Cohort profile

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Cohort Profile: The Chongqing Longitudinal Twin Study (LoTiS)

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Key Features

- We aim to define the relative contribution of genetic factors and the environment to early ~~onset illness of infancy~~health from ~~and to birth~~identify and to identify specific biomarkers and critical time points from pregnancy onwards for emergence of ~~differences in illness in infancy~~childhood-associated phenotypes ~~by~~ through the establishment of a longitudinal twin pregnancy birth cohort.
- The LoTiS cohort was established in January 2016 in~~at~~ Chongqing, China. ~~with aim of~~The aim was to enroll~~ing~~ 300 ~~twin pregnant~~ women, aged 20-40 years, pregnant with twins ~~aged 20-40 years~~ and their offspring. ~~The women~~Participants were ~~followed up~~assessed in early, mid, late pregnancy and at delivery, ~~and their~~with offspring ~~were followed up~~measures at birth, 6 weeks and 3, 6, 12, 18, 24, 30, 36 months of age. Currently, there ~~were~~ are 439 participants recruited ~~and~~ of which 333 have completed ~~the visits during~~ pregnancy assessments.
- Maternal social demography, laboratory ~~examination~~data, perinatal outcomes, neonatal outcomes, and a range of ~~infantile~~ offspring measures, including ~~growth data and assessment~~anthropometry ~~were~~ are being collected.
- The ~~The study was collaborated with~~LoTiS was established in collaboration with the Murdoch Children's Research Institute, ~~Royal Children's Hospital~~, Australia and Fetal Medicine Centre, Birmingham Women's & Children's Foundation Trust, UK. Data ~~was assessed~~are stored and accessible at ~~at the Website:~~ https://www.medscinet.com/Lotis/empty.aspx.

Why was the cohort set up?

The Fetal Programing Hypothesis/Fetal Origins of Adult Disease Hypothesis (FOAD), first proposed in the 1980's,[1] stated that a suboptimal intrauterine environment has potential to negatively impact fetal developmental in a manner that ultimately results in disease in

adulthood. This hypothesis covered many different tissue and organ systems and was particularly relevant to metabolic and cardiovascular conditions in adulthood. After considerable supporting evidence arising from both direct animal experimentation and also observational human studies, the FOAD theory gradually evolved to that of "The developmental origins of health and diseases" (DOHaD), still widely used today.[2]

Current knowledge around the molecular processes playing roles in DOHaD-related phenomena strongly implicates the interaction of a range of both genetic and environmental influences over time. Such complex interactions between genes and environment are particularly prevalent in outbred and diverse human populations, making exploration of NCD ~~an~~ etiology challenging, often requiring very large cohorts ~~of,~~ commencing early in life, with detailed exposure and outcome data, plus ~~multiple~~ a range of biospecimens.

Twins show the same rates of complex disease in adulthood as singletons and have proved an invaluable tool for investigating disease origins.[3] They provide the additional advantage of facilitating control for variation in genetics, birth mother, age and shared upbringing. Clinically and therefore prospectively, it is only possible to classify twin pregnancies according to their ultrasound features of di- or monochorionicity (double or single placenta) and number of amniotic sacs (double or single). All monochorionic twins are monozygotic (MZ); whilst 80% of dichorionic twins are dizygotic (DZ) and 20% ~~are~~ MZ. ~~Dizygotic (~~DZ~~)~~ twins, that arise from two separate fertilization events, share half their genetic variation on average, while ~~monozygotic (~~MZ~~)~~ twins, which result from the splitting of a single embryo, are generally considered genetically identical. MZ and DZ twins provide a natural study design for determining the contribution of nature (heredity) and nurture (environment) to the variation of complex phenotypes including ~~illnesses in infancy~~a range of early life adverse health outcomes. Classic twin studies enable partitioning of phenotypic variance within a population into additive genetic (also known as heritability), shared environmental and non-shared environmental effects. By comparing the similarity of DZ twin pairs as a group

relative to MZ twins, it is thus possible to gain insights into the role of genetic factors in specifying individual phenotypes. This approach has allowed estimates of heritability for a variety of human diseases.[4-8] Although highlighting the role of genetic variation in contributing to disease, the low rates of heritability for many disorders also emphasizes the importance of environmental factors in disease risk.[9, 10]

In twin pregnancies, shared environment can be viewed as maternal *in-utero* factors (such as maternal diet, smoking, *in vitro* fertilization), and non-shared factors specific to each baby, such as placental volume/area or umbilical cord insertion site. By controlling for genetic variation and shared environment, MZ twin studies have the unique advantage of providing insights into the importance of such risk factors unique to the individual; for example, birth weight discordance within MZ twins is more likely to reflect differences in placental function or blood and nutrient supply to the individual fetus rather than shared maternal factors.

