Overcoming Obstacles in Epigenetic Analysis of Human Twins: The Baylor Infant Twin Study

Date: June 1, 2015

PROTOCOL

Overall design. We will use the existing infrastructure of the Baylor Infant twins Study (BITS). BITS initiates contact with twins in the postpartum recovery room and at well-child check-ups up to the age of 4 months. Samples for the current proposal will be collected in the postpartum recovery room, or at the first BITS visit, depending on mode of recruitment. The current proposal adds the parent study 1) additional biological sample collection, and 2) permission to review medical records for chorionicity during the contact. For the current proposal, we will recruit 40 monochorionic MZ twin pairs and 40 dichorionic MZ twin pairs to the study either prenatally, at birth, or at the 2-week well-child check-up. From all of these twins we will collect buccal swabs, urine, and nail samples to be used for DNA isolation and monochorionic/dichorionic comparisons in Aim 2. From a subset (at least 23 individuals, monochorionic or dichorionic) we will also collect hair follicles from both twins to be used for the tissue comparisons in Aim 1.

Participants.

Cohort description: A cohort of 40 twin pairs will be recruited. MEs are established early on in embryonic development and are not altered after gastrulation. Therefore we do not have exclusion criteria based on mode of delivery, gestational age or birth weight. *Inclusion criteria*: Infants from a twin birth less than 4 months of age, with available medical records from pregnancy, from a predominantly English- or Spanish- speaking family. *Exclusion criteria*: Infants from a pregnancy with twin-to-twin transfusion syndrome. Infants with major congenital anomalies (assessed by medical records and parent-report at the time of recruitment). Parents with inadequate English or Spanish to understand the study protocol and give informed consent. Infants from mothers < 18 years of age (mothers must be at least 18 to give consent for the infants' participation in Texas).

Recruitment. We will recruit twins through one of 6 routes:

(1) Medical records for women seeking prenatal care at TCH will be searched. For twin pregnancies meeting the criteria above, women will be sent an informational package to their home address giving details on the study, at approximately 30 weeks gestation. This will be followed by a single follow-up phone call from the study coordinator inviting their participation. Their name and address will be recorded. Participants will also be sent a stamped postcard to return after the twins' birth. If we have not heard from them by their due date, a follow-up 'phone call will occur.
(2) Birth records from TCH will be examined daily. Where two birth codes are recorded to the same mother, on the day of discharge, the study coordinator will visit the woman and invite her to participate in the study.
(3) Advertisements inviting study participation will be posted on 'Bellaire Moms of Multiples' support group pages. Brief study details will be given and interested women invited to contact the study coordinator. Initial consent will be given over the phone. The first study visit will be scheduled and written consent obtained.
(4) Lactation consultants at TCH will be asked to pass out study flyers to mothers of twins under 3 weeks. Interested women will be invited to contact the study coordinator. Initial consent will be given over the phone. The first study visit will be scheduled and written consent obtained.
(5) TCP pediatricians will be asked to hand out flyers to mothers of multiples at well child checks for children between the ages of 2 weeks to 3 months. The flyer will invite interested women to contact the study coordinator. In addition, it will invite interested parties to provide their contact information in a box at the reception. A follow-up phone call will occur. Initial consent will be given over the phone. The first study visit will be scheduled and written consent obtained then.

Procedure.

Initial consent is either in-person, or on the phone. At recruitment, study exclusion criteria will be screened for by the research coordinator, Dr. Stuff. At initial recruitment, Dr. Stuff will also obtain permission to access pregnancy medical records, which will be recorded in writing at the first study visit.
For recruitment via deliveries at TCH, upon receiving consent on the day of discharge, the study coordinator will return to the room and collect buccal swab, urine, nail, and (from a subset) hair follicle samples (termed "the first study visit" for this proposal).
For participants recruited prenatally or at well-child checkups a visit will be scheduled at the Children's Nutrition Research Center (CNRC) metabolic research unit (MRU) when the infants are ~4 months of age (this coincides with the initial BITS visit).
For all participants, informed consent forms will be offered in both English and Spanish due to the high volume of native Spanish speakers in Houston. It is prohibitive to offer consent in other languages. Informed consent will cover accessing medical records, and both the current epigenetic analysis, as well as a separate consent section covering the deposition of data from potential future genomic and epigenomic analyses in public databases such as dbGaP. Participants will receive $50 compensation per twin pair, as well as commemorative photographs of their children.

