A life course approach to the relationship between fetal growth and hypothalamic-pituitary-adrenal axis function

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Abstract

Context:

Human and animal studies suggest that hypothalamic-pituitary-adrenal axis (HPA-A) function may be programmed in utero; however, these findings are inconsistent. Given the powerful metabolic actions of cortisol, it is important to clarify the influence of early life on adult HPA-A function.

Objective:

To determine the relationship between fetal growth and HPA-A stress response to a psychosocial stressor in young adults.

Design:

Multigenerational, prospective cohort study (The Raine Study) conducted between 1989 and 1991.

Setting:

King Edward Memorial Hospital, Perth, Western Australia, Australia.

Participants:

917 participants aged 18 from Gen 2 of the Raine Study.

Main Outcome Measures:

Measures of Hypothalamic-Pituitary-Adrenal-Axis function before and after exposure to the Trier Social Stress Test.

Results:

In fully adjusted models, an inverse linear relationship was observed between birth weight and and plasma measures of 1) baseline cortisol (β = -0.90%, 95% CI: -1.73 to -0.07; p = 0.03); 2) peak cortisol (β = -0.78%, 95% CI -1.51 to -0.06; p=0.03); 3) AUC_g (β = -0.89%, 95% CI -1.60 to -0.18; p=0.01); and 4) adrenal sensitivity (β = -1.02, 95% CI: -1.85 to -0.18; p=0.02). Similar results were demonstrated for per cent optimal birth weight. No consistent quadratic relationships were identified. No associations were found between measures of fetal adiposity and HPA-A function at age 18, or fetal growth and HPA-A response pattern.

Removal of anticipatory responders from the models substantially attenuated the observed relationships.

Conclusion:

We observed an inverse linear relationship between fetal growth and HPA-A function at age 18. This differs from the inverse parabolic relationship (inverted U curve) reported in adults of advanced age. Altered adrenal sensitivity may underlie this relationship.

Keywords: DOHaD, HPA, Hypothalamic-pituitary-adrenal axis, Trier Social Stress Test, fetal growth, developmental origins

Introduction

Non-communicable disease (NCD) is the biggest killer of our species. In 2017, NCD's accounted for 41 million deaths or 71% of annual global mortality (1); with 42% being under the age of 70 (2). While the risk factors for these diseases are traditionally considered to be behavioural or genetic, there is a growing recognition that adult disease susceptibility may be determined during development, a hypothesis known as the Developmental Origins of Health and Disease (DOHaD) (3-5). Over the last three decades, an abundance of animal and human evidence has emerged in support of DOHaD (6). In particular, strong associations have been observed between suboptimal fetal growth and aberrant HPA Axis (HPA-A) function in later life (7,8). Given the well-established associations between HPA-A dysfunction and both cardiometabolic (9) and neuropsychiatric (10) disorders, adverse programming of the fetal HPA-A may present a vital pathway linking adult NCD's to early-life events.

Human studies suggest that measures of fetal growth predict both baseline(11) and dynamic HPA activity, in addition to adrenal glucocorticoid sensitivity(12); however, many of these studies exhibit conflicting findings. For instance, in a pooled study of 670 people from three different populations and age groups, low birth weight was associated with a high fasting plasma cortisol (11), suggesting a negative relationship. Conversely, in a separate study of 6470 adults, birth weight was associated with a high waking salivary cortisol, indicating a positive relationship, once adjusted for gestational age (13). Many such conflicting findings exist, with multiple studies finding either no association (14-17), inverse quadratic associations (12), or associations limited to subject subgroups (18-20).

The heterogeneity in previous publications is likely due to differences between studies which include sample size, age of subjects (children vs middle-aged adults vs elderly) and HPA-A assessment protocols (fasting cortisol vs CO2 inhalation vs Trier Social Stress Test). Together, these differences cloud the overall relationship between fetal growth and HPA-A function, as all of these factors significantly affect the HPA-A response to an experimental stressor. Additionally, many of the *in utero* exposures known to confound the relationship between fetal growth and HPA-A function are absent from analyses in these studies. This makes it difficult to determine whether there is genuinely an association between fetal growth and HPA-A function, or whether early life exposures confound their relationship. Further, measures of fetal growth are historically limited to birth weight and ponderal index. While these measures are useful, they often require further adjustment for gestational age to convey information about growth potential, while offering no information about fetal adiposity. To address these uncertainties, a study testing the association between fetal growth and HPA-A function is needed that is sufficiently powered, appropriately adjusts for confounders and uses a single gold standard HPA-A assessment protocol.

The aim of this study was to evaluate the relationship between measures of fetal growth and dynamic HPA-A function in a large population of young adults. To achieve this aim, we used unique data from the Raine Study, an Australian longitudinal cohort study which recruited 2900 women between 1989 and 1991. From this cohort, we used a combination of maternal and offspring data collected at birth, as well as data from a Trier Social Stress Test (TSST) administered on the Raine Study Gen2 participants at age 18. We hypothesised that we

would observe an inverse relationship between measures of fetal growth and HPA-A activity. We structured our findings as per the STROBE statement (21).

Methods

Data Source

The Raine Study is a longitudinal cohort study, which actively recruited 2900 pregnant women between 1989 and 1991. Women were recruited between 16-18 weeks' gestation from the public antenatal clinic at King Edward Memorial Hospital (KEMH), as well as surrounding private clinics. Additional inclusion criteria for enrolment in the cohort were sufficient English language skills to participate in the study, an expectation that they would deliver at KEMH, and that they were likely to remain in Western Australia, to allow follow up of their offspring.

Parental socioeconomic, anthropomorphic, and medical information was collected using questionnaires at 18 and 34 weeks, as well as data regarding pregnancy, ultrasound and Doppler outcomes. A total of 2868 live births occurred during this period, from 2730 mothers. Both Gen1 and Gen2 of the Raine Study were contacted and invited for follow-ups at ages one, two, three, five, fourteen, seventeen and eighteen. A variety of data was collected at these follow-ups, including behavioural, environmental, genetic and anthropomorphic information. Over the past 30 years, this has resulted in the generation of more than 85,000 phenotypic variables. The Human Research Ethics Committees at the University of Western Australia, King Edward Memorial Hospital and Princess Margaret Hospital in Perth, Australia, granted ethics approval for each follow-up in the study. Parents, guardians and adolescent participants provided written informed consent either before enrolment or at data collection at each stage of follow-up. Further technical details about the Raine Study are available elsewhere (22).