Although generally higher than that of DZ twins, the phenotypic concordance (including age of onset) for most human conditions in MZ twins is rarely 100%.[11-14] These individuals within a pair therefore provide an incredibly valuable model for understanding the mechanistic link between environmental exposures and complex phenotypic outcomes, including those in early life. Despite this, longitudinal twin studies commencing in pregnancy are relatively rare[15, 16] and as such the generalizability of findings in these valuable studies, particularly across different geographic and ethnic groups, remains unclear.

The Chongqing Longitudinal Twin Study (LoTiS), recruited in pregnancy, was designed to facilitate an unparalleled investigation of the link between genetic risk, early life environment, potential mediating mechanisms, and early life phenotypes. Further, it aimed to identify potentially predictive biomarkers present in childhood associated with later development of a range of adverse outcomes of neurodevelopmental, cardiovascular and/or metabolic origin.

Non-invasive risk phenotypes, reproducibly and readily quantified in children, often track from mid-childhood into adulthood, and therefore act as intermediate phenotypes toward adult disease.

The overarching aims of Chongqing LoTiS cohort study are to; (i) more fully understand ~~more about~~ the health challenges of twin pregnancies in a Chinese ethnic population, (ii) explore the relative roles of genetic and environmental factors to a range of early life outcomes and, (ii) identify specific within-pair differences in outcome in response to shared and non-shared environmental exposures. The longer-term goal is to define potentially modifiable environmental determinants of early life phenotypes and critical time points for intervention. Specific aims are:

(1) To establish a cohort of 600 twins (300 twin pairs) and their parents, recruited early in pregnancy, with longitudinal assessment of environmental and phenotypic measures and collection of a detailed panel of biospecimens.

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(2) By examining dizygotic and monozygotic twins in combination, to define the relative contribution of genetic factors and the environment (the exposome) to early life health measures, such as allergic conditions (eczema and food allergy) and to identify biomarkers of later onset phenotypes of neurodevelopmental, cardiovascular and metabolic health.

(3) By examining phenotypically discordant monozygotic twin pairs, to identify specific biomarkers and cellular pathways (epigenetic, metabolomic, microbial etc), and critical timing~~,~~ from pregnancy onwards, of differences in early life phenotypes, largely independent of underlying genetic variation. This will inform future interventional strategies by defining key periods in early life amenable to intervention in addition to biomarkers to monitor any changes to risk trajectory.

Who is in the cohort?

The LoTiS cohort study recruited twin pregnant women from multiple pregnancy clinics at the two major public hospitals in Chongqing, namely the First Affiliated Hospital of Chongqing Medical University and the Chongqing Health Centre for Women and Children, between January 2016 and September 2018. Following ethics approval provided by the Medical Ethics Committee of The First Affiliated Hospital of Chongqing Medical University (No. 201530), women aged 20-40 years and diagnosed in the first trimester as a twin pregnancy by ultrasound were invited to participate. Women pregnant with twins who agreed to donate biological samples including, maternal peripheral blood, urine, hair, and buccal mucosa, in addition to cord blood and placenta at birth, were included. The maximum gestational age at recruitment was 16 weeks. Informed written consent was obtained from each participant.

Our aim was to collect data and samples from 300 mothers and their twin offspring. Despite MZ twins making up only ~1/3 of all twin pregnancies, we aimed to recruit approximately even numbers of MZ and DZ pregnancies. Thus, the cohort was enriched for women most likely to be carrying MZ pairs, namely those identified as monochorionic (MC) by ultrasound scan in first trimester (11-14 gestational weeks). Zygosity of dichorionic (DC) twin pregnancies was ascertained after delivery. In total, 439 twin pregnant women were recruited at first follow-up. The first twin babies were born on 9 April 2016 and the last twin babies were born on 25 January 2019. After excluded 16 abortions, 11 deaths of one of twin fetuses, 10 preterm deliveries before the third visit and 69 participants lost to follow up, we achieved our target of 300 with 143 MC and 190 DC twin pregnancies. The participants ~~included ther~~remaining at the delivery visit ~~remains~~ showed similar characteristics compared to participants enrolled at first ~~follow up~~assessment (Supplementary Table 1). After zygosity testing, the final sample were 173 pairs of MZ and 160 pairs of DZ twins. Figure 1 shows the participant flow of the LoTiS cohort.