All participant will be enrolled into the parent study (BITS) and so be contacted for follow-up visits at 4 months (if the first visit was postnatal), 1 and 2 years (by questionnaire and use of medical records) and 3 years (in person follow-up at the CNRC).

Measures.

Assessment of Chorionicity. At the referring centers for this study, chorionicity is routinely determined by ultrasound by experienced sonographers at 11-18 weeks gestation, as standard medical practice for multiple gestations. Chorionicity is recorded in the medical record and will be retrieved by our study coordinator.

Samples for DNA methylation analysis.

Buccal swab: A buccal swab is collected using an Isohelix's SK-2 kit. A research assistant swabs the child's mouth for 30 seconds using firm pressure. The swab is placed in a sterile tube, which contains 500 ul Lysis buffer. 20 uL of proteinase K is added, and the tube is capped. The tube is immediately frozen and stored at -80 C until DNA isolation by our standard method.
Hair follicles: For this procedure, as adapted from our previous studies,[1] the mother is offered the choice of a lidocaine numbing spray or the application of ethyl chloride spray to numb a small area of the scalp. The chosen local anesthetic is applied by the research coordinator, who then plucks hairs from this area using sterile tweezers. The hairs are placed in a sterile, clear tube labelled with the de-identified study ID. Each hair is inspected, and when 10 hairs are collected with a visible follicle, the procedure is complete. Our previous studies suggest this requires the removal of 10-15 hairs. When possible, this is conducted while the infant is sleeping. Hair follicles are stored at -80 C until DNA isolation by our standard method.
Urine: Upon arrival at the MRU, or in the mother's birth room, a 200 ml McKesson Polyproplene pediatric urine collection bag, with adhesive closure, is placed over the genitals of each infant and remains in place until sample collection is complete. From our experience, in birth rooms, the research coordinator often has to return within 1 hour to collect the bag. Urine is immediately combined with urine conditioning buffer, after which it is stable for up to 6 days until DNA isolation (Extract-ALL Urine DNA Kit, Zyme Research).
Peripheral Blood Cells (PBCs): A small heel prick is administered by the study coordinator. A 903 Proteinsaver Snap-Apart card (GE Healthcare Life Sciences) is used to collect a single sample of blood. Each well holds 75-80 µl of sample; we anticipate needing <50 µl. A new card will be used for each participant. The QiaAmp DNA Blood Mini Kit (Qiagen) will be used to isolate DNA from blood.
Nail clippings: A standard safety clipper is used to clip the infants' fingernails (preferentially) or toenails (if necessary). As a service to our parents, all fingernails (or toenails) are clipped as appropriate – we do not stop when a suitable quantity of sample has been collected. The clippings are stored in a sterile tube -80 C, then washed several times in EDTA before DNA isolation.

Analysis of DNA methylation by bisulfite-pyrosequencing. Genomic DNA is bisulfite-modified (EZ DNA Methylation-Direct kit, Zymo Research), amplified by one-round of PCR using locus-specific primers, and sequenced on a Qiagen MD Pyrosequencer, by our standard protocol.[1,2] Prior to use, we validate accuracy and sensitivity of all our pyrosequencing assays using human genomic methylation standards.[3] We will focus our analyses on at least ten validated human ME loci[2,4] that are proximal to genes of interest, including for

example, *PAX8*, *DUSP22*, *VTRNA2-1*, etc. Final selection of the ME panel will be based on ongoing ME-discovery in the Waterland laboratory and review of the current CVD literature.

Statistical Plan

Aim 1: Validate nail clippings as acceptable for the study for human MEs.
In a subset of the infants recruited for this study, in which both hair follicle and nail DNA is obtained from infants (n=23), we will study the correlation between ME methylation in nail and hair follicle DNA. At each ME, % methylation values for multiple CpG sites will be averaged to yield a mean % methylation value for each sample. The correlation between % methylation at 10 MEs in nail and hair follicle DNA will be assessed using Pearson correlations. At ME loci with non-normal methylation distributions, data will be transformed to improve normality prior to correlation analysis.

Aim 2: Compare epigenetic discordance at MEs in monochorionic vs. dichorionic MZ twins.

Analysis: At each ME, we will compare the intra-class correlation between monochorionic and dichorionic twin pairs. Given the small sample size, we will likely report results which compare the intra-class correlation for % methylation averaged over 10 CpG sites. We will compare the results for the tissue types: fingernail /urine -, buccal swab and PBC.

REFERENCES

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