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BLOOD LEVELS OF MATERNAL SERUM CORTICOTROPIN-RELEASING HORMONE (CRH) AT MID-PREGNANCY: WHICH MATERNAL CHARACTERISTICS ARE INFLUENTIAL?

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ABSTRACT

BLOOD LEVELS OF MATERNAL SERUM CORTICOTROPIN-RELEASING HORMONE (CRH) AT MID-PREGNANCY: WHICH MATERNAL CHARACTERISTICS ARE INFLUENTIAL?

By

Yumin Chen.

The objective of this study was to examine the influence of maternal demographics, anthropometrics, behavioral and psychological factors on mid-pregnancy blood CRH levels by focusing only on women with uncomplicated pregnancies (401 non-Hispanic White, 345 African-American women) in the Pregnancy Outcomes and Community Health (POUCH) Study. Maternal serum at 15-27 weeks' gestation and maternal factors were obtained at the time of enrollment. Regression models, weighted for the subcohort sampling scheme, were constructed to evaluate the associations between maternal factors and log transformed CRH pg/ml levels (dependent variable). Race, pre-pregnancy BMI, maternal education, maternal age, Medicaid status, smoking status during pregnancy and maternal depressive symptoms/psychotropic medication were significantly associated with CRH levels after adjusting for gestational week at blood sampling. Multivariate analyses demonstrated substantially lower CRH levels in African-American women vs White women (mean difference=-0.41, $P<0.01$), women with top quartile vs bottom quartile BMI (mean difference=-0.25 $P<0.01$), and women with $\leq$ 12 years vs > 12 years education (mean difference=-0.13, $P<0.05$). Women with either high levels of depressive symptoms or exposure to psychotropic medications exhibited significantly lower CRH levels (mean difference=-0.14, $P<0.01$).

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KEY TO ABBREVIATIONS

Abbreviation	Meaning
CRH	Corticotropin-releasing hormone
ACTH	Corticotropin
HPA	Hypothalamic-pituitary-adrenal
CRHBP	Corticotropin binding proteins
CRH1α	Corticotropin-releasing hormone type 1 receptor
NO	Nitric oxide
DHEAS	Dehydroepiandrosterone sulfate
COX-2	Cyclooxygenase 2
PTD	Preterm delivery
PTL	Spontaneous preterm labor
PROM	Spontaneous preterm premature rupture of membranes
MIND	Medically indicated preterm birth
White	Non-Hispanic White
BMI	Body mass index
SGA	Small for gestational age
AGA	Birth weight appropriate for gestational age
POUCH	Pregnancy Outcomes and Community Health
EPDS	Depression Scale
MSAFP	Maternal serum alpha-fetoprotein
MOM	Multiples of the median
CES-D	Center for Epidemiological Studies-Depression Scale
LMP	Last menstrual period
US	Ultrasound

Literature review

HPA axis

Corticotropin-releasing hormone (CRH), a polypeptide hormone, is a 41-amino acid neurotransmitter involved in stress responses to stressors[1]. It is produced by neuroendocrine cells in the paraventricular nucleus of the hypothalamus and then travels to the pituitary gland, where it stimulates the secretion of corticotrophin (ACTH), which in turn stimulates the production of glucocorticoid hormones (mainly cortisol in humans) in the adrenal cortices [1]. Release of CRH from the hypothalamus is influenced by stress and negatively controlled by cortisol and ACTH levels in blood. A complex set of direct and indirect connections between the hypothalamus, the pituitary gland and the adrenal gland is referred to as the HPA axis (hypothalamic-pituitary-adrenal axis) [1]. The HPA axis is a major neuroendocrine system that involves in the reaction to stress and regulates various body processes including digestion, the immune system, mood, and energy usage [1].

Placental CRH levels and functions

Although the HPA axis is a feature of other vertebrates as well as mammals, the production of CRH by the placental syncytiotrophoblast is a distinct characteristic of primates, with maternal blood CRH levels primarily of placental origin [2]. During pregnancy, most circulating placental CRH is attached to CRH binding proteins (CRHBP), and their bioactivity is restricted. Placental CRH, which enters maternal blood, functions the same way in the HPA axis as does hypothalamic CRH. A distinct characteristic which dramatically differentiates placental CRH from hypothalamic CRH is the positive feed-forwards system of placental CRH in pregnant women [2, 3]. Cortisol

induced from the fetal and maternal adrenal glands by placental CRH stimulates further placental CRH production. This positive feed-forward loop has been demonstrated through in vitro studies[4, 5]. CRH levels have been investigated in maternal plasma and amniotic fluid, showing that maternal plasma CRH levels increase from 51 pmol/l at 25-32 weeks' gestation to 375 pmol/l at 33-40 weeks. Levels decrease markedly after delivery, indicating that the exponential increase of maternal CRH with advancing pregnancy peaks at the time of delivery [6]. In women who deliver preterm, the exponential increase is rapid, whereas for women who deliver after the estimated due date, the rise is slower [7-9]. Additionally, CRHBP levels in plasma drop markedly at the end of the pregnancy, thereby increasing the bioactivity of CRH [2, 10, 11]. Even though all the above findings suggest that a placental derived CRH may be involved in determining the onset of delivery, the mechanisms by which CRH leads to delivery are still incompletely understood. Furthermore, CRH levels in maternal plasma cannot explain the whole story of preterm birth, which may result through many suspected pathways occurring independently or collaboratively.

