First Trimester Maternal Cortisol Signatures in Small-for-gestational Age

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Abstract

Background: Abnormal maternal hypothalamus-pituitary-adrenal axis is associated with fetal growth, and we hypothesized that the alteration in metabolic signatures of cortisol might be detectable during early pregnancy. The objective of this study was to identify predictable maternal serum signatures of cortisol metabolism during the first trimester of women who are expected to deliver small-for-gestational age (SGA) neonates.

Methods: This prospective cohort study included 112 pregnant women (with and without SGA, n = 56 each). Maternal serum samples were collected at 10~14 gestational weeks to quantify the levels of cortisol and its precursors and metabolites by liquid chromatography-mass spectrometry.

Results: Increased maternal serum levels of tetrahydrocortisol (THF, 11.82 ± 8.16 ng/mL vs. 7.51 ± 2.90 ng/mL, $P < 0.005$) and decreased 21-deoxycortisol (21-deoxyF, 2.98 ± 1.36 ng/mL vs. 4.33 ± 2.06 ng/mL, $P < 0.0001$) were observed in pregnant women carrying SGA fetus. In conjunction with individual steroid levels, metabolic ratios corresponding to the activity of related enzymes were calculated. In addition to increased THF/cortisol ratio ($P < 0.006$), the SGA group showed a significant increase in the two metabolic ratios including cortisol/11-deoxycortisol (F/11-deoxyF; $P < 0.03$) and cortisol/21-deoxycortisol (F/21-deoxyF; $P < 0.0003$) indicating cortisol biosynthesis. The ROC curve generated in combination with three variables of 21-deoxyF concentration and two metabolic ratios of F/21-deoxyF and THF/F resulted in AUC = 0.824 (95% confidence interval, 0.713 ~ 0.918).

Conclusions: A significant decrease in maternal serum levels of 21-deoxyF and an increase in two metabolic ratios of F/21-deoxyF and THF/F, indicating cortisol biosynthetic rate, represent a reliable biomarker for the prediction of SGA in the first trimester.

Introduction

Small-for-gestational age (SGA) neonates are defined as small babies with birth weights below the 10th percentile for babies in the same gestational condition. The SGA is associated with not only an increased risk of stillbirth and neonatal mortality, but also adverse lifelong health-related outcomes [1, 2]. Pregnant women carrying SGA fetus warrant clinical attention such as intensive fetal surveillance to reduce the risk of fetal compromise. Therefore, the accurate prediction of SGA is essential for clinical management. Several predictable biomarkers have been suggested, but they provide poor sensitivities and/or specificities [3]. It is difficult to distinguish between pathological and constitutional SGA, although it is an important issue in the management of SGA condition [4].

The maternal hypothalamic-pituitary-adrenal (HPA) axis is activated during pregnancy, resulting in increased cortisol production. The intense activity of placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which metabolizes cortisol into cortisone, ensures the control of biologically active glucocorticoid levels in the fetus compared with those of the pregnant female, and protects the fetus from excessive cortisol levels [5, 6]. Despite the altered adrenal function of newborns with intrauterine

growth restriction (IUGR) within the first several weeks of life [7], increased cord blood levels of cortisol were detected in SGA fetus and newborn [8, 9], while low-birth weight preterm infants had decreased cortisol levels compared with full-term normal babies [10, 11]. Cortisol metabolism in pregnant women carrying SGA fetus was altered, but the increased levels of maternal serum cortisol were not statistically significant [12, 13].

Since antenatal overexposure to glucocorticoids is one of the key factors underlying early life programing of disease [14], maternal abnormalities involving the HPA axis can influence fetal development and birth outcomes. Among the currently available diagnostic procedures [4], non-invasive serum analysis for predictable detection of cortisol levels at an early stage of pregnancy enables intrauterine homeostasis of cortisol metabolism. Here, liquid chromatography-mass spectrometry (LC-MS)-based quantitative profiling [15] was extensively used to identify metabolic signatures of cortisol in maternal serum samples obtained from SGA group in the first trimester. The main objective of this study was to establish whether altered cortisol metabolism in maternal serum during the first trimester was an appropriate tool for assessing the risk of SGA infant delivery.

