The agreement between the two methods was evaluated by correlating results from patient samples and control solutions of the two assays, and a Bland-Altman plot was constructed to visualize the differences (Fig. 1). Despite the fairly good correlation, the trend observed in the Bland–Altman plot together with the large SD (2.19 mg/L for the samples) prevent direct comparison of the results. The Bland-Altman plot (Fig. 1) further indicates that the difference is composed of both constant and proportional components.

To further evaluate the nature of the differences, we performed a Deming regression analysis because there are neither commutable primary calibrators nor reference methods available to evaluate the trueness of results of adiponectin measurements. In the Deming regression analysis, both patient and control samples were included, and it yielded a slope of 0.7670 (SE analysis, both patient and control samples were included, adiponectin measurements. In the Deming regression proportional components.

Despite the fairly good correlation, the trend observed in well-controlled multivariate or longitudinal study settings would be imperative.

The Fujirebio Inc. (Tokyo, Japan) made this study possible with the practical disadvantages associated with the use of radiolabeled reagents, this may be interpreted as an advantage for the ELISA method over the RIA-based method in the research laboratory.

In conclusion, this study confirmed that the enzyme immunometric assay evaluated here is a robust and an easily implementable tool for measuring adiponectin concentrations on a standard platform in clinical laboratory research.

The Fujirebio Inc. (Tokyo, Japan) made this study possible by kindly donating the reagents needed to perform the adiponectin ELISA assay.

References


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Proteinuria and Hypertension Are Independent Factors Affecting Fetal DNA Values: A Retrospective Analysis of Affected and Unaffected Patients, Akihiko Sekizawa,1 Antonio Farina,1,2 Yumi Sugito,1 Ryu Matsuoka,1 Mariko Iwatsuki,1 Hiroshi Saito,1 and Takashi Okai1

Cell-free fetal DNA circulates in the plasma of pregnant women (1–3). Increased concentrations of fetal DNA have been reported in the plasma of pregnant women with various complications of pregnancy, including pre-eclampsia (PE) (4–6), preterm labor (7), invasive placenta (8), hyperemesis gravidarum (9), and aneuploidy (10–12). We recently reported that fetal DNA in maternal plasma is derived primarily from villous trophoblasts bordering the intervillous spaces, which are filled with maternal blood (13). Because trophoblast damage may be involved in the pathogenesis of PE, it may be possible to use fetal DNA as a marker to monitor the severity of PE. In this study, we assessed the relationship between fetal DNA concentrations in maternal plasma and clinical evidence of PE, such as proteinuria and/or hypertension.

We conducted a retrospective study in which the control group included 116 women with uncomplicated male pregnancies between 28 and 40 weeks of gestation. Controls were matched with 45 consecutive pregnancies in 50

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which different grades of proteinuria and/or hypertension were observed at the time of blood sampling (28–40 weeks). PE was defined as gestational hypertension (systolic pressure >140 mmHg or diastolic blood pressure >90 mmHg on at least two occasions after 20 weeks of gestation) with proteinuria (>0.3 g/day). Severe PE (SPE) was defined as severe gestational hypertension (systolic pressure >160 mmHg or diastolic blood pressure >95 mmHg on at least two occasions after 20 weeks of gestation) with severe proteinuria (>3 g/day). Fetal growth restriction was defined as an estimated fetal weight 2.0 SD below the mean expected weight for gestational age (GA), as determined by ultrasonographic evaluation. None of the pregnant women had fetal aneuploidy, abnormalities of cord insertion, and/or maternal complications such as systemic lupus erythematosus, diabetes mellitus, or hyperthyroidism. All samples were obtained before the onset of labor. In the present study, 24 cases had PE, 8 had gestational hypertension, and 13 had proteinuria alone, the symptoms of which occurred after 20 weeks of gestation. All women presented at Showa University Hospital between August 2001 and December 2002. Fetal growth restriction was observed in 15 of 24 preeclamptic women. All study participants provided written informed consent for the use of their biological specimens for research purposes. The ethics committee of Showa University School of Medicine approved this protocol.