CRH acts primarily by binding to the CRH type 1 receptor (CRH1α), a member of the seven-transmembrane G protein receptor family [2]. CRH receptors are present in the pituitary and the adrenal glands in both the mother and the fetus, the myometrium and perhaps the lungs of the fetus. Therefore, rising levels of CRH can act at multiple sites in the mother and fetus to initiate the changes for parturition. Although CRH has no direct effects on the myometrial contraction, once bound to CRH1α at term it may change CRH1α to a form that is less effective in activating relaxation pathways in myometrium [12, 13]. In addition, CRH has been reported to potentiate the contractile effects of

several uterotonins, such as oxytocin and prostaglandin F2α, which is proposed as an alternative pathway for CRH to affect the myometrium [14, 15].

In vitro studies have demonstrated that exogenous CRH significantly promotes human trophoblast proliferation in first-trimester primary cultures. In vitro studies also suggest that uterine CRH acts in local immune processes associated with early pregnancy [16-18]. Thus, it has been postulated that CRH might have an important role in early placental development and early pregnancy tolerance. High levels of CRH in mid-pregnancy have been associated with some obstetric complications, such as fetal growth retardation and preeclampsia [19-21]. In vitro studies have deeply explored these associations and concluded that CRH functions as a potent vasodilator in human fetal-placental circulation, and that increased levels of CRH may result from low oxygen perfusion in the placenta[22]. However, CRH-induced vasodilation of fetal-placental vascular system, an effect partially mediated by nitric oxide (NO) [22], was completely blocked during low oxygen perfusion, and thus couldn't compensate for a poor placental oxygen perfusion in those complications [22, 23].

Placental CRH is secreted predominately into maternal blood, but it also enters the fetal circulation. Although the concentrations are lower in the fetal circulation than in the maternal circulation, CRH plays a very important role in fetal growth. With advancing pregnancy, the increased levels of CRH stimulate the maturation of the fetal pituitary and the adrenal gland, resulting in increases in ACTH and cortisol production. It also stimulates the fetal adrenal zone to produce dehydroepiandrosterone sulfate (DHEAS), which is converted to estrogen by the placenta [2]. In turn, the rising concentrations of cortisol in the fetus further increase the placental CRH production in in vitro studies [4].

Meanwhile, increased CRH levels in the fetus may promote the maturation of fetal lungs by promoting the production of surfactant protein A and phospholipids [24]. Increased cortisol and surfactant proteins activate inflammation pathways in the amnion, leading to both cervical softening and myometrial activation involving progesterone withdrawal and increased cyclooxygenase 2 (COX-2) production [2, 25, 26]. Fetal growth and myometrial activation combined with progesterone withdrawal further promote uterine contraction.

Maternal serum CRH levels and PTD

Preterm delivery (PTD) is often categorized into spontaneous preterm labor (PTL), spontaneous preterm premature rupture of membranes (PROM) and medically indicated preterm birth (MIND). PTD resulting from spontaneous PTL and PROM are usually considered together as spontaneous PTD. Although extensively studied, efficient methods for predicting spontaneous PTD remain undiscovered. Many biomarkers have been linked with PTD and positive associations have been found, but due to the undisclosed complicated mechanisms behind the disease, almost no biomarker can provide both high sensitivity and high specificity. Among all the predictors, CRH has been repeatedly studied, and multiple studies report a strong and consistent association between CRH and PTD [9, 12, 13, 18, 24, 27-30]. In the majority of the studies, CRH was measured in maternal blood collected during the second or third trimester. In some studies, CRH levels predicted preterm birth only at certain intervals of gestational weeks [30], while other studies reported a much wider span of gestational age for prediction; the largest from 18 to 36 weeks' gestation [28]. Maternal CRH levels in Wadhwa *et al.*'s study were found to be a prognostic indicator of spontaneous PTD or PTL. However, in MIND, CRH

levels were a marker of antepartum risk (e.g. urinary tract infection, vaginal bleeding, placenta previa, hypertension, preeclampsia/eclampsia), but not an independent predictor of gestational length [21]. Levels of CRHBP were also measured in several studies. A positive association of CRHBP with PTD was observed in early second trimester, but a negative association was found in the third trimester [10, 11].

Maternal serum CRH levels and fetal growth restriction

CRH is also associated with fetal growth restriction. One study of levels of CRH in maternal plasma and fetal growth restriction was conducted by Wadhwa, *et al.* They reported that high levels of CRH at 33 weeks' gestation were associated with preterm small for gestational age (SGA) births [21]. Another study by Goland, *et al.*, also reported that CRH levels in umbilical cord blood were significantly higher in the growth-retarded fetuses than in normal growth fetuses. They further noted that this pattern tended to happen in cesarean section versus vaginal deliveries, or in those with antecedent labor before cesarean section compared to those without labor [31]. Furthermore, CRH has also been linked with preeclampsia in some studies [19, 20, 32]. Although positive associations have been shown between the birth outcomes and levels of CRH in maternal blood, the underlying mechanisms are still not clearly understood.

