Cytochrome P450-mediated metabolic alterations in preeclampsia evaluated by quantitative steroid signatures

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ABSTRACT

Although preeclampsia has been suggested potential risk factors including placental and systemic inflammation, oxidative stress, and abnormal steroid metabolism during pregnancy, the pathogenesis of preeclampsia has not fully been elucidated, particularly in steroid metabolism. The association between various cytochrome P450 (CYP)-mediated steroid metabolic markers and preeclampsia risk was therefore investigated. The serum levels of 54 CYP-mediated regioselective hydroxysteroids and their substrates were quantitatively evaluated from both pregnant women with preeclampsia ($n = 30$; age, 30.8 ± 4.5 years) and normotensive controls ($n = 30$; age, 31.0 ± 3.5 years), who were similar with respect to maternal age, gestational age, and body mass index. The levels of 6β-, 7α-, and 11β-hydroxymetabolites of androgens and corticoids were significantly increased in women with preeclampsia. In addition, the levels of oxysterols, including 7α-, 7β-, 4β-, 20α-, 24S-, and 27-hydroxycholesterol, were markedly higher, while the levels of 16α-OH-DHEA, 16α-OH-androstenedione, and cholesterol were significantly decreased in patients. The 6β-hydroxylation of androgens and corticoids by CYP3A4 ($P < 0.01$), the activation of 20,22-desmolase (a cholesterol side-chain cleavage enzyme) by CYP11A1 ($P < 0.00001$), and the multi-hydroxylation of cholesterol at C-4β, C-7α, C-7β, C-24S, C-27, and C-20α ($P < 0.0001$) by catalytic or enzymatic reaction (e.g. CYP3A4, CYP7A1, CYP27A1, and CYP46A1) were differed between preeclamptic women and control subjects. In particular, an increased oxysterols (induction > 2.0-fold) were positively correlated with the conditions of preeclampsia. Our metabolic profiling suggests the CYP-mediated alterations in steroid metabolism and hydroxylation in pregnancy-induced hypertension. These multiple markers could serve as background information for improved clinical diagnosis and management during pregnancy.

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1. Introduction

Preeclampsia is referred to as pregnancy-induced hypertension. This pregnancy disorder is characterized by a widespread maternal endothelial dysfunction, and is a leading cause of maternal and fetal morbidity and mortality [1]. Several factors, including placental and systemic inflammation, oxidative stress, and abnormal steroid metabolism during pregnancy, have been regarded as potential risk factors. However, the pathogenesis of preeclampsia has not fully been elucidated [1]. In particular, there is no definitive evidence for a role of steroid metabolism.

Steroid hormones regulate physiological and pharmacological processes in the body. They are divided into androgens, estrogens, corticoids, progestins, and sterols. In biosynthetic pathways, steroid hormones are produced from cholesterol via cytochrome P450 (CYP) enzymes (CYP1A/B, CYP2B, CYP3A, CYP7A/B, CYP11A/B, CYP17A, CYP19A, CYP21A, CYP27A, and CYP46A isoforms), aided by 3β-, 11β-, and 17β-hydroxysteroid dehydrogenases (Fig. 1) [2–5]. The CYP enzymes are primarily involved in NADPH-dependent hydroxylation, which is irreversible in target tissues such as the kidneys, liver, ovaries, testes, and placenta [3–5]. The activities of CYP enzymes have been elucidated in vitro and in vivo, using a number of steroid substrates [6–8]. However, abnormalities in steroid-dependent diseases remain poorly understood. Here, we report the metabolic profiling of preeclamptic CYP-mediated steroid hydroxylation in human serum.

The evaluation of CYP activity can be used to describe the functional diversities of biological systems, which depend on

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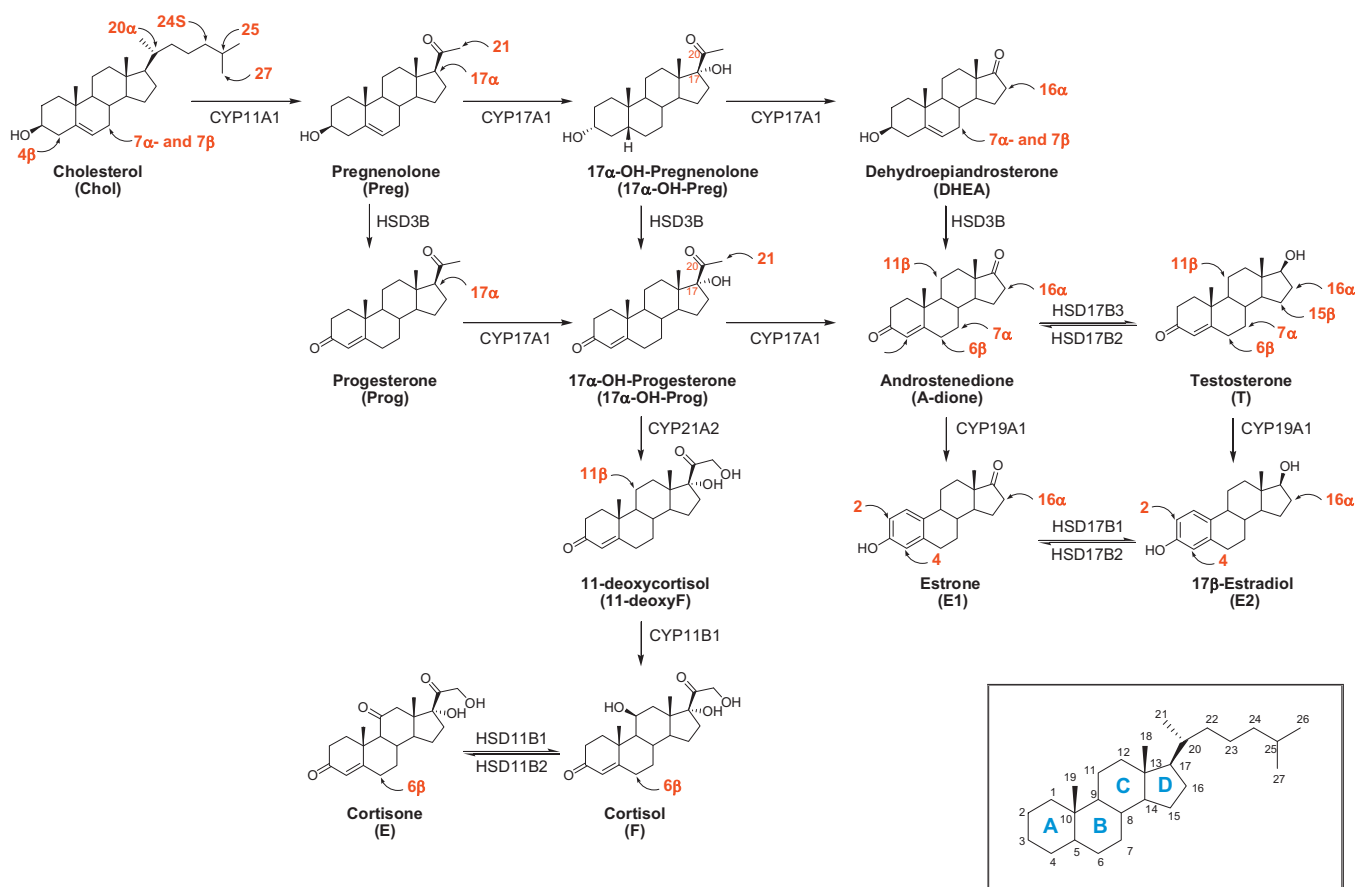*E-mail address:* mh_choi@kist.re.kr (M.H. Choi).