Maternal blood samples (7 mL) were collected into tubes containing EDTA. After separation, the plasma samples were stored at −20°C until use. DNA was extracted from 1.5-mL samples of plasma supernatant by use of a QIAamp Blood Mini Kit (Qiagen). The Y-chromosome-specific DYS14 sequence was subsequently quantified by PCR in a LightCycler (Roche Diagnostics), as described in previous reports (1, 6).

Strict precautions were taken to avoid contamination, and water blanks were used as negative controls. A female staff member performed all procedures, including sample preparation, DNA extraction, and PCR amplification.

Descriptive analysis of the available variables was performed by routine testing. Data were first stratified according to the presence of PE: 0 = absent; 1 = mild PE; and 2 = SPE. In addition, stratification based on the presence of proteinuria and/or hypertension was performed, including those cases affected by proteinuria alone or hypertension alone. Symptoms of proteinuria and hypertension were classified as follows: 0 = absent; 1 = mild; and 2 = severe. Two different \( \log_{10} \) linear regressions were performed, the first to estimate the effect of mild and severe PE on fetal DNA concentrations, and the second to estimate the value of proteinuria and hypertension as independent predictors of increased fetal DNA. In the first regression analysis, we included only those cases with evidence of PE. All data (PE cases + hypertension-alone cases + proteinuria-alone cases) were included in the second regression analysis. The regressions were done with fetal DNA as the dependent variable and GA as a quantitative independent variable. Further data with regard to PE (for the first regression) and proteinuria and hypertension (for the second regression) were placed in the linear regression analyses as “dummy” variables; thus, they assumed values of 0 = absent, 1 = mild, or 2 = severe. The mean effect on fetal DNA was considered log-linear at any week and then estimated exactly halfway (mean) through the study interval (28–40 weeks of gestation).

The patient demographics for each group included in the regression analyses are shown in Table 1. The GAs of the pregnancies in this study ranged from 28 to 40 weeks, with a mean (SD) GA of 34 (3.28) weeks. We thus used 34 weeks as a reference point by which to estimate the effect of various variables on fetal DNA values. GA was associated with an increase of ~12% in fetal DNA per week.

The following equation was derived to estimate fetal DNA values according to severity of PE: \( \log_{10}\text{DNA} = 0.44 + 0.035 \times \text{GA} + 0.355 \times \text{PE} \) (see the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue1/ for details). At 34 weeks, PE was associated with a 2.25-fold increase in the fetal DNA concentration, whereas SPE was associated with a 5.06-fold increase.

Both proteinuria and hypertension showed a strong independent association with fetal DNA values, including in the presence of GA, which represents a well-known source of variation in fetal DNA values. The following equation was derived to estimate fetal DNA values as a function of proteinuria (P) and hypertension (H): \( \log_{10}\text{DNA} = 0.62 + 0.045 \times \text{GA} + 0.259P + 0.201H \) (see the online Data Supplement for details). Proteinuria was associated with greater increases in fetal DNA than hypertension (see the online Data Supplement for details). In fact, fetal DNA was 1.82 and 3.32 times greater in patients with mild and severe proteinuria, respectively, compared with controls at 34 weeks. The presence of hypertension was associated with mean increases of 1.58- and 2.52-fold in patients with mild and severe hypertension, respectively, compared with controls. Fig. 1 shows DNA estimations based on the second linear regression scale. It should be noted that similar fetal DNA values were calculated for the control group by the first and the second regression.