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How often have they been followed up?

In accordance with international and Chinese guidelines for the management of twin pregnancies[17], pregnant women underwent examination every two or three weeks to assess fetal growth. Four detailed interviews were conducted in the clinic: at recruitment (11^{+0}-15^{+6} weeks of gestation), in the second trimester (22^{+0}-25^{+6} weeks of gestation), in the third trimester (30^{+0}-33^{+6} weeks of gestation) and at delivery. Biological samples were collected at each time point. During infancy, the twin offspring were reviewed at 6 weeks, 3 months, and 6 months of age, and then and 6-monthly until 36 months of age. Specifically, the follow up at 6 weeks, 3 months, 6 months and 12 months were based on corrected age, (calculated as the chronological age (weeks) minus the difference between 40 weeks and chronological gestational weeks at delivery).

What has been measured?

Detailed data and biological sample collection and processing are shown in Table 1 and Table 2.

(1) Maternal questionnaire, examination and biospecimens

At the recruitment visit, a questionnaire was administered to the participants to obtain parental demographic and socio-economic data, and details regarding parental lifestyle, obstetric history and current pregnancy parameters including mode of conception and chorionicity, medical and family history data. Chorionicity was identified by ultrasound scan in first trimester and confirmed by placenta examination at delivery.

Maternal psychological stress in the first and third trimesters was assessed using the 36-item Pregnancy Stress Rating Scale (PSRS36) revised by Chen et al. in 2015,[18] which was originally constructed in Chinese and has been validated for Chinese women.[19, 20] Maternal dietary intake was recorded by a food frequency questionnaire (FFQ) and 24-hour diet recall in the face-to-

face interview at 23-26 weeks of gestation. The FFQ was originated from the GUSTO study[21] and has been validated in a study on food intake and metabolism in our laboratory.[22]

Routine antenatal clinical and laboratory data were abstracted from the hospital case notes, including measurements of body weight, blood pressure and abdominal circumference. Hematological, biochemical and microbial results were obtained following standard antenatal blood and urine tests, and tests of oral glucose tolerance (OGTT), and liver, renal and thyroid function. Pregnancy outcomes including maternal complications (gestational diabetes, gestational hypertension disorder) and perinatal outcomes (such as preterm delivery, postpartum hemorrhage) were derived from the hospital medical records.

Maternal biological samples were collected in first, second and third trimester, including maternal peripheral blood, urine, hair and buccal swab. Whole blood samples were centrifuged to purify serum, plasma, red and white blood cell fractions before cryopreservation at -80℃. The biological samples were collected for direct measurement of a range of maternal exposures and biomolecules (eg. gene and protein expression, epigenetic variation). Recently, the concept of the "exposome" (the totality of an individual's exposures) has emerged.[23-25] and our team have pioneered the use of metabolomic approaches to directly measure biological intermediates of a range of exposures and associated outcomes.[26-28] Metabolomics is the global analysis of the complete set of low molecular weight metabolites that is the result of our body's metabolic function. It is a powerful strategy for investigating low molecular weight biochemicals present in a cell, tissue or biofluid and their associations with clinical outcomes.

(2) Fetal biometry and biospecimens

The assessment of fetal intrauterine growth was mainly through ultrasound examination every two or three weeks in twin pregnancies. The indices including biparietal diameter, femur length, head circumference, abdominal circumference, amniotic fluid deep and estimated fetal weight.

Neonatal outcomes, including gestational age, birthweight, birth length, complications and NICU admission, were deriving from hospital medical records.

Cord blood and placenta tissues were collected at delivery according to the optimising sample collection for placental research[29]. These biological samples were collected in order to assess the epigenome, transcriptome, proteome and metabolome in order to potentially identify early life biomarkers to predict illness in infancy and childhood. Zygosity was identified by short tandem repeat-PCR (STR-PCR) using DNA from cord blood (conducted by Chongqing forensic examination, China)

