Title: The effect of in utero hyperglycaemia, maternal overnutrition and interaction with postnatal lifestyle on cardiometabolic risk at young adulthood – extension of HAPO follow-up study

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The prevalence of diabetes mellitus (DM) and cardiovascular disease (CVD) escalate remarkably worldwide and obesity becomes an epidemic disease. The main contributing factors are undoubtedly the contents of our food and lifestyle. These primarily adult diseases now extend across to the younger generation including children. Using Hong Kong as an example, 20% of type 2 DM were diagnosed as young onset below the age of 40 years.[1] Moreover, type 2 DM in children had increased by 10 times between 1997 and 2007, and likewise, the prevalence of childhood overweight and obesity has risen steadily over time in the last decade.[2,3]

The concept of fetal origin of disease was proposed by David Barker, who had challenged the traditional belief that the causes of non-communicable diseases, like DM and CVD, were predominantly explained by a combination of bad genes inherited from our parents and unhealthy lifestyles. Barker proposed that their origins could lie in the earliest stages of one's life. This Barker's hypothesis was subsequently supported by work on animal models and epidemiology studies. The most cited work was the long-term outcome of the Dutch famine birth cohort, and their persistent epigenetic changes in association with prenatal exposure of undernutrition.[4,5] Furthermore, these persistent epigenetic changes can be transmitted to the next generation.[6]

Over the last 2 decades, the model of Developmental Origin of Health and Disease (DOHaD) has extended from the spectrum of *in-utero* undernutrition to overnutrition. Through the follow-up study of mother-child pair from the "Hyperglycemia and Adverse Pregnancy Outcome" (HAPO) study participants, our group had demonstrated that maternal gestational diabetes mellitus (GDM) increased offspring's risk of DM, obesity and hypertension at 7 years of age, independent of various confounding factors, like maternal obesity and maternal DM status.[7] Similar findings were reproduced by the multi-centre HAPO follow-up study funded by NIH and NIDDK, where our group shared our study design and was also one of the 10 study centres, when the children were 11-14 year of age.[8] At the latest HAPO follow-up visit, 18% of 740 children assessed in the HK centre were diagnosed with prediabetes (based on their OGTT and HbA1c level) at their early teens.

The prevalence of maternal obesity and GDM are increasing worldwide. Up to 20-25% of pregnant mothers were affected by GDM in Hong Kong. A recent nation-wide study covering 31 provinces in mainland China discovered that 1.4% and 13% of women planning to get pregnant were diagnosed DM and prediabetes respectively, of whom, 8% were either overweight or obese.[9] Taken together, the DOHaD model might help to explain the worldwide escalating prevalence of these metabolic diseases on top of our gene and lifestyle. The key concept of DOHaD is that the fetal and early infancy period is a window of plasticity, whereby the in utero programming can alter our gene expression.

Early studies in the 1980's on Pima Indian women had shown that the children and adolescent offspring of women with abnormal glucose tolerance during pregnancy were more obese and glucose intolerant, independent of their birth weight.[10,11] It, therefore, suggested that maternal GDM was associated with long-term effects on obesity and glucose tolerance in the offspring. It has also been shown that offspring of type 1 DM mothers have an increased risk of developing DM in later life.[12] Study of amniotic fluid insulin by amniocentesis has demonstrated that excessive insulin secretion in utero was a strong predictor of impaired glucose tolerance (IGT) in the children

of DM mothers.[13] This may explain the relationship between in-utero overstimulation of pancreatic cells and the subsequent reduction in insulin secretion in their later life.[14]

The findings on the relationship between the *in-utero* exposure to hyperglycaemia and the future development of childhood overweight or obesity are still inconsistent due to the heterogeneous methodology of confounders adjustment, importantly the pre-conceptional maternal or paternal BMI.[15,16] A multinational study including 7,372 children of 9-11 ages from 12 countries shows that maternal GDM is positively associated with increased risk of children's obesity, but results are attenuated when adjusted by maternal BMI. Maternal obesity is considered as an essential confounding factor for the association between maternal hyperglycaemia and the obesity in offspring that predispose to chronic cardiometabolic disorders in the future.[17]