Materials And Methods

Study design and etiology of SGA

Current case-control study population included 56 pregnant women who provided fasting serum samples at 10–14 weeks of gestation and subsequently delivered SGA neonates between 2015 and 2018. SGA was defined as birth weight less than 10 percentile for each gestational age at delivery according to Korean birth weight standard of reference [16]. The control group included 56 pregnant women with a fasting serum sample drawn at 10–14 weeks and who delivered non-SGA neonates. Control subjects were matched for gestational age at sampling and clinical variables, which affected fetal growth (maternal age, parity, maternal height and weight) based on propensity score analysis. Patients with gestational diabetes, preeclampsia and preterm birth were excluded.

This study was approved by the Institutional Review Board of the Seoul National University Hospital (approval number 1606-051-770). Patients provided written informed consent for the sample collection and use of biologic materials for research purposes. All experiments were performed in accordance with relevant guidelines and regulations.

Chemicals

Cortisol and its 2 precursors and 7 metabolites (Fig. 1) were purchased from Sigma (St. Louis, MO, USA), Steraloids (Newport, RI, USA), and Wako (Osaka, Japan). An internal standard of 9,11,12,12,-d_4-cortisol was obtained from C/D/N isotopes (Pointe-Claire, QC, Canada). The stock solutions of 10 reference standards were prepared at a concentration of 1 mg/mL in a mixture of high-performance liquid chromatography (HPLC)-grade methanol and chloroform (9:1, *v/v*; Burdick & Jackson, Muskegon, MI, USA). Working solutions were prepared at concentrations ranging from 0.02 to 1 μg/mL. All standard

solutions were stored at −20°C until required. A commercially available steroid-free serum sample (SCIPAC, Sittingbourne, UK) was further processed for both calibration and quality control (QC) as described in our previous method [17] and used after confirming the lack of endogenous steroids.

Analytical parameters

LC-MS was performed using a Nexera UHPLC system (Shimadzu Corp., Kyoto, Japan) interfaced with an LCMS-8050 triple-quadrupole-mass spectrometer (Shimadzu Corp.). All analytes were separated using a Hypersil Gold C18 column (50 mm × 2.1 mm i.d., 1.9-µm particle size; Thermo Scientific, Waltham, MA, USA) at a flow rate of 250 µL/min using a mobile phase consisting of eluant A [0.1% formic acid (MS-grade, 98% purity; Sigma) in 5% acetonitrile (Burdick & Jackson)] and eluant B (0.1% formic acid in 95% acetonitrile) at ambient temperature.

All adrenal steroids were analyzed and quantified in MRM mode using electrospray ionization (ESI) coupled with polarity switching with a high-speed scan rate of 30,000 u/s. Among the 10 steroids tested, cortisol (F), 6β-hydroxycortisol (6β-OHF) and cortisone (E) were detected in negative ion mode with the MRM screening method, and the remaining 7 steroids were analyzed in the positive ion mode. All peaks were identified via comparison of retention times and matching the area ratios of characteristic ions.

Sample pretreatment

Sample preparation was performed as described previously [15]. Briefly, 200 µL serum was spiked with 20 µL of d_4-F, (0.2 µg/mL) and diluted with 1.8 mL phosphate buffer (0.2 M, pH 7.2). The sample was incubated with 50 µL of β-glucuronidase extracted from *Escherichia coli* (aqueous solution stabilized with 50% glycerol; Roche Diagnostics GmbH, Mannheim, Germany) for 1 h at 55°C. The hydrolyzed sample was loaded on an Oasis HLB cartridge (3 mL, 60 mg; Waters, Milford, MA, USA) preconditioned with 4 mL of methanol, followed by 4 mL of distilled water (Burdick & Jackson) for solid-phase extraction (SPE). The cartridge was washed twice with 10% methanol (0.7 mL). Serum adrenal steroids were then eluted with absolute methanol (1 mL · 2). Combined eluates were evaporated under a stream of nitrogen at 40°C. The dried extract was reconstituted with 50 µL of methanol and centrifuged using an Ultrafree-MC Centrifugal Filter (polyvinylidene fluoride, pore size: 0.1 µm; Millipore, Billerica, MA, USA) for 5 min at 14,000 rpm. Thereafter, 50 µL of 10% dimethyl sulfoxide (DMSO) was added to the Ultrafree-MC filter and centrifuged for 5 min at 14,000 rpm. Finally, an aliquot (5 µL) was injected into the LC-MS system.