(3) Infant follow-up to 36 months of age

Infants' visits were carried out in two centers: the First Affiliated Hospital of Chongqing Medical University and the Chongqing Health Centre for Women and Children. Anthropometric assessments of child growth trajectories are conducted in the Child Health Care center at each visit. Health status, drug use, feeding patterns of dairy products (formula feeding, breastfeeding and mixed feeding) including volume and duration were also recorded via interviews. As only one sonographer was available for this study, cardiovascular assessments were performed only for infants assessed at the First Affiliated Hospital of Chongqing Medical University. Aortic intima media thickness (aIMT) and aortic diameter (AoD) of offspring were measured at the corrected age of 6 weeks in 172 pairs of twins; aIMT can be reliably measured from fetal life onward via transabdominal ultrasound,[30-32] and used to quantify early changes related with atherosclerosis.[33, 34] Since the rising rate of infants lost to follow-up, skin prick testing was carried out in 244 pairs of twins to assess food allergy at the corrected age of 6 months and 200 pairs of twin offspring were administered the Bayley scales of Infant Development-Chinese Revision at 18 months of age. Body composition of children was assessed by bioimpedance spectroscopy device at 12, 24, 36 months of age; the measured

parameters included the percentage of total body water, extracellular fluid, intracellular fluid, fat mass and fat-free mass.

Serial questionnaires were used to assess infant development. The Brief Infant Sleep Questionnaire (BISQ) was conducted at 6 months of age to assess sleep duration and quality, and 186 pairs of twins completed this investigation. Infant nutritional status questionnaire was conducted at 12 months of age to assess eating behavior, food intake and diet problem; a total of 172 pairs of twins completed this investigation. Carey's toddler temperament questionnaire (TTQ) was conducted at 24 months of age; 166 pairs of twins completed this investigation. The Child behaviour checklist was conducted at 30 months of age, and this investigation is in progress. A detailed history of the twin offspring's health, and nutrition was obtained through a caregiver interview at 36 months of age.

What has it found?

Table 3 provides data about maternal and twin pair-specific factors of the 333 women who completed all the follow-up assessments during pregnancy. In brief, the median maternal age at recruitment was 29 years, and the median pre-pregnancy BMI was 21.2. 93.7% of the participants were Chinese Han, 73.6% had an education level of university and above, 73.9% were in employment, 74.2% were primiparous, 61.3% conceived naturally and 98.2% underwent cesarean section. The median gestational age at birth was 36.8 weeks and over one-fifth of the twins were born preterm. Zygosity was representative of twins, nearly half of the pairs were monozygotic, higher than the average. Birthweight discordance was another important representative of twins, with a maximum birthweight discordance of 47.2%. With reference to birthweight percentiles for twin neonates in China,[35] 13.5% neonates were small-for-gestational age (SGA) and 2.9% were large-for-gestational age (LGA).

Maternal stress

Using data from 215 pregnant women who completed the pregnancy stress rating scale in both early and late pregnancy, we found that the predominant prenatal stressor in both early and late pregnancy was concern about the pregnancy and childbirth safety; higher stress in late pregnancy was associated with a higher risk of premature rupture of membranes (OR 1.02, 95% CI 1.01-1.03).[36]

Gestational weight gain

Gestational weight gain (GWG) during the second trimester may be the determinant of total GWG and profoundly impact the perinatal outcomes since weight gain was minimal in the first trimester, then accelerated in the second trimester and decelerated in late pregnancy. Using data on 177 mothers with dichorionic twin pregnancies, gestational weight gain during 12 to 20 gestational weeks was found to be negatively associated with higher risk of SGA (defined as neonatal birth weight was below the 10th percentile for gestational age and sex) at least of one in a twin pair (OR 0.76, 95% CI 0.59-0.99).[37]

Gestational diabetes

The incidence of GDM (29.7% or 99 pregnancies) was consistent with GDM being higher among women with twin pregnancies due to increased insulin resistance.[38] Accumulating evidence indicates that maternal dietary intake is involved in the development of GDM in singleton pregnancy[39], while the evidence very limited in twin pregnancies. Using dietary data collected by a 93-item food frequency questionnaire in the second trimester from 324 mothers, we identified four distinct dietary patterns, however, no dietary patterns correlated with later GDM risk [40]. This may have been due to the high incidence of GDM in our cohort, such that environmental exposure factors had limited influence on the blood glucose levels.[40] Previous studies have reported conflicting results regarding the influence of GDM on perinatal outcomes in twin pregnancies. We therefore studied the impact of GDM on perinatal outcomes as well as

offspring growth profile. We found that GDM did not correlate with adverse perinatal outcomes and only positively associated with a higher risk of childhood overweight at 6 months of age (OR 1.86, 95% CI 1.19-2.90).[41]