Assessment of HPA Function: Trier Social Stress Test (TSST)

Participants for the TSST Protocol were recruited from Gen2 at the 18-year follow-up of the Raine Study. Of the original Raine Study Gen2 participants (n=2868), 17% had withdrawn from the study, died, or been lost to follow up (n=701). This attrition left 2167 Raine Study Gen2 participants eligible to be contacted for the 18-year review, of which 1137 agreed to participate in the TSST protocol. From the 1137 eligible participants, 220 were excluded from analysis (see Figure 1) resulting in a final study population of 917.

Methods: Trier Social Stress Test Experimental Protocol

Before attending the protocol, participants were asked to refrain from eating, smoking, engaging in physical activity or drinking anything other than water for an hour before their arrival. Study participants arrived between 1200 and 1600 to minimise the effect of their circadian rhythm on HPA function. On arrival, participants were consented, and those that agreed (n=921) had a single intravenous cannula inserted under local anaesthetic by an anaesthetist. They then rested over the next 45 minutes, during which they filled out a questionnaire regarding use of oral contraceptives, medication, recent illness, levels of physical activity, the character of their menstrual cycles and smoking habit. Measures of height and weight were also taken during this time. Following the rest period and before

commencing the Trier Social Stress Test (TSST) protocol, blood and saliva were collected to measure baseline cortisol levels (time 0 minutes).

The TSST protocol (~15 minutes) involved three minutes of preparation time in isolation, a five-minute mock job interview, and a five-minute arithmetic task, all of which occurred in front of a non-responsive group of adult assessors. As per validated protocols (23-25), environmental additions such as formal dress and use of mock audio-visual equipment were used to add authenticity to the scenarios. Assessors were asked to report on each participant's behavioural response to the interview and arithmetic challenge using a standardised scoring form. All participants were debriefed at the end of the protocol about the rationale behind the study and the nature of the stressor. In consenting participants, blood was drawn at multiple time points after the TSST (16, 25, 35, 45, 60, 75 and 105 minutes). Saliva was collected before the commencement of the TSST protocol and subsequently at 16, 35 and 105 minutes.

Methods: Laboratory Analysis

Blood collection was done using BD Vacutainers, containing ethylenediaminetetraacetic acid (Becton, Dickinson and Company, Franklin Lakes, NJ). All samples were stored on ice for the duration of the test, after which they were centrifuged, aliquoted and frozen at -80°C until assays were completed. Quantification of plasma and salivary cortisol was conducted using the GammaCoatTM 125I cortisol radioimmunoassay (RIA) kit (DiaSorin, Stillwater, MN). Plasma ACTH was quantified using 125I immunoradiometric (IRMA) assay (DiaSorin, Stillwater, MN). All samples were quantified as per manufacturer's instructions. Concentrations were measured in micrograms per decilitre, which were converted to nanomoles per litre by multiplying results by 27.59. All samples were assayed in duplicate against an appropriate standard curve. Additional dilutions were repeated where required. Intra and inter-assay variability was <10% for all assays. Coefficients of inter-assay variation for total and free cortisol were 6.6% and 4.52% respectively and were used as a part of the criteria for assigning participants to particular response groups.

Early-life variables

Data regarding maternal characteristics were obtained from questionnaires at 18- and 34-weeks' gestation. Variables in our analyses included: maternal pre-pregnancy BMI, weight gain as a percentage of pre-pregnancy weight, smoking status and cumulative antenatal exposure to stressful life events at 18 weeks' gestation (26), as well as diagnoses of diabetes and hypertension at any point in the mother's life.

Data about fetal characteristics were collected at birth. These included fetal sex (male or female), gestational age at delivery (weeks), birth weight (g) and fetal adiposity measured through skinfold thickness (cm) taken on the triceps, parascapular and infrascapular regions. As part of a previous work within the Raine Study, a measure of fetal attainment of growth potential - percent optimal birth weight (POBW), has been developed. POBW estimates fetal growth potential using a formula which adjusts for fetal sex, maternal height, maternal parity and gestational age(27), and was used as a metric of fetal growth. In addition, we also used ponderal index (PI), a measure of fetal weight adjusted for length, which was calculated using the formula PI = [(Birth weight (g) x 100)/(Crown heel length)^3] (see Supplementary Table 1; uon:36340)(28).

HPA Axis variables

Blood samples were collected at eight times points (0, 16, 25, 35, 45, 60, 75 and 105 minutes); saliva was collected at four time points (0, 16, 35 and 105 minutes). Cortisol concentration was measured in all blood and saliva samples. ACTH concentration was measured in plasma samples at 0, 16, 25 and 105 minutes. Together, these measures generated cortisol and ACTH response curves spanning 105 minutes. From these curves, well-defined variables were created based on established definitions (29). Variables used in our analyses included salivary and plasma measures of baseline cortisol concentration (C_{BL}), maximum concentration after the TSST (C_{MAX}) and area under the curve with respect to ground (C_{AUCg}). Plasma ACTH area under the curve with respect to ground ($ACTH_{AUCg}$), maximum concentration after the TSST ($ACTH_{MAX}$) and baseline concentration ($ACTH_{BL}$) were also evaluated. We derived the variable for adrenal sensitivity (ADSEN) by dividing plasma C_{AUCg} by $ACTH_{AUCg}$(12).

TSST Pattern variables

The collection of blood at different time points in the TSST generated characteristic stress response curves when measures were plotted over time. Several studies have observed distinct morphologies in these response curves. These findings have led to the proposition of three well-established patterns (30): “reactive-responders” (RR) (31) who mount an appropriate cortisol rise in response to stress; “anticipatory-responders” (AR) (31) who begin the TSST with high levels of baseline cortisol in anticipation of a stressful event; and “non-responders” (NR) (32) who do not show a change in baseline HPA activity following the TSST. Given that NR and AR status has been shown to be associated with neuropsychiatric and cardiometabolic disease (33-35), we evaluated the relationship between fetal growth and TSST response pattern in young adults. The protocol for assigning trier patterns in this cohort is available elsewhere (30).

Covariates

Covariates for inclusion in our multivariable models were based on theoretical relationships between exposures and outcomes. A direct acyclical graph (DAG) was constructed to clarify these relationships (Figure 2). On this basis, we generated three adjusted models. Model 1 *adjusted for coexistent participant factors* that may have affected HPA-A function included smoking status, sex and oral contraceptive (OCP) use at age 18. Sex and oral contraceptive use were combined into a single, three category variable; group one was females not taking the OCP, group two was males, and group three was females taking the OCP. Model 2 adjusted for *only antenatal factors* that may have theoretically altered either fetal growth or adult HPA-A function including maternal smoking, pre-pregnancy BMI, maternal weight gain during pregnancy, diabetes, hypertension and exposure to severely stressful life events (26) during pregnancy. Model 3 *combined covariates from Model 1 and Model 2.* Gestational age was based on the date of the mother’s last menstrual period, unless there was discordance of seven or more days with the estimated due date based fetal biometry at the 18-20 week morphology scan, in which case gestational age was based on fetal biometry. Corrected gestational age was included as a covariate in all models of fetal growth except for POBW, as the derivation of this measurement already included adjustment for gestational age.

