Title: Protocol – Analysis of Environmental Chemicals in Dried Blood Spots
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Summary of procedures

Two types of procedures will be performed: 1) a finger or heel stick of approximately five drops of blood and 2) the collection of an extra tube of blood on a current blood draw. This study will recruit 100 children, from infancy to age 21, from Children's Memorial Hospital Outpatient Center Laboratories: at the Main Hospital (Halsted and Fullerton) and at the Lincoln Park Outpatient Center (Clark and Deming). The number 100 was selected to generate a comparison to previous study findings, while remaining small enough to be collected quickly and as conveniently as possible. GCSC staff from Northwestern University will recruit and formally consent participants for this study. Families who present for a previously scheduled blood draw will be approached for participation. CMH staff will verify the volumes of blood that have already been drawn from each child to ensure that no monthly blood draw limits are approached. Once consented, a phlebotomist will draw one 10 mL lavender top tube of blood, as well as collect five dried blood spots dropped on a single Whatman 903 Protein Savor Card from each participant. Each participant will fill out a 2-question survey asking for their age and sex. The CMH SSPC lab staff will process and store the specimens. Please see the below detailed protocol describing the specimen processing and laboratory procedures.

Blood Processing

Dried Blood Spots (DBS): Heavy items should not be placed on top of the filter papers before they are used. This will compress the papers and prevent them from absorbing blood evenly. The papers should be transported in plastic containers for protection. After the samples are collected and the blood has dried, compression is not a concern, and papers can be transported in plastic ziplock bags.

Gloves should be used during DBS collection. Clean the participant's finger with 70% isopropyl alcohol. Prick the middle or ring finger just off the center of the tip of the finger with a sterile, single-use micro-lancet. Immediately dispose of the lancet in a sharps container. Wipe away the first drop of blood with a kimwipe or gauze pad. Apply subsequent drops to the filter paper (Whatman #903). It is absolutely vital that blood from the participant's finger be drawn onto the paper via capillary action. DO NOT BLOT the finger on the paper-- the finger should never touch the paper. The paper should be brought up to the finger and touched to the blood, allowing the paper to absorb the blood away from the finger without actually touching it. If this is done correctly, the spot should look identical on both sides of the filter paper once the spot has dried. Collect 5 spots of blood (approximately 60 µL each). DO NOT PLACE BLOOD ON TOP OF BLOOD.

Once the blood has been spotted on the paper, another drop of blood should never be placed on top of it, even if the previous spot is small. After collection, place a bandage on the participant's finger. Allow the filter papers to air dry for at least 4 hours (overnight is fine). Do not stack the samples until they are dry. After drying, close and stack the specimen collection cards and seal them in plastic bags with desiccant prior to transport to the Laboratory for Human Biology Research at the Anthropology Department, Northwestern University.

Venous Blood: Venous blood will be collected in a 10 mL vacutainer containing EDTA as an anticoagulant. The tubes should be gently inverted 8-10 times after blood collection to insure proper mixing. Blood samples can be stored on ice or at 4° C for up to four hours before processing. Blood should not be frozen until processed.

Separation of the red blood cells from the plasma will be performed by centrifugation at 800g for 15 minutes. The top plasma layer will be transferred using a Pasteur pipet into a 15 ml polypropylene centrifuge tube. Caution should be taken to avoid transferring the thin buffy coat layer located between the plasma and the red blood cell fractions.

Red blood cells will be washed with an equal volume of saline solution (0.9% NaCl) by gently inverting the tubes. The top wash layer will be removed, along with the buffy coat layer, using a Pasteur pipet and discard. This process will be repeated three times. After the final wash, the red blood cells will be frozen, and the plasma and red blood cells will be transported to the Laboratory for Human Biology Research at Northwestern University.

All plasma, red blood cells, and DBS specimens will be stored at -80°C.

Laboratory Procedures

Aim 1: Analysis of lead, cadmium, mercury, and arsenic in DBS: Our initial protocol for analysis of toxic metals in DBS will be based on our preliminary pilot work which focused on lead, cadmium, mercury, and arsenic in neonatal dried blood spots. DBS specimens will be analyzed for heavy metals using inductively coupled plasma-mass spectrometry (ICP-MS). DBS will be excised using a ceramic blade on a acid leached Teflon surface, and extracted in 10 ml of extraction solution (9% acetic acid; 1% nitric acid; 0.1% Triton X-100; 200ppb gold to amalgamate mercury; In, Bi and Y as internal standards). An equal volume of blood-free filter paper will be excised adjacent to each DBS sample, and will be used to assess background levels of each metal. All extractions will be performed in 15 ml polypropylene centrifuge tubes that have been acid leached for one week in 1% nitric, 9% acetic acid. All solution and sample volumes will be determined gravimetrically to avoid potential contamination arising from sample transfer and contact with pipet tips. Samples will be briefly vortexed and sonicated for ten minutes followed by 30 minutes incubation at room temperature. Prior to analysis using ICP-MS, extracted samples will be centrifuged for 15 minutes.