Statistical analysis

The data were analyzed using SPSS (v 22.0; SPSS Inc., Chicago, IL, USA) and GraphPad Prism (v. 8.2; GraphPad Software Inc; San Diego, CA, USA). Based on the quantitative results of individual steroids, the ratios of metabolite to corresponding precursor, which indicate enzyme activities, were examined. A non-parametric Mann-Whitney *U* test was used to evaluate the group differences between sterol signatures and laboratory findings. The predictive performance was evaluated according to the receiver operating characteristic (ROC) curves for the 50% training/discovery set split. All quantitative results are expressed as means ± SD and statistical significance was considered at $P < 0.05$.

Results

Clinical characteristics

General characteristics of the study population are listed in Table 1. Clinical parameters such as maternal age, parity, and gestational age/BMI at sampling were not different between the two groups analyzed. The two groups had similar gestational age at delivery, but the birth weight varied significantly between the two groups of cases (2.7 ± 0.2 kg vs. 3.2 ± 0.3 kg, $P < 0.001$).

Table 1
General characteristics of maternal women studied

Variables	SGA (n = 56)	non-SGA (n = 56)	*P* value
Age (year)	32.5 ± 3.8	32.3 ± 3.1	0.659
Nulliparity	26 (46%)	25 (45%)	0.850
GA at sampling (weeks)	13.1 ± 1.5	13.2 ± 1.8	0.411
BMI[a] (kg/m^2) at sampling	21.3 ± 3.0	21.6 ± 3.3	0.623
GAD[b] (weeks)	39.3 ± 1.1	39.1 ± 1.2	0.331
Birth weight (kg)	2.7 ± 0.2	3.2 ± 0.3	< 0.001

[a]Body mass index.

[b]Gestational age at delivery.

*All parameters were measured at the time of blood sampling on 10 ~ 14 gestational weeks.

Serum metabolic signatures of cortisol

Comparative serum levels were observed in SGA samples with increased THF (11.82 ± 8.16 ng/mL vs. 7.51 ± 2.90 ng/mL, $P < 0.005$) and decreased 21-deoxycortisol (2.98 ± 1.36 ng/mL vs. 4.33 ± 2.06 ng/mL, $P < 0.0001$), whereas cortisol and other steroids were not statistically significant (Fig. 1). Metabolic ratios of cortisol and its precursors and metabolites were also evaluated to reflect enzyme activities. One of cortical reduction metabolic ratios, THF/F, was also remarkably increased in the SGA group ($P < 0.006$) (Fig. 2A). Two metabolic ratios, indicating cortisol biosynthesis, of cortisol to 11-deoxycortisol (F/11-deoxyF; corresponding to 11β-hydroxylase, $P < 0.03$) and F/21-deoxyF (corresponding to 21-hydroxylase, $P < 0.0003$) were significantly increased in the SGA group (Fig. 2B and 2C). Other metabolic ratios did not differ between groups studied (data not shown).

Based on quantitative signatures, the ROC curves for discriminate accuracies between SGA and non-SGA groups yielded the highest predictive performance in the area under the ROC curve (AUC) if the three

statistically significant variables of 21-deoxyF and two metabolic ratios of F/21-deoxyF and THF/F were combined (AUC = 0.824, 95% confidence interval, CI, 0.713 ~ 0.918, Fig. 3). Individual signatures of 21-deoxyF, F/21-deoxyF, F/11-deoxyF and THF/F in the ROC curves resulted in 0.711 (0.570 ~ 0.837), 0.693 (0.580 ~ 0.824), 0.624 (0.461 ~ 0.766) and 0.656 (0.517 ~ 0.802), respectively. Other signatures of combinations analyzed via multivariate analysis of 11-deoxyF + 21-deoxyF and 21-deoxyF + F/21-deoxyF were 0.694 (0.559 ~ 0.832) and 0.706 (0.574 ~ 0.829), respectively.