Fig. 1. The regioselective oxidation of steroid metabolism induced by human cytochrome P450 (CYP) enzymes. CYP isoforms with either high activity or a distinct regioselectivity for catalyzing a given metabolic reaction are indicated in red. See Supplementary Table 1 for the full names of steroids analyzed.

genetic variations, environmental factors, or individual variability [9]. Some steroids for measuring CYP enzyme activities have been monitored using immunochemical methods, such as radioimmunoassay (RIA) or enzyme immunoassay (EIA). However, these techniques have limited applicability, because of overestimation with the cross-reaction [10,11]. In contrast, mass spectrometry-based profiling offers enhanced quantitative reproducibility, and can be applied to large-scale clinical studies for biomarker discovery and diagnostic applications [12,13]. In particular, gas chromatography–mass spectrometry (GC–MS) profiling constitutes a powerful technique, which is widely used for multi-targeted steroid analysis in biological samples [14–17].

In the present study, we validated the GC–MS-based profiling of regioselective hydroxysteroids catalyzed by CYP enzymes in human serum, including 26 androgens, 9 estrogens, 5 progestins, 6 corticoids, and 8 sterols. Our objective was to evaluate the possible role of CYP enzymes in preeclamptic steroid metabolism.

2. Experimental

2.1. Materials

Reference standards of the 54 steroids examined (Supplementary Table 1) were obtained from Sigma (St. Louis, MO) or Steraloids (Newport, RI). The internal standards (ISs) 16,16,17-d_3-testosterone for the 26 androgens, 2,4,16,16-d_4-17β-estradiol for the nine estrogens, 9,11,12,12-d_4-cortisol for the six corticoids, 2,2,4,6,6,17α,21,21,21-d_9-progesterone and 2,2,4,6,6,21,21,21-d_8-17α-hydroxyprogesterone for the five progestins, and 25,26,26,26,27,27,27-d_7-4β-hydroxycholesterol

and 25,26,26,26,27,27-d_6-27-hydroxycholesterol for eight sterols were purchased from NARL (Pumble, Australia), C/D/N isotopes (Pointe-Claire, Quebec, Canada), and Avanti Polar lipids (Alabaster, AL). Sodium acetate (reagent grade), acetic acid (glacial, 99.99+%) and L-ascorbic acid (reagent grade) were obtained from Sigma. The trimethylsilylating (TMS) agents N-methyl-N-trifluorotrimethylsilyl acetamide (MSTFA), ammonium iodide (NH$_4$I), and dithioerythritol (DTE) were purchased from Sigma. All organic solvents used were of analytical or HPLC grade and were purchased from Burdick & Jackson (Muskegan, MI). Deionized water was prepared using a Milli-Q purification system (Millipore, Billerica, MA).

2.2. Standard solution and quality-control (QC) samples

Each stock solution of the reference standard was prepared at a concentration of 1 mg/mL by dissolving in 10 mL of methanol and stored at −20 °C. L-Ascorbic acid (1 mg/mL) was used to prevent oxidation of the catechol estrogens including 2-OH-E1, 2-OH-E2, 4-OH-E1, and 4-OH-E2. The calibration solutions were made by mixing each standard in methanol at various concentrations of 0.01–10 μg/mL, except cholesterol (0.1–100 μg/mL). All working solutions were stable for a minimum of 3 months at 4 °C in a teflon-sealed amber-glass vial.

For calibration and quality control (QC) purposes, steroid-free serum was produced in-house by ultra-centrifuging charcoal/dextran-treated fetal bovine serum (FBS) (HyClone, Logan, UT) through Amicon® Ultra 0.5 mL filters of 3 kDa molecular weight cut-off (MWCO) (Millipore, Billerica, MA) at 12,000 rpm for 20 min. The absence of various steroids, especially sterols,

was confirmed in blank samples prior to running multiplexed CYP assays. The calibration samples were made up at 15 different concentrations, while the QC samples were prepared at 10 different concentrations, using in-house steroid-free serum according to the sensitivity and reference values in human plasma.

2.3. Subjects and sample collection

This study was approved by the medical ethics committee of the University Hospital (Inje University, Busan, Korea). Preeclamptic patients and control subjects were recruited from the antenatal ward and clinic of the Department of Obstetrics, Inje University School of Medicine. According to the recommendations by the American College of Obstetricians and Gynecologists, preeclampsia was defined as abrupt onset hypertension (i.e., a systolic blood pressure of ≥140 mm Hg or a diastolic blood pressure of ≥90 mm Hg on 2 occasions) and proteinuria (>0.3 g/L in a 24 h collection on 2 consecutive dipsticks) after 20 weeks of gestation [1]. After obtaining informed consent, serum samples were collected from 30 control subjects and 30 preeclamptic patients without preexistent hypertension, diabetes, renal dysfunction, immune diseases, or intrauterine fetal death at the time of sampling. All samples were stored at −80 °C until required.

2.4. Assay procedure

A total of 54 steroids were quantitatively analyzed using a mixture of 7 internal standards (ISs): 16,16,17-d_3-testosterone (0.5 μg/mL) for the 26 androgens; 2,4,16,16-d_4-17β-estradiol (0.5 μg/mL) for the 9 estrogens; 9,11,12,12-d_4-cortisol (1 μg/mL) for the 6 corticoids; 2,2,4,6,6,17α,21,21,21-d_9-progesterone (2 μg/mL); 2,2,4,6,6,21,21,21-d_8-17α-hydroxyprogesterone (1 μg/mL) for the 5 progestins; and 25,26,26,26,27,27,27-d_7-4β-hydroxycholesterol (10 μg/mL) and 25,26,26,26,27,27-d_6-27-hydroxycholesterol (10 μg/mL) for the 8 sterols. For solid-phase extraction, an Oasis HLB cartridge (3 mL, 60 mg; Waters, Milford, MA) was preconditioned with 3 mL of methanol followed by 3 mL of deionized water. Serum samples (0.4 mL) were then subjected to Oasis HLB^{TM} SPE and methyl t-butyl ether (MTBE) extraction, according to a previously described procedure with minor modifications (16). After trimethylsilylation (TMS), 2 μL of the resulting mixture was subjected to GC–MS in the selected-ion monitoring (SIM) mode.

2.5. Instrumental conditions

GC–SIM/MS was performed with an Agilent 6890 Plus gas chromatograph interfaced with a single-quadrupole Agilent 5975 MSD (Agilent Technologies, Palo Alto, CA). The electron energy was 70 eV and the ion source temperature was 230 °C. Each sample (2 μL) was injected in split mode (8:1) at an injector temperature of 280 °C, and separated through an Ultra-1 capillary column (25 m × 0.2 mm i.d., 0.33-μm film thickness; Agilent Technologies). The oven temperature was initially set at 220 °C, then ramped to 245 °C at 1 °C/min, and finally increased to 315 °C at 5 °C/min. Ultrahigh purity helium was used as the carrier gas, with a column head pressure of 213.0 kPa (column flow: 1.0 mL/min at an oven temperature of 220 °C). For quantitative analysis, the characteristic steroid ions were determined as their TMS derivatives. Peak identification was achieved by comparing the retention times and matching the peak height ratios of the characteristic ions (Supplementary Table 1).

2.6. Assay validation

The method validation was achieved based on previous reports [17]. The detailed validation parameters, including the limit of detection (LOD), limit of quantification (LOQ), recovery (%), calibration linearity (r^2), precisions expressed as coefficients of variation (% CV) and accuracies as percent relative errors (% bias) for the intra- and inter-day assays, and stability of the analyte during sample collection and handling.

2.7. Statistical analysis and steroid signatures

Clinical and biological variables and the concentrations of the individual steroids and their metabolic ratios were calculated by dividing the concentration of substrate by that of its oxidation products (as an indicator of enzyme activity). The data for the plasma samples obtained from preeclamptic patients and control subjects are reported as means ± SD. Group comparisons were made using the unpaired two-tailed Student's t-test. Differences with $P < 0.05$ are regarded as statistically significant.