Metal concentrations in the blood extracts will be quantified using an X Series 2 ICP-MS (Thermo Fisher Sci.) located in the Northwestern Quantitative Bioelemental Imaging Center. The X Series 2 ICP-MS is capable of detecting most elements in the low ppb concentration range. Metal concentrations will be quantified based on a five-point calibration curve for each analyte. For lead, three isotopes will be scanned and summed (*m/z*: 206, 207, and 208). While for cadmium, mercury, and arsenic, only single isotopes will be scanned with *m/z* of 111, 202, and 75, respectively. In addition to the samples and paired filter paper blanks, five quality control samples will be run along with each batch, which will consist of a matrix blank, calibration standard, quality control standard, lab control standard, and a spiked lab control standard. This is consistent with standard US Environmental Protection Agency practices (EPA ICP-MS methods 200.8 and 6020a).

Additional studies will be performed to expand on our previous work. These studies have two specific sub-aims:

(1) We will consider inclusion of additional elements for analysis in DBS that might be of interest to the National Children's Study. Additional elements will be considered based on their toxicity, the potential for exposures to occur within the study population, and measurement feasibility. Additional elements will be considered based on an extensive literature review. Specimen collection cards will be evaluated for background contamination for elements that meet the above criteria.

(2) We will develop methods to minimize contamination introduced through sample collection. This goal will be addressed using two approaches. First, alternative blood collection media will be evaluated. The specimen collection cards currently being used were developed for screening neonates for metabolic disorders, and are not intended for trace-elemental analysis. Currently, polypropylene filters are being evaluated to determine their potential to replace the specimen collection cards. Second, we will evaluate the potential to assess metal concentrations in DBS using protein-bound metals. In previous studies we have developed protocols for isolating proteins in DBS [1]. Unbound metals in the blood extracts will be removed using micro-spin filters that retain the proteins. Metals bound to the protein will then be liberated using acidic conditions, and analyzed using the ICP-MS procedures outlined above.

Aim 2: Assessment of Oxidative Stress in DBS: Protein carbonylation will be measured in DBS specimens as biomarkers of oxidative stress associated with exposure to environmental toxicants. Extracted proteins will be assayed for total carbonyl content using ELISA protocols that have been previously developed for venous blood assays (Cell Biolabs, Inc.). The Laboratory for Human Biology Research at Northwestern has extensive experience and expertise in developing DBS ELISA assays based on methods that have been developed for serum [2 3 4 5]. A DBS blood extraction procedure will be

optimized using a variety of extraction and incubation conditions. Extracted DBS, and reduced/oxidized BSA standards, will be diluted to10 µg/ml in 1X PBS. One hundred µl of each will be transferred to a 96-well protein binding plate. The plate will be incubated at 37°C for a minimum of 2 hours (incubation time will be optimized). Following incubation, wells will be aspirated and washed three times with 250 µl 1X PBS. One hundred µl of dinitrophenylhydrazine working solution will be added to each well and incubated for 45 minutes at room temperature in the dark. Wells will be washed with 250 µl of 1X PBS/ethanol (1:1, v/v) on an orbital shaker for 5 minutes, repeated a total of five times. A final two washes with 250 µl of 1X PBS will be made prior to the addition of 200 µl of blocking solution. The blocking solution will be incubated for 1-2 hours on an orbital shaker. The wells will be washed following incubation, and 100 µl of diluted anti-DNP antibody will be added to each well, incubated for one hour on an orbital shaker. The wells will be washed 3 times as described above, and 100 µl of diluted secondary antibody-HRP conjugate will be added to each well and incubated for 1 hour at room temperature on an orbital shaker. The substrate solution will be warmed to room temperature and added to each well in 100 µl aliquots, and incubated at room temperature for 5-20 minutes on an orbital shaker. The reaction will be stopped once optimal color has developed using 100 µl of stop solution, and the plate will be read on a microplate reader using 450 nm as the primary wavelength.

The DBS assays will be validated for sensitivity, accuracy, precision, and reliability. Paired venous blood and DBS specimens will be used to compare the conventional, gold-standard, venous blood collection methods to our new DBS assays using specimens collected from consented participants (described above).

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