Discussion

The performance of cortisol immunoassays is diminished by allo-THF, 11-deoxyF, 21-deoxyF, 6β-OHF and synthetic glucocorticoids [18]. Therefore, the LC-MS-based profiling was conducted to generate serum cortisol signatures of SGA at 10 ~ 14 gestational weeks and quantify individual glucocorticoid levels. Based on the individual quantities, the metabolic ratios of precursors to their corresponding metabolites were also determined for indirect assessment of comparative enzyme activities.

Progesterone slowly increases from week 9 until week 32 of pregnancy, whereas the serum cortisol level increases significantly in the first trimester and peaks in the second week, followed by a decline in the third trimester [19]. Biologically active free cortisol is generally decreased by the increased corticosteroid-binding globulin; however, in pregnant women the cortisol is replaced by a large quantity of progesterone [20]. Maternal stress is one of the major risk factors for spontaneous abortion during the earliest gestational stages and the increased levels of maternal cortisol are correlated with a higher risk of miscarriage during pregnancy [21]. However, the serum levels of cortisol in the SGA group at early stage of the first trimester were not statistically significant (P = 0.337; Fig. 1), consistent with previous studies involving women in the first and third trimesters [12, 13].

However, the metabolic signatures of cortisol, in this study, showed remarkable changes in SGA (Fig. 1). Among those signatures, 11β-HSD2 metabolizes cortisol to inactive cortisone, which is reactivated by 11β-HSD1. Both cortisol and cortisone are then reduced to dihydro-, tetrahydro- and allo-tetrahydrometabolites catalyzed by different reductases. In addition to antenatal glucocorticoid treatment [14], the decreased activity of placental 11β-HSD2 is caused by higher levels of maternal cortisol [5, 6], which also results in birth weight reduction [22]. However, the maternal blood levels of cortisone and its 5β-reduced metabolite THE remained unchanged in SGA and non-SGA groups (Fig. 1). The metabolic ratios of E/F, indicating 11β-HSD2 activity, and THE/E were also unchanged (data not shown). In contrast, the metabolite of cortisol catalyzed by 5β-reductases, THF, was significantly increased (Fig. 1), while its metabolic ratio relative to cortisol, THF/F, was also higher in women carrying SGA fetus (Fig. 2A), similar to previous findings in pregnant women in third trimester [13]. Our study demonstrated that these changes may also represent predictive biomarkers in the first trimester. The level of 5α-reduced metabolite of cortisol, allo-THF, tended to increase in the SGA group (Fig. 1), while its metabolic ratio relative to cortisol (allo-THF/F) was not significant (data not shown). Another reduced metabolite of cortisol monitored in this study, 20α-DHF, which is catalyzed by 20α-reductase, was also not significant between the groups (Fig. 1).

Cortisol is derived from two different precursors, 11-deoxyF and 21-deoxyF, catalyzed by 11β- and 21-hydroxylases, which are key enzymes in cortisol biosynthesis (Fig. 1). In contrast to suppression of cortisol biosynthesis under low 11-deoxyF levels in cord blood obtained from very low birth weight babies [10], maternal 11-deoxyF was decreased ($P = 0.098$, Fig. 1), which may be comparable to the apparent decrease in intrauterine fetal death and anencephalic fetus [23]. The maternal F/11-deoxyF metabolic ratio was increased (Fig. 2B), indicating activated 11β-hydroxylase in cortisol synthesis, whereas a reduced 11β-hydroxylase was found in low birth weight neonates [10]. The 21-Hydroxylase deficiency results in increased 21-deoxyF, which represents an alternative diagnostic marker in late-onset congenital adrenal hyperplasia [24], with similar activity between preterm and term infants [25].