Ethnic and social-class disparities of obstetrical outcomes

Ethnic and social-class disparities have been found to be associated with adverse pregnancy outcomes. The difference of prevalence of preterm delivery (PTD) among different race groups has been noted. The rate of PTD among African-American women is twice that of any other racial group of women, and prevalence of PTD in Hispanic women is a little higher than non-Hispanic whites and Asian women in the U.S. [27, 33].

Socio-economic status also plays a role in disparity of PTD. As a proxy of socio-economic status, maternal education has been reported to be associated with PTD. Lower education groups were more likely to deliver preterm compared with more highly educated women in Norway [34]. Marital status also appears to be a risk factor for PTD. Rates of PTD among unmarried mothers versus married mothers were significantly higher. In the unmarried group, non-cohabitating mothers had an even higher rate than cohabitating mothers [27].

Race disparities also exist in other obstetrical outcomes, such as low birth weight. African-American women have a substantially higher risk of delivering low birth weight infants than their white and Hispanic counterparts [35]. US-born African-American mothers tended to have an elevated risk of fetal growth retardation compared to foreign-born Black mothers, but the difference was not significant. However, after controlling for socio-economic and medical characteristics, the racial disparities in fetal growth disappeared [35]. The above associations indicate that demographic factors may partially contribute to ethnic differences of these complications (e.g., PTD, fetal growth retardation, preeclampsia), but the casual pathway leading from demographic factors to these medical conditions remain unclearly understood. Recent studies have disclosed race discrepancies in maternal serum CRH levels, leading us to question whether or not the association between demographic factors and obstetrical outcomes were mediated by placental CRH during pregnancy.

Ethnic differences in maternal serum CRH levels

Race disparities in maternal serum CRH levels have been found in several studies. Holzman, *et al.*, first described ethnic differences in CRH levels in a nested case-control

study [36]. In this study, the association between second trimester maternal plasma CRH levels and preterm delivery was assessed. Maternal blood samples were collected at 15-19 weeks' gestation in 423 pregnant women (97 delivered at less than 35 weeks' gestation, 144 delivered at 35-36 weeks and 244 delivered at term). Ethnic disparities in CRH levels were observed, such that levels were significantly lower in African-American women compared to white women within case and control groups. The results were adjusted for gestational week at prenatal screen.

Significant ethnic differences in plasma CRH levels were also demonstrated by Ruiz, *et al* [37]. In this prospective study, the Hispanic group exhibited lower CRH levels compared with a Caucasian group at two gestational time periods (15-19 weeks and 23-26 weeks' gestation). No differences between African-American and Caucasian women were found because African-American women only accounted for 5% of the study population. The small sample size couldn't provide sufficient power to assess any difference between these two groups. Moreover, the potential confounders, such as maternal age, parity, pre-pregnancy BMI, income and medical risk for PTD were not adjusted in the analysis, which might lead to a doubtable conclusion. In addition, the authors only described the racial differences and didn't discuss potential mechanisms for the discrepancy [37].

Siler-Khodr, *et al.* ,also found race differences in maternal plasma CRH levels between Hispanic and white populations. In this prospective study, significantly lower CRH levels in Hispanic women as compared with non-Hispanic whites were found at 14-18 weeks' gestation. The conclusions were quite reliable due to the large sample size (N=1069), but the analysis only adjusted for maternal weight at sampling. Other

obstetrical factors that may be potential confounders and explain the racial disparities, such as maternal age at sampling, pre-pregnancy BMI, education, and parity were neglected in the study. Furthermore, differences among other ethnic groups couldn't be assessed because African-American women were absent in this study [38].

Ethnic disparities in CRH levels were further investigated by Glynn, *et al* [39]. In this study, maternal serum CRH, ACTH, and cortisol levels were measured at three time points during pregnancy: 18-20 weeks, 24-26 weeks and 30-32 weeks gestation. This study demonstrated a significant ethnic effect of mean CRH levels between African-American women and non-Hispanic whites at two visits (18-20 and 30-32 weeks gestation), with lower levels of CRH exhibited in African-American women. However, differences in levels of CRH between Hispanics and non-Hispanic whites were not reproduced in this study. In addition, ethnic differences of ACTH and cortisol levels were observed at specific time points, and higher levels of cortisol at 18-20 weeks gestation were associated with higher levels of CRH at 30-32 weeks gestation among the African Americans and Hispanic women, but not among non-Hispanic white women. Compared with previous studies, this study had an advantage in that the relationships among multiple measurements of the three biomarkers (serum CRH, ACTH and cortisol) were assessed in analysis such that the evolution of each biomarker could be observed. Moreover, demographic variables such as income, education, maternal age, pre-pregnancy BMI, parity and medical risk for preterm birth, were adjusted in the analysis, suggesting that the conclusions of this study were more convincing. Even though it provided a very detailed conclusion and adjusted analyses, the sample size of this study (N=310) was still not large enough to deeply evaluate the casual pathway of the disparity.