Statistical Analyses

Our study investigated whether measures of fetal growth were associated with HPA-A function in adulthood. First, we log-transformed all cortisol and ACTH measures to approximate normality. We then assessed the relationship between markers of fetal growth and measures of HPA-A function by creating generalised linear models (GLM) with Gaussian reference distributions. Measures of fetal growth (exposures) included birth weight, POBW, tricep skin fold thickness, parascapular skin fold thickness and infra-scapular skin fold thickness. HPA-A function outcome measures were salivary and plasma measures of baseline cortisol, maximum cortisol and cortisol area under the curve above ground. Measures of baseline ACTH, maximum ACTH and ACTH area under the curve above ground were also used as outcomes. Finally, we used the derived variable adrenal sensitivity as an outcome. Covariates were included in the models to control for potential confounding; non-linearity of associations was tested by adding the second order polynomial form (exposure x exposure) of the term into regression equations. Interactions between fetal growth and sex were also assessed.

We then generated linear mixed models with unstructured covariance and exponential residual structure for exposures exhibiting or approaching statistical significance in our linear regression models. An exponential residual structure was used due to the uneven distances between sampling times. We then added sampling time as an interaction term, and split exposures into tertiles.

For variables included in our models, missing data ranged from 0.33% to 10.36% (Supplementary Table 1; uon:36340)(28). We handled missingness with complete case analysis. Sensitivity analyses were performed to assess the internal validity of our models. This was done by removing or including participants from specific subgroups in our models. Finally, we calculated E-values for relationships between birth weight, POBW and plasma cortisol measures to quantify the association any unmeasured confounder would need with both exposure and outcome to nullify any associations observed. All estimates, regression coefficients and 95% confidence intervals (CI) presented are unstandardised. Estimates represent the expected percent change in an outcome measure for a given increase in the exposure. We set the significance level (alpha) to 0.05 which was not modified for multiple testing, as the primary purpose of our study was hypothesis generation.

Results

Description of Cohort

Demographic characteristics of the study population are presented in Table 1. Within the TSST participants, males had the highest birth weight and were most likely to be smokers at age 18. There were no significant differences between genders in measures of fetal adiposity. Compared to non-TSST participants (see Supplementary Table 2; uon:36340)(28), those completing the TSST had mothers who were older, experienced less stressful life events in pregnancy and were less likely to smoke in pregnancy. TSST participants were also more advanced in their gestational age at birth and all measures of fetal growth.

Outcome measures

Outcomes are presented in Table 1, stratified by gender and oral contraceptive pill (OCP) use. Female participants taking the OCP had the highest plasma cortisol levels at baseline and during the TSST. Overall, males had the highest salivary cortisol levels. Measures of serum ACTH were lowest in female OCP users and highest in males. Information detailing the effects of the TSST on our participants' cortisol and ACTH levels, as well as the impact of smoking, body weight, phase of menstruation and gender have been published elsewhere (30). In brief, females on the oral contraceptive pill had the highest baseline levels of circulating cortisol, while males demonstrated a larger increase from baseline in response to stress; participants that were obese and currently smoking had lower total plasma cortisol concentrations; no relationship was observed between phase of menstruation and HPA-A responsiveness.

Relationships between fetal growth and HPA-A Function at 18 years

Individual participant data for baseline cortisol concentration, cortisol AUCg and adrenal sensitivity are plotted against POBW in Figure 3. We observed inverse linear relationships between POBW and plasma measures of both baseline and dynamic HPA-A function. TSST response profiles and mixed models analyses of the lowest, middle and upper tertiles of POBW are presented in Figure 4, and supplementary table 9 (uon:36340)(28). We observed no significant differences in the cortisol or ACTH response profiles between the three tertiles of POBW.

Tables 2 and Supplementary Tables 3 to 6 (uon:36340)(28) present the results of the multivariable linear regression analyses describing the association between various measures of fetal growth and adult HPA-A function. In our fully adjusted model a 10% increase in POBW was associated with: 1) a 3.53% reduction in baseline cortisol (95% CI -6.46 to -0.59; p=0.02); 2) a 3.40% reduction in cortisol area under the curve (95% CI -5.91 to -0.89; p=0.01); 3) a 3.02% reduction in peak cortisol concentration (95% CI -5.57 to -0.46; p=0.02); 4) and a 3.69% reduction in adrenal sensitivity to ACTH (95%CI -6.65 to -0.73; p=0.01). Similar results with smaller effect sizes were observed with associations to birth weight In our fully adjusted model, a 100g increase in birth weight was associated with: 1) a 0.90% reduction in baseline cortisol (95% CI -1.73 to -0.07; p=0.03); 2) a 0.89% reduction in cortisol area under the curve (95% CI -1.60 to -0.18; p=0.01); 3) a 0.789% reduction in peak cortisol (95% CI -1.51 to -0.06; p=0.03); and 4) a 1.02% reduction in adrenal sensitivity (95%CI -1.85 to -0.18; p=0.02).

Associations between salivary cortisol and fetal growth were only significant when POBW was the exposure. There were few consistent relationships between ponderal index or measures of neonatal adiposity and each of the HPA function measures in our study

population (see Supplementary Tables 3 to 6; uon:36340)(28). Furthermore, we found no association between measures of fetal growth and a participant's TSST pattern (Supplementary Table 7; uon:36340)(28).

Effects of early and later life exposures on HPA function

We observed significant associations between multiple early- and later-life exposures and HPA-A function at 18 years. Adjusting for covariates present at the time of the TSST (Model 1 including smoking and oral contraceptive use) had a significant effect on the results, accounting for almost 20% of the variance across each of our models (Data not presented). Further, analyses highlighted associations between measures of fetal growth and BMI at 18 years, suggesting that BMI at 18 may be a mediator of the relationship. Accordingly, we did not include this variable in any of our models.

Of the antenatal covariates in Model 2, maternal diabetes, maternal BMI and maternal smoking were significantly associated with several measures of fetal growth, and rarely with adult HPA-A function. In contrast, maternal weight gain in pregnancy and maternal stressful life events were significantly associated with both measures of fetal growth and HPA-A function. Overall, adjustments in Model 2 accounted for approximately 2% of the variance across each of our models.