3. Results

3.1. GC–MS characteristics of CYP-mediated hydroxysteroids

To evaluate differences in CYP-mediated steroid hydroxylation between control subjects and preeclamptic patients, we fully validated and applied the quantitative profiling technique. The major CYP-mediated metabolites of the steroid substrates were as follows (Fig. 1): testosterone (6β-, 7α-, 11β-, 15β-, and 16α-OH-testosterone); androstenedione (4-, 6β-, 7α-, 11β-, and 16α-OH-androstenedione); DHEA (7α-, 7β-, and 16α-OH-DHEA); estrone (2-, 4-, and 16α-OH-estrone); 17β-estradiol (2-, 4-, and 16α-OH-estradiol [estriol]); pregnenolone (17α- and 21-OH-pregnenolone); progesterone (17α-OH-progesterone); cortisol (6β-OH-cortisol); cortisone (6β-OH-cortisone); and cholesterol (4β-, 7α-, 7β-, 20α-, 24S-, 25-, and 27-OH-cholesterol) [2–7]. The devised steroid profiling may indicate CYP activities of the CYP1A/B, CYP2B, CYP3A, CYP7A/B, CYP11A/B, CYP17A, CYP19A, CYP21A, CYP27A, and CYP46A isoforms.

Peak identification was achieved by retention times, characteristic ions, and mass spectra, and the latter were compared with those of reference standards (Supplementary Table 1). The GC oven program resulted in a good chromatographic separation for all steroids with retention times from 12.56 min (androsterone) to 38.07 min (27-hydroxycholesterol) (Fig. 2). Some partially overlapped compounds (e.g., 7α-OH-DHEA and epitestosterone, 7α-OH-testosterone and 7β-OH-DHEA, and 16α-OH-DHEA and 4-OH-estradiol) were differentiated by their different characteristic ions. Due to 7α-OH-cholesterol formed 2 peaks (at 31.96 min and 32.58 min) in the total-ion chromatogram and the first peak overlapped with cholesterol in serum samples, the second peak (with approximately one-third height intensity of the first peak) was selected for exact quantification (Supplementary Fig. 1).

Most steroids were monitored with their molecular ions as the base peaks. The following exceptions were made: 7α-OH-androstenedione and 7α-OH-testosterone, which had an abundant [M−90; M−OTMS]$^+$ ion at m/z 428 and m/z 430, respectively; 7α-OH-DHEA and 7β-OH-DHEA with an [M−90−15; M−OTMS−CH$_3$]$^+$ ion at m/z 415; 16α-OH-DHEA with an [M−15; M−CH$_3$]$^+$ ion at m/z 505; 16-keto-17β-estradiol and 16α-OH-estrone with an [M−15]$^+$ ion at m/z 487; pregnenolone with an [M−15]$^+$ ion at m/z 445; 11-deoxycortisol, cortisol, and 6β-OH-cortisol with an [M−90]$^+$ ion at m/z 544, m/z 632, and m/z 720, respectively; and cortisone and 6β-OH-cortisone with an [M−90−15]$^+$ ion at m/z 615 and m/z
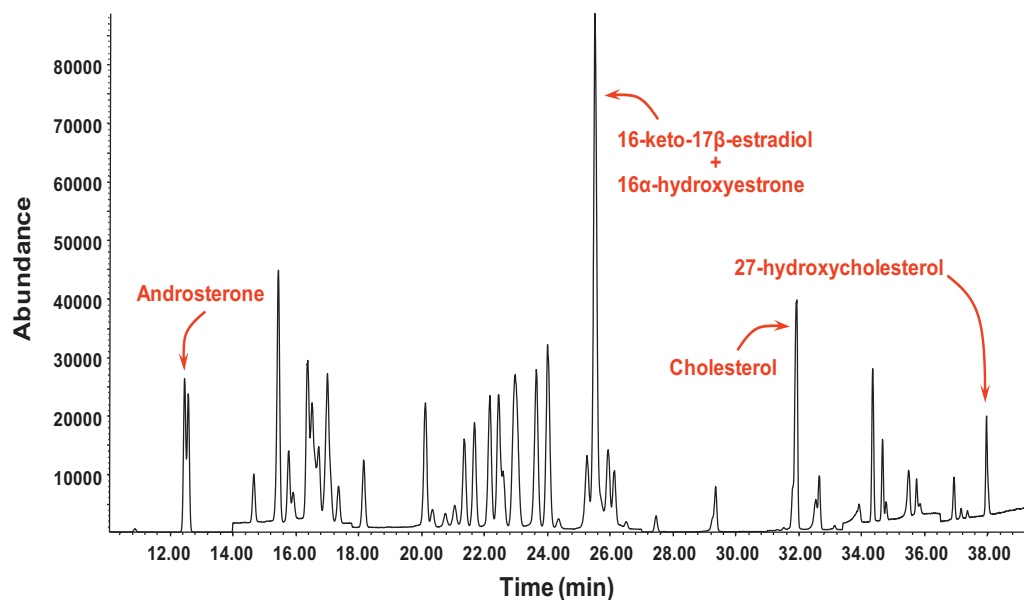
Fig. 2. Total-ion chromatogram obtained from a healthy female subject for detection of 54 steroids in serum. The substrates and their CYP-mediated regioselective hydroxysteroids were included 26 androgens, 9 estrogens, 5 progestins, 6 corticoids, and 8 sterols.

703, respectively. The 19-OH-DHEA had an $[M−90−103]^+$ ion at m/z 327, as major fragments formed by a typical loss of 90 Da (−OTMS) from the molecular ion (m/z 520), and elimination of 103 Da (TMS–O–CH$_2{}^\bullet$) from the C-19 primary alcohol. In the case of cholesterol, a less intense fragment ion at m/z 443 $[M−15]^+$ was chosen instead of intense characteristic ion at m/z 368 $[M−90]^+$, because of the high concentration of cholesterol in serum samples.

In addition, hydroxycholesterols as their TMS derivatives showed different fragmentation patterns depending on the −OH positions of their moiety (Supplementary Figs. 2A–G). Two oxysterols with 7α- or 7β-hydroxylation at the B-ring of the cholesterol nucleus were characterized by an intense fragment ion at m/z 456 $[M−90]^+$, because of easy elimination of the −OTMS group at C-7. In comparison with the simple fragment patterns of 7α- and 7β-OH-cholesterols, the characteristic ions of 4β-OH-cholesterol were observed at m/z 147 $[(CH_3)_2Si{=}OTMS]^+$ as a base peak, m/z 456 $[M−90]^+$, m/z 366 $[M−180; M−2OTMS]^+$, m/z 417 $[M−129; M−(CH_3)_3Si−O^+{=}CHCH{=}CH_2]^+$, m/z 327 $[M−129−90]^+$, m/z 441 $[M−90−15]^+$, and m/z 546 $[M]^+$. Meanwhile, the characteristic ions of 27-OH-cholesterol were monitored at m/z 129, m/z 456, m/z 417, m/z 441, and m/z 546. Among these fragments, m/z 456 was chosen instead of the base peak m/z 147 (4β-OH-cholesterol) or m/z 129 (27-OH-cholesterol), to enhance selectivity from serum interference. The fragment ion at m/z 129 (consisting C-1–C-3 and 3-OTMS) characterizes 3β-hydroxy-Δ5 compounds as TMS ether, while the rearrangement ion at m/z 147 is common in electron-impact ionization (EI) mass spectra of compounds with 2 (CH$_3$)$_3$Si groups of the vicinal diol type. The terminal alcohol 25-OH-cholesterol, in the TMS form, was monitored using a less intense fragment at m/z 456 $[M−90]^+$, and not the base peak at m/z 131 resulting from the cleavage at side chain C24–C25, the (CH$_3$)$_2$C$=$O$^+$–Si(CH$_3$)$_3$ group. In addition, 24S-OH-cholesterol showed prominent peaks at m/z 145, m/z 129, m/z 159, m/z 413 $[M−90−43]+$, and m/z 503 $[M−43]^+$. Ions at m/z 145 and m/z 159, and loss of a fragment of mass 43 from the molecular ion, arise from fragmentation along the side chain and are induced by the presence of a 24-OTMS group. Among these fragments, m/z 456 and m/z 413 were monitored instead of the base peak m/z 131 (25-OH-cholesterol) and m/z 145 (24S-OH-cholesterol), respectively, to enhance selectivity. The 20α-OH-cholesterol was identified on the basis of the

fragment at m/z 201 as a base peak (C$_8$H$_{16}$OTMS$^+$, arising from cleavage between C-17 and C-20) and m/z 461 as a quantitative ion (M$^+$−C$_6$H$_{13}$, arising from cleavage between C-20 and C-22).