The only null results of ethnic disparity occurred in Mancuso's study [40]. In this prospective study, no significant race difference was found in serum CRH levels in the study participants. The reason for this result is still unclear, but one possible explanation is the small sample size. Of the 282 participants in this study, 43% were African Americans, 32% were Latin Americans and 24% were European Americans. Most likely, the power is not sufficient to differentiae the mean of plasma CRH levels for different ethnic groups [40].

Nonetheless, several studies consistently observed ethnic differences in CRH levels, with African-American pregnant women exhibiting significantly lower plasma CRH concentrations compared to non-Hispanic whites. In contrast, the PTD and SGA rates are substantially higher among African-American women than in any other race group in the U.S. [23, 27, 35]. If higher PTD rates or SGA rates can be explained by high levels of placental CRH concentration, how can we interpret the reversed difference in CRH levels among ethnic groups? Few in vivo studies have addressed functional differences in placental CRH production and release, making it difficult to explain the race/ethnic discrepancy.

Maternal serum CRH levels and depressive symptoms

As a part of the HPA axis, elevated levels of hypothalamic CRH in cerebrospinal fluid have been reported to be associated with melancholic depression [41, 42]. But, the association between placental CRH and prenatal depressive symptoms remains scarcely studied. To our knowledge, there are only two published studies addressing this association. In a prospective study performed by Susman, *et al.* [43], low CRH in early pregnancy was associated with more depressive symptoms in early and late pregnancy,

which is in contrast to the generally accepted knowledge. However, the inverse results were obtained in a special population consisting mainly of low-income adolescents (58 white and 1 African American). CRH concentrations were measured in 9-21 weeks' gestation, which is earlier than the time point in the Pregnancy Outcomes and Community Health (POUCH) study. Depressive symptoms and conduct disorders were evaluated at two time points during pregnancy and once at postpartum, using different measurement criteria from those of the POUCH study. Complicated pregnancies were not identified and thus couldn't be excluded from this study. The author speculated the inverse finding to be a phenomenon unique to adolescent pregnancy, but didn't explore the reasons.

On the contrary, a positive conclusion that elevated placental CRH levels in mid-pregnancy are associated with risk of prenatal but not postpartum depressive symptoms was reported by Rich-Edwards, et al. [44]. In this prospective study, maternal serum CRH levels were assessed at a mean 27.9 weeks' gestation, which is later compared with the blooding sampling in the POUCH study. A 10-item Edinburgh Postpartum Depression Scale (EPDS) was used to assess depressive symptoms at mid-pregnancy and postpartum. As compared with the POUCH study population, participants in this study were older, better educated, and more likely to be White women with relatively low prevalence of obesity and preterm delivery. As compared with Susman's study, one advantage of this study is that women with complicated pregnancies (e.g., preeclampsia, gestational diabetes or gestational hypertension) were excluded and PTD status was controlled in the analysis, making the results more convincible. Although the results of this study are consistent with the currently accepted knowledge about cerebrospinal CRH

levels and major depression in non-pregnant adults [41, 42], it is in contrast to Susman's study. The authors suggested that conflicting results could be to due to chance, different populations, different measures of depression, or possibly different types of depression measured, but didn't identify specific reasons. Furthermore, the authors didn't exclude from their study women with obstetric complications associated with high CRH levels, and as a result couldn't exclude a spurious conclusion.

Conclusion

Given the importance of this biomarker, relatively little is known about maternal characteristics and pregnancy circumstances that might influence CRH levels during pregnancy. With the exceptions of race/ethnic and maternal affect, no other maternal characteristics have been carefully examined in relation to maternal blood CRH levels. Further examining the potential pathways contributing to the variation of CRH levels is absolutely necessary in modeling CRH levels in pregnancy and risk of adverse outcomes.

Blood levels of maternal serum corticotropin-releasing hormone (CRH) at mid-pregnancy: Which maternal characteristics are influential?

Introduction

Corticotropin-releasing hormone (CRH), produced by the hypothalamus, is a component of the HPA axis (hypothalamic-pituitary-adrenal axis). Hypothalamic CRH stimulates the secretion of corticotropin (ACTH) which in turn increases cortisol production and release from the adrenal glands. ACTH and cortisol levels exert a negative feedback on hypothalamic CRH. The HPA axis is a major neuroendocrine system involved in the stress response and in various body processes including digestion, the immune system, mood, and energy usage [1].

The production of CRH by the placenta is a distinct characteristic of primates, and maternal blood CRH levels during pregnancy are primarily of placental origin. Evidence suggests that cortisol from fetal and maternal adrenal glands stimulates placental CRH production in contrast to cortisol's negative feedback effects on hypothalamic CRH [2, 4, 5]. Levels of CRH increase exponentially with advancing pregnancy and peak at the time of delivery [6]. In women who deliver preterm, the exponential increase is rapid, whereas for those who deliver after the estimated due date, the rise is slower. Some investigators hypothesize that CRH plays a critical role in the onset of parturition [7, 9]. Elevated maternal serum CRH levels in the 2nd and 3rd trimesters have been positively associated with adverse obstetrical outcomes such as preterm delivery (PTD), low birth weight and preeclampsia [8, 11, 18-20, 23, 27, 28, 31, 44]. Given the importance of this biomarker,

relatively little is known about maternal characteristics and pregnancy circumstances that might influence its levels in uncomplicated as well as complicated pregnancies.

