four distinct regions of each placenta were collected from every participant (umbilical cord, peripheral membrane on the fetal side, central tissue, and tissue from the maternal side). An additional tissue sample was collected from any pathological finding. Despite the 30-year history of this birth cohort, the sampling strategy employed at the inception of the SGA study is still in-line with contemporary scientific opinion on the optimal sample collection procedures for placental research,[16] thus speaking to the scientific rigor upon which this birth cohort was built. Previous research showed differential gene expression between intra-individual sampling sites in human placenta at term,[17] as well as systematic differences in gene expression between maternal, fetal and intermediate layers of the human placentas from successful full-term pregnancies.[18] Taking this into account, this pilot study will look at miRNA expression in tissue samples from four distinct reasons of one placenta from one mother as opposed to single tissue samples from single regions for multiple mothers. Given the historical nature of these samples, our first priority is to test the integrity of our placenta samples for further use in a cohort wide analysis, and we believe our proposed intra-individual analysis will provide meaningful site-specific and sample-specific information.

Measuring maternal serum EDCs

Measurement of a comprehensive set of endocrine disrupting chemicals (EDCs) is available for a sub-set of participating mothers from the SGA Study.[13,14] Analyses of maternal serum per- and polyfluoroalkyl substances (PFAS) and organocholoride (OC) concentrations in maternal serum collected during the 2nd trimester (gestational week 17-20) will be used in this pilot study.

Measuring PFAS

PFAS analyses were performed at the Norwegian Institute for Air Research, Tromsø, Norway (NILU). Maternal serum samples were quantified for two target analytes including perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS). Detailed information about the sample preparation, extraction method, analytical method, reagents and instrumentation has been previously reported.[19,20] PFAS concentrations were determined using sonication-facilitated liquid-liquid extraction, activated ENVI-carb clean-up,[21] and analyzed by ultrahigh pressure liquid chromatography triple-quadruple mass-spectrometry (UHPLC-MS/MS). Participation in the AMAP Ring Test ensures that the uncertainties of the analysis are within ± 15-20% of the assigned values.[22]

Measuring OCs

The OC analyses were performed at the Institut National de Santé Publique du Quebec, Centre Toxicologie, Quebec. Several OCs were measured, including hexachlorobenzene (HCB), oxychlordane, poly- chlorinated biphenyl (PCB) 52, 101, 118, 153, 156, 170, and 180, p,p'-dichlorodiphenyldichloroehylene (p,p'- DDE), p,p'-dichlorophenyltrichloroethane (p,p'-DDT), β- hexachlorohexane (β-HCH) and trans-nonachlor (t-NC). In short, 0.5-1 mL serum sample was extracted using hexane (2×6 mL), ethanol (2 mL) and saturated ammonium sulphate solution (2 mL). This method is a slight modification of the one described by Sandanger *et al*.,[23] where

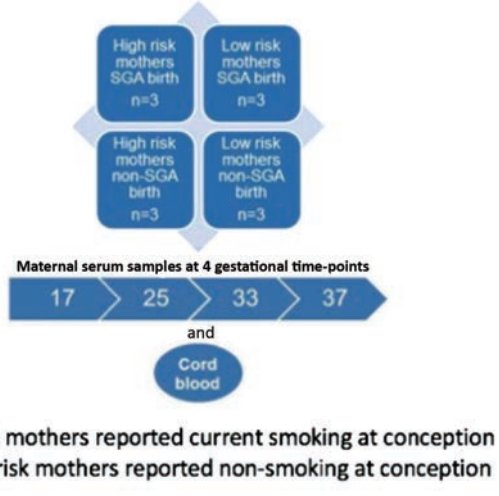

Figure 1. Participants sample selection for pilot study.

the samples were cleaned using 1 g of activated fluorisil on an automated liquid handler system before GC-MS analysis.[24]

Measuring miRNAs

Total RNA isolation

Total RNA will be isolated from 200 µL plasma by using the QIAGEN miRNeasy serum/serum kit, as previously described.[25] Briefly, the kit uses phenol-chloroform extraction to isolate RNA from DNA and proteins. A spin column is then used to separate RNA from organic contaminants and high-quality total RNA is eluted from the column into RNase free water. RNA quality and presence of small RNAs will be assessed by using the Eukaryote total RNA pico assay on the 2100 Bioanalyzer. In contrast to total RNA from cells, ribosomal RNA (rRNA) is depleted in cell-free serum, so sample quality will be determined by detecting the presence of a small RNA peak and depletion of 18S and 28S rRNA peaks in the Bioanalyzer trace.

miRNA sequencing

Total RNA will be used to prepare each sample for sequencing using the NEXTflex small RNA Seq Kit. We will use standard protocols that include spike-in RNAs for normalization and all work will be performed at the Norwegian University of Science and Technology (NTNU) Genomics Core Facility (GCF). This facility has extensive expertise in performing both the capture and next generation sequencing via the lllumina Genome Analyzer II and lllumina HiSeq platforms. One major advantage of using GCF is the use of robotics in the facility. Samples will be barcoded and multiplexed with 24 samples sequenced per lane for miRNA. We will use single-end sequencing and we expect to achieve 20 million reads per sample.

miRNA expression in maternal serum and cord blood

The small RNA sequencing data will be analysed according to a previously described protocol.[25] Briefly, sequence adapters are trimmed from the raw sequencing,[17,26] trimmed reads are collapsed into single unique reads,[27] and collapsed reads along with their total read counts, are aligned by using bowtie2.[28] MicroRNA processing variants (isomiRs) will be detected by using SeqBuster.[29] The calcNormFactors function from the Bioconductor package edgeR will be used to compute sample-specific normalization factors based on reads mapping to the spike-in RNAs.[30] The Bioconductor package limma combined with the voom transformation will be used to create miRNA expression profiles and detect differentially expressed miRNAs and isomiRs.[31,32]

miRNA expression in placenta tissue

The placenta samples to be used in this study are formalin fixed and paraffin embedded (FFPE). Given that miRNA expression profiles from frozen and FFPE samples are highly correlated, we expect the miRNA profiles from these samples to be representative of the original placentas. In our hospital, formalin-fixed and paraffin embedded placenta samples were analyzed with the qPCR technique, and phenotypes of preeclampsia were identified by the miRNA expression.