The present study shows a significantly increased metabolic ratio of cortisol to 21-deoxyF in women carrying SGA fetus ($P < 0.0003$, Fig. 2C) with a remarkable decrease in serum levels of 21-deoxyF ($P < 0.0001$, Fig. 1). Both cortisol precursors are generally converted from 17α- and 11β-hydroxyprogesterones (17α-OHP and 11β-OHP), respectively. As 21-deoxyF may also be alternatively catalyzed by 11β-hydroxylase from 17α-OHP directly [24], we compared and found decreased metabolic ratios of 11-deoxyF/17α-OHP ($P = 0.058$) and 21-deoxyF/17α-OHP ($P < 0.0005$) in the SGA group (data not shown). Altered maternal serum levels of 11-deoxyF and 21-deoxyF may not only be derived from placenta, which lacks 17α- and 21-hydroxylase activities, but also from fetal adrenal glands entirely [23]. The maternal 11-DeoxyF level generally increases with progressive pregnancy and peaks at 39 weeks [23], whereas 21-deoxyF is not elevated in premature infants [24]. However, competitive metabolic pathways to produce 11- and 21-deoxyF were not identified because of many outliers.

The activity of 11β-HSD isoenzymes and its metabolic capacity for glucocorticoids play a pivotal role in regulating fetal growth. The placental mRNA level of 11β-HSD 1 is significantly increased during the late gestation period (38 ~ 40 weeks), while the levels of 11β-HSD 2 are decreased, corresponding to fetal maturation and labor [26]. In contrast, pregnant women in their first trimester showed a compartmental distribution of 11β-HSD 1 and 2 at the feto-maternal interface, both of which were upregulated to possibly coordinate the interaction between isoenzymes [27]. Excessive glucocorticoid levels inhibit fetal growth and are expressed by increased cortisol metabolite THF and the THF/F ratio [13, 28]. The finding was in accordance with the negative association of maternal serum THF levels with birth weight of babies (Fig. 4).

To the best of our knowledge, this is the first study to evaluate metabolic signatures of cortisol in the first trimester. Although the present work was designed to provide detailed information regarding cortisol metabolism in serum obtained from pregnant women with SGA fetus, this prospective cohort study has several limitations. First, the incidence of adverse fetal outcomes may reflect placental and maternal abnormalities, but the cortisol signatures in placenta were not measured in pregnant women in their first trimester. The quantitative results from mothers could be indirectly evaluated based on those obtained from babies due to protective mechanism against excessive glucocorticoid levels. However, no comparative experiment was performed. Third, the longitudinal data related to metabolic changes during pregnancy in this study were intended to identify metabolic changes in early stages.

In summary, a decreased serum 21-deoxyF combined with increased F/21-deoxyF combined with higher THF/F ratio, indicating fetal growth inhibition [13, 28], represents a potentially reliable biomarker for predicting SGA in the first trimester (Fig. 3). Studies are needed to investigate the molecular mechanism of enzymes related to cortisol metabolism and their association with lipid profiles.

Abbreviations

SGA = small-for-gestational age; HPA = hypothalamic-pituitary-adrenal; 11β-HSD = 11β-hydroxysteroid dehydrogenase; IUGR = intrauterine growth restriction; LC-MS = liquid chromatography-mass spectrometry; ROC = receiver operating characteristic

Declarations

Authors' contributions

C.L. performed steroid analysis, data collection, and drafting the manuscript. S.M.L. contributed to clinical sampling and interpretation of quantitative results. D.J.B. was done in statistical analysis based on steroid quantities. S.Y.K. performed the correlation of clinical parameters with steroid signatures. D.Y.L. investigated the predictive biomarkers using various statistical equations. Y.M.J interpreted the results obtained from both lab findings and clinical observations. H.I.K. confirmed mass spectrometric data in quantification. C.W.P. was involved in manuscript editing. J.S.P. conceived the idea for this study. M.H.C. supervised analytical platform and finalized the manuscript. First draft was prepared by C.L., S.M.L and M.H.C. All authors read the manuscript, edited it and participated in the final version.

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Availability of data and materials

The raw and processed data used and analyzed in the current study available from the corresponding author.

Ethics approval and consent to participate

This study was approved by the Ethics Committees of Seoul National University Hospital. All participants signed an informed consent form prior to the study.

Consent for publication

Not applicable.

Competing interests

All authors have read the journal's authorship agreement and policy on conflicts of interest and have no conflicts of interest to declare.