3.2. Optimization of multiplexed CYP assay

Based on the chemical and physical diversities within androgens, estrogens, corticoids, progestins, and sterols, we optimized our metabolic profiling for simultaneous quantification in a single extract. After solid-phase extraction, the extraction efficiency and matrix interference were compared using 2 different organic extraction solvents—MTBE, and mixtures of ethyl acetate and n-hexane (2:3, v/v). MTBE was selected for further use based on the overall recoveries and chromatographic properties (data not shown). Using optimized conditions, all steroids were extracted with good yields (≥69.1%, with the exception of 46.2% for 6β-OH-cortisol) and remained detectable when 0.4 mL of serum was prepared (Supplementary Table 2).

3.3. Assay validation

For calibration and QC purposes, it is important to employ a negative control sample, comprising an appropriate sample of the authentic matrix with few or no analytes. Initially, we used commercially available steroid-free serum [16], but this was not sufficient for the analysis of sterols, especially cholesterol. The steroid-low serum resulting from stripping with activated carbon may have contained high concentrations of some of the compounds (e.g., cholesterol) bound to serum lipoproteins [18]. In the present study, we selected the *in-house* steroid-free serum as a suitable matrix for the calibration sample of all 54 steroids, including cholesterol. Thus, the commercially available steroid-free charcoal/dextran-treated FBS, which did not completely remove cholesterol, 7α-OH-cholesterol or 7β-OH-cholesterol, was additionally ultra-filtered through micro-porous membranes to obtain a matrix containing negligible levels of these sterols (Supplementary Fig. 3). When the *in-house* blank serum was compared using carbon-stripping and ultra-filtered steroid-free serum, all of the monitored steroids were effectively removed to yield a relatively low LOQ for cholesterol, 7α-OH-cholesterol and 7β-OH-cholesterol.

Table 1
Clinical characteristics of the control subjects and preeclampsia patients.

Variable	Control subjects ($n = 30$)	Preeclampsia patients ($n = 30$)	P
Maternal age (years)	30.8 ± 4.5 (24–43)	31.0 ± 3.5 (24–38)	0.874
Gestational age at sampling (weeks)	34.2 ± 3.0 (24.1–37.6)	33.8 ± 3.9 (22.2–40.5)	0.646
Gestational age at delivery (weeks)	34.7 ± 2.9 (25.3–38.0)	34.4 ± 3.7 (22.3–40.5)	0.783
Systolic blood pressure (mm Hg)	124.8 ± 7.4 (120–140)	165.3 ± 15.7 (130–200)	<0.001
Diastolic blood pressure (mm Hg)	77.6 ± 5.8 (70–80)	104.7 ± 9.0 (90–120)	<0.001
Proteinuria (g/24 h)[a]	ND	4.3 ± 4.9 (0.2–11.6)	NC
BMI at pre-pregnancy (kg/m^2)	22.4 ± 3.5 (17.6–31.6)	22.6 ± 4.1 (16.0–32.7)	0.870
BMI at sampling (kg/m^2)	26.8 ± 3.2 (21.0–34.4)	28.1 ± 4.2 (19.2–36.4)	0.183
Baby's weight (kg)	2.4 ± 0.7 (0.9–3.6)	2.1 ± 0.7 (0.3–3.4)	0.093
Cesarean delivery (%)	50.0	56.7	0.619

Data are expressed as mean ± standard deviation (range) or a percentage, as indicated.

[a] Proteinuria was not quantified because the dipstick was negative. ND: not detectable; NC: not comparable.

Assay validation required the evaluation of accuracy, precision, selectivity, sensitivity, reproducibility, and stability, using spiked samples prepared with in-house steroid-free serum. The LOD and LOQ, the corresponding sample concentrations required to provide signal-to-noise (S/N) ratios of >3 and >10, respectively, were also evaluated (Supplementary Table 2). The resulting LOQs of the TMS derivatives were 0.5–5.0 ng/mL for the 26 androgens, 0.5–2.0 ng/mL for the 9 estrogens, 1.0–2.0 ng/mL for the 5 progestins and 6 corticoids, and 5.0–10.0 ng/mL for the sterols (with the exception of 50 ng/mL for cholesterol). The calibration curve consisted of a blank sample (a matrix sample processed without an IS), a zero sample (a matrix sample processed with an IS), and 13 samples from the LOQ to expected range in the sample. The devised method was found to be linear with the correlation coefficient ($r^2 \geq 0.994$) for all of the analyzed steroids, with the exception of cholesterol ($r^2 > 0.988$). Assay precisions and accuracies were determined by analysis of the QC samples acquired for the intra- and inter-day assays at 3 different levels of the individual CYP-mediated steroids (among 2 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL, and 500 ng/mL; and 200 ng/mL, 5000 ng/mL, and 20,000 ng/mL for cholesterol) with in-house steroid-free serum, according to the sensitivity and reference values in human serum. Intra-day ($n = 5$) precisions (expressed as % CV) were 3.2–23.3%, whereas accuracies (expressed as % bias) were 94.8–115.3%. Inter-day ($n = 5$) precisions (% CV) and accuracies (% bias) were 3.7–15.9% and 95–110.0%, respectively (Supplementary Table 2).

The stability test was evaluated for the reliable quantification of the CYP-mediated steroids. The parameters assessed included standard solution storage, short-term storage (bench-top, room temperature), freeze/thaw cycles, and the analytical process described in Section 2.1. Stock solutions and QC samples were freshly prepared, and L-ascorbic acid (1 mg/mL) was added to prevent oxidative degradation of the catechol estrogens. The standard solutions were stable at −20 °C for 3 months and at room temperature for 6 h. The short-term stability, which was tested by thawing the QC samples at 25 °C and leaving them to stand for 6 h, showed no significant changes in concentration within 24.5% deviation under the tested conditions. The freeze/thaw stability was evaluated using the aliquot concentration not subjected to freeze/thaw cycles as a reference. The stability of the compounds was further demonstrated in serum samples subjected to 3 freeze/thaw cycles. The overall differences between the cycles were not significant in the 3.2–25.5% RSD range in all of the compounds studied. Three repeated freeze/thaw cycles had no apparent effect on the concentration of the substrates and their hydroxysteroids. It is possible for instability to occur in the sample matrix, and also in the prepared samples. Hence, it is important to test the post-preparative stability under the analysis conditions, including the auto-sampler for the anticipated maximum time of an analytical run, to determine whether the analytical run can be reanalyzed in the case of instrumental failure. The TMS derivatives of hydroxysteroids were quite stable when the prepared samples were injected 40 h after being placed in the sample trays (≤12.8% RSD) (Supplementary Table 3).

3.4. Assay application

The main clinical and biological characteristics from the patients with pathologic outcomes are presented in Table 1. All 60 pregnant women with a mean maternal age of 31 years examined in this study were similar with respect to gestational age at sampling and delivery, body mass index (BMI) at pre-pregnancy and during pregnancy, baby's weight, and delivery method. In comparison with control subjects, preeclamptic patients had significantly higher systolic and diastolic blood pressure, and higher proteinuria, each of which constitutes a classification criterion for preeclampsia [1].