Excessive maternal gestational weight gain (GWG), which reflects the in-utero overnutrition environment, is found to be positively associated with the adiposity in the offspring,[18] while some studies observe a U-shaped relationship.[19,20] A few studies also demonstrated that excessive GWG predicted obesity-related metabolic factors including fasting plasma glucose (PG), insulin resistance and blood pressure (BP) in both childhood and adulthood.[21,22]

In the early 2000's, our group has reported an 8-year follow-up study on a cohort of women with previous history of GDM diagnosed between 1991 and 1994.[23] We found that offspring exposed to mild GDM had significantly higher BP and worse lipid profile compared to offspring of mothers with normal glucose tolerance.[23] Elevated umbilical cord insulin and C peptide levels at birth was associated with increased risk of glucose intolerance in children at 8 years, obesity and metabolic syndrome at 15 years, as well as increased arterial stiffness in adolescents, highlighting the persistent effects of hyperglycaemia and hyperinsulinaemia in utero on subsequent obesity, glucose intolerance and cardiovascular risk in the offspring.[23-25]

Since women with GDM in the cohort had received dietary and/or insulin treatment during pregnancy, the data from this cohort cannot address whether this intervention had led to any improvement in the risks of future DM and metabolic syndrome amongst the offspring.

Through the follow-up study of nearly 970 mother-child pairs from the HAPO study, our group discovered that maternal GDM increased their children's risk of prediabetes and DM by 3-fold, overweight and obesity by 50%, and higher BP by 10% at 7 years of age.[7] Moreover, we also demonstrated a graded effect of increasing risks of prediabetes/DM, overweight/obese and prehypertension/hypertension in association with maternal PG level at pregnancy.[7] These associations were independent of BMI before pregnancy, childhood obesity or status as large for gestational age.

The above study had sparked off an international multicentre HAPO follow-up study in 2013-2016, supported by NIH and NIDDK. Our research team was one of the 10 study centres collaborating this project. Similar associations discovered by our team could be reproduced by the multicentre HAPO follow-up study result, showing that maternal GDM increases childhood obesity by 50% after adjusting for maternal obesity and other confounders in 4,775 children.[8] A graded effect of maternal glucose level in pregnancy on childhood obesity was also reiterated. The association of maternal GDM and glycaemic level at pregnancy with children's glycaemic level and abnormal

glucose regulation were also presented at the ADA meeting in 2017 with the manuscript submitted for publication.

In the HAPO follow-up study, we also discovered that both inadequate and excessive GWG, which could be surrogate indicators of maternal undernutrition and overnutrition respectively, increased children's risk of overweight/obesity at 7 years of age. We also demonstrated that children had higher BP, greater insulin resistance and compensatory rise in insulin secretion in association with either inadequate or excessive GWG.[26]

Our research team has just completed measuring maternal and umbilical cord serum Vitamin D level in the HAPO cohort funded by GRF 2016-2017. Preliminary analysis on the 7-year-old follow-up data showed that low umbilical cord blood vitamin D level was independently associated with children's hyperglycaemia as well as arterial stiffness, which is the precursor of hypertension in later life. Two abstracts on the preliminary analysis were accepted in 2 international conferences.[27,28]

The available data would have great potential to explore the effect of in utero vitamin D status and long-term cardiometabolic risk at early teens and young adulthood. Our preliminary finding is consistent with the previous report showing vitamin D insufficiency in adult was associated with increase arterial stiffness and endothelial dysfunction. [29] Experimental studies did suggest that vitamin D modulated the endothelial cell function by reducing the endothelium-dependent contraction and regulating the calcium influx. [30,31] Vitamin D may affect the vascular wall through the renin-angiotensin-aldosterone axis and takes part in the lymphocyte and monocyte/macrophage differentiation and secretion of inflammatory cytokines which determines the monocyte infiltration and cholesterol retention in the vascular wall. [32,33]

Our research team has been investigating the effect of maternal hyperglycaemia, under- and overnutrition on the children's long-term cardiometabolic risk in the HAPO study cohort over the last 10 years. We have the most comprehensive data among all HAPO study centres, including offspring's BP, BMI, skinfold thickness, 5 point OGTT with insulin levels to explore their trajactories, plus the pre-pregnant BMI and information on GWG

Now that the HAPO cohort children are reaching young adulthood. We have archived important antenatal, childhood data, maternal serum and cord blood serum, and cord blood DNA material to determine these associations and their possible mechanism. Epigenome-wide association study (EWAS) was already performed to identify differentially methylated loci and regions in cord blood DNA in offspring which was supported by previous GRF.