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Figures

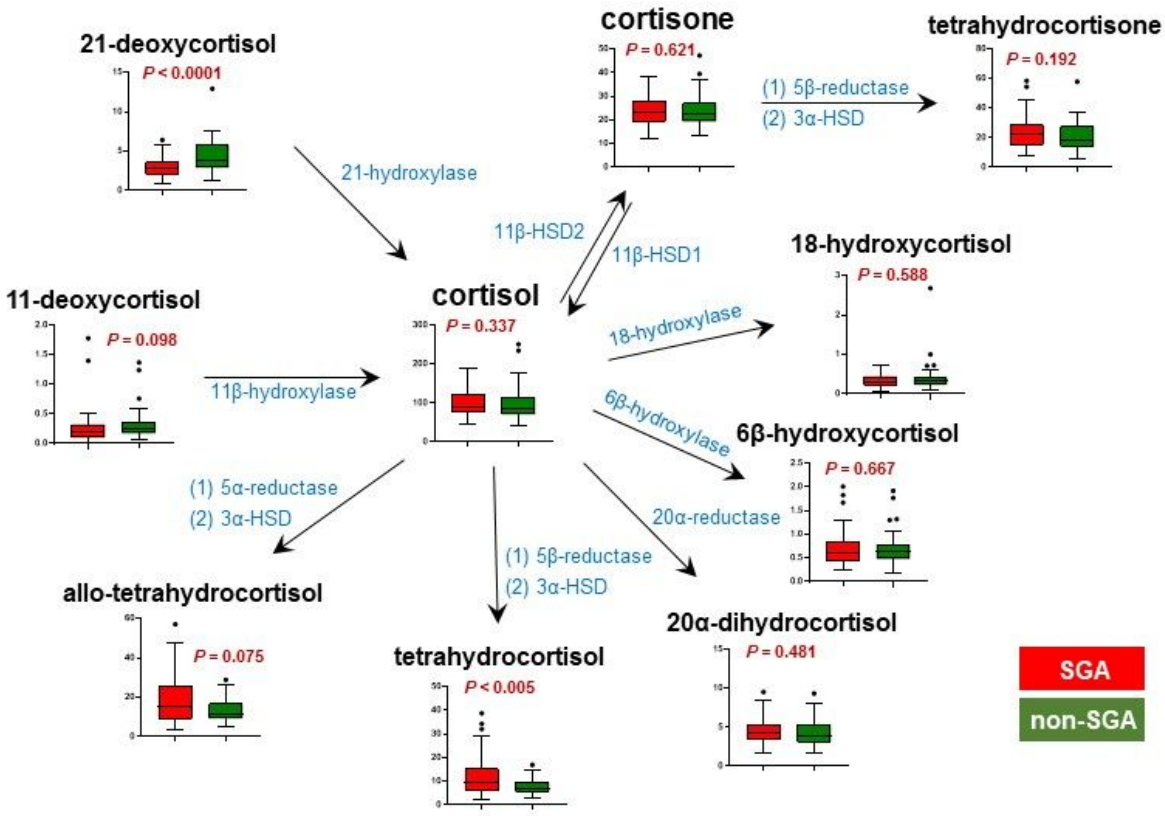

Figure 1

Figure 1

Cortisol metabolism and comparative serum levels of individual steroids between SGA and non-SGA pregnant women. As a cortisol precursor, 21-deoxycortisol was significantly decreased ($P < 0.0001$), whereas one of the reductive metabolites of cortisol, tetrahydrocortisol (THF) was remarkably increased in the SGA group ($P < 0.005$). Serum levels are expressed in ng/mL. The horizontal lines represent the mean value and error bars indicate 95% confidence intervals.