The levels of endogenous steroids in serum samples from preeclamptic patients and age-matched control subjects were measured using the validated multiplexed CYP assay. A totally 48 steroids, including 21 androgens, 9 estrogens, 4 progestins, 6 corticoids, and 8 sterols, were detected quantitatively (Table 2). Among the steroids used as substrates, pregnenolone (Preg) and progesterone (Prog) were significantly increased ($P < 0.001$, >2.0-fold) in preeclamptic patients compared with control subjects, while cholesterol (Chol) was significantly decreased ($P < 0.001$, 1.4-fold). In contrast, the concentrations of androgens, estrogens, and corticoids, such as DHEA, testosterone (T), androstenedione (A-dione), estrone (E1), estradiol (E2), cortisol (F), and cortisone (E), did not differ significantly between the 2 groups. In CYP-mediated hydroxysteroids, the levels of 6β-, 7α-, 11β-, or 17α-hydroxymetabolites of some substrates, e.g., 6β-OH-A-dione ($P < 0.001$), 6β-OH-T, 6β-OH-E, 6β-OH-F ($P < 0.02$), 7α-OH-DHEA, 7α-OH-A-dione, 11β-OH-A-dione ($P < 0.005$), 11β-OH-T, 17α-OH-Preg ($P < 0.02$), and 17α-OH-Prog ($P < 0.001$), were increased in patients compared to the controls, while the levels of 16α-OH-DHEA ($P < 0.001$) and 16α-OH-A-dione ($P < 0.05$) were decreased. In addition, preeclamptic patients showed significantly higher levels of hydroxylated metabolites of cholesterol, e.g., 7α-OH-Chol ($P < 0.01$), 7β-OH-Chol ($P < 0.01$), 4β-OH-Chol ($P < 0.01$), 20α-OH-Chol ($P < 0.001$), 24S-OH-Chol ($P < 0.01$), and 27-OH-Chol ($P < 0.001$).

Based on the quantitative results, the metabolic ratio of hydroxymetabolite to substrate was demonstrated (Fig. 3. and Supplementary Table 4). In comparison with control subjects, preeclamptic patients showed lower 16α-hydroxylation of DHEA and A-dione by CYP3A7 and CYP2B6, respectively (Fig. 3A, $P < 0.02$, 2.0-fold; Fig. 3B, $P < 0.05$, 1.6-fold). However, the 17α-hydroxylase of Preg and Prog by CYP17A1 did not differ significantly between the 2 groups. The 17,20-lyase of Preg and Prog by CYP17A1, and the ratios of 17α-OH-Preg to DHEA and 17α-OH-Prog to A-dione were reduced in preeclamptic patients (Fig. 3C, $P < 0.02$, 1.7-fold;

Table 2
Serum steroid concentrations altered significantly in the control subjects and preeclamptic patients.

Compound (trivial name)	Control subjects ($n = 30$)	Preeclampsia patients ($n = 30$)	Fold difference[a]	P
Androgens				
Androsterone	0.7 ± 0.3	1.0 ± 0.8	↑ 1.5	<0.05
Etiocholanolone	1.3 ± 0.3	1.6 ± 0.6	↑ 1.2	<0.05
6β-OH-androstenedione	0.7 ± 0.2	1.3 ± 0.8	↑ 1.8	<0.001
11β-OH-androstenedione	3.5 ± 2.3	5.1 ± 1.6	↑ 1.5	<0.005
16α-OH-dehydroepiandrosterone	1.5 ± 0.9	0.9 ± 0.6	↓ 1.7	<0.001
16α-OH-androstendione	3.5 ± 1.1 (17)[b]	2.6 ± 0.7 (13)[b]	↓ 1.3	<0.05
Progestins				
Pregnenolone	6.5 ± 4.3	15.1 ± 11.7	↑ 2.3	<0.001
Progesterone	288.1 ± 184.8	597.2 ± 395.7	↑ 2.1	<0.001
17α-hydroxypregnenolone	9.2 ± 11.2	23.2 ± 27.2	↑ 2.5	<0.02
17α-hydroxyprogesterone	17.1 ± 13.8	43.3 ± 31.8	↑ 2.5	<0.001
Corticoids				
6β-hydroxycortisol	17.3 ± 16.5	41.1 ± 50.5	↑ 2.4	<0.02
Sterols				
CHOLESTEROL	396,297.4 ± 69009.3	290,126.8 ± 69,007.1	↓ 1.4	<0.001
7α-Hydroxycholesterol	1136.8 ± 649.1	2644.8 ± 2557.7	↑ 2.3	<0.01
7β-Hydroxycholesterol	2039.3 ± 1923.2	6088.2 ± 7572.5	↑ 3.0	<0.01
4β-Hydroxycholesterol	114.4 ± 55.0	186.7 ± 138.1	↑ 1.6	<0.01
20α-Hydroxycholesterol	11.1 ± 2.8	16.8 ± 5.8	↑ 1.5	<0.001
24S-Hydroxycholesterol	26.9 ± 11.7	39.0 ± 17.4	↑ 1.4	<0.01
27-Hydroxycholesterol	20.9 ± 5.3	30.2 ± 9.1	↑ 1.4	<0.001

Data are expressed as mean ± standard deviation (ng/mL) and were analyzed by Student's *t*-test.

[a] Fold differences were calculated by diving the mean level of preeclampsia by that of control, or vice versa (↑, increase; ↓, decrease).

[b] Numbers in parentheses indicate the number of subjects in which the corresponding steroids were detected. ND, not detectable; NC, not comparable; NS, not significant.

Fig. 3D, $P < 0.02$, 2.4-fold). In addition, the ratio of 11-deoxyF to 17α-OH-Prog by 21-hydroxylase (CYP21A2) was lower in preeclamptic patients than in control subjects [Fig. 3E, $P < 0.05$, 1.5-fold]. The 6β-hydroxylase (an indicator of CYP3A4 activity) and the ratios of 6β-OH-A-dione to A-dione (Fig. 3F, $P < 0.01$, 1.4-fold) and 6β-OH-F to F (Fig. 3G, $P < 0.01$, 2.3-fold) were significantly increased in preeclamptic patients. The hydroxylation of cholesterol at C-7α, C-7β, C-4β, C-24S, C-27, and C-20α by enzymatic or non-enzymatic oxidation was significantly higher $P < 0.001$, (>2.0-fold), and the 25-hydroxylase of cholesterol was significantly upregulated ($P < 0.05$, 1.5-fold) in preeclamptic patients compared with control subjects (Fig. 3H-N). The 20,22-desmolase production by CYP11A1 (cholesterol side-chain cleavage) was significantly increased (Fig. 3O, $P < 0.001$, 3.2-fold). However, other enzymes, including aromatase (CYP19A1) and hydroxysteroid dehydrogenase types (e.g., 3β-HSD, 17β-HSD, and 11β-HSD), did not differ significantly between the 2 groups.

4. Discussion

Based on steroid profiling, the associations between various CYP-mediated steroid metabolic markers and preeclampsia risk were evaluated. Initially, the high maternal serum levels of progesterone were associated with an increased risk of preeclampsia, which was consistent with previous studies [19,20]. Progesterone is essential for the maintenance of human pregnancy. However, elevated progesterone concentrations could suppress the production of the potent vasodilator, prostacyclin [20–22]. The level of 20,22-desmolase, which converts cholesterol to pregnenolone by CYP11A1, was significantly increased in preeclamptic women compared to control subjects, and this may be related to higher pregnenolone and progesterone synthesis in the preeclamptic placenta.

Findings concerning androgens and estrogens in the blood of preeclamptic women are controversial. The increased androstenedione and testosterone concentrations were detected in preeclampsia [23,24], which is not concurred with our present data and others [25,26]. In addition, a lower estradiol level in preeclamptic patients [27] was observed, but it was not differentiated in this study. The level of aromatase, which can transform androstenedione and testosterone into the estrogens, estrone and estradiol, respectively, by CYP19A1, has also been inconsistently reported. Some studies have indicated a reduction in aromatase levels during pregnancy [27], whereas our data revealed no significant difference in aromatase levels. Among corticoids, several studies have reported no difference in plasma cortisol. These findings are in accordance with the present data, which were collected from hypertensive patients at a number of time points during the 3rd trimester. However, the analysis based on gestational age from the 16th week of pregnancy until delivery indicated that maternal cortisol concentrations were lower from the 36th week onwards [28]. Conflicting results between preeclamptic patients and control subjects may be because of differences in the clinical characteristics of the study population (e.g., gestational age and BMI) [29,30] and also technical limitations with the RIA [11].