Since the mothers have not been subjected to antenatal treatment on the various degree of maternal hyperglycaemia in pregnancy, this is a unique cohort that enables us to determine the effect of various degree of maternal hyperglycaemia below the level of overt DM, on children's cardiometabolic risk in Chinese population.

Our research team has successfully followed up more than 70% children from the original HAPO cohort, even though we only started to re-contact them a few years after the completion of the HAPO study; we are keeping them in contact for future assessment. Among those we have been in

touch with, we expect a higher response rate among those who have attended the last two follow-up studies and estimate a response rate of 80% in the proposed study.

Objectives

1. To assess maternal glycaemic levels, gestational weight gain and pre-pregnancy BMI in the prediction of offspring's risk of DM, hypertension and obesity at 18-20 years of age.
2. To determine the trajectory of insulin resistance and pancreatic beta cell functions at 7 years and 11-14 years of age in association with corresponding BMI to the development of young-onset type 2 DM at 18-20 years of age.

Research plan and methodology

i) Subjects

Our unit is one of the centres contributing to the International multicentre HAPO study. We recruited 1760 Chinese pregnant women between 2000 and 2005. The HAPO study investigated whether any adverse outcome was associated with mild degree of GDM. All mothers underwent a 75-gram OGTT at 24-32 weeks gestation, but clinicians were blinded to the results as long as the fasting PG was ≤ 5.8 mmol/L & 2-hour PG ≤ 11.1 mmol/L. The maternal serum C-peptide and HbA1c, cord serum C-peptide and early neonatal PG, pregnancy outcome and the neonatal anthropometric parameters are available for future study. This is so far the largest cohort in a Chinese population who has been investigated for glycaemia during pregnancy, but with OGTT results remained undisclosed to subjects and clinicians. This unique cohort can allow us to study the effect of in-utero hyperglycemia on the cardiometabolic risks at childhood, adolescence and adulthood.

Eligible subjects are all mother-child pairs participating in the original HAPO study. Children born preterm before 37 weeks of gestation, non-Chinese and whose mother's OGTT result were unblinded during pregnancy will be excluded.The family (the child and the mother) will be invited for a third follow-up assessment.

ii) Data collection

Questionnaire, anthropometry and BP measurement

Detail clinical information on maternal characteristics during pregnancy (mothers' smoking history, alcohol use and smoking habit, pregnancy complications, mode of delivery and gestational age at delivery), children's characteristics at birth, at 7 and 11-14 years of age were available in our dataset from the original HAPO study and the two follow-up studies.[7,8]

In the present study, the mother-child pairs will be asked to complete a questionnaire to review any significant medical history, family history of DM and hypertension, dietary pattern and activity level. We will also capture the menstrual history and endocrine information in both mother and female offspring, as well as reproductive history of mother. Signs of androgen excess in female offspring and mothers will be noted in the physical examination and graded according to the

Ferriman-Gallwey score or the frequency of shaving per week. Offspring's dietary pattern will be assessed by using a newly derived short version food frequency questionnaire (FFQ), which was designed on the basis of previously validated FFQ used by the local population, as well as expert opinions from dietitians and nutritionists experienced in nutritional epidemiology.[34] Research staff will administer the FFQ to capture the participant's usual intakes over the past one year on the food groups and type of cooking style.

For female offspring, their skinfold will be assessed by skinfold calipers at 4 sites (biceps, triceps, subscapular and suprailiac) in order to predict the amount of body fat. In all offspring, body fat percentage will be evaluated by electrical bioimpedance. A subset of offspring will be further evaluated for non-alcoholic fatty liver disease (NAFLD) by using Fibroscan® (Echosens).

Offspring's physical activities will be recorded and analysed by short form of International Physical Activity Questionnaire (IPAQ-S) developed for the physical activity surveillance.[35] Metabolic Equivalents (METs) will be used for the analysis of IPAQ data to express the intensity of physical activities.