Interactions and non-linearities

We assessed the interaction between fetal growth and Sex/OCP-use as a combined predictor of HPA-A function in fully adjusted (model 3) linear regression models, where POBW was set as the exposure. In linear regression models, we found no interactions between POBW and participant sex, which was replicated in subgroup analyses (data not presented). These findings suggest that the relationship between fetal growth and HPA-A function at 18 does not differ by sex or oral contraceptive use. We found no consistent evidence of quadratic relationships between fetal growth and different HPA-A measures (data not presented).

Mixed models

We generated mixed models to determine whether participants' overall HPA-A response profiles differed by tertiles of fetal growth. We limited these analyses to POBW, because in linear regression models, this was the only measure of fetal growth that demonstrated consistent relationships between plasma and salivary measures of cortisol. In fully adjusted mixed models, there were no significant differences in plasma cortisol, salivary cortisol or ACTH response profiles between participants in the lower, middle or upper tertiles of POBW (all $p>0.06$).

Sensitivity analyses

Sensitivity analyses were conducted through the omission and reintroduction of specific participant subgroups. Removal of participants from the lowest *and* highest deciles of birth weight and POBW increased the effect of these exposures on salivary and plasma cortisol by up to 30%. In contrast, removal of the highest decile alone had no impact on effect size. Removal of anticipatory responders from our models also had a substantial impact, attenuating effect sizes by up to 27% in linear regression models. Removal of participants

who only provided saliva (did not consent to blood collection) also enhanced effect sizes in salivary cortisol models by 18 to 32%. Other sensitivity analyses conducted included the re-introduction of participants who were born preterm and removal of non-responders from the models, neither of which had any impact on effect sizes.

Finally, given the observational nature of our study, there is likely a degree of unmeasured confounding that our models could not account for. We estimated the E-Value (36) to determine the necessary strength of the association that any unmeasured confounder would need to have with both exposure and outcome, *independent* of our measured covariates, to fully account for the relationship in question. For computation, the E-value requires the exposure to confer a risk ratio for the outcome. We thus converted our exposures into dichotomous outcomes of highest fetal growth tertile versus remaining tertiles. Our estimates ranged between E-values of 1.6 – 1.82 depending on the exposure and outcome (Supplementary Table 10; uon:36340)(28). These results suggest that a confounding variable, independent of all other covariates, would need to be associated with *both* exposure and outcome by an odds ratio of at least 1.6 to 1.82, to explain away the relationships observed in our study.

Discussion

This study has demonstrated that both baseline and dynamic HPA-A function in response to a standardised psychosocial stressor at age 18 is associated with birth weight and POBW. Specifically, we showed that adults of higher birth weights had attenuated HPA-A responses to an acute psychosocial stressor. We also observed attenuated adrenal sensitivity in these participants, such that they produced less plasma cortisol per picogram of plasma ACTH in response to a psychosocial stressor. Additionally, we found that measures of neonatal adiposity were not predictive of HPA-A function in adulthood. This may be due to limited variance within our adiposity measures or skinfold thickness being a poor estimator of fetal adiposity; we note that novel approaches such as air displacement plethysmography are more accurate estimators of fetal body fat percentage.Alternatively, neonatal adiposity may be an inaccurate marker of HPA-A function in later life.

Our findings are consistent with a meta-analysis of 11 studies using 2301 subjects which demonstrated an inverse relationship between birth weight and circulating cortisol (37). Our results also replicated the meta-analysis' finding of no interaction between fetal growth and gender as a combined predictor of HPA-A function, as well as no modifying effect of prematurity on the relationship between fetal growth and adult HPA-A function.

The results of this study are also similar to those published by Kajantie et al. (12), in which subjects from the Helsinki Birth Cohort aged 61-70 underwent the TSST. In that study, there was an inverse parabolic association between multiple measures of fetal growth and HPA-A reactivity. In comparison, our study found an inverse linear relationship. A few important differences in participant characteristics may account for these differences. Firstly, *the TSST participants in the Helsinki Birth Cohort were, by design, from the lowest tertile of birthweight in the cohort*. Participants in this study were across the whole birthweight range. Repeating this study with adequate numbers of low birth weight babies may replicate the inverse parabolic relationship observed in the Helsinki cohort. Alternatively, hypoactive HPA-A function at extremes of birthweight may simply require advanced age to manifest, as the

participants from the Helsinki TSST were between 61 and 70 compared to the 18 year-old participants in our study.

There are a number of possible explanations for inverse relationship between birth weight and circulating cortisol. First, the inverse relationship may be mediated by attenuated adrenal sensitivity to ACTH in babies with high birth weight. Conversely, individuals with low birth weight may have increased sensitivity to ACTH. Animal studies have documented altered adrenal responsiveness to stress in the offspring of mothers exposed to antenatal glucocorticoids(38,39); this is usually associated with low, rather than high birth weight. This is supported by our estimates of adrenal sensitivity, which exhibited an inverse relationship to fetal growth. Alternatively, fetal growth may be associated with changes in adult levels of cortisol binding globulin (CBG). We could not assess this using our data-set, as CBG was not measured as a part of the TSST protocol. A third possibility could be that fetal growth is associated with altered CRH sensitivity. This explanation seems unlikely as, to our knowledge, there is no evidence demonstrating an association between birth weight and CRH (40) in humans. Further, ACTH levels were similar across the range of birth weights in our study.

Our study is also novel in several ways. First, we demonstrated that the relationship between fetal growth and adult HPA-A function exists despite adjustment for multiple antenatal factors. To the best of our knowledge, this has not been studied in detail in any other cohort. Further, we have shown that, in addition to birth weight, HPA-A function can be predicted by novel measures such as percent of optimal fetal growth (POBW). This is important as the use of birthweight (rather than POBW) requires adjustment for multiple confounders. In contrast, POBW as a measure of growth potential has already made adjustments for several of these factors, such as markers of maternal constraint and gestational age. This enables the preservation of statistical power when interrogating the relationships between fetal growth and HPA-A function.