In the present study, the significant metabolic changes in 4β-OH-Chol, 6β-OH-A-dione, and 6β-OH-F (≥1.6-fold increase), and also in 6β-OH-T and 6β-OH-E (>50% incidence detection in preeclamptic patients) were observed because these hydroxysteroids are highly affected by CYP3A4. These data are in accordance with that pregnancy hypertension may be associated with CYP3A activity [31,32]. Consistent with these results, the metabolic ratios of 6β-OH-A-dione/A-dione, 6β-OH-F/F, and 4β-OH-Chol/Chol as indicators of CYP3A4 activity showed a positive association with preeclampsia. These compounds may therefore serve as indicators of preeclampsia. In contrast to 6β-hydroxylase, there is a lack of data on the up-regulated patterns of 7α-OH-DHEA and 7α-OH-A-dione, and also of 11β-OH-A-dione and 11β-OH-T (formed by the 7α-hydroxylase and 11β-hydroxylase of androgens, respectively) during pregnancy. Similar to progesterone in relation to preeclampsia, the increased concentrations of 17α-OH-Preg and 17α-OH-Prog in preeclamptic patients were detected; this may have been linked to a lower level of 17,20-lyase. In particular, a significant increase in 17α-OH-Prog (>2.5-fold) may be related to deficiencies in 17,20-lyase (required for sex steroid synthesis) and 21-hydroxylase (required for cortisol synthesis); these deficiencies may interfere partially or completely with the hydroxylation of 17α-OH-Prog. Although these differences between preeclamptic patients and control subjects have not been identified, measurement of these factors could be used to indicate
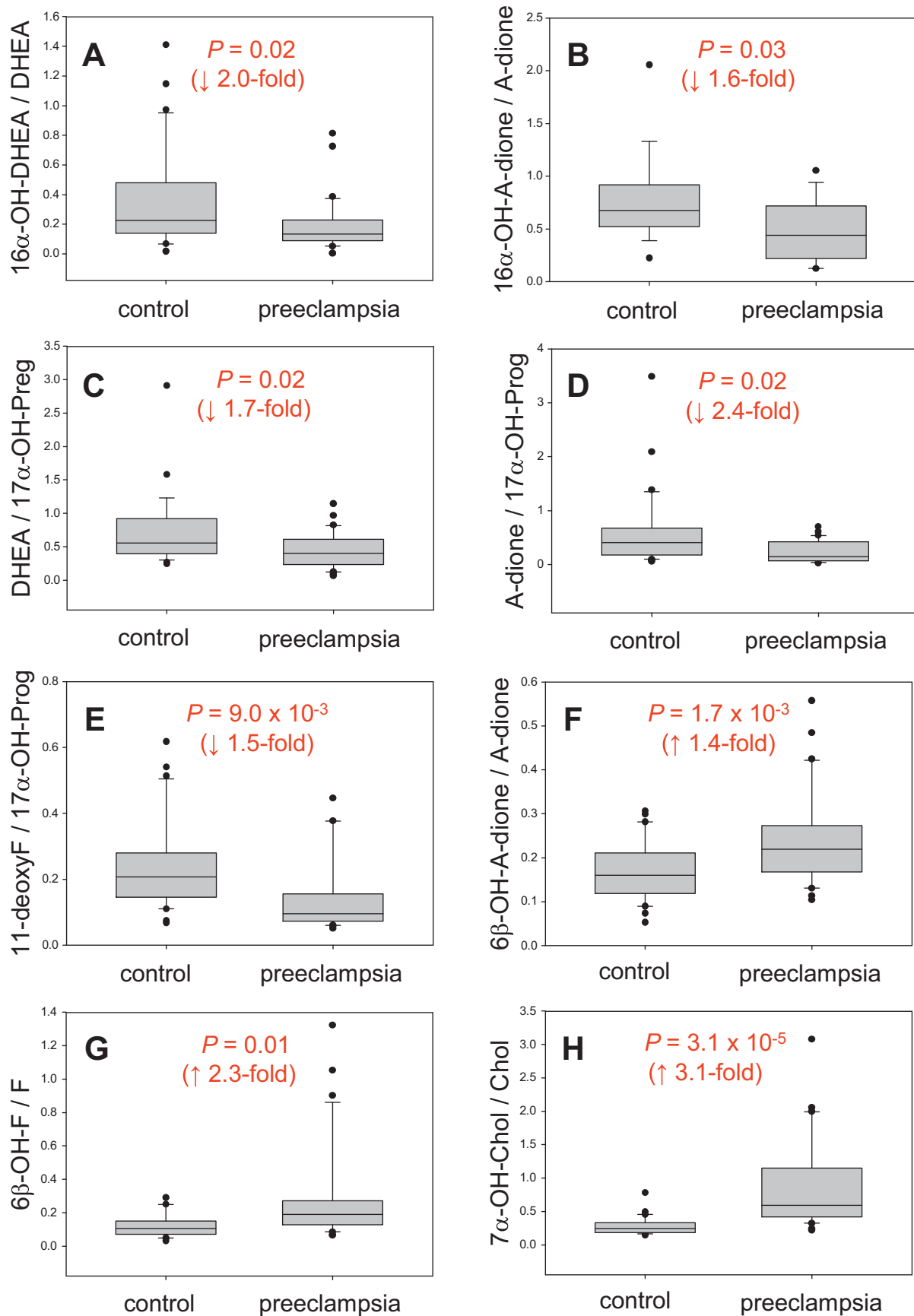
Fig. 3. Comparison of the metabolite/substrate ratio as enzyme activity in control (n = 30) and preeclamptic (n = 30) serum samples. The ends of the boxes define the 25th and 75th percentiles, and the lines inside the boxes define the medians. Dots above and below indicate the plot putliers with the 10th and 90th percentiles. The fold changes are presented as the mean ratios of preeclampsia to control levels, which was indicated in parentheses. Statistical comparisons were made using Student's t-test.
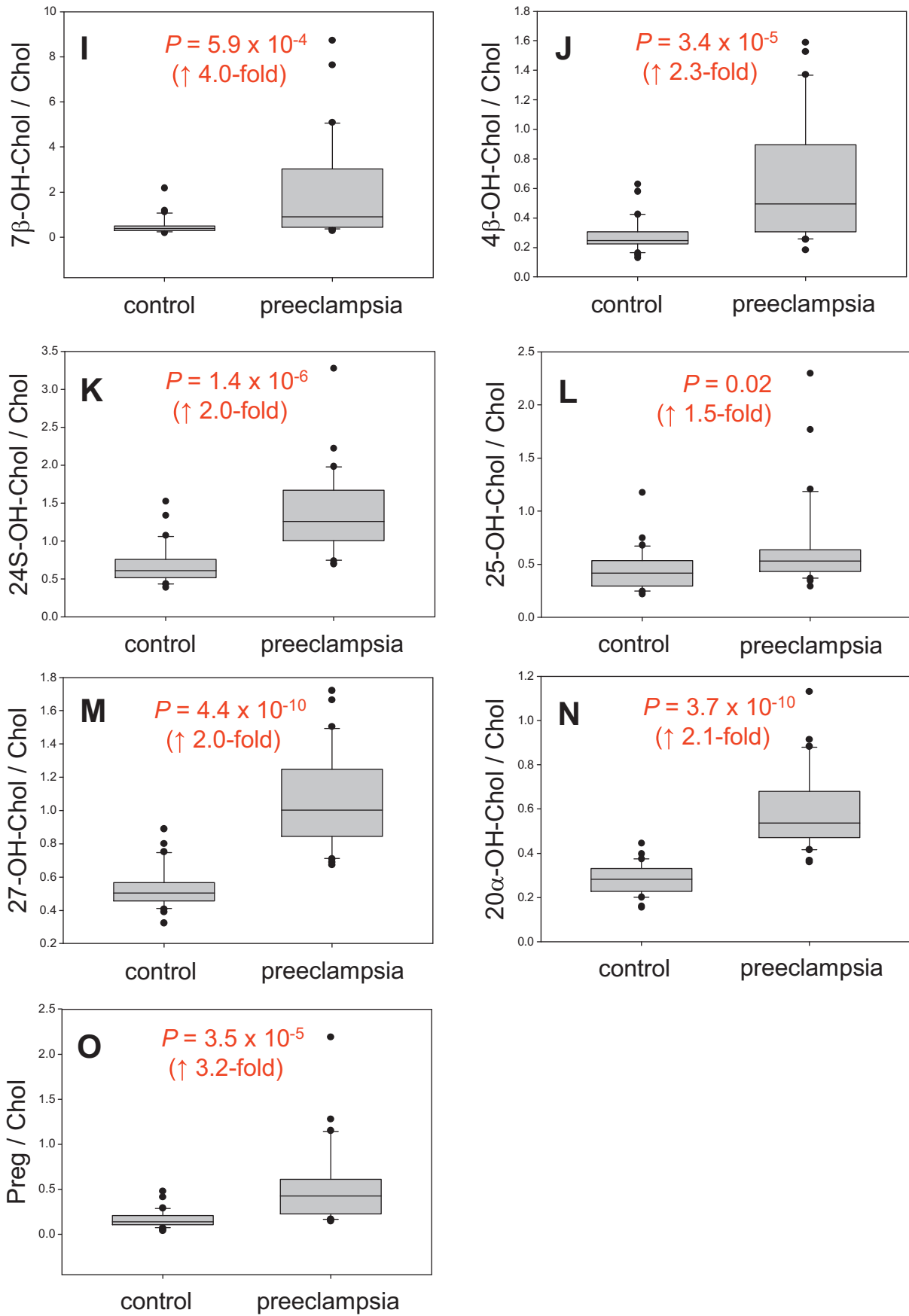
I — 7β-OH-Chol / Chol; $P = 5.9 \times 10^{-4}$ (↑ 4.0-fold); control, preeclampsia