In the few studies that have examined race/ethnicity in relation to maternal blood CRH levels, African-American[36] and Hispanic women were found to have significantly lower levels of CRH [37, 38]. However within each racial/ethnic group, high CRH levels remained associated with adverse pregnancy outcomes. Maternal stress/mood disorders with resultant HPA axis dysregulation may influence blood CRH levels in pregnancy, but again this association has received minimal investigation. Currently there are reports of both positive and inverse relations between levels of depressive symptoms and/or pregnancy-related anxiety and CRH levels in pregnancy [40, 43-45]. Data from one study suggested that smoking might be linked to higher levels of blood CRH in the 2nd trimester, but in the final adjusted analysis there was no significant association [46].

The aim of this study was to investigate maternal demographics, anthropometrics, behavioral and psychological factors that might influence maternal CRH levels in mid-pregnancy. To separate these influences from pathologic conditions, the focus was on women with uncomplicated pregnancies i.e., term deliveries (>=37 weeks gestational age), birth weight appropriate for gestational age (AGA), and no maternal hypertension before/during pregnancy.

Material and Method

Study design and population

The Pregnancy Outcomes and Community Health (POUCH) study recruited pregnant women from August 1998 to June 2004 in 52 clinics from five Michigan communities.

The inclusion criteria were maternal age >15 years, English-speaking, singleton pregnancy without known congenital anomaly, screening for maternal serum alpha-fetoprotein (MSAFP) between 15-22 weeks' gestation and no history of pre-pregnancy diabetes. Women were enrolled in the 15^{th}-27^{th} week of pregnancy. Those with unexplained high MSAFP ($\geq$ 2 multiples of the median (MOM)) were oversampled (i.e. 7% of the cohort), because MSAFP was a biomarker of particular interest to the POUCH study. Of the 3,038 women enrolled, 3,019 participants (99.4%) who had consented completed the study.

Gestational age was determined by the first day of the last menstrual period (LMP) or early ultrasound (US) data. US data was used only when a gestational age based on LMP differed from that estimated by US examination. PTD was defined as deliveries before completion of 37 weeks' gestation.

Data from POUCH study participants were compared with data recorded on birth certificates from the five study communities in 2000, POUCH Study women were similar to community mothers with respect to factors such as age, parity, education levels, the proportions with Medicaid insurance, and prevalence of preterm delivery, previous stillbirth and previous low birth weight infants. The only exception was that the percentage of African-American women over 30 years of age in the POUCH study was lower than that found in birth certificates.

Cohort women were stratified by ethnicity (i.e. African-American and White/Other), and by MSAFP levels (i.e. normal < 2 MOM, unexplained high $\geq$ 2 MOM). A sub-cohort was sampled from the cohort by including all the women who delivered preterm, all the women with high MSAFP, and a random sample of women with term deliveries and

normal MSAFP with over-sampling of African-Americans in this latter group. Among the 1371 sub-cohort women, 30 were excluded from this analysis due to either a missing or a low volume blood sample. The focus of this study was on maternal factors that influence CRH other than pregnancy complications; therefore, another 535 sub-cohort women were excluded who had PTD (delivery at <37 completed weeks), preeclampsia, chronic hypertension, gestational hypertension, or an infant small for gestational age (SGA). SGA was defined as <10th percentile birthweight for gestational week based on gender-specific birthweight distributions in singletons [47]. Of the remaining 806 participants, 33 Hispanics, 15 Asians and 12 women of 'other race/ethnic' background were excluded due to the small numbers and because their CRH levels appeared unlike those of African-Americans and Whites (Figure 1). After all exclusions the analyses included 345 African-American women and 401 White women who delivered term, non-SGA infants.

Maternal factors

Cohort women were interviewed at enrollment by study nurses in their respective communities, and information about demographics, current pregnancy, reproductive history, health behaviors, social and psychological factors were collected. Maternal race was determined by self-report. Depressive symptoms were assessed using the Center for Epidemiological Studies-Depression Scale (CES-D), a well-established screening tool [48]. The CES-D score was cut at $\geq$ 16, the typical cut point for a positive depression screen. Women also reported types of psychotropic medicines taken during pregnancy (up through the time of enrollment) by responding yes or no to three separate categories of medicines, psychiatric meds, tranquilizers/sedatives, and sleeping pills. The CES-D

dichotomous variable was combined with information on psychotropic medication use to create a two-level variable: Low CES-D (<16) and no psychotropic medication versus High CES-D (>16) and/or use of psychotropic medication. This combined variable was created to increase ascertainment of women with negative affect disorders. Screening tools measure mood over a brief period and psychotropic medication use reflects chronic affect conditions. Maternal weight and height were determined at enrollment and body mass index (BMI) was calculated as Kg/m^2.