Childhood overweight

Recently, the prevalence of childhood overweight and obesity has dramatically increased, and a growing body of evidence has shown that there are few effective treatments for obesity in childhood. We calculated z-scores for BMI-for-age Z-score (BAZ) based on the World Health Organization Child Growth Standards (https://www.who.int/childgrowth/software/en/). Overweight was defined as BAZ above the 85th percentiles.[42] In our cohort, 24.7%, 13.4%, 9.5% and 10.5% of infants were overweight at 6, 12, 18 and 24 months of age, respectively. Through comparisons between monozygotic and dizygotic twins, we calculated the heritability of being overweight at 6, 12, 18 and 24 months of age using Holzinger formula, a simple calculation formula of heritability proposed by Holzinger in 1929. As shown in Table 4, the results demonstrated that the heritability of being overweight increases with age, which is consistent with previous reports that the greatest variation in rates of weight gain is seen in the first 2 years of life, potentially as a compensatory mechanism for intrauterine restraint or enhancement of fetal growth. After two years of age. growth appears to be more influenced by genetic factors. [43] Nevertheless, it is also clear that environmental factors are important in early life. A total of 12 potential risk factors in early life (including maternal age, pre-pregnancy BMI, maternal education (≤high school or ≥ Bachelor degree), occupation (unemployed, employed), mode of conception (naturally conceived, ART), GDM (no or yes), GHD (no or yes), gestational age, birthweight, born with VLBW (no or yes), born to IUGR (no or yes), catch-up growth (no or yes)) were investigated to study the relationship with childhood overweight and obesity at 24 months of age. The results further confirmed that catch-up growth (defined as BMI gain >0.67 z-score from birth to 24 months of age) correlated with an increased risk of

development of overweight and obesity in childhood by using the twins' model (*manuscript submitted*).

Childhood neuro-development

Associations between adverse intrauterine environment and poor neuro-development in childhood have been reported by numerous reports. We measured the cognitive function of the twin offspring at 18 months of age via Bayley scales of Infant Development-Chinese Revision and examined the association between low birthweight or fetal growth restriction and cognitive function. Our findings suggest that IUGR affected the mental development index (MDI), rather than psychomotor development index (PDI) (manuscript submitted).

Omics studies

Selective intrauterine growth restriction (sIUGR), is one of the most serious complications of MC twins with co-twins estimated fetal weight (EFW) or birthweight discordance greater than 20%. sIUGR is a typical intrauterine phenotypic discordance in MZ twins, and an ideal model to explore the impact of intrauterine growth restriction on fetal health and long-term growth development after birth. Therefore, we conducted a study of metabolic biomarkers of sIUGR in cord plasma and placenta using samples collected from 15 pairs of sIUGR, 24 pairs of uncomplicated MC twins and 14 singletons diagnosed with intrauterine growth restriction (IUGR). The cord blood metabolome demonstrated better separation of sIUGR and normal twins compared to placenta tissues via the partial least squares discriminant analysis (PLS-DA). Disrupted fatty acid and amino acid metabolism, as well as exposure to high level of environmental xenobiotics were significantly associated with the smaller co-twin of sIUGR, supporting the theory that intrauterine and extrauterine environmental factors, rather than by genetics, play a major role in fetal growth discordance in twin pregnancies.[44]

What are the main strengths and weaknesses?

This longitudinal prospective study recruited twin pregnant women from early pregnancy and until approximately three years after birth, which covers "the first 1000 days in early life". We have collected detailed data on mothers and infants prospectively. A strength of this study is the depth of sampling both in terms of data and samples collected, and the frequency of assessment. Biological samples were collected and stored from early pregnancy, enabling the direct measurement of maternal exposures and their association with pregnancy and offspring outcomes. Additionally, to our best of knowledge, this study was the first to assessed the aIMT and skin prick testing among twin offspring recruited prior to birth.

Women pregnancy with twins were recruited from two clinical centers in Chongqing, a city located in Southwest China and are thus an urbanised population. The overall sample size of 300 twin pregnancies is moderate for a prospective cohort in which serial biospecimens are taken repeatedly, and the sample size is relatively small compared to larger twin cohort or registry studies that collect primarily data with limited biological sampling. The high rate of twin pregnancies loss and the high rate of 'lost to follow-up' resulted in significant challenges in obtaining complete data and biospecimens from each participant.

Can I get hold of the data? Where can I find out more?

The Chongqing Longitudinal Twin Study welcomes collaboration with investigators interested in this research field. Interested investigators should contact the lead principal investigator Prof. Qi Hongbo (qihongbo728@163.com) and/or Assoc Prof. Tong Chao (chaotongcqmu@163.com). LoTiS has an online database that details the information about the participants and their offspring (https://www.medscinet.com/Lotis/empty.aspx).

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Conflict of interest

None declared.

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