J — 4β-OH-Chol / Chol; $P = 3.4 \times 10^{-5}$ (↑ 2.3-fold); control, preeclampsia

K — 24S-OH-Chol / Chol; $P = 1.4 \times 10^{-6}$ (↑ 2.0-fold); control, preeclampsia

L — 25-OH-Chol / Chol; $P = 0.02$ (↑ 1.5-fold); control, preeclampsia

M — 27-OH-Chol / Chol; $P = 4.4 \times 10^{-10}$ (↑ 2.0-fold); control, preeclampsia

N — 20α-OH-Chol / Chol; $P = 3.7 \times 10^{-10}$ (↑ 2.1-fold); control, preeclampsia

O — Preg / Chol; $P = 3.5 \times 10^{-5}$ (↑ 3.2-fold); control, preeclampsia

Fig. 3. (*Continued*).

altered steroid metabolism during preeclampsia. In addition, the levels of 16α-OH-A-dione and 16α-OH-DHEA were significantly lower in preeclamptic patients, suggesting a lack of fetal liver 16α-hydroxylase required for estriol synthesis in the placenta. The relationship between 16α-hydroxylated C19 steroid precursors and preeclampsia remains unclear. However, 16α-hydroxylation of DHEA during pregnancy may reflect the function not only of the fetus, but also of the placenta. Further studies are required to identify the effect of preeclampsia on 16α-hydroxylase.

The cholesterol level of preeclampsia patients was also reduced through multi-hydroxylation by enzymatic or catalytic reaction, e.g., 7α-hydroxylation, 7β-hydroxylation, 4β-hydroxylation, 20α-hydroxylation, 24S-hydroxylation, or 27-hydroxylation. However, based on a significantly higher expression of cholesterol hydroxylation in preeclampsia patients than in control subjects ($P < 0.001$, >2-fold), hydroxylated cholesterol metabolites (oxysterols) showed a positive association with preeclampsia risk. These findings are consistent with those of previous studies [33–35]. Oxysterols have potent biological activities, including regulation of cholesterol and steroid hormone biosynthesis, lipid homeostasis, inflammation, and cytotoxic effects [5,33]. The increase levels of oxidized low-density lipoproteins carrying oxysterols during pregnancy and higher oxysterol concentrations in preeclamptic patients may explain a possible link with oxidative stress and inflammation [34]. In addition, cholesterol oxidation products in blood and aortic tissue may be derived from endogenous free radical activity, which can enhanced under specific pathological conditions in hypertensive rabbits [36].

The observed increase in oxysterols levels in the present study may be explained by several factors. Firstly, exposure to oxysterols during pregnancy (because of increased oxidative stress) may impair differentiation and fusion of term trophoblast cells through liver X receptor (LXR). This differentiation and fusion is a prerequisite for normal fetal growth and development, and therefore, impairment may disrupt the formation of tight placental endothelial cells, which are responsible for the exchange of gases, nutrients, and waste; production of hormones; and immune tolerance [33]. Secondly, an increase in oxysterols levels under the conditions associated with oxidative stress may promote secretion of proinflammatory cytokines (e.g., IL-6, MIP-1β, and TNF-α) in placental trophoblast cells, via the activation of the TLR4-signaling and cholesterol-sensitive NF-kB pathways. These pathways play important roles in the placental inflammatory response and innate immune response, respectively. Thus, their activation may contribute toward placental inflammation [34]. Further studies are required to confirm the effect of CYP enzymes on oxysterol levels according to gestational age, and therefore whether increased oxysterols levels represent a risk factor for early diagnosis of preeclampsia.

5. Conclusions

The metabolic profiling reveals a broad spectrum of CYP-mediated steroid hydroxylations during pregnancy-induced hypertension. To our knowledge, this is the first report to evaluate a range of CYP enzyme activities affected by preeclampsia, and to indicate that pregnancy-induced hypertension may be caused not by a single effect on an isolated enzyme system, but by multiple effects on a variety of enzyme systems. In comparison with conventional CYP assays, the devised GC–MS-based steroid assay enables the simultaneous assessment of a diverse range of CYP enzymes. The multiple markers could serve as background information for improved clinical diagnosis during pregnancy, which may minimize the risk of preeclampsia and prenatal death. Thus, our validated multiplexed CYP assay represents a valuable monitoring tool for predicting the activities of steroid-metabolizing CYP enzymes, and for identifying multiple CYP enzyme markers.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2013.02.014.