To our knowledge, this study is first using the TSST to demonstrate the relationship between fetal growth and adult HPA-A function in young adults. Confirming this relationship in young adults is important, as HPA-A function is known to stabilise in young adulthood (41), thereby setting an individual's cumulative lifetime exposure to glucocorticoids. Finally, we have shown that in our participants that there is no relationship between fetal growth and the risk of developing a particular HPA-A stress response pattern, although our data did suggest that the relationship between fetal growth and HPA-A function may be stronger in anticipatory responders than reactive responders and non-responders. This was determined through the sensitivity analyses which demonstrated that removal of anticipatory responders from our models attenuated the relationship between fetal growth and measures of plasma cortisol by up to 27%. This observation is important, as both anticipatory and non-responsive stress patterns are associated with important chronic illnesses. A lack of association between fetal growth and these patterns thus suggests that these patterns themselves may not mediate the relationships between fetal growth and adverse health outcomes in adulthood, but rather, that they may confer increased risk of HPA-A dysfunction and subsequent development of non-communicable disease in babies with low birth weight. Sensitivity analyses also revealed that the relationship between fetal growth and measures of salivary cortisol were enhanced by 18 to 32 per cent when those who provided salivary cortisol *only* were removed from models. This is most likely due the effects of gender, as these participants were less likely to have been male, and more likely to have been females on the OCP.

There are several important limitations in our study. First, participants in this study were generally young and healthy. Although elevated plasma cortisol is associated with multiple chronic diseases, it is difficult to establish temporal order in these associations. As such, the association between fetal growth and HPA-A function may merely be a representation of normal, with no attendant consequences for long-term health. The participants in our study were also predominantly Caucasian. This racial homogeneity inevitably limits the generalisability of our findings. We also observed some important differences between participants who participated in the TSST and those who did not. TSST participants generally had more favourable early life phenotypes; they were heavier at birth, had longer gestations, had mothers who reported fewer stressful life events and were less likely to smoke during pregnancy. We would expect this to diminish the strength of the observed associations among our cohort, however, our findings persisted among our TSST Participant group.

Another important limitation of our study was the way we estimated adrenal sensitivity, which was calculated by dividing plasma cortisol area under the curve by ACTH area under the curve. This approach may have introduced bias into our estimates, as cortisol area under the curve was calculated using eight time points, whereas ACTH was calculated using four. To address this issue, we imputed new cortisol AUCg values using sampling times that were identical to ACTH (0, 16, 25 and 105). Although we anticipated this would improve the accuracy of our adrenal sensitivity measure, the newly calculated areas under the curve were substantially different (up to 39.5% difference between measures; see supplementary figure one; uon:36340)(28) to the original AUCg measures, which used all eight plasma sampling points. Use of these newly imputed estimates would likely have introduced further bias into assessments of adrenal sensitivity; therefore, we opted to use the most complete and accurate representations of each hormonal curve in our calculation of adrenal sensitivity.

Given the observational nature of our study, there is likely a degree of residual confounding in our models. Our estimates ranged between E-values of 1.6 – 1.82 suggesting that a confounding variable, independent of all other covariates, would need a risk ratio of 1.6 to 1.82 to fully account for the relationships observed in our study. The risk of a type I error in our study was also high; our significance levels were not adjusted to account for multiple testing as the primary purpose of the study was hypothesis generation. Timing of menstrual cycle or level of adiposity were also not taken into account, and although our findings were confirmed using birth weight, per cent optimal birth weight is a measure of fetal growth unique to the Raine study. Finally, our findings should be interpreted with caution, as they demonstrate that substantial changes in fetal growth are necessary to create subtle variations in adult HPA-A function.

While our study offers valuable insight into the mechanisms underpinning the relationship between fetal growth and HPA-A function at age 18, further studies are required to fully elucidate underlying mechanisms. For example, although we found that a higher birthweight was associated with reduced adrenal sensitivity, our sample was underpowered to detect this effect among growth tertiles using mixed models. Additionally, fetal adiposity was measured through skinfold thickness in our participants; novel, gold-standard tools have emerged since the birth of our cohort which provide more accurate measurements of neonatal adiposity (42). Use of these tools to assess the fetal growth-HPA-A relationship may provide clearer estimates than the ones used in our study. Finally, the implications of

our findings need further investigation. For example, while the associations between low birth weight, elevated HPA-A function and resting systolic blood pressure have been established (6,43-46), how much of this relationship is *causally mediated* by growth-driven changes to HPA-A function remains unknown. Understanding this relationship will be fundamental to determining how the early life environment may contribute to the development of important non-communicable diseases.

In conclusion, this study found an association between increased fetal growth and blunted HPA-A response to an acute psychosocial stressor at the age of 18. Our findings suggest that HPA-A function in adulthood may have its origins in early life.

Acknowledgements

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Data Availability

Restrictions apply to the availability of data generated or analysed during this study to preserve patient confidentiality. The corresponding author will, on request, detail the restrictions and any conditions under hich access to some data may be provided.

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Tables: Legend

Table 1: Descriptive Statistics of study participants

Table 2: Relationship between birthweight, per cent optimal birthweight (POBW), and HPA-A response to acute psychosocial stress

Figures*: Legend (see attached files)

Figure 1: Inclusion and exclusion criteria from the TSST Protocol

Figure 2: Direct Acyclical Graph (DAG)

Figure 3: Linear multivariate regression

Figure 4: Mixed Models

	Females on the OCP (n=184)		Females off the OCP (n=257)		Males (n=476)	
	Mean	**sd or %**	**Mean**	**sd or %**	**Mean**	**sd or %**
Maternal Characteristics						
BMI, kg/m^2	21.9	3.7	22.4	4.3	22.1	4.2
Weight Gain[a]	14.6	6.6	14	5.6	14.35	6.4
Stressful life events[b]						
1 to 3	81	18.3	134	30.3	228	51.5
More than 3	4	15.4	8	30.8	14	53.9
Diabetes[c]	2	6.1	11	33.3	20	60.6
Hypertension[d]	35	15.6	61	27.1	129	57.3
Nulliparous	90	20.1	123	52.5	235	27.5
Smoking in Pregnancy (yes/no)	38	20.1	61	32.3	90	47.6
Fetal characteristics						
Corrected gestational Age, weeks	39.8	1.2	39.9	1.3	39.8	1.3
Birthweight, grams	3389.8	450.7	3389.4	465.7	3493.3	469.5
Skin fold thickness, cm						
Parascapular	4.6	1.1	4.6	1.1	4.5	1.1
Triceps	4.3	0.9	4.4	0.9	4.3	0.9
Infrascapular	4.4	1.1	4.4	1	4.2	1
Percent optimal birthweight	98.8	11.5	98.4	12.3	97.9	12.3
Characteristics at age 18						
Currently smoking	20	15.4	31	23.9	79	60.8
BMI, kg/m^2	23.3	4.0	24.6	5.8	23.8	4.4
HPA-A Measure	**Mean[e]**	**sd**	**Mean[e]**	**sd**	**Mean[e]**	**sd**
Plasma Cortisol (nmol/litre)						
Baseline	518.5	303.3	273.2	140.5	339.5	179
Peak	636.7	346	381.9	160.1	479.4	198.1
Area under the curve[f]	53280	29567	29238.8	11879.9	36522.6	15082.3
Adrenal sensitivity[g] (nmol/pg)	34.2	25.6	14.9	8.2	14.8	9.2
Salivary Cortisol (nmol/litre)						
Baseline	11.1	9.1	10.3	9.8	12.8	10.6
Peak	13.4	10.3	14.3	10.9	17.2	11.3
Area under the curve	1085.3	724.9	1083.7	661.7	1321.5	731.2
Plasma ACTH (pg/mL)						
Baseline	13.9	9.5	17.3	8.4	22.2	13.1
Peak	19	13.5	24	13.5	30.7	16.1
Area under the curve[a]	1555.9	763.8	1967.7	772.5	2457.2	972.7