Blood collection and laboratory assay

Plasma samples were collected in EDTA tubes at enrollment and then chilled briefly before being processed in a cooled centrifuge (-4C), aliquoted, and stored at -80C until assayed. Methanol (3.5ml) was used to extract CRH from serum (0.5ml). The precipitate was separated by centrifugation and the methanol-extracted CRH was dried. The residue was suspended in assay diluent (250ul) before the assay. CRH was measured using a specific and sensitive radioimmunoassay similar to that described by Siler-Khodr et al for GnRH [49], except CRH in these samples was first extracted free of the CRH binding protein as described. Aliquots of resuspended extract were assayed in duplicate. Antiserum to CRH (TS-6) (100uL) and CRH samples or a standard CRH (100uL) were preincubated for 2 days at 4 C, and then 125I-CRH (10pg/mL) was added. Tyr-CRH was radioiodinated by the method of Hunter and Greenwood [50]. After addition of a label, incubation was continued for 3 days at 4C. Separation of bound and free CRH was done with antirabbit gamma globulin conjugated to magnetic beads. Assay sensitivity was 5 pg/tube or approximately 30pg/mL when corrected for extraction loss. Intra-assay and interassay coefficients of variation were 3% and 10% respectively [36].

Analytic strategy

The overall analytic strategy was to first examine each maternal characteristic in relation to CRH levels, and then build relevant multivariate models. Sampling weights were applied to account for over-sampling of women with unexplained high MSAFP into the cohort, and the sampling scheme used to construct the sub-cohort. Gestational week at blood sampling was included as a covariate in all analyses to remove its effect on CRH levels.

CRH levels were transformed using the nature log to correct for positive skewing. All the maternal factors were modeled as categorical variables. Regression models with sub-cohort sampling weights were constructed in which log CRH levels were considered as the dependent variable. Maternal characteristics (i.e., race, maternal education, Medicaid Insurance status, maternal age, BMI, smoking status during pregnancy, parity/PTD history, depressive symptoms/psychotropic medicine use) were evaluated one by one in association with log CRH levels using the SAS Surveyreg procedure [SAS 9.01; SAS Institute, Cary, NC]. Adjusted (for gestational age at sampling) mean log CRH levels were calculated for categories of each maternal factor. Maternal characteristics related to log CRH levels (i.e., a criterion of $P \leq 0.20$) entered into a multivariate analysis. Criteria for eliminating variables included: 1) P value > 0.10 in the likelihood ratio test; and 2) removal of the variable did not affect adjusted mean of retained variables by more than 15%. Three variables were strongly correlated (e.g., maternal age, Medicaid Insurance status and maternal education), and when included in the same model were not statistically significant. Further modeling (stepwise and backwards elimination) showed that maternal education was the only demographic variable of the three to be retained,

and the reduced models produced the best fit (assessed based on AIC and BIC values). We also assessed all the potential covariate interactions.

Results

The distributions of selected characteristics for the sample are provided in Table 1. Log CRH levels were normally distributed with a range of 1.95-6.59 CRH pg/ml and a mean of 4.12 pg/ml (SD=0.81 pg/ml). The mean log CRH was significantly associated with race, maternal education, Medicaid status, maternal age, smoking during pregnancy, BMI and depression symptoms and psychotropic medications, after adjusting for gestational age at blood sampling (Table 1). A subgroup analysis was conducted with women who had the week of pregnancy determined mainly according to ultrasound examinations to test whether race/ethnic differences in CRH levels might be explained by misclassification of gestational age at blood sampling. Results showed that the CRH-Race association persisted and was not attenuated (data not shown).

In multivariate analyses the adjusted mean log CRH levels remained significantly higher in White/Asian versus African-American women, but there was a 20% decrease in the race difference (Final Model) from that observed in the previous model that adjusted only for gestational age at blood sampling (Table 2). The race difference in mean log CRH went from 0.49 to 0.41pg/ml (Table 2) after adjusting for maternal factors. BMI was inversely associated with adjusted mean log CRH levels. The most extreme example was the comparison between the highest and lowest quartiles of BMI (difference in adjusted means=-0.25pg/ml, $P<0.0001$). Women with less than or equal to 12 years of education exhibited lower CRH levels than those of women with more than 12 years of education (difference in mean log CRH= -0.13pg/ml, $P<0.05$). Compared to women with

lower levels of depressive symptoms and no psychotropic medicine use in the first half of pregnancy, women with either high levels of depressive symptoms or exposure to psychotropic medications had lower CRH levels (difference in mean log CRH= -0.13pg/ml, $P<0.05$). Medicaid Insurance status, maternal age, and smoking were no longer significantly associated with blood CRH levels after adjusting for other maternal factors.

For depressive symptoms/psychotropic meds and log CRH levels there was a suggestion of an interaction by race ($P=0.12$). The inverse association was stronger for White women (difference in mean log CRH= -0.20pg/ml, $P=0.01$) compared with African-American women (Mean difference=-0.02pg/ml, $P>0.05$). All other covariate interactions had P value great than 0.20.

Discussion

Previous studies have mainly concentrated on maternal CRH levels in association with pregnancy complications [8, 11, 18-20, 23, 27, 28, 31, 44] or as a potential mediator of parturition [7-9, 12-15]. We found that mid-pregnancy CRH levels were also related to maternal characteristics in uncomplicated pregnancies. As reported in two other studies [36, 39], African-American women in this sample had significantly lower mid-pregnancy CRH levels. The race differences in maternal blood CRH levels were attenuated but not eliminated after adjusting for other maternal factors such as education, BMI, depressive symptoms, and psychotropic medication use in pregnancy.