### Table 1. Demographic characteristics of the patients.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Maternal age, years</th>
<th>Weeks after onset</th>
<th>GA, weeks</th>
<th>LogDYS14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>116</td>
<td>31.4 (4.87)</td>
<td>34.2 (3.28)</td>
<td>2.12 (0.42)</td>
<td></td>
</tr>
<tr>
<td>Mild PE</td>
<td>7</td>
<td>34.0 (6.27)</td>
<td>31.4 (4.39)</td>
<td>36.0 (3.01)</td>
<td>2.72 (0.26)</td>
</tr>
<tr>
<td>SPE</td>
<td>17</td>
<td>36.0 (3.99)</td>
<td>32.1 (3.14)</td>
<td>35.0 (2.47)</td>
<td>2.83 (0.25)</td>
</tr>
<tr>
<td>P* + PE</td>
<td>37</td>
<td>34.8 (4.50)</td>
<td>32.9 (3.50)</td>
<td>35.7 (2.65)</td>
<td>2.81 (0.35)</td>
</tr>
<tr>
<td>H + PE</td>
<td>32</td>
<td>34.2 (4.91)</td>
<td>32.0 (3.33)</td>
<td>35.4 (2.55)</td>
<td>2.84 (0.33)</td>
</tr>
<tr>
<td>P</td>
<td>13</td>
<td>33.7 (4.02)</td>
<td>34.2 (2.79)</td>
<td>36.4 (2.65)</td>
<td>2.96 (0.50)</td>
</tr>
<tr>
<td>H</td>
<td>8</td>
<td>30.5 (3.58)</td>
<td>32.2 (3.05)</td>
<td>35.8 (2.50)</td>
<td>2.83 (0.49)</td>
</tr>
</tbody>
</table>

* P, proteinuria; H, hypertension.
The pathogenesis of PE is poorly understood, but it is likely associated with failure of the uterine vasculature to undergo adequate physiologic remodeling by extravillous trophoblasts in women with PE (14). Because there is insufficient invasion of extravillous trophoblasts into the uterine vasculature, placental vascular resistance is not reduced; there thus is inadequate oxygenation of blood within the placental intervillous spaces that border villous trophoblasts. This might damage the villous trophoblasts (15), leading to the release of DNA into the intervillous spaces as a result of cell damage or apoptosis (6, 13), after which it might enter the maternal circulation. Thus, both hypoxia and increased amounts of circulating fetal DNA might occur with insufficient trophoblast invasion. Lo et al. (15) have reported that the half-life of circulating fetal DNA (placental DNA) is 16.3 min. However, a fourfold increase in the clearance half-life of fetal DNA has been observed in patients with PE compared with controls (114 vs 28 min) (16). The rapid turnover of fetal DNA in women with PE might make fetal DNA a useful marker to monitor trophoblast damage over time.

In the present study, estimated fetal DNA concentrations in patients with mild and severe PE were 2.25 and 5.06 times greater than those in controls at 34 weeks (mean gestational age of the line of regression). We assumed a log-linear relationship between severity of PE and maternal fetal DNA concentrations. At the very least, our findings indicate that fetal DNA values increase with increasing severity of PE. This is likely attributable to more extensive villous trophoblast damage in patients with SPE than in those with milder PE. If there is indeed a correlation between fetal DNA concentrations and trophoblast damage, hypoxia within the intervillous spaces might be more pronounced in cases of SPE than cases of mild PE.

We also examined the relationship between proteinuria and hypertension and fetal DNA values. We found (a) that both proteinuria and hypertension were independently and strongly associated with increased concentrations of fetal DNA in maternal plasma, the extent to which depended on the severity of each symptom; and (b) that proteinuria was associated with greater increases in fetal DNA than hypertension. Again, we assumed a log-linear relationship between severity of proteinuria and hypertension and maternal fetal DNA values. We speculated that the pathogenesis of proteinuria might differ from that of hypertension and that proteinuria in pregnant women might be more closely related to hypoxic damage to villous trophoblasts than hypertension. On the basis of our results, it appears that proteinuria is a better indicator of damage to villous trophoblasts or placental hypoxia than hypertension. We believe that further studies may be needed to help clarify these findings.