Table 1: Descriptive statistics of study participants. [a]Weight gain as a percentage of pre-pregnancy body-weight. [b]Stressful life events measured by the Tennant and Andrews stressful life events in pregnancy scale. [c]Any diabetes (Pre-existing or Gestational Diabetes Mellitus). [d]Any Hypertension or hypertensive complication of pregnancy (Pre-existing, gestational, pre-eclampsia, eclampsia). [e]Geometric mean. [f]Area under the curve above ground. [g]Ratio of plasma cortisol area under the curve above ground to plasma ACTH area under the curve above grou

	Birth Weight		Percent Optimal Birth Weight	
PLASMA CORTISOL				
	Beta (95% CI)	**p**	**Beta (95% CI)**	**p**
Baseline	-0.90 (-1.73 to -0.07)	0.03	-3.53 (-6.46 to -0.59)	0.02
AUCg	-0.89 (-1.6 to -0.18)	0.01	-3.4 (-5.91 to -0.89)	0.01
Peak	-0.78 (-1.51 to -0.06)	0.03	-3.02 (-5.57 to -0.46)	0.02
Adrenal Sensitivity	-1.02 (-1.85 to -0.18)	0.02	-3.69 (-6.65 to -0.73)	0.01
SALIVARY CORTISOL				
Baseline	-0.84 (-1.73 to 0.04)	0.06	-3.77 (-6.9 to -0.63)	0.02
AUCg	-0.82 (-1.57 to -0.07)	0.03	-3.49 (-6.14 to -0.83)	0.01
Peak	-0.74 (-1.57 to 0.09)	0.08	-3.45 (-6.39 to -0.51)	0.02
PLASMA ACTH				
Baseline	-0.27 (-1.05 to 0.52)	0.50	-1.02 (-3.79 to 1.75)	0.47
AUCg	0.10 (-0.64 to 0.85)	0.79	0.13 (-2.5 to 2.77)	0.92
Peak	-0.29 (-1.14 to 0.56)	0.51	-1.42 (-4.42 to 1.58)	0.35

Table 2: Relationship between birthweight, per cent optimal birthweight (POBW), and HPA-A response to acute psychosocial stress. Beta coefficients, 95% confidence intervals and p-values of multivariate regression models predicting the % change in HPA-A function for every 100g change in birth weight and 10% change in per cent optimal birthweight. Models adjusted for maternal diabetes, hypertension, anxiety, BMI, weight gain during pregnancy, smoking status, participant sex and participant smoking status. Birth weight models additionally adjusted for gestational age.