Few in vivo studies have addressed functional differences in placental CRH production and release, making it difficult to explain obscured race differences in maternal blood CRH levels. Some studies have examined the race differences in HPA

axis function and found that among non-pregnant women exposed to physical exercise or administered ovine CRH, African-Americans had higher levels of ACTH than that in Whites [51, 52]. However, race differences in ACTH levels did not result in race differences in cortisol levels, suggesting variation in regulation points along the HPA axis, perhaps in response to chronic stressors. At least one study has noted race differences in a CRH related gene [53]. The relevancy of these studies to race/ethnic differences in placental CRH levels remains uncertain.

In this study obese women exhibited lower blood CRH levels when compared to leaner women. The inverse association between BMI and CRH levels may represent a dilution effect, a phenomenon observed with other pregnancy biomarkers such as MSAFP [54]. The dilution effect could be underestimated given that high BMI increases the prevalences of certain placental vascular problems [55, 56] which in turn have been linked to increases in maternal blood levels of CRH [28, 32] (Figure 2). Though we excluded women with clinical signs of vascular complications, women remaining in our analyses may have had subclinical placental vascular pathology that influenced CRH levels and was related to BMI. Our findings emphasize the need to consider BMI as a confounding element as well as a potential component in a causal pathway when evaluating CRH levels.

Maternal education was the socio-economic measure most strongly associated with maternal CRH levels in mid-pregnancy. It followed the same direction as that of race, both suggesting that social disadvantage is aligned with lower CRH levels. Having also examined delivered placentas as part of the POUCH study protocol, we looked to see if a decrease in placental size might link low education or race to lower CRH levels but found

no support for this hypothesis (data not shown). Previous studies have noted a smoking-HPA association in non-pregnant populations, but this correlation was established based on the measurement of cortisol and/or ACTH [57, 58]. Our results agreed with the one previous study [46] of smoking and CRH levels in pregnant women, showing no association in final models.

Two studies have assessed the association between maternal depressive symptoms and blood CRH levels during pregnancy [43, 44]. Our results are most consistent with that of Susman et al showing lower CRH levels among women with more depressive symptoms. In contrast Rich-Edwards et al noted a positive correlation between maternal CRH levels in mid-pregnancy and prenatal depressive symptoms [44]. The contrasting findings may be due to variations in populations studied, exclusion criteria, tools used to measure depressive symptoms, gestational age at blood sampling, and covariates used in modeling. Susman's study included 59 low-income, predominantly white adolescents. Rich-Edwards's et al's sample had a large percentage of older, well-educated, White women with relatively low prevalences of obesity and preterm delivery. Complicated pregnancies were either controlled by or excluded from the analysis in Rich-Edwards's study, but not mentioned by Susman et al. The hypothesis that higher maternal blood CRH levels will be detected in pregnant women who are depressed stems from a series of observations. Elevated levels of hypothalamic CRH in cerebrospinal fluid have been reported to be associated with melancholic depression [41, 42]. In addition, there appears to be a change in HPA axis regulation in some people experiencing depression which is marked by either unusually high [59, 60] or unusually low [61] cortisol levels. But these depression-cortisol associations have been difficult to demonstrate in non-clinical

samples [60]. Finally, in vitro studies have found that cortisol can stimulate CRH production in placental tissue [4, 5]. With so many factors influencing depressive symptoms, cortisol levels and CRH levels in pregnancy, the interrelations between these measures will be difficult to unravel. Add to this the influence of psychotropic medicines used to treat depressive symptoms and the picture becomes even more complicated. Two studies have reported that women with high levels of prenatal anxiety or stress, conditions that often overlap with depressive symptoms, show increases in CRH levels during pregnancy [40, 45]. But women can have prenatal anxiety due to complications associated with elevated CRH; disentangling the order of these factors presents a challenge.

A major strength of our study was the composition of the cohort, which was sampled from diverse settings representing a wide range of socioeconomic backgrounds. In addition, maternal CRH levels, depression symptoms and BMI were measured at the same time in pregnancy. By focusing only on women without pregnancy complications we reduced the likelihood that pathology and maternal pregnancy-related concerns would operate as confounders. The range of available variables allowed for a deeper probing of potential explanations for previously observed race differences in CRH levels during pregnancy.

One limitation is that maternal CRH levels and depressive symptoms were measured only once, thereby precluding the chance to examine time-order and changes in the association across the span of pregnancy. Our assessment of depression was limited to a screening tool, the CES-D, which lacks specificity and represents a very brief period of symptoms (the past week). To address this brief period we also included any woman who

used psychotropic medications during pregnancy prior to enrollment, a potential indicator of treatment for affect disorders. However, we did not have detailed data on timing of use or self-reports of specific anti-depressant medications used. We included a broad variety of psychotropic medications give description to enhance sensitivity at the expense of specificity.