References

[1] E.A. Steegers, P. von Dadelszen, J.J. Duvekot, R. Pijnenborg, Pre-eclampsia, Lancet 376 (2010) 631–644.
[2] D.W. Nebert, D.W. Russell, Clinical importance of the cytochromes P450, Lancet 360 (2002) 1155–1162.
[3] A.H. Payne, D.B. Hales, Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones, Endocrine Reviews 25 (2004) 947–970.
[4] Y.Y. Zhang, L. Yang, Interactions between human cytochrome P450 enzymes and steroids: physiological and pharmacological implications, Expert Opinion on Drug Metabolism and Toxicology 5 (2009) 621–629.
[5] I.A. Pikuleva, Cholesterol-metabolizing cytochromes P450, Drug Metabolism and Disposition 34 (2006) 513–520.
[6] K.K. Miller, J. Cai, S.L. Ripp Jr., W.M. Pierce, T.H. Rushmore, R.A. Prough, Stereo- and regioselectivity account for the diversity of dehydroepiandrosterone (DHEA) metabolites produced by liver microsomal cytochromes P450, Drug Metabolism and Disposition 32 (2004) 305–313.
[7] C.C. Peng, I. Templeton, K.E. Thummel, C. Davis, K.L. Kunze, N. Isoherranen, Evaluation of 6 beta-hydroxycortisol, 6 beta-hydroxycortisone, and a combination of the two as endogenous probes for inhibition of CYP3A4 in vivo, Clinical Pharmacology and Therapeutics 89 (2011) 888–895.
[8] A.K. Goodenough, J.M. Onorato, Z. Ouyang, S. Chang, A.D. Rodrigues, S. Kasichayanula, S. Huang, W. Turley, R. Burrell, M. Bifano, M. Jemal, F. LaCreta, A. Tymiak, D. Wang-Iverson, Quantification of 4-beta-hydroxycholesterol in human plasma using automated sample preparation and LC–ESI-MS/MS analysis, Chemical Research in Toxicology 24 (2011) 1575–1585.
[9] D.W. Nebert, T.P. Dalton, The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis, Nature Reviews Cancer 6 (2006) 947–960.
[10] T. Wong, C.H. Shackleton, T.R. Covey, G. Ellis, Identification of the steroids in neonatal plasma that interfere with 17α-hydroxyprogesterone radioimmunoassays, Clinical Chemistry 38 (1992) 1830–1837.
[11] A.W. Hsing, F.Z. Stanczyk, A. Bélanger, P. Schroeder, L. Chang, R.T. Falk, T.R. Fears, Reproducibility of serum sex steroid assays in men by RIA and mass spectrometry, Cancer Epidemiology Biomarkers and Prevention 16 (2007) 1004–1008.
[12] O.J. Semmes, Defining the role of mass spectrometry in cancer diagnostics, Cancer Epidemiology Biomarkers and Prevention 13 (2004) 1555–1557.
[13] W.J. Griffiths, T. Koal, Y. Wang, M. Kohl, D.P. Enot, H.P. Deigner, Targeted metabolomics for biomarker discovery, Angewandte Chemie-International Edition 49 (2010) 5426–5445.
[14] N.B. Krone, A. Hughes, G.G. Lavery, P.M. Stewart, W. Arlt, C.H. Shackleton, Gas chromatography/mass spectrometry (GC/MS) remains a pre-eminent discovery tool in clinical steroid investigations even in the era of fast liquid chromatography tandem mass spectrometry (LC/MS/MS), Journal of Steroid Biochemistry and Molecular Biology 121 (2010) 496–504.
[15] J.-Y. Moon, H.-J. Jung, M.H. Moon, B.C. Chung, M.H. Choi, Heat-map visualization of gas chromatography–mass spectrometry based quantitative signatures on steroid metabolism, Journal of the American Society for Mass Spectrometry 20 (2009) 1626–1637.
[16] Y.W. Ha, J.-Y. Moon, H.-J. Jung, B.C. Chung, M.H. Choi, Evaluation of plasma enzyme activities using gas chromatography–mass spectrometry based steroid signatures, Journal of Chromatography B 877 (2009) 4125–4132.
[17] J.-Y. Moon, K.J. Kim, M.H. Moon, B.C. Chung, M.H. Choi, A novel GC–MS method in urinary estrogen analysis from postmenopausal women with osteoporosis, Journal of Lipid Research 52 (2011) 1595–1603.
[18] N.C. Van de Merbel, Quantitative determination of endogenous compounds in biological samples using chromatographic techniques, Trends in Analytical Chemistry 27 (2008) 924–933.
[19] R. Tamimi, P. Lagiou, L.J. Vatten, L. Mucci, D. Trichopoulos, S. Hellerstein, A. Ekbom, H.O. Adami, C.C. Hsieh, Pregnancy hormones, pre-eclampsia, and implications for breast cancer risk in the offspring, Cancer Epidemiology Biomarkers and Prevention 12 (2003) 647–650.
[20] S.W. Walsh, Progesterone and estradiol production by normal and preeclamptic placentas, Obstetrics and Gynecology 71 (1988) 222–226.

[21] S.W. Walsh, S. Coulter, Increased placental progesterone may cause decreased placental prostacyclin production in preeclampsia, American Journal of Obstetrics and Gynecology 161 (1989) 1586–1592.

[22] S.W. Walsh, Eicosanoids in preeclampsia, Prostaglandins Leukotrienes and Essential Fatty Acids 70 (2004) 223–232.

[23] M.T. Acromite, C.S. Mantzoros, R.E. Leach, J. Hurwitz, L.G. Dorey, Androgens in preeclampsia, American Journal of Obstetrics and Gynecology 180 (1999) 60–63.

[24] R. Troisi, N. Potischman, J.M. Roberts, R. Ness, W. Crombleholme, D. Lykins, P. Siiteri, R.N. Hoover, Maternal serum oestrogen and androgen concentrations in preeclamptic and uncomplicated pregnancies, International Journal of Epidemiology 32 (2003) 455–460.

[25] N.R. Miller, D. Garry, H.W. Cohen, R. Figueroa, Serum androgen markers in preeclampsia, Journal of Reproductive Medicine 48 (2003) 225–229.

[26] R. Troisi, N. Potischman, C.N. Johnson, J.M. Roberts, D. Lykins, G. Harger, N. Markovic, P. Siiteri, R.N. Hoover, Estrogen and androgen concentrations are not lower in the umbilical cord serum of pre-eclamptic pregnancies, Cancer Epidemiology Biomarkers and Prevention 12 (2003) 1268–1270.

[27] A. Hertig, P. Liere, N. Chabbert-Buffet, J. Fort, A. Pianos, B. Eychenne, A. Cambourg, M. Schumacher, N. Berkane, G. Lefevre, S. Uzan, E. Rondeau, P. Rozenberg, M.E. Rafestin-Oblin, Steroid profiling in preeclamptic women: evidence for aromatase deficiency, American Journal of Obstetrics and Gynecology 203 (477) (2010) e1–e9.

[28] J.T. Ho, J.G. Lewis, P. O'Loughlin, C.J. Bagley, R. Romero, G.A. Dekker, D.J. Torpy, Reduced maternal corticosteroid-binding globulin and cortisol levels in pre-eclampsia and gamete recipient pregnancies, Clinical Endocrinology (Oxf) 66 (2007) 869–877.

[29] S.P. Salas, G. Marshall, B.L. Gutiérrez, P. Rosso, Time course of maternal plasma volume and hormonal changes in women with preeclampsia or fetal growth restriction, Hypertension 47 (2006) 203–208.

[30] J.T. Murai, E. Muzykanskiy, R.N. Taylor, Maternal and fetal modulators of lipid metabolism correlate with the development of preeclampsia, Metabolism: Clinical and Experimental 46 (1997) 963–967.

[31] A.G. Frantz, F.H. Katz, J. W, Jailer, 6 beta-hydroxy-cortisol: high levels in human urine in pregnancy and toxemia, Proceedings of the Society for Experimental Biology and Medicine 105 (1960) 41–43.

[32] C.O. Watlington, L.B. Kramer, E.G. Schuetz, J. Zilai, W.M. Grogan, P. Guzelian, F. Gizek, A.C. Schoolwerth, Corticosterone 6 beta-hydroxylation correlates with blood pressure in spontaneously hypertensive rats, American Journal of Physiology 262 (1992) F927–F931.

[33] I.L. Aye, B.J. Waddell, P.J. Mark, J.A. Keelan, Oxysterols inhibit differentiation and fusion of term primary trophoblasts by activating liver X receptors, Placenta 32 (2011) 183–191.

[34] I.L. Aye, B.J. Waddell, P.L. Mark, J.A. Keelan, Oxysterols exert proinflammatory effects in placental trophoblasts via TLR4-dependent, cholesterol-sensitive activation of NF-κB, Molecular Human Reproduction 18 (2012) 341–353.

[35] P. Bodzek, B. Janoszka, T. Wielkoszyński, D. Bodzek, A. Sieroń, Concentration of chosen oxycholesterols in plasma of pregnant women with pregnancy-induced hypertension, Biomedical Chromatography 16 (2002) 13–18.

[36] H.N. Hodis, S. Hashimoto, W.J. Mack, A. Sevanian, Probucol reduces oxysterol formation in hypertensive rabbits, Hypertention 36 (2000) 436–441.