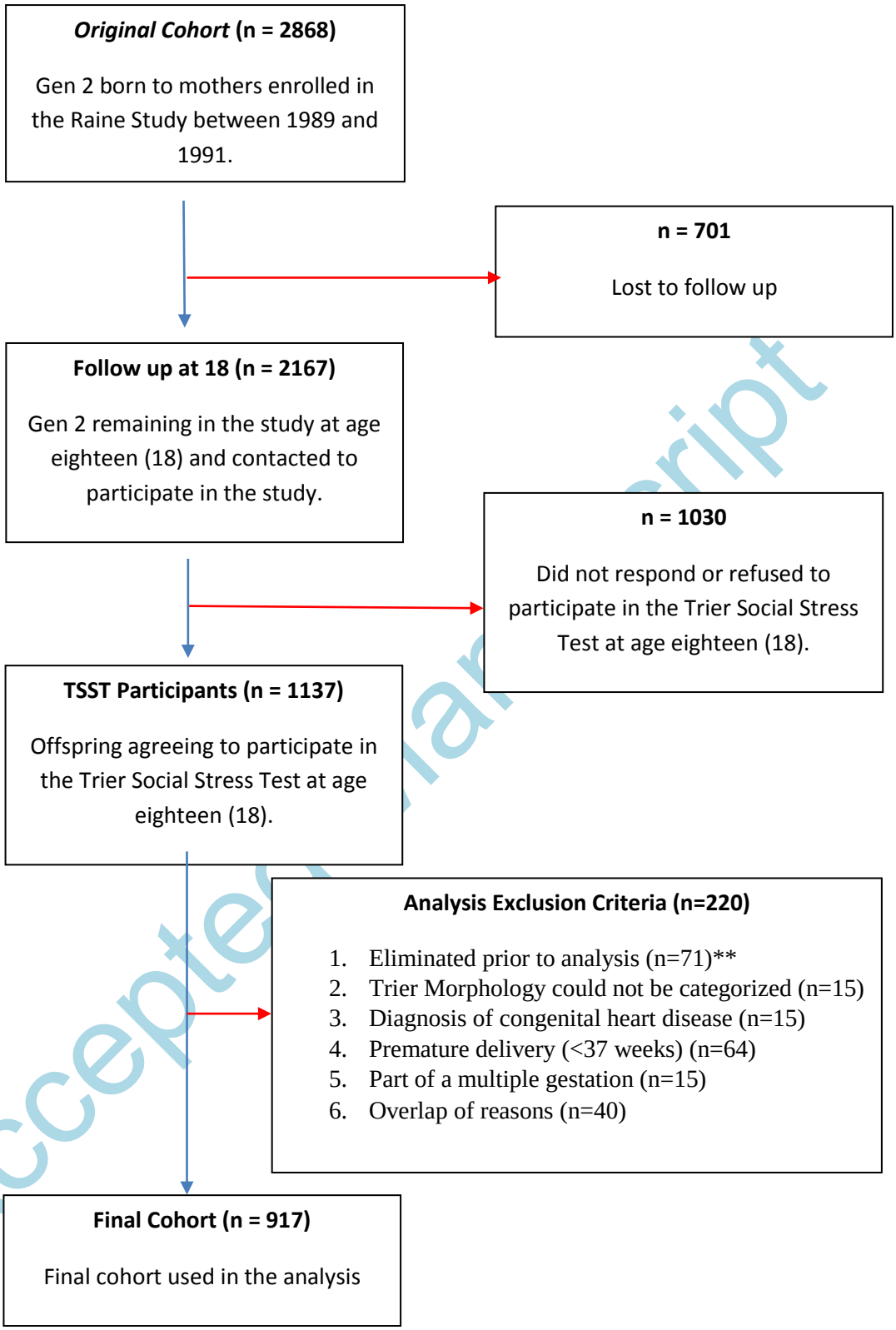


Figure 1: Flowchart describing inclusion and exclusion criteria for participation in the TSST. A total of 220 participants were excluded from analysis; 71 did not complete the Trier protocol. **Reasons for this included include: 1) Unable to complete TSST (n=2), samples unusable (n=3), severe menstrual pain (n=1), pregnant or suspected to be pregnant (n=2), lactating or suspected to be lactating (n=2), diagnosis of type 1 diabetes (n=4), taking exogenous steroids (n=7), taking neuroactive medication (n=22), taking anti-depressants (n=19), taking other medications affecting HPA-Axis function (n=2) and fainted during the Trier Social Stress Test (n=15).

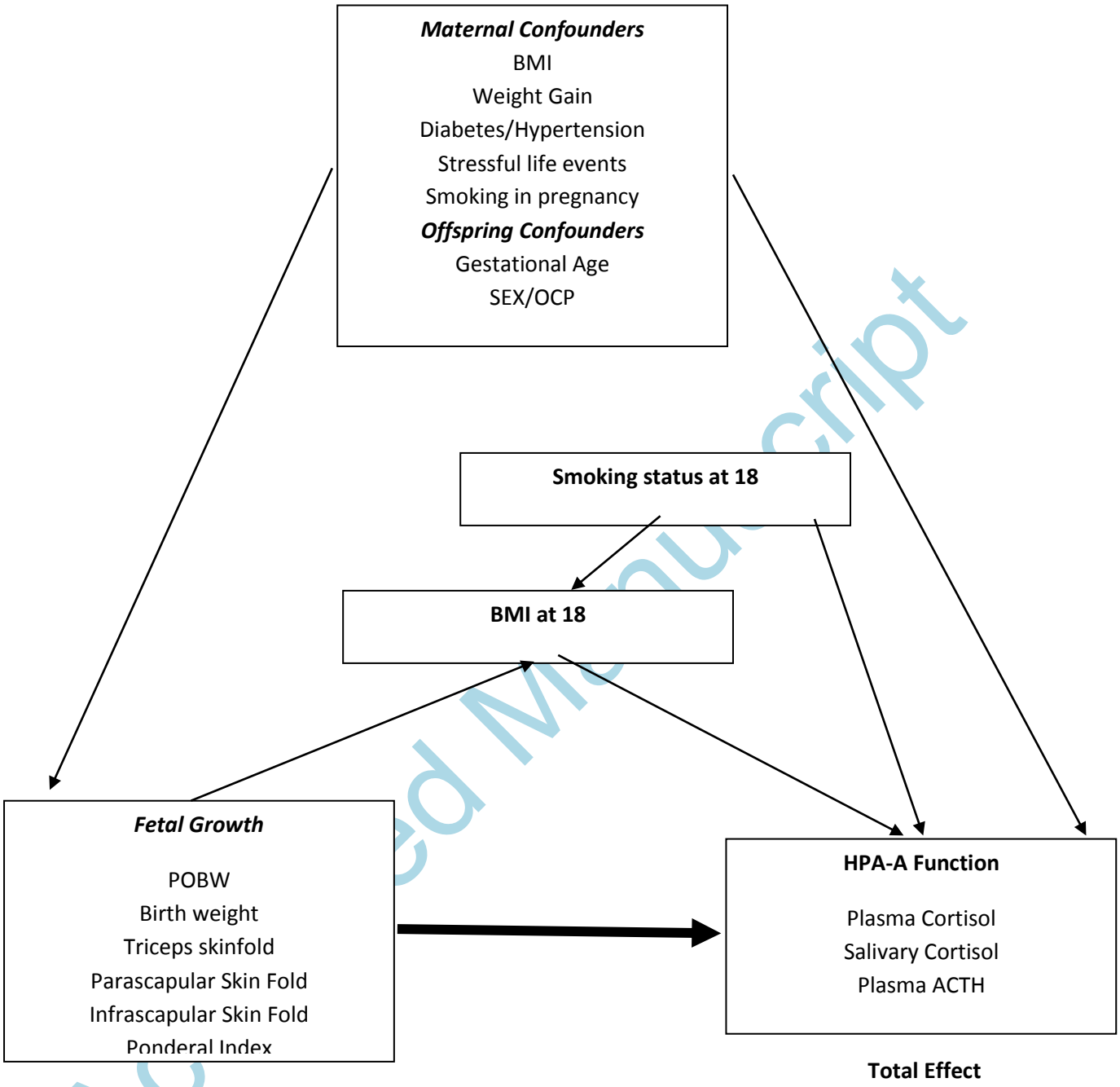
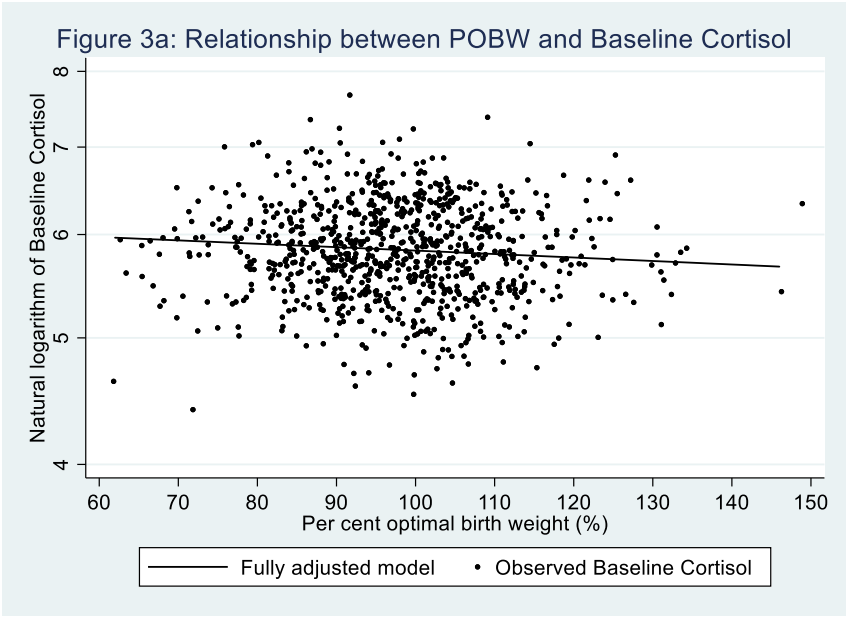
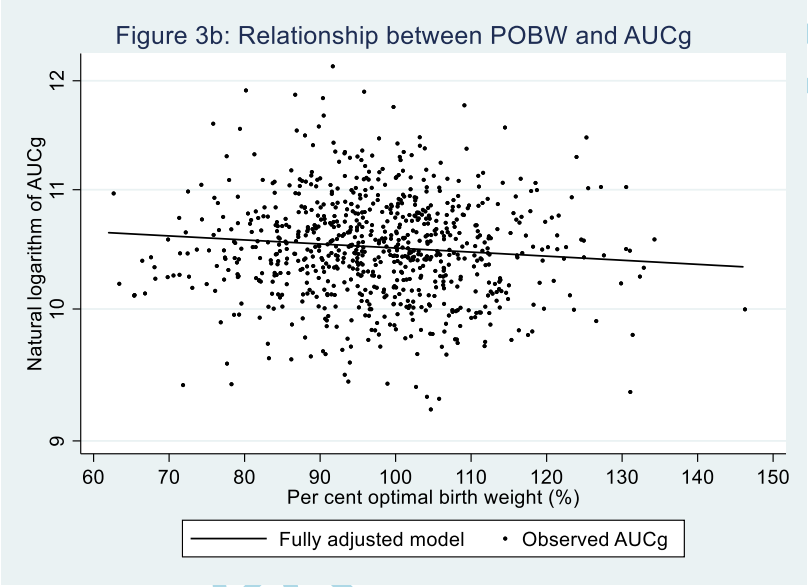