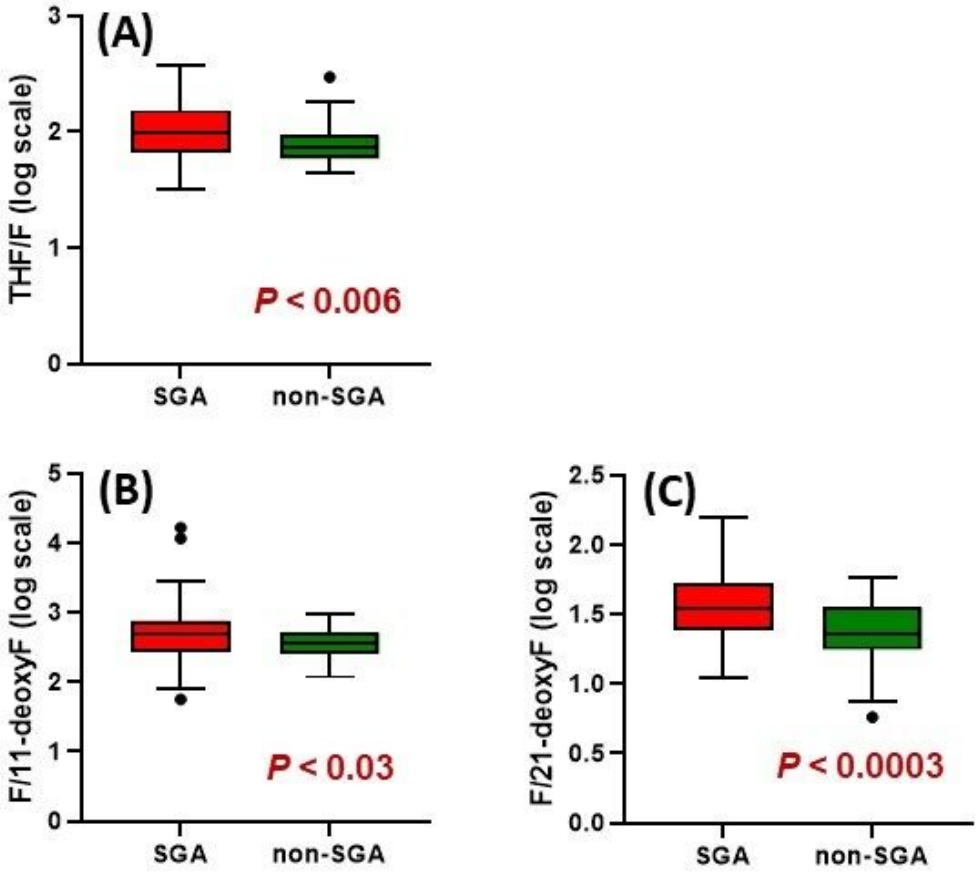

Figure 2

Figure 2

Comparative maternal serum metabolic ratios between SGA and non-SGA groups. Metabolic ratios of (A) tetrahydrocortisol to cortisol (THF/F), (B) cortisol to 11-deoxycortisol (F/11-deoxyF), and (C) cortisol to 21-deoxycortisol (F/21-deoxyF) were significantly increased in the SGA group compared with the non-SGA group. All data were converted to log values and the horizontal lines represent the mean value and error bars indicate 95% confidence intervals.