Because CRH in pregnancy has received considerable attention, it is important to identify factors such as race/ethnicity, education level, BMI, and maternal affect that are associated with maternal levels of this biomarker. This allows for more careful modeling of CRH in relation to hypothesized pathways involving pregnancy complications. In addition it offers clues to the complexity of interpreting CRH levels and motivates future studies. The observed lower CRH levels in relation to social disadvantage (race, education), when in fact it is high CRH that marks elevated risk, remains an enigma that deserves further exploration.

Table 1.Maternal characteristics and log CRH levels in women delivered at term†

	N (%)	Adjusted Mean log CRH(pg/ml)**
Race*		
Whites	401 (53.8)	4.32
African-Americans	345 (46.2)	3.83
Maternal Education (years)*		
<12 years	164 (21.0)	3.94
>=12 years	582 (78.0)	4.25
Medicaid*		
Yes	411 (55.1)	4.04
No	335 (44.9)	4.34
Maternal Age (years)*		
<20	120 (16.1)	3.99
20-29	433 (58.0)	4.19
>=30	193 (25.9)	4.32
BMI Quartiles*		
Q1 (Bottom)	175 (23.5)	4.31
Q2	185 (24.8)	4.33
Q3	194 (26.0)	4.18
Q4 (Top)	192 (25.7)	3.98
Smoking during pregnancy*		
Did not smoke during pregnancy	610 (81.8)	4.05
Smoked during pregnancy	136 (18.2)	4.23
Depressive symptoms and psychotropic meds history*††		
CES-D<16 and Meds: No	440 (59.4)	4.30
CES-D>=16 or Meds: Yes	301 (40.6)	4.04
Parity/PTD History		
No Previous Live Birth	291 (39.0)	4.22
Previous Live Birth no PTD	431 (57.8)	4.20
Previous Live Birth PTD	24 (3.2)	4.13

*P<0.05 ANOVA test of mean log CRH levels in women with term delivery

* Weighted for sample scheme and adjusted for gestational week at blood sampling

† Excludes women with preeclampsia, gestational hypertension, and infants small for gestational age

†† Data missing for 5 women

Table 2: Mean differences of log CRH (95%CI) by maternal characteristics in women delivered at term†

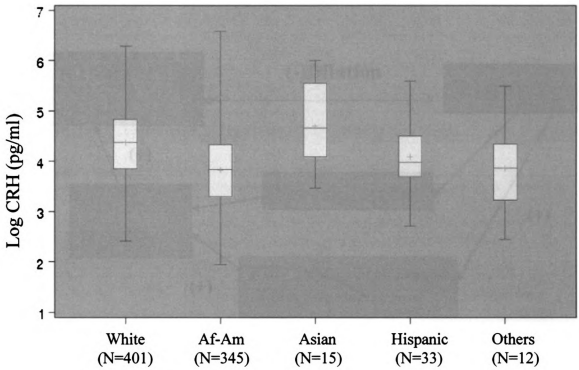
Independent Variables		**Final Model††**
	Adjusted mean difference in log CRH pg/ml#	Adjusted mean difference in log CRH pg/ml
Race (White vs Af-Am)	0.49 (0.39, 0.59)*	0.41 (0.30, 0.51)*
Maternal education (<12 vs >=12)	-0.31 (-0.44, -0.18)*	-0.13(-0.26, -0.005)*
BMI (Q_2 vs Q_1)	-0.02 (-0.18, 0.14)	-0.03(-0.18, 0.12)
(Q_3 vs Q_1)	-0.14 (-0.29, 0.01)	-0.13(-0.28, 0.01)
(Q_4 vs Q_1)	-0.35 (-0.50, -0.19)*	-0.25(-0.40, -0.10)*
CES-D>=16 or Meds:yes vs CES-D<16 and Meds:no	-0.26(-0.37, -0.15)*	-0.16 (-0.27, -0.05)*
Medcaid (Yes vs No)	-0.30(-0.41, -0.19)*	
Maternal age (< 20 vs 20-29)	-0.20(-0.37, -0.03)*	
(≥30 vs 20-29)	0.14(0.01, 0.26)*	
Smoking During Pregnancy (Yes vs No)	-0.19(-0.32, -0.05)*	
Parity/PTD History (Previous live birth no PTD vs No birth)	-0.02(-0.13, 0.09)	
(Previous live birth PTD vs No birth)	-0.09(-0.62, 0.44)	

* P<0.05

Adjusted for gestational age at blood sampling

† Excludes women with preeclampsia, gestational hypertension, and infants small for gestational age

†† Adjusted for gestational age at blood sampling and all other variables listed in the table



Graph1: Distribution of log CRH in Ethnic groups

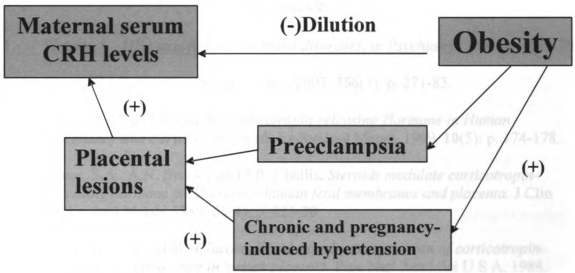


Figure 2: Causal pathway from Obesity to maternal serum CRH levels

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