In summary, this is the first report to describe a relationship between fetal DNA in maternal plasma and severity of PE, as well as an association between fetal DNA and proteinuria and hypertension. Because fetal DNA is thought to come from villous trophoblasts and is associated with the severity of each of these findings, fetal DNA might represent a molecular marker that can be used to evaluate trophoblast damage and monitor the status of pregnancies affected by PE in clinical practice.

This work was supported in part by two Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (Grants 14770870 and 15591163), as well as the Takeda Science Foundation, Japan, and the Fondazione CARISBO Progetto Triennale–Molecular Genetics of Fetal DNA, Italy.

References
2. Lo YMD, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al.
Arachidonic acid can be metabolized by cytochrome P450 enzymes to a range of compounds that play a central role in the regulation of vascular tone, renal function, and blood pressure (1, 2). In the vasculature, smooth muscle cells produce 20-hydroxyeicosatetraenoic acid (20-HETE) as a major product of CYP450 metabolism. 20-HETE can cause vasoconstriction by inhibition of potassium chan-
nels and is thought to contribute to the vasoconstrictor action of hormones such as angiotensin II and endothelin (3, 4). Despite the physiologic importance of CYP450 metabolites of arachidonic acid, very little is known about the regulation of the concentration of 20-HETE in biological fluids or the relationship of these concentrations with physiologic state in healthy individuals. This has in part been attributable to the lack of reliable sensitive and specific assays to measure endogenous concentrations of these compounds. Gas chromatography–mass spectrometry (GCMS) has been used successfully to measure 20-HETE in biological samples. However, available methods rely on one or more thin-layer chromatography steps (5, 6), and for human urine the presence of interfering peaks can be a problem (7). An alternative procedure has recently been reported that uses a sensitive fluorescent HPLC assay (8), although this may lack the specificity of MS.

We have developed a simplified and reliable method for the analysis of urinary 20-HETE and analyzed 20-HETE concentrations in 24-h urine samples from a group of 30 healthy individuals. Our method involves the use of a single solid-phase extraction cartridge containing both reversed-phase and strong anion-exchange packing followed by HPLC separation before derivatization and GCMS analysis. We have found that preparation of the tert-butylidimethylsilyl derivative (tBDMs), as originally used by Prakash et al. (6), gives better chromatographic separation from interfering peaks present in urine.

20,20-[2H$_2$]-20-HETE was prepared according to previously published procedures (9). Unlabeled 20-HETE was purchased from Cayman Chemicals. β-Glucuronidase (Escherichia coli), pentafluorobenzyl bromide (PFB Br), N,N-diisopropylethylamine, and tert-butylidimethylsilyl-N-methyltrifluoroacetamide were purchased from Sigma-Aldrich. Pyridine was purchased from Fluka. Bond Elut-Certify II (200 mg, 3 mL) columns were purchased from Varian Inc. Volunteers were recruited from the general population. We monitored 24-h blood pressures (BP) by use of an ambulatory device (Spacelabs 90207). All studies with samples from humans were approved by the Human Ethics Committee of Royal Perth Hospital.

The internal standard [2H$_2$]-20-HETE (2 ng) was added to urine (2 mL). Each sample was left at room temperature for 10 min to equilibrate before incubation with 0.2 mg of β-glucuronidase from E. coli (in 0.075 mol/L potassium phosphate buffer, pH 6.8, containing 1 g/L bovine serum albumin) for 2 h at 37 °C. After hydrolysis, samples were diluted with 2 mL of 0.1 mol/L sodium acetate solution (pH 7) containing 50 mL/L methanol, and the pH was adjusted to 6.0 with 100 mL/L acetic acid.

Bond Elut-Certify II columns were preconditioned with 2 mL of methanol, followed by 2 mL of 0.1 mol/L sodium acetate solution (pH 7) containing 50 mL/L methanol before application of the urine samples. The columns were washed with 2 mL of methanol–water (1:1 by volume), and urinary 20-HETE and internal standard were eluted with 2 mL of hexane–ethyl acetate (75:25 by volume) containing 10 mL/L acetic acid. The organic