Overall goal and study aims

Epigenetic regulation of gene expression may be involved in the developmental origins of health and disease (DOHaD).[33] The overall goal of this pilot project is to evaluate the integrity of our samples, optimize our approach and test the feasibility of a larger five-year project. The research aims of the current pilot study are the following ones.

Aim 1

To investigate the intra-individual stability of miRNA expression profiles in maternal serum samples at four gestational time-points (weeks 17, 25, 33 and 37), and in the corresponding cord blood sample at birth (5 samples for each mother-child pair; n=60 samples from 12 mother-child pairs)

Aim 2

To test the integrity of our placenta samples using four samples from four discrete regions of one placenta from one mother.

Aim 3

To obtain an overview of the data accounting for possible differential miRNA expression profiles across sample type (serum and cord blood) and over time (gestational weeks 17, 25, 33, 37, birth) in high- and low-risk mothers, across SGA and non-SGA birth.

Aim 4

To observe maternal serum concentrations of EDCs at gestational week 17 in relation to repeated measures of miRNA.

Statistical analysis

For the purpose of the pilot, we will conduct an exploratory factorial analysis to describe variability among correlated variables with the ultimate aim being variable reduction based on interdependencies between observations. The larger 5-year project will include 1009 mother-child pairs. The statistical approach for the larger project will consider results from this pilot study.

Ethics

All participants provided informed written consent to participate in the SGA study. In 2012, renewed consent for continued use of bio-samples was sought, and research ethics approval for use of these samples was granted until June 2020 (2014/496/REK midt). The current ethics approval includes this pilot project, and the larger 5-year project provided bio-samples are analysed before June 2020, after which time new consent will be needed. The bio-samples for mothers who have not provided renewed consent are not available for continued analysis. After June 2020, any unused bio-samples will be destroyed in accordance with Institutional and National biobanking regulations.

Discussion and Conclusions

Endocrine disrupting chemicals (EDCs) such as synthetic pesticides, metals, or contaminants in food, place the mother and fetus at high risk of environmental injury during the prenatal period. Maternal exposure to EDCs is known to result in abnormal fetal growth patterns, later neurodevelopmental delays in children, and may increase susceptibility to non-communicable diseases later in life.[7,8] Molecular fingerprints from EDCs and resulting epigenetic modifications can be observed via miRNA expression profiles.

The overall goal of this pilot study is to evaluate the integrity of our samples, optimize our approach and test the feasibility of a larger five-year project. In so doing, we will refine our operations including sample selection, laboratory methods, bioinformatics pipeline, and overall organization for the larger cohort-wide analysis. Our sample size for the current pilot study is limited to 12 mother child-pairs with maternal serum samples from four gestational time-points, and corresponding cord blood sample. We recognize that we lack statistical power to provide definitive conclusions on associations between exposure and outcome. However,

our observations will provide some overview of the data including sample integrity, and some measures of reproducibility that would need to be validated in the larger cohort of 1009 mother-child pairs. Moreover, results from this pilot study may provide us an indication of biological significance that may further help us with temporal sample selection for the cohort-wide study. Given our repeated measures (five samples for each pair) and the sophistication of our laboratory methods, the cost of the analysis was also a factor in selecting a sample size of 12 mother-child pairs.

In addition to maternal serum and corresponding cord blood, we also have placenta tissue samples available for analysis. These historic samples are FFPE fixed and have been stored at room temperature for many years. The integrity of these samples, and our ability to extract RNA from these samples is unknown. For each mother we have four tissue samples, each from a distinct region of the placenta. Given the anatomical and functional heterogeneity of this organ,[34] four tissue samples per placenta are required to generate representative data.[16] We therefore anticipate that miRNA expression from different regions of the same placenta will provide us more information than single samples from multiple participants. With careful use of finite biological material, we have an unprecedented opportunity to study the intergenerational impact of epigenetic modifications throughout pregnancy, and across sample types. This pilot study is longitudinal from both intra-individual and inter-generational perspectives. Results from this pilot study will form the basis for a larger 5-year project proposal with a focus on neuro- and metabolic development of offspring in relation to environmental exposures during pregnancy.

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Key words: environmental health, endocrine disrupting chemicals, fetal growth, miRNA.
Acknowledgements: the authors would thank all participants of the Scandinavian Successive Small for Gestational Age Study. The authors also thank the editors and anonymous reviewers for their feedback.
Contributions: TLL and MTS are co-Principal Investigators of the pilot study. GWJ is the PI of the cohort for which this pilot study is based. TLL, PS, GS, CM, and MTS wrote the methods and designed the pilot study. All authors contributed to, read and approved the final manuscript.
Conflict of interest: the authors declare no potential conflict of interest.
Funding: this work is supported by a grant received from the Peder Sather Center for Advanced Study, University of California, Berkeley in collaboration with the Norwegian University of Science and Technology, Trondheim, Norway.
Conflict of interests: the authors declare no conflict of interests.
Received for publication:11 January 2019.
Revision received: 10 May 2019.
Accepted for publication: 10 May 2019.

Journal of Public Health Research 2019;8:1550
doi:10.4081/jphr.2019.1550

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