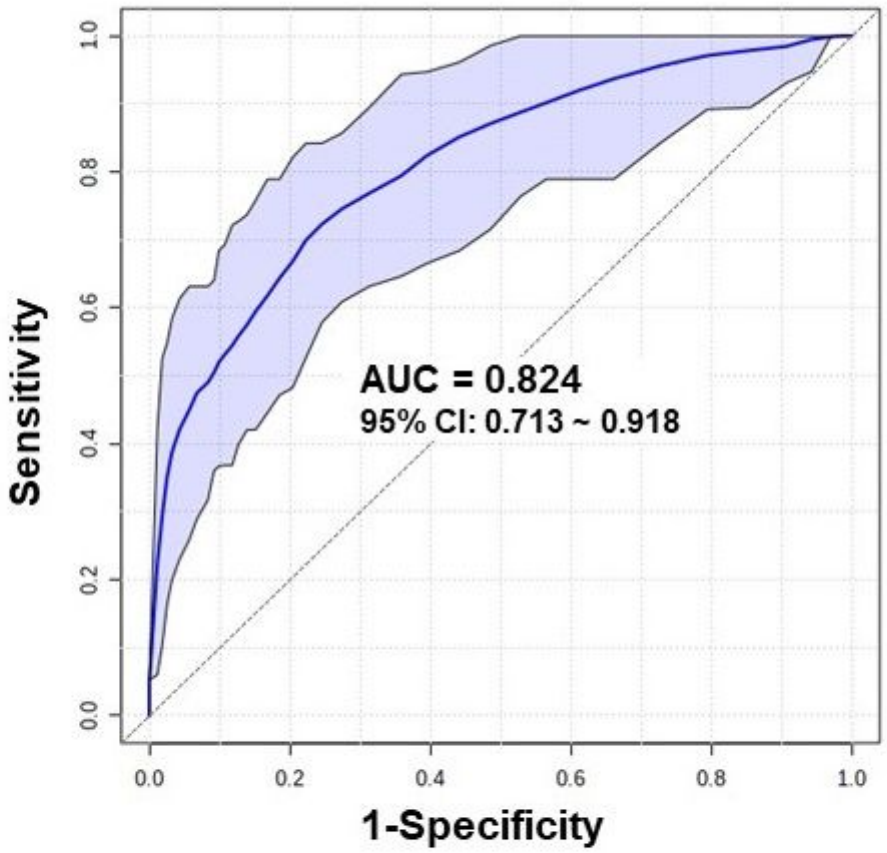

Figure 3

Figure 3

The ROC curve of maternal signatures in cortisol metabolism of the SGA group. A logistic regression model using a combination of serum 21-deoxyF and two metabolic ratios of F/21-deoxyF and THF/F was selected. Predictive probabilities of sensitivity and specificity were 0.734 (0.696 ~ 0.773) and 0.817 (0.784 ~ 0.851), respectively. Blue area indicates 95% CI.

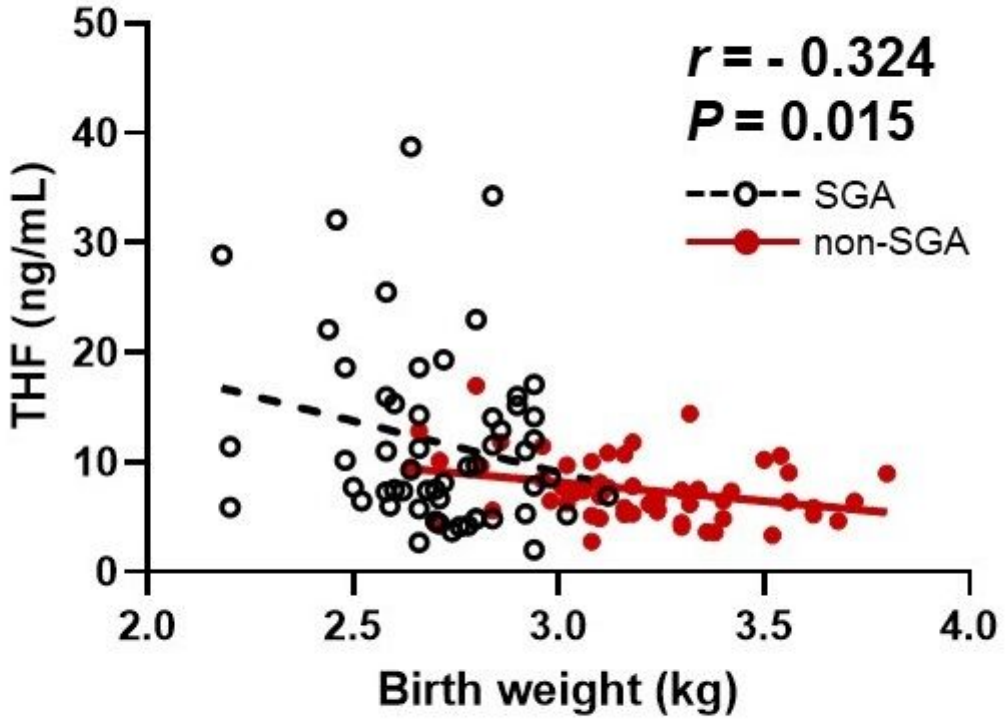

Figure 4

Figure 4

The scatter plots with linear fitted curves and Pearson correlation coefficients between tetrahydrocortisol (THF) and maternal characteristics. Serum levels of THF were negatively correlated with birth weight. The correlations were statistically significant (P < 0.02 for all) in only non-SGA pregnant women (red colored).