Figure 2: Direct Acyclical Graph. Demonstrates assumptions and relationships between covariates assumed in model building. The minimum sufficient adjustment set to estimate the total effect was corrected gestational age, Maternal BMI, Maternal Diabetes, Maternal Hypertension, Maternal Smoking, SEX/OCP use, Stressful life events, and Weight gain in pregnancy.





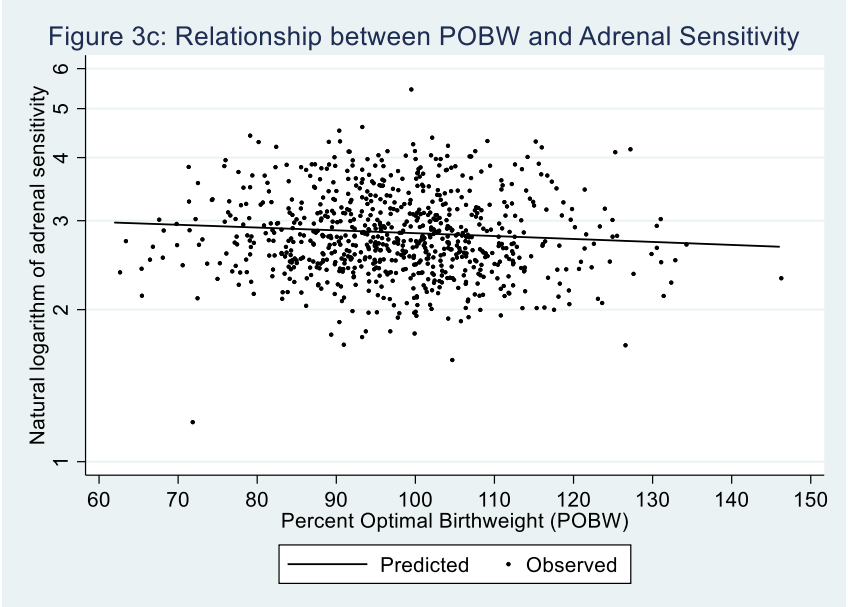


Figure 3a, 3b and 3c: Linear associations between per cent optimal birthweight (%) and measures of HPA-Axis function in adulthood (nmol/L). Blue solid lines represent fully adjusted models. Overlaid maroon circles represent univariate relationships between per cent optimal birthweight and the natural logarithms of a) Baseline cortisol (β=-0.0034, CI: -0.0064 to -0.0005, p=0.02) b) cortisol area under the curve above ground (β=-0.0033, CI: -0.0059 to -0.0009, p=0.01) and c) adrenal sensitivity (β=-0.0039, CI: -0.0068 to -0.0010, p=0.01).

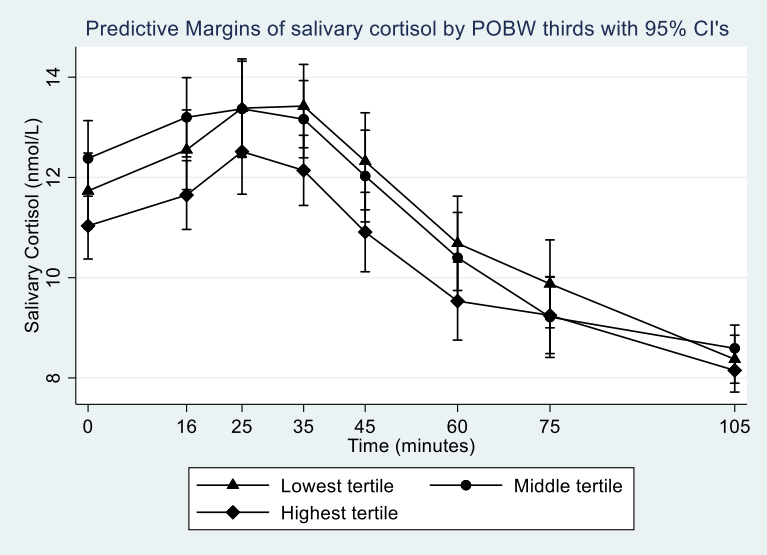


Figure 4a, 4b and 4c: Mixed models partitioned by per cent optimal birthweight tertiles, demonstrating changes in salivary and plasma cortisol as well as serum ACTH, before and after the administration of the Trier Social Stress Test. Concentrations predicted based on fully adjusted models. Bars represent 95% confidence intervals. No significant differences were observed between POBW tertiles in the cortisol response to stress (all p>0.06 for interactions; see table 4).