Their body height and weight will be measured to the nearest 0.1 cm and nearest 0.1 kg respectively. BP will be measured three times in the non-dominant arm using an automated BP monitor, at one minute intervals, after five minutes of rest. The average readings will be used for analysis.

Arterial stiffness of mothers

Mothers will be lying on their back with their head supported and their arms relaxed by their sides. Three ECG electrodes will be applied to their chest walls. Augmentation index and pulse wave velocity will be acquired by using applanation tonometry (SphygmoCor® PVx).

- Augmentation index: Tonometer will be placed and gently compressed onto the radial artery with the subject's wrist in neutral position. A series of peripheral blood pressure waveforms are then captured by holding the tonometer for 10 seconds. Central aortic blood pressures and augmentation index will be computed by SphygmoCor® PVx after using the mathematical transfer function.
- Pulse wave velocity: Tonometer will be applied to carotid artery, radial artery and femoral artery in the same manner to obtain the peripheral blood pressure waveforms. The speed of BP wave which travels along the carotid, radial and femoral arteries are determined with reference to the ventricular contraction recorded from the ECG. The distances between the suprasternal notch (the notch at the top of the sternum) to the various tonometry points, namely carotid, radial femoral in order to compute the carotid-radial and carotid-femoral PWV. The acquisition of the data is non-invasion and the device has been approved by FDA.

Biochemical assay

Mothers will have fasting blood collected for the measurement of glucose, lipid profile, HbA1c, female hormonal profile (LH, FSH, estradiol, total testosterone, androstenedione, 17-OH and AMH) and renal function profile. The offspring will undergo a 75-gram OGTT with PG and insulin at 0, 30 and 120 minutes. Fasting blood will be taken from all offspring for lipid profile and renal function profile, whereas female hormonal profile will be assessed in female offspring only. HbA1c and lipid profile of the offspring will also be assessed by fasting blood. Blood for extraction of DNA will also be collected from offspring for genetic and epigenetic studies, including validation of established genetic risk scores for cardiometabolic traits.

Primary and secondary outcomes

The primary outcome is the rate of DM in the young adult offspring.

The secondary outcomes are their rate of impaired glucose regulation, obesity and adiposity, hypertension and various insulin indices (insulin sensitivity, pancreatic β-cell function, oral disposition indices).

iii) Statistical analysis

Between-group comparison of outcomes between offspring of mothers with GDM and those of mothers with normal glucose tolerance will be made by using the Student's *t*-tests and the Chi-square/Fisher's exact tests, as appropriate. Group comparison of outcomes among offspring of mothers with inadequate, appropriate and excessive GWG will be made by using ANOVA test. Linear and logistic regression analyses with the adjustments for potential confounders will be used to assess the associations for continuous and binary outcome variables, respectively. Any sex difference in the associations will be performed in subgroup analysis. Data will be presented as beta with standard error for continuous outcomes, and odds ratios (ORs) with 95% confidence intervals for binary outcomes.

The data from FFQ will be analysed by using the nutrition analysis software Food Processor Nutrition analysis and Fitness software version 8.0 (ESHA Research, Salem, USA) with specific focus on local foods consumption from Mainland and Hong Kong. Dietary patterns of the studied population will be generated by principal component analysis.

iv) Sample size and power calculation

We estimate the sample size and calculate the power using the objective 1.

Assuming an 50% response rate of the eligible subjects, around 900 mother-child pairs will be recruited. Our previous findings detected an adjusted OR of 1.8-2.0 in children's abnormal glucose regulation with per SD rise in maternal fasting, 1-hour and 2-hour PG at OGTT during pregnancy. We estimate the overall prevalence rate of type 2 DM as 2% in this young age group, the sample size will be able to detect a similar odds ratio of 1.8 per SD rise in glycaemic levels, at 80% power and a significance level of 0.05 by using logistic regression analysis.[36]

v) Duration of study

We estimate two and half year to complete the recall of all the HAPO mother-child pairs Another 6 months will be required for the data analysis.

This protocol is in compliance with the Declaration of